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ARTICLE

Pulsed Electric Field-Assisted Vinification of Aglianico and Piedirosso Grapes

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Pulsed electric field (PEF) treatments were applied to increase the polyphenolic content of fresh red wines made from Aglianico and Piedirosso grapes. Prior to the fermentation/maceration step, the grape skins were treated at different PEF intensities (field strengths from 0.5 to 1.5 kV/cm and energy inputs from 1 to 50 kJ/kg), with their permeabilization being characterized by electrical impedance measurements. Furthermore, the release kinetics of the total polyphenols and anthocyanins were characterized during the maceration stage by spectroscopic and Folin−Ciocalteu colorimetric methods, respectively. Finally, the fresh wine, obtained after pressing, was characterized for total acidity, pH, reducing sugar, color intensity, total polyphenols, anthocyanins content, antioxidant activity, and volatile compound composition. PEF treatment on Aglianico grapes induced a significantly higher release of polyphenols (+20%) and anthocyanins (+75%), thus improving the color intensity (+20%) and the antioxidant activity of the wine (+20%) while preserving the other organoleptic characteristics. In contrast, there was only a minor impact on the polyphenolic release kinetics of Piedirosso grapes, despite the significant degree of cell membrane permeabilization.

KEYWORDS: Pulsed electric fields; red wine; polyphenols; anthocyanins; antioxidant activity; volatile compounds

INTRODUCTION

Pulsed electric field (PEF) technology has stimulated intensive research as a nonthermal treatment for attaining microbial inactivation (*1*, *2*). However, in recent years, PEF application on the permeabilization of cell membranes, with the aim of improving mass transfer, has increased in interest. In fact, membrane permeabilization of animal and plant cells normally requires lower electric field intensities, due to their being larger than bacterial cells, which is reflected in lower energy consumption (*3*).

Because permeabilization of plant cells induces a reduction of the resistances to mass transfer, PEF technology can be used as a pretreatment to increase the yield of fruit juices and accelerate the transfer of water during drying operations as well as improve the extraction of valuable compounds (such as antioxidants, colorants, or flavors) from the inner core of the cells (*4*−*7*).

In particular, the use of PEF to improve the extraction of antioxidants appears to be especially advantageous in winemaking to enrich the wine in polyphenols released from the grape skins.

The traditional winemaking process can extract only a fraction of the large amounts of different phenolic compounds, located in the grape skin, due to the resistance to the mass transfer of cell walls and cytoplasmatic membranes. Phenolic compounds in red wine, such as anthocyanins, tannins, and their polymers, are responsible for both the color and the body of the wine (*8*). However, the presence of phenolic compounds is also responsible for the health-beneficial properties (*9*).

The phenolic content and composition of wines depends on the initial content in the grapes, which is a function of both the variety and the cultivation factors, as well as on the winemaking techniques, such as a higher fermentation temperature or the use of maceration enzymes, aimed at permeabilizing the grape skin cells to improve the extraction of phenolic compounds (*10*). However, traditional techniques for high phenolic content wines consist of extending the maceration time beyond the time required for fermentation, by up to 3 or 4 weeks (*11*). Moreover, either higher energy costs, worsening of the wine quality, or long production times represent significant drawbacks that push toward the application of PEF as a viable option for improving the extraction of phenolic compounds from the skin cells during the maceration steps, without altering the quality of the wine and with moderate energy consumption (*12*, *13*).

As an alternative to thermovinification, a PEF-based process and corresponding electroporation device were recently patented (*14*) and the PEF treatment was tested on Pinot noir mash, achieving a complete opening of the cells with an applied electric field strength of approximately 40 kV/cm and a specific energy of >35 kJ/kg (*15*, *16*), which was considerably lower than thermovinification and obtained a similar polyphenolic content (*17*).

In addition, as a pretreatment to maceration, PEF offered significant advantages in terms of improving the polyphenols content of red wines. For example, when PEF treatment (10 kV/cm and 6.7 kJ/kg) was applied to Tempranillo grape skins prior to

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maceration, a significant improvement of the release rate of the phenolic compounds during maceration was observed. Furthermore, PEF did not affect the other wine characteristics such as alcohol content, total acidity, pH, reducing sugar concentration, and volatile acidity (*18*). However, the application of a similar PEF treatment (2−10 kV/cm and 0.4−7 kJ/kg) to three different Spanish grape varieties (Garnacha, Mazuelo, and Graciano) showed that the efficiency of the treatment depends on the grape variety (*19*). The higher efficiency observed for Mazuelo was explained in terms of worse extractability of the phenolic compounds from the Mazuelo grapes than from the other two grape varieties, suggesting that PEF treatment could be more useful when the extraction of the phenolic compounds from the grape skins is more difficult (*19*).

PEF treatment also was found to be more effective than commercial enzyme preparations. For example, after 3 months of storage, color intensity, anthocyanins content, and total polyphenol index were higher in the PEF-treated wine than in the control wine, whereas the increase due to enzyme preparations was lower (*20*).

Over 12 months of aging in bottles, red wine made from PEF-treated Cabernet Sauvignon grapes (5 kV/cm and 4 kJ/kg) exhibited a higher polyphenolic concentration and color intensity. Furthermore, there were no significant differences from the control wine in both the monomeric anthocyanins content and the quality parameters. However, the content of flavan-3-ols, flavonols, and hydroxycinnamic acids and derivatives was higher in the PEF-treated wine (*21*). Similar results were also obtained for aging in oak barrels (*22*).

Recently, the feasibility of PEF-assisted vinification was proved in a continuous system for Cabernet Sauvignon (*23*) as well as in a pilot-scale plant on Cabernet Sauvignon, Syrah, and Merlot (*24*), using a collinear treatment chamber. The polyphenols release kinetics were affected by the grape variety (the effect of PEF was more evident for Cabernet Sauvignon than for Syrah and Merlot) and significantly depended on the electric field applied as well as the energy delivered (*24*).

It is worth noting that until now the reported studies on PEF as a pretreatment to maceration for the production of red wine have been carried out only by the research group of the University of Zaragoza (*19*−*27*). Therefore, the originality of this study is in the application of PEF treatment to two Italian grape varieties (Aglianico and Piedirosso) to increase the polyphenols content of the red wine obtained after maceration/fermentation, using both different PEF equipment and treatment conditions. It has the aim of contributing to the calibration of the optimal PEF process conditions in dependence of the grape variety as well as maximizing the yield of the extracts with minimum energy consumption. In particular, insights on the mechanisms of polyphenols release are obtained by comparing the polyphenols extraction kinetics under electric fields of mild intensity (0.5−1.5 kV/cm) with the electrical characterization of the degree of permeabilization of the grape skins. In addition, the effect of the PEF treatment is evaluated in terms of the improvement of the antioxidant activity of the wine obtained from grape skins treated by PEF.

MATERIALS AND METHODS

Plant Material. Two different grape varieties were tested, Piedirosso and Aglianico, which were both manually harvested at optimum maturity during 2008 from vineyards in the province of Avellino (Italy). The grapes were refrigerated at 4 °C until vinification, which was conducted within 1 week of harvesting.

The grapes of each variety were weighed, crushed in a roller crusher (Enologica Meola, Italy), and then subjected to manual destemming, with potassium metabisulfite (Enologia Balducci, Italy) subsequently being added as recommended by the manufacturer (5 g/100 kg). Batches of the crushed grapes with a total weight of 650 g were placed in 750 mL flasks to carry out the fermentation and maceration processes.

Table 1. PEF Treatments and Matrices Where Applied

	treatment conditions	impedance measurements	vinification
control		Aglianico, Piedirosso	Aglianico, Piedirosso
enzyme	2 g/100 kg of grapes		Aglianico, Piedirosso
PEF1	0.5 kV/cm 10^3 pulses 1 kJ/kg		Piedirosso
PEF2	1.0 kV/cm 10^3 pulses 5 kJ/kg		Piedirosso
PEF3	1.0 kV/cm 10^4 pulses 50 kJ/kg	Aglianico, Piedirosso	Aglianico
PEF4	1.5 kV/cm 10^3 pulses 10 kJ/kg	Aglianico, Piedirosso	Aglianico, Piedirosso
PEF5	1.5 kV/cm 2.5×10^3 pulses 25 kJ/kg	Aglianico, Piedirosso	Aglianico
PEF6	1.0 kV/cm 5×10^3 pulses 25 kJ/kg		Piedirosso

The must and skins were separated and weighed after crushing to perform the PEF pretreatment on the grape skins. The skins were subjected to PEF treatment and then readded to the must in the original proportions in the fermentation flasks. Furthermore, other batches of the product were treated with a commercial enzyme preparation added to the crushed grapes prior to the maceration phase.

PEF Treatment. The PEF treatment was carried out by means of a high-voltage pulse generator developed by Diversified Technology, Inc. (Bedford, WA), which consists of a power supply, a modulator, and a pulse control unit.

The system, designed to provide both monopolar and bipolar square wave pulses, allows for the independent setting of the applied voltage (0−25 kV/cm), pulse width (1−10 μs), and pulse repetition rate (1−1000 Hz), limited only by the average power of 25 kW.

The grape skins (160 g) were treated in a batch process chamber, made of two plane parallel electrodes of stainless steel separated by a Teflon spacer, which also acted as a container of the product to be treated. The distance between the two electrodes was 2 cm, and their area was 75 cm^2.

The actual voltage and current signals of the treatment chamber were measured, respectively, by a high-voltage probe (Tektronix, P6015A, Wilsonville, OR) and a Rogowsky coil (2-0.1 Stangenes, Inc.) connected to a 300 MHz digital oscilloscope (Tektronix, TDS 3034B).

The maximum electric field intensity (E, kV/cm) was evaluated as the peak voltage divided by the interelectrode gap. The specific energy input per pulse (W, kJ/kg/pulse) was calculated according to eq 1

$$W = \frac{1}{m}\int_0^\infty U(t) \times I(t) \times \mathrm{d}t \quad (1)$$

where $U(t)$ and $I(t)$ represent, respectively, the voltage across the electrodes and the current intensity through the product at time t and m is the mass of the treated product. The total specific energy (W_T, kJ/kg) was calculated by multiplying W and the number of pulses applied.

For both grape varieties tested, PEF treatments with monopolar square wave pulses of different field strengths (0.5−1.5 kV/cm) and energy inputs (1−50 kJ/kg) at a frequency of 1 Hz and a pulse width of 10 μs were applied, as summarized in **Table 1**.

In all of the experiments, the final temperature of the samples never exceeded 30 °C.

Enzyme. Prior to the maceration phase, for both the Piedirosso and Aglianico grapes, pectolytic enzymes (Everzym Color, Everintec, Italy) were added to the crushed grapes at a concentration of 2 g/100 kg, as recommended by the manufacturer.

Winemaking. The applied vinification protocol was the same for all of the samples tested, independent of the treatment applied and grape variety.

The fermentation and maceration processes of both the control samples as well as the PEF- and enzyme-treated samples with the same proportion of skins and grape juice were carried out in 750 mL flasks for three replicates. Alcoholic fermentation was carried out with a selected yeast (Zymaflore F15, Laffort Oenologie, France), which was dosed as recommended by the manufacturer (20 g/100 kg). Fermentation temperature was kept at 25 ± 3 °C. During fermentation, the skins and must were mixed at least once a day. Measurement of the residual sugars or soluble solids was routinely used to monitor the progress of the fermentation. The refraction index (°Brix level) and densitometric measurements of the grape solids, presumed to be mainly sugars, were carried out using a portable density meter, Densito 30 PX (Mettler Toledo, Columbus, OH).

Furthermore, for each day of maceration, 10 mL of must was sampled to monitor the evolution of the color intensity as well as the phenol compounds.

The end of the fermentation period for both the Aglianico and Piedirosso samples was fixed as the time required to obtain a concentration of residual sugars of approximately 1 °Brix.

After the fermentation period, the samples were pressed in a manually operated basket press (Enologica Meola, Italy) to obtain fresh wine, which was then stored in glass demijohns.

The vinification was carried out in triplicate for all of the experimental conditions.

Impedance Measurement. The measurements of the electrical complex impedance of the grape skins in frequency sweep were used to characterize the tissue permeabilization after PEF treatment. These measurements were carried out by loading the grape skins of intact or treated samples of each variety (**Table 1**) into a test vessel between the two parallel plate cylindrical electrodes (2.5 cm diameter) up to a 10 mm thickness. For the measurements, the electrodes were connected to an impedance analyzer (1260, Solartron, U.K.) consisting of a generator and an analyzer.

The generator produces a sinusoidal voltage at a fixed effective voltage value of 1 V peak to peak for a frequency ranging between 1 kHz and 10 MHz. The analyzer provides a frequency response of the sample and calculates the electrical impedance as the ratio of the voltage drop across the sample and the current crossing it during the test.

The results were plotted as the absolute value of the complex impedance $|Z(j\omega)|$ and the phase angle θ as a function of the frequency as well as for different treatment conditions.

To quantify the cellular degree of permeabilization attained by each treatment, a coefficient Z_p, the cell permeabilization index (*28*), was evaluated on the basis of the measurement of the absolute values of the complex impedance of the intact $|Z_{untr}|$ and PEF-treated tissues $|Z_{tr}|$ in the low-frequency (1 kHz) and high-frequency (10 MHz) ranges:

$$Z_p = \frac{|Z_{untr(1kHz)}| - |Z_{tr(1kHz)}|}{|Z_{untr(1kHz)}| - |Z_{tr(1MHz)}|} \quad (2)$$

The value of this index varies between 0, for the intact tissue, and 1, for the fully permeabilized tissue.

Evolution of Color and Polyphenols. For every day of maceration, each container was sampled, with the extracts being centrifuged at 5000 rpm for 5 min at 10 °C to remove any undesired solids in preparation of measurement of the sugar content by refractometer analysis.

The evolution of color intensity (CI) was measured during fermentation, as the sum of the optical density at 420 nm (yellow), 520 nm (red), and 620 nm (blue), obtained by spectrometric analysis:

$$CI = DO_{420} + DO_{520} + DO_{620} \quad (3)$$

In general, color intensity varies between 3 and 18 (*25*).

The concentration of free anthocyanins and total polyphenols in the must obtained from Aglianico and Piedirosso grapes during maceration and fermentation was measured using appropriate spectrometer kits (Biogammma, Italy). The kit for the determination of total polyphenols uses the Folin−Ciocalteu colorimetric method (*29*). The total polyphenols concentration is reported as grams per liter of gallic acid equivalents.

The kits for the determination of the anthocyanin compounds in wine are based on the principle that these compounds are ionized in an acid environment reacting with a controlled ionic strength buffer (*30*). Obviously, this method can measure only the ionizable anthocyanins and not those polymerized with tannins.

The effect of sampling from the fermentation flasks was taken into account by compensation of the final values of color intensity as well as the polyphenols and anthocyanins concentrations.

Wine Analysis. After the must was pressed at the end of the primary fermentation, which separated the skins and other solid matter from the liquid, the fresh wine obtained was analyzed for total acidity, pH, reducing sugar, color intensity, total polyphenols and anthocyanins contents, and antioxidant activity as well as volatile compounds composition.

The total acidity, that is, the sum of the titratable acidity when the wine is brought to pH 7 with the addition of a standard alkaline solution, was determined by a titrimetric method, using bromothymol blue as an indicator of the end of the reaction (*31*), and was expressed in grams per liter of tartaric acid.

The pH was measured by using a Crimson pH-meter, calibrated with reference buffer solutions with a pH of 4, 7, or 9 (*32*).

The amount of sugar that is reduced by fermentation in wine, called reducing sugar, was determined by a titrimetric analysis in which reducing sugars, acting as reducing agents, reduce a cupro-alkaline solution (*31*).

The glucose and fructose content was measured with enzymatic kits (Biogammma, Italy). The enzymatic analysis, extremely selective because it is not influenced by the other reducing sugars, was carried out according to the method recommended in the enzymatic kit, with the absorbance measurements being carried out in a V-670 UV−vis spectrophotometer (Jasco Instruments, USA) with a 1 mm glass cell at 340 nm, corresponding to the peak of absorbance of reduced nicotinamide adenine dinucleotide phosphate (NADPH).

The antioxidant capacity of wines, strictly related to the amount of phenolic compounds, was determined by the DMDP method (*33*). This method is based on the color change of a chromogenic substrate of *N*,*N*-dimethyl-*p*-phenylenediamine (DMDP) upon the addition of the sample. This change is then measured using a V-670 UV−vis spectrophotometer (Jasco Instruments, USA) with a 1 mm glass cell at 505 nm. The antioxidant activity was expressed as milligrams per liter of ascorbic acid equivalents antioxidant capacity, using the calibration curve plotted with different amounts of ascorbic acid.

The analyses relating to the content of total polyphenols and anthocyanins as well as the color intensity for the evaluation were carried out according to the methods described above.

A solid phase extraction-gas chromatographic method was used to determine the middle-range volatility compounds as well as the analytes, which are present in a low concentration (*34*).

The extraction/concentration step, necessary due to the low levels of the aromatic compounds in wines, was carried out using styrene−divinylbenzene cartridges. The cartridges (Supelco Park, Bellefonte, PA) were conditioned by sequentially rinsing them with 4 mL of dichloromethane (Aldrich), 4 mL of methanol (Aldrich), and finally 4 mL of a water/ethanol mixture (12% v/v). All of the solvents were of HPLC grade. Fifty milliliters of the sample (wine or synthetic wine) was rinsed through the cartridge by vacuum suction at a flow rate of 2 mL/min. The cleanup was obtained by flushing the cartridge with 10 mL of water. Subsequently, the adsorbent was dried by letting air pass through it for 10 min. The analytes were recovered by elution with 2 mL of dichloromethane.

The analysis of the extract was carried out in a GC-MS Finnigan-Focus (Thermo-Fisher Scientific, U.K.). An RTX-5 SIL MS capillary column (30 m × 0.25 mm i.d. and 0.25 μm film thickness) with a cross-linked stationary phase of polyethylene glycol (Restek) was used. The chromatographic conditions were as follows: He as the carrier gas; the injector in split mode with a split flow of 20 mL/min and a temperature of 230 °C; the temperature of the ion source was 200 °C; the temperature of the transfer line was 280 °C. The middle-range volatile compounds were separated using a temperature program with an initial oven temperature of 40 °C for 5 min and a temperature gradient of 2 °C/min to a final temperature of 230 °C, which was maintained for 1 min. Three microliters of the sample

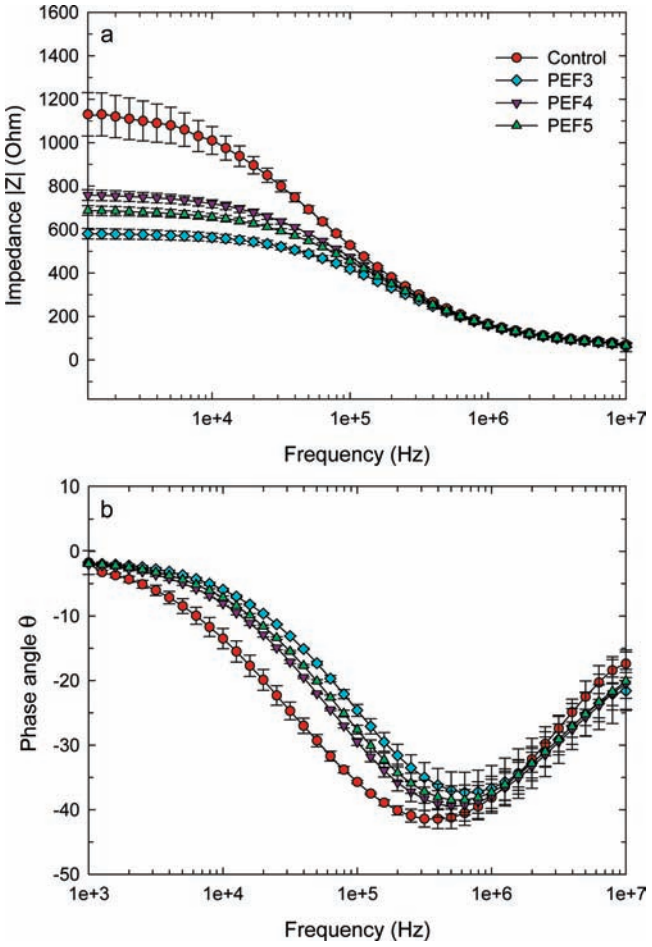

Figure 1. Absolute value |Z| (a) and phase angle θ (b) of the complex impedance as a function of frequency for the control and PEF-treated skins of Aglianico grapes.

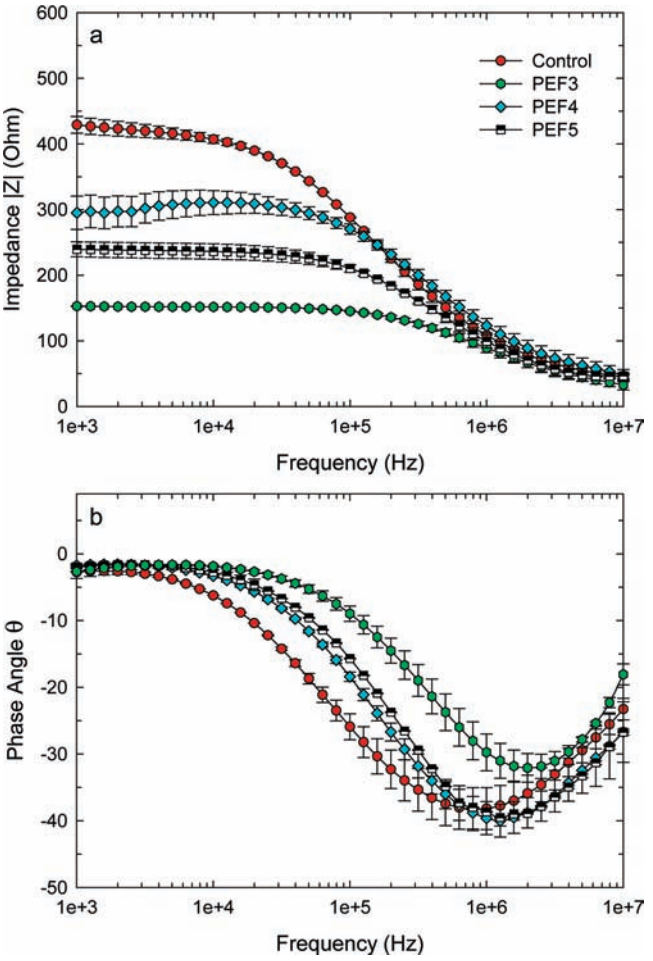

Figure 2. Absolute value |Z| (a) and phase angle θ (b) of the complex impedance as a function of frequency for the control and PEF-treated skins of Piedirosso grapes.

was injected, using the split technique. The ionization was produced by electronic impact at 70 eV.

The eluted compounds were identified using the retention times and by comparing their mass spectra with a spectral library of known standard compounds. The identification was carried out in full scan mode between 40 and 400 amu. The quantification for all of the compounds was carried out by peak area comparisons with the area of a known amount of internal standard (3-octanol).

Statistical Analysis. All of the tests were carried out in triplicate on samples prepared and treated independently. All of the results reported are the average of the measurements taken, plus or minus the standard deviation. The reproducibility of the chemical analysis was expressed as a coefficient of variation (CV).

RESULTS

Tissue Permeabilization. The extent of tissue permeabilization due to the PEF treatment on the two matrices studied was evaluated through impedance measurements in frequency sweep on untreated and treated grape skins. As shown in **Table 1**, the impedance measurements were carried out on both grape varieties under the same PEF treatment conditions (PEF3, PEF4, and PEF5). The results are reported in **Figure 1** for Aglianico and in **Figure 2** for Piedirosso. **Figures 1** and **2** report the frequency-impedance spectra and the transition from an intact to ruptured state in the frequency range investigated for the grape skins of Aglianico and Piedirosso, respectively.

The results show that the absolute impedance value of the intact biological tissue is strongly frequency dependent. This is because in the low-frequency field the cell membrane acts as a capacitor preventing the flow of the electric current in the intracellular medium (ohmic-capacitive behavior). When the frequency is increased, the cell membrane becomes less and less resistant to the current flow. At very high-frequency values (5−10 MHz), the membrane is totally shorted out and the absolute value of the complex impedance is representative of the contribution of both the extra- and intracellular medium (pure ohmic behavior). Therefore, tissue permeabilization, induced by an external stress such as PEF treatment, can be detected in the low-frequency range by comparing treated and untreated samples (*35*, *36*). The observed decrease of the impedance values in the low-frequency range can be explained as the result of the increased concentration of ionic species in the extracellular space, due to PEF-induced membrane permeabilization. Furthermore, the results also show that the higher the PEF treatment intensity, that is, the higher the field strength and/or energy input, the higher the degree of membrane permeabilization (*7*).

Increasing the intensity of the PEF treatment also increased the value of the measured phase angle θ (**Figures 1** and **2**). The phase angle of the control samples is in general negative, due to the capacitive behavior of the cell membranes. When the samples are PEF treated, the capacitance of the cell membranes becomes more and more shortened and, consequently, the phase angle increases, suggesting that the vegetable tissue exhibits an increased resistive behavior, due to cell membrane permeabilization.

According to eq 2, the extent of permeabilization can be quantified by evaluating the cell permeabilization index Z_p.

Table 2. Permeabilization Index Z_p of Aglianico and Piedirosso Grape Skins under Different of Treatments

PEF treatment	Z_p, Aglianico	Z_p, Piedirosso
PEF3	0.59	0.71
PEF4	0.41	0.44
PEF5	0.48	0.57

Table 2 reports the Z_p values for Aglianico and Piedirosso as a function of PEF treatment.

Interestingly, in the range of electric fields investigated (1.0−1.5 kV/cm), the permeabilization degree appears to depend mainly on the energy input rather than on the applied electric field, as previously discussed (*7*). For both varieties, the lowest value of Z_p was observed for PEF4 (1.5 kV/cm and 10 kJ/kg), followed by PEF5 (1.5 kV/cm and 25 kJ/kg), whereas the highest value was observed for PEF3 (1.0 kV/cm and 50 kJ/kg), characterized by a higher energy input but lower electric field. Moreover, at parity of PEF treatments, the Z_p values for Piedirosso are always higher than for Aglianico, by up to 20%.

On the basis of these results, during the vinification process, the PEF3, PEF4, and PEF5 treatments were applied to Aglianico, whereas to Piedirosso, which can be apparently more easily permeabilized, it was decided to apply, in addition to PEF4, which served as a comparison with Aglianico, less intensive treatments such as PEF1, PEF2, and PEF6 (**Table 1**).

Evolution of Fermentation. The evolution of the sugar content during fermentation/maceration was monitored for the musts obtained from both Aglianico and Piedirosso grapes, by °Brix measurements using a portable density meter in situ. In both cases, 9 days is required to reduce the sugar content to approximately 1 °Brix from 22 °Brix for Aglianico and from 20 °Brix for Piedirosso and, therefore, complete the fermentation process (results not reported).

It is worth noting that no difference at all was observed between the control samples and the samples treated with both pectolytic enzymes as well as PEF, suggesting that the permeabilization of the cell membrane is not a rate-limiting step in the fermentation process. In addition, the fermentation rate of the two matrices differed only slightly in the first days, when Piedirosso exhibited a faster initial rate of fermentation reactions.

Color Intensity. The evolution of color intensity during fermentation/maceration depends on the release of polyphenolic compounds from the skin cells and can therefore be affected by the degree of permeabilization of their membranes. **Figure 3** shows the evolution of the color intensity in the must during fermentation/maceration for both grape varieties.

For Aglianico (**Figure 3a**), the color intensity increased at a constant rate in the first 6 days of fermentation, before stable values were attained after a small decrease in the remaining 3 days. The control sample exhibited the lowest color intensity (9.5), whereas PEF3, enzyme, and PEF4 were significantly higher, ranging from 10.0 to 10.8. PEF5 treatment induced at the end of the fermentation the highest color intensity, reaching a value of 11.5. It is worth noting that the color intensity of the enzyme-treated samples at day 1 was comparable with the control sample, as expected. Over the fermentation time, its color intensity progressively increased, exceeding the color intensity of the sample treated by the PEF3 treatment. On the other hand, the color intensity of the PEF-treated samples was always higher than the control from the first day of fermentation/maceration, due to the immediate permeabilization due to the PEF treatment.

In contrast, the PEF and enzyme treatments did not accelerate or improve the evolution of the color intensity in the case of Piedirosso, as shown in **Figure 3b**. No significant differences were

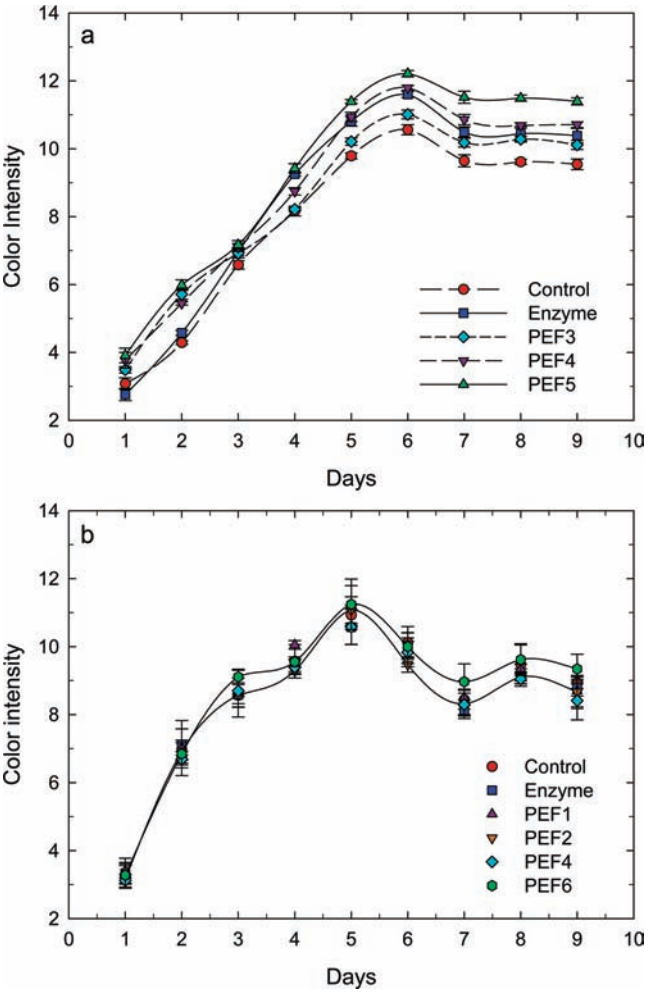

Figure 3. Evolution of the color intensity index during the fermentation of the control, enzyme-treated, and PEF-treated Aglianico (**a**) and Piedirosso (**b**) grapes.

observed during the 9 days of maceration between the color intensity of the control samples and the treated samples. The color intensity of Piedirosso also increased in the first 5 days and then stabilized around an asymptotic value, between 8.5 and 9.5 for all samples.

Total Polyphenols Content. The evolution of the total polyphenols concentration in the must during fermentation/maceration depends on the release rate of the polyphenols from the skin cells. **Figure 4** compares the control samples with the enzyme- and PEF-treated samples for both Aglianico (**Figure 4a**) and Piedirosso (**Figure 4b**).

The trend of the concentration of the total polyphenols during the maceration of the Aglianico grapes was similar to that of the color intensity, with a constant increase in the first 6 days up to a maximum value, followed by a decrease, probably due to the oxidation of a fraction of the polyphenols as well as the attaining of constant values in the last days of fermentation/maceration. In the control samples, the total polyphenols concentration reached a maximum value of 2.2 g/L, before decreasing to 1.8 g/L at the end of fermentation. Compared to the control sample, all of the enzyme- and PEF-treated samples showed higher concentrations of polyphenols, with higher concentrations when the intensity of the treatment was increased. The PEF5 treatment, which resulted the most efficient in improving color intensity, was also the one that increased the most the release of total polyphenols, the concentration of which reached a peak of 2.6 g/L on day 6 and

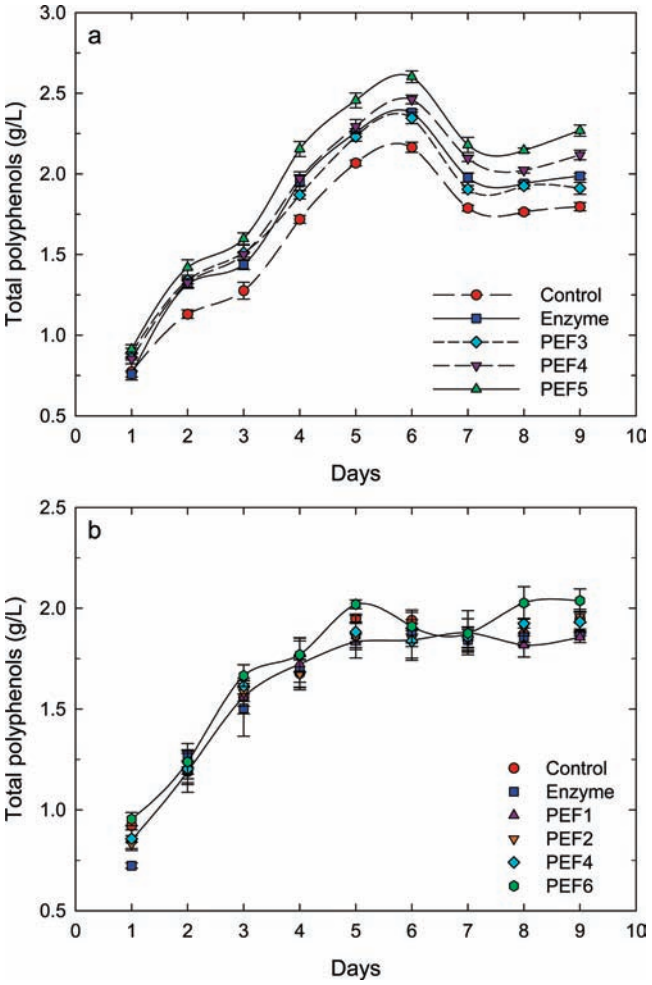

Figure 4. Evolution of the total polyphenols content during the fermentation of the control, enzyme-treated, and PEF-treated Aglianico (**a**) and Piedirosso (**b**) grapes.

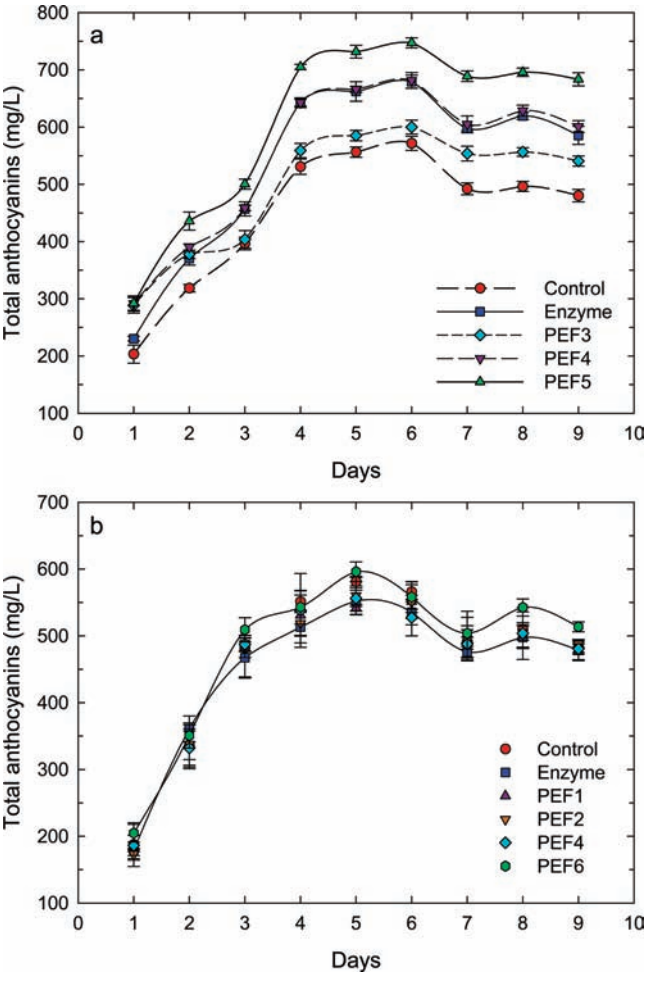

Figure 5. Evolution of the total anthocyanins content during the fermentation of the control, enzyme-treated, and PEF-treated Aglianico (**a**) and Piedirosso (**b**) grapes.

afterward decreased and stabilized at 2.3 g/L. This trend was previously observed for the evolution of the total polyphenols and anthocyanins of Spanish grapes such as Garnacha, Mazuelo, and Graciano (*19*).

The permeabilization effect of the PEF3 treatment was indistinguishable from the effect of the added enzyme (**Figure 4a**), resulting in a total polyphenols concentration at the end of fermentation between 1.9 and 2.0 g/L, whereas the PEF4 treatment increased the final total polyphenols concentration to 2.1 g/L.

In the case of Piedirosso (**Figure 4b**), no significant differences could be observed between the control and treated (either PEF or enzyme) samples. Only for the most intense PEF treatment (PEF6) was there a slight increase in the polyphenols concentration. Despite concentration values similar to Aglianico being attained at the end of the fermentation (between 1.8 and 2.1 g/L), the total polyphenols concentration did not go through a maximum; a constant value was reached after 5 days and remained constant until the end of fermentation/maceration. It is worth highlighting that the enzyme treatment was also not able to improve the release of polyphenols in comparison to the control, suggesting that for Piedirosso the mass transfer of polyphenols from the cells is not limited by the membrane resistances.

Total Anthocyanins. The anthocyanins are known to contribute not only to the red color of wine but also to its health-beneficial properties. The evolution of the concentration of the total anthocyanins during fermentation/maceration of the treated and untreated samples is reported in **Figure 5**. Coherently with the trends observed for color intensity and the total polyphenols concentration, for Aglianico (**Figure 5a**) the increase of the total anthocyanins concentration induced by the PEF and enzyme treatments could be clearly observed, whereas for Piedirosso (**Figure 5b**) only a marginal improvement was caused by the most intense PEF treatment.

In the Aglianico musts, the PEF-treated samples always exhibited a higher anthocyanins content, which was about 100 mg/L higher than the control and enzyme-treated samples on day 1, and for the PEF5 sample about 200 mg/L higher than the control at the end of fermentation (day 9).

For all of the samples, after an initial rapid increase until reaching a maximum value on day 6, the total anthocyanins concentration underwent a certain decrease (around 100 mg/L) to a value that was then maintained constant. The decrease was previously observed (*19*) and was attributed to the oxidative polymerization of monomeric anthocyanins as well as the formation of complexes with other phenols (*37*).

When considering the final value reached in the Aglianico musts by the anthocyanins, which was constant in the last 3 days of fermentation/maceration, it is worth noting that in comparison to the control samples (480 ± 11 mg/L) the PEF treatment caused a significant increase in the anthocyanins content, which was more consistent with increasing treatment intensity. For example, PEF3 induced a significant increase (541 ± 9 mg/L); PEF4 treatment determined a final anthocyanins concentration of

Table 3. Characteristics of Fresh Wine from Untreated (Control), Enzyme-Treated, and PEF-Treated Aglianico Grapes

		control	enzyme	PEF3	PEF4	PEF5
alcohol content (v/v %)	χ	11.8	11.7	11.9	12.0	11.8
	CV[a] (%)	1.0	1.1	1.0	1.1	1.1
pH	χ	3.2	3.2	3.2	3.2	3.2
	CV (%)	0.6	0.6	0.6	0.6	0.6
total acidity (g/L tartaric acid)	χ	11.2	11.2	11.3	11.1	11.0
	CV (%)	2.0	2.0	2.1	2.1	2.3
glucose and fructose (mg/L)	χ	610	690	650	600	620
	CV (%)	0.4	0.5	0.4	0.4	0.4
reducing sugars (g/L)	χ	2.1	2.1	2.3	2.2	2.2
	CV (%)	10.0	10.5	11.0	12.0	11.5
color intensity	χ	9.8	10.8	10.4	11.0	11.7
	CV(%)	0.4	0.6	0.5	0.5	0.5
total polyphenols (g/L)	χ	1.6	1.9	1.8	2.1	2.2
	CV (%)	0.6	0.5	0.6	0.6	0.7
free anthocyanins (mg/L)	χ	477	705	522	734	839
	CV (%)	1.6	2.0	1.6	1.7	1.4
antioxidant activity (mg/mL ascorbic acid)	χ	65.2	70.3	69.9	73.3	78.5
	CV (%)	3.0	3.2	3.1	2.7	2.6

[a] The reproducibility of the results was expressed as a coefficient of variation (CV).

601 ± 10 mg/L and PEF5 a concentration of 683 ± 12 mg/L. The enzyme treatment increased in efficiency over time, and whereas on day 1 no significant difference was observed with respect to the control samples, at the end of fermentation/maceration, the anthocyanins concentration was statistically undistinguishable from the PEF4-treated sample (585 ± 16 mg/L).

For the Piedirosso musts, at the end of the fermentation only the PEF6 sample exhibited an anthocyanins concentration slightly higher than the control sample (514 ± 7 vs 483 ± 8 mg/L). The samples treated with pectolytic enzymes did not induce any increase in the anthocyanins concentration (479 ± 16 mg/L).

PEF-Treated Wine Characteristics. The fresh wine obtained after the must pressing at the end of fermentation/maceration was analyzed to identify the different characteristics induced by the PEF treatment in comparison to both the control and the enzyme addition samples. The analysis was carried out only for the Aglianico, where there was a significant effect of the PEF treatment on the polyphenolic content during fermentation/maceration.

The results of the analysis of the characteristics of the Aglianico wines are given in **Table 3**. The PEF and enzyme treatments did not significantly alter the alcohol content (which changed at most by 3%), pH, which is the most important analytical parameter for a wine due to its implications relating to the stability and sensory characteristics (*32*), total acidity (which changed by $<1\%$), and reducing sugars (useful in determining the dryness of a wine), the variability of which is always within the uncertainty of measurement. The glucose and fructose concentration was mainly affected by the enzyme addition (+13% in comparison to the control), whereas it was substantially unaffected by the PEF treatments (+7% for PEF3, −2% for PEF4, and +2% for PEF5). A moderate effect of the PEF treatment on the wine characteristics was also reported for Tempranillo wine, when more intense electric fields were applied (*25*).

More significant differences among the wines were observed in terms of color intensity and total polyphenols and free anthocyanins content. The color intensity increased by 10% due to the enzyme treatment, which was higher than the increase induced by PEF3 (+6%) but lower than the other PEF treatments (+12% for PEF4 and +19% for PEF5). The effect of the permeabilization treatments on the polyphenols concentration was even more evident. The enzyme addition caused an increase of 19%, PEF3 13%, PEF4 31%, and PEF5 38%, in comparison to the control sample. For the free anthocyanins concentration, the same trend was observed, with the enzyme addition inducing an increase in relation to the control of 48%, higher than the increase due to PEF3 (+9%) but lower than PEF4 (+54%) and PEF5 (+76%).

The increased phenols concentration also resulted in an improved antioxidant activity. Phenolic compounds in wines can be classified in subgroups such as phenolic acids, flavanols, and anthocyanins. The structural differences of these compounds affect their antioxidant activity; for example, flavanols generally have higher antioxidant activity than phenolic acids. Differences in antioxidant activity between the samples treated in a different way could be linked to their phenolic composition, following polymerization or decomposition reactions.

In fact, the antioxidant activity of enzyme-treated wine is comparable to that of PEF3 wine (70.3 and 69.9 mg/mL ascorbic acid, respectively), even though the total polyphenols and free anthocyanins concentrations of PEF3 wine are always lower, whereas PEF4 and PEF5 wine exhibited a further increase in the antioxidant activity, which increased by 12 and 20%, respectively, in comparison to the control.

The reported analysis suggests that adequate PEF treatments can significantly improve the polyphenols and anthocyanins contents of wine, thus improving its antioxidant properties, while preserving the other characteristics, such as acidity and sugar content. In comparison to the enzyme treatment, PEF treatments can not only prevent the addition of undesired compounds to the wine and the alteration of its characteristics but also improve the antioxidant activity of the wine.

Gas chromatography−mass spectrometry (GC-MS) analysis of the treated and untreated Aglianico wine samples led to the identification of 30 volatile compounds, the most abundant of which were, in order of elution, 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl butyrate, 2-hexanol, isohexyl alcohol, 3-ethyl-1-butanol, 1-hexanol, isoamyl acetate, 2-methylbutyl acetate, heptyl alcohol, hexanoic acid, ethyl hexanoate, ethyl isovalerate, 2-phenylethanol, ethyl succinate, octanoic acid, ethyl octanoate, phenethyl acetate, amyl methacrylate, decanoic acid, ethyl decanoate, ethyl 2-isocyanato-2-phenylpropanoate, tryptophol, 3,4,5-trimethoxyphenylacetic acid, and methyl 3-(indol-3-yl)propionate. These compounds can be classified into three classes of characteristic volatile wine compounds, such as esters, alcohols, and acids (*38*).

Esters such as isoamyl acetate and esters of higher alcohols, formed primarily during fermentation, are considered to be important contributors to young wine aroma because they exhibit floral and fruity odors. The group of alcohols is composed of aliphatic and aromatic alcohols, which include 2-heptanol, 1-hexanol, 2-nonanol, 2,3-butanediol isomers, benzyl alcohol, and 2-phenylethanol. The alcohols occur in various amounts, and they can be recognized by their strong and pungent smell and taste. Most of these compounds, products of yeast fermentation, have intense odors, with a key role in wine aromas. 2-Phenylethanol, which is usually one of the most abundant compounds, is important for the quality of the final product because it gives a rose aroma to the wine and positively contributes to the global aroma. The fatty acids, which are enzymatically formed during fermentation, constitute an important group of aroma compounds

Table 4. Relative Concentration of the Compounds Constituting the Volatile Fraction of Fresh Wine from Untreated (Control) and PEF-Treated Aglianico Grapes

		composition (%)		
no.	compound	control	PEF4	PEF5
1	isoamyl alcohol (3-methyl-1-butanol)	43.72	37.24	29.19
2	2-methyl-1-butanol	9.34	12.50	10.20
3	ethyl butyrate	0.11	0.10	0.08
4	2-hexanol	0.30	0.27	0.28
5	isohexyl alcohol	0.06	0.11	0.06
6	nonyl alcohol	0.05	0.06	0.06
7	3-ethyl-1-butanol	0.26	0.37	0.25
8	1-hexanol	3.48	4.02	3.46
9	Isoamyl acetate	0.87	1.54	0.87
10	2-methylbutyl acetate	0.11	0.21	0.13
11	heptyl alcohol	0.07	0.06	0.05
12	hexanoic acid	0.34	0.72	0.46
13	ethyl hexanoate	0.44	0.67	0.49
14	ethyl isovalerate	0.40	0.47	0.28
15	2-phenylethanol	37.27	34.95	45.45
16	ethyl succinate	0.36	0.26	0.10
17	octanoic acid	0.48	0.76	0.67
18	ethyl octanoate	0.33	0.51	0.30
19	phenethyl acetate	0.32	0.32	0.27
20	amyl methacrylate	0.19	0.22	0.17
21	decanoic acid	0.21	0.14	0.11
22	ethyl decanoate	0.08	0.09	0.04
23	ethyl 2-isocyanate-2-phenylpropanoate	0.28	0.62	0.21
24	tryptophol	2.82	3.29	2.09
25	3,4,5-trimethoxyphenylacetic acid	0.15	0.14	0.15
26	methyl 3-(indol-3-yl)propionate	0.11	0.08	0.06
	total	99.22	99.55	95.42

that can contribute fruity, cheesy, fatty, and rancid notes to the sensory properties of the wine. In addition to the above-mentioned compounds, there are also aliphatic acids (C2−C6, C8, and C10) and phenylacetic acid.

The GC-MS analysis was aimed at evaluating any changes in the volatile composition of Aglianico wine as a consequence of the PEF treatment.

Volatile compounds (alcohols, esters, acids, etc.) play an important role in the aroma of wines. The flavor of a wine is very complex, due to the large number of compounds present with different polarities and volatilities and in a wide range of concentrations (*39*).

The aroma of red wines, which is also the product of a biochemical and technological sequence (grape destemming, crushing, and pressing technology), is mainly influenced by the alcoholic fermentation procedure. However, all of the technological factors, even PEF treatment, can influence the complexity of wine aroma (*40*).

The results, reported in **Table 4**, showed that intense PEF treatments (PEF4 and PEF5) did not appreciably alter the aromatic profile of the wine, with the concentration of only a few volatile compounds being changed to a significant extent. Because the aroma of wine consists of thousands of compounds (*41*) and only a subset of them is likely to actively contribute to flavor (*42*), depending on their concentration and the threshold value of human perception (*43*), it is extremely difficult to identify if the small changes in the aromatic profile may alter the flavor of the wine obtained from PEF-treated grapes. Recent studies, aimed at establishing a correlation between instrumental analysis and sensory perception of wine constituents (*43*, *44*), may help in obtaining some hints on the effect of PEF treatment.

The reduction of isoamyl alcohol in PEF4 and PEF5 wines in comparison with control wine appears to positively affect the overall flavor, isoamyl alcohol being associated with alcohol and cheese notes (*44*). The other most abundant volatile compound, 2-phenylethanol, which together with isoamyl alcohol accounts for 80% of the volatile compounds, is instead increased by the PEF treatment of the grapes. Because 2-phenylethanol is associated with rose flavor and sweet notes (*43*), which usually develop during aging (*44*), also in this case a positive impact on the global aroma of PEF wines is expected. Similarly, the increase in PEF wines of isoamyl acetate and 2-methylbutyl acetate may potentially have a positive effect on global flavor, such molecules being associated with fruity and floral notes (*43*, *44*). In contrast, the increase in PEF wines of hexanoic and octanoic acid, with their sweaty and cheesy notes (*43*, *44*), may likely have a negative impact on the global wine flavor. Despite these considerations, the impact of the odor-active compounds already identified requires further GC−olfactometric studies to be definitely confirmed.

DISCUSSION

The different kinetics of the total polyphenols and anthocyanins release observed for Aglianico grapes upon variation of the PEF treatment are in apparent contradiction with the results of tissue permeabilization. As shown in **Table 2**, the degree of permeabilization, as measured through electrical impedance, clearly depends on the energy input and is not significantly affected by the intensity of the applied electric field (PEF3 > PEF5 > PEF4), as previously reported by other authors (*7*, *45*). On the other hand, the polyphenols extraction kinetics seem to principally depend on the electric field and only secondarily on the energy input (PEF5 > PEF4 > PEF3).

In addition, the two grape varieties, upon PEF treatment, exhibited a significantly different behavior in terms of polyphenols release. For example, PEF4 treatment, which caused a measurable permeabilization of both matrices (Z_p = 0.41 for Aglianico and Z_p = 0.44 for Piedirosso), determined a quantifiable improvement of the polyphenols release only for Aglianico, with no measurable effects for Piedirosso.

Moreover, the observation that the polyphenols release kinetics of the Piedirosso grape skins, which are measurably permeabilized by the electric treatments, are instead substantially unaffected by both the PEF treatment and the enzyme addition, raising further questions on the effect of any kind of permeabilization treatment (enzymatic or electric) on polyphenols extraction.

Polyphenols and anthocyanins are mainly contained within the vacuoles of the cells, and therefore their extraction encounters two main resistances to mass transfer, which are formed, respectively, by the vacuole membrane and the cell membrane. PEF treatment causes permanent membrane permeabilization provided that a critical transmembrane potential is induced across the membrane by the externally applied electric field (*46*). Because for a given external electric field the transmembrane potential increases with cell size (*47*), the critical value of the external electric field E_{cr} required for membrane permeabilization will be lower for larger systems. Therefore, it can be assumed that the critical electric field for cell membrane permeabilization, E_{cr1}, will be lower than the one for vacuole membrane permeabilization, E_{cr2}.

Therefore, in agreement with our results, it can be assumed that the applied electric field $E > E_{cr1}$ already at E = 1 kV/cm and that the extent of cell membrane permeabilization depends only on the energy input, whereas, in the case of the vacuole membrane permeabilization, the critical value E_{cr2} is probably in the range of the applied electric field, and the increase of the intensity of

E (from 0.5 to 1.5 kV/cm) can also increase the permeabilization of the membrane of smaller vacuoles.

On the basis of the same considerations, it can also be assumed that for Aglianico grapes, the mass transfer through the vacuole membrane represents the limiting step in polyphenols extraction.

On the other hand, for Piedirosso grapes, it appears that neither the cell membrane nor the vacuole membrane is the rate-limiting step in polyphenols extraction. In fact, despite the measured electric permeabilization of the cell membrane or the pectolytic effect of the enzymes, no significant improvement of the rate of polyphenols extraction was observed.

The scarce effect of PEF on the extraction of polyphenols from Piedirosso grapes, in comparison with Aglianico grapes, can be explained also in terms of a different polyphenols extractability, for example, due to different grape maturity or simply different biological structure. The replication of the experiments on Aglianico and Piedirosso grapes after 1 year gave similar results (not reported): even if the final concentrations of polyphenols and anthocyanins were different in value from those reported in the present work, the PEF treatment had a measurable and significant effect only on increasing the polyphenols release from Aglianico grapes, whereas it had only a minor impact on Piedirosso grapes. Further studies are needed to clarify this issue.

In summary, PEF treatments of the grape skins can significantly affect the content of polyphenols in the wine after maceration, depending on the grape variety. For Piedirosso grapes, neither the PEF treatment nor the use of pectolytic enzymes was able to increase the release rate of polyphenols, probably due to biological factors. On the other hand, PEF treatment had significant effects on Aglianico grapes: the most effective PEF treatment induced, in comparison with the control wine, a significantly higher content of polyphenols (+20%) and anthocyanins (+75%), thus improving the color intensity (+20%) and the antioxidant activity of the wine (+20%), while preserving the other organoleptic characteristics. Moreover, in comparison with the use of a pectolytic enzyme, the most effective PEF treatment resulted not only in the increase of 15% of the total polyphenols, of 20% of the anthocyanins, of 10% of the color intensity, and of 10% of the antioxidant activity but also in lower operational costs. In fact, the cost for the enzyme treatment is of about 4 €/ton of grapes (the average cost of the enzyme is about 200 €/kg, and the amount used is 2 g/100 kg of grapes), whereas the energy cost for the PEF treatments, calculated as the electric power consumption (in kWh/t of product) times the energy cost (assumed to be 0.12 €/kWh), was estimated at about 0.8 €/ton of grapes in the case of the most effective treatment (PEF5).

Obviously, the evaluation of the industrial feasibility of the integration of the PEF technology in wine production should take into account also other parameters, such as the investment costs, the risks associated with a novel technology, the need for qualified technicians to run and maintain it, and the estimation of the market driving forces as well as the optimization of the PEF treatment for grapes characterized by difficult extractability of the polyphenols.

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