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Marcos Andrés Maza

Implementación de la Tecnología de los Pulsos Eléctricos de Alto Voltaje en Bodega

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TESIS DOCTORAL

MARCOS ANDRÉS MAZA

UNIVERSIDAD DE ZARAGOZA

2019

Directores:
Dr. Ignacio Álvarez Lanzarote
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A mi familia, y a mi hijos…

RESUMEN

El vino es una de las bebidas de mayor complejidad tanto en su composición como todo su proceso de elaboración. Cerca de 292,3 Mhl se elaboran anualmente y entre ellos más del 70% de esta producción es destinada a vino tinto. Durante el proceso de maceración/fermentación se produce una extracción de polifenoles de hollejos y semillas dando a los vinos tintos sus propiedades de color y sabor, y su capacidad de envejecimiento. En la actualidad, nuevos procesos y tecnologías desempeñan un papel importante con el fin de alcanzar mayor competitividad a través de la optimización de los procesos productivos y la mejora de la calidad del vino. En este sentido, el uso de los Pulsos Eléctricos de Alto Voltaje (PEAV) ha demostrado ser una novedosa tecnología no térmica capaz de mejorar el proceso de maceración y con mayor potencial respecto a tecnologías usadas en la actualidad gracias a la capacidad de formación de poros en las envolturas celulares (electroporación), con la consiguiente mejora en la extracción de los diferentes compuestos del interior de la célula mejorando de este modo la transferencia de masa. El objetivo general de esta Tesis Doctoral fue estudiar la implementación técnica de los PEAV a una escala próxima a bodega y optimizar el proceso de elaboración del vino tinto, utilizando las instalaciones y equipamientos normales de una vinificación con grandes volúmenes de uva. De este modo evaluar, se pretende evaluar la mejora de la extracción de los compuestos fenólicos a partir de los hollejos de la uva durante el proceso de maceración/fermentación del vino tinto y reducir los tiempos de maceración favorecidos por el aumento en la velocidad de extracción de dichos compuestos. Los resultados obtenidos, han demostrado que la aplicación de PEAV en flujo continuo a escala semi-industrial (2500 $kg.h^{-1}$) aumenta la extracción de los compuestos fenólicos y redujo los tiempos de maceración entre un 25 a un 50%. Tratamientos PEAV de 4 $kV.cm^{-1}$ y 4-5 $kJ.kg^{-1}$ resultaron los más adecuados para su aplicación en bodega para la elaboración de vinos tintos de la variedad *Garnacha*. Los vinos obtenidos por PEAV poseen mejores características aromáticas, cromáticas y compuestos fenólicos que mejoran las características sensoriales y a su vez al proceso de envejecimiento. Los vinos obtenidos con uvas tratadas con PEAV obtuvieron mejores características fenólicas, incluso, después de 24 meses de conservación.

Por otro lado, los PEAV podrían llegar a reducir el tiempo de maceración hasta solo 24 horas, optimizando el proceso de maceración/ fermentación y reduciendo al

mismo tiempo los costes energéticos. De hecho y para la obtención de vinos tintos, la tecnología de los PEAV considerando un tiempo de amortización de 10 años en una bodega con una capacidad de procesado de 10 millones de $kg.año^{-1}$ supondría un incremento de menos de 1 céntimo de euro por litro de vino obtenido.

ÍNDICE

INTRODUCCIÓN

Pulsed electric Field Applications for winemaking

Marcos Andrés Maza, Ignacio Álvarez and Javier Raso

Book Chapter: *Pulsed Electric Fields Technology for the Food Industry: Fundamentals and Applications* (2nd *Edition*).

1. Abstract

Wineries may take advantage of the ability of pulsed electric field (PEF) to electroporate cell membrane of grape skins and microbial cells to improve different operations. PEF permeabilization of red grape skin cells permits reducing the duration of the maceration or to increasing the color and concentration of polyphenolic compounds in the wine without impairing its sensorial attributes. The capability of PEF to inactivate spoilage microorganisms preserving physicochemical and sensorial properties of must and wines may contribute to enhance the quality of wine ensuring reproducible fermentations reducing or replacing the use of SO_2 for wine stabilization. Finally, it has been demonstrated that PEF trigger yeast autolysis accelerating the release of mannoproteins from the cell wall and decreasing the duration of aging on the lees process.

2. Introduction

Grape production represents the most important fresh fruit crop. Worldwide grape production in 2017 was about 73.3 million metric tons and approximately 52 % of the production was fermented into wine (OIV, 2018). Global wine production in 2018 (excluding juice and musts) was around 279 mhl (OIV, 2018). More than the 70 % of this total production corresponded to red wine.

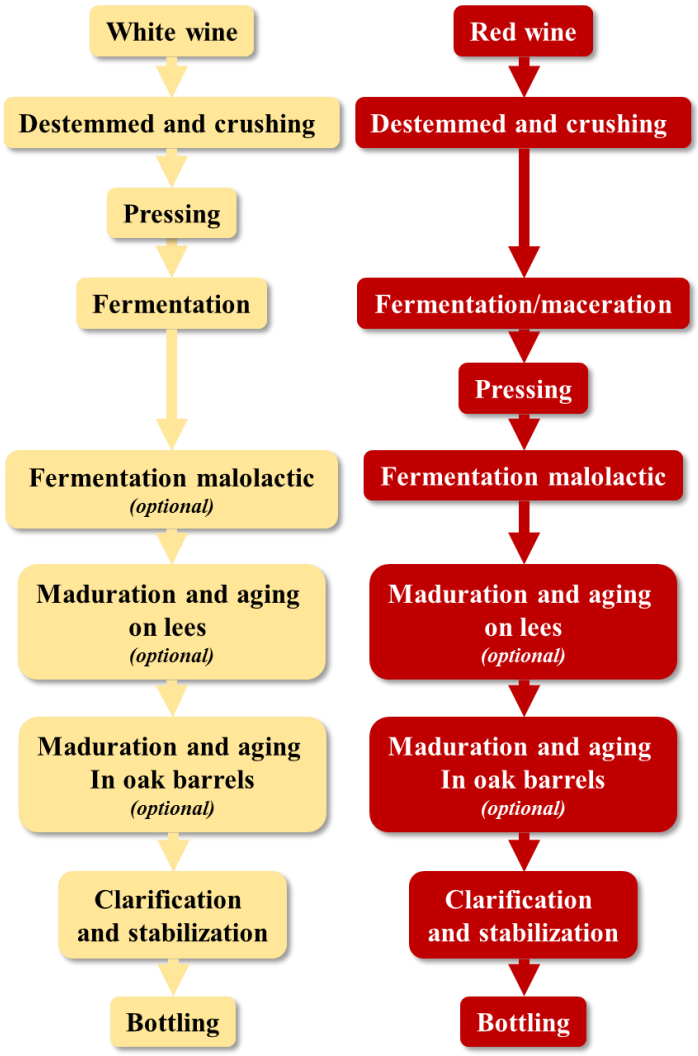

Figure 1: Red and white winemaking steps

The main step of with and red winemaking is shown in Figure 1. The first step of winemaking once the grapes reach the winery involves removing the leaves and the steems of the bunch of grapes. For red and rosé wines the fruit is then crushed to release the juice and the process of maceration of the resulting juice with pomace (skins and seeds) begin. In the case of white wine althought in some cases maceration is kept for a

few hours, fermentation is always restricted to the juice extracted from the grapes immediately upon crushing and pressing.

Red winemaking is characterized for prolonged macerations ranging from several days to weeks that occur simultaneously with alcoholic fermentation. The alcohol generated during fermentation enhances the extraction of polyphenols from the seeds and skins that give red wines their properties of appearance, taste, and flavor, and their capability of aging.

Rosé wines are obtained from red grapes exposed at short pre fermentative maceration. Grape pomace is maintained in contact with the juice for a short period of time (12-24 hours) at cool temperatures until the sufficient color has been extracted. The juice is subsequently drawn and fermented similarly to that of white wine.

Although traditionally wine fermentation was conducted by yeast derived from the grapes or present in the winery, currently the standard practice is to inoculate commercial preparation of yeasts of know characteristics. During fermentation, yeast not only transforms the sugar of the juice in ethanol also produce a wide array of aromatic compounds that contribute to generate the flavor attributes of the wines. Alcoholic fermentation is considered to be completed once sugar levels drop to a low undetectable level, generally less than 2 $g.L^{-1}$. After completing alcoholic fermentation the wine may be treated to foster a second fermentation called malolactic. In this fermentation that generally occurs in red wines, the grape acid malic is converted to lactic by lactic acid bacteria that can be indigenous to the winery or proceed from commercial preparations.

After several weeks of finishing fermentation step the wine is racked. In this operation, the wine is separated from solids (yeast and bacterial cells, precipitated tannins, proteins, tartrate crystals) that settle out during spontaneous or induced clarification. Prior to bottling, wines are commonly chilled and filtered to further enhance clarification and stability. Bottled wines are normally aged at the winery for several months. Some red wines are aged prior bottling in oak barrels for a period of time ranging from few months to several years. This aging period allows the important chemical transformation of the wine that leads to flavor modification and integration. Following in-barrel maturation, the wines typically receive further in-bottle aging at the winery before release.

Another type of aging that traditionally has been conducted in the production of sparkling wines but that more recently is used in the production of white and red wines is aging on the lees. This technique consists in keeping the wine in contact with the yeast once the fermentation process has finished. During this process, yeast autolysis occurs

and polysaccharides of the yeast cell wall, mainly mannoproteins, are released. These compounds improve the aromatic and gustatory complexity of the wine.

In summary, winemaking is a multistep process involving a series of the microbial and biochemical process wherein components of the grape are transformed into taste and aroma characteristic of the wine. Wineries may take advantage of the application of PEF in different steps with the purpose of impact processes involving in winemaking. Irreversible electroporation of the cytoplasmic membrane of the grape skin cells caused by PEF may facilitate the release of polyphenols located into the skin cells during the process of maceration improving extraction yields or reducing maceration times. On the other hand, the irreversible electroporation of the cytoplasmic membranes of bacteria yeast and molds causes the loss of their selective permeability, leading to microbial death. The capability of PEF to inactivate microbial cells at temperatures that avoid the harmful effect of heat on wine characteristics may result in an alternative to the use of SO_2 and filtration for controlling microbial spoiling in the wineries. Finally, electroporation accelerates the release of mannoproteins from the cell wall of the yeast during "aging on the lees".

3. Application of PEF for improving red winemaking

Phenolic compounds play a highly significant role in red winemaking. Sensory characteristics such as color and taste and aging properties of red wines are strongly dependent on their polyphenolic composition (Cheynier et al., 2006). On the other hand, in vitro, and in vivo studies have shown that some phenolic compounds of red wine have antioxidant properties which may play a positive role in human health including protection against cardiovascular diseases and cancer (Nichenametla et al., 2006).

Wine phenolic compounds can be divided into non-flavonoid (phenolic acids and stilbenes) and flavonoid (anthocyanins, flavan-3-ols, and flavonols). The monomeric forms of anthocyanins are the main responsible of the red color of young red wines and they also contribute to the development of red polymeric pigments during wine aging by the association between anthocyanins pigments and other polyphenols especially tannins and phenolic acids (Salas et al., 2003). Flavan-3-ols (tannins) are a large family of polyphenolic compounds which are mainly responsible for the astringency, bitterness, and structure of wines. The last group of flavonoids is flavonols that contribute to bitterness, display antioxidant activity and affect red wine colour acting as copigments of

anthocyanins. Between non-flavonoid compounds, stilbenes have been also recognized as compounds with antioxidant and anti-inflammatory properties (Tomera, 1999).

The aroma is also an important quality attribute of wines. The aroma of young wine emerges mainly in the process of fermentation. Although grapes and must from non-floral grape varietals do not possess intense or explicit flavors, fresh fermented wine has pleasant aromas which can be related to the varietal origin (Delfini et al., 2001). It is estimated that more than 40 different aromatic molecules such as acids, alcohols, esters, aldehydes, terpenols, lactones, norisoprenoids, and volatile phenols are formed or released from precursors during fermentation (Loscos et al., 2007).

Juice of red grapes has a low content of phenolic compounds and aromatic molecules. Therefore, maceration is required for enriching the fermenting must with polyphenols and aromatic precursors located in the pomace. While tannins are located into the cells of grape skins and seeds, anthocyanins and aromatic precursors are situated inside the cells of the grape skins (Amrani-Joutei and Glories, 1995). Therefore maceration is one of the most critical technological operations in wineries not only for their influence in the wine quality but also because represent the stage with the highest requirements in energy and manpower (Togores, 2011).

Generally, during traditional maceration, the solid parts of the grape berries remain in contact with the fermenting must during the entire fermentation process (7-10 days) or even maceration time is extended for several days after fermentation in order to obtain a wine with adequate polyphenol content. This required long period of contact is due to the presence of cellular barriers such as the cytoplasmic membrane and the cell walls that difficult the mass transfer of the compounds of interest from the inner of the cells to the fermenting musts.

Maintaining the grape pomace in contact with the must during fermentation involves several drawbacks for the wineries. During maceration, approximately the 20 % of the fermentation tanks is occupied by the solid parts of the grapes, thereby reducing the effective volume of the tanks and, as a consequence, the wineries' production capacity. This problem becomes especially significant during the peak of harvesting because for reducing maceration time with the purpose of increasing production capacity may compromise wine quality. Other negative side-effects are related with the difficulty of controlling the fermentation temperature when the solid parts are present in the fermentation tanks, as well as with the workforce and energy consumption required to

periodically pump the wine over the skin mass that rises to the top of the fermentation tanks.

Current techniques used in the wineries for improving extraction of polyphenols and to reduce maceration time are enzymatic treatments or procedures based on heating such as thermovinification and flash expansion. The use of commercially pectolytic enzymes that act on the wall of the grape skin cells is a widespread practice in the wineries to improve the color and aroma of wines. However, some contradictory results attributed to the different nature and activities of the commercial enzymes preparations and the presence of unwanted enzymes in the preparations such as *β*-glucosidase have been reported (Bautista-Ortín et al., 2005). On the other hand, thermal techniques present a series of problems, such as the difficulty involved in stabilizing the extracted phenolic compounds (Morel-Salmi et al., 2006; Samoticha et al., 2017), the loss of varietal aromas (Geffroy et al., 2015) and the consumption of high quantities of energy.

Effect of PEF on the extraction of phenolic compounds during the maceration of red winemaking

The application of PEF to accelerate and/or increase the extraction of phenolic compounds during the maceration of red winemaking has been deeply investigated. In some of these studies, it has also been observed that the treatment also improves the extraction of aromatic compounds (Delsart et al., 2012; Garde-Cerdán et al., 2013). The treatment is generally applied to the crushed and destemmed grapes with the objective of cause irreversible electroporation of the cytoplasmic membrane of the cells of the grape skins that contain the majority of the compounds required for obtaining red wine of quality. Pores formed in the membrane improve mass transfer rates of the compound of interest without the necessity of the complete disintegration of cell envelopes.

The potential of PEF for improving extraction of polyphenols during red winemaking was initially evaluated using batch treatment chambers of parallel electrodes with a capacity from few grams to few kilograms. The benefits of the electroporation were demonstrated for different varieties of grapes such as *Tempranillo, Grenache, Graciano, Mazuelo, Merlot, Aglianico, Cabernet Sauvignon, and Cabernet Franc* harvested in different countries such as Spain, France, Lebanon, and Italy. Main results obtained in these experiments in term of improvement in color intensity (CI), total anthocyanins content (TAC) and total polyphenol index (TPI) are shown in Table 1.

Table 1 Improvements achieved in colour intensity (ΔCI), anthocyanin content (ΔTAC) and total polyphenols index (ΔTPI) in PEF wines obtained in a batch PEF system at the end of alcoholic fermentation.

Grape variety	PEF treatment conditions	Δ CI %	Δ TAC %	Δ TPI %	Ref.
Tempranillo	5 kV.cm^{-1}; 50 pulses; 1.8 kJ.kg^{-1}	13	15	18	(López et al., 2008b)
	10 kV.cm^{-1}; 50 pulses; 6.7 kJ.kg^{-1}	23	26	24	
Grenache	2 kV.cm^{-1}; 50 pulses; 0.4 kJ.kg^{-1}	26	11	18	(López et al., 2008a)
	5 kV.cm^{-1}; 50 pulses; 1.8 kJ.kg^{-1}	21	32	14	
	10 kV.cm^{-1}; 50 pulses; 6.7 kJ.kg^{-1}	29	24	16	
Graciano	2 kV.cm^{-1}; 50 pulses; 0.4 kJ.kg^{-1}	19	18	14	
	5 kV.cm^{-1}; 50 pulses; 1.8 kJ.kg^{-1}	12	14	13	
	10 kV.cm^{-1}; 50 pulses; 6.7 kJ.kg^{-1}	6	7	12	
Mazuelo	2 kV.cm^{-1}; 50 pulses; 0.4 kJ.kg^{-1}	29	16	14	
	5 kV.cm^{-1}; 50 pulses; 1.8 kJ.kg^{-1}	51	35	31	
	10 kV.cm^{-1}; 50 pulses; 6.7 kJ.kg^{-1}	62	38	36	
Cabernet Sauvignon	5 kV.cm^{-1}; 50 (exp. pulses); 2.1 kJ.kg^{-1}	48	43	45	(López et al., 2009)
Aglicano	1.0 kV.cm^{-1}, 10^4 pulses; 50 kJ.kg^{-1}	6	9	13	(Donsì et al., 2010)
	1.5 kV.cm^{-1}, 10^3 pulses, 10 kJ.kg^{-1}	12	54	32	
	1.5 kV.cm^{-1}, 2.5 x 10^3 pulses, 25 kJ.kg^{-1}	19	76	38	
Merlot	0.5 kV.cm^{-1}, 100 ms.	ns	25	18	(Delsart et al., 2012)
Cabernet Franc	0.8 kV.cm^{-1}; 100 ms, 42 kJ.kg^{-1}	20	49	51	(El Darra et al., 2013)
	5 kV.cm^{-1}; 1 ms, 53 kJ.kg^{-1}	23	60	62	

ns: no significant difference.

The improvements obtained in different oenological parameter depending on the polyphenol extraction by application of PEF treatments ranged from 20 to 60 % as compared with the wine obtained with untreated grapes. This effect seemed to depend on the intensity of the treatment, the grape variety, and the grape maturity. Generally the PEF treatments were more effective at higher electric field strengths although in some varieties such as *Graciano* the most suitable treatment was at the lowest intensity applied (in the range of 2 to 10 kV.cm^{-1}) and in *Cabernet Sauvignon* it was an intermediate intensity (5 kV.cm^{-1}) (López et al., 2008a). These results indicate that for some grape varieties, an intense electroporation of the skin cells may promote a very intense release of phenolic compounds that precipitate rather than keep stabilized in the wine. The different effect of PEF on the extraction of polyphenols for different grape varieties has been explained in terms of polyphenol extractability that depends on grape maturity, cell morphology, and composition of the skin cell wall (López et al., 2008a). Results obtained suggest that the electroporation of the cells of the grape skin was more useful in those situations in which the extraction of phenolic compounds from the grape skins results more difficult (López et al., 2008a).

The implementation of the PEF technology for improving polyphenolic extraction requires the application of the treatments in a continuous flow to be able to process de hundreds or thousands of tons of grapes that are processed annually in a winery. The first studies conducted in continuous flow at pilot plant scale (120 $kg.h^{-1}$) permitted to validate the results obtained in batch but more important to be able to produce sufficient amount of wine to evaluate the sensory characteristics of the wine obtained with grapes treated by PEF and the evolution of this wine during aging **(**Puértolas et al., 2010e).

An overview of the main results reported on the benefits of the application of PEF in a continuous flow in terms of improvement color intensity (CI), total anthocyanin content (TAC) and total polyphenol index (TPI) are shown in Table 2. This table shows results obtained at pilot plant scale in which it was processed hundreds of $kg.h^{-1}$ but also results obtained a semi-industrial scale with flows of tons per hour. Studies obtained in continuous flow confirm those previously obtained at lab scale in batch with few amounts of grape. Differences observed in the global effect were unimportant despite the different treatment chamber configuration used in continuous flow. While batch treatments were conducted with parallel electrode treatment chamber, in continuous flow the configuration of the treatment chamber was colinear.

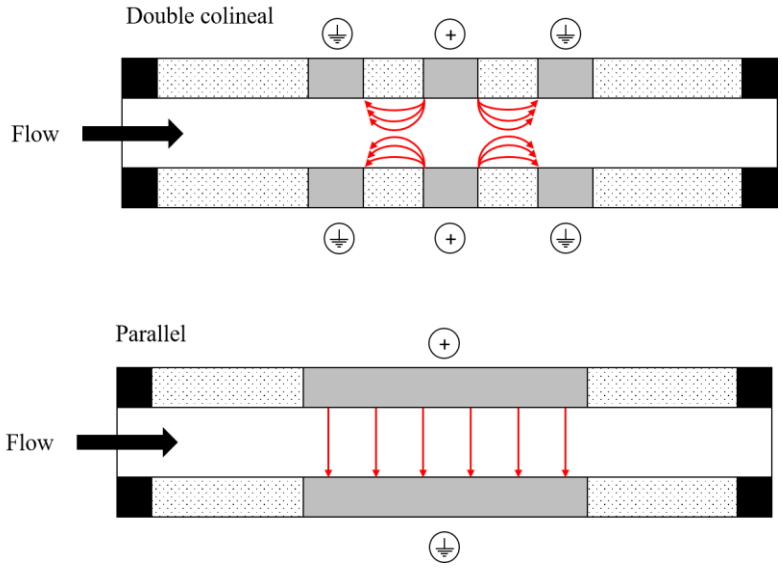

Figure 2: Treatment chamber designs for PEF processing in continuos flow

As compared with a parallel electrode treatment chamber configuration (Figure 2) the colinear configuration is characterized for its lower energetic requirement due to its highest load resistance and because the circular section of the chamber facilitates the flow of the crushed grapes (van den Bosch, 2007). However, the inhomogeneity in the distribution of the electric field in this configuration could cause a proportion of cells of

the grape skins were unaffected of affected by an insufficient electric field to cause electroporation.

Table 2: Improvements achieved in colour intensity (ΔCI), anthocyanin content (ΔTAC) and total polyphenols index (ΔTPI) in PEF wines obtained in continuous flow system at the end of alcoholic fermentation.

Grape variety	PEF treatment conditions	Δ CI%	Δ AC%	Δ TPI%	Ref.
Cabernet Sauvignon	5 $kV.cm^{-1}$; 3.67 $kJ.kg^{-1}$	34	38	23	(Puértolas et al., 2010e)
Grenache	4 $kV.cm^{-1}$; 1.5$kJ.kg^{-1}$	13	25	24	(Luengo et al., 2014)
Graciano (2012)	4.6 $kV.cm^{-1}$, 720 µs	103	84	51	
Tempranillo (2012)	4.6 $kV.cm^{-1}$, 720 µs	184	184	93	(López-Giral et al., 2015)
Grenache (2012)	4.6 $kV.cm^{-1}$, 720 µs	230	458	98	
Grenache	4.0 $kV.cm^{-1}$ 370 µs	52	18	29	(Maza et al., 2019)

López-Giral et al. (2015) studied the effect of PEF applied in continuous flow (400 $kg.h^{-1}$) on improving the phenolic compound extraction from three grape varieties (*Graciano, Tempranillo, Grenache*) during two vintages. Results showed that the PEF effect depends on the grape variety but also that grape physicochemical composition, that it was different for each vintage, influenced PEF effect. The improvement in the extraction of polyphenols from the grapes treated by PEF was more important when the concentration of phenolic compounds in the skins was lower. Therefore, the application of the PEF technology in the wineries for improving extraction of polyphenols would result particularly interesting in those vintages in which the concentration of these compounds in the grape skins is poor.

Potential of PEF for reducing maceration time has been also investigated in studies conducted in continuous flow at pilot plant scale and semi-industrial scale. Grapes of *Cabernet Sauvignon* treated by PEF at a flow of 120 $kg.h^{-1}$ were vinified in tanks of 80 L **(**Puértolas et al., 2010e). Grape pomace was separated from the fermenting must after 96 hours while in the control the grape skins were in contact with the fermenting must until the end of fermentation (144 h). Although the maceration time for wine obtained from *Cabernet Sauvignon* grapes treated by PEF (5 $kV.cm^{-1}$, 150 µs, 3.67 $kJ.kg^{-1}$) was 48 h shorter, the wine at the end of the alcoholic fermentation presented higher CI, TAC, and TPI than the control wine.

Trials conducted a semi-industrial scale in wineries confirmed results obtained at laboratory and pilot plant scale (Luengo et al., 2014; Maza et al., 2019). Luengo et al., (2014) processed 3,000 kg of grapes of *Grenache* variety by PEF treated (4.3 $kV.cm^{-1}$, 60 μs) using a co-linear treatment chamber at a flow of 1,900 $kg.h^{-1}$. The wine obtained from PEF-treated grapes with a maceration time of 7 days was compared with wine obtained from untreated and PEF-treated grapes with the current maceration time used in the winery (14 days). After 7 days of maceration, the CI, TAC, and IPT of the wine obtained from grapes treated by PEF were a 13 %, a 25 % and a 24 % higher respectively, than in wine obtained from untreated grapes after 14 days of maceration. These results confirm the potential of PEF to obtain a wine with a sufficient concentration of polyphenolic compounds with moderate maceration times.

More recently twelve tons of *Garnacha* grapes were processed at a flow rate of 2,500 $kg.h^{-1}$, and wines obtained from untreated and PEF-treated (4.0 $kV.cm^{-1}$) grapes after 3 and 6 days of maceration were compared (Maza et al., 2019). The pre-treatment of grapes by PEF permitted to reduce maceration time from 6 to 3 days without decreasing the color or the concentration of polyphenolic compounds in red wine; alternatively, it permitted to increase those parameters when maceration time was extended to 6 days. The higher tannin concentration observed in PEF treated wines was consequence of a higher degree of extraction of tannins located in the grape skins rather than in the seeds.

Evolution of wine obtained with grapes treated by PEF during aging in bottles and oak barrels.

The wine obtained after finishing the fermentation-maceration step require a stabilization stage followed by an aging period in a bottle or oak barrels before consumption Figure 1. In the aging stage, polyphenols participate in subsequent reactions that have the greatest influence on the overall sensory quality of the finished wine (Setford et al., 2017). These modifications are a consequence of precipitations, degradation reactions, or polymerization reactions that lead to the formation of new stable compounds, and they help bring about important changes in the wine's sensory properties (Guadalupe and Ayestarán, 2008a). Since these reactions depend to a great extent on the type and concentration of polyphenols obtained during fermentation, it is important to know the evolution of wines produced from grapes treated with PEF during aging.

Evolution of wine obtained with grapes treated by PEF during aging in bottled and oak barrels was investigated for the first time by Puértolas et al., (2010c). Evolution of

the main wine indexes depending on polyphenolic extraction along aging of Cabernet Sauvignon wine obtained with grapeS treated by PEF follow the same trend than evolution of control wine in terms of development of color intensity, anthocyanin content, total phenolic content, and tannins content during wine aging in bottle, or in oak barrels (6 months) followed by bottle (8 months). The better chromatic characteristics and higher polyphenolic content obtained from the PEF treatment after the fermentation process was maintained after aging.

Grenache wine obtained with PEF treated grapes were aged during 24 months of aging in bottles, as well as during 6 months of aging in oak barrels followed by 18 months of aging in a bottle (Maza et al., 2019). Similar conclusions on the evolution of indexes depending on polyphenolic extraction were obtained even after longer aging periods. In terms of color intensity, phenolic families (anthocyanins, hydroxycinnamic acids, flavonols, and flavanols) and individual phenolics, the *Grenache* wine obtained with grapes treated by PEF with different maceration times follow an evolution similar to the wine obtained with untreated grapes during aging in bottle, or in oak barrel followed by the bottle. In all cases, a decrease in the concentration of those compounds was observed across time but the differences between the wine obtained from untreated and PEF-treated grapes were maintained at the end of aging.

Sensory properties of wines obtained with grapes treated by PEF

Aromatic molecules also play a highly significant role in red winemaking, owing to their contribution to sensory properties. In this sense, several investigations on the effect of PEF treatments on the wine aroma compounds have been conducted.

The effect of PEF on the volatile composition of wine was investigated for the first time by (Garde-Cerdán et al., 2013). The volatile composition of *Tempranillo* and *Graciano* wines was not improved by treating the grapes with PEF, but that the aromatic characteristics of *Grenache* wine were improved by increasing the concentration of monoterpenoids, *β*-ionone, total esters, and volatile phenol compounds.

Other study conducted with the same grape variety in which control wine was compared with wine obtained from grapes treated by PEF after 3 and 6 days of maceration showed that the content of all major aromatic compounds was greater in the wines that had undergone longer maceration (Maza et al., 2019). Small differences were observed, among the wines obtained with untreated and PEF-treated grapes subjected to the two maceration times investigated. In the case of aroma compounds liberated from the

precursors in the grapes are present in "trace" concentrations ($\mu g.L^{-1}$) in the wine, the concentration of *β*-ionone associated with the floral aroma of "violets" that was undetected in the control wines, exceeded the odor threshold in the wines obtained from grapes treated by PEF.

Therefore, PEF could contribute to the extraction of some individual aroma precursors present in grapes, thereby increasing the concentration of some molecules which, once liberated from the precursors, can exert a positive effect on wine aroma.

The effect of PEF on the organoleptic characteristics of the wine has been also assessed by sensory evaluation.

A panel of professional testers preferred *Merlot* wine obtained by grapes treated by PEF at 0.5 and 0.7 $kV.cm^{-1}$ for 40 ms than control wine or wine obtained by grapes treated at more intense PEF treatment (0.7 $kV.cm^{-1}$, 100 ms) (Delsart et al., 2012). While after applying the most intense treatment the diffusion of tannins to the wine was probably excessive, in the case of less intense PEF treatments the diffusion of these compounds was lower and the wines were more aromatic and fruity suggesting that PEF treatments promote an additional diffusion of aromatic compounds from the cell skins.

Results of a triangle and preference showed significant sensory differences between the wines obtained with untreated or PEF-treated grapes after 3 and 6 days of maceration when aged either in a bottle or in oak barrels. All panelists were able to differentiate the wines obtained with untreated or PEF-treated grapes and a majority of panelists (86 %) preferred wines elaborated with grapes treated with PEF. Therefore, these results indicate that the improvement in polyphenolic extraction brought about by the application of a PEF treatment before maceration permits to obtain wines that are sensory different and that were preferred by panelists.

4. Application of PEF in wineries for microbial inactivation

Microbial processes play a fundamental role in the final quality flavor and aroma characteristics of the wine. While the yeast *Saccharomyces cerevisiae* conducts alcoholic fermentation, malolactic fermentation is conducted by lactic acid bacteria. Microbial control in the wineries it is necessary for preventing wine spoilage for the microorganisms involved in both fermentations once the process is finished but also to prevent the growth of other microorganisms that are not involved in the fermentation process such as *non-Saccharomyces* yeasts or acetic acid bacteria (Drysdale and Fleet, 1988).

The development of in the wine may cause great economic loses in wineries (Zuehlke, 2013). The growth of undesirable yeast during alcoholic fermentation may impair the development of the inoculated yeast and also may produce important sensorial changes in the wine just after fermentation. On the other hand, lactic acid bacteria are responsible for the alteration named "piqûre lactic", acetic acid bacteria are able to spoilage the wine by ethanol acidification, and yeast from the genus *Brettanomyces* are involved in the formation of unpleasant odors in the wine which are described as "leather", "animal" and "horse sweat"

The most common practice used in wineries to decrease the risk of microbial spoilage during the winemaking process is the addition of sulphur dioxide (SO_2) due to its effectivity for inhibiting the development of *non-Saccharomyces* yeast and spoiling bacteria. Although currently the SO_2 is considered indispensable in the wineries because combined antimicrobial and antioxidant activities some drawbacks are associated with this practice. It has been reported that these compound may cause organoleptic deviations and unpleasant flavors when it is added in excess and in the other hand may results hazardous for health producing allergic reactions in sensitive people (Lester, 1995; Yang and Purchase, 1985). Consequently, one of the oldest challenges of the wineries is to find alternatives that permit to reduce or to eliminate the use of SO_2 in winemaking.

Recently, one of the alternatives that have been evaluated for obtained additive-free wine is PEF due to its ability for inactivating vegetative cells of microorganisms at lower temperatures that those used in thermal processing.

The application of PEF to must of *Parellada* white grape variety before fermentation as an alternative to the addition of SO_2 for controlling the developed of spoilage microorganisms was investigated by first time by Garde-Cerdán et al. (Garde-Cerdán et al., 2008). In this study, it was demonstrated a reproducible fermentation of PEF treated must without modifying significantly the composition of volatile compounds responsible for the typical flavor of wines through alcoholic fermentation and aging of white wine. Therefore, the SO_2 concentration could be reduced to safer levels or even eliminated.

Puértolas et al., (2009a) investigated the PEF resistance of wine spoilage microbiota such as *Dekkera anomala*, *Dekkera bruxellensis*, *Lactobacillus plantarum*, and *Lactobacillus hilgardii*. PEF treatments permitted obtaining substantial inactivation in all the microorganism investigated being yeasts were more PEF sensitive than bacteria.

The most complete study on the inactivation of wine-associated microbiota by PEF was conducted by González-Arenzana et al. (2015). Four different PEF treatments in continuous process were tested in a continuous-flow system for inactivating 25 species of yeast, lactic acid bacteria and acetic acid bacteria associated with winemaking. The inactivation obtained was highly dependent on the microorganism investigated. Overall, the level of inactivation obtained varied from 0.64 to 4.94 log cycles. Results obtained in this study were posteriorly validated on different stages of red winemaking of *Tempranillo* Rioja wines: after alcoholic fermentation, after malolactic fermentation and during aging in oak barrels.

The application of PEF to the wine after alcoholic fermentation aimed to reduce the microbial population to facilitate the growth of a commercial strain *Oenococcus oeni* for conducting the malolactic fermentation (Delsart et al., 2016). The PEF applied (33 $kV.cm^{-1}$, 105 ms, 158 $kJ.kg^{-1}$) caused significant reduction in the yeast population, completely eliminated the acetic acid bacteria but a lower inactivation was observed in the case of lactic acid bacteria in the four different wines assayed. In three of the four wines, it was observed a slight shortening of the malolactic fermentation duration monitored trough malic lactic acid consumption after inoculating *O. oeni* and a better sensorial quality after the PEF treatment.

A common practice in the wineries is the addition of SO_2 to the wine after malolactic fermentation to prevent spoilage of wine and to decline the activity of enzymes that cause wine oxidation. The effect of the current stabilization process used in the winery (addition of 30 $mg.L^{-1}$ SO_2) was compared with the effect of a PEF treatment (33 $kV.cm^{-1}$, 105 ms, 158 $kJ.kg^{-1}$) and the combination of the addition of 15 $mg.L^{-1}$ SO_2 plus a PEF treatment (Garde-Cerdán et al., 2008). Four days after the treatment it was observed that PEF treatment was more effective in reducing the microbial population of the three wines investigated than the addition of SO_2. After 6 months of aging, yeast and acetic bacteria were not detected in any of the wines independently of the previous treatment applied and some of the lactic acid bacteria were still viable in some of the wines. After these observations and evaluation of the physicochemical and sensory properties of the assayed wines, the authors concluded that PEF constituted an alternative to SO_2 in terms of microbial stabilization as well as a physicochemical and sensory quality.

One of the main problems of aging wine in oak barrels is the difficulty of disinfecting the barrels due to the porous structure of the wood. As a consequence, some microorganisms that survive in the wood may spoilage the wine contained in the barrels.

The main cause of wine spoilage during aging in oak barrels is the growth of *Brettanomyces/Dekkera* yeast. These yeasts that are characterized for tolerating the wine ethanol content and commonly used doses of SO_2 produce ethyl-phenols responsible of unpleasant odors in the wine. The capability of PEF for decontamination of wines contained *Brettanomyces/Dekkera* yeast was investigated by González-Arenzana et al., (2015). After the PEF treatment, it was observed a significant microbial inactivation of the most important spoilage microorganism of aged wines including *Brettanomyces/Dekkera* that led to a significant reduction of volatile phenols as compared with the untreated wine.

In conclusion studies conducted to demonstrate the ability of PEF to inactivate wine spoilage microorganisms and validation of the technique for microbial decontamination in different stages of winemaking together with the demonstration that PEF did not negatively affected physicochemical and sensory properties of the wine support the potential of using PEF as an alternative to reduce or eliminate the use of SO_2 in the wineries.

5. Application of PEF in wineries for accelerating aging on the lees.

Aging on the lees is a technique traditionally used in the wineries in the production of sparkling wines but more recently has been extended in the production of white wine and even red wine (Pérez-Serradilla et al., 2008). The technique consists of keeping the wine after fermentation in contact with the lees for a period of time. The lees are the residues in the bottom of the fermentation tanks after alcoholic fermentation. Although the composition of the lees is variable, they are mainly composed of yeast (Guilloux-Benatier & Chassagne, 2003).

During the aging of wine on the lees, autolysis of the yeast occurs. Yeast autolysis is a lytic event in the cells caused by intracellular yeast enzymes. As a consequence of this degradation of the yeast certain yeast compounds such as proteins, nucleic acids, lipid, and polysaccharides are transferred to the wine (Charpentier, Dos Santos, & Feuillat, 2004). One of the transferred compounds that have an effect on physicochemical and sensory properties of wine are mannoproteins. Mannoproteins are highly glycosylated proteins which constitute the major component of the cell wall in yeast. Their presence in wine produces positive effects such as reduction of haze formation, prevention of tartaric salt precipitation, diminution of astringency, and the improvement of mouthfeel, aroma intensity, and color stability (Pérez-Serradilla and de Castro, 2008).

Traditional aging on the lees is a long process lasting from few months to years, requires investment in equipment (tanks, barrels), entails elevated labor costs (periodic stirring – bâtonnage – and sensorial analyses), and implies immobilization of winery stocks. Furthermore, this practice may negatively affect wine quality increasing the risk of wine oxidation and microbial contamination. Due to the drawbacks associated with aging on the lees, wineries have a great interest in reducing the duration of this process.

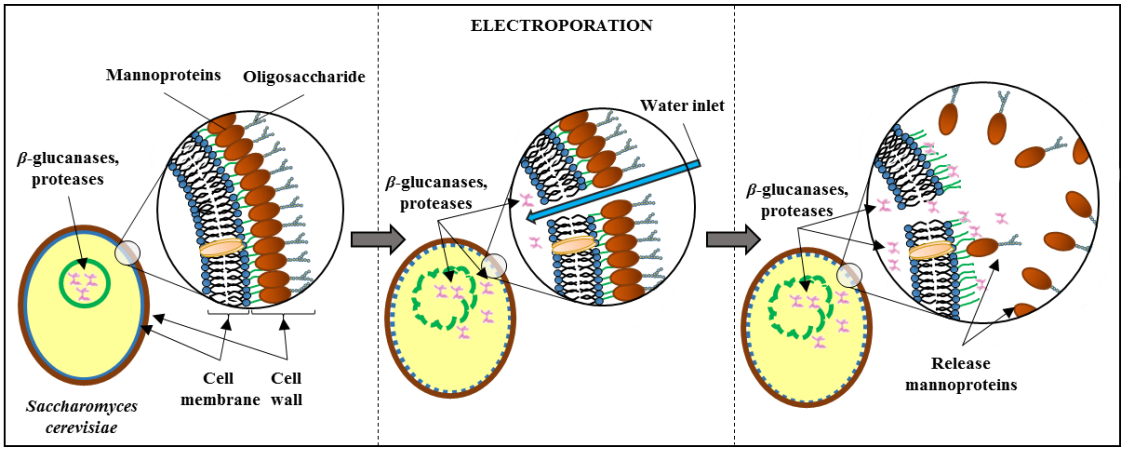

Figure 3: Release of mannoproteins from *S. cerevisiae* cells treated by PEF treatments.

It has been recently proven that PEF treatment triggers the autolysis of *S. cerevisiae* and accelerates the release of mannoproteins (Martínez et al., 2016). Several mechanisms related to electroporation could be involved in the induction of autolysis by PEF. On the one hand, electroporation would permit water from the surrounding media enters to the cytoplasm where there is a higher solute concentration The decrease of osmotic pressure within the cytoplasm as a consequence of the water inlet could lead to plasmolysis of the organelles and the release of the enzymes involved in cell wall degradation (proteases and *β*-glucanases). On the other hand, the electroporation of the cytoplasmic membrane by PEF could facilitate the contact of those released enzymes with the outermost layer of the yeast cell wall, where the mannoproteins are located Figure 3. This effect that was initially observed in yeast suspended in the buffer has also been validated during aging on the lees of white wine of *Chardonnay* variety (Martínez et al., 2019).

Chardonnay aging in the presence of *S. cerevisiae* yeast treated by PEF was compared with traditional aging on the lees (Martínez et al., 2019). Mannoprotein release increased drastically in *Chardonnay* wine containing PEF-treated (5 and 10 $kV.cm^{-1}$, 75 µs) yeasts.

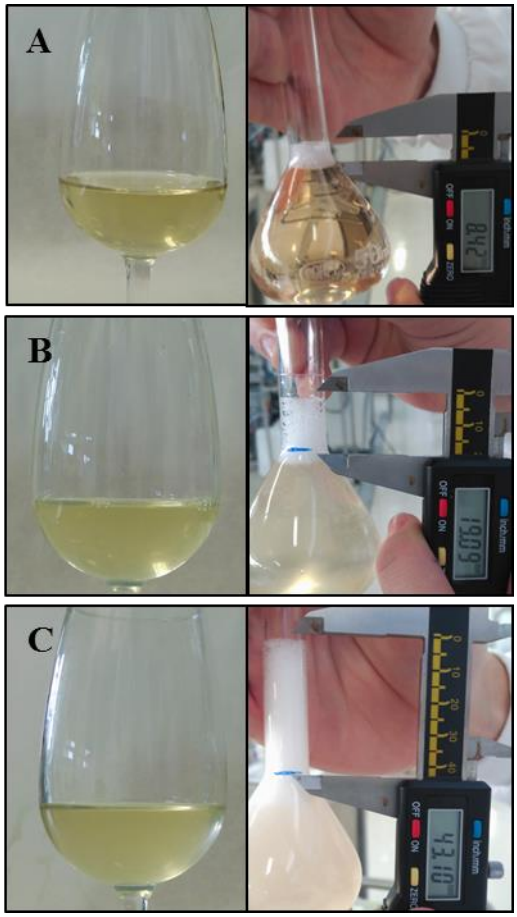

Figure 4: The photograph shows the turbidity and foam measure of *Chardonnay* wine containing *S. cerevisiae* cells either untreated (A) or treated by PEF of different intensity: 5 kV.cm^{-1} 75 μs (B) or 10 kV.cm^{-1} 75 μs (C) after 30 days of stored.

While no mannoprotein release was observed in the first ten days of aging on the lees in wine containing untreated yeast; mannoprotein concentration increased by 40 and 60 % in wines containing yeast treated by PEF at 5 and 10 kV.cm^{-1}, respectively. After 30 days of incubation, the mannoprotein concentration in wines containing yeast treated under the most intense PEF conditions reached the maximum value. Control cells, on the other hand, required six months to reach that maximum level. Chromatic characteristics, total polyphenol index, total volatile acidity, pH, ethanol, and CIELAB parameters of the wine were not affected during aging on the lees with PEF-treated yeast. On the other hand, mannoproteins released from yeast treated by PEF decreased wine turbidity after centrifugation and showed foaming properties as mannoproteins released during the traditional aging on the lees (Figure 4).

6. Conclusions

PEF could be considered as a universal technology for the wineries because its ability for improving different operations conducted in wineries. Effects of PEF in different stages of winemaking respond to some of current demands of food industry such as improving energy efficiency and sustainability, and reducing the use of chemicals.

The current physical techniques used in the wineries to reduce the duration of the maceration-fermentation stage in red winemaking such as thermovinification and flash expansion are based on heating (Morel-Salmi, Souquet, Bes, & Cheynier, 2006). Big efforts have been conducted to optimize energy consumption and heat recovery in processes based on heating, however, PEF may yet provide a further potential to help reduce energy consumption and operational costs while improving food production sustainability. Table 3 compares the energy requirements for grape processing by thermovinification, flash expansion and PEF to obtain an equivalent effect in terms of polyphenol extraction in red winemaking.

Table 3**:** Estimation of the energetic requirements of thermovinification, flash expansion and PEF for improving polyphenol extraction during winemaking

Technology	Specific energy delivered to the grape ($kJ.kg^{-1}$)	Additional specific energy * ($kJ.kg^{-1}$)	Total specific energy ($kJ.kg^{-1}$)
Thermovinification	161.92	40.68	202.6
Flash-release	251.88	45.86	297.7
PEF	6.70	-	6.70

* The energy required for the complete operation of the thermal system (pumps, refrigeration and condensation systems)

The energy required to increase the temperature of grapes is much higher than the energy required to electroporate grape skin cells by PEF. As the PEF treatment only cause a temperature increment of a few degrees, an additional advantage of this technique is that it is not necessary to cool the grapes after heating to initiate fermentation. From an energetic point of view, another important issue when comparing thermal and PEF is that, in the latter processes, energy is delivered directly to the product, thus making such methods much more efficient than heating techniques where thermal energy is transferred through an intermediate medium (water, water vapor, or oil). While thermal techniques require water, PEF permit to obtain similar objectives without increasing water consumption in a winery. Consequently, PEF is considerably more sustainable: by reducing the use of resources as well as CO_2 emissions.

Table 4: Fixed and variable costs of different PEF equipment in the cellars

	Small winery	Medium winery	Large winery
Production.year^{-1}	**500 ton**	**3,000 ton**	**15,000 ton**
Colinear chamber	**Ø4 x 2.5 GAP**	**Ø5 x 3 GAP**	**Ø10 x 10 GAP**
Imput voltaje (kV)	10	15	40
Production capacity (ton.h^{-1})	5	10	40
Investment (€)	60,000	80,000	250,000
Depreciation range (years)	10	10	10
Installation (€)	5,500	6,500	20,000
Replacement value (€)	72,000	96,000	300,000
Interest (%)	6	6	6
Depreciation range (€.annum^{-1})	7200	9,600	30,000
Interest (€.annum^{-1})	3,600	5,760	18,000
Maintenance (€.annum^{-1})	500	1,000	2,500
Fixed costs (€.annum^{-1})	**11,300**	**16.360**	**50.500**
Total specific energy (kJ.kg^{-1})	6 - 8	7 - 8	7 - 8
Specific energy imput	4.5 kWh	13.8 kWh	69.0 kWh
	0.90 kWh.ton^{-1}	1.38 kWh.ton^{-1}	1.73 kWh.ton^{-1}
Power price	0.13 €.kWh^{-1}	0.13 €.kWh^{-1}	0.13 €.kWh^{-1}
	0.58 €.h^{-1}	1.78 €.h^{-1}	8.90 €.h^{-1}
	0.12 €.ton^{-1}	0.18 €.ton^{-1}	0.22 €.ton^{-1}
Variable costs (€.annum^{-1})	**58**	**534**	**3,338**
Total costs	11,358 €.annum^{-1}	16,894 €.annum^{-1}	53,838 €.annum^{-1}
Total costs per ton	**22.72 €.ton^{-1}**	**5.63 €.ton^{-1}**	**3.59 €.ton^{-1}**
Total costs per liter (wine)	**0.03495 €.L^{-1}**	**0.00866 €.L^{-1}**	**0.00552 €.L^{-1}**

Another issue to be considered concerning the implementation of PEF is the ease of installation of the unit in the winery and the possibility of the same generator for different application (Figure 5). The required space for the installation of thermovinification or flash expansion is much greater and generally, renovation is required in the winery to install these units with associated auxiliary units. PEF technology differs from other techniques in view of its portability. The pulse generator unit may be separate from the treatment chamber, thereby allowing a rapid adaptation of the process, depending on the product to be treated. This could permit that the PEF generator used for electroporation of the grape skins used for the other application of the technology in a winery such as microbial inactivation or treatment of lees.

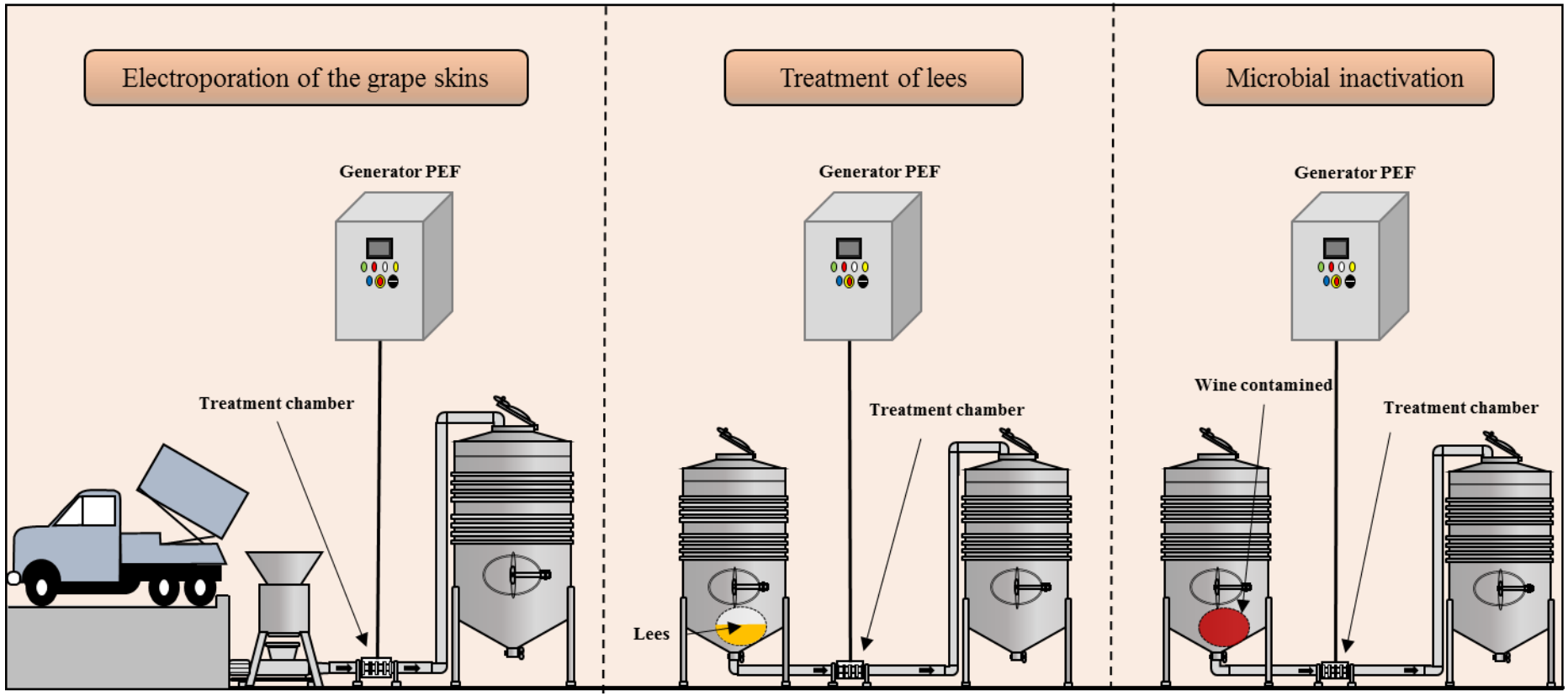

Figure 5: Diagram showing the pulse generator that can be used in different applications and allows a quick adaptation of the process, depending on the product to be treated.

Table 4 shows the estimated costs for the introduction of a PEF unit in a winery according to its production capacity. Although the same unit could be used for different applications, the total cost per liter of wine produced has been calculated considering only the treatment of the grapes before maceration-fermentation.

In conclusion, the development of pulse power systems able to respond to the processing capacity requirements of the wineries, the low energy consumption for the different applications of the PEF treatment for winemaking and the easy implementation of the treatment chambers into the existing processing lines of wineries are solid arguments the successful transfer of PEF technology to the wineries in a next future.

Thermal and non-thermal physical methods for improving polyphenol extraction in red winemaking.

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1. Abstract

Maceration-fermentation is a critical stage in the elaboration of high quality red wine. During this stage, the solid parts of the grape berries remain in contact with the fermenting must in order to extract polyphenols mainly located in the grape skin cells. Extracted polyphenols have a considerable impact on sensory properties (color, flavor, astringency, and bitterness) and on the aging behavior of red wine. In order to obtain wines with a sufficient proportion of those compounds, long maceration times are required. The presence of the solid parts of the grapes during red wine fermentation involves several problems for the wineries such as production capacity reduction, higher energy consumption for controlling the fermentation temperature and labor and energy consumption for periodically pump the grape must over the skin mass. Physical techniques based on heating such as thermovinification and flash expansion are currently being applied in wineries to improve the extraction of polyphenols and to reduce maceration time. However, these techniques present a series of problems derived from the heating of the grapes that affect wine quality. A series of recent studies have demonstrated that non-thermal innovative technologies such as pulsed electric fields (PEF) and ultrasound may represent effective alternatives to heating for assisting polyphenol extraction. In terms of general product quality and energetic requirements, this review compares these thermal and non-thermal physical technologies that aim to reduce maceration time.

2. Introduction

Red wine is obtained from the must of red grapes that undergoes fermentation together with the solid parts of the grape berries. In this step, known as maceration-fermentation, sugars of the must are converted into ethanol by yeast, and polyphenolic compounds are extracted mainly from the grape skin and the seeds.

Maceration-fermentation is the most critical stage in the red winemaking process. It is essential for obtaining high quality red wines, but is also the one that requires the most energy and workforce. It is estimated that about 64.3% of the total energy needed to produce a liter of wine is consumed during the maceration fermentation stage (Genc et al., 2017). Polyphenols are key actors in red wine, since they are involved in its sensory properties (color, flavor, astringency, and bitterness) (Arnold and Noble, 1978), in its aging behavior, and in beneficial health effects attributed to moderate wine consumption (Perez-Vizcaino and Fraga, 2018). In traditional red winemaking, in order to obtain a final product with high polyphenol content, the solid parts of the grape pomace remain in contact with the must during the entire alcoholic fermentation process (7–10 days), or even over a longer period of time. Although maximum anthocyanin content and color intensity is already achieved during the first days of maceration (Boulton et al., 2013; Zanoni et al., 2010), the extraction of procyanidins and other flavonoids, which have significant impact on other sensory attributes such as astringency and mouthfeel, requires longer maceration periods (Cerpa-Calderón and Kennedy, 2008; Hernández-Jiménez et al., 2012). As these compounds are mainly located in the seeds, its extraction required the presence of ethanol to disorganize the outer lipidic cuticle surrounding the seeds (Setford et al., 2017). On the other hand, in red winemaking: aromatic precursors responsible for the varietal aromas in wines are extracted from the solid parts of grape barriers, along with polyphenolic compounds.

The necessity of maintaining the solid parts of the grape berries in contact with the fermenting must leads to several issues faced by wineries in the red winemaking process (Marais, 2003). It is estimated that approximately 20% of the fermentation tanks are occupied by the solid parts, resulting in a reduction of the effective volume of the tanks and, as a consequence, of a winery's production capacity. This issue becomes especially significant at the peak of harvesting, when the fermentation-maceration tanks' production capacity may be exceeded. Other negative side effects of longer maceration periods are related with the difficulty of controlling the temperature increment as a

consequence of the fermenting activity of the yeasts when the solid parts are present in the fermentation tanks, as well as with the labor force and energy consumption required to periodically pump the wine over the skin mass that rises to the top of the fermentation tanks (Togores, 2011).

Different strategies have been adopted in wineries to enhance the extraction of phenolic compounds and to reduce the duration of the maceration-fermentation stage in red winemaking (Lowe et al., 1976; Pezzi et al., 2013). Physical technologies based on heating, such as thermovinification and flash expansion, are currently being applied in wineries for this purpose (Morel-Salmi et al., 2006). They present a series of problem such as the difficulty involved in stabilizing the color, the loss of varietal aromas through temperature increment, and the consumption of high quantities of energy (Atanacković et al., 2012; Fischer et al., 2000). A series of studies have recently demonstrated that non-thermal innovative technologies such as pulsed electric fields and ultrasound may represent effective alternatives to heating in the attempt to improve polyphenol extraction (Delsart et al., 2012; El Darra et al., 2013; Leong et al., 2016; López et al., 2008a). This review compares thermal and non-thermal physical technologies that aim to reduce maceration time in terms of equipment complexity, energetic requirements, and overall quality of the red wine.

3. Thermal technologies for improving polyphenol extraction

Although the heating of red grapes in order to reduce maceration has been investigated since the early 20th century, the process was not commercially adopted until the 1970s, when industrial heating systems were developed for that purpose (Rankine, 1973).

In general terms, the process consists in heating grapes to over 70 °C for a period of time ranging from a few minutes to several hours. As a consequence of heating, the cell envelopes of the grape skins are braked down, thereby facilitating the subsequent release of polyphenols (mainly anthocyanins) that are located inside the cells into the liquid phase (Girard et al., 2001). Heating also denatures enzymes such as polyphenol oxidase, thereby preventing browning. In fact, heating was originally used to prevent laccase activity in grapes contaminated with the mold *Botrytis cinerea* (Ribéreau-Gayon et al., 2006).

Although generally heating of the grapes before fermentation is called “thermovinification”, different pre-fermentation heating processes are currently being

applied in wineries. These techniques can be classified into two groups, depending on whether the cooling of the grapes, similarly to heating, is conducted using heat exchangers, or whether the cooling is conducted into a vacuum chamber. The first kind of process is designated as "thermovinification", along with its variations, known as "pre-fermentation hot maceration" (MPC), and "short-time-high-temperature treatment with warm maceration" (KZHE). The second group involves the technique called thermo-flash, flash détente or flash-release.

Thermovinification, MPC, and KZHE

Description of the techniques

Thermovinification, MPC, and KZHE are pre-fermentative heating techniques; they all have in common that the temperature of the grape mash does not increase above 85 °C, and that heating and cooling are conducted in heat exchangers (Nordestgaard, 2017).

In thermovinification, heating up to around 70 °C is conducted for a period of time of less than one hour, after which the grape mash is pressed to separate the solid parts and perform fermentation as for white wine. If heating at the same temperature is extended for a longer period of time (up to 24 h), and the fermentation is conducted in the presence or absence of the solid phase, the process is called MPC ("pre-fermentation hot maceration"). A variation of MPC is the process developed in Germany called KZHE ("short-time-high-temperature treatment with warm maceration"). In the latter, fermentation is conducted in the absence of solids after maintaining the grapes at around 45 °C for 6–10 h after having heated them to around 85 °C for 2 min.

Equipment

The simplest and most inexpensive heat exchangers used to heat grapes before fermentation are tube-in-tube heat exchangers. To prevent blocking problems in this heat exchangers, it is required the application of the treatment to the entire mix of juice and solid parts. To save energy, it is recommended to treat the solid parts after pre-draining in order to minimize the quantity of material that needs to be heated and cooled. In this case, it is recommended to use a scraped surface heat exchanger with a rotating shaft that improves heat transfer to the product. This approach permits to process the grape mash with a moderate degree of pre-draining while avoiding blocking issues.

Different approaches have been developed to save energy in the heating of the grape mash by recovering heat. In such systems, incoming well mixed crushed grapes without any pre-draining are pre-heated together with the crushed grapes that have already been heated. In these systems, and in order to avoid blocking, spiral heat exchangers or heat exchangers with a section of rectangular or parallel rectangular channels are preferred.

An alternative to the above-described continuous single pass method is to heat the grape mash with a tube-in-tube heat exchanger while recirculating them on a tank. This approach, generally used in smaller wineries, results in slower and more heterogeneous heating.

For transformation the sugar of must into ethanol by yeasts during fermentation, temperatures between 20 and 30 °C are required. Therefore, after the heating period, it is necessary to cool down the grape mass prior to fermentation. The cooling step is conducted with heat exchangers similar to those that are used for heating.

Fluids used in this type of equipment are hot water or steam for heating, and cold water or glycol for cooling.

In general, such installations used for pre-fermentative heating occupy a considerable area within the winery. The space is required for the heat exchanger systems as well as for the facilities designed to heat and cool the fluids.

Impact of the treatment in the composition of wine

The main objective in using these pre-fermentation heating techniques is to speed up the extraction of polyphenols from the grape skins with the purpose of eliminating or reducing the maceration stage. However, the characteristics of the final wine obtained with such heated grapes may be affected (Auw et al., 1996; de Andrade Neves et al., 2014).

As a consequence of heating, wild yeast populations are inactivated, thus requiring the addition of microbial starters to trigger fermentation. Generally, alcoholic fermentation is initiated without problems after pre-fermentation heating. Occasionally a more abrupt fermentation than in traditional fermentation is observed, probably related to the release of nutrients from the solid parts of the grapes as a consequence of heating (Martinière and Ribéreau-Gayon, 1969). A significant increase in sugar concentration, pH, amino acids, and ammonium in thermovinified Carignan must was reported (Geffroy et al., 2018). Bacterial populations of lactic as well as acetic bacteria are also inactivated,

resulting in wines with low volatile acid content. Total acidity of wine is not usually affected by pre-fermentation heating. Although a more elevated extraction of cations and anions as a consequence of grape heating has been described, they precipitate as salts of tartaric acid, thus ultimately leaving wines thus obtained in the same condition as untreated wines (Niculaua et al., 2017).

Pre-fermentation heating, in which the solid parts of the grapes are pressed and fermentation is conducted in the liquid phase, has the main objective of enhancing the extraction of color from the skins. The color increment is a consequence of the rapid extraction of anthocyanins.

While anthocyanins are extracted since the first moments of fermentation, flavanols require the presence of ethanol to be extracted.

Piccardo and González-Neves (Piccardo and González-Neves, 2013) reported that the extraction of anthocyanins after thermovinification was practically immediate. As consequence the anthocyanin concentration and the color intensity in the first days of fermentation were 21% and 45% higher, respectively, than in control. Most studies of the thermovinification technique have been conducted with Pinot noir due to the difficulty of extracting anthocyanins from that grape variety. It has been reported that the anthocyanin quantity in the Pinot noir variety reached a maximum at the onset of fermentation, with a concentration 2 to 3 times higher than in traditional fermentation. A drastic decrease in anthocyanins was observed, however, towards the end of fermentation (Gao et al., 1997). Studies conducted at laboratory scale have demonstrated the degradation of anthocyanins due to temperature (Geffroy et al., 2018, 2015). Anthocyanin content was affected by thermovinification when the treatment was very prolonged, or above 70 °C.

Concerning the effect of pre-fermentation heating on aroma, it has been reported that wines have a standardized sensory profile often described by oenologists as “banana yogurt” (Girard et al., 1997). For example, varietal aromatic compounds with green pepper aromas (methoxypyrazines) decreased in Cabernet Sauvignon wines when they were thermo-treated (Boubée et al., 2002). Geffroy et al. (2015) reported that a heat treatment at 70 °C for two hours induced a significant loss of several grape-derived aroma compounds (terpenols, norisoprenoids and some phenols) associated with an increase in *α*-terpineol, guaiacol and 2,6-dimethoxyphenol, suggesting thermal degradation. When thermovinification was applied to Carignan wine at two different temperature levels, 50 °C and 75 °C, and within two different time intervals, 30 min and 3 h, the effect of temperature on aroma composition was greater than that of heating time. Wines obtained

from grapes treated at 50 °C had higher concentrations of geraniol, *β*-citronellol, *β*-damascenone, and 3-mercaptohexanol, in most cases (Geffroy et al., 2018).

Although thermovinification reinforces anthocyanin extraction, the wines thereby obtained are known to lack color stability and structure. Anthocyanins can decrease due to enzymatic hydrolysis (Markakis, 1982), to combination with proteins, or to re-fixation with solid parts such as the skin (Kelebek et al., 2006) and yeasts (Morata et al., 2003). Since no alcohol is present at the time of heating, the wine does not contain sufficient levels of tannin to stabilize unstable anthocyanins and to provide structure. As a consequence, wines obtained by thermovinification are not usually used for aging, but commercialized as table wine for everyday use.

Finally, since tannin extraction is much more dependent on increasing ethanol content to encourage its solubilization, one approach to obtain a higher extraction of polyphenolic compounds consists in fermenting grapes after heating with solid parts of the grapes, as in standard vinification with shorter maceration time. This alternative was found to increase total phenolic index, color intensity and anthocyanins content in wine 58%, 25% and 45%, respectively (Piccardo and González-Neves, 2013).

Flash release

Description of the technique

The process called “flash release” or “flash détente” consists in rapidly heating the grapes at temperatures between 85–95 °C by a direct injection of steam. Grapes are then introduced into a vacuum that instantly vaporizes the water, thereby cooling the treated grapes and weakening their skin cell envelopes by boiling the water inside the cells (Ageron et al., 1995). This effect on the skin cells enhances extractability in subsequent fermentation process that may be conducted with or without the solid parts of the grapes. A modification of this process is called “half” flash détente (Doco et al., 2007). It uses a weaker vacuum to cool the grape mash to around 50 °C instead of 30 °C.

Equipment

Flash release or flash expansion equipment consists of a heat exchanger and a vacuum chamber. In the heat exchanger, the steam is directly injected to the grape mash. Grape mash is continuously moved by two hollow stem augers through which the steam enters into the vacuum chamber. Since the chamber is under negative pressure (20–25 hPa), the water instantly evaporates, while the grape mash is simultaneously cooled. The

estimated amount of evaporated water ranges between 6 to 10% (Baggio, 2013). It is condensed in a condenser connected with the vacuum chamber, and reincorporated into the grape mash totally or partially, depending on the amount of water in a gaseous state added to the grape mash during the heating process. The flash release system requires a boiler to produce water vapor for rapid heating.

Impact of the treatment in the composition of wine

It has been reported that the yeast population lag phase before starting fermentation is slightly shorter when the grape mash is treated by flash release, probably because the treatment has triggered the release of some yeast nutrients (Vernhet et al., 2016).

Characteristics of wines obtained by flash release can be modulated by conducting fermentation in liquid phase, or by keeping the solid parts of the grapes in contact with the liquid phase for different periods of time. It has been observed that flash release increases the extraction of flavanols and flavonols from skins rather than from seeds. Therefore, when fermentation is carried out without the skins, the concentration of tannins with respect to anthocyanins is low, as in wines obtained via traditional pre-fermentation heating. The destabilization of grape skin cell envelopes seems to facilitate the extraction of tannins located in the vacuoles of the hypodermal cells of the grape skins. However, the proportion of those tannins in the resulting wine is low compared with the tannins coming from the seeds, which require the presence of ethanol to be extracted and also a more maceration time (Escudier et al., 2006).

Morel-Salmi et al. (Morel-Salmi et al., 2006) investigated the phenolic extraction kinetics during the maceration-fermentation of *Grenache* must previously treated by flash release. They observed that the amount of various families of phenolic compounds was higher at the beginning of the fermentation process in the flash release treated must than in control. On the other hand, while the levels of catechins, flavonols, and proanthocyanidins increased during fermentation of flash release treated musts, the concentration of hydroxycinnamic acids remained constant and anthocyanins decreased during the first day, and then they remained constant. The increment in concentration of galloylated units increased throughout fermentation, reflecting the gradual extraction of seed tannins as the ethanol level increased. Therefore, although the effect of flash-release on grape skin cell envelopes is more drastic than that of other pre-fermentation heating techniques, a contact period of the solid parts of the grapes with the must during

fermentation after treatment is required in order to obtain structured wines with large amounts of polyphenols. At the of the vinification process, the wine obtained with *Grenache* grapes treated by flash release had a total phenolic index and a colour intensity 14% and 9% higher than the control wine respectively.

The effect of flash release on the extraction of aromatic compounds and aroma precursors has been also investigated (Kotséridis et al., 2002). As compared to wines obtained by other pre-fermentation heating techniques, wines obtained with flash release maintain their varietal aromatic profile. The treatment increases the levels of fatty acid ethyl esters and *β*-ionone in *Grenache* wines. On the other hand, it has been observed that flash release may reduce the content of C6 compounds responsible for herbaceous aromas (Razungles, 2010). This effect is especially interesting when the wines are elaborated with grapes that have not reached their optimal stage of maturity.

Wines of different varieties such as *Grenache, Carignan, Syrah*, and *Mourvedre* obtained with flash expansion technique were preferred to control wines in a sensory analysis, especially when the contact time of the solid parts of the grapes with the fermenting must was extended (Besnard et al., 2018).

4. Non-thermal techniques for improving polyphenol extraction

Non-thermal technologies have been one of the most frequently investigated topics in the field of food processing over the last decades (Toepfl et al., 2006b The "non-thermal" concept refers to a group of technologies whose effects in foods are similar to those caused by heating, albeit at temperatures lower than the ones used in thermal processing. Some of these treatments may involve heat due to the generation of internal energy (e.g., resistive heating during PEF). However, they are classified as non-thermal, because they can eliminate or significantly reduce the application of high temperatures in food processing, thereby avoiding the deleterious effects of heat on the flavor, color, and nutritive value of foods.

The emergence of non-thermal technologies can lead to high quality products while saving energy by improving heating efficiency. Most of these technologies are locally clean processes and therefore appear to be more environment friendly, with less environmental impact than traditional ones (Chemat et al., 2017b). Novel processing technologies are increasingly attracting the attention of food processors, since they can provide food products with improved quality and a reduced environmental footprint, while reducing processing costs and improving the products' added value.

Due to their special mechanism of action, pulsed electric fields and high-intensity ultrasound are among the non-thermal technologies that have been most investigated with the purpose of improving polyphenol extraction in wineries.

Pulsed electric fields (PEF)

Description of the technique

PEF processing consists in the intermittent application of short duration pulses (ms-μs) of high voltage (kV) to a product located between two electrodes. The applied external voltage generates an electric field whose strength depends not only on voltage intensity, but also on the distance between the electrodes. When exposed to a sufficiently strong electric field, the cell membrane undergoes a phenomenon called electroporation, consisting in the increment of cell envelope permeability as a consequence of the formation of pores in the cytoplasmatic membrane (Tsong, 1989).

If the intensity of the electric field is not high enough, or if the exposure to the electric field is sufficiently brief, the membrane can spontaneously return to its initial state and remains viable (reversible electroporation). However, intense electric fields or longer exposures can cause irreversible electroporation (Weaver and Chizmadzhev, 1996). Reversible electroporation is a procedure that is typically used in molecular biology and in clinical biotechnological applications to gain access to the cytoplasm for the introduction or delivery in vivo of drugs, oligonucleotides, antibodies, plasmids, etc. However, the main applications of PEF in the food industry aim to cause irreversible electroporation of the cell membranes. It has been demonstrated that irreversible modification of the permeability of cell membranes can inactivate vegetative cells of microorganisms, enhance mass transfer in different operations of the food industry (e.g., extraction of intracellular components of interest, dehydration, infusion of compounds into the cells, etc.), and modify food structure (Puértolas et al., 2012; Toepfl, 2012).

Equipment

Basic components of an apparatus for the application of PEF are a pulse generator and a treatment chamber. The pulse generator is a Marx generator of square waveform pulses with a direct current power supply which converts alternating current to direct current line that is used, in turn, to charge a set of capacitors at high voltage. When the high voltage switch (a high-power solid-state switch) is opened, the capacitors are charged. If the high power switch is then closed, all the electrical energy stored in the

capacitors is delivered to the treatment chamber. The switching system permits the controlled discharge of the capacitor in the form of pulses of very short duration at very high frequencies (reaching hundreds of pulses per second).

During PEF processing, a liquid food or pumpable product is passed through a treatment chamber where it is subjected to short pulses of high voltage. The treatment chamber consists of two electrodes made of a conducting material such as stainless steel or titanium; they are separated by an insulating material, which forms an enclosure containing the food material. Different types of treatment chambers have been designed to minimize the effect of electrolysis as well as corrosion. The two most important treatment chamber designs that are presently considered for the commercial application of PEF are parallel electrode and co-linear configurations. The latter configuration is the one habitually used for processing crushed grapes after destemming, with the purpose of electroporating the cytoplasmic membrane of grape skin cells to facilitate the extraction of polyphenols during the maceration-fermentation stage. The co-linear treatment chamber consists of an electrically insulating tube through which the grape mash flows. The electrodes are located in the middle (High voltage) and on either side of the chamber (ground). They consist of two metal pipes that also serve as the entrance and exit for the fluid. The circular section of this co-linear configuration facilitates its installation in winery circulation pipes used to transport crushed and destemmed grapes to the fermentation-maceration tanks (Figure 6) (Luengo et al., 2014).

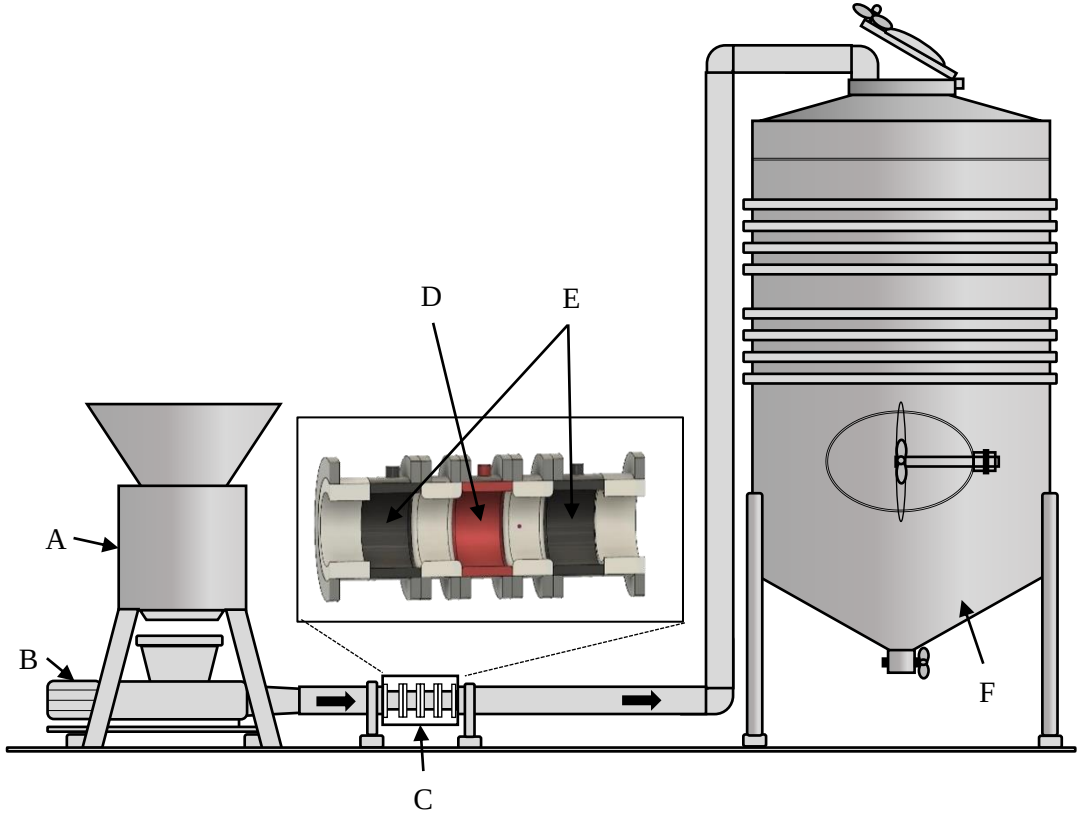

Figure 6: Flow chart of grape processing with PEF technology. A: destemming; B: progressive cavity pump; C: co-linear treatment chamber; D: high voltage electrode; E: ground electrode; F: fermentation tank.

The lack of reliable and viable industrial-scale equipment has limited the commercial exploitation of PEF in the food industry for many years. However, recent developments in pulse power generators have enabled the design of PEF equipment with characteristics that can meet industrial standards in terms of reliability and workloads (Toepfl, 2011).

Impact of the treatment in the composition of wine.

As compared with heating techniques, of the improvement of extraction of polyphenols by PEF requires to maintain the solid parts of the grapes in contact with the liquid phase for different periods of time (Puértolas et al., 2010a). Therefore, the effect of PEF treatment on cell skin envelopes seems to be less aggresive than that of techniques based on heating (Cholet et al., 2014). Tests carried out by different authors on different grape varieties agree that PEF treatment neither affects the fermentation process nor the physicochemical properties of the resulting red wine. Ethanol content, pH, volatile acidity and total acidity in the wines obtained with grapes treated by PEF were similar to control wines (Puértolas et al., 2010a; Saldaña et al., 2017).

The electroporation of cell grape skins by the application of PEF accelerates and increases the extraction of phenolic compounds during the maceration-fermentation stage in the vinification of red grapes (Ricci et al., 2018). Different studies have shown that, after the same maceration time than in control wine, PEF treatment reinforces oenological parameters by a rate of 10% to 60%, depending on the extraction of polyphenols (color intensity, total anthocyanin content, and total polyphenol content) in the maceration-fermentation stage (Puértolas et al., 2010a).

Puértolas et al. (2010) showed that PEF technology can help reduce maceration times. *Cabernet Sauvignon* wine obtained from PEF-treated grapes (5 $kV.cm^{-1}$, 150 μs, and 3.67 $kJ.kg^{-1}$) presented higher color intensity, total anthocyanin content, and total polyphenol content values, although the duration of the maceration of the grapes treated by PEF was 48 h shorter than for control wines. Evolution during aging of the wine obtained from grapes treated by PEF was similar to control wine. The differences in color intensity, total anthocyanin content, and total polyphenol content observed at the end of fermentation between control wine and the wine obtained from PEF-treated grapes were maintained after aging the wine in bottle or oak barrels (Puértolas et al., 2010d). Determination of individual polyphenols by means of high-performance liquid chromatography (HPLC) highlighted that the wines obtained by PEF treatments did not

show differences in terms of the proportion of different polyphenols, thus indicating that PEF treatment did not selectively extract phenolic compounds from grape skins. López-Alfaro et al. (2013) reported that the content of resveratrol, one of the most researched phenols in wine due to its beneficial properties, increased by a proportion of 200, 60 and 50% in *Tempranillo, Grenache* and *Graciano*, respectively, when the grapes were treated with PEF before maceration-fermentation.

Energetic requirements for the electroporation of cells of grape skins are lower than 10 $kJ.kg^{-1}$; as a consequence, the treatment causes an increment of less than 2 °C in grape mash temperature. This low impact allows the obtained wines to maintain their varietal character (Puértolas et al., 2010a). Some experiments have shown that PEF treatments encourage the diffusion of aromatic compounds found in the skin, as well as of aromatic precursors (Garde-Cerdán et al., 2013). PEF treatment did not increase the concentration of C6 family compounds associated with herbaceous aromas in wines obtained from *Grenache, Tempranillo,* and *Graciano* varieties (Garde-Cerdán et al., 2013). The treatment significantly increased monoterpenoid compounds, and a had positive effect on the concentration of *β*-ionone, total esters, and benzenoid compounds in *Grenache* wine. However, the volatile composition of *Tempranillo* and *Graciano* wines was not affected by PEF.

Sensory analysis did not detect any drawbacks in *Cabernet Sauvignon* wines obtained with grapes treated by PEF. Luengo et al. (Luengo et al., 2014) compared *Grenache* wines featuring similar enological parameters in terms of polyphenol content obtained, on the one hand, with PEF treated grapes and 7 days of maceration and, on the other hand, with untreated grapes and 14 days of maceration. Compared with control wine, panelists preferred the wine obtained with grapes treated by PEF and a shorter maceration period.

Ultrasound

Description of the technique

Acoustic waves of a specific frequency lying above the detection threshold of human hearing (i.e., over 16–18 kHz) are designated as ultrasound. Ultrasound is divided into two categories, according to the frequency range and the intensity of ultrasonic waves. The first group, commonly known as high-intensity ultrasound, features low frequency and high intensity (20–100 kHz; >10 $W.cm^{-2}$). The second group, commonly

called diagnostic ultrasound, uses high frequency and low power (>100 kHz; <1 W/cm^{-2}).

When high-intensity ultrasound passes through a liquid medium, a phenomenon called acoustic cavitation occurs (Cravotto and Cintas, 2006). Cavitation consists in the implosion of bubbles formed in liquid media when the local pressure in the expansion phase falls below vapor pressure. During the implosion, it is estimated that high temperatures and pressures are reached in very small spots and very short periods of time: liquid jets of up to 280 $m.s^{-1}$ are likewise generated. These phenomena brought about by cavitation are responsible for effects attributed to high-intensity ultrasound, such as the increment of mass transfer, or the breakage of cells of microorganisms, or of plant or animal tissues (Chemat et al., 2017b). Ultrasound may therefore enhance the extraction of polyphenols from the solid parts of grapes in red winemaking by breaking up the cells, and by facilitating the diffusion of polyphenols from the cells to the must (González-Centeno et al., 2014).

Equipment

An apparatus for the generation of ultrasound consists in a power supply and a transducer. The power supply converts alternating current line voltage to frequencies of over 20 kHz electrical energy. This high-frequency electrical energy is fed to a transducer, where it is converted to mechanical vibrations at the same frequency as the transformed electrical current. The physical concept underlying the transducer is the piezoelectric effect: the property of certain materials causes them to change shape when an electric current is applied to them. An ultrasound transducer contains a thin disk, square, or rectangle of piezoelectric ceramic placed between two electrodes which expand and contract when subjected to alternating voltage. The converter vibrates in a longitudinal direction and transmits the motion to the solution, thereby causing cavitation (Cravotto and Cintas, 2006).

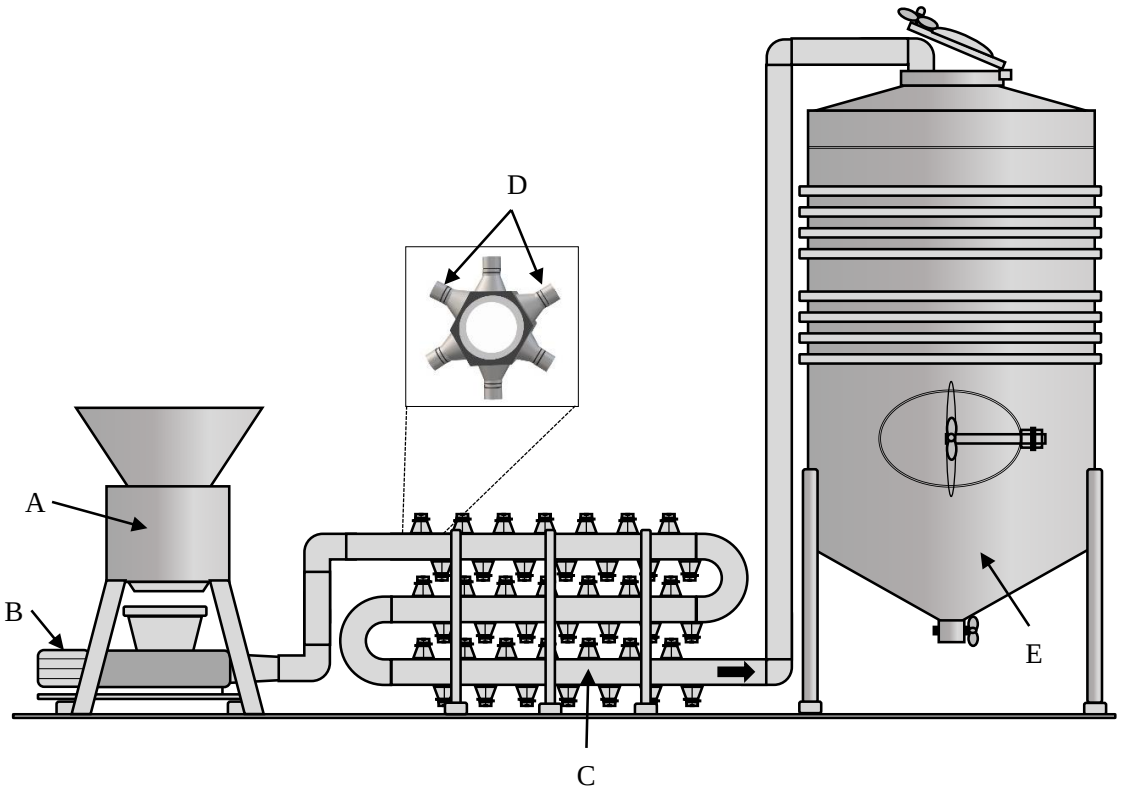

Figure 7: Flow chart of grape processing with ultrasound technology. A: destemming; B: progressive cavity pump; C: ultrasound treatment zone; D: transducer; E: fermentation tank.

A power ultrasound system has recently been developed for processing destemmed and crushed grapes in continuous flow. The equipment consists of a hexagonal stainless-steel pipe into which the transducer is welded (Figure 7). The length of the pipes containing the transducers is variable, depending on the installation's processing capacity, which can reach up to ten tons per hour. The cavitation caused by the ultrasound treatment provokes the destruction of the cells of the solid parts of the grapes, thereby leading to the release of polyphenols.

Impact of the treatment in the composition of wine

The use of high-power ultrasound (US) to improve the extraction of phenolic compounds from grapes has been recently studied (Celotti and Ferraretto, 2016; Zhang et al., 2016) As in the case of PEF technology, an ultrasonic treatment applied at different frequencies (45, 80, and 100 kHz) with the purpose of improving polyphenolic extraction did not modify the physicochemical properties of wine. Total acidity and pH of *Cabernet Sauvignon* wine obtained from ultrasound-treated grapes did not show significant differences with respect to control, although electrical conductivity was slightly higher (4 %). This increment in conductivity could be associated with the release of ions located inside the cells of the solid parts of the grapes to the must (Zhang et al., 2016).

El Darra et al. (2013) investigated the effect of ultrasound on the extraction of polyphenols from Cabernet Sauvignon grapes at laboratory scale using an US probe in a flask containing 400 ± 5 g of must and grape skins. Results showed an increment in the phenolic, anthocyanin, and tannin contents of the wines obtained from grapes treated by ultrasound. A greater color intensity compared with the untreated samples was likewise observed in the wines after ultrasonication treatment, whereby the highest values of those parameters were achieved by the samples that had been subjected to the most intense treatment (363 $kJ.kg^{-1}$).

Monastrell wines obtained after different maceration times with grapes treated by a continuous flow pilot-scale power ultrasound system (2500 W, 28 kHz, 8 $W.cm^{-2}$) were compared with wines obtained from untreated grapes (Bautista-Ortín et al., 2017). Results showed an increase in the chromatic characteristics of the wines obtained with ultrasonicated grapes. The values for these chromatic characteristics were higher in wines obtained with ultrasonicated grapes and 3 days of maceration than in control wines with a longer maceration period (5 days). After two months of aging, the wines obtained with grapes treated by US contained between 20 and 35% more total polyphenols than control

wines (Bautista-Ortín et al., 2017). The ultrasound treatment also encouraged the extraction of tannins from the seeds, although to a lesser extent than tannins from the skins. As a consequence, the wines elaborated with ultrasonicated grapes and 3 days of maceration presented twice the concentration of proanthocyanidins than that of control wines obtained with 8 days of maceration.

Concerning the effect of ultrasonication treatment on the volatile composition of wines, no significant differences were observed in the total concentration of those compounds between control and wine obtained from grapes treated by ultrasound, regardless of maceration time (González-Centeno et al., 2014).

5. Discussion

Novel non-thermal processing technologies have been developed in the last years with the aim of preventing problems associated with thermal processing, and with the purpose of improving energy efficiency and food production sustainability. The introduction of a new technology on the market requires that it must perform at least as well as existing commercial processes. Table 5 compares, as an example, the improvements derived of application of different thermal and non-thermal physical methods to the grapes before vinification in terms of polyphenolic extraction. It is observed that PEF and ultrasound permits attaining similar enhancements in total anthocyanin content, color intensity and total polyphenol content than techniques based in the heating of the grapes. However, as it is shown in Table 6 thermovinification and flash release present certain drawback related with the wine quality, energy consumption etc. that would support the implementation of non-thermal physical techniques to improve polyphenol extraction.

Although in the past decades the food industry has carried out immense efforts to optimize energy consumption and heat recovery in conventional processes, the introduction of non-thermal technologies may yet provide a further potential to help reduce energy consumption and operational costs while improving food production sustainability.

Table 5: Improvements derived of grape treatment before vinifications with different thermal and non-thermal physical methods in terms of increment in total polyphenolic content, color intensity and total anthocyanin content.

Technology	Treatment	Variety	Total polyphenolic content	Colour Intensity	Total Anthocyanin Content	Ref.
Thermovinification	82ºC 1 hour Flow rate: 500 $kg.h^{-1}$ Maceration time: 5 days	*Merlot*	36 %	N/A	26 %	(Niculaua et al., 2017)
Flash Release	95 °C for 6 min Strong vacuum (>100 mbar) Flow rate: N/A Maceration time: 5 days	*Carignan*	11 %	30 %	30 %	(Morel-Salmi et al., 2006)
PEF	5 kV/cm, 150 µs (50 pulses 3 µs, 3.67$kJ.kg^{-1}$) Flow rate: 118 $kg.h^{-1}$ Maceration time: 4 days	*Cabernet Sauvignon*	23 %	38 %	34 %	(Puértolas et al., 2010a)
Ultrasound	2500 W; 28 kHz; 8 $W.cm^{-2}$ Flow rate: 400 $kg.h^{-1}$ Maceration time: 4 days	*Monastrell*	32 %	31 %	13 %	(Bautista-Ortín et al., 2017)

N/A: information not available.

Table 6: Advantages and disadvantages of different thermal and non-thermal technologies for improving polyphenol extraction in red winemaking for grape pre-fermentation treatments.

Technology	Advantage	Disadvantages	Ref.
Thermovinification	Possibility of obtaining red wines without maceration For obtaining table wines. Permits to inactive enzymes and microorganisms Approved by OIV	Poor color stability Possible degradation of anthocyanins Loss of varietal aromatic compounds Wines not usually used for aging Addition of starter cultures for initiating fermentation required High energetic requirement. Supplies of methane or diesel oil required	(de Andrade Neves et al., 2014; Geffroy et al., 2018; Niculaua et al., 2017; Pezzi et al., 2013; Piccardo and González-Neves, 2013)
Flashrelease	Mainly for obtaining table wines. Permits to inactive enzymes and microorganisms Obtaining of more complex sensory characteristics Approved by OIV	Possible degradation of anthocyanins Wines not usually used for aging Addition of starter cultures for initiating fermentation required High energetic requirement. Supplies of methane or diesel oil required Renovations are required in the winery for installation (Large facilities: >100m^2)	(Baggio, 2013; Besnard et al., 2018; Morel-Salmi et al., 2006)
PEF	Demonstrated the ability of aging of the wines in oak barrels Easy implementation in the winery (small facilities:<10 m^2) Possibility of renting the PEF unit Possibility of conducting fermentations with wild yeast Possibility of using for other applications in winery (microbial inactivation or accelerating aging on the lees) Low energy requirements	Maceration of few days is required for obtaining red wines Approval for the OIV in process. No enzymatic inactivation.	(Delsart et al., 2012; Luengo et al., 2014; Puértolas et al., 2010c, 2010b)
Ultrasound	Easy implementation in the winery (small facilities:<10 m^2) Possibility of renting the ultrasound unit Possibility of conducting fermentations with wild yeast Possibility of using for other applications in winery (accelerating aging on the lees) Low energy requirements	Maceration of few days is required for obtaining red wines Approval for the OIV in process. No enzymatic inactivation.	(Bautista-Ortín et al., 2017; Del Fresno et al., 2019; Singleton and Draper, 1963)

Table 7 compares the energy delivered to grapes (after destemming and crushing) by several thermal (with final treatment temperatures between 50 to 85 °C before fermentation) and non-thermal processes (with temperature increases lying under 5 °C) to obtain an equivalent effect in terms of polyphenol extraction in red winemaking. One can observe that the energy required to increase the temperature of grapes is much higher than the energy required to electroporate grape skin cells by PEF, or to disrupt skin and seed cells by ultrasound. From an energetic point of view, non-thermal techniques present an additional advantage, since the low energy delivered to the product does not substantially increase its temperature. As compared with themovinification or flash expansion in the case of winemaking, this implies that it is not necessary to waste energy to cool the grape mash to the temperature required to initiate fermentation. According to Table 7, the average specific energy of thermal treatments is 17.6-fold higher than that required for non-thermal processes being the specific energy required for PEF treatment lies 3.2-fold lower than that required by ultrasound treatment. Consequently, considering that the energy source is different for thermal and non-thermal processes, lower operational costs are required for PEF and ultrasound processing.

Table 7**:** Estimation of the energetic costs of thermal and non-thermal physical methods for improving polyphenol extraction during winemaking

Technology	**Specific energy delivered to the grape ($kJ.kg^{-1}$)**	**Additional specific energy * ($kJ.kg^{-1}$)**	**Total specific energy ($kJ.kg^{-1}$)**	**$kWh.ton^{-1}$**	**€$.ton^{-1}$ [a]**
Thermo-vinification	161.92	40.68	202.6	56.28	7.32
Flash-release	251.88	45.86	297.7	82.70	10.75
PEF	6.70	-	6.70	1.86	0.24
Ultrasound	21.60	-	21.60	6.0	0.78

[a] Energy cost: Electricity : 0.13€ $kW.h^{-1}$

* The energy required for the complete operation of the thermal system (pumps, refrigeration and condensation systems) .

From an energetic point of view, another important issue when comparing thermal and non-thermal technologies is that, in the latter processes, energy is delivered directly to the product, thus making such methods much more efficient than heating techniques where thermal energy is transferred through an intermediate medium (water, water vapor, or oil). While thermal techniques require water, non-thermal techniques permit to obtain similar objectives without increasing water consumption in a winery. As a consequence, non-thermal technologies are considerably more sustainable: they reduce the use of resources as well as CO_2 emissions.

Another aspect that differentiates thermal from non-thermal techniques is related with the installation of the unit in the winery. The required space for the installation of thermovinification or flash expansion is much greater than that required for the installation of ultrasound or PEF units. Generally, considerable renovation is required for a winery to introduce a thermovinification or a flash expansion unit with associated auxiliary units. PEF technology differs from other techniques in view of its portability. The pulse generator unit is separate from the treatment chamber, thereby allowing a rapid adaptation of the process, depending on the product to be treated. Moreover, these units are small enough to be easily integrated into existing production lines without requiring major factory overhaul.

To summarize, non-thermal techniques such as PEF and ultrasound are now increasingly attracting the attention of wineries as an alternative to techniques based on grape heating in order to reduce the duration of maceration time and/or to avoid the purchase of maceration-fermentation tanks. These techniques can encourage the production of wine with improved quality and a reduced environmental footprint, while at the same time decreasing processing costs.

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Y

OBJETIVOS

Los particulares efectos que ejercen los tratamientos de pulsos eléctricos de alto voltaje (PEAV), provocan que esta tecnología pueda tener diversas aplicaciones en el proceso de elaboración del vino (Donsì et al., 2010). El vino se puede definir como el producto obtenido de la fermentación alcohólica total o parcial del mosto de la uva. En el proceso de elaboración de vinos blancos, el mosto se separa de los hollejos y la fermentación de sus azúcares del mosto se realiza en ausencia de los hollejos. El vino rosado fermenta del mismo modo que el blanco, adquiriendo, su color característico, en una etapa de maceración pre-fermentativa en la que se mantienen los hollejos en contacto con el mosto durante unas horas. La fermentación del mosto durante la elaboración del vino tinto se realiza en presencia de los hollejos. En esta etapa, además de transformarse los azúcares en alcohol por acción de las levaduras, se produce una salida de sustancias, fundamentalmente compuestos fenólicos, que juegan un papel determinante en sus propiedades sensoriales. Los fenoles son los principales responsables del color y de la mayoría de las propiedades que definen la calidad del vino tinto (Moreno-Arribas and Polo, 2005). Además, en los últimos años se ha demostrado que estos compuestos tienen potenciales efectos beneficiosos sobre la salud debido a su actividad antioxidante (Rice-Evans et al., 1996). Por todo ello, el paso de compuestos fenólicos al mosto es una de las principales etapas en la elaboración de vinos tintos yademás, condiciona de forma muy importante su proceso de producción al determinar la duración del proceso de maceración/fermentación (Sacchi et al., 2005). Con objeto de obtener una cantidad suficiente de compuestos fenólicos, en el proceso de elaboración de vinos tintos se dificulta el control de la temperatura del mosto con el riesgo de que se detenga la fermentación (Luengo et al., 2012). Con objeto de mejorar el proceso de extracción de los compuestos fenólicos, para acortar esta etapa, se han propuesto distintas alternativas al proceso de vinificación de los vinos tintos como la termovinificación o el empleo de enzimas (Sacchi et al., 2005). A pesar de las aparentes ventajas que podría presentar el proceso de termovinificación, se observa que los vinos elaborados con esta tecnología presentan problemas de tipo sensorial (aromas extraños y perdida de frescor y astringencia), y dificultades para su clarificación por la destrucción de enzimas pectolíticas. El empleo de preparados enzimáticos para favorecer la extracción de los componentes de la piel es una práctica cada vez más utilizada en las bodegas. Sin embargo, presenta algunos inconvenientes como su elevado precio o la poca reproducibilidad en los resultados obtenidos.

La capacidad de los PEAV para permeabilizar las membranas celulares hace que esta tecnología pueda tener diferentes beneficios en el proceso de elaboración del vino. La permeabilización de los hollejos por los PEAV, podría facilitar la extracción de los componentes fenólicos lo que permitiría evitar o acortar el periodo de maceración pre-fermentativa en la elaboración de vinos rosados y de maceración en la elaboración de vinos tintos. En vinos tintos jóvenes, que requieren una maceración más corta, podría acortarla o incluso los procesos de maceración y de fermentación se podrían realizar separadamente, con lo que se mejoraría el control de la temperatura durante la fermentación. Finalmente, un incremento en la extracción de estos componentes podría contribuir a la mejora de las propiedades beneficiosas del vino sobre la salud (Donsì et al., 2010; Puértolas et al., 2012). Los resultados obtenidos indican que, efectivamente, la permeabilización de los hollejos por un tratamiento de PEAV (hasta 10 $kV.cm^{-1}$ y tiempo de 100 μs) permite extraer los compuestos fenólicos con tiempos de maceración y fermentación mucho más cortos; los tiempos se pueden reducir a la mitad,en comparación conlos requeridos en los procesos de vinificación tradicionales (López et al., 2008a; Puértolas et al., 2010a). Además, los vinos obtenidos muestran características sensoriales similares a los no tratados por PEAV e incluso con aromas más intensos. Sin embargo, los resultados obtenidos hasta el momento han sido desarrollados en vinificaciones que van desde los mililitros a unos pocos litros debido a que se trabajó con un equipo de PEAV que sólo permitía su aplicación a escala de laboratorio o en planta piloto, pero no en bodega. Es por ello, que sería de gran interés evaluar la aplicación de la tecnología PEAV en un flujo continuo que permitiera trabajar con mayores volúmenes para realizar vinificaciones a escala de planta piloto y, sobre todo a escala de bodega, para verificar los resultados previos obtenidos. Concretamente, se pretende confirmar la posibilidad de reducir los tiempos y temperaturas de maceración de un proceso normal de vinificación observada a menor escala, así como el incremento en la extracción de polifenoles. Todo ello conllevará la confirmación y cuantificación en la obtención de vinos de mayor calidad sensorial, una mayor productividad al aprovechar mejor la capacidad de los depósitos de maceración/fermentación, al permitir los PEAV reducir el tiempo de contacto entre los hollejos y el mosto; e incluso la mayor extracción de polifenoles, podría permitir obtener nuevos productos como vinos con menor grado alcohólico, pero con características más parecidas al vino tinto. La participación en el proyecto de una empresa fabricante de equipos va a permitir abordar estos aspectos a escala de bodega y valorar el potencial de la tecnología en condiciones reales de trabajo.

Por todo ello, el objetivo principal de esta investigación es:

Evaluar a escala de bodega el efecto de los PEAV en la extracción de compuestos fenólicos, color y antocianos durante la etapa de maceración/fermentación en el proceso de obtención de vino tinto. Para la consecución de este objetivo se plantearon los siguientes objetivos parciales:

- Determinar el efecto de los principales parámetros de procesado de los PEAV en flujo continuo en la extracción de compuestos fenólicos a partir de uva y en el tiempo de maceración del proceso de obtención de vino tinto.

- Evaluar el efecto de los tratamientos PEAV en el aroma y en las características sensoriales de vinos tintos obtenidos a partir de uva tratada por PEAV.

- Estudiar la influencia de los PEAV en el envejecimiento de vinos tintos.

- Investigar el impacto de la aplicación de tratamientos.

PEAV de alta energía en el tiempo de maceración y en la calidad del vino tinto.

- Realizar un análisis de costes de la implementación de la tecnología de PEAV en una bodega para el tratamiento de uva.

MATERIAL

Y

MÉTODOS

1. UVA

Para la realización de esta Tesis Doctoral, se utilizó uva tinta (*Vitis vinífera* L.) de las variedades *Garnacha* y *Caladoc*, cultivadas en viñedos ubicados en la Denominación de Origen Campo de Borja pertenecientes a la cooperativa "Bodegas Aragonesas" (Fuendejalón, España) durante las añadas 2016 al 2018. Las vides se cultivan en viñedos en espaldera y sin riego (secano).

2. Tratamiento PEAV

Para la realización de los estudios de esta Tesis Doctoral, se utilizaron distintos montajes que se describirán específicamente más adelante para cada investigación. En todos los casos, fue requerido el uso de un generador de PEAV utilizándose 2 generadores distintos dependiendo de la intensidad del campo eléctrico que se fuera aplicar en las pruebas realizadas.

3. Generador PEAV

Un generador de PEAV está formado principalmente por un "Generador" de corriente eléctrica continua, condensadores, interruptor y, en algunos casos, un transformador del voltaje del pulso. El generador de corriente eléctrica continua transforma la corriente alterna (CA) de la red eléctrica en corriente continua (CC). Los condensadores almacenan la energía eléctrica para luego descargarla en forma de pulso discontinuo y controlado en la cámara de tratamiento. El interruptor o *switch* regula de forma muy precisa la duración el pulso y el paso de la corriente eléctrica desde el condensador a la cámara de tratamiento. Los generadores de última generación, incluso, no disponen de condensador ya que el transformador puede generar directamente la corriente eléctrica a los voltajes requeridos. El esquema sencillo de un equipo de PEAV se puede muestra en la Figura 8. Este esquema varía dependiendo de la electrónica que emplea cada uno de los generadores utilizados.

Como se ha indicado, en esta Tesis Doctoral, se han empleado 2 generadores PEAV dependiendo de la intensidad del campo eléctrico aplicado: un generador desarrollado por la empresa Scandinova PG y otro por Energy Pulse Systems (EPS).

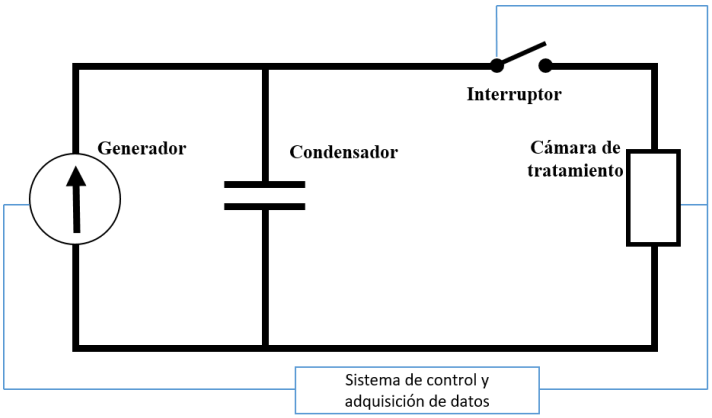


Figura 8: Esquema simplificado de un sistema de generación de pulsos eléctricos de alto voltaje.

Generador ScandiNova (SCA)

El generador ScandiNova PG, ScandiNova, Uppsala, Suecia (Figura 9), al que llamaremos a partir de ahora SCA, consta de un transformador (DCPS D10-400, ScandiNova) que convierte la corriente de alta potencia alterna (380V, 16A) en corriente continua de 1 kV, la cual es trasferida a 6 interruptores IGBT (Switch rack SR6, ScandiNova) conectados en serie. Una señal eléctrica externa (TTL, 5 V), regida por un generador de funciones (Tektronik, AFG 3022, Wilsonville, Oregon, EE.UU.) que controla la apertura y cierre de los módulos IGBT, provocando la descarga de la corriente de 1 kV en una primera señal pulsante de onda cuadrada. Finalmente, un transformador de pulsos (*Pulse Transformer ScandiNova*) convierte esa primera señal pulsante en la señal de alto voltaje deseada.

Este equipo está diseñado para trabajar con cámaras de tratamiento con una resistencia eléctrica óptima de 100 a 170 Ω. En estas condiciones, se consigue un pulso totalmente cuadrado en el cual el voltaje aumenta hasta el valor establecido a una velocidad de 48 $kV.\mu s^{-1}$; una vez terminado el pulso, la velocidad de descenso es de 56 $kV.\mu s^{-1}$. A lo largo de toda la duración del pulso, el voltaje oscila menos de un 2 %. El tiempo de duración del pulso es fijo y su valor es de 3 µs, siendo pulsos cuadrados unipolares positivos. La intensidad del pulso puede llegar a 30 kV y 200 A y una frecuencia máxima de 300 Hz.

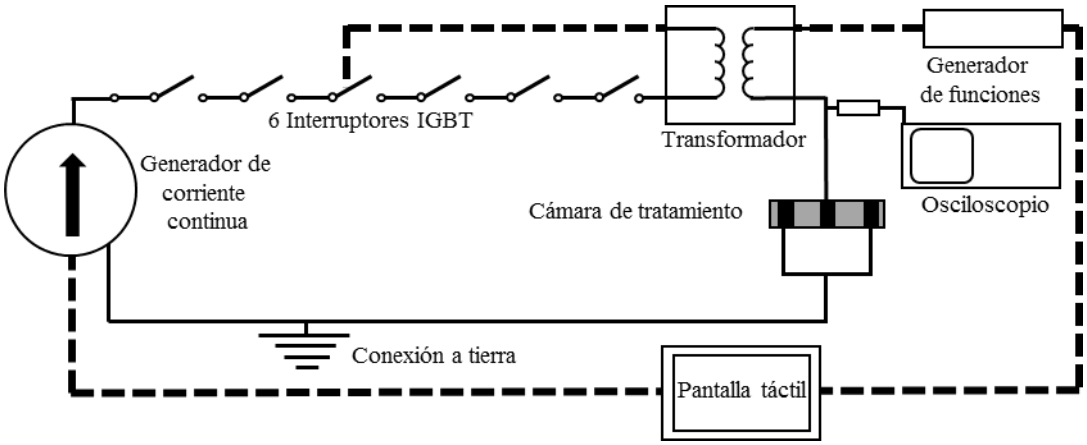


Figura 9. Esquema del circuito eléctrico básico del generador Scandinova.

Durante la aplicación de los tratamientos, parte de la energía eléctrica generada se disipa en forma de calor (hasta 1 kW). Para evitar el sobrecalentamiento del sistema, el generador posee un sistema de refrigeración líquido con aceite dieléctrico (de baja conductividad eléctrica) que a si vez es refrigerado por un primer sistema de refrigeración con agua, cuyo sistema de bombeo se encuentra en el exterior del generador y necesita un caudal mínimo de agua de 9 $L.min^{-1}$ y una presión de 3 a 8 bares. La temperatura del agua debe estar comprendida entre los 10 y los 40ºC. Para que las condiciones de trabajo del equipo sean estables, la temperatura del agua durante la aplicación de los tratamientos debe ser igual a su temperatura inicial ±2,5ºC.

El control del equipo se realiza mediante un software específicamente diseñado por el fabricante (K1-15m, ScandiNova) accesible mediante pantalla táctil (Simantic panels, Siemens, Múnich, Alemania) en donde se selecciona la intensidad del pulso, estando conectado al equipo mediante una clavija tipo RS-232 (RS-Amidata). Además, y debido a que el equipo aplica altos voltajes e intensidades de corriente, dispone de un interruptor o pulsador externo de seguridad de rápido accionamiento (RS Amidata) que permite la desconexión manual del circuito eléctrico en caso de emergencia. Al ser accionado corta el circuito y cierra el sistema impidiendo el paso de corriente a través de él, de modo que la manipulación de sus componentes en la zona de la cámara de tratamiento es segura.

Para determinar y verificar que el voltaje y amperaje realmente aplicados y, conocer si las condiciones efectivas de tratamiento, el sistema se completa con una sonda de alto voltaje de 75 Mhz (P6015A, Tektronix, Wilsonville, Oregón, EEUU) y una sonda de amperaje interna del propio equipo (Stangenes Industries, Palo Alto, California, EE.UU.) conectada al electrodo de alto voltaje de la cámara de tratamiento, cuya lecturas

son registradas en un osciloscopio digital de dos canales (TDS 220, Tektronix, Wilsonville, Oregón, EEUU) como se muestra en la Figura 10 B y C.

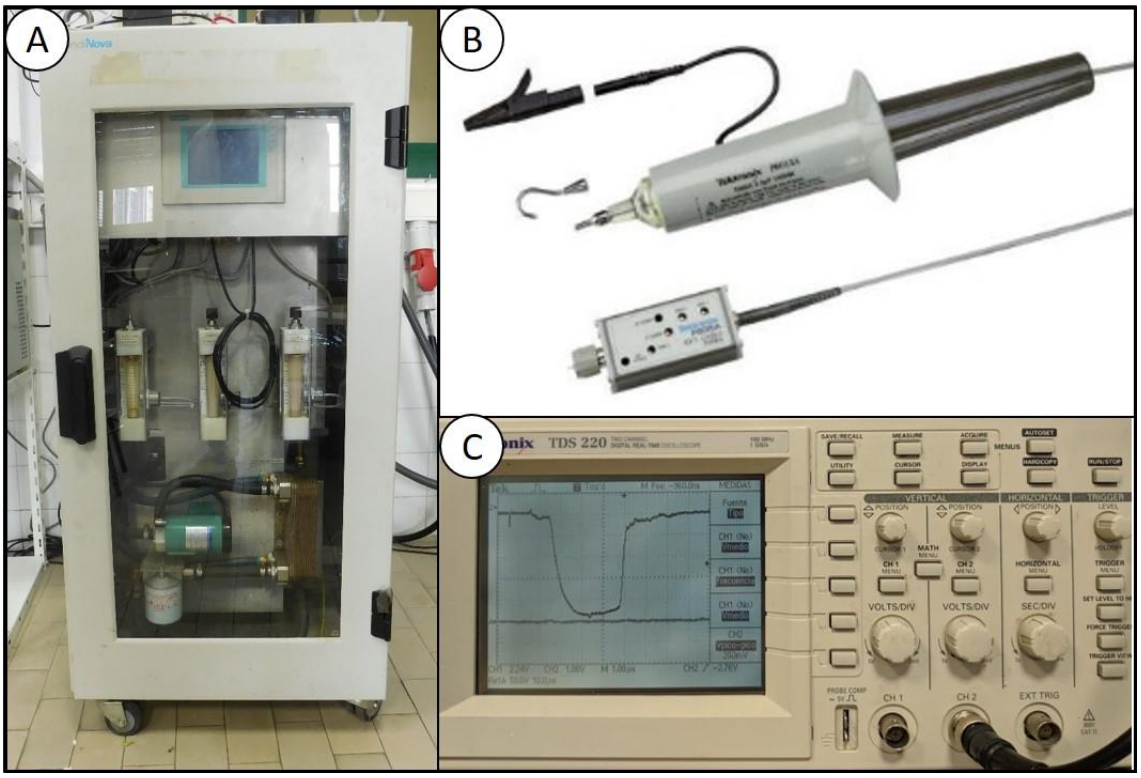


Figura 10: Imagen del generador de pulsos eléctricos de alto voltaje Scandinova (A), Sonda de alto voltaje P6015A (B) y osciloscopio TDS 220 (C).

Modulador Energy Pulse Systems (EPS)

El segundo generador de pulsos eléctricos de alto voltaje utilizado en los distintos experimentos se trata del equipo EPULSUS® PM1-10 (Energy Pulse Systems LDA, Lisboa, Portugal) cuyo esquema eléctrico se muestra en la Figura 11. Es un equipo modular compacto de dimensiones y peso reducidos (60 kg) comparados con SCA (350 kg). Consta 10 semiconductores de 1200 V cada uno, que están disponibles en el mercado de forma estándar lo que hace a este equipo más económico respecto al resto de los distintos generadores. Estos proporcionan en conjunto un voltaje máximo de 10 kV y 240 A.

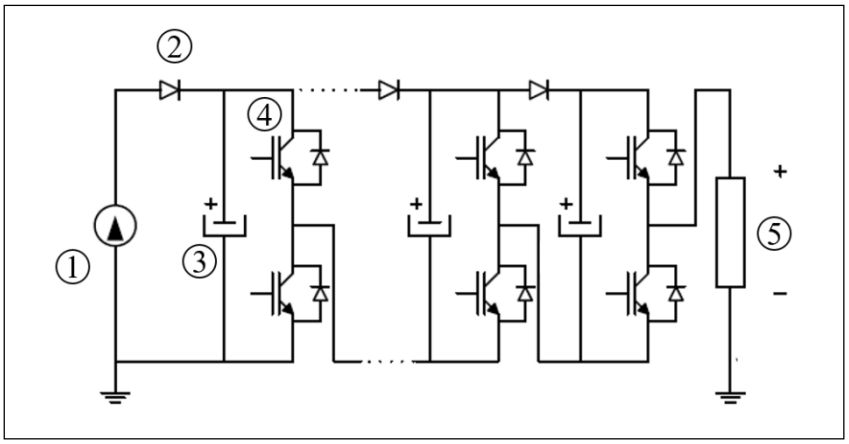


Figura 11: Esquema del circuito básico del generador de tipo Marx en estado sólido. Fuente de alimentación (1), diodos (2), condensadores (3), interruptores (4), cámara de tratamiento (5).

Este tipo de generador posee una ventaja considerable en el momento de realizar la descarga del pulso: la intensidad del pulso no depende de la impedancia de la carga siempre que la energía almacenada en el condensador sea lo suficientemente alta en comparación con la energía suministrada en cada pulso. El resultado es que esta ventaja mejora la estabilidad del pulso cuando se procesan alimentos donde la densidad y la conductividad eléctrica del alimento que pasa por la cámara de tratamiento varían mucho. Es decir, si la conductividad del medio cambia, el generador compensa la intensidad del pulso para mantener el voltaje necesario del mismo, aplicando siempre el campo eléctrico seleccionado (Redondo, Andrade, Santos, Barros, & Pereira, comunicación personal).

El sistema se configura mediante pantalla táctil (Figura 12B) y se puede programar para proporcionar un pulso eléctrico unipolar positivo cuadrado (Figura 12C) y de ancho variable de 2 a 100 µs con una frecuencia máxima de 200 Hz. Proporciona una potencia máxima de salida del pulso de 240 A con una fuente de alimentación ECAPACITAS®3kW5 Energy Pulse Systems LDA, alimentada con 220 V para un consumo máximo de 3,5 kW. También, posee un interruptor de seguridad externo y frontal, de rápido accionamiento conectado al equipo internamente que cierra y boquea automáticamente el funcionamiento de modulador, y poder así, manipular sus componentes de manera segura.

El generador posee refrigeración forzada de aire con 4 ventiladores internos. El voltaje de salida se verifica mediante una sonda de alto voltaje a 75 MHz (P6015A, Tektronix, Wilsonville, Oregón, EEUU) conectada al electrodo de alto voltaje que se encuentra ubicado en la cámara de tratamiento y, cuyas lecturas son registradas en un osciloscopio digital (TBS 1102B-EDU, Tektronix) como se muestra en la Figura 12C.

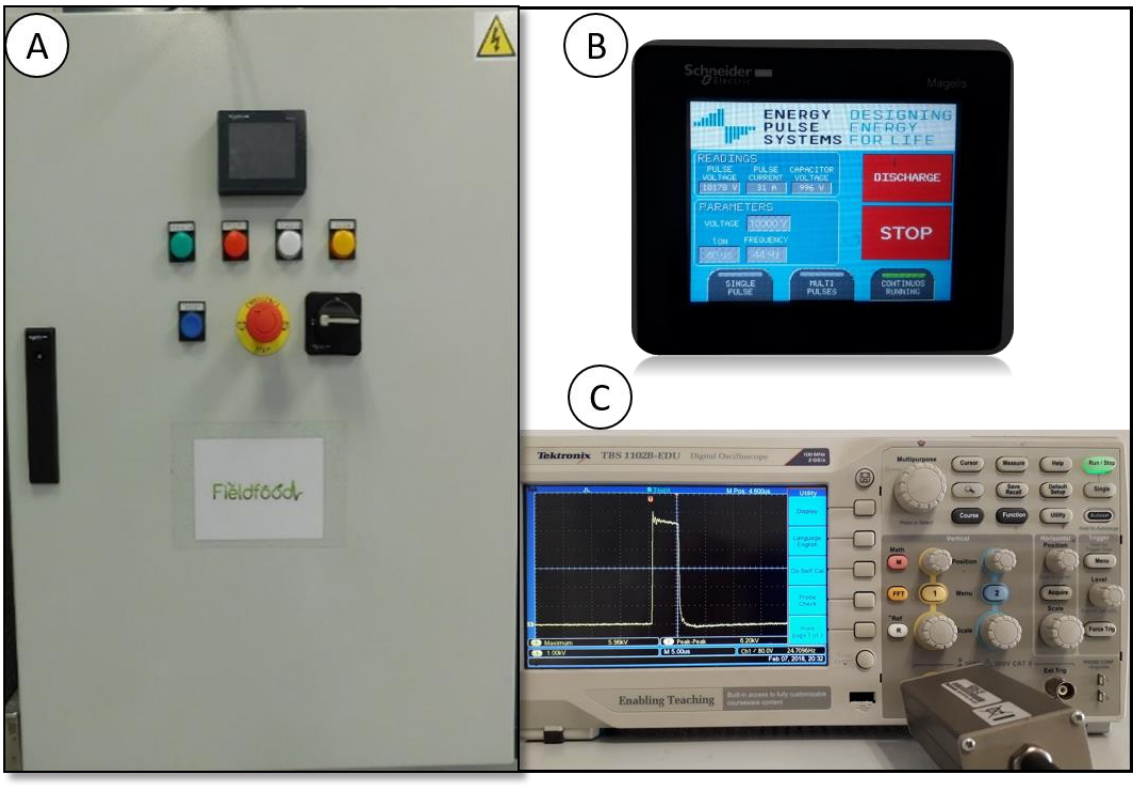


Figura 12: Generador modular de pulsos eléctricos de alto voltaje EPULSUS® PM1-10 (A), pantalla táctil para introducir las especificaciones del pulso (B), osciloscopio TBS 1102B y detalle de un pulso de 5 µs (C).

Parámetros y condiciones de los tratamientos PEF

Intensidad de Campo Eléctrico (E)

El campo eléctrico corresponde a la relación entre la intensidad del voltaje aplicado entre los dos electrodos de la cámara de tratamiento y la distancia que existe entre los mismos.

$$E = V/d$$

Donde E expresa la intensidad de campo eléctrico expresado en $V.m^{-1}$, V es la intensidad de voltaje aplicado en V y d es la distancia entre los dos electrodos expresada en m. De forma general, en la tecnología de los PEAV, el campo eléctrico se suele expresar en $kV.cm^{-1}$.

Es el parámetro más importante y su valor determina la eficacia del proceso de electroporación del alimento por el uso de PEF. Para que la electroporación sea efectiva es necesario superar un determinado umbral del campo eléctrico crítico (Toepfl, 2011). Estudios confirman que para células eucariotas y procariotas existe tanto un nivel mínimo de campo eléctrico para que la electroporación se produzca como también existe un valor máximo en el cual ya no se producen mejoras sustanciales del tratamiento (Álvarez et al.,

2003; Barsotti et al., 1999; Barsotti and Cheftel, 1999). Por otro lado, la efectividad del campo eléctrico estará garantizada por su distribución dentro de la cámara de tratamiento. Este es un aspecto fundamental en cámaras en las que los electrodos no son paralelos como la utilizada en esta Tesis Doctoral, de tipo colineal que se describirá más adelante. En este tipo de cámaras, de un punto determinado de la zona de tratamiento el valor del campo eléctrico es función de su posición dentro dela misma. Además, dada la imposibilidad de realizar una medida real del campo eléctrico aplicado, todo cálculo del mismo en esta clase de cámaras se basa en métodos matemáticos como la simulación numérica. Por todo ello, es complicado definir el tratamiento de PEAV aplicado cuando se utilizan diseños colineales. En esta Tesis Doctoral, de acuerdo a las directrices marcadas por (Puértolas et al., 2009b), se decidió utilizar el campo eléctrico estimado en el punto central de la zona de tratamiento como el valor de campo eléctrico a utilizar para definir los tratamientos calculándose en base a la ecuación anteriormente señalada para facilitar los cálculos.

Forma, ancho y tiempo del pulso.

Todos los equipos generadores de PEAV poseen características específicas que dependen, en general, de la empresa que los construye y de los distintos componentes que incluye. Los dos equipos utilizados en nuestras investigaciones poseen características similares, pero no iguales que influyen al momento de su utilización como se comentará en cada una de las investigaciones realizadas. La forma, ancho y tiempo del pulso son factores de suma importancia para un determinado tratamiento.

Los dos equipos aplican pulsos de forma cuadrada y unipolar como se muestra en la Figura 13. Estos pulsos se caracterizan, porque, tras el rápido incremento de voltaje, éste se mantiene constante para luego, descender rápidamente. Por lo tanto, en este caso la descarga de los condensadores está completamente regulada con el objetivo de mantener el voltaje seleccionado constante a lo largo de toda la duración del pulso.

El ancho del pulso puede variar considerablemente dependiendo el equipo usado. El equipo EPS posee la ventaja respecto a SCA de poder variar el ancho del pulso de 2 a 100 µS, además de que su voltaje no está tan condicionado por la variación de la conductividad eléctrica del producto tratado como se ha comentado.

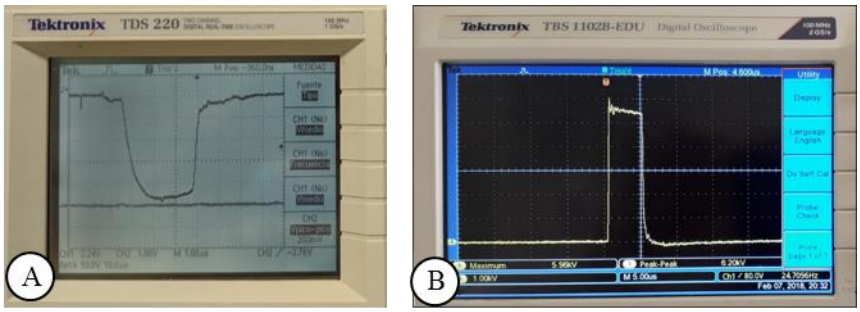


Figura 13: Pulso de onda cuadrada observada en osciloscopio aplicada con el equipo SCA (A) (5kV, 3µs, onda invertida) y EPS (B) (6kV, 5µs y 25Hz).

Resistencia eléctrica de la cámara de tratamiento (R)

Esta resistencia eléctrica depende de las dimensiones geométricas de cámara de tratamiento (superficie de los electrodos y separación entre ellos) y de la conductividad eléctrica del medio a tratar.

$$R = \frac{\rho * d}{A}$$

Donde la resistencia *R*, expresada en ohmios **Ω** es directamente proporcional a la resistividad del medio de tratamiento ρ expresada en **Ω** por metro (Ωm) y a la distancia entre los electrodos d expresada en metros e inversamente proporcional a su área A en m^2. Como se ha indicado, en el equipo SCA la resistencia debe estar comprendida entre 100 y 170 **Ω** con el fin de que la forma del pulso sea lo más cuadrada posible. Fuera de este rango, el pulso deja de ser cuadrado. En el caso del equipo EPS, esta circunstancia no se produce obteniéndose pulsos cuadrados.

Energía específica del pulso (Q o W)

Es la energía eléctrica necesaria para mantener la diferencia de potencial entre los electrodos de la cámara de tratamiento cuando la corriente eléctrica pasa a través del alimento. De forma simplificada, la energía eléctrica se puede calcular teóricamente a partir del voltaje, la anchura del pulso y la resistencia de la cámara de tratamiento aplicando la siguiente ecuación.

$$W = \frac{(V^2 * \tau)}{R}$$

Donde V es la diferencia de potencial entre los electrodos de la cámara de tratamiento (expresada en Voltios); τ es la anchura del pulso (en segundos) y R es la resistencia que ofrece la cámara de tratamiento al paso de la corriente (Ω).

También la energía del pulso se puede calcular a partir de la siguiente ecuación:

$$W = \int_0^{\infty} V(t) * I(t) * dt$$

Donde V es el voltaje (Voltios) e I es la intensidad de la corriente eléctrica (Amperios).

La energía eléctrica total aplicada durante un tratamiento de PEAV es igual a:

$$W(total) = W * n$$

Donde W es la energía del pulso, y n es el número de pulsos.

La energía por unidad de masa o energía específica (W´), se calcula a partir de la energía total aplicada y de la masa de producto procesado (m), expresándose generalmente en $kJ.kg^{-1}$: La energía específica es la energía aplicada en cada pulso por unidad de masa.

$$W' = \frac{W(total)}{m}$$

Donde m es la masa del producto tratado (expresado en kg).

En resumen, y como un factor de importancia, esta energía específica depende del voltaje, de la intensidad de la corriente, del número de pulsos como también de su anchura, de la conductividad y la masa del alimento. Toda esta información aportada por este parámetro hace que el mismo, junto con la intensidad del campo eléctrico y el tiempo de tratamiento sean los parámetros a indicar para caracterizar un tratamiento PEAV.

Frecuencia (F)

Es el número de pulsos aplicados por una unidad de tiempo. La frecuencia utilizada por los equipos de generación de PEAV puede ser variada, pudiendo oscilar entre 1 y 5.000 Hz, aunque en los equipos que utilizados en esta Tesis Doctoral la frecuencia máxima seleccionable es de 200 Hz. En tratamientos en flujo continuo, la frecuencia de los pulsos dependerá del tiempo de permanencia del producto en la cámara de tratamiento y de la cantidad de pulsos que se quiera aplicar, es decir, la frecuencia dependerá del caudal del producto.

4. Cámara de tratamiento

La cámara de tratamiento es el lugar físico en donde se aplican los pulsos eléctricos de alto voltaje. Su construcción, forma y diseño, está condicionado por numerosos aspectos incluyendo el tipo de producto a tratar, o si el tratamiento es estático o en flujo continuo, etc., todo ello considerando que se intenta conseguir la máxima eficiencia del pulso en la cámara (van den Bosch, 2007). Existen estudios en los que se evalúa la eficiencia de cámaras para un determinado producto, sobre todo en aquellas de flujo continuo donde se hace necesario la utilización de herramientas de simulación numérica para optimizar los diseños de dichas cámaras para conseguir la máxima uniformidad del campo eléctrico (Huang et al., 2013).

En estudios previos a esta Tesis Doctoral, se utilizaron cámaras de tratamiento estáticas de electrodos paralelos para evaluar el efecto de los PEAV en la extracción de compuestos fenólicos de la uva (López et al., 2008b).Sin embargo, este tipo de cámaras solo puede usarse a escala de laboratorio y su aplicación industrial es limitada. Posteriores estudios evaluaron el comportamiento de cámaras colineales en flujo continuo (E. Puértolas et al., 2010b) cuyos diseños se basaron en los trabajos de Toepfl et al. (Toepfl et al., 2007). La cámara utilizada en los experimentos de Puértolas et al. (2009b) tenía una distribución simétrica entre el diámetro de la cámara y la distancia entre los electrodos (2 cm x 2 cm).

Debido a que todos los experimentos realizados en esta Tesis Doctoral tenían el objetivo de evaluar el comportamiento de los PEAV en escala semi-industrial, se utilizó una cámara de flujo continuo de tipo colineal.

Cámara colineal

La cámara colineal que se usó en todos los tratamientos es una cámara de sección circular. El líquido o mosto circula por el centro de la misma y posee 2 zonas de tratamiento delimitadas entre los 3 electrodos cilíndricos de acero inoxidable configurados como muestran las Figura 14, Figura 15 y Figura 16. El electrodo de alto voltaje (Figura 14, nº 1) se encuentra en el centro de la cámara y separado por un material aislante (Figura 14, nº 2) de polioximetileno (poliacetal o Delrin®) de los dos electrodos de tierra (Figura 14, nº 3).

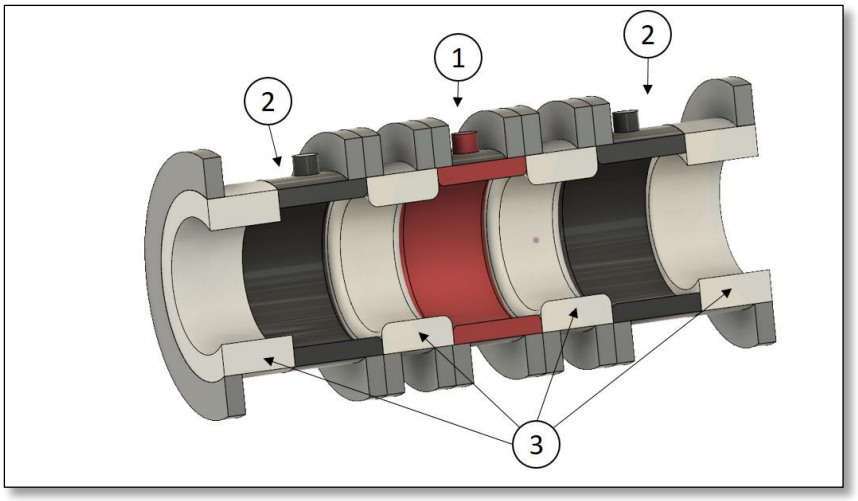


Figura 14: Esquema de una cámara de tratamiento PEAV colineal. Electrodo de alto voltaje (1), electrodos de tierra (2) y material aislante (3).

Tanto el diámetro de la cámara como también la distancia entre los dos electrodos son de suma importancia para que la distribución del campo eléctrico sea lo más homogénea posible en toda la zona de tratamiento (Toepfl et al., 2007). De forma general y como una norma sencilla, la distribución del campo eléctrica más uniforme se consigue cuando el diámetro y la separación entre los electrodos (*GAP*) tienen la misma longitud (Toepfl et al., 2007).

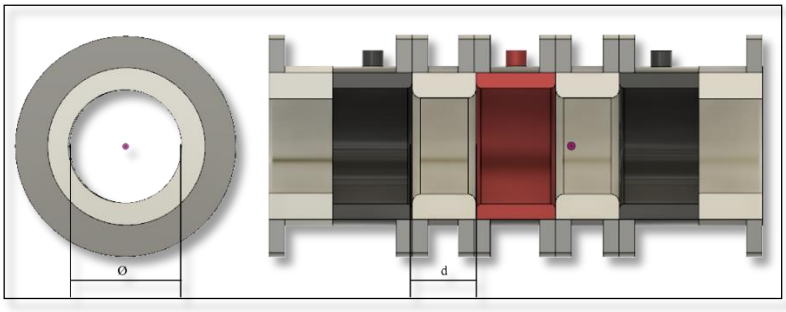


Figura 15: Esquema de cámara de tratamiento de PEAV colineal: vista frontal y lateral. Diámetro de la cámara (Ø) y distancia entre los electrodos (d).

Sin embargo, y con el objetivo de maximizar la capacidad de procesado y trabajar en las instalaciones de una bodega, las dimensiones de la cámara utilizada no siguieron esta norma general. Así, en una de las cámaras utilizadas el diámetro interno del electrodo fue de 3,5 cm y la distancia de los electrodos era de 2,5 cm con el objetivo de aumentar la intensidad del campo eléctrico. Esta configuración permitió la utilización de equipos generadores como el EPS cuya potencia de voltaje máxima es limitada para alcanzar campos eléctricos más elevados si se hubiera mantenido la relación diámetro/*GAP*.

En el caso de los tratamientos de termovinificación, debido a que fue requerida la aplicación de tratamientos de mayor energía que los utilizados en las aplicaciones semi-

industriales, se utilizó una cámara colineal de 2 cm de diámetro y 2 cm de distancia entre los electrodos

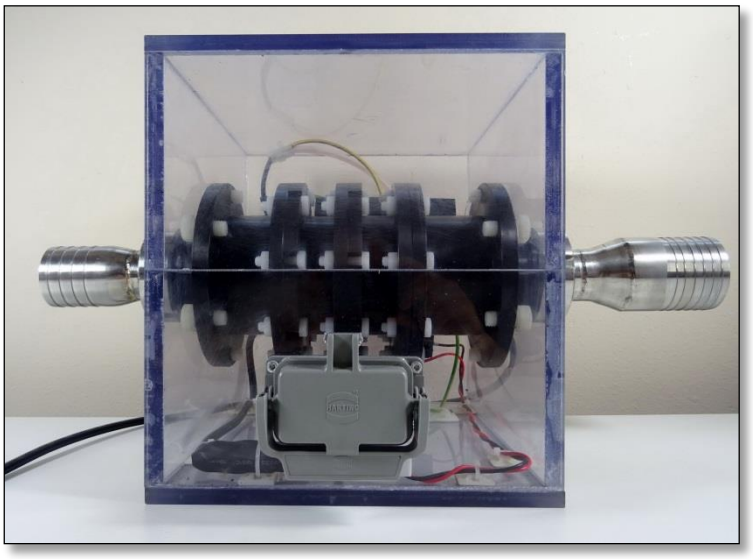


Figura 16: Imagen de una de las cámaras colineales utilizada en esta Tesis Doctoral para el tratamiento en flujo continuo a escala semi-industrial con 3,5 cm de diámetro y 2,5 cm de separación entre los electrodos.

5. Caracterización general de los mostos y vinos

Con el fin de caracterizar los mostos y el vino obtenido en los distintos momentos de su elaboración, se evaluaron distintos parámetros que se describen a continuación.

Grado de alcohol probable

El grado de alcohol probable del vino se calculó a partir de la determinación de los ºBrix del mosto. Los ºBrix indican los gramos de sólidos solubles en 100 mL de solución. Se determinaron mediante refractometría (Digital refractometer PR-101 Palette, Atago Ltd., Japón). Para ello, se depositó 1 mL de mosto sobre el detector de refractómetro posterior a su calibración con agua destilada. A partir de los ºBrix, se puede calcular el grado alcohólico probable del vino en base a la siguiente ecuación:

$$ºA = (0{,}6757 * ºB) - 2{,}0839$$

Donde $ºA$ es el grado alcohólico probable y $ºB$ son los ºBrix.

pH

A temperatura ambiente el pH del vino se encuentra entre 2,9 a 3,9. Valores inferiores dan lugar a vinos ácidos y superiores a vinos "apagados". Además, el pH afecta a la estabilidad tanto microbiológica como físico-química del vino.

El pH del mosto del vino se determinó por medición directa utilizando un pH-metro (Crisol Instrumentes, S.A. Barcelona España) siguiendo el método oficial de la Organización International de la Viña y del Vino (OIV) (2009).

Densidad

La densidad de los mostos durante la fermentación se realizó utilizando un densímetro calibrado a 20ºC (Veraxa), siguiendo la metodología propuesta por la OIV (2009).

Acidez total

La acidez es un parámetro crítico en el vino ya que modifica el sabor (a mayor acidez, el vino tiene un sabor más fresco), el color (a mayor acidez, color más intenso) y la estabilidad microbiológica (a mayor acidez, mayor dificultad para el desarrollo de las bacterias). De forma general, todos los vinos tienen un carácter ácido. La acidez total, o también denominada acidez titulable, es la suma de los ácidos volátiles y fijos, valorables por alcalimetría - acidimetría hasta pH 7,0. Los ácidos fijos que más influyen son el ácido tartárico, el ácido málico y el ácido cítrico. Mientras que el ácido acético, el ácido láctico y el ácido succínico corresponden a la serie de ácidos volátiles.

La acidez se determinó por valoración volumétrica con hidróxido de sodio 0,1 N en presencia de azul de bromo timol o potenciométricamente según el método oficial según la OIV (2009). Los resultados se expresaron en $g.L^{-1}$ de ácido tartárico. Los gramos de ácido tartárico por litro se calculan a partir de la siguiente ecuación:

$$Acidez\ total\ (\mathrm{g}.L^{-1}) = \frac{(V * N * pm)}{V´v}$$

donde $g.L^{-1}$son los gramos de ácido tartárico por litro de mosto o vino, Ves el volumnen en mL de hidróxido de sodio 0,1N; N es la normalidad del hidróxido de sodio; pm es el peso molecular del ácido tartárico (150); $V´$es el volumen de muestra utilizado (10 mL) y v es la valencia del ácido tartárico (2).

Acidez volátil

Para la determinación de la acidez volátil se utilizó el método de García-Tena et al. (García Barceló et al., 1976). Para ello, los ácidos volátiles se separan mediante arrastre con vapor de agua y posteriormente se condensan. Los ácidos condensados se valoran con hidróxido de sodio en presencia de fenolftaleína como indicador. Para la determinación de la acidez volátil, se introdujeron 11 mL de vino en un matraz esférico. El matraz se conecta al sistema de destilación y se calienta con un mechero de alcohol. Los vapores condensados se recogen en una probeta y se desecha los primeros 5,1 mL. A continuación, se coloca otra probeta donde se recogen los siguientes 3,2 mL. El destilado recogido se vierte en un matraz erlermeyer y se valora con hidróxido de sodio 0,02 M en presencia de fenolftaleína. La acidez volátil, expresada en $g.L^{-1}$ de ácido acético, se determina en base a la siguiente ecuación:

$$Acidéz\ volátil\text{: (g.}L^{-1}\text{)}de\ acético\ =\ V_{NaOH}\ x\ 0{,}366$$

donde V son los mL de NaOH 0,02 M utilizados en la valoración. El valor 0,366 es un factor de corrección que tiene en cuenta las interferencias en la medida causadas por el SO_2 y el ácido láctico presente en el vino.

Nitrógeno fácilmente asimilable (FAN)

El mosto contiene diferentes formas de nitrógeno, como aminoácidos, péptidos y proteínas (Gobert et al., 2019; Sablayrolles et al., 1996). Se considera que las concentraciones mínimas para conseguir una fermentación adecuada deben ser de alrededor de 150 $mg.L^{-1}$ (Paladino et al., 2004).

El procedimiento de análisis utilizado para la determinación del nitrógeno fácilmente asimilable fue propuesto por Sanhueza (1999). Para ello, se añadieron 50 mL de mosto en un vaso de precipitados ajustándose el pH a 8,5 con hidróxido de sodio 1 N. Posteriormente, en otro vaso de precipitados se añaden 20 mL de formaldehido al 40 % ajustándose a 8,5 el pH con NaOH 0,1 N. Ambas soluciones ajustadas a pH 8,5 se mezclan observándose una disminución del pH. Finalmente, se valora la mezcla con hidróxido de sodio 0,1 N hasta alcanzar nuevamente un pH de 8,5. La concentración de nitrógeno utilizable por las levaduras se calculó a partir de la siguiente ecuación:

$$Nitrógeno\ mg.L^{-1} = N * 28$$

donde N son los mL de hidróxido de sodio 0,1 N gastados en la valoración.

Extractabilidad de compuestos fenólicos

El método utilizado para determinar la extractabilidad de los antocianos y polifenoles fue el propuesto por Glories (1984). El método consiste en comparar la extracción de los polifenoles de la uva en unas condiciones que simulan el proceso de maceración durante la elaboración del vino con unas en las que se provoca la completa desintegración en las envolturas de las células de los hollejos. La diferencia entre la extracción obtenida en ambas condiciones refleja la mayor o menor fragilidad de la membrana celular.

Se trituraron con Ultraturrax (Ikalabertechink, Staufen Br, Alemania) 200 gramos de uva que posteriormente se dividieron en dos porciones de 50 gramos cada una. A una de ellas se le añadieron 50 mL de HCl 0,1 N hasta alcanzar un pH de 1 y, a la otra, una solución tampón de pH 3,2. Dicha solución se obtuvo disolviendo 5 gramos de ácido tartárico en 10 mL de NaOH 2 N en un matraz aforado de 1 litro.

Después de la maceración de 4 horas, el pH de la muestra a la que se añadió el ácido clorhídrico se incrementó hasta 3,2 adicionando NaOH. A continuación, se centrifugaron las dos muestras a 6000 g durante 10 minutos y se determinó en el sobrenadante el contenido de antocianos, y de polifenoles totales mediante la técnica que se describe en el siguiente apartado. Los valores obtenidos de esos tres índices de la muestra macerada en presencia de ácido clorhídrico se denominan contenido en antocianos, polifenoles e intensidad de color potencial.

La extractabilidad de los antocianos o índice de madurez celular (EA o IMC) y la extractabilidad de los polifenoles (EP) se calcularon a partir de la siguiente ecuación:

$$\% = \frac{(A1 - A2)}{A1} * 100$$

donde $A1$ es la absorbancia de la determinación de antocianos del macerado a pH 1 y *A2* es la absorbancia del macerado a pH 3,2 en el caso de la extractabilidad de Antocianos (EA) medidos a 520 nm. Para la determinación de la extractabilidad de los polifenoles (EP), *A1* es la absorbancia de la determinación del índice de polifenoles del macerado a pH 1 y *A2* es la absorbancia del macerado a pH 3,2 medidos a 280 nm. En ambos casos a las absorbancias obtenidas se les aplicó el factor de corrección debido a la dilución realizada. Ambos parámetros se expresaron en tanto por litro.

Grado alcohólico

El *volumen real* de un líquido alcohólico es el volumen que ocupa dicho líquido a la temperatura de 15ºC. Si la temperatura difiere de la indicada, el volumen que ocupa el líquido se llama *aparente*. EL grado alcohólico de un líquido es el porcentaje de alcohol absoluto (etanol y sus homólogos) contenido en un volumen real. Su valor se determinó mediante la destilación del vino alcalinizado y la posterior medida de la densidad del destilado por aerometría (OIV, 2009). A 200 cm^3 de vino se le añaden 10 mL de una solución de hidróxido cálcico a una concentración de 2 moles.L^{-1} (Vinikit). De la mezcla se destilan aproximadamente las ¾ partes de su volumen, el cual se completa con agua destilada hasta alcanzar los 200 mL iniciales. Finalmente, la mezcla se homogeniza y se mide su grado alcohólico mediante un alcoholímetro (Verexa).

Azúcares reductores

El método utilizado fue propuesto por Cobos et al. (2017) y para ello en un una probeta de 100 mL se realiza la decoloración del vino tinto hasta obtenerlo totalmente cristalino. Para ello, se mezclan 90 mL de vino con 1,0 g de carbón activo y 10 mL de agua destilada que posteriormente se dejan reposar durante 10 minutos. Tras este tiempo, el vino decolorado se filtra. El filtrado se usa para realizar la titulación durante la ebullición en 15 mL de Licor de Fehling–Causse–Bonnans. Para ello, se coloca el vino decolorado en la bureta y se procede a la titulación mientras el Licor de Fehling–Causse–Bonnans se encuentra en ebullición. Se utiliza azul de metileno como indicador hasta la desaparición del color azul en toda la masa del líquido.

$$Azúcares\ reductores: (\mathrm{g}.L^{-1}) = \frac{45{,}1}{V}$$

Donde V es el volumen de vino decolorado expresado en ml.

6. Determinaciones espectrofotométricas en mostos y vinos

En los mostos y vinos, se analizaron los diversos índices espectrofotométricos que se detallan a continuación. Previamente a su análisis, las muestras fueron centrifugadas (6000g.10 min^{-1}) para eliminar los sólidos en suspensión y el CO_2 disuelto que pudiera estar presente. Las medidas se realizaron en un espectrofotómetro Biochron Libra S12 (Unicam, Cambridge, Reino Unido), utilizando cubetas de cuarzo (Hellma, Müllheim, Alemania) o de plástico (Sarstedt, Nümbrecht, Alemania) de 1 y 10 mm de paso óptico.

Características cromáticas

Determinación del índice de color (CI)

El color del vino se debe a los pigmentos que contiene, pero no hay una proporcionalidad directa entre la cantidad de pigmentos y el color. También, intervienen otros factores físico-químicos, como el pH, el potencial óxido-reducción, el SO_2 libre y la presencia de co-pigmentos, entre otros.

Los vinos tintos jóvenes o vinos de consumo diario presentan un máximo de absorción a 520 nm responsables del color rojo definido, debido a los antocianos de la uva. Cuando el vino envejece, el máximo de 520 nm tiende a desaparecer. Esto se corresponde con un aumento de color amarillo (absorbancia a 420 nm) en relación con el rojo (absorbancia a 520 nm) que explica la evolución del color rojo definido, hacia un tinte rojo anaranjado (Glories, 1984). En los vinos blancos, la medida del color puede dar indicios sobre el estado de oxidación, un vino más oxidado presenta una lectura mayor a 420nm.

Las medidas en las que se basa la intensidad y matiz o tinte son las originadas por Sudraud (Sudraud, 1958), quien definió estos conceptos del color del vino de la siguiente forma:

Intensidad **(I)** = Absorbancia a 420 nm + Absorbancia a 520 nm.

Matiz o Tinte **(T)** = Absorbancia a 420 nm / Absorbancia a 520 nm.

Gloríes (1984) considera en sus índices la lectura a 620 nm, para evaluar los tonos azules utilizando para ello la siguiente expresión con la que se defina el índice de color **(CI)**:

$$\boldsymbol{CI} = A420\,nm + A520\,nm + A620\,nm$$

Estas medidas se refieren a cubetas de 1 cm, utilizando agua destilada como blanco.

Tono y porcentajes de componentes amarilla, roja y azul

A partir de las 3 absorbancias obtenidas para la determinación de la intensidad de color, se calculó el tono, el porcentaje de componente amarilla, roja y azul en base a las siguientes ecuaciones:

$$T = \frac{A_{520}}{A_{420}}$$

$$\%Amarillo = \frac{A_{420}}{CI}$$

$$\%Rojo = \frac{A_{520}}{CI}$$

$$\%Azul = \frac{A_{620}}{CI}$$

Donde T es el tono, *A* la absorbancia a 420, 520 y 620 nm según el subíndice. CI es la intensidad de color

Parámetros CIELAB

El color de los vinos no solo informa de posibles defectos o evolución sino que también marca un factor importante en el momento de su aceptación por parte del consumidor.

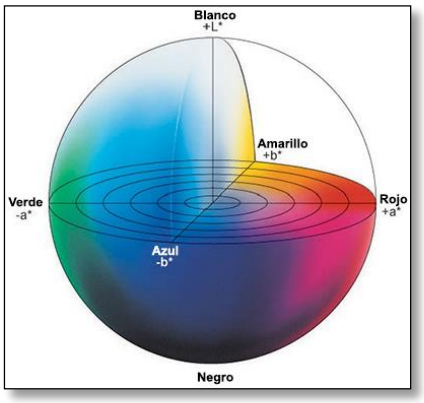


Figura 17: Gráfica espectral con las coordenadas L*, a* y b.*

Los parámetros CIELAB se calculan a partir de las absorbancias comprendidas entre 380 y 780 nm haciendo referencia a un observador de 10 grados de campo visual y un iluminante D65, que permite el cálculo de los valores triestímulo (X, Y, Z) y que define cada color a partir de unas coordenadas denominadas L* (luminosidad), a* y b* (Negueruela et al., 1995). Los parámetros C* (croma métrico o saturación) y H* (tonalidad o tono) se calculan a partir de a* y b*, y junto con L* definen las coordenadas de un espacio cilíndrico que contiene los tres atributos básicos del color (luminosidad, saturación y tonalidad) (Figura 17). Desde el año 2001 y a partir del trabajo conjunto entre la Universidad de Zaragoza y la de La Rioja se desarrolló un software informático para el cálculo de los parámetros del espacio CIELAB llamado “MSCV” (Ayala et al., 2001, 1997). El software utiliza para el cálculo de los parámetros del CIELAB las medidas

espectrofotométricas de la absorbancia del mosto o del vino realizadas con los valores de 450, 520, 570 y 630 con cubetas de 0,1 cm de paso óptico y realizando el blanco con agua destilada.

Compuestos fenólicos

Determinación de antocianos totales (TAC)

El contenido de antocianos totales se determinó por el método de Puissant-León descrito por Ruíz-Hernández (2004). Para ello, se mezcla el vino o el mosto diluido en agua destilada (1/10) en una proporción de 1/10 con ácido clorhídrico al 1 %. A continuación, se mide la absorbancia a 520 nm de dicha mezcla introducida en una cubeta de 1 cm de paso óptico, tras realizar el blanco con ácido clorhídrico al 1 %. A partir de la absorbancia de la muestra, se calcula el contenido en antocianos utilizando la siguiente ecuación:

$$\text{TAC } (mg.L^{-1}) = A_{520} * 22{,}76 * f$$

donde $mg.L^{-1}$ son los miligramos de antocianos expresados como malvidina-3-glucósido por litro de solución; A_{520} es la absorbancia obtenida a 520 nm; 22,76 es el factor de corrección aplicado para obtener los antocianos como malvidina-3-glucósido en $mg.L^{-1}$; y *f* el factor de dilución (100).

Determinación del índice de polifenoles totales (TPI)

Existen deferentes métodos para medir el índice de polifenoles totales. Sin duda el más utilizado debido a su fácil medida es el índice que se obtiene por la medida de la absorbancia del mosto o del vino a 280 nm, debido a que el núcleo bencénico característico de los compuestos polifenólicos tiene su máximo de absorbancia a esta longitud de onda. La medida se hace del mosto o vino diluido 100 veces con agua destilada, utilizando una cubeta de cuarzo de 1 cm de paso óptico (Ribéreau-Gayon et al., 2006). El valor TPI se obtiene multiplicando la absorbancia obtenida (A_{280}) por el factor de dilución:

$$TPI = A(280)\, x\, 100$$

Determinación de Taninos condensados totales (TC)

En este trabajo, se usó el método propuesto por Sarneckes et al. (2006) que consiste en la determinación de los taninos condensados por medio de la precipitación con metilcelulosa. En forma simultánea a 2 tubos de ensayo de10 ml (A y B) se agrega la siguiente preparación: En el tubo A, se colocan 0,25 mL de vino, 2 mL de solución saturada de $SO_4(NH_4)_2$, y 7,75 mL de agua destilada y al tubo B, se agrega 0,25 mL de vino, 3 mL de metilcelulosa al 0,04 % (Sigma M-0387, 1500 cP viscosity at 2 %), 2 mL de solución saturada de $SO_4(NH_4)_2$ y 4,75 mL de agua destilada. Se dejan en reposo 10 minutos antes de centrifugarlos 5 minutos a 10.000g. Luego se toma el sobrenadante y se lee a 280 nm, en cubeta de 1 cm de cuarzo usando agua destilada como blanco.

A280 Tanino= (A280 tubo A) – (A280 tubo B)

Para expresar los resultados como (-)-epicatequina se realizó un curva de calibración (10, 25, 50, 75, 100, 150, y 200 $mg.L^{-1}$ de epicatequina).

7. Determinación de polifenoles individuales por HPLC

El contenido de polifenoles individuales presente del vino se determinó mediante cromatografía líquida de alta resolución (HPLC). El protocolo seguido fue el descripto por (Puértolas et al., 2010b) y se utilizó un HPLC Varian ProStar (Varian Inc., Walnut Creek, CA) para cromatografía líquida de alta resolución con un módulo de bombeo ternario ProStar 240, un muestreador automático de 84 muestras ProStar 410 y un detector con arreglo de diodos ProStar 335. La fase de separación de la muestra se realizó con la columna de fase reversa LC Luna® 100 Å C18 250 x 4.6 mm; 5 µm de tamaño de partícula (Phenomenex) que a su vez tenía instalada una pre-columna del mismo material LC Luna 50 mm x 4,6 mm x 5 µm (Phenomenex) de tamaño de partícula. La temperatura tanto de la columna como de la precolumna se mantuvo a 40ºC durante la realización de los análisis gracias al horno que incorpora el inyecto automático.

Se realizó un gradiente de elución (Tabla 8) consistente en ácido fórmico al 5 % (A) y acetonitrilo (B) a un flujo de 1 $mL.min^{-1}$. Previamente las muestras fueron filtradas utilizando filtros de jeringa estériles de acetato de celulosa de 0,2 µm de tamaño de poro (VWE, West Chester, Pensilvania EE.UU.) El volumen de la inyección fue de 10 µL. Cada muestra se inyectó por duplicado.

Tabla 8: Tiempo de elución de ácido fórmico (A) y acetonitrilo (B).

Tiempo (min)	%A	%B
0	98	2
25	94	6
40	85	15
52	80	20
70	60	40
80	0	100
83	98	2
103	98	2

Se registraron 4 canales o longitudes de onda (280, 320, 360 y 520 nm). Estas longitudes de onda sirvieron para identificar los distintos polifenoles: A 280 nm se registraron los flavan-3-ols; a 320 nm los ácidos hidroxycinnamicos y sus derivados; a 360 nm los flavonoles; y a 520 nm los antocianos. Los distintos compuestos fenólicos fueron individualizados por medio de patrones puros y también fueron identificados según la bibliografía y los tiempos de retención presentes en la literatura (Cantos et al., 2002; Hermosín-Gutiérrez et al., 2005; Monagas et al., 2005; Puértolas et al., 2011).

La cuantificación de los compuestos de los cuales se disponía estándar se llevó a cabo mediante las respectivas curvas de calibración. Estas se obtuvieron utilizando las concentraciones habituales presentes en el vino. En el caso de los compuestos de los cuales no se disponía estándar , la cuantificación se realizó mediante las curcas de calibración del estándar más similar: cloruro de malvidina (Sigma-Aldrich) para los antocianos monómero, la quercitina-3-glucósido para las miricetina-3-glucósido, el ácido cafeico para el ácido *t*-caftárico, y el ácido *p*-cumárico para el ácido *t*-cutárico. La concentración de los distintos compuestos fue expresada en $mg.L^{-1}$.

8. Determinación de aromas individuales

El análisis cuantitativo de los compuestos mayoritarios se llevó a cabo utilizando el método propuesto y validado por Ortega et al. (2001). Para el desarrollar el método, primero se mezclaron 3 mL de vino con 7 mL de agua. Posteriormente, se le agregaron 4,5 g de sulfato de amonio y 200 µL de diclorometano. A continuación, el extracto fue analizado por cromatografía gaseosa (GC) con detección de FID usando las condiciones descritas en (Ortega et al., 2001). Los datos cuantitativos se obtuvieron mediante la interpolación de las áreas relativas en los gráficos de calibración construidos previamente por el análisis de vinos sintéticos que contienen cantidades conocidas de los analitos. Se utilizaron 2-butanol, 4-metil-2-pentanol, 4-hidroxi-4-metil-2-pentanona y 2-octanol, a una concentración de 200 $\mu g.g^{-1}$ en diclorometano, como estándares internos.

El análisis de los compuestos minoritarios se llevó a cabo utilizando el método propuesto y validado por López et al. (López et al., 2002) con los siguientes cambios en el procedimiento anterior: los cartuchos SPE estándar (1 mL de volumen total) llenos con 200 mg de resina LiChrolut EN se colocaron en el sistema de extracción de vacío (Varian) y el solvente se acondicionó enjuagando los cartuchos con 4 mL de diclorometano, 4 mL de metanol y, finalmente, con 4 mL de una mezcla de agua y etanol (12 %, $v.v^{-1}$). Posteriormente, los cartuchos se cargaron con 50 mL de muestra de vino y 26 µL de solución patrón que contenía 3-octanona, *β*-damascona y ácido heptanoico (todos a 200 $\mu g.g^{-1}$ de etanol). Esta mezcla se pasó a través de los cartuchos SPE (2 $mL.min^{-1}$), seguido de una etapa de lavado con 5 mL de una solución de agua al 30 % en metanol y 1 % de $NaHCO_3$. A continuación, se secaron las resinas dejando pasar aire a través de ellas (presión negativa de 0,6 bar, 10 min). Los analitos se recuperaron en un vial de 2 mL por elución con 1,6 mL de diclorometano. Se agregaron 34 µL de una solución estándar interna (300 $mg.L^{-1}$ de 4-hidroxi-4-metil-2-pentanona y 2-octanol) a la muestra eluida. El extracto se analizó por GC con detección de MS de trampa de iones (Cromatógrafo gaseoso GC-450 MS con captura de iones Varian Saturn 2200).

9. Análisis sensorial

Después de 2 años de conservación y añejamiento de los vinos, estos fueron evaluados mediante un análisis sensorial. Este se realizó en las instalaciones del Consejo Regulador D.O. Campo de Borja ubicada en calle Subida San Andrés, 6, Ainzón, Zaragoza (España). La evaluación se realizó por siete enólogos (4 hombres y 3 mujeres de entre 40 a 59 años) pertenecientes al panel oficial de la D.O. Campo de Borja. Los vinos se encontraban a temperatura ambiente (20±ºC) y 20 mL exactos fueron agregados a cada copa con ayuda de un dosificador especialmente preparado para tal fin. Los panelistas se distribuyeron en cabinas individuales, se les informó sobre las muestras a analizar y se les recordó el procedimiento de llenado de la planilla confeccionada para tal fin según las normas preestablecidas.

Inicialmente, los vinos se evaluaron mediante un análisis discriminatorio triangular utilizando un diseño completamente al azar asociado a una prueba de preferencia. El objetivo de esta primera prueba fue determinar si después de envejecer en botella o en barricas de roble más botella, el panel podría distinguir entre los vinos obtenidos de uvas sin tratar y las tratadas con PEAV con distintos días maceración. Después de seleccionar la muestra que se consideró diferente, también se pidió a los

panelistas que indicaran la muestra preferida. El resultado del análisis de preferencias sólo se tuvo en cuenta cuando los panelistas identificaron correctamente la muestra diferente.

Figure 18: Consejo regulador de la D.O. Campo de Borja. Panel sensorial de vino.

También, el mismo panel, llevó a cabo una evaluación sensorial descriptiva de los vinos obtenidos de uvas no tratadas y tratadas con PEAV después de seis meses de envejecimiento en barricas de roble más seis meses de envejecimiento en botellas. El protocolo de evaluación estaba compuesto por seis descriptores sensoriales, cuatro de los cuales podrían verse afectados por el contenido polifenólico de los vinos: astringencia, cuerpo, persistencia e intensidad de color. La magnitud de los descriptores sensoriales se midió en una escala entre 0 (muy baja intensidad) y 9 (muy alta intensidad). Los resultados mostrados corresponden al promedio de las puntuaciones informadas para cada panelista.

10. Análisis estadístico

Cuando se indica en el texto, las curvas de extracción de polifenoles totales obtenidas en las vinificaciones fueron descritas matemáticamente con el fin de determinar el efecto de los parámetros de tratamiento PEAV (intensidad del campo eléctrico, tiempo de tratamiento y energía específica) en la extracción de polifenoles y así determinar las condiciones óptimas de tratamiento. Para ello, se utilizó la siguiente ecuación matemática:

$$Y = \alpha + (\beta * t) * (t < \gamma) + (\beta * \gamma) * (t \geq \gamma)$$

Donde Y es la concentración de polifenoles totales extraídos a distintos tiempos (días), α es el valor inicial estimado de polifenoles totales; β es la pendiente de la regresión lineal

de la primera parte de la curva; y γ es el número de días en el que la curva alcanza la asíntota que corresponde al máximo valor de extracción de polifenoles.

Con el fin de determinar la influencia de los parámetros de tratamiento PEAV con la pendiente de la parte lineal de las curvas de extracción de los polifenoles, se utilizó la metodología de superficie respuesta desarrollándose una ecuación polinomial mediante regresión múltiple que permitiera establecer las relaciones entre los parámetros indicados.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \beta_{ij} X_i X_j$$

Donde Y es la variable de respuesta (pendiente); X_i y X_j son factores independientes (campo eléctrico y energía específica); β_0 es la ordenada en el origen; β_i son los coeficientes de las relaciones lineales; β_{ii} los coeficientes de las relaciones cuadráticas; β_{ij} los coeficientes de las relaciones de interacción; y k el número total de factores independientes. Los términos de la ecuación final obtenida fueron analizados estadísticamente en base a los valores F de cada termino para un nivel de significancia de p<0,05. Para la obtención de los parámetros del modelo, se utilizó el método de regresión por "paso hacia atrás", que consiste en comenzar con el modelo incluyendo todas las variables e interacciones y, a continuación, se van eliminando aquellos factores independientes cuya presencia no mejora la calidad del modelo según el criterio especificado (p<0,05). El modelo de regresión fue realizado con la macro de Excel Essential Regression 2.220 desarrollado por Steppan, Werner y Yeater (Steppan et al., 2013).

Los análisis de la varianza (ANOVA), el análisis de componentes principales y las curvas de regresión lineal y plateau realizados durante los distintos experimentos se llevaron a cabo con el software Infostat en la versión 2011 (Di Rienzo et al., 2011). Los datos presentados en las tablas y las figuras de esta Tesis Doctoral se representan los valores medios ± desviaciones estándar al 95 %. Así mismo, los gráficos realizados durante las fermentaciones o los periodos de conservación y añejamiento fueron realizados con GraphPad PRISM (GraphPad Software, Inc., San Diego, CA) y Excel Package (Microsoft, Redmond, Washington, EE.UU.).

11. Aplicación de PEAV en las distintas elaboraciones de vinos tintos

Como se ha indicado, para las distintas vinificaciones se usaron uvas tintas (*Vitis vinífera* L.) provenientes de la D.O. Campo de Borja elaborándose los vinos en Bodegas

Aragonesas (Fuendejalón) o en las instalaciones de la Planta Piloto de Ciencia y Tecnología de los Alimentos de la Universidad de Zaragoza. Dependiendo de la investigación se utilizó uva *Garnacha* o *Caladoc* vendimiadas en las campañas de 2016 al 2018.

El esquema de la Figura 19 resume los estudios realizados, indicados con números en el esquema, que se describirán a continuación así como los análisis llevados a cabo en cada investigación.

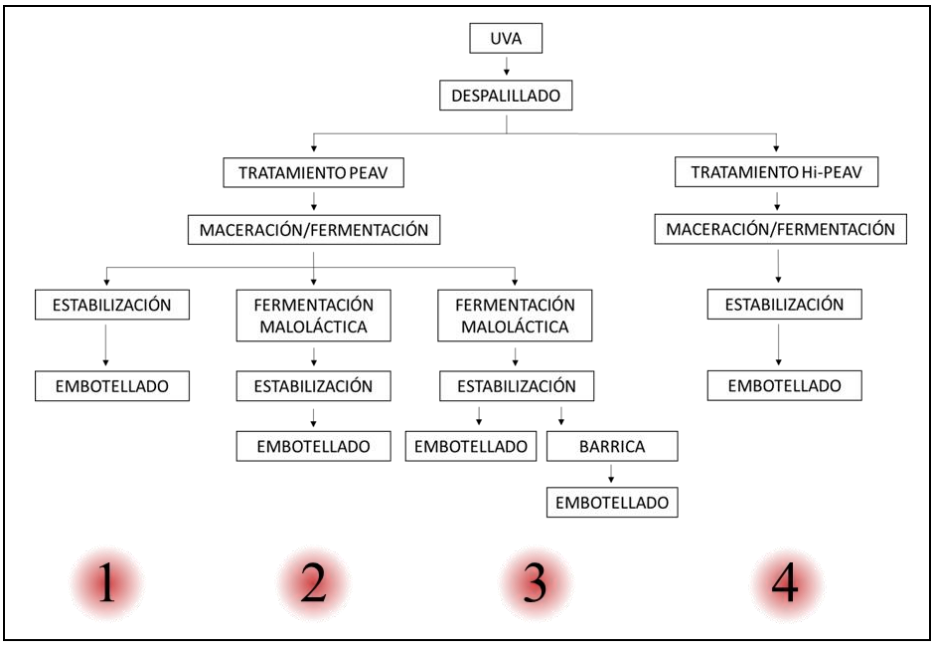


Figura 19: Protocolo general del proceso de elaboración de vino tinto utilizado en esta Tesis Doctoral aplicando tratamientos PEAV.

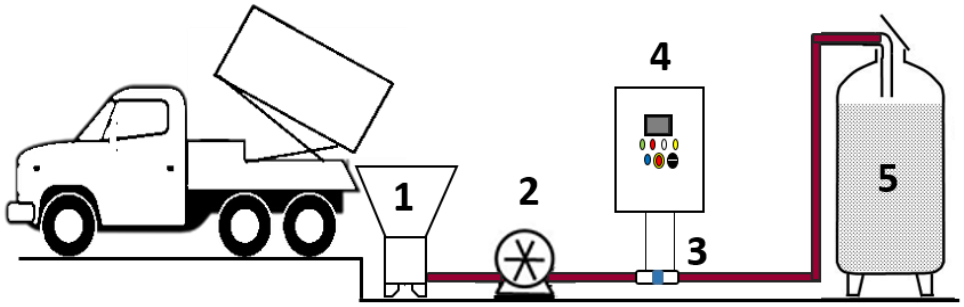
Estudio 1: Influencia de los tratamientos PEAV en la extracción de polifenoles durante la maceración/fermentación de uva Garnacha ("PEF-dependency of polyphenol extraction during the maceration/fermentation of Grenache grapes").

Elaboración del vino tinto

Con la finalidad de evaluar la influencia del campo eléctrico a igualdad de energías específicas aplicadas, 5 tratamientos con PEAV fueron realizadas con micro-vinificaciones por duplicado a nivel de planta piloto en las instalaciones de Bodegas Aragonesas.

Se utilizaron aproximadamente 1.120 kg de uva *Garnacha* para este estudio. Las uvas fueron cosechadas manualmente y transportadas a la bodega donde a la recepción se

llevó a cabo su pesado y los correspondientes controles de calidad de la uva. Las uvas se descargaron automáticamente al lagar y, posteriormente, con un tornillo sin-fin se transportaban al mínimo flujo posible del tornillohasta la despalilladora (ENO50, Enomundi, Zaragoza, España). En este experimento, las uvas estrujadas eran bombeadas con una bomba peristáltica (Rotho MS1, Ragazzini, Faenza, Italia) hacia la cámara de tratamiento colineal a un caudal de 600 $kg.h^{-1}$ donde recibía los tratamientos PEAV descritos en el siguiente apartado (Figura 20).



1: Despalilladora; 2: Bomba; 3: cámara de tratamiento; 4: generador PEF; 5: contenedor

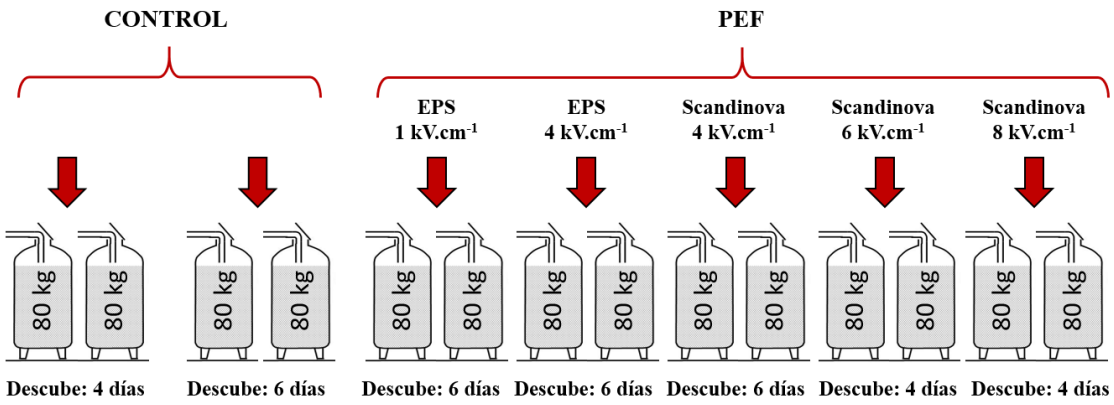


Figura 20: Esquema de la línea de procesado para la aplicación de PEAV en la bodega (imagen superior) y de la distribución de la uva procesada con los distintos tratamientos PEAV en depósitos así como el tiempo de descube utilizados en este ensayo. CONTROL: no tratado.

Tras los tratamientos, las uvas fueron distribuidas uniformemente en 10 contenedores de acero inoxidable de 80 litros cada uno con las uvas procesadas con cada uno de los tratamientos PEAV (2 por cada tratamiento) más 4 contenedores con uva sin tratar (14 contenedores en total). Una vez llenos los depósitos, se trasladaron a una cámara a temperatura controlada a 22ºC ubicada en la Planta Piloto de Ciencia y Tecnología de los Alimentos en la Facultad de Veterinaria de la Universidad de Zaragoza.

Una vez los depósitos en la planta piloto, se adicionaron 10 $mg.kg^{-1}$ de $K_2S_2O_5$ y tras 12 horas se inocularon en los tanques las levaduras comerciales (15 $mg.kg^{-1}$ OenoFrance, La

Marquise E491, Epernay, France). La temperatura de fermentación fue de 22ºC controlada durante todo el tiempo de maceración/fermentación. El control de la fermentación se realizó mediante la medida de la densidad del mosto dos veces al día. Como se ha indicado, se utilizaron dos tiempos de maceración en este ensayo (4 y 6 días) dependiendo de las intensidades de los tratamientos PEAV aplicados como se indicará posteriormente. Por cada tiempo de maceración, fueron descubados además de los contenedores con uva procesada con PEAV, 2 de uva sin tratar. Una vez descubados, se realizó el prensado de los vinos en fermentación con una prensa neumática PE150 (Pera, Pera-Pellenc, Francia). Tras el prensado, los mostos siguieron su normal fermentación en fase líquida hasta que terminó la fermentación de los azúcares (12 días). El final de la fermentación se determinó mediante el análisis de los azúcares reductores de todos los vinos hasta que los mismos tuvieron <2 $g.L^{-1}$ de estos compuestos.

Tratamientos PEAV

Debido a que con un mismo equipo de PEAV no se podían aplicar los tratamientos PEAV en el rango objeto de estudio, se utilizaron los dos generadores de PEAV descritos en los apartados anteriores. Para los tratamientos en los que se aplicaron los tratamientos a mayores campos eléctricos, se utilizó el generador Scandinova (SCA): 4, 6 y 8 $kV.cm^{-1}$. Para aplicar los tratamientos a campos eléctricos bajos (1 $kV.cm^{-1}$) y con el fin de aplicar el nivel energético más parecido al de los tratamientos más elevados, se utilizó el generador EPS que permitía generar pulsos de anchura de hasta 100 µs. En el caso del SCA, debido a que el ancho de pulso de 3 µs era fijo resultaba imposible al caudal empleado y con la frecuencia del generador aplicar el número de pulsos necesario para alcanzar la energía específica de los tratamientos a mayores campos eléctricos. Para completar el diseño experimental y con el fin de comparar el efecto de la anchura de los pulsos y de los generadores utilizados, con el EPS, se aplicó un tratamiento a 4 $kV.cm^{-1}$ con pulsos de 100 µs comparable a nivel energético con los tratamientos más intensos realizados con el generador SCA.

La Tabla 9 muestra los parámetros PEAV de los tratamientos investigados que incluye el voltaje seleccionado en cada generador, la intensidad del campo eléctrico aplicado, la frecuencia, anchura y número de pulsos, así como el tiempo y energía específica aplicados. Debido a la enorme dificultad de aplicar exactamente el mismo tratamiento con los diferentes generadores, solo con 3 tratamientos de distintos campos eléctricos se alcanzó una energía específica de 6 $kJ.kg^{-1}$ y 2 tratamientos tuvieron energías

específicas menores de 4 $kJ.kg^{-1}$. Las uvas procesadas con los tratamientos SCA6 y SCA se descubaron tras 4 días así como 2 depósitos control. El resto se descubó tras 6 días de maceración.

Tabla 9: Parámetros y condiciones de tratamiento aplicados en este estudio con los generadores SCA y EPS

Tratamiento	Voltaje (kV)	Campo eléctrico ($kV.cm^{-1}$)	Frecuencia (Hz)	Pulsos	Ancho de pulso (µs)	Tiempo de tratamiento (µs)	Energía específica total ($kJ.kg^{-1}$)
EPS1	1,60	0,70	200	50	100	5000	2,87
EPS4	10,00	4,00	12	3	100	300	6,72
SCA4	8,50	3,40	290	72	3	216	3,52
SCA6	14,00	5,60	187	46	3	138	6,16
SCA8	19,70	7,80	103	26	3	78	6,72

La cámara de tratamiento utilizada en este experimento fue de tipo colineal anteriormente descrita de 3,5 cm de diámetro y 2,5 cm de separación entre los electrodos (Figura 16).

Estabilización, conservación y añejamiento

Después de la fermentación alcohólica (12 días), se procedió al control de la fermentación malolática mediante test enzimático (L-Malic acid, R-Biopharm AG, Darmstadt, Alemania). Tras esta, los vinos fueron ajustados con 100 $mg.L^{-1}$ de $K_2S_2O_5$ e introducidos en una cámara 4±1ºC para que su estabilización durante 30 días. Posteriormente, los vinos fueron envasados en botellas de 750 mL, tapados con corcho sintético y colocados en una cámara a temperatura controlada a 16±1ºC durante 12 meses hasta que se realizó los análisis correspondientes.

Análisis realizados

En las microvinificaciones, se realizaron los análisis que se muestran en la Tabla 7 durante la fase de maceración/fermentación y el añejamiento.

En la Tabla 10 observan los distintos análisis realizados en cada etapa y las técnicas de cada uno de ellos se han desarrollado en el apartado anterior.

Tabla 10: Análisis realizados en este estudio.

Etapa en la que se realizaron los distintos análisis		
Mosto	**Maceración/Fermentación-**	**Vino (añejamiento)**
Alcohol probable	*TPI*	*Alcohol*
Acidez total	*IC*	*Acidez Volátil*
IC	*Taninos condensados*	*pH*
Antocianos	*Antocianos totales*	*CIELab*
IPT	*Densidad*	*TPI*
ºBrix		*CI*
pH		*Antocianos totales*
Extractabilidad		*Taninos condensados*

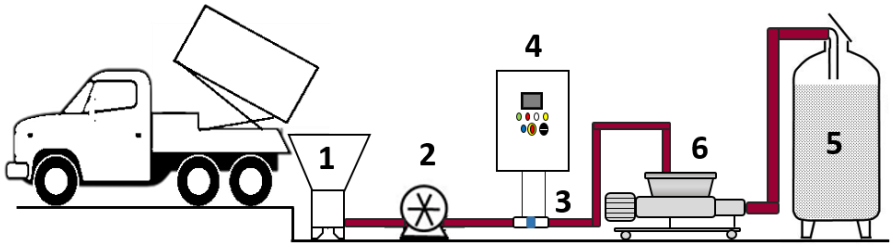
Estudio 2: Influencia de los pulsos eléctricos de alto voltaje sobre la composición de aromas y polifenoles del vino tinto elaborado con uvas Grenache ("Influence of Pulsed Electric Fields on aroma and polyphenolic compounds of Grenache wine").

Elaboración de vino tinto en bodega

En este ensayo, se utilizó uva *Garnacha*. 12.000 kg de uvas fueron cosechadas manualmente y transportadas a la bodega (Bodegas Aragonesas, Fuendejalón, España) en 4 remolques de 3.000 kilogramos. En la recepción, se pesaron las uvas y se tomaron muestra para realizar los correspondientes controles de calidad de la uva. Posteriormente, las uvas se descargaron automáticamente al lagar y con un tornillo sin-fin se transportaron hasta la despalilladora (ENO50, Enomundi, Zaragoza, España). En este experimento, las uvas estrujadas eran bombeadas con una bomba peristáltica (Rotho MS1, Ragazzini, Faenza, Italia) hacia la cámara de tratamiento colineal a un caudal de 2.500±100 $kg.h^{-1}$ donde recibía el tratamiento PEAV (Figura 21). Tras el tratamiento, las uvas se impulsaban con una bomba de tornillo (Delta I-MV) hasta los tanques de fermentación ubicados en el interior de la bodega a unos 25 metros de distancia.

Tras los tratamientos, las uvas se distribuyeron en 4 tanques de 5.000 litros de capacidad caca uno. Dos tanques se llenaron con uvas tratadas con PEAV y 2 con uvas sin tratar Figura 21. Con motivo de uniformizar la uva de la vendimia durante el ensayo en la bodega, ésta se distribuyó uniformemente entre los 4 tanques de fermentación. Tras el llenado de los tanques, se adicionó en cada tanque 10 $mg.kg^{-1}$ de $K_2S_2O_5$ y, tras 12 horas, se inocularon las levaduras comerciales (15 $mg.kg^{-1}$ OenoFrance, La Marquise E491, Epernay, France). La temperatura de fermentación fue seleccionada y controlada

automáticamente a 25±2ºC mediante el sistema de refrigeración instalado en la bodega. El mismo consta de agua previamente enfriada a 4ºC que circula (por acción de dos electroválvulas programadas para que se accione a dicha temperatura) por una doble camisa o chaqueta alrededor de los tanques en fermentación. Además se realizaron dos remontes diarios manualmente.



1: Despalilladora; 2: Bomba; 3: cámara de tratamiento; 4: generador PEF; 5: contenedor; 6: bomba de tornillo

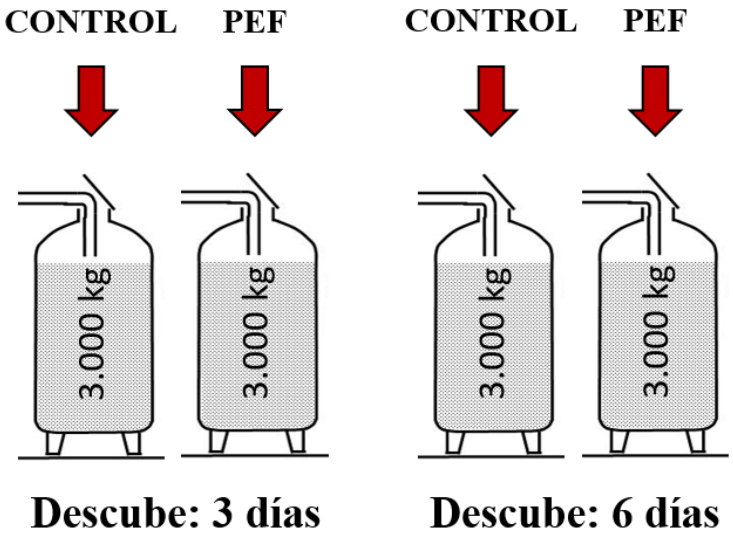


Figura 21 : Esquema general del proceso de elaboración de vino tinto en Bodegas Aragonesas (imagen superior) y distribución de las uvas en los depósitos llevados a cabo en el ensayo (imagen inferior).

El control de la fermentación se realizó mediante la medida de la densidad del mosto dos veces al día. Con el fin de evaluar los tiempos de maceración, se descubaron 2 tanques al tercer día, un tanque con uva tratada por PEAV y otro con uva sin tratar denominándolos PEF-3 y C-3, respectivamente. Los otros dos depósitos, con uva tratada con PEAV (PEF-6) y uva control (C-6) fueron descubados al sexto día. En el tercer y sexto días, se realizó el prensado de los vinos en fermentación con una prensa neumática PE150 (Pera, Pera-Pellenc, Francia). Tras el prensado, los mostos siguieron su normal fermentación en fase líquida hasta que terminó la fermentación de los azúcares (10 días). El final de la fermentación se determinó mediante el análisis de azúcares reductores de todos los vinos hasta un contenido <2 gr.L^{-1} de azúcares reductores.

Tratamientos PEAV

Para la aplicación de PEAV, se utilizó el generador EPS. Como se ha indicado, la uva despalillada fue impulsada por la bomba peristáltica hacia la cámara de tratamiento. Ésta era de tipo colineal según se muestra en el esquema de la Figura 14, con un diámetro de 3,5 cm y una distancia entre los electrodos de 2,5 cm constituyendo un área de tratamiento de 9,62 cm^2. Se aplicó un tratamiento de 370 µs (pulsos de 100 µs de anchura) a un campo de 4 $kV.cm^{-1}$, lo que corresponde a una energía específica total aplicada de 8,2 $kJ.kg^{-1}$ para una conductividad eléctrica del mosto de 1,4 $mS.cm^{-1}$ a 25ºC. Para el caudal utilizado (2.500 $kg.h^{-1}$) y las dimensiones de la cámara de PEAV, el tiempo de residencia de la uva en la zona de tratamiento era de 0,09 segundos.

Conservación y envejecimiento

Tras la fermentación (10 días), se extrajeron 200 litros de cada tanque y el vino fue trasegado a 2 contenedores de acero inoxidable de 100 litros cada uno, hasta la terminación de la fermentación maloláctica (8 contenedores en total). Después de la fermentación maloláctica, los vinos fueron ajustados con 100 $mg.L^{-1}$ de $K_2S_2O_5$ e introducidos en una cámara a 4ºC para su estabilización durante un tiempo de 30 días. Posteriormente, los vinos fueron embotellados en botellas de 750 ml, tapados con corcho sintético y colocados en una cámara con temperatura controlada a 16±1ºC hasta la realización de los análisis correspondientes.

Análisis realizados

Durante el desarrollo de este estudio, se realizaron los análisis indicados en la Tabla 11 durante las fases de maceración/fermentación y durante la de envejecimiento. Los análisis durante la etapa de maceración/fermentación se realizaron diariamente mientras que durante la etapa de conservación, los análisis fueron realizados cada 2 meses.

Tabla 11: Análisis realizados en el estudio 2.

Etapa en la que se realizó el análisis		
Mosto	**Fermentación-Maceración**	**Vino/envejecimiento**
Alcohol probable	*TPI*	*Alcohol*
Acidez total	*IC*	*Acidez Volátil*
IC	*Taninos condensados*	*pH*
Antocianos	*Antocianos totales*	*Azucares reductores*
IPT	*Densidad*	*CIELab*
ºBrix		*TPI*
pH		*CI*
Extractabilidad		*Antocianos totales*
		Taninos condensados
		Aromas minoritarios
		Aromas mayoritarios
		Polifenoles individuales

Estudio 3: Evolución de los compuestos fenólicos durante el envejecimiento en botellas y barricas de vino obtenido a partir de uvas de la variedad Garnacha tratadas por Pulsos Eléctricos de Alto Voltaje ("Evolution of polyphenolic compounds during aging in bottles and oak barrels of Grenache wine obtained from grapes treated by Pulsed Electric Fields").

Elaboración de vino tinto en bodega

Este estudio constituyó una segunda parte del anterior. El proceso de obtención del vino a partir de uva *Garnacha* fue el descrito en el estudio anterior así como el tratamiento de PEAV según se ha mostrado en la Figura 19. En la Figura 22, se presenta el esquema general del estudio.

Estabilización, conservación y envejecimiento

Tras de la fermentación (12 días), se extrajeron 100 litros de vino de cada tanque que se trasegaron a 2 contenedores de 80 litros de acero inoxidable cada uno. En este momento, se analizaron los vinos en relación a los azúcares reductores ya que el control de azúcares residuales era crítico en este estudio. Cuando los vinos registraron menos de 2 $g.L^{-1}$ de azúcares reductores, se llevó a cabo la fermentación maloláctica, tras la cual se estabilizaron los vinos durante 30 días a 4ºC en una cámara de refrigeración de la Planta Piloto de Ciencia y Tecnología de los Alimentos de la Universidad de Zaragoza.

Tras esta etapa, una parte del vinos fue embotellada en botellas de 750 mL, cerradas con corcho sintético y colocados en una cámara a temperatura controlada de 16±1ºC durante toda la etapa de conservación (24 meses) hasta la realización de los análisis correspondientes. Paralelamente, el otro volumen de vino se utilizó para llenar barricas (por duplicado) de 16 litros de roble americano (*Quercus alba*) con un tostado medio, adquiridas (Tonelería Los Pinos, Córdoba, España) específicamente para el envejecimiento del vino. Tras 6 meses de envejecimiento en las barricas, los vinos fueron embotellados en botellas de 750 mL, cerradas con tapón sintético y conservadas en la misma cámara (16±1ºC) donde se encontraban las botellas que no tenían añejamiento en botella durante 18 meses. En resumen, Los dos tipos de vino ("Vino con Barrica" y "Vino sin Barrica") fueron almacenados en la cámara a 16±1ºC durante un tiempo de 18 meses, de forma que los "Vinos con Barrica" estuvieron 6 meses en barrica y 18 meses en botella y los "Vinos sin Barrica", 24 meses en botella (Figura 22).

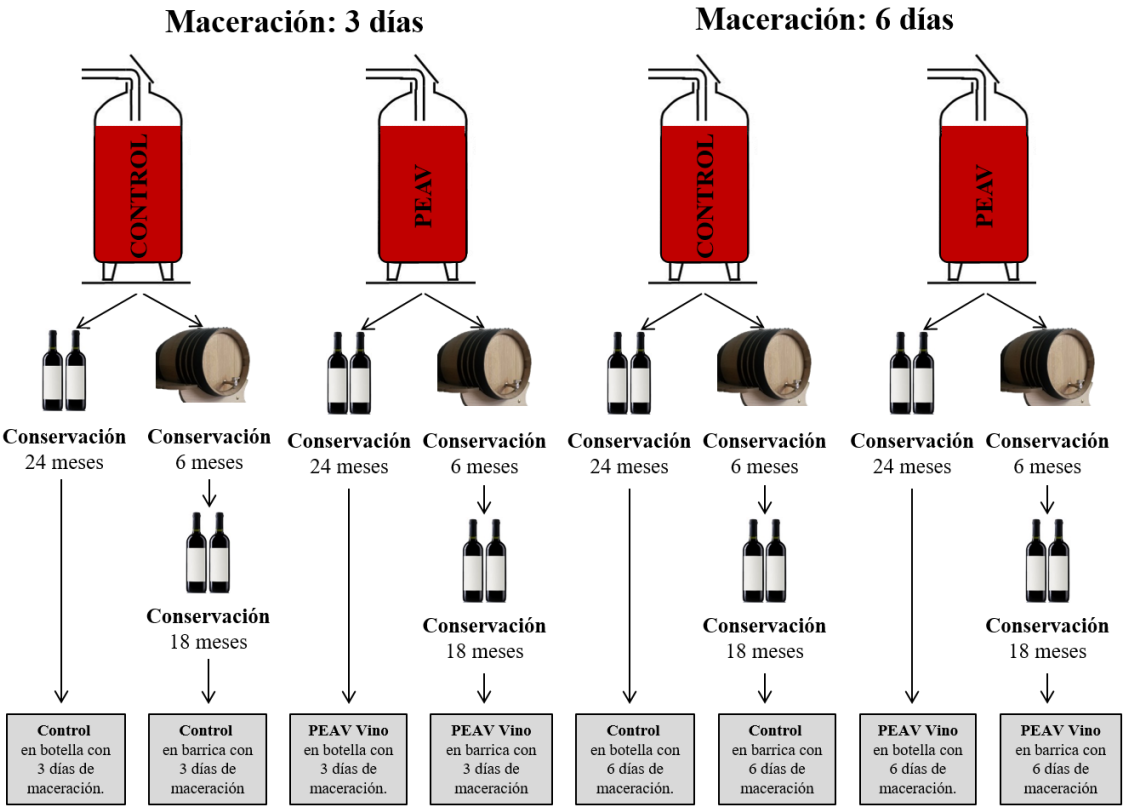


Figura 22: Esquema general del proceso de elaboración de vino tinto de uva de la variedad *Garnacha* en Bodegas Aragonesas tratado o no con PEAV y su conservación en la Planta Piloto de Ciencia y Tecnología de los Alimentos de la Facultad de Veterinaria de la Universidad de Zaragoza.

Análisis realizados

Durante el desarrollo de este estudio, se realizaron distintos análisis tras la finalización de la fermentación, durante la fermentación maloláctica y durante la etapa de envejecimiento y conservación. En la Tabla 12, se indican los análisis realizados en estas etapas.

Tabla 12: Análisis realizados en este estudio 3.

Vino
Alcohol
Acidez Volátil
pH
CIELAB
TPI
CI
Antocianos totales
Taninos condensados
Polifenoles individuales HPLC
Análisis sensorial

ESTUDIO 4: El incremento de la energía específica aplicada en los tratamientos de PEAV permite reducir el tiempo de maceración para obtener vinos tintos de las variedades de uva Caladoc y Garnacha ("Increasing total specific energy of PEF treatments permits reducing maceration time during vinification of Caladoc and Grenache wine").

Elaboración del vino tinto

En este estudio, se realizaron micro-vinificaciones en la Planta Piloto de Ciencia y Tecnología de los Alimentos (PPCTA) de la Facultad de Veterinaria de la Universidad de Zaragoza con dos variedades de uva, *Garnacha* y *Caladoc*, que fueron cosechadas manualmente de viñedos ubicados en la D.O. Campo de Borja (España) durante la campaña 2018. La uva fue transportada hasta la PPCTA en cajas de 15 kg cada una. Las uvas fueron pesadas y despalilladas (Master E-10, Enomundi, Zaragoza, España). La mezcla obtenida era recogida en la tolva de alimentación de una bomba de desplazamiento positivo de tornillo helicoidal (Rotor-MT, Bominox, Gerona, Spain). Con esta bomba, se impulsó la uva despalillada a un caudal constante de 140 $kg.h^{-1}$ hacia la cámara de tratamiento de PEAV. Los tratamientos PEAV aplicados (de alta y baja energía) se

describen en el siguiente apartado. Tras los tratamientos, las uvas fueron distribuidas en 14 contenedores de acero inoxidable de 80 litros cada uno (8 contenedores fueron utilizados para los tratamientos con las uvas de la variedad *Caladoc* y 6 para los de la uva *Garnacha*) donde se llevó a cabo la maceración/fermentación. En el caso de los tratamientos PEAV de "Alta Energía Específica" (I-PEF) se adicionaron 300 g de nieve carbónica en cada depósito con el fin de disminuir la temperatura de la uva tratada lo más rápidamente posible e igualarla a la del tratamiento PEAV de moderada energía específica (M-PEF).

En este estudio, se evaluaron tres tiempos de maceración para la uva *Caladoc*: 4 y 24 horas para los tratamientos de I-PEF y 4 días para los tratamientos M-PEF de baja energía específica. En el caso de la uva *Garnacha*, los tiempos de maceración fueron de 24 horas para los tratamientos de PEAV I-PEF y de 4 días para los de baja energía (M-PEF). Tras los correspondientes tiempos de maceración, los hollejos fueron prensados con una prensa neumática de 50 L de capacidad (Grifo PEA100, Italia). Los vinos continuaron la fermentación sin los hollejos (en fase líquida) hasta que el contenido de azúcares reductores fue inferior a 3 $g.L^{-1}$. Una vez finalizada la fermentación, los vinos fueron estabilizados mediante su almacenamiento en una cámara a 2ºC durante 30 días.

Tratamientos PEAV

Con el objetivo de evaluar el efecto del aumento de la temperatura debido a la energía específica aplicada directamente a las uvas, se aplicó un tratamiento de PEAV de mayor duración (5,75 veces superior) y energía (52,9 $kJ.kg^{-1}$) denominado "I-PEF" ("Intenso PEF") respecto a uno de menor energía específica (8,8 $kJ.kg^{-1}$) denominado "M-PEF" ("Moderado PEF") que fue el nivel energético normalmente utilizado en los anteriores estudios en bodega (Tabla 13). En este tratamiento, se usó el generador EPS que permite aplicar pulsos de anchura superior al SCA como se ha indicado anteriormente. La energía aplicada en el tratamiento AEE, con un tiempo de tratamiento PEAV total mayor, provocó un aumento instantáneo de la temperatura de la uva desde los 20±1ºC hasta casi 38ºC a la salida de la cámara. La temperatura fue medida con un sensor de temperatura termocupla conectado a un datalogger Almeno 2590 (Ahlaborn, Holzkirchen, Alemania) colocado a unos 10 cm de la salida de la cámara de tratamiento de PEAV.

La cámara de tratamiento utilizada fue de tipo colineal de 2 cm de diámetro por 2 cm de separación entre los electrodos. La conductividad del mosto fue medida previa al

tratamiento con una sonda de conductividad eléctrica FYA641LFP1 (Ahlaborn, Holzkirchen, Alemania) conectada a un datalogger Almeno 2590 (Ahlaborn, Holzkirchen, Alemania).

Tabla 13 Parámetros de procesado de los tratamientos de PEAV aplicados en este estudio.

Tratamiento	**Voltage (kV)**	**Campo eléctrico ($kV.cm^{-1}$)**	**Temperatura después del tratamiento ºC**	**Número de pulsos**	**Ancho del pulso (µs)**	**Tiempo total del tratamiento (µs)**	**Energía Específica total ($kJ.kg^{-1}$)**
I-PEF	10,0	**5,0**	37,2±0,6	46,0	40,0	1840,0	**52,9**
M-PEF	10,0	**5,0**	22,1±0,5	8,0	40,0	320,0	**8,8**

Conservación

Una vez finalizada la fermentación, los vinos fueron estabilizados mediante su almacenamiento en una cámara a 2ºC durante 30 días. Tras este tiempo, el vino fue embotellado en botellas de 750 mL, cerradas con corcho sintético y depositadas en una cámara a temperatura controlada de 16±1ºC durante 3 meses o hasta si correspondiente análisis.

Análisis realizados

El control de los vinos durante la maceración/fermentación fue realizado diariamente llevándose a cabo los análisis indicados en la Tabla 14, así como cuando se indica los correspondientes a su almacenamiento tras la fermentación.

Tabla 14: Análisis realizados durante el desarrollo del estudio 4.

Etapa en la que se realizó el análisis		
Mosto	**Fermentación-Maceración**	**Vino**
Alcohol probable	*TPI*	*Alcohol*
Acidez total	*IC*	*Acidez Volátil*
IC	*Taninos condensados*	*pH*
Antocianos	*Antocianos totales*	*Azucares reductores*
IPT	*Densidad*	*CIELab*
ºBrix		*TPI*
pH		*CI*
Extractabilidad		*Antocianos totales*
		Taninos condensados

RESULTADOS

Y

DISCUSIÓN

ESTUDIO 1: PEF-dependency of polyphenol extraction during the maceration/fermentation of *Grenache* grapes.

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1. Abstract

The influence of the application of different electric field strengths (E) and specific energies (W) applied with Pulsed Electric Fields (PEF) was studied on the rate of extraction of phenolic compounds during maceration/fermentation of *Grenache* grapes at industrial environment and on the 12-months wine aging. A response surface model was established between the extraction rate of the Total Polyphenol Index (TPI) and the E and W of the PEF treatments which permitted to define PEF processing conditions to achieve a certain TPI. To achieve a TPI of 50, treatments ranged from high intensity and short time (8 $kV.cm^{-1}$ and 45 µs) to low intensity and long time (1 $kV.cm^{-1}$ and 2,800 µs) for an optimum total specific energy in all cases of 4 $kJ.kg^{-1}$. The application of these PEF treatments reduced the maceration time between 25 to 37 % with respect to the untreated control without affecting quality characteristics (color intensity, tannins and total anthocyanins) neither after the fermentation nor during 12 months of aging in bottles of the obtained red wines.

2. Introduction

Pulsed electric field (PEF) treatments consist in the application of very short electric pulses (in the range from μs to ms) at field strengths greater than 0.5 $kV.cm^{-1}$, resulting in an increment of cell membrane permeabilization, a process called electroporation (Cholet et al., 2014). This phenomenon that explains the potential of PEF for improving mass transfer rates in processes which aim to extract intracellular components, including phenolic compounds, from grapes (Delsart et al., 2012; Donsì, et al., 2011; Leong, et al, 2016; López, et al., 2008a; López-Giral et al., 2015). This improvement in phenolic extraction has made it possible to shorten the maceration step (the time of contact of grape skins with the must). This can produce logistic benefits for wineries, including gains in productivity and the reduction of fermentation deposits. Although these results have been observed at the lab and pilot plant scale, different PEF treatment conditions were applied, and the influence of the main PEF parameters such as electric field strength, treatment time, and energy, on polyphenol extraction has not been clearly evaluated (Delsart et al., 2012; Donsì et al., 2011; López et al., 2008a; Luengo, et al., 2014; Puértolas, et al., 2010a). That would be a necessary step for the implementation of the PEF technology in wineries, since they work with large flow rates (from 10 to 50 $ton.h^{-1}$ depending on production capacity). The application of high electric field strengths makes it possible to reduce treatment time. However, the PEF generator requires an increased amount of input voltage and power, and this factor could limit the technology's application. Moreover, in large treatment chambers which mainly have colinear configurations, electric field strength is not uniform (Gerlach et al., 2008; Jaeger, et al., 2009), although the effectiveness of PEF treatment on the electroporation of plant cells is associated with the amount of applied pulses (Delsart et al., 2014; López-Alfaro et al., 2013). This problem could play a certain role when applying high field strengths. Conversely, the use of low electric field strengths requires the application of long treatment times and a great number of short pulses, or less pulses but of longer width. Short pulses mainly require the use of high frequencies for large flows, and this can be a limiting factor for PEF generators. Long pulses, on the other hand, can result in unwanted electrochemical reactions in the electrodes, resulting in fouling, corrosion, and the release of metals which can affect the safety and quality of the wine (Pataro et al., 2014; Roodenburg, et al., 2005). Therefore it is necessary to carry out a systematic study of the effect of the main PEF processing parameters on the extraction of polyphenols from

grapes under comparable conditions. This kind of study can play an essential role in helping to decide upon the specific PEF treatment conditions to be applied to extract the greatest amount of phenolic compounds within the shortest maceration time, and also in order to determine the characteristics of the PEF generator to be used in a winery.

The objective of this study was to evaluate the effect of the intensity of the electric field strength, the total specific energy, and the treatment time of PEF treatments on the extraction of polyphenols and other compounds associated with the chromatic and quality characteristics of *Grenache* wines during maceration/fermentation, and also during aging in bottles of the obtained red wines.

3. Material and methods

Samples

Grapes of *Vitis vinifera* var. *Grenache* (D.O. Campo de Borja, Fuendejalón, Spain), manually harvested from the 2016 vintage in optimal ripening stage and in good sanitary conditions, were used in this investigation. Grapes were transported to the winery on 3,000-kg trailers. Physical and chemical characteristics of grapes and must are shown in Table 15.

Table 15**:** Physico-chemical characteristics of grapes at time of harvesting. Mean values and standard deviations.

Probable alcohol (% $v.v^{-1}$)	15.6±0.04
pH	3.45±0.35
Total acidity ($g.L^{-1}$)[a]	4.99±0.16
ºBrix	26.30±0.12
Total phenols (OD 280 nm)	12.45 ±0.04
Colour intensity	3.05±0.07
Conductivity (mS.cm)	1.38±0.04

[a] Expressed as tartaric acid

PEF equipment

Two PEF generators were used in order to apply different PEF treatment conditions working at the same flow rate (600 $kg.h^{-1}$). In order to evaluate the effect of different electric field strengths at the same total specific energy (around 6 $kJ.kg^{-1}$), a Scandinova PEF generator was used. It has a high input voltage (30 kV), which makes it possible to apply high electric field strengths; however, due to its configuration, the pulse width is constant (3 µs), thereby limiting the treatment time to achieve a certain energy level when applying low field strengths. To obtain low electric fields and high total

specific energies, a second PEF generator (EPS system) with variable pulse width was used.

The Scandinova PEF generator (SCA) has a maximum output voltage and current of 30 kV and 200 A, respectively (Pulse Generator PG, ScandiNova, Uppsala, Sweden). It generates square waveform pulses of 3 µs pulse width, with a frequency of up to 300 Hz. The voltage applied in the treatment chamber was measured with a high voltage probe (Tektronix P6015A, Wilsonville, OR, USA) connected to an oscilloscope (Tektronix, TDS 220, Wilsonville, OR, USA).

For PEF treatments of low and moderate electric field strengths (0.7 to 4.0 $kV.cm^{-1}$), a PEF generator (EPS) of maximum output voltage and current of 10 kV and 200 A, respectively, was used (EPULSUS® PM1-10, Energy Pulse Systems LDA, Lisbon, Portugal). The apparatus generates monopolar square waveform pulses ranging from 2 to 200 µs with a frequency up to 200 Hz. The applied voltage was measured with a high voltage probe (Tektronix, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronix, TBS 1102B-EDU, Wilsonville, Oregon, USA).

Treatments were applied in continuous flow in a co-linear treatment chamber that consists of three stainless steel cylindrical electrodes separated by two polyoxymethylene insulators. Whereas the central electrode is connected to the high voltage, the electrodes of both extremes are grounded. This co-linear configuration defined two cylindrical treatment zones of 2.5 cm between the electrodes with an inner diameter of 3.5 cm. The indicated electric field strength corresponds with the field strength in the mid position of the central axis of the treatment zone (Toepfl, et al., 2007). A peristaltic Rotho MS1 pump (Ragazzini, Faenza, Italy) was used to pump the grape mass to the treatment chamber. The flow rate was regulated to 600 $kg.h^{-1}$ for both PEF systems. The obtained flow provided a residence time of the medium in the PEF treatment zone of 0.14 sec.

PEF treatments

After crushing and destemming, grapes were treated with the PEF generators previously described (EPS and SCA). The applied PEF treatment conditions for each parameter (electric field strength, number of pulses, treatment time, and specific energy) are indicated inTable 16. The voltage, frequency, and pulse width used in the treatments with each generator are also indicated. Of the five PEF treatments, three were applied at total specific energy greater than 6 $kJ.kg^{-1}$, and two treatments were applied at less than 6 $kJ.kg^{-1}$. In all experiments, the increment of temperature induced by the PEF treatment

never exceeded 2 ºC. Apart from the PEF treatments, two control samples were also obtained. In total, seven treatment conditions were evaluated.

Table 16**:** Parameters and conditions of the treatments applied in the study.

Treatment	Voltage (kV)	Electric field ($kV.cm^{-1}$)	Frequency (Hz)	Pulses	Pulse width (µs)	Treatment time (µs)	Specific energy ($kJ.kg^{-1}$)
EPS1	1.60	0.70	200	50	100	5000	2.87
EPS4	10.00	4.00	12	3	100	300	6.72
SCA4	8.50	3.40	290	72	3	216	3.52
SCA6	14.00	5.60	187	46	3	138	6.16
SCA8	19.70	7.80	103	26	3	78	6.72

Winemaking

After the PEF treatments, grapes were distributed in 100 kg stainless steel tanks. Two batches per PEF treatment and four additional ones of untreated grapes were used as control (Control 1 and Control 2), as depicted in Table 16. In total, 14 batches of 80 kg each were fermented, corresponding to a total of 1,120 kg of grapes. Initially, 20 $mg.kg^{-1}$ of $K_2S_2O_5$ were added to each tank, followed by 15 $g.hl^{-1}$ of commercial culture of Saccharomyces cerevisiae (OenoFrance La Marquise E491, Epernay, France). Pilot-plant scale fermentations were performed, and temperature was maintained at 22±1 ºC. Maceration time was 4 days when high field strengths were applied (SCA6 and SCA8), and 6 days for the other field strengths (EPS1, EPS4, and SCA4). To compare results, one control batch (Control 1) was macerated for 4 days, and the other (Control 2) for 6 days. The different maceration times were decided upon in function of polyphenol extraction as verified during vinification. During the fermentation period, temperature and must density were monitored daily, and the cap was punched down once a day. The concentration of residual sugars at the end of fermentation (13 days) was always lower than 3 $g.L^{-1}$. The wines were subsequently racked and stabilized for a period of one month at 2 ºC and then bottled and stored at 16±1 ºC. To study the evolution of phenolic compounds, samples were taken after 0, 3, 6, 9, and 12 months of bottling.

Physic-chemical analysis of enological parameters

In order to characterize the grapes used in this study, total acidity, pH, ºBrix, color intensity, total polyphenol index, and electrical conductivity were analyzed in the must. Total acidity was measured according to the methods prescribed by the OIV (Organization Internationale de la Vigne et du Vin, 2009); ºBrix was measured with a digital refractometer (PR-101 Palette digital refractometer, Atago Ltd. Japan).

In order to monitor the wine's evolution during maceration/fermentation and aging, 1 mL samples of the musts and wines were centrifuged in an Eppendorf AG centrifuge for 15 min at 3000 rpm (Eppendorf, Hamburg, Germany). Absorbance of the musts and wines was then directly measured at 420, 520, and 620 nm in a Biochrom LibraS12 spectrophotometer (Biochrom Limited, UK) with Hellma® Analytics QS Quartz SUPRASIL® 300 Precision cells (light path 1 mm) (Hellma Analytics, Müllheim, Germany). Color Intensity (CI) was calculated as the sum of 420, 520, and 620 nm absorbance, according to Glories (1984). Total Polyphenol Index (TPI) was determined by a direct reading of the absorbance at 280 nm of diluted wine 1/100 ($v.v^{-1}$) with Hellma® QS quartz SUPRASIL® 300 cuvettes (light path 10 mm) (Hellma Analytics, Müllheim, Germany). TPI was calculated by multiplying the absorbance measured at 280 nm by one hundred. Total Content of Anthocyanins (TCA) expressed in milligrams per liter of malvidin-3-glucoside was analyzed by determining the absorbance at 520 nm of diluted wine 1/100 ($v.v^{-1}$) with 1% ($v.v^{-1}$) HCl using the corresponding calibration curve (Ruiz-Hernández, 2004). Condensed tannins (TC) were determined according to Sarneckis et al. (2006). Aqueous (-)-epicatechin solutions (10, 25, 50, 75, 100, 150, and 200 $mg.L^{-1}$ epicatechin) were used in order to obtain the calibration curve. All values are reported in $mg.L^{-1}$ of epicatechin equivalents of the original sample (corrected for assay dilution). All analyses were carried out in triplicate on each 80-kg batch

Statistical analysis

A linear plateau regression was performed on the experimental TPI data with Infostat software (Di Rienzo et al., 2011) according to the following equation:

$$Y = \alpha + (\beta * t) * (t < \gamma) + (\beta * \gamma) * (t \geq \gamma) \qquad \text{(Eq 1)}$$

in which Y is the concentration of TPI extracted at different times (days); α is the estimated initial value of TPI; β is the slope of the line in the first regression sector; and γ is the number of days in the course of which the TPI value was transformed into a plate, i.e. when it achieved the maximum value.

Response surface methodology (RSM) was used to study the possible advantages of PEF application for TPI extraction. To determine the influence of electric field strength (E) and specific energy (W) on the extraction rate of TPI (β values), the results were analyzed by multiple regression. They were fitted to a general quadratic equation that accounted for the influence of the individual factors, E (X_1), W (X_2), interaction effects,

(X_1 x X_2), and quadratic effects (X_1^2 , X_2^2) of the investigated factors on the response (Eq. 2), in which Y is the response variable to be modeled.

$$Y = \beta_1 + \beta_1 X_1 + \beta_2 X_2 + \beta_{1x2} (X_1 \text{ x } X_2) + \beta_1 X_1^2 + \beta_2 X_2^2 \text{ (Eq. 2)}$$

A backward regression procedure was used, systematically removing the effects that were not significantly associated (p>0.05) with the response until a model with only a significant effect was obtained. The regression coefficients obtained with the coded factors were then used to make statistical calculations to generate three-dimensional representations of the general regression model. The surface response function and the corresponding analysis of the data were carried out using the software package Design–Expert 6.0.6 (Stat-Ease Inc. Minneapolis, MN, USA).

Data presented in tables and figures represent mean values ±95 % confidence level. Analysis of variance (ANOVA) was carried out using InfoStat statistical software in the 2018 version. Graphics were obtained using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA) and Excel Package (Microsoft, Redmond, Washington, USA).

4. Results and discussion

The potential of PEF treatments for the extraction of phenolic compounds from grapes and possible benefits for wineries are all very well described in the literature (Delsart et al., 2012; Donsì et al., 2011; Leong et al., 2016; López et al., 2008a; López-Giral et al., 2015). However, these results have been observed at lab and pilot plant scale only, using static and continuous treatment chambers, and with different grape varieties (Delsart et al., 2012; Donsì et al., 2011; López et al., 2008a; Luengo et al., 2014; Puértolas, et al., 2010a). Probably due to practical limitations, no investigation has hitherto studied the effect of the main PEF parameters (field strength, time, and energy) on this process at a pilot plant scale. Certain studies have been carried out at lab scale, but using generators of different configuration, square and exponential decay pulses (El Darra et al., 2016), evaluating a narrow range of conditions, or evaluating the effect of electric field strength and treatment time but applying different energy levels (López et al., 2008a; Luengo et al., 2014; Puértolas, et al., 2010d). This makes it difficult to determine the treatment conditions most adequate for an implementation of the PEF technology at a large scale, especially taking possible equipment requirement limitations into account (high voltage, number of pulses, frequency, etc). No study has hitherto evaluated how to optimize treatments designed to be applied at different field strengths, times, and specific

energies in order to extract the greatest amount of phenolic compounds as well as to reduce maceration time with respect to traditional winemaking. In this study, the same energy level has been applied with different electric field strengths, or the same electric field strength with different energies in order to evaluate the individual effect of each parameter. Due to the difficulties of using large flows of grapes to apply a wide range of PEF treatment conditions, two PEF generators were used as described above, while fixing the greatest possible number of variables in order to enable direct comparison of results.

Effect of the electric field strength during maceration/fermentation

In order to identify the advantages of PEF treatments, vinification processes carried out upon grapes treated with PEF at different electric field strengths and total specific energies (EPS1, EPS4, SCA4, SCA6, and SCA8) were compared with other vinification processes carried out upon untreated grapes (Control 1 and Control 2). Treatments of low field strength (EPS1 and EPS4) were applied with long pulses (100 µs) in order to achieve the highest specific energies comparable with treatments of greater field strengths at the flow rate used in the study (Table 16). These treatments could only be applied with the EPS system. Even with those pulse widths, in the case of 0.7 $kV.cm^{-1}$, the maximum specific energy that could be released was the lowest of those applied (2.87 $kJ.kg^{-1}$). In order to compare the possible influence of pulse width, treatments at 4 $kV.cm^{-1}$ (EPS4 and SCCA4) were applied with both generators. Finally, to directly compare the influence of electric field strength, treatments of the same total specific energy (around 6 $kJ.kg^{-1}$) were applied with both generators (EPS4, SCA6 and SCA8).

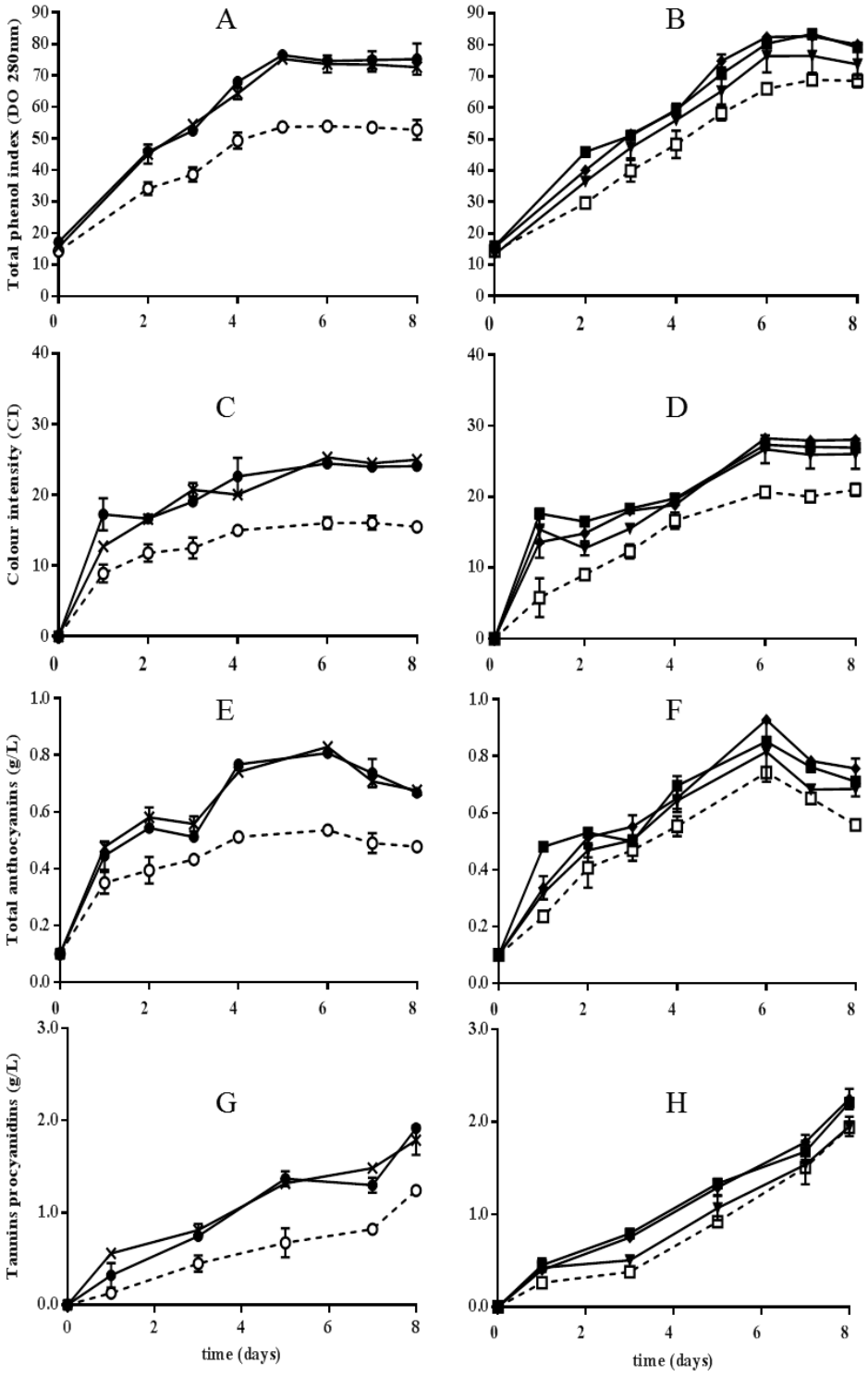

Figure 23: Evolution of total polyphenol content (TPI) A and B, color intensity (CI) C and D, Total anthocyanins (TAC) E and F, Tannins condensed (TC) G and H, along maceration time during vinification of PEF-treated (EPS 1▼, EPS4 ■ SCA4 ♦, SCA6 ●, SCA8 X) and untreated wines (Control 1 ○ and Control 2 □).

Figure 23 shows the TPI (23A and 23B), CI (23C and 23D), TAC (23E and 23F) and TC (23G and 23H) of the must obtained during the maceration/fermentation stage (8 days) from grapes subjected to PEF treatments as indicated in Table 16 and from untreated grapes. Figures 23A, 23C, 23E and 23G show the results for the PEF treatments applied with the highest electric field strengths (SCA6, SCA8) and for untreated grapes (Control 1). In these cases, skins were removed after 4 days of maceration. Figures 23B, 23D, 23F, and 23H display the results of the fermentation process for the less intense treatments (EPS1, EPS4, and SCA4) and for the corresponding untreated grapes (Control 2). In these treatments, skins were removed after 6 days of maceration. As observed, the evolution of the four oenological parameters was similar in all wines, and the values thereof were always higher at any maceration/fermentation time when PEF treatments were applied, independently of their intensity. Similar observations in terms of the effect of PEF have already been indicated in literature (Corrales et al., 2008; López et al., 2008a; Puértolas et al., 2010d).

When comparing the results of the most intense PEF treatments (SCA6 and SCA8), independently of maceration time, the differences in terms of the four oenological parameters (TPI, IC, TAC, and TC) were practically negligible (Figures 23A, 23C, 23E and 23G). For less intense field strengths (Figures 23B, 23D, 23F and 23H), treatments at 0.7 $kV.cm^{-1}$ showed slightly lower values of oenological parameters, but no differences were observed for the treatments applied at around 4 $kV.cm^{-1}$, independently of specific energy, pulse width, or number of pulses.

Since different maceration times were applied, if one wants to compare the effect of different PEF intensities on the investigated wine parameters (Figure 23 ACEG versus Figure 23 BDFH), results have to be compared for the same maceration time (up to 4 days). After that period, the longer the time in contact with the skins, the higher were the values of those parameters, even when lower field strengths were applied. Regarding electric field strength and comparing treatments of the same specific energy around 6 $kJ.kg^{-1}$ (EPS4, SCA6 and SCA8), TPI of 59.35±2.75, 65.3±2.54 and 68.05±0.77 were achieved with field strengths of 4 (EPS4), 6 (SCA6), and 8 $kV.cm^{-1}$ (SCA8), respectively. These results indicated that, of those three treatments, the one at 6 $kV.cm^{-1}$ would be the most adequate, since the highest TPI would be obtained with the same specific energy after the same maceration time. Similar conclusions apply for the other wine quality parameters. These results are in line with previous publications, which reported that

electric fields greater than 5 $kV.cm^{-1}$ showed no significant differences in terms of extraction of polyphenolic compounds (López et al., 2008a).

When comparing treatments of the same electric field strength around 4 $kV.cm^{-1}$ (EPS4 vs SCA4), but varying specific energy (6.7 and 3.5 $kJ.kg^{-1}$), no significant differences were observed in terms of TPI (SCA4=59.00±0.14 and EPS4= 59.35±2.75) or CI, TAC, and TC. This would indicate that field strength is a key parameter in extraction, but the pulse protocol could be affecting the results. Thus, for the treatment at 3.5 $kJ.kg^{-1}$, 72 pulses of 3 µs were applied, whereas only 3 pulses of 100 µs were applied for the one at 6.7 $kJ.kg^{-1}$. As previously indicated, the application of a reduced number of pulses could ultimately have less effect on electroporation, particularly when electric field strength inside the treatment zone is not uniform, as occurs with colinear configurations (Gerlach et al., 2008; Jaeger et al., 2009). Thus, some reports indicated a significant increase in the concentration of TPI when the number of applied pulses to *Grenache* (López-Alfaro et al., 2013) or *Cabernet Sauvignon* grapes (Delsart et al., 2014) was greater. These results suggest that an interaction among the number of pulses, pulse width, specific energy, and electric field strength play a role in ascertaining which is the most adequate PEF treatment to be applied for the extraction of a certain compound. Thus, a more detailed study of the effect of each PEF processing parameter is required, not only within a specific time of maceration, but in the course of the entire process.

Analysis of the evolution of total polyphenol index

In order to further analyze the influence of the PEF treatment parameters on the winemaking process, the evolution of the extraction of the total phenolic index (TPI) was used as a reference wine quality parameter. The TPI value was selected as an indicator, not only due to the fact that the analysis is simple to perform, but also because all wineries use it as a guide to determine the moment during maceration at which the skins should be removed (Togores, 2011). For wines that are to be aged for long periods, longer maceration times are required in order to achieve higher TPI values, contrary to young wines destined for everyday consumption, and which require lower TPI.

Each treatment carried out on the grapes registered an extraction rate (variation of TPI within a certain extraction time) of the different phenolic compounds during the maceration stage. In order to determine the influence of PEF treatments on the extraction rate, Equation 1 was used to describe the TPI curves of Figure 24.

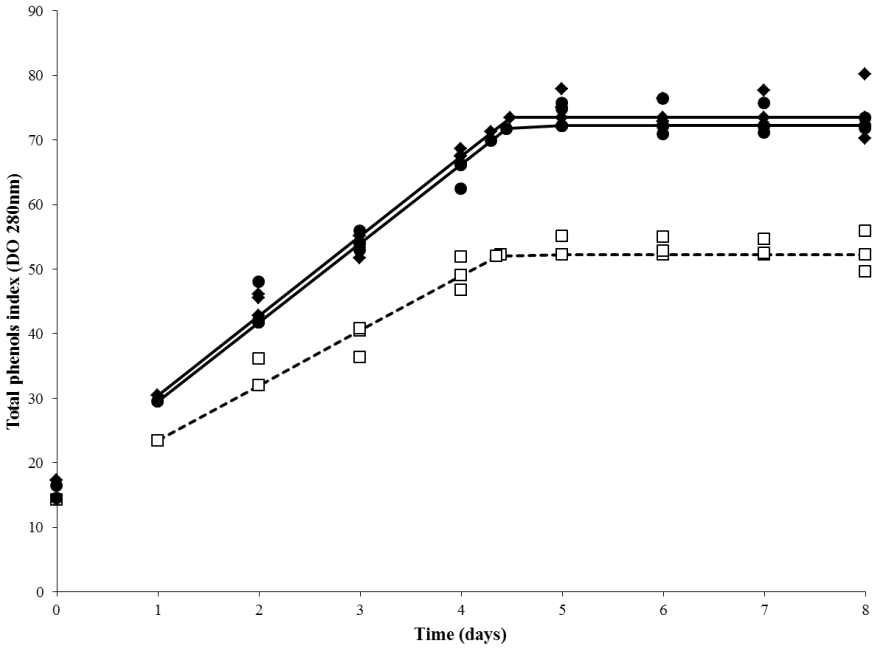

Figure 24: Fit of Equation 1 to the TPI values during maceration/fermentation of wines obtained from untreated (□) and PEF-treated grapes at 6 $kV.cm^{-1}$ and 6.2 $kJ.kg^{-1}$ (●) and 8 $kV.cm^{-1}$ and 6.7 $kJ.kg^{-1}$ (♦).

As an example, Figure 24 shows the result of the nonlinear and plateau regression performed on the TPI data obtained in grapes treated with PEF (SCA6, SCA8) and untreated grapes (Control 1). As observed, predicted values (continuous line) adequately described the TPI values obtained under different investigated conditions. The TPI values increased (slope) at a constant rate until the moment of removal of skins of the must (4 days in the example), after which the values remained constant until the end of fermentation. The same procedure was carried out for the other PEF treatments (data not shown). To compare results, values from the fitting of Equation 1 to TPI curves of Figure 24 are shown in Table 17. In this table, the slope (extraction rate in the linear portion of the TPI curves expressed as $TPI.day^{-1}$), the TPI maximum values, and the days required to achieve the maximum TPI as estimated by *Equation 1* are indicated for each treatment. To show the goodness of fits for each treatment, real maceration time (days in contact between skin and must), along with R^2 and RMSE, are also included.

Table 17: Values from the fit of Equation 1 to TPI curves showed in Figure 23.

Treatment	Maceration time (days)	TPI (máx. estimated)	Slope, β (TPI.day^{-1})	R^2	RMSE
EPS 1	6	73.49±6.58bcd	10.41±0.69b	0.93	4.57
EPS 4	6	79.19±0.46cd	10.53±0.22b	0.94	3.51
SCA 4	6	79.61±0.94d	11.5±0.29bc	0.96	3.08
SCA 6	4	72.24±1.76bc	12.24±0.91c	0.93	3.29
SCA 8	4	73.55±3.43cd	12.37±0.07c	0.93	3.70
CONTROL 1	4	52.31±2.20a	8.53±0.54a	0.92	2.82
CONTROL 2	6	66.30±0.81bc	8.99±0.66a	0.96	3.03

Conclusions similar to those previously indicated can be reached from the analysis of the fitted values. Maximum estimated TPI values depended on the type of PEF treatment, but to a greater extent on maceration time. Thus, control samples showed the lowest values compared to samples treated with PEF (with a difference ranging from 10 to 34 %). We investigated the existence of an effect of PEF intensity on the slope, as well as relationships between the PEF parameters and the slopes. These correlations are of interest in order to elucidate the significant effect of each PEF parameter on phenol extraction. For example, it would be useful to determine if the treatment at 6 kV.cm^{-1} and 6.16 kJ.kg^{-1} with 4 days of maceration was more effective than the one at 4 kV.cm^{-1} and 3.5 kJ.kg^{-1} with 6 days of maceration. On the other hand, the evaluation of this relationship makes it possible to establish the minimum PEF treatment that produces an increase in phenol extraction. For example, as described in Table 17 the EPS1 treatment, of which the electric field intensity was 0.7 kV.cm^{-1}, resulted in a slope (10.41 TPI.day^{-1}) higher than the one for untreated grapes (8.99 TPI.day^{-1}), and significantly similar to the SCA4 at 4 kV.cm^{-1} (11.5 TPI.day^{-1}). Therefore, in view of future attempts to scale up the process, it is of great interest to determine the interactions of the main PEF parameters with the extraction of polyphenols. For this purpose, relationships between field strength, specific energy, and slopes shown in Table 17 were evaluated by a multiple regression analysis based on response surface modelling (RSM).

Response surface modelling

RSM is an excellent tool for the extraction of more complex information: it saves significant experimental time, analysis of materials, and personnel costs (Zhang et al., 2007). Table 18 shows the terms and coefficients of the obtained polynomial *Equation 2*, which relates the TPI extraction slope with the electric field strength (E) and the specific

energy (W) applied in the PEF treatments. The obtained R^2 and R^2-adj, RMSE, and an equation *F*-value of 1,556.2, indicated that the equation was significant ($p<0.01$) and accurately described the observed slopes. As shown, the TPI extraction rate depended on both parameters, but mainly on energy, whereby W and W^2 were the main parameters which exerted an influence on the slope (lowest *p* values). The interaction between both PEF parameters also exerted a noticeable effect.

Table 18: *p*-values for significant variables and their interactions for polynomial equations regarding the effect of PEF treatments (field strength, E, and specific energy, W) on the extraction rate (slope) of polyphenols (TPI).

Term	Coefficients	**-95 %**	**95 %**	***p*-value**
	-0.00458	-1.225	1.216	0.9922
E	-0.432	-0.589	-0.274	0.0016
W	5.365	4.725	6.005	<0.0000
W^2	-0.608	-0.677	-0.539	<0.0000
E*W	0.135	0.111	0.159	0.0001
R^2				0.99
R^2-adj				0.99
RMSE				0.0335
F-value				1,556.2

Figure 25 displays the three-dimensional response surface graph that describes the relationship between all the aforementioned parameters during the maceration/fermentation of PEF treated grapes based on Table 18. At low energy levels, the extraction rate was independent of field strength. However, the slope increased with the field strength when the specific energy augmented. On the other hand, the extraction rate varied notably with the specific energy, mainly at the greatest field strengths. Maximum extraction rates were observed at energy levels of around 4 to 5 $kJ.kg^{-1}$, ranging from 4.4 $kJ.kg^{-1}$ at 1 $kV.cm^{-1}$ (11.9 $TPI.day^{-1}$) to 5.3 $kJ.kg^{-1}$ at 8 $kV.cm^{-1}$ (13.6 $TPI.day^{-1}$), which would correspond with the maximum extraction rate. Based on these results, these energy levels would be the ones most suitable to maximize TPI extraction via PEF at any of the applied electric field strengths.

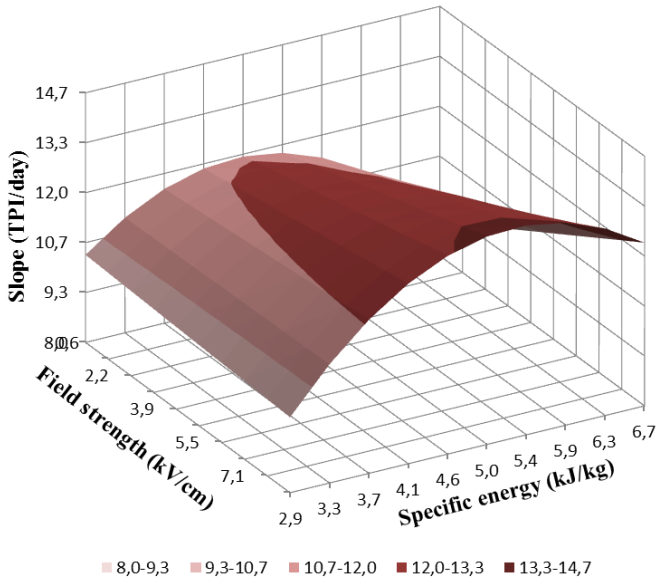

Figure 25: Response surface plots of the influence of electric field strength and specific energy on the extraction rate (slope) of TPI during the maceration/fermentation period.

One of the advantages of RSM is that it enables researchers to establish the values of the factors to obtain a certain level of response. Thus, aiming for a final TPI of 50 (a value selected because it was achieved in all the treatments in the present study including control samples,thereby making results comparable with one another) and aiming for an energy level of 4 $kJ.kg^{-1}$, for example, maceration time could be reduced from 4.1 days (untreated grapes) to 3 days in grapes treated with PEF at 1 $kV.cm^{-1}$ (28 %), to 2.9 days (30 %) at 4 $kV.cm^{-1}$, or 2.7 days (32 %) at 8 $kV.cm^{-1}$ (Figure 26A). If the specific energy were of 5 $kJ.kg^{-1}$, reduction of maceration time would vary from 28 % at 1 $kV.cm^{-1}$ to 32 % at 4 $kV.cm^{-1}$ and up to 37 % at 8 $kV.cm^{-1}$. Additionally, based on *Equation 2*, if one wanted to obtain a TPI of 60, at least 5 days of maceration for control samples could be estimated, again enabling a 32 % time reduction when applying PEF of 4 $kV.cm^{-1}$ and 5 $kJ.kg^{-1}$. That is, in order to achieve a certain TPI value when applying PEF treatments such as those that achieved a TPI of 50, a similar percentage of time reductions in maceration could be determined. From a logistic and economical point of view, it is very important for wineries to be able to reduce maceration time during fermentation without affecting wine quality, as previously mentioned. Such time reduction can allow them to reduce viz. optimize cooling energy and to carry out a number of necessary operations during fermentation, since the number of deposits used for purposes of fermentation would be reduced (Aranda et al., 2005; Genc et al., 2017; Palacios et al., 2009).

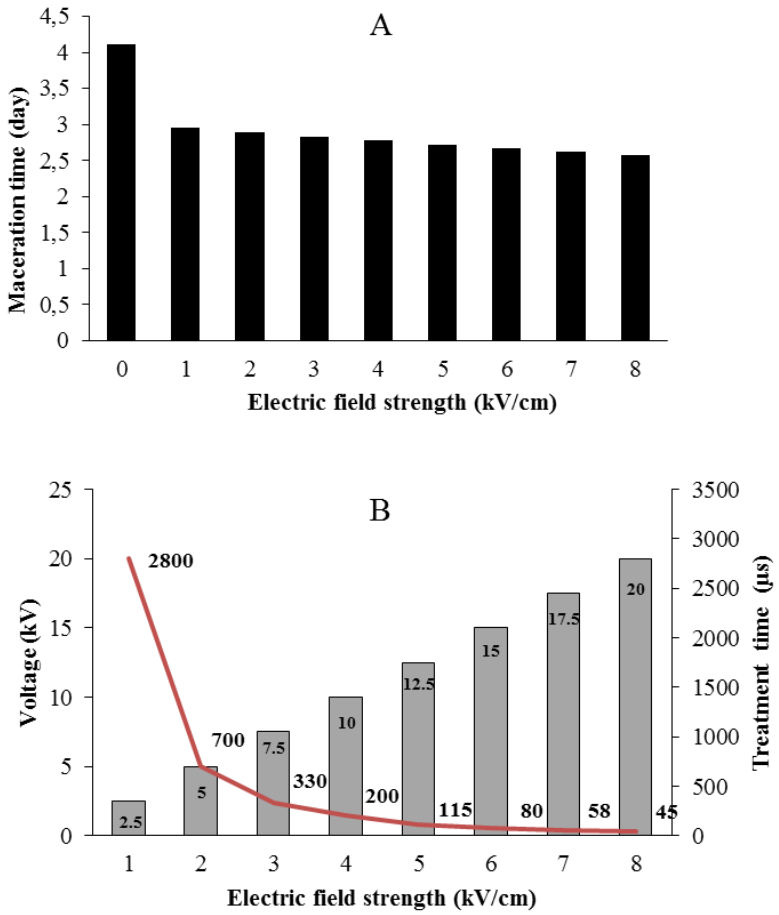

Figure 26: Influence of electric field strength on maceration time (A), and on voltage and treatment time (B) to achieve 50 TPI with PEF treatments of 4 kJ.kg^{-1}.

Independently of the reduction in maceration time, the intensity of the PEF treatments determines the characteristics of a PEF generator, and thereby the cost of the PEF system. Based on the previously determined energy conditions (4 kJ.kg^{-1}) that aim to achieve a TPI of 50 at different field strengths, Figure 26B shows the required working conditions to be applied with a PEF generator in a treatment chamber similar to the one used in this investigation. One can observe that the application of more intense electric field strengths requires higher input voltages and more powerful PEF generators, but with the advantage of shortening the treatment times and therefore the number of pulses as well as pulse frequency for a certain flow. On the contrary, the lower the voltage to obtain a smaller electric field strength, the longer the treatment time to obtain the desired results. Therefore, depending on the PEF treatment conditions one wants to apply in a winery, the PEF generator's intrinsic construction properties will vary and vice versa, as previously indicated. Based on the results shown in Figure 26B, generator voltage can be increased 2 fold by merely applying the treatment at 1 or 2 kV.cm^{-1}, and up to 8 fold if it

is applied at 8 $kV.cm^{-1}$. Regarding treatment time, by increasing field strength from 1 to 2 $kV.cm^{-1}$ time is reduced 4 fold (and up to 62 fold at 8 $kV.cm^{-1}$). This last point is associated with a frequency reduction from 560 Hz to 9 Hz at field strengths varying from 1 to 8 $kV.cm^{-1}$, respectively, if pulses of 5 microseconds are used. In a situation more similar to real circumstances, and using a chamber of 10 cm GAP and 10 cm diameter for a flow of 40 $ton.h^{-1}$ of grapes, the frequency would be 1867 at 1 $kV.cm^{-1}$, 467, 220, 133, 77, 53, 39 and 30 Hz at 2, 3, 4, 5, 6, 7 and 8 $kV.cm^{-1}$, respectively, applying pulses of 10 µs. This is the same reduction as in the previous case. Thus, depending on the power of the PEF generator, PEF treatment conditions could be adapted for effective use in a winery. According to this data, and in order not to require an extremely powerful PEF generator or to apply very few of pulses (thereby limiting treatment homogeneity), treatments of 4 $kV.cm^{-1}$ and 4-5 $kJ.kg^{-1}$ would be the optimal PEF conditions suitable for application in a winery with high flow rates (up to 40-50 $ton.h^{-1}$), using short pulses (i.e. 10 µs). This would also limit electrolysis and electrode wear (Ho and Mittal, 2000; Pataro et al., 2014) and reducing the maceration time 30-32 %.

Effect of PEF on the evolution in bottle.

The potential to reduce maceration time and/or increase the chromatic characteristics of wine at the end of fermentation is a great advantage offered by PEF technology. However, due to the influence of these parameters on the sensory and nutritive quality of red wine, it is essential that they be maintained during aging, or at least evolve similarly to non-PEF treated wines. Previous results in studies on *Cabernet Sauvignon* wines have shown this (Puértolas et al., 2010d). However, no results have been obtained for other grape varieties such as *Grenache*, nor under conditions where the effect of the intensity of PEF treatments observed just after the fermentation process continued during the aging period. Thus, in this investigation, the analytical parameters of wines obtained from untreated grapes and from grapes treated with PEF at different intensities were analyzed after 12 months.

Table 19: TPI, CI, TAC, TC values for PEF-treated and non-PEF-treated wines at the beginning and after 12 months of aging in bottles.

	TPI		IC		Anthocyanins[a]		Condensed tannins[b]	
Time of aging	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**
CONTROL 1	43.70 ± 2.97 a	44.3 ± 0.85a	11.33 ± 0.88a	12.60 ± 1.41a	0.51 ± 0.02a*	0.26 ± 0.03 a	1.21 ± 0.13 a	1.55 ± 0.12a
CONTROL 2	61.10 ± 0.28b*	57.95 ± 0.78b	15.83 ± 0.43b	16.95 ± 0.07b	0.49 ± 0.08a*	0.27 ± 0.03 a	1.82 ± 0.12b*	2.72 ± 0.26b
EPS 1	65.25 ± 6.72bc	61.9 ± 6.93bc	20.75 ± 3.05c	19.15 ± 2.90bc	0.68 ± 0.04b*	0.37 ± 0.02bc	1.88 ± 0.03b*	2.84 ± 0.05b
EPS 4	73.15 ± 3.61d	68.6 ± 1.98cd	21.93 ± 0.54c	20.70 ± 0.28c	0.77 ± 0.04bc*	0.39 ± 0.01c	2.36 ± 0.04c*	3.00 ± 0.16b
SCA 4	71.45 ± 0.78cd	69.55 ± 0.92d	22.44 ± 0.37c	21.80 ± 0.14c	0.79 ± 0.01c*	0.39 ± 0.01c	2.28 ± 0.03c*	2.83 ± 0.01b
SCA 6	65.20 ± 0.71bc	62.10 ± 1.13bc	20.28 ± 1.00c	20.60 ± 0.99c	0.70 ± 0.03bc*	0.35 ± 0.01bc	1.91 ± 0.02b*	2.7 ± 0.28b
SCA 8	64.75 ± 1.34bc	61.30 ± 1.98bc	20.28 ± 0.23c	20.95 ± 0.21c	0.69 ± 0.01b*	0.33 ± 0.02b	1.81 ± 0.28b*	2.65 ± 0.07b

Values represent mean with their standard deviation (n=2)
Different letters within the same file indicate significant differences ($p \leq 0.05$); * indicate significant difference between 0 and 12 months for aging in bottle.
CI: color intensity; TPI: total polyphenol index.
[a] Expressed as malvidin-3-glucoside ($g.L^{-1}$).
[b] Expressed as procyanidin ($mg.L^{-1}$).

The Table 19 shows the TPI, CI, TAC, and TC values of resulting wines at the beginning of bottling and after 12 months aging in bottles. The TPI and CI values remained more or less constant during 12 months of bottle aging.

Anthocyanins decreased during storage, and the value of polymerized tannins (TC) increased, thereby indicating an augmentation of tannin polymerization over time. Independently of the evolution of these parameters, the differences in TPI, CI, TAC, and TC observed between PEF and non-PEF treated samples at the end of the fermentation (8 days) were maintained over a 12-month period. Thus, comparing these results with those shown in Figure 27, positive correlations (R^2>0.80) can be observed between TPI, CI, TAC, and TC at the end of maceration/fermentation with values of wines at 0 and 12 months of storage in bottles (Figure 27).

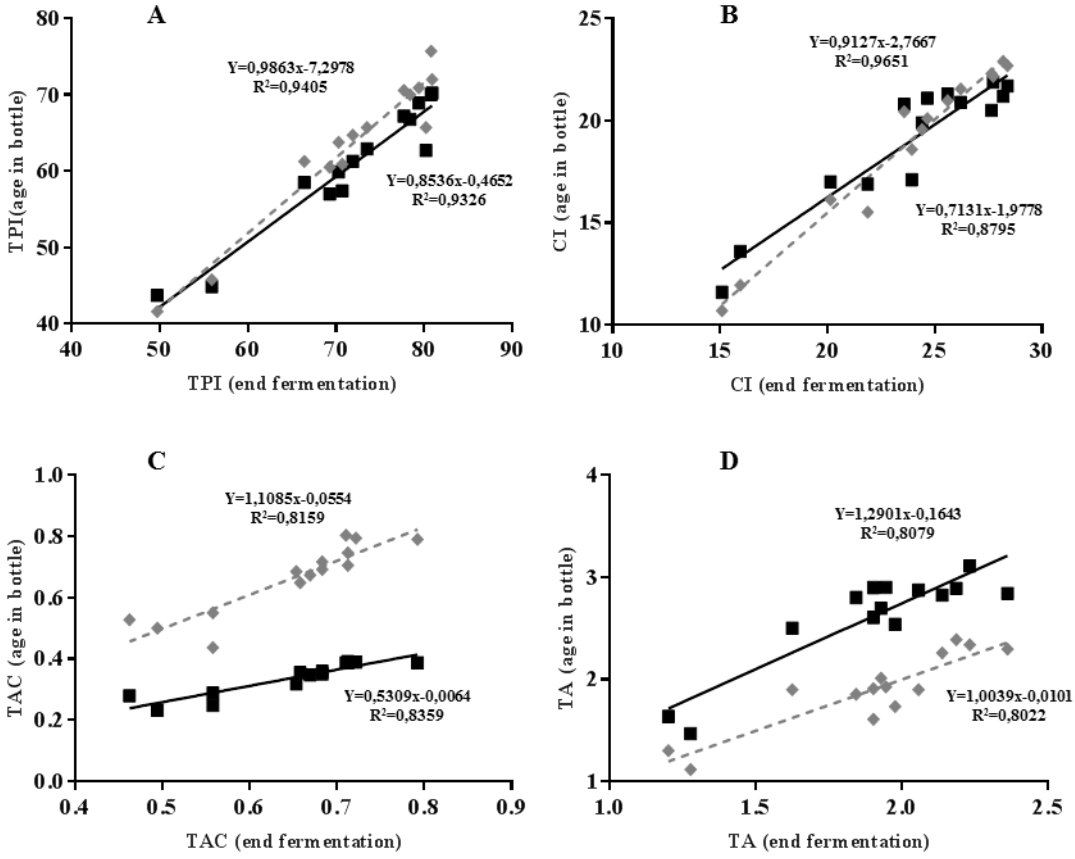

Figure 27: Correlation between TPI (A), CI (B), TAC (C), and TC (D) of PEF-treated wines at the end of the fermentation process with those of PEF-treated wines at the beginning of bottling (♦) and after 12 months of aging in bottle (■).

These results indicate that the conclusions regarding the effect of PEF treatments on the extraction of polyphenols, tannins, color, and anthocyanins during maceration can be extrapolated to the end of the aging period in bottles. Specifically: Control samples showed the lowest values; maceration time exerted a notable influence on wine quality

parameters, and any PEF treatment, even at 0.7 $kV.cm^{-1}$, increased the values of those quality parameters. Based on these results, a treatment of 4 $kV.cm^{-1}$ and 4 $kJ.kg^{-1}$ applying pulses of 10 µs and 4 days of maceration would permit to obtain wines with 12 months of aging in bottles with a TPI of around 62-69, 21 of CI, 0.37 TAC, and 2.8 of TC, e.g. values that lie between the wines obtained from grapes treated with SCA4 and SCA6 after 4 and 6 days of maceration, respectively. Therefore, this relationship would make it possible to determine the optimal PEF treatment conditions to achieve certain final characteristics of a wine. However, more data need to be obtained in order to quantitatively define the effect of PEF in the final colorimetric characteristics of aged wines.

5. Conclusions

The results obtained in this research reveal the need to optimize the process of grape treatment with PEF. Electric field, treatment time, pulse duration, and total specific energy are conditions that play a critical role in obtaining wines with a high concentration of polyphenolic compounds. The extraction rate thereof is determined by intensity of treatment, treatment time, and the number of pulses to which the grapes are subjected. Thus, higher extraction rates of polyphenolic compounds from *Grenache* grapes were achieved with treatments ranging from 4 to 5 $kJ.kg^{-1}$, and varying from 11.9 $TPI.day^{-1}$ at 1 $kV.cm^{-1}$ to 13.6 $TPI.day^{-1}$ at 8 $kV.cm^{-1}$ which resulted in the highest extraction rate in the investigated range of PEF treatment conditions. That PEF energy level reduced maceration time by a rate ranging from 25 to 37 % depending on field strength, without impairing the quality of the wine obtained after fermentation or during the aging process. PEF treatments of 1 $kV.cm^{-1}$ exerted a clear effect; however, they would necessitate very long treatment times, thus requiring PEF generators of very high frequency. Greater field strengths, on the other hand, limit those requirements but require more powerful PEF generators. According to this study, PEF treatments of 4 $kV.cm^{-1}$ and 4-5 $kJ.kg^{-1}$ applied to grapes prior to maceration would be recommendable for wineries that seek to reduce maceration time while achieving adequate red wine quality parameters at the end of the fermentation process and after 12 months of aging. Finally, the effect of PEF intensities observed at the end of the maceration/fermentation step can be extrapolated to the end of the aging period, thereby making it possible to determine optimal PEF processing conditions to obtain a wine with certain desired characteristics.

ESTUDIO 2: Influence of Pulsed Electric Fields on aroma and polyphenolic compounds of *Grenache* wine.

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1. Abstract

The potential of pulsed electric fields (PEF) for improving polyphenol extraction, decreasing maceration and increasing aroma compounds in the course of the winemaking process were investigated. Twelve tons of *Grenache* grapes were processed at a flow rate of 2,500 $kg.h^{-1}$, and wines obtained from untreated and PEF-treated (4.0 $kV.cm^{-1}$) grapes after 3 and 6 days of maceration were compared. The highest values of total anthocyanin, total polyphenolic, tannin content, and color intensity were obtained with PEF-treated grapes as well as when the maceration period was extended. The higher tannin concentration observed in PEF treated wines was a consequence of a higher degree of extraction of tannins located in the grape skins rather than in the seeds. Finally, PEF contributed to the extraction of some individual precursors positive for aroma.

These results, obtained at conditions very close to requirements at industrial scale, may encourage the adoption of PEF technology by wineries.

2. Introduction

Polyphenols and volatile molecules are the main compounds responsible for the quality of red wine. Phenolic compounds play a highly significant role in red winemaking, owing to their contribution to sensory properties (color, flavor, astringency, and bitterness), aging behavior, and their beneficial effect associated with moderate wine consumption (Gawel, 1998; Powers et al., 1988; Ribéreau-Gayon et al., 1983; Singleton, 1992). While anthocyanins are the main factor responsible for the color of red wine, flavan-3-oles and tannins are responsible for its astringency and bitterness (Pinelo et al., 2006). The aroma of young wine emerges mainly in the process of fermentation. Although grapes and must from non-floral grape varietals do not possess intense or explicit flavors, fresh fermented wine has pleasant aromas which can be related to the varietal origin (Delfini et al., 2001). It is estimated that more than 40 different aromatic molecules such as acids, alcohols, esters, aldehydes, terpenols, lactones, norisoprenoids, and volatile phenols are formed or released from precursors during fermentation (Loscos et al., 2007).

As the content of phenolic compounds and aromatic molecules in the juice of red grapes is low, the maceration of the solid parts of the grape berries with the fermenting must is required for the extraction of polyphenols and aromatic precursors. Generally, the solid parts of the grape berries remain in contact with the fermenting must during the entire fermentation process (7-10 days) or even maceration time is extended for several days after fermentation in order to obtain a wine with adequate polyphenol content. However, certain problems regarding the maceration process in wineries have been identified. During maceration, 20 % of the fermentation tanks is occupied by the solid parts of the grapes, thereby reducing the effective volume of the tanks and, as a consequence, the wineries' production capacity. This problem becomes especially significant during the peak of harvesting because reducing maceration time with the purpose of processing all harvested grapes may compromise wine quality. Other negative side-effects of longer maceration periods are related with the difficulty of controlling the fermentation temperature when the solid parts are present in the fermentation tanks, as well as with the workforce and energy consumption required to periodically pump the wine over the skin mass that rises to the top of the fermentation tanks.

Several different physical techniques based on heating such as thermovinification and flash expansion are currently being applied in wineries in order to improve extraction of polyphenols and to reduce maceration time. However, these techniques present a series

of problems, such as the difficulty involved in stabilizing the extracted phenolic compounds during aging (Morel-Salmi et al., 2006; Samoticha et al., 2017), the loss of varietal aromas (Geffroy et al., 2015) and the consumption of high quantities of energy.

Pulsed electric field treatment (PEF) consists of the application of very short electric pulses (in the range from µs to ms) at electric field intensities higher than 1 $kV.cm^{-1}$. These treatments cause the non-thermal irreversible permeabilization of plant tissue cells facilitating the extraction of cytoplasmic content. Different studies have demonstrated that the application of a PEF treatment to the grapes increases the extraction rate of phenolic compounds during the maceration-fermentation step in red winemaking (Cholet et al., 2014; Donsì et al., 2011; El Darra et al., 2016; López et al., 2008b; Luengo et al., 2014; Puértolas et al., 2010a).

However, these investigations have generally been conducted on a laboratory or pilot plant scale in batch or in continuous flows, under processing conditions far removed from the requirements of wineries. Furthermore, very few investigations on the effect of PEF treatments on the wine aroma compounds have been conducted until now (Garde-Cerdán et al., 2013).

The objective of this investigation was to conduct a semi-industrial study in a winery in order to evaluate the effect of PEF on the extraction of polyphenols during maceration-fermentation step of the elaboration of *Grenache* wine and on the volatile composition of obtained wine.

3. Material and methods

Samples

Grapes of *Vitis vinifera* var. *Grenache* (D.O Campo de Borja, Fuendejalon, Spain 41º 43´58" N 1º23´37´´O) harvested manually from the 2016 vintage in their optimal ripening stage and in good sanitary conditions were used in this investigation. Grapes were transported to the winery on 3,000-kg trailers. Physical and chemical characteristics of grapes and must are shown in Table 20.

Table 20**:** Mean values and standard deviations of physicochemical characteristics of grapes at harvesting time.

Probable alcohol (% $v.v^{-1}$)	18.1±0.04
pH	3.45±0.35
Total acidity ($g.L^{-1}$)[a]	4.99±0.16
^{o}Brix	27.9±0.17
Total phenols (OD 280 nm)	12.45 ±0.04
Colour intensity	3.05±0.07
Conductivity ($mS.cm^{-1}$)	1.38±0.04

[a] Expressed as tartaric acid

PEF equipment

PEF equipment consisting of a EPULSUS® PM1-10 modulator (supplied by Energy Pulse Systems LDA, Lisbon, Portugal), with a maximum output voltage and current of 10 kV and 240 A, respectively, was used in this investigation. The apparatus generates monopolar square waveform pulses of 1 to 200 μs with a frequency of up to 200 Hz. The voltages applied were measured with a high voltage probe (Tektronix, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronix, TBS 1102B-EDU). The treatment chamber consisted of three cylindrical stainless steel electrodes separated by two polyoxymethylene insulators. Whereas the central electrode is connected with high voltage, the electrodes of the two extremes are grounded. This colinear configuration delimited two cylindrical treatment zones of 2.5 cm of gap and 3.5 cm of diameter.

Vinification and PEF treatment

This study was conducted in the facilities of the winery "Bodegas Aragonesas" (Fuendejalon, Spain). After destemming and crushing, the grape mass was pumped through the colinear treatment chamber by a peristaltic pump (Rotho MS1, Ragazzini, Faenza, Italy) at a mass flow rate of 2,500±100 $kg.h^{-1}$. This flow corresponds with a residence time of 0.09 s in the treatment zone. The PEF treatments consisted in 3.7 pulses of a width of 100 μs at electric field strengths of 4 $kV.cm^{-1}$. After treatment, the grape mass was pumped over to the fermentation maceration tanks. A total of 12 tons of grapes were processed: they were distributed among 4 fermentation tanks. Two of the four tanks contained 3,000 kg of PEF treated grapes, and the two other tanks were filled with untreated grapes that had passed through the PEF treatment chamber with the PEF modulator off. After the grapes were introduced in the tanks, they were immediately treated with a dose of 20 $mg.kg^{-1}$ of $K_2S_2O_5$ and, after 12 h, the tanks were inoculated

with commercial *Saccharomyces cerevisiae* yeast strain (15 mg.kg^{-1} OenoFrance, La Marquise E491, Epernay, France). Fermentation temperature was controlled at 25±2ºC, and must density was monitored once daily. Maceration times assayed for untreated and PEF treated grapes were 3 days (C-3, PEF-3) and 6 days (C-6, PEF-6). The mash was subsequently drawn off to remove the skins, and free-run musts were left to conclude the fermentation at 20±2ºC. At the end of fermentation, which occurred after 10 days, the wine was treated with sulfites (100 mg.L^{-1} $K_2S_2O_5$) and kept at 4ºC for 1 month for purposes of tartaric stabilization. Finally, the wine was bottled and stored in a dark chamber at 16±1ºC.

General wine analysis

The pH of must was determined by a pHmeter (Crison Basic 20, Crison Instruments, Spain). The total soluble matter (ºBrix) of must was measured with an Atago PR101 refractometer (Atago, Japan). Total acidity and alcohol were determined according to procedures described in the methods of the Organisation Internationale de la Vigne et du Vin (2009). Residual sugar was determined according to Cobos et al, (2017).

Spectrophotometric determinations

All samples were centrifuged in an Eppendorf AG centrifuge for 15 min at 3000 rpm (Eppendorf, Hamburg, Germany), and absorbance measurements were carried out in a Biochron Libra S12 spectrophotometer (Biochron, Cambridge, UK).

Colorimetric indexes

The absorbance of the musts was measured at 420, 520, 620 nm using quartz cells of 1 mm path length. The following variables were calculated: color intensity (CI) as the sum of 420 nm, 520 nm and 620 nm absorbance; hue (Tint) as the proportion of the absorbance measured at 420 nm and 520 nm; proportion of yellow colour (%Ye) as the relation between 420 nm absorbance and colour intensity; proportion of red color (%Rd) as the relation between 520 nm absorbance and color intensity; and proportion of blue color (%Bl) as the relation between 620 nm and color intensity according to Glories (1984).

CIELAB parameters

The wine color was further assessed with CIELAB parameters. The absorbance of the musts was measured at 450, 520, 570 and 630 nm using quartz cells of 1 mm path

length. Lightness (L*), chroma (C*), hue (h*), red-greenness (a*) and yellow-blueness (b*) were determined according to the methods of Ayala et al. (1997), and data processing was performed with MSCV software (Ayala et al., 2001).

Total polyphenol index

The total polyphenol index (TPI) was determined by direct reading of the absorbance at 280 nm of diluted wine 1/100 ($v.v^{-1}$) using quartz cells of 1 cm path length. TPI was calculated by multiplying the absorbance measured at 280 nm by 100.

Total anthocyanins

Total anthocyanins (TAC) expressed in milligrams per liter of malvidin-3-glucoside were analyzed by determining the absorbance at 520 nm of diluted wine 1/100 ($v.v^{-1}$) with 1 % ($v.v^{-1}$) HCl (Ruiz-Hernández, 2004).

Determination of proanthocyanidins

Determination of tannins condensed (TC) in wine samples was determined according to Sarneckis et al., (2006). Determination of the apparent mean degree of tannin polymerisation (mDP), concentration and % of epigallocatechin, and percentage of galoylation were conducted according to (Busse-Valverde et al., 2010). Briefly, the analyses were carried out by depolymerizing the molecule using a phloroglucinol reagent. The depolymerized samples (10 µL injection volume) were analyzed by HPLC (Waters 2695 system, Waters, Milford, USA). Tannin cleavage products were estimated by applying their response factors relative to (+)-catechin, which was used as the quantitative standard.

Analysis of volatile compounds

Quantitative analysis of major compounds was carried out using the method proposed and validated by Ortega (Ortega et al., 2001). In accordance with this method, 3 mL of wine and 7 mL of water were added with 4.5 g of ammonium sulfate and extracted with 200 µL of dichloromethane. The extract was then analyzed by GC with FID detection, applying conditions described elsewhere (Ortega et al., 2001). Quantitative data were obtained by interpolation of relative peak areas in the calibration graphs built by the analysis of synthetic wines containing known amounts of the analytes. Internal standards used were 2-Butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone,

and 2-octanol dissolved in dichloromethane at a concentration of 200 μg.g^{-1}. The extract was analyzed by GC with FID detection.

Analysis of minor volatile compounds was carried out using the method proposed and validated by López et al, (2002), with the following modifications: standard SPE cartridges (1 mL total volume) filled with 200 mg of LiChrolut EN resins were placed in the vacuum manifold extraction system (Varian Sample preparation products), and the sorbent was conditioned by rinsing the cartridges with 4 mL of dichloromethane, 4 mL of methanol, and, finally, with 4 mL of a water-ethanol mixture (12 %, v.v^{-1}). The cartridges were then loaded with 50 mL of wine sample and 26 μL of a surrogate standard solution (recovery standard) containing 3-octanone, *β*-damascone and heptanoic acid (all at 200 μg.g^{-1} of ethanol). This mixture was passed through the SPE cartridges (2 mL.min^{-1}), followed by a wash step using 5 mL of 30 % water-methanol, 1 % $NaHCO_3$ solution. The resins were then dried by letting air pass through them (negative pressure of 0.6 bar, 10 min). Analyses were recovered in a 2 mL vial by elution with 1.6 mL of dichloromethane. Thirty-four microliters of an internal standard solution (300 mg.L^{-1} of 4-hydroxy-4-methyl-2-pentanone and 2-octanol) were added to the eluted sample. The extract was then analyzed by GC with ion trap MS detection (GC-450 gas chromatograph fitted to a Varian Saturn 2200 ion trap MS).

Statistical analysis

Analysis of variance (ANOVA) and principal component analysis were carried out using InfoStat statistical software in the 2011. The statistical significance of each selected attribute was calculated according to Tukey's test ($p \leq 0.05$).

4. Results

Effect of PEF on oenological parameters of Grenache wines obtained with 3 and 6 days of maceration.

Oenological parameters after wine stabilization before bottling of the control wine and the wine obtained from grapes treated by PEF with a maceration of 3 and 6 days are shown in Table 21 Neither the duration of the maceration, nor the PEF treatment applied to the grapes before fermentation/maceration affected the ethanol content, pH and total volatile acidity significantly. Most studies conducted with different grape varieties have shown that these oenological parameters are not affected by PEF (Ricci et al., 2018).

Generally, when it has been reported that pH and total acidity were significantly affected by PEF, the differences observed were so low that they did not have practical implications (Garde-Cerdán et al., 2013).

Table 21: Oenological parameters of control wine and wines obtained with grapes treated by PEF after 3 and 6 days of maceration.

	3 maceration days		6 maceration days	
	Control	**PEF**	**Control**	**PEF**
TPI (A.U)	39.6±0.71 a	51.3±0.14 c	46.9±0.07 b	64.3±0.07 c
CI (A.U.)	8.0±0.22 a	12.1±0.11 c	11.6±0.14 b	14.4±0.02 d
TAC (mg.L^{-1}) [a]	657.8±7.17 a	777.5±1.57 b	814±17.38 c	840.3±11.22 c
TC (mg.L^{-1}) [b]	987.6±25.03 a	1408.8±250.3 ab	1313.3±20.02 b	1458.4±155.2 b
Hue (A420/A520)	0.543±0.02 c	0.508±0.01 b	0.485±0.03 a	0.483±0.05 a
(%Ye)= (A420/CI x 100)	31.8±0.01 c	30.4±0.20 b	29.9±0.24 ab	29.7±0.39 a
(%Rd) = (A520/CI x 100)	58.6±0.63 a	59.9±0.52 b	61.6±0.22 c	61.5±0.04 c
(%Bl) = (620/CI x 100)	9.6±0.62 a	9.7±0.32 a	8.5±0.47 a	8.8±0.43 a
L*	76.2±0.14 a	65.7±0.07 b	67.7±0.28 c	61.6±0.14 d
C*	30.2±0.37 a	41.6±0.01 b	42.2±0.19 b	48.5±0.41 c
a*	30.2±0.34 a	41.6±0.01 b	42.1±0.19 b	48.4±0.39c
b*	-1.5±0.73 a	-1.3±0.29 a	-2.5±0.01 a	-1±1.03 a
pH	3.26±0.01	3.28±0.01	3.28±0.01	3.29±0.01
Alcohol (%v.v^{-1})	17.75±0.07	17.85±0.07	17.45±0.07	17.95±0.07
Total acidity (gr.L^{-1})[c]	4.4±0.15	4.2±0.1	4.2±0.2	4.1±0.15

Values represent mean with their standard deviation (n=2)
Different letters within the same file indicate significant differences ($p \leq 0.05$).
TPI: total polyphenol index; CI: color intensity; TAC: total anthocyanins content; TC: tannins condensed; %Ye, %Rd, %Bl: percentages of yellow, red, and blue colors respectively; (L*): lightness, (C*). chroma, (a*): red-greenness and (b*): yellow-blueness; A.U.: absorbance units.
[a] Expressed as malvidin-3-glucoside.
[b] Expressed as epicatechin.
[c] Expressed as tartaric acid

Oenological parameters related with the extraction of polyphenols such as TAC, TPI, TC and CI were significantly affected by both PEF treatment and maceration time. Wines obtained from untreated grapes after 6 days of maceration and from grapes treated by PEF after 3 and 6 days of maceration had a similar or even higher value on these indexes than other *Grenache* wines reported in the literature (López et al., 2008a; López-Alfaro et al., 2013). However, these indexes were lower for the wine obtained with untreated grapes after 3 days of maceration.

In general terms, the highest values of TAC, TPI, TC and CI were observed when the grapes were treated by PEF, as well as when the maceration periods were extended from 3 to 6 days. TPI for the wine obtained from grapes treated by PEF with 6 days of maceration were around 64 % higher than in the control wine with the shorter maceration, and around 35 % higher than in the control wine with the longer maceration. On the other

hand, the TPI of the PEF wine with 3 days of maceration was around 30 and 10 % higher than the untreated wine with 3 and 6 days of maceration, respectively. The highest TC and CI levels were also observed in the wine obtained from grapes treated by PEF after 6 days of maceration. The TC and CI values of this wine were around 11 and 24 % greater, respectively, than the wine obtained after the same maceration time with untreated grapes, and more than 50 % greater than the control wine with 3 days of maceration. Smaller differences for these indexes were observed among the wines obtained with grapes treated by PEF. No significant differences were observed between the TC of the two wines, whereas the CI of the control wine with 3 days of maceration was 19 % lower.

Different maceration times and the application of a PEF treatment to the grapes did not result in any important influence on tint %Ye, %Rd and %Bl. Similar values were obtained in the control wines and in the PEF wines that means that PEF increased the extraction of those components responsible for color intensity, but the proportion of the extracted compounds did not change.

CIELAB parameters are correlated with CI. The C-3 wine featuring the lower CI had the highest L* (lightness) value, and the lowest a*(redness) and c*(chroma) values. On the other hand, no significant differences were observed in the h* (hue) and b*(yellow) values of the four wines. Finally, although the CI of the PEF-3 wines was slightly higher than the CI of wine obtained from untreated grapes with 3 days more of maceration, no statistically significant differences were observed among the CIELAB parameters of the two wines. Therefore, PEF treatment allowed for a reduction of maceration time from 6 to 3 days without negatively affecting chromatic parameters.

Determination of tannin polymerization

The mean degree of polymerisation of tannins, the percentage of galloylation, and the concentration of epigallocatechin for the four wines are shown in Table 22. Epigallocatechin content permits to ascertain the proportion of skin tannins in wine, since this compound is only contained in the tannins extracted from grape skin but is absent in seed tannins.

The average degree of tannin polymerization (mDP) was neither affected by PEF treatment, nor by maceration time. Similar values of this index were found in the four wines obtained in this investigation. On the other hand, although certain differences were observed in the percentage of galloylation between wines obtained from untreated and PEF treated grapes, such differences were very small and did not have practical

implications. These two indexes would indicate that the higher tannin concentration observed in both wines obtained from grapes treated by PEF was brought about by a greater extraction of tannins located in the grape skins rather than in the seeds. This observation is supported by the higher epigallocatechin content in the wines obtained from grapes treated by PEF than in the control wines.

Table 22: Concentration of proanthocyanidins of control wine and wines obtained with grapes treated by PEF after 3 and 6 days of maceration.

	3 maceration days				6 maceration days			
	Control		PEF		Control		PEF	
mDP	4.25 ±0.07 a		4.25 ±0.07 a		4.05 ±0.07 a		4.25 ±0.07 a	
%EGC	22.30 ±1.60 a		20.75 ±0.92 a		20.05 ±0.49 a		21.80 ±0.28 a	
%Galloylation	3.75 ±0.07 a		4.3 ±0.1 b		4.3 ±0.00 b		4.85 ±0.07 c	
EGC (µM)	396.9 ±16.4 a		539.5 ±24.1 b		458.1 ±14.4 a		578.3 ±20.8 b	

Values represent mean with their standard deviation (n=2)
Different letters within the same file indicate significant differences ($p \leq 0.05$).
mDP: mean degree of polymerisation; EGC: epigallocatechin.

Effect of PEF on volatile aroma compounds of Grenache wines obtained with 3 and 6 days of maceration

Concentration of aroma compounds in the *Grenache* wines obtained with untreated and PEF treated grapes with a maceration of 3 and 6 days are displayed in Table 23 and features the major aroma compounds generated during alcoholic fermentation as a consequence of yeast metabolism, Table 23 and Table 24 displays the minor aroma compounds liberated from the precursors present in the grapes.

Table 23: Concentration of major aromatic compounds (mg.L^{-1}) of control wine and wines obtained with grapes treated by PEF after 3 and 6 days of maceration.

	Odor threshold	3 maceration days		6 maceration days	
		Control	PEF	Control	PEF
CARBONYL					
Acetaldehyde	0.5	0.52a	0.53a	0.47a	0.56a
Diacetyl	0.1	0.9a	0.99a	1.19a	1.14a
Acetoine	150	4.86b	5.64c	3.27a	3.44a
Sum of carbonyl		*6.37*	*7.16*	*4.93*	*5.14*
ACETATES					
Ethyl acetate	12.3	50.4b	54.8b	43.9a	43.6a
Isoamyl acetate	0.03	1.87c	1.79c	1.33b	1.01a
Hexyl acetate	1.5	0.04a	0.05a	0.04a	0.04a
Sum of acetates		*52.3*	*56.7*	*45.3*	*44.7*
LINEAL ETHYL ESTERS					
Ethyl butyrate	0.125	0.18b	0.18b	0.17ab	0.14a
Ethyl hexanoate	0.062	0.53b	0.38ab	0.44a	0.39a
Ethyl octanoate	0.58	0.53b	0.27ab	0.43a	0.27a
Ethyl decanoate	0.2	0.04	0.03	0.02	0.02
Sum of lineal ethyl esters		*1.29*	*0.86*	*1.06*	*0.80*
ALCOHOLS					
Isobutanol	40	22.4a	20.6a	21.6a	20.7a
1-Butanol	150	3.17a	3.39a	3.30a	3.37a
Isoamyl alcohol	30	274c	258bc	248ab	235a
1-Hexanol	8	1.83b	1.95b	2.47a	2.65a
C-3-Hexenol	0.4	0.03b	0.03ab	0.02ab	0.02a
Metionol	1	1.50b	0.83a	0.73a	0.72a
Benzylic alcohol	200	0.36a	0.36a	0.42a	0.35a
β-Phenylethanol	14	40.0c	30.9b	29.2ab	27.6a
Sum of alcohols		*344*	*316*	*306*	*290*
MISCELLANEOUS ESTERS					
Ethyl lactate	154	8.86b	9.83ab	8.14a	7.61a
Diethyl succinate	200	0.76	0.79	0.59	0.44
Sum of miscellaneous esters		*9.6*	*10.6*	*8.74*	*8.05*
ACIDS					
γ-Butyrolactone	35	12.4b	12.1b	7.57a	7.31a
Acetic acid	300	619c	849b	433a	455a
Butyric acid	0.17	1.37a	1.24a	1.18a	1.18a
Isobutyric acid	3.3	1.16a	1.17b	0.92a	1.03a
Isovalerianic acid	0.03	1.73a	1.64a	1.60a	1.66a
Hexanoic acid	0.42	2.02d	1.44c	1.98b	1.83a
Octanoic acid	0.5	3.52d	2.26c	2.82b	2.49a
Decanoic acid	1	0.51b	0.28a	0.24a	0.22a
Sum of acids		*10.3*	*8.0*	*8.7*	*8.4*
Sum of major aromatic compounds		**437,25**	**412,13**	**382,88**	**365,69**

Values represent mean (n=2)
Different letters within the same file indicate significant differences ($p \leq 0.05$).

Table 23 shows that the content of all major aromatic compounds was greater in the wines that had undergone longer maceration; very small differences were observed, however, among the wines obtained with untreated and PEF treated grapes subjected to the two maceration times investigated. On the other hand, regarding the content of the different groups of major aromatic compounds, statistically significant differences were

only observed between the control wine with 3 days of maceration and the other wines in the total content of lineal ethyl esters, alcohols, and acids. Ethyl hexanoate, ethyl octanato, and ethyl decanoate were the aroma compounds of the lineal ethyl esters group that were more abundant in this wine but the concentration of ethyl octanate nevertheless lay below the odor threshold. Isoamyl alcohol, metionol, and *β*-phenylethanol were the molecules that exceeded the olfactory threshold responsible for the highest content of superior alcohols in the control wine with 3 days of maceration. While the odor descriptor of *β*-phenylethanol is roses, the descriptors for the other molecules are related to "cheesy" foot odor, solvent, and baked potato. For the remainder of the analyzed compounds, significant differences between the control wine with the lowest maceration time and the rest of wines were only observed in the case of ethyl hexanoate and ethyl lactate. Therefore, in general terms, it can be concluded that the differences among aroma compounds generated in the course of alcoholic fermentation are minimal in the four wines. The differences are especially small among the wines obtained with grapes treated by PEF and the control wine with 6 days of maceration, which were also the wines with the highest index values related to polyphenol extraction. Apparently, therefore, PEF treatment did not affect the normal development of fermentative aromas during vinification.

Aroma compounds liberated from the precursors in the grapes are present in "trace" concentrations ($\mu g.L^{-1}$) in the wine. As displayed Table 24 the total content of minor aromatic compounds was around 15 % higher for the wines obtained with grapes treated by PEF at both maceration times in comparison with control wines. The content of the different groups of minor aromatic compounds is manifest at a higher concentration of branched ethyl esters in the control wine with 3 days of maceration, but the latter displayed the lowest concentration of norisopreoids and volatile phenols. On the other hand, despite the fact that the content of acetates was significantly higher in 3 day macerated wines, their concentration was far below the odor threshold. Regarding the individual molecules analyzed, significant differences in the concentration of 8 molecules were ascertained when the control wines were compared with PEF treated, 3 day macerated wine

Table 24: Concentration of minor aromatic compounds ($\mu g.L^{-1}$) found in control wines and wines obtained with grapes treated by PEF after 3 and 6 days of maceration.

	Odor threshold	3 maceration days		6 maceration days	
		Control	PEF	Control	PEF
BRANCHED ETHYL ESTERS					
Ethyl isobutyrate	15	29.64b	15.38a	16.14a	15.48a
Ethyl 2-methylbutyrate	18	4.03c	3.34b	2.84ab	2.79a
Ethyl isovalerate	3	11.1b	13.04c	9.37a	9.97ab
Sum of branched ethyl esters		*44.8*	*31.8*	*28.3*	*28.2*
ACETATES					
Isobutyl acetate	1600	14.4b	11.43a	11.64a	11.01a
Butyl acetate	1800	6.68a	6.81a	5.2a	5.5a
Phenylethyl acetate	250	102.65d	92.11c	58.77b	45.74a
Sum of acetates		*123.7*	*110.3*	*75.6*	*62.2*
MONOTERPENOLS					
Benzaldehyde	2000	1.16a	1.4a	1.25a	1.14a
Linalool	25	7.48a	8.07a	8.3a	7.96a
Linalool acetate	-	1.54b	1.29b	1.24b	1.42ab
α-Terpineol	250	3.24a	3.06a	3.08a	2.96a
Geraniol	20	10.78a	10.56a	11.92a	11.01a
Sum of monoterpenols		*23.0*	*22.9*	*24.5*	*23.3*
NORISOPRENOIDS					
β-Damascenone	0.05	0.47a	0.52a	0.94a	0.58a
β-Ionone	0.09	nd	0.51	nd	0.47
Sum of norisoprenoids		*0.4*	*1.0*	*0.9*	*1.0*
VOLATILE PHENOLS					
Guaiacol	9.5	13.14a	18.49a	18.4a	24.6a
o-Cresol	31	3.31a	3.06a	2.84a	2.61a
4-Ethylguaiacol	33	0.4a	0.84a	0.83a	0.78a
Eugenol	6	2.97a	2.03a	1.87a	2.07a
4-Ethylphenol	35	0.48a	0.89b	0.79b	0.87b
4-Vinylguaiacol	40	109.7a	223.05ab	211.7ab	299.35b
E-Isoeugenol	6	19.7a	15.2a	13.72a	15.33a
2.6-Dimethoxyphenol	570	45.56a	57.88ab	50.63ab	70.79b
4-Vinylphenol	180	98.59a	141.88b	145.35b	215.67c
4-Alyl-2.6-dimethoxyphenol	1200	7.22 b	5.28a	6.14ab	5.28a
Sum of volatile phenols		*301.1*	*468.6*	*452.2*	*637.4*
CINAMATES					
Ethyl dihidrocinnamate	1.6	0.93a	0.83a	0.85a	0.75a
Sum of cinamates		*0.9*	*0.8*	*0.8*	*0.7*
LACTONES					
γ-Nonalactone	25	22.59a	30.53c	23.78a	26.18b
Sum of nonalactone		*22.6*	*30.5*	*23.8*	*26.2*
VANILLIN DERIVATIVES					
Vanillin	995	30.85ab	21.93a	33.04b	25.52ab
Methyl vanillinate	990	35.11b	33.22b	26.1a	24.93a
Ethyl vanillate	3000	60.51a	54.81a	91.82b	103.44b
Acetovanillone	1000	213.17a	212.79a	249.91a	243.89a
Sum of vanillin derivatives		*339.6*	*322.8*	*400.8*	*397.8*
Sum of minor aromatic compounds		**857.9**	**990.2**	**1009.1**	**1178.3**

Values represent mean (n=2)
Different letters within the same file indicate significant differences ($p \leq 0.05$).
n.d.: not available

Significant differences in the concentration of 2 molecules were likewise ascertained when the control wines were compared with the PEF treated, 6-day macerated wine. It is notable that, in the wines obtained with PEF treated grapes, much higher concentrations of 4-vinylguaiacol (clove and curry descriptors) and *γ*-nanolactone (coconut and peach descriptors) were detected. The concentration of 4-vinylguaiacol in the wine obtained with grapes treated by PEF with only 3 days of maceration (223 $mg.L^{-1}$) was similar to control wine with 6 days of maceration (212 $mg.L^{-1}$), but twice greater than the concentration in the control wine with the shorter maceration (110 $mg.L^{-1}$), and 34 % lower than the wine obtained with grapes treated by PEF with 6 days of maceration (299 $mg.L^{-1}$). On the other hand, while the concentration of *γ*-nanolactone in the control wines lay below the odor threshold, the higher concentration of that molecule in the wines obtained with PEF treated grapes allowed it to exceed the minimum odor threshold. Therefore, PEF could contribute to the extraction of some individual aroma precursors present in grapes, thereby increasing the concentration of some molecules which, once liberated from the precursors, can exert a positive effect on wine aroma.

Principal component analysis of wines obtained with untreated and PEF treated Grenache grapes after 3 and 6 days of maceration

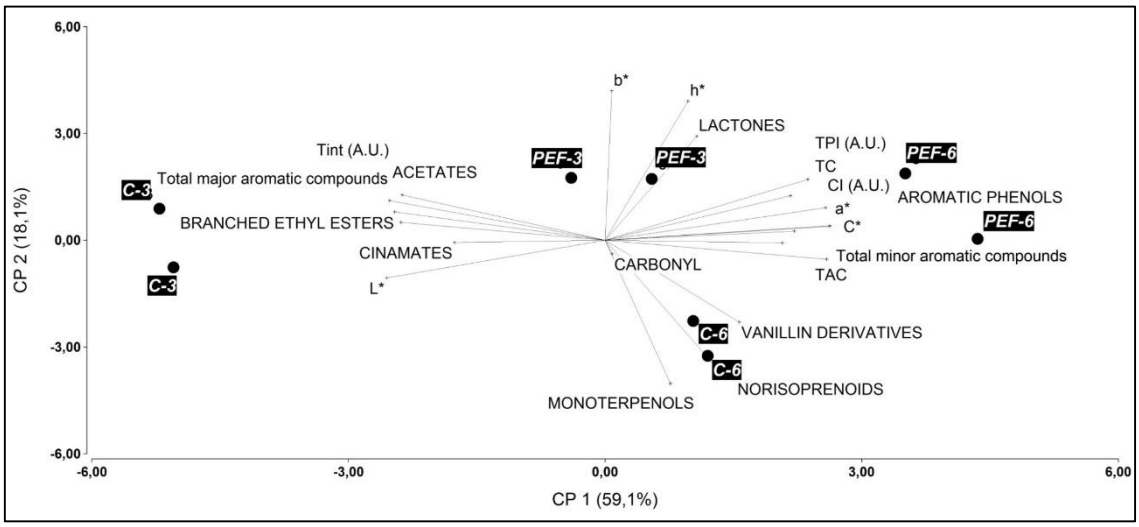

Figure 28: Biplot representation of the principal component analysis, showing the distribution of the wine samples along components 1 and 2. (C-3): control wine with 3 days of maceration, (C-6): control wine with 6 days of maceration, (PEF-3): wines obtained from grapes treated by PEF with 3 days of maceration, (PEF-6): wines obtained from grapes treated by PEF with 6 days of maceration.

A principal component analysis was conducted in order to differentiate the wines elaborated with non-treated and PEF treated grapes as a function of wine parameters depending on polyphenol extraction (TAC, TPI, CI, TC, Tint), the sum of the total major and minor aromatic compounds, and the sum of the major families of minor aromatic

compounds. Given the large number of volatile compounds identified, and in order to simplify analysis, only families of minor aromatic compounds were used, since the release of precursors of volatile molecules located in the grape skin could be affected by both PEF treatment and maceration time. Principal component analysis showed that two principal components explained 77.2 % of the variability of the data.

Figure 28 illustrates the differences among the four wines: the wine obtained from untreated grapes and 3 days of maceration (which correspond with the wine with the lowest chromatic parameters) was clearly separated along PC1 from the other 3 wines, but especially from the PEF-6 wine, which featured the highest chromatic parameters. On the other hand, the PEF-3 wine was closer to the other two wines, especially to the C-6. The typical duration of maceration in the traditional process of winemaking is 6 or even more days. The application of a PEF treatment to the grapes permitted to obtain a wine with chromatic and aromatic characteristics similar to a wine with typical maceration conducted in a winery, but reducing maceration time by 3 days. In the case of extending the maceration of the wine obtained from PEF treated grapes from 3 to 6 days, the PEF treatment can help deliver a wine with a higher content of polyphenols and tannins i.e. compounds that need to be in high concentration in order to obtain quality aged wines in oak barrels.

5. Discussion

The effect of electroporation induced by PEF with the purpose of accelerating and/or increasing the extraction of phenolic compounds during red winemaking has been deeply investigated. Studies conducted with different grape varieties (*Cabernet Sauvignon, Merlot, Syrah, Grenache, Mazuelo, Graciano, Aglianico, Piedirosso*) harvested in different countries (Spain, France, Lebanon, Italy, and New Zealand) have concluded that PEF improves the permeability of the cytoplasmic membranes of the hypodermal cells of grapes, there by facilitating the release of polyphenols located in the cytoplasm (Puértolas et al., 2016). However, most of those investigations were conducted by applying PEF treatments in batch or in continuous processing at few $kg.hour^{-1}$. These are processing conditions far removed from the actual requirements of wineries. In this research, the positive effects reported on the enhancement of polyphenol extraction during red winemaking under processing conditions close to those required for wineries using one of the most widely cultivated grapes worldwide (*Grenache*) have been validated.

Results obtained in this investigation confirm that the application of a PEF treatment at flows of tons per hour brought about the electroporation of the grapes and improved the extraction of polyphenols during maceration-fermentation in tanks containing three tons of grapes. The pre-treatment of grapes by PEF permitted to reduce maceration time from 6 to 3 days without decreasing the color or the concentration of polyphenolic compounds in red wine; alternatively, it permitted to increase those parameters when maceration time was extended to 6 days. Similar effects on improving polyphenol extraction have been recently reported by treating grapes by ultrasound before fermentation, a study featuring Monastrel grapes at 400 $kg.h^{-1}$ (Bautista-Ortín et al., 2017). While the effect of ultrasound was attributed to a disruption of grape cell structures, PEF only brought about the formation of pores in the cytoplasmic membrane but left the cell structure intact.

It is remarkable to note that PEF not only improved the extraction of anthocyanins and total polyphenols, but also the extraction of tannins, even when the maceration period was shorted to 3 days. In typical vinification, most of the wine tannins are extracted from the seeds that are also in contact with the fermenting must, since the tannin concentration in the skins is lower (Pinelo et al., 2006). The presence of tannins in red wine is essential, not only because of its contribution to taste, but also in view of the development of red polymeric pigments by association with anthocyanins that stabilize wine color during aging. One of the drawbacks associated with processes aiming to reduce maceration time is that the short period of contact of the solid parts of the grapes with the fermenting must does not result in wines with elevated tannin content because the extraction of tannin from the berry seed requires the presence of ethanol. The high level of tannins in the PEF-3 wine, as observed in our analysis, was due to the fact that PEF treatment considerably increased the extraction of tannins contained in the grape skin. The improvement of tannin extraction from grape skin could have a beneficial effect on wine quality. While seed tannins are associated with “green” or “hard” sensory descriptors, skin tannins are associated with more desirable sensory descriptors, such as “soft” or “ripe”.

The effect of PEF on the volatile composition of wine has only been previously investigated by Garde-Cerdán et al. (Garde-Cerdán et al., 2013). Those authors reported that the volatile composition of *Tempranillo* and *Graciano* wines was not improved by treating the grapes with PEF, but that the aromatic characteristics of *Grenache* wine were improved by increasing the concentration of monoterpenoids, *β*-ionone, total esters, and volatile phenol compounds. In our case, no increment in the concentration of

monoterpenoids and total esters by application of a PEF treatment was observed. However, the concentration of β-ionone associated with the floral aroma of "violets" that had gone undetected in the control wines was indeed observed at concentrations greatly exceeding the odor threshold in the wines obtained from grapes treated by PEF. Therefore, similarly to the extraction of polyphenols, we observed that the electroporation of grape skins may contribute to enhance the extraction of precursors of aromatic molecules contained in grape skins, thereby helping to improve the flavor profile of wines obtained from grapes treated by PEF.

6. Conclusions

The present investigation has validated, on a semi-industrial scale, the benefits of the application of PEF to grapes before maceration/fermentation by either reducing the maceration time or by increasing the concentration of polyphenols previously demonstrated at laboratory or pilot plant scale. On the other hand, this technique's potential for improving aromatic profile was likewise observed. The result obtained in this investigation, conducted at conditions very close to the processing requirements of wineries, may encourage wine producers to introduce this novel technology as an alternative to current techniques or procedures such as thermo-vinification or the use of enzymes, which are applied to achieve the same objective. PEF, will give wineries a clear competitive edge by increasing the production capacity without the need to invest in the acquisition of more fermentation tanks and by reducing energy input in the maceration-fermentation step that represents the stage of red wine production with the greatest energy consumption.

7. Acknowledgments

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ESTUDIO 3: Evolution of polyphenolic compounds during aging in bottles and oak barrels of *Grenache* wine obtained from grapes treated by Pulsed Electric Fields.

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1. Abstract

The evolution of polyphenolic compounds and sensory properties of wines obtained from untreated and Pulsed electric field (PEF)-treated *Grenache* grapes during 24 months of aging in bottles, as well as during 6 months of aging in oak barrels followed by 18 months of aging in bottles, are compared in this investigation. At the time of aging, enological parameters that depend on phenolic extraction during skin maceration increased when grapes were treated with PEF, or when skin maceration was extended from 3 to 6 days. Greater differences were found for color intensity (51%) and total anthocyanin content (37%) between the wines obtained from untreated and PEF-treated grapes when maceration time was shorter. The total polyphenol index and tannin content were 30 to 40 % higher for the wines obtained from grapes treated with PEF at both maceration times. In terms of color intensity, phenolic families (anthocyanins, hydroxycinnamic acids, flavonols, and flavanols) and individual phenolics, the wine obtained with grapes treated by PEF with different maceration times follows an evolution similar to the wine obtained with untreated grapes during aging in bottle, or in oak barrels followed by bottles. In all cases, a decrease in the concentration of those compounds was observed across time. Sensory discriminative analysis revealed that the application of a PEF treatment results in wines that are sensory different from those obtained with untreated grapes; panelists preferred wines obtained from grapes treated with PEF. Physicochemical and sensory analyses show that grapes treated with PEF are suitable for obtaining wines of the highest quality that require aging in bottle or in oak barrels.

2. Introduction

Maceration-fermentation is the most critical stage in the red winemaking process. Sugars are transformed into ethanol through the action of yeast, but the phenolic composition, aroma, flavor, and aging capacity of the final product largely depend on the extraction of specific compounds from the grape skins (Monagas, et al., 2005).

Improving the degree of extraction of polyphenols during the maceration-fermentation stage of red winemaking is one of the applications of pulsed electric fields (PEF) that has been most investigated in recent years. Several research groups working with different grape varieties have demonstrated that a PEF treatment applied to grapes before the maceration-fermentation stage allows for a reduction of the contact time of grape skins with the fermenting must, or helps obtain wines with higher polyphenolic content (Cholet et al., 2014; Donsì et al., 2011; El Darra et al., 2016; Leong et al., 2016; Puértolas et al., 2010d). This effect is attributed to a phenomenon called electroporation, which consists in the alteration of the membrane permeability of the cells as a consequence of the application of external electric field strength (Cholet et al., 2014). Although some polyphenolic are located in the cell wall of grape skins and the seed, the majority of phenolic compounds responsible for the color stability and sensory properties of red wine are located into the vacuoles of the cytoplasm of grape skin cells (Pinelo et al., 2006). Therefore, the electroporation of membrane cell of grape skin improves the mass transfer of these compounds to the fermenting must during maceration. This effect, which permits an earlier removal of grape skins from the fermentation tanks, could help reduce the demand for expensive fermentation tanks and save on labor costs associated with the process, or it could help obtain wines with a higher concentration of polyphenols. PEF treatment has proven beneficial in obtaining fresh fermented red wine which, however, is not the wine with the best sensorial quality. After the maceration-fermentation stage, an aging period is required in bottle or in oak barrels, a period in which polyphenols participate in subsequent reactions that have the greatest influence on the overall sensory quality of the finished wine (Setford, et al., 2017).

These modifications are a consequence of precipitations or reactions of degradation polymerization and copigmentation that lead to the formation of new stable compounds, and they help bring about important changes in the wine's sensory properties (Guadalupe and Ayestarán, 2008a). Since these reactions depend to a great extent on the type and concentration of polyphenols obtained during fermentation, it is important to

obtain more precise knowledge regarding the evolution of wines produced from grapes treated with PEF during aging.

The objective of this investigation was to compare the evolution and sensory properties of wines obtained from untreated and PEF-treated *Grenache* grapes during aging in bottle, as well as in oak barrels and bottles, for 24 months.

3. Material and methods

Samples and vinification

Around 15,000 kg of *Grenache* grapes *(Vitis vinifera L.*) cultivated in the Campo de Borja Appellation of Origin (A.O.) located in the Spanish region of Aragón were harvested in October 2016. The winemaking process was the one shown in Figure 29. Grape mass was distributed in four fermentation tanks of 5,000 liters capacity each. Two of the tanks contained ca. 3,000 kg of untreated grapes, and the other two tanks contained ca. 3,000 kg of PEF-treated grapes.

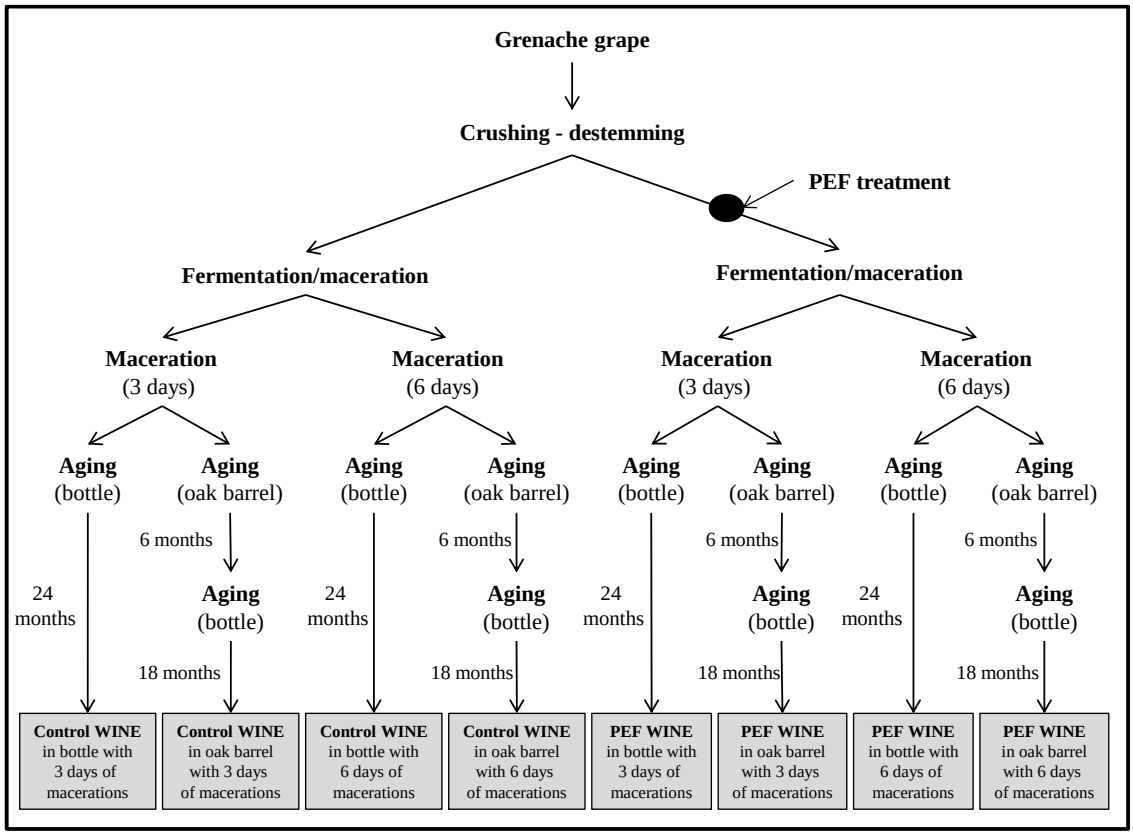

Figure 29: Schematic diagram of vinification and aging

Maceration times assayed for untreated and PEF treated grapes were three days (Control-3, PEF-3) and six days (Control-6, PEF-6). At the end of fermentation, which

occurred after ten days, the wine was treated with sulfites (100 mg of $K_2S_2O_5.L^{-1}$) and kept at 4ºC for a period of three months for purposes of stabilization. The wine was subsequently separated into two portions. One part was racked and aged in bottles for 24 months in a conditioned room maintained at 18±1°C. The other portion was racked and aged in new medium-toast American Oak barrels of 16L (Tonelería los Pinos, Cordoba, Spain for six months, then racked again, bottled, and stored in bottles for a further 18 months.

PEF equipment

For this investigation, an EPS® PM1-10 PEF modulator (Energy Pulse Systems LDA, Lisbon, Portugal) with a maximum output voltage of 10 kV, an output current of 240 A, and a frequency up to 200 Hz of monopolar square waveform pulses of 1 to 200 µs was used. A high-voltage probe (Tektronix, P6015A, Wilsonville, Oregon, USA) and an oscilloscope (Tektronix, TBS 1102B-EDU) were used to record and measure the shape and intensity of the pulse. The treatment chamber consisted of three cylindrical stainless steel electrodes separated by two insulators. The end electrodes were connected to ground, and the central electrode was connected to high voltage. This colinear configuration formed two cylindrical treatment zones of 2.5 cm length and 3.5 cm diameter.

PEF processing

Grape mass was driven through the treatment chamber at a flow rate of 2,500 ± 200 $kg.h^{-1}$ using a peristaltic pump (Rotho MS1, Ragazzini, Faenza, Italy). The residence time of the grape mass between the electrodes was 0.09 s. PEF treatment consisted of 3.7 pulses of a width of 100 µs at electric field strength of 4 $kV.cm^{-1}$. Total specific energy (6.2 $kJ.kg^{-1}$) was calculated according to Martínez et al., (2019). The grape mash temperature was measured before and after the treatment, and it never increased by more than 2ºC. Control samples consisted of untreated grapes that passed through the PEF treatment chamber with the PEF modulator turned off.

General analysis of enological parameters

Total acidity, alcohol content, and pH were determined according to the methods prescribed by the Organisation Internationale de la Vigne et du Vin (OIV 2009).

For spectrophotometric determination, all samples were centrifuged in an Eppendorf centrifuge for 15 min at 3000 rpm (Eppendorf AG, Hamburg, Germany), and absorbance measurements were carried out in a Biochron Libra S12 spectrophotometer (Biochron, Cambridge, UK).

Colorimetric indexes

The absorbance of the musts was measured at 420, 520, and 620 nm, using quartz cells of 1 mm path length. Color intensity (CI) was calculated as the sum of 420, 520, and 620 nm absorbance, and hue (Tint) was calculated as the proportion of the absorbance measured at 420 nm and 520 nm according to Glories (1984).

Phenolic determinations

Total polyphenol index (TPI) was determined by a direct reading of the absorbance at 280 nm of diluted wine 1/100 ($v.v^{-1}$) using a quartz cuvette of 1 cm path length. TPI was calculated by multiplying the absorbance measured at 280 nm by 100 (Ribéreau-Gayon et al., 2006).

Total anthocyanins (Anthocyanin) expressed in milligrams per liter of malvidin-3-glucoside were analyzed by determining the absorbance at 520 nm of diluted wine 1/100 ($v.v^{-1}$) with 1 % ($v.v^{-1}$) HCl using the corresponding calibration curve (Ruiz-Hernández, 2004).

Tannins (condensed tannins) were determined according to Sarneckis et al. (2006). Aqueous (-)-epicatechin solutions (10, 25, 50, 75, 100, 150, and 200 $mg.L^{-1}$ epicatechin) were used in order to obtain the calibration curve. All values are reported in $mg.L^{-1}$ of epicatechin equivalents of the original sample (corrected for assay dilution).

High-Performance Liquid Chromatography (HPLC)

Analyses were performed according to the chromatographic conditions described by Puértolas et al. (2010d). An HPLC Varian ProStar (Varian Inc., Walnut Creek, CA) high-performance liquid chromatography equipped with a ProStar 240 ternary pump, a ProStar 410 autosampler and a ProStar 335 photodiode array detector were used. Separation was achieved on a reverse-phase column (LC Luna® 100 Å C18 250 x 4.6 mm; 5 μm particle size, Phenomenex) with a pre-column of the same material (LC Luna 50 x 4.6 mm; 5 μm particle size, Phenomenex). Chromatograms at 280 nm (flavan-3-ols), 320 nm (hydroxycinnamic acids and derivatives), 360 nm (flavonols), and 520 nm

(anthocyanins) were recorded. The analyzed phenolic compounds were tentatively identified according to the retention time and the UV-vis spectra of pure standards and following the UV-vis spectral characteristics published in the literature (Cantos et al., 2002; Hermosín-Gutiérrez et al., 2005; Monagas et al., 2005). The concentrations of all studied compounds were expressed in $mg.L^{-1}$.

Sensory Analysis

Wines after twelve months of aging in bottle, as well as six months of aging in oak barrels plus six months of aging in bottle, were sensory evaluated by seven winemakers (4 men and, 3 women ranging from 40 to 59 years) from the official panel of the A.O. Campo de Borja. Samples of 20 ml of wine at room temperature were presented in clear wine glasses (ISO NORM 3591, 1997) labelled with 3-digit random codes. Panelists were distributed in individual booths and they were not informed about the samples to be tested.

Initially the wines were evaluated by triangular discriminative analysis using a completely randomized design associated to a preference tests. The objective of this first test was to determine if after aging in bottle, or in oak barrels plus bottle, the panel could distinguish between the wines obtained from untreated and PEF-treated grapes with 3 and 6 days of maceration. After selecting the sample that was considered different, the panelists were also asked to indicate the preferred sample. The result of the preference analysis was only taken into consideration when the panelists correctly identify the different sample.

On the other hand, a descriptive sensory evaluation of the wines obtained from untreated and PEF-treated grapes after six months of aging in oak barrels plus six months of aging in bottles was conducted. The evaluation protocol was composed of six sensory descriptors, four of which could be affected by the polyphenolic content of the wines: astringency, body, persistence, and color intensity. Magnitude of the sensory descriptors was measured in a scale between 0 (very low intensity) and 9 (very high intensity). Results showed correspond to average of the scores reported for each panelist.

Statistical analysis

For wines aged in bottles, three samples corresponding to different bottles were analyzed for each condition. In the case of aging in barrel samples from two barrels were

analyzed for each condition and them during the aging in bottle two samples from each barrel were analyzed.

The data presented in tables and figures represent mean values ± 95 % confidence level. Analysis of variance (ANOVA) was carried out using InfoStat statistical software in the 2018 version. The statistical significance of each selected attribute was calculated according to Tukey's test ($p \leq 0.05$). The significant difference for triangular tests was determined using statistical tables reported by Roessler et al, (Roessler et al., 1948).

4. Results and discussion

Physicochemical analysis of wine at the time of aging in bottles and oak barrels

Table 25 shows the enological parameters of wines obtained with 3 and 6 days of skin maceration of untreated and PEF-treated grapes. Data correspond to the wines at the time of bottling and aging in oak barrels. Parameter values lay within the range usually observed in *Grenache* variety wines (Garde-Cerdán et al., 2013; Pascual et al., 2016). No statistically significant differences were found in the pH, and the total acidity of the four wines and the differences in ethanol content between the wine with the lowest value and the highest value were lower than one unit of ethanol (%v.v^{-1}). These differences can be attributed to the varying fermentation processes brought about by the yeast in the separate tanks rather than to the PEF treatment. A significant effect of PEF on the alcoholic content of wine has not been reported in the literature (López-Giral et al., 2015).

Table 25: Physico-chemical parameters of wines obtained with 3 and 6 days of skin maceration from untreated and PEF-treated grapes at the time of bottling and aging in oak barrels.

		pH	Ethanol (%, v.v^{-1})	Titratable acidity[a]	CI (A.U.)	TPI (A.U.)	Tannin[b] content	Anthocyanins[c]
3 days of maceration	Untreated	3.2±0.01a	17.75±0.6a	4.4±0.1a	8.0±0.2a	38.6±0.2a	900.8±12.5a	308.4±11.2a
	PEF	3.2±0.03a	17.85±0.1a	4.2±0.2a	12.1±0.1b	51.0±0.3c	1232.7±78.8b	421.1±9.6b
6 days of maceration	Untreated	3.2±0.01a	17.45±0.1a	4.2±0.1a	11.6±0.1b	45.5±1.4b	1040.7±10.0a	477.9±6.4c
	PEF	3.2±0.01a	17.90±0.1a	4.1±0.1a	14.4±0.1c	61.9±0.9d	1457.9±6.8c	513.2±8.0c

Different letters within the same column represent significant differences according to one-way ANOVA analysis ($p < 0.05$)
CI color intensity, TPI total polyphenol index, A.U. absorbance units.
a Expressed as tartaric acid (g.L^{-1}).
b Expressed as procyanidin (mg.L^{-1}).
c Expressed as malvidin-3-glucoside (mg.L^{-1}).

For the remaining enological parameters that depend on phenolic extraction during skin maceration, values increased when grapes were treated with PEF, or when skin maceration was extended from 3 to 6 days. Statistically significant differences were found between the wines obtained from untreated grapes and PEF-treated grapes after 3

or 6 days of skin maceration. Greater differences were found for color intensity (51%) and total anthocyanins (37%) between the wines obtained from untreated and PEF-treated grapes when maceration time was shorter. However, the total polyphenol index and the tannin content were 30 to 40 % higher for the wines obtained from grapes treated with PEF at both maceration times. These effects are attributed to the electroporation caused by PEF that facilitates the release of intracellular compounds (Puértolas et al., 2010a).

Evolution of color intensity, anthocyanin content, total phenolic content, and tannin content during aging in bottles, and oak barrels.

Figure 30 and Figure 31 show the evolution of wine parameters depending on the polyphenols extracted throughout the maceration stage during aging in bottle for 24 months vs. aging in oak barrels for 6 months plus subsequent aging in bottle for 18 months.

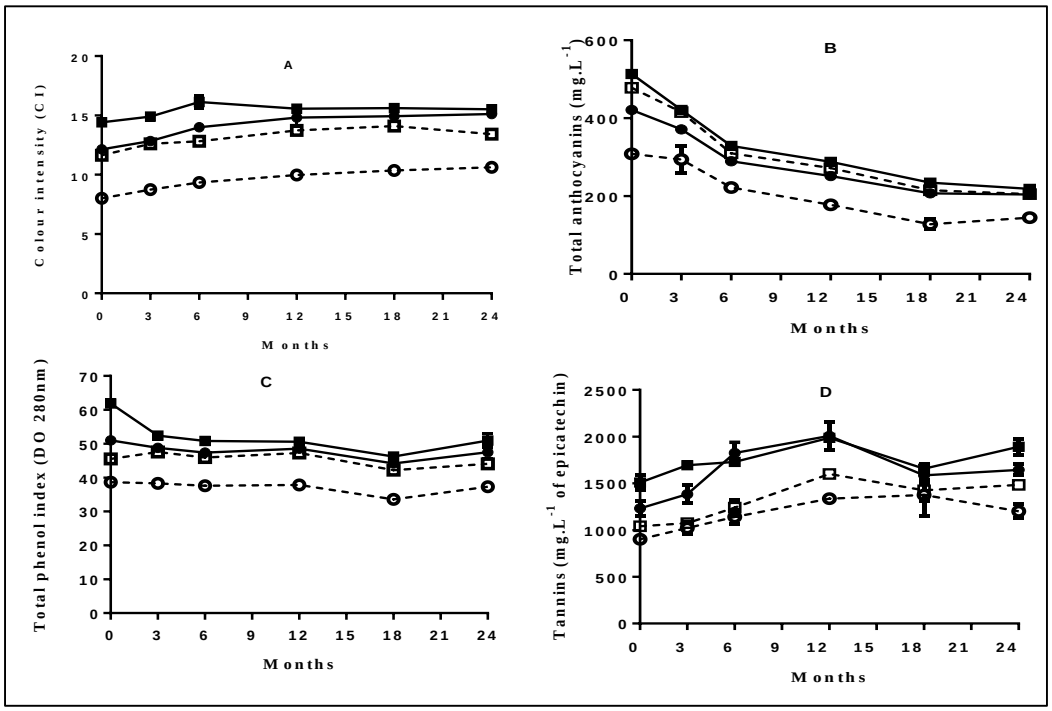

Figure 30: Evolution of color intensity (A), total anthocyanins (B), total phenol index (C) and tannins (D) of wines during 24 months of aging in bottle. (○) wine obtained from untreated grapes with 3 days of maceration; (●): wine obtained from PEF treated grapes with 3 days of maceration; (□): wine obtained from untreated grapes with 6 days of maceration; (■): wine obtained from PEF treated grapes with 6 day of maceration.

In general, the application of a PEF treatment to grapes prior to the maceration-fermentation stage did not affect the subsequent development of color intensity, anthocyanin content, total phenolic content, and tannin content during wine aging in bottle, or in oak barrels followed by bottle. In all cases, at the end of the aging process

the values for those indexes were lowest for the wine obtained from untreated grapes with 3 days of maceration. However, the differences in color intensity, total anthocyanins, total phenolic content, and tannin content at the end of aging between the wine obtained from untreated and PEF-treated grapes with 3 days of maceration were maintained in the wines aged in bottles and barrels. It has been reported that wines obtained with techniques such as thermovinification or flash-expansion, which greatly accelerate polyphenol extraction, produce wines that often have poor stability and little structure (Gao et al., 1997). This effect has been associated with the fact that these techniques promote the extraction of anthocyanins, while the extraction of other polyphenols that take longer to extract are not on a sufficient level to provide much structure and to stabilize anthocyanins in order to maintain wine color (Morel-Salmi et al., 2006). According to our results, the evolution of the wine obtained with grapes treated by PEF followed the typical pattern reported for wine aging in the literature (Guadalupe and Ayestarán, 2008b).

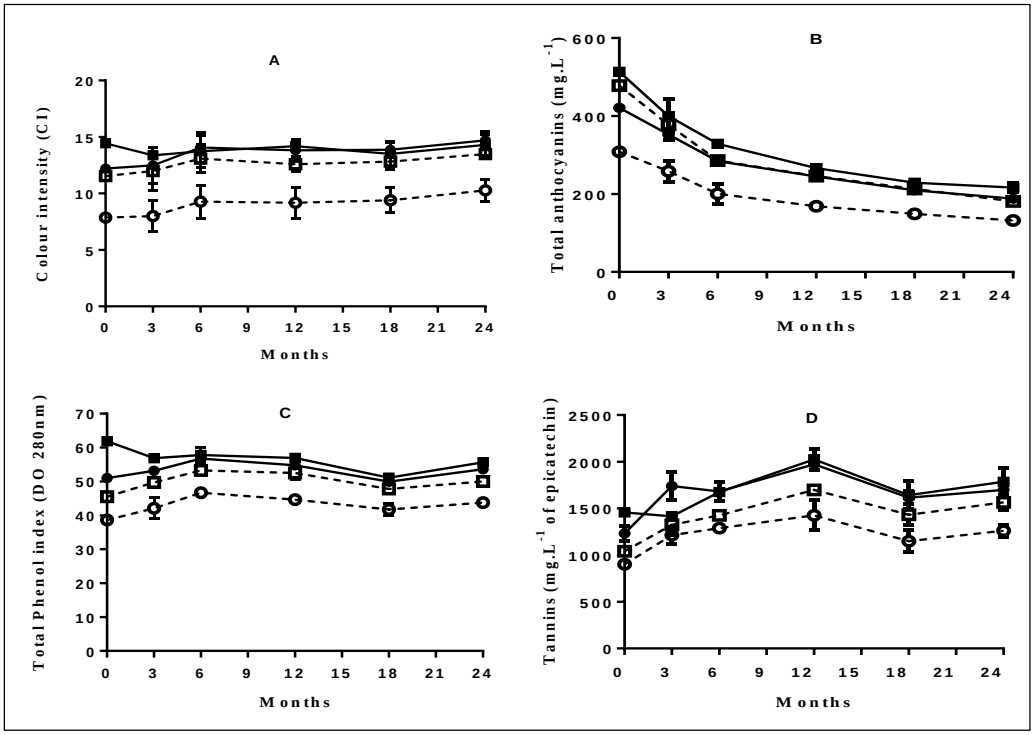

Figure 31: Evolution of color intensity (A), total anthocyanins (B), total phenols index (C) and tannins (D) of wines during 6 months of aging in oak barrels plus 18 months of aging in bottle. (○) wine obtained from untreated grapes with 3 days of maceration; (●): wine obtained from PEF treated grapes with 3 days of maceration; (□): wine obtained from untreated grapes with 6 days of maceration; (■): wine obtained from PEF treated grapes with 6 day of maceration.

Color intensity values of wines aging in bottle (Figure 30A) or oak barrels plus bottles (Figure 31A) obtained from untreated viz. PEF treated grapes did not change significantly after 24 months of storage. However, aging caused a significant decrease in

total anthocyanin content for the four wines (Figure 30B and Figure 31B). For both aging procedures, the reduction in anthocyanin content was more rapid during the first six months of aging. The decrease in anthocyanins during wine aging has been attributed to precipitation and oxidation reactions (Boulton, 2001; He et al., 2012; Mateus and de Freitas, 2001). They seem to occur to the same extent in wine obtained from untreated grapes than in wine obtained from grapes treated with PEF. Although anthocyanins are the compounds that mainly account for the red and purple color of wine, the reduction of those compounds during wine aging did not affect the color intensity. This preservation of color during aging is attributed to the formation of polymeric pigments ranging from anthocyanins to other wine components, mainly tannins, and of derived pigments formed by condensation, which consist in non-covalent links of anthocyanins with colorless molecules, or with a series of anthocyanins (Dueñas et al., 2006; Salas et al., 2003). Therefore, similarly to the wine obtained from untreated grapes, the wine obtained with grapes treated by PEF contained the molecules that participate in those reactions responsible for color stabilization.

The total phenol index (TPI) of the wines with 3 days of maceration aged in bottle remained practically constant (Figure 30C). In the case of the wine obtained from PEF-treated grapes with 6 days of maceration, a decrease in this index was observed after the 3 first months of aging, after which the TPI value remained practically constant (Figure 30C). This decay in TPI could be due to the precipitation of a proportion of polyphenols as a consequence of their high initial concentration at the point of bottling. The evolution of TPI followed a similar pattern when the same wine aged in oak barrels (Figure 31C). However, for the remainder of the wines, the TPI increased during aging in barrels (6 months), after which it slightly decreased during aging in bottle. Therefore, the extraction of phenolic compounds from the wood responsible for the TPI increment occurs in wines obtained with untreated and with PEF-treated grapes. In the case of the wine obtained with PEF-treated grapes after 6 days of maceration, an increment in TPI was not observed. This was probably because the precipitation of polyphenols exceeded the degree of phenolic extraction from the wood.

The tannin content represented in Figure 30D and Figure 31D is known as proanthocyanidins. They are formed by the polymerization of the polyphenolic flavan-3-ol monomers catechin and epicatechin (Dueñas et al., 2006). An increment in tannin content up to 12 months of aging was observed as a consequence of the formation of polymer chains with a different degree of polymerization for the four wines aged in bottle

or in barrels. After 12 months, this index tended to decrease slowly. Similarly as in TPI, no differences in tannin content were observed at the end of the aging process between the wine obtained from untreated grapes with six days of maceration and the wine obtained from grapes treated by PEF with 3 days of maceration. These results indicate that the concentration of alcohol after 3 days of maceration encourages an elevated rate of extraction from the electroporated grape skins of the polyphenols that form the tannins. These polyphenols, which have a low degree of water solubility, require the presence of ethanol in order to be extracted (Zamora, 2003). As a consequence, they are mainly extracted when the fermentation process is well-advanced.

Evolution of the content of phenolic families and individual phenolics during aging in bottle and in oak barrels

The concentration of phenolic families (anthocyanins, hydroxycinnamic acids, flavonols and flavanols) and the individual phenolics of the four wines after 6, 12, and 24 months of aging in bottle viz. in oak barrels, then bottled, are shown in Table 26 and Table 27, respectively.

During the 2-year aging period, the total content of phenolic families tended to decrease, independently of PEF treatment or maceration time, for both wines: for those aged only in bottle, and for those aged in oak barrels plus bottle. Similar results have been observed in the aging of *Cabernet Sauvignon* wine obtained from grapes treated with PEF (Puértolas et al., 2010d).

As in the evolution of the parameters described above, a higher concentration of individual phenolic compounds was generally observed in the wines obtained from PEF-treated grapes than in those obtained from untreated grapes after the same amount of maceration time. The differences between the wines obtained from untreated grapes after 6 days of maceration and the wines obtained from PEF-treated grapes with 3 or 6 days maceration tended to disappear in the course of aging, whereby the polyphenolic content of the wines obtained from untreated grapes with 3 days of maceration was always lower. In all cases, no evidence of a particular effect of the PEF treatment on the extraction of a specific family or individual phenolic compound was observed.

Monomeric anthocyanins were the predominant polyphenols in all the wines. Among all polyphenolic families, anthocyanins were considerably more reduced in all four wines, as reported above, either due to reactions associated with the formation of new stable polymeric pigments, or due to degradation reactions. As the color of all four

wines remained stable during aging, the loss of monomeric anthocyanins was due to their transformation into more stable pigments in terms of color, rather than to their degradation. Anthocyanin degradation was more pronounced in the wines aging only in bottles than in the wines aging for 6 months in oak barrels, then bottles. After 24 months of aging, the total individual anthocyanins were 20 to 40 % higher for wines aged in barrels.

Table 26: Evolution of individual phenolic compounds ($mg.L^{-1}$) in wines obtained from untreated and PEF treated grapes with 3 and 6 days of maceration after 24 months of aging in bottle.

	6 months				12 months				24 months			
	3 maceration days		6 maceration days		3 maceration days		6 maceration days		3 maceration days		6 maceration days	
	Control	PEF	Control	PEF	Control	PEF	Control	PEF	Control	PEF	Control	PEF
delphinidin-3G	6.07±0.35	11.66±3.50	12.58±5.96	15.61±3.35	3.27±0.04	9.62±2.61	0.47±0.05	13.6±0.91	3.75±0.25	8.64±2.83	11.73±0.18	10.01±0.21
cyanidin-3G	1.63±0.50	8.50±1.98	3.27±0.78	3.41±0.72	3.18±1.41	5.39±0.11	9.43±1.41	3.92±2.86	0.76±0.54	0.94±0.58	1.21±0.45	0.78±0.65
petunidin-3G	12.02±1.23	16.14±2.19	19.18±3.39	20.61±0.19	2.51±0.43	9.05±0.18	10.33±7.86	10.18±1.99	1.99±0.66	2.91±0.73	1.29±1.01	1.55±0.24
peonidin-3G	8.67±0.07	13.92±1.45	15.19±4.62	17.54±0.90	5.02±0.80	10.98±0.96	5.97±0.81	17.03±2.15	3.39±0.99	7.98±0.01	6.79±0.04	10.29±2.02
malvidin-3G	104.87±0.50	136.18±7.43	134.58±15.89	185.13±6.71	32.47±1.43	50.25±2.63	60.04±10.04	68.07±10.84	9.63±0.36	26.11±0.76	29.56±3.22	34.27±4.53
delphinidin-3G-Ac	9.23±0.52	13.51±2.14	20.75±4.49	14.53±1.80	4.65±0.06	9.56±4.82	0.10±0.07	11.07±1.04	2.10±1.05	3.12±0.25	1.48±1.83	4.19±0.90
cyanidin-3G-Ac	1.32±0.58	2.36±0.28	2.43±0.42	3.02±0.41	3.71±0.24	2.87±1.99	14.11±0.94	2.21±1.06	1.01±0.35	0.98±0.78	1.20±0.48	0.72±0.25
petunidin-3G-Ac	3.25±1.08	10.35±0.25	13.48±6.01	7.74±1.93	6.89±0.61	12.61±4.79	8.74±1.91	11.31±0.24	1.05±0.61	1.87±1.41	3.52±0.02	2.01±1.05
malvidin-3G-Ac + peonidin-3G-Ac	7.35±1.55	26.33±3.39	23.95±0.01	30.62±3.04	10.28±0.88	18.33±0.34	17.19±6.70	27.00±1.07	4.50±0.01	9.05±1.48	11.45±4.01	11.8±2.56
delphinidin-3G-Cm	1.42±1.72	9.17±1.32	10.51±1.09	12.64±2.52	3.44±0.64	8.07±3.90	8.58±0.42	7.59±5.26	1.11±0.17	1.67±0.30	1.15±0.47	1.07±0.35
cyanidin-3G-Cm	0.85±0.54	1.08±0.43	1.35±0.98	1.65±0.11	1.07±0.41	1.88±0.05	2.03±0.36	2.48±0.68	0.78±0.18	1.10±0.06	1.39±0.08	1.17±0.24
petunidin-3G-Cm	2.01±0.77	7.71±5.63	11.44±0.15	6.49±1.11	2.97±0.50	2.48±2.58	3.64±0.66	3.17±2.34	0.66±0.03	0.75±0.27	0.69±0.03	2.26±2.42
peonidin-3G-Cm	4.14±2.08	3.96±1.71	8.99±7.73	8.09±0.07	5.43±0.73	7.68±0.94	5.61±0.03	5.16±1.52	1.42±0.09	2.02±0.01	3.29±0.11	2.56±0.48
malvidin-3G-Cm	7.94±4.70	7.72±3.42	14.27±2.7	14.19±1.57	4.69±1.42	6.29±1.95	8.72±1.79	7.86±0.63	1.54±0.18	3.46±0.47	4.93±0.48	7.28±1.43
Total individual anthocyanins	**170.78**	**268.61**	**291.96**	**341.26**	**89.56**	**155.06**	**154.96**	**190.63**	**33.66**	**70.59**	**79.67**	**89.93**
t-caftaric acid	8.70±0.99	16.6±2.55	13.05±1.48	23.90±1.98	7.85±0.07	13.50±0.57	14.30±0.28	16.95±1.06	2.80±0.14	8.40±0.28	8.50±0.85	8.45±0.92
p-coumaric acid	2.90±0.28	2.95±1.06	3.90±0.42	4.45±0.21	1.65±0.35	2.70±0.14	2.55±0.35	3.35±0.49	0.30±0.01	1.65±0.64	2.60±0.14	3.70±0.14
caffeic acid	0.50±0.01	1.55±0.35	0.70±0.42	1.40±0.28	0.30±0.01	0.50±0.14	0.40±0.28	1.30±0.01	1.05±0.07	1.05±0.21	0.65±0.07	1.45±0.07
Total individual hydrocynnamic ac	**12.10**	**21.10**	**17.65**	**29.75**	**9.80**	**16.70**	**17.25**	**21.6**	**4.15**	**11.10**	**11.75**	**13.60**
myricetin-3G	5.65±0.07	8.35±1.91	7.50±0.14	9.15±1.20	2.40±0.57	6.01±0.28	5.25±0.35	6.95±1.06	0.50±0.01	4.60±0.57	3.15±0.35	4.25±1.06
myricetin	0.45±0.21	3.05±0.21	0.55±0.07	2.65±0.78	0.20±0.01	1.05±0.21	0.45±0.07	1.60±0.01	0.10±0.01	0.20±0.14	0.40±0.14	0.40±0.14
isorhamnetin-3O-glucoside	0.30±0.14	1.05±0.07	1.40±0.28	1.30±0.71	0.40±0.57	0.15±0.21	0.60±0.14	3.30±0.14	ND	0.15±0.21	0.40±0.01	0.15±0.07
quercetin-3G	4.60±1.98	7.30±0.42	7.75±1.34	8.90±0.99	2.05±0.49	5.2±0.42	5.50±0.14	6.40±1.27	2.60±0.42	5.30±0.28	5.90±0.28	7.25±2.33
quercetin	1.30±0.57	1.35±0.07	0.70±0.28	1.01±0.42	0.70±0.14	0.65±0.07	0.65±0.07	0.55±0.07	0.35±0.07	0.40±0.14	0.30±0.28	0.70±0.28
Total individual flavonols	**12.30**	**21.10**	**17.90**	**23.00**	**5.75**	**13.05**	**12.45**	**18.80**	**3.55**	**10.65**	**10.15**	**12.75**
(+)-catechin	10.15±0.07	18.05±0.92	17.35±1.91	20.75±2.19	7.05±2.76	7.20±1.27	11.50±0.71	13.01±1.41	6.20±0.57	6.95±1.48	7.85±0.92	10.10±1.27
(-)-epicatechin	7.25±0.49	13.55±2.33	14.80±1.27	15.20±1.13	10.95±1.48	14.5±0.71	13.50±0.71	17.50±2.12	5.45±1.20	8.00±1.13	10.25±1.06	10.55±2.05
Total individual flavanols	**17.40**	**31.60**	**32.15**	**35.95**	**18.00**	**21.70**	**25.00**	**30.51**	**11.65**	**14.95**	**18.10**	**20.65**

[a] Nd: not detected. G: glucoside. Ac: acylated. Cm: coumarylated

Table 27: Evolution of individual phenolic compounds (mg.L^{-1}) in wines obtained from untreated and PEF treated grapes with 3 and 6 days of maceration after 6 months of aging in oak barrels plus 18 months of aging in bottle.

	6 months				12 months				24 months			
	3 maceration days		6 maceration days		3 maceration days		6 maceration days		3 maceration days		6 maceration days	
	Control	PEF	Control	PEF	Control	PEF	Control	PEF	Control	PEF	Control	PEF
						Anthocyanins						
delphinidin-3G	7.14±0.06	12.13±1.91	9.44±0.98	11.57±2.11	5.64±0.74	8.19±0.63	14.80±0.03	16.33±1.46	4.07±0.24	10.67±1.1	11.85±0.06	11.17±0.21
cyanidin-3G	1.49±0.86	6.31±3.88	4.41±0.13	3.56±0.61	3.30±1.69	2.80±0.38	5.61±0.52	3.76±1.37	1.61±0.01	3.53±2.16	3.13±0.12	3.12±1.53
petunidin-3G	13.79±0.30	16.41±1.28	14.29±2.51	18.42±1.80	6.46±0.79	14.24±1.50	7.76±1.99	11.31±0.60	0.72±0.01	3.11±0.07	4.77±1.16	7.87±1.37
peonidin-3G	8.03±2.04	13.69±3.54	23.74±6.39	15.52±3.09	8.21±1.74	10.64±3.93	11.54±0.23	19.74±0.18	10.65±1.07	8.54±0.60	8.98±0.81	9.90±1.79
malvidin-3G	75.39±4.80	144.36±12.05	127.93±19.59	182.36±12.39	25.2±1.77	52.24±2.90	50.59±2.61	59.18±1.78	12.68±3.01	23.27±2.63	31.51±3.84	40.63±6.67
delphinidin-3G-Ac	8.46±0.49	10.85±4.07	15.87±1.51	21.21±0.45	2.64±0.24	7.68±0.09	9.26±0.58	11.56±5.75	1.33±0.03	4.69±1.31	10.22±0.03	7.61±0.77
cyanidin-3G-Ac	2.86±0.58	3.36±0.34	2.80±0.06	3.71±0.22	0.62±0.18	1.14±0.12	1.34±0.09	2.82±2.03	0.59±0.36	0.02±0.04	2.29±0.10	2.45±0.05
petunidin-3G-Ac	2.87±0.23	7.85±2.81	7.97±0.88	13.68±2.79	3.50±1.86	9.42±2.41	7.96±2.09	13.44±2.77	1.82±0.14	2.45±1.25	2.58±1.10	3.00±0.50
malvidin-3G-Ac + peonidin-3G-Ac	13.38±1.16	34.85±7.99	21.14±0.78	36.58±2.54	10.67±2.48	16.96±3.01	22.01±0.27	20.09±5.04	5.73±0.59	10.93±1.43	16.77±1.36	17.38±5.19
delphinidin-3G-Cm	2.68±1.21	5.10±0.23	8.78±3.46	10.09±2.01	2.52±0.53	5.28±0.80	5.43±0.88	3.98±0.19	1.10±0.18	4.14±0.35	2.17±0.23	3.73±0.07
cyanidin-3G-Cm	1.11±0.04	1.96±0.22	3.03±1.44	1.29±2.65	0.57±0.02	1.88±0.72	1.46±0.34	2.44±0.05	1.04±0.42	2.50±0.02	1.34±0.09	0.55±0.20
petunidin-3G-Cm	1.75±0.65	6.56±3.70	7.22±0.95	8.45±0.01	1.15±0.70	3.01±0.97	4.13±2.30	3.82±1.97	1.55±0.39	2.30±0.91	1.81±0.29	1.12±0.23
peonidin-3G-Cm	2.99±0.87	14.58±0.37	4.38±1.64	12.71±1.41	1.42±0.35	4.74±0.41	7.62±5.27	5.36±0.33	1.81±0.73	3.29±0.38	1.37±0.52	5.60±0.04
malvidin-3G-Cm	5.09±0.89	7.43±0.73	19.32±0.01	12.72±1.43	3.86±1.09	8.60±2.43	5.23±2.71	9.12±1.53	1.89±0.26	5.03±0.75	5.28±0.10	10.11±0.54
Total individual anthocyanins	**147.04**	**285.43**	**270.32**	**351.88**	**75.74**	**146.80**	**154.74**	**182.96**	**46.60**	**84.48**	**104.07**	**124.23**
						Hydroxycinnamic acids						
t-caftaric acid	9.90±1.84	16.80±2.12	13.85±0.21	23.60±4.53	7.40±1.41	14.55±1.20	13.60±0.14	17.50±0.85	3.60±0.85	8.60±0.85	9.10±0.28	10.70±0.14
p-coumaric acid	2.65±0.49	3.20±0.57	3.35±0.07	3.60±0.28	2.20±0.14	2.75±0.35	3.10±0.28	3.10±0.57	0.20±0.14	1.60±0.01	1.90±1.84	1.50±0.57
caffeic acid	0.55±0.07	1.25±0.92	0.50±0.14	0.65±0.21	0.50±0.02	0.60±0.02	0.55±0.07	1.40±0.01	0.75±0.07	1.25±0.07	0.75±0.07	1.50±0.42
Total individual hydrocynnamic ac	**13.10**	**21.25**	**17.70**	**27.85**	**10.10**	**17.90**	**17.25**	**22.00**	**4.55**	**11.45**	**11.75**	**13.70**
						Flavonols						
myricetin-3G	4.90±0.28	7.75±0.92	7.40±0.57	11.45±1.63	2.60±0.71	6.80±0.85	5.35±0.35	7.01±1.13	0.85±0.21	5.05±0.35	3.25±0.35	4.95±0.07
myricetin	0.35±0.35	2.65±0.78	0.90±0.01	2.70±0.99	0.20±0.14	1.05±0.07	0.45±0.07	1.60±0.14	nd	0.30±0.14	0.45±0.07	0.90±0.14
isorhamnetin-3O-glucoside	1.15±0.64	2.75±0.07	0.95±0.92	3.85±2.47	0.15±0.21	0.20±0.28	0.70±0.42	0.20±0.01	0.15±0.21	0.20±0.28	0.45±0.07	0.20±0.01
quercetin-3G	3.10±0.42	7.35±2.47	6.90±0.85	8.45±0.92	2.45±0.21	5.45±0.64	5.75±0.07	6.85±0.92	2.55±0.35	4.35±0.21	5.65±0.78	7.55±0.92
quercetin	0.75±0.64	0.05±0.07	1.01±0.14	1.35±0.64	0.60±0.14	0.35±0.35	0.75±0.07	0.75±0.35	nd	0.20±0.28	0.32±0.14	0.75±0.35
Total individual flavonols	**10.25**	**20.55**	**17.15**	**27.80**	**6.00**	**13.85**	**13.00**	**16.40**	**3.50**	**10.10**	**10.10**	**14.35**
						Flavanols						
(+)-catechin	12.55±0.64	22.3±1.84	19.85±0.21	29.01±0.99	10.01±0.01	8.45±0.49	8.50±3.54	10.75±3.18	4.45±2.05	9.05±0.49	11.00±1.41	8.90±1.56
(-)-epicatechin	8.90±2.12	19.25±2.62	14.55±1.06	18.1±1.84	5.85±0.07	23.5±0.71	17.01±2.83	24.5±4.95	5.55±0.78	10.50±0.71	10.50±0.71	13.5±3.54
Total individual flavanols	**21.45**	**41.55**	**34.40**	**47.10**	**15.85**	**31.95**	**25.50**	**35.25**	**10.00**	**19.55**	**21.50**	**22.40**

[a] Nd: not detected. G: glucoside. Ac: acylated. Cm: coumarylat

Malvidin-3-glucoside was the major anthocyanin that accounted for color stability. It represented practically half of all monomeric anthocyanins in wines aging in bottles and barrels for 6 months. As in other studies on wine aging, the observed decrease in total monomeric anthocyanins was mainly due to the high decrease of this individual polyphenol (Guadalupe & Ayestarán, 2008b). After 24 months of aging, the concentration of this anthocyanin decreased significantly in all wines, representing approximately one third of all monomeric anthocyanins. This decrease was observed in the same proportion in the wines obtained from untreated and from PEF-treated grapes. Thus, after the same maceration time, wines obtained with untreated grapes had a lower content of monomeric anthocyanins compared with the wines obtained from PEF-treated grapes after 24 months in both aging processes.

It was assumed that glucoside, acetylated and coumarylated anthocyanins evolved in a similar way, decreasing in the course of aging in both wines obtained with untreated and PEF-treated grapes.

A total of three hydrodynamic acids, five flavonols, and two flavanols were identified and quantified in all wines. The evolution of these polyphenolic families in wines obtained from untreated and PEF-treated grapes was similar in the course of aging, either in bottle, or in oak barrels with subsequent bottling. In all cases, a progressive decrease throughout aging was observed. In general terms, by the end of the aging process, the highest value of these families was observed in the wine obtained from grapes treated with PEF after 6 days of maceration, and the lowest values thereof in the wines obtained after 3 days of maceration with untreated grapes.

Similar results as those discussed regarding different polyphenol families were observed for the individual polyphenols of each family as well. The evolution of individual polyphenols was similar in the two wines obtained from untreated and PEF-treated grapes after aging in bottle, or oak barrels with subsequent bottling. In all cases, a decrease in the concentration of these compounds was observed through time. The wine obtained from grapes treated with PEF after 6 days of maceration presented the highest content of hydroxycinnamic acids in the course of aging, mainly due to a higher content of t-caftaric acid. This wine also presented the highest content of flavonols, whereby myricetin-3- glucoside was the most abundant flavonols in all the wines. In the case of flavanols, after 6 months of aging their content tended to be higher in the wines in oak barrels than in the wines exclusively aged in bottle. This higher content is related to the extraction of flavanols from oak wood (Guadalupe & Ayestarán, 2008b). Whereas after

6 months of aging the content of (+)-catechin was higher than the content of (-)-epicatechin in all wines, after 24 months of aging the content of both flavanols was similar.

Sensory evaluation

Table 28 shows the percentages of correct responses identifying the odd sample in the triangle test and the results of the preference test. Significant sensory differences were detected by the panelists in the wines obtained with untreated or PEF-treated grapes after 3 and 6 days of maceration when aged either in bottle or in oak barrels.

Table 28: Triangle test and percentage of preference for each of the comparisons among wines obtained from untreated and PEF treated grapes with 3 and 6 days of maceration after aging in bottles (12 months), and in oak barrels (6 months) followed by bottle (6 months).

		Triangle test (percentage of correct responses)[a]	Preference test (percentage of preference)[b]			
			Control-3 days	PEF-3 days	Control-6 days	PEF-6 days
Bottles	**Untreated-3 days / PEF-3 days**	100***	14	86	-	-
	Untreated-6 days / PEF-6 days	71*	-	-	29	71
	PEF-3 days / PEF-6 days	86**	-	29	-	71
	PEF-3 days / Untreated-6 days	86**	-	57	43	-
Oak barrels	**Untreated-3 days / PEF-3 days**	100***	14	86	-	-
	Untreated-6 days / PEF-6 days	100***	-	-	43	57
	PEF-3 days / PEF-6 days	86**	-	14	-	86
	PEF-3 days / Untreated-6 days	86**	-	71	29	-

[a] Significant differences between the samples *,**,*** are statistically significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.
[b] Proportion of preferences statistically different to 50 % according to chi-square test.

All panelists were able to differentiate the wines obtained with untreated or PEF-treated grapes after 3 days of maceration for both types of aging (bottles vs. oak barrels plus bottles). In both cases, a majority of panelists (86%) preferred wines elaborated with grapes treated with PEF. When the wines obtained with untreated and PEF-treated grapes with 6 days of maceration were compared, panelists had more difficulty in differentiating them (71% success) when they had aged in bottle, but all panelists were able to differentiate them when they had aged in barrels. In both cases, panelists likewise preferred the wine obtained from grapes treated with PEF. Finally, independently of the type of aging, panelists were able to differentiate the wines obtained with grapes treated by PEF with 3 days of maceration from the wines obtained with 6 days of maceration with untreated or PEF-treated grapes with a success rate of 86%.

In the preference test, panelists preferred the wines obtained from grapes treated with PEF with longer maceration times than with shorter maceration times when they had

aged in bottle (71%) or in oak barrels (57%). Smaller differences were observed in the panelists' preferences between the wine obtained from grapes treated with PEF and 3 days of maceration (57%) and the wine obtained from untreated grapes and 6 days of maceration (43%), but 71% of the panelists preferred the wine obtained from PEF-treated grapes after six months of aging in oak barrels.

In summary, these results indicate that the improvement in polyphenolic extraction brought about by the application of a PEF treatment prior to maceration permits to obtain wines that are sensorily different from those obtained with untreated grapes.

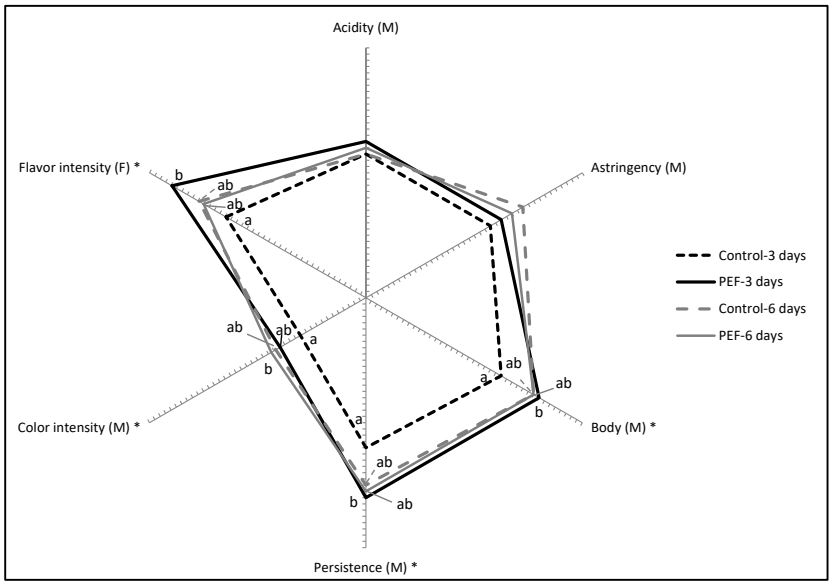

Figure 32: Cobweb diagram of the mean sensory scores (n=7) for the significant mouthfeel (M) and flavor (F) attributes of wines obtained from untreated and PEF treated grapes with 3 and 6 days of maceration after in aging oak barrels (6 months) plus bottles (6 months). Attributes identified with * indicate statistical significance at $p<0.05$.

In all cases, panelists preferred wines obtained from grapes treated with PEF after aging in bottle, or in oak barrels followed by bottles. These results support conclusions previously obtained in the comparison of physicochemical wine parameters. The application of a PEF treatment to the grapes permitted to reduce maceration time from 6 to 3 days without negatively affecting the wines' physicochemical and sensory parameters. When comparing wines obtained with untreated and PEF-treated grapes after longer maceration periods, smaller differences were observed in parameters depending on polyphenol extraction, but from a sensory point of view the wine obtained from grapes treated by PEF was preferred by panelists especially after it had aged 6 months in barrels.

Figure 32 displays the sensory profiles of the wines obtained from untreated and PEF-treated grapes with 3 and 6 days of maceration after six months of aging in oak barrels and 12 months of aging in bottle. This evaluation confirmed differences among

the wines already observed through physicochemical chemical analysis. Wine obtained from untreated grapes and 3 days of maceration was clearly distinct from the rests of the wines. This wine showed a lower intensity in flavor, and lower descriptors directly related with polyphenol content such as colour intensity, body, astringency, and persistency.

On the other hand, smaller differences in sensory descriptors were obtained between the other three wines, thereby confirming the potential of Pulsed Electric Fields for the reduction of maceration time without impairing physicochemical parameters and sensory properties of wine, even after aging.

5. Conclusions

Results obtained in this study reveal that the extraction of different families of polyphenols and individual polyphenols were significantly affected by PEF treatments, resulting in wines possessing a higher content of those compounds when compared with wines obtained from untreated grapes after the same amount of maceration days. However, the wine obtained from grapes treated by PEF with different maceration times followed an evolution similar to the wine obtained from untreated grapes in the course of 24 months of aging in bottles or in oak barrels plus bottles.

Physicochemical and sensory analysis showed that grapes treated by PEF can result in wines not only suitable for everyday consumption, but also in certain high-quality wines that require aging in bottle or in oak barrels.

6. Acknowledgments

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ESTUDIO 4: Increasing the total specific energy of PEF treatments permits the reduction of maceration time during vinification of *Caladoc* and *Grenache* wines.

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1. Abstract

The effect of moderate PEF treatment (M-PEF) (5 $kV.cm^{-1}$, 8,8 $kJ.kg^{-1}$) and intense PEF treatment (I-PEF) (5 $kv.cm^{-1}$, 52,9 $kJ.kg^{-1}$) on the reduction of maceration time during vinification of *Caladoc* and *Grenache* grapes was investigated.

In both grape varieties, M-PEF treatment combined with 4 days of maceration was the most effective treatment in achieving high anthocyanin content, color intensity and total phenol index at the end of fermentation. The I-PEF treatment promoted a rapid release of anthocyanins and phenolic compounds, along with a fast increment in the color intensity of the must after 24 h of maceration. Although color intensity and anthocyanin content decreased significantly throughout fermentation when grape pomace was removed after 24 h, these parameters were similar, after 3 months of bottling, in the case of *Caladoc* and slightly lower in *Grenache* than the control wine (10 days of maceration). Therefore, results obtained in this investigation are the first to demonstrate the potential of I-PEF for the reduction of maceration time to 24 hours in red winemaking.

2. Introduction

Over the last decades, considerable research efforts have been devoted to the development of non-thermal processing technologies (Zhang et al. 2011). The main goal of these technologies is to prevent the deterioration of foods that have a heat labile chemical or physical structure, thereby providing the food industry with processes that are more sustainable and eco-friendly in terms of energy requirements (Chemat et al. 2017).

PEF technology is regarded as a promising alternative to thermal processing with the purpose of improving microbial inactivation (Wang et al. 2018) and mass transfer (Puértolas et al. 2012), and modify food structure (Oey et al. 2017). The treatment generates a high intensity electric field between two electrodes by applying pulses of high voltage and short duration. The effects of PEF on foods are attributed to a presumed structural rearrangement of the cell membranes called electroporation, which consists in the formation of local defects or pores (Kotnik et al. 2019). The electroporation of grape skin cells with the purpose of improving the extraction of phenolic compounds during the maceration-fermentation step in red winemaking is one of the most widely investigated applications of PEF in recent years (Ricci et al. 2018). Polyphenols play an essential role in the quality attributes of red wine (Cheynier et al. 2006). Therefore, the optimization of the extraction of these compounds is of key importance in red winemaking. In order to obtain red wine, a contact period of grape pomace with the fermenting must is required to extract anthocyanins, which are located in the skin cells, and tannins, which are not only present in the skin cells, but also in the seeds (Bautista-Ortín et al. 2014; Busse-Valverde et al. 2010).

Different studies conducted in the laboratory (Delsart, et al. 2014), but also at pilot plant and semi-industrial scale, have demonstrated that PEF treatments can allow winemakers to reduce maceration time and/or obtain a wine with a greater amount of phenolic compounds (Maza et al. 2019). In view of such effects, PEF could become an alternative to techniques such as thermovinification or “flash release”, currently used in wineries to improve polyphenol extraction based on the heating of grapes. Thermovinification consists in heating grapes at temperatures between 70 and 75º C for a period ranging from 30 min to 24 hours (Sacchi et al. 2005). On the other hand, “flash release” consists in a rapid heating of grapes (85-95 C) with direct steam injection, after which grapes are exposed to a vacuum that induces instant vaporization of the water they

contain, thereby cooling them and weakening their skin cell envelopes (Moutounet and Escudier 2000). As compared with PEF, these techniques share the feature that solid parts of the grapes are removed after treatment, and fermentation is usually conducted in liquid phase, as in white wine. The benefits of fermenting in liquid phase include a better use of the effective volume of the tanks, an improved control of fermentation temperature, and savings in labor as well as in the energy consumption required to periodically pump the wine over the skin mass that rises to the top of the fermentation tanks.

Although it has been demonstrated that electroporation of grape skins by PEF significantly improves the extraction of polyphenols such as anthocyanins and tannins, a certain maceration time is required to obtain wines with a sufficient amount of these compounds (López et al. 2009). Typical maceration times reported by different authors for wines obtained with grapes treated by PEF range from 3 to 6 days (Maza et al. 2019). This maceration period required to obtain a sufficient amount of polyphenols could be due to the moderate PEF intensity ($\leq$5 kV.cm^{-1} and <10 kJ.kg^{-1}) applied in the studies conducted on the topic, which might be causing an incomplete electroporation of the grape skin cells.

In the present study, intense PEF treatments in terms of specific energy were applied to electroporate grape skins of two grape varieties (*Caladoc* and *Grenache*) in order to evaluate whether the maceration step could thereby be reduced to just a few hours.

3. Material and methods

Grape samples

Seven hundred kilograms of *Caladoc* (21.1°Brix, titratable acidity: 6.1 g.L^{-1} tartaric acid) and Grenache (26.9° Brix, titratable acidity: 4.8 g.L^{-1} tartaric acid) red grapes (Fuendejalón, Spain) were manually harvested in 2018. Harvesting was carried out in the first week of September for *Caladoc* grapes and in the first week of October for Grenache grapes. Prior to the PEF treatments, electrical conductivity was measured with a FYA641LFP1 conductivity probe (Ahlaborn, Holzkirchen, Germany) connected to an Almeno 2590 data logger (Ahlaborn, Holzkirchen, Germany)..

PEF equipment and processing

An EPULSUS® PM1-10 PEF-generator (Energy Pulse Systems LDA, Lisbon, Portugal) was used. This apparatus, with an output voltage and current of 10 kV and 200 A, respectively, generates monopolar square waveform pulses of 2 to 200 µs with a frequency up to 200 Hz. The applied voltage was measured with a high voltage probe (Tektronix, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronix, TBS 1102B-EDU, Wilsonville, Oregon, USA).

The treatment chamber consisted of three stainless steel cylindrical electrodes, separated by two methacrylate insulators based on a previous design by Toepfl et al. (Toepfl et al. 2007). Whereas the central electrode is connected to the high voltage, the electrodes of the two extremes are grounded. Two cylindrical treatment zones of 2.0 cm between the electrodes and an inner diameter of 2 cm were defined as a colinear configuration. The electric field strength used to characterize the PEF treatments corresponds to the field strength in the middle position of the treatment zone's central axis, which is almost equivalent to the field strength calculated by dividing the applied voltage and the gap between the electrodes (Toepfl et al. 2007). Mass flow was 140 $kg.h^{-1}$, providing a residence time of the medium in the treatment zone of 0.32 s. Temperature was measured before and after the PEF treatments by means of a type K thermocouple (Ahlborn, Holzkirchen, Germany) connected to an Almeno 2590 data logger (Ahlborn, Holzkirchen, Germany). The characteristics of the applied PEF treatments and the outlet temperature of the grape pomace are shown in Table 29.

Table 29: Parameters of the applied PEF applied to the red grape pomace

Treatment	Voltaje (kV)	Electric field ($kV.cm^{-1}$)	Temp after treatment ºC	Number of pulses	Pulse width (µs)	Treatment time (µs)	Specific energy ($kJ.kg^{-1}$)
High PEF	10.00	**5.00**	37.2±0.6	46.00	40.00	1840.00	**52.90**
PEF	10.00	**5.00**	22.1±0.5	8.00	40.00	320.00	**8.80**

Winemaking

The red grapes were weighed, crushed and destemmed with a Master E-10 destemmer (Enomundi, Zaragoza, Spain). Then, must was pumped by a progressive cavity pump (Rotor-MT, Bominox, Gerona, Spain) to the colinear treatment chamber. After PEF treatment, the must was distributed into fourteen stainless steel tanks (eight for Caladoc and six for Grenache grapes). Two additional batches of untreated grapes were used as control for each variety. In each tank, $K_2S_2O_5$ (10 $mg.kg^{-1}$), was added, and then 15 $g.hl^{-1}$ of a commercial suspension of the yeast Saccharomyces cerevisiae (OenoFrance

La Marquise E491, Epernay, France) were added. All treatments were fermented in duplicate at 22±1º C. Maceration times depending on the intensity of the applied PEF treatment were: 4 hours for *Caladoc* grapes treated with the intense PEF treatment (I-PEF), 24 hours for *Caladoc* and *Grenache* grapes treated with the I-PEF treatment, 4 days for *Caladoc* and *Grenache* grapes treated with moderate PEF treatment (M-PEF), and 9 days for untreated *Caladoc* and *Grenache* grapes. During the fermentation process, enological parameters, temperature, and must density were monitored daily, and the cap was punched down once a day. The concentration of residual sugars at the end of fermentation (13 days) was always lower than 3 $g.L^{-1}$. After fermentation, the wines were racked and stabilized for a period of one month at 2º C, and finally racked again, bottled, and stored in a conditioned room kept at 18 ± 1º C until analyzed..

General wine analysis

During fermentation, all wines were analyzed according to the methods prescribed by the OIV (Organization Internationale de la Vigne et du Vin, 2009). At the end of fermentation, alcohol content, total acidity, and pH were measured. The pH was determined with a Crison Basic20 pH-meter (Crison Instruments, SA, Barcelona).

Colorimetric indexes

All samples were centrifuged in an Eppendorf AG centrifuge for 15 min at 3000 rpm (Eppendorf, Hamburg, Germany). The absorbance of the musts was measured at 420, 520, and 620 nm by a Biochrom LibraS12 spectrophotometer (Biochrom Limited, UK) with Hellma® Analytics QS Quartz SUPRASIL® 300 Precision cells (light path 1 mm) (Hellma Analytics, Müllheim, Germany). Color intensity (CI) was calculated as the sum of 420, 520, and 620 nm absorbance, and Hue was calculated as the proportion of the absorbance measured at 420 nm and 520 nm according to Glories (1984). Total polyphenol index (TPI) was determined by a direct reading of the absorbance at 280 nm of diluted wine 1/100 ($v.v^{-1}$) with a Hellma® QS quartz SUPRASIL® 300 cuvette (light path 10 mm) (Hellma Analytics, Müllheim, Germany). TPI was calculated by multiplying the absorbance measured at 280 nm by 100. Total anthocyanins (AC) expressed in milligrams per liter of malvidin-3-glucoside were analyzed by determining the absorbance at 520 nm of diluted wine 1/100 ($v.v^{-1}$) with 1 % ($v.v^{-1}$) HCl (Ruiz-Hernández 2004).

Determination of condensed Tannins

Condensed Tannins (TC) were determined according to Sarneckis et al. (2006). The determination was carried out by precipitation with methylcellulose. All values are reported in mg.L-1 of epicatechin equivalents according to a calibration curve obtained from aqueous solutions of (-)-epicatechin (10, 25, 50, 75, 100, 150, and 200 $mg.L^{-1}$ of epicatechin).

High-Performance Liquid Chromatography (HPLC)

Anthocyanins were analyzed under the chromatographic conditions described by Puértolas, et al. (2010d). An HPLC Varian ProStar high-performance liquid chromatograph (Varian Inc., Walnut Creek, CA) equipped with a ProStar 240 ternary pump, a ProStar 410 autosampler, and a ProStar 335 photodiode array detector were used. Separation was achieved on a reverse-phase column (LC Luna® 100 Å C18 250 x 4.6 mm; 5 μm particle size, Phenomenex) with a pre-column of the same material (LC Luna® 50 x 4.6 mm; 5 μm particle size, Phenomenex). Chromatograms at 520 nm were recorded. The analyzed phenolic compounds were identified according to the retention time and the UV-vis spectra of pure standards, and according to the UV-vis spectral characteristics published in the literature (Puértolas et al. 2011). The concentrations of all studied compounds were expressed in $mg.L^{-1}$.

Statistical analysis

The data presented in tables represent mean values ± 95% confidence level. Analysis of variance (ANOVA) was carried out using InfoStat statistical software in the 2018 version. The graphics were carried out using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA).

4. Results

Effect of PEF treatments of different intensities on the extraction kinetics of color intensity, anthocyanins, and total phenolic compounds after different maceration times.

The evolution of color intensity, anthocyanin content, and total phenolic compounds during the maceration-fermentation stage of *Caladoc* grapes treated by I-PEF after 4 and 24 hours of maceration are shown in Figure 33.

Evolution of the same oenological indexes during maceration-fermentation of untreated and moderate PEF treated *Caladoc* grapes after 10 and 4 days of maceration, respectively, is also shown in Figure 33 for comparison. Considerable differences were observed between vinifications conducted with PEF electroporated grapes and with untreated grapes from the earliest moments of the maceration-fermentation stage onward. The I-PEF treatment led to a rapid release of anthocyanins and phenolic compounds, along with a rapid increment in the color intensity of the must from the onset of the maceration-fermentation step. After 4 hours of maceration, the color intensity, anthocyanin content, and total phenolic index of the must containing grapes treated with I-PEF were much higher than those of the fermenting must containing M-PEF treated *Caladoc* grapes and control grapes, and the same difference could still be observed even after 24 hours of maceration. However, a pronounced decrease in anthocyanin content and color intensity was observed when the grape skins were removed after 4 hours of maceration. At the end of fermentation, as a consequence of that tendency, wines obtained with grapes treated by I-PEF with a maceration of only 4 hours had the lowest value for the three indexes analyzed. Figure 33 also shows that color intensity, anthocyanins, and total phenolic index increased when maceration time for the grapes treated by I-PEF was extended to 24 h. Although these two indexes also declined after removing the grape pomace, the wine obtained at the end of fermentation had higher anthocyanin content and a similar color intensity and total phenol index to that of the control wine in which grape pomace remained in contact with fermenting must for 10 days.

Wine obtained with M-PEF treated grapes after 4 days of maceration was the one with the highest anthocyanin content, color intensity, and total phenolic index at the end of fermentation. Although the values of anthocyanin content and color intensity of the fermenting must containing the grapes treated by M-PEF after 4 days of maceration were similar to those of the fermenting must containing *Caladoc* grapes treated by I-PEF after 24 hours of maceration, the decline in anthocyanin content and color intensity after the removal of grape pomace was less pronounced. The stabilization of these two indexes was probably related to the presence of a higher concentration of tannins in the wine after 4 days of maceration. It is well known that tannins are required to stabilize unstable anthocyanin, and that the presence of ethanol is necessary for the extraction of tannins from the seeds (Busse-Valverde et al. 2010; Hernández-Jiménez et al. 2012). Since ethanol content in the first 24 hours of maceration-fermentation is too low, no presence of seed tannins in the seeds of wines obtained from such short maceration is expected.

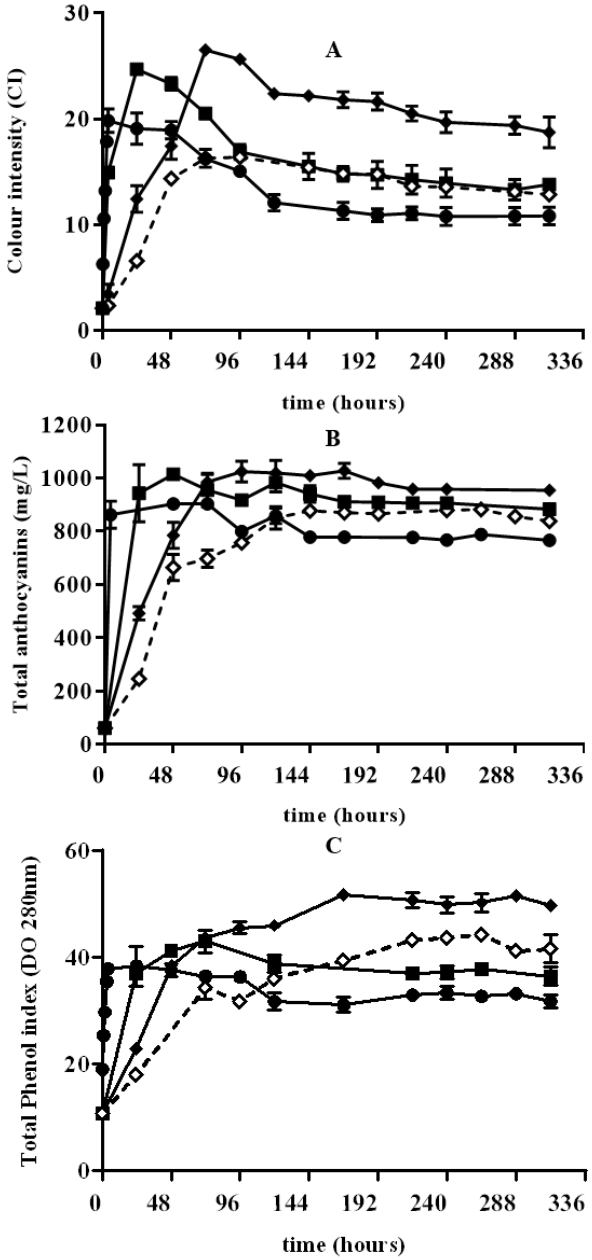

Figure 33: Evolution of *Caladoc* color intensity (CI) ***A,*** Total anthocyanins (TAC) **B**, and total polyphenol index (TPI) **C** along maceration time during vinification of PEF wine (♦), control wine (◊), vinification with the Hi-PEF treatment with 24 hours of maceration (■) and 4 hours of maceration (●).

The evolution of color intensity, anthocyanin content, and total phenolic compounds during the maceration-fermentation stage of Grenache grapes treated by I-PEF after 24 hours of maceration is compared with the evolution of the same oenological indexes during maceration-fermentation of untreated and M-PEF treated *Grenache* grapes after 10 and 4 days of maceration in Figure 34. Since a considerably pronounced decline in color intensity and anthocyanin content in *Caladoc* was observed when the

maceration time of the grapes treated by I-PEF was reduced to 4 hours, this combination was not evaluated when the study was conducted on Grenache grapes. Similarly to the case of *Caladoc* grapes, the application of an I-PEF treatment prior to vinification caused a rapid increment of the three indexes in the first 24 hours of maceration-fermentation. Anthocyanin content and color intensity obtained after only 24 hours of maceration were similar to the indexes obtained in control wine after 10 days of maceration. However, as in the case of *Caladoc*, the significant decrease observed in anthocyanin content and color intensity after the removal of grape skins entailed that those indexes were lower at the end of fermentation than those of control wine. Although the total phenol index did not decrease significantly in the wine obtained with grapes treated with I-PEF after 24 hours of maceration, the value of that index in the wine after fermentation was lower than in control wine, due to the fact that polyphenol extraction was more elevated when maceration time was extended. In the case of *Grenache*, the low concentration of ethanol could also have been the reason for the lower total polyphenol index and the observed decrease in color intensity and anthocyanin content when grape pomace was removed after 24 hours of maceration.

As in the case of *Caladoc*, the moderate PEF treatment combined with 4 days of contact of grape skins with the fermenting must was the most effective treatment in terms of anthocyanin content, color intensity, and total phenol index at the end of fermentation.

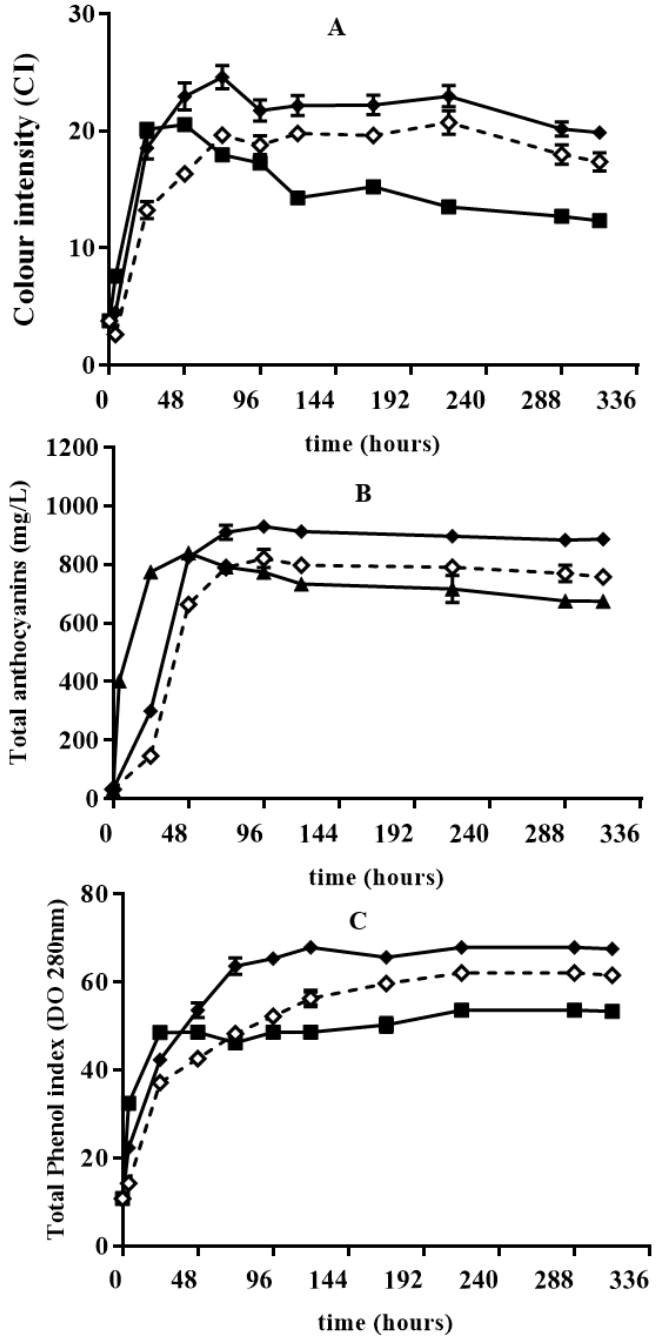

Figure 34: Evolution of *Grenache* color intensity (CI) ***A***, Total anthocyanins (TAC) **B**, and: total polyphenol content (TPI) **C**, along maceration time during vinification of PEF wine (♦), control wine (◊), and vinification with the Hi-PEF treatment with 24 hours of maceration (■).

Effect of PEF treatments of different intensities on oenological parameters

Table 30 compares the oenological parameters of the four *Caladoc* wines and the three *Grenache* wines after 3 months of bottling. As previously reported by other authors, pH, alcoholic content, and total acidity of the wines obtained with grapes treated by PEF

did not significantly differ from control wines even in those obtained with the most intense PEF treatments (Garde-Cerdán et al. 2013).

The combination most effective in obtaining *Caladoc* wine with the highest CI, AC, and TPI consisted in the application of a moderate electric field prior to vinification with 4 days of maceration. The wine obtained with this approach displayed AC, CI, and TPI values that were 25, 81, and 26 % higher, respectively, than control wine with 10 days of maceration. Similar results have been reported in studies conducted with other grape varieties, which have demonstrated the benefit of the application of a PEF treatment for increasing polyphenol content or reducing maceration time (Maza et al. 2019). Although the TPI and AC of the control wine was around 23% and 50% higher than the wine obtained with *Caladoc* grapes treated with I-PEF after only 4 hours of maceration, no statistically significant differences were found in AC and CI for the two wines. The lower TPI amd AC values obtained in the wines with only 4 hours of maceration significantly increased when maceration was extended to 24 hours. After prolonging the maceration time of the grapes treated by I-PEF for 24 hours, the obtained wine was not significantly different from control wine in terms of AC, TPI, and TC, whereby CI was slightly higher. As ethanol concentration after 24 hours of maceration is very low, tannins of the wine obtained after that short maceration period should come from the grape skins rather from seeds (Zamora 2003).

Similarly to *Caladoc*, the wine obtained with *Grenache* grapes treated with M-PEF after 4 days of maceration displayed the highest index values depending on polyphenol extraction: its TPI, TC, and CI values were significantly higher compared to those of the other two wines. The wine obtained with I-PEF treated grapes and short maceration (24 h) contained values that were lower than control for the 4 indexes associated with polyphenol extraction. However, the wine obtained with PEF treated grapes displayed TPI and AC indexes similar to control (less than 10% lower).

The application of M-PEF treatments of different intensity to *Caladoc* and *Grenache* grapes prior to vinification did not significantly affect the %Ye, %Rd, and %Bl of the obtained wines. No statistically significant differences were found in these values for wines after three months of aging.

Table 30: Oenological parameters of *Caladoc* and *Grenache* wines after three months of bottling.

	Caladoc				*Grenache*		
	Control	**High-PEF (4 hours)**	**High-PEF (24 hours)**	**PEF (4 days)**	**Control**	**High-PEF (24 hours)**	**PEF (4 days)**
pH	3.36 ± 0.02 a	3.34 ± 0.01 a	3.37 ± 0.04 a	3.37 ± 0.05 a	3.28 ± 0.02 ab	3.26 ± 0.02 a	3.29 ± 0.01 b
Alcohol	12.00 ± 0.14 a	12.05 ± 0.07 a	12.05 ± 0.21 a	11.95 ± 0.07 a	16.75 ± 0.07 a	16.65 ± 0.21 a	16.6 ± 0.28 a
Total acidity (gr.L^{-1})[c]	5.91 ± 0.07 a	5.82 ± 0.03 a	5.84 ± 0.06 a	5.80 ± 0.14 a	4.37 ± 0.22 a	4.32 ± 0.14 a	4.21 ± 0.14 a
IC (A.U.)	12.66 ± 0.44 a	11.97 ± 0.66 a	14.98 ± 0.71 b	22.90 ± 0.47 c	15.77 ± 0.52 b	12.36 ± 0.85 a	18.57 ± 0.74 c
TAC (mg.L^{-1}) [a]	766.26 ± 35.37 a	746.61 ± 26.83 a	799.26 ± 35.30 a	954.38 ± 23.46 b	837.25 ± 15.49 b	749.00 ± 11.03 a	883.74 ± 14.67 c
Hue (420/520)	0.38 ± 0.02 a	0.37 ± 0.01 a	0.37 ± 0.01 a	0.36 ± 0.01 a	0.49 ± 0.01 a	0.59 ± 0.05 b	0.48 ± 0.01 a
TPI (A.U.)	38.90 ± 0.28 c	30.00 ± 0.85 a	34.75 ± 1.77 b	49.20 ± 1.13 d	51.55 ± 1.34 b	47.40 ± 1.41 a	58.85 ± 0.49 c
TC (mg.L^{-1}) [b]	1077.88 ± 27.53 b	545.14 ± 170.21 a	831.86 ± 25.03 b	1472.57 ± 80.09 c	1649.56 ± 240.29 ab	1207.08 ± 100.12 a	2015.93 ± 52.57 b
(%Y)= (A420/CI x 100)	24.19 ± 1.29 a	24.76 ± 0.46 a	24.82 ± 0.56 a	24.34 ± 0.32 a	29.66 ± 0.12 a	32.99 ± 1.43 b	29.27 ± 0.13 a
(%R) = (A520/CI x 100)	64.69 ± 0.45 a	67.33 ± 1.20 ab	67.46 ± 1.34 ab	67.94 ± 0.91 b	60.27 ± 0.08 a	55.87 ± 2.64 a	60.47 ± 0.12 a
(%B) = (6420/CI x 100)	11.13 ± 0.84 b	7.92 ± 0.74 a	7.73 ± 0.79 a	7.74 ± 0.59 a	10.07 ± 0.20 a	11.14 ± 1.21 a	10.26 ± 0.01 a

Values represent mean with their standard deviation (n=2)

Different letters within the same file and variety of grape indicate significant differences ($p \leq 0.05$).

TPI: total polyphenol index; CI: color intensity; TAC: total anthocyanins content; TC: tannins condensed; %Ye, %Rd, %Bl: percentages of yellow, red, and blue colors respectively; A.U.: absorbance units.

[a] Expressed as malvidin-3-glucoside.

[b] Expressed as epicatechin.

[c] Expressed as tartaric acid

Therefore, although the PEF treatments improved the extraction of those components of grapes responsible for the color of wine, the proportion in which these compounds were extracted was similar to that of the untreated grapes. In all cases, the values obtained in this study for the %Ye, %Rd, and %Bl were within a range considered as optimal (Glories 1984).

Effect of PEF treatments of different intensities on anthocyanin composition

Individual anthocyanins of the obtained wines were identified and quantified. It is well known that anthocyanins extracted from the skins of red grapes are the principal components responsible for the red wine color in young wines.

Table 31 compares the anthocyanin content of *Caladoc* and *Grenache* wines obtained from I-PEF treated grapes and short maceration time (4 and 24 hours) with the wines obtained from M-PEF treated grapes and longer maceration time (4 days), as well as with untreated grapes. On general terms, similar anthocyanin profiles were observed for all wines obtained with each grape variety. Therefore, even when maceration time was reduced to 24 hours or even less, an M-PEF treatment did not produce a selective effect on any anthocyanin compound.

Table 31 shows that monoglucoside derivates of anthocyanins (Unacylated) predominated in all cases. Unacylated anthocyanins represented 70-80% and 85 95% of total anthocyanins for *Caladoc* and *Grenache* wines, respectively. These differences in the proportion of unacylated anthocyanins may be attributed to the grape variety, as has been ascertained by other authors (Puértolas, et al. 2011). In the wines from two varieties obtained with different procedures, malvidin-3-glucoside was the most dominant monomeric anthocyanin; nevertheless, significant amounts of petudin-3-glucoside and delphinidin-3-glucoside were likewise found. Similar results have also been reported for wines obtained from other grape varieties. Regarding acylated and coumroylated compounds, conjugates of malvidin were the ones most detected in all the wines. These results agree with those reported by other authors concerning the composition of anthocyanin derivates in red wine (Cacho et al. 1992; Puértolas et al. 2011).

Table 31**:** Anthocyanic content after the fermentation of *Caladoc* and *Grenache* wines.

	Caladoc				*Grenache*		
	Control	**High-PEF (4 hours)**	**High-PEF (24 hours)**	**PEF (4 days)**	**Control**	**High-PEF (24 hours)**	**PEF (4 days)**
Delphinidin-3G	28.22 ± 1.41 ab	19.53 ± 1.73 a	34.28 ± 8.42 b	52.71 ± 3.93 c	37.76 ± 0.95 b	27.00 ± 4.29 a	48.00 ± 2.83 c
Cyanidin-3G	3.93 ± 5.25 a	1.45 ± 0.27 a	3.63 ± 2.63 a	6.31 ± 2.23 a	5.87 ± 4.91 a	1.45 ± 0.66 a	7.45 ± 0.70 a
Petunidin-3G	47.01 ± 16.57 ab	37.33 ± 60 ab	20.92 ± 15.25 a	57.83 ± 7.28 b	47.26 ± 7.86 ab	37.49 ± 5.78 a	60.00 ± 1.41 b
Peonidin-3G	10.84 ± 1.52 a	7.81 ± 0.21 a	11.22 ± 2.88 a	18.19 ± 1.13 b	41.16 ± 0.30 b	24.96 ± 0.70 a	49.11 ± 1.87 c
Malvidin-3G	501.19 ± 14.16 b	387.47 ± 8.65 a	489.00 ± 25.34 b	682.66 ± 7.30 c	547.36 ± 3.39 b	436.66 ± 32.85 a	603.09 ± 3.95 b
Delphinidin-3G-Ac	3.94 ± 1.64 ab	2.59 ± 0.16 a	4.30 ± 0.59 ab	6.44 ± 0.38 b	5.50 ± 1.41 ab	4.00 ± 0.71 a	7.30 ± 0.71 b
Cyanidin-3G-Ac	4.25 ± 2.07 a	1.48 ± 1.33 a	4.45 ± 0.73 a	3.88 ± 0.65 a	1.75 ± 0.78 a	2.35 ± 0.71 a	2.60 ± 0.57 a
Petunidin-3G-Ac	7.28 ± 1.96 a	5.73 ± 0.95 a	6.11 ± 0.72 a	8.26 ± 0.29 a	4.95 ± 0.64 ab	0.87 ± 0.56 a	6.70 ± 2.40 b
Malvidin-3G-Ac + peonidin-3G-Ac	54.11 ± 5.98 a	54.20 ± 5.89 a	72.04 ± 3.17 b	74.04 ± 3.12 b	11.97 ± 1.19 b	1.71 ± 0.18 a	11.75 ± 2.47 b
Delphinidin-3G-Cm	6.64 ± 7.15 a	1.86 ± 0.52 a	1.43 ± 0.44 a	2.71 ± 0.37 a	3.15 ± 0.07 a	1.83 ± 0.01 a	3.80 ± 0.99 a
Cyanidin-3G-Cm	1.99 ± 1.43 a	1.05 ± 0.21 a	1.96 ± 0.48 a	0.27 ± 0.13 a	0.41 ± 0.01 b	nd	1.75 ± 0.35 c
Petunidin-3G-Cm	11.83 ± 0.70 b	3.65 ± 0.04 a	5.22 ± 3.46 a	8.53 ± 0.67 ab	5.45 ± 1.34 b	0.32 ± 0.16 a	5.55 ± 0.07 b
Peonidin-3G-Cm	7.05 ± 0.65 bc	1.89 ± 0.37 a	4.89 ± 1.68 b	8.29 ± 1.12 c	6.36 ± 0.34 b	0.39 ± 0.09 a	7.95 ± 0.21 c
Malvidin-3G-Cm	24.13 ± 0.66 ab	16.22 ± 50 a	24.98 ± 0.66 b	33.75 ± 3.19 c	9.98 ± 1.36 a	7.80 ± 0.48 a	15.20 ± 1.16 b
Unacylated	**591.17 ± 10.58 b**	**453.57 ± 4.31 a**	**559.03 ± 24.03 b**	**817.7 ± 2.82 c**	**679.4 ± 8.12 b**	**527.55 ± 22.83 a**	**767.65 ± 5.62 c**
Acetylated	**69.57 ± 0.31 a**	**63.99 ± 5.35 a**	**86.9 ± 3.74 b**	**92.62 ± 2.57 b**	**24.17 ± 0.08 b**	**8.93 ± 0.74 a**	**28.35 ± 4.74 b**
Coumarylated	**51.63 ± 7.73 bc**	**24.66 ± 4.37 a**	**38.47 ± 1.07 ab**	**53.53 ± 5.22 c**	**25.35 ± 3.13 b**	**9.41 ± 1.85 a**	**34.25 ± 0.33 c**
Total anthocyanic	**727.09 ± 25.25**	**549.98 ± 2.33**	**801.47 ± 22.89**	**1109.26 ± 58.76**	**759.35 ± 14.31**	**549.3 ± 25.22**	**897.93 ± 36.44**

Values represent mean with their standard deviation (n=2)
Different letters within the same file and grape variety indicate significant differences ($p \leq 0.05$)
Anthocyanins: mean ± SD, gr.L^{-1} as malvidin-3-O-glucoside
nd: not detected. G: glucoside, Ac: acetylated, Cm: coumarylated

5. Discussion

Although certain phenolic compounds present in red wine, such as polymeric tannins and monomeric flavanols, come from the seeds of the grapes, most of them, especially anthocyanins, are located in the grape skin cells (Amrani-Joutei and Glories 1995; Río Segade et al. 2017). Polyphenol extraction from the grape skins to the fermenting must during the maceration-fermentation step is a diffusion process. An intact cytoplasmic membrane of the skin cells acts as a barrier that prevents components of the cellular interior from leaving the cell. Therefore, diffusion rate and extraction yield are both highly dependent on the integrity of grape skins' cytoplasmic membrane (Cerpa-Calderón and Kennedy, 2008; Pinelo et al., 2006). Several investigations have demonstrated that the application of PEF treatments of very low energy (<10 $kJ.kg^{-1}$) to grapes prior to the maceration-fermentation step can accelerate the extraction of polyphenols (Delsart et al., 2014; López et al., 2008b; López-Giral et al., 2015). This effect has been attributed to electroporation, which causes the loss of the selective permeability of the cytoplasmic membrane (Saulis 2010). Although it has been widely demonstrated that PEF treatments facilitate polyphenol release from grape skins, several days of maceration are required to obtain a sufficient amount of phenolic compounds (Luengo et al., 2012; Puértolas et al., 2010e). This research is the first to investigate the potential of PEF for reducing the contact time of grape pomace with the fermenting must to just a few hours. The strategy consisted in incrementing the total specific energy delivered by the PEF treatment to the grapes by increasing the total number of pulses. The rapid increment observed in the indexes that depend on polyphenol extraction may be attributed to an increment in the number and/or size of the pores created in the cytoplasmic membrane of the grape skin cells, or it could be associated with the increment in the number of electroporated cells in grape skin tissues (Weaver and Chizmadzhev 1996). As compared with a parallel electrode treatment chamber configuration, the colinear configuration used in this investigation has lower energetic requirements, thanks to its greater load resistance, and the chamber's circular section facilitates the flow of crushed grapes. However, inhomogeneity in the distribution of the electric field in this configuration could entail that a proportion of cells of the grape skins may have been unaffected or insufficiently affected by an electric field to cause electroporation (Huang et al. 2013; van de Bosh 2007). An increment in specific energy delivered to the treatment chamber by increasing the number of the applied pulses could increase the proportion of

cells affected by the critical electric field required for electroporation to take place. This effect would be reflected in an increment in the amount of polyphenols released to the must within a shorter time frame.

The intense PEF treatment applied here was especially effective in releasing anthocyanins and, as a consequence, in increasing color intensity in the first moments of maceration. The major role of anthocyanins in the initial color of red wine is well known (Setford et al. 2019). However, similarly to the data reported on evolution in wines obtained with thermovinification or flash expansion techniques with or without very short maceration periods (Gao et al. 1997), wines obtained with grapes treated by PEF and short macerations exhibited a considerable decrease in anthocyanin concentration when grape pomace was removed from the fermenting must. A decrease in anthocyanin concentration is generally observed during the maceration phase in red winemaking as a consequence of several reactions that involve anthocyanins, such as oxidation, copigmentation, or adsorption by yeast (Hermosín-Gutiérrez et al. 2005; Morata et al. 2003; Setford et al. 2017; Shenoy 1993; Wesche-Ebeling & Montgomery 1990). Generally, when grape pomace is in contact with the fermenting must, a decrease in anthocyanin content during the first days of maceration is not observed because the amount of anthocyanins extracted from the grape skins is higher than the amount of anthocyanins affected by reactions which cause them to decrease (Boulton 2001; Mateus and de Freitas 2001).

One of the drawbacks associated with oenological techniques aiming to eliminate or reduce maceration time is that the wines thereby obtained have poor color stability due to their low tannin content, since the extraction of tannins from the berry seed requires the presence of ethanol (Alcalde-Eon et al. 2014). Ethanol molecules not only contribute to astringency and mouthfeel, but they also participate in condensation reactions with anthocyanins that ensure a stabilization of wine color after bottling. It is remarkable to note that the I-PEF treatment applied in this investigation also encouraged the extraction of tannins, even when the maceration period was shortened to 24 hours. Those tannins, therefore, helped maintain the color intensity of *Caladoc* and *Grenache* wines after three months of aging in bottle, and helped ensure that the CI values remained within the range of those reported for other young wines obtained with longer maceration periods.

Conclusions

In this investigation, the potential of the application of PEF for obtaining red wine with a maceration time of only 24 hours has been demonstrated for the first time.

Although color intensity and anthocyanin content decreased significantly throughout fermentation when grape pomace was removed, oenological parameters after 3 months of bottling were similar to and slightly lower than control wine in the case of *Caladoc* and *Grenache* wines, respectively.

Therefore, PEF could well become an alternative to current techniques used in wineries to improve polyphenolic extraction and, as a consequence, to eliminate or reduce maceration time associated with the heating of grapes. PEF could solve several problems associated with thermal methods such as the loss of varietal aromas through temperature increment, the consumption of high quantities of energy, and space requirements.

6. Acknowledgments

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DISCUSIÓN GENERAL

Si bien los resultados de esta Tesis Doctoral ya se han discutido parcialmente en los manuscritos que se adjuntan en la sección de Resultados, en este apartado se presenta una discusión conjunta de todos ellos con el objetivo de obtener conclusiones generales de los estudios realizados.

La tecnología de los pulsos eléctricos de alto voltaje (PEAV) ha sido investigada para mejorar distintos procesos de la industria alimentaria como la inactivación microbiana, la extracción de compuestos de interés como el aceite, el azúcar, colorantes, etc. (Abenoza et al., 2013; El-belghiti and Vorobiev, 2005; Mahnič-Kalamiza et al., 2014; Puértolas et al., 2012), la deshidratación de alimentos (Wiktor et al., 2014) o cortado y pelado de frutas y hortalizas (Arnal et al., 2018; Oey et al., 2017). En la actualidad, la tecnología ya se ha implantado en más de 100 empresas con dos objetivos fundamentales la pasteurización de zumos de frutas y el tratamiento de patatas para la posterior fabricación de patatas fritas congeladas y snacks. Mientras que para la primera aplicación los equipos más potentes son capaces de procesar hasta 5.000 litros de zumo/ hora, en el caso de las patatas se pueden alcanzar capacidades de procesado de hasta 50 toneladas/hora.

Comprender con exactitud el efecto e impacto de los distintos parámetros de esta tecnología en los diferentes procesos es de vital importancia para implementación industrial y más concretamente en las bodegas. Aunque este sector siempre se ha considerado muy tradicional, desde hace unos años está incorporando numerosos avances tecnológicos con el objetivo final de obtener un producto de mayor calidad y de reducir costes de procesado. Aunque la tecnología PEAV puede ofrecer numerosas ventajas en el sector enológico, para su implantación no solo es necesario demostrar su capacidad para mejorar el vino y/o su proceso de elaboración, sino que, además, debe de resultar más competitiva que otras tecnologías que actualmente se utilizan para el mismo objetivo y debe de ser aceptada por las bodegas

Los PEAV actúan a nivel de la membrana citoplasmática de las células modificando su permeabilidad selectiva debido a la formación de poros. (Saulis, 2010; Tsong, 1989; Weaver and Chizmadzhev, 1996). La formación de estos poros facilita la extracción de distintos componentes del interior celular del hollejo en el proceso de elaboración del vino (Vorobiev and Lebovka, 2006). La mayoría de los estudios realizados en esta Tesis Doctoral se han llevado a cabo a escala de bodega experimental. Los resultados han corroborado resultados previos obtenidos a escala de laboratorio o piloto que indicaban que los PEAV aumentan la velocidad de difusión de estos

compuestos, especialmente los polifenoles, acortando la etapa de maceración/fermentación con todos los beneficios que ello conlleva para las bodegas.

1. Extracción de los compuestos fenólicos

Consideraciones generales

Los compuestos fenólicos pertenecen a una gran clase de metabolitos secundarios de plantas, que muestran una diversidad desde estructuras simples como los ácidos fenólicos, a estructuras extremadamente complejas generalmente polimerizadas, denominadas flavonoides (Cheynier, 2012). Los compuestos fenólicos son importantes en la industria alimentaria ya que son responsables del color de los frutos rojos, zumos y vinos, son sustratos en las reacciones de pardeamiento enzimático, contribuyen al sabor de los alimentos y están involucrados en los efectos beneficiosos para la salud que tienen algunos alimentos debido a su poder antioxidante. En las uvas, el contenido fenólico depende de muchos factores siendo la variedad de la uva uno de los principales. Otros factores como la genética de la variedad, la localización geográfica del cultivo, las condiciones macro y micro-climáticas, las propiedades edáficas, el sistema de riego así como, y no menos importantes, las distintas técnicas de manejo de cultivo condicionan dicho contenido (Arozarena et al., 2002; Cantos et al., 2002; Jackson and Lombard, 1993; Sipiora and Granda, 1998). La variabilidad en la concentración de los polifenoles individuales, también, le confieren al vino sus características de calidad (Boulton, 2001; Fischer et al., 2000; Glories, 1984; Robichaud and Noble, 1990; Vidal et al., 2004). Entre ellas, los polifenoles determinan en gran medida el color, la astringencia, el amargor y los aromas de los vinos, características que condicionan la aceptación del vino por parte del consumidor. Además, se considera que contribuyen a los beneficios para la salud al igual que el consumo de frutas y verduras en la dieta debido a la reducción del riesgo de diversas enfermedades degenerativas gracias a la capacidad de los polifenoles de eliminar radicales libres (Estruch, 2000; Nichenametla et al., 2006; Perez-Vizcaino and Fraga, 2018, p.; Stoclet et al., 2004; Tomera, 1999).

En la uva *Vitis vinífera* la mayor concentración de los polifenoles que pasarán a formar parte del vino se encuentran en el interior de las células del hollejo. Para que exista una extracción de estos compuestos, tiene que haber un periodo de contacto entre las partes sólidas de la uva y el mosto durante el proceso de fermentación. Es decir, que la difusión de los compuestos fenólicos desde los hollejos al mosto durante la maceración-

fermentación es una etapa fundamental en el proceso de elaboración del vino tinto, de ahí que haya sido uno de los procesos más estudiados y tecnificados (Busse-Valverde et al., 2010; Gao et al., 1997; Kelebek et al., 2009, 2006; Koyama et al., 2007; Sacchi et al., 2005). La modificación que producen los PEAV en la membrana citoplasmática del hollejo de la uva favorece la extracción de estos compuestos y ofrece numerosas posibilidades en este sector.

Implementación de los PEAV

El uso de los PEAV para fomentar el proceso de transferencia de masa en vinificaciones en tinto se ha estudiado desde hace varios años en variedades como *Mazuelo* (López et al., 2008a), *Tempranillo* (López et al., 2008b), *Garnacha* (Luengo et al., 2014), *Cabernet Sauvignon* (Delsart et al., 2014), *Merlot* (Delsart et al., 2012), *Cabernet Franc* (El Darra et al., 2013), *Aglianico, Piedirosso* (Donsì et al., 2010) y *Graciano* (López-Giral et al., 2015) cosechadas en distintos países (España, Francia, Líbanmo, Italia y Nueva Zelanda) (Puértolas et al., 2016). En todos estos trabajos y variedades de uva se ha comprobado la eficiencia del uso de los PEAV en la extracción de los compuestos fenólicos, permitiendo de forma general incrementar hasta en un 20 % el índice de polifenoles totales (IPT), el índice de color (IC) y los antocianos totales (AT). A pesar de todos estos resultados, no había ningún trabajo en el que se realizara un estudio sistemático del efecto de la intensidad del campo eléctrico en la extracción de compuestos fenólicos aplicando en los tratamientos PEAV la misma cantidad de energía específica total. Este planteamiento permitía comparar, a igualdad de condiciones, el efecto del campo eléctrico. Por otro lado, incluir la energía específica aplicada permite evaluar y comparar los PEAV con otros procesos y tecnologías en lo referente al coste del tratamiento como se discutirá más adelante. La ausencia de trabajos en este sentido probablemente es debido a una limitación técnica ya que no existen equipos que permitan evaluar, a caudales de planta piloto, un amplio rango de condiciones de tratamientos. La realización de este estudio requería que se pudiera tratar la uva en un rango amplio de tiempos de tratamiento (número de pulsos x anchura del pulso) para poder aplicar a bajos campos eléctricos (1 kV/cm) y la misma energía específica que la aplicada a campos eléctricos más elevados (8 kV/cm). Como se discutirá, la posibilidad de disponer de dos generadores de características complementarias permitió realizar este estudio.

Resultados previos a los mostrados en esta Tesis Doctoral evaluados en condiciones estáticas indicaban que tratamientos de PEAV de las uvas a campos

eléctricos superiores a los 5 $kV.cm^{-1}$ no producían un mayor incremento del nivel de polifenoles extraídos (López et al., 2008b). Esto indicaría que por encima de un determinado campo eléctrico no se consiguen mejoras en esta aplicación y tratamientos más intensos únicamente conducen a un mayor gasto energético del proceso. Esta afirmación se confirmó con uvas de la variedad *Garnacha* en las pruebas realizadas en el ESTUDIO 1 de esta tesis, a escala de planta piloto (600 $kg.h^{-1}$) en las que al aplicar intensidades de 6 y 8 $kV.cm^{-1}$ del mismo nivel energético (6 $kJ.kg^{-1}$), no se determinaron diferencias entre estos tratamientos en los valores del índice de polifenoles totales, intensidad de color, antocianos y taninos condensados (TC) obtenidos a distintos tiempos de la maceración-fermentación (Figure 23). Es decir, 6 $kV.cm^{-1}$ con una energía de 6 $kJ.kg^{-1}$ podría considerarse como el tratamiento máximo a aplicar. Si bien 6 $kV.cm^{-1}$ es un valor relativamente bajo comparado con los tratamientos utilizados para inactivar microorganismos (>15$kV.cm^{-1}$), cuando estas intensidades se aplican en instalaciones industriales con caudales elevados de la uva de hasta 40-50 $ton.h^{-1}$, se requiere de equipos extremadamente potentes y por lo tanto muy caros que, limitarían la aplicación de la tecnología en las bodegas. Es por ello que la optimización de los tratamientos de PEAV a intensidades de campo más bajas es de suma importancia para facilitar su implementación.

En este sentido se complementó el estudio con pruebas a intensidades de campo menores con el mismo objetivo de evaluar la extracción de los polifenoles a intensidades del campo eléctrico más bajas manteniendo la intensidad de energía específica total aplicada.

Es necesario recalcar que, mientras menor sea el campo eléctrico utilizado para el proceso de extracción de los polifenoles, menor será la potencia necesaria del equipo a utilizar.

Para poder aplicar tratamientos de la misma energía específica que la que se aplicaba a campos eléctricos más elevados (6 $kJ.kg^{-1}$) a campos eléctricos más bajos fue necesario utilizar dos generadores de PEAV distintos Los tratamientos a campos eléctricos más elevados (6 y 8 kV/cm) se realizaron con un equipo que permite aplicar un voltaje máximo de 30 kV (Scandinova) y pulsos de una máxima anchura de 3 µs. El tratamiento a 1 kV/cm se aplicó con un generador que si bien su máximo voltaje es 10 kV permite aplicar pulsos de una anchura de hasta 100 µs. De esta manera, con estos pulsos de mayor anchura se pudo aplicar a bajo campo eléctrico un tratamiento con una energía específica próxima a la aplicada con los tratamientos a campos eléctricos elevados.

Finalmente se aplicó un tratamiento a 4 kV/cm con cada uno de los dos equipos a efectos comparativos.

Con el objetivo de establecer las mejores condiciones de tratamiento de PEAV y al mismo tiempo determinar que tratamiento es el más efectivo en términos de extracción para reducir los tiempos de maceración, se analizó la velocidad de extracción de los polifenoles (Table 17). Gracias a este estudio, se determinó que en las uvas sin tratar por PEAV la velocidad de extracción fue la menor de todos los tratamientos (8,7 IPT.día^{-1}), un 40 % más lento si se compara con los tratamientos de 8 kV.cm^{-1} cuyo valor fue de 12,3 (IPT.día^{-1}). Para los tratamientos obtenidos a intensidades de campo eléctrico más bajas (1 y 4 kV.cm^{-1}) las velocidades fueron un 19 y 31 % superiores respecto al control. Es de destacar el tratamiento de 1 kV.cm^{-1} que aceleró la extracción reduciendo en un 20 % el tiempo de extracción comparado con el proceso control ya que desde un punto de vista de escalado es una opción muy interesante al requerirse equipos de menor potencia para generar dicho campo eléctrico. Sin embargo, y como se ha destacado anteriormente, si bien el generador de PEAV sería menos potente y probablemente más económico, requeriría altas frecuencias de pulsos para aplicar tratamientos más largos. Esta cantidad de tiempo podría resultar excesiva para su aplicación a nivel de bodega sobre todo para trabajar con grandes volúmenes. En cualquier caso, es un aspecto de gran interés a valorar en un futuro es la viabilidad de tratamientos de baja intensidad, pero de suficiente nivel energético sobre todo con el fin de utilizar cámaras de mayores dimensiones donde conseguir altos campos eléctricos a día de hoy puede resultar una limitación. Independientemente de estas consideraciones, el estudio realizado permitió determinar que los tratamientos con los que se conseguía la mayor velocidad de extracción para conseguir un determinado nivel de IPT (en el estudio, por ejemplo, 50) se producirían al aplicar niveles energéticos entre 4 y 5 kJ.kg^{-1} (Figure 25), dependiendo a su vez del campo eléctrico aplicado. Así como se observa en la Figura 35, los niveles energéticos (círculos blancos en la Figura 35) para una velocidad de 12 IPT.día^{-1} variarían entre 4,4 kJ.kg^{-1} a 1 kV.cm^{-1} hasta los 3,7 kJ.kg^{-1} a 8 kV.cm^{-1}.

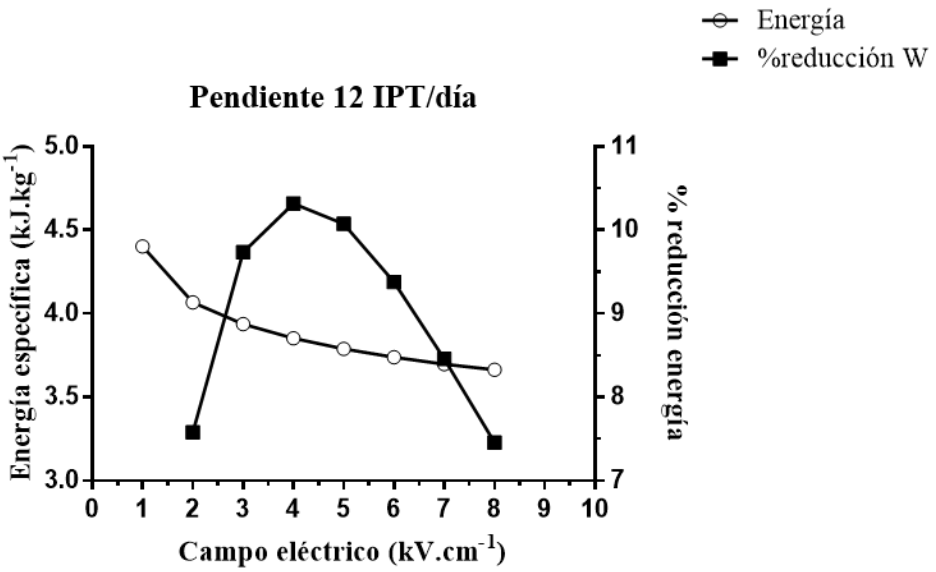

Figura 35: Relación entre la intensidad del campo eléctrico y la energía específica (○) para conseguir una velocidad de extracción de 12 IPT.día^{-1} al aplicar PEAV (eje OY izquierda) y el porcentaje de reducción de la energía específica aplicada por cada incremento de 1 kV.cm^{-1} (■).

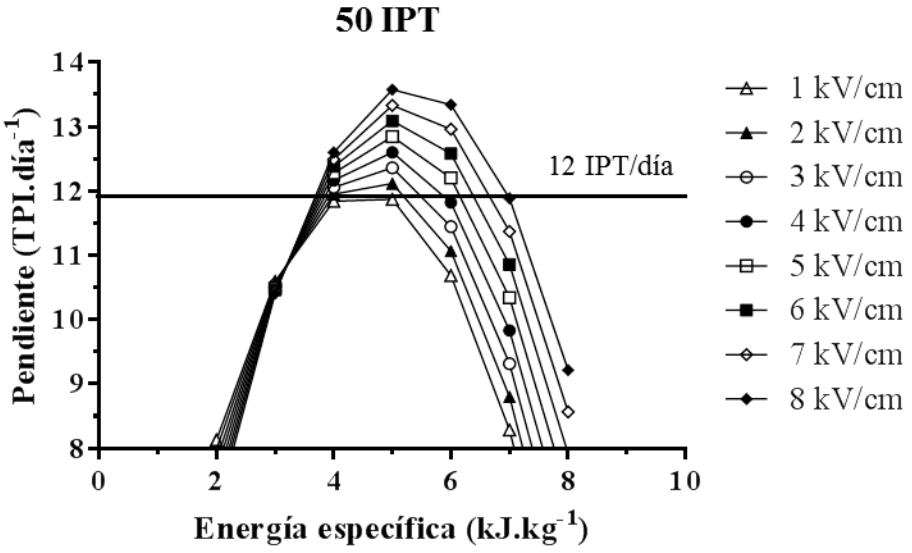

Figura 36: Relación entre la velocidad de extracción de IPT por día y la energía específica necesario para lograrlo a distintos campos eléctricos (kV.cm^{-1}).

Considerando esta relación entre la energía aplicada y el campo, se puede determinar que cada incremento del campo de 1 kV.cm^{-1} supone un determinado porcentaje de reducción de la energía específica que haría falta aplicar (cuadrados negros en la Figura 35). Por ejemplo, pasar de 1 a 2 kV.cm^{-1} supondría reducir de 4,4 kJ.kg^{-1} a 4,1 kJ.kg^{-1} es decir una reducción de un 7,6 %; de 2 a 3 kV.cm^{-1}, esa reducción sería de un 9 %. Según este análisis, para una velocidad de extracción de 12 IPT.día^{-1}, los tratamientos de alrededor de 4 kV.cm^{-1} son los que permitirían conseguir el mayor

porcentaje de reducción. En conclusión, tratamientos de 4 $kV.cm^{-1}$ con un nivel energético de 4-5 $kJ.kg^{-1}$ serían los más adecuados a aplicar para conseguir las mayores velocidades de extracción de polifenoles. Además, la aplicación de estos tratamientos permitiría reducir el tiempo de maceración hasta un 30 % que era uno de los objetivos a evaluar en este trabajo (Figure 26).

Implementación de los PEAV en vinificaciones en Bodega

Los ensayos preliminares obtenidos nos indicaron que los campos eléctricos utilizados deberían estar en torno a los 4 $kV.cm^{-1}$. Sin embargo, la aplicación de PEAV a caudales mayores a los de un ensayo de planta piloto simulando ya una producción a escala semi industrial (2500 $kg.h^{-1}$) hizo necesario una configuración de PEAV utilizando pulsos anchos (100 µs) reduciendo el número de estos (3,7 pulsos), para lograr obtener la energía específica total de 6,7 $kJ.kg^{-1}$. El estudio anterior, se observó que el efecto de la extracción de polifenoles era más eficiente en la medida que aumentábamos el número de pulsos. Este hecho probablemente era debido a que la distribución del campo eléctrico en la cámara colineal utilizada no es uniforme por lo que era posible que una parte de la uva procesada no recibiera el tratamiento estimado. Este fenómeno quizás es menos marcado cuando se aplican el mismo tratamiento con un mayor número de pulsos más estrechos. Ello implica aumentar la frecuencia de tratamiento y como consecuencia la probabilidad de que un mayor volumen de uva sea afectado por el tratamiento.

Independientemente de estas consideraciones, la implementación de los tratamientos PEAV a escala de bodega fue comprobada en la zona experimental de "Bodegas Aragonesas" llegando a procesar 12.000 kg de uva *Garnacha*. La Figura 37 muestra una fotografía de la instalación de la línea de PEAV en la bodega trabajando a un caudal de 2500 $kg.h^{-1}$ y la Figura 44 de los Anexos un esquema de una instalación. Como se ha descripto en la sección de "Material y Métodos", tras la recepción de la uva en una tolva (Figura 37, número 1), ésta era impulsada por un tornillo sin-fin (nº 2) hasta la despalilladora (nº 3). La mezcla del mosto y los hollejos era aspirada por una bomba peristáltica (nº 4) que impulsaba la uva a través de la cámara PEAV (nº 5) hasta una segunda bomba de tornillo (nº 6) que llevaba la mezcla pulsada hasta los depósitos de 5000 kg.

Figura 37: Equipos instalados y utilizados en "Bodegas Aragonesas" para realizar la implementación de los PEAV a escala de bodega (foto Marcos Maza). (1) Tolva de recepción. (2) Tornillo sin-fin. (3) Despalilladora. (4) Bomba peristáltica. (5) Cámara de PEAV. (6) Bomba de tornillo helicoidal.

Figura 38: Imagen obtenida del mosto tras 60 minutos de contacto con hollejos de uva *Garnacha* tratados (vaso izquierdo) y sin tratar (vaso derecho) por PEAV.

El tratamiento aplicado en la bodega confirmó los resultados obtenidos en los tratamientos a escala planta piloto. Es más, a los 60 minutos tras el tratamiento de la uva, ya era visible el efecto de los PEAV observándose un aumento de la intensidad del color del mosto en contacto con los hollejos tratados (Figura 38). En el caso de los vinos

obtenidos a partir de uva tratada con PEAV, estos mostraron un 30 % más de IPT que los vinos sin tratar (Table 21) y entre un 15 a un 30 % para los otros parámetros (IC, AT y TC). Es de destacar que el efecto de los PEAV era mayor para los vinos obtenidos con menos tiempo de maceración que aquellos con mayor tiempo. Así por ejemplo, el índice de color de los vinos tratados por PEAV con 3 días de maceración fue un 50 % superior que los no tratados y de un 27 % cuando el tiempo de maceración fue de 6 días. Estos resultados fueron similares a los descritos en la literatura, pero aplicados a menores caudales (López-Giral et al., 2015; Puértolas et al., 2010e). Los resultados por tanto obtenidos indicarían que el efecto de los PEAV observados a escala de laboratorio en relación a la extracción de compuestos de la piel de la uva se confirma a escala de bodega en tanto en cuanto se consiga alcanzar aplicar las condiciones óptimas de tratamiento a los caudales demandados en la bodega.

Influencia del tiempo de maceración

Uno de los objetivos de esta Tesis Doctoral era demostrar si el tratamiento PEAV lograba reducir el tiempo de maceración. Las vinificaciones tradicionales poseen un tiempo de maceración tradicional de 10-14 días, que conlleva a obtener vinos aptos para envejecer o cortes de vinos para consumo diario. Reducir, aún más, los tiempos de maceración beneficiarían directamente en una reducción de los costes operativos traducidos en energía y un aumento en la capacidad de procesado de uva en la bodega durante la época de vendimia. Sin embargo, reducir el tiempo de contacto de los hollejos de la uva con el mosto durante la maceración-fermentación podría ocasionar una disminución en la calidad del vino obtenido. Los estudios desarrollados durante esta Tesis Doctoral demostraron el efecto de los PEAV aumentando la velocidad de extracción de los compuestos polifenólicos (Table 17 y Table 21). Una mayor velocidad de extracción ayudaría a reducir los tiempos de maceración si el objetivo es obtener un vino de una determinada concentración de polifenoles. En el estudio 2, se observó que el vino obtenido con PEAV y 3 días de maceración obtuvo mejores características analíticas que el vino control con el doble de tiempo de maceración. Así mismo, el vino resultante con 6 días de maceración obtenido con uvas tratadas con PEAV mostró valores analíticos un 10 % superior de promedio para IPT, IC, AT y TC, nuevamente marcando una diferencia entre los vinos tratados de los no tratados. Es decir la aplicación de tratamientos PEAV de 4 $kV.cm^{-1}$ y 6,7 $kJ.kg^{-1}$ permitió reducir hasta en un 50 % el tiempo de maceración para obtener vinos con unas características similares al proceso control a escala de

bodega. Es más, en térmicos de intensidad de color, compuestos fenólicos (antocianos, ácidos hidroxicinámicos, flavonoles, etc.) y polifenoles individuales, los vinos obtenidos de uvas tratados por PEAV independientemente del tiempo de maceración evolucionaron de forma similar a los obtenidos a partir de uvas sin tratar durante su envejecimiento durante 2 años en botella o en barrica y posterior almacenamiento en botella (Figure 30, Figure 31, Table 26 y Table 27).

Es de destacar que la aplicación de tratamientos PEAV no sólo produjo el incremento de la extracción de antocianos y polifenoles totales, sino también el de los taninos incluso con maceraciones de 3 días. Como ya se ha indicado, la presencia de taninos en el vino tinto es esencial, no solo por su contribución al sabor, sino también por el desarrollo de pigmentos poliméricos rojos por asociación con antocianinas que estabilizan el color del vino durante el envejecimiento. El alto nivel de taninos en el vino obtenido de uvas tratadas por PEAV (Table 22) se debió a una extracción de los taninos de la piel y no a partir de las semillas como sucede en otros vinos con largas maceraciones. En este caso, además, mientras que los taninos de semilla se asocian con descriptores sensoriales "verdes" o "duros", los de la piel se asocian con descriptores sensoriales más deseables, como "suave" o "maduro", aspecto que quedó reflejado en los análisis sensoriales como se comentará más adelante.

Influencia en los compuestos aromáticos y análisis sensorial

A diferencia de otras técnicas de vinificación utilizadas para incrementar la extracción de compuestos a partir de la piel de la uva como el termo-maceración, los PEAV no producen una pérdida de los aromas varietales en los vinos obtenidos. Al contrario, los PEAV mantienen los compuestos aromáticos propios de la variedad (ESTUDIO 2). Esto fue demostrado al analizar y evaluar los compuestos aromáticos mayoritarios y minoritarios entre los vinos obtenidos. Los resultados de la mayoría de los compuestos mayoritarios no manifestaron diferencias significativas o sus concentraciones fueron menores que el umbral olfativo (Table 23). Igualmente, algunos compuestos superaron el umbral olfativo como el alcohol isoamilo, el metionol y el β-feniletanol de los alcoholes superiores y el hexanoato de etilo, el octanato de etilo y el decanoato de etilo del grupo de ésteres etílicos con mayor concentración. Sin embargo, no mostraron diferencias estadísticamente significativas entre los vinos obtenidos de uvas tratadas por PEAV de los de uvas control. Estos resultados estaban en línea con lo que habían

observado Garde-Cerdán et al. (Garde-Cerdán et al., 2013) en uvas de las variedades *Tempranillo* y *Graciano*.

Por otro lado, en el análisis de los compuestos volátiles minoritarios, se observaron algunas diferencias como en el caso de la *β*-ionona (molécula asociada con el aroma florar a "violeta") que solo fue encontrada en los vinos provenientes de uvas tratadas con PEAV indistintamente del tiempo de maceración aplicado (Table 24). También, la γ-nonalactona superó los umbrales olfativos en aquellos vinos que provenían de uvas tratadas con PEAV. Estos resultados son de gran interés ya que apenas se ha investigado este aspecto en los vinos tintos. En el caso de los vinos blancos, sí que se ha destacado la mejora en la extracción de precursores aromáticos en vinos obtenidos de uvas tratadas con PEAV (Comuzzo et al., 2018). Según los resultados obtenidos y de forma similar a la extracción de polifenoles, la electroporación de las pieles de uvas puede contribuir a mejorar la extracción de moléculas precursoras aromáticas de la piel, permitiendo potenciar el perfil aromático de los vinos obtenidos a partir de uvas tratadas por los PEAV. Esta circunstancia que se determinó analíticamente, se confirmó en las valoraciones sensoriales llevadas a cabo con un panel de catadores.

Si bien ya se han publicado estudios preliminares sobre el efecto de los PEAV en la calidad sensorial de los vinos, estos resultados procedían de vinos jóvenes con cortos periodos de conservación obtenidos en vinificaciones a escala de planta piloto (Puértolas et al., 2010e). En esta Tesis Doctoral y debido a la importancia que tiene para el vino el periodo de conservación, ya sea en botella o en barricas, se realizó un estudio de la evolución del vino durante 2 años para determinar el efecto de los PEAV sobre la composición fenólica y su influencia en las características sensoriales de los vinos envejecidos (ESTUDIO 3). La valoración llevada a cabo por enólogos de la "D.O. Campo de Borja", especialistas en la elaboración de vinos de la variedad *Garnacha* (Table 28), determinó que los vinos no manifestaron defectos o alteraciones que pudieran indicar que el tratamiento de PEAV afectara en la calidad de los vinos obtenidos con esta técnica (Figura 24). Es más, los vinos obtenidos de uvas tratadas por PEAV fueron siempre seleccionados como preferido respecto a los vinos control, independientemente que el tiempo de maceración fuera de 3 o 6 días. Es decir, estos resultados, junto con los análisis físico-químicos, indicarían que las uvas tratadas por PEAV pueden dar como resultado vinos no solo adecuados para el consumo diario, sino también para ciertos vinos de mayor calidad que requieren envejecimiento en botella o en barricas de roble.

Extracción rápida de polifenoles con tratamiento de PEAV elevados

El objetivo de incrementar la extracción de compuestos fenólicos en la maceración-fermentación ha sido uno de los aspectos más investigados en la elaboración de los vinos. De ahí que en los últimos años se hayan incorporado distintas procesos industriales o tecnológicos con esta finalidad (de Andrade Neves et al., 2014; El Darra et al., 2016; Lowe et al., 1976; Niculaua et al., 2017) como la termo-maceración, la termo-flash o flash-reléase, maceración pre-fermentativa en caliente (MPC) o la técnica de cortos tiempos de maceración altas temperaturas (KZHE) (Nordestgaard, 2017). Estas técnicas se basan en el efecto del calor sobre la permeabilidad de las células del hollejo, facilitando y/o aumentando la velocidad de intercambio entre el interior y exterior de la célula de los distintos compuestos (Amrani-Joutei and Glories, 1995). Algunos trabajos indican mejoras en la extracción del 10 al 20 % dependiendo de la técnica utilizada hasta un máximo del 58 % para IPT y IC, solo entre un 1 y un 5 % en la concentración de antocianos totales (Geffroy et al., 2015). Piccardo and González-Neves, (2013) indicaron que estas técnicas también poseen una serie de desventajas que deben ser tenidas en cuenta a la hora de considerarse para el tratamiento de las uvas, como la pérdida de aromas varietales o la desnaturalización de antocianos, entre otros. La utilización de los PEAV con tratamientos a mayores intensidades podría mejorar la extracción de compuestos fenólicos, disminuyendo los tiempos de maceración a escasas horas y continuar el proceso fermentativo normal del mosto en fase líquida. La singular ventaja de trabajar en fase liquida radica especialmente en la posibilidad de trabajar con recipientes completamente llenos durante la fermentación optimizando la capacidad de fermentación de una bodega aproximadamente un 25% debido a la eliminación de los hollejos de la maceración. En los estudios realizados a escala de planta piloto para conseguir este objetivo se aplicó un tratamiento de PEAV de energías específicas de alrededor de 52 kJ/kg^{-1} durante 0,3 segundos, con lo que la temperatura final de la uva fue de 38 ± 2ºC. En términos de energía específica aplicada (Table 29 de la página 164), la de los PEAV fue 5,6 veces menor si la comparamos con los tratamientos termo-flash y 3,8 veces menor que una termo-vinificación a 80ºC. Se sabe que el uso del incremento de la temperatura en la uva trae aparejado una serie de inconvenientes ligados sobre todo a la estandarización de los aromas perdiendo el carácter varietal y la generación de compuestos indeseables (Dennis et al., 2012; Fischer et al., 2000), así como una pérdida de color debido a la baja concentración de taninos y a la desnaturalización de los antocianos por efecto del calor

(Geffroy et al., 2015). En esta Tesis Doctoral apenas se observaron estos efectos debido al corto tiempo de tratamiento y la temperatura final alcanzada, muy inferior como se ha comentado a la de dichos procesos.

Tanto en los tratamientos con la uva *Caladoc* como con la *Garnacha*, se observó una extracción prácticamente instantánea de los compuestos polifenólicos manifestándose en un aumento del índice del color, de los antocianos y del IPT tan solo a las 24 horas de haber realizado en tratamiento PEAV. La rápida extracción (Figure 33) de los compuestos fenólicos sería debido fundamentalmente a una electroporación más intensa de los hollejos de la uva ya que un incremento de la temperatura de 38 ºC durante 0,3 segundos no debería mejorar la extracción. Durante la fermentación los vinos manifestaron un valor máximo de IPT e IC a las 24 horas coincidente con el tiempo de maceración y posterior a ello, se observa una disminución del IC durante el tiempo que demora la fermentación. Así mismo, los resultados muestran que los tratamientos de PEAV a mayor intensidad mostraron al final de la fermentación índices iguales o levemente inferiores a los vinos control sin tratamiento de PEAV. Similares resultados fueron obtenidos con el análisis de los antocianos individuales analizados en todos los vinos. Si bien los vinos en general podrían no ser aptos para un periodo de añejamiento prolongado, podrían serlo para vinos destinados al consumo como vino de mesa, al igual que los vinos obtenidos por termo-maceración (Pezzi et al., 2013).

En resumen, a diferencia de las técnicas con tratamientos con calor, la aplicación de PEAV de 8 $kJ.kg^{-1}$ solo incrementó la temperatura entre 1 a 2ºC (Table 29). En el caso del tratamiento Hi-PEAV de temperaturas moderadas (52 $kJ.kg^{-1}$) aumentó 18ºC. Este aumento de la temperatura es bajo comparado con los 60ºC que debe incrementar la uva en los tratamientos térmicos. Es decir, en comparación, la cantidad de energía necesaria para realizar un tratamiento Hi-PEAV a temperatura moderada es significativamente menor obteniendo buenos resultados en lo que respecta a la calidad del vino y considerables ventajas respecto a la disminución del tiempo de maceración.

Análisis económico de la implementación de PEAV en una bodega

Con los resultados obtenidos en esta Tesis Doctoral, queda demostrado que, todas las ventajas derivadas de la introducción de la tecnología PEAV en el proceso de vinificación de los vinos tintos descritas en la literatura a nivel de laboratorio y planta

piloto, se confirman a una escala semi-industrial. Es decir la mayor velocidad de extracción de los compuestos fenólicos permite acortar los tiempos de maceración hasta en un 50 % lo que supone un mayor aprovechamiento de los depósitos, favoreciendo la rotación de los mismos y, como consecuencia, la capacidad de producción de la bodega. Esto se debe, a la posibilidad de realizar la etapa de fermentación en ausencia de hollejos, permitiendo al mismo tiempo, un mayor control de la temperatura durante esta etapa y por ende mayor eficiencia energética. Sumado a ello, permite realizar el resto de las operaciones comunes durante la fermentación de forma más eficiente. Sin embargo, todas estas ventajas no son implementables si la tecnología resulta extremadamente costosa desde un punto de vista económico. Ya se ha mostrado (Figura 37) que la introducción de los PEAV en bodega resulta sencilla sin requerir grandes modificaciones de sus instalaciones y líneas de procesado por lo que esta no sería una dificultad. Es por ello que en este apartado de la Tesis Doctoral, se ha realizado un análisis de costes de dicha implementación donde se detallan los parámetros más importantes a tener en cuenta, así como una comparativa con otras tecnologías utilizadas para acelerar la extracción de polifenoles.

En la introducción de esta Tesis Doctoral, se compararon distintos procedimientos térmicos y no térmicos que se utilizan o se pueden utilizar en las bodegas para acelerar la extracción de compuestos fenólicos. En general, todas las tecnologías tenían incrementos medios de un 30 % en los principales parámetros cromáticos respecto a la uva sin tratar. Sin embargo, si comparamos los requerimientos energéticos de los PEAV con las otras técnicas (ultrasonidos, la termo-maceración o la termo-flash), los PEAV es la técnica que menos energía consume por kg de producto tratado.

Cuando se realizó la comparación del coste operacional, únicamente se ha tenido en cuenta el coste directo del tratamiento; independientemente del precio de adquisición del equipo y de su instalación. En el caso de los PEAV y los ultrasonidos, el coste está directamente relacionado con el consumo de electricidad y, en el caso de los tratamientos térmicos, el combustible utilizado para el calentamiento de la masa de la uva para elevar la temperatura, sumado a ello, la electricidad necesaria en los distintos componentes que forman parte del proceso (bombas de impulsión). En ninguno de los casos, se ha tenido en cuenta la energía requerida para la refrigeración (en caso de que exista). Como se muestra en la Tabla 3 en las que se presenta los costes energéticos calculados por cada uno de los tratamientos pre-fermentativos evaluados, existen notables diferencias sobre todo debido a las temperaturas alcanzadas en cada uno de ellos. Algunos elevan la

temperatura 40ºC (60ºC de temperatura final de la uva) y otros como la termo-flash incrementan hasta 60ºC (80ºC de temperatura final de la uva) por lo que el gasto debido por el calentamiento de las uvas hasta la temperatura indicada representa el costo de mayor incidencia operacional del proceso (Pezzi et al., 2013). Así, tanto los tratamientos de PEAV (6,7 $kJ.kg^{-1}$) como los tratamientos de ultrasonidos (21,6 $kJ.kg^{-1}$) son los tratamientos de los analizados más económicos en comparación con la termo-maceración, (202,6) y la; termo-flash, (297,7 $kJ.kg^{-1}$). Ello supone unos costes de procesado de tan sólo 0,24 $€.ton^{-1}$ para los PEAV, 0,78 $€.ton^{-1}$ para los ultrasonidos incrementando hasta los 7,3 $€.ton^{-1}$ para la termo-maceración y casi 11 $€.ton^{-1}$ para la termo-flash. Es decir hasta 44 y 10 veces menos coste energéticamente hablando con los PEAV y ultrasonidos que con el procesado térmico.

Por otro lado, tomando como referencia un tratamiento PEAV de los utilizados en esta Tesis Doctoral de 4 $kV.cm^{-1}$ de intensidad del campo eléctrico y 6,7 $kJ.kg^{-1}$ de energía específica total aplicada, se podría inferir el coste de la aplicación de los PEAV en una bodega. Para ello, y en base a las disponibilidades en el mercado de generadores PEAV ofertadas por distintas empresas (Energy Pulse Systems, Elea GmbH, Pulsemater, ScandiNova, Kea-Tec GmbH, Pure Pulse Technologies, Wek-Tec, Hazemeyer, etc.), se ha realizado un análisis de los principales costes que tendría la implementación de los PEAV, que implica por un lado los costes fijos (costes de amortización, coste del equipamiento, etc.) y por otro lado los costes variables (costes de energía utilizada, entre otros). La Tabla 32 recoge todos estos datos donde se ha considerado que el coste fijo correspondería a adquirir un generador y su instalación para un tratamiento de uva a un caudal estimado de 40 $ton.h^{-1}$.

Tabla 32: Análisis de costes fijos y variables para la instalación de un generador PEAV en una bodega de grandes dimensiones (10.000 ton.año^{-1} y una cámara de tratamiento de Ø10 cm x 10 cm de GAP).

Voltaje de entrada	40 kV
Caudal de producción	40 ton.h^{-1}
Inversión	250.000 €
Tiempo de amortización	10 años
Costo de instalación	20.000 €
Valor residual	-
Reemplazo	300.000 €
Interés	6 %
Depreciación	30.000 €.año^{-1}
Interés	18.000 €.año^{-1}
Mantenimiento	2.500 €.año^{-1}
Sub-total costes fijos	**50.500 €.año^{-1}**
Energía especifica total	6 - 8 kJ.kg^{-1}
Energía consumida	69,0 kWh
	1,73 kWh.ton^{-1}
Coste de la energía	0,13 €.kWh^{-1}
	8,90 €.h^{-1}
	0,22 €.ton^{-1}
Sub-total costes variables	**2.225 €.año^{-1}**
Coste Total	52.725 €.año^{-1}
Coste por tonelada	**5,27 €.ton^{-1}**
Coste por litro (vino)	**0,00811 €.L^{-1}**

Este análisis está considerado para una bodega de grandes dimensiones, lo que favorece a disminuir los costes fijos rápidamente por la cantidad de uva procesada. Según este análisis, se puede concluir que los costes de aplicar esta tecnología por litro de vino obtenido serían inferiores a 1 céntimo de euro, obteniendo vinos con las características indicadas además de las posibles ventajas logísticas y de producción señaladas que no se han considerado en este estudio

Otra ventaja de los PEAV en relación a las otras técnicas es que el equipo se podría utilizar en la bodega para otras aplicaciones. En la actualidad, se está estudiando está tecnología para inactivar microorganismos indeseables en distintas etapas del proceso de elaboración del vino lo que podría contribuir a reducir el uso de SO_2 (Garde-Cerdán et al., 2008; González-Arenzana et al. 2015). Por otro lado, se ha demostrado que los PEAV aceleran la autolisis de las células acelerando la liberación de manoproteínas y acortando de este modo el periodo de crianza sobre lías (Martínez et al., 2019, 2016).

Tanto la inactivación microbiológica como la liberación de manoproteínas son dos actividades que pueden desarrollarse con el mismo generador de PEAV utilizado para la extracción de compuestos fenólicos. Es decir que el costo del equipo generador de PEAV podría dividirse en estas actividades, logrando así, disminuir o acortar el tiempo de amortización del generador. En estos dos últimos casos, únicamente habría que cambiar la cámara de tratamiento por una de menor dimensión y ajustar el tratamiento y caudal adecuado para ese propósito. Sin embargo, estas otras aplicaciones de los PEAV no se han valorado en el análisis de costes realizados en este trabajo.

Los PEAV, por tanto, es una tecnología de procesado de los alimentos con multitud de aplicaciones en la industria alimentaria, pero especialmente en las bodegas. A diferencia de otras tecnologías que en la actualidad se están instalando en las bodegas para mejorar alguna etapa específica del proceso de elaboración de vino, los PEF podrían utilizarse con distintos objetivos (mejora de la extracción de polifenoles, la reducción de la duración de la crianza sobre lías o la inactivación de microorganismos indeseables). El reciente desarrollo de generadores de PEAV con potencia suficiente para cumplir con los requerimientos de procesado de las bodegas, la fácil instalación de las cámaras de tratamiento en las líneas de procesado ya existentes y el bajo consumo de energía de la tecnología son las claves para que los PEAV sea una tecnología viable en las bodegas en la actualidad.

CONCLUSIONES

I. En el desarrollo de esta tesis doctoral se ha validado a escala semi-industrial (2.500 kg/hora) el potencial de los pulsos eléctricos de alto voltaje (PEAV) en la mejora de la extracción de compuestos fenólicos durante la etapa de maceración-fermentación en el proceso de elaboración de vino tinto.

II. Un estudio sistemático de la influencia de la intensidad de campo eléctrico y la energía específica de los tratamientos PEAV en flujo continuo permitió establecer que el tratamiento óptimo para la extracción de polifenoles de uva la variedad *Garnacha* fue de 4 kV/cm y 4-5 kJ/Kg

III. El tratamiento de PEAV a escala semiindustrial a uva de la variedad *Garnacha* permitió reducir a la mitad el tiempo de maceración o incrementar la concentración de compuesto fenólicos y el color del vino a igualdad de tiempo de maceración debido fundamentalmente a la electroporación de las células de la piel de la uva.

IV. La mayor concentración de β-ionona detectada en los vinos obtenidos con uva de la variedad *Garnacha* tratada con PEAV indicaría que la electroporación de la piel de la uva podría contribuir a la obtención de vinos con mejores perfiles aromáticos debido a una mayor extracción de precursores de aromas.

V. El envejecimiento del vino de la variedad *Garnacha* durante 24 meses en botella o 6 meses en barrica y su posterior almacenamiento en botella durante 18 meses afectó de manera similar a las características cromáticas y fenólicas de los vinos obtenidos a partir de uva tratada y sin tratar por PEAV, manteniéndose al final del envejecimiento las diferencias observadas al final de la fermentación.

VI. Un panel sensorial diferenció en un análisis triangular los vinos obtenidos a partir de uva *Garnacha* tratada por PEF de los vinos control independientemente del tiempo de maceración y del tipo de envejecimiento. En el análisis de preferencia asociado los catadores prefirieron los vinos obtenidos a partir de uva tratada por PEAV

VII. Se ha demostrado por primera vez que el incremento de la intensidad del tratamiento PEAV podría permitir obtener un vino tinto con uva de las variefades *Caladoc* y *Garnacha* con un tiempo de maceración de 24 horas por lo que estos tratamientos podrían considerarse como una alternativa a las actuales técnicas utilizadas actualmente en las bodegas para mejorar de la extracción de polifenoles basadas en el incremento de la temperatura

VIII. Se ha demostrado el potencial de la tecnología de los PEAV para reducir el tiempo de crianza sobre lias en el vino tinto.

IX. Los datos obtenidos en esta tesis doctoral han permitido hacer una evaluación económica de la implantación de la tecnología PEAV. La implantación de la tecnología con un tiempo de amortización de 10 en una bodega con una capacidad de procesado de 10 millones de kilogramos/año supondría un incremento de menos de 1 céntimo de euro por litro de vino obtenido.

ANEXOS

Pulsos Eléctricos de Alto Voltaje (PEF), una tecnología innovadora en el proceso de elaboración de vinos

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http://www.interempresas.net/Flipbooks/VN/6/html5forpc.html

1. Introducción

El constante aumento de la producción de vino en países históricamente no productores y la bajada de su consumo en países tradicionalmente consumidores han provocado que las bodegas deban mejorar la calidad de los vinos reduciendo sus costes de elaboración para seguir siendo competitivas. La introducción de nuevas tecnologías de procesado es una de las estrategias para conseguir este objetivo.

En las últimas décadas, se ha realizado un gran esfuerzo en el desarrollo de algunas tecnologías que permiten procesar los alimentos a temperaturas inferiores a las habitualmente utilizadas con el objetivo de evitar los efectos adversos que el calor ejerce sobre las propiedades nutritivas y sensoriales de los alimentos. Estas técnicas se denominan genéricamente tecnologías no térmicas de procesado y entre ellas se encuentran las altas presiones hidrostáticas, los ultrasonidos, las radiaciones ultravioleta o los pulsos eléctricos de alto voltaje (PEF, del inglés, *Pulsed Electric Fields*). De entre estas tecnologías no térmicas la que más ha sido investigada en el campo de la enología ha sido los PEF. Estos tratamientos provocan un fenómeno denominado electroporación que consiste en el incremento de la permeabilidad de la membrana citoplasmática tanto de las células vegetales como microbianas. Estos tratamientos aplicados a distintas intensidades, facilitan la extracción de compuestos fenólicos de la piel de la uva durante el proceso de fermentación-maceración, permiten inactivar microorganismos indeseables del mosto o del vino y recientemente se ha demostrado que aceleran el proceso de autolisis de las levaduras durante la crianza del vino sobre lías. Todos estos efectos pueden resultar de mucho interés para mejorar la competitividad de las bodegas.

2. Pulsos eléctricos de alto voltaje (PEF)

La tecnología de los pulsos eléctricos de alto voltaje consiste en la aplicación intermitente de una diferencia de potencial con una duración del orden de la millonésima parte de un segundo (μs) a un producto colocado entre dos electrodos. Como consecuencia, se genera un campo eléctrico (E) cuya intensidad depende tanto de la diferencia de potencial (V) como de la distancia entre los electrodos (d): $E=V/d$

Cuando el campo eléctrico aplicado supera un determinado valor umbral se produce un fenómeno denominado electroporación que consiste en el incremento de la

permeabilidad de las células debida a la formación de poros en su membrana citoplasmática. El campo eléctrico externo que hay que aplicar para electroporar las células depende, entre otros factores, de ciertas características propias de la célula, entre los que destaca su tamaño. Mientras que para electroporar las células de tejidos vegetales se requieren campos inferiores a 10 $kV.cm^{-1}$, la formación de poros en las membranas citoplasmáticas microbianas requiere campos eléctricos superiores (por encima de 15 $kV.cm^{-1}$).

Los efectos que provocan los PEF sobre las células resultan de mucho interés para la mejora de multitud de procesos de la industria alimentaria, biotecnológica e incluso en el campo de la medicina Figura 39.

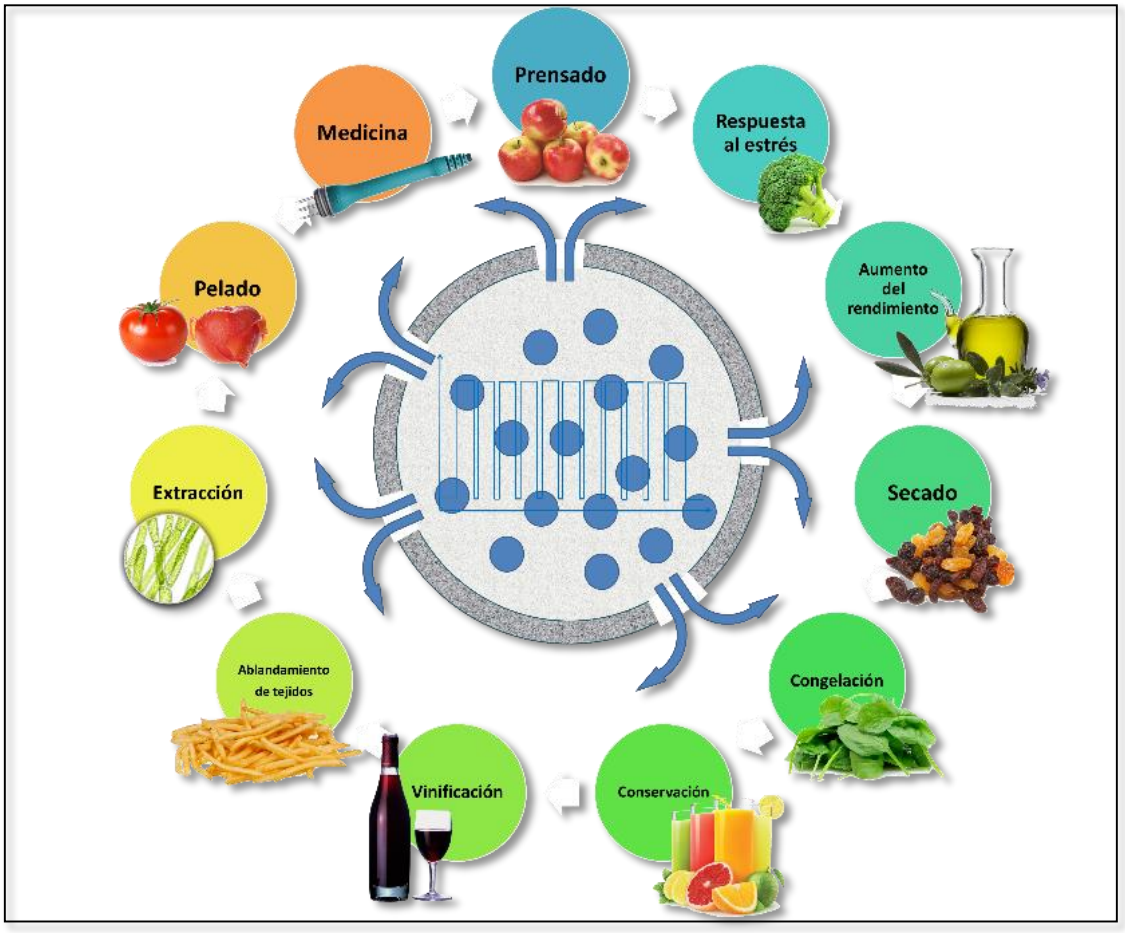

Figura 39. Esquema de múltiples campos de acción y estudio de la Tecnología PEF (Pulsed Electric Fields) en la actualidad.

El incremento de la permeabilidad de las células de tejidos vegetales favorece la extracción de compuestos intracelulares mejorando el rendimiento y/o acortando el tiempo en procesos de extracción, por ejemplo, de azúcar de remolacha azucarera, de betanina de remolacha roja, de antocianos de patata morada o de zumo de manzana.

Además, este efecto también facilita la salida del agua intracelular acelerando los procesos de deshidratación de los alimentos. Por otro lado, el incremento de la permeabilidad de la membrana citoplasmática de los microorganismos provoca su inactivación por lo que estos tratamientos permiten pasteurizar alimentos sensibles al calor, como los zumos de frutas, a temperaturas inferiores a las utilizadas en el procesado térmico. Otras aplicaciones derivadas de los efectos que provoca esta tecnología son el ablandamiento de frutas, hortalizas y tubérculos disminuyendo la fuerza necesaria para su corte y facilitando su pelado (Puértolas et al., 2016).

Las cámaras de tratamiento que se utilizan para el procesado de productos líquidos o partículas sólidas de pequeño tamaño suspendidas en un medio líquido como el caso de la vendimia tras su despalillado y estrujado es una cámara con sección circular (configuración colineal) que se puede instalar en las propias conducciones que se utilizan en las empresas para transportar el producto. Para el tratamiento de piezas enteras de fruta u hortaliza se utilizan cámaras de tratamiento de una sección rectangular a través de las cuales circula el producto transportado con una cinta sinfín sumergido en un líquido de gobierno que permite el paso de la energía (Figura 40).

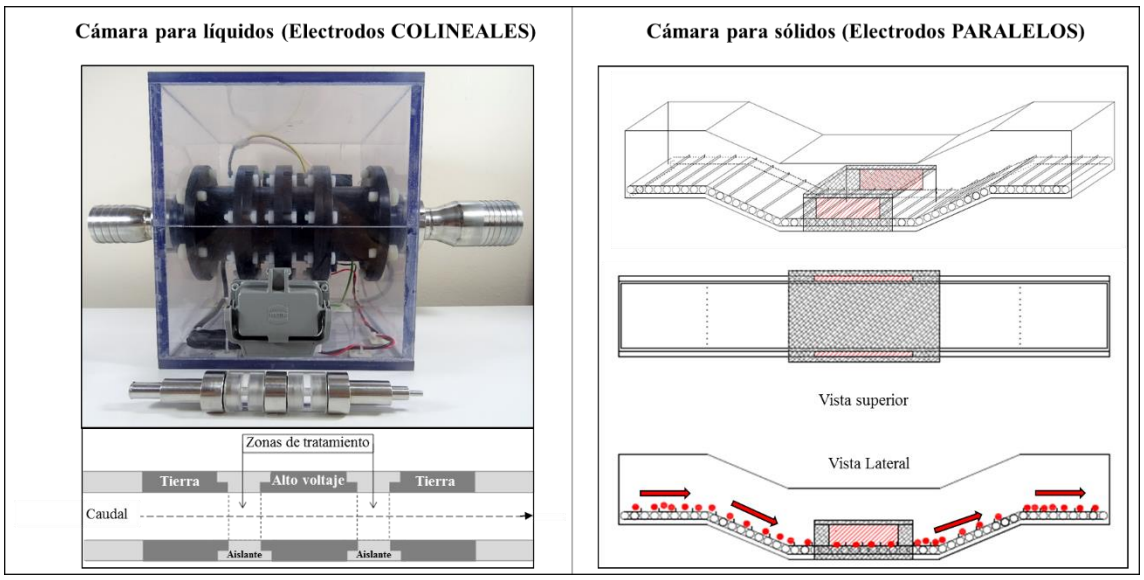

Figura 40. Esquema de las distintas cámaras de tratamiento PEF utilizadas según el producto a tratar.

En la actualidad, las principales aplicaciones de los PEF transferidas a la industria alimentaria son la pasteurización de zumos de frutas y el tratamiento de patatas para la fabricación de patatas fritas congeladas. Estas instalaciones son capaces de procesar hasta 3000 $L.h^{-1}$ en el caso de los zumos y hasta 50 $ton.h^{-1}$ en el caso de las patatas.

Los efectos que provocan los PEF resultan de mucho interés para la mejora de distintas operaciones que ocurren en las bodegas durante el proceso de elaboración de vino. A continuación se describen las principales aplicaciones de los PEF en este sector.

3. Mejora de la extracción de compuestos fenólicos en el proceso de elaboración de vino tinto.

El proceso de elaboración de vino tinto se caracteriza porque la fermentación del mosto se realiza en contacto con la piel de la uva, comúnmente conocida como hollejos. En esta etapa denominada fermentación-maceración, además de transformarse la sacarosa del mosto en etanol por acción de las levaduras, se produce la transferencia de compuestos fenólicos localizados en las células de la piel de la uva al mosto que está fermentando. Estos compuestos fenólicos juegan un papel fundamental en el color, propiedades sensoriales, capacidad de envejecimiento e incluso en los efectos beneficiosos para la salud derivados del consumo de vino tinto.

Aunque la concentración de compuestos fenólicos que posee un vino tinto depende principalmente de la variedad de uva y de su calidad, las prácticas enológicas que se realizan en la bodega durante la fase de fermentación-maceración también juegan un papel importante.

El procedimiento tradicional para conseguir vinos con un elevado contenido en compuestos fenólicos es mantener los hollejos en los tanques de fermentación-maceración incluso una vez que el proceso de fermentación ha terminado. Esta práctica, que en algunos casos se puede prolongar hasta 30 días, provoca que un porcentaje del volumen de los depósitos esté ocupado por los hollejos lo que dificulta la rotación de los depósitos y, como consecuencia, la capacidad de producción de la bodega. Esta pérdida de capacidad en los depósitos de maceración-fermentación resulta especialmente problemática en la época de vendimia pues en momentos puntuales la bodega puede quedarse sin capacidad para procesar más uva. Esta situación se puede solventar con la adquisición de más depósitos, o intentando acelerar la extracción de los compuestos fenólicos con objeto de reducir el tiempo de maceración. Incrementar el número de depósitos de fermentación-maceración supone una importante inversión en unas instalaciones que se necesitan solo en momentos puntuales de la vendimia y que no serán necesarios una vez terminado el proceso de fermentación-maceración.

En los últimos años, se han propuesto distintas técnicas para facilitar la extracción de compuestos fenólicos de la piel de la uva como la termovinificación, o la "flash-expansión". En términos generales, estas tecnologías requieren elevadas inversiones iniciales, altos costes operativos, ocupan una superficie importante de la bodega donde son instalados y, como se produce un calentamiento de la vendimia, suelen dar lugar a vinos en los que se pierde su carácter varietal.

Frente a estas tecnologías, los PEF permiten mejorar la extracción de compuestos fenólicos con unos costes de inversión de la instalación más bajos, con menores costes energéticos, sin aumentar la temperatura de la vendimia y, por lo tanto, manteniendo el carácter varietal de los vinos, sin necesidad de que la instalación ocupe permanentemente un espacio en la bodega (Puértolas, et al., 2010; Saldaña, et al , 2017).

La aplicación de tratamientos de PEF a la uva una vez despalillada y estrujada provoca la formación de poros en las membranas de las células de los hollejos facilitando la extracción de los compuestos fenólicos localizados en el interior de las células durante la etapa de fermentación-maceración (Figura 41).

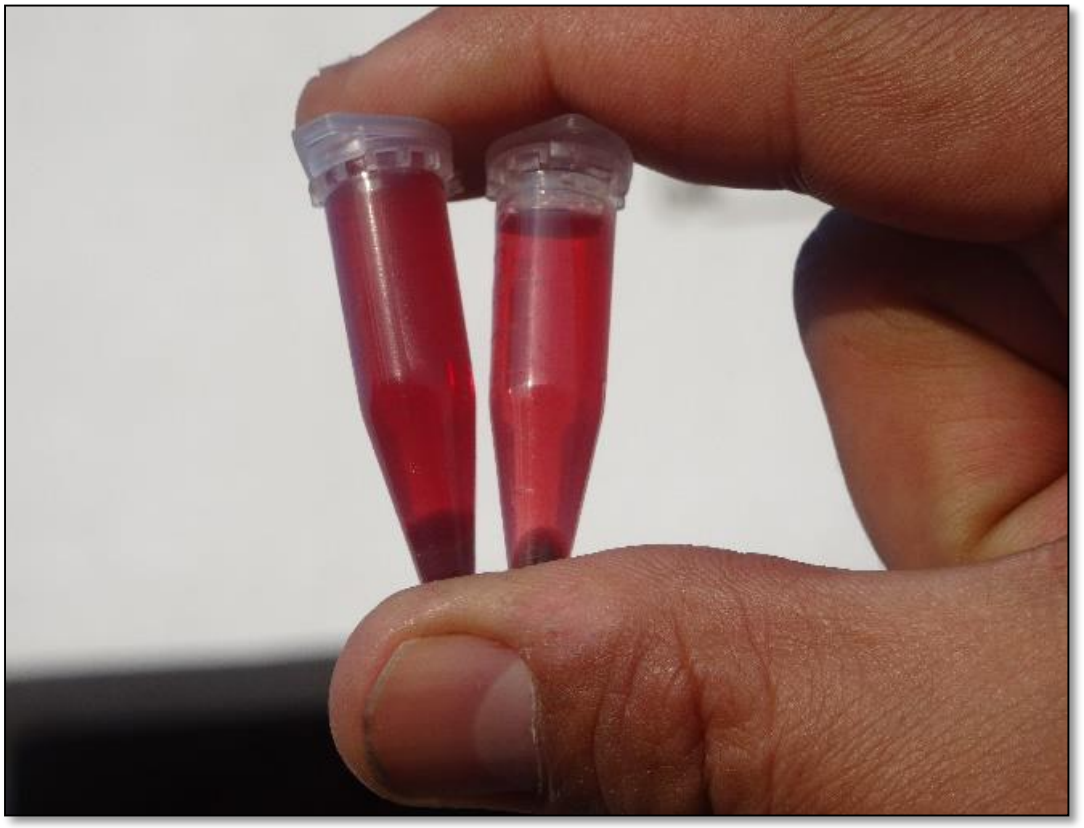

Figura 41. Imagen comparativa, respecto a la intensidad colorante, en mosto obtenido a partir de hollejos tratados por PEF (izquierda) y no tratados (derecha).

Con estos tratamientos se pueden conseguir vinos con un mayor contenido en polifenoles o reducir el tiempo de maceración sin afectar al contenido final de polifenoles en el vino (López, et al., 2009). Aunque nuestro grupo de investigación de la Universidad de Zaragoza es pionero en las investigaciones sobre la aplicación de los PEF para este objetivo, este efecto ha sido demostrado por distintos grupos de investigación de otros países como Francia, Italia, Alemania o Libia, con variedades de uva típicas de estos países. Hay que destacar que en ninguno de los estudios realizados se ha detectado que el tratamiento afecte negativamente a las propiedades sensoriales del vino obtenido con uva previamente tratada por PEF.

Tabla 33: Resultados analíticos promedios, una vez finalizada la maceración-fermentación de vino tinto de la variedad *Grenache* (Vendimia 2016).

Tratamiento (Campo Eléctrico)	Maceración (días)	Energía Específica ($kJ.kg^{-1}$)	IPT	Antocianos ($mg.L^{-1}$)	Taninos ($mg.L^{-1}$)
Sin tratar	12	-	68,6	558	1822
8 $kV.cm^{-1}$	4	6,72	75,2	668	1809
6 $kV.cm^{-1}$	4	6,16	72,7	676	2068
4 $kV.cm^{-1}$	6	6,72	80,2	711	2362
1 $kV.cm^{-1}$	6	2,87	73,9	685	1876

A modo de ejemplo, la Tabla 33 muestra los resultados obtenidos durante la maceración-fermentación de uva de la variedad *Grenache* tratada por tratamientos PEF de distinta intensidad de campo eléctrico. Se observa que con los tratamientos más intensos (6 y 8 $kV.cm^{-1}$) se consiguió con un tiempo de maceración de 4 días un vino con una mayor intensidad de color y un contenido mayor de polifenoles, antocianos y taninos que el vino control en el que la maceración se extendió a los 12 días. Resultados similares se obtuvieron en los vinos elaborados con uva tratada a intensidades de campo eléctrico inferiores (1 y 4 $kV.cm^{-1}$) cuando el tiempo de maceración se prolongó hasta los 6 días. Por lo tanto, el tratamiento PEF permitió reducir el tiempo de contacto de los hollejos con el mosto entre 6 y 8 días obteniéndose vinos al final de la fermentación con un contenido en polifenoles, antocianos y taninos que el vino control con 12 días de maceración. La energía específica de todos los tratamientos aplicados fue inferior a 7 $kJ.kg^{-1}$ lo que indica que si el tratamiento se hubiera aplicado al agua su temperatura hubiera aumentado menos de 2ºC.

4. Crianza sobre lías

La crianza sobre lías es una práctica enológica que tiene como objetivo principal enriquecer el vino con manoproteínas procedentes de la pared celular de las levaduras. Las manoproteínas contribuyen positivamente a la calidad del vino ya que favorecen la estabilidad tartárica, proteica y de la materia colorante además de mejorar la percepción organoléptica del vino contribuyendo a mejorar la sensación de cuerpo y volumen.

Aunque durante el proceso de fermentación alcohólica se produce cierta liberación de manoproteínas, es durante la autolisis de las levaduras en la crianza del vino con las lías cuando los vinos se enriquecen en estos componentes. La autolisis que ocurre tras la muerte de las levaduras es un proceso complejo que implica la destrucción de las membranas de los orgánulos celulares de la membrana citoplasmática y la liberación al espacio periplasmático de enzimas citoplasmáticos que provocan la desintegración de la pared celular y la liberación de manoproteínas al medio.

El enriquecimiento del vino con manoproteinas bien exógenas o mediante contacto prolongado de los vinos con sus lías es una operación frecuente en las bodegas. La adición de manoproteínas exógenas supone un coste adicional al proceso de elaboración de vino mientras que la crianza sobre lías requiere tiempos de contacto de al menos tres meses con remontados frecuentes lo que implica riesgos de crecimiento de flora indeseable en el vino y costes de mano de obra.

Resultados obtenidos en nuestro laboratorio han demostrado que la electroporación de las células de las levaduras mediante PEF acelera la autolisis de las levaduras en el proceso de crianza sobre lías.

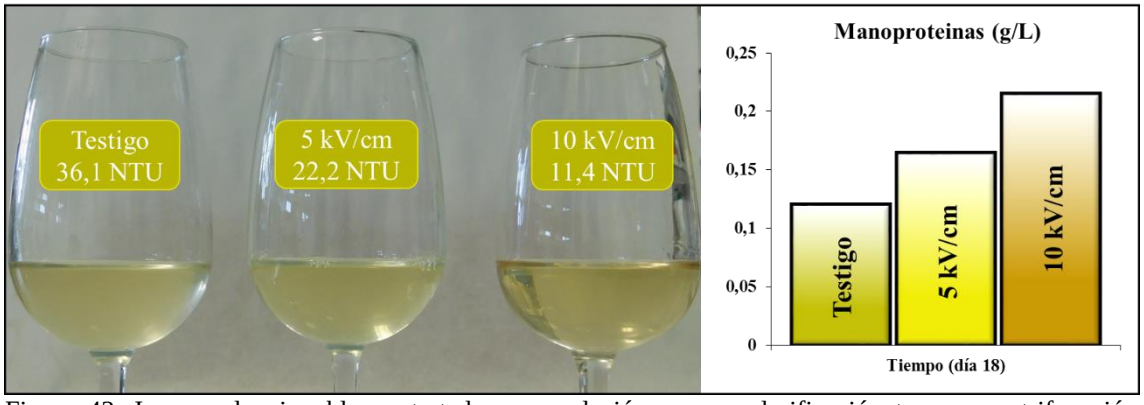

Figura 42. Imagen de vino blanco tratado y su relación con su clarificación tras su centrifugación (izquierda). Gráfica comparativa de liberación de manoproteínas en vino blanco a los 18 días (derecha.)

Se observó que los niveles de manoproteínas que se obtenían tras tres meses de crianza sobre lías en vino blanco de la variedad *Chardonnay* se conseguían en 18 días

cuando las levaduras habían sido previamente tratadas por PEF. La Figura 42 muestra como la mayor concentración de manoproteínas en el vino que contenían levaduras tratadas por PEF facilitaba su clarificación tras su centrifugación (Martínez et al., 2016).

5. Inactivación microbiana

Al igual que en cualquier otra industria alimentaria, el desarrollo de microorganismos alterantes es un problema que puede causar grandes pérdidas económicas en las bodegas. Debido a que el tratamiento térmico afecta a las propiedades sensoriales del vino, el principal procedimiento utilizado en las bodegas para evitar los riesgos de alteraciones microbiológicas es la adición de SO_2 en distintas etapas del proceso de elaboración del vino y la filtración esterilizante antes de su embotellado. Aunque existe mucha controversia en relación al uso del SO_2 en el proceso de elaboración del vino debido a que puede causar efectos negativos en la salud de los consumidores, especialmente de aquellos que tienen una sensibilidad especial, por el momento, debido a los múltiples efectos beneficiosos que este compuesto ofrece en el proceso de elaboración del vino no existe ninguna alternativa. Por otro lado, la filtración esterilizante se ha mostrado como un proceso eficaz de estabilización microbiológica del vino antes de su embotellado. Sin embargo, se trata de un proceso costoso económicamente y que puede afectar negativamente a las propiedades sensoriales del vino.

La capacidad de los PEF de inactivar microorganismos a temperaturas inferiores a las utilizadas en el procesado térmico es un efecto muy atractivo no solo para la industria alimentaria en general sino también para las bodegas. Distintos estudios han demostrado la inactivación de levaduras (*Dekkera anómala*, *Dekkera bruxellensis*) y bacterias (*Lactobacillus plantarum*, *Lactobacillus hilgardii*) responsables de la alteración del vino mediante tratamientos de PEF aplicados tanto en el mosto como en el vino. Debido a que el tamaño de los microorganismos es inferior al de las células de la piel de la uva, para conseguir inactivar entre 99,9 y 99,99 % de la población de estos microorganismos se requieren tratamientos más intensos que para favorecer la extracción de los polifenoles (Puértolas et al., 2009).

A la vista de los resultados obtenidos, el tratamiento del mosto antes de la fermentación podría además de contribuir a reducir o eliminar la adición de SO_2 permitiría una fermentación más reproducible por parte de los cultivos iniciadores al no estar presentes o en menor concentración las levaduras salvajes en el mosto. Por otro lado, un

tratamiento de PEF al vino antes de su embotellado podría evitar la última filtración que se le hace al vino antes de su embotellado preservando sus propiedades sensoriales.

6. El proyecto FieldFOOD

El proyecto FieldFOOD (www.fieldfood.eu) es un proyecto financiado por la UE dentro del programa H2020 que está coordinado por el grupo de Nuevas Tecnologías de Procesado de la Universidad de Zaragoza. El objetivo del proyecto es promover la implantación de la tecnología de PEF en distintas sectores de la industria alimentaria: vino, aceite, tomate, zumos de frutas y sidra. En el proyecto, participan 5 centros de investigación, 1 empresa fabricante de equipos de PEF, 5 empresas potenciales usuarias de la tecnología, entre las que se encuentran dos empresas españolas (Agrinarsa y Bodegas Aragonesas), y EFFOST (Federación Europea de Ciencia y Tecnología de los Alimentos (Figura 43).

Figura 43. Países y Empresas participantes del proyecto FieldFood.

Durante el primer año del proyecto, se identificaron los requerimientos específicos de cada industria con objeto de diseñar equipos de PEF modulares, portátiles y de bajo coste adaptados a las necesidades específicas. Durante el segundo año del proyecto, estos equipos se instalaron en las líneas de producción de las empresas participantes en el

proyecto y se demostraron los beneficios de la tecnología aplicando los tratamientos a los mismos requerimientos de procesado o muy próximos a los que habitualmente se utilizan en la empresa. Los resultados obtenidos durante el segundo año de procesado se confirmarán en un tercer año en el que se tratará de satisfacer todos los requerimientos de procesado de todas las industrias.

7. Análisis de costes

Uno de los mayores impedimentos para la implementación de los PEF en la industria alimentaria ha sido el elevado coste de los equipos disponibles. Sin embargo, con el avance de la tecnología, se han conseguido desarrollar equipos de PEF con potencia suficiente como para ser instalados en las industrias y a costes cada vez más reducidos. La Tabla 34 muestra los costes por tonelada de uva procesada que supondría la implantación de la tecnología en una bodega cuyo objetivo fuese mejorar la extracción de compuestos fenólicos. Para el cálculo de la amortización, se ha considerado una vida útil de 5 años con un valor residual del 40 % al cabo de ese tiempo. El valor del equipo ha sido considerado en 60.000 € con una capacidad de procesado máxima de 3.600 ton por temporada de vendimia.

Tabla 34. Costes operativos, mantenimiento y amortización estimados en el tratamiento de uvas con PEF.

Ítem	€	cent€
Amortización	0,0033 **€.kg^{-1}**	0,33 **cent€.kg^{-1}**
Mantenimiento	0,00002 **€.kg^{-1}**	0,002 **cent€.kg^{-1}**
Electricidad	0,00021 **€.kg^{-1}**	0,021 **cent€.kg^{-1}**
Total (x kg)	0,00356 **€.kg^{-1}**	0,356 **cent€.kg^{-1}**
Total (x ton)	3,56 **€.ton^{-1}**	356 **cent€.ton^{-1}**
Total Vino (x Litro)	0,00475 **€.L^{-1}**	0,475 **cent€.L^{-1}**

En los cálculos de amortización, no se ha considerado el uso del equipo en otras aplicaciones como el tratamiento de levaduras para acelerar la crianza sobre lías o para la inactivación microbiana, lo que podría suponer un ahorro adicional tanto energético como de material y de mano de obra. Los tratamientos permitirían reducir el tiempo de maceración entre 50 y el 60 % del tiempo, obteniéndose vinos con un contenido en polifenoles similar o incluso superior.

8. Conclusiones

Los PEF es una tecnología de procesado de los alimentos con multitud de aplicaciones en la industria alimentaria, pero especialmente en las bodegas. A diferencia

de otras tecnologías que en la actualidad se están instalando en las bodegas para mejorar alguna etapa específica del proceso de elaboración de vino, los PEF podrían utilizarse con distintos objetivos como la mejora de la extracción de polifenoles, la reducción de la duración de la crianza sobre lías o la inactivación de microorganismos indeseables.

El reciente desarrollo de aparatos de PEF con potencia suficiente para cumplir con los requerimientos de procesado de las bodegas, la fácil instalación de las cámaras de tratamiento en las líneas de procesado ya existentes y el bajo consumo de energía de la tecnología son las claves para que los PEF sea una tecnología viable en las bodegas en la actualidad (Figura 44).

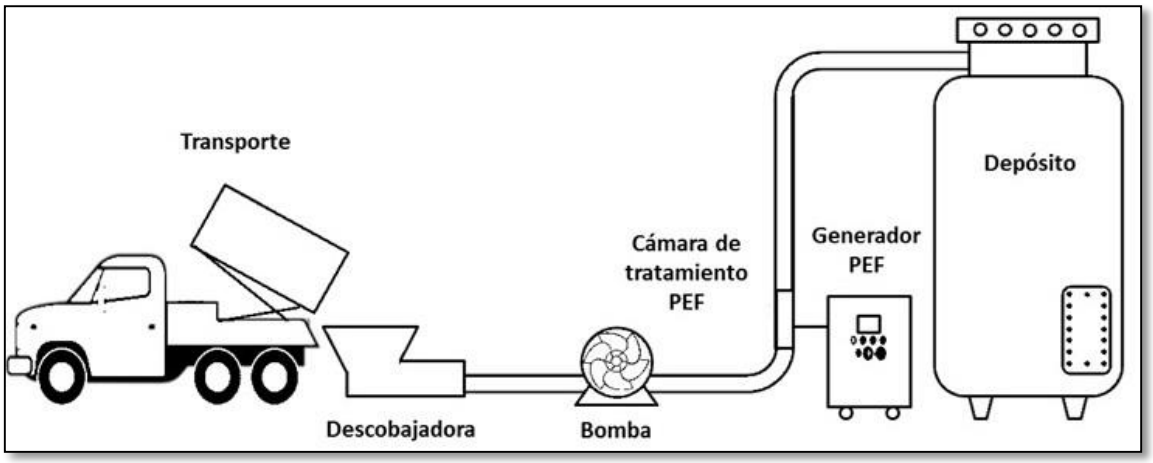

Figura 44. Esquema de trabajo para el tratamiento de uvas con la tecnología PEF.

Pulsed electric fields shorten aging on lees time to achieve a release of mannoproteins from *Saccharomyces cerevisiae* in red wine that provides improved mouthfeel and astringency

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1. Abstract

The potential of PEF for triggering autolysis of *Saccharomyces cerevisiae* yeast and accelerating the release of mannoproteins during aging on lees of *Caladoc* red wine was evaluated. Release of mannoproteins in red wine increased rapidly in samples containing PEF-treated yeasts in comparison to untreated. After one month of aging on lees, the mannoproteins concentration in wines containing PEF-treated yeast attained the maximum value (223 $mg.L^{-1}$), whereas the wines containing untreated yeast required three months of aging to reach that maximum level. The analysis of enzymatic activity during aging demonstrated that PEF treatments triggered β-glucanase and protease activities in the extracellular media where yeast were aged, enzymes that similarly act during natural autolysis which is produced slower. The functional properties of mannoproteins released from PEF-treated yeast were similar to that of those released from untreated yeast. The sensorial analysis confirmed that panelists did not found differences between the wine obtained through PEF-induced autolysis in shorter time and the wine obtained after natural autolysis process in three-fold more aging time. This technique therefore permits to accelerate the aging on lees step while avoiding or reducing the problems associated with it representing great saves and advantages for the wineries.

2. Introduction

Although wine elaboration is an ancestral practice, wine technology is evolving and reinventing itself continuously in order to fit with the trends of modern consumers. Aging on lees is a technique that consists of left deliberately the wine in contact with the lees sediment (mainly composed by yeast) after the fermentation (Charpentier et al., 2004). This is a traditional practice in the manufacture of white wines fermented in barrels, natural sparkling wines (cava, champagne) and in flor sherry wine (Palomero et al., 2007). However, aging on lees have recently attracted the attention of enologists for the making of red wines and especially due to its positive effect on different sensorial properties (Guadalupe and Ayestarán, 2008b). During this step it is produced the autolysis of yeast that causes disorganization of membranous systems and thus permits the release of endogenous enzymes such as glucanase and protease, thereby leading to the degradation of the cell wall and the subsequent release of mannoproteins into the wine (Alexandre and Guilloux-Benatier, 2006). The presence of these highly glycosylated proteins in the wines improves color stability (Escot et al., 2001), reduces astringency (Guadalupe et al., 2007; Vidal et al., 2004) and increases body and mouthfeel (Pérez-Serradilla and de Castro, 2008; Wolz, 2005). However, autolysis process during aging on lees is very slow when it is produced naturally, lasting from months to years implying immobilization of winery stocks, increased microbial spoilage risk and loss of sensorial quality of wines (Alexandre and Guilloux-Benatier, 2006). Furthermore, this technique requires considerable investment on the part of wineries in equipment (tanks, barrels) and entails elevated labor costs (periodic stirring-*bâtonnage*-and sensorial analysis) that impact in augmented production costs (Pérez-Serradilla and de Castro, 2008).

Different strategies such as addition of enzymes to hydrolyze *β*-glucans, thermolysis or mechanical methods for large-scale disruption of microbial cells have been suggested for the acceleration of yeast autolysis. However, these methods present numerous disadvantages that avoid their implementation and nowadays the most used alternative is the addition of commercial mannoproteins preparations despite the enormous costs that represents for wineries.

Pulsed electric fields (PEF) is a technology that causes the increment of the permeability of the cytoplasmic membrane of cells (electroporation) by applying intermittent electric fields of high intensity and extremely short duration (from μs to ms) (Puértolas and Barba, 2016). It has been recently demonstrated the potential of PEF for

accelerating the release of mannoproteins during the aging on lees of white *Chardonnay* wine (Martínez et al., 2019).

The aim of the present study was to prove the potential of PEF for shortening the aging on lees step in red wine and to study the mechanisms implied in the autolysis of *Sacharomyces cerevisiae* yeast induced by PEF.

Material and methods

Winemaking

Caladoc grapes (100 kg) were received in our pilot plant, destemmed and crushed and 10 $mg.kg^{-1}$ of $K_2S_2O_5$ were added. The grapes were inoculated with 15 $g.hl^{-1}$ of the commercial yeast *Saccharomyces cerevisiae* (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland) and fermented in 100-kg-stainless steel tanks. Alcoholic fermentation and maceration (contact of grape skins and must) was conducted at 20±2ºC during 10 days until the concentration of residual sugars was lower than 3 $g.L^{-1}$. After that, the wines were pressed, racked and stabilized for a period of one month at 2ºC and stored at 16±1ºC.

Characterization of the initial wine

Ethanol concentration, pH, volatile acidity and total acidity were analyzed in the initial wine according to the specifications established by the (Organization Internationale de la Vigne et du Vin, 2009), and the results are shown in Table 35.

Aging on lees step

The yeast used for the aging on lees derived from a strain of *S. cerevisiae* from an industrial preparation (Levuline Sélection C.I.V.C. France, Bahnhofstrasse, Switzerland) which participated in the fermentation of white wine. Once fermentation had taken place, the yeasts were collected by removing the wine after sedimentation of the lees. A concentrated yeast suspension (1.0×10^9 $cells.mL^{-1}$) was directly used for the PEF treatments or maintained untreated, and then mixed with the *Caladoc* wine for aging-on-lees. Control *Caladoc* wine, *Caladoc* wine containing untreated yeast (10^8 $cells.mL^{-1}$) and *Caladoc* wine containing PEF treated yeast (10^8 $cells.mL^{-1}$) were dispensed in 250 mL bottles and aged during three months at 18°C.

PEF treatment

The PEF equipment used in this investigation was the commercial model EPULSUS®-PM1-10 (Energy Pulse System, Lisbon, Portugal). It consists of a Marx generator of square waveform pulses with 10 kV of maximum voltage, 180 A of maximum current, and 3.5 kW of power. The concentrated yeast suspension (1.34 $mS.cm^{-1}$) was PEF-treated in a parallel electrode continuous chamber of 3 cm length and 0.50 cm width. The gap between electrodes was 0.40 cm, resulting in a total treatment volume of 0.6 mL. The flow rate was set at 2.7 $L.h^{-1}$ and the calculated mean residence time in the treatment chamber was 0.80 seconds. Frequency was calculated by dividing the number of pulses by the residence time.

A heat exchanger consisting in a coil submerged in a thermostatic batch was used to set the initial temperature of the suspension before the treatment. Temperature of the lees suspension was measured with thermocouples located before and after the heat exchanger, and just after the PEF treatment chamber.

The specific energy input (*W*) per pulse expressed in $kJ.kg^{-1}.pulse^{-1}$ was calculated by the following equation (Eq. 1):

$$W = m \cdot V \cdot I \cdot t \quad (1)$$

where *m* (kg) is the mass of the lees suspension contained in the volume of the treatment chamber; *V* is the input voltage (kV); *I* is the current intensity (A); and *t* is the pulse width (μs). The total specific energy was calculated by multiplying the specific energy input per pulse by the number of pulses.

Based on results of yeast inactivation reported by Martínez et al. (Martínez et al., 2019) using the same yeast strain, PEF treatment consisting of 25 pulses of 3 μs (75 μs) at 15 $kV.cm^{-1}$ electric field was selected ensuring the inactivation of 99 % of population. The total specific energy of the treatment was 22.61 $kJ.kg^{-1}$.

Monitoring of mannoproteins release

Release of mannoproteins from untreated and PEF-treated *S. cerevisiae* yeast was monitored along the aging-on-lees storage period. Samples were periodically collected and centrifuged (3000_x g 5 minutes), and the mannoprotein concentration in the supernatant of the wine was determined following the protocol described in Martínez et al. (Martínez et al., 2019).

Evaluation of the enzymatic activity in yeast supernatants during aging

Determination of protease activity

Protease enzymatic activity in the supernatants of the untreated and PEF-treated *S. cerevisiae* cells was determined periodically during incubation time after treatments by the EnzChek® Peptidase/Protease Assay Kit (E33758, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Determination of *β*-glucanase activity

β-glucanase enzymatic activity in the supernatants of the untreated and PEF-treated *S. cerevisiae* cells was determined using the azo-barley glucan commercial method (malt and bacterial *β*-glucanase assay procedure, Megazyme International, Wicklow, Ireland).

Analysis of wines subjected to aging-on-lees periods of varying length

After different times of aging on lees, wines samples containing untreated or PEF-treated yeasts were collected and centrifuged (3000 g x 5 minutes) to remove the lees and the supernatants were bottled. The analyses were performed on the wines obtained.

Total polyphenol index and total tannin content were determined using a Libra S12 spectrophotometer (Biochrom, UK). The total polyphenol index (TPI) was measured by directly reading the absorbance of diluted wine 1/20 ($v.v^{-1}$) at 280 nm (Ribéreau-Gayon et al., 2006).

The chromatic characteristics of the wines were determined by directly measuring their absorbance at 420, 520, and 620 nm using a spectrophotometer (Libra S12, Biochrom, UK) with a 10-mm path-length quartz cuvette. Color intensity (CI) was calculated as the sum of absorbance at 420, 520, and 620 nm. Hue was determined as the proportion of absorbance measured at 420 and 520 nm proportion, yellow colour (%Ye) as the relation between 420 nm absorbance and colour intensity; proportion of red color (%Rd) as the relation between 520 nm absorbance and color intensity; and proportion of blue color (%Bl) as the relation between 620 nm and color intensity (Glories, 1984; Sudraud, 1958).

Quantification of condensed tannins was measured by precipitation with methyl cellulose according to (Sarneckis et al., 2006), and results were expressed as epicatechin equivalents.

Astringency analysis of wines of different aging on lees periods

Astringency of wines which contained untreated or PEF treated yeast during different aging on lees periods and of control wine was evaluated. The method used determines astringency by using ovalbumin as the precipitation agent and tannic acid solutions as standards (Llaudy et al., 2004).

Sensorial analysis of aged on lees wines and control wine

Control wine and wines obtained after one month of aging on PEF-treated lees or after three months of aging on untreated lees were sensory evaluated by twenty panelists (five males, fifteen females; aged 23 to 55 years) that were recruited based on availability and willingness to participate from faculty staff and Ph.D. students from the Food Technology department at University of Zaragoza. Recruited panelists took part in two one-hour training sessions over two weeks, followed by two 10-min evaluation sessions over two weeks. The evaluations were conducted in the morning, with all sessions scheduled from 10:00 to 14:00. Samples of 20 ml of wine at room temperature were presented in clear wine glasses (ISO NORM 3591, 1977) labelled with 3-digit random codes. Panelists were distributed in individual booths and they were not informed about the samples to be tested.

The wines were evaluated by triangular discriminative analysis using a completely randomized design associated to a preference tests. The objective of this first test was to determine if the panel could distinguish between the wines obtained containing untreated or PEF-treated yeast obtained after different aging on lees periods but containing similar concentration of mannoproteins. In addition two triangular discriminative analysis was performed to evaluate differences between aged on PEF-treated lees wines and control wine and between aged on untreated lees wines and control wine. After selecting the sample that was considered different, the panelists were also asked to indicate the preferred sample. The result of the preference analysis was only taken into consideration when the panelists correctly identified the different sample.

Statistical data treatment

The results represent the mean ± standard error of the mean of three replicates of treatments analyzed in triplicate. A one-way ANOVA test was conducted to assess significant differences between treatments. The differences were considered significant

at $p<0.05$. The significant difference for triangular tests was determined using statistical tables reported by (Roessler et al., 1948).

3. Results and discussion

Release of mannoproteins from untreated and PEF-treated lees in red wine

Release of mannoproteins during yeast autolysis was monitored by determining the concentration of mannose in the extracellular media after acid hydrolysis as previously was reported (Dallies et al., 1998; Martínez et al., 2016). Figura 45 shows the release of mannoproteins to the wine from untreated and PEF-treated *S. cerevisiae* cells during the aging on lees of red wine. Mannoprotein concentration in the initial wine previous to the aging on lees was 33 $mg.L^{-1}$, which was probably due to the release of mannoproteins during the course of alcoholic fermentation (Domizio et al., 2017; Escot et al., 2001).

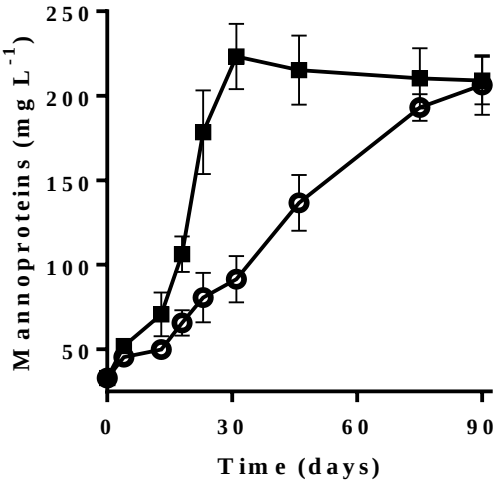

Figura 45: Release of mannoproteins in *Caladoc* wine from *S. cerevisiae* cells untreated (○) and PEF-treated (15 kV cm^{-1}, 75 μs) (■) along aging on lees time

Along aging on lees step, release of mannoproteins in red wine increased rapidly in samples containing PEF-treated yeast as compared with those containing untreated lees. After three weeks of aging on lees the concentration of mannoproteins in wines which contained untreated lees was 80.5 $mg.L^{-1}$; however, after the same period of time, mannoproteins concentration increased to 178.5 $mg.L^{-1}$ in wine which contained PEF-treated yeast. Mannoproteins release occurs after the cell death during the process of yeast autolysis. This phenomenon of self-degradation of the cell constituents requires that the endogenous enzymes be released and come in contact with the cell wall where the

mannoproteins are located. While naturally this process is very slow, the triggering of yeast autolysis by PEF treatment permitted the release of 80 % of the total of mannoproteins after only three weeks of aging on lees. After one month of aging on lees, the mannoproteins concentration in wines containing PEF-treated yeast attained the maximum value (223 $mg.L^{-1}$), whereas the wines containing untreated yeast required three months of aging on lees to reach that maximum release of mannoproteins. These results confirm that natural autolysis in red wine is indeed a slow process and that PEF treatment drastically reduces the time required for the release of mannoproteins from yeast.

The release of mannoproteins is associated to the activity of the cytoplasmic enzymes in the cell wall and it has been demonstrated in previous studies that this enzymatic activity is greatly dependent on the environmental conditions in which autolysis occurs (Martínez et al., 2017). Mannoproteins release, both from untreated yeast and PEF-treated yeast was found to be slower during the aging on lees of red wine in comparison to the incubation in buffer (Martínez et al., 2017, 2016). The presence of ethanol and the acidic pH were reported to delay the release of mannoproteins (Martínez et al., 2017). However, in the present research the release of mannoproteins from PEF-treated cells in red wine was similar than the release of mannoproteins previously reported in white wine (Martínez et al., 2019); in both cases the wines containing PEF-treated yeast reached the maximum values of mannoproteins concentration (c.a. 220 $mg.L^{-1}$) after approximately one month of aging on lees. However, as it is shown in Figura 45, the release of mannoproteins from untreated yeast in red wine reached the maximum after three months while in the case of *Chardonnay* wine natural autolysis required 6 months to attain the maximum. This difference might be explained by the lower concentration of ethanol and higher pH of *Caladoc* red wine of the present study in comparison to the characteristics of *Chardonnay* wine of previous study (Martínez et al., 2019).

Although the usual conditions of winemaking are not the most suitable for autolysis performance (Fornairon-Bonnefond et al., 2002), a PEF treatment that inactivated the majority of yeast population (99 %) was demonstrated to speed up the release of mannoproteins in red wine as previously had been demonstrated in white wine.

Enzymatic activity in the extracellular media containing untreated and PEF-treated yeast during aging on lees

In order to go deeper into the mechanisms of PEF-induced autolysis, it was investigated if enzymatic activity was detected in the extracellular medium containing untreated and PEF-treated yeast. According to literature, the main enzymes involved in *S. cerevisiae* autolysis process are *β*-glucanases and proteases (Alexandre and Guilloux-Benatier, 2006). Figura 46 shows the evolution along the time of *β*-glucanase (A) and protease (B) enzymatic activity in the extracellular medium of untreated or PEF-treated *S. cerevisiae* cells. The release of both enzymes to the extracellular media was higher when cells were previously PEF-treated. After one week of incubation, the *β*-glucanase activity in the medium containing PEF-treated yeast was 2-fold the activity in the medium containing untreated yeast (Figura 46A). On the other hand, in the case of protease, the activity in the extracellular medium after one week of incubation containing untreated yeast was undetectable, while the activity in medium containing PEF-treated yeast was higher from the beginning of incubation and increased drastically during the first three days rising 17-fold more activity than medium containing untreated yeast after one week of incubation (Figura 46B).

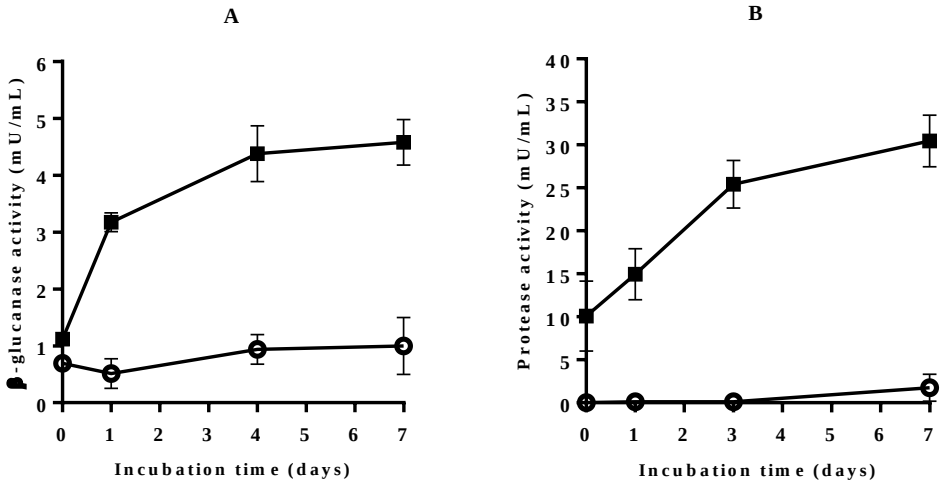

Figura 46: Evolution of β-glucanase (A) and protease (B) enzymatic activity in the extracellular media of model wine of untreated (○) and PEF-treated (15 kV cm^{-1}; 75 µs) *S. cerevisiae* (■) during incubation.

When kinetic of mannoprotein release is compared with kinetics of enzymatic activity in aging media it seems that both phenomena are strongly linked. These results support the hypothesis proposed in previous studies (Martínez et al., 2019, 2017, 2016). The loss of selective permeability of cytoplasmic membrane after electroporation due to PEF-treatment would permit the uncontrolled water inlet to the cytoplasm during

incubation. It would lead to the decrease of the cytoplasmic osmotic pressure, the plasmolysis of vacuoles which contain the hydrolytic enzymes (β-glucanase and protease) and their subsequent breakage producing the release of these enzymes. In parallel the electroporation of the cytoplasmic membrane would facilitate the contact of these enzymes with the outermost layer of cell wall where mannoproteins are located and the release of these glycoproteins to the extracellular medium.

The high enzymatic activity detected in media in which autolysis was been producing confirms that the triggering effect of PEF is performed by similar mechanisms to that of natural autolysis, although the phenomenon it is produced faster thanks to the PEF-induction.

Analysis of wines after aging on untreated and PEF-treated lees

The effect of mannoproteins in wine stability and their positive influence on a great number of technological and quality properties of red wine have been widely described (Guadalupe et al., 2010). However, the studies evaluating the characteristics that the addition of different extracts of mannoproteins or the application of techniques to accelerate their release from yeast provide to the wines are very heterogeneous and diverse. The addition of commercial mannoprotein to red wine did not act as stabilizing colloids, did not modify the content of anthocyanins or phenols and did not affect color (Guadalupe and Ayestarán, 2008b). However, these compounds clearly modified the sensorial properties of red wines, improving the sweetness and roundness (Guadalupe et al., 2007). The addition of commercial dry yeast preparations reduced green tannins increasing the softness on the palate, and managed to stabilize the color (Del Barrio-Galán et al., 2012). Similarly, the method used to accelerate yeast autolysis may exert an influence on the ability of mannoproteins released to improve the characteristic of wines (Núñez et al., 2006). For instance, mannoproteins obtained enzymatically resulted more effective in avoiding protein haze in white wines than mannoproteins obtained from heat-treated yeast (Dupin et al., 2000; Moine-Ledoux and Dubourdieu, 1999).

In order to ascertain the effect on red wine of mannoproteins obtained by PEF-induced autolysis, physico-chemical characteristics of the wines obtained were analyzed in the course of aging on lees process. Futhermore, since reduction of astringency and improved mouthfeel have been described as some of the most interesting benefits of yeast mannoproteins presence in wines, it was analyzed the astringency and the sensorial properties of wines containing untreated and PEF-treated yeast in order to evaluate the

functional properties of mannoproteins released from yeast whose autolysis was accelerated by PEF.

Physicochemical characteristics of the wines

Total polyphenol index (TPI), color intensity (CI), hue, total anthocyanins content (TAC), tannins condensed (TC) and chromatic characteristics of the red wine before aging on lees and after 1 and 3 months of aging on lees with untreated and PEF-treated lees are shown in Table 35.

Among these parameters, except for CI, TAC and percentage of blue color (%Bl), significant statistical differences were not found between the treatments, neither did they occur between aging on lees time intervals. It would therefore seem that such physicochemical characteristics of wines are not a reflection of different levels of mannoproteins release or of presence of yeast during aging.

On the other hand, CI increased as the aging time was prolonged for all the conditions and this is attributed to the formation of polymeric pigments ranging from anthocyanins to other wine components, mainly tannins, and of derived pigments formed by condensation, which consist in non-covalent links of anthocyanins with colorless molecules, or with a series of anthocyanins (Dueñas et al., 2006; Salas et al., 2003). In the case of TAC, values decreased as the aging time was prolonged and this reduction was greater for wines which contained lees in comparison to control without lees, thus it seems that the yeast cell walls interact adsorbing anthocyanins as it has been previously described (Morata et al., 2003). Finally, %Bl after 2 months of aging was greater for wines which contained lees in comparison to control without lees, indicating that the tones associated to young wines were maintained due to the presence of yeast. It is described that capacity of yeast to consume oxygen during aging prevents oxidation of wines (Salmon et al., 2000).

Table 35**:** Physico-chemical characteristics of the initial wine, control wine without lees, wine containing untreated and PEF-treated (15 kV cm^{-1}, 75 µs) *S. cerevisiae* yeast after 1 or 3 months of aging.

	Initial wine	**1 month aging**			**3 months aging**		
		Control	PEF-treated lees	Untreated lees	Control	PEF-treated lees	Untreated lees
Alcohol (% v.v-^{1})	11.60±0.28						
pH	3.37 ± 0.03						
Volatile acidity (g.L^{-1})*	0.23 ± 0.02						
Total acidity (g.L^{-1})**	5.82 ± 0.13						
TPI (A.U)	33.56 ± 0.59 a	33.5 ± 2.12 a	30.4 ± 40 a	33.28 ± 3.92 a	32.85 ± 0.49 a	28.48 ± 0.48 a	28.53 ± 0.40 a
CI (A.U.)	8.94 ± 0.04 a	10.93 ± 1.31 ab	10.65 ± 0.99 ab	10.49 ± 0.71 ab	11.53 ± 0.04 b	10.4 ± 0.53 ab	9.71 ± 0.07 ab
Hue (A420/A520)	0.45 ± 0.03 a	0.43 ± 0.02 a	0.46 ± 0.03 a	0.43 ± 0.01 a	0.44 ± 0.02 a	0.47 ± 0.03 a	0.44 ± 0.03 a
TAC (mg.L^{-1}) ***	700.06 ± 12.18 b	691.10 ± 8.81 ab	683.49 ± 8.35 ab	673.11 ± 2.12 ab	681.35 ± 16.94 ab	634.55 ± 4.49 a	634.55 ± 4.49 a
TC (mg.L^{-1}) ****	1045.23 ± 282.18 a	1047.80± 180.17 a	911.70 ± 20.65 a	926.75 ± 19.3 a	1190.3 ± 132.65 a	971.50 ± 33.09 a	1016.3 ± 93.62 a
(%Ye)= (A420/CI x 100)	27.77 ± 1.33 a	27.19 ± 0.15 a	28.36 ± 0.70 a	26.71 ± 0.54 a	27.51 ± 0.04 a	28.44 ± 0.25 a	26.82 ± 0.33 a
(%Rd) = (A520/CI x 100)	62.27 ± 1.43 a	63.8 ± 0.01 a	61.11 ± 2.11 a	61.63 ± 1.45 a	62.97 ± 0.13 a	60.52 ± 1.64 a	61.51 ± 1.05 a
(%Bl) = (620/CI x 100)	9.96 ± 0.46 ab	9.01 ± 0.15 a	10.53 ± 1.41 ab	11.66 ± 0.91 b	9.52 ± 0.09 a	11.04 ± 1.39 ab	11.66 ± 0.72 b

Values represent mean ± standard deviation (n=2).
Different letters within the same row indicate significant differences ($p \leq 0.05$).
TPI: total polyphenol index; CI: color intensity; TAC: total anthocyanins content; TC: tannins condensed; %Ye. %Rd. %Bl: percentages of yellow. red. and blue colors respectively; A.U.: absorbance units.
*Expressed as acetic acid
**Expressed as tartaric acid
***Expressed as malvidin-3-glucoside.
**** Expressed as epicatechin.

Therefore, differences observed in physico-chemical characteristics seems to be mainly related to the presence of the yeast rather than to the concentration of mannoproteins released and it was demonstrated that aging on PEF-treated lees did not negatively affect these characteristics of wines.

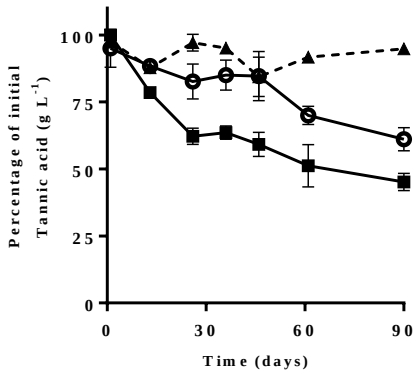

Figura 47: Evolution of astringency during the aging on lees of wines expressed as percentage of initial tannic acid. Control wine (▲), wine containing untreated (○) and PEF-treated (15 kV cm^{-1}; 75 µs) *S. cerevisiae* (■).

Astringency measurements

Astringency measurements of control wines without aging on lees and containing untreated or PEF-treated lees after centrifugation (conducted with the purpose of removing yeast) after different aging on lees periods are shown in Figura 47. Results were obtained after precipitation with ovalbumin of wines and are expressed as the percentage of initial tannic acid, which was used as standard. It is observed that the indirect measurement of astringency by this method was maintained during the three months of aging in the case of control wine without lees while the astringency values decreased in both wines aged on lees. However, this gradual decrease of astringency measurement along the aging on lees was faster for wines aged on PEF-treated yeast in comparison to those aged on untreated lees. For example, after one month of aging on lees the astringency measurement of wine which contained PEF-treated and untreated cells was 62 % and 82 % respectively of the value of the wine before aging step. At the end of the three-months aging on lees these values decreased to 45 and 61 % respectively for wines containing PEF-treated and untreated yeast. This evolution of the indirect measurement of astringency along aging on lees seems to be associated with the evolution of mannoprotein concentration along time in the different wines assayed. It is well known that mannoproteins can combine with tannins in wine, thereby decreasing the amount of free tannins (Escot et al., 2001; Escot and Fuster, 2002; Feuillat, 2003; Vidal et al., 2004). The method used determines astringency by using ovalbumin as the precipitant agent,

thus the higher amount of polymeric tannins formed by the interactions of mannoproteins and monomeric tannins would facilitate the precipitation caused by the ovalbumin resulting in lower measurement of astringency.

Sensorial analysis of wines

Finally the sensorial properties of the control wine without lees and two wines with similar concentration of mannoproteins, one which contained untreated yeast during three months of aging on lees and another which contained PEF-treated yeast during one month of aging on lees, were evaluated by twenty panelists in order to evaluate the perceived astringency and the mouthfeel. As it is shown in Table 36 , significant differences were found between the control wine without lees and the wine containing PEF-treated lees and between the control wine and the wine containing untreated lees. On the other hand, no significant differences were found between the wine containing PEF-treated lees and the wine containing untreated lees. However, it was required three months of aging on lees to obtain the wines in the case of untreated lees while the wine aged on PEF-treated lees was obtained after only one month of aging time. The differences detected by the panelists were associated to the different concentration of mannoproteins of wines Figura 45. Astringency phenomenon is thought to be due to the interaction between polyphenols and salivary proteins leading to the formation of insoluble aggregates that precipitate in the mouth and modify saliva lubrication (Ramos-Pineda et al., 2018). Although astringency perception can differ widely between individuals (González-Royo et al., 2017), the majority of panelists distinguished the wines with greater concentration of mannoproteins. It is known that polysaccharides of cell wall, such as mannoproteins, could disrupt protein-tannin interaction, can retain tannins or can limit available proanthocyanidins reducing astringency of wines (Escot et al., 2001; García-Estévez et al., 2017; Hanlin et al., 2010; Riou et al., 2002; Rodrigues et al., 2012). In addition, among the panelist that distinguished the wines in the triangular tests, the majority preferred the wine containing higher concentration of mannoproteins, thus indicating that presence of glycoproteins is a desired characteristic.

Table 36: Triangle test and preference test comparing control wine without lees, wine after 3 months of aging on untreatred lees and wine after 1 month of aging on PEF-treated lees +2 months of aging without lees. The two wines aged on the lees contained the same mannoprotein concentation.

	Triangle test (percentage of correct responses)	**Preference test (percentage of preferred wine)**
Wine without lees (C) Vs Wine aged on PEF-treated lees (PL)	60*	66 % PL
Wine without lees (C) Vs Wine aged on untreated lees (UL)	60*	60 % UL
Wine aged on PEF-treated lees (PL) Vs Wine aged on untreated lees (UL)	40	-

* Significant differences between the samples, ($p \leq 0.05$).

These results support the hypothesis that mannoproteins released from yeast treated by PEF have functional properties in terms of reduction of astringency and contribution to the mouthfeel of wines similar to those of mannoproteins released from natural autolysis, whereby the differences observed reflect the varying concentration of mannoproteins in the wine.

4. Conclusions

This study demonstrates the potential of PEF to accelerate the release of mannoproteins from *S. cerevisiae* yeast during the aging on lees of *Caladoc* red wine. The analysis of enzymatic activity during aging demonstrated that PEF treatments triggered *β*-glucanase and protease activities in the extracellular media where yeast were aged, enzymes that similarly act during natural autolysis which is produced slower. Autolysis induced by PEF did not negatively affect the wines´ physico-chemical properties, while the aging on lees procedure protected wine from oxidation. The mannoproteins released in a shorter time from PEF-treated cells featured similar functional properties in wines than mannoproteins released during natural autolysis from untreated yeast. The sensorial analysis confirmed that panelists did not found differences between the wine obtained through PEF-induced autolysis in shorter time and the wine obtained after natural autolysis process in three-fold more aging time. This technique therefore permits to accelerate the “aging on lees” step while avoiding or reducing the problems customarily associated with it representing great saves and advantages for the wineries.

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