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Impact of Frost on the Morphology and Chemical Composition of *cv.* Santulhana Olives

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Abstract: Frost events and extreme weather phenomena greatly affect several characteristics of the olive fruit. This study evaluated the impact of frost on the morphology, composition (moisture, fat, fatty acids, tocopherols, and total phenolic contents), and antioxidant activity of olives of *cv.* Santulhana. A total of 14 trees from the same geographical region (Santulhão, northeast of Portugal) were chosen, including trees subjected or not subjected to frost conditions (n = 7 each). The results showed that frost led to morphological changes in olive fruits, particularly in terms of weight and diameter, which were imposed by a huge decrease in the moisture content (−20%). Fat relative content increased as a consequence of the water loss (+29% in fresh pulp weight), with a slight reduction of the relative abundance of saturated fatty acids (−4%) and tocopherol contents in the fat (−17%). However, the total phenolic contents and antioxidant activity were severely affected (−70% and −42%, respectively), with potential consequences for the olive oil stability and sensorial attributes. Principal component analysis showed that both morphological and chemical parameters could be used as biomarkers to identify olives subjected or not subjected to frost. The overall negative impact of frost on the minor antioxidant contents of *cv.* Santulhana olives may anticipate a quality loss of olive oils extracted from olives affected by frost.

Keywords: frost; *cv.* Santulhana; fruit morphology; fatty acids profile; bioactive compounds

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1. Introduction

Olive oil, the main source of fat in the Mediterranean diet, is a food with an exceptional balance between saturated and unsaturated fatty acids, containing other important minor compounds such as polyphenols, tocopherols, carotenoids, sterols, and chlorophylls. Fruit cultivar, agronomic practices, fruit ripeness, weather, and geographical origin (soil conditions, latitude, and altitude) greatly influence the physicochemical and sensory profiles of the olive oils [1–6].

Establishing the "right time" to harvest is of utmost significance. During the ripening process of the fruit, several chemical and enzymatic reactions change the color, pulp-to-stone ratio, weight, oil content, and phenolic compound concentration, reflecting the final olive oil composition [7,8] and conditioning its yield, sensory characteristics, shelf-life, and color. Olive color can be used to assess the ripeness index of the olive fruit because the photosynthetic activity decreases along with ripeness, reducing the concentration of chloroplast pigments, and changing the green olive fruit coloration to purple shades [8]. Later harvest will also deliver olives with a higher percentage of oil per mass of the fruit (lower moisture content), increasing the olive oil extraction yield per fruit mass, although not necessarily per tree. However, these olive oils have low bitterness and lack green fruit aromas compared to oils extracted from olives at earlier ripeness stages, showing a shorter shelf-life imposed by a reduction on its natural antioxidants and being, therefore, more

prone to the appearance of defects such as rancidity [9]. On the other hand, too early a harvest leads to reduction of the extraction yield and, occasionally, to the extraction of unacceptable oils from an organoleptic point of view, due to an excessive bitterness imposed by its high concentration of polyphenols [7,8,10]. Thus, olive farmers and olive oil producers face a dilemma in establishing the best harvest time-period, which would allow farmers to obtain a satisfactory yield without compromising the olive oil physicochemical quality.

In addition to all these expected biological evolutions of the plant, the weather conditions are of utmost importance for the ripening evolution, and consequently, the definition of harvest. Climate change and unexpected weather events, particularly unpredicted early frosts or other extreme weather phenomena, have increased the complexity of these decisions. While natural weather variations are known to influence the composition of olive oils, with a higher proportion of monounsaturated fatty acids at lower temperatures [11,12], among other known variations, the effect of frost on olive oil yield and quality has only been recently addressed [13–16].

Frost occurs every year across southern and eastern agricultural geographical regions. Mediterranean countries like Portugal are also affected by frost. This phenomenon is particularly problematic in the Trás-os-Montes region (northeast of Portugal), the second-largest Portuguese production area of olive trees for oil extraction. Olive oils from the Trás-os-Montes region are well-known for their high physicochemical quality and distinctive sensory characteristics. One example is the olive oils extracted from Santulhana cultivar, whose olives have medium-high weight, are large and symmetric, with an interesting oil yield and high levels of antioxidants [17]. Unfortunately, olive groves with Santulhana trees are mainly located in the northeast of Portugal, a geographical area with optimal conditions (i.e., clear, cold nights, without wind or at higher altitudes) for frost occurrences.

In this sense, and to truly understand the frost impact on the quality of *cv*. Santulhana olives, several morphological and chemical parameters were evaluated in olives subjected or not subjected to frost conditions, aiming to provide a decision basis regarding its harvest and quality impact.

2. Materials and Methods

2.1. Experimental Design

Olives were harvested during the crop year of 2018–2019, in one olive grove located in Santulhão village (41°33′57.103″ N 6°37′28.834″ W, and 663 m high; district of Bragança, northeast of Portugal). The trees (plant density is 7 × 7 m) were approximately 60 years old and belonged to the *cv*. Santulhana. The olive grove followed the guidelines for organic production and, during the year under study, it was neither tilled nor sprayed with insecticides. The mean temperatures recorded during the months of October, November, and December 2018 were, respectively, (i) $T_{minimum}$: 7.2, 4.4 and 3.1 °C; (ii) $T_{average}$: 13.5, 8.1 and 7.1 °C; and (iii) $T_{maximum}$: 19.8 °C; 11.8 and 11.2 °C. Although the positive mean minimum temperatures were recorded for three months, on several nights the minimum temperature reached negative values, between −3 and −1 °C. Fourteen different trees were chosen, seven that are usually subjected to frost and seven others that are not affected by frost issues due to their location. On 7 January 2019, approximately 2 kg of olives from each olive tree were harvested in with a maturation index of 6, then analyzed to evaluate the fruit morphology and chemical composition.

2.2. Sampling and Olive Measurements

From each of the 14 olives trees, 100 olives affected by frost and not affected were randomly selected to perform each type of analysis. All samples were morphologically characterized, determining the length (mm), maximum diameter (mm) and weight (g) for the fruit and the endocarp of the same fruit according to the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV) for *Olea europea* [18]. Briefly, the fruit fresh weight was registered, and then each fruit was manually separated into flesh and stone. The fruit-to-stone ratio was calculated as the ratio between the fruit and stone

weights. The fresh fruit pulps were frozen and freeze-dried (SCANVAC Coolsafe 110-4, Bjarkesvej, Denmark) and crushed (Moulinex 327, 700 W) until a homogenous sample was obtained and were then kept in a desiccator until further analysis (i.e., total phenols content and antioxidant activity).

2.3. Moisture (%)

The moisture (%) was determined according to the AOAC method [19]. For each sample, approximately 5 g of olive pulp was weighed and dried in an oven (BINDER, Model ED 400, Tuttlingen, Germany) at 100 °C until reaching a constant weight. The assays were performed in triplicate.

2.4. Fat Content

The total fat content was determined using a Soxhlet apparatus (PSelecta®). For each sample, 5 g of olive pulp was placed on a filter paper (Whatman n° 4), which was folded and closed tightly using a cotton wire. Each sample was placed in previously weighted Soxhlet beakers and was extracted with petroleum ether. The solvent remains were evaporated using a rotary evaporator (Stuart®, RE300DB, Stone, UK) at 35 °C. After that, the samples were put in an oven at 50 °C until they reached a constant weight, and they were then stored in a desiccator. The extracted fat was packaged refrigerated for analysis of the lipid constituents.

2.5. Total Phenols Content and Antioxidant Activity

2.5.1. Extract Preparation

The total phenol content and antioxidant activity were assessed on methanolic extracts prepared from the freeze-dried olive samples (1.5 g). The mixture was left under stirring for 1 h with 50 mL of methanol in the dark. After filtration, the extraction was repeated twice under similar conditions. At the end of the extraction process, the methanol was evaporated on a rotary evaporator at 35 °C. From the extract obtained, a subsample was made in methanol with a concentration of 0.5 mg/mL, which was filtered with a Whatman Nylon (0.20 µm) filter and stored in dark flasks at refrigerated temperature until further analysis.

2.5.2. Total Phenols Content (TPC)

The TPC was assessed from the combined extract for the sample analyzed. Briefly, 1 mL of extract was added to 1 mL of Folin–Ciocalteu reagent (Aldrich Chemistry) and vortexed for 3 s and allowed to react for 3 min. Then, 1 mL of a saturated sodium carbonate solution (Na_2CO_3) and 7 mL of deionized water were added, vortexed for 3 s, and further allowed to react for 90 min in the dark at room temperature. This procedure was carried out in triplicate for each extract obtained. All samples were spectrophotometrically analyzed (UV-VIS/UV-1280 Shimadzu spectrophotometer, Kyoto, Japan) at 725 nm, and the TPC was determined using a calibration curve established between the absorbance values recorded for standard methanolic solutions with different concentrations of gallic acid ($R^2 \geq 0.9999$), with the concentrations expressed as mg of gallic acid equivalents (GAE) per kg of fresh pulp (mg GAE/kg fresh pulp).

2.5.3. Radical Scavenging Activity

The radical scavenging activity of each olive extract was evaluated by spectrophotometrically assessing the absorbance at 517 nm (UV-VIS/UV-1280 Shimadzu spectrophotometer) [20]. For that, 0.3 mL of the extract were mixed with 2.7 mL of DPPH solution (0.06 mM), vortexed for 3 s, and left in the dark for 1 h. Then, the absorbance was read. The DPPH radical scavenging was expressed as the percentage of reduction of DPPH activity.

2.6. Fat Chemical Characterization

2.6.1. Extraction Process

In order to determine the fatty acid profile and tocopherol composition, lipids were extracted using the procedure described by Fernandes et al. [21] with few alterations. For each condition, 5.0 g of olive fruits were crushed in Moulinex equipment, and anhydrous sodium sulfate was added to remove moisture remains. The lipid fraction was obtained by Soxhlet extraction with petroleum ether for a 6 h period. To prevent oxidation during the extraction process, 0.01% (*w*/*v*) of BHT (2,6-di-*tert*-butyl-4-methylphenol, Sigma, Madrid, Spain) was added to the solvent. Then, the solvent was removed with a rotatory evaporator at a temperature of 35 °C (Stuart®, RE300DB), and the samples were stored at −20 °C until analysis.

2.6.2. Determination of Fatty Acid Profile

From the extracted fat, fatty acids were evaluated by gas chromatography (Chrompack CP 9001 apparatus with flame ionization detection (FID), Varian, Middelburg, The Netherlands) after conversion to methyl esters using cold alkaline transesterification with a methanolic potassium hydroxide solution following the recommended method for olive oil analysis [22]. The fatty acid profile was accomplished using a fused silica capillary column (50 m × 0.25 mm i.d. × 0.25 µm; SelectFAME, Agilent, Santa Clara, CA, USA) with helium as the carrier gas at 110 kPa. The temperatures of the detector and injector were 250 and 230 °C, respectively, with an optimized temperature gradient for fatty acid complete separation. The fatty acid composition is expressed in the relative percentage of each fatty acid regarding the total fatty acid methyl esters eluting between myristic and lignoceric methyl esters [21]. A certified fatty acid methyl ester standard mixture (Supelco 37 Component FAME Mix) was used for identification and FID calibration purposes (Sigma).

2.6.3. Determination of Tocopherols Profile

Tocopherols were evaluated as previously described [23]. Tocopherol standards (α, β, γ, and δ) were purchased from Sigma, and 2-methyl-2-(4,8,12-trimethyltridecyl) chroman-6-ol (tocol), used as the internal standard, was from Matreya Inc. (Pleasant Gap, PA, USA). Filtered oil (50 mg) plus 10 µL of the internal standard solution (tocol, 100 µg/mL prepared with n-hexane) were mixed and then centrifuged for 5 min at 13,000 rpm, and the obtained supernatant was analyzed by high-performance liquid chromatography (HPLC). A Jasco integrated system (Tokyo, Japan) equipped with an LC–NetII/ADC data unit, a PU-1580 Intelligent Pump, and a FP-920 fluorescence detector ($\lambda_{excitation}$ = 290 nm and $\lambda_{emission}$ = 330 nm) was used. A Luna Silica column (3 µm, 100 × 3.0 mm from Phenomenex, USA) was used for separation at constant room temperature (23 °C). The eluent was a mixture of n-hexane and 1,4-dioxane (97.5:2.5) at a 0.7 mL/min flow rate. Data were analyzed with the ChromNAV Control Center–JASCO Chromatography Data Station (Tokyo, Japan). The compounds were identified using standards, co-elution, and evaluation of the UV spectra. The internal standard method was applied for quantification, using the fluorescence signal response and individual calibration curves for each tocopherol. Total vitamin E was quantified as the sum of the individual tocopherol contents and expressed as mg/kg of extracted fat.

2.7. Statistical Analysis

All data were expressed as the mean value ± standard deviation of at least three independent replicates. Results were analyzed using the t-Student test, which was performed at a significance level of 0.05 using the software IBM® SPSS® Statistics 23 for Windows (SPSS Software, 2015, New York, NY, USA). Additionally, principal component analysis (PCA) was used as an unsupervised pattern recognition multivariate technique aiming to evaluate the possibility of using subsets of the experimental data to differentiate the studied *cv*. Santulhana olives according to their exposure or non-exposure to frost. PCA

was performed using the Sub-select and MASS packages of the open-source statistical program R (version 3.6.2).

3. Results and Discussion

3.1. Morphological Characterization

Olive fruits can be damaged by frost, i.e., when the air temperature decreases to 0 °C or lower, promoting freezing of the fruits' pulp water, reducing enzymatic and biochemical reactions, as well inhibiting microbial activity [24,25]. Thus, olive fruits and endocarps subjected or not subjected to natural freezing–defrosting cycles before harvest were morphologically characterized according to the UPOV parameters [18], and the data are listed in Table 1. The values obtained for size (length and diameter) and weight for the olives not affected by frost are in accordance with previous data for this cultivar [17], strengthening *cv.* Santulhana size attributes in comparison with other typical cultivars from the Trás-os-Montes region, such as *cvs*. Cobrançosa, Verdeal Transmontana, and Madural. Frost promoted a significant reduction in the diameter and weight of the studied olives (minus 6 and 13%, p-value < 0.05) but not their length. This decrease could be related to the significant loss of moisture observed in olive fruits affected by frost (minus 20%, p-value < 0.05), associated with a reduction of the "firmness" of olive fruits (not evaluated). In fact, water crystallization inside the fruit pulp cellular structures is followed by defrosting cycles, creating cellular damage that facilitates water loss, which leads to water evaporation and loss of weight and, consequently, a weight and diameter reduction. Since the endocarp is not affected by water crystallization as the pulp, the pulp-to-stone weight ratio significantly decreased for olives subjected to frost (minus 20%, p-value < 0.05). These results are in accordance with previous studies, which reported that the weight of olives grown at lower temperatures (higher altitudes) was lower than the weight of fruits grown at higher temperatures [26,27]. In opposition, the total fat content of olives affected by frost was significantly greater compared to the weight of unfrosted olives (plus 29%, p-value < 0.05). This can be explained by the loss of water, as stated above, which is also indicative that the potential cellular damage imposed by water crystals does not affect the oil content.

Table 1. Morphological characteristics of olive fruit and stone, moisture, and fat contents of *cv.* Santulhana of olive fruits affected or not affected by frost (mean ± standard deviation; minimum and maximum values in brackets).

	No Frost	**With Frost**	***p*-Value**
Olive Fruit			
Length (mm)	24.8 ± 1.0 (23.4 ± 1.6 to 25.9 ± 1.6)	24.3 ± 0.9 (23.1 ± 0.7 to 25.4 ± 1.4)	0.333
Diameter (mm)	17.8 ± 0.6 (16.7 ± 1.1 to 18.5 ± 0.8)	16.7 ± 0.8 (15.2 ± 0.7 to 17.5 ± 0.8)	0.014
Weight (g)	4.5 ± 0.4 (4.0 ± 0.7 to 5.1 ± 0.7)	3.9 ± 0.5 (3.1 ± 0.3 to 4.4 ± 0.5)	0.029
Stone			
Length (mm)	18.0 ± 0.8 (16.4 ± 1.2 to 18.5 ± 0.6)	18.3 ± 0.5 (17.3 ± 1.0 to 18.8 ± 1.1)	0.362
Diameter (mm)	9.2 ± 0.3 (8.7 ± 0.5 to 9.6 ± 0.5)	9.1 ± 0.3 (8.6 ± 0.5 to 9.6 ± 0.4)	0.631
Weight (g)	0.9 ± 0.1 (0.7 ± 0.1 to 1.0 ± 0.1)	0.9 ± 0.1 (0.8 ± 0.1 to 1.0 ± 0.1)	1.000
Weight ratio Pulp/Stone	4.4 ± 0.3	3.5 ± 0.4	0.001
Moisture (%)	59.1 ± 6.2	47.5 ± 3.3	0.001
Fat (%)	25.9 ± 3.6	33.4 ± 3.8	0.001

The feasibility of using the moisture and fat contents as differentiating parameters to recognize/distinguish *cv*. Santulhana olives subjected or not subjected to frost were further evaluated using a PCA. As can be seen from the 2D PCA biplot (Figure 1, explaining the first two principal components, PCs, 100% of the data variability), the two variables allowed the unsupervised differentiation of olives affected (lower moisture and higher total fat content) or not affected by frost. The first PC (PC1) accounted for the largest possible variance in the data set (i.e., 88.8%) and PC2 accounted for the remaining variance (11.2%). This finding confirms the significant impact of frost on these two parameters.

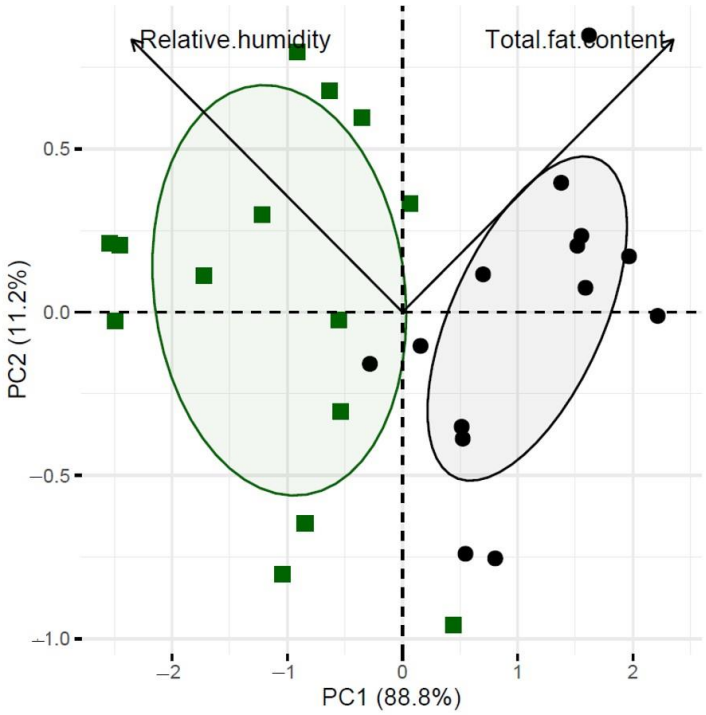

Figure 1. Differentiation of *cv*. Santulhana olives not subjected to frost (■) and subjected to frost (●): 2D PCA biplot based on the relative humidity and total fat content.

3.2. Fatty Acid Composition

The impact of the frost phenomenon on the olive fruits' fatty acid profiles from *cv*. Santulhana, related to the freezing–defrosting cycles to which the olives may be subjected before harvest, was evaluated. For all samples, 13 fatty acids could be detected and quantified, including seven saturated, four monounsaturated, and two polyunsaturated fatty acids (SFA, MUFA and PUFA, respectively). The mean relative percentages are listed in Table 2 for myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), heptadecanoic acid ($C_{17:0}$), heptadecenoic acid ($C_{17:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidic acid ($C_{20:0}$), eicosenoic acid ($C_{20:1}$), behenic acid ($C_{22:0}$), and lignoceric acid ($C_{24:0}$). Oleic acid (67–71%), linoleic acid (12–15%), palmitic acid (12–13%), and stearic acid (2–3%) were the most abundant fatty acids for both groups.

Table 2. Fatty acids profile (%) of olives from *cv*. Santulhana subjected or not to frost (mean ± standard deviation).

Fatty Acid Profile (%)	No Frost	With Frost	*p*-Value
Myristic acid ($C_{14:0}$)	0.04 ± 0.04	0.05 ± 0.05	0.669
Palmitic acid ($C_{16:0}$)	12.21 ± 0.39	11.58 ± 0.29	0.001
Palmitoleic acid ($C_{16:1}$)	0.83 ± 0.06	0.71 ± 0.06	0.001
Heptadecanoic acid ($C_{17:0}$)	0.08 ± 0.01	0.07 ± 0.01	0.999
Heptadecenoic acid ($C_{17:1}$)	0.10 ± 0.01	0.09 ± 0.01	0.999
Stearic acid ($C_{18:0}$)	2.72 ± 0.18	2.79 ± 0.23	0.420
Oleic acid ($C_{18:1}$)	68.35 ± 0.91	69.47 ± 1.74	0.043
Linoleic acid ($C_{18:2}$)	13.26 ± 0.68	13.05 ± 1.50	0.643
Linolenic acid ($C_{18:3}$)	1.19 ± 0.09	1.06 ± 0.15	0.016
Arachidic acid ($C_{20:0}$)	0.40 ± 0.02	0.39 ± 0.02	0.327
Eicosenoic acid ($C_{20:1}$)	0.27 ± 0.02	0.26 ± 0.02	0.637
Behenic acid ($C_{22:0}$)	0.14 ± 0.01	0.13 ± 0.01	0.153
Lignoceric acid ($C_{24:0}$)	0.06 ± 0.00	0.05 ± 0.01	0.001
ΣSFA	15.66 ± 0.38	15.07 ± 0.44	0.001
ΣMUFA	69.54 ± 0.89	70.53 ± 1.72	0.068
ΣPUFA	14.45 ± 0.66	14.12 ± 1.40	0.423

In general, the results agree with those obtained by Malheiro et al. [28] when studying the fat fraction of green table olives produced with *cv*. Santulhana (oleic acid (66.9%), linoleic acid (10.3%) palmitic acid (13.0%), and stearic acid (2.8%).

The statistical analysis showed that although the olives subjected to frost have a significantly lower relative abundance of saturated fatty acids (in general, p-value < 0.05), from a practical point of view, the levels found were of the same order of magnitude. This finding demonstrated that the relative abundance of the fatty acids is frost tolerant for the studied *cv*. Santulhana olives. Overall, the results shown in Table 2 are not in line with the main findings of Morelló et al. [14] or Asheri et al. [29], who concluded that the fatty acid composition of oils was not significantly influenced by olives subjected to frost or stored under frozen conditions, respectively. However, the study of Asheri et al. [29] also pointed out that for some cultivars (e.g., *cvs*. Arbequina and Mission), the relative abundance of fatty acids of oils extracted from frozen olives (at 4 °C during one or three weeks) was significantly influenced (increased or decreased, depending on the fatty acid and on the olive cultivar), which in turn is in line with the findings of the present study. On the other hand, oleic acid, the most abundant fatty acid, showed a slight increase in its relative abundance in oils extracted from olives affected by frost. The observed increase of oleic acid could be tentatively attributed to a possible partial inhibition of the activity of oleate desaturase enzyme, which is responsible for the transformation of oleic into linoleic acid [30]. The lower temperatures during frost, as well as the slightly lower abundance of linoleic acid in the oils obtained after frost, can justify the hypothesized enzymatic inhibition. The higher abundance of oleic acid in the oils from olives subjected to frost are in accordance with the findings of Asheri et al. [29] for *cvs*. Arbequina and Mission oils extracted from frozen olives, as well as with the results of Mafrica et al. [31] and Piravi-Vanak et al. [32], demonstrated an increase in unsaturated fatty acid percentage in oils from olives grown at lower temperatures. Furthermore, the results showed no significant effect of frost on the MUFA and PUFA relative abundance, which is in line with the findings of Asheri et al. [29] for olive oils extracted from olives grown at low temperatures. Finally, the results showed no significant effect of frost on the MUFA and PUFA relative abundance, which is in line with the findings of Asheri et al. [29] for olive oils extracted from olives grown at low temperatures. However, the slight but significant reduction in the relative proportion of linolenic acid (18:3), known to be the more sensitive to oxidation due to the presence of three double bonds, is a strong indicator for the presence of higher oxidative stress in the oil extracted from the olives subjected to frost. Water loss

and water crystallization might induce the formation of electrolyte concentrated spots, thereby increasing oxidative stress.

A PCA was also performed to infer the unsupervised differentiation of the studied *cv*. Santulhana olives, subjected or not subjected to frost, based on the relative contents of the 13 fatty acids identified. Figure 2 shows the 2D-PCA biplot, with the two first PCs explaining 60.5% of the data variability. As can be seen, the initial 13 variables under study and the respective large dataset (i.e., relative abundance of the 13 fatty acids) could be dimensionally reduced into a smaller dataset (comprising the 2 first PCs), which still accounted for more than 60% of the information of the large set. Figure 2 further shows that the differences in the relative composition of the fatty acids allowed a satisfactory differentiation of the studied olives, confirming that frost affected the fatty acids' relative abundance depending on whether the olives were exposed or not exposed to frost. In fact, with the exception of oleic acid ($C_{18:1}$) and stearic acid ($C_{18:0}$), all the remaining fatty acids' relative abundance slightly decreased when the olives were subjected to frost. From both nutritional and technological perspectives, these changes have a minor impact.

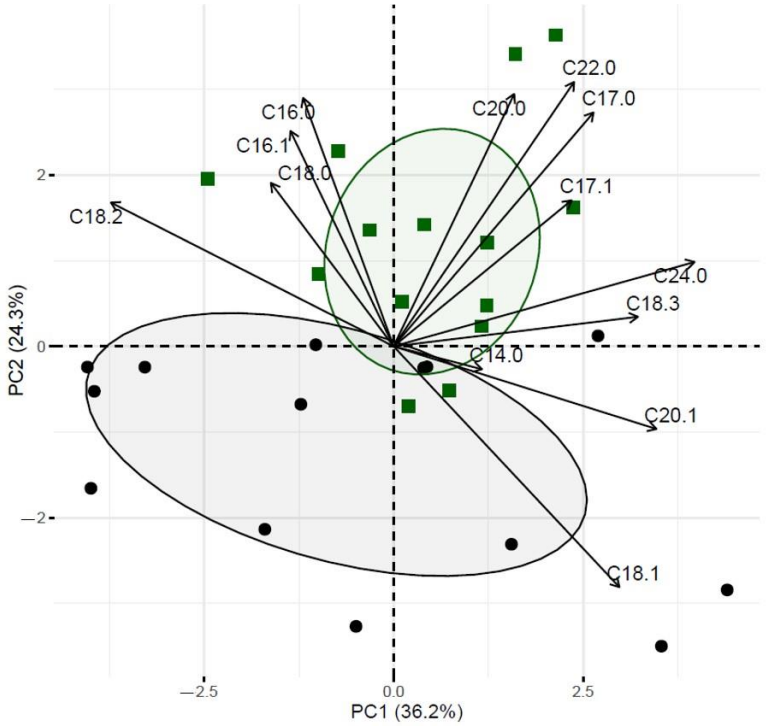

Figure 2. Differentiation of *cv*. Santulhana olives not subjected to frost (■) and subjected to frost (●): 2D PCA biplot based on the fatty acids profile ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$, $C_{18:3}$, $C_{20:1}$, $C_{22:0}$ and $C_{24:0}$).

3.3. Tocopherols, Total Phenols Content, and Antioxidant Activity

Table 3 shows the mean concentrations of tocopherols in the oil extracted from the pulp and for both TPC and DPPH inhibition percentages (antioxidant activity) in the pulp methanolic extracts. The results showed that frost had a significant negative effect (p-value < 0.05) on the majority of the contents of the olives' lipophilic and hydrophilic antioxidant tocopherols and TPC, as well as on the radical scavenging activity (antioxidant activity). In fact, frost imposed a decrease in the mean concentrations of α-, β-, and γ-tocopherols (an average reduction of 18, 12, and 6%, respectively). This slight reduction could be explained by the minor oxidative stress observed in the fatty acid profile [15,16]. Contrarily, the TPC concentration was severely affected by the frost events. An average reduction of 70% was observed (Table 3). These results are in accordance with those reported by Morelló et al. [14], who also observed a decrease in the concentration of phenolic compounds in olive oils extracted from olives collected after frost. The phenols'

marked decrease can be partially attributed to the olive tissue injuries due to the water crystallization and the abrupt decrease of temperatures during the frost event, followed by a temperature rise after frost, which promotes the oxidative activity of enzymes, thereby leading to a degradation of phenolic compounds [15]. The potentially direct exposure of the inner pulp to oxygen during the frosting periods due to the micro-fissures created from crystal damage cannot be excluded, with a direct impact on phenolics' oxidative damage. Additionally, due to their water solubility, the phenolic compounds can also be affected by the water movements in the fruit pulp, increasing their probability of damage. The decrease of the antioxidant activity of the olives' extracts due to the frost phenomenon can be easily correlated with the decrease in the bioactive contents in the olives after frost, with an average significant reduction of 42% on the DPPH inhibition being observed.

Table 3. Tocopherols contents, total phenols content, and DPPH radical scavenging inhibition of olives affected by frost and not affected by frost (mean ± standard deviation).

Parameters	No Frost	With Frost	*p*-Values
	Tocopherols content (mg/kg_{oil})		
α-tocopherol	248 ± 38	203 ± 34	0.003
β-tocopherol	2.5 ± 0.4	2.2 ± 0.5	0.098
γ-tocopherol	18 ± 2	17 ± 2	0.199
Σ tocopherols	268 ± 40	222 ± 34	0.003
Total Phenols content (TPC) (mg GAE/$kg_{fresh\ pulp}$)	4861 ± 657	1469 ± 536	<0.001
DPPH assay (inhibition%)	55 ± 7	32 ± 8	<0.001

Finally, the data on the bioactive compounds and the related antioxidant activity were used to evaluate the possibility of being used as biomarkers for differentiating *cv.* Santulhana olives subjected or not subjected to frost. Figure 3 (2D-PCA biplot, with the two first PCs explaining 81.1% of the data variability) clearly shows that the tocopherols and TPC together with the DPPH inhibition percentages enabled an almost full differentiation of the olives affected or not affected by frost, pointing out visually that olives not subjected to frost had a richer composition of the evaluated bioactive compounds and a higher antioxidant activity.

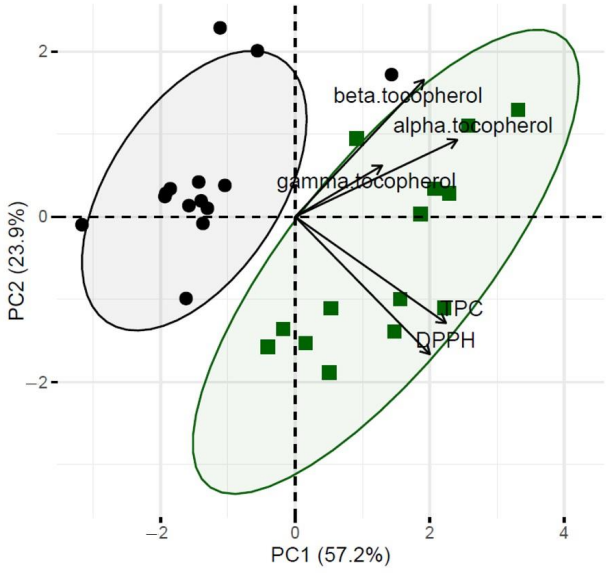

Figure 3. Differentiation of *cv.* Santulhana olives not subjected to frost (■) and subjected to frost (●): 2D PCA biplot based on the tocopherols content (α-, β- and γ-), total phenol content (TPC), and DPPH radical scavenging inhibition.

4. Conclusions

Lower temperatures may cause physiological damage to the olive fruit due to the formation of ice crystals during a frost event. The injuries caused could break the olive cell walls since the different olive compounds are prone to different biochemical reactions that could affect the subsequent olive oil quality. In this study, it was observed that frost events had a negative impact on the chemical composition of olive fruits of *cv*. Santulhana. Olives subjected to frost showed a lower relative humidity; however, since no fat loss was observed, a final increase in the relative proportion of fat per fruit was observed. As to the fat quality, only a slight reduction in the saturated fatty acids' abundance was observed. However, frost had a more negative impact on the contents of the minor bioactive antioxidants, with a pronounced reduction of the total phenol content as well as of the tocopherol contents from olives *cv*. Santulhana, thereby leading to a decrease of the antioxidant activity that could influence the oxidative stability and shelf-life of olive oils extracted from olives subjected to frost. This research focused on one olive cultivar from the Trás-os-Montes region in Portugal, i.e., *cv*. Santulhana, which, due to their geographic location, are more susceptible to frost events. The extent of the damage caused by a frost event or events depends on the olive's variety and chemical composition, as well as on the maturation stage of the olives at harvest. Therefore, further studies are needed, including studies of other olive cultivars, as well as further studies at a microscopic level, to understand the global impact of frost events in olive oil yield and quality.

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