Pulsed electric field processing as an alternative to sulfites (SO_2) for controlling *Saccharomyces cerevisiae* involved in the fermentation of *Chardonnay* white wine

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ABSTRACT

The use of sulfites (SO_2) for microbial control in the winemaking process is currently being questioned due to its potential toxicity. Pulsed Electric Fields (PEF) are capable of inactivating microorganisms at low temperatures, thus avoiding the negative effects of heat on food properties. In this study, the capacity of PEF technology for the decontamination of yeasts involved in the fermentation process of *Chardonnay* wine from a winery was evaluated. PEF treatments at 15 kV/cm of low (65 µs, 35 kJ/kg) and higher intensity (177 µs 97 kJ/kg) were selected for evaluating the microbial stability, physicochemical and volatile composition of wine. Even with the least intense PEF-treatment, *Chardonnay* wine remained yeast-free during 4 months of storage without sulfites. PEF-treatments did not affect the wine's oenological parameters or its aroma during storage. This study, therefore, reveals the potential of PEF technology as an alternative to sulfites for the microbiological stabilization of wine.

Keywords: *White wine, Pulsed Electric Fields, Yeasts, Decontamination, Sulfites, Shelf-life.*

1. Introduction

Winemaking is a complex, large-scale process in which a series of chemical, physical, and microbiological reactions are involved. As wine is a fermented beverage, microorganisms play an important role in different winemaking steps. However, they can also represent a serious problem in wine spoilage, leading to considerable economic losses for the wine industry (Marcobal et al., 2006). Indigenous yeast and bacteria strains present in grapes or in winery facilities may contaminate the must or the wine, thereby causing spoilage. Additionally, the yeast and lactic acid bacteria that are technologically applied in the winemaking process for alcoholic or malolactic fermentation are also considered spoilage microorganisms (Loureiro & Malfeito-Ferreira, 2003). Yeast strains that have been involved in the alcoholic fermentation may cause wines with traces of sugars to referment generating or may make it difficult for acidolactic bacteria to perform malolactic fermentation

Currently, the most widely used control method to achieve microbial stability in wine is sulfur dioxide (SO_2), which is dosed during almost every step in the winemaking process (Ribéreau-Gayon et al., 2021). Apart from its antimicrobial properties, SO_2 is the most effective additive used in wineries to prevent oxidation (Andrew L. Waterhouse, Gavin L. Sacks, 2016). However, as in other food industries, there is a general trend in the winemaking industry to reduce or eliminate the use of chemical preservatives such as SO_2 (Lisanti et al., 2019). In 2009, the World Health Organization (WHO) recommended to reduce or replace the use of SO_2 with other techniques because of its potentially toxic effects on human health (WHO, 2009). Consequently, when the concentration of SO_2 is higher than 10 ppm, it is compulsory to indicate on the bottle label that the wine “contains sulfites”. Furthermore, high sulfur dioxide concentrations may impart an unpleasant odour and taste in young wines but its most serious drawback is the inhibition of the malolactic fermentation or the alcoholic fermentation performed by non-saccharomyces strains (Ribéreau-Gayon et al., 2021; Vaquero et al., 2021).

In recent years, several emerging physical procedures such as high hydrostatic pressure, ultrasound, ultraviolet light, ionizing radiation, ultra-high pressure homogenization, and pulsed

electric fields (PEF) have been proposed to ensure the microbiological stability of wine (Błaszak et al., 2019; Jiranek et al., 2007; Morata et al., 2012; Rizzotti et al., 2015; Zamora & Guamis, 2015). These technologies have generally been shown to be quite useful for controlling microbial activity in wine. However, the main issue with the above-mentioned studies is that they have generally been conducted at laboratory scale under conditions that would not be reproducible on an industrial scale (Lisanti et al., 2019).

The ability of pulsed electric fields to inactivate vegetative cells of microorganisms, the recent development of pulse power systems capable of responding to the processing capacity demands of wineries, and the easy incorporation of treatment chambers into existing processing lines of the wineries supports that PEF is one of the most promising alternatives for microbial control in the winemaking industry. PEF treatment is based on the application of brief pulses of high voltage to a product located between two electrodes. The voltage generates an electric field which, if it is intense enough, causes the formation of pores in the cytoplasmic membrane of microbial cells (electroporation). Thus, PEF modifies the selective permeability of the cytoplasmic membrane, which, in turn, affects microbial homeostasis, leading to microbial death. The resistance to PEF exhibited by wine-associated microbiota including yeast and bacteria has been investigated by different authors in batch (Puértolas et al., 2009) and continuous process (Delsart et al., 2015a; Delsart et al., 2015b; González-Arenzana et al., 2015). Results presented by those studies demonstrated the potential of PEF for microbial control in wine. However, most of that research was performed with artificially contaminated wine rather than with microorganisms growing in the wine itself. This approach could affect microbial PEF resistance, which is well known to be dependent on growth conditions (Delso et al., 2022). Furthermore, in the reported studies, the content of free or total SO_2 in the wines was usually not specified, although it could be a key parameter that influences microbial sensitivity to PEF. Finally transferring PEF technology to wineries for purposes of microbial stabilization requires that treatments be optimized, aiming to achieve the desired antimicrobial efficacy without affecting wine quality.

For this reason, our study's aim was to evaluate the effect of PEF alone or in combination with moderate doses of SO_2 on the yeast population that performed the alcoholic fermentation of a *Chardonnay* white wine, and the impact of PEF treatments on that wine's microbial stability as well as on its physicochemical and volatile composition along time.

2. Material and methods

2.1. White wine sampling

50 liters of *Chardonnay* wine were provided by Cooperativa San Juan Bautista (Fuendejalon, Aragón, Spain) immediately after finishing the process of alcoholic fermentation performed by the commercial starter culture *Saccharomyces cerevisiae var. bayanus* CHP (LEVULINE OenoFrance, Magenta, France). Oenological parameters of the wine immediately after fermentation are shown in Table 1. SO_2 content in the wine corresponded to the SO_2 added to the must prior to alcoholic fermentation with the purpose of preventing oxidations and repressing non-saccharomyces yeasts and promoting the growth of sulfite-tolerant saccharomyces that are used for wine fermentation. To characterize the resistance of yeasts to PEF treatments at different intensities, an aliquot of 10 liters was taken after resuspending the full yeast population in the wine by gentle agitation. For the long-term experiment aiming to evaluate the impact of PEF technology on microbial stability as well as on physicochemical and volatile composition, the clarified wine after 5 days of sedimentation at 4 ºC was used. Immediately after PEF processing, different amounts of SO_2 were dosed into control and PEF-treated samples; samples were subsequently distributed in sterilized glass bottles of 500 mL and stored at 18 ºC for 4 months. Microbiological and physicochemical analyses were performed during storage.

2.2. PEF processing and storage

A commercial PEF generator (Vitave, Prague, Czech Republic) that applies monopolar square waveform pulses at a maximum voltage of 20 kV and current up to 500 A was used in this study. The actual voltage of each treatment was measured by a high voltage probe (Tektronik, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronik, TDS 220). PEF

treatments were applied in a continuous flow system pumped at 10 L/h by means of a peristaltic pump (BVP, Ismatec, Wertheim, Germany) through a parallel titanium electrode chamber of 3 cm length, 0.5 cm width, and a gap of 0.4 cm with a residence time of 0.22 s. White wine was tempered with a coil heat exchanger before entering the PEF treatment chamber. Immediately after the PEF treatment, the wine was cooled down in a cooling coil exchanger. Inlet and outlet temperatures were measured using a type K thermocouple (Ahlborn, Holzkirchen, Germany).

In order to identify optimal PEF conditions for inactivation of *Saccharomyces cerevisiae CHP*, cumulative treatment times using pulses of 10 µs width were applied by delivering various frequencies (10-117 Hz) at different electric fields of 15, 20 and 25 kV/cm. *Chardonnay* wine with a conductivity of 1.8 mS/cm entered the treatment chamber at 20 °C ±2.0 ºC. Exit temperatures were 30, 35, 40, 45 and 50 ±2.0 ºC, corresponding to total specific energies ranging from 35 to 117 kJ/kg. Immediately after the chamber treatment, the wine was cooled down to under 15 ° C in less than 5 s before bottling. For the storage experiment, two PEF treatments at 15 kV/cm were selected: one at low total specific energy (43 kJ/kg), and another at higher total specific energy (97 kJ/kg). These treatments corresponded with exit temperatures of 30 ±2.0 ºC and 45 ±2.0 ºC, respectively.

2.3. Sulfite dosage

A solution of 25 g/L of SO_2 was prepared by dissolving the appropriate amount of potassium bisulfite (Sigma, Burlington, MA, United States) in distilled water. From that solution, the amounts for obtaining 5 and 20 ppm of SO_2 were added to the control wine and to the wine treated at two PEF intensities. The total free SO_2 in the samples at the moment of starting the storage experiments was the sum of the initial SO_2 content (12 ppm) plus the SO_2 added in that phase.

2.4. Microbial analysis

Viable yeast populations were quantified in the wine before PEF treatments, after PEF treatments, after 1, 7, 15, 30 days, and after 4 months of storage. For each sample, adequate

dilutions in peptone water (Oxoid, Basingtok, Hampshire, UK) were made and 0.1 or 1 mL were plated onto Potato Dextrose Agar (Oxoid) and incubated at 25 ºC for 48 h. After incubation, the number of colonies counted corresponded with the number of viable cells expressed as colony-forming units per milliliter of wine (CFU/mL). The survival fraction was calculated by dividing the number of microorganisms that survived the treatment (N_t) by the initial number of viable cells (N_0).

2.5. Oenological parameter analysis

Basic oenological parameters (pH, glucose-fructose, % ethanol, total acidity, volatile acidity, and malic acid) of *Chardonnay* white wine were measured by FTIR spectroscopy using MIURA 200 and BACCHUS 3 MultiSpec models (TDI, Barcelona Spain).

The color of wine samples was determined by measuring absorbance at 420 nm using a Biochron Libra S12 spectrophotometer (Biochron, Cambridge, UK).

The determination of total and free SO_2 sulfur dioxide was carried out with the Ripper method, which is based on an oxidation-reduction titration using iodine as a reagent in an acid medium in the presence of starch. Briefly, 1 mL of starch (1 %) and 2 mL of sulfuric acid 1/3 w/v vinikit (PanReac, Barcelona, Spain) were added to 15 mL of wine. This solution was titrated with an iodine solution (0,01N) until a blue color appeared.

2.6. Browning effect

The susceptibility of white wines to browning was measured by modifying the POM-test previously described by (Müller-Späth, 1992). Wines were centrifuged at 6700*g* for 10 min and five milliliters of the supernatant were mixed with 25 µL of a 3 % hydrogen peroxide solution and heated at 60 ºC for one hour. Browning was determined as the percentage increment in the absorbance of wines at 420 nm.

2.7. Analysis of volatile compounds

Major and minor volatile compounds were measured after 4 months of storage by solid-phase extraction and gas chromatography with ionization detection or mass spectrometric detection, respectively, based on the reference method established by López et al. (2002) and Ortega et al. (2001). For purposes of comparison, we selected the untreated wine (control) with the highest concentration of added SO_2 (20 ppm), along with the wine treated at the highest PEF intensity (97 kJ/kg) without extra sulfur dioxide dosage.

2.8. Statistical analyses

For each condition, 3 samples were analyzed and data are expressed as the mean ± the standard deviation. One-way analysis of variance (ANOVA) and Tukey tests using GraphPad Prism (Graph-Pad Software, San Diego, California, United States) were performed to evaluate the significance of differences among the mean values using a significant level $\alpha = 0.05$.

3. Results and discussion

3.1. Resistance of *Saccharomyces cerevisiae CHP* cells to PEF treatments of different intensities

In a first step, inactivation experiments were performed to determine the PEF resistance of the *S. cerevisiae CHP* cells involved in the fermentation of *Chardonnay* wine. Typical survival curves were obtained at different electric field strengths at an inlet temperature of 20 ºC. As all the experiments were conducted at a constant flow rate (10 L/h) and the wine's residence time in the treatment chamber was thereby constant, frequency of pulse application was modified to obtain corresponding curve dots at different treatment times (Figure 1A) or total specific energies (Figure 1B). Numbers near the dots in Figure 1A indicate the outlet temperature achieved after the different treatments. Under the tested conditions, microbial inactivation did not occur at outlet temperatures below 29 ºC (total specific energy lower than 50 kJ/kg) under the electric field assayed. Above that threshold, microbial inactivation followed a first-order inactivation kinetics rising lethality by increasing total treatment time or the total specific energy applied. For lower electric field strengths, longer treatment times were required to achieve similar lethality: for

example, to inactivate 4.0 log cycles the yeast population, 162 and 65 µs were required at 15 and 25 kV/cm, respectively. Results obtained in this study disagree with those obtained previously by Puértolas et al (2009), who reported concave upward survival curves for PEF inactivation of *S. cerevisiae* in wine. The change in the kinetics of inactivation from concave upwards to a linear response might be explained by the fact that the study by Puértolas et al. was conducted in batch, applying pulses at low frequencies (1 Hz). That protocol of pulse application facilitates the dissipation of heat generated in the treatment zone through the electrodes, and prevents a temperature increment of the sample above 30 ºC even after the application of a high number of pulses. However, in our study, which was conducted in continuous flow, an increment in the number of pulses applied to the product inevitably led to a rise in temperature, because the short residence time of the wine in the treatment zone (0.22 s) prevented the dissipation of heat through the electrodes. The greater lethality of PEF at higher temperatures which has been previously observed (Saldaña et al., 2012; Timmermans et al., 2019), along with the progressive increment of temperature when treatment time increases (figure 1A) might explain the change in inactivation kinetics from concave upwards to linear behavior. This change in kinetics as a consequence of the application of PEF in continuous flow has a significant practical implication, since it allows for the achievement of high inactivation levels with moderate treatments (low total specific energy, low electric field, and moderate exit temperatures). For example, whereas Puértolas et al. reported that a total specific energy of 300 kJ/kg at 31 kV/cm was required to achieve 5.0 log cycles of yeast inactivation in batch, the same degree of inactivation was obtained in our study conducted in continuous flow with a total specific energy of 120 kJ/kg at only 15 kV/cm.

Regarding the influence of total specific energy on microbial inactivation, similar inactivation was obtained when applying the same total specific energy at different electric field strengths. For example, when the total specific energy delivered was 101 kJ/kg corresponding to 45 ±2.0 ºC of exit temperature, yeast inactivation lay around 4.0 log cycles for all electric field strengths. These results agree with those obtained by Huang et al. (2014) and Puértolas et al.

(2009), who reported that the electric field did not influence lethality of PEF on different saccharomyces strains suspended in must or wine.

Currently it is well established that PEF, similarly to other inactivation techniques, causes sublethal injury under certain treatment conditions (Cebrián et al., 2016; García et al., 2003, 2005). Sublethally injured microbial cells are cells that have been damaged, but not sufficiently to be inactivated. As compared with dead cells, injured cells might be able to repair the damages they have incurred and survive when recovery conditions after the treatment are optimal. However, if recovery conditions are not appropriate, such cells are not capable of recovering from their injuries, and die (Saulis, 2010). As compared with a microbial growth media such as PDA (used for counting the number of survivors after a PEF treatment), the low acidity of wine along with the presence of ethanol and free SO_2 make wine an unsuitable medium for the recovery of sublethally injured yeasts after PEF. Therefore, in order to detect whether a proportion of the yeast cells treated by PEF was sublethally injured, the number of survivors in the treated wines after the application of PEF and after 24 h of incubation were compared. Figure 2 compares the log cycles of inactivation obtained by plating the samples of the treated wine at 15 kV/cm for different treatment times just after the treatment, as well as after 24 hours of storage. The differences in log cycles of inactivation correspond to the amount of sublethally injured cells. Here, the occurrence of sublethal injuries is evident for treatments of shorter duration. For example, the treatment at 65 µs (35 kJ/kg; 29 ºC), which hardly had any lethal efficacy whatsoever immediately after the PEF treatment (white bar), achieved a lethality of 2.6 Log_{10} cycles when plating was carried out 24 hours after treatment. This behavior, however, was less pronounced with longer treatment times: indeed, it was negligible above 127 µs (77.8 kJ/kg; 40 ºC). Sublethal injury caused by PEF has been associated with the reversibility and irreversibility of electroporation (García et al., 2003; Somolinos et al., 2007). The manifestation of sublethal injury at lower treatment intensities could be related to the presence of a proportion of yeasts that have been reversibly electroporated. Those reversibly electroporated yeast cells would be able to close their pores when they are immediately plated, and hence survive. However, when they are

maintained in wine, recovery does not take place, and the damage caused by electroporation becomes irreversible, leading to their death. In the case of more intense treatments where incubation after PEF had no effect, the synergetic effect of sublethal PEF injuries and increased temperatures might raise the lethality during PEF treatment, thereby only inducing irreversible electroporation in the full population affected by the electric fields. From a practical point of view, the increment in lethality of PEF treatments because of the impossibility of cells recovering from sublethal injuries should be taken into consideration for the optimization of PEF treatments, since it could allow for a decrease of the treatment intensities required for the microbiological stabilization of wine.

In view of the results obtained regarding the resistance of *S. cerevisiae* to PEF and the occurrence of sublethal injury, the PEF treatment with the lowest intensity (PEF_1: 15 kV/cm. 65 µs, 35 kJ/kg), along with another, more intense treatment (PEF_2: 15 kV/cm, 177 µs 97 kJ/kg), were selected with the purpose of evaluating the effect of PEF on microbial stability, physicochemical composition, and volatile composition of wine during 4 months of storage. As microbial inactivation achieved with treatments of different total specific energies was independent of electric field strength, the lowest electric field (15 kV/cm) was selected in both cases. In terms of practical application, as the highest voltage of industrial PEF generators is limited to around 30-40 kV, lower electric field requirements are desirable, because they allow to apply the required electric field strength using treatment chambers with a greater separation between the electrodes. Consequently, the risk of arching is reduced, and the volume of the treatment chamber is greater, thereby allowing to increase the installation's production capacity.

3.2. Evolution of yeast population in *Chardonnay* wine treated by PEF along 4 months of storage

Untreated and PEF-treated *Chardonnay* wines subjected to the two selected intensities (see above) were bottled and stored at 18 ºC for 4 months. An aliquot of untreated wine and an aliquot of PEF-treated wine were bottled without adding SO_2, and two further aliquots were prepared by adding 5 and 20 ppm of SO_2. This experimental design aimed to check whether PEF

could reduce or prevent the addition of sulfites after white wine fermentation. The evolution of the yeast population (Log_{10} CFU/mL) in the bottled wines during storage is shown in Table 2. In order to simulate the white winemaking process, this study was conducted with Chardonnay wine after several days of yeast decanting. Consequently, the initial yeast population (3.44 Log_{10} CFU/mL) was lower than in the previous assay, where the full yeast population that had participated in the fermentation process was resuspended in the wine for purposes of evaluating yeast resistance to PEF. Yeast population of the untreated wine without added SO_2 was over 3.0 Log_{10} CFU/mL for 4 months of storage. Therefore, the free SO_2 (12 ppm) present in the wine after fermentation did not have antimicrobial activity for the untreated yeast. However, a significant reduction of the yeast population was observed after only 1 day of incubation in the untreated wine added with 5 and 20 ppm of SO_2. After 15 days of incubation, the yeast population in those wines lay below the detection limit (< 30 CFU/mL). Yeast population in wine treated by PEF_2 was below the detection limit (< 30 CFU/mL) immediately after the application of the treatment. Considering the initial yeast population in the *Chardonnay* wine after 5 days of decanting (3.4 Log_{10} cycles), this result agrees with the previous results we had obtained, in which PEF_2 had caused an inactivation of around 4.0 Log_{10} cycles. On the other hand, as was expected from our previous results, the PEF treatment applied at the lowest intensity (PEF_1) had no significant lethal effect on yeast population (< 0.5 Log_{10} cycles) just after the application of the treatment (0 days). However, 24 hours after the treatment, the yeast population in the wine without or with added sulfites lay below the detection limit (< 30 CFU/mL). Generally, as the existence of a sublethally injured population has not been previously considered, PEF treatments of higher intensity than those featured in this investigation have been applied for the inactivation of yeasts in wine. For example, treatments of 320 kJ/kg at 20 or 40 kV/cm were required to inactivate 3.0 and 4.5 log cycles of saccharomyces yeasts, respectively, in sweet white wine after alcoholic fermentation (Delsart et al., 2015a). Our results show that comparable lethal effects can be obtained in white wine with PEF treatments applied at sublethal intensities (15 kV/cm, 35 kJ/kg), which are able to damage a large proportion of the population, which dies when it remains in the wine. This study therefore confirms the efficacy of PEF, even applied at very low intensity (PEF_1), as an alternative

to sulfites for decontamination of yeasts that have participated in the fermentation of *Chardonnay* wine. Microbial control of the yeast population that remains in the wine after fermentation is required to prevent refermentation, especially in sweet and semi-sweet wines containing sugar residues (Loureiro & Malfeito-Ferreira, 2003).

3.3. Effects of PEF processing on physicochemical parameters and aroma composition of *Chardonnay* wine

White wines are extremely sensitive to oxidative degradation, leading to a loss of the characteristic floral and fruity aromas typical of young wines, as well as to the development of a brownish color (Li et al., 2008). SO_2 is extensively used in winemaking due to its antimicrobial properties, but it is also the most effective additive used for the prevention of oxidation (Ribéreau-Gayon et al., 2021). Therefore, when a new technology or process is introduced in winemaking in order to eliminate or reduce SO_2, the preservation of the product's quality parameters is one of the main concerns for wineries, since its commercial value and consumer acceptability depend on them.

Table 3 shows the oenological parameters of the untreated and PEF-treated *Chardonnay* wines with and without the addition of 5 and 20 ppm of SO_2. According to the differences observed among them, basic oenological quality parameters were not affected by the PEF treatment or by the presence of SO_2. Total SO_2 in wine is split into free SO_2 and bound SO_2. At normal wine pH, bisulfite form is the predominant free SO_2 form (>90%) that is responsible for antimicrobial and antioxidant effects. Table 3 shows that free and total SO_2 content in the samples depended on the amount of SO_2 added to the wine before bottling, and was not affected by the PEF treatment applied to them. The decrease of the free SO_2 form along time in all wines is because that bisulfite ion tends to form covalent adducts with carbonyl compounds, whereby this fraction of SO_2 is bound to other compounds that are poorly available for wine protection. Results in Table 3 also show that PEF treatments did not affect wine color either. No significant differences were found in the absorbance of the samples measured at 420 nm. These results agree with the observations made by Delsart et al., (2015a), who reported no significant browning in

white wine treated at 20 kV/cm as compared to a treatment at 40 kV/cm that increased the wine's Abs_{420nm}. A POM-test used to evaluate the wines' predisposition toward browning in the presence of oxidative species and high temperatures did not show meaningful differences between untreated and PEF-treated wines. These results on the Abs_{420nm} and the POM-test indicate that PEF treatments did not trigger any oxidation process during 4 months of storage. PEF has been called into question for generating a series of electrochemical reactions and for the formation of reactive species which might lead to non-desirable oxidative reactions in food processing (Pataro & Ferrari, 2020). However, the mild PEF conditions applied (15 kV/cm) for white wine processing in this study might prevent such electrochemical reactions, which are usually related to electric fields above 30 kV/cm and to long pulse widths (Morren et al., 2003; Zhao et al., 2012).

It is well established that aroma is one of the main quality attributes of white wine. Therefore, in order to detect if PEF treatment can affect wine quality, major and minor volatile compounds of control wine added with 20 ppm of SO_2 were compared with those of wine treated by PEF at the highest intensity (PEF_2) after 4 months of storage in bottle (Table 4). For most of the major and minor volatile compounds evaluated, there were no statistical differences between the values detected in untreated and PEF treated wine. The only detected significant differences were for the values of diethyl succinate and phenylethyl acetate, which were higher in PEF_2-treated wine. Those volatile compounds, described as fruity and flowery, respectively, are associated with yeast autolysis (Martínez-Rodríguez & Pueyo, 2009; Peinado et al., 2004). The higher concentration of these compounds in the wine treated by PEF could be caused by the electroporation of the yeasts, which has been shown to trigger yeast autolysis (Martínez et al., 2019). Results from aroma analysis evidence the negligible effect of PEF on the volatile profile and final aroma of *Chardonnay* white wine. These results support observations made by other authors, who reported that testers did not detect odor/flavor differences between untreated wines and wines treated by PEF for purposes of microbial stabilization (Abca & Akdemir Evrendilek, 2015; González-Arenzana et al., 2019; van Wyk et al., 2018). These findings are particularly relevant due to the observed decrease in certain acetate esters and a number of ethyl esters in

oxidative storage conditions of wines with low sulfites (Pati et al., 2019). Furthermore, this corroborates the general data obtained regarding the non-detrimental effect of pulsed electric fields on the quality attributes of white wines, even under the most intense conditions we evaluated.

4. Conclusions

Although thermal processing is a thoroughly effective treatment for microbial inactivation, it has been avoided in wineries due to the thermo-sensitivity of wine quality parameters, especially regarding color and volatile/aromatic compounds. PEF is regarded as a non-thermal technology; however, the electrical energy that is required to generate the electric field responsible for microbial electroporation in the treatment zone does cause an increment in temperature. Results obtained in this study have demonstrated that increments of temperature below 50 ºC for a short period of time (< 0.5 s) during PEF treatments did not significantly affect the wine quality parameters of *Chardonnay* wine, while managing to successfully control yeast populations for 4 months. Therefore, PEF could represent an innovative physical technology for microbial stabilization of wine as a complement to SO_2 for obtaining wines with reduced SO_2 content. SO_2 can even be avoided altogether if PEF is combined with other chemical additives that have antioxidant properties but do not have antimicrobial properties.

Declaration of 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.

Author Contributions

Conception and design of the study: C.D., I.A. and J.R. Acquisition of data: C.D., A.B. and J.S. Analysis and interpretation of the data: C.D., I.A. and J.R. Drafting of the article C.D. A.B. and J.S. Critical revision of the article I.A. and J.R. All authors have read and agreed to the published version of the manuscript.

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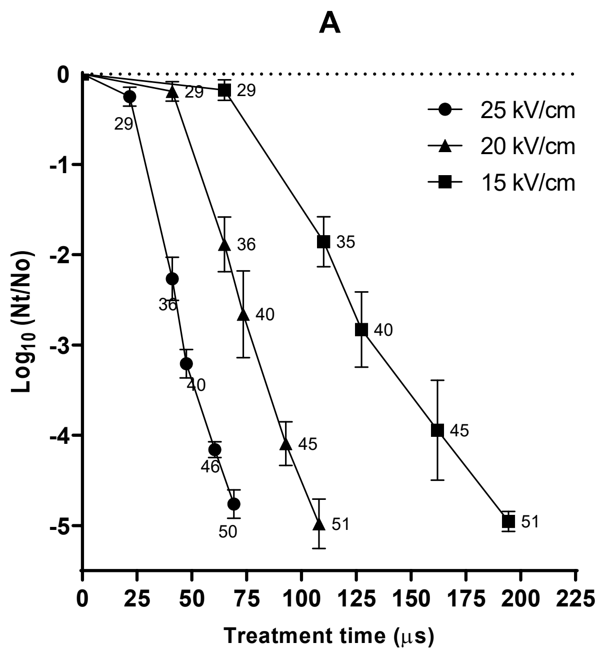
Table 1. Oenological parameters of *Chardonnay* white wine after alcoholic fermentation.

	Chardonnay white wine
pH	3.34 ± 0.02
Glucose-Fructose (g/L)	0.31 ± 0.01
% Ethanol (v/v)	13.32 ± 0.18
Total acidity (g/L)[a]	5.70 ± 0.25
Volatile acidity (g/L)[b]	0.1 ± 0.0
Malic acid (g/L)	1.93 ± 0.01
Free SO_2 (mg/L)[c]	12.1 ± 3.2
Total SO_2 (mg/L)[c]	22.4 ± 3.2
Abs_{420nm}	0.188 ± 0.011

[a] Expressed as tartaric acid
[b] Expressed as acetic acid
[c]Expressed as the mean ± the deviation of the analytical method

Figure 1. Influence of treatment time (μs) **(A)** and total specific energy (kJ/kg) **(B)** on the inactivation of *Saccharomyces cerevisiae* CHP after alcoholic fermentation at different electric field strengths (15, 20 and 25 kV/cm). Numbers near the dots indicate the outlet temperature achieved during treatments.



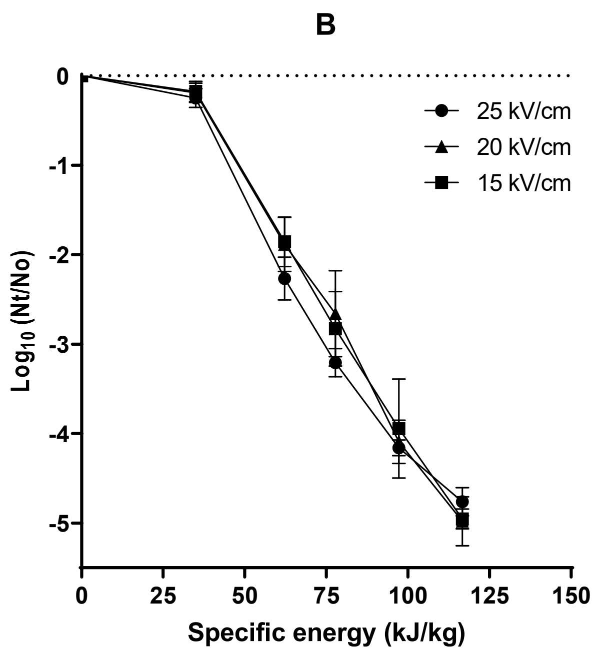


Figure 2. Comparison of Log_{10} cycles of inactivation of *Saccharomyces cerevisiae* CHP in samples plated immediately after PEF treatment (white bars) or in samples plated 24 hours after PEF treatment (grey bars). PEF treatments at 15 kV/cm for different treatment times (μs) corresponding to different specific energies (kJ/kg) and outlet temperatures.

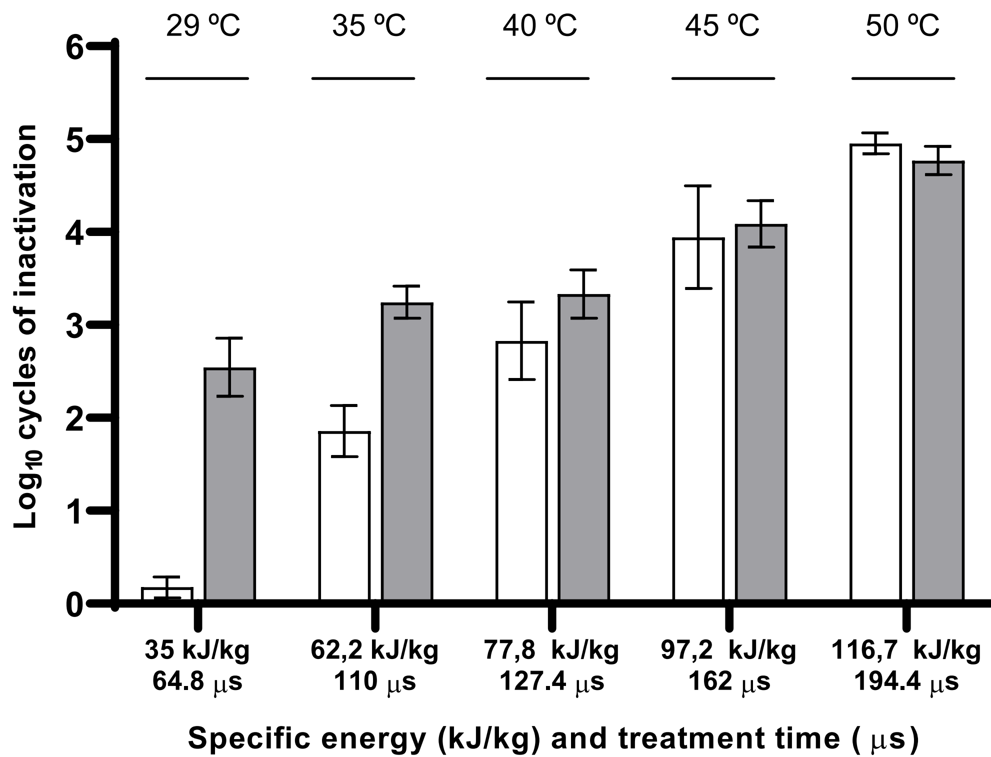


Table 2. Evolution of yeast population (Log_{10} CFU/mL) along storage time for untreated wines, for wine subjected to treatment PEF_1 (15 kV/cm, 65 µs, 35 kJ/kg, 29ºC) and for wine subjected to treatment PEF_2 (15 kV/cm, 177 µs, 97 kJ/kg, 45 ºC), without or with the addition of 5 and 20 ppm of SO_2.

	0 days	1 day	7 days	15 days	1 month	4 months
Control	3.44 ± 0.14[a]	3.50 ± 0.01[a]	3.03 ± 0.02[a]	2.88 ± 0.01[a]	3.28 ± 0.42[a]	3.25 ± 0.07[a]
Control 5 ppm SO_2	3.42 ± 0.00[a]	2.13 ± 0.07[b]	1.74 ± 0.03[b]	< 1.5[b]	< 1.5[b]	< 1.5[b]
Control 20 ppm SO_2	3.15 ± 0.01[a]	1.75 ± 0.02[b]	1.58 ± 0.07[b]	< 1.5[b]	n.d.[c]	n.d.[c]
PEF_1	3.39 ± 0.07[a]	< 1.5[c]	< 1.5[c]	< 1.5[bcd]	n.d.[c]	n.d.[c]
PEF_1 5 ppm SO_2	3.38 ± 0.06[a]	< 1.5[c]	< 1.5[c]	< 1.5[cd]	n.d.[c]	n.d.[c]
PEF_1 20 ppm SO_2	3.06 ± 0.02[a]	< 1.5[cd]	< 1.5[cd]	< 1.5[cd]	n.d.[c]	n.d.[c]
PEF_2	< 1.5[b]	< 1.5[cd]	n.d.[d]	< 1.5[d]	n.d.[c]	n.d.[c]
PEF_2 5 ppm SO_2	< 1.5[b]	< 1.5[cd]	n.d.[d]	< 1.5[d]	n.d.[c]	n.d.[c]
PEF_2 20 ppm SO_2	< 1.5[b]	n.d.[d]	n.d.[d]	< 1.5[d]	n.d.[c]	n.d.[c]

n.d. = non detected. < 1.5 log CFU/mL = below the quantification limit (30 CFU/mL). For each column, values with different letters are significantly different among samples ($p \leq 0.05$).

Table 3. Oenological parameters of *Chardonnay* white wines after 4 months of storage depending on the PEF treatment and the sulphite dose. PEF_1 (15 kV/cm, 65 µs, 35 kJ/kg, 29ºC), PEF_2 (15 kV/cm, 177 µs, 97 kJ/kg, 45 ºC).

	Control			**PEF_1**			**PEF_2**		
	SO_2 0 ppm	*SO_2 5 ppm*	*SO_2 20 ppm*	*SO_2 0 ppm*	*SO_2 5 ppm*	*SO_2 20 ppm*	*SO_2 0 ppm*	*SO_2 5 ppm*	*SO_2 20 ppm*
pH	3.33 ±0.02	3.32 ±0.01	3.33 ±0.05	3.35 ±0.04	3.37 ±0.07	3.36 ±0.02	3.34 ±0.02	3.34 ±0.04	3.35 ±0.01
Glucose-Fructose (g/L)	0.32 ±0.01	0.32 ±0.02	0.32 ±0.02	0.31 ±0.03	0.32 ±0.02	0.31 ±0.01	0.3 ±0.02	0.3 ±0.03	0.29 ±0.01
% Ethanol (v/v)	13.41 ±0.15	13.44 ±0.09	13.43 ±0.11	13.46 ±0.17	13.44 ±0.19	13.45 ±0.08	13.07 ±0.11	13.07 ±0.13	13.09 ±0.06
Total acidity (g/L)[a]	5.67 ±0.22	5.52 ±0.15	5.45 ±0.19	5.6 ±0.10	5.6 ±0.09	5.45 ±0.17	6.12 ±0.22	6.04 ±0.15	5.85 ±0.08
Volatile acidity (g/L)[b]	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Free SO_2 (ppm)[c]	3.2	6.4	9.6	3.2	6.4	6.4	3.2	3.2	9.6
Total SO_2 (ppm)[c]	22.4	22.4	35.2	22.4	25.6	32.0	22.4	25.6	35.2
Abs_{420nm}	0.126 ±0.016	0.132 ±0.011	0.127 ±0.001	0.134 ±0.008	0.136 ±0.012	0.136 ±0.010	0.135 ±0.018	0.123 ±0.009	0.129 ±0.006
POM-test	77.49 ± 4.62	77.46 ± 6.79	77.10 ± 9.93	75.22 ±10.74	72.86 ±12.20	64.37 ±5.35	68.47 ± 5.61	68.15 ±6.30	74.37 ± 2.22

Results are expressed as the mean and standard deviation of three independent replicates (n=3).
[a] Expressed as tartaric acid
[b] Expressed as acetic acid
[c] Expressed as the mean ± the deviation of the analytical method

Table 4. Values of major (mg/L) and minor (μg/L) volatile compounds detected after 4 months of storage in control wine with addition of 20 ppm of SO_2 and in wine treated by PEF_2 without addition of SO_2

Mayor volatile compounds (mg/L)	**Odor threshold**	**Control 20 ppm SO_2**	**PEF_2**
Carbonyl compounds			
Acetaldehyde	0.5	16.93 ± 3.78	21.87 ± 4.73
Acetates			
Ethyl acetate	12.3	66.37 ± 4.03	69.00 ± 1.97
Isoamyl acetate	0.03	3.92 ± 1.11	6.20 ± 1.36
Hexyl acetate	1.5	0.04 ± 0.02	0.07 ± 0.02
Lineal ethyl esters			
Ethyl propanoate	5.5	0.10 ± 0.02	0.11 ± 0.00
Ethyl butyrate	0.125	0.18 ± 0.01	0.23 ± 0.03
Ethyl hexanoate	0.062	0.63 ± 0.03	0.73 ± 0.19
Ethyl octanoate	0.58	0.77 ± 0.05	0.83 ± 0.14
Ethyl decanoate	0.2	0.40 ± 0.05	0.45 ± 0.12
Alcohols			
Isobutanol	40	14.90 ± 1.15	15.50 ± 1.93
Isoamyl alcohol	30	175.67 ± 7.51	169.67 ± 4.73
1-hexanol	8	0.60 ± 0.00	0.64 ± 0.03
C-3-Hexenol	0.4	0.03 ± 0.01	0.02 ± 0.01
Metionol	1	1.31 ± 0.15	1.23 ± 0.11
Benzyl alcohol	200	0.17 ± 0.02	0.19 ± 1.85
β-Phenylethanol	14	33.87 ± 1.72	34.40 ± 1.85
Acids			
Acetic acid	300	60.73 ± 5.55	55.57 ± 17.16
Butyric acid	0.173	0.77 ± 0.10	0.65 ± 0.08
Isobutyric acid	0.05	1.14 ± 0.17	1.00 ± 0.14
Isovaleric acid	0.033	1.21 ± 0.13	0.82 ± 0.11
Hexanoic acid	0.42	3.51 ± 0.09	3.58 ± 0.04
Octanoic acid	0.5	4.89 ± 0.60	6.16 ± 0.37
Decanoic acid	1	1.26 ± 0.19	1.81 ± 0.53

Minor volatile compounds (μg/L)	**Odor threshold**	**Control 20 ppm SO_2**	**PEF_2**
Branched ethyl esters			
Ethyl isobutyrate	15	82.90 ± 3.68	83.84 ± 1.93
Ethyl 2-methylbutyrate	18	6.01 ± 0.39	6.32 ±0.07
Ethyl isovalerate	3	7.21 ± 0.70	8.29 ± 1.81
Acetates			
Isobutyl acetate	1600	130.60 ± 4.06	143.45 ± 2.28
Butyl acetate	1800	17.48 ± 0.27	17.37 ± 1.11
Phenylethyl acetate	250	672.09 ± 6.06[a]	736.90 ± 9.19[b]
Monoterpenols			
Linalool	25	6.50 ± 0.08	6.67 ± 0.18
Linalool acetate		0.14 ± 0.01	0.12 ± 0.01
α-Terpineol	250	2.07 ± 0.10	2.10 ± 0.12
β-Citronelol	100	1.95 ± 0.18	1.72 ± 0.06
Geraniol	20	9.33 ± 0.08	8.17 ± 2.03
Norisoprenoids			
β-Damascenone	0.05	14.44 ± 1.19	14.95 ± 1.20
α-Ionone	2.60	0.61 ± 0.04	0.65 ± 0.02
β-Ionone	0.09	0.23 ± 0.10	0.25 ± 0.15
Phenols			
Eugenol	6	1.27 ± 0.08	1.17 ± 0.05
4-Vinylguaiacol	40	350.20 ± 8.20	406.33 ± 16.28
4-Vinylphenol	180	311.08 ± 10.30	345.49 ± 4.22
Cinamates			
Ethyl dihidrocinnamate	1.6	0.43 ± 0.08	0.43 ± 0.03
Ethyl cinnamate	1.1	9.35 ± 0.42	9.10 ± 0.43

*All parameters are listed with mean (n=3) and standard deviation. For each row, values with different letters are significantly different between the samples ($p \leq 0.05$). Odor threshold: estimated odor threshold in a synthetic wine. < DL: below the detection limit.