Universitat de Lleida

Conservación de mosto de uva con pulsos eléctricos de alta intensidad de campo

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Tesis doctoral

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Dedico aquest treball a tots aquells que m'han sofert.

Índice general

Resum

S'ha realitzat l'estudi de l'efecte del processat de suc de raïm amb polsos elèctrics d'alta intensitat de camp (PEAIC) amb la intenció d'aconseguir la seva conservació. Els seus efectes s'han mesurat en diferents propietats característiques del suc de raïm com el contingut en sòlids solubles, pH, acidesa, conductivitat elèctrica, activitat microbiològica i enzimàtica, alguns compostos bioactius com el contingut en polifenols o vitamina C i capacitat antioxidant. Els valors obtinguts s'han comparat amb els obtinguts del tractament conservant de referència actual, el processat tèrmic. També s'ha determinat l'evolució de components específics durant el procés de vinificació des de l'obtenció del suc de raïm fins a l'envelliment del vi.

L'estudi s'ha dut a terme en 4 fases en les que s'ha determinat que els PEAIC redueixen el contingut microbià del suc de raïm, tant l'inoculat com el natural, i l'activitat enzimàtica oxidativa. Així mateix, s'ha constatat que es possible optimitzar el procés tenint en compte les característiques de l'equipament i el medi. La comparació dels resultats de les variables estudiades amb aquesta tecnologia i amb tractament tèrmic a permès evidenciar que els efectes dels PEAIC són menys dràstics, fet que permet la seva utilització en l'elaboració de suc de raïm i vi sense que afecti negativament al procés o a l'envelliment del producte.

La principal conclusió de l'estudi amb les dades obtingudes de tres varietats habitualment utilitzades a Catalunya és que el processat amb PEAIC és una eina de conservació del suc de raïm viable des del punt de vista tecnològic. L'adquisició de dades d'altres varietats i temporades fora de gran utilitat per ampliar aquesta conclusió.

Summary

The effect of pulsed electric fields as a preservation method on grape juice was studied. Its effects were measured on typical grape juice properties such as brix degrees, pH, acidity, electrical conductivity, microbial and enzymatic activities, and some bioactive compounds like vitamin C and polyphenol contents or antioxidant activity. The obtained values were compared with the current preservative reference technology, which is the heat processing. It was also analysed the effect of PEF on the evolution of specific compounds throughout the wine making process from juice to ageing.

The study was carried out in 4 phases. It was observed a clear reduction of the microbial populations of both, inoculated and natural flora, as well as the oxidant enzymatic activities of grape juice. Besides, these effects were optimised taking into account the PEF processing device and the food product. The comparison of both treatments revealed that the PEF treatment yielded milder values of the variables than the thermal treatment. Therefore it could be used in juice and wine making without a negative impact on the quality of the product.

The main conclusion inferred from the study is that on three commonly used Catalan varieties PEF processing is a feasible technology to preserve grape juice. New data coming from different varieties and years should broaden this conclusion.

Resumen

Se ha realizado el estudio del efecto del procesado de zumo de uva con pulsos eléctricos de alta intensidad de campo (PEAIC) para su conservación. Los efectos se han medido en distintas propiedades características del zumo de uva como el grado brix, pH, acidez, conductividad eléctrica, actividad microbiológica y enzimática, algunos compuestos activos biológicamente como el contenido en polifenoles o vitamina C y la capacidad antioxidante. Los valores obtenidos se han comparado con los del tratamiento conservante de referencia, es decir, el tratamiento térmico. También se ha determinado la evolución de compuestos específicos durante el procesado desde el zumo de uva hasta el envejecimiento del vino.

El estudio se ha realizado en 4 fases en las que se ha observado que los PEAIC reducen claramente la micro flora del zumo de uva, tanto la inoculada como la natural, así como la actividad enzimática oxidante. Se ha confirmado, además, que es posible optimizar esta reducción teniendo en cuenta las características del equipo de procesado y el medio. La comparación de resultados de las variables estudiadas tras el procesado PEAIC con el tratamiento térmico ha permitido constatar que los efectos de los PEAIC son menos drásticos lo que permitiría su uso como tratamiento en la elaboración del zumo y vino sin que afecte negativamente al proceso de vinificación o a su envejecimiento.

La principal conclusión del estudio con los datos de tres variedades habitualmente utilizadas en Cataluña es que el procesado mediante PEAIC para la conservación de zumo de uva es técnicamente viable. La obtención de nuevos datos de otras variedades y distintas vendimias seria útil para poder generalizar esta conclusión.

1 Introducción

1.1. Uva, mosto y vino

Las frutas han tenido una especial relevancia en la alimentación desde el inicio de la humanidad. Esa importancia se ha traducido en un gran interés por lograr su conservación evitando su estacionalidad. Hasta el siglo pasado, el único modo para consumir fruta fuera de temporada se basaba en reducir su actividad de agua para garantizar la estabilidad ya fuese por secado, edulcoración, salado o una combinación de estos procesos para dar lugar a un producto aceptable sensorialmente. La rápida evolución tecnológica que se produjo durante el siglo XX ha permitido que, en la actualidad, la fruta pueda consumirse en fresco durante prácticamente durante todo el año.

Los avances tecnológicos también han facilitado el auge de los zumos de las frutas como productos comerciales producidos industrialmente. La publicidad comercial y la idiosincrasia humana han acuñado la imagen de que los zumos industriales son como los caseros en cuanto a sabor y características nutritivas. En consecuencia, su elaboración debe incidir mínimamente en sus características y habitualmente la elaboración de zumos de frutas se considera un proceso de conservación de la fruta.

Un caso aparte entre las frutas lo constituye la uva. Aunque su consumo en fresco y como zumo en el conjunto de frutas sea tan significativo como el de manzanas o naranjas (Fig. 1.1), su principal uso siempre ha sido la vinificación. En el período desde 1999 hasta 2008 la producción mundial estuvo alrededor de los 28 millones de toneladas. Ello significa alrededor de 30 veces más que la cantidad de zumo de uva producido o 15 más que la producción de zumo de naranja, siendo, a la vez, uno de los productos agrícolas elaborados mas exportado después del conjunto de aguas minerales y sus derivados [74].

El vino, desde siempre, ha sido considerado un artículo con entidad propia y no un simple derivado de la uva. Consecuentemente, su obtención a partir de la fruta tampoco se entiende como un mero tratamiento conservante y en la actualidad llega a considerarse prácticamente un arte, dado que su elaboración es más compleja que la requerida por otros alimentos y puede durar varios años. Para poder garantizar al consumidor un producto aceptable, la legislación vigente estipula los requisitos mínimos de calidad y las prácticas de elaboración permitidas [44, 45, 46, 118].

Una breve descripción de las etapas de elaboración de los distintos derivados de la uva permite una visión de las relaciones entre ellos (Fig. 1.2). Como ocurre con cualquier proceso realizado por fases, la eficiencia y buen hacer en cada una

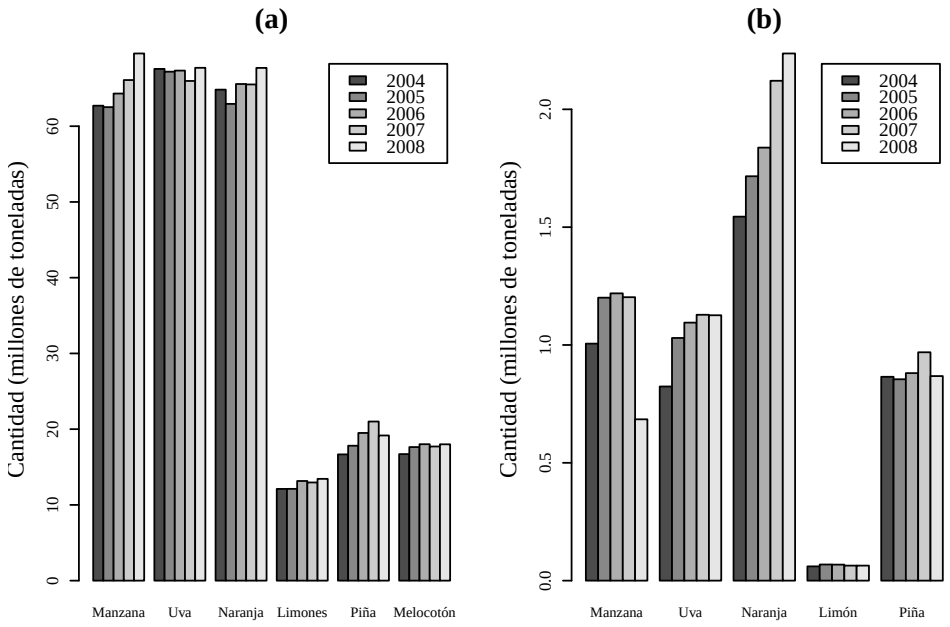


Figura 1.1: Evolución de la producción mundial de (a) frutas y (b) zumos [74].

de ellas reducen la pérdida de calidad del producto final.

El primer paso consiste en la recolección de las uvas en el punto de madurez adecuado teniendo en cuenta la existencia de variedades específicas para cada tipo de producto final [105]. La selección y lavado son las únicas etapas para la comercialización en fresco. El secado posterior produce las *uvas pasas*.

Para obtener derivados líquidos de la uva, el prensado es una etapa crítica del proceso. Esta fase define el rendimiento contabilizado exclusivamente como cantidad de producto final a partir del inicial. Por este motivo se han desarrollado un gran número de técnicas industriales que incrementan notablemente su eficiencia. El control de la temperatura, el diseño de las prensas y la presión por etapas son algunas de ellas aunque la más utilizada sigue siendo la rotura de los granos de uva o *crushing*.

En función del producto final, tarde o temprano, se requiere la separación del zumo de las partes sólidas de los frutos y nuevamente existen diferentes métodos para lograrlo como la clarificación, filtrado, centrifugación o una combinación de ellos. También es necesario un proceso de eliminación o reducción del contenido en ácido tartárico y sus derivados que con el tiempo precipitan dando turbidez al producto final. Existen distintas técnicas que siguen diversas leyes físicas o químicas para lograr este objetivo como el enfriamiento, el reposo, la criogénesis localizada, la siembra con cristales de tartratos o la sustitución del ácido tartárico por especies más estables mediante la adición de compuestos químicos o

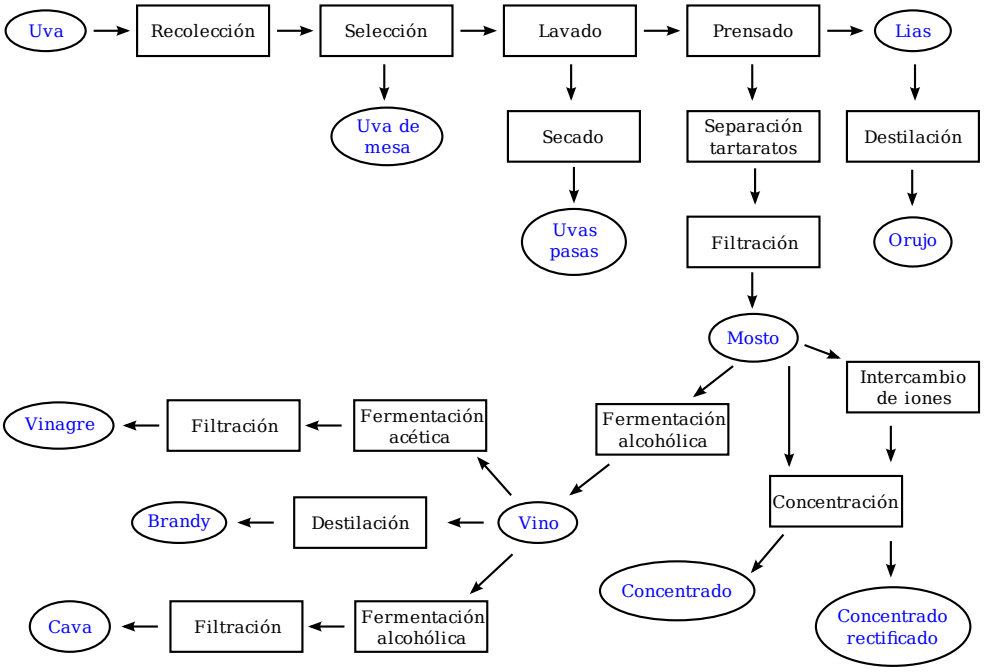


Figura 1.2: Esquema genérico de la elaboración de los derivados de uva.

utilizando membranas selectivas de iones [214, 105, 147].

Llegados a este punto, la flora microbiana presente en las uvas, los equipos industriales o en el ambiente, probablemente ha iniciado la etapa de crecimiento exponencial, excepto si se han introducido agentes antimicrobianos en alguna de las etapas anteriores. El inicio de la fermentación se cuida con celo en la industria vitivinícola. Se utilizan incluso cepas seleccionadas resistentes a los inhibidores microbianos utilizados que pueden sustituir a las nativas. Estas se añaden directamente o tras eliminar las cepas previas con un tratamiento térmico [214, 105].

Una vez finalizada la fermentación, la estabilidad del vino habitualmente está garantizada pudiendo envejecer durante un período mas o menos largo a fin de modificar sus características organolépticas. Posteriormente pueden incorporarse más azúcares y ser sometido a otra etapa fermentativa para obtener *cava* o *champán*. Un proceso de destilación conduce a productos con un elevado contenido alcohólico conocidos como *brandy* y una fermentación acética permite obtener *vinagre* de vino.

Para el resto de derivados, la fermentación alcohólica reduce drásticamente la calidad de producto obtenido y debe evitarse mediante un tratamiento térmico conocido como pasteurización. El mosto así tratado es envasado directamente como *zumo de uva*. Puede concentrarse eliminando el agua de constitución como se hace en otros zumos de fruta obteniéndose *zumo concentrado* cuya principal

ventaja consiste en abaratar los costes de transporte en los intercambios comerciales. Si previamente a la concentración se eliminan los iones que contiene se convierte en un jarabe edulcorante natural de aplicación en otros ámbitos de la industria alimentaria como la bollería, pastelería o en procesos biotecnológicos que tiene el nombre de *concentrado rectificado* [147].

Es evidente que la obtención del mosto de uva es una etapa intermedia ineludible en la elaboración del vino o el conjunto de derivados líquidos de la uva y, aunque las condiciones para obtener uno u otro producto no son iguales, si presentan similitudes. Como se ha comentado el zumo de uva tiene su propia importancia en el ámbito de la industria alimentaria (Fig. 1.1) y cuenta con su propia legislación para satisfacer unos requisitos mínimos de seguridad y calidad [157, 199, 156, 158, 153]. Sin embargo, considerado como producto intermedio, cualquier tratamiento que suponga mejoras en su obtención o gestión es susceptible de ser utilizado puesto que beneficiará también a sus derivados.

1.2. Conservación de alimentos, pasteurización

La gran diversidad de tratamientos disponibles para cada etapa del procesado descrito surge, obviamente, de necesidades específicas de cada uno de los productos derivados de la uva. No obstante, sólo existe un tratamiento aceptado actualmente que permite estabilizar física, química y microbiológicamente el mosto independientemente del procesado posterior al que sea sometido, la pasteurización.

Esta tecnología constituye el referente en toda la industria de alimentos. El tratamiento térmico en zumos, típicamente, oscila entre los 85 °C y 95 °C durante 15 s y 2 s respectivamente para eliminar los microorganismos. Si el objetivo son los enzimas vegetales, son corrientes tratamientos entre 86 °C y 99 °C durante hasta 40 s [133]. Así pues, la combinación específica de temperatura y tiempo de tratamiento se escoge en función del agente pernicioso más resistente que se desee destruir. Sin embargo la acción del calor no es específica e influye en el resto de componentes presentes en el zumo. El grado de afectación será mayor al aumentar la potencia del tratamiento o cuanto mayor sea la susceptibilidad térmica del componente en cuestión. En el caso de los zumos de frutas el grupo de constituyentes más lábiles son las vitaminas hidrosolubles y los componentes antioxidantes.

Con el efecto de reducir la degradación de estos componentes de las frutas, el tratamiento térmico habitualmente se combina con otros para mitigar en la medida de lo posible la severidad de esta etapa. Así, en el caso de los zumos, es típico un rápido enfriamiento después del procesado térmico y un inmediato envasado en un ambiente aséptico con lo que la vida útil puede alcanzar meses [133]. En el caso de los zumos concentrados, el tratamiento térmico se produce durante la etapa de concentración que a la vez reduce la actividad de agua del producto resultante. En vinificación, como se ha descrito, la práctica habitual

consiste en la adición de compuestos químicos inhibidores del crecimiento microbiano como los sulfitos. Sin embargo, el mismo cambio de mentalidad de los consumidores que ha promovido el aumento del consumo de derivados de frutas ha suscitado el rechazo por aquellos alimentos con sustancias añadidas. Ésta es una de las razones por las que, en las últimas décadas, ha habido una fuerte auge en la búsqueda de métodos alternativos de procesado para la conservación de alimentos

Se han estudiado técnicas utilizadas en otras áreas industriales y científicas distintas de la conservación de alimentos como las atmósferas protectoras que se usan, desde hace mucho, en las cámaras de frigo-conservación de productos vegetales; los recubrimientos con compuestos activos provenientes del envasado o, la radiación ultravioleta utilizada en laboratorios microbiológicos para la esterilización de superficies. Otras tecnologías térmicas, como el calentamiento óhmico o la irradiación mediante microondas, aprovechan también el calor como agente pero intentan minimizar sus inconvenientes produciéndolo directamente en el interior del propio alimento. Otras se basan en el uso de procesos físicos completamente distintos al tratamiento térmico como las altas presiones. El presente trabajo se centra en el estudio de los pulsos eléctricos de alta intensidad de campo (PEAIC) para la conservación del zumo de uva.

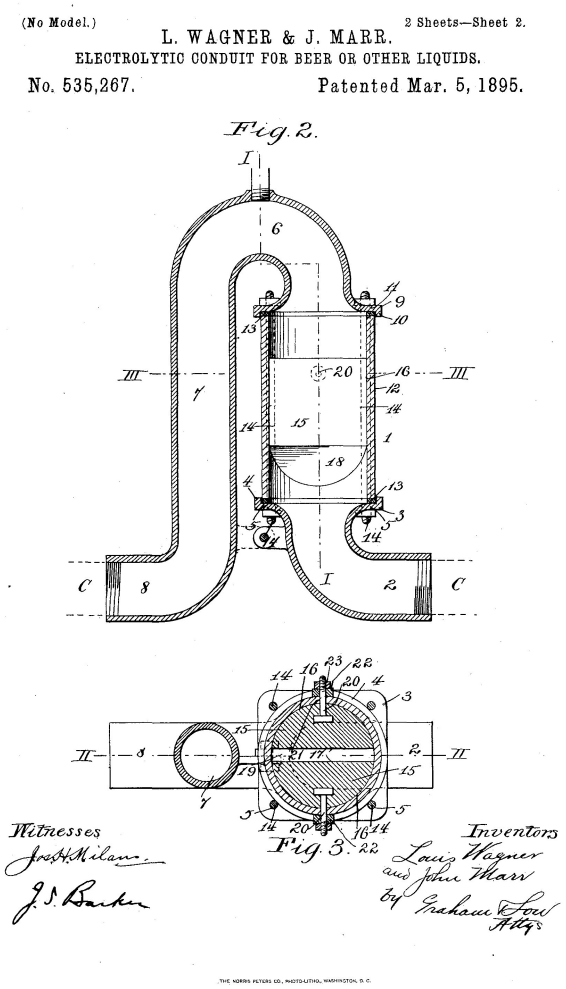
1.3. Alternativas para la conservación de alimentos

La utilización de electricidad para estabilizar alimentos es muy antigua [258, 233, 247]. Hay patentes de equipos para tratar líquidos desde finales del siglo XIX e inicios del XX (Fig. 1.3). Sin embargo los estudios en los que se basan los diseños están realizados por los propios autores sin aportar datos que puedan ser comprobados.

En los años 20 y 30 se describen efectos contrastables de la aplicación de electricidad a zumo de uva y leche a temperaturas controladas por debajo de las letales (46 °C) [249, 163]. En esta época ya se sospechaba que los efectos destructivos sobre los microorganismos eran debidos al calor producido y/o a la generación de especies químicas tóxicas de las corrientes eléctricas continuas a través del producto.

Esta tecnología, sin embargo, quedó prácticamente en el olvido en la década de los 40 [187, 144]. En los años 50 surgió un tratamiento denominado tratamiento *electro-hidráulico*, el cual consistía en aplicar descargas de alta intensidad de campo y provocar arcos eléctricos, produciéndose así, la destrucción de microorganismos y enzimas pero, al mismo tiempo, aparecían productos de electrólisis causantes de cambios en la composición de los alimentos [187].

Sale y Hamilton en 1967 aplicando la electricidad en forma de pulsos demostraron que el efecto producido en los microorganismos era debido a la electricidad y no a la electrólisis ni al calor, puesto que los tratamientos no se realizaron a temperaturas elevadas [223]. Pero fue a finales del siglo XX, cuando se realiza-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L. WAGNER & J. MARR.

ELECTROLYTIC CONDUIT FOR BEER OR OTHER LIQUIDS.

No. 535,267. Patented Mar. 5, 1895.

Fig. 2.

Fig. 3.

Witnesses

Inventors

Figura 1.3: Extracto de una patente del año 1895 de los Estados Unidos de América en la que se detalla un sistema de procesado en continuo de cerveza y otros líquidos mediante electridad [258].

ron muchos estudios para mejorar esta técnica de conservación y se patentaron varios sistemas de aplicación de pulsos eléctricos de alta intensidad de campo (PEAIC) [39, 268, 265]. Con estos sistemas se han descrito buenos resultados en la inactivación de microorganismos y enzimas describiéndose además una modificación mínima de las propiedades nutricionales y sensoriales de los alimentos [22, 27, 63].

1.4. Factores influyentes en el procesado mediante PEAIC

Los factores que influyen en la aplicación de esta tecnología son muy diversos. Para un mejor entendimiento y estudio habitualmente son clasificados en:

- características del agente a tratar u objetivo,
- características del producto a tratar y
- características del equipo de procesado.

1.4.1. Objetivo

El principal objetivo siempre ha sido garantizar la seguridad del consumidor. Ello exige la eliminación de los microorganismos patógenos. La leche o sus derivados, por su importancia y sus características, eran más propensos a contenerlos y muchos estudios PEAIC iniciales estudiaron como diana los microorganismos habituales de la leche [143, 212, 69].

En los zumos de fruta el valor de su pH impide, habitualmente, la proliferación de patógenos. Por lo tanto, el área de estudio se centro en microorganismos causantes de alteraciones en su calidad hasta que se confirmaron casos de intoxicaciones por patógenos en zumos, mayoritariamente causados por *Escherichia coli* O157:H7. Ello se unió a la utilización de frutas con valores de pH más elevados y mezclas con leche promovidas para diversificar la producción. Ello supuso que los productos derivados de frutas fuesen más susceptibles de contener agentes patógenos [164, 239].

Hasta la fecha, todos los estudios conducen al resultado de que es posible conseguir una reducción significativa de levaduras y bacterias mediante el uso de PEAIC aunque el grado de destrucción alcanzado difiere [48, 59, 58, 54, 97, 99, 211]. Estas diferencias se han relacionado con características microbianas como el tamaño, la forma, la estructura de la membrana, el estado de crecimiento o de agregación del microorganismo [112, 266, 194, 15, 16, 79, 77].

Gracias a dichos estudios, se ha podido determinar la causa de su efectividad. Es aceptado que el cambio de potencial eléctrico somete a las membranas celulares a un esfuerzo de adaptación a las condiciones externas que no son capaces de soportar, provocando su rotura con la consecuente pérdida de la homeostasis

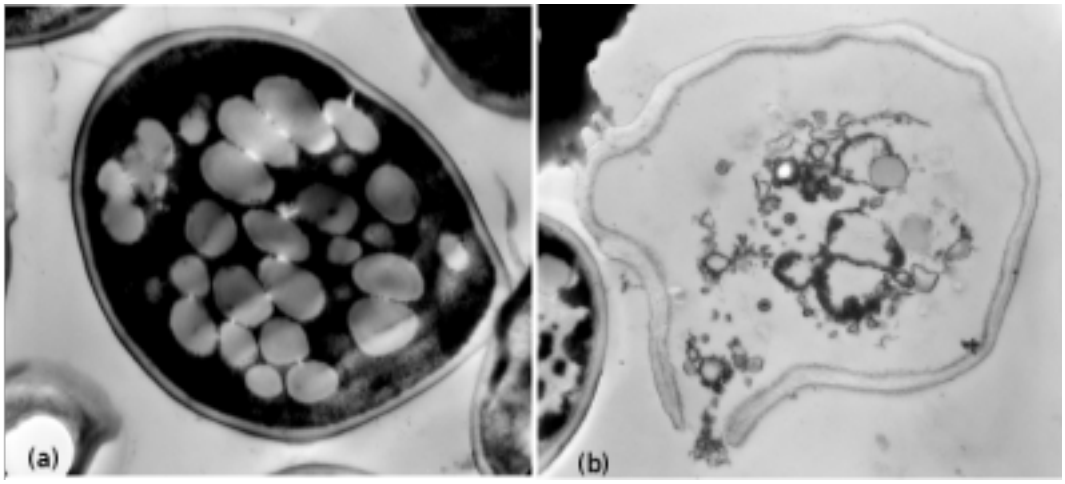

Figura 1.4: Micrografías (×16000) obtenidas con un microscopio de transmisión de electrones de células de *S. cerevisiae* en zumo de naranja (a) antes y (b) después de un tratamiento mediante PEAIC [59].

y, en la mayoría de los casos, la muerte celular [229, 228]. Las etapas del proceso consisten en:

1. La generación de un potencial entre la capa interna y externa de la membrana celular debido al efecto del campo eléctrico externo aplicado.

2. La creación de pequeños poros hidrofílicos metaestables.

3. La evolución en tamaño y numero de estos poros por el tratamiento eléctrico.

4. La evolución de los poros después del tratamiento (consolidación, reparación, unión entre ellos, ...) condiciona el efecto sobre las células.

En cuanto a las formas resistentes de los microorganismos, las esporas, los datos obtenidos no permiten ni siquiera generalizar el efecto de los PEAIC. Estudios sobre la especie *Bacillus* [234, 48] no mostraron destrucción significativa alguna. En cambio, en esporas de levaduras de la especie *Botrytis* [68] o de la especie *Alicyclobacillus*, muy resistente a los tratamientos térmicos, los resultados son muy positivos [193, 94, 250].

Menos estudios se han realizado en virus transmitidos por alimentos. Sus resultados no permiten suponer que este tipo de procesado les afecte. El poco efecto de los tratamientos mediante PEAIC se achaca a las diferencias estructurales entre las membranas celulares y las cápsides víricas [123].

Una vez asentada y aceptada en la comunidad científica el hecho de su efecto en microorganismos algunos equipos de investigación han expandido sus estudios

para abarcar como objetivo de los PEAIC a cualquier componente de los alimentos capaz de modificar el producto durante su vida útil centrando la atención en los enzimas [28, 63, 160, 14].

Los resultados obtenidos muestran unas grandes diferencias, que según el enzima estudiado pueden ser la inactivación total o un aumento de su actividad [106, 134]. En consecuencia, no existe un mecanismo aceptado sobre el modo de actuación de los PEAIC como ocurre con los microorganismos. La disparidad de resultados parece que podría deberse a las diferencias estructurales existentes entre enzimas como resultado de su diversa funcionalidad. Es decir, los enzimas no poseen un elemento en común como la membrana celular microbiana. Luego los efectos del campo eléctrico influyen de forma diferente sobre los enzimas dependiendo de sus conformaciones espaciales [24, 32, 61]. Algunos efectos sugeridos son cambios conformacionales en la estructura enzimática o la promoción de reacciones químicas internas que generan nuevos enlaces o rompen los que existían [24, 270].

La investigación en este campo esta abierta a nuevos descubrimientos, aunque sí es aceptado que para lograr un efecto sobre los enzimas es necesario un tratamiento más enérgico que sobre microorganismos [61].

1.4.2. Producto

El producto a procesar es importante puesto que constituye la matriz en la que se encuentran tanto las especies biológicas o químicas que interesa destruir como las que se pretende preservar. A priori, la presencia de interacciones entre ellas es una suposición razonable puesto que ambas están sometidos al mismo tratamiento. Sin embargo, el hecho de que los tratamientos mediante PEAIC sean más localizados que los tratamientos térmicos y afecten con la misma intensidad a todo el volumen de producto tratado sugiere no obstante que dichas interacciones sean menores espacial y temporalmente que en el procesado térmico.

Inicialmente estos estudios fueron realizados en matrices simples y controladas como disoluciones acuosas o medios simulados para minimizar las posibles interacciones y discernir claramente entre factores pertenecientes a los objetivos y, factores pertenecientes al medio [16, 251, 29]. Así, se ha observado que la conductividad del medio o la viscosidad condicionan la magnitud del campo eléctrico aplicado. Cuanto mayor es la conductividad de un producto mayor debe ser la diferencia de potencial aplicada para obtener resultados similares. Además, la conductividad eléctrica y la viscosidad de un producto son a su vez modificados por la temperatura que, poco o mucho, cambia durante el procesado. También se ha observado que el pH influye de un modo más significativo sobre los efectos en microorganismos que en enzimas o que la presencia de partículas o gases disueltos modifica el campo eléctrico local al que se encuentran sometidos los componentes del alimento [257, 6, 16, 79, 77].

Últimamente, se han destinado muchos recursos a la investigación en produc-

tos más complejos. Por el momento hay descritos efectos en muchos productos líquidos de consumo como zumos de naranja [59, 58], de tomate [172], de fresa [178], de manzana [66], leche [239], vino [200], cerveza [251, 67], huevo líquido [225].

Dada la gran complejidad de la composición de los medios de tratamiento el estado de conocimiento actual es similar al de los factores microbianos. Es decir, se conocen los factores que influyen pero no existe una tendencia general de cada factor a un efecto concreto. Esto es debido en parte a que sólo pueden medirse características generales del medio y los valores locales de esas características donde existe la interacción deben suponerse.

Sin embargo, estos estudios han permitido discernir que ocurre con algunos de los componentes de los alimentos. Esta es la fuente de información más novedosa en cuanto a que una de las afirmaciones iniciales de este procesado es que no afecta al resto de las propiedades del alimento. Hasta finales del siglo pasado no existían los medios separativos y analíticos necesarios para estudiar los efectos en componentes específicos de los alimentos. Actualmente, además de la determinación del efecto de los factores de la matriz en las especies a destruir, en muchos de ellos se analizan sus componentes nutricionales y características organolépticas [240, 244, 172, 178, 4]. También se han realizado comparaciones entre los tratamientos mediante PEAIC y térmicos. Todos ellos confirman en mayor o menor medida las afirmaciones de que los efectos de los PEAIC son comparables a los del procesado térmico pero sin muchas de las repercusiones en el resto de los componentes del alimento [122].

Existe una dificultad añadida en este área. En los estudios científicos es común modificar el medio para adaptarlo a las características del equipo de procesado. Las conclusiones de los estudios se supone que no se ven comprometidas por estos cambios. Sin embargo, la implementación industrial de esta tecnología exigiría adaptar los equipos al producto a tratar. Consecuentemente, una de las etapas necesarias debe ser confirmar que los efectos descritos en una matriz modificada son comparables a los que se producen en un producto industrial o alternativamente realizar estos estudios con productos sin modificar.

1.4.3. Parámetros de procesado

Las investigaciones realizadas hasta ahora muestran sin lugar a dudas los parámetros de procesado a controlar para lograr modular los efectos del tratamiento aplicado.

El campo eléctrico ($\vec{E}$), especialmente el valor de su módulo o intensidad (E), es junto con el tiempo de tratamiento, la pareja de parámetros con más influencia en los tratamientos mediante PEAIC. Dicho tiempo de tratamiento se ha contabilizado, habitualmente, como la suma de pulsos recibidos [261, 230]. Se ha descrito y contrastado que para *S. cerevisiae* el mínimo valor de intensidad de campo que produce efectos letales se encuentra alrededor de $8\,\mathrm{kV{\cdot}cm^{-1}}$. Una vez alcanzado ese valor, una mayor duración del tratamiento provoca una mayor

destrucción [266, 230].

Desde los inicios de procesado de alimentos mediante PEAIC se sabe que el tratamiento con polaridad alterna reduce el calor generado y las reacciones de electroquímicas en los electrodos [249]. En estas reacciones intervienen los electrodos y el alimento en contacto con ellos con la posibilidad de formación de especies químicas tóxicas o de descomposición de componentes nutritivos. Así, aunque algunos de los estudios mencionados han experimentado con tratamientos monopolares parece observarse una mayor eficiencia de los campos eléctricos alternos [71].

Otras características del campo eléctrico son también importantes como la forma del pulso. Se han utilizado formas ondulatorias, de caída exponencial [268, 143], de carga inversa instantánea [262] pero de momento parece haber un cierto grado de consenso en que los pulsos cuadrados son más eficientes que los pulsos de caída exponencial o sinusoidales puesto que mantienen una diferencia de potencial constante durante más tiempo [202].

En pulsos de campo eléctrico cuadrado o de caída exponencial, el tiempo que debe durar el pulso es otro de los factores que afecta al procesado. Hasta el momento son típicos los valores de entre 1 µs y 10 µs. Debe tenerse en cuenta que cuanto mayor sea este valor más acusada es la generación de calor en el seno del producto. Por lo tanto se tiende a usar los valores más bajos que proporcionen resultados adecuados [59, 58].

Otros parámetros como la frecuencia de los pulsos o el flujo de producto a través del equipo influyen en cuanto se relacionan con el tiempo que el producto permanece en el equipo, es decir, el tiempo de tratamiento. De este modo condicionan la cantidad de energía que recibe un volumen determinado por unidad de tiempo puesto que una mayor frecuencia o un flujo más lento aumentan la energía recibida por el producto. Así pues, están relacionados con la eficacia del procesado [230].

1.5. Equipos de procesado mediante PEAIC

Los equipos de tratamiento mediante pulsos eléctricos han variado a medida que el conocimiento adquirido en la investigación de los factores influyentes ha ido avanzando. También ha tenido mucha importancia el desarrollo de los sistemas eléctricos y electrónicos a lo largo del siglo pasado.

Los elementos indispensables son [226, 110]:

- una fuente de alimentación que proporciona la energía necesaria,
- un lugar donde almacenar dicha energía, habitualmente, un condensador,
- un interruptor para liberarla a voluntad y
- un lugar donde liberarla en forma de diferencia de potencial. Ese lugar debe además contener a la muestra a tratar y normalmente se conoce

como *cámara de tratamiento*. Dependiendo de si la muestra se mantiene en el interior de la cámara o se va sustituyendo durante el tratamiento se obtienen los dos tipos de equipos fundamentales, por lotes o en continuo, respectivamente.

Los diseños para el procesado por lotes se han utilizado mucho en investigación puesto que su simplicidad permite un mayor control de los factores en estudio. Los experimentos iniciales y estudios de transformación celular se han realizado en este tipo de cámaras de tratamiento [249, 260].

El procesado por lotes está siendo considerado como una forma de tratar el producto una vez envasado puesto que la generación de campos eléctricos no requiere el contacto con el producto. A la vez, este modo de trabajo eliminaría el reprocesado del producto por contaminación posterior [129]. El principal problema que plantea este uso es que los valores de campo eléctrico deberían ser aún mayores que los utilizados en la actualidad, hecho que aumenta el gasto en inversión y el consumo energético.

Por el momento, la opción más viable desde el punto de vista técnico es la aplicación en continuo, puesto que aumenta en gran medida la capacidad de producción respecto al procesado por lotes. Este tipo de procesado exige el uso de sistemas de seguimiento con el objetivo de garantizar la calidad y seguridad del producto. Dichos sistemas pueden ser menos costosos si se puede asegurar la uniformidad del tratamiento. El mejor modo de conseguirlo es mediante un buen diseño del equipo de procesado y en especial de las cámaras de tratamiento. Este es uno de los motivos de la existencia un considerable número de patentes relacionadas con el diseño de equipos de procesado mediante PEAIC.

En esencia, existen dos tipos de cámaras de tratamiento que se fundamentan en la naturaleza vectorial de la tecnología. Aquellas en las que el campo eléctrico es perpendicular al flujo de producto o *cámaras de campo normal* y aquellas en las que el campo eléctrico coincide con el flujo o *cámaras de campo paralelo*. Ambos tipos de cámaras tienen diversas variantes esquematizadas en la figura 1.5. Según algunos estudios, los diseños en que los flujos de materia y de campo son paralelos permiten tratar de un modo más uniforme productos no homogéneos, ya sea por la presencia de partículas como por tener diferencias de viscosidad. Es decir, permiten mayor variabilidad del producto. Además, según los diseñadores, el sistema *co-flow* reduce notablemente las posibilidades de arco eléctrico. No obstante, este tipo de cámara requiere utilizar generadores de pulsos de altos voltajes que no son tan asequibles desde el punto de vista electrotécnico actual [35].

El análisis mediante modelos de elementos finitos indica que en ambos tipos de cámaras de tratamiento las eficiencias superan el 50 % si bien es más alta en aquellas en las que el flujo de materia y de campo eléctrico coinciden [36]. Este hecho favorece que la cantidad de energía disipada como calor sea menor y, en consecuencia, también la temperatura alcanza valores más bajos en este tipo de cámaras. Sin embargo, estos cálculos fueron realizados suponiendo la

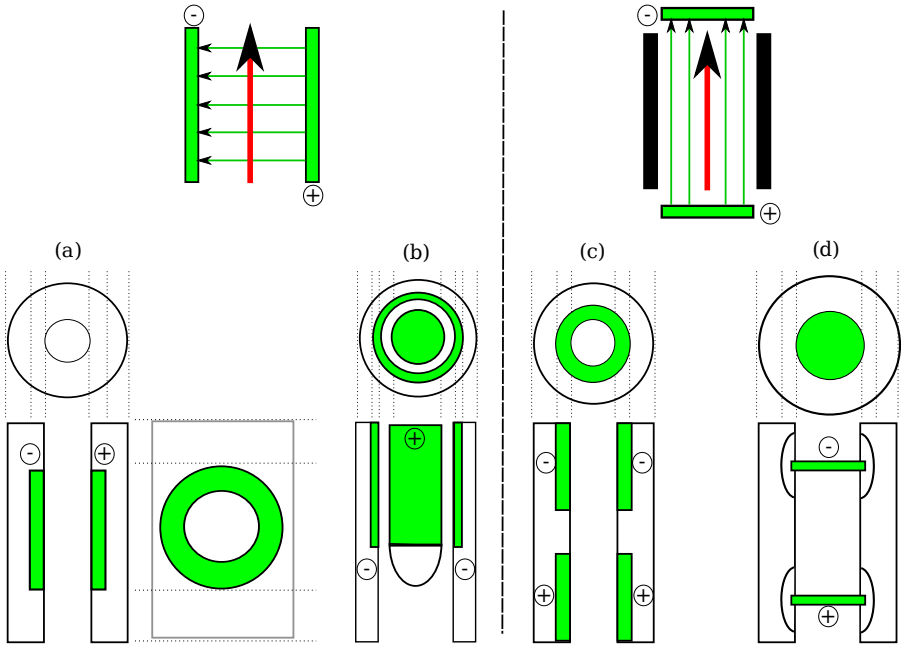

Figura 1.5: Esquemas de los tipos de cámaras de tratamiento (a) *cross-field*, (b) *co-axial*, (c) *co-field* y (d) *co-flow*. Las líneas rojas representan el flujo de producto, las verdes el sentido del campo eléctrico.

homogeneidad completa del producto tratado. El procesado real de zumos de frutas o leche es de esperar que produzca valores locales distintos.

Junto con estos condicionantes, la cámara debe diseñarse teniendo en cuenta que la distribución del tiempo de residencia del producto en ellas depende de un caudal de flujo determinado. Se ha observado experimentalmente que los flujos turbulentos favorecen la homogeneidad del tratamiento y permiten incluso triplicar los efectos sobre los microorganismos del tratamiento mediante PEAIC [230]. Hay modelizaciones para el cálculo de la eficiencia que la rebajan hasta el 30 % en función del régimen de circulación del material en el interior de la cámara [36].

El producto puede ser sometido al tratamiento completo mediante un único paso por la cámara de tratamiento. Muchos de los estudios se han realizado con este esquema con la argumentación de que es el método óptimo o que permite controlar perfectamente parámetros esenciales como el tiempo de tratamiento [1, 36]. Sin embargo, si no es posible obtener intensidades de campo muy elevadas, el tiempo de tratamiento debe aumentarse pudiendo no ser posible realizarlo de una sola vez. En ese caso hay dos posibles soluciones, diseñar el equipo con cámaras de tratamiento en serie o procesar el producto mas de una vez hasta garantizar el tratamiento adecuado. Trabajos realizados en equipos con más de una cámara [203] o haciendo pasar el producto varias veces por la misma

cámara [59, 58], muestran mejores resultados que cuando el producto se procesa una única vez cuando se comparan los resultados en el mismo equipo [230].

Otros puntos que debe tenerse en cuenta en el diseño del equipo de procesado mediante PEAIC son los relacionados con la seguridad tanto para el consumidor como para los operarios del equipo de procesado.

Las reacciones electroquímicas más probables de los componentes del producto procesado en los electrodos son claras. Puesto que el componente mayoritario es el agua, los principales productos probables son el hidrógeno y/o el oxígeno. La obvia complejidad de los alimentos procesados no permite conocer si alguna de las especies presentes presentará una mayor o menor tendencia a la reducción u oxidación. No obstante, los estudios teóricos que han analizado este problema concluyen que los tratamientos mediante PEAIC bipolares o incluso los tratamientos monopolares de frecuencia baja evitan este problema. Se ha observado experimentalmente la aparición de especies químicas nuevas pero cuantitativamente no son mayores que las que se obtienen al tratar los productos mediante calor [162, 148].

En cuanto a las reacciones electroquímicas que implican la disolución de las partes metálicas de las cámaras de tratamiento, se ha demostrado en soluciones acuosas y en zumo de naranja que la buena selección de materiales de las cámaras, un correcto diseño y selección de los parámetros de procesado garantiza una larga vida útil de las mismas y, en consecuencia, una baja disolución de los metales que contienen. Este proceso de disolución es tan bajo que los niveles de hierro, níquel, cromo y manganeso, los materiales mayoritarios del acero 316, que es el material habitualmente usado en la industria alimentaria, se encuentran muy por debajo de los límites autorizados para el agua de consumo humano [162, 67, 220, 221].

1.6. Uso de la tecnología PEAIC

Del mismo modo que se han realizado estudios comparativos de los efectos del procesado de productos mediante PEAIC y calor, también se ha estudiado la viabilidad económica del mismo [107]. Se concluye que la inversión en el equipo PEAIC es superior y el coste energético del funcionamiento del equipo ligeramente mayor al no poder aprovechar el calor generado por el equipo [107, 51]. Es en la limpieza, mantenimiento y reparaciones del equipo donde los costes son mucho menores y compensan con creces la inversión inicial.

En la actualidad se están distribuyendo equipos de procesado industriales por parte de empresas relacionadas con la investigación en este campo en los Estados Unidos de América [43, 52] y en Alemania [247, 85]. En Estados Unidos se puede usar la tecnología de los tratamientos mediante PEIAC desde que lo aprobase la Food and Drug Administration (FDA) en 1996. En ese país algunos productos ya se han comercializado siendo la principal baza comercial la superior calidad del producto puesto que al no haber sido sometido a tratamientos térmicos sus

Figura 1.6: Zumos comerciales procesados mediante PEAIC por Genesis Juice Corp. (Eugene, Oregon, USA) desde 2006 (imagen tomada del artículo [43]).

propiedades organolépticas y nutritivas no han disminuido (Fig. 1.6). En Europa aún se esta pendiente de una legislación clara puesto que la actual sobre nuevos productos alimentarios es ambigua en cuanto al uso de esta tecnología [247, 188].

Hay autores que creen que uno de los motivos que esta retrasando la implementación industrial de esta tecnología es precisamente este vacío legislativo. Aun así, la investigación y el desarrollo en este área no deja de crecer. De continuar los resultados positivos, las posibilidades de que termine siendo aceptada como alternativa o complemento a los tratamientos térmicos también aumenta [238].

2 Objetivos

El propósito inicial del presente trabajo fue el estudio de la viabilidad técnica de la aplicación de los pulsos eléctricos de alta intensidad de campo (PEAIC) para procesar y conservar el zumo de uva de forma que mantenga las características del producto fresco. Para la consecución de esta finalidad los objetivos pormenorizados planteados fueron el estudio:

- del efecto de los tratamientos mediante PEAIC sobre la población microbiana habitual del zumo de uva y determinación de las mejores condiciones para el procesado,
- del efecto de los tratamientos mediante PEAIC sobre los principales enzimas oxidantes presentes en el zumo de uva y determinación de las mejores condiciones para el procesado,
- de la evolución de una selección de compuestos indicativos de calidad durante la vinificación y envejecimiento del vino procedente de zumo procesado mediante PEAIC, y
- de las repercusiones fisicoquímicas, microbiológicas, sensoriales y nutricionales del zumo de uva natural de distintas variedades procesado mediante PEAIC y su comparación con un tratamiento térmico conservante tradicional.

3 Métodos y material

3.1. Fases del estudio

El estudio se realizó en fases coincidentes con cada uno de los objetivos planteados. Es decir:

Fase 1 Determinación de las condiciones óptimas de procesado mediante PEAIC para la eliminación de los microorganismos mayoritarios presentes en zumo de uva variedad Parellada.

Fase 2 Determinación de las mejores condiciones de procesado mediante PEAIC para la eliminación de las actividades enzimáticas oxidantes más relevantes presentes en zumo de uva variedad Parellada.

Fase 3 Análisis de la evolución del proceso de vinificación de zumo de uva variedad Parellada procesado mediante PEAIC condicionado por la presencia o ausencia de dióxido de azufre.

Fase 4 Determinación y análisis de los efectos del procesado mediante PEAIC en las características fisicoquímicas, microbiológicas, enológicas, nutricionales y sensoriales de distintas variedades de zumo de uva en comparación con un tratamiento térmico típico. Se utilizaron zumos de las variedades Parellada, como mosto blanco, Moscatell como mosto aromático y Mazuelo como mosto tinto.

Los métodos y materiales descritos en este capítulo son una recopilación resumida de la descripción más detallada que se encuentra en aquellos capítulos en que fueron utilizados.

3.2. Diseño experimental

3.2.1. Fase 1 y 2

El alcance de los objetivos planteados las fases 1 y 2 debían conducir a las mejores condiciones de tratamiento mediante PEAIC para la eliminación de microorganismos y enzimas perniciosos para el zumo de uva. En consecuencia, se uso una herramienta estadística dirigida a la optimización de procesos como es la *superficie de respuesta* [124, 167, 171]. Esta se aplicó a los parámetros de procesado puesto que los otros dos factores, objetivo y producto, descritos en la sección 1.4 están claramente definidos.

Se escogió, pues, optimizar la intensidad de campo eléctrico (E), el tiempo de tratamiento (t_t) y la frecuencia de procesado (F) de la que no existe mucha información. La anchura de pulso (τ) se fijó en un valor medio entre los descritos en la literatura, 4 µs en la fase 1 µs y 5 µs en la fase 2. En cuanto al diseño del equipo y las cámaras de tratamiento estuvieron definidas dado el equipo disponible (Sec. 3.7.2).

El rango de valores de las variables en estudio fue seleccionado de entre los citados en la bibliografía y de acuerdo con la experiencia en este tipo de procesado en el equipo. Así, los valores del campo eléctrico se fijaron entre $20\,\mathrm{kV{\cdot}cm^{-1}} \leq E \leq 35\,\mathrm{kV{\cdot}cm^{-1}}$, la frecuencia de los pulsos entre $100\,\mathrm{Hz} \leq F \leq 1000\,\mathrm{Hz}$ y el tiempo de tratamiento máximo fue de 1 ms y 5 ms en la fase 1 y 2 respectivamente.

De los posibles diseños se escogió el diseño conocido como *diseño central compuesto de Box-Wilson* o más brevemente *diseño central compuesto (CCD)* en su variante centrada en las caras [171]. Los principales motivos fueron que permite tratamientos en el rango de trabajo del equipo PEAIC y que reduce el número de experimentos puesto que sólo se necesitan tres niveles para cada factor. Como tercer valor para cada factor se escogió un valor central en el rango de valores citados en el párrafo anterior. El modelo estadístico de este diseño se expresa en la siguiente ecuación:

$$S = k_0 + k_1 \cdot E + k_2 \cdot F + k_3 \cdot t_t + k_4 \cdot E^2 + k_5 \cdot F^2 + k_6 \cdot {t_t}^2 + \\ + k_7 \cdot E \cdot F + k_8 \cdot E \cdot t_t + k_9 \cdot F \cdot t_t + \varepsilon\,, \quad (3.1)$$

donde S representa cualquiera de las medidas obtenidas, k_i son los coeficientes desconocidos de la ecuación y ε el error experimental.

3.2.2. Fase 3

En la fase 3 se realizaron entre dos y cuatro repeticiones de los tratamientos PEAIC para poder realizar comparaciones entre los resultados. El tratamiento mediante PEAIC consistió en pulsos cuadrados de polaridad alterna de 4 µs de anchura y una amplitud de 10.2 kV para conseguir en las cámaras un campo eléctrico de $35\,\mathrm{kV{\cdot}cm^{-1}}$ (Ec. (3.8)). La frecuencia de los pulsos fue de 1 kHz durante 1 ms.

3.2.3. Fase 4

En esta fase se utilizó un diseño en bloques que permite el estudio de la variable *tratamiento* considerando otra variable con una supuesta variabilidad intrínseca como la *variedad* del zumo de uva tratado. El hecho de realizar repeticiones de los tratamientos para cada variedad permite además, obtener información de la posible influencia de la variedad en el tratamiento del zumo de uva, es decir, de la interacción de las dos variables. El modelo estadístico genérico de este diseño

viene dado por la siguiente ecuación:

$$Y_{ijk} = P_i + V_j + (P \cdot V)_{ij} + \epsilon_{ijk} \tag{3.2}$$

donde

Y representa el conjunto de datos de cada variable medida,

P es la variable tipo de tratamiento. Fue considerado como un factor fijo con i-niveles

1. zumo no procesado (NT),
2. zumo tratado mediante PEAIC (PEF), y
3. zumo procesado térmicamente (TT),

V es la variable variedad, considerada un factor aleatorio con j-representantes

1. Mazuelo,
2. Moscatel, y
3. Parellada

que se supone que es independiente y sigue una distribución normal con media cero y varianza σ_V^2,

$P \cdot V$ es la interacción entre las dos variables y, consecuentemente, otro factor aleatorio con las mismas propiedades que el anterior. Sus términos reflejan las posibles desviaciones de los tratamientos según la variedad. Se ha supuesto como simplificación que estas desviaciones de producirse eran idénticas con un valor σ_{PV}^2, y finalmente,

ϵ es el error asociado con las medidas experimentales. Es también una variable aleatoria supuestamente independiente, idéntica y normalmente distribuida con una media cero y una varianza σ^2.

En los casos en que no se trataron distintas variedades el modelo aplicado fue el mismo pero con el primer (P_i) y último términos únicamente (ϵ_{ik}).

3.3. Análisis de los resultados

Los datos obtenidos en la fase 1 y 2 se ajustaron al modelo (3.1) mediante el método de mínimos cuadrados. El ajuste se verificó mediante el análisis de los residuos y fueron validados con nuevos experimentos en el rango de condiciones tratadas. Se utilizó la correlación lineal (r) entre los valores calculados y medidos como parámetro para medir la calidad del ajuste considerando mejores las previsiones cuanto mayor era su valor.

Con la finalidad de comparar predicciones proporcionadas por distintos modelos, los datos de actividad enzimática residual relativa (RA) de la fase 2 se

ajustaron a modelos de primer orden para cada valor de intensidad de campo eléctrico y frecuencia según las ecuaciones:

$$\frac{RA}{RA_0} = e^{-k_{\mathrm{PPO}} \cdot t_t} \qquad \text{para PPO y} \tag{3.3}$$

$$\frac{RA - RA_\infty}{RA_0 - RA_\infty} = e^{-k_{\mathrm{POD}} \cdot t_t} \qquad \text{para POD.} \tag{3.4}$$

Los valores de las constantes fueron obtenidos mediante métodos iterativos de regresión no lineal (algoritmo de Marquardt) tomando como valores iniciales los obtenidos en la literatura [87, 89, 151]. Se escogió el valor del coeficiente de determinación (R_A^2) como medida del ajuste de los modelos cinéticos de inactivación enzimática a los datos obtenidos de forma que cuanto más cercano a la unidad estuviese este valor mayor es el grado de similitud entre los datos y una cinética de inactivación de primer orden.

Las comparaciones entre resultados de zumos de uva tratados con PEIAC y los zumos de referencia en la fase 3 fueron analizados mediante el análisis de varianza (ANOVA) utilizando el método de mínimos cuadrados y las diferencias establecidas mediante test de comparación de medias.

Los resultados sobre las características fisicoquímicas, microbiológicas, enológicas, sensoriales y nutricionales obtenidos en la fase 4 fueron comparadas mediante ANOVA [53]. El modelo estadístico mixto (Ec. (3.2)) se ajustó mediante el método de probabilidad máxima restringida (*restricted maximum likelihood, REML*) o probabilidad máxima (*maximum likelihood, ML*). En esta ocasión la determinación de diferencias entre tipos de tratamiento se realizó mediante la selección de los contrastes adecuados [191]. Cuando no se ajustó un modelo mixto el proceso seguido fue el mismo que el utilizado en la fase 3.

3.4. Preparación de la muestra

3.4.1. Fase 1

Se utilizó zumo de uva variedad Parellada clarificado y pasteurizado. Fue adquirido a un productor vitivinícola (Baixas Lehnberg CSP, Santes Creus, Tarragona) y procedía del mismo lote de fabricación.

Dicho zumo de uva fue inoculado con las especies microbianas, tanto de levaduras como de bacterias, más comunes presentes en los zumos de uva de acuerdo con la experiencia de los colaboradores y la bibliografía [113]:

S. cerevisiae levadura procedente de la cepa P29 de la colección vinícola de levaduras del Instituto Catalán de la Viña y el Vino (INCAVI), Villafranca del Penedès, Barcelona.

Kloeckera apiculata también conocida como *Hansenioaspora uvarum*, levadura 11105 de la colección española de cultivos tipo de la Universidad de Valencia, Valencia.

Lactobacillus plantarum bacteria procedente de la cepa C11 de la colección vinícola del INCAVI, Villafranca del Penedès, Barcelona.

Lactobacillus hilgardii bacteria número 4786 de la colección española de cultivos tipo de la Universidad de Valencia, Valencia.

Gluconobacter oxydans bacteria número 1408 del Laboratorium voor Microbiologie, Universiteit de Gent, Gent, Bélgica.

El proceso de inoculación fue el mismo para cada uno de los microorganismos. Consistió en obtener una población elevada de cada especie, aclimatarlas al mosto y posteriormente unirlas para obtener poblaciones microbianas similares en todas las repeticiones realizadas.

3.4.2. Fase 2

En esta fase sólo se utilizó la variedad Parellada. Las uvas fueron proporcionadas por un productor vitivinícola local (Raimat, Lleida). Los racimos fueron seleccionados aleatoriamente del campo en el momento en que estaban siendo recogidos para su vinificación. El mismo día fueron lavados con agua potable, secados, envasados en bolsas de polietileno hasta aproximadamente 1 kg de peso y congelados a −20 °C hasta su procesado.

Para el procesado mediante PEAIC, se descongelaron, durante 24 horas a 5 °C, las bolsas necesarias en función del volumen a tratar previsto. Posteriormente las uvas se prensaron y filtraron a través de un tamiz de acero inoxidable de 1 mm de diámetro de poro. Se eliminó el aire disuelto agitando el mosto mientras se provocaba el vació mediante una bomba de agua durante 15 min. El zumo se procesó en alícuotas de entre 50 mL y 100 mL en función de los análisis a realizar.

3.4.3. Fase 3

En esta fase sólo se utilizó la variedad Parellada, adquirida y preparada según lo descrito en la fase anterior siendo la única diferencia que se procesó en lotes de 1 L.

3.4.4. Fase 4

Agricultores de la denominación de origen Penedès vendimiaron y transportaron uvas de las variedades *Mazuelo* (Cariñena), *Moscatel* (Moscatel de Alejandría o Moscatell) y *Parellada* a la planta del INCAVI en Vilafranca del Penedès donde fueron procesadas hasta la obtención del zumo escurrido y destartarizado. Posteriormente el zumo de uva fue transportado a las instalaciones de la Universitat de Lleida (UdL) donde se realizó el procesado en lotes de como máximo 1 L.

Varios racimos de cada variedad fueron conservados a 5 °C para ser procesados del mismo modo que en la fase 1 con el fin de evitar la natural reducción de la actividad enzimática una vez los granos se han roto.

3.5. Procesado de la muestra

3.5.1. Tratamientos conservantes

Los equipos de tratamiento mediante PEAIC y térmico fueron acondicionados previamente a su uso (Sec. 3.7.2). En las fases 1, 3 y 4 se recirculó una disolución de hipoclorito sódico (NaClO) al 10 % durante 5 min, seguido por un enjuagado con agua esterilizada. En la fase 2 el acondicionamiento consistió en la circulación de hidróxido sódico (NaOH) al 20 % a 40 °C durante 5 min y posterior enjuagado con agua des-ionizada. El punto final del enjuagado fue determinado con papel de pH.

Las condiciones de tratamiento fueron las correspondientes a las indicadas por el diseño experimental. En el procesado térmico y mediante PEAIC la temperatura de los baños calefactores y refrigerantes fue medida mediante termómetros de inmersión.

Además se controlaron mediante un osciloscopio digital el voltaje e intensidad de corriente eléctrica entre electrodos, la forma y anchura del pulso y la frecuencia de tratamiento. La temperatura del producto se midió a la entrada y salida de cada par de cámaras de tratamiento con termopares (tipo T).

Durante el procesado térmico, se tomo como valor de temperatura de tratamiento el valor del agua en que estaba sumergido el intercambiador de calor. Dado el diseño del equipo, el tiempo de tratamiento (t_t) del procesado térmico coincide con el tiempo de residencia (t_r) en el intercambiador de calor. Éste se calcula a partir de su volumen (vol_c) y del caudal volumétrico del producto (q) proporcionado por la bomba peristáltica según la fórmula:

$$t_r = \frac{vol_c}{q} = t_t. \tag{3.5}$$

Este valor es una fracción del tiempo de procesado (t_p) de todo el volumen de producto a tratar (vol_s) medido como

$$t_p = \frac{vol_s}{q}\,, \tag{3.6}$$

y como el caudal es el mismo resulta que se puede obtener una relación entre el valor del tiempo de tratamiento y el tiempo de procesado según la ecuación:

$$t_t = \frac{vol_c}{vol_s} \cdot t_p. \tag{3.7}$$

En el tratamiento mediante PEAIC la intensidad de campo eléctrico (E) se calculó a partir de la diferencia de potencial entre electrodos (V) y la distancia

entre ambos (d) (Ec. (3.8)). Experimentos previos permitieron conocer la diferencia de potencial necesaria para conseguir la intensidad de campo eléctrico para el mosto dada su conductividad eléctrica. Por el diseño del equipo la dirección de este vector $\vec{E}$ coincidió con el flujo de producto mientras que su sentido fue alternando en cada cámara de tratamiento. Por el mismo motivo todas las cámaras poseían el mismo valor de campo eléctrico dado por la expresión

$$E = \frac{V}{d}. \tag{3.8}$$

En este caso el tiempo de procesado de la muestra también viene dado por la ecuación (3.6). Como en el equipo anterior es posible obtener una relación entre el tiempo de procesado de la muestra y su tiempo de tratamiento pero con la diferencia de que el tiempo de tratamiento, definido por la anchura de los pulsos (τ) y el número de pulsos (n) según la ecuación

$$t_t = \tau \cdot n \tag{3.9}$$

no coincide con el tiempo de residencia (3.5). La relación entre ambos viene dada por el cálculo del número de pulsos mediante la fórmula

$$n = F \cdot t_r\,, \tag{3.10}$$

y uniéndolo todo se obtiene como expresión final

$$t_t = \tau \cdot F \cdot \frac{vol_c}{vol_s} \cdot t_p\,, \tag{3.11}$$

muy similar a la ecuación (3.7) y que fueron las que se utilizaron para contabilizar el tiempo de tratamiento.

Después del procesado en cualquiera de los equipo los restos de producto eran eliminados mediante un enjuague con agua des-ionizada. Finalmente, el equipo fue limpiado con la circulación de hidróxido sódico (NaOH) al 20 % a 40 °C durante 5 min y posterior enjuagado con agua des-ionizada. El punto final del enjuagado fue determinado con papel de pH.

3.5.2. Vinificación

Durante la fase 3 fue necesario obtener vino a partir del zumo procesado. Cada repetición se realizó con 3 L de zumo de uva.

A las alícuotas destinadas a la vinificación tradicional se les añadió metabisulfito potásico ($K_2S_2O_5$) hasta llegar a una concentración de 20 mg·L^{-1} de dióxido de azufre. También se adicionó fosfato diamónico ($(NH_4)_2HPO_4$) hasta que el contenido en nitrógeno del mosto fue aproximadamente de 55 mg·L^{-1}. Posteriormente se inoculó la cepa Na33 de *S. cerevisiae* seleccionada de la Estación de Viticultura y Enología de Navarra (Olite) en una proporción de 0.2 g·L^{-1}. El cultivo con más de 2×10^9 cfu·mL^{-1} se obtuvo después de hidratar 0.65 g de

extracto de la levadura seco con 7.5 mL de agua conteniendo 70 mg de sacarosa y agitar durante 30 min a 35 °C.

La fermentación tuvo lugar en matraces de doble boca de 3.5 L de capacidad sellados mediante un séptum y una trampa de dióxido de carbono. Los matraces fueron agitados magnéticamente y mantenidos a una temperatura constante de 18 °C mediante un incubador. Después de la fermentación, en caso de ser preciso, el vino fue clarificado por decantación a −4 °C durante 48 h, envasado, sellado con tapones de corcho y mantenido a temperatura controlada ambiental o 5 °C.

3.6. Determinaciones analíticas

Los equipos y materiales utilizados se describen en la sección 3.7.1.

3.6.1. Parámetros físicos y químicos

Se agrupan en esta sección aquellos parámetros que se utilizan típicamente para la caracterización de zumos. Se realizaron como mínimo dos medidas de

acidez total medida en moles de hidróxido sódico necesarios para obtener pH = 8.1 por litro de zumo (M) según el procedimiento descrito en la legislación [154]. Se utilizo el equipo de medida de pH para detectar el punto final de la valoración ácido-base,

conductividad eléctrica medida a 20 °C y en reposo con un conductímetro. En algunos casos se midió entre 5 °C y 50 °C para evaluar los efectos de su variación durante los tratamientos PEAIC,

densidad medida siguiendo la metodología descrita en la legislación vigente excepto en que el picnómetro utilizado fue de 10 mL debido a la poca cantidad de muestra disponible [152],

grados brix medidos según la legislación vigente [152],

pH medido en unidades de pH según la legislación actual [152],

3.6.2. Parámetros bioquímicos

Durante la fase 2 y 4 se realizaron al menos dos medidas de

actividad polifenoloxidasa (PPO) obtenida gracias a su reacción con el substrato que fue seguida mediante la evolución de absorbancia durante 2 min. La enzima fue purificada con algunas modificaciones respecto al método descrito [131, 255].

La pendiente del tramo recto inicial fue tomada como la velocidad de reacción de la polifenoloxidasa y fue asociada a su actividad enzimática medida como la variación de unidades de absorbancia (±0.001) por minuto

y mililitro A. Para cada serie de medidas se realizaron determinaciones en blanco que fueron sustraídas del valor de las mediciones de absorbancia. El resultado se expresó como actividad residual relativa RA calculado mediante la expresión:

$$RA = \frac{A}{A_0} \tag{3.12}$$

actividad peroxidasa (POD) también medida siguiendo la reacción con su substrato, peróxido de hidrógeno con algunas modificaciones respecto al método original [195]. Los resultados fueron expresados en actividad residual relativa según la ecuación (3.12).

3.6.3. Parámetros enológicos

Se agrupan en esta sección aquellos parámetros considerados de utilidad en el proceso de vinificación. Se realizaron como mínimo dos medidas de

azúcares reductores a partir del índice de refracción y medidas enzimáticas mediante un analizador multiparamétrico,

amonio medido mediante reacciones enzimáticas y colorimétricas con un analizador multiparamétrico,

ácido acético medido mediante reacciones enzimáticas y colorimétricas con un analizador multiparamétrico,

acetaldehido a partir de medidas enzimáticas y colorimétricas mediante un analizador multiparamétrico,

ácidos hidroxicinámicos mediante la medida de la absorbancia a 320 nm de una muestra de zumo de uva [272],

ácidos grasos mediante su separación y análisis de fragmentos en un cromatógrafo de gases con detector de espectrometría de masas tras extraer la parte lipídica de los zumos con cloroformo y aumentar su volatilidad transformándolos en ésteres metílicos según el Código Alimentario Español [198]. Se usaron undecanoato y heptadecanoato de metilo disueltos en hexano como patrones internos.

aminoácidos libres mediante la separación por HPLC y detección ultravioleta tras la clarificación de la muestra y adición de L-norleucina y sulfona de L-metionina como patrones internos.

alcohol etílico determinado a partir del punto de ebullición mediante un ebullómetro,

catequinas libres mediante el índice DMAC, un modo indirecto de conocer el contenido en taninos condensados. El método se basa en la medida de la absorbancia a 640 nm de una muestra de zumo de uva tras una reacción de polimerización [168].

compuestos volátiles el análisis se realizó mediante cromatografía de gases usando métodos distintos en función de la volatilidad y abundancia de las especies químicas.

dióxido de azufre analizado mediante un analizador multiparamétrico,

polifenoles que se midieron de 3 modos distintos dependiendo del equipo que realizase la medición

- a partir de mediciones colorimétricas mediante un analizador multiparamétrico.
- según un método colorimétrico basado en la reacción de los fenoles con el reactivo de Folin-Ciocalteu en presencia de carbonato de sodio (Na_2CO_3) [236, 237].
- mediante la medición de la absorbancia a 280 nm de una muestra de zumo de uva ultra filtrada [91].

3.6.4. Parámetros microbiológicos

En general, cada muestra se analizó por duplicado. Durante la fase 1 y 4 las muestras procesadas se diluyeron en disoluciones decimales con solución Ringer. Cada dilución fue filtrada a través de filtros de nitrocelulosa (Whatman International Ltd, Maidstone, Inglaterra) de 0.45 µm de diámetro de poro para recoger los microorganismos. Los filtros fueron depositados en placas con medios de crecimiento y atmósferas selectivas.

Durante la fase 3 se determinó el recuento total de microorganismos aerobios mesófilos según la norma ISO 4833 [115] y de mohos y levaduras según la norma ISO 7954 [114].

Los resultados de las muestras procesadas y sin procesar se expresaron a partir de la fracción sobreviviente (S) o de la población (P) según las expresiones:

$$S = \log\left(\frac{N}{N_0}\right) \tag{3.13}$$

$$P = \log N \tag{3.14}$$

donde N y N_0 es el número de unidades formadoras de colonias de las placas que contenían entre 25 y 250 unidades multiplicado por la dilución decimal correspondiente a dichas placas.

3.6.5. Parámetros nutricionales

Se agrupan en esta sección aquellas características de los zumos consideradas habitualmente como relacionadas con la nutrición. Así, se realizaron medidas de

capacidad antioxidante analizada mediante la reducción de la absorbancia de una disolución metanólica del radical 2,2-difenil-1-(2,4,6-trinitrofenil)hidrazilo (DPPH$^{\cdot}$). El método se basa en que al descomponerse el radical DPPH$^{\cdot}$ en presencia de compuestos antioxidantes la coloración de su disolución alcohólica disminuye [38, 245]. La capacidad antioxidante expresada en porcentaje se calculo según la siguiente ecuación:

$$CA = \left(1 - \frac{\mathrm{DPPH}^{\cdot}{}_{s}}{\mathrm{DPPH}^{\cdot}{}_{c}}\right) \times 100. \tag{3.15}$$

contenido en proteínas determinada utilizando el método de Bradford [37, 185, 259]. Este método se fundamenta en la unión de las proteínas con un colorante denominado azul brillante de Coomassie G-250 que pasa de color rojo en estado libre a azul en estado combinado. Las absorbancias fueron comparadas con las obtenidas al realizar el mismo análisis utilizando proteína de suero bobino (BSA) hasta $150\,\mathrm{mg{\cdot}L^{-1}}$.

vitamina C analizada siguiendo mediante la reducción de todas la formas vitamínicas a ácido dehidroascórbico mediante la reacción con 2,3-dimercapto-1-propanol (BAL) y acido metafosfórico [241]. Esta disolución se separa mediante un equipo HPLC-UV y se cuantifica la cantidad de ácido dehidroascórbico por comparación con patrones.

3.7. Materiales y equipos

Los materiales y reactivos utilizados fueron adquiridos en los proveedores habituales del Departamento de tecnología de los alimentos como Scharlau, S.L., Sigma-Aldrich, Across entre otros descritos detalladamente en los capítulos correspondientes.

3.7.1. de análisis

Los equipos utilizados para el análisis de los distintos parámetros medidos durante la realización de los experimentos fueron

agitador de tubos TK3S (Kartell Spa., Noviglio, Italia),

agitador magnético Ikamag RTC Basic (Millian SA, Geneve, Switzerland),

analizador multiparamétrico Enochem (Tecnología Difusión Ibérica, Barcelona). Equipo que permite el análisis de varias características de mostos y vinos basándose en medidas enzimáticas y espectrofotométricas,

centrifuga AVANTI™ J-25 (Beckman Instruments Inc., Fullerton, CA, USA) y Selecta (J.P. Selecta, Barcelona),

colorímetro Macbeth Colour-Eye 3000 (Bacbeth-Kollmorgen Inst. Corp., Newburg, NY, USA),

conductímetro Testo 240 (Testo AG, Lenzkirch, Alemania),

columna cromatográfica de gases la columna de separación fue una DB-WAX de 30 m de longitud, 0.25 mm de diametro interno y de 0.25 µm de espesor de polietilenglicol (Cromlab, Barcelona)

columna cromatográfica de líquidos una columna de fase inversa PicoTag de 300 mm y 3.9 mm de diámetro interno conteniendo polidimetiloctadecilsililo como fase estacionaria unida a sílice amorfa,

cubetas espectrofotométricas de 1 cm de recorrido óptico de plástico (Kartell Spa., Noviglio, Italia), de cristal 100-OS y cuarzo 100-QS (Hellma, Hellma Hispania, S.L., Badalona),

ebullómetro Dujardin-Salleron (París, Francia),

equipo de cromatografía de gases (GC) Shimadzu GC-14B (Shimadzu, Kyoto, Japan) con un detector de ionización de llama (FID),

equipo GC-MS equipo formado por la combinación de un cromatógrafo de gases y un detector de masas Finnigan (San Jose, CA, USA) y Shimadzu QP 5000 con inyector AOC-20i (Shimadzu, Kyoto, Japón),

espectrofotómetro UVA-Vis CE 2021 (Cecil Instruments Ltd., Cambridge, England),

homogeneizador Ultra-Turrax T25 (Rose Scientific, Edmonton, Canadá),

incubador J.P. Selecta (Barcelona),

indice de refracción ABBE modelo 325 (Misco, Cleveland, OH, USA),

Membranas de ultrafiltración Ultrafree MC (Millipore, Ballerica, MA, USA).

pH-metro Crison 2000 (Crison, S.A., Barcelona) y Metrohm 702 (Metrohm, Erisau, Alemania) con un electrodo para medida de pH,

refractómetro Atago RX-1000 (Atago Co. Ltd, Tokyo, Japón) con corrección de temperatura,

sistema de extracción en fase sólida Vac Elut 20 station (Varian, CA, USA) con cartuchos de extracción en fase sólida de 3 cm^3 de volumen rellenos de 200 mg de resina LiChrolut EN (Merck, Darmstadt, Alemania),

sistema HPLC-UV formado por dos bombas modelo 510, un inyector automático 717 Plus Autosampler, un detector de matriz de diodos (PAD) 996 (Waters, Milford, MA, USA) y una precolumna de derivatización de fenilisotiocianato (Pierce Biotechnology, Rockford, IL, USA).

3.7.2. de procesado

Prensa hidraulica Vaslin-Bucher X Pro 5 (Chalonnes S/Loire, Francia).

Equipo PEAIC consta de un generador de pulsos de alto voltaje (1 kV–12 kV) OSU-4F (Ohio State University, Columbus, OH, USA), un disparador o interruptor 9412A (1 Hz–2000 Hz) (Quantum Composers Inc., Bozeman, MT, USA), ocho cámaras de tratamiento del tipo *co-field* (Sec. 1.5) de 12 mL^3 y 2.9 mm de separación entre electrodos mediante plástico Delrin® (Fig. 3.1). Las propias cámaras y el sistema de tuberías del equipo PEAIC son de acero inoxidable lo que permite la formación de serpentines de refrigeración cada par de cámaras. Existen sondas de temperatura tipo termopar cada par de cámaras. Este equipo puede desarrollar una potencia máxima de 2 kW con pulsos rectangulares mono o bipolares de hasta 10 µs.

El equipo permite el procesado en continuo de productos líquidos al estar unido mediante tubos de silicona a una bomba peristáltica 75210-25 (Cole Palmer Inc., Vernon Hills, IL, USA) con un caudal de agua máximo de aproximadamente 8 $cm^3 \cdot s^{-1}$.

Osciloscopio digital THS720 que permite el control en continuo de la forma, la polaridad, la amplitud, la anchura y frecuencia de los pulsos de voltaje además la forma, el sentido y la amplitud de la corriente eléctrica residual (Tektronix Inc., Beaverton, OR, USA).

Equipo de tratamiento térmico consta de una bomba peristáltica, modelo D-21V (Dinko, Barcelona), que impulsa el producto a través de un sistema de tuberías compuesto de dos secciones (Fig. 3.2). La primera sección se introduce en el baño termostatado a la temperatura deseada. La segunda sección se introduce en un baño de agua y hielo permitiendo la rápida refrigeración del producto tratado. Las tuberías son de acero inoxidable tipo 304 de 0.085″ de diámetro interno y 0.125″ de diámetro externo (Resteck stainless steel ref: 21513, Teknokroma S. Coop C. Ltda, Sant Cugat del Vallès). El tiempo de tratamiento viene condicionado por el caudal proporcionado por la bomba que fue de 40 $mL \cdot min^{-1}$.

Figura 3.1: Vista general del equipo de procesado mediante PEAIC.

Figura 3.2: Vista general del equipo de procesado térmico.

4 Optimising the inactivation of grape juice spoilage organisms by pulse electric fields

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Abstract

The effect of some pulsed electric field (PEF) processing parameters (electric field strength, pulse frequency and treatment time), on a mixture of microorganisms (*K. apiculata*, *S. cerevisiae*, *L. plantarum*, *L. hilgardii* and *G. oxydans*) typically present in grape juice and wine were evaluated. An experimental design based on response surface methodology (RSM) was used and results were also compared with those of a factorially designed experiment. The relationship between the levels of inactivation of microorganisms and the energy applied to the grape juice was analysed. Yeast and bacteria were inactivated by the PEF treatments, with reductions that ranged from 2.24 to 3.94 logarithmic units. All PEF parameters affected microbial inactivation. Optimal inactivation of the mixture of spoilage microorganisms was predicted by the RSM models at 35.0 $kV \cdot cm^{-1}$ with 303 Hz pulse width for 1 ms. Inactivation was greater for yeasts than for bacteria, as was predicted by the RSM. The maximum efficacy of the PEF treatment for inactivation of microorganisms in grape juice was observed around 1500 $MJ \cdot L^{-1}$ for all the microorganisms investigated. The RSM could be used in the fruit juice industry to optimise the inactivation of spoilage microorganisms by PEF.

4.1. Introduction

The food industry aims to satisfy consumer expectations of products with unmodified natural taste and nutritional values that are microbiologically safe. New processing technologies such as high intensity pulsed electric fields (PEF) have been developed in response to these requirements. A lot of research has dealt with the effects of PEF on microbes in water, or models solutions with various physical, chemical and biochemical properties, resulting in information on the fundamentals of this technology [223, 112, 257]. However, a necessary

step to promote industrial application of this technology is experimentation on real foods such as fruit juices.

Studies of microbial responses to PEF treatments of juices have been carried out with various juices including orange juice [59, 58], apple juice [95, 78], carrot-orange juice mix [216, 232] and berry juice [96]. In all the reported work the degree of inactivation microorganisms by PEF varied with the species and strain, the juice, the design of the PEF device and the processing parameters. However, there is little information about the effect of PEF treatments on microorganism that spoil grape juice, although it is one of the most widely traded juice commodities.

Jaya [117] and Wu [262] applied PEF to grape juice, but without reporting the species that were studied. Other studies investigated the effects of PEF treatments on oxidative enzymes of grape juice [141], its nutritive components [82], and on specific sensory characteristics of wine obtained from PEF processed grape juice [84, 83].

The aim of the study reported in this paper was to obtain reliable data about the behaviour of typical flora (yeasts and bacteria) under PEF processing conditions similar to those likely to be used in the industry. Microbial inactivation data were used to obtain a predictive model for each microorganism investigated, which after an optimisation process should allow the identification of the best PEF processing parameters to reduce the content of spoilage microorganisms in grape juice.

4.2. Materials and methods

4.2.1. Experimental designs

The response variable (S) in experimental designs was calculated according to the formula:

$$S = -\log_{10}\left(\frac{N}{N_0}\right), \tag{4.1}$$

where N and N_0 are the number of viable cells of each microorganism on treated and untreated juice samples, respectively. Therefore, all the results were positive numers, and the higher the P-value the more effective the PEF treatment because more microorganisms were inactivated.

Response surface design

A central composite design was used to evaluate the effect of each factor on the response analysed, and to develop a mathematical relationship between the response and the independent factors [124, 167]. This relationship allows estimation of the optimum response and the values of the factors needed to achieve such a response. Table 4.1 shows the experimental design with 20 experiments randomly distributed among factorial, star and central points.

Treatment time (t), electric field (E), and pulse frequency (F) were taken as the continuous independent factors to be optimised. The experimental design required three values of each factor under study so, 0, 0.500 ms and 1.000 ms were selected as the treatment time values, 20.0 $kV \cdot cm^{-1}$, 27.5 $kV \cdot cm^{-1}$ and 35.0 $kV \cdot cm^{-1}$ were chosen for the electric field strength values, and 100 Hz, 200 Hz and 600 Hz were selected as the pulse frequencies. The highest values correspond to the upper values for the operations conditions that could be achieved in previous studies [82, 141]. The minimum values were chosen to cover a wide range of experimental conditions. The central values were arbitrarily chosen to look for non-linear behaviour [86].

Factorial design

The predictions obtained with RSM were compared with the results of a factorial set of experiments using electric field strengths of 20.0 $kV \cdot cm^{-1}$, 25.0 $kV \cdot cm^{-1}$, 27.5 $kV \cdot cm^{-1}$, 30.0 $kV \cdot cm^{-1}$ and 35.0 $kV \cdot cm^{-1}$ and pulse frequencies of 100 Hz, 200 Hz and 600 Hz. Microbial populations were measured after 0, 0.125 ms, 0.250 ms, 0.500 ms, 0.750 ms and 1.000 ms treatment times using the same equipment and analytical methods. Four grape juice batches were used and the microorganisms were inoculated from the same stock cultures as in the previous experimental design.

The same data allowed study of the responses of each microbial species subjected to PEF treatments. A possible relationship between the energy per volume unit delivered to grape juice and the inactivation of the microorganisms was also investigated.

4.2.2. Sample preparation

Pasteurised grape juice (*Vitis vinifera* variety Parellada) was supplied by a local wine maker (Baixas Lehnberg SCP, Santes Creus, Tarragona, Spain). Microorganisms selected as type specimens of the most common yeast and bacterial species occurring in grape juice [113] were used to inoculate the juice. These organisms were *S. cerevisiae* strain P29 (yeast wine collection, Instituto Catalán de la Viña y el Vino (INCAVI), Vilafranca del Penedès, Barcelona, Spain); *K. apiculata* (*H. uvarum*) CECT 11105 (Spanish Type Culture Collection, Universidad de Valencia, Valencia, Spain); *L. plantarum* strain C11 (INCAVI); *L. hilgardii* CECT 4786 (Universidad de Valencia) and *G. oxydans* LMG 1408 (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium).

The initial culture of each microorganism was propagated in three steps. The stock culture of each species was inoculated in 10 mL of broth. When the microbial population reached the stationary phase, 10 mL of fresh broth were added. After again reaching the stationary phase, 80 mL of grape juice were added to obtain 100 mL of microbial culture. Microbial growth was monitored from the absorbance measured at 600 nm. The five 100 mL inocula were mixed to-

gether into 500 mL of grape juice to obtain a final volume of 1 L. The numbers of each microorganism ranged between 1×10^6 cfu·mL^{-1} and 1×10^7 cfu·mL^{-1}. Finally, 50 mL aliquots were PEF treated according to the experimental designs. Microbial culture manipulations were performed under aseptic conditions.

G. oxydans was grown at 28 °C, under aerobic conditions, in a broth containing 0.5% yeast extract, 0.3% peptone and 2.5% mannitol. Lactic acid bacteria were grown at 30 °C, under an aerobic atmosphere containing 5% carbon dioxide, in *Oenococcus oeni* medium of pH = 4.8, mixed with 30% tomato juice. *K. apiculata* and *S. cerevisiae* were grown at 30 °C, on an aerobic atmosphere with 5% carbon dioxide in a broth containing 1% yeast extract, 2% peptone and 2% glucose. Obtaining the inocula for the grape juice preparation took 7, 6, 5 and 3 days, respectively.

Yeast extract, mannitol and *O. oeni* medium were provided by Scharlau Chemie, S.A., Barcelona, Spain. Glucose and soy peptone were provided by Panreac Quimica, S.A., Barcelona, Spain. Tomato juice was obtained from tomatoes, which were purchased in a local market, choped and pressed through a sieve of 1 mm diameter. Presurised carbon dioxide was obtained from Abelló Linde, S.A., Barcelona, Spain.

4.2.3. Assessment of viable microbial populations

Serial decimal dilutions the PEF treated and untreated samples were prepared with Ringer solution. Each dilution was filtered through nitrocellulose filters (Whatman International Ltd, Maidstone, England) of 0.45 µm pore diameter to collect the microorganisms, and the filters were placed on plates of the appropriate selective growing environment.

G. oxydans were incubated in an aerobic atmosphere at 28 °C for seven days, on agar made of glucose (5%), yeast extract (2%), nystatin (50 mg·L^{-1}), penicillin G (3 unit·mL^{-1}) and pH = 4.5. Lactic acid bacteria were incubated under an anaerobic atmosphere at 30 °C for five days, on a 70% *O. oeni* agar of pH = 4.8 suplemented with 30% tomato juice, nystatin at 50 mg·L^{-1} and chloramphenicol at 500 mg·L^{-1}. *K. apiculata* was grown in an aerobic atmosphere containing 5% carbon dioxyde at 30 °C for four days on peptone (2%), glucose (2%), yeast extract (1%), cycloheximide (50 mg·L^{-1}), chloramphenicol (500 mg·L^{-1}) agar. *S. cerevisiae* was incubated in an aerobic atmosphere containing 5% carbon dioxyde at 30 °C for seven days, on peptone (2%), glucose (2%), yeast extract (1%), chloramphenicol (500 mg·L^{-1}), ethyl alcohol (13% v/v) agar.

Chloramphenicol, cycloheximide, nystatin and penicillin G were purchase from Sigma-Aldrich Inc. (Stinheim, Germany). Ethanol was obtained from Panreac Quimica, SA.

4.2.4. Grape juice characterisation

Parellada grape juice was characterised by analysing the soluble solid content (degrees brix), density ($g \cdot mL^{-1}$), pH and total acidity measured as the volume of NaOH 0.1 M (Scharlau) required to reach pH=8.1 per litre of grape juice. The analyses were performed following the Spanish regulation about grape juice [155]. A digital refractometer (Atago RX-1000, Atago Co. Ltd, Tokyo, Japan) with temperature correction and a precision of ± 0.1% was used to measure the soluble solid content of grape juice. Density was assessed as the ratio between the weight (±0.1 mg) of 10 mL of grape juice contained in a pycnometer (±0.1 mL, Afora, S.A., Barcelona, Spain). Total acidity and pH were measured using a pH-meter (Crison 2000, Crison, S.A., Barcelona, Spain) with a precision of ±0.02 pH units. The measurements of density, pH and acidity were carried out at 20 °C. In addition, electrical conductivity of grape juice at temperatures from 10 °C to 45 °C was determined with a conductivity meter (Testo 240, Testo AG, Lenzirch, Germany) with a precision of $\pm 1\ \mu S \cdot cm^{-1}$. A mathematical relationship between the electrical conductivity and the temperature was obtained using linear regression. For all the analyses, six samples of untrated juice were used and each measurement was made in triplicate.

4.2.5. PEF equipment and treatments

An OSU-4F bench-scale continuous unit manufactured by Ohio State University (Columbus, OH, USA) was used to treat the grape juice samples [141]. Eight co-field chambers, each with a volume of $12\ mm^3$ and a gap distance of 2.9 mm between electrodes were connected in series. Four cooling coils were connected before and after each pair of chambers and submerged in a water-ice mixture to maintain the grape juice temperature below 35 °C. The device was sterilised before each treatment by circulating a 10% sodium hypochlorite (Scharlau) solution throught it for 3 min, followed by rinsing with a continuous flow of sterile water for 10 min.

Temperatures were recorded by thermocouples (T type, ±0.1 °C) located before and after each pair of treatment chambers. Pulse waveform, voltage, intensity, pulse width and pulse frequency delivered in the treatment chambers were recorded using a digital oscilloscope (Tektronix THS720A, Tektronix, OR, USA) to check the accuracy of the equipment with a precision of ±reading×3%. Measured temperature and electrical values were recorded in a computer. The flow rate was set with a peristaltic pump (Millipore, Bedford, MA, USA) calibrated with grape juice before each use.

Grape juice was processed with pulses of 5 µs in bipolar mode, with the values of treatment time, electric field strength and pulse frequency of the experimental designs. Grape juice temperature at the inlet of the first chamber was kept at 15 °C. The maximum temperature of 30.4 °C was reached at the last pair of treatment chambers with the treatment that delivered the maximum electrical

power to juice circulating at a flow rate of $3.33\,\mathrm{mL{\cdot}s^{-1}}$.

The PEF treatment time of grape juice samples (t_t, ms) were calculated as the product of the average pulse width (τ, ms) and the number of pulses (n delivered in the treatment chambers. That is:

$$t_t = \tau \cdot n \tag{4.2}$$

The number of pulses can be obtained from the pulse frequency (F, Hz) and the average residence time (t_r, s) of the sample in the treatment chambers as:

$$n = F \cdot t_r\,, \tag{4.3}$$

and the residence time is calculated as the relationship between the volumetric capacity of the treatment chambers (vol_c, mL) and the average flow rate (q, $\mathrm{mL{\cdot}s^{-1}}$):

$$t_r = \frac{vol_c}{q} \tag{4.4}$$

The pulse voltage difference (V, kV) between electrodes divided by the electrode gap (d, cm) of the treatment chambers was taken as the electric field strength value (E, $\mathrm{kV{\cdot}cm^{-1}}$):

$$E = \frac{V}{d} \tag{4.5}$$

The energy per volume unit, also known as the energy density (E_d, $\mathrm{MJ{\cdot}L^{-1}}$) applied to the grape juice was calculated using the equation [1, 224]:

$$E_d = 10^{-6} \cdot E^2 \cdot \sigma \cdot t_t\,, \tag{4.6}$$

were E ($\mathrm{V{\cdot}m^{-1}}$) is the electric field strength, σ ($\mathrm{S{\cdot}m^{-1}}$) is the electrical conductivity of the sample and t_t (s) is the treatment time.

4.2.6. Data analysis

All data were organised using an electronic spreadsheet (OpenOffice.org Calc, OpenOffice 2.0, Sun Microsystems Inc. Santa Clara, CA, USA). All statistical assessments and tests with a 95% confidence level, numerical optimisations and graphic preparations were performed using a statistical software application [206].

Response surface design

A polynomial model was used to fit the experimental S-values of each microorganism according to the least squares method (Eq. (4.7)). The terms of the polynomial model come from a Taylor development up to second order of the investigated factors [124, 167]:

$$\begin{aligned} S = k_0 + k_1 \cdot E + k_2 \cdot F + k_3 \cdot t_t + k_4 \cdot E^2 + k_5 \cdot F^2 + k_6 \cdot {t_t}^2 + \\ + k_7 \cdot E \cdot F + k_8 \cdot E \cdot t_t + k_9 \cdot F \cdot t_t + \varepsilon \end{aligned} \tag{4.7}$$

where k_i are the coefficients of the polynomial equation with the corresponding units to obtain a dimensionless value of the response (S); E, F and t_t are the continuous factors under study; and ϵ is the experimental error.

The least squares method provided the coefficients of the polynomial models and a F-test was used to discriminate the terms of the model that produced statistically significant changes of the response variables. The presence of outliers and influential data was checked for, by means of the studentised residuals and the Cook's distance, respectively. The goodness of fit and linearity of the fitted model, the independence, normal distribution and constant variance of the residuals, which are the common assumptions in Normal distributions, were analysed using residual plots [70].

The response surface equation for each microorganism was optimised to obtain the highest value of S. After this, the optimum treatment conditions for maximum inactivation of all the microorganisms in the mixture wer identified.

Factorial design

The predictions of the equations obtained from the response surface analysis were compared with the results obtained from the factorial design experiments, to assess the accuracy of the predictions. This was performed for each microorganism calculating the linear correlation (r) between both data sets. Moreover, the factorial data set allowed study of the realionship between the S-values and the type of microorganism, to confirm the S-values obtained with the response surface methodology. The statistical model used was:

$$S = \mu + m + \varepsilon\,, \tag{4.8}$$

were μ is the average S-value if the type of microorganism does not affect the microbial populations after PEF processing, m is the species of microorganism, and ϵ is the random variation of the experimental S-values. Significant differences were assessed using the Tukey honest difference test.

Finally, a graphical distribution of the S-values from the factorial data set against the energy density delivered to the grape juice samples was proposed. The electric conductivity values of equation (4.6) were achieved using the experimental electrical conductivity values for the grape juice at the highest temperature reached during each experiment.

4.3. Results

The general characteristics of Parellada grape juice were soluble solid content, (14.9 ± 0.3) brix; density, $(1.060 \pm 0.002)\,\mathrm{kg{\cdot}L^{-1}}$; pH, (3.79 ± 0.06) , and acidity, $(3.7 \pm 0.3) \times 10^{-3}\,\mathrm{M}$ of NaOH. Its electrical conductivity (σ, $\mathrm{S{\cdot}m^{-1}}$) was given by the expression:

$$\sigma = (0.12 \pm 0.01) + (6.0 \pm 0.5) \cdot 10^{-3} \times T\,, \tag{4.9}$$

Table 4.1: Response surface design and experimental results[a].

Experiment	E^b	F^c	${t_t}^d$	$S_{K.\ apiculata}{}^e$	$S_{S.\ cerevisiae}{}^e$	$S_{\text{L.bacteria}}{}^e$	$S_{G.\ oxydans}{}^e$
1	27.5	200	0.500	3.69	3.79	1.98	0.41
2	27.5	200	1.000	3.88	3.90	2.82	0.37
3	35.0	600	0	0.00	0.00	0.00	0.00
4	35.0	600	1.000	2.57	3.28	2.90	2.24
5	20.0	600	0	0.00	0.00	0.00	0.00
6	20.0	600	1.000	3.22	3.24	0.00	0.00
7	35.0	100	0	0.00	0.00	0.00	0.00
8	35.0	100	1.000	3.36	3.90	3.54	1.20
9	27.5	100	0	0.00	0.00	0.00	0.00
10	27.5	100	0.500	2.74	3.63	0.66	0.55
11	20.0	100	0	0.00	0.00	0.00	0.00
12	20.0	100	1.000	3.08	3.94	0.00	0.07
13	20.0	200	0.500	2.98	2.93	0.28	0.07
14	35.0	200	0.500	3.06	3.68	2.86	0.96
15	27.5	600	0.500	2.49	2.68	1.97	1.38
16	27.5	200	0.500	3.00	2.88	2.67	0.01
17	27.5	200	0.500	3.01	3.25	1.20	0.21
18	27.5	200	0.500	3.22	2.54	2.52	0.01
19	27.5	200	0.500	2.67	2.79	1.96	0.20
20	27.5	200	0.500	3.00	2.68	1.55	0.00

[a] Average of three measurements.
[b] Electric field strength in $\text{kV}\cdot\text{cm}^{-1}$.
[c] Pulse frequency in Hz.
[d] Treatment time in ms.
[e] Inactivation level in logarithmic units.

where T (°C) is the grape juice temperature. The positive slope value indicates a gradual increase of grape juice electric conductivity within the range of temperatures found in the study.

The highest S-value for each microorganism were 3.88, 3.94, 3.54 and 2.24-log reductions for *K. apiculata*, *S. cerevisiae*, the mixture of lactic acid bacteria and *G. oxydans*, respectively (Tab. 4.1). There were experiments that yielded a null response ($S = 0$) as a consequence of its definition (Eq. (4.1)). The least squares method applied to data of table 4.2 provided estimates of the unknown parameters of the statistical models. Table 4.2 also shows which factors affected the measured response of each microorganism.

With *K. apiculata* the S-values were affected by the treatment time and the pulse frequency (t_t, F, ${t_t}^2$ and F^2). The significance of the squared factors suggests that the trend of their influence was not linear. The response surface (Fig. 4.1) displays graphically the effects of these processing parameters. The significance (p-values) of the k_i-estimates of *S. cerevisiae* show that it was affected by only the treatment time (t_t and ${t_t}^2$). Again, the statistical significance of the squared factor showed a non linear effect of the treatment time (Fig. 4.1).

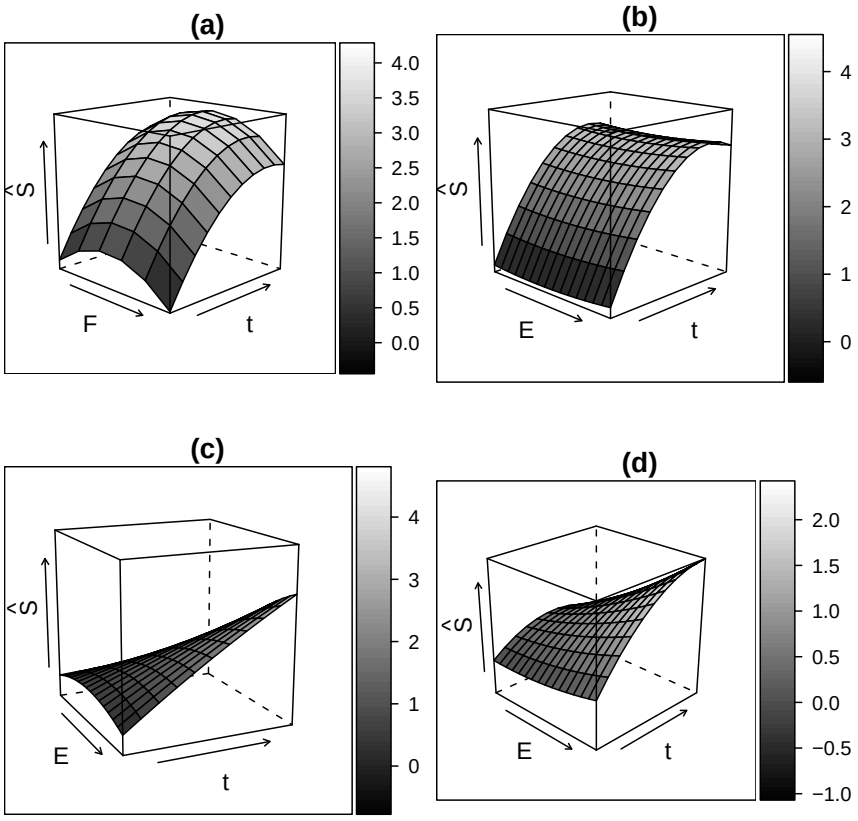

Figure 4.1: Response surface plot of (a) *K. apiculata*, (b) *S. cerevisiae*, (c) Lactic acid bacteria and (d) *G. oxydans*.

The inactivation of bacteria was affected by the values of the three factors studied. Pulse frequency by itself and treatment time and electric field strength as a part of an intereaction term had statistically significant effects (Tab. 4.2). The effect of pulse frequency on lactic acid bacteria was very close to statistical significance. The presence of significant interaction between the two factors $E \times t_t$ with both species of lactic and acetic acid bacteria makes it difficult to drawn general conclusions from the data, since information about the degree of inactivation of each microorganism would depend on the specific values of the factors in the interaction term. This can be seen from figure 4.1 in which the response surfaces for bacteria are more complex than these for yeasts.

The percentages of P-variability accounted for by each developed model as well as their maximum random errors are also shown in table 4.2. The mathematical models explain more than 90% of the data variability with a maximum error of 0.53-log for lactic acid bacteria. After constructing the models for prediction of the degree of inactivation with a specific PEF treatment, the accuracies of the predictions were determined. The linear correlations between predicted and measured data sets were 0.89, 0.97, 0.49 and 0.52 for *K. apiculata*, *S. cerevisiae*, lactic acid bacteria and *G. oxydans*. The high r-values for yeast indicate that their models gave good predictions for S-values.

The ANOVA analysis of the factorial data set indicated that the average S-values after PEF processing depended on the organisms that was studied. A Tukey honest difference test showed that the S-values for the organisms were significantly different from each other except for those for the yeasts ($S_{K.\ apiculata} = 2.0 \pm 0.3$, $S_{S.\ cerevisiae} = 2.2 \pm 0.3$). There was greater inactivation of lactic acid bacteria ($S = 0.9 \pm 0.3$) than of acetic acid bacteria ($S = 0.6 \pm 0.2$) although the difference was small. This independent analysis confirms that yeast are more sensitive to PEF than bacteria as the response surfaces indicated.

After the models for prediction of the population of individual organisms remaining following application of specific PEF treatment were constructed the optimum treatment conditions for inactivation of as many microorganisms as possible was calculated. The optimisation process indicated that 4.0 ± 0.8 logarithmic reductions of *K. apiculata* population could be reached with a PEF treatment of $24.9\,\mathrm{kV{\cdot}cm^{-1}}$ at 330 Hz for 0.921 ms. The best treatment conditions for *S. cerevisiae* were $35\,\mathrm{kV{\cdot}cm^{-1}}$ at 100 Hz and 0.878 ms. The statistical model predicted a reduction of 4 ± 1 logarithmic units for this treatment. Lactic acid bacteria numbers would be reduced by 4 ± 2-log units using $35.0\,\mathrm{kV{\cdot}cm^{-1}}$, at 333 Hz for 1 ms, and *G. oxydans* numbers would be reduced by 1.1 ± 0.8 logarithmic units with a treatement of $27.5\,\mathrm{kV{\cdot}cm^{-1}}$, at 600 Hz and 1 ms. If all the microorganisms were together in the juice, the optimum treatment would be $35.0\,\mathrm{kV{\cdot}cm^{-1}}$, at 303 Hz for 1 ms, which would give reductions of 3.8 ± 0.9, 4 ± 1, 4 ± 2 and 0.9 ± 0.8 logarithmic units for *K. apiculata*, *S. cerevisiae*, lactic acid bacteria and *G. oxydans*, respectively.

Figure 4.2 shows the S-values obtained for each microorganism versus the energy delivered to the grape juice. Inactivation of all the microorganisms is

Table 4.2: Estimated parameters[a] of the model[b] fitted to experimental data.

	K. apiculata		*S. cerevisiae*		L.bacteria		*G. oxydans*	
Parameter	Estimate	p-value	Estimate	p-value	Estimate	p-value	Estimate	p-value
k_0	-2 ± 5	0.215	1 ± 7	0.724	-7 ± 9	0.118	3 ± 4	0.218
k_1 ($\mathrm{cm \cdot kV^{-1}}$)	$(1 \pm 3) \cdot 10^{-1}$	0.426	$(-0.9 \pm 5) \cdot 10^{-1}$	0.712	$(4 \pm 7) \cdot 10^{-1}$	0.218	$(-1 \pm 3) \cdot 10^{-1}$	0.421
k_2 ($\mathrm{Hz^{-1}}$)	$(1 \pm 0.8) \cdot 10^{-2}$	0.014[c]	$(-0.2 \pm 1) \cdot 10^{-2}$	0.754	$(2 \pm 2) \cdot 10^{-2}$	0.048[c]	$(-1.1 \pm 0.8) \cdot 10^{-2}$	0.011[c]
k_3 ($\mathrm{ms^{-1}}$)	7 ± 2	< 0.001[c]	9 ± 4	< 0.001[c]	-3 ± 5	0.134	-0.6 ± 2	0.561
k_4 ($\mathrm{cm^2 \cdot kV^{-2}}$)	$(-2 \pm 6) \cdot 10^{-3}$	0.507	$(2 \pm 9) \cdot 10^{-3}$	0.660	$(-0.7 \pm 1) \cdot 10^{-2}$	0.252	$(-2 \pm 6) \cdot 10^{-3}$	0.497
k_5 ($\mathrm{Hz^{-2}}$)	$(-1 \pm 1) \cdot 10^{-5}$	0.021[c]	$(0.3 \pm 2) \cdot 10^{-5}$	0.690	$(-2 \pm 2) \cdot 10^{-5}$	0.065	$(1 \pm 1) \cdot 10^{-5}$	0.015[c]
k_6 ($\mathrm{ms^{-2}}$)	4 ± 2	< 0.001[c]	-5 ± 2	< 0.001[c]	-0.5 ± 3	0.754	-2 ± 2	0.025[c]
k_7 ($\mathrm{cm \cdot kV^{-1} \cdot Hz^{-1}}$)	$(-0.6 \pm 1) \cdot 10^{-4}$	0.224	$(-0.2 \pm 2) \cdot 10^{-4}$	0.815	$(-0.7 \pm 2) \cdot 10^{-4}$	0.492	$(0.7 \pm 1) \cdot 10^{-4}$	0.182
k_8 ($\mathrm{cm \cdot kV^{-1} \cdot ms^{-1}}$)	$(-1 \pm 6) \cdot 10^{-2}$	0.634	$(0 \pm 8) \cdot 10^{-2}$	1.000	$(2 \pm 1) \cdot 10^{-1}$	0.002[c]	$(11 \pm 5) \cdot 10^{-2}$	< 0.001[c]
k_9 ($\mathrm{Hz^{-1} \cdot ms^{-1}}$)	$(-0.8 \pm 2) \cdot 10^{-3}$	0.303	$(-1 \pm 2) \cdot 10^{-3}$	0.216	$(-0.8 \pm 3) \cdot 10^{-3}$	0.573	$(1 \pm 2) \cdot 10^{-3}$	0.199
R^2	0.9809		0.9635		0.9075		0.9047	
Adjusted-R^2	0.9637		0.9307		0.8243		0.8189	
ε[d]	0.27		0.40		0.53		0.26	

[a] Expressed as a value and its 95% confidence interval.
[b] $S = k_0 + k_1 \cdot E + k_2 \cdot F + k_3 \cdot t_t + k_4 \cdot E^2 + k_5 \cdot F^2 + k_6 \cdot {t_t}^2 + k_7 \cdot E \cdot F + k_8 \cdot E \cdot t_t + k_9 \cdot F \cdot t_t + \varepsilon$.
[c] $p \leq 0.05$.
[d] Random error.

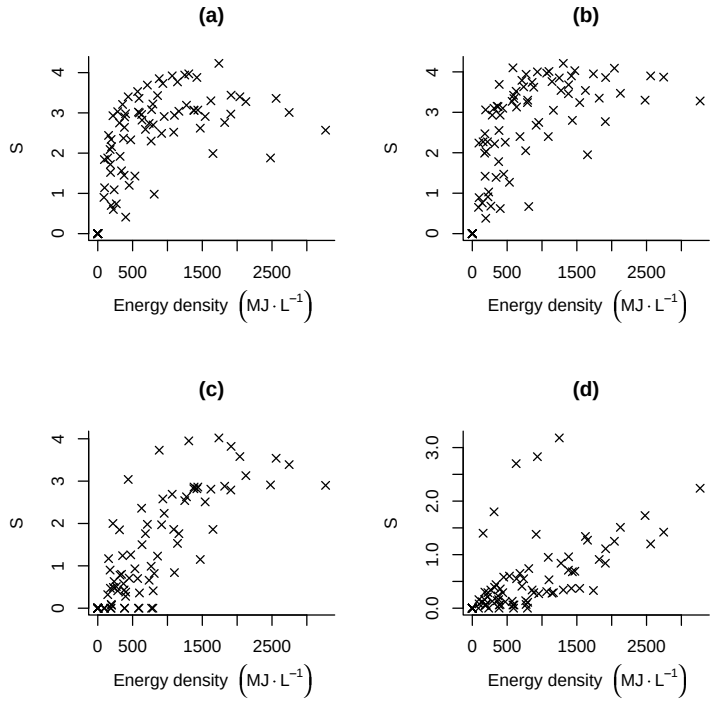

Figure 4.2: Effect of PEF energy density on (a) *K. apiculata*, (b) *S. cerevisiae*, (c) Lactic acid bacteria and (d) *G. oxydans*.

maximum around 1500 $MJ \cdot L^{-1}$. Moreover, all inactivation patterns, except that for *G. oxydans*, seem to be similar with the degree of inactivation increasing rapidly with increasing energy density at first, then decreasing slowly as the energy density increases beyond the point of maximum inactivation.

4.4. Discussion

The reductions obtained in the current study are similar those reported by other researchers who applied PEF to grape juice. In a study of the effect of PEF processing on the free amino acid and fatty acid contents of Parellada grape juice, *S. cerevisiae* was used as the target microorganism for confirmation of the effectiveness of the treatment [82]. Four logarithmic reductions for yeast populations were obtained. The treatment conditions and equipment were similar to those used in our study. Wu [262] achieved a reduction of spoilage flora of up to 4.2 logarithms using PEF at 50 °C, and they achieved higher reductions when they combined PEF with the addition of various antimicrobials. The same authors confirmed that below 44 °C there was no evidence of lethal thermal effects of PEF treatments on microorganisms. In all the experiments carried out in the

current study the temperature was below 31 °C, so any lethal effects of heating during these treatments can be discounted. Jaya [117] reported final bacterial counts of 400 cfu·mL^{-1} and complete inactivation of the yeasts in grape juice. The authors did not report the initial microbial load or the species present in the juice, so comparison of the findings of that and the current work is difficult.

Although there has been no report of the effects of PEF on specific bacteria in grape juice, information of that sort is avalaible for other juices. Elez [58] obtained a 5.8 logarithmic reduction of *Lactobacillus brevis* suspended in orange juice. Rodrigo [216] and Sampedro [224] obtained 2.5 logarithmic reductions of *L. plantarum* in an orange-carrot juice and an orange juice-milk mixture, respectively. Both used similar PEF conditions with electric field strengths around 35 kV·ms^{-1} and pulses of between 2.5 µs and 5.0 µs. They concluded that electric field strength and treatment time were the most important parameters affecting the survival of the microorganisms. The disparity of data probably reflects the numerous differences between the conditions used in the various studies.

In our study, the electric field strength had no effect upon yeast populations, although all previous work on PEF indicates that is a key parameter of this technology [48]. This discrepancy could be explained by the difference of PEF sensitivity of yeast and bacteria. The critical electric fields strength needed for any effect on bacteria is reported to be between 15 kV·cm^{-1} and 20 kV·cm^{-1} [100]. Most of the experiments performed at this level in our study gave a null response for bacterial populations. However, the reported lethal threshold value for yeast, specifically for *S. cerevisiae*, is around 7.5 kV·cm^{-1} [267, 159]. It is then possible that at 20 kV·cm^{-1}, which was the lowest level used in the current study, yeasts can be so severely damaged that any further increase of electric field strength would not enhance the lethal effects of PEF treatments.

Analysis of the results also gave information about how microorganism respond to pulse frequency. Some authors have claimed that frequency do not have any biological effect [230]. However, pulse frequency allows modulation of the way the electrical energy is applied to food being processed, so it could affect the efficacy of PEF treatments. In thermal processing, heat transfer into a medium depends only on the medium's heat transmission properties, whereas with PEF the rate at which energy is delivered to the product can be selected. The higher the pulse frequency, the higher the rate of energy input. Moreover, a high alternating pulse polarity migth stress microbial membranes which would have to adapt to a continuously changing external electric field.

K. apiculata was maximaly inactivated at intermediate pulse frequencies whereas low and high frequencies reduced the effectiveness of the treatments. Elez [59] reported decreased inactivation of *S. cerevisiae* in orange juice as the pulse frequency of the applied PEF treatments increased. In our study, the pulse frequency of the treatments also affected the inactivation of *G. oxydans*. The effect on the lactic acid bacteria was not so evident and more experiments are needed to clarify the situation with these organisms. Elez [58] reported a decrease

of PEF effects on *L. brevis* as pulse frequency increased. More information is needed for explanation of these discrepancies.

The factorial data set showed that the mathematical models that were developed could be used for accurate prediction of yeast but not bacterial S-values. Consequently, the optimum treatments identified from the models would be more appropiate for yeast than for bacteria.

The species most commonly found in grape juice are *K. apiculata*, *S. cerevisiae*, lactic acid bacteria, that are mainly *L. plantarum* and *L. hilgardii*, and acetic acid bacteria that are mainly *G. oxydans*. Typical populations contain yeasts in the range $(1 \times 10^4$–$1 \times 10^6)\,\mathrm{cfu{\cdot}mL^{-1}}$, lactic acid bacteria $(1 \times 10^2$–$1 \times 10^4)\,\mathrm{cfu{\cdot}mL^{-1}}$, and acetic acid bacteria at about $1 \times 10^6\,\mathrm{cfu{\cdot}mL^{-1}}$ [105, 213]. When comparing these values with the model predictions, it is evident that the optimised PEF treatment of $35.0\,\mathrm{kV{\cdot}cm^{-1}}$ with pulses of 303 Hz for 1 ms can reduce microbial populations although not enough for adequate pasteurisation. In wine making, a PEF treatment of such characteristics could greatly reduce the natural flora without the adverse effects on juice quality of heating [82]. The treated juice could be then inoculated with a selected yeast strain.

The optimisation process aimed at identifying the best treatment for reducing microbial populations. The response of microbial populations to the energy density applied to grape juice seemed to have the same trend for all the microorganisms. Other authors [224] have studied the effects of energy density, but only at low energy ranges and with only single microorganisms, which precludes useful comparison of their findings with ours.

Evidently the natural flora of grape juice can be greatly reduced using PEF treatments. Increasing the treatment time increased the level of microbial inactivation; and the electric field strength and pulse frequency should be carefully selected for each microorganism. The response surface methodology seemed to be suitable tool for defining optimum PEF treatment conditions for real food commodities.

5 Optimization of PEF processing conditions to inactivate oxidative enzymes of grape juice

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Abstract

White grape juice was processed using high intensity pulsed electric fields (PEF). The effect of this preservative technology on polyphenoloxidase (PPO) and peroxidase (POD) as well as on refractometric index, sugar content, pH, acidity and density was studied. In addition, the use of the response surface methodology as a tool to obtain accurate information and provide predictions of enzymatic depletion on real foods was assessed.

The studied PEF factors were electric field strength, pulse frequency, pulse width and total treatment time. Inactivation values of 100% for PPO and up to 50% for POD were achieved after different PEF treatments. The activity depletion was strongly dependent on the evaluated factors, mainly PEF treatment time. A predictive equation for each residual activity covering the whole range of experimentation was developed and their results were confirmed against a set of validation experiments. In addition, predictions obtained from the developed response surfaces and exponential kinetic models were comparable.

In conclusion, PEF treatments depleted PPO and POD activities of grape juice although it was observed that grape POD was less sensible than PPO to PEF technology. Response surface methodology allows analysing, optimizing the PEF treatments and predicting their results with very few experiments.

5.1. Introduction

Electricity has been studied as a preservative technology in food processing since beginning of the previous century [25], though lately the research has been increased due to the motivating demand of high quality products by consumers. The research subject has been focused on food microbiology overlooking the effect of PEF processing on enzyme activities. Nevertheless, it has been established that enzymes are less sensible to PEF than microbes and that both seem to have the same critical factors [63, 145, 146].

Electric field strength, treatment time, pulse frequency, pulse polarity or pulse shape are among the most important PEF operational parameters affecting microbial and enzymatic inactivation. The effects of these factors have been studied on several types of enzymes such as polyphenoloxidases, peroxidases and pectinmethylesterases of some fruits and vegetables [106, 88, 87, 89, 264, 56, 62, 271] as well as on several microbial enzymes [106, 29, 31, 32]. Some authors have reported the observed effects and others have tried to fit kinetic models to the data obtained as a way to deduce the enzymatic depletion mechanism. In any case, this has been usually performed in aqueous solutions or simulated media to avoid interferences.

However, it is generally accepted that the effectivity of PEF treatments varies depending on the enzyme surrounding environment and, as fas as we know, there is not information available on the effect of PEF technology on industrially unwanted enzymes of grape juice, which is one of the most internationally traded juices [23]. Examples of damaging enzymatic activities on grape juice are polyphenoloxidases (PPO) and peroxidases (POD), which are oxidoreductases involved in severe colour, flavour and taste modifications [149, 215, 105].

Thereby, the aim of this research was to study whether PPO and POD activities of grape juice are affected by PEF treatments and to assess the processing conditions in terms of treatment time, electric field strength, pulse frequency and pulse width to achieve the lowest PPO and POD residual activities of Parellada grape juice. In addition, the use of the response surface methodology allowed analysing, optimizing and making predictions of the process under study.

5.2. Materials and methods

5.2.1. Sample preparation

White grape bunches (*V. vinifera* cv. Parellada) were kindly supplied by a local wine making manufacturer (Raimat, Lleida, Spain). Once randomly harvested, they were washed with drinking water, drained, split off from bunches and frozen into polyethylene bags at −20 °C until processing within the following two months. Grape juice was obtained a few minutes prior PEF processing by pressing grapes thawed for 24 h at 5 °C. Squeezed grape juice was filtered using a stainless steel sieve with an approximate mesh of 1 mm and collected in an opaque bottle at 5 °C from which aliquots (100 mL) were taken to be processed with PEF.

PEF processing device

PEF treatments were performed using a high voltage generator (OSU-4F, Ohio State University, Columbus, OH, USA) and a pulse trigger (model 9412A, Quantum Composers, Inc., Bozeman, MT, USA). This equipment allows continuous grape juice processing (maximum 7.8 $cm^3 \cdot s^{-1}$) as it flows through eight

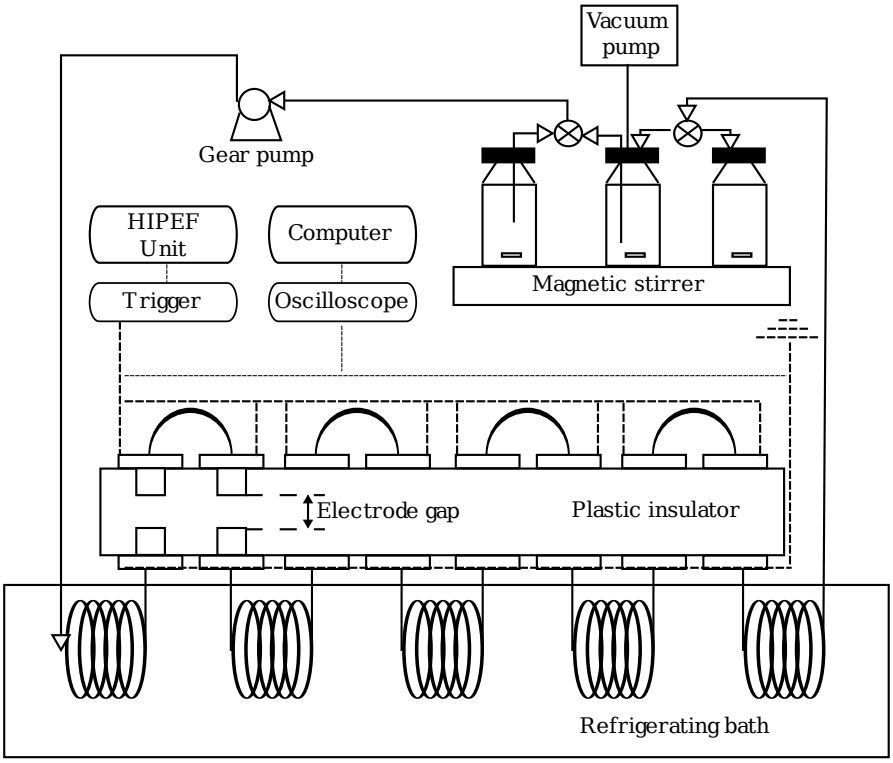

Figure 5.1: Sketch of the PEF equipment used at the University of Lleida.

colinear treatment chambers arranged in series (Fig. 5.1). Each treatment chamber has a volume of 0.012 cm^3 with a the distance between electrodes of 0.29 cm. Grape juice was refrigerated between each pair of chambers by passing it through to tubular stainless steal heat exchangers submerged in an iced water bath. The maximum grape juice temperature for all the treatments was kept between 35 °C to 40 °C combining the refrigerating system and the fluid flow between 1 $cm^3 \cdot s^{-1}$ and 1.5 $cm^3 \cdot s^{-1}$ with a gear pump (Cole Palmer Inc., Vernon Hills, IL, USA). Temperature was checked just before and after treatment chambers using temperature thermocouples (±0.1 °C) attached to the pipes and a mercury bulb thermometer (±0.25 °C) in the reservoir bottle. The temperature of grape juice at the inlet of treatment chambers and into the reservoir bottle was always between 12 °C and 15 °C.

This equipment allows grape juice processing using squared-wave pulses in bipolar mode. The maximum power of the device is 2 kW. The characteristics of the electric pulses delivered such as shape, polarity, width, difference of potential as well as the electric current generated across the electrodes and the pulse frequency were monitored using a digital oscilloscope (THS720, Tektronix

Inc., Beaverton, OR, USA) plugged into the treatment chamber sockets. Total treatment time was calculated as the product of the average pulse width and the average number of pulses delivered in all treatment chambers taking into account pulse frequency and resident time of each volume unit of grape juice

5.2.2. Grape juice characterisation

Characterization of fresh and processed Parellada grape juice was carried out taking aliquots of untreated and PEF treated juice of three randomly selected runs in each replication. The aliquots were analysed for soluble solids content, density, total acidity and pH following the Spanish regulation [155]. Soluble solids measured as degrees brix were determined using a digital refractometer with temperature correction (±0.1%, Atago RX-1000, Atago Co. Ltd., Tokyo, Japan). Density was obtained from the ratio between a weight of grape juice and its volume using a (10.0 ± 0.1) mL pycnometer. A pH-meter (Crison 2000, Crison S.A., Barcelona, Spain) allowed taking measures of pH (±0.02 pH units) and total acidity which was recorded as mol of NaOH 0.1 M needed to reach pH = 8.1 per litre of grape juice. Both analyses were carried out at 20 °C. The dependence of grape juice specific electric conductivity ($\pm 1\,\mu S \cdot cm^{-1}$) with temperature was analysed with a conductivity meter (Testo 240, Testo AG, Lenzkirch, Germany), heating non-processed grape juice from 5 °C to 50 °C.

Polyphenoloxidase activity determination

Polyphenoloxidase extraction and activity measurement were performed following a methodology described by Valero [253] with some modifications. Each aliquot of 10 mL of fresh and PEF treated grape juice was combined in a tube with 10 mL of sodium phosphate buffer obtained mixing monosodium phosphate (Riedel-de-Haën, Seelze, Germany) and disodium phosphate (Scharlau, Barcelona, Spain) at pH = 7.2 ± 0.1. This buffered solution also contained $12\,mmol \cdot L^{-1}$ of ascorbic acid (Prolabo, Fonteneau-Blois, France). The resulting suspension was separated at $24000 \times g$ for 10 min in a centrifuge (Avanti J-25, Beckman Coulter Inc., Fullerton, CA, USA). Afterwards, 5 mL of a sodium phosphate buffered suspension containing Triton® X-100 1.5% (w/v) (Prolabo), polyvinylpirrolidone (PVP) 2% (w/v) (Across Organics, Geel, Belgium) and calcium chloride 50 mM (Prolabo) was added to the pellet. After vigorously shaking for two minutes with a mixer (TK3S, Kartell Spa., Noviglio, Italy) the suspension was centrifuged again at $24000 \times g$ for 15 min. The liquid of the centrifuge tube was decanted and filtered through Whatman nº1 filter paper obtaining a transparent greenish solution which was taken as the enzyme extract. The full process was carried out keeping samples and solutions below 5 °C.

Afterwards, 0.05 mL of enzyme extract was added to a 1 cm optic path polystyrene cuvette (Kartell) which contained 2.45 mL of catechol $20\,mmol \cdot L^{-1}$

(Sigma-Aldrich, Germany) in a buffered McIlvine solution (pH = 4.75 ± 0.02) and mixed gently up to complete homogenization avoiding, though, foam formation. The reaction was conducted at 25 °C and the absorbance evolution was monitored at 400 nm nm of wavelength each 20 s for two minutes using an UV-visible spectrophotometer (±1% absorbance units, CE 2021, Cecil Instruments Ltd., Cambridge, England). The sample absorbance was referred to a blank sample which contained the same reactants and distilled water instead of enzyme extract. Polyphenoloxidase activity from enzyme extract was determined from the linear part of the absorbance-time curve. The variation of 0.001 absorbance units per minute and millilitre of enzymatic extract was defined as a PPO enzymatic activity unit. Each PPO spectrophotometric measurement was carried out twice and averaged.

5.2.3. Peroxidase activity determination

Peroxidase activity of Parellada grape juice was measured following the process reported by Poux [195] with some modifications. Each aliquot of 10 mL of fresh and PEF treated grape juice was added to a tube with 10 mL of sodium phosphate buffer (pH = 7.2 ± 0.1). After removing solid parts by centrifugation at $2460 \times g$ for 10 min, decantation and filtration through Whatman nº1 paper filter a greyish-brown liquid was obtained, which was taken as the enzymatic extract. The process was performed keeping samples and solutions below 5 mL.

Afterwards, 0.1 mL of enzymatic extract was added into a 1 cm optical path glass cuvette (100-OS, Hellma GmbH & Co KG., Müllheim, Germany) containing 2.7 mL of acetic acid (Riedel-de-Haën) and sodium acetate (Prolabo) solution buffered at pH = 5.4 ± 0.1 and 0.1 mL of 1,4-diphenilendiamine 0.20 mol·L^{-1} (Merck, Hoenbrunn, Germany). The reaction started when 0.1 mL of hydrogen peroxide 1.8 mol·L^{-1} (Merck) was added and homogenized. The temporal evolution of absorbance at 420 nm was recorded each 20 s for 2 min. The absorbance values were referred to a sample blank containing all reagents except for hydrogen peroxide which was substituted by distilled water. The initial linear slope of the absorbance-time curve was taken as the peroxidase activity and one unit of POD activity was defined as the variation of 0.001 absorbance units per minute and millilitre of enzymatic extract. Each POD spectrophotometric measurement was performed twice and averaged.

5.2.4. Experimental design

A face centred central composite design was used to determine the effect of electric field strength (E), pulse frequency (F) and treatment time (t_t) applied to Parellada grape juice as well as their optimum values to process grape juice with PEF technology. Three (maximum, minimum and central) values of each factor were considered what led to 16 experiments (Tab. 5.2). The experimental design was performed twice for each enzyme considering each replication a different

block to control the unknown effects of different experimental units. Assays within blocks were randomly carried out. At this stage, pulse width (τ), was kept constant at 4 µs in all experiments. The effect of pulse width on natural PPO and POD activities of Parellada grape juice was evaluated later using a set of experiments performed at the optimum values of electric field strength, treatment time and pulse frequency.

Three samples of 10 mL of fresh and treated grape juice were taken and analysed for PPO and POD activities after circulating through the PEF equipment for the same period of time. The residual activity of each enzyme relative to the untreated sample (Eq. (5.1)) was selected as the response variable of PEF treatments:

$$RA = \frac{A}{A_0} \tag{5.1}$$

where A_0 and A are the enzymatic activity averages of fresh and PEF processed grape juice samples respectively. Thus, RA-values are dimensionless figures ranging from 0, if no residual activity was measured after treatment, up to 1, when the measured residual activity after treatment corresponded to the original activity.

The design was analysed fitting a second order polynomial model to the observed data according to the response surface methodology [167]:

$$\begin{aligned} RA = k_0 + k_1 \cdot E + k_2 \cdot F + k_3 \cdot t_t + k_4 \cdot E^2 + k_5 \cdot F^2 + k_6 \cdot {t_t}^2 + \\ + k_7 \cdot E \cdot F + k_8 \cdot E \cdot t_t + k_9 \cdot F \cdot t_t + \varepsilon\,, \end{aligned} \tag{5.2}$$

where RA is the residual enzymatic activity of PPO or POD, E (kV·cm^{-1}) is the electric field strength, F (Hz) is the pulse frequency, t (ms) is the treatment time and ε the experimental error.

The regression coefficients ($k_0 - k_9$) were evaluated by the least squares method. The significance of the regressions and also the significance of their individual terms were determined using an analysis of variance ($\alpha = 0.05$). The coefficient of determination (R^2) provided information about the rate of the observed variance explained by the models. The adequacy of the fitted model was assessed by means of a lack of fit test ($\alpha = 0.05$) and an analysis of residuals which permitted confirming the validity of the assumptions regarding the independence and normal distribution of the errors. Predictive models were developed from the information of the analysis of variance taking only those terms that affected significantly the response variables and maintaining the hierarchy of the variables into the models.

An analysis of variance ($\alpha = 0.05$) was also carried out to assess the significance of pulse width effect on enzymatic behaviour and a Fisher-Newman-Keuls test was selected to compare the means of the different pulse widths.

5.2.5. Validation of the predictive polynomial equations

A second set of experiments was performed to validate the developed predictive equations. Fresh grape juice aliquots were randomly treated with electric pulses between 25 kV·cm^{-1} and 35 kV·cm^{-1} for treatment times ranging from 1 ms to 5 ms, at frequencies between 200 Hz and 1000 Hz using the procedure described previously. Each combination of electric field strength and pulse frequency values was tested twice using squared-wave pulses of 4 µs width in bipolar mode.

The correlation coefficient between the polynomial model predictions and the experimental data was taken as a measure of the prediction accuracy.

5.2.6. Kinetic models

A description of the behaviour of PPO and POD enzymatic activities with the PEF treatment time has usually been performed using first order kinetic models (Eqs. (5.3), (5.4)) such as:

$$\frac{RA}{RA_0} = e^{-k_{\mathrm{PPO}} \cdot t_{\mathrm{t}}} \tag{5.3}$$

$$\frac{RA - RA_\infty}{RA_0 - RA_\infty} = e^{-k_{\mathrm{POD}} \cdot t_{\mathrm{t}}} \,. \tag{5.4}$$

In these equations, RA_0 and RA are the residual enzymatic activities of fresh and processed grape juice whereas RA_∞ is the expected asymptotic value of the enzymatic activity, k_{PPO} and k_{POD} are constants, which provide information of the reaction rate (ms^{-1}), and t_{t} is the treatment time (ms).

A Marquardt algorithm was used to determine the parameters of the mathematical equations adjusted by non-linear regression. The starting values of the parameters to perform iterations were taken from literature [87, 89, 151]. The goodness of adjust was assessed by the adjusted coefficient of determination (R_A^2) along with an analysis of residuals.

5.3. Results and discussion

5.3.1. Grape juice characterization

As can be observed in table 5.1, the values of some properties of PEF processed grape juice were not statistically different from those obtained from fresh grape juice, thus meaning that any physicochemical analysis would not let discern between both types of juice. A similar behaviour of soluble solids content, total acidity and pH has been described when apple juice was processed with PEF [274]. The reduced size of the processed samples did not allow performing sensory tests to check differences between both fresh and PEF treated juices.

The electric conductivity is an important property that should be kept in mind when foods are treated with any electrical device because it determines

Table 5.1: Physicochemical properties of fres and PEF-processed Parellada grape juice[a].

Measured parameter	Fresh grape juice	Processed grape juice
Refractometric index (brix)	13.7 ± 0.4	13.7 ± 0.3
Sugar content ($g \cdot L^{-1}$)[b]	121.8	121.8
pH	3.81 ± 0.06	3.80 ± 0.06
Total acidity ($mmol \cdot L^{-1}$)	3.5 ± 0.4	3.5 ± 0.4
Density ($kg \cdot L^{-1}$)	1.054 ± 0.005	1.055 ± 0.005

[a] Average of six samples.
[b] Obtained from density values using a conversion table of OIV [179].

the intensity of the electric current through the material. Electrical conductivity evolution of different fruit and vegetable juices and egg derivatives over a range of temperatures has been studied [187, 8] although not yet in grape juice. Parellada grape juice electrical conductivity followed a linear trend (Fig. 5.2) within the temperature range, from 5 °C up to 50 °C, with intercept $(0.10 \pm 0.01)\,S \cdot m^{-1}$ and slope $(0.0071 \pm 0.0005)\,S \cdot m^{-1} \cdot °C^{-1}$. The small slope value, similar to that reported for other fruit juices, suggests that variations of grape juice conductivity should not influence PEF treatment effects.

5.3.2. Response surfaces and analysis of the effect of the treatment time, electric field strength and pulse frequency

The lack of fit test produced a p-value large enough ($F_{\mathrm{PPO}} = 13.44\,, p = 0.0714\,; F_{\mathrm{POD}} = 10.16\,, p = 0.0933$) to accept the second order polynomial model (Equation 2) as suitable for the observed data. The analysis of variance also showed that the polynomial models containing up to second order terms, including interactions and quadratic factors, explained the variability of raw data ($R^2_{\mathrm{PPO}} = 0.9147\,; R^2_{\mathrm{POD}} = 0.9010$) better than models with only main factors. The tests on the individual parameters of PPO model revealed that only six terms (t_t, F, $E \cdot F$, $E \cdot t$, F^2 and t_t^2) had a significant effect on the PPO relative residual activity (Fig. 5.3). The polynomial model including only these factors was selected as the predictive equation for PPO residual activity (Tab. 5.3). Such equation providing the lowest standard deviation of the regression coefficients and an adjusted determination coefficient that was higher than the one obtained when the complete polynomial model was fitted to experimental data.

As far as POD is concerned, only five parameters were statistically different from zero which means that the corresponding terms (t_t, F, E, $E \cdot F$ and E^2) had a significant influence on POD relative residual activity (Fig. 5.3). Following the same criteria, the non-significant effects were removed from the general POD model. Thus, a predictive model for the residual POD activity with narrower confidence intervals than the those obtained using the general

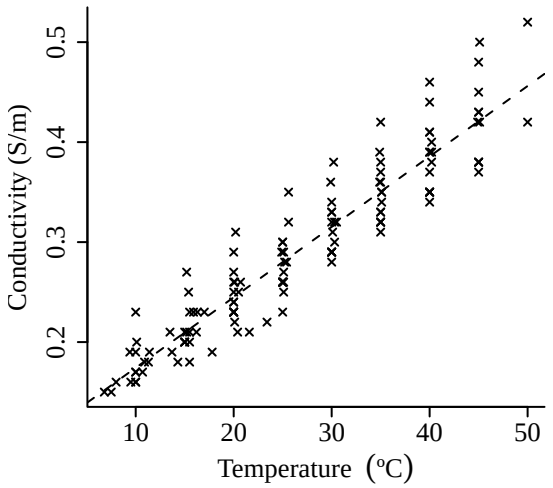

Figure 5.2: Effect of Parellada grape juice temperature on its electrical conductivity. The dotted line correspons to the linear trend fitted to the 16 measurements performed.

model was obtained and, again, this was achieved without substantial reduction of the adjusted determination coefficient (Tab. 5.3).

Therefore all main factors affected depletion of PPO and POD enzymatic activities on natural grape juice (Fig. 5.3). The negative values of the coefficients of treatment time, electric field strength and pulse frequency mean that both enzymatic activities lessened as the values of these parameters increased. Figure 5.4 displays the response surfaces of PPO and POD at the longest treatment time. These graphics are an example of the different behaviour of both enzymes when processed with PEF. PPO activity could be eliminated completely whereas the best result for POD would allow only reaching around 50% of its original activity. It seems clear that grape PPO is more susceptible than POD to PEF processing.

Several authors have reported similar outcomes in aqueous solutions [106, 88, 87, 89] and even in ready to eat products. Thus, Elez [56, 62] reported the absence of POD activity and a reduction of 80% of PME activity in orange juice after a continuous PEF processing at $35\,\mathrm{kV{\cdot}cm^{-1}}$, 400 Hz of 4 µs and a total treatment time of 1.5 ms. Yeom [264], who also studied orange juice PME, achieved a 17% of residual activity after a PEF treatment of $35\,\mathrm{kV{\cdot}cm^{-1}}$, 700 Hz of 2.2 µs after a total treatment time of 184 µs. Finally, the different PEF susceptibility of PPO and POD, from mushroom and horseradish respectively, in buffered aqueous solutions has been recently described [271].

Less research has been conducted on the effect of pulse frequency although

Table 5.2: Face centred central composite design followed to process Parellada grape juice with different PEF treatments[a].

Experiment	E (kV·cm^{-1})	F (Hz)	t_t (ms)	RA_{PPO}		RA_{POD}	
				Block 1	Block 2	Block 1	Block 2
1	35	1000	1	0.7139	0.7490	0.6247	0.6796
2	25	600	3	0.2255	0.3247	0.7000	0.7153
3	35	600	3	0.1829	n.d.[b]	0.6242	0.6266
4	30	1000	3	0.1135	0.0516	0.5212	0.5791
5	30	600	1	0.5789	0.4665	0.7410	0.7285
6	25	1000	5	0.1552	0.0794	0.5064	0.4953
7	35	200	1	0.9433	0.9462	0.8102	0.6997
8	25	200	5	0.8760	0.9076	0.8387	0.7948
9	35	200	5	0.1329	0.1867	0.5431	0.4954
10	30	600	3	0.2377	0.1507	0.5020	0.5419
11	25	200	1	0.9524	0.9684	0.9494	0.9296
12	25	1000	1	0.4460	0.5729	0.7128	0.7941
13	30	200	3	0.7537	0.5645	0.6088	0.6025
14	35	1000	5	0.3755	0.2753	0.5224	0.5251
15	30	600	5	n.d.	n.d.	0.4942	0.5188
16	30	600	3	0.2782	0.2035	0.5283	0.5613

[a] All treatments were performed at 4 µs of pulse width in bipolar mode.
[b] Not detected.

Table 5.3: Coefficient values of the whole and predictive polynomial models of the PPO and POD residual activities of Parellada grape juice processed by PEF[a].

Equation parameter	PPO polynomial mode		POD polynomial model	
	Whole	Predictive	Whole	Predictive
k_0	4 ± 2	2.5 ± 0.4	4.9 ± 0.8	5.4 ± 0.6
k_1 (kV·cm^{-1})	$(-1 \pm 1) \cdot 10^{-1}$	$(-3 \pm 1) \cdot 10^{-2}$	$(-24 \pm 5) \cdot 10^{-2}$	$(-28 \pm 4) \cdot 10^{-2}$
k_2 (Hz^{-1})	$(-46 \pm 6) \cdot 10^{-4}$	$(-46 \pm 6) \cdot 10^{-4}$	$(-9 \pm 2) \cdot 10^{-4}$	$(-10 \pm 2) \cdot 10^{-4}$
k_3 (ms^{-1})	$(-1 \pm 100) \cdot 10^{-3}$	$(-5 \pm 10) \cdot 10^{-2}$	$(-12 \pm 5) \cdot 10^{-2}$	$(-47 \pm 5) \cdot 10^{-3}$
k_4 (kV·cm^{-1}·Hz^{-1})	$(7 \pm 1) \cdot 10^{-5}$	$(7 \pm 1) \cdot 10^{-5}$	$(26 \pm 6) \cdot 10^{-6}$	$(26 \pm 6) \cdot 10^{-6}$
k_5 (kV·cm^{-1}·ms^{-1})	$(-9 \pm 3) \cdot 10^{-3}$	$(-9 \pm 3) \cdot 10^{-3}$	$(4 \pm 10) \cdot 10^{-4}$	n.s.[b]
k_6 (Hz^{-1}·ms^{-1})	$(6 \pm 40) \cdot 10^{-6}$	n.s.[b]	$(-4 \pm 100) \cdot 10^{-7}$	n.s.[b]
k_7 (kV^2·cm^{-2})	$(2 \pm 2) \cdot 10^{-3}$	n.s.[b]	$(35 \pm 8) \cdot 10^{-4}$	$(42 \pm 7) \cdot 10^{-4}$
k_8 (Hz^{-2})	$(16 \pm 3) \cdot 10^{-7}$	$(16 \pm 3) \cdot 10^{-7}$	$(-1 \pm 10) \cdot 10^{-8}$	n.s.[b]
k_9 (ms^{-2})	$(3 \pm 1) \cdot 10^{-2}$	$(4 \pm 1) \cdot 10^{-2}$	$(10 \pm 5) \cdot 10^{-3}$	n.s.[b]
R^2_{Adjusted}	0.8789	0.8848	0.8605	0.5604

[a] Parameters are given as a estimation and 95% confidence intervals
[b] Non-significant effect using a α-value of 0.05

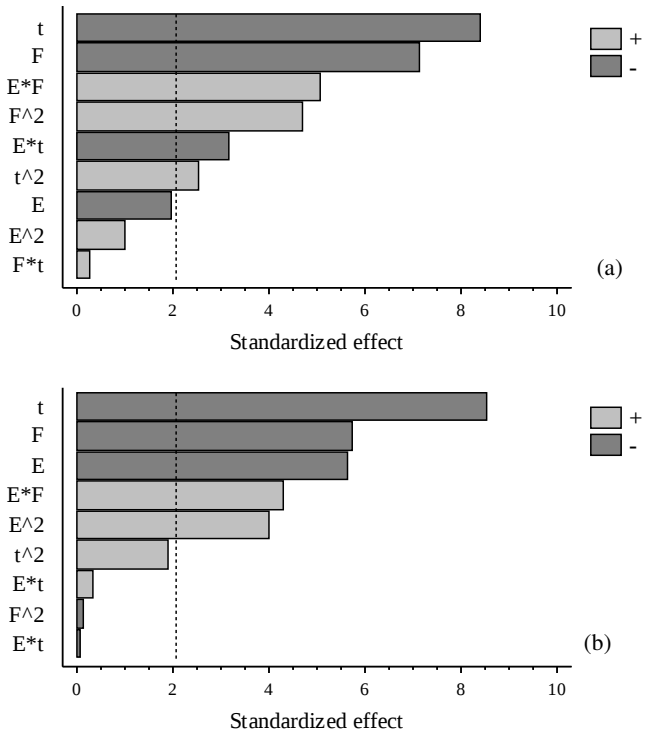

Figure 5.3: Pareto charts displaying graphically the effect of each term of the general polynomial model on the relative residual activity of PPO (a) and POD (b). Each model term is represented by a bar whose lenght is proportional to its standardized estimated effect. Bars longer than the vertical dotted line mean that their corresponding factors exert a significant indluence on the response variables ($p \leq 0.05$). Clear bars indicate that an increase of the facotr value will increase the relative residual enzymatic activity. Dark bars mean that an increase variation of the factor value will produce a decrease of the measured response. t: treatment time; E: electric field strength; F: pulse frequency.

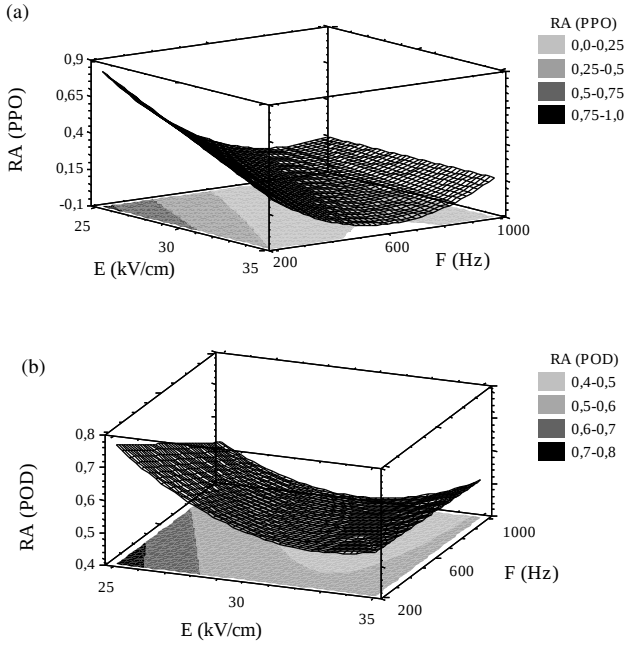

Figure 5.4: Estimated response surfaces of PPO (a) and POD (b) at 5 ms of treatment time.

some studies claim that it also has influence on the effects of PEF on food products. An increase in orange juice peroxidase and pectinmethylesterase inactivation was reported when pulse repetition rate was increased [56, 62]. A lipase in milk and a protease in simulated milk ultrafiltrate also showed the same behaviour when the pulse frequency was gradually raised and the remaining treatment conditions were kept constant [31, 29]. The data of the current study seem to agree with these studies although data also evidence a strong interaction between electric field strength and pulse frequency for both enzymatic activities. This fact means that it is not possible to make general conclusions about the effect of one variable without taking into account the other parameter. In other words, the effect of electric field strength on enzyme activities of grape juice can be different depending on the value of the pulse frequency or viceversa. So any application of PEF to grape juice should analyse thoroughly the combination of these variables to optimize the degree of enzyme inactivation.

Table 5.4: Optimal processing PEF factors to reach the lowest PPO and POD relative residual activity.

Factor	PPO[a]	POD[b]	PPO / POD[c]
t_t (ms)	5.000	5.000	4.9363
E (kV·cm^{-1})	35.0	30.2	33.5
F (Hz)	630	1000	677
Predicted RA[d]	-0.0045	0.4187	-0.0216 / 0.4877

[a] $RA = 2.5 - 0.03 \times E - 0.0046 \times F - 0.05 \times t_t + 0.00007 \times E \times F - 0.0091 \times E \times t_t + 0.0000016 \times F^2 + 0.04 \times t_t^2$.

[b] $RA = 5.4 - 0.028 \times E - 0.0010 \times F - 0.047 \times t_t + 0.000026 \times E \times F + 0.0042 \times E^2$.

[c] Optimal processing PEF parameters predicted by the combination of both predictive polynomial equations in order to achieve the smallest relative residual activity of both enzymes at once.

[d] Residual enzymatic activity calculated using the predictive equations and the optimal values of the processing factors presented in this table.

5.3.3. Assessing the critical values of the studied factors and validating de predictive models

The response surfaces of PPO and POD activities did not show any absolute minimum in the experimental region. According to the previous section it is reasonable to think that it should occur at a longer treatment time. It would be more difficult to predict the electric field strength necessary to achieve the highest inactivation for each enzyme since it depends on the working pulse frequency. However, it was possible to determine the coordinates of the points that should lead to the lowest relative enzymatic activities within the studied range (Tab. 5.4). The polynomial equation for PPO behaviour pointed towards a major lessening of enzymatic activity as electric field strength conditions become severe using moderate pulse frequencies. The POD model showed the maximum inactivation at medium electric field strength values combined with high pulse frequencies. The superposition of both equations allows predicting the best treatment conditions to achieve the largest activity reduction of both enzymatic activities simultaneously.

To complete the study, a set of experiments were carried out to validate the prognostics of the developed predictive models. The data comparison (Fig. 5.5) showed that the proposed predictive expressions were accurate enough to fit experimental results. The correlation coefficients between observed and predicted RA-data were 0.7769 and 0.7012 for PPO and POD models respectively. This results show that response surface methodology could be used to obtain valuable quantitative data about PEF processing with really few experiments.

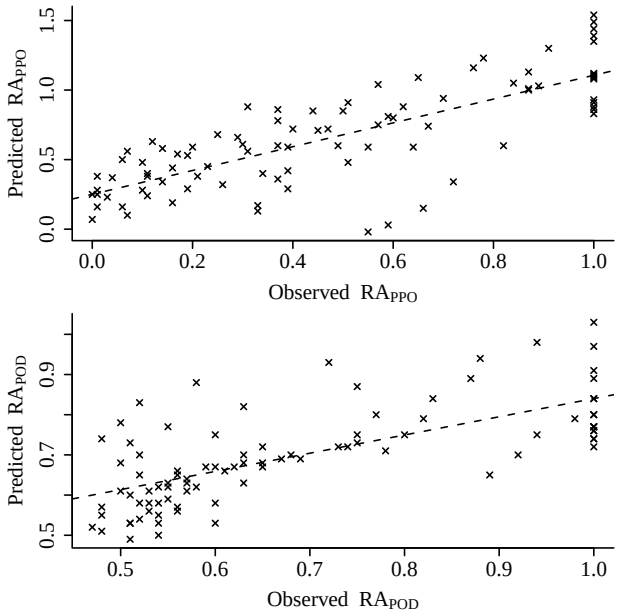

Figure 5.5: Scatter plots of the observed and predicted data of PPO (a) and POD (b) residual activities of the validation trials. The straight line indicates the correlation between both groups of data.

5.3.4. Effect of the pulse width of PEF tratments on the enzymatic activities

The effect of pulse width on the activity of PPO and POD was evaluated at the optimal values of treatment time, electric field strength and pulse frequency (Tab. 5.4) and are displayed in table 5.5. Total inactivation of PPO was achieved at any tested pulse width, so it was impossible to get information of the effect of this factor on PPO activity after PEF processing. Significant differences among the different pulse widths were observed for POD activity. Nevertheless these differences neither were very large (around 5%) nor showed a clear trend so it was not possible to extract any clear conclusion of the effect of PEF pulse width on POD activity. Conversely, several authors have reported an increase in PEF processing effectiveness on several enzymes such as PPO, POD, PME of different fruits like apple, pear, peach and orange when pulse width increase [87, 89, 56, 62].

5.3.5. Kinetic models

Relative enzymatic activity data obtained from validation experiments were used to fit equations (5.3) and (5.4). The use of a different equation for each enzymatic activity was motivated by the different behaviour of the residual PPO and POD activities of grape juice (Fig. 5.6).

Table 5.5: Effect of pulse width on the relative activity of PPO and POD of grape juice after PEF processing at the optimal conditions for each enzyme. Values are expressed as a mean of three experiments and 95% conficence intervals. Different number of asterisks between two values means significant differences.

τ (µs)	RA_{PPO}	RA_{POD}
2	not detected	$0.483 \pm 0.003^{*}$
4	not detected	$0.509 \pm 0.002^{**}$
6	not detected	$0.5365 \pm 0.0008^{***}$
8	not detected	$0.512 \pm 0.002^{**}$

Equation (5.3) was used to fit PPO relative residual activity reduction and it describes a typical first order kinetic behaviour. This mathematical model was reported as suitable to fit the effects of PEF processing on PPO activity of apple, peach and pear extracts [87, 89] and lipoxygenase (LOX) activity of tomato juice [88, 151].

Equation (5.4) was adjusted to the POD relative residual activity of grape juice after PEF treatments. This mathematical model was proposed by Levenspiel [132] and it is a modified first order kinetic model which usually allows fitting the behaviour of enzymes whose residual activities follow an asymptotic depletion up to a non-zero value. The fractional conversion model, as this equation is also known, was successfully used to describe the inactivation of a commercial pectin enzyme formulation by PEF treatments [64].

These equations described well the behaviour of grape residual PPO and POD enzymatic activities as a function of the treatment time (Tab. 5.6). Thus, the whole mechanism of enzymatic inactivation, although still unknown, could be summarized through a first order kinetic model. In such a case, the k-coefficient of the mathematical models provides information about the rate at which enzymatic depletion occurs. Thereby, it can be stated that the rate of PPO inactivation depends more on the treatment pulse frequency than on the electric field strength applied (Tab. 5.6). Nevertheless, the highest POD k-mean values always occurred at 1000 Hz and 35 $\mathrm{kV \cdot cm^{-1}}$. Consequently, these seem to be the best conditions to eliminate quickly at least a half of the total POD activity of grape juice.

Exponential expressions gave, in general, slightly more accurate predictions than the predictive polynomial equations (Tab. 5.7). The fact that the data used to fit the exponential equations were the same set of data used to make predictions could explain so good results. The main drawbacks of kinetic models when compared with response surface models are the huge quantity of experiments necessary to obtain a suitable fit and the reduced range of application since an equation is needed for each level of the remaining factors.

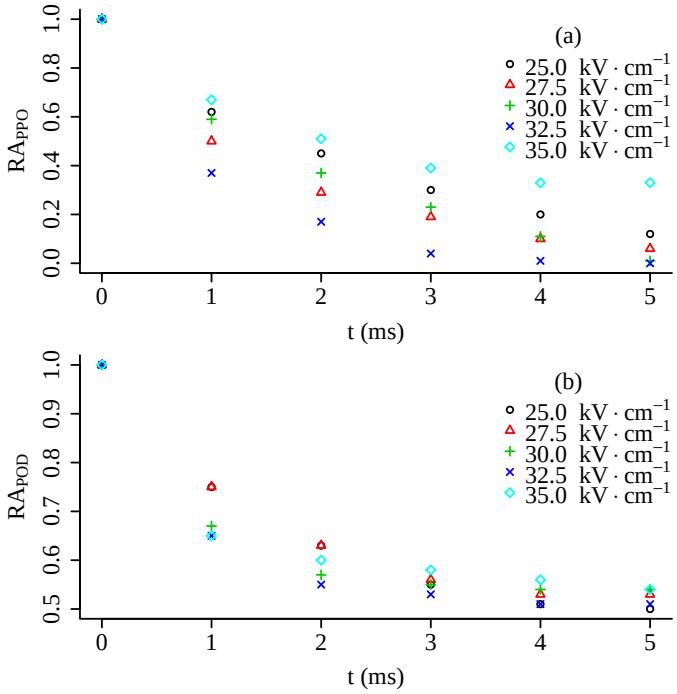

Figure 5.6: Relative residual activity of PPO at 600 Hz (a) and (b) POD at 1000 Hz at different treatment times.

Table 5.6: Values of the estimated equation parameters of the first order kinetic models adjusted to PPO and POD residual activities after PEF processing[a].

Electric conditions		PPO[b]			POD[c]			
E (kV·cm^{-1})	F (Hz)	RA_0	k_{PPO} (ms^{-1})	$R_A^{2\,d}$	RA_0	RA_∞	k_{POD} (ms^{-1})	$R_A^{2\,d}$
25.0	200	1.0 ± 0.3	0.04 ± 0.01	0.9401	1.00 ± 0.02	0.77 ± 0.07	0.3 ± 0.2	0.9904
25.0	600	1.0 ± 0.2	0.5 ± 0.1	0.9607	1.00 ± 0.02	0.47 ± 0.02	0.8 ± 0.1	0.9990
25.0	1000	1.0 ± 0.1	0.5 ± 0.1	0.9800	1.00 ± 0.02	0.47 ± 0.02	0.63 ± 0.09	0.9989
27.5	200	0.98 ± 0.09	0.29 ± 0.06	0.9805	1.02 ± 0.05	0.7 ± 2	0.08 ± 0.7	0.8699
27.5	600	1.00 ± 0.02	0.82 ± 0.04	0.9935	1.01 ± 0.07	0.47 ± 0.08	0.7 ± 0.3	0.9866
27.5	1000	1.0 ± 0.1	0.6 ± 0.2	0.9854	1.00 ± 0.02	0.51 ± 0.02	0.7 ± 0.1	0.9988
30.0	200	1.0 ± 0.1	0.29 ± 0.06	0.9347	1.00 ± 0.03	0.53 ± 0.04	0.6 ± 0.1	0.9971
30.0	600	1.00 ± 0.09	0.6 ± 0.1	0.9919	1.00 ± 0.03	0.49 ± 0.02	0.8 ± 0.1	0.9980
30.0	1000	1.00 ± 0.06	0.9 ± 0.1	0.9961	1.00 ± 0.01	0.537 ± 0.008	1.3 ± 0.1	0.9995
32.5	200	1.00 ± 0.05	0.59 ± 0.05	0.9978	1.00 ± 0.03	0.41 ± 0.06	0.5 ± 0.1	0.9973
32.5	600	1.00 ± 0.07	0.9 ± 0.1	0.9960	1.000 ± 0.008	0.468 ± 0.007	0.86 ± 0.04	0.9998
32.5	1000	0.99 ± 0.08	0.7 ± 0.1	0.9936	1.00 ± 0.02	0.51 ± 0.01	1.2 ± 0.2	0.9988
35.0	200	1.1 ± 0.2	0.3 ± 0.1	0.9189	1.000 ± 0.005	0.503 ± 0.005	0.67 ± 0.02	0.9999
35.0	600	1.0 ± 0.1	0.7 ± 0.1	0.9910	1.00 ± 0.02	0.60 ± 0.01	1.0 ± 0.1	0.9987
35.0	1000	0.97 ± 0.07	0.23 ± 0.04	0.9854	1.00 ± 0.06	0.56 ± 0.03	1.5 ± 0.7	0.9890

[a] Parameters are given as a mean value and 95% confidence intervals calculated using the asymptotic standard errors of estimations.
[b] $RA = RA_0 \cdot e^{-k_{\mathrm{PPO}} \cdot t_{\mathrm{t}}}$
[c] $RA = RA_\infty - (RA_0 - RA_\infty) \cdot e^{-k_{\mathrm{POD}} \cdot t_{\mathrm{t}}}$
[d] Adjusted coefficient of determination.

Table 5.7: Coefficients of determination of the predictive polynomial and firs order kinetic fitted equations.

Electric conditions		PPO models		POD models	
E (kV·cm^{-1})	F (Hz)	Polynomial[a]	Kinetic[b]	Polynomial[c]	Kinetic[d]
25.0	200	0.9162	0.8786	0.9711	0.9970
25.0	600	0.9730	0.9974	0.8849	0.9997
25.0	1000	0.9879	0.9997	0.9179	0.9997
27.5	200	0.9759	0.9968	0.9717	0.9601
27.5	600	0.9575	0.9934	0.9012	0.9957
27.5	1000	0.9959	0.9960	0.9017	0.9996
30.0	200	0.9674	0.9891	0.9263	0.9991
30.0	600	0.9928	0.9938	0.8832	0.9994
30.0	1000	0.9689	0.9982	0.8015	0.9998
32.5	200	0.9979	0.9904	0.9428	0.9992
32.5	600	0.9633	0.9993	0.8712	0.9999
32.5	1000	0.9869	0.9891	0.8128	0.9996
35.0	200	0.9809	0.9827	0.9104	0.9999
35.0	600	0.9878	0.9980	0.8445	0.9995
35.0	1000	0.9961	0.9940	0.8012	09967

[a] PPO predictive polynomial model with parameters of table 5.3.
[b] Equation (5.3) with parameters of table 5.6.
[c] POD predictive polynomial model with parameters of table 5.3.
[d] Equation (5.4) with parameters of table 5.6.

5.4. Conclusions

PEF processing of grape juice below 40 ℃ reduced the activity of natural PPO and POD enzymes. Treatment time, electric field strength and pulse frequency had a significant influence on the relative residual activity of the selected enzymes, which was generally reduced as these parameters increased. Pulse width also affected POD inactivation although without showing a clear trend. According to the predictive expressions developed, no PPO activity would be observed after 5 ms of 35 $kV \cdot cm^{-1}$ pulses at 630 Hz regardless the pulse width whereas up to 58.13% of POD depletion would be achieved using 30.2 $kV \cdot cm^{-1}$ and 1000 Hz for the same treatment time. In this case, the shortest pulse width gave the lowest POD activity, although differences among treatments using different pulse widths were small.

Response surface methodology is a suitable tool to analyse and optimize the inactivation of PPO and POD enzymatic activities of grape juice using PEF technology since it can supply information about critical factors and their optimum values. In addition, the predictive equations produced accurate values of enzymatic activity over the whole range of evaluated conditions.

6 Effects of thermal and non-thermal processing treatments on fatty acids and free amino acids of grape juice

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Abstract

Common thermal and pulsed electric field (PEF) treatments have been evaluated to assess their effect on fatty acids and free amino acids contents of Parellada grape juice. These compounds have great importance in winemaking as nutritive compounds for yeasts growth. The effect of thermal and PEF treatments has also been determined on several physicochemical properties and a population of inoculated *Saccharomyces cerevisiae*. Both technologies reduced the population of the spoilage microorganism inoculated in grape juice. No viable cells were observed after thermal processing of grape juice whereas PEF treatment achieved four logarithmic reductions of the microbial viability. No significant changes were noticed on physicochemical properties measured such as reducing sugar content, total acidity and pH. Neither thermal nor PEF treatments modified the total content of fatty acids and free amino acids of Parellada grape juice. However, the concentration of lauric acid diminished after PEF processing and the concentration of some amino acids varied after both treatments.

6.1. Introduction

Lately fruit juices have become very popular commodities because consumers associate them to healthy products, so their commercialisation has increased in the last years. Among them, grape juice is one of the most important because it is consumed directly either as a final product or as a raw material (i.e., winemaking). In both manufacturing processes, grape juice is undergone to different treatments such as thermal processing which can affect its components. From the point of view of human consumption, lipid and nitrogen contents of grape juice are low and they have hardly nutritional interest in human diet. However, they are related with sensorial quality losses of grape juice and wine. Such sensory defects come from collateral reactions during the manufacturing process in which are involved amino acids and unsaturated fatty acids. These side reactions

acquire more significance when the product temperature increases. Thus, amino acids produce reduced sulphur compounds like hydrogen sulphide [101], ethyl carbamate from urea [181], and they also participate in non-enzymatic browning of grape juice. Unsaturated fatty acids, by their side, can yield some volatile compounds by enzymatic splitting such as aldehydes, ketones and alcohols that confer undesirable flavour and taste to final products [209].

In addition, both lipids and nitrogen compounds play an important role in the fermentative steps of winemaking. Fatty acids and sterols have a great influence on the growth of fermentative yeast and thus, on the development of alcoholic fermentation. They have a remarkable influence on the transport of amino acids through the microbial membrane and the activity of the membrane-linked enzymes such as ATPase [197]. Moreover, the lack of unsaturated fatty acids decreases the yeast tolerance to ethanol [189]. Nitrogen compounds of grape juice are also essential in the metabolism of yeasts because nitrogen is, after carbon, the second element utilized during their growth. The content of nitrogen compounds also affects the kinetics of fermentation. Thus, a lack of nitrogen was related with some *sluggish* and *stuck* fermentation [127]. In addition, amino acids participate in aroma development and health-related metabolic by-products [13].

It is of general knowledge that thermal treatments modify not only the sensorial characteristics of food but also it could affect its composition and some physicochemical properties. A non-thermal processing such as pulsed electric fields (PEF) is an emerging technology efficient enough to preserve some foodstuffs [89, 151], especially fruit and vegetable juices, with no significant changes in their natural quality [201, 263]. However, few studies exist on the effects of these two sterilizing treatments on the composition of grape juice. For that reason, the aim of this research was to evaluate and compare the influence of thermal and pulsed electric fields (PEF) treatments on fatty acids and free amino acids contents of grape juice.

6.2. Material and methods

6.2.1. Grape juice preparation

A local wine manufacturer, Raimat (Lleida, Spain), kindly provided grapes (*Vitis vinifera* variety Parellada). They were harvested at ripeness, washed, drained, split from bunches and frozen at −20 °C until processing during the following two months. About 18 kg of frozen grapes were thawed at 5 °C for 24 h, afterwards they were manually pressed leading to approximately 9 L of grape juice that were divided in three aliquots, which were de-aerated by stirring under reduced pressure for 30 min. One of the aliquots was kept as control sample, a second aliquot was thermally processed and the remaining aliquot was treated by PEF. This procedure was performed twice and four samples of control and of processed grape juice were taken to analyse. After thermal and PEF processing,

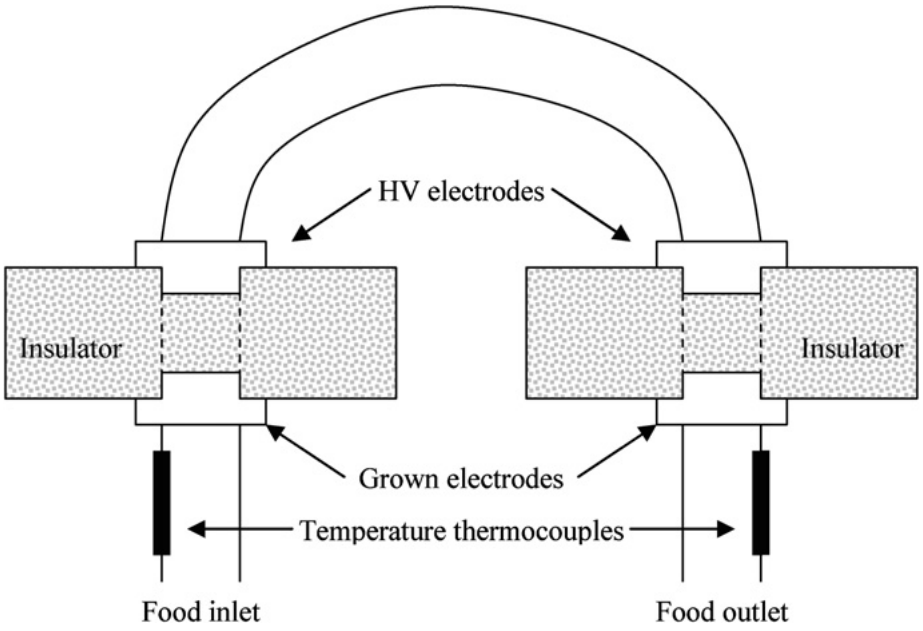

Figure 6.1: Scheme of a pair of treatment chambers of the PEF equipment used.

grape juice was bottled and immediately frozen at −20 °C until analysis. All recipients and materials, which were in contact with the samples, were previously sterilized.

6.2.2. Devices and treatment conditions of thermal and PEF processing

Typical conditions to pasteurize young wines range from 60 °C for some minutes until half minute at 90 °C [105]. However, juices usually are processed with higher treatment times because they have more carbohydrates and less alcohol content than wines. Thus, in this research grape juice was thermally treated using a bench scale continuous pasteurizer made of two stainless steal tubular heat exchangers, a peristaltic pump model D-21V (Dinko, Barcelona, Spain), and a stainless steel tubing system. The first heat exchanger raised and maintained grape juice temperature at 90 °C whereas the second one refrigerated the processed juice below 5 °C. Both heat exchangers were submerged in separate water baths where water temperatures were 90 °C and 0 °C, respectively. Grape juice flow was adjusted to 0.67 mL·s^{-1} to obtain a total thermal treatment of 1 min.

A laboratory scale PEF unit (Ohio State University, Columbus, OH) was used to treat grape juice with PEF. The pulse generator module consists of a high voltage generator (OSU-4F), which can produce potential differences of up to 12 kV, and a pulse generator unit model 9412A (Quantum Composers,

Inc., Bozeman, MT), which can render square wave pulses of up to 10 µs and 2000 Hz. The chamber module has eight equal treatment chambers composed of two stainless steel connectors, which act as electrodes, screwed into a Delrin® insulator and separated 0.292 cm (Fig. 6.1). The total volume of each chamber is 0.012 cm^3 and the generated electric field between the electrodes had the same direction of the juice flow. A tubing system and a gear peristaltic pump model 75210-25 (Cole Palmer, Vernon Hills, IL), allowed a continuous mode processing of grape juice (3.5 $mL \cdot s^{-1}$). Temperature probes attached just before and after each pair of chambers measured inlet and outlet juice temperatures. After each two chambers, grape juice was refrigerated in a tubular heat exchanger immersed into a water–ice bath to avoid temperatures higher than 40 °C, which was the highest temperature recorded by the thermocouples located at the exit of the treatment chambers. PEF treatment was performed with bipolar electric field pulses of 4 µs width and with a field strength of 35 $kV \cdot cm^{-1}$. The pulse repetition rate was 1000 Hz and the total PEF treatment time was 1 ms, which was calculated as the product of the pulse width and the number of pulses delivered to grape juice.

6.2.3. Microbial culture and inoculation

To evaluate the effects on microbial population of both treatments, 100 mL of freshly squeezed grape juice was inoculated with *S. cerevisiae* CECT 1383 (Spanish type culture collection, University of Valencia, Valencia, Spain). First, the yeast was cultured on dishes containing chloramphenicol glucose agar (CGA) (Biokar Diagnostics, Beauvais, France) for 48 h at 30 °C to obtain cells in the stationary phase. Afterwards, cells were suspended in saline peptone solution (Biokar Diagnostics) and collected by centrifugation (JP Selecta, Barcelona, Spain) at 4000 revolutions per minute (r/min) for 10 min at 4 °C. Cell pellet was added to the juice providing an initial yeast load of 2.7×10^7 $cfu \cdot mL^{-1}$. Microbial manipulation was performed with sterile material in a biological safety cabinet (Telstar, SA, Terrassa, Spain).

6.2.4. Determination of the microbial inactivation

After sterile sampling, control and processed grape juice were serially diluted and 1 mL of each dilution was pour plated with chloramphenicol glucose agar (Biokar Diagnostics) for 5 days at 25 °C. The yeast surviving fraction logarithm of each treatment was used to make comparisons

$$S = \log\left(\frac{C}{C_0}\right) \tag{6.1}$$

where C and C_0 are the recovered yeast concentration ($cfu \cdot mL^{-1}$) of the processed and non-processed grape juice, respectively, calculated as the average of the four dishes with a count of colony forming units ranging from 25 to 250.

6.2.5. Physicochemical determinations

Some characteristic parameters of grape juice were determined before and after processing such as sugar content measured as reducing sugars, total acidity measured as tartaric acid and pH. Quantification of reducing sugars was performed by enzymatic measurement (reactives from Chema Italia, Rome, Italy) using a multi-parametric analyser Enochem (Tecnología Difusión Ibérica, Barcelona, Spain). The pH was determined by using a pH-meter Metrohm 702 (Metrohm, Herisau, Germany). The total acidity was determined by the method described by the OIV [179]. Of each one of the samples, the analysis of these parameters was carried out twice.

6.2.6. Extraction and analysis of fatty acids

The lipid fraction of grape juice was extracted using the procedure of Darne [49]. Thus, 10 mL of ethanol (Merck, Darmstadt, Germany), and 10 mL of distilled water were added to 20 mL of sample and homogenised for 2 min at 8000 r/min using an UltraTurrax T25 (Rose Scientific, Edmonton, Canada). Afterwards, fatty acids were extracted 5 times with 20 mL of chloroform (Merck) at 0 °C by homogenising at 8000 r/min for 1.5 min with the Ultra-Turrax T25. All chloroform aliquots were combined before fatty acid derivatization, which was done following the method recommended in the Codigo Alimentario Espanol [198]. This method consists of generating methyl esters of the naturally occurring fatty acids by transesterification or esterification. Afterwards, these methyl esters were analysed with a GC–MS Shimadzu QP 5000 (Shimadzu, Kyoto, Japan), equipped with an automatic injector Shimadzu AOC-20i.

Helium (99.999% of purity) was the carrier gas (41.1 $cm \cdot s^{-1}$). Samples of 1 µL were injected into an inlet at 230 °C where they were splitted using a 24:1 ratio. A DBWAX capillary column (Cromlab, Barcelona, Spain) of 30 m length, 0.25 mm of internal diameter and 0.25 µm of film thickness with a bonded stationary phase of cross-linked polyethylene glycol was used to perform the separation of methyl esters. Oven temperature was raised from 40 °C up to 111 °C with a slope of 2 $°C \cdot min^{-1}$ that was immediately followed by other constant increase of 3 $°C \cdot min^{-1}$ up to 220 °C. This temperature was maintained for 5 min before the oven program returned to the initial conditions. The temperature of the detector was set at 230 °C. The ionisation of fatty acid methyl esters was achieved by electronic impact at 70 eV. The fragments analysed had a mass/charge in the range between 40–298. Undecanoate and heptadecanoate methyl esters (Aldrich, Gillingham, UK), were used as the internal standards. Identification of fatty acid methyl esters was made using the retention times and comparing either their mass spectra with a spectral library of known standard compounds. The quantification of the compounds was made using the full scan screening method. For the quantification of the compounds, the area of the corresponding peaks was normalized by that of the corresponding internal

Table 6.1: Gradient elution program[a].

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
13.5	97	3
24.0	94	6
30.0	91	9
50.0	66	34
62.0	66	34
62.5	0	100
66.5	0	100
67.0	100	0
87.0	100	0

[a] Flow rate of 1 mL·min^{-1}

standard and was interpolated in a calibration graph made through the analysis of standard solutions in hexane. Of each one of the samples, fatty acids analysis was carried out four times.

6.2.7. Preparation and HPLC analysis of free amino acids

Analysis were performed with a Waters high-pressure liquid chromatography (Milford, MA) equipped with two 510 pumps, a 717 Plus Autosampler, and a 996 Photodiode Array Detector used at 254 nm. A PicoTag reverse phase column (300 mm-length × 3.9 mm of internal diameter) with a stationary phase of dimethyl octadecylsilyl bonded to amorphous silica was used. Amino acid derivatization was performed using a Waters PicoTag workstation. The PicoTag method used for amino acid analysis is described in Ancin [10]. Samples were cleaned by ultra filtration with a Millipore Ultrafree MC cartridge (Billerica, MA), and then L-norleucine and L-methionine sulfone (Aldrich) were added as internal standards. Afterwards, a precolumn derivatisation was carried out with phenyl isothiocyanate (Pierce Biotechnology, Rockford, IL). A Millennium 32 software package (Waters) was employed for chromatographic control. The amount of sample injected was 10 µL. The column was set at 46 °C.

Mobile phase A was a solution of 2.5% of acetonitrile (Scharlau, Barcelona, Spain) and 97.5% of a solution of sodium acetate (70 mM), with pH adjusted to 6.55 with acetic acid (10%) (Merck). Mobile phase B was made of acetonitrile, water and methanol (Scharlau) with a volumetric proportion 45:40:15, respectively. The mobile phases used were filtered through a 0.45 µL Millipore filter and deaerated. Table 6.1 shows the elution gradient used to perform amino acid separation. Amino acids determination was repeated four times for each sample.

6.2.8. Ammonium determination

Ammonium nitrogen was quantified using enzymatic kits (Chema Italia, Rome, Italy), according to the manufacturer's instructions, in a multi-parametric analyser Enochem. Of each one of the samples, the measurements were done four times.

6.2.9. Statistical analysis

All the results are expressed as mean and standard deviation. Means between treatments were compared using the least significant difference (LSD) method with a significance level of 5%. All data processing was performed with a SPSS software package (SPSS Inc., Chicago, IL).

6.3. Results and discussion

6.3.1. Effects of processing on *Saccharomyces cerevisiae*

S. cerevisiae was not recovered when grape juice was processed thermally at 90 °C for 1 min. However, the average of *S. cerevisiae* recovered from Parellada grape juice after 1 ms of square waved pulses of 4 µs-width and 35 $kV \cdot cm^{-1}$ electric field strength in bipolar mode using a continuous PEF equipment was 2.7×10^3 $cfu \cdot mL^{-1}$ that means 4.0 ± 0.4 decimal logarithmic reductions at a maximum temperature of 40 °C. The lessening of survival fraction of yeast obtained after PEF processing is within the range of values reported in other juices inoculated with *S. cerevisiae*. A similar PEF treatment was effective enough to reach up to 5.1 and 5.8 logarithmic reductions of *S. cerevisiae* and *Lactobacillus brevis* population in orange juice using the same equipment [59, 58]. Cserhalmi [48] achieved a maximum reduction of 3.4 logarithms when apple juice was treated with square wave pulses of 2 µs and 28 $kV \cdot cm^{-1}$ for 100 µs in a similar PEF device because they used only six treatment chambers. These results also show how the microbial destruction and its rate might vary with a large number of factors such as the processing conditions, the media and the microorganism characteristics.

The effect of the use of several combination of treatments (thermal and PEF treatments and antimicrobials) to reduce yeast population of red and white grape juice have recently been published. Wu [262] reported 3.9 logarithmic reductions combining a thermal treatment of 50 °C for 30 s with a PEF treatment of 20 adjacent triangular shaped pulses of 1 µs-width and 40 $kV \cdot cm^{-1}$ in bipolar mode using a batch PEF equipment. They also reported up to 5.2 logarithmic reductions when these treatments were combined with 4 $g \cdot L^{-1}$ of lysozyme. In addition, they proved that no significant reduction of yeast population was achieved heating grape juice at 44 °C for 15 min. Thus, as far as yeast destruction is concerned, it seems that a typical thermal treatment provides more stable

Table 6.2: Physicochemical properties measured on fresh and processed Parellada grape juice.

Measured properties[a b]	Control	Heat	PEF
pH	3.84 ± 0.02^{x}	3.835 ± 0.006^{x}	3.83 ± 0.02^{x}
Reducing sugars ($g \cdot L^{-1}$)	131 ± 2^{x}	130 ± 2^{x}	130 ± 2^{x}
Total acidity ($g \cdot L^{-1}$)[c]	2.37 ± 0.04^{xy}	2.33 ± 0.01^{x}	2.43 ± 0.05^{y}

[a] Values were reported as a mean and standard deviation of 8 measures.
[b] Values with different letters are statistically different of each other.
[c] Expressed as tartaric acid.

grape juice than a PEF treatment defined as a proper treatment because allow reaching 5 logarithmic reductions.

6.3.2. Effects of processing on the measured physicochemical properties

Table 6.2 shows the measured properties of the fresh squeezed and processed Parellada grape juice. Reducing sugar content in all the samples was below the usually values for this parameter in the grape juices, between $180\,g \cdot L^{-1}$ and $288\,g \cdot L^{-1}$. Therefore, grape juice from this variety is usually used to obtain sparkling low alcohol wines or fizzy ones with pale colour and a freshly and floral taste [105]. There were no statistically significant changes in the measured physicochemical properties of grape juice when Parellada grape juice was thermally processed at 90 °C for 60 s. In the same way, reducing sugar content, pH and total acidity were not significantly affected by PEF processing. These results widen the outcome obtained when tomato, orange and apple juices were treated by PEF, as they did not show significant changes of these parameters [151, 263].

6.3.3. Effects of thermal and PEF processing on grape juice fatty acids

The fatty acid concentrations of control and processed grape juice are showed in table 6.3. Comparing Parellada grape juice with other varieties such as Cortese and Garnacha, the total fatty acid values are similar or quite lower respectively [9, 50] evincing that composition of grape juice is variety dependent. The total fatty acid content in the PEF treated grape juice ($51\,mg \cdot L^{-1}$) was slightly lower than the control juice ($54.1\,mg \cdot L^{-1}$) and the thermally processed grape juice ($52.3\,mg \cdot L^{-1}$). These values show that both processing treatments have little effect on total fatty acid content. The thermal treatment did not affect the concentration in the grape juice of any fatty acid while the PEF treatment made that the concentration of lauric acid in the grape juice diminished (Tab. 6.3). The presence of short chain fatty acids (C12:0, C14:0) in the

Table 6.3: Concentration of fatty acids measured on fresh and processed Parellada grape juice.

Fatty acid[a b]	Control	Heat	PEF
Caprilic acid (C8)	n.d.[c]	n.d.	n.d.
Capric acid (C10)	n.d.	n.d.	n.d.
Lauric acid (C12)	0.32 ± 0.01^x	0.32 ± 0.02^x	0.19 ± 0.03^y
Myristic acid (C14)	0.37 ± 0.05^x	0.35 ± 0.01^x	0.38 ± 0.03^x
Palmitic acid (C16)	14.55 ± 0.08^x	13.1 ± 0.1^x	13.8 ± 0.4^x
Palmitoleic acid (C16:1)	n.d.	n.d.	n.d.
Stearic acid (C18)	1.07 ± 0.01^x	1.0 ± 0.1^x	1.11 ± 0.09^x
Oleic acid (C18:1)	2.296 ± 0.003^x	2.3 ± 0.4^x	2.3 ± 0.1^x
Linoleic acid (C18:2)	25.38 ± 0.04^x	24.6 ± 0.3^x	24.2 ± 0.8^x
Linolenic acid (C18:3)	10.073 ± 0.009^x	9.9 ± 0.1^x	9.4 ± 0.4^x
Total fatty acid	54.1 ± 0.1^x	52.3 ± 0.5^{xy}	51 ± 1^x

[a] Values were reported as a mean and standard deviation in $mg \cdot L^{-1}$ of 16 measures.
[b] Values with different letters are statistically different of each other.
[c] Not detected.

yeasts is only given in occasions and in trace levels. On the other hand, linolenic and oleic acid contents were unaffected by the type of treatment. These are the most necessary fatty acids during the normal growth of yeasts. In addition, both fatty acids act as a survival factors at the end of fermentation [204]. In a similar way, the processing treatment did not modify the long-chain saturated fatty acids (C14 and C16), which affect the degree of saturation of the plasma membrane of yeast [169]. Actually, grape juice treatments should not decrease the total concentration of fatty acids since a low level could lead to slow fermentations and an increase of volatile acidity of wine because of an incorrect development of the yeast along the fermentative process. Some of these troubles have been reported when other treatments have been carried out on other varieties of grapes.

6.3.4. Effects of thermal and PEF processing on grape juice ammonium and free amino acids

Table 6.4 shows the concentrations of ammonium and free amino acids found in Parellada grape juice before and after processing. The ammonium concentration was not affected by either thermal or PEF grape juice treatments. The ammonium content of Parellada grape juice was in the range for this cation in grape juices, between $19\,mg \cdot L^{-1}$ and $240\,mg \cdot L^{-1}$. Its importance comes from the fact that it is the first inorganic source of nitrogen used by yeast. Furthermore, its presence in the media also affects the sequence in which amino acids are taken by yeast [18].

Table 6.4: Concentration of nitrogen compounds measured on fresh and processed Parellada grape juice.

Nitrogen species[a b]	Control	Heat	PEF
Ammonium cation (NH_4^+)	47.5 ± 0.6^x	44.5 ± 0.6^x	43.5 ± 0.5^x
Histidine (His)	26.5 ± 0.9^x	10.9 ± 0.5^y	33 ± 4^z
Lysine (Lys)	5.2 ± 0.5^x	4.8 ± 0.9^x	8 ± 2^x
Tryptophan (Trp)	30 ± 2^x	37 ± 2^x	46 ± 3^y
Arginine (Arg)	774 ± 69^x	798 ± 71^x	772 ± 52^x
Serine (Ser)	29 ± 3^x	38 ± 6^x	29 ± 4^x
Asparagine (Asn)	24 ± 2^x	30 ± 3^{xy}	37 ± 3^y
Glycine (Gly)	3.5 ± 0.3^x	3.3 ± 0.3^x	3.2 ± 0.4^x
Threonine (Thr)	16 ± 1^x	17 ± 3^x	14 ± 3^x
Alanine (Ala)	82 ± 7^x	82 ± 6^x	87 ± 6^x
Tyrosine (Tyr)	8.6 ± 0.3^x	7.4 ± 0.4^x	11 ± 2^x
Valine (Val)	22 ± 2^x	21 ± 2^x	30 ± 5^x
Methionine (Met)	28 ± 3^x	28 ± 5^x	20 ± 4^x
Isoleucine (Ile)	7.3 ± 0.2^x	8.4 ± 0.7^x	6.3 ± 0.9^x
Leucine (Leu)	28 ± 2^x	28 ± 3^x	25 ± 2^x
Phenylalanine (Phe)	12.9 ± 0.7^x	14 ± 1^{xy}	18 ± 1^y
Proline (Pro)	119 ± 11^x	140 ± 13^x	101 ± 6^x
Aspartic acid (Asp)	26 ± 2^x	24 ± 3^x	23 ± 2^x
Glutamic acid (Glu)	31 ± 2^x	33 ± 3^x	31 ± 2^x
Ornithine (Orn)	9 ± 1^x	8 ± 1^x	18 ± 3^y
γ-Aminobutiric acid (GABA)	38 ± 3^x	35 ± 5^x	32 ± 2^x
Creatinine (Creat)	13 ± 1^x	11 ± 2^x	12 ± 2^x
Phosphoserine (Pser)	8.3 ± 0.4^x	11.0 ± 0.6^y	8.1 ± 0.5^x
Hydroxyproline (Hyp)	16 ± 2^x	16 ± 2^x	20 ± 2^y
Cystathionine (Cyst)	37 ± 1^x	37 ± 4^x	39 ± 12^x
Total amino acids	1394 ± 71^x	1443 ± 74^x	1424 ± 54^x

[a] Values were reported as a mean and standard deviation in $mg \cdot L^{-1}$ of 16 measures.

[b] Values with different letters are statistically different of each other.

All Parellada grape juice samples, either treated or untreated, showed a higher amino acid content (control 1394 $mg \cdot L^{-1}$, thermal 1443 $mg \cdot L^{-1}$, PEF 1424 $mg \cdot L^{-1}$) than the described for Garnacha (678 $mg \cdot L^{-1}$), Cabernet Sauvignon (782 $mg \cdot L^{-1}$), and Pinot Noir (857 $mg \cdot L^{-1}$). The treatments applied to the grape juice did not affect the total concentration of amino acids (Tab. 6.4). On the other hand, both preservative treatments showed few effects on free amino acids content. In pasteurized grape juice, the histidine concentration was lower than in the control grape juice and the phosphoserine concentration was lightly superior in the pasteurized grape juice than in the control. In PEF treated grape juice, the concentrations of histidine, tryptophan, asparagine, phenylalanine and ornithine were superiors than in the control. These amino acids probably were released through the pores formed in the plasmatic membrane of the grape juice indigenous yeasts [246]. The conditions of PEF treatment could cause organelle disruptions, so vacuoles were destroyed too allowing the proteases to have a free access to the cytoplasmic enzymes [99]. These proteases could cause cellular protein degradation into smaller peptides and amino acids. It is very important that thermal and PEF treatments at least do not change significantly the natural amino acid content because, as noted previously, it is directly linked with the fermentation rate, the yeast population and with the global quality of the resulting wine.

6.4. Conclusions

The main outcome of the current work evidence that both types of processing technologies, thermal and PEF treatments, let diminish microbial population although the thermal treatment was more effective than the PEF treatment. None of the two treatments affected to the total concentration of fatty acids and amino acids of grape juice. This is important since these compounds are essential for the development of the yeasts during fermentation.

7 A comparison of the effects of pulsed electric field and thermal treatments on grape juice

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Abstract

The work was aimed at comparing the effect of pulsed electric field (PEF) and thermal technologies on physical, chemical, microbiological, and nutritional properties of freshly squeezed grape juice of different varieties when they are applied using continuous flow systems. It has been evidenced that grape variety is a factor to be taken into account when comparing the processing effects of PEF and heat treatments. Nevertheless, results of general and specific microbial populations were not affected by it and followed a very similar trend with each processing treatment. Soluble solids, pH, acidity, and the electrical conductivity of grape juice were not affected by PEF processing. On average, PEF treatment reduced the radical scavenging activity a 9% in front of the 13% of the heat treatment whereas both treatments halved the protein content. Data of vitamin C, total polyphenol, cinnamic acid, free catechin and non-flavonoid content were not enough to show differences between both treatments. However, all these nutritionally related properties share the same trend, PEF processing yielded milder values than heat processed samples.

Industrial relevance: The use of the pulsed electric field technology as a preservative method in the fruit juice industry can be promoted providing accurate information of its effects on common products. In this study this was achieved comparing the results with the data of a typical thermal treatment, which is the current technological reference this area.

7.1. Introduction

Pulsed electric field (PEF) technology has roughly a hundred of years of history [25]. However, in the last three decades it has been gathered enough infor-

mation to consider it an alternative to thermal processing in several areas of the food industry. Grounded on the different susceptibility of the food components when interact with an electric field, the technology has evolved from the application of direct electric current to foodstuffs in a container to the use of trains of alternating pulses in a continuous devices with several treatment chambers. Even though the increased efficiency, the main influent factors are the same, the strength of the field and the treatment time [242, 41].

All the evidences support that PEF treatments reduce microbial populations [98, 223] and enzymatic activities of fruit juices or milk [19, 32] without degrading excessively the taste [125], quality attributes [119, 274] and healthy components [30] of different food products. In addition, first studies suggested that can be efficient from an economical point of view [107]. As a way to promote the use of PEF processing in food industry it has to be compared with the reference technology in preservation which is thermal processing. Such studies have been carried out on diverse fruit juices such as tomatoes [2], longan [269], apple cider [20] or milk products [239] just to cite a few recent examples.

On grapes, this technology has been studied as a way to extract nutritional components to elaborate wines with a higher nutritive content, and to increase the final yield [136, 135, 93, 200]. Others have studied the technology as a preservation tool reporting the effects of PEF treatments on specific components such as fatty and amino acids [82], microorganisms [249, 117, 262, 142] and, enzymes [141]. However, until the moment there is not any study that compares the effect of a preservative PEF treatment with a typical thermal processing of grape juice. This study was aimed at studying how both treatments, PEF and thermal, affect physical, chemical, microbiological and, several nutritional properties of grape juice.

7.2. Material and methods

7.2.1. Grapes, juice elaboration and processing

Three varieties of *V. vinifera* were selected as representatives of the wide diversity of the crop, keeping in mind their industrial use though. The chosen varieties were *Mazuelo* also known as Cariñena in Spain or Carignan in France, which is a typical red grape. *Moscatel* or Muscat of Alexandria, which is a white aromatic variety, and finally, a common white grape used in Cava elaboration, which is the Spanish Champagne, *Parellada*. They were kindly supplied by local farmers from the Denominació d'Origen Penedès (D.O. Penedès, Catalonia, Spain). After harvesting, 150 kg of grapes were processed at the INCAVI pilot plant.

Mazuelo grapes were destemmed, crushed and the juice was macerated with the skins in stainless steel tanks of 200 L of capacity at 18 °C for 5 h. After this stage, the pomace was pressed in a Vaslin-Bucher X Pro 5 (Chalonnes S/Loire, France), applying different pressing cycles up to 2 bar of pressure according

to maturity characteristics of the grapes and instructions of the press manufacturer's. White varieties Parellada and Moscatel were crushed and directly pressed following the same protocol. Grape juice obtained after pressing was gravity settled in stainless steel tanks at temperatures near the freezing point for at least 24 h.

Afterwards, the supernatant part made of grape juice free of tartarates was placed in plastic containers of 25 L at low temperature. The containers of the different grape juice varieties were transported to the pilot plant of the University of Lleida where they were processed in aliquots of 500 mL.

7.2.2. Thermal device

It consists of a metallic pipe forming two consecutive sections, a peristaltic pump (model D-21V, Dinko, Barcelona) and a grape juice repository. The tube was made of 304 stainless steal with 0.085″ internal diameter and 0.125″ external diameter (Resteck stainless steel ref: 21513, Teknokroma S. Coop C. Ltda, Sant Cugat del Vallès) and each section was arranged as a coil submerged in a water bath at controlled temperature.

The length of the section coil in the heated bath was calculated to provide a treatment of 1 min with a grape juice flow of 40 mL·min^{-1}. The temperature of the treatment was 90 °C. The following coil was placed in an iced water bath to achieve a sharp refrigeration of the processed grape juice.

All processing devices were sterilised before each treatment by means of a 10% sodium hypochlorite (Scharlab, S.L., Sentmenat, Spain) solution that flowed through it for 5 min and a rinse with a continuous flow of sterile water.

After each treatment the devices were cleaned with deionized water, a circulation of a 4% sodium hydroxide (Panreac Química, S.A.U, Castellar del Vallès, Spain) solution at 40 °C for 5 min at the highest flow rate, and a new rinse with deionized water. In both cases, cleaning and disinfecting, the final point of water rinsing was monitored with pH strips.

7.2.3. PEF device

An OSU-4F bench-scale continuous unit manufactured by the Ohio State University (Columbus, OH, USA) was used to treat the grape juice samples. Eight co-field chambers, each with a volume of 12 mm^3 and a gap distance of 2.9 mm between electrodes were connected in series. Four cooling coils were connected before and after each pair of chambers and submerged in a water-ice mixture to maintain the grape juice temperature below 45 °C. The tubing system was made of the same material of the thermal device.

Grape juice was processed with pulses of 4 µs in bipolar mode for 2.5 ms of treatment time with an electric field strength of 35 kV·cm^{-1} and a pulse frequency of 400 Hz. These processing parameters were obtained from previous optimisation studies [141, 142].

Grape juice temperature at the inlet of the first chamber was kept at 15 °C. The maximum temperature of 44.4 °C was reached at the last pair of treatment chambers.

The PEF treatment time of grape juice samples (t_t, ms) were calculated as the product of the average pulse width (τ, ms) and the number of pulses (n) delivered in the treatment chambers. That is

$$t_t = \tau \cdot n. \tag{7.1}$$

The number of pulses can be obtained from the pulse frequency rate (F, Hz) and the average residence time (t_r, s) of the sample in the treatment chambers with the expression

$$n = F \cdot t_r, \tag{7.2}$$

and the residence time supposing a turbulent flow is calculated as the relationship between the volumetric capacity of the treatment chambers (vol_c, mL) and the average flow rate (q, $\mathrm{mL \cdot s^{-1}}$):

$$t_r = \frac{vol_c}{q} \tag{7.3}$$

The flow rate was maintained with a peristaltic pump (model 75210-25, Millipore, Bedford, MA, USA) that allows a maximum flow rate of approximately $8\,\mathrm{cm^3 \cdot s^{-1}}$ of grape juice.

The pulse voltage difference (V, kV) between electrodes measured with the oscilloscope divided by the electrode gap (d, cm) of the treatment chambers was taken as the electric field strength value (E, $\mathrm{kV \cdot cm^{-1}}$):

$$E = \frac{V}{d} \tag{7.4}$$

Temperatures were taken using thermocouples (T type, ±0.1 °C) located before and after each pair of treatment chambers. Pulse waveform, voltage, intensity, pulse width and pulse frequency delivered in the treatment chambers were measured using a digital oscilloscope (Tektronix THS720A, Tektronix, OR, USA). Their values were recorded in a computer.

7.2.4. Physical and chemical properties

Soluble solids were measured as brix degrees that were determined using a digital refractometer with temperature correction (Atago RX-1000, Atago Co. Ltd., Tokyo, Japan). The pH was taken using a pH-meter (Crison 2000, Crison S.A., Barcelona, Spain). The same device was used to measure the total acidity that was expressed as grams of tartaric acid per litre of grape juice.

All were measured following the Spanish regulation [152]. The electrical conductivity of the grape juices was also determined with a conductivity meter (Testo 240, Testo AG, Lenzkirch, Germany) at 20 °C.

7.2.5. Microbiology

Each sample of grape juice was analysed to measure the content of aerobic and anaerobic microorganisms, lactic bacteria, yeast and moulds. Grape juice samples were 10-fold serially diluted with a Ringer solution and plated on a selective medium and growing conditions.

Aerobic microorganisms determination was carried out on Plate Count Agar (PCA, Scharlau Chemie S.A., Barcelona, Spain) plates and counted after 48 h at 28 °C [17]. Anaerobic microorganisms were also measured on PCA plates by incubation into anaerobic jars (BBL™ GasPak™ Anaerobic System, Becton Dickinson and Company, USA) for 96 h at 30 °C. Samples to measure mould and yeast counts were plated on Bengal Rose agar with chloramphenicol (Scharlau) and incubated at 28 °C for 72 h [256, 17]. Samples to determine lactic bacteria population were plated on a *O. oeni* medium (Scharlau) with 2% agar-agar and 50 mg·L^{-1} of nystatin (Sigma-Aldrich Inc., Steinheim, Germany) at 30 °C for 6 days into an atmosphere containing 5% CO_2 [273].

The population of *K. apiculata*, *S. cerevisiae*, *L. plantarum*, *L. hilgardii*, and *G. oxydans* as specific species of grape juices were measured as described in Marsellés-Fontanet [142] where each specie grows in a specific environment that hinders the development of the remaining species.

Microbiological data were expressed as the decimal logarithm of the colony forming units (cfu) per millilitre of juice of the dilution that provided counts ranging 25–250.

7.2.6. Nutritional properties

Protein content was analysed using the Bradford method [37]. In this analytical method food proteins bind specifically a red coloured dye (Coomassie Brilliant Blue G-250) in a methanolic solution. This protein-dye complex has a strong blue coloration. Ultra filtered (0.45 µm) grape juice samples of 100 µL were added to a solution with 2.9 mL of Bradford dye (Sigma-Aldrich). The absorbance at 595 nm was recorded after homogenisation. This values were compared with the absorbances of standards of bovine serum albumin (BSA) up to 150 mg·L^{-1}.

Radical-scavenging activity was measured following the reduction of the content of the stable free 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical (DPPH$^{\cdot}$) described in Brand[38] as an estimation of the antioxidant activity. The analysis was carried out mixing 100 µL of ultra filtered grape juice into a glass cuvette of 1 cm-optical path containing 2.9 mL of DPPH$^{\cdot}$ methanolic solution of 25 mg·L^{-1}. After 30 min at room temperature the absorbance at 515 nm was measured (DPPH$^{\cdot}{}_s$). A sample blank using water was also measured (DPPH$^{\cdot}{}_0$). The radical scavenging activity (RSA)

was expressed as percentage calculated using the expression

$$RSA = \frac{1 - \mathrm{DPPH^{\cdot}}_s}{\mathrm{DPPH^{\cdot}}_0} \times 100. \tag{7.5}$$

Vitamin C content was determined as ascorbic acid following the process described in Odriozola [173] where all the forms of vitamin C from the sample (1.25 mL) were converted to, and stabilised as, ascorbic acid with a solution of 2,3-dimercapto-1-propanol (BAL) 0.050 M (Acros Organics, NJ, USA) and metaphosphoric acid 10% (Sigma-Aldrich Química SA, Madrid). Separation and detection of ascorbic acid was performed with a chromatographic device HPLC-UV (Waters, Milford, MA, USA). Quantification was carried out comparing the area of the signal with the area of standards from 2.5 $\mathrm{mg{\cdot}kg^{-1}}$ to 105 $\mathrm{mg{\cdot}kg^{-1}}$.

Total polyphenol content was assessed by two methods

- measuring the absorbance or optical density at 280 nm as described in Glories [91]. Grape juices were filtered through a nitrocelullose filter of 0.45 µm and white ones were diluted with water ten times. Red grape juice was 25-fold diluted.
- measuring the absorbance of the filtered grape juice at 760 nm following the Folin-Ciocalteu method [237].

Non-flavonoid content as a Folin-Ciocalteu index after precipitating the flavonoid content of the grape juice with a formaldehyde solution at low pH following the method described by Kramling [126]. The difference between the Folin-Ciocalteu index and the current value provided an estimation of the flavonoid content of the juices.

Cinnamic acid derivatives content as an index obtained from the optical density at 320 nm following the method described by Zoecklein [272].

Free catechin content as DMAC index was determined measuring the compounds that absorb at 640 nm following Nagel [168]. As a measure of the free catechin content it decreases as the tanin polymerization increases.

All the optical densities were taken in an spectrophotometer Lambda 25 UV-Vis (Perkin-Elmer, USA).

7.2.7. Experimental design and data analysis

A replicated blocked design was performed to determine whether the studied variables were affected by the process treatment as well as the grape variety. These hypotheses were formalised in the general model given by the following equation:

$$Y_{ijk} = P_i + V_j + (P \cdot V)_{ij} + \epsilon_{ijk} \tag{7.6}$$

where Y represents the data of each dependent variable described in the precedent sections, which was measured twice and averaged. Each treatment was also replicated twice (k) on grape juice of each variety.

P is the processing treatment of the grape juice, which was considered a fixed factor, with i-levels *unprocessed (NT)*, *electrically processed (PEF)*, and *thermally processed (TT)*.

V is the grape variety factor, which was taken as a random variable, with the previously cited j-representatives *Mazuelo*, *Moscatel*, and *Parellada*. It was supposed to be independent and normally distributed, with mean zero and variance σ_V^2.

$P \cdot V$ stands for the interaction between both factors, and consequently, it is also a random factor accounting for the deviations from the average treatments by different varieties. In the current study, these differences were assumed to be equals with a value σ_{PV}^2.

Finally, ϵ is the variability associated with the data and is assumed independent and identically normally distributed, with mean zero and variance σ^2, and also independent of the random effects.

The experimental data was managed using an electronic spreadsheet (OpenOffice.org Calc, OpenOffice 2.4.1, Sun Microsystems Inc., Santa Clara, CA, USA). Afterwards, it was statistically analysed using a mixed model regression analysis that provided estimates of the treatment (P) and variety (V) factors as well as the experimental error (ϵ) [191].

The best fitted model based on the equation (7.6) was selected using the Akaike Information Criterion (AIC) [222], the Bayesian Information Criterion (BIC) [231] and, the analysis of residuals. The significance of the levels of the treatment factor when there was difference were assessed using the suitable contrasts in the fixed-effects matrix of the model. A confidence level of 95% was considered for all the comparisons. When the interaction term was considered influent an interaction plot of the factors was displayed to get some insight into their relationship. Statistical analysis, mixed model regression [190], and graphics [227] were performed using the R software suit [206].

7.3. Results and discussion

7.3.1. Effects on the physical and chemical properties

The statistical analysis of the physical and chemical characterising variables of grape juice (Tab. 7.1) showed that the degrees brix and the electrical conductivity were affected by the type of processing. The analysis by means of contrasts revealed that only the thermal treatment increased the mean values of both variables. The change was small though, 0.5 brix units and 0.01 $\mathrm{S{\cdot}m^{-1}}$ respectively. There was not any difference between both treatments for the soluble solids measure but it was for the electrical conductivity.

Table 7.1: Physical, chemical, and microbiological data of unprocessed (NT) grape juice varieties and after being processed with pulsed electric fields (PEF), or with heat (TT).

Variety	Treatment	Brix	pH	Acidity[a]	Conductivity[b]	Aerobic[c]	Anaerobic[c]	Lactic bacteria[c]	Yeast & Mould[c]
Mazuelo	NT	20.9	3.21	5.4	0.1923	7.20	6.26	7.50	7.04
Mazuelo	NT	19.7	3.18	6.2	0.209				
Mazuelo	PEF	21.1	3.23	4.7	0.204	3.70	2.11	2.16	3.18
Mazuelo	PEF	19.9	3.22	6.1	0.204				
Mazuelo	TT	21.1	3.26	5.0	0.227	0.18	0.88	0.00	0.00
Mazuelo	TT	20.3	3.24	6.0	0.209				
Moscatel	NT	18.6	3.18	6.2	0.215	7.00	3.81	4.41	4.95
Moscatel	NT	17.8	3.16	7.2	0.213				
Moscatel	PEF	18.5	3.18	6.0	0.215	4.72	1.63	1.36	1.43
Moscatel	PEF	18.3	3.18	6.3	0.214				
Moscatel	TT	18.8	3.18	5.1	0.212	0.00	0.00	0.00	0.00
Moscatel	TT	18.6	3.18	6.2	0.215				
Parellada	NT	18.0	3.25	6.2	0.1977	9.30	6.68	5.08	6.48
Parellada	NT	17.5	3.25	6.8	0.1967				
Parellada	PEF	18.0	3.25	5.8	0.206	3.68	4.38	2.87	2.83
Parellada	PEF	17.9	3.25	6.0	0.202				
Parellada	TT	18.2	3.25	5.8	0.220	0.00	1.26	0.00	0.00
Parellada	TT	18.2	3.25	5.8	0.215				

[a] in $g \cdot L^{-1}$ of tartaric acid.
[b] in $S \cdot m^{-1}$
[c] in $cfu \cdot mL^{-1}$

As expected the variety was found to influence the soluble solids measures by 1.3 brix on average even taking into account the clear different variability of each variety data. It is obvious that a different selection of grape varieties could have provided a different estimated effect. The mean effect of the variety over the pH measures was 0.04 units, which is close to the device precision. There was not any interaction effect for none variables.

These results agree with the current knowledge. Several authors reported that the soluble solids content, pH and, acidity of apple, orange and, even grape juices were not modified by the PEF processing treatments [274, 58, 82]. The higher electrical conductivity of the heat processed grape juices means a higher ionic content than the unprocessed product and could be the result of a release of the content of the broken grape cells. As far as the soluble solids, is not unusual a slight augment of its value after a heat treatment due to the evaporation of some water.

7.3.2. Effects on general and specific grape juice microorganisms

The results of the analysed microbiological variables were categorical (Tab. 7.1). There was not detected any interaction effect and the microbial counts were drastically trimmed by both processing treatments despite of the grape variety. Thermal processing achieved a consistently higher degree of microbial destruction than the PEF treatment. No viable cells were found after heat processing except for the anaerobic counts where an average of 0.7 logarithms remained. PEF achieved reductions of 3.8 ± 0.8, 2.9 ± 0.6, 3.5 ± 0.8, and 3.6 ± 0.5 logarithms of aerobic, anaerobic, lactic bacteria, yeast and mould counts respectively.

As a complementary information, more experiments were also carried out on a different batch of the same grape juice varieties analysing, though, several typical grape juice microorganims such as *K. apiculata* and *S. cerevisiae* representing the yeast, *L. hilgardii* and *L. plantarum* as lactic bacteria and, to extend the study, *G. oxydans* as a representative of the acetic bacteria.

The statistical analysis of these new data (Tab. 7.2) allowed the validation of the previous general results confirming that the processing treatment PEF reduced in average 2.0 ± 0.4, 2.78 ± 0.08, 3.3 ± 0.5 and 2.2 ± 0.3 logarithms the populations of *K. apiculata*, *S. cerevisiae*, lactic bacteria and *G. oxydans*. The final lactic bacteria average values of the two experiments were similar whereas the logarithmic reductions of *K. apiculata* were smaller than the general yeast and mould result. Acetic bacteria value was lower than the obtained for lactic but according with what has previously reported in Parellada grape juice [142]. The difference in yeast destruction could be explained by the different initial microbial counts [111] although this issue is controversial because other authors reported that the PEF effect on bacterial populations was independent of the initial values [6].

Table 7.2: Results of the processing treatments of several specific grape juice microbial species

Variety	Treatment[a]	*K. apiculata*[b]	*S. cerevisiae*[b]	Lactic bacteria[b]	*G. oxydans*[b]
Mazuelo	NT	3.97	6.94	4.17	5.71
Mazuelo	NT	3.91	6.94	6.67	6.29
Mazuelo	PEF	2.77	3.88	1.49	3.47
Mazuelo	PEF	1.16	3.90	3.24	3.60
Mazuelo	TT	0.00	0.00	0.00	0.00
Mazuelo	TT	0.00	0.00	0.00	0.00
Moscatel	NT	5.40	7.07	6.40	5.43
Moscatel	NT	4.36	7.07	7.28	6.41
Moscatel	PEF	3.73	4.14	2.26	2.74
Moscatel	PEF	1.86	4.57	3.58	3.72
Moscatel	TT	0.00	0.00	0.00	0.00
Moscatel	TT	0.00	0.00	0.00	0.00
Parellada	NT	5.68	6.75	4.85	4.30
Parellada	NT	4.60	6.75	5.86	5.20
Parellada	PEF	3.16	4.12	1.96	2.74
Parellada	PEF	2.88	4.11	2.94	3.69
Parellada	TT	0.00	0.00	0.00	0.00
Parellada	TT	0.00	0.00	0.00	0.00

[a] fresh (*NT*), electrically (*PEF*), and thermally (*TT*) processed grape juice.
[b] in cfu·mL^{-1}

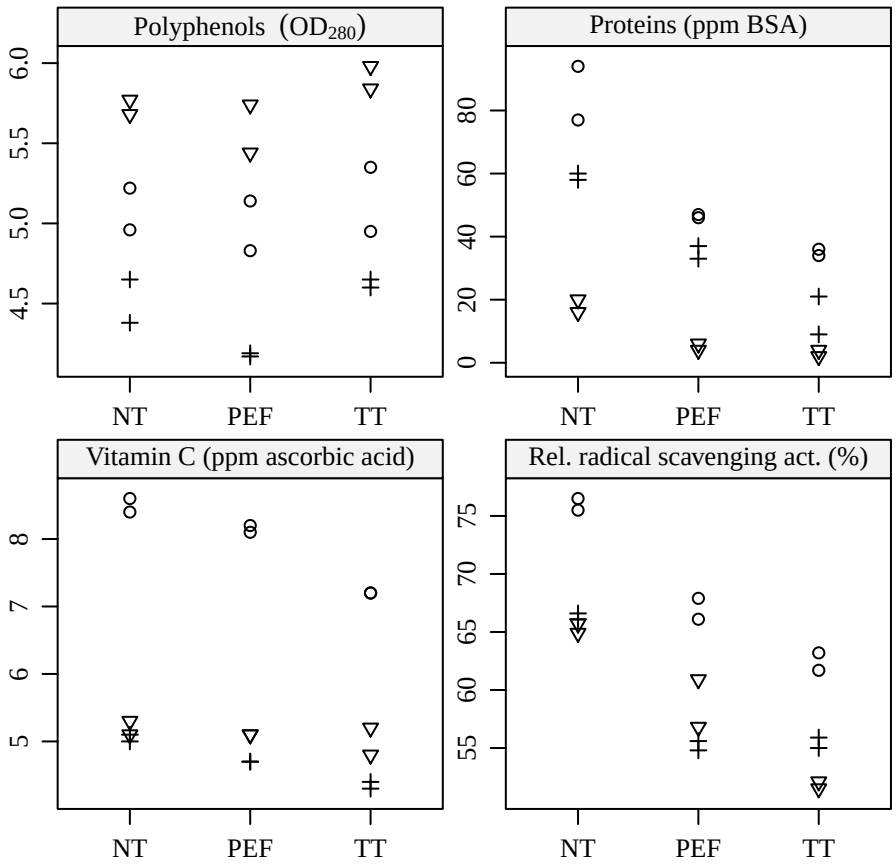

Figure 7.1: Results of several nutritional properties of Mazuelo (○), Moscatel (+), and Parellada (▽) grapes of unprocessed (NT) or electrically (PEF), and thermally (TT) processed juices.

7.3.3. Effects on nutritional properties

The results of the nutritional variables studied are summarised in figures 7.1 and 7.2. Following the framework provided by the experimental design, they should be considered values produced by the fitted model (Eq. (7.6)). In this context, grape juice variety was found to be a significant factor for all the dependent properties. As a consequence of considering the variety a random factor it was possible to obtain an estimation of its influence (Tab. 7.3). Until now, it was commonly accepted that grape variety could influence the results of the processing treatments even though there are not any reported value. The range of the estimations could be reduced using more varieties.

The statistical analysis also confirmed that protein content and radical scav-

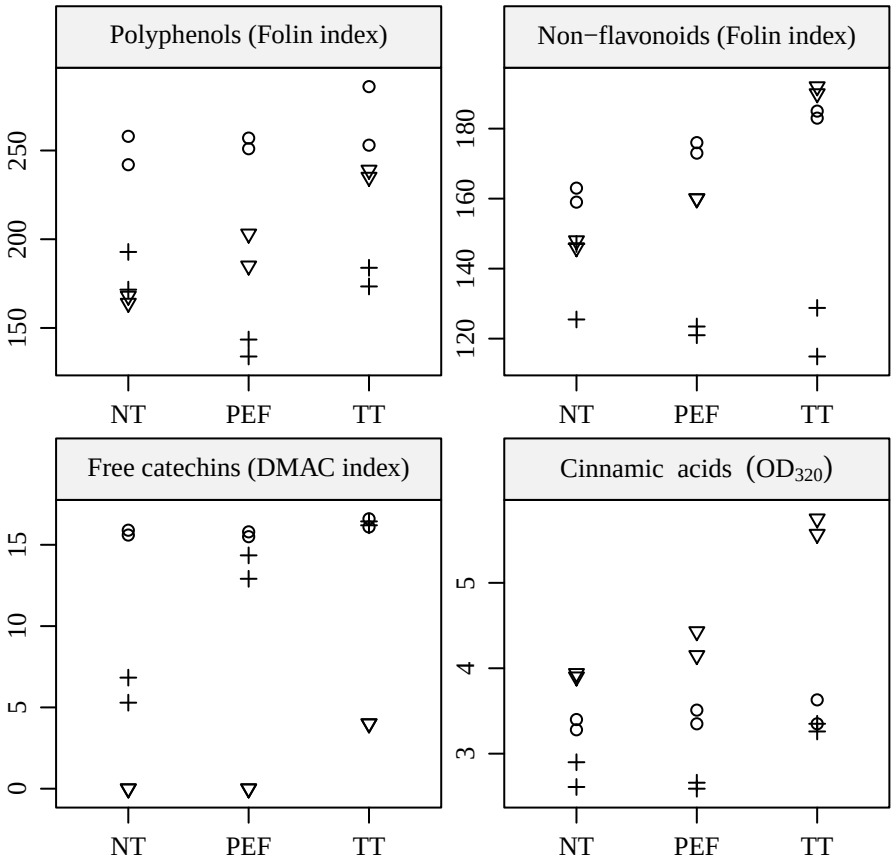

Figure 7.2: Results of several polyphenol fractions of Mazuelo ($\circ$), Moscatel ($+$), and Parellada ($\bigtriangledown$) grapes grapes of unprocessed (NT) or electrically (PEF), and thermally (TT) processed juices.

enging activity were clearly affected by the processing treatments (Fig. 7.1). Protein mean values for each level of processing NT, PEF and TT were 54 ± 13, 25 ± 5 and 18 ± 5 ppm respectively. There was not a statistical difference between both treatment processes probably due to the great variability within each variety.

These results do not mean that proteins disappeared from the juice. It could be that the treatment had changed the sites that binds with the dye used in the analysis. Such a structural modification would agree with the current proposed mechanism of interaction between PEF and enzymes that suggests changes of the secondary or tertiary structures [270]. Nutritionally speaking, grape juice is not a good source of proteins although they play a role in wine-making industry were nitrogen fractions of grape juice are related with the final wine aromatic profile [84].

The reduction of the antioxidant activity was higher for the grapes thermally processed ($13\,\% \pm 2\,\%$) than for the PEF treated juice ($9\,\% \pm 2\,\%$). Other authors that also compared PEF and heat treatments on tomato juice did not observe any effect on its antioxidant activity [175]. However, performing a similar study on strawberry juice it has been confirmed a statistical reduction of the antioxidant capacity in the processed juice even though the authors could not detect any difference between both treatments [177]. In orange juice and gazpacho soup there were no differences in antioxidant capacity between PEF-treated and untreated products, whereas heat-treated foods showed lower values of antioxidant capacity [60]. These apparently different behaviours could be merged if the antioxidant capacity is considered an index that gathers the contributions of several factors including among them the phenolic profile and the ascorbic acid content [109, 108], which are variable among species and even among varieties of the same specie [42].

In this case, the interaction between the processing treatment factor and the variety factor was significant. That means that the results produced by each processing treatment depend on the grape juice variety. It can be seen in figure 7.1 where it seems clear that Mazuelo always have the highest antioxidant capacity. However, the relative radical scavenging activity of each treatment changes with each variety of the white grape juices. This suggests that more experiments should be performed on other varieties so that it could be understood the specific effect of PEF on this topic.

The analysis of the polyphenol content of the grape juices corresponding to the optical density measured at 280 nm indicated that the statistical significance of the treatment factor was on the boundary ($p = 0.05$). In addition, the data shown the unusual fact that the polyphenol content of Parellada grape juice was higher than the content of a red variety such as Mazuelo (Fig. 7.1).

It was measured the Folin-Ciocalteu index to contrast these data. The statistical analysis indicated that the processing treatment factor had no effect on the results and at the same time produced a more common polyphenol profile (Fig. 7.2). It is thought that Mazuelo grape juice macerating time was so short

Table 7.3: Statistical significance and estimation values of the analysed factors corresponding to the nutritional variables of grape juice.

Variable	Processing effect (P)	Variety effect (V)[a]
Protein (ppm BSA)	Yes	23
Radical scavenging activity (%)	Yes	5
Vitamin C (ppm ascorbic acid)	No	0.3
Polyphenol content (OD_{280})	No	0.6
Polyphenol content (Folin index)	No	21
Non-flavonid content (Folin index)	No	24
Free catechin content (DMAC index)	No	7.4
Cinnamic acid content (OD_{320})	No	0.8

[a] Obtained as variances but here expressed as standard deviations to facilitate the comparison and comprehension.

that the polyphenol content of the juice was lower than the usual and very close to the typical values for white grape varieties.

The results of the remaining nutritional studied variables (vitamin C, non-flavonoids, catechins, and cinnamic acid derivatives contents) show a dependency with the combination of both factors as occurred with the antioxidant activity. Consequently, the variety factor hinders the adequate evaluation of the effects of the processing treatment factor, so it can no be ruled out without getting new data to clarify the effect of the processing treatments [53]. Conversely, if this interaction term had been non-significant it would confirm the absence of any treatment effect which was the main reason to replicate the experiments.

However, it is possible to observe some trends with the current information. Vitamin C content loses seem higher for Mazuelo and Moscatel varieties after the heat treatment than after PEF treatment (Fig. 7.1). This supposed behaviour would be supported by works performed on other juices such as strawberry [177], tomato [175], orange and a vegetable soup [60] have shown that usually PEF treatments preserve the vitamin C better than heat treatments.

Regarding the different constituents of the grape polyphenols, the results show that the non-flavonoid species are the main polyphenol fraction (Fig. 7.2). Their mean values after each processing treatment shows certain similitude with the cinnamic acids content probably due to the fact that the cinnamic acid derivatives are the main non-flavonoid compounds of the juices [126]. Even so, the trend of the Mazuelo grape juice with the processing treatment did not match at all. Cinnamic acid derivatives play an important role in wine colour as the precursors of the pyranoanthocyanins. This chemical species contribute to the red-orange colour of aged white wines. Besides, they reduce the colour loss due to pH and oxidative degradation stabilising [21, 161].

The free catechin content provided by the DMAC index is inversely related with the condensed tannic compounds of the grape juice. It seems clear that any processing effect raises its content in the Moscatell variety, although apparently the effects of the heat treatment were higher. Mazuelo free catechin content was not influenced by any treatment whereas in Parellada the values of the heat processed juice look higher than the obtained for the unprocessed and the PEF processed juices.

Then despite the fact that it was not possible to confirm these tendencies with the current results, it can be stated that, in general, the PEF treatment yield intermediate values among the non-processed and the thermally processed grape juices.

7.4. Conclusions

The PEF processing treatment yielded milder effects on any variable when compared with the thermal processing treatment.

Soluble solids, pH, acidity, electrical conductivity of grape juice were not af-

fected by PEF processing treatment. Microbial populations were clearly reduced by both treatments with a very similar pattern for all the tested varieties. As far as the remaining studied variables more experimentation should be done to confirm the suggested tendencies showed by the current work.

In addition, it can be drawn that grape variety has a strong influence on the results and that it should be taken into account when studying the effects of PEF on grape juice. In this context, it would be very informative similar PEF and thermal processing comparison of other grape varieties to enhance the scope of the obtained data.

8 Influence of SO_2 on the consumption of nitrogen compounds through alcoholic fermentation of must sterilized by pulsed electric fields

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Abstract

The aim of this work was to study the influence of SO_2 on the use of nitrogenous compounds by yeast through wine alcoholic fermentation. Thus Parellada must was sterilized by a pulsed electric field treatment and inoculated with *S. cerevisiae* Na33 strain. The fermentations were carried out with SO_2 ($20\,mg{\cdot}L^{-1}$) and without SO_2. The obtained results shown that yeast better consumed the aminoacids in the first half of fermentation with the presence of SO_2. The final concentration of amino acids in the obtained wine was greater when the must fermented without SO_2 than when the latter compound was present. Therefore, it could be stated that the presence of SO_2 facilitated the consumption of amino acids and, hence, the wine should have more complex flavour and microbiological stability than that obtained from fermentation without SO_2.

8.1. Introduction

The nitrogen fraction of must is complex, variable and is involved in the kinetics of fermentation and aroma production [26, 248] because nitrogen compounds are essential to the growth and metabolism of yeast. However, unless they are completely consumed after the fermentation process they can promote microbiological instability because of the bacterial growing, and the production of ethyl carbamate, which is a carcinogenic compound [182]. Some factors affecting the yeast assimilation of nitrogen compounds from the must such as must composition [120], must clarification [18], yeast strain [183], pH, temperature and ethanol content [102] have already been studied. Nevertheless, the effect of a widespread preservative like sulphur dioxide on the nitrogen metabolism of yeasts has not been reported yet.

Sulphur dioxide is used in vinification as an antiseptic against undesirable microorganisms and as an antioxidant against the effects of the oxygen. It has also some effect on the activity of certain grape enzymes that promote the loss of quality of the juice and its derivatives. It is the case of polyphenoloxidases, including tyrosinase and peroxidase. These enzymes occur in the must, arising from the grape itself, or from fungi that have infected the grapes. SO_2 inactivates these enzymes by reducing their copper cofactor [219]. Once dissolved in water, sulphur dioxide is a weak acid that renders other inorganic compounds called generically SO_2-free forms. In addition, each form reacts with several components of the must to produce SO_2-combined forms. The concentrations of each product depend mainly on the temperature, pH and the initial concentration of sulphur dioxide since these factors have a strong influence on the equilibrium of the chemical reactions. In general, at low concentrations and high pH, sulphur dioxide has a fungistatic effect whereas at high concentrations and low pH acts as a fungicide. It is well accepted that yeast are mainly affected by SO_2-free forms that disturb essential biological paths after entering the yeast cells. Macris [138] proposed that *S. cerevisiae* uptakes SO_2 by active transport although other study [243] suggested that diffusion could be bound to occur. Once inside the cell, SO_2 would induce changes in enzymatic 3D-conformations and would cause depletion in the yeast's cellular ATP content due to its effects on glycolysis and respiratory chain phosphorylation [139]. Thus, the SO_2 could influence in the utilization of amino acids by the yeasts. Regarding bacteria, all SO_2-compounds have antibacterial activity and so it is possible to act selectively on the different microorganisms present in must [105]. However, sulphur dioxide could have negative effects on human health [219]. For that reason, several international organizations (WHO, FAO, OIV) have set down maximum limits for the vinification ($350\,mg{\cdot}L^{-1}$) as well as promoted a reduction of its concentration in foodstuffs, specifically in wines.

Pulsed electric field (PEF) technology has been used to preserve fruit juice and to delay the spoilage by microorganisms [59, 58, 96]. Recently, this technology has been implemented for the production of commercial fruit juices in the USA whereas some important food processors of the EU are trying this technology at a pilot plant level. A previous study showed that a PEF treatment did not affect to the content of nitrogen compounds and fatty acids of must [82]. This is an important issue since these compounds are essential for the development of the yeasts during fermentation. It has also been reported that PEF treatments also decreases the activity of enzymes as peroxidases and polyphenoloxidases in apple and pear extracts [87], peach puree [89] and orange juice [57]. The proposed mechanism of enzymatic inactivation might be related with the change of specific structures of the enzymes [271]. However, as far as we know, the combination of both PEF and SO_2 has not been considered previously in grape juice processing.

Due to the fact that a PEF treatment allow reducing the level of sulphur dioxide to guarantee the biochemical and microbiological stability of the must, the aim of this study was to assess the effect of the sulphur dioxide content on

the consumption of nitrogen compounds throughout the alcoholic fermentation of must processed by PEF. Thus this study could be a starting point leading to an effective reduction of the sulphur dioxide content in wines.

8.2. Materials and methods

8.2.1. Must sterilization

A local wine manufacturer, Raimat (Lleida, Spain), kindly provided grapes (*V. vinifera* variety Parellada). They were harvested at ripeness and then, washed, drained, split from bunches and frozen at −20 °C until processing. The grapes were thawed at 5 °C for 24 h, afterwards they were manually pressed obtaining 12 L of must that was de-aerated by stirring under reduced pressure for 30 min.

A laboratory scale PEF unit (Ohio State University, Columbus, OH, USA) was used to treat must. The pulse generator module consists of a high voltage generator (OSU-4F), which can supply differences of electric potential between the electrodes of the treatment chambers of up to 12 kV, and a pulse generator unit model 9412A (Quantum Composers, Inc., Bozeman, MT, USA), which can render square wave pulses of up to 10 µs and 2000 Hz. PEF treatment was performed with bipolar electric field pulses of 4 µs width and with an electric field strength of 35 $kV \cdot cm^{-1}$. The pulse repetition rate was 1000 Hz and the total PEF treatment time was 1 ms, which was calculated as the product of the pulse width and the number of pulses delivered to must [82].

8.2.2. Vinification

The PEF-processed must was divided into four batch of 3 L. Diammonium phosphate (DAP) was added to must until reaching approximately 55 $mg \cdot L^{-1}$ of nitrogen due to the low ammonium content of the must. Two lots of must were kept without any preservative and the other two lots were supplied with potassium metabisulphite up to a concentration of 20 $mg \cdot L^{-1}$ of total SO_2. After that, all musts were inoculated with an active dry of the Na33 strain of *S. cerevisiae* subspecie cerevisiae selected from the Estación de Viticultura y Enología de Navarra (Olite, Spain) and commercialized by Lallemand España. Na33 strain was inoculated in the musts in a proportion of 0.2 $g \cdot L^{-1}$ rehydrating 0.65 g of dry in a sterile flask with 7.5 mL of distilled water containing 0.07 g of sucrose for 30 min at 35 °C that provide more than 2×10^9 viable cells per millilitre.

The fermentations took place in glass fermentors with a capacity of 3.5 L and with a burnished lid with two outlets, one of them for sample extractions and the other with a CO_2 trap to eliminate it from the fermentative environment and prevent the entrance of air during fermentation. The hole for sample extraction was covered with a septum during the fermentation. The fermentors

were placed over magnetic stirrers (Ikamag RCT basic, Milian SA, Geneve, Switzerland) at 630 r/min, to ensure a homogenous fermentation. The fermentations were carried out in a hot-cold incubator (Selecta, Barcelona, Spain) at a controlled temperature of 18 °C. The fermentations were daily measured for sugar concentration through their refractive index at 20 °C, using a refractometer ABBE model 325 (Misco, Cleveland, OH, USA) and through enzymatic measures (reagents from Chema Italia, Rome, Italy) using a multi-parametric analyzer Enochem (Tecnología Difusión Ibérica, Barcelona, Spain). This is an automated device where the appropriate reactions take place. It automatically provides the necessary reactants and also performs the spectrophotometric measurement of the absorbance changes after the programmed incubation time. Samples were taken before the beginning of the fermentation, at 50% of consumed sugars and at the end of fermentation when reducing sugars were below 2.5 $g \cdot L^{-1}$. All recipients and materials, which were in contact with the samples, were previously sterilized.

8.2.3. Analysis of free amino acids

Analysis were performed with a Waters high-pressure liquid chromatography (Milford, MA, USA) equipped with two 510 pumps, a 717 Plus Autosampler, and a 996 Photodiode Array Detector used at 254 nm. A Pico.Tag reverse phase column (300 mm-length × 3.9 mm i.d.) with a stationary phase of dimethyl-octadecylsilyl (ODS) bonded to amorphous silica was used. Amino acid derivatization was performed using a Waters Pico.Tag workstation. The Pico.Tag method used for amino acid analysis is described in Ayestaran [18]. Samples were cleaned by ultra filtration with a Millipore Ultrafree MC cartridge (Billerica, MA, USA), and then L-norleucine and L-methionine sulfone (Aldrich, Gillingham, England) were added as internal standards. Afterwards, a precolumn derivatization was carried out with phenyl isothiocyanate (Pierce Biotechnology, Rockford, IL, USA). A Millennium 32 software package (Waters) was employed for chromatographic control. The amount of sample injected was 10 µL. The column temperature was set at 46 °C.

Mobile phase A was a solution of 2.5% of acetonitrile (Scharlau, Barcelona, Spain), and 97.5% of a solution of sodium acetate (70 mM) with pH adjusted to 6.55 with acetic acid (10%) (Merck, Darmstadt, Germany). Mobile phase B was made of acetonitrile, water and methanol (Scharlau) with a volumetric proportion of 45:40:15, respectively. The mobile phases used were filtered through a 0.45 µm Millipore filter.

Amino acids were eluted at 1 $mL \cdot min^{-1}$ of flow rate with linear gradients from 0% to 3% of eluent B in 13.5 min, from 3% to 6% of eluent B in 10.5 min, from 6% to 9% of eluent B in 6 min, from 9 to 34% of eluent B in 20 min, maintained during 12 min, from 34% to 100% of eluent B in 0.5 min, maintained during 4 min, followed by washing and reconditioning the column. Amino acids determination was repeated four times for each sample. The concentrations of

amino acids are given as the mean value and the standard deviation of 8 analyses since the fermentations were carried out in duplicate.

8.2.4. Nitrogenous fractions and oenological parameters

The ammonium content was analysed by enzymatic measurement of the ammonium cation present in the samples using the multi-parametric analyzer Enochem, using reactives from Chema Italia. The free amino acid content measured by HPLC was taken as the amino nitrogen value whereas the assimilable nitrogen was calculated as the sum of the ammonium and the amino nitrogen without taking into account the proline concentration. All the measurements were performed four times.

Determinations of acetic acid, total SO_2, acetaldehyde and total polyphenols were made in the multi-parametric analyzer Enochem by enzymatic and colorimetric methods. The pH was determined by using a pH-meter Metrohm 702 (Metrohm, Herisau, Germany). The total acidity was determined following the method described by the OIV [179]. The alcoholic level of the final wine was determined by using a Salleron-Dujardin ebulliometer (Paris, France). All the fermentations and analyses were made in duplicate so that the values shown on table 8.1 are the mean value of 4 analyses.

8.3. Results and discussion

8.3.1. Oenological parameters

The wines obtained from the inoculated must fermented with or without SO_2 did not show the presence of acetic acid (Tab. 8.1). This result could be due to the fact that the inoculated yeast (*S. cerevisiae* Na33 strain) produces a very low concentration of acetic acid [75]. In general, *Saccharomyces* yeasts produce less acetic acid than other yeasts [217].

The concentration of acetaldehyde was higher at the beginning of the fermentation in the samples fermented in presence of SO_2 than in those fermented without SO_2, which is know to enhance the production of acetaldehyde [103]. At the end of the fermentation, higher concentration of acetaldehyde was observed in the wine obtained from the fermentation without SO_2 (Tab. 8.1).

During the alcoholic fermentation, the concentration of total polyphenols decreased in the studied samples. Polyphenols (including catechins, proanthocyanidins, cinnamic acids and their derivatives) are subject to oxidation so that the initial straw yellow colour of white wines turns into the deep golden yellow typical of browned wines [140]. The wines obtained from both fermentations with or without SO_2 presented similar levels of total polyphenols (Tab. 8.1), therefore the SO_2 content did not affect to the final polyphenol concentration.

Table 8.1: Oenological parameters at the beginning of the fermentation process, at 50% of consumed sugars and of the final wines obtained from fermentation with and without SO_2.

Measured property[a]	Must	50% of consumed sugar		Wine	
		with SO_2	without SO_2	with SO_2	without SO_2
Alcohol (%)[b]	n.d.[c]	4.2 ± 0.2	4.1 ± 0.1	8.3 ± 0.2	8.5 ± 0.5
Sulphur dioxide ($mg \cdot L^{-1}$)	19.5 ± 0.2	13 ± 1	n.d.	8 ± 1	n.d.
pH	3.83 ± 0.02	3.22 ± 0.04	3.17 ± 0.02	3.24 ± 0.02	3.25 ± 0.01
Total acidity ($g \cdot L^{-1}$)[d]	2.43 ± 0.05	3.5 ± 0.2	4.1 ± 0.1	4.1 ± 0.1	4.33 ± 0.07
Acetic acid ($g \cdot L^{-1}$)	n.d.	n.d.	n.d.	n.d.	n.d.
Acetaldehyde ($mg \cdot L^{-1}$)	4 ± 0	17.5 ± 0.2	11 ± 1	1.7 ± 0.5	8 ± 1
Total polyphenols ($mg \cdot L^{-1}$)	250 ± 3	203 ± 4	189.5 ± 0.6	122 ± 2	122 ± 4

[a] All parameters are given with their standard deviations (n = 4).
[b] Expressed as volumetric proportion.
[c] Not detected.
[d] Expressed as tartaric acid.

Table 8.2: Nitrogenous fractions of the initial PEF treated must, at 50% of consumed sugars and of the final wines obtained from fermentation with and without SO_2.

Nitrogen species[a]	Must	50% of consumed sugar		Wine	
		with SO_2	without SO_2	with SO_2	without SO_2
Ammonium group ($mg \cdot L^{-1}$)	57.9 ± 0.5	3.7 ± 0.7	5 ± 1	2.3 ± 0.6	3.7 ± 0.7
Amino group ($mg \cdot L^{-1}$)	300 ± 1	129 ± 1	175 ± 4	71 ± 3	100 ± 6
Assimilable ($mg \cdot L^{-1}$)	347 ± 1	27 ± 1	68 ± 8	18.8 ± 0.6	31.7 ± 0.7

[a] The concentration are shown with their standard deviations (n = 8).

8.3.2. Nitrogenous fractions

The ammonium nitrogen of the initial must represented 17% of the assimilable nitrogen (Tab. 8.2). This fact could promote an increase of higher alcohols because the yeasts are forced to use the amino acids of must as nitrogen source [252]. The highest uptake of all nitrogen fractions occurred in the first half of the fermentation likely due to the exponential growth phase of yeast where nitrogen is used for biomass production [186]. The ammonium nitrogen was almost entirely consumed either with SO_2 (96%) or without SO_2 (94%). Regarding amino nitrogen, it was reduced up to a 76% when the fermentation was carried out with sulphur dioxide and a 67% in absence of SO_2 (Tab. 8.2). Thus, the total consumption of assimilable nitrogen was $328\,mg \cdot L^{-1}$ (95%) during the fermentation carried out with SO_2 and $315\,mg \cdot L^{-1}$ (91%) when SO_2 was not present (Tab. 8.2).

8.3.3. Must composition and utilization of amino acids during the fermentation

Arginine and proline, together with alanine were the most abundant amino acids in must (Tab. 8.3). They accounted for 68% of the total amino acids content of must. This fact agrees with data reported by other authors [102, 12]. The concentration of arginine in the initial must is the greatest by far accounting for 56% of total amino acids. This result is similar to those reported by other authors [254]. Parellada variety had a low proline/arginine ratio, what means that the must contained a high quantity of assimilable nitrogen for the yeasts. This ratio is influenced mainly by the grape variety, and the grape ripeness [26].

It is well known that the availability and metabolism of nitrogen compounds is among the key components of both, fermentation development and final wine quality. As can be seen in table 8.3 and table 8.4, amino acids are taken up by *S. cerevisiae* mainly during the first half of the fermentation. Besides, in this phase, the consumption of amino acids by yeasts was greater in the fermentation carried out with SO_2 ($1018\,mg{\cdot}L^{-1}$) than in that performed without this substance ($867\,mg{\cdot}L^{-1}$). The most consumed amino acid was the most abundant, that is, arginine; this amino acid presented a higher consumption in the fermentation carried out in presence of SO_2 ($689\,mg{\cdot}L^{-1}$) than in the fermentation without SO_2 ($596\,mg{\cdot}L^{-1}$). Arginine and ammonium ions were the yeasts' principal source of nitrogen during fermentation. Alanine was consumed in a great extension by yeasts during both fermentations (with SO_2, $54.2\,mg{\cdot}L^{-1}$; without SO_2, $50.8\,mg{\cdot}L^{-1}$). Leucine, threonine, tryptophan, tyrosine, isoleucine, γ-amino butyric acid, and citrulline were completely consumed in this phase of fermentation in both cases since they are suitable nitrogen sources [102]. Glutamic acid was not consumed in none of the two fermentations though this amino acid is a preferred source for the yeasts (Tab. 8.3). This phenomenon could be attributed to the fact that the arginine consumption was very high and glutamate anion is among the final products of arginine metabolism, so yeasts did not need to take glutamic acid from the medium. The lysine and glycine contents did not decrease in this step of the fermentations because these amino acids are not considered good nitrogen sources for *S. cerevisiae* although they could be metabolised by microorganisms in other fermentations [47]. Yeasts did not consume proline during the initial stage of fermentation because this amino acid is uptaken by the yeasts only under severe nitrogen stress conditions and the presence of ammonium in the medium will inhibit or repress the uptake of proline [184]. The liberation of proline to the medium might be due to the metabolism of arginine since it is an intermediate product in the degradation of arginine [182]. It is thought that, at the beginning of fermentation, *S. cerevisiae* obtain the necessary nitrogen compounds for cellular division from the medium without performing any modification on them. Once the first necessities of each amino acid are satiated, yeasts usually take those amino acids in excess in the medium to use them as nitrogen source [34].

Table 8.3: Concentration of protein amino acids in the initial PEF treated must, at 50% of consumed sugars and in the final wines obtained from fermentation with and without SO_2.

Amino acids[a]	Must	50% of consumed sugar		Wine	
		with SO_2	without SO_2	with SO_2	without SO_2
Arginine (Arg)	692 ± 21	3.0 ± 0.1	96 ± 7	3.03 ± 0.09	13 ± 1
Proline (Pro)	89 ± 3	874 ± 33	919 ± 40	446 ± 12	591 ± 25
Alanine (Ala)	58 ± 2	3.8 ± 0.1	7.2 ± 0.2	8.7 ± 0.2	11.9 ± 0.7
Leucine (Leu)	30 ± 1	n.d.[b]	n.d.	n.d.	1.2 ± 0.1
Serine (Ser)	28 ± 2	3.1 ± 0.1	2.7 ± 0.2	2.4 ± 0.4	2.5 ± 0.6
Threonine (Thr)	26 ± 4	n.d.	n.d.	n.d.	n.d.
Thryptophan (Trp)	23.5 ± 0.2	n.d.	n.d.	n.d.	n.d.
Glutamic acid (Glu)	22.5 ± 0.8	21 ± 1	34 ± 2	22.1 ± 0.2	22 ± 1
Aspartic acid (Asp)	21.7 ± 0.8	3.2 ± 0.1	8.2 ± 0.5	4.2 ± 0.2	5.3 ± 0.3
Valine (Val)	21 ± 2	n.d.	1.7 ± 0.1	n.d.	1.5 ± 0.2
Asparagine (Asn)	20.4 ± 0.8	2.8 ± 0.1	7.4 ± 0.3	3.7 ± 0.3	7.2 ± 0.2
Tirosine (Tyr)	$16..3 \pm 0.6$	n.d.	n.d.	n.d.	2.0 ± 0.1
Histidine (His)	16 ± 1	1.7 ± 0.3	n.d.	1.9 ± 0.1	2.6 ± 0.2
Methionine (Met)	15 ± 1	n.d.	5.3 ± 0.3	n.d.	4.0 ± 0.2
Phenylalanine (Phe)	14.8 ± 0.3	0.81 ± 0.01	n.d.	n.d.	0.45 ± 0.03
Isoleucine (Ile)	10.3 ± 0.3	n.d.	n.d.	n.d.	n.d.
Lysine (Lys)	5 ± 1	8 ± 1	4.8 ± 0.4	3.0 ± 0.1	3.1 ± 0.2
Glycine (Gly)	3.7 ± 0.3	4.8 ± 0.2	16 ± 2	3.0 ± 0.2	7.0 ± 0.7

[a] The concentration are shown in $mg \cdot L^{-1}$ with their standard deviations (n = 8).
[b] Not detected.

In the second half of fermentation, the amino acid uptake was the same in both types of fermentation ($466\,mg \cdot L^{-1}$), so that the concentration of SO_2 did not affect to the amino acids consumption in this phase of fermentation. The total decrease of amino acids was lower than the achieved in the first part of the fermentation (Tab. 8.3). Conversely to the first stage, proline was the most consumed amino acid accounting for 49% ($428\,mg \cdot L^{-1}$) and 36% ($328\,mg \cdot L^{-1}$) of the total in the fermentation with and without SO_2, respectively (Tab. 8.3). The yeasts could use this amino acid in this phase of the fermentation since there were less good nitrogen sources in the medium than at the beginning of the fermentation (Tab. 8.3). When the good nitrogen sources depleted, the general amino acid permeases and the specific permeases such as proline permease allow the accumulation of the poorer nitrogen sources as proline [26]. The remaining amino acids underwent few variations in their concentrations except arginine, glutamic acid and glycine, which were significantly reduced their concentrations in the fermentation without SO_2, and alanine, which was excreted in both types of fermentation (Tab. 8.3). Non-proteic amino acids were consumed in greater extension through the second half of the fermentation (Tab. 8.4).

At the end of the fermentation, high ethanol concentration usually alters the structure and permeability of the plasmatic cell membrane accelerating the passive entry of protons as the electrochemical gradient between both membrane faces decreases in a similar way [40]. While this process takes place, some yeasts

Table 8.4: Concentration of non-protein amino acids in the initial PEF treated must, at 50% of consumed sugars and in the final wines obtained from fermentation with and without SO_2.

Amino acids[a]	Must	50% of consumed sugar		Wine	
		with SO_2	without SO_2	with SO_2	without SO_2
γ-Aminobutyric acid (Gaba)	35 ± 3	n.d.[b]	n.d.	n.d.	n.d.
Cystathionine (Cyst)	30 ± 1	15 ± 1	21 ± 2	11 ± 1	13 ± 1
Creatinine (Creat)	19 ± 2	27 ± 1	36 ± 1	18.7 ± 0.1	27 ± 1
Citrulline (Cit)	11.1 ± 0.4	n.d.	n.d.	n.d.	n.d.
Ornithine (Orn)	10.2 ± 0.7	1.9 ± 0.1	11 ± 1	1.0 ± 0.1	8 ± 1
Hydroxyproline (Hyp)	6.9 ± 0.7	22 ± 2	18 ± 1	0.76 ± 0.03	2.6 ± 0.1
Phosphoserine (Pser)	6.1 ± 0.2	6.7 ± 0.1	5.9 ± 0.1	2.9 ± 0.1	2.4 ± 0.2

[a] The concentration are shown in $mg{\cdot}L^{-1}$ with their standard deviations (n = 8).
[b] Not detected.

also release amino acids into the wine by a passive process of desorption. All these processes are the physiological response to the exhaustion of sugars [33]. However, the yeast strain used in our study was resistant to the presence of ethanol, which is a specific strain characteristic [121]. Thus, the yeasts did not release amino acids at the end of the fermentation but they continued consuming amino acids. At the end of the vinification process, the wine obtained from the fermentation with SO_2 had less concentration of amino acids ($532\,mg{\cdot}L^{-1}$) than the wine obtained from the fermentation without SO_2 ($728\,mg{\cdot}L^{-1}$).

8.4. Conclusions

The consumption of amino acids was higher when the fermentation of the must was performed with sulphur dioxide. SO_2 content affected to the consumption of total amino acids in the first phase of fermentation. Arginine was the most abundant amino acid in the must and it was also the most consumed amino acid in the fermentation. Its consumption was favoured in the fermentation carried out in presence of sulphur dioxide. Throughout the second part of fermentation the most consumed amino acid was proline and the presence of SO_2 also enhanced its consumption. The final content of amino acids in the wine obtained from the fermentation without SO_2 was greater than that measured in the wine obtained from the fermentation with this compound. Therefore, it could be stated that the presence of sulphur dioxide promoted the consumption of total amino acids and hence the wine obtained from the fermentation with SO_2 should have more complex flavour and microbiological stability than that obtained from the fermentation without SO_2.

9 Influence of SO_2 on the evolution of volatile compounds through alcoholic fermentation of must stabilized by pulsed electric fields

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Abstract

The aim of this work was to study the influence of sulphur dioxide (SO_2) on the formation of volatile compounds by yeast through wine alcoholic fermentation. Thus Parellada must was microbiologically stabilized using a pulsed electric field (PEF) treatment and inoculated with *S. cerevisiae* Na33 strain. Fermentation was carried out with or without SO_2 and the results showed that the evolution of the volatile compounds profile throughout the process was similar. The content of volatile acids in wine obtained by using sulphur dioxide was not significantly different from that fermented without adding the compound. However, the final content of total alcohols and esters was significantly different even thought the differences were small. Consequently, when grape must is treated by PEF the sulphur dioxide concentration usually used in winemaking could be reduced to safer levels or even eliminated without an important effect on the volatile compounds content of the final product. Therefore, the absence of sulphur dioxide should not have a negative impact on the sensory characteristics of wine.

9.1. Introduction

The compounds formed during alcoholic fermentation have a decisive influence on the volatile composition of wine. The major volatile products of yeast metabolism such as ethanol, glycerol, and carbon dioxide have a relatively small but fundamental contribution to wine flavour. The main groups of compounds that form the fermentation bouquet are esters, alcohols and acids [128]. The formation of these compounds depends on several factors where yeast strain,

fermentation conditions and type of vinification are the most important parameters [130, 55, 11, 12].

Sulphur dioxide (SO_2) is used in vinification as an antiseptic against undesirable microorganisms as well as an antioxidant to avoid the effects of the dissolved oxygen. It has also some effect on the activity of certain grape enzymes that promote the loss of quality of the must and its derivatives. It is the case of polyphenoloxidases, including tyrosinase, and peroxidase. These enzymes occur in the must, arising from the grape itself, or from fungi that have infected the grapes. SO_2 inactivates these enzymes by reducing their copper cofactor [219]. It is well accepted that yeast is mainly affected by SO_2-free forms that disturb essential biological paths after entering the yeast cells. Macris [138] proposed that *S. cerevisiae* uptakes SO_2 by active transport although other studies [243] suggested that diffusion was bound to occur. However, SO_2 could have negative effects on human health and consequently several international organizations such as the World Health Organization (WHO), the Food and Agriculture Organization of the UN (FAO) or the Organisation Internationale de la Vigne et du Vin (OIV) have set down the maximum limits for vinification to promote a reduction of its concentration in foodstuffs, specifically in wines.

Pulsed electric field (PEF) technology has been used to preserve fruit juice and delay the spoilage by microorganisms [59, 58, 96]. A previous study showed that a PEF treatment did not affect the content of nitrogen compounds and fatty acids of must [82]. This is an important issue since these compounds are essential for the development of the yeasts during fermentation. It has been reported that PEF treatment also decreases the activity of polyphenoloxidase in apple and pear extracts [87], peach puree [89] and peroxidase in orange juice [57]. The proposed cause of enzymatic inactivation might be related with the change of specific structures of the enzymes [271].

Therefore the aim of this study was to evaluate the effect of the SO_2 content on the formation and evolution of volatile compounds throughout the alcoholic fermentation of must processed by PEF which could lead to a reduction of the sulphur dioxide content of wines.

9.2. Materials and methods

9.2.1. Measurement of enological parameters

The evolution of the fermentation process was followed by sampling daily and measuring the sugar concentration through their refractive index at 20 °C using a refractometer ABBE model 325 (Misco, Cleveland, OH, USA). The other studied parameters were monitored at the beginning of the fermentation, at 25, 50 and 75% of consumed sugars and at the end of fermentation when the content of reducing sugars was below $2.5\,g{\cdot}L^{-1}$.

Sugar, acetic acid, SO_2, acetaldehyde and total polyphenol contents were analysed using a multi-parametric analyser Enochem (Tecnología Difusión Ibérica,

Barcelona, Spain). This is an automated device to perform spectrophotometric measurement of the samples after the addition of the necessary reactants (Chema Italia, Rome, Italy) and a programmed incubation time to complete enzymatic reactions. The pH was determined by using a pH-meter Metrohm 702 (Metrohm, Herisau, Germany). The total acidity was determined following the method described by the Office International de la Vigne et du Vin [179]. The alcoholic level of the final wine was determined by using a Salleron-Dujardin ebulliometer (Paris, France). All analyses were made in duplicate.

9.2.2. Analysis of volatile compounds by gas chromatography

For the analyses of the compounds of high volatility and high concentration (ethyl acetate, n-propanol, isobutanol, and isoamyl alcohols) the method outlined by Fraile [76] was used. These compounds were analysed by direct injection of 0.5 µL of sample in a gas chromatograph Shimadzu GC-14B (Shimadzu, Kyoto, Japan) with a flame ionisation detector (FID). A DB-WAX capillary column (30 m-length × 0.25 mm of internal diameter) with a crosslinked stationary phase (0.25 µm of film thickness) of polyethylene glycol (Cromlab, Barcelona, Spain) was used. Chromatographic conditions were as follows, He as carrier gas (30.9 $cm \cdot s^{-1}$); injector and detector temperature, 180 °C; oven temperature, 80 °C. The standards were prepared with reagents from Aldrich (Gillingham, England) at concentrations between 1 $mg \cdot L^{-1}$ and 400 $mg \cdot L^{-1}$.

For the analyses of the middle-range volatility and, in general, present in lesser concentrations than the former ones the method outlined by Garde [80] was used. To extract middle-range volatile compounds, solid-phase extraction (SPE) with cartridges LiChrolut EN resins (Merck, Darmstadt, Germany) was used. The analysis of the extract was carried out in a GC– MS Finnigan (San Jose, CA, USA) using the same DBWAX capillary column. The chromatographic conditions were, He as carrier gas (30.9 $cm \cdot s^{-1}$); injector temperature, 240 °C; temperature of the transfer line, 240 °C. The middle-range volatile compounds were separated using a temperature program with initial oven temperature of 40 °C for 5 min, a temperature gradient of 2 °C·min^{-1} to a temperature of 50 °C, maintained during 10 min, followed by a gradient temperature of 2 °C·min^{-1} to a final temperature of 240 °C, and a final time of 20 min (total run time = 135 min). The sample injected was 2.5 µL, using the splitless technique. The ionisation was produced by electronic impact at 70 eV. The operation mode was Full Scan, between 35 and 300 u. The dissolutions of the standards were prepared in dichloromethane HPLC quality (Panreac) from Aldrich reagents in concentrations between 0.1 $mg \cdot L^{-1}$ and 250 $mg \cdot L^{-1}$, to which heptanoic acid (Aldrich) was added as internal standard in the same concentration as in the samples. The identification of these compounds was made using the retention times and comparing either their mass spectra with a spectral library of known standard compounds. The standards of each compound were injected individually and thus a library was made of the characteristic mass spectra of each one of

the compounds. The quantification of these compounds was made in Full Scan. For the quantification, the area of the corresponding peaks was normalised by that of the internal standard and was interpolated in a calibration graph made through the analysis of standard solutions in dichloromethane. The recovery values were always higher than 78%.

The compounds of the middle-range volatility and their retention time (in minutes) were: isoamyl acetate (11.44), ethyl hexanoate (21.14), ethyl lactate (31.23), *n*-hexanol (32.55), ethyl octanoate (38.40), ethyl 3-hydroxybutyrate (44.06), butyric acid (51.01), ethyl decanoate (52.07), diethyl succinate (54.00), 3-(methylthio)-1-propanol (56.09), 2-phenylethyl acetate (61.14), hexanoic acid (63.18), benzyl alcohol (64.38), 2-phenylethanol (66.41), internal standard (heptanoic acid, 68.48), diethyl malate (73.05), octanoic acid (74.14), 4-methoxyacetophenone (76.48), decanoic acid (83.49), ethyl acid succinate (88.10), dodecanoic acid (92.42), tetradecanoic acid (100.58), hexadecanoic acid (108.42), tyrosol (111.54), octadecanoic acid (115.56), 9,12-octadecadienoic acid (116.56), vanillic acid (126.39), and tryptophol (129.44).

Each determination was performed four times and the results averaged. Since the fermentations were carried out in duplicate, the results, which appear in the figures, are the mean average of eight analyses.

9.2.3. PEF processing of grape must

A local wine manufacturer, Raimat (Lleida, Spain), kindly provided grapes (*V. vinifera* variety Parellada). They were harvested at ripeness and then, washed, drained, split from bunches and frozen at −20 °C until processing. The grapes were thawed at 5 °C for 24 h, after which they were manually pressed obtaining 12 L of must that was deaerated by stirring under reduced pressure for 30 min. A laboratory scale PEF unit (Ohio State University, Columbus, OH, USA) was used to treat the must. The pulse generator module consists of a high-voltage generator (OSU-4F), which can supply differences of electric potential between the electrodes of the treatment chambers of up to 12 kV, and a pulse generator unit model 9412A (Quantum Composers, Inc., Bozeman, MT, USA), which can render square wave pulses of up to 10 µs and 2000 Hz. PEF treatment was performed with bipolar electric field pulses of 4 µs-width and an electric field strength of 35 $kV \cdot cm^{-1}$. The pulse repetition rate was 1000 Hz and the total PEF treatment time was 1 ms, which was calculated as the product of the pulse width and the number of pulses delivered to the must [82].

9.2.4. Fermentation of must

Diammonium phosphate (DAP) was added to the must up to approximately 55 $mg \cdot L^{-1}$ to increase its low ammonium content. Two aliquots of 3 L were kept without the preservative and the remaining two were provided with potassium metabisulphite up to a final concentration of 20 $mg \cdot L^{-1}$ of SO_2. An active dry of

the Na33 strain of *S. cerevisiae* subspecie cerevisiae selected from the Estación de Viticultura y Enología de Navarra (Olite, Spain) and commercialised by Lallemand España was selected to perform the fermentation process. The Na33 strain was inoculated into the musts in a proportion of $0.2\,g{\cdot}L^{-1}$ rehydrating 0.65 g of active dry in a sterile flask with 7.5 mL of distilled water containing 0.07 g of sucrose for 30 min at 35 °C which yielded more than 2×10^9 cfu·mL^{-1}.

The must fermentation took place anaerobically in glass fermentors with a capacity of 3.5 L placed on hot-cold incubators (Selecta, Barcelona, Spain) to keep the temperature constant at 18 °C. The fermentors had several orifices to extract samples and to eliminate carbon dioxide from the environment. Magnetic stirrers (Ikamag RCT basic, Milian SA, Geneva, Switzerland) at 630 r/min ensured a homogeneous fermentation.

9.2.5. Statistical analysis

All the results shown in the table 9.1 and figures 9.1, 9.2, and 9.3 are expressed as mean and standard deviation. The data of the volatile compounds in the wines at the end of fermentation shown in figures 9.1, 9.2, and 9.3 were analysed statistically using the analysis of variance. The differences between means were compared using the least significant differences (LSD) test at 0.05 probability level.

9.3. Results and discussion

9.3.1. PEF processing

The PEF technology was used to remove the naturally occurring flora from grapes before the must fermentation so that the following inoculation with the selected yeast strain led to a reproducible fermentation process. The low standard deviations obtained between fermentation replicates (Tab. 9.1) could be a proof of the success of such an approach. This sterilization system is used in some food industries where fermentation takes place. The brewery industry eliminates native flora of raw materials just before inoculating the mix liquor with one or several selected strains of yeasts. By doing so, it is possible to keep reasonably constant the taste and remaining sensory properties of the final product independently of the raw material batch. However, in winemaking this method is avoided since the status of wine is grounded on the *traditionally made* slogan. The PEF treatments have the advantage of processing food products at low temperatures, thus avoiding the side effects of a thermal process, or at least they occur in less extension. Some studies have reported that apple and grape juices retained their properties and composition after a PEF treatment [82, 274].

Table 9.1: Enological parameters at the beginning PEF treated must, at 25, 50 and 75% of consumed sugars and of the final wines obtained from fermentation with and without SO_2.

Measured property[a]	Must	25% of consumed sugar		50% of consumed sugar		75% of consumed sugar		Wine	
		with SO_2	without SO_2	with SO_2	without SO_2	with SO_2	without SO_2	with SO_2	without SO_2
Alcohol (%)[b]	n.d.[c]	2.2 ± 0.2	2.1 ± 0.2	4.2 ± 0.2	4.1 ± 0.1	6.3 ± 0.5	6.1 ± 0.1	8.3 ± 0.2	8.5 ± 0.5
Sulphur dioxide ($mg \cdot L^{-1}$)	19.5 ± 0.2	10 ± 0	n.d.	13 ± 1	n.d.	10.8 ± 0.5	n.d.	8 ± 1	n.d.
pH	3.83 ± 0.02	3.19 ± 0.04	3.17 ± 0.03	3.22 ± 0.04	3.17 ± 0.02	3.20 ± 0.04	3.17 ± 0.03	3.24 ± 0.02	$3.25 \pm .01$
Total acidity ($g \cdot L^{-1}$)[d]	2.43 ± 0.05	3.48 ± 0.09	4.67 ± 0.04	3.5 ± 0.2	4.1 ± 0.1	3.88 ± 0.06	4.21 ± 0.06	4.1 ± 0.1	4.33 ± 0.07
Acetic acid ($g \cdot L^{-1}$)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetaldehyde ($mg \cdot L^{-1}$)	4 ± 0	28 ± 1	7.3 ± 0.5	17.5 ± 0.2	11 ± 1	9.8 ± 0.5	12 ± 1	1.7 ± 0.5	8 ± 1
Total polyphenols ($mg \cdot L^{-1}$)	250 ± 3	243 ± 3	192 ± 4	203 ± 4	189.5 ± 0.6	180 ± 8	190.5 ± 0.6	122 ± 2	122 ± 4

[a] All parameters are given with their standard deviations (n = 4).
[b] Expressed as volumetric proportion.
[c] Not detected.
[d] Expressed as tartaric acid.

9.3.2. Enological parameters

The wines obtained from the inoculated must fermented with or without SO_2 did not show the presence of acetic acid (Tab. 9.1). This result could be due to the fact that the inoculated yeast (*S. cerevisiae* Na33 strain) produces a very low concentration of acetic acid [80]. The concentration of acetaldehyde was higher at the beginning of the fermentation in the samples fermented in presence of SO_2 than in those fermented without SO_2, which is known to promote the production of acetaldehyde, as Herraiz [103] observed. At the end of the fermentation, a higher concentration of acetaldehyde was observed in the wine obtained from the fermentation without SO_2 (Tab. 9.1).

During the alcoholic fermentation, the concentration of total polyphenols decreased in the studied samples. Polyphenols, including catechins, proanthocyanidins, cinnamic acids and their derivatives, are subjected to oxidation so that the initial straw-yellow colour of white wines turns into deep golden yellow typical of browned wines [140]. The wines obtained from both fermentations with or without SO_2 displayed similar levels of total polyphenols (Tab. 9.1), therefore the SO_2 content did not affect the final polyphenol concentration.

9.3.3. Volatile compounds

Figure 9.1 shows the evolution of alcohols through the fermentation. It is clear that the formation of alcohols was higher during the first half of the fermentation, because the consumption of amino acids was higher in this stage of the fermentation [84]. The process with presence of sulphur dioxide hardly modified its content after middle fermentation whereas the increase of alcohols in samples without preservative was smooth from the beginning to the end (Fig. 9.1). Final alcohol content ($160\,mg{\cdot}L^{-1}$) of wines fermented without sulphur dioxide was around a 20% lower than in those fermented with it. This value corresponds to a half of the threshold for affecting adversely wine aroma that has been settled above $300\,mg{\cdot}L^{-1}$ [210].

The total alcohol concentration followed the same trend that isoamyl alcohol content since it is the main alcohol of wines [248]. The isoamyl content found was well above $60\,mg{\cdot}L^{-1}$, which is the sensory detection level of this compound [235]. A similar pattern was observed for 2-phenylethanol and tyrosol whereas the behaviour of *n*-propanol, tryptophol, 3-methyltio-1-propanol and *n*-hexanol was practically the same either using or not sulphur dioxide. Benzyl alcohol was the only compound whose content was higher in the fermentation without preservative than with its presence even though its effect on sensory properties is not important due to its high sensory threshold ($200\,mg{\cdot}L^{-1}$) [92].

As for volatile esters produced during fermentation, their evolution throughout the process is gathered in figure 9.2 where there is a plot that collects the general behaviour except for ethyl acetate. Ethyl acetate is considered separately due to its different contribution to wine flavour. Anyway, it is obvious

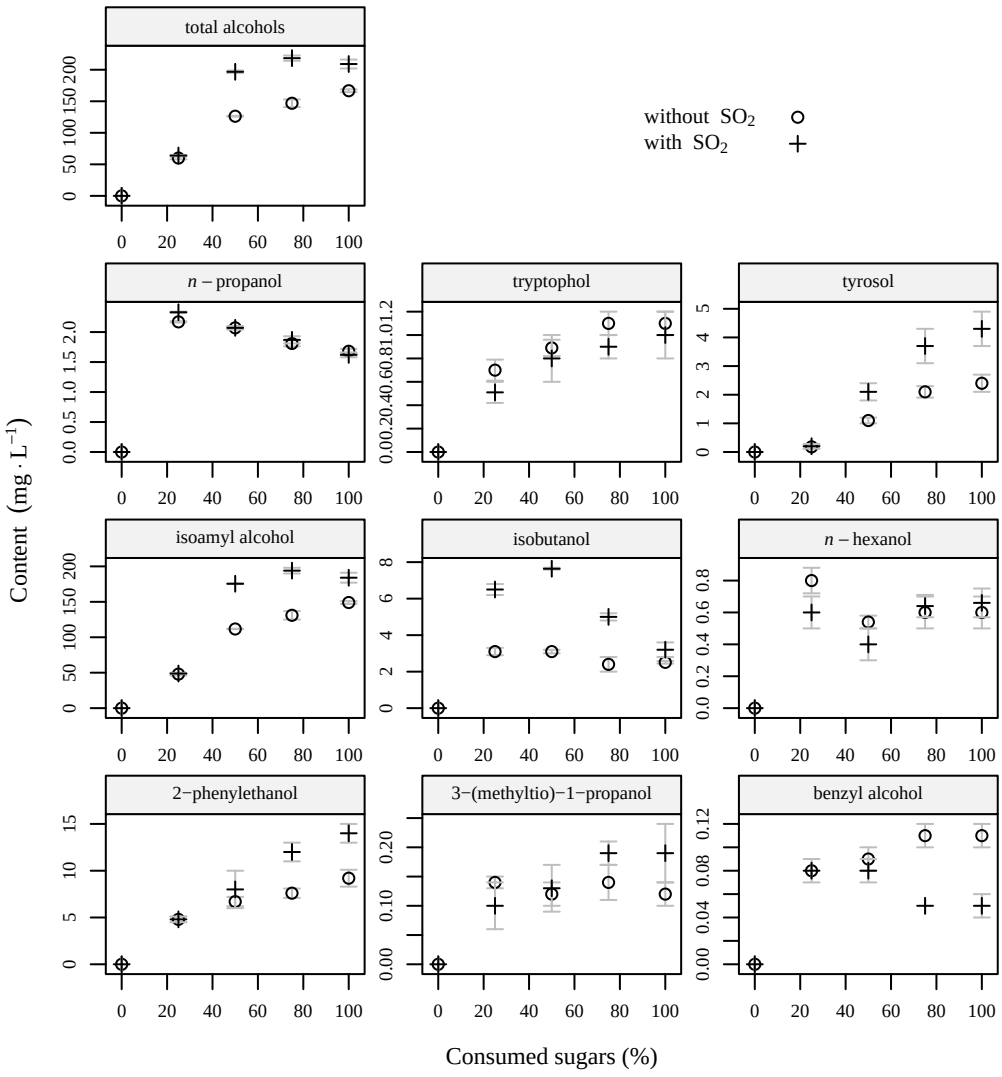

Figure 9.1: Evolution of several alcohol levels of Parellada grape juice through the fermentation process conditioned by the SO_2 content.

that, independently of the use of sulphur dioxide, the evolution profile for all of them was very similar having a substantial rise after 25% of consumed sugars followed by a gradual increase up to reaching the maximum at the end of the fermentation. This fact agrees with Jackson [116] who reported that ester production by yeast is inhibited by the presence of oxygen that could be dissolved in some degree at the beginning of the fermentation. Besides, in general, must fermented without SO_2 had higher total ester content than must with it. However, only ethyl hexanoate showed significant differences between both types of fermentation, which agrees with the result obtained by Herraiz [104]. This author found that ethyl hexanoate was formed in greater quantity in the fermentation of Verdejo must made without SO_2 than in the fermentation with SO_2.

Ethyl acetate confers complexity to the wine fragrance below $50\,mg{\cdot}L^{-1}$, whereas it is bound to add vinegar off-odours to the wine above $150\,mg{\cdot}L^{-1}$ [7]. Both types of fermentation yielded a similar final concentration, which was less than $50\,mg{\cdot}L^{-1}$, despite being the most abundant ester produced [128]. Isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate concentrations, which play a significant role in wine flavour, were above their sensory threshold levels ($1\,mg{\cdot}L^{-1}$, $0.08\,mg{\cdot}L^{-1}$, $0.58\,mg{\cdot}L^{-1}$ and $0.51\,mg{\cdot}L^{-1}$, respectively) [235, 65]. These data suggest that the absence of sulphur dioxide should not decrease the quality of wine.

Fatty acids were the third group of substances analysed. They showed a more homogeneous behaviour between fermentations with and without sulphur dioxide than the previous compounds, since both the trend and the measured concentrations were practically the same throughout the process (Fig. 9.3). Similar outcomes have been reported by other authors [76, 84].

The final concentration of fatty acids was $15\,mg{\cdot}L^{-1}$ regardless of the presence of sulphur dioxide. These compounds produce changes in the wine flavour depending on their concentrations and it has been described that the limit of perception is around $20\,mg{\cdot}L^{-1}$ [196]. At this level, fatty acids add a fresh flavour to wine increasing the appreciation of other taste sensations whereas higher values confer an unpleasant flavour. Consequently, the obtained results indicate that the elimination of the preservative during fermentation did not produce any damage to the typical notes that fatty acids give to the wine flavour when the must is stabilized by PEF. Consequently, PEF treatments together with the inoculation of a selected strain of yeast may lead to a reproducible fermentation process of must to wine without modifying significantly the composition of volatile compounds responsible for the typical flavour of wines. Besides, this approach should allow reducing or even eliminating the use of sulphur dioxide.

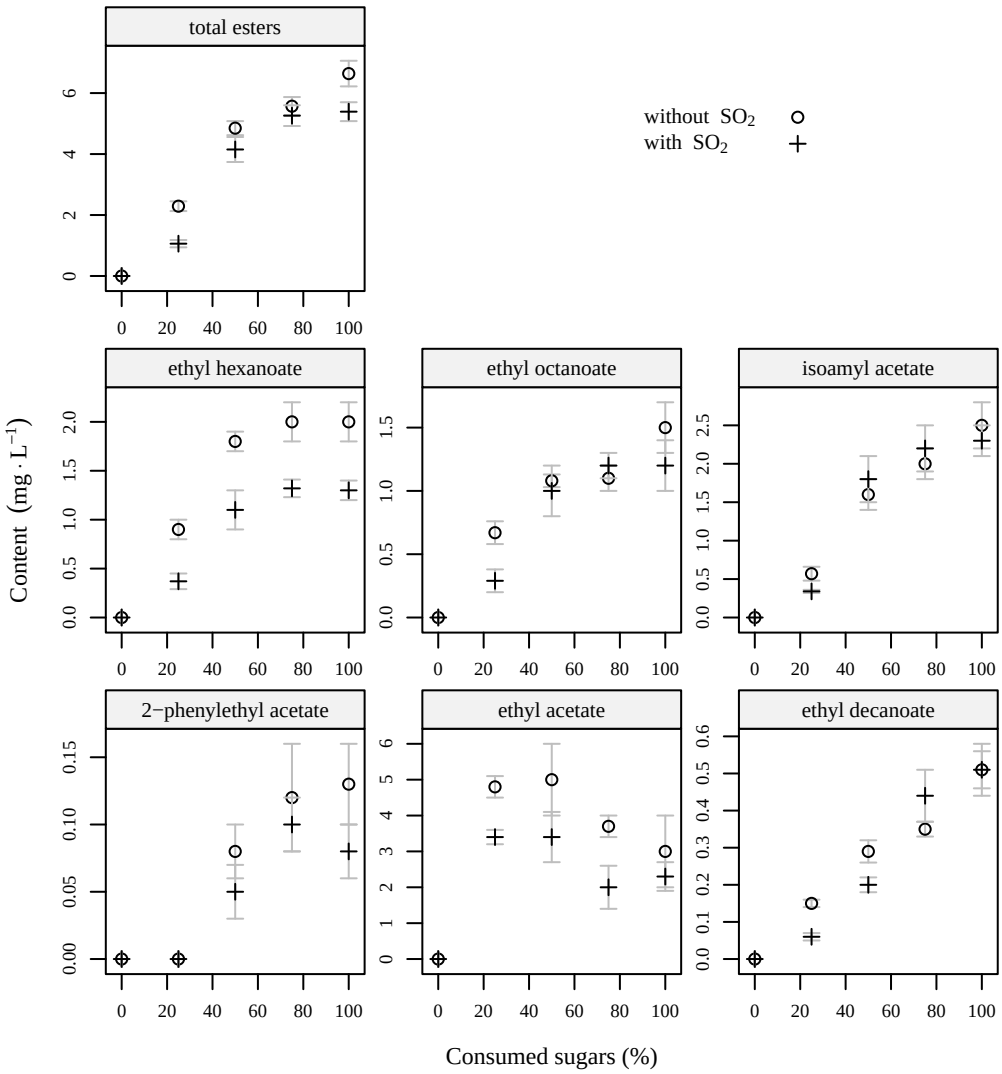

Figure 9.2: Evolution of several ester levels of Parellada grape juice through the fermentation process conditioned by the SO_2 content.

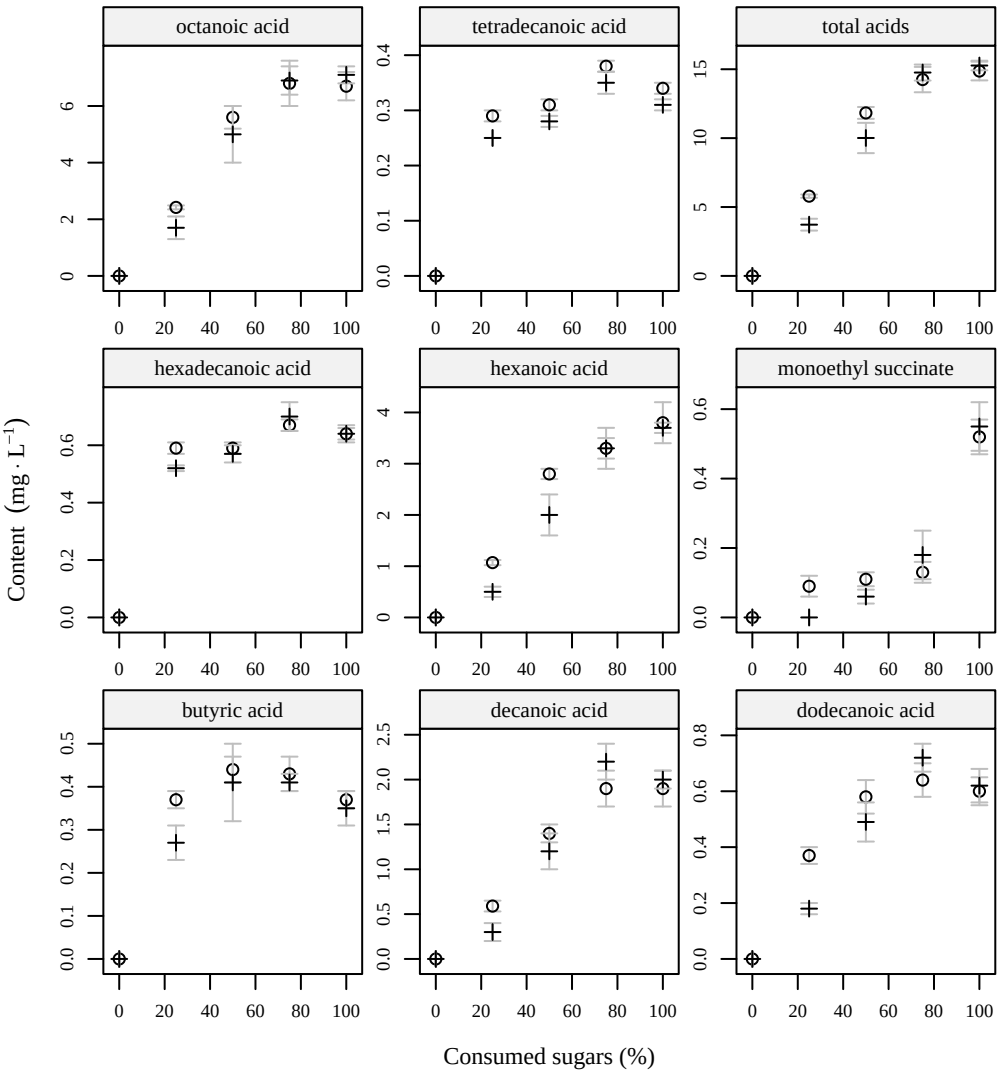

Figure 9.3: Evolution of several acid levels of Parellada grape juice through the fermentation process conditioned by the SO_2 content (circles stands for no-SO_2).

10 Effect of storage conditions on the volatile composition of wines obtained from must stabilized by PEF during ageing without SO_2

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Abstract

The aim of this work was to study the effect of the storage conditions on the evolution of volatile composition of white wines aged in bottles without the addition of SO_2. Therefore Parellada must was stabilized by pulsed electric fields (PEF) and fermented without the employment of SO_2 and later on the wine was aged in bottles, without addition of this preservative, at low and controlled temperature and at room temperature. The obtained results showed that the concentration of some important compounds for wine aroma such as isoamyl acetate, and ethyl esters of fatty acids was higher in the wines stored at low and controlled temperature than in those aged at room temperature. However, the temperature favoured the formation of total alcohols during the aging of wines in the bottles. Consequently, from the point of view of the aromatic quality, the conservation of white wines obtained from fermentation of must processed by PEF and aged in bottles without the addition of SO_2 was improved under controlled storage conditions than at room temperature.

Industrial relevance: SO_2 is used as a preservative agent in wine due to its multi-action in the wine conservation. Although neither carcinogenicity nor mutagenic effects have been found in SO_2, this compound has an influence on human health. For that reason, several competent international organizations (WHO, FAO, OIV) have set down maximum limits for wines as well as promote a reduction of its concentration in wines. Therefore, potential industrial applications of this work include the possibility to produce and store wines without SO_2. This has been achieved by stabilizing the musts with PEF. The wines produced under these conditions can be conserved without this additive when used under controlled conditions of storage.

10.1. Introduction

The compounds formed during alcoholic fermentation have a decisive influence on the volatile composition of wine. The major volatile products of yeast metabolism, ethanol, glycerol and carbon dioxide make a relatively small yet fundamental contribution to wine flavour. The main groups of compounds that form the *fermentation bouquet* are esters, alcohols and acids and, to a lesser extent, aldehydes [128]. After the alcoholic fermentation the white wine is bottled for its commercialisation and later consumption. There is a tendency among an important sector of the market to demand white wines that are organoleptically characterized for their noticeable fruity and fresh aroma, pale colour and elegant acid taste. Therefore, the evolution of the product in the bottle before its consumption is very important. The wines in the sale places remain at room temperature and once acquired by the consumer they stay, generally, at room temperature too. For this reason, it is important to study how a bottle of wine evolves at room temperature and compare it with the evolution at low and controlled temperature.

Pulsed electric field (PEF) technology has been used to preserve fruit juice and to delay the spoilage by microorganisms [59, 58, 96]. Recently, this technology has been implemented for the production of commercial fruit juices in the USA whereas some important food processors of the EU are trying this technology at a pilot plant level. A previous study showed that, when grape must is treated by pulsed electric fields (PEF), the sulphur dioxide concentration could be reduced to safer levels or even eliminated without an important effect on the volatile compound content of the final product [83]. It has also been reported that PEF treatments also decrease the activity of enzymes such as peroxidases and polyphenoloxidases in grape juice [141], in apple and pear extracts [87], peach purée [89] and orange juice [57]. The proposed mechanism of enzymatic inactivation might be related with the change of specific structures of the enzymes [271].

Sulphur dioxide is widely used in food industry since it is a good antioxidant agent and an excellent antimicrobial compound. It is known that it has an influence on human health although neither carcinogenicity nor mutagenic effects have been found [219]. Nevertheless it would be important to reduce the dose of SO_2 in wine to avoid an accumulative effect due to the fact that it is a very common additive in many food products being also present in the atmosphere as a consequence of the industrial activity. For that reason, several competent international organizations (WHO, FAO, OIV) have set down maximum limits for wines as well as promote a reduction of its concentration in foodstuffs, specifically in wines.

Until now, we have not found any study with a relationship between wine aged without SO_2 in bottles with different storage conditions and its volatile composition. Consequently, the aim of this work was to study and compare the evolution of the volatile composition of white wines aged in bottles at room

temperature and at low and controlled temperature, without the addition of sulphur dioxide. To do it, must of the variety Parellada was stabilized by pulsed electric fields (PEF) and inoculated with a *S. cerevisiae* yeast strain and it was fermented without SO_2. Afterwards the obtained wine was aged at room temperature (weighted average temperature, 23 °C) and at low and controlled temperature of 5 °C during 6 months without the employment of SO_2.

10.2. Materials and methods

10.2.1. Samples and vinification

The grape variety used was *V. vinifera* variety Parellada. The grape was de-stemmed, crushed, pressed and finally filtered. The must was processed by pulsed electric fields (PEF), as described by Garde [82]. A laboratory scale PEF unit (Ohio State University, Columbus, OH, USA) was used to treat the must. The pulse generator module consists of a high voltage generator (OSU-4F), which can supply differences of electric potential between the electrodes of the treatment chambers of up to 12 kV, and a pulse generator unit model 9412A (Quantum Composers, Inc., Bozeman, MT, USA), which can render square wave pulses of up to 10 µs and 2000 Hz. PEF treatment was performed with bipolar electric field pulses of 4 µs width and with an electric field strength of 35 $kV \cdot cm^{-1}$. The pulse repetition rate was 1000 Hz and the total PEF treatment time was 1 ms, which was calculated as the product of the pulse width and the number of pulses delivered to must.

The fermentations were made in duplicate with two aliquots of PEF processed grape juice (3 L). Each one was inoculated with the active dry *S. cerevisiae* subspecie cerevisiae (Na33 strain). This yeast was selected by the Estación de Viticultura y Enología de Navarra (Olite, Spain) and commercialised by Lallemand España. The Na33 strain was inoculated in the musts in a proportion of 0.2 $g \cdot L^{-1}$ using 0.65 g of dry yeast hydrated in a sterile flask with 7.5 mL of distilled water and 0.07 g of sucrose that yielded, at least, 2×10^9 $cfu \cdot mL^{-1}$. This suspension was inoculated after 30 min at 35 °C and the must was vigorously mixed to obtain a homogeneous distribution. The fermentations took place in spheric glass fermentors of 3.5 L with two outlets, one for sample extractions capped with a plastic septum and the other with a CO_2 trap to eliminate carbon dioxide. The fermentors were placed over magnetic stirrers (Ikamag RCT basic, Milian SA, Geneve, Switzerland) to ensure a homogeneous fermentation. The fermentations were carried out in a hot–cold incubator (Selecta, Barcelona, Spain) at a controlled temperature of 18 °C. The fermentations were measured daily for sugar concentration through a refraction index at 20 °C, using a refractometer ABBE model 325 (Misco, Cleveland, USA) and through an enzymatic measure (reactives from Chema Italia, Rome, Italy) using a multi-parametric analyser Enochem (Tecnología Difusión Ibérica, Barcelona, Spain). All recipients and materials, which were in contact with the samples, were previously

sterilized.

The wine was stabilized and clarified by decantation during 48 h at −4 °C. The obtained wine was stored for 6 months in 500 mL bottles, which were sealed with corks, in a dark place at room temperature (highest temperature, 27 °C; lowest temperature, 15 °C; weighted average temperature, 23 °C) and at controlled temperature of 5 °C in a hot–cold incubator (Selecta). Sampling was done at the end of the fermentation when the reducing sugar content was below 2.5 $g \cdot L^{-1}$, after 3 and at 6 months of ageing. Two samples were taken from different fermentors and bottles.

10.2.2. Standard wine making process

Several bottles of wine were elaborated following a standard process. It was performed using the same grape variety and fermenting conditions although as the grape juice was not PEF treated the fermentation process begun spontaneously by the native yeast species. As in the standard wine making method it was added 50 $mg \cdot L^{-1}$ of sulphur dioxide to grape juice to avoid the spoilage before fermentation. Bottle ageing was carried out in the same place that the PEF processed the samples. The sampling procedure was also the same.

10.2.3. Enological parameters

Enological parameters The reducing sugar and volatile acidity analyses were made in a multi-parametric analyser Enochem by enzymatic methods. The pH was determined by using a pH-meter Metrohm 702 (Metrohm, Herisau, Germany). The total acidity was determined by the method described by the OIV [179]. The alcoholic level of the final wine was determined by using a Salleron-Dujardin ebulliometer (Paris, France). The analytical measure and the fermentation process were performed twice. Therefore, the values shown in table 10.1 are the average of 4 analyses.

10.2.4. Analysis of volatile compounds by gas chromatography

The analysis was carried out using two different methods because of the great difference of volatilities of the studied compounds and the wide interval of concentrations [76]. This method was used to analyse the compounds of high volatility and high concentration such as ethyl acetate, *n*-propanol, isobutanol, and isoamyl alcohols. In this method the sample of 0.5 µL is injected (split ratio 1:50) into a gas chromatograph device Shimadzu GC-14B (Shimadzu, Kyoto, Japan) and the separated analytes quantified with a flame ionisation detector (FID). A DB-WAX capillary column (30 m-length × 0.25 mm of internal diameter) with a cross-linked stationary phase (0.25 µm of film thickness) of polyethylene glycol (Cromlab, Barcelona, Spain) was used. The chromatographic conditions were helium (purity of 99.999%) as carrier gas (30.9 $cm \cdot s^{-1}$). The injector and detector temperature was 180 °C whereas oven temperature was 80 °C. The standards

were prepared with reagents from Aldrich at concentrations between 1 mg·L^{-1} and 400 mg·L^{-1}. Four chromatographic analyses were made of each sample. Therefore the results are the average of 8 analyses.

For the analysis of the middle-range volatility compounds and the analytes present in low concentration, the method described by Garde [80] was used. This method extracts middle-range volatile compounds using solid-phase extraction (SPE) cartridges. They are pre-packed cartridges of 3 mL filled with 200 mg LiChrolut EN resins (Merck, Darmstadt, Germany). The cartridges are placed in an extraction system (Vac Elut 20 station from Varian, CA, USA) and conditioned by rinsing sequentially with 4 mL of dichloromethane (Panreac, Barcelona, Spain), 4 mL of methanol (Scharlau, Barcelona, Spain) and, finally, with 4 mL of water–ethanol mixture (12% v/v). All the solvents were HPLC grade. Wine samples were centrifugated at 3000 r/min during 30 min and 50 mL of the supernatant phase was passed through a SPE cartridge at a flow rate of 2 mL·min^{-1}. Afterwards, the adsorbent was dried letting air pass through it for 20 min. Analytes were recovered by elution with 1.3 mL of dichloromethane and 50 µL of the internal standard (heptanoic acid) solution was added over the eluted sample. The mixture was then sealed and stored at −30 °C until analysis.

The analysed compounds of the middle-range volatility were, in order of elution: diacetyl, isoamyl acetate, ethyl hexanoate, acetoin, ethyl lactate, *n*-hexanol, ethyl octanoate, ethyl 3-hydroxybutyrate, trans-2,3-butanediol, cis-2,3-butanediol, γ-butyrolactone, butyric acid, ethyl decanoate, diethyl succinate, 3-(methylthio)-1-propanol, 2-phenyl ethyl acetate, hexanoic acid, benzyl alcohol, 2-phenyl ethanol, diethyl malate, octanoic acid, 4-methoxyacetophenone, decanoic acid, ethyl acid succinate, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, tyrosol, octadecanoic acid, 9,12-octadecadienoic acid, vanillic acid, and tryptophol.

The analysis of the extract was carried out in a GC-MS Finnigan (San Jose, CA, USA). The same DB-WAX capillary column was used. The chromatographic conditions were, He as carrier gas (30.9 cm·s^{-1}); injector temperature, 240 °C; and temperature of the transfer line, 240 °C. The middle-range volatile compounds were separated using a temperature program with an initial oven temperature of 40 °C for 5 min, a temperature gradient of 2 °C·min^{-1} to a temperature of 50 °C, maintained during 10 min, followed by a gradient temperature of 2 °C·min^{-1} to a final temperature of 240 °C, and a final time of 20 min (total run time of 135 min). The sample injected was 2.5 µL, using the splitless technique. The ionisation was produced by electronic impact at 70 eV.

The standards were prepared in dichloromethane HPLC quality (Panreac) from Aldrich reagents in concentrations between 0.1 mg·L^{-1} and 250 mg·L^{-1}, to which heptanoic acid (Aldrich) was added as the internal standard in the same concentration as in the samples. Identification of the eluted compounds was made using the retention times and comparing their mass spectrum with a spectral library of known standard compounds. The standards of each compound were injected individually and thus a data base of the characteristic mass

Table 10.1: Enological parameters of Parellada wine obtained by different methods.

Measured property[a]	PEF-processed	Standard
Alcohol (%)[b]	8.4 ± 0.3	8.5 ± 0.2
pH	3.23 ± 0.01	3.56 ± 0.03
Reducing sugars ($g \cdot L^{-1}$)	1.52 ± 0.02	1.66 ± 0.01
Total acidity ($g \cdot L^{-1}$)[c]	4.32 ± 0.08	3.32 ± 0.04
Volatile acidity ($g \cdot L^{-1}$)[d]	n.d.[e]	0.50 ± 0.01

[a] Expressed as average value and standard deviation ($n = 4$).
[b] As volumetric proportion.
[c] As tartaric acid.
[d] As acetic acid.
[e] Not detected.

spectra of each one of the compounds was made. Both, identification and quantification, were carried out in full scan mode with mass-charge ratios between 35–300.

The quantification process was performed normalising the area of the corresponding peak with the area of the internal standard and, the result was interpolated in a calibration graph of the standard solutions in dichloromethane. The recovery values were always higher than 78%. Two extractions were made from each sample and from each extraction there were two chromatographic analyses which means that the results correspond to the mean average of 8 analyses, as the fermentations were made in duplicate and two samples were taken from two bottles from each type of wine aged in bottle.

10.3. Results and discussion

10.3.1. Characteristics of wine

Table 10.1 shows the pH of the wines is within the range considered normal for this product (3.1–3.6). The wine obtained from must treated by PEF did not show any volatile acidity, since the *Saccharomyces* yeasts produce less acetic acid than the non-*Saccharomyces* yeasts [217], and because the inoculated yeast (*S. cerevisiae* Na33 strain) produces a very low concentration of acetic acid [80]. The total acidity of the wine obtained from standard fermentation was lower than the wine obtained from must treated by PEF.

10.3.2. Esters

The concentration of total esters except ethyl acetate hardly suffered modifications in the wine that remained in bottles at low and controlled temperature

(5 °C), while it increased in a significant way in the wine aged at room temperature (Fig. 10.1). This increase was due fundamentally to the contribution of the monoethyl succinate to the total concentration. The evolution of esters in the standard wine making process was very similar.

The concentration of ethyl acetate, which was measured apart because it has a different contribution to the wine aroma, diminished in both wines during their permanency in bottles , in such a way that, at the end of the ageing, the concentration of this compound was similar in both wines and very inferior to the concentration beyond which this compound affects in a negative way the wine aroma ($150\,mg{\cdot}L^{-1}$)[7]. Ethyl acetate production was larger in the standard fermentation, probably due to the fact that non-*Saccharomyces* yeasts are greater producers of this compound than *Saccharomyces* yeasts [192].

2-Phenyl ethyl acetate was only formed in the standard fermentation. Rojas [217] found that non-*Saccharomyces* yeasts have a high production of this ester during alcoholic fermentation.

The concentration of the esters that exert a high influence in the wine aroma (isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate) diminished with the time of ageing more in the samples that remained in bottle at room temperature than in those that stayed at low and controlled temperature (5 °C), with the exception of ethyl octanoate whose evolution was similar in both wines. Isoamyl acetate, ethyl hexanoate and ethyl octanoate were formed in much higher quantities in the fermentation of stabilized and inoculated must than in the standard fermentation.

Acetate esters of higher alcohols and ethyl esters of fatty acids are considered important contributors to young wine aroma because they exhibit floral and fruity odours. However, the concentration of ethyl esters of organic acids (ethyl acetate, diethyl succinate, monoethyl succinate, and diethyl malate), which seem to play only a limited role in the organoleptic qualities of healthy wines [214], increased in an important way in the wines aged in bottle at room temperature whereas in the wines that remained at 5 °C, the concentration of these compounds increased lightly or did not change. These esters, with the exception of monoethyl succinate, were not detected in the wine obtained from standard fermentation.

As far as ester contents are concerned, the equilibrium concentrations of each ester and its respective hydrolysis products were different. The ethyl esters of fatty acids and the acetate ester of higher alcohols were hydrolysed with the time of ageing while the ethyl esters of organic acids were formed during the permanency of the wines in bottle. These results coincide with what was found by other authors [207, 72]. Both processes, esterification of fatty acids and hydrolysis of acetate esters, were favoured by the temperature.

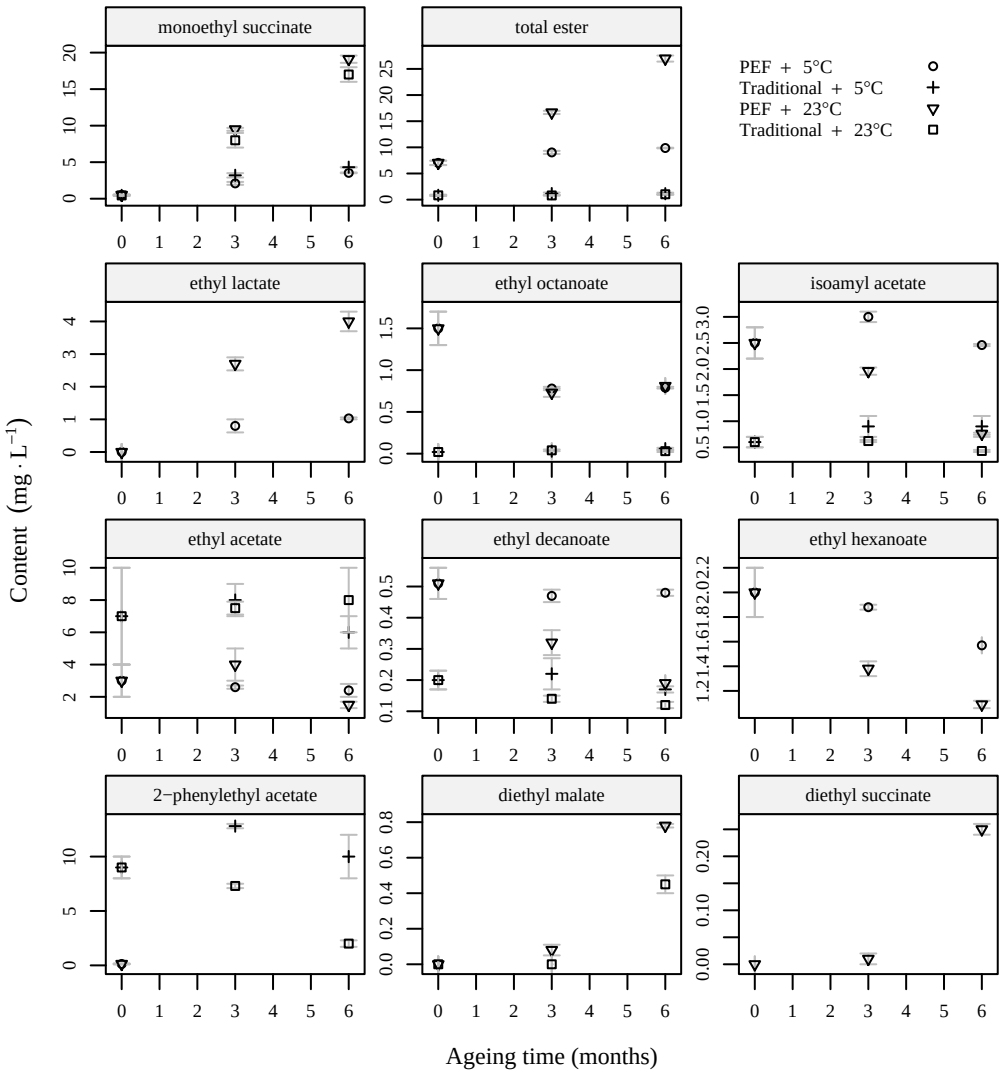

Figure 10.1: Evolution of ester contents in the wines aged at different temperatures depending on their processing.

10.3.3. Alcohols

The concentration of total alcohols increased lightly in the wines aged in bottle at room temperature while it did not change in the wines that remained at 5 ℃ (Fig. 10.2). The wine, after the alcoholic fermentation, had a residual concentration of amino acids [84], which could be transformed into the corresponding alcohols at moderate room temperature. Alcohols have intense odours that play a role in wine aromas. At low concentrations, typically less than $300\,mg{\cdot}L^{-1}$, they contribute to the wine's aromatic complexity [210]. At higher levels, their penetrating odours mask the wine's aromatic finesse. The concentration of total alcohols in our samples was always below this threshold, so it is expected that these compounds enhance the wine aroma.

The concentration of isobutanol increased in the wine aged at room temperature while its concentration diminished in the wine that remained at 5 ℃. The concentration of *n*-propanol and isoamyl alcohols hardly changed during the permanency of the wines in bottle. The levels of these compounds were lightly superior in the wine that remained at room temperature than in the wine conserved at low and controlled temperature. Isoamyl alcohols are the majority alcohols in the wine and were above its perception threshold ($60\,mg{\cdot}L^{-1}$)[235]. Isoamyl alcohols were found in higher concentrations in wine obtained from inoculated fermentation than in wine obtained from the standard fermentation. This is probably because *Saccharomyces* yeasts are good producers of these sort of alcohols [218].

The concentration of 2-phenyl ethanol increased in a similar way in both wines during the ageing in bottle reaching a value beyond $14\,mg{\cdot}L^{-1}$, which is the threshold level of sensory perception [73]. This compound is important for the quality of the final product since it gives a rose aroma to the wine [65] contributing in a positive way to the global aroma of the wine. The concentration of 2-phenyl ethanol was higher in the wine obtained from the standard fermentation than the wine obtained from the inoculated fermentation due to the fact that the non-*Saccharomyces* indigenous yeasts are great producers of this alcohol [80].

The concentration of tyrosol and tryptophol diminished in both wines, although in a different way; at the end of the ageing phase, the concentration of tyrosol was higher in the wines that remained at room temperature while the concentration of tryptophol was higher in the wines conserved at low and controlled temperature (5 ℃). Nevertheless these alcohol concentrations augmented throughout the ageing of the wine coming from the standard fermentation process. The concentration of 3-(methylthio)1-propanol, *n*-hexanol, and benzyl alcohol evolved in a similar way in both wines; at the end of the ageing, their concentration in the wines was lightly above the initial concentration.

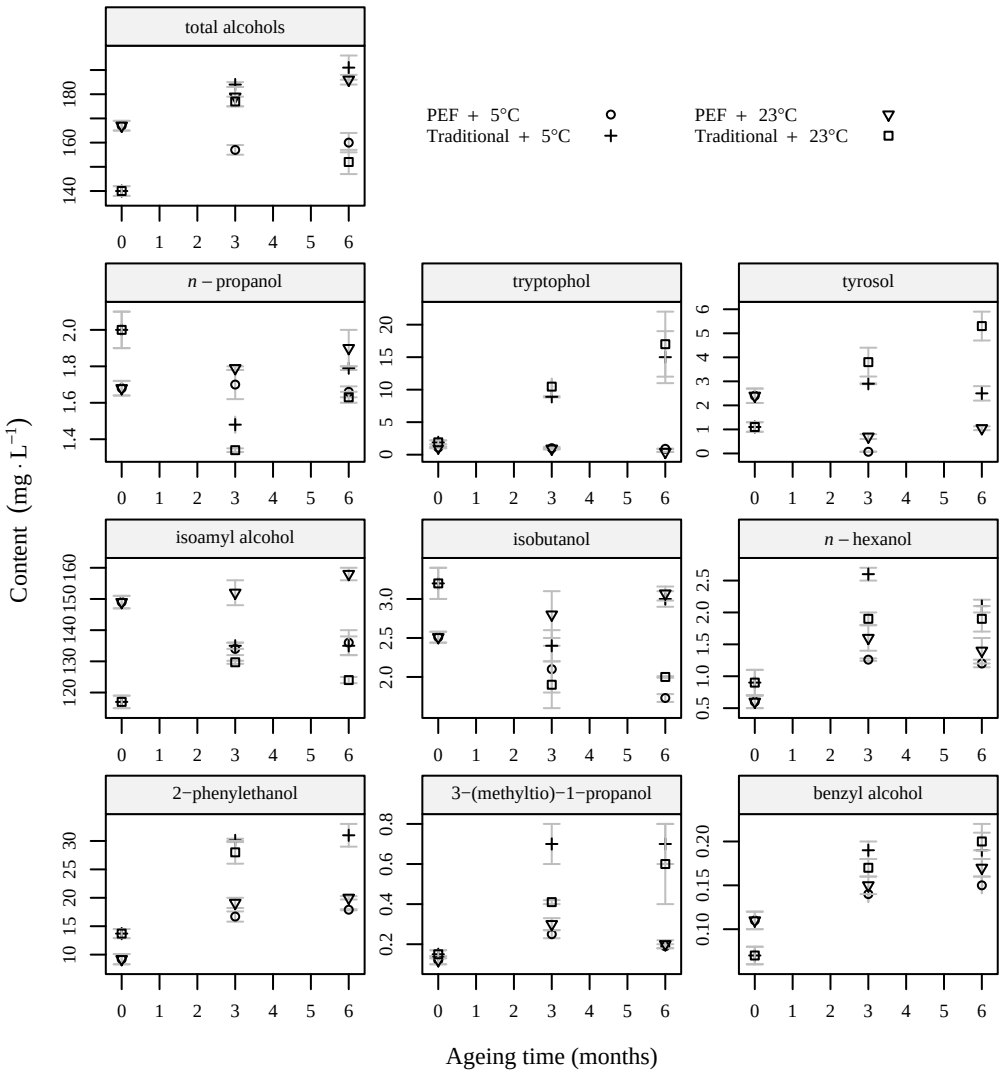

Figure 10.2: Evolution of the alcohol contents in the wines aged at different temperatures depending on their processing.

10.3.4. Acids

The concentration of total acids increased during the ageing of the wines in bottle, being higher the increase of the concentration in the wines that remained at 5 ℃ than in the wines aged at room temperature and figure 10.3. Fatty acids confer a fresh flavour to wine, which can be transformed in an unpleasant flavour if fatty acid concentrations are high, and contribute to modify the perception of other taste sensations [214]. The total concentration of fatty acids at the end of the ageing in the wine that remained in the bottles at room temperature was 17 $mg \cdot L^{-1}$ while in the wine aged in bottle at 5 ℃ was 22 $mg \cdot L^{-1}$. Both concentrations were close to the concentration stated as negatively influential in wine aroma (20 $mg \cdot L^{-1}$) [196]. The concentrations of total acids, hexanoic acid, octanoic acid and decanoic acid in the wine obtained from standard fermentation were much lower than the concentrations of these compounds found in the wine made from stabilized and inoculated must. This could have been due to the fact that the *Saccharomyces* inoculated yeasts are good producers of these compounds [81].

Butyric and hexanoic acids increased their concentration with the time of ageing in a similar way in both wines. Thus, at the end of the study, the concentration of butyric acid was lightly superior in the wines aged at room temperature while the hexanoic acid did not present changes in its concentration in function of the conditions of ageing of the wines.

Octanoic and decanoic acid increased their concentrations in both wines until 3 months of permanency in bottle and later on their concentrations diminished in such a way that the wines aged at low and controlled temperature presented higher concentration of these compounds than the wines that remained at room temperature.

The concentration of dodecanoic acid changed little in the wine aged at room temperature and increased in the wine that remained at 5 ℃. The concentration of tetradecanoic acid diminished lightly in the wine aged at room temperature and changed little in the wine that remained at low and controlled temperature. The concentration of hexadecanoic acid increased in both wines although in a superior way at the end of the ageing in the wine that stayed at 5 ℃. Dodecanoic, tetradecanoic and hexadecanoic acids were not found in wines obtained from the standard wine making process.

10.4. Conclusions

The obtained results showed that the concentration of some important compounds for wine aroma such as isoamyl acetate, and ethyl esters of fatty acids was higher in the wines stored at low and controlled temperature than in those aged at room temperature. The same trend was observed with the concentration of the majority of fatty acids in the wines. However, the temperature favoured the formation of total alcohols during the ageing of wines in the bottles. Con-

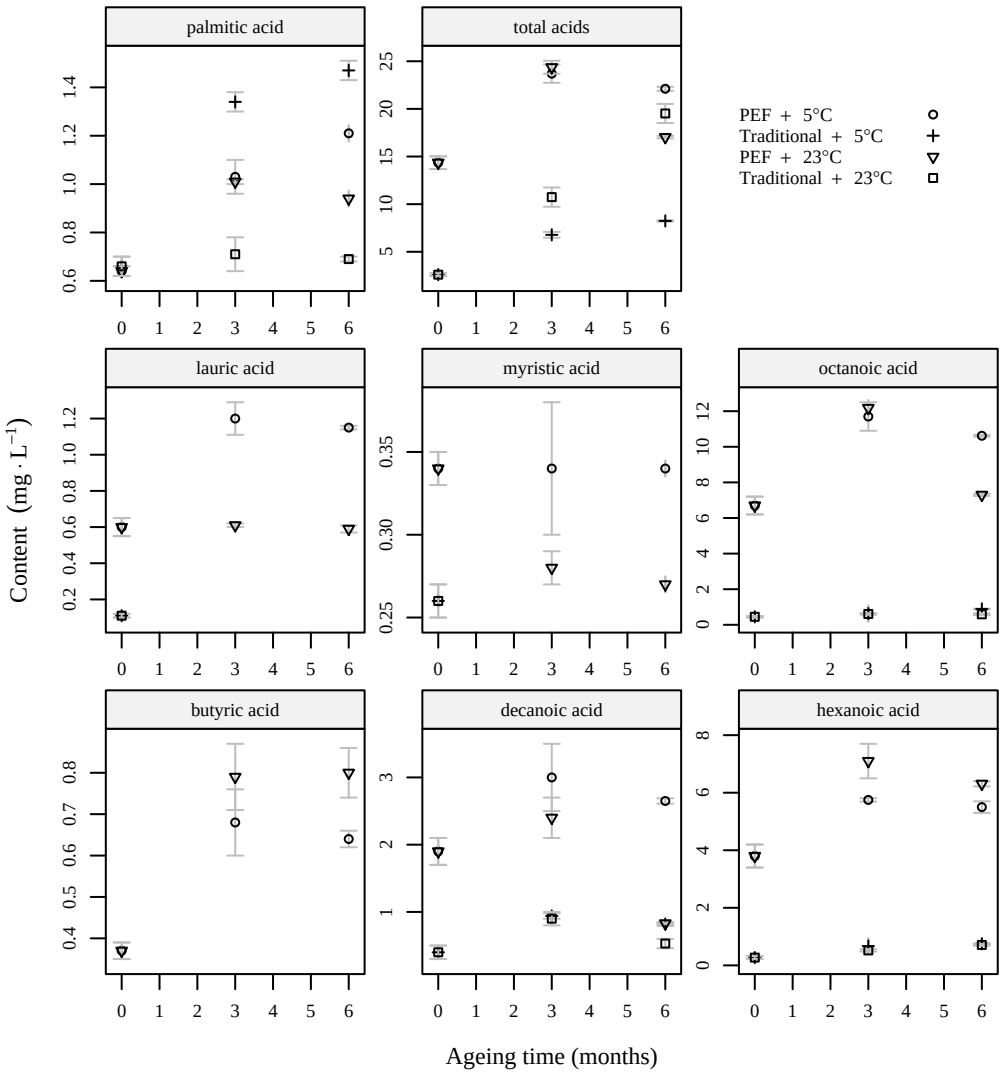

Figure 10.3: Evolution of the acid contents in the wines aged at different temperatures depending on their processing.

sequently, from the point of view of the aromatic quality, the conservation of white wines obtained by fermentation of grape juice processed with PEF and aged in bottles without the addition of SO_2 was better under controlled storage conditions than at room temperature.

11 Discusión general

11.1. Microbiología del zumo de uva

Según los datos obtenidos durante esta investigación, la población microbiana general en zumo de uva ha sido reducida con la aplicación de PEAIC. Los microorganismos aerobios entre 3 y 6 reducciones logarítmicas y los anaerobios entre 2 y 4. Similares resultados (3–5 reducciones) se han obtenido cuando se han analizado especies de poblaciones más concretas como microorganismos anaerobios facultativos como las bacterias lácticas. En mohos y levaduras se han obtenido resultados sistemáticamente reproducibles alrededor de 4 reducciones decimales. Los resultados en aquellas especies típicas de los zumos de uva como *K. apiculata* o *S. cerevisiae* entre las levaduras o bacterias como *L. hilgardii*, *L. plantarum* y *G. oxydans* se encuentran entre los rangos descritos.

Se ha observado que las variaciones dentro de dichos márgenes son consecuencia principalmente de diferencias en el tiempo de procesado. Asimismo, se ha constatado que el grado de destrucción microbiana no esta sujeto a variaciones varietales. La intensidad de campo eléctrico también se ha confirmado como uno de los parámetros mas influyentes y su efecto aumenta con su valor al menos hasta el límite del equipo con el que se han realizado los estudios. Se ha determinado que la frecuencia de los pulsos eléctricos influye significativamente en la eliminación microbiana aunque su valor óptimo es especifico para cada combinación del resto de parámetros incluyendo el tipo de microorganismo. Lo mismo sucede con la amplitud de los pulsos eléctricos. Así pues ambos factores pueden supeditarse a otras necesidades del procesado de zumo de uva como la temperatura o la eficiencia energética. Los datos sugieren una posible influencia del nivel de contaminación inicial. Sin embargo esta efecto podría ser debido a los distintos estados de agregación del zumo que no se han controlado exhaustivamente.

El mosto o zumo de uva ha sido poco estudiado hasta la actualidad a pesar de ser uno de los productos alimentarios más comercializados [74]. Hasta el momento se había constatado una reducción de flora microbiana de 4.2 logaritmos decimales aunque no se especificó que tipo de microorganismos estaban presentes [262]. Este valor coincide con el rango hallado en el presente estudio. En otros zumos de consumo habitual como el de naranja [58] o tomate [150] o más exóticos como el de cerezas ácidas [5] se dan cifras parecidas, entre 3 y 5 reducciones logarítmicas en bacterias y entre 4 y 6 en levaduras. Es decir, en general, los datos de destrucción microbiana obtenidos son similares a los descritos por investigaciones en otros productos usando la misma tecnología.

En este aspecto, el procesado del zumo de uva mediante PEAIC no alcanza el nivel de los resultados proporcionados por el tratamiento térmico habitual. De todos modos, se puede afirmar que los PEAIC pueden ser una alternativa al procesado tradicional del zumo de uva en particular.

Uno de los motivos para realizar esta afirmación es que, dada su naturaleza, la presencia de microorganismos patógenos no supone un problema trabajando en entornos correctamente gestionados de acuerdo con las normas de calidad e higiene. Aun así, sus poblaciones se encontrarían en el margen de valores que permiten ser eliminados mediante PEAIC como esta descrito para *E. coli* O157:H7 y *Salmonella enteritidis* en zumo de manzana o naranja [69, 165]. Incluso en zumos no tan habituales y más susceptibles de contener patógenos como el zumo de melón o sandía se han llegado a describir reducciones de entre 4–5 logaritmos decimales [166].

Los microorganismos que degradan el zumo de uva representan el verdadero problema aunque sólo afecte a la calidad del producto. Su presencia puede alcanzar niveles más elevados que la de los patógenos. En este caso, pueden utilizarse varios tratamientos PEAIC en distintas etapas del procesado puesto que su incidencia en otros aspectos de la calidad del producto es menor que el del procesado térmico como ha quedado patente. Además, como se ha comentado anteriormente, los primeros estudios sobre viabilidad económica sugieren que el uso de esta tecnología podría ser más rentable que el procesado mediante calor [107]. Algunas áreas de aplicación serían la reducción de la carga microbiana previa a la fermentación con levaduras especificas o la elaboración de zumo de uva de alta calidad organoléptica entre otras aplicaciones.

A pesar de todo ello, algunos aspectos deben ser corregidos o mejorados para favorecer el uso industrial de esta tecnología. Uno es la estandarización de los procedimientos de procesado. Se ha advertido, durante la realización de este trabajo, bastante disparidad en, por ejemplo, los tiempos de tratamiento de los productos alimentarios y que, no obstante, rinden grados de destrucción microbiana similares.

A modo de ejemplo se describen dos casos concretos. Altunas [5] indica que el equipo utilizado en su estudio del procesado mediante PEAIC en zumo de cerezas ácidas es un OSU-4A. Es un equipo del mismo fabricante que el utilizado en la Universitat de Lleida. La única diferencia destacable según su descripción es que posee 6 cámaras de tratamiento respecto a las 8 del equipo de la UdL. Los resultados microbiológicos descritos coinciden con los rangos del presente trabajo si bien el zumo fue tratado durante 131 µs que es un tiempo de tratamiento unas 20 veces menor que el usado en la UdL. Del mismo modo, Evrendilek [69] describe la práctica eliminación, aproximadamente 5 logaritmos, de *E. coli* en zumo de manzana con el mismo tipo de equipo utilizando apenas 200 µs.

No se ha investigado la causa de esta diferencia como tampoco su posible repercusión puesto que no es uno de los objetivos de este trabajo. En nuestro caso además, la reproducibilidad de los resultados obtenidos no exigía profundizar en ello. En los casos particulares descritos algunas diferencias entre las

investigaciones, aparte de la naturaleza del producto, son la presentación de los mismos y el modo de procesado. Los resultados de Evrendilek [69] y Altunas [5] se llevaron a cabo en zumos prácticamente clarificados y fueron sometidos a todo el tratamiento en un solo paso por las cámaras de tratamiento. No obstante, Schirive (2006) [230] confirmó que la metodología del procesado del zumo en continuo utilizado en la UdL rendía mejores resultados microbiológicos. Eso si, el equipo PEAIC a partir del cual se obtuvo esa conclusión constaba de una sola cámara de tratamiento del tipo *cross-field* en el que el flujo del producto es perpendicular al campo eléctrico aplicado (Fig. 1.5, Sec. 1.5).

Así pues, en cuanto a la parte microbiológica, técnicamente el procesado de zumo de uva mediante PEAIC es viable y rinde resultados reproducibles a pesar de la existencia de ciertas anomalías. Además, la considerable cantidad de resultados microbiológicos obtenidos a lo largo de la investigación pasa a engrosar el conjunto de información disponible para garantizar la seguridad alimentaria en productos procesados con tecnologías alternativas al calor.

11.2. Actividad enzimática del zumo de uva

El conocimiento de los efectos de los PEAIC en los enzimas vegetales ha sido posterior a los efectos microbiológicos. Sin embargo, en la industria frutícola nacional, los efectos de las actividades enzimáticas son, como poco, tan perniciosos como los que suponen los microorganismos. Esto es debido a que son capaces de degradar muy rápidamente la calidad de los productos, bien sea en fresco o como producto elaborado.

Basta con analizar las primeras etapas de elaboración de cualquier producto derivado de la fruta o las verduras. La mayor parte de ellas o las condiciones bajo las que se realizan están dirigidas a evitar o minimizar la pérdida de calidad de la materia prima provocada por la liberación de los enzimas celulares debido al procesado.

Pese a ello, aún no existe una explicación de los efectos observados de la interacción entre el campo eléctrico de los pulsos aplicados y los enzimas. Debe tenerse en cuenta que esta tarea no es sencilla. Las enzimas, o proteínas en general, como componentes celulares pueden parecer menos complejos que las propias células y en consecuencia sus mecanismos de interacción con el entorno más fáciles de entender. No obstante, dada su actividad específica, la diversidad estructural de los enzimas es muy variada. Esas marcadas diferencias estructurales son las que dificultan el descubrimiento del mecanismo de actuación de los pulsos eléctricos.

En las células típicas existe una estructura común sobre la que actúan, siempre del mismo modo, los pulsos eléctricos, la membrana celular. Ese efecto puede ser mayor o menor pero existe siempre. Sin embargo, basta modificar la estructura celular, incluso simplificándola como ocurre con las partícula víricas [123], y los efectos habitualmente observados provocados por los tratamientos PEAIC

desaparecen o cambian.

Hasta el momento se ha confirmado que los PEAIC modifican la estructura proteica tridimensional en algunos enzimas como la PPO o la lipooxigenasa (LOX). Mayoritariamente afectan a la secundaria modificando el porcentaje de α-hélices o laminas-β presentes [270, 271]. Recientes estudios parecen confirmar el efecto sobre la estructura proteínica terciaria en LOX [137]. Ahora bien, esa modificación puede manifestarse como una disminución o como un aumento de su actividad o, también, en una ausencia de efecto dependiendo de si el centro activo enzimático se ve influenciado por estos cambios [106, 134]. En este sentido se ha descrito tanto la eliminación práctica de la actividad poligalacturonasa (PG) de preparados comerciales [90] como la ausencia de efecto sobre la misma en zumo de tomate [170]. De ser comparables ambos tratamientos, estos hechos supondrían una incongruencia excepto si se acepta que la actividad PG es debida a enzimas lo suficientemente distintos en su estructura como para que se puedan apreciar estos sorprendentes resultados.

Se ha observado que los PEAIC reducen la actividad de enzimas oxidantes del zumo de uva independientemente de la variedad, concretamente la actividad de la polifenoloxidasa y la peroxidasa. También se ha constatado que el tiempo de procesado es la variable que más afecta a esta reducción junto con el valor de la intensidad del campo eléctrico. El campo eléctrico necesario para obtener dichos efectos es mayor que el necesario para actuar sobre los microorganismos. Además, como ocurre en el caso de los microorganismos, los efectos de la anchura y frecuencia de los pulsos, aunque influyentes, pueden ajustarse en función de las necesidades del procesado.

Se ha comparado la reducción de actividad enzimática con la conseguida con el tratamiento térmico tradicional y se ha observado que el grado de reducción de la actividad polifenoloxidasa fue similar, logrando su eliminación. En cuanto a la actividad peroxidasa ninguno de los dos tratamientos la eliminó si bien en ambos casos se alcanzó una reducción de su actividad igual o superior a la mitad de la inicial, un 50 % mediante PEAIC y el 70 % del tratamiento térmico respectivamente. Los valores obtenidos se encuentran en el rango obtenido por otros estudios realizados en zumos de fresa, tomate o sandía [3].

Con toda esta información se puede deducir que las condiciones de procesado necesarias para la reducción de las actividades enzimáticas en zumos en general, y específicamente en zumo de uva, son mas enérgicas que las que se utilizan para la eliminación de los microorganismos presentes. Ello sugiere el uso de enzimas vegetales como objetivos a monitorizar para garantizar la efectividad de los tratamientos mediante PEAIC. En la industria láctea se ha utilizado la fosfatasa alcalina para este fin cuando se procesa la leche térmicamente.

11.3. Vinificación

Este tema fue incluido en el estudio con el fin de elucidar si esta tecnología no térmica afectaba de algún modo al vino que, cuantitativa y económicamente, representa el producto más importante derivado de la uva.

En este sentido se analizaron las variables que se pensó que podrían influir en mayor medida en la calidad del producto final. Por este motivo, se escogieron familias de componentes presentes en el zumo que permiten a las levaduras llevar a cabo su misión o cuya presencia o magnitud modificase la calidad del vino elaborado. Se decidió además, ampliar el estudio investigando su evolución tras el tratamiento. Los compuestos escogidos fueron las fracciones nitrogenadas y las sustancias volátiles en concreto alcoholes, ácidos y sus derivados los ésteres.

Debido al creciente interés de la sociedad en la obtención de productos alimentarios con un bajo contenido en sustancias ajenas a su propia composición en esta etapa se tuvo en cuenta la presencia del mayor conservante utilizado en enología, el dióxido de azufre (SO_2).

Se observó que el contenido en acetaldehído fue mayor en zumos procesados con PEAIC que contenían dióxido de azufre. Lo mismo ocurrió cuando se analizó el contenido en nitrógeno cuyo valor corresponde a la suma de las tres familias de compuestos nitrogenados consideradas el total asimilable, el amoniacal y la fracción proteínica que incluye a su vez a los aminoácidos. La diferencia entre el zumo de uva con conservante y sin el no fue muy elevada ya que en ambos casos el contenido final de nitrógeno asimilable fue inferior al 10 % del inicial. Pese a ello, varias investigaciones han relacionado un mayor consumo de compuestos nitrogenados por parte de las levaduras con un aumento de la complejidad aromática del vino [26, 248].

En este sentido, se analizaron los compuestos volátiles obtenidos durante la fermentación de zumo de uva procesado mediante PEAIC conteniendo dióxido de azufre y sin el. Se evidenció que sus perfiles evolutivos fueron similares a pesar del hecho que el contenido final de alcoholes y ésteres fue significativamente distinto en los dos casos. En esta ocasión, las diferencias tampoco fueron exageradas. No obstante, el resultado más significativo fue que esas diferencias no siguieron ningún patrón concreto en cuanto a la presencia o ausencia de conservante (SO_2). Luego el menor consumo de compuestos nitrogenados en los mostos procesados mediante PEAIC sin dióxido de azufre no implica un aumento ni de la cantidad ni de la complejidad del aroma del vino. Por ello, es de suponer que en caso de utilizar este tipo de tecnología, la ausencia de dióxido de azufre no supondría, a priori, cambios en el aroma del producto final.

Finalmente se estudió la evolución de estos mismos componentes volátiles a medida que el vino elaborado a partir de zumo tratado con PEAIC fue envejeciendo a dos temperaturas distintas, una controlada (5 °C) y la otra ambiental. Se observó como el contenido en ésteres y alcoholes en el vino conservado a temperatura ambiente fue mayor. Por otra parte, el contenido en ácidos, específicamente los ácidos grasos, del vino conservado durante 6 meses a temperatura

controlada fue superior.

Sin embargo, dentro del conjunto de ésteres, los que aumentaron su presencia fueron aquellos que mejoran la calidad organoléptica del vino como el acetato de isoamilo y los ésteres etílicos. Este hecho, junto a un mayor contenido en ácidos grasos del vino conservado a una temperatura controlada sugiere que no es necesaria la adición del conservante si se puede garantizar una conservación a temperatura baja.

Debe tenerse en cuenta que esta linea de investigación es hasta el momento el reto más original y en ella sólo fue estudiada una única variedad de uva. Consecuentemente los resultados tienen aplicación limitada aunque un mayor número de datos en este campo podría aportar nuevos usos a esta tecnología.

11.4. Comparación de tratamientos conservantes sobre zumo de uva

Como se ha sugerido al finalizar la sección anterior, la transferencia de esta tecnología para su uso en la industria alimentaria sólo puede basarse en el conocimiento de las ventajas e inconvenientes que posee. Para ello se requiere la recopilación de información, cuanto mayor sea la cantidad y más diversa mejor.

El hecho de que actualmente el procesado térmico sea la tecnología de referencia en el área de la conservación de alimentos tanto por su uso como por la eficacia de sus resultados la hace idónea para tomarla como tratamiento de comparación.

En este momento existen numerosos estudios describiendo los efectos del procesado mediante PEAIC en zumos de frutas y verduras. Desde los mas tradicionales de naranja, manzana o tomate [19, 150, 175, 274, 66, 180] hasta los no tan habituales como los de sandía, melón o gazpacho [172, 177, 178, 4, 60]. En varios de ellos incluso se realiza la comparación directa con el procesado térmico como ocurre en el presente trabajo.

Del conjunto de información existente se extrae que muchos de los parámetros que caracterizan física o químicamente estos productos no cambian substancialmente al utilizar los PEAIC como el contenido en solidos solubles, el pH, la acidez o la densidad. No obstante, el procesado térmico realizado correctamente tampoco las modifica en exceso puesto que son características fáciles de medir que se utilizan para garantizar la calidad en las transacciones comerciales. En este sentido cualquier alteración exagerada de alguna de ellas supone importantes pérdidas económicas. En el presente estudio se ha observado un comportamiento similar acorde con lo descrito en otras frutas, es decir, el zumo de uva tratado mediante PEAIC y calor no modifica sus características fisicoquímicas.

El efecto particular de los PEAIC sobre los microorganismos y enzimas presentes en zumo de uva ha sido ya comentado en previos apartados (Secs. 11.1, 11.2). La comparación directa entre ambas tecnologías conservantes indica que el procesado mediante PEAIC no es tan efectivo como el calor aunque podría ser útil

en determinadas aplicaciones. Este hecho tampoco supone nada nuevo e, incluso, parece razonable puesto que la tecnología de procesado térmico está muy optimizada después de más de 50 años de uso industrial a escala mundial. Por su parte la tecnología PEAIC, si bien es conocida desde hace un siglo, no ha evolucionado hasta la actualidad donde han aparecido los primeros equipos a nivel industrial y los primeros productos comerciales.

Además de todas estas propiedades, se ha comparado el efecto sobre algunos componentes del zumo de uva de creciente interés en la sociedad actual. Concretamente se ha determinado el efecto sobre algunos componentes y propiedades que están relacionados en mayor o menor medida con efectos beneficiosos en nuestro organismo como son el contenido en ácidos grasos, aminoácidos, proteínas, vitamina C, polifenoles o la capacidad antioxidante entre otros.

En cuanto al contenido en general de cada uno de esos componentes, se ha constatado que en caso de existir diferencias entre los zumos de uva procesados mediante PEAIC y los recién obtenidos, en general, son de menor magnitud que las obtenidas entre el zumo sin procesar y el tratado térmicamente. Esta información coincide con los resultados obtenidos con estudios en otros zumos de fruta como los de fresa o zanahoria por citar algunos de los más novedosos, recientes y realizados con un equipamiento comparable [177, 205].

En el único caso en que el zumo de uva procesado con PEIAC resulto afectar más que el procesado térmico fue en el contenido total de ácidos grasos de la variedad Parellada. No obstante, la diferencia fue de sólo 2 ppm frente al valor del zumo no procesado.

Los casos más claros son los obtenidos para las proteínas que se redujeron a prácticamente la mitad en el zumo procesado mediante PEAIC y unos dos tercios del valor inicial en el zumo tratado térmicamente. No se ha identificado el mecanismo de actuación del procesado sobre las proteínas puesto que el contenido en aminoácidos no se vio incrementado. La suposición que se realizó fue que los tratamientos modificaron la distribución espacial de las macromoléculas de tal modo que les impidió la reacción con el reactivo colorante en que se basaba el análisis.

Por su parte, la reducción de capacidad antioxidante fue mucho mayor en zumos tratados con calor (13 %) que eléctricamente (9 %). En este caso no se ha podido atribuir este hecho a ninguno de los componentes habitualmente relacionados con la capacidad antioxidante pese a que se ha descrito la correlación entre la capacidad antioxidante y el contenido en polifenoles o vitamina C [174]. En frutas de grano o bayas, como uvas o arándanos, o verduras, como la espinaca, también ha sido documentada la correlación entre su capacidad oxidante y el contenido en polifenoles o en alguno de sus integrantes [42, 108, 109]. Sin embargo, no se ha encontrado ninguna evidencia que sostenga esa afirmación en este estudio, es decir, la variación de la capacidad antioxidante del zumo en función del procesado no parece coincidir con la variación del contenido en polifenoles o de vitamina C.

Una novedad del presente trabajo ha sido el considerar la variedad de la fruta

como una variable aleatoria para así poder estimar su efecto. En este sentido, conviene aclara que la utilización de la variedad como un factor a tener en cuenta en la realización de estudios en frutas y verduras es habitual [176, 109]. Sin embargo lo que se planteó en este trabajo fue intentar obtener una estimación del efecto de la variedad para poder compararlo con los efectos del procesado tanto térmico como eléctrico. De este modo se pretendió obtener información sobre la posible generalización de los resultados. Naturalmente dadas las pocas variedades utilizadas frente a la enorme diversidad varietal de uvas existentes hacen que los valores obtenidos sólo puedan considerarse una estimación.

Así pues, la obtención de nueva información con otras variedades de uvas seria conveniente para incrementar la precisión de dichas estimaciones. Seria interesante también disponer de un mayor número de información relativa a diferentes temporadas para elucidar si los resultados descritos son sostenidos con el tiempo. A su vez, estos nuevos datos podrían clarificar el efecto del procesado en el contenido en polifenoles y la vitamina C.

12 Conclusiones

El conjunto de información recopilada durante esta investigación sobre el procesado de zumo de uva mediante PEAIC ha permitido obtener las siguientes conclusiones:

- La intensidad de campo eléctrico y el tiempo de tratamiento son los factores más decisivos sobre los agentes perniciosos estudiados de modo que, en general, cuanto mayores sean los valores utilizados mayores son los efectos observados.
- El resto de factores, ya sean las características propias del zumo u otras variables del equipo de procesado, condicionan también el efecto de los pulsos eléctricos en enzimas y microorganismos dependiendo de su combinación concreta, aun así, su influencia es netamente inferior a los anteriores.
- Los efectos observados sobre las características fisicoquímicas y nutricionales estudiadas del zumo de uva debidos a los PEAIC son, en general, menos drásticos que los producidos por el tratamiento térmico habitual.
- La obtención de zumo de uva comercial procesado mediante PEAIC es viable tecnológicamente teniendo en cuenta que la elección del tratamiento óptimo debe elegirse considerando los objetivos a conservar o eliminar, el equipo de procesado y el propio producto. Sin embargo, para aumentar el margen de seguridad deberían adoptarse precauciones antes y durante el procesado del zumo de uva para evitar un excesiva carga microbiana. La combinación de un tratamiento PEAIC con otras medidas conservantes sería otra alternativa.
- En el procesado mediante PEAIC de zumo de uva para vinificación puede reducirse o eliminarse el uso de dióxido de azufre sin perjuicio para las características sensoriales del vino estudiadas, pese a que se han observado ligeros cambios cuantitativos en la composición del vino.
- Es conveniente ampliar el ámbito de estudio a un mayor número de variedades de uva, de vendimias y equipos de procesado para poder generalizar la validez de estas conclusiones.

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