UNIVERSIDAD DE CASTILLA-LA MANCHA

Facultad de Ciencias y Tecnologías Químicas

Departamento de Química Analítica y Tecnología de los Alimentos

DIFERENTES ESTRATEGIAS PARA DISMINUIR EL EMPLEO DE SO_2 EN LA FASE PREFERMENTATIVA. INFLUENCIA EN LA CALIDAD DE LOS VINOS.

Memoria presentada por

Lourdes Marchante Cuevas

para optar al título de Doctora en Enología, Viticultura y Sostenibilidad por la Universidad de Castilla-La Mancha

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Ciudad Real, 2021

Departamento de Química Analítica y Tecnología de Alimentos

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CERTIFICA:

Que Lourdes Marchante Cuevas, con DNI 70585080N, ha realizado con aprovechamiento la estancia de investigación con duración total de 3 meses, desde el 5 de noviembre de 2018 hasta el 1 de febrero de 2019, en el Centro de Biotecnología, Universidad de Concepción, Chile.

Las actividades llevadas a cabo durante la estancia han sido las siguientes: "Determinación de radicales libres en el vino por Resonancia Paramagnética Electrónica (EPR)" y "Determinación de actividad antioxidante de extractos naturales mediante EPR para reducir la reacción de Fenton".

Y, para que así conste y tenga los efectos oportunos, firmo este certificado, en Concepción (Chile), a 1 de febrero de 2019.

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FINANCIACIÓN

Esta Tesis Doctoral está enmarcada dentro del Proyecto de Investigación fundamental orientada y acciones complementarias dentro del Programa Estatal de I+D+I orientada a los Retos de la Sociedad, del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016: RTA2014-00055-C03-01, titulado "Estudio de la actividad antimicrobiana de extractos obtenidos a partir de residuos vitivinícolas y su aplicación en la elaboración de vinos de calidad", financiado por el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER). Además, esta Tesis Doctoral se ha realizado con la ayuda para la formación de personal investigador en agroalimentación en centros de investigación agraria y alimentaria INIA-CCAA (FPI-INIA: CPD-2016-019) cofinanciada con el Fondo Social Europeo (FSE) y la Junta de Comunidades de Castilla-La Mancha.

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AGRADECIMIENTOS

Al llegar al final del camino, de esta etapa formativa, me encuentro con los sentimientos, emociones y recuerdos del principio. Un principio lleno de ilusión, con personas maravillosas que me dieron la oportunidad de formar parte de un proyecto y me guiaron en este reto que es la Tesis Doctoral.

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Lourdes Marchante Cuevas

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ÍNDICE GENERAL

Abreviaturas

a*	Coordenada rojo-verde CIELab/ Red-green CIELab coordinate
AAPH	2,2′-azo-bis(2-amidinopropano) diclorhidrato / 2,2′-azo-bis (2-amidinopropane) dihydrochloride
ABTS	Ácido 2,2′-azino-bis (3-etilbenzotiazolin-6-sulfónico) / 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
acglc	Acetil-glucósido / Acetyl-glucoside
a_H	Constante de acoplamiento hiperfina para el hidrógeno / Hyperfine coupling constants for hydrogen
a_N	Constante de acoplamiento hiperfina para el nitrógeno / Hyperfine coupling constants for nitrogen
ANOVA	Análisis de varianza / Analysis of variance
ASE	Extracción acelerada con disolventes / Accelerated Solvent Extraction
b*	Coordenada amarillo-azul CIELab/ Yellow-blue CIELab coordinate
C*	Cromaticidad CIELab / Chroma CIELab
ºC	Grado centígrado / Degree centigrade
CE	Comisión Europea / European Commission
CFU	Unidades formadoras de colonias / Colony Forming Units
CI	Intensidad de color CIELab/ Color intensity CIELab
cm	Centímetro / Centimeter
cmglc	Cumaroil-glucósido / Coumaroyl-glucoside
cfglc	Cafeoil-glucósido / Caffeoyl-glucoside
CSC	Complejo de plata coloidal / Colloidal Silver Complex
DAD	Detector ultravioleta-visible de matriz de fotodiodos / Diode Array Detector
dB	Decibelio / Decibel
DEEMM	Dietiletoximetilenmalonato / Diethylethoxymethylenemalonate

DMACA	4-(dimetilamino) cinamaldehído / 4-(dimethylamino) cinnamaldehyde
DMDC	Dicarbonato de dimetilo / Dimethyl dicarbonate
DPPH	1,1-difenil-2-picrilhidracilo / 1,1-diphenyl-2-picrylhydrazyl
DW	Peso seco / Dry Weight
EPR	Resonancia paramagnética electrónica / Electron Paramagnetic Resonance
ESI	Ionización por electrospray / Electrospray ionization
eV	Electronvoltio / Electronvolt
g	Gramo / Gram
G	Gauss / Gauss
GABA	Ácido γ-aminobutírico / γ-aminobutyric acid
GAE	Equivalentes de ácido gálico / Gallic Acid Equivalents
GC-MS	Cromatografía de gases acoplada a espectrometría de masas / Gas Chromatography-Mass Spectrometry
glc	Glucósido / Glucoside
GRP	Producto de reacción de la uva / Grape Reaction Product
GSH	Glutatión / Glutathione
H*	Ángulo de tono CIELab / Hue angle CIELab
h	Hora / Hour
1-HER	Radical 1-hidroxietilo / 1-hydroxyethyl radical
hL	Hectolitro / Hectoliter
HPLC	Cromatografía líquida de alta eficacia / High Performance Liquid Chromatography
HPLC-DAD-ESI-MS	Cromatografía líquida de alta eficacia acoplada a detector de matriz de fotodiodos y espectrometría de masas con ionización electrospray / High Performance Liquid Chromatography-Diode Array Detector-Electrospray Ionization-Mass Spectrometry

ISO	Organización Internacional de Normalización / International Organization for Standardization
KHz	Kilohercio / Kilohertz
L	Litro / Liter
L*	Luminosidad CIELab / Lightness CIELab
LAB	Bacterias lácticas / Lactic acid bacteria
M	Molar / Molar
mB	Milibar / Milibar
m/z	Relación masa-carga / Mass-to-charge ratio
mg	Miligramo / Milligram
min	Minuto / Minute
mL	Mililitro / Milliliter
mm	Milímetro / Millimeter
mM	Milimolar / Millimolar
MS/MS	Espectrometría de masas en tándem / Tandem mass spectrometry
MSD	Detector selectivo de masas / Mass Selective Detector
NAD	Dinucleótido de nicotinamida y adenina / Nicotinamide Adenine Dinucleotide
NADH	Dinucleótido de nicotinamida y adenina reducido / Nicotinamide Adenine Dinucleotide Reduced
N.D.	No detectado / Not Detected
nm	Nanómetro / Nanometer
OIV	Organización Internacional de la Viña y el Vino / International Organisation of Vine and Wine
ORAC	Capacidad de absorción de radicales oxígeno / Oxygen Radical Absorbance Capacity
PBN	α-fenil-*N*-tert-butilnitrona / α-phenyl-*N*-tert-butylnitrone

PC	Componente principal / Principal Component
PCA	Análisis de componentes principales / Principal Component Analysis
POBN	*α*-(4-Piridil-1-óxido)-*N*-tert-butilnitrona / *α*-(4-pyridyl-1-oxide)-*N*-tertbutylnitrone
PPO	Polifenoloxidasa / Polyphenoloxidase
Q	Cuadrupolo / Quadrupole
s	Segundo / Second
SPE	Extracción en fase sólida / Solid Phase Extraction
spp.	Especie / Species
T	Tonalidad CIELab / Tonality CIELab
TPC	Contenido de polifenoles totales / Total Phenolic Content
Trolox	Ácido 6-Hidroxi-2,5,7,8-tetrametilcromann-2-carboxilico / 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
µg	Microgramo / Microgram
µL	Microlitro / Microliter
µM	Micromolar / Micromolar
µmol	Micromol / Micromole
UE	Unión Europea / European Union
UV	Ultravioleta / Ultraviolet
UV–vis	Ultravioleta-visible / Ultraviolet-visible
v/v	Volumen/volumen / Volume/volume
W	Vatio / Watt
mW	Milivatio / Milliwatt
YAN	Nitrógeno fácilmente asimilable / Yeast Assimilable Nitrogen

Abreviaturas vinos

Artículo 1. Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO_2 for white wines: Effects on chemical composition and sensorial properties.

SO2	Vino control con SO_2 (50 mg/L)
E1	Vino con extracto de semilla de uva (0.5 g/L)
E2	Vino con extracto de raspón (0.5 g/L)
E1Ag	Vino con extracto de semilla de uva (0.5 g/L) y CSC (1 g/L)
E2Ag	Vino con extracto de raspón (0.5 g/L) y CSC (1 g/L)

Artículo 2. Oenological potential of extracts from winery and cooperage by-products in combination with colloidal silver as natural substitutes to sulphur dioxide.

SO2	Vino control con SO_2 (50 mg/L)
SC1	Vino con extracto de semilla de uva (0.5 g/L)
SC1+CSC	Vino con extracto de semilla de uva (0.5 g/L) y CSC (1 g/L)
SC2	Vino con extracto de semilla de uva (1 g/L)
QC1	Vino con extracto de madera de roble (0.5 g/L)
QC1+CSC	Vino con extracto de madera de roble (0.5 g/L) y CSC (1 g/L)

Artículo 3. Impact of oenological antioxidant substances on the formation of 1-hydroxyethyl radical and phenolic composition in SO_2 free red wines.

CW	Vino control sin SO_2
SO2W	Vino control con SO_2 (50 mg/L)
STW	Vino con extracto de raspón (2 g/L)
SHW	Vino con extracto de sarmiento (2 g/L)
DYW	Vino con levadura seca inactiva (0.2 g/L)
CHW	Vino con quitosano (0.2 g/L)

Artículo 4. Potential of different natural antioxidant substances to inhibit the 1-hydroxyethyl radical in SO_2-Free wines.

CRW	Vino tinto control sin SO_2
CWW	Vino blanco control sin SO_2
CFR	Control reactivos Fenton
SEW	Vino con extracto de semilla de uva (0.5 g/L; 2 g/L)
CHW	Vino con quitosano (0.5 g/L; 2 g/L)
DYW	Vino con levadura seca inactiva (0.5 g/L; 2 g/L)
GSW	Vino con glutatión (0.5 g/L; 2 g/L)
ASW	Vino con ácido ascórbico (0.5 g/L; 2 g/L)

Artículo 5. Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO_2-free red wines.

SO2W	Vino control con SO_2 (50 mg/L)
CHW	Vino con quitosano (0.2 g/L)
CHW+SO2W	Vino con quitosano (0.2 g/L) y SO_2 (25 mg/L)

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I. INTRODUCCIÓN

IV. RESULTADOS Y DISCUSIÓN

RESUMEN

Actualmente, el sector enológico busca productos naturales o tecnologías emergentes que permitan ser utilizadas como alternativas al uso de SO_2 en vinificación, debido a los efectos negativos que este tiene sobre la salud y, consecuentemente, cumplir con una legislación cada vez más exigente. Además, otra de las causas por las que se persigue sustituir o disminuir la concentración de SO_2 utilizada en vinificación es para satisfacer la demanda de los consumidores, que en los últimos años se dirige hacia el consumo de alimentos y bebidas libres de conservantes, y cuya producción sea respetuosa con el medio ambiente.

Por otro lado, existe una tendencia creciente hacia la revalorización de los subproductos de la industria vitivinícola, ya que son una fuente importante de compuestos bioactivos que los hace ser materias primas de alto valor añadido, con diferentes usos en la industria alimentaria, farmacéutica o agrícola. En particular, los residuos de *Vitis vinifera* L. y *Quercus alba* se han presentado como una alternativa prometedora al uso de conservantes por su actividad antimicrobiana y antioxidante. Del mismo modo, otros productos naturales como el quitosano y la levadura seca inactiva han mostrado tener actividad antimicrobiana y/o antioxidante en diferentes matrices de alimentos.

Debido a la importancia que tiene la composición volátil y fenólica sobre la calidad del vino, uno de los objetivos principales de esta Tesis Doctoral fue evaluar la influencia de la adición prefermentativa de diferentes alternativas naturales al SO_2 sobre los compuestos volátiles y fenólicos de los vinos. Además de su efecto en otros parámetros fisicoquímicos que influyen en la calidad del mismo, como son los parámetros del color, ácidos orgánicos y productos secundarios de fermentación. Para ello, se realizaron diferentes vinificaciones, a las cuales se les añadió, antes de realizar la fermentación alcohólica, las sustancias antioxidantes y/o antimicrobianas (extractos acuosos liofilizados de subproductos de vid o de residuos de tonelería, quitosano, levadura seca inactiva, plata coloidal o SO_2).

El diseño experimental de las vinificaciones realizadas en esta Tesis Doctoral se realizó en base a la previa caracterización de los extractos, objeto de Tesis Doctorales anteriores o paralelas a este trabajo, además de los hallazgos publicados anteriormente en bibliografía sobre la actividad antimicrobiana y/o antioxidante de los productos comerciales: plata coloidal (colloidal silver complex, CSC), quitosano y levadura seca inactiva.

Con estas premisas, se realizaron diferentes vinificaciones con la adición prefermentativa de los productos naturales a evaluar como alternativas al SO_2:

(I) Vinificaciones de uva Airén, con adición prefermentativa de: SO_2, extracto de semilla de uva y extracto de raspón. Las vinificaciones realizadas con extractos se llevaron a cabo con el extracto solo, así como en combinación con CSC.

(II) Vinificaciones de uva Cabernet Sauvignon, con adición prefermentativa de: SO_2, extracto de semilla de uva y extracto de madera de roble. Las vinificaciones realizadas con extractos se llevaron a cabo con el extracto solo, así como en combinación con CSC.

(III) Vinificaciones de uva Cabernet Sauvignon, con la adición prefermentativa de: SO_2, quitosano, levadura seca inactiva, extracto de raspón y extracto de sarmiento.

Según el objetivo perseguido en cada tipo de vinificación realizada, se utilizaron diferentes técnicas analíticas para aportar evidencia científica que ayude a esclarecer el efecto de las diferentes alternativas al SO_2 sobre la calidad del vino. Se analizaron los compuestos volátiles por cromatografía de gases-espectrometría de masas (GC-MS) y los principales compuestos fenólicos del vino, antocianos, flavonoles, flavan-3-oles, ácidos hidroxicinámicos y estilbenos por cromatografía líquida de alta eficacia con doble detección por espectrofotometría de diodos y espectrometría de masas con ionización electrospray (HPLC-DAD-ESI-MS^n). Las características organolépticas de los diferentes vinos fueron descritas mediante el análisis sensorial.

Por otro lado, para evaluar el efecto de las alternativas al SO_2 sobre la actividad antioxidante de los vinos se llevaron a cabo diferentes ensayos "in vitro", concretamente los métodos ABTS, DPPH y ORAC. Además, en las últimas vinificaciones realizadas, se evaluó la actividad antioxidante del vino mediante resonancia paramagnética electrónica (EPR). Esta es una técnica analítica con gran aplicabilidad en el estudio de la oxidación química del vino, ya que permite la detección directa de radicales libres. Sin embargo, a día de hoy, son pocos los estudios publicados acerca de los radicales formados durante la oxidación del vino en matrices de vino real y el efecto que ejercen los diferentes antioxidantes utilizados en vinificación en la propagación de los radicales. Por ello, otro de los objetivos principales de esta Tesis Doctoral fue evaluar la capacidad de diferentes sustancias naturales para inhibir uno de los radicales mayoritarios formados durante la oxidación del vino, concretamente el radical 1-hidroxietilo.

Adicionalmente, en los vinos en los que se observaron diferencias en los productos secundarios de fermentación, se analizó el contenido de aminoácidos y aminas biógenas por HPLC-DAD, así como el análisis de carbamato de etilo por GC-MS, dada la importancia que dichos compuestos poseen en la estabilidad microbiana y salubridad del vino.

Los resultados obtenidos en la realización de la presente esta Tesis Doctoral se encuentran publicados en 5 artículos científicos de revistas internacionales de alto impacto, dando respuesta al efecto que ejercen las diferentes alternativas al SO_2 estudiadas, en la etapa prefermentativa, sobre la calidad del vino. Esta Tesis Doctoral forma parte del proyecto RTA2014-00055-C03-01, financiado por el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER). Además, se ha realizado con la ayuda para la formación de personal investigador en agroalimentación en centros de investigación agraria y alimentaria INIA-CCAA (FPI-INIA: CPD-2016-019) cofinanciada con el Fondo Social Europeo (FSE).

I. INTRODUCCIÓN

I.1. Procesos oxidativos involucrados en la calidad del vino

Los procesos oxidativos que ocurren durante la vinificación y almacenamiento del vino se pueden dividir en dos tipos: enzimáticos y químicos. La oxidación enzimática ocurre principalmente en el mosto de uva, mientras que la oxidación química, también denominada oxidación no enzimática, predomina en el vino fermentado y consta de reacciones más lentas que las anteriores, pero con relevante implicación en la calidad del vino debido a la producción de especies reactivas de oxígeno. En ambos procesos oxidativos, los compuestos fenólicos son los principales sustratos de oxidación y dan como resultado subproductos denominados quinonas. Las quinonas formadas durante la oxidación del vino son altamente reactivas con otros compuestos nucleófilos, como son los fenoles, tioles, aminoácidos y SO_2, generando importantes cambios en la composición química del vino y en sus características sensoriales. Todo ello es de enorme importancia por su relación con la estabilidad y evolución de los vinos pre y post-embotellado y por tanto en su comercialización.

I.1.1. Oxidación enzimática

El estrujado y prensado de la uva conllevan la liberación de enzimas y la incorporación de oxígeno al mosto, siendo los compuestos fenólicos sustratos altamente susceptibles a la oxidación enzimática. Estas reacciones implican la hidroxilación en la posición *orto* de monofenoles para dar el correspondiente *o*-difenol y la consecutiva oxidación de estas moléculas a *o*-quinonas. Las quinonas formadas pueden polimerizarse y condensarse con otros compuestos y dar lugar a la formación de pigmentos marrones (Wang, 1990). Además, las *o*-quinonas pueden causar la oxidación de otros sustratos con menor potencial redox, como son los compuestos fenólicos, ácido ascórbico y SO_2, mientras se reducen a *o*-difenol (Robards et al., 1999) (Figura I.1).

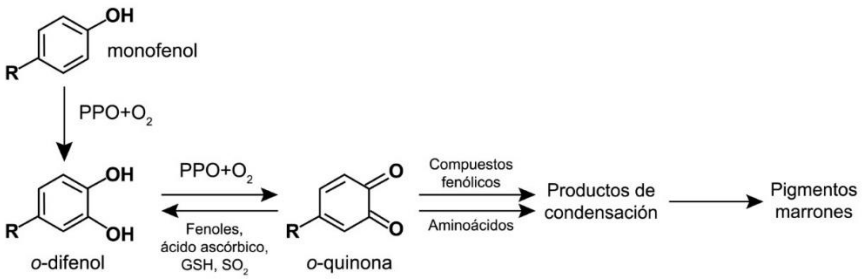


Figura I.1. Oxidación enzimática (Adaptado de Li et al., 2008).

Las principales enzimas responsables de los procesos oxidativos en el mosto durante el procesado de la uva son las polifenoloxidasas (PPO): tirosinasa, catecol oxidasa y lacasa; y en menor medida la peroxidasa (Salgues et al., 1986). La tirosinasa y la catecol oxidasa se encuentran de manera natural en la uva. La tirosinasa puede catalizar tanto la oxidación de monofenoles como de *o*-difenoles (Singleton, 1987), a diferencia de la catecol oxidasa que únicamente puede catalizar la oxidación de catecoles (Decker et al., 2006) (Figura I.2). Por otro lado, la lacasa suele estar presente en los mostos procedentes de vendimias con podredumbre, debido a que esta enzima es producida por mohos como *Botrytis cinerea* (Du Toit et al., 2006). Además, entre las PPO, la lacasa se considera particularmente perjudicial ya que es más resistente a la acción inhibidora del SO_2 (Du Toit et al., 2006).

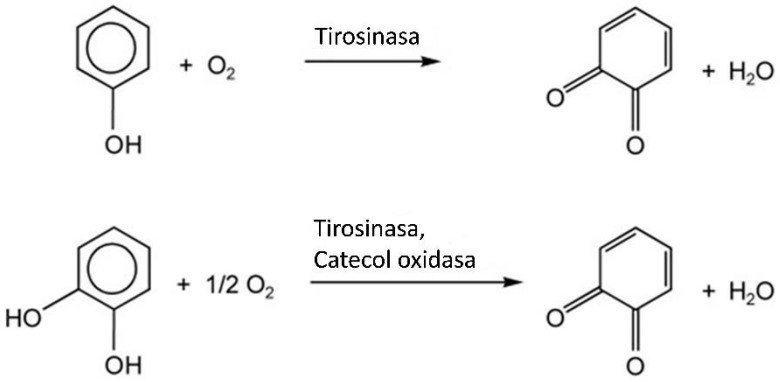


Figura I.2. Actividad enzimática de la tirosinasa y de la catecol oxidasa (Adaptado de Decker et al., 2006).

En el mosto de uva, los principales sustratos de la oxidación enzimática son los ácidos hidroxicinamoiltartáricos (ésteres de ácidos hidroxicinámicos con ácido tartárico), principalmente ácido caftárico (ácido cafeoiltartárico) y ácido cutárico (ácido *p*-cumaroiltartárico) (Cheynier et al., 1989; Gunata et al., 1987). Las quinonas formadas, a partir de estos fenoles susceptibles a la oxidación enzimática, pueden ser reducidas mediante la oxidación acoplada de ácido ascórbico, SO_2 u otros compuestos fenólicos, como los flavonoides, los cuales se oxidan a quinonas secundarias (Cheynier y Ricardo Da Silva, 1991). Los compuestos fenólicos regenerados, en la oxidación acoplada, pueden volver a oxidarse mediante enzimas PPO, propagando la cadena de oxidación (Cheynier et al., 1990; Singleton, 1987). Las quinonas, procedentes de las oxidaciones enzimáticas y acopladas, polimerizan y forman pigmentos pardos (Cheynier et al., 1990) (Figura I.3).

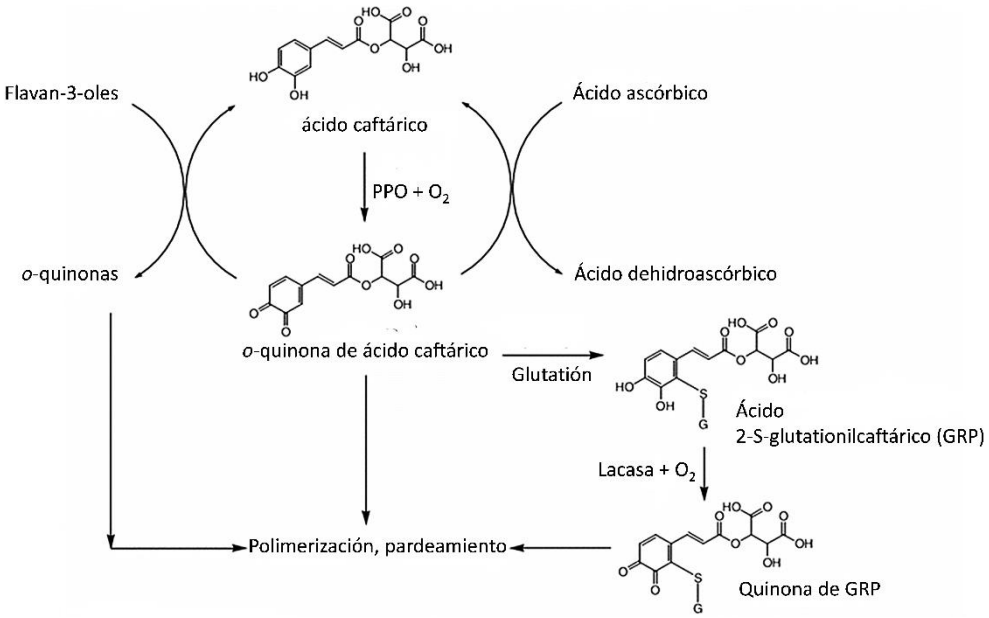


Figura I.3. Mecanismo de oxidación del ácido caftárico, formación de GRP, polimerización y pardeamiento en mosto (Adaptado de Comuzzo y Zironi, 2013).

El pardeamiento enzimático del mosto puede ser controlado o ralentizado por antioxidantes exógenos como el SO_2 (que inhiben la actividad enzimática o regeneran las quinonas), así como por sustancias que se encuentran de manera natural en la uva como son los tioles y que pueden bloquear las reacciones de las *o*-quinonas (Cheynier y Van Hulst, 1988). Este es el caso de la oxidación del ácido caftárico y la posterior unión

de la quinona formada con el glutatión (GSH), dando lugar a un producto incoloro denominado producto de reacción de la uva (ácido 2-S-glutationilcaftárico, GRP –Grape Reaction Product–) (Figura I.3) que no es un sustrato de oxidación para la tirosinasa (Salgues et al., 1986). Ello implica que, si existe suficiente cantidad de GSH en el mosto de uva, este puede atrapar a las quinonas y disminuir la formación de pigmentos pardos (Singleton y Cilliers; 1995; Singleton et al., 1985; Du Toit et al., 2006). Por ello, la susceptibilidad del mosto al pardeamiento enzimático depende tanto del contenido inicial de ácidos hidroxicinámicos como de su riqueza en glutatión (Cheynier et al., 1990). No obstante, otros compuestos del mosto (ácido ascórbico y compuestos fenólicos) también reaccionan con la quinona del ácido caftárico, compitiendo así con el glutatión y limitando la formación del GRP, mientras aumentan las quinonas secundarias y la formación de polímeros pardos (Rigaud et al., 1991).

En presencia de lacasa, en mostos procedentes de uvas con un déficit de estado sanitario por contaminación de *Botrytis cinerea*, el GRP es oxidado a su correspondiente *o*-quinona (Salgues et al., 1986) (Figura I.3). Además, el GRP puede ser hidrolizado lentamente durante el envejecimiento en botella (Cheynier et al., 1986) o ser oxidado por la quinona del ácido caftárico después de haberse agotado el glutatión, dando como resultado un pardeamiento intenso (Cheynier y Van Hulst, 1988).

Durante la etapa prefermentativa, los productos de condensación, formados como resultado de la oxidación de los compuestos fenólicos, evolucionan dando como resultado formas insolubles que son eliminadas fácilmente con el desfangado (Cheynier et al., 1989). Por ello, uno de los tratamientos que se realiza con el objetivo de disminuir la concentración de compuestos fenólicos y, por tanto, para mejorar la estabilidad de los vinos blancos es la hiperoxidación, que consiste en insuflar oxígeno a los mostos para acelerar la oxidación de los compuestos fenólicos y separar los productos insolubles. La hiperoxidación es una técnica especialmente interesante en el caso de mostos que han tenido una maceración con las partes sólidas de la uva o un prensado fuerte, los cuales tienen un contenido elevado en flavan-3-oles. Por el contrario, es menos efectiva en los mostos con altas concentraciones en glutatión, en los que las reacciones de oxidación acoplada y condensación están bloqueadas (Cheynier et al., 1989).

I.1.2. Oxidación química

La oxidación química comienza cuando el oxígeno disuelto en el vino reacciona con los compuestos fenólicos en presencia de metales de transición como el hierro (Fe II) o el cobre (Cu I), los cuales actúan como catalizadores. Estas reacciones de oxidación dan como resultado subproductos de semiquinona y quinona, mientras el O_2 es reducido a peróxido de hidrógeno (H_2O_2) (Danilewicz et al., 2008; Danilewicz y Wallbridge, 2010). El H_2O_2 junto con el ion ferroso (Fe II) genera el radical hidroxilo ($^{\bullet}OH$), capaz de oxidar cualquier molécula orgánica del vino, y es conocida como la reacción de Fenton (Waterhouse y Laurie, 2006). Entre los radicales libres producidos durante la oxidación del vino, el radical 1-hidroxietilo es el más abundante y podría ser el principal agente responsable de otras oxidaciones del vino (Elías et al., 2009).

I.1.2.1. Activación del oxígeno disuelto en el vino, formación de quinonas y especies reactivas de oxígeno

Durante la oxidación química del vino se producen quinonas y especies reactivas de oxígeno, como resultado de la oxidación de los compuestos fenólicos. Estas reacciones de oxidación comienzan con la activación del oxígeno disuelto en el vino, debido a que el oxígeno posee una configuración electrónica en estado normal (triplete) que no reacciona directamente con los polifenoles. Aunque la activación del oxígeno puede darse por exposición a la luz, la forma más común de activación del oxígeno en el vino es la catálisis por metales de transición, que aumenta considerablemente su reactividad (Danilewicz, 2003). De este modo, la limitación de la reactividad del oxígeno en estado triplete es superada mediante la transferencia de un electrón por parte de los iones de metales de transición reducidos, específicamente Fe (II) y Cu (I) (Oliveira et al., 2011).

La velocidad de activación del O_2 es más elevada a mayor disponibilidad del Fe (II) para actuar como catalizador. El estado de oxidación del Fe depende de la composición química del vino. Generalmente, a mayor concentración de compuestos fenólicos, capaces de reducir el Fe (III) a Fe (II), la velocidad de activación del O_2 es más elevada. Sin embargo, a medida que avanza la oxidación y la polimerización fenólica, la disponibilidad del Fe (II) para actuar como catalizador disminuye, debido a que una gran

fracción de Fe (III) está unida a la fracción orgánica del vino como tartratos (Weber,1991) y taninos (Paleologos et al., 2002). Por lo tanto, la disponibilidad del Fe (II), que influye en la velocidad de oxidación, depende de la composición inicial del vino y de cómo reaccionen los metales de transición con los compuestos de la matriz del vino a lo largo de la oxidación y envejecimiento del mismo.

Durante la oxidación química del vino, el O_2 es reducido a H_2O en una secuencia de reacciones consecutivas y durante este proceso se generan especies reactivas de oxígeno que son agentes altamente oxidantes. El potencial redox de estas especies reactivas de oxígeno al pH del vino se muestra en la Tabla I.1. Entre ellas, destacan el radical hidroxilo ($^{\bullet}OH$) y el radical hidroperoxilo ($HOO^{\bullet}$) con potenciales redox lo suficientemente altos para oxidar otras moléculas orgánicas (Tabla I.1).

Tabla I.1. Potencial Redox de las especies reactivas de oxígeno a pH 3.6 (Danilewicz, 2012).

Especies reactivas de oxígeno	**Potencial Redox (V)**
Estado normal oxígeno (O_2)	-0.09
Radical hidroperoxilo ($HOO^{\bullet}$)	1.22
Peróxido de hidrógeno (H_2O_2)	0.59
Radical hidroxilo $^{\bullet}OH$	2.53

Como se ilustra en la Figura I.4, la formación de estas especies se produce de forma secuencial y comienza cuando el oxígeno, en presencia de Fe (II) o Cu (I), acepta un electrón formando el anión superóxido que en el vino se encuentra en la forma de radical $HOO^{\bullet}$. Los compuestos fenólicos son oxidados por el radical $HOO^{\bullet}$ a quinona, mientras que el radical se reduce a H_2O_2. Consecutivamente, el H_2O_2 en presencia de Fe (II) da lugar a la reacción de Fenton. A partir de esta reacción se genera el radical $^{\bullet}OH$, un radical altamente reactivo que puede oxidar cualquier molécula orgánica del vino y produce H_2O, producto final de la reducción del oxígeno (Danilewicz, 2003; Waterhouse y Laurie, 2006). Al ser el etanol uno de los compuestos mayoritarios de la matriz del vino, el $^{\bullet}OH$ reacciona rápidamente con el etanol oxidándolo al radical 1-hidroxietilo, el cual es responsable de la oxidación de otras sustancias orgánicas en vino (Elias et al. 2009).

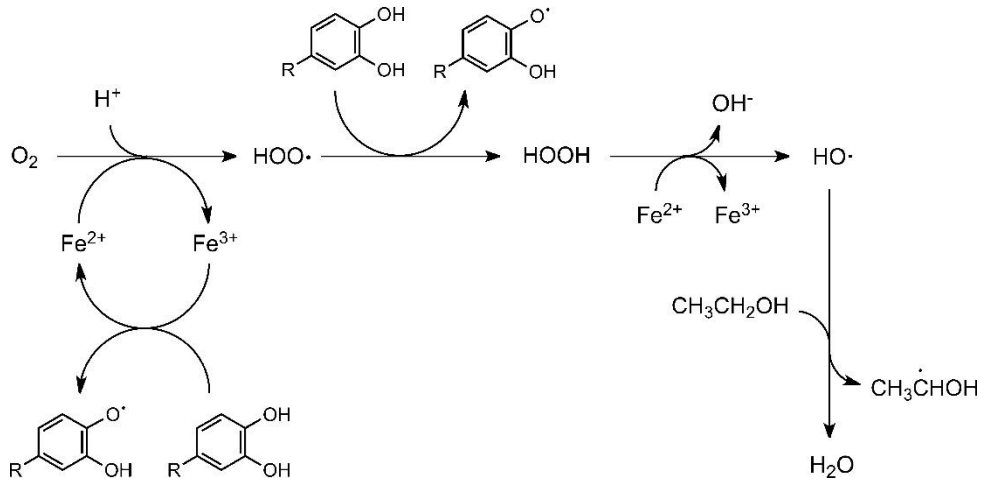


Figura I.4. Mecanismos de oxidación química en vino (Kreitman et al., 2013).

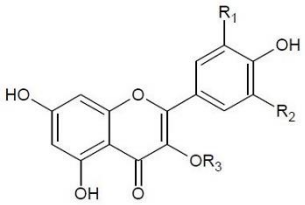
I.1.2.2. Mecanismos de oxidación de los compuestos fenólicos del vino

Los compuestos fenólicos se caracterizan por tener al menos un anillo aromático (núcleo bencénico) con uno o varios grupos hidroxilos. En la Tabla I.2. se muestran los principales compuestos fenólicos presentes en las uvas, clasificados en flavonoides y no flavonoides. Los flavonoides (C6-C3-C6) de la uva incluyen a los antocianos, flavan-3-oles y flavonoles. Por otro lado, los no flavonoides engloban estilbenos (C6-C2-C6) y ácidos fenólicos, estos últimos clasificados en ácidos hidroxicinámicos (C6-C3) y ácidos hidroxibenzoicos (C6-C1).

Tabla I.2. Clasificación de los compuestos fenólicos de uvas y vinos *Vitis vinifera.*

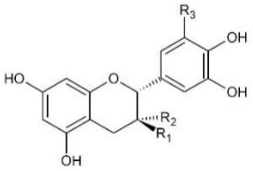
Flavonoides

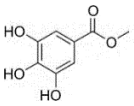
Flavonoles



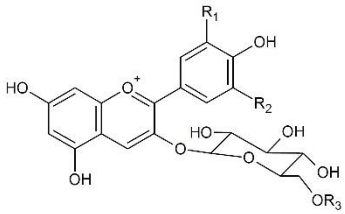
	R_1	R_2	R_3	Sustituyente R_3
Kaempferol	H	H	H	
Quercetina	OH	H	H	Glucósido
Miricetina	OH	OH	H	Galactósido
Isoramnetina	OCH_3	H	H	Ramnósido
Laricitrina	OCH_3	OH	H	Rutinósido
Siringetina	OCH_3	OCH_3	H	Glucurónido

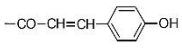
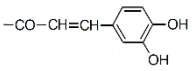
Flavan-3-oles



	R_1	R_2	R_3	Sustituyente R_3
Catequina	OH	H	H	
Epicatequina	H	OH	H	
Galocatequina	OH	H	OH	
Epigalocatequina	H	OH	OH	Galato

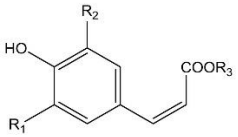
Antocianos

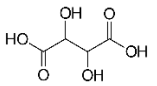


	R_1	R_2	R_3	Sustituyente R_3
				$-CO-CH_3$ Acetil
Cianidina	OH	H	H	
Delfinidina	OH	OH	H	
Peonidina	OCH_3	H	H	Cumaroil
Petunidina	OCH_3	OH	H	
Malvidina	OCH_3	OCH_3	H	
				Cafeoil

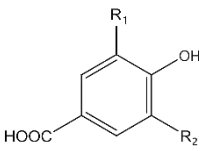
No Flavonoides

Ácidos hidroxicinámicos



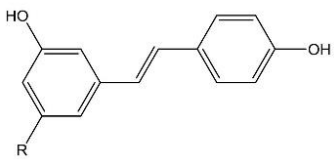
	R_1	R_2	R_3	Sustituyente R_3
p-Cumárico	H	H	H	
Caféico	OH	OH	H	
Ferúlico	OCH_3	H	H	
Fertárico	OCH_3	H	$C_4H_6O_6$	Ácido tartárico ($C_4H_6O_6$)
p-Cutárico	H	H	$C_4H_6O_6$	
Caftárico	OH	OH	$C_4H_6O_6$	

Ácidos hidroxibenzóicos



	R_1	R_2
p-Hidroxibenzoico	H	H
Protocatéquico	H	OH
Gálico	OH	OH
Vanílico	OCH_3	H
Siríngico	OCH_3	OCH_3

Estilbenos



Resveratrol R: OH

Resveratrol-Glucósido R: O-$C_6H_{12}O_6$

(Isómero *trans*)

Los compuestos fenólicos se encuentran distribuidos en el raspón, hollejo, pulpa y semillas de la uva (Figura I.5). La composición fenólica de las uvas está condicionada por la variedad de *Vitis vinifera*, además de las prácticas agronómicas y las condiciones climáticas que afectan el desarrollo de la uva. Por otro lado, numerosos factores de las técnicas de vinificación influyen en gran medida en la extracción y en la estabilidad de los compuestos fenólicos en el vino (como son el tiempo y la temperatura de fermentación y maceración de los hollejos y semillas, el prensado, la estabilización, clarificación y el envejecimiento).

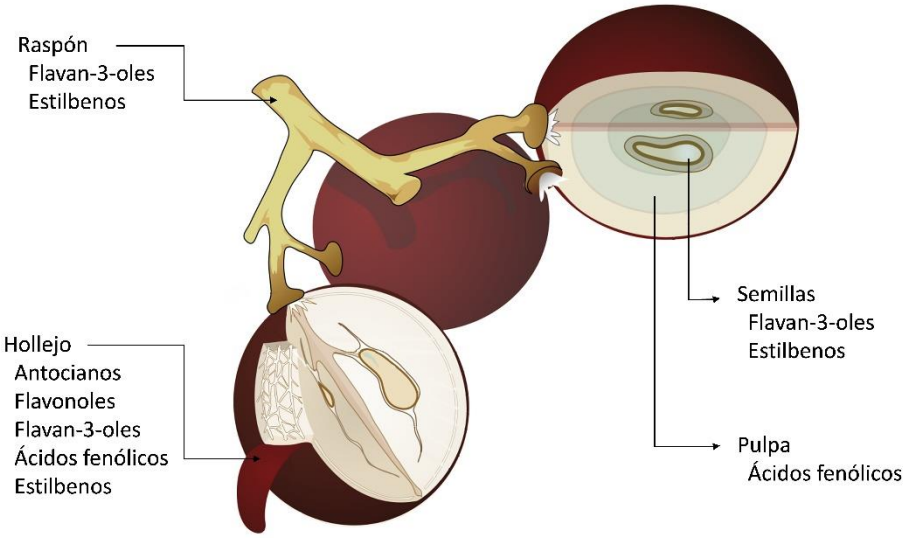


Figura I.5. Distribución de los compuestos fenólicos en el racimo de uva.

Debido a su estructura química, los compuestos fenólicos se consideran antioxidantes ya que pueden neutralizar radicales libres directamente mediante la donación de átomos de hidrógeno y estabilización del compuesto resultante por resonancia (Vanderhaegen et al., 2006). Sin embargo, en el vino, su capacidad de ceder electrones rápidamente aumenta la formación de H_2O_2 y, a consecuencia de ello, propagan la oxidación. Como se ha comentado en la sección anterior (1.1.2.1), el Fe (II) cataliza la reacción de activación del O_2 al radical $HOO^{\bullet}$. Consecutivamente, los compuestos fenólicos al ser sustancias muy reductoras le ceden un hidrógeno al radical $HOO^{\bullet}$ formando H_2O_2 y radicales de semiquinona (Waterhouse y Laurie, 2006) (Figura I.6).

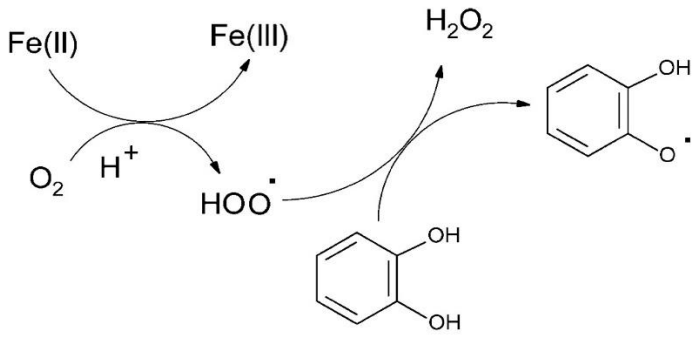


Figura I.6. Oxidación de los compuestos fenólicos por el radical hidroperoxilo (HOO•) (Adaptado de Danilewicz, 2014).

Otra vía de oxidación de los compuestos fenólicos es la catálisis por metales de transición, al reducir el Fe (III) a Fe (II) los fenoles son oxidados a semiquinonas (Elias y Waterhouse, 2010). Posteriormente, la semiquinona resultante es capaz de reducir el Fe (III) oxidándose simultáneamente a quinona (Figura I.7)

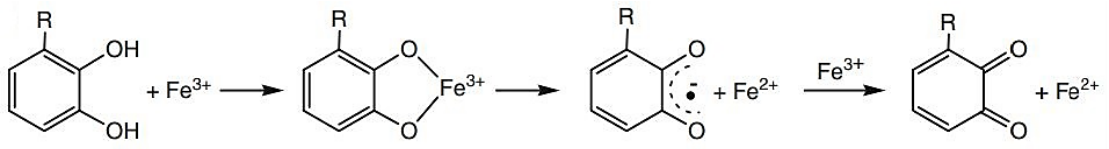


Figura I.7. Oxidación de los compuestos fenólicos por Fe (III) (Danilewicz, 2007).

Además, otros radicales, como el radical •OH, el radical peroxomonosulfato ($SO_5^{\bullet-}$) y el radical sulfato ($SO_4^{\bullet-}$) también pueden oxidar los compuestos fenólicos (Danilewicz, 2007). En la Figura I.8 se muestra la reacción de oxidación de los compuestos fenólicos por el radical $SO_5^{\bullet-}$ (Danilewicz, 2007).

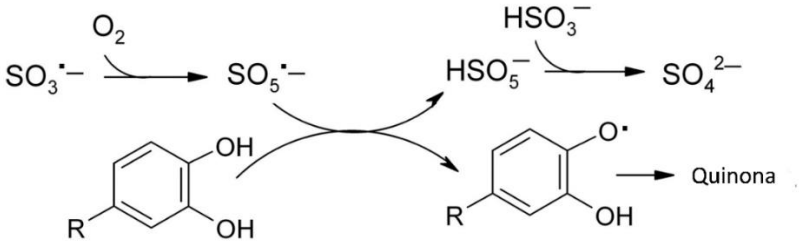


Figura I.8. Oxidación de los compuestos fenólicos por el radical peroxomonosulfato ($SO_5^{\bullet-}$) (Danilewicz, 2007).

La velocidad de reacción de cada compuesto fenólico con los radicales depende de su capacidad para formar un radical estable (Danilewicz, 2011). Es decir, aquellos compuestos que tras la formación del radical semiquinona pueden ser estabilizados por resonancia (deslocalización electrónica). Los compuestos fenólicos que contienen un grupo catecol (1,2-difenol) como son la catequina, epicatequina, ácido caftárico o taninos condensados, y un grupo galato (1,2,3-trifenol o también denominado pirogalol) los cuales incluyen el ácido gálico, galocatequina y epigalocatequina, son los más fáciles de oxidar porque el radical semiquinona puede estabilizarse formando una *o*-quinona (Figura I.9). Por otro lado, los monofenoles, meta-difenoles y fenoles sustituidos (especialmente los derivados metoxi) no se oxidan tan fácilmente porque no producen radicales semiquinona estabilizados (Waterhouse y Laurie, 2006).

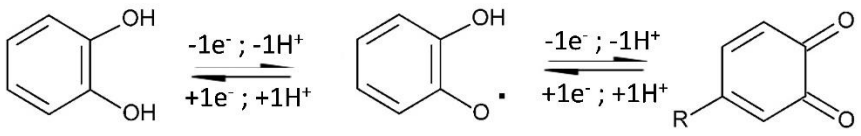


Figura I.9 Mecanismos de oxidación de catecol, formación de un radical semiquinona y una *o*-quinona (Chiorcea-Paquim et al., 2020).

Además, cuanto menor es el potencial redox del compuesto fenólico más fácilmente se oxida (Danilewicz, 2003). Kilmartin et al. (2001) mostraron que los compuestos fenólicos más fácilmente oxidables, debido a su bajo potencial redox, son aquellos que, como se ha indicado anteriormente, contienen en su estructura un grupo catecol y un grupo galato. Estos autores demostraron que la quercetina tiene un potencial redox

menor que la mayoría de los compuestos fenólicos del vino, ya que al igual que la catequina y la epicatequina, tiene una conjugación extendida con el anillo heterocíclico que proporciona una estabilización adicional, lo cual contribuye a su rápida pérdida en el vino (Castellari et al., 2000; Danilewicz, 2003). Entre los flavan-3-oles, la epicatequina se oxida a un potencial más bajo que la catequina (Kilmartin et al., 2001; Yang et al., 2001), y normalmente se esperaría que, en condiciones idénticas, la epicatequina pudiera oxidarse a mayor velocidad (Sioumis et al., 2006). Otros compuestos fenólicos, como el ácido ferúlico, *t*-resveratrol, ácidos vainílico y *p*-cumárico, necesitan un mayor potencial para oxidarse (Kilmartin et al., 2001). Por ello, estos compuestos no participan en los primeros pasos de la oxidación del vino con la misma facilidad que aquellos compuestos que contienen un grupo catecol o galato. Del mismo modo, la malvidina-3-glucósido, el antociano principal del vino tinto, no se oxida fácilmente mientras que la cianidina 3-O-6"-*p*-cumaroil-glucósido y delfinidina 3-O-glucósido son más sensibles a la oxidación (Cheynier y Fulcrand, 2003).

Los antocianos también pueden ser degradados por H_2O_2 (Özkan et al., 2002; Sondheimer y Kertesz, 1952) y actuar como secuestradores del radical $^{\bullet}OH$ en vinos tintos (Rivero-Pérez et al., 2008). La estructura conjugada de las antocianinas permite la deslocalización de los electrones y da como resultado productos radicales muy estables (Van Acker et al., 1996). La actividad antioxidante de los antocianos depende del número y posición de los grupos hidroxilo, metoxilación en el anillo B (Pereira et al., 1997) y grado de glicosilación (Kähkönen y Heinonen, 2003).

Las quinonas formadas tras la oxidación de los compuestos fenólicos, debido a su carácter electrófilo, pueden combinarse con compuestos nucleofílicos del vino incluidos compuestos fenólicos, tioles y aminoácidos (Sección I.1.2.3). Las reacciones entre las quinonas y los compuestos fenólicos se denominan polimerización regenerativa (Figura I.10), en las que se producen oligómeros y polímeros de mayor tamaño (Singleton 1987).

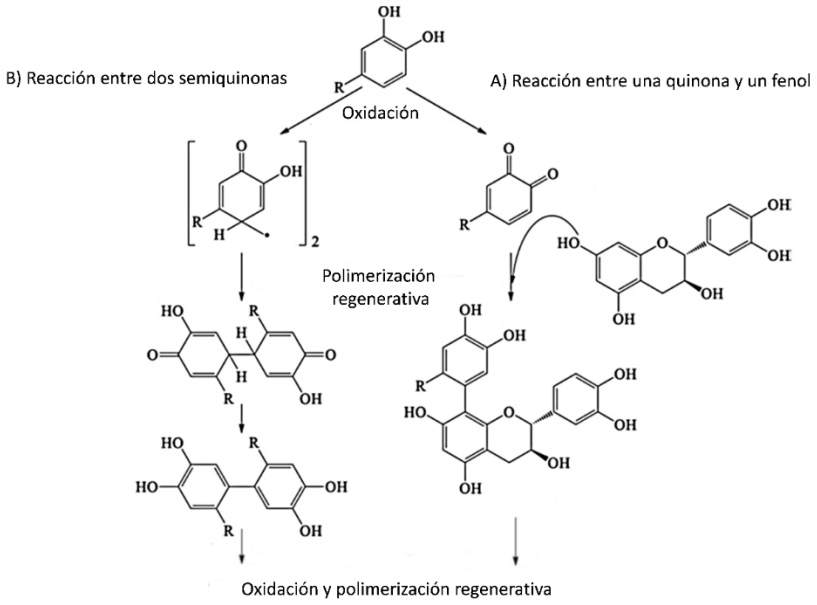


Figura I.10. Oxidación y polimerización regenerativa. A) Regeneración de oligómeros por reacción entre una quinona y un compuesto fenólico. B) Regeneración de oligómeros por reacción entre dos semiquinonas (Adaptado de Li y Duan, 2019).

Un dímero de quinona-fenol se puede convertir en un difenol (Figura I.10, A), más susceptible a la oxidación por tener potenciales redox más bajos (Boulton et al., 1996; Li et al., 2005). De la misma manera, dos radicales libres de semiquinona también puede unirse (Figura I.10, B), formando un dímero reoxidable (Singleton, 1987). Por lo tanto, los difenoles regenerados aceleran las reacciones de polimerización, ya que pueden ser de nuevo oxidados, produciendo oligómeros o polímeros más grandes hasta que finalmente precipitan (Boulton et al., 1996; Zhai et al., 2001).

I.1.2.3. Productos intermedios de oxidación y consecuencias en la calidad del vino

Durante los procesos oxidativos se forman productos intermedios muy reactivos que originan importantes cambios en la composición química y en la calidad del vino. Especialmente, las quinonas, y los radicales $^{\bullet}$OH y 1-hidroxietilo son moléculas muy inestables que pueden reaccionar con los compuestos de la matriz del vino. Algunos ejemplos son las diferentes reacciones de quinonas con tioles, fenoles y aminoácidos, la oxidación de etanol por la reacción de Fenton, además de las reacciones de los

compuestos carbonilos, formados durante la oxidación, con diferentes moléculas orgánicas del vino.

Una oxidación controlada del vino puede ser beneficiosa para disminuir aromas vegetativos (Cejudo-Bastante et al., 2011) o aromas indeseables de reducción (Nikolantonaki y Waterhouse 2012), mientras que una oxidación excesiva degrada compuestos aromáticos de importante impacto sensorial y genera aromas relacionados con la oxidación del vino, como el metional y el fenilacetaldehído (con aromas a patata cocida y a miel, respectivamente) (Escudero et al., 2000; Ferreira el al., 2002). Por otro lado, las reacciones de oxidación pueden llevar a efectos deseados o perjudiciales en el color según el tipo de vino. En el caso de vinos tintos, la oxidación de los compuestos fenólicos estabiliza el color (Boulton et al., 1996) y disminuye la sensación de amargor y astringencia en boca (Castellari et al., 1998). Por el contrario, la oxidación de los compuestos fenólicos en vinos blancos provoca un pardeamiento indeseable (Singleton y Kramling, 1976; Sioumis et al., 2006).

A continuación, se describen los mecanismos de acción de los productos intermedios de oxidación y su implicación en la calidad del vino.

❖ Mecanismos de acción de las quinonas

Las *o*-quinonas, productos de oxidación de los compuestos fenólicos, son moléculas electrofílicas que pueden reaccionar con varios nucleófilos del vino como son los tioles, los antioxidantes utilizados en vinificación, los compuestos fenólicos y en menor medida los aminoácidos (Figura I.11). En presencia de HSO_3^- y ácido ascórbico, antioxidantes ampliamente utilizados para prevenir la oxidación del vino, las quinonas reaccionan rápidamente y se reducen a su forma fenol (Barril et al., 2012; Danilewicz, 2008; Oliveira 2016). Las reacciones, entre las quinonas y los compuestos nucleófilos del vino, dan lugar a cambios importantes en la calidad del vino, incluidas modificaciones del color, disminución de compuestos volátiles varietales, pérdida de aromas desagradables (compuestos volátiles de reducción) o formación de compuestos volátiles asociados a la oxidación del vino (Oliveira, 2017).

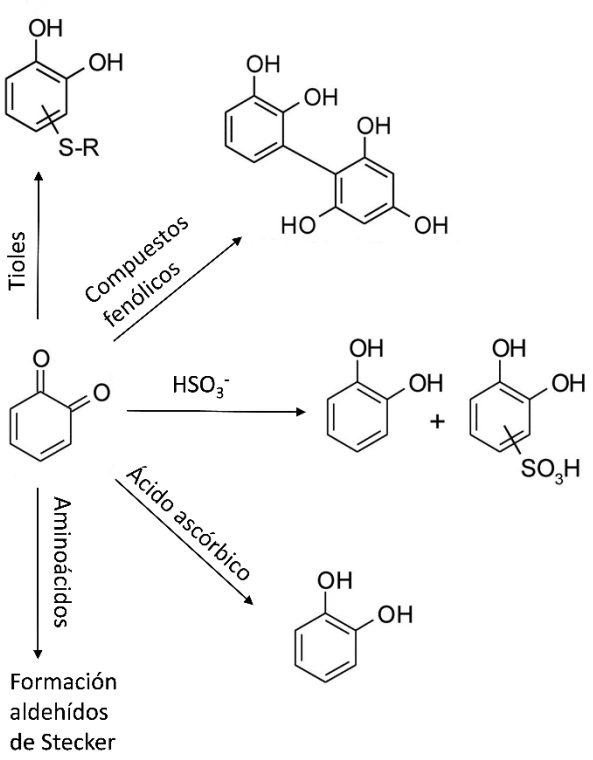


Figura I.11. Mecanismos de acción entre quinona y nucleófilos del vino (Adaptado de Nikolantonaki y Waterhouse, 2012).

Uno de los productos más conocidos como consecuencia de la reacción entre una quinona y un tiol es el producto de reacción de la uva (GRP). Formado tras la unión de la quinona del ácido caftárico con el tripéptido glutatión (comentado anteriormente en la oxidación enzimática, sección I.1.1). Otros tioles volátiles como el 3-mercaptohexanol, relacionado con el aroma afrutado de algunas variedades de uva como Sauvignon Blanc, Cabernet Sauvignon, Merlot y Cabernet Franc, también pueden reaccionar con la quinona procedente de la oxidación de la catequina, dando como resultado una pérdida del carácter varietal afrutado (Blanchard et al., 2004). Del mismo modo, la disminución de aromas indeseables a reducción se atribuye a la formación de quinonas que reaccionan fácilmente con los compuestos de azufre volátiles, dando lugar a compuestos estables en condiciones normales de almacenamiento (Nikolantonaki et al., 2012). Sin embargo, posteriormente Vela et al. (2018) demostraron que los compuestos de azufre volátiles, en particular los sulfhidrilos de bajo peso molecular como el sulfuro de hidrógeno (H_2S) y el metanotiol (MeSH) se pierden con la microoxigenación, pero

pueden regenerarse después durante un envejecimiento acelerado anóxico (oxígeno disuelto agotado) a alta temperatura (50 °C). Por lo tanto, la estabilidad de las quinonas unidas a compuestos de azufre volátiles se ve comprometida según las condiciones de almacenamiento a las que se someta el vino.

Las quinonas también pueden polimerizar con los flavan-3-oles dando como resultado importantes cambios en el color y en la sensación gustativa (Guyot y Vercauteren, 1996; Li et al., 2008). La reacción entre quinonas y taninos condensados origina una nueva molécula de mayor tamaño molecular, con enlaces estables al pH del vino, aumentando así la formación de estructuras fenólicas poliméricas (Cheynier et al., 2002; Danilewicz, 2003; Singleton, 2001). Estas reacciones de polimerización en el vino terminado pueden llevar a efectos deseados o perjudiciales según el tipo de vino. En el caso de vinos tintos, la oxidación de los compuestos fenólicos estabiliza el color del vino por polimerización entre los taninos condensados y los antocianos, dando como resultado vinos con tonalidades de color teja persistente (Boulton et al.,1996). Además, la polimerización de los flavan-3-oles y posterior precipitación de los taninos disminuye la sensación de amargor y astringencia en boca (Castellari et al., 1998). Mientras que la oxidación de compuestos fenólicos en vinos tintos da lugar a una suavización del carácter tánico, no se conocen mejoras en la sensación en boca que surjan de la oxidación del vino blanco. Además, las reacciones de polimerización de los flavan-3-oles en vinos blancos provocan un pardeamiento indeseable (Sioumis et al., 2006; Singleton y Kramling, 1976).

Otra de las vías de acción de las *o*-quinonas es la formación de aldehídos de Strecker como el fenilacetaldehído y metional, los cuales poseen un bajo umbral de detección y son asociados con aromas de oxidación a miel y patata cocida, respectivamente (Escudero et al., 2000; Ferreira et al., 2002). Estos aldehídos a bajas concentraciones pueden aumentar la complejidad de un vino, pero a niveles más altos, son responsables de la pérdida de frescor (San-Juan et al., 2011) y el desarrollo de compuestos volátiles indeseables relacionados con la oxidación (Culleré et al., 2007). En aquellos vinos en los que los tioles varietales no son compuestos aromáticos clave, la formación de estos aldehídos es la principal causa del deterioro del aroma del vino (Bueno et al., 2016). Varios autores han demostrado la formación de fenilacetaldehído y metional a partir de

quinonas de catequina, ácido cafeico, ácido gálico, y los aminoácidos fenilalanina y metionina (Grant-Preece et al., 2013; Monforte et al., 2020; Oliveira et al., 2017).

Los mecanismos de acción de las quinonas, comentados anteriormente, se desarrollan a diferentes velocidades según la composición química del vino. Nikolantonaki y Waterhouse (2012), cuantificaron las tasas de reacción de la 4-metil-1,2-benzoquinona con nucleófilos relevantes en el vino como son los tioles volátiles (4-metil-4-sulfanilpentan-2-ona, 3-sulfanilhexan-1-ol, 2-furanmetanotiol y sulfuro de hidrógeno); el glutatión; SO_2; ácido ascórbico; aminoácidos (metionina y fenilalanina); y floroglucinol. Los resultados de este estudio mostraron que los antioxidantes del vino (SO_2, glutatión y ácido ascórbico) y el sulfuro de hidrógeno fueron los nucleófilos más reactivos con la quinona estudiada. También observaron que los tioles volátiles mostraron velocidades de reacción crecientes a menor impedimento estérico. Por su parte, el floroglucinol reaccionó con la quinona a menor velocidad que los tioles volátiles, mientras que los aminoácidos, metionina y fenilalanina, presentaron una velocidad de reacción limitada con la *o*-quinona en el tiempo estudiado. En la misma línea, Oliveira et al. (2016) estudiaron las reacciones entre las quinonas de ácido gálico, ácido caféico y catequina, con distintos nucleófilos del vino, como el SO_2, el ácido ascórbico, los tioles varietales y los aminoácidos fenilalanina y metionina, y confirmaron que la reactividad más baja de estos compuestos con las quinonas correspondía a los aminoácidos. Los mismos autores demostraron que la quinona de catequina produce mayor concentración de fenilacetaldehído, seguida de las quinonas procedentes de los ácidos gálico y cafeico.

- Mecanismos de acción del radical $^{\bullet}OH$

Entre las especies reactivas de oxígeno, el radical $^{\bullet}OH$ es uno de los oxidantes más reactivos, con una vida útil muy corta, capaz de oxidar rápidamente la mayoría de las sustancias orgánicas (Choe y Min, 2006; Zhao, 1999). El radical $^{\bullet}OH$ no es selectivo y reacciona únicamente con moléculas adyacentes por poseer un radio de acción pequeño (Waterhouse y Laurie, 2006; Zhao, 1999). Por ello, el etanol, al ser el compuesto mayoritario, de la matriz del vino es el sustrato más susceptible a la oxidación por dicho radical. Mediante resonancia paramagnética electrónica, se confirmó que el radical $^{\bullet}OH$ extrae un hidrógeno del etanol dando lugar a la formación del radical 1-hidroxietilo,

también denominado 1-HER, y en menor proporción se forma el radical 2-hidroxietilo (Elias et al., 2009).

Como se ilustra en la Figura I.12, además del etanol, el ácido tartárico y el glicerol, debido a sus concentraciones relativamente altas en el vino, también pueden ser oxidados por el radical •OH dando lugar al ácido glioxílico y al gliceraldehído, respectivamente (Es-Safi et al., 1999; Fulcrand et al., 1997; Laurie y Waterhouse, 2006a).

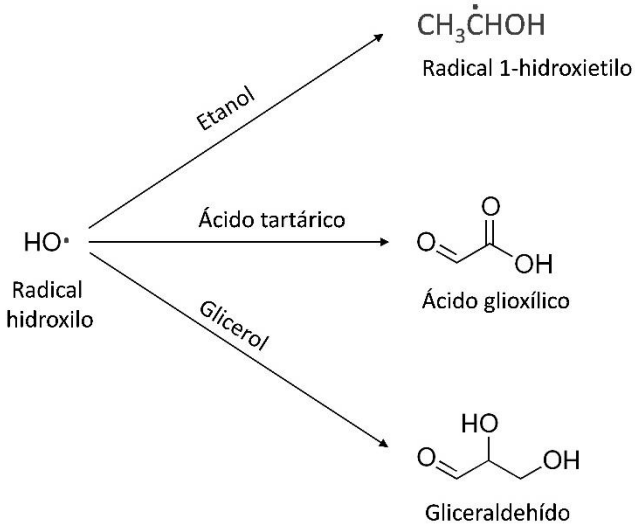


Figura I.12. Reacción del radical •OH con los compuestos mayoritarios del vino.

Laurie y Waterhouse (2006a) estudiaron la oxidación del glicerol por la catálisis de H_2O_2 y Fe (II) en vino modelo, e identificaron el gliceraldehído y la dihidroxiacetona como los principales compuestos tras la reacción de Fenton. Además, observaron un notable aumento de color tras adicionar estos derivados de oxidación a un vino tinto joven. Ante estos resultados, analizaron la reacción del gliceraldehído con (+)-catequina, (-)-epicatequina y malvidina-3-glucósido, en soluciones modelo, y demostraron la formación de aductos flavan-3-ol-flavan-3-ol y antocianina-flavan-3-ol unidos con gliceraldehído (Laurie y Waterhouse, 2006b).

Por otro lado, el ácido tartárico, al igual que el acético y oxálico, forman complejos con el Fe (III) que no son atacados por el radical •OH (El-Ghenymy et al., 2012). Por ello, la reactividad del radical •OH con el ácido tartárico puede ser impedida si se da la unión

del ácido tartárico con el Fe (III), antes de que el Fe (III) sea reducido de nuevo a Fe (II) por los compuestos fenólicos. Además, otros autores han demostrado que la degradación oxidativa del ácido tartárico y la producción de ácido glioxílico no tiene lugar en ausencia de luz (Clark et al., 2007; Clark et al., 2011), por lo que en vinos conservados en botellas opacas o en oscuridad se evita la oxidación del ácido tartárico. No obstante, en el caso de que el vino esté expuesto a condiciones desfavorables durante el transporte o almacenamiento, como es exposición a la luz o a temperaturas altas, el ácido glioxílico puede reaccionar con dímeros de catequina o epicatequina, formándose el catión xantilo y ocasionando pardeamientos no deseados en vinos blancos (Clark et al., 2007; Clark et al., 2011; Dias et al., 2012).

A pesar de que los compuestos fenólicos se encuentran en el vino en menor concentración (que el etanol, el glicerol o el ácido tartárico) pueden ser oxidados por el radical $^{\bullet}OH$ (Perron y Brumaghim, 2009). Por ello, el radical $^{\bullet}OH$ puede reaccionar con los fenoles dando lugar a radicales de semiquinona y finalmente formarse quinonas en presencia de otros radicales o Fe (III) (Danilewicz, 2008).

❖ Mecanismos de acción del radical 1-hidroxietilo

El radical 1-hidroxietilo, producido a consecuencia de la oxidación del etanol por el radical $^{\bullet}OH$, se oxida a acetaldehído en presencia de Fe (III) u O_2 (Danilewicz, 2003; Elias y Waterhouse, 2010). En la figura I.13 puede observarse que la formación del radical 1-hidroxietilo y su posterior oxidación a acetaldehído es un proceso autocatalítico, el cual tendrá lugar mientras exista etanol, Fe (III) u oxígeno disponible para participar en la reacción. Así pues, durante la oxidación del 1-hidroxietilo a acetaldehído el Fe (III) es reducido simultáneamente a Fe (II). Por otro lado, en presencia de oxígeno, el 1-hidroxietilo forma el radical 1-hidroxietil peroxilo el cual se descompone formando acetaldehído y radical $HOO^{\bullet}$ (Elias et al., 2009). El radical $HOO^{\bullet}$ es reducido por los compuestos fenólicos del vino a H_2O_2 (Waterhouse y Laurie 2006). Por ello, durante la oxidación del 1-hidroxietilo a acetaldehído se regeneran los sustratos de la reacción de Fenton, amplificando así la formación del radical $^{\bullet}OH$, el cual está disponible de nuevo para oxidar el etanol y formar el radical 1-hidroxietilo.

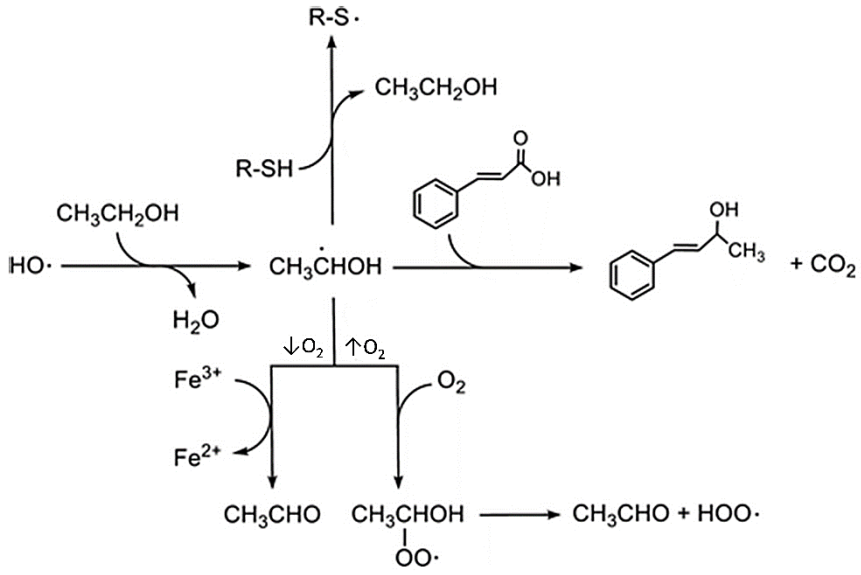


Figura I.13. Formación y mecanismo de acción del radical 1-hidroxietilo (Adaptado de Kreitman et al., 2013).

Gislason et al. (2011) demostraron que el 1-hidroxietilo puede reaccionar con los ácidos hidroxicinámicos, formando alcohol alílico (1-propen-3-ol) y CO_2. Posteriormente, Kreitman et al. (2013), confirmaron que los ácidos hidroxicinámicos reaccionan con el 1-hidroxietilo contribuyendo de este modo a disminuir la formación del radical 1-hidroxietilo. Además, estos autores informaron que el 1-hidroxietilo reacciona de manera directa con los tioles, por lo que su concentración en el vino disminuye con la formación del radical 1-hidroxietilo.

❖ Mecanismos de acción de los compuestos carbonilos

Como se ha comentado anteriormente, durante la oxidación química del vino se producen quinonas, radical $^{\bullet}OH$ y radical 1-hidroxietilo que originan, entre otras consecuencias, un incremento de aldehídos (acetaldehído, gliceraldehído y aldehídos de Strecker). El carácter electrófilo de los aldehídos los hace reactivos a otros nucleófilos del vino (Figura I.14).

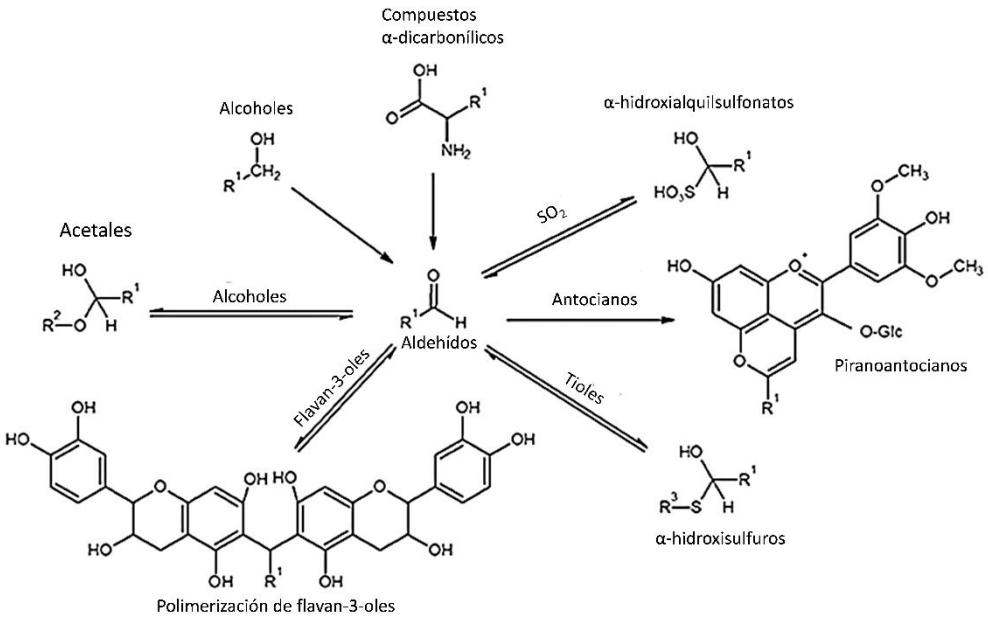


Figura I.14. Formación y reacción de aldehídos en vino (Adaptado de Grant-Preece et al., 2013; Waterhouse et al., 2016).

Algunas de las reacciones más importantes de los aldehídos en el vino son aquellas con los compuestos fenólicos, que estabilizan el color de los vinos tintos (Laurie y Waterhouse, 2006b). Es conocido que el acetaldehído y el gliceraldehído pueden unir dos flavan-3-oles, un flavan-3-ol y un antociano, o dos antocianos (Escribano-Bailón et al., 2001; Laurie y Waterhouse, 2006b). La unión de los flavonoides tiene lugar en el anillo A de cada compuesto fenólico, mediante un puente de etilo producido entre el acetaldehído o el gliceraldehído y dichos compuestos fenólicos. Los pigmentos formados, con puentes de etileno, muestran un desplazamiento batocromático, es decir, poseen tonalidades más violáceas y son menos susceptibles al ataque nucleofílico por el SO_2 o por una molécula de H_2O evitando así la pérdida de color. Sin embargo, esta reacción es reversible y los antocianos podrían liberarse (Escribano-Bailón et al., 2001).

El acetaldehído, también puede reaccionar con la malvidina 3-glucósido y formar visitina B. La vitisina B y la vitisina A (formada por la reacción del ácido pirúvico y la malvidina 3-glucósido), son pigmentos denominados piranoantocianos por tener un anillo de pirano adicional (Figura I.14). Estos pigmentos poseen una tonalidad anaranjada y son más estables a las variaciones de pH y al almacenamiento prolongado (Bakker y Timberlake, 1997).

Los aldehídos también pueden unirse reversiblemente al SO_2 formando α-hidroxialquilsulfonatos (Bueno et al., 2014; de Azevedo et al., 2007; Grant-Preece et al., 2013). Los α-hidroxialquilsulfonatos de acetaldehído y los aldehídos de Strecker pueden actuar como una reserva de aldehídos y ser liberados durante la oxidación del vino (Bueno et al., 2016). Los aldehídos también pueden unirse de forma reversible a tioles como el glutatión o la cisteína para dar α-hidroxisulfuros (Baert et al., 2015; Lienhard y Jencks, 1966; Sonni et al., 2011a). Así como reaccionar con alcoholes dando como resultado acetales (Câmara et al., 2003).

I.2. Alteración microbiológica del vino

Las fermentaciones alcohólica y maloláctica son procesos bioquímicos complejos a controlar durante la vinificación para evitar alteraciones en la calidad del vino. La susceptibilidad del vino al deterioro microbiológico depende de diferentes parámetros, incluidas las especies de levaduras y bacterias presentes en la uva o las instalaciones de vinificación, las características físico-químicas del mosto y del vino (pH y concentración de ácidos orgánicos, azúcar, etanol, entre otros), los tratamientos de estabilización realizados, así como el tipo y la concentración de aditivos utilizados como antimicrobianos (Bartowsky, 2009; Du Toit y Pretorius, 2000; Loureiro y Malfeito-Ferreira, 2003).

Determinadas especies de levaduras y bacterias pueden provocar deterioro microbiológico y pérdida de la calidad del vino. La alteración microbiológica del vino engloba defectos organolépticos como la formación de turbidez, incremento en la formación de ácido acético, aumento de viscosidad y aparición de aromas indeseables debido a compuestos volátiles como acetato de etilo, fenoles volátiles y otros volátiles con bajo umbral de detección (Bartowsky, 2009; Du Toit y Pretorius, 2000). Así como, la producción de compuestos tóxicos para la salud humana, como son las aminas biógenas (Lonvaud-Funel, 2001; Marcobal et al., 2006), la acroleína (Bauer et al., 2016) y el carbamato de etilo (Ryu et al., 2015).

Las levaduras responsables del deterioro microbiológico pueden ser clasificadas en: (i) levaduras fermentativas (*Saccharomyces cerevisiae*), únicamernte cuando se refieren a vinos embotellados con residuos de azúcar; (ii) *Zygosaccharomyces bailii*, que causan turbidez o sedimentos en los vinos embotellados; (iii) especies de *Hansenula*, *Kloeckera*, *Pichia*, *Metchnikowia*, *Debaryomyces*, que son formadoras de velo; y (iv) levaduras que producen mal sabor o aromas indeseables (*Brettanomyces* spp., *Schizosaccharomyces pombe* y *Saccharomycodes ludwigii*) (Kunkee y Bisson,1993; Loureiro y Malfeito-Ferreira, 2003). Entre ellas, *Brettanomyces bruxellensis*, *Zygosaccharomyces bailii* y *Saccharomyces cerevisiae* (solo en el caso de vinos embotellados con azúcar residual) se consideran generalmente como las levaduras alterantes en el sentido más estricto (Loureiro y Malfeito-Ferreira, 2003).

Por otro lado, las bacterias lácticas que se encuentran en el mosto de uva y el vino incluyen cuatro géneros: *Lactobacillus*, *Leuconostoc*, *Oenococcus* y *Pediococcus*. Algunas especies de bacterias lácticas poseen aplicaciones tecnológicas de interés en la elaboración del vino, como por ejemplo la fermentación maloláctica llevada a cabo por *Oenococcus oeni* o por *Lactobacillus plantarumn* para mejorar las características organolépticas de los vinos tintos, mientras que en los vinos blancos, en los cuales se pretende mantener la acidez y la frescura, esta fermentación no es deseada. Además, el crecimiento de *Lactobacillus, Pediococcus* e incluso de algunas cepas de *Oenococcus* en el vino pueden producir compuestos volátiles indeseables, y también aminas biógenas, acroleína y carbamato (Bartowsky, 2009; Ribéreau-Gayon et al., 2006a).

Respecto a las bacterias acéticas que se pueden encontrar en el vino, *Acetobacter*, *Gluconobacter* y *Gluconacetobacter*, son consideradas bacterias alterantes que pueden conducir a un aumento excesivo de acidez volátil (Bartowsky, 2009; Sengun y Karabiyikli, 2011).

I.3. Uso del SO_2 en vinificación

El SO_2 es uno de los productos enológicos más utilizados en vinificación, por su actividad antioxidante, antioxidásica y antimicrobiana. El SO_2 se adiciona principalmente en la etapa prefermentativa sobre la uva o mosto, una vez finalizada la fermentación y antes de embotellar los vinos. Sin embargo, debido a las reacciones adversas que pueden desarrollarse a consecuencia de su ingesta, el sector enológico tiene como propósito disminuir la concentración de SO_2 utilizada en mostos y vinos.

I.3.1. Formas químicas y principales funciones del SO_2 en el vino

En el mosto o el vino, el SO_2 puede encontrarse en distintas formas químicas (según el pH, composición y temperatura), en función de las cuales depende su actividad antioxidásica, antioxidante y antimicrobiana. El SO_2 libre es la fracción presente en forma gaseosa o inorgánica (SO_2 molecular, anión bisulfito: HSO_3^-, ion sulfito: SO_3^{2-}), y la fracción combinada es aquella que se encuentra unida a diferentes compuestos orgánicos de la matriz vínica, denominándose SO_2 total a la suma de ambas fracciones (Figura I.15).

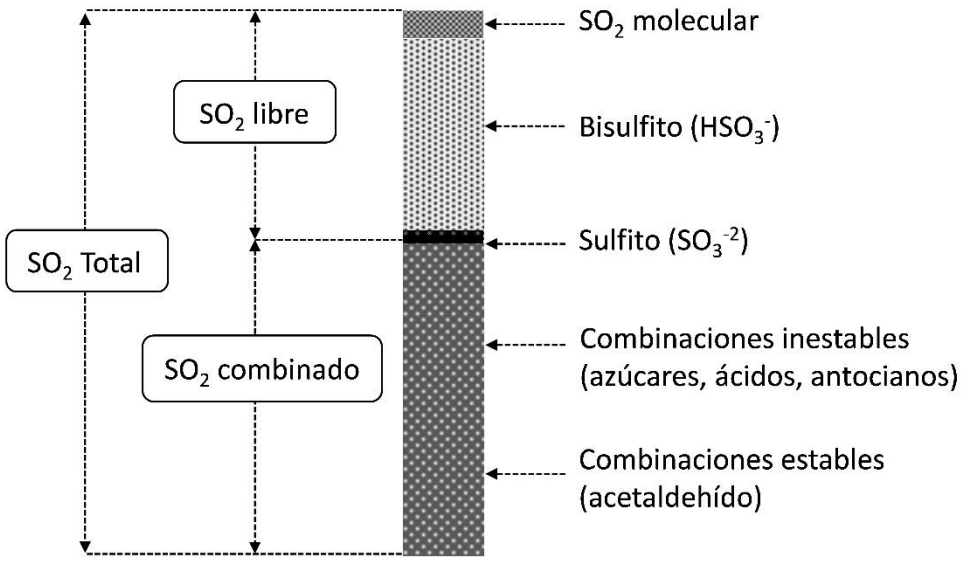


Figura I.15. Formas químicas del SO_2 en el vino.

El SO_2, en forma de HSO_3^-, es capaz de producir inactivación enzimática y prevenir el pardeamiento oxidativo de los mostos (Sección I.1.1). Su efectividad varía dependiendo del tipo de polifenoloxidasas (PPO) presentes en el mosto. Entre ellas, la tirosinasa es más sensible a la inactivación por el SO_2 que la lacasa, por ello esta última requiere concentraciones más altas de SO_2 y puede estar presente en el vino final (Du Toit et al., 2006; Li et al., 2008).

Por otro lado, el HSO_3^- ejerce una importante actividad antioxidante durante la oxidación química del vino (Sección I.1.2). El HSO_3^- actúa sobre las quinonas, reduciéndolas de nuevo a su forma fenol y forma aductos de fenol-ácido sulfónico mediante adición nucleófila (Danilewicz, 2007; Danilewicz, 2008) (Figura I.16, A). Además, el HSO_3^- también reacciona con el H_2O_2 (Danilewicz, 2003; Waterhouse y Laurie, 2006). Al eliminar el H_2O_2 a mayor velocidad que la reacción de Fenton, el HSO_3^- previene eficazmente la formación del radical $^{\bullet}OH$ y la oxidación del etanol (Danilewicz, 2007; Figura I.16, B).

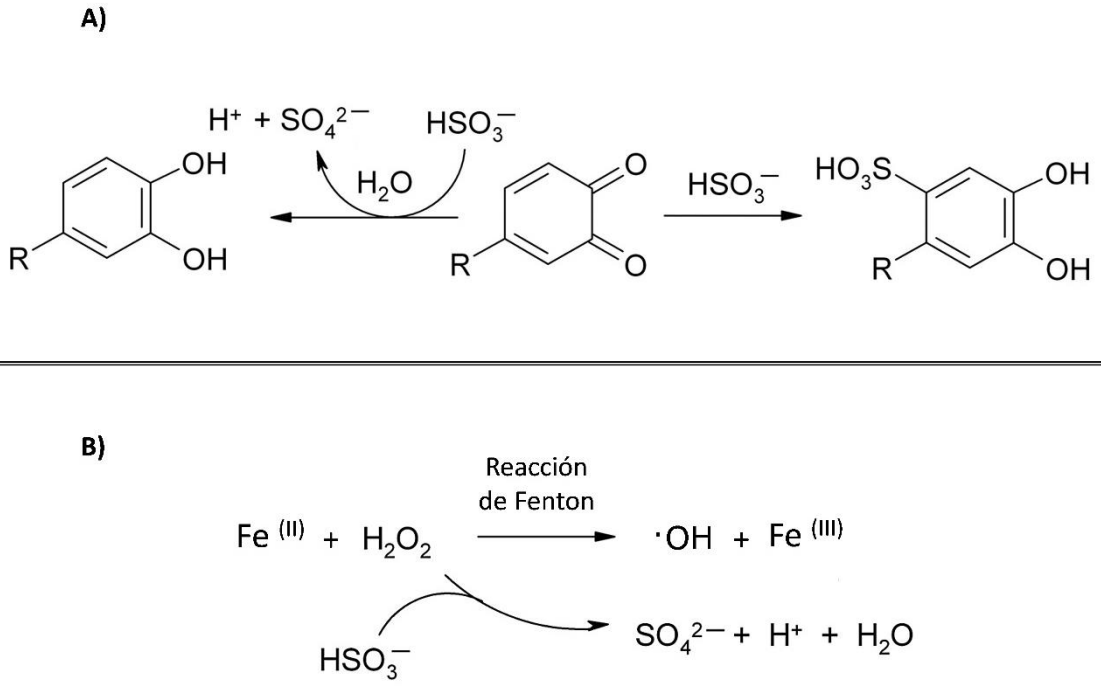


Figura I.16. Actividad antioxidante del bisulfito (HSO_3^-). A) Reducción de las quinonas por el HSO_3^-; B) Reacción del HSO_3^- con el peróxido de hidrógeno (H_2O_2) (Adaptado de Danilewicz, 2007).

Los compuestos fenólicos y el HSO_3^- se complementan entre sí y su equilibrio tiene un papel importante en la velocidad de oxidación química del vino. En presencia de oxígeno, la autooxidación del HSO_3^- es catalizada por el Fe (III) promoviendo la propagación de la cadena radical (Figura I.17, A). Esta secuencia de reacciones se inicia cuando el HSO_3^- se oxida con Fe (III) al radical sulfito ($SO_3^{\bullet-}$), que luego reacciona rápidamente con el oxígeno para producir el radical peroxomonosulfato ($SO_5^{\bullet-}$), el cual es capaz de oxidar el HSO_3^- directamente, aumentando la formación del radical $SO_3^{\bullet-}$ y promoviendo las reacciones en cadena (Brandt et al., 1994; Connick et al., 1995). En el proceso de oxidación del HSO_3^-, por el radical $SO_5^{\bullet-}$, también se forma el radical sulfato ($SO_4^{\bullet-}$), el cual es muy reactivo, con potencial redox similar al radical $^{\bullet}OH$ y, por lo tanto, también oxidará el etanol a acetaldehído (Danilewicz, 2007) (Figura I.17, B). De este modo, en ausencia de compuestos fenólicos, el HSO_3^- generará un sistema altamente oxidante en presencia de oxígeno. Sin embargo, en el vino, los compuestos fenólicos evitan la oxidación del HSO_3^- al interceptar el radical $SO_5^{\bullet-}$ y, por su parte, el HSO_3^-, como se ha comentado anteriormente, es capaz de reducir las quinonas de nuevo a su forma fenol y eliminar el H_2O_2 producido tras la oxidación de los compuestos fenólicos (Danilewicz y Wallbridge 2010).

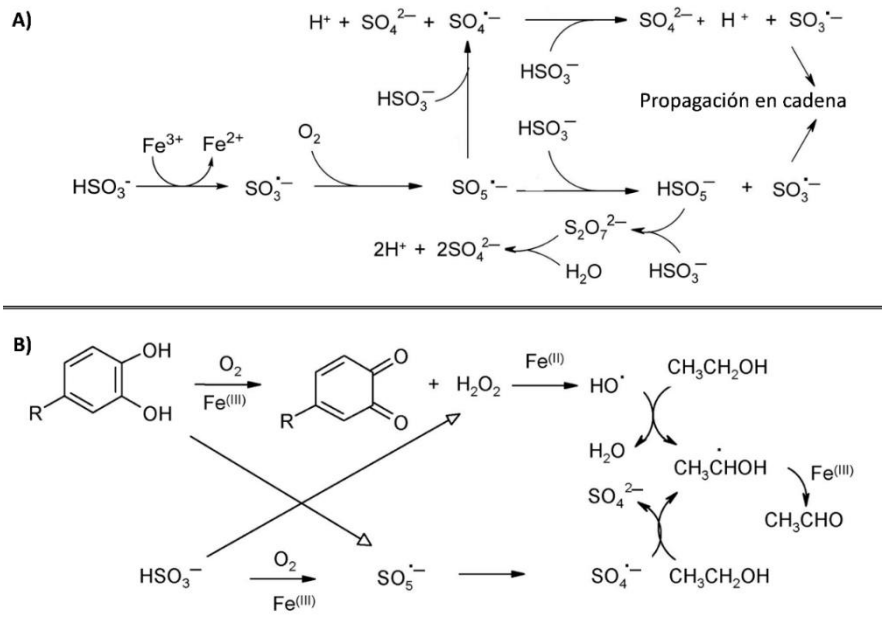


Figura I.17. Autooxidación del bisulfito (HSO_3^-) catalizada por Fe (III). A) Reacción en cadena de la formación del radical sultito ($SO_3^{\bullet-}$). B) Inhibición de formación de radicales sulfitos por los compuestos fenólicos (Adaptado de Danilewicz, 2007; Danilewicz y Wallbridge 2010).

Respecto a la actividad antimicrobiana, el SO_2 molecular es más activo que el HSO_3^- mientras que el SO_2 combinado solo ejerce una baja acción antibacteriana (Ribéreau-Gayon et al., 2006a). El SO_2 molecular penetra en las células por difusión simple, más que por transporte activo (Macris y Markakis, 1974), y se disocia en el citoplasma a bisulfito ($HSO_3^- + H^+$) y sulfito ($SO_3^{-2} + H^+$) (Divol et al., 2012). Esta disociación genera un aumento de la concentración de protones, lo que provoca una rápida acidificación y un desequilibrio redox. Una vez dentro de la célula, el SO_2 reacciona con diversos metabolitos y enzimas, interfiriendo sobre los procesos intracelulares e inhibiendo el crecimiento microbiano (Rose, 1989).

La actividad antimicrobiana del SO_2 se ha aprovechado para controlar el crecimiento de bacterias lácticas, bacterias acéticas y diferentes levaduras que deterioran el vino (Bartowsky, 2009; Oelofse et al., 2008). El SO_2 ejerce una acción antimicrobiana selectiva (bacterias > levaduras no *Saccharomyces* > levaduras *Saccharomyces*), ya que no todos los microorganismos presentan el mismo grado de sensibilidad hacia el mismo (Ribéreau-Gayon et al., 2006a). Esto permite el control de fermentaciones indeseables, favoreciendo una correcta realización de la fermentación alcohólica (Ribéreau-Gayon et al., 2006a). La eficacia antimicrobiana del SO_2 sobre las bacterias lácticas está más influenciada por el pH que sobre las levaduras. Y entre las bacterias lácticas, generalmente, las cepas de *Oenococcus oeni* son más sensibles al SO_2 que *Lactobacillus* y *Pediococcus* (Ribéreau-Gayon et al., 2006a).

Como puede observarse en la Figura I.18 el equilibrio químico entre cada forma de SO_2 depende del pH del vino. El SO_2 molecular predomina en rangos de pH de 0 a 2, el HSO_3^- de pH 2 a 7 y el SO_3^{2-} de pH 7 a 10. En general, el pH de los vinos suele tener valores entre 3 y 4, por lo tanto, la forma química de SO_2 predominante en el vino es el HSO_3^-, seguida del SO_2 molecular, mientras que el ion SO_3^{2-} se encuentra en concentraciones insignificantes (Waterhouse et al., 2016). Por ello, normalmente el termino SO_2 libre es utilizado para hacer referencia al HSO_3^- y al SO_2 molecular.

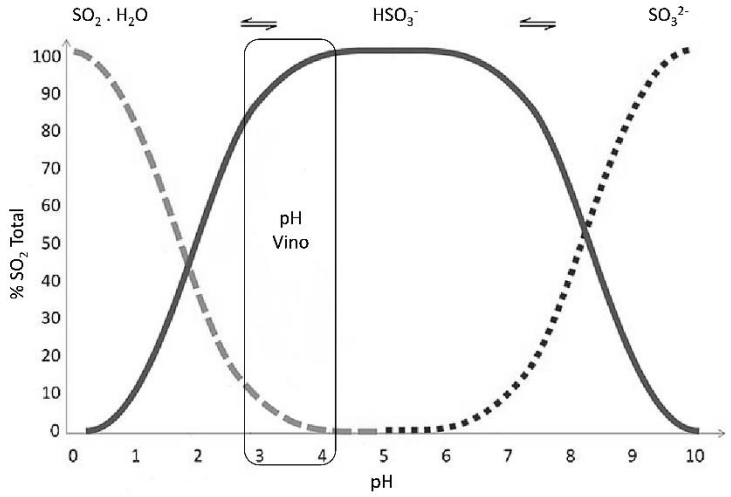


Figura I.18. Formas químicas del SO_2 en función del pH (Adaptado de Divol et al., 2012).

Las concentraciones de SO_2 libre utilizadas para evitar la oxidación se encuentran en el rango de 20-40 mg/L, y al menos 0,6 mg/L o 0,8 mg/L de SO_2 molecular para evitar el deterioro microbiano de los vinos secos o vinos dulces, respectivamente. La ecuación de Henderson-Hasselbalch, [SO_2 molecular] = [SO_2 libre] / $(1 + 10^{(pH-pKa1)})$, se puede utilizar para determinar la cantidad de SO_2 libre necesaria para lograr diferentes concentraciones de SO_2 molecular en función del pH (Waterhouse et al., 2016). Según esta ecuación, cuanto menor es el pH del vino mayor es el porcentaje de SO_2 en forma molecular. En la Figura I.19 se representan las curvas de concentración del SO_2 molecular, en función del pH, para diferentes concentraciones de SO_2 libre (Waterhouse et al., 2016). Es preferible que la concentración de SO_2 molecular no sea superior a su umbral sensorial (2 mg/L). Sin embargo, mantener el SO_2 molecular por debajo de este nivel es complejo en vinos con pH bajos. Para lograr una concentración de SO_2 molecular menor de 2 mg/L en un vino con pH 2.9 se necesitaría tener un nivel de SO_2 libre menor de 20 mg/L, existiendo en este caso un riesgo de oxidación. Además, otros parámetros como la temperatura y el grado alcohólico del vino influyen en el porcentaje de SO_2 molecular, de forma que este será más elevado cuanto mayores sean ambos parámetros (Ribéreau-Gayon et al., 2006a).

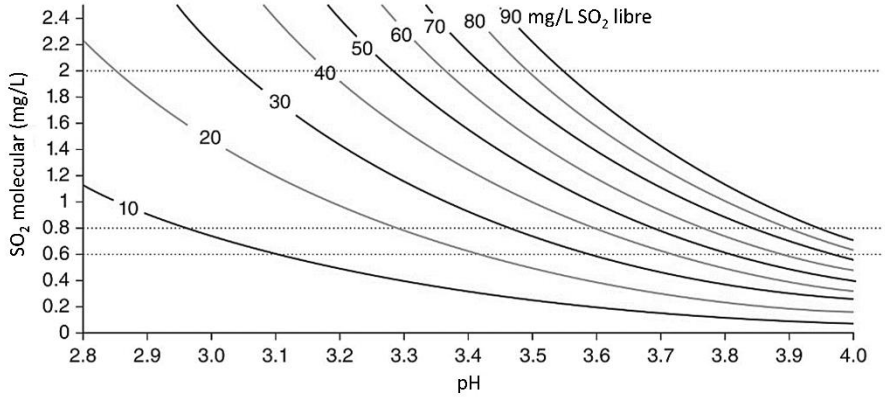


Figura I.19. Curvas de isoconcentración de SO_2 molecular en función del pH para diferentes concentraciones de SO_2 libre, determinadas a partir de la ecuación de Henderson-Hasselbalch (Waterhause, 2016).

Por otra parte, la mayor parte del SO_2 adicionado al mosto o al vino está combinado con otros compuestos orgánicos formando aductos covalentes (sulfonatos) (Ribéreau-Gayon et al., 2006a). En la Figura I.20, puede observarse que la fracción de SO_2 unido al acetaldehído es muy estable, por ello no está disponible para proteger al vino de la oxidación. A diferencia de este, los compuestos unidos débilmente al SO_2, como son el ácido pirúvico, el α-cetoglutarato, el ácido galacturónico y la glucosa y fructosa (en vinos dulces), pueden actuar como reserva de SO_2 libre después de la oxidación del vino (Waterhouse et al., 2016).

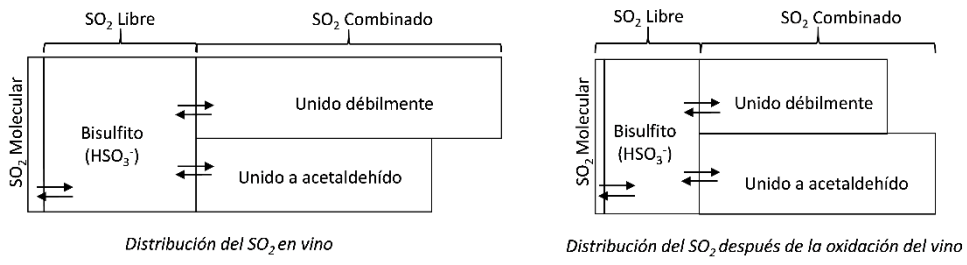


Figura I.20. Esquema de las diferentes formas de SO_2 antes y después de la oxidación del vino (Adaptado de Waterhouse, 2016).

I.3.2. Inconvenientes del uso de SO_2. Legislación en la Unión Europea

La concentración utilizada y el momento de adicionar el SO_2 es una decisión clave para el control de la vinificación. Sin embargo, concentraciones de SO_2 muy altas, o valores de pH demasiado bajos (que causan una mayor proporción de SO_2 molecular; Sección I.3.1), pueden producir alteraciones organolépticas en la calidad del vino. El SO_2 molecular está en equilibrio entre su forma acuosa y su forma gaseosa (Coelho et al., 2015). El SO_2 en forma gaseosa o volátil puede producir una sensación de irritación y ardor en la nariz y su umbral sensorial en el vino es de 2 mg/L con aroma a fósforo quemado (Ribéreau-Gayon et al., 2006b).

Además, la unión del SO_2 con otros compuestos orgánicos puede causar modificaciones en el perfil aromático del vino. Los compuestos carbonílicos, son compuestos volátiles con impacto sensorial, que pueden reaccionar con el SO_2 formando sulfonatos no volátiles. La formación de estos compuestos disminuye la intensidad aromática de compuestos volátiles con aromas agradables, como por ejemplo la β-damascenona con aroma afrutado (Daniel et al., 2004), mientras que en el caso de la unión del acetaldehído y otros aldehídos con aromas oxidativos puede ser favorable. No obstante, durante el proceso de oxidación, el HSO_3^- se libera de los aldehídos y los aromas desagradables a oxidación pueden ser percibidos (Bueno et al., 2016). El SO_2 también puede promover la formación de compuestos de azufre volátiles, como el H_2S con aroma indeseable a reducción o huevo podrido. Bekker et. al. (2016), mostraron que la adición de SO_2 antes del embotellado aumenta las concentraciones de H_2S, y destacaron que es importante mantener las concentraciones de Cu^{2+} residual lo más bajas posible antes de añadir SO_2 en el vino terminado, debido a que la combinación de SO_2 y Cu^{2+} puede aumentar la concentración de H_2S después del embotellado.

Por otro lado, el uso de SO_2 en la industria alimentaria ha generado una creciente preocupación como consecuencia de las reacciones adversas sobre la salud que pueden desarrollarse tras su ingesta. La exposición tópica y la ingesta del SO_2 provoca diferentes efectos nocivos en individuos sensibles, que van desde dermatitis, urticaria, rubor, hipotensión y síntomas gastrointestinales, hasta reacciones anafilácticas y asmáticas potencialmente mortales (Vally et al., 2009).

Como consecuencia del efecto perjudicial que el SO_2 puede tener sobre la salud y según las recomendaciones de la Organización Mundial de la Salud (OMS), el uso de SO_2 está regulado por la Unión Europea (UE). De este modo, de acuerdo con el Reglamento de la UE, el SO_2 y las diversas formas de sulfitos deben etiquetarse con los códigos E 220-228 (Reglamento UE 1129/2011). Además, cuando la concentración de SO_2 en los productos alimenticios, incluido el vino, sea superior a 10 mg/L o 10 mg/kg es obligatorio indicar "contiene sulfitos" (Reglamento UE 1129/2011). Para el sector enológico, la legislación europea establece la concentración límite de SO_2 total hasta 150 mg/L en vinos tintos y 200 mg/L en vinos blancos y rosados que contienen un máximo de 5 g/L de azúcares (Reglamento UE 2019/934). Estos límites se incrementan en 50 mg/L si las concentraciones de azúcares son iguales o superiores a 5 g/L (Reglamento UE 2019/934) y se reducen de 30 a 50 mg/L, según la categoría de vino, en vinos ecológicos (Reglamento UE 203/2012).

I.4. Alternativas para disminuir o reemplazar el uso de SO_2 en vinificación

Uno de los retos del sector enológico es disminuir la concentración de SO_2 utilizada en mostos y vinos, debido a los problemas de salud asociados con su consumo o exposición, con el fin de satisfacer la demanda de los consumidores y cumplir con una legislación cada vez más estricta sobre los conservantes alimentarios. Para lograr este objetivo, se buscan alternativas adecuadas que permitan controlar los procesos oxidativos y los microorganismos que deterioran el vino. A continuación, se presentan alternativas comerciales y extractos naturales de subproductos vitivinícolas enfocados a ser aplicados como alternativas o coadyuvantes al uso de SO_2 en vinificación.

I.4.1. Alternativas comerciales

Entre los productos enológicos que pueden ser utilizados como alternativas al SO_2, el dicarbonato de dimetilo, la lisozima y el ácido sórbico están permitidos como antimicrobianos en mostos y vinos, de acuerdo con la legislación de la UE (Reglamento 2019/934), y admitidos por la OIV. Otros coadyuvantes permitidos en vinificación, utilizados con diferentes aplicaciones a la estabilización microbiológica (UE y OIV), como el quitosano, glutatión, derivados de levadura y taninos también han sido propuestos como alternativas al SO_2 por su actividad antimicrobiana y/o antioxidante.

- Dicarbonato de dimetilo

El dicarbonato de dimetilo (DMDC), E-242, es un compuesto químico capaz de inhibir los microorganismos al reaccionar irreversiblemente con las enzimas alcohol deshidrogenasa y gliceraldehído-3-fosfato deshidrogenasa (Daudt y Ough, 1980). Según el Código Internacional de Prácticas Enológicas de la OIV (OENO 5/01, OENO 421-2011) la adición de DMDC en vinificación tiene como objetivo obtener la estabilidad microbiológica del vino embotellado que contiene azúcares fermentables, prevenir el desarrollo de levaduras y bacterias lácticas no deseadas, y bloquear la fermentación de vino dulce. La adición de DMDC no debe superar los 200 mg/L y solo debe agregarse poco antes embotellado. Además, no debe aumentar la concentración de metanol en el

vino por encima del contenido máximo recomendado por la OIV, y el vino no debe comercializarse mientras sea detectable.

El uso del DMDC por sí solo no es suficiente para sustituir completamente al SO_2, debido a que no protege el vino de la oxidación. No obstante, gracias a la sinergia entre el SO_2 y DMDC en la acción antimicrobiana (Ough et al., 1988; Terrell et al., 1993) podría ser útil para disminuir la concentración de SO_2 empleada en vinificación. Varios estudios han demostrado que el DMDC es más eficaz contra las levaduras que contra las bacterias, bajo los límites de concentración autorizados (Costa et al., 2008; Delfini et al., 2002; Divol et al., 2005). Por lo que la adición del DMDC no es aconsejable antes de que finalice la fermentación (Divol et al., 2005; Renouf et al., 2008). Otro de los inconvenientes del DMDC es que se convierte rápidamente en metanol y CO_2, a las 12-24 horas de su adición al vino, por lo que su efecto es limitado y solo es recomendable su uso en el mismo momento del embotellado (Costa et al., 2008; Divol et al., 2005).

- Lisozima

La lisozima, E-1105, también conocida como muramidasa o N-acetilmuramida glicanhidrolasa, es una enzima aislada de la albúmina del huevo. Su acción antimicrobiana se debe a la capacidad de esta enzima para hidrolizar los enlaces 1,4-β entre el ácido N-acetilmurámico y la N-acetil-D-glucosamina de las paredes celulares de bacterias Gram positivas (Liburdi et al., 2014). A diferencia del SO_2, la actividad de la lisozima es mayor cuando aumenta el pH del vino, por lo que puede reforzar la actividad antimicrobiana del SO_2 en mostos y vinos con pH elevado (Ribéreau-Gayon et al., 2006a). Según el Código Internacional de Prácticas Enológicas de la OIV (OENO 6/97, OENO 10/97), cuando el mosto y el vino se tratan con lisozima, la concentración acumulada no debe exceder los 500 mg/L.

La actividad antimicrobiana de la lisozima depende de la estructura de la pared celular, debido a que rompe el enlace β-glucosídico de mureína. Por consiguiente, es muy activa contra las bacterias Gram positivas, mientras que tiene baja actividad contra las bacterias Gram negativas y es inactiva contra las células eucariotas (McKenzie y White, 1991). Por ello, la lisozima no puede controlar las bacterias acéticas que son Gram negativas y levaduras alterantes, como *Dekkera/Brettanomyces* (McKenzie y White,

1991). Como consecuencia de estas diferencias, una de las principales aplicaciones de la lisozima es la inhibición de la fermentación maloláctica en vinos blancos, cuando esta no es deseada (Gerbaux et al., 1999; Ribéreau-Gayon et al., 2006a), o el retraso de la misma en elaboración de vino tinto, lo que permite realizar maceraciones más largas o la aplicación de microoxigenación antes de la fermentación maloláctica (Gerland, 2006; Pilatte et al., 2005). Aunque hay que tener en cuenta que la actividad antimicrobiana de la lisozima puede verse reducida por su combinación con coloides de mosto y sólidos suspendidos (Azzolini et al., 2010; Delfini et al., 2004), así como por las proantocianidinas de bajo peso molecular en vinos tintos (Guzzo et al., 2011; Liang et al., 2013).

Varios trabajos han estudiado el efecto de la lisozima en la composición volátil del vino. Sonni et al., (2009) encontraron que la adición conjunta de lisozima y taninos enológicos durante la fermentación del mosto blanco dio vinos con menor concentración de acetaldehído respecto a los vinos con SO_2. Además, los vinos blancos elaborados con lisozima mostraron una mayor concentración de ésteres y menor cantidad de alcoholes superiores que los vinos con SO_2 (Sonni et al., 2011b). En la misma línea, Nieto-Rojo et al. (2015) demostraron que las adiciones de lisozima (250 mg/L) o DMDC (200 mg/L) con una dosis complementaria de SO_2 (25 mg/L), al mosto de Garnacha, da lugar a vinos con concentraciones más altas de compuestos volátiles que aportan notas aromáticas positivas, y con mayor calidad sensorial con respecto a la única adición de SO_2 (50 mg/L). Por otro lado, entre los efectos no deseados que el uso de la lisozima puede ocasionar sobre el vino tinto se encuentra la disminución del color y el contenido de compuestos fenólicos (Bartowsky et al., 2004). También, la lisozima induce la turbidez en vinos blancos y es necesario la estabilización de proteínas después del tratamiento (Bartowsky et al., 2004).

- Ácido sórbico

El ácido sórbico es un ácido graso insaturado de cadena corta que se utiliza como agente antifúngico en la industria alimentaria (Lück, 1990). Su acción antimicrobiana no se conoce bien, aunque se considera que se basa en la acidificación intracelular (Bagar et al., 2009; Plumridge et al., 2004), lo que conduce a la interrupción de las vías metabólicas (Mira et al., 2010). La adición de ácido sórbico (E-200) o de sorbato de

potasio (E-202) en el mosto y en el vino no debe superar los 200 mg/L, expresada en ácido sórbico, según el Código Internacional de Prácticas Enológicas de la OIV (5/88).

Debido a su actividad antimicrobiana contra las levaduras, se utiliza generalmente en el embotellado de vinos dulces para evitar refermentaciones por *Saccharomyces cerevisiae* (Fugelsang, 1997; Zoecklein et al.,1995). Además, el ácido sórbico inhibe el desarrollo de las levaduras filmógenas (*Candida* spp.) en la superficie de los vinos (Ribéreau-Gayon et al., 2006a). Sin embargo, no es un inhibidor eficaz de bacterias lácticas, bacterias acéticas y levaduras alterantes del vino, como *Brettanomyces*, *Saccharomycodes* y *Zygosaccharomyces* (Du Toit y Pretorius, 2000). La eficacia del ácido sórbico depende del pH del vino, el nivel de alcohol, la concentración de SO_2 y el número y naturaleza de las levaduras (Zoecklein et al., 1995). Debe usarse junto con el SO_2 para prevenir las reacciones oxidativas y la alteración microbiológica derivada de la actividad bacteriana (Ribéreau-Gayon et al., 2006a). Otra desventaja del ácido sórbico es que puede ser degradado por las bacterias lácticas presentes en el vino, produciendo compuestos volátiles responsables del aroma a geranio (principalmente debido al 2-etoxi-3,5-hexadieno) (Ribéreau-Gayon et al., 2006b).

- Quitosano

El quitosano es un biopolímero de N-acetil-2-amino-2-desoxi-D-glucopiranosa y 2-amino-2-desoxi-D-glucopiranosa derivado de la quitina, con actividad antimicrobiana reconocida (Raafat y Sahl, 2009). El uso de quitosano, procedente de *Aspergillus niger,* está permitido en vinificación como clarificante (con dosis máxima de 100 g/hL), quelante de metales pesados (con dosis máxima de 100 g/hL), para reducir la concentración de ocratoxina A (con dosis máxima de 500 g/hL) o para disminuir la población de *Brettanomyces* spp. (con dosis máxima de 10 g/hL) según el Código Internacional de Prácticas Enológicas de la OIV (OENO 336A/2009, OENO 337A/2009, OENO 338A/2009).

Se han propuesto varias teorías para explicar el modo de acción que conduce a la actividad antimicrobiana del quitosano. Aunque aún no se ha aclarado el mecanismo exacto, la hipótesis de que el quitosano ocasiona la salida de componentes intracelulares es ampliamente aceptada (Jeon et al., 2014). En este mecanismo, el quitosano cargado positivamente se une a la superficie bacteriana cargada negativamente modificando la

permeabilidad de su membrana, lo que da lugar a la salida de componentes intracelulares que causan la muerte celular (Raafat et al., 2008). En bacterias Gram negativas, Jeon et al. (2014) demostraron que las micropartículas de quitosano interactúan específicamente con una proteína de la superficie bacteriana (la proteína A de la membrana externa). En bacterias Gram positivas, Raafat et al. (2008) propusieron que la unión del quitosano a los ácidos teicoicos de la pared celular, junto con una posible extracción de los lípidos de la membrana, da como resultado una secuencia de efectos, que en última instancia conduce a la muerte bacteriana.

En vinificación, el quitosano es eficaz frente a bacterias acéticas (Valera et al., 2017), bacterias lácticas y levaduras indeseables como *Brettanomyces spp*. (Bağder Elmaci et al., 2015; Ferreira et al., 2013, Gómez-Rivas et al., 2004; Nardi et al., 2014; Petrova et al., 2016) permitiendo el crecimiento de especies de *Saccharomyces* (Bağder Elmaci et al., 2015). Además, el tratamiento con quitosano es efectivo para disminuir el impacto sensorial indeseable de los fenoles volátiles, subproductos de *Brettanomyces,* responsables principalmente del mal olor fenólico (Filipe-Ribeiro et al., 2018). El quitosano también se ha presentado como posible alternativa al SO_2 por su actividad antioxidante, previniendo la oxidación del vino (Chinnici et al., 2014; Nunes et al., 2016). Chinnici et al. (2014) informaron que el uso de quitosano disminuyó la oxidación de tioles, y sugirieron que este aditivo podría contribuir a mantener el carácter varietal de los vinos y evitar el pardeamiento, debido a su capacidad quelante que permite limitar la disponibilidad del Fe (II).

- ❖ Nanopartículas de plata y complejos de plata coloidal

La plata metálica posee actividad antimicrobiana frente a un gran número de bacterias Gram positivas y Gram negativas. Los mecanismos de acción antimicrobiana aceptados son: (1) la captación de iones de plata libres seguida de la interrupción de la producción de ATP y la replicación del ADN, (2) generación de iones de plata de especies reactivas de oxígeno, y (3) daño directo de las nanopartículas de plata a las membranas celulares (Marambio-Jones y Hoek, 2010). Este elemento, en forma de nanopartículas de plata y complejo de plata coloidal (CSC), no está autorizado en vinificación. Sin embargo, la OIV permite el uso de cloruro de plata (AgCl), para eliminar o disminuir los defectos

de aroma debidos a los compuestos de azufre volátiles. El límite legal establecido por la OIV es de 1g/hL y el contenido máximo de plata debe ser inferior a 0,1 mg/L en el vino terminado. Además, el AgCl debe de aplicarse mediante un soporte inerte, como el caolín (OENO, 145/2009).

El CSC es un producto comercial bajo patente (PCT/ES2015/070532; Laboratorios Argenol, SL, Zaragoza, España; www.laboratorios-argenol.com), con tamaño de partícula menor de 10 µm e insoluble en etanol y agua, compuesto por un material inerte inorgánico (caolín) utilizado como soporte, sobre cuya superficie se depositan las nanopartículas de plata metálica (≤10 nm). El CSC es capaz de controlar el desarrollo de bacterias lácticas y acéticas durante la elaboración de vino blanco y tinto (Garde-Cerdán et al., 2014; Izquierdo-Cañas et al., 2012). Posteriormente, Izquierdo-Cañas et al. (2018) evaluaron el efecto de CSC sobre vinos contaminados con bacterias acéticas y *Brettanomyces bruxellensis*. Estos autores mostraron que el CSC (1 g/L) es efectivo para disminuir la población de bacterias acéticas y *Brettanomyces bruxellensis* en vino, aunque no eliminan completamente dichos microorganismos. Respecto a su efecto en la composición química del vino, en comparación al uso de SO_2, los vinos elaborados con CSC poseen menor grado alcohólico y menor concentración de compuestos fenólicos (Garde-Cerdán et al., 2014; Izquierdo-Cañas et al., 2012).

También se ha estudiado la actividad antimicrobiana de las nanopartículas de plata estabilizadas con polietilenglicol y con glutatión. García-Ruiz et al. (2015) informaron que este tipo de nanopartículas eran eficaces en el control de la fermentación maloláctica e inhibición del crecimiento de bacterias acéticas. Gil-Sánchez et al., (2019) confirmaron la efectividad de las nanopartículas de plata, recubiertas con polietilenglicol y glutatión, contra las bacterias lácticas y acéticas después de la fermentación alcohólica. Además, estos autores demostraron que las nanopartículas de plata recubiertas con glutatión se mantienen inalteradas tras el proceso de digestión in vitro, mientras que las nanopartículas recubiertas con polietilenglicol sufren aglomeración. Por lo tanto, es necesario investigar en profundidad los posibles problemas de seguridad relacionados con las aplicaciones de nanopartículas de plata “in vivo".

❖ Glutatión

El glutatión (GSH) es un tripéptido, compuesto por ácido glutámico, cisteína y glicina, que se encuentra de manera natural en bajas concentraciones en el vino (Kritzinger et al., 2013; Meister, 1988). Las propiedades nucleofílicas y reductoras del GSH se deben al grupo tiol de la cisteína (Elskens et al., 1991). Es conocido que el glutatión se une a *o*-quinonas (Cheynier y Van Hulst, 1988) y aldehídos reactivos en el vino (Sonni et al., 2011a), y forma aductos de glutationil vinil fenol durante la oxidación en presencia de ácidos hidroxicinámicos (Bouzanquet et al., 2012). La adición de GSH como antioxidante está permitida en mosto y vino, hasta un máximo de 20 mg/L, según el Código Internacional de Prácticas Enológicas de la OIV (OENO 445/15, OENO 446/15). También, se puede agregar GSH al mosto o al vino mediante la adición de otros aditivos permitidos (como son los derivados de levadura), que permiten aumentar la concentración de GSH (Pozo-Bayón et al., 2009a; Reglamento UE 2019/934).

El GSH tiene un papel importante en la prevención de la oxidación del vino (Sección I.1.1), evitando la pérdida de ésteres, terpenos y tioles y protegiendo al vino contra el pardeamiento oxidativo (Kritzinger et al., 2013; Roussis et al., 2007). Okuda y Yokotsuka (1999) sugirieron que el GSH puede eliminar el H_2O_2 y otros peróxidos y/o radicales del mosto. Posteriormente, Makhotkina y Kilmartin (2009) mostraron que cuando se agrega GSH a soluciones modelo que contienen polifenoles, su comportamiento es similar al SO_2 y plantearon la hipótesis de que el GSH, además de reaccionar directamente con las *o*-quinonas (adición nucleofílica), puede reducir las *o*-quinonas a catecoles. Por su actividad antioxidante, el GSH se ha propuesto como alternativa al SO_2 en vinificación (Makhotkina et al., 2014). A pesar de ello, Ugliano et al. (2011) demostraron que, aunque el glutatión disminuye la degradación del 3-mercaptohexanol (contribuyendo así a mantener el aroma varietal), también induce la producción de sulfuro de hidrógeno durante el envejecimiento del vino en condiciones de bajo oxígeno (es decir, promueve el aroma indeseado de reducción).

Algunos autores han evaluado la combinación de diferentes métodos enológicos con el uso de GSH para prevenir la oxidación del vino. Sonni et al. (2011c) compararon la actividad antioxidante del GSH solo o con ácido ascórbico en vinos modelo y

mostraron que la presencia de ácido ascórbico con altas concentraciones de glutatión puede retrasar la degradación oxidativa del vino. Por su parte, Ferrer-Gallego et al. (2017) informaron que la combinación de levadura seca inactiva enriquecida en GSH y taninos de orujo de uva supone un método eficaz para incrementar la vida útil de los vinos Tempranillo y Albariño.

Por otro lado, varios estudios discrepan sobre la eficacia antioxidante del GSH durante el almacenamiento del vino. En este sentido, Panero et al. (2015) observaron que la adición de GSH (a una concentración de 20 mg/L) no evitó la oxidación de los vinos embotellados durante el almacenamiento. Estos resultados no fueron apoyados por Webber et al. (2017), los cuales demostraron que el GSH (a concentraciones de 10, 20 y 30 mg/L) reduce el pardeamiento y la formación de acetaldehído, de vinos espumosos, hasta 12 meses. Las discrepancias existentes sobre la efectividad del GSH pueden estar sujetas a las diferencias de composición y condiciones de almacenamiento de los vinos, pues Nikolantonaki et al. (2018) proporcionaron evidencia de que la eficiencia del GSH contra la oxidación durante el envejecimiento en botella depende de la composición química del vino, específicamente de los compuestos que contienen nitrógeno y azufre, como aminoácidos, compuestos aromáticos y péptidos, los cuales poseen un fuerte carácter nucleofílico y también reaccionan con los polifenoles oxidados.

- Derivados de levadura

Los derivados de levadura (YD) son considerados una estrategia para controlar la oxidación del vino por su capacidad para consumir oxígeno y liberar antioxidantes de su composición (Comuzzo et al., 2015, Pozo-Bayón et al., 2009a). Se obtienen a partir de *Saccharomyces cerevisiae*, una vez eliminada su capacidad fermentativa, y pueden incluir en su composición compuestos solubles (aminoácidos, péptidos, monosacáridos, etc.) e insolubles (membranas y paredes de levadura). Los YD son clasificados en cuatro grupos, según el proceso de fabricación: (1) levadura inactiva (obtenida por inactivación térmica seguida de secado), (2) autolisados de levadura (además de la inactivación térmica hay una etapa de incubación que permite que las enzimas se liberen de las vacuolas, degradando así parte del contenido celular intracelular), (3) paredes de levadura (fracción insoluble formada por paredes de levadura sin contenido citoplasmático) y (4) extractos

de levadura (fracción soluble después de la degradación total del contenido citoplasmático) (Pozo-Bayón et al., 2009a).

Los YD son usados en vinificación por su capacidad para corregir las fermentaciones estancadas o lentas (Bisson et al., 2000), y para mejorar o modificar el aroma y las características sensoriales del vino (Comuzzo et al., 2006; Comuzzo et al., 2011; Pozo-Bayón et al., 2009b; Pozo-Bayón et al., 2009c). Entre los compuestos liberados por los YD en el vino, el glutatión (GSH) recibe la mayor parte de la atención científica (Bahut et al., 2019, Kritzinger et al., 2013), en aquellos YD que han sido enriquecidos con GSH durante su fabricación. Aun así, muchos autores coinciden en destacar el efecto combinado del GSH con otros compuestos liberados por los YD. En este sentido, Andujar-Ortiz et al. (2010) sugirieron que la menor pérdida de compuestos volátiles durante el almacenamiento del vino con YD es consecuencia de la actividad antioxidante del GSH, la cual es promovida por otros componentes presentes en las YD. Estos hallazgos fueron respaldados por Gabrielli et al. (2017), que mostraron que el GSH puro, añadido a la misma concentración que el liberado por la levadura inactivada, ejerce una menor protección sobre los tioles volátiles. Recientemente, Bahut et al. (2020) observaron, en un estudio de actividad antioxidante de diferentes YD, que en presencia de otros compuestos nucleófilos procedentes de las YD, la disminución del GSH tras la oxidación química es menor.

Ácido ascórbico

El ácido ascórbico (E-300) es un potente captador de oxígeno, por lo que se utiliza especialmente durante el estrujado de la uva o justo antes del embotellado, cuando se produce una alta disolución de oxígeno (Comuzzo y Zironi, 2013). La dosis de ácido ascórbico permitida es de 250 mg/L, en uva, mosto o vino, y la concentración acumulada no debe superar 300 mg/L, según el Código Internacional de Prácticas Enológicas de la OIV (OENO 10/01, OENO 11/01, OENO 12/01). Al igual que el SO_2, el ácido ascórbico reduce fácilmente las *o*-quinonas a su estado fenólico original (Bradshaw et al., 2011; Waterhouse y Laurie 2006) y, además, reacciona rápidamente con el radical $^{\bullet}OH$ y otros radicales inhibiendo su propagación (Bradshaw et al., 2011).

No obstante, dependiendo de la composición química del vino y de la concentración de ácido ascórbico usada, este también puede actuar como prooxidante (Bradshaw et al., 2003; Bradshaw et al., 2001). En presencia de catalizadores metálicos, la oxidación del ácido ascórbico conduce a la producción de ácido deshidroascórbico y H_2O_2 (Bradshaw et al., 2011). Por esta razón, el ácido ascórbico se usa normalmente en combinación con SO_2, debido a que el SO_2 es capaz de eliminar el H_2O_2, evitando la oxidación del etanol por reacción de Fenton (Danilewicz, 2007). Además, en ausencia de SO_2, la actividad antioxidante del ácido ascórbico depende de la concentración utilizada, actuando como prooxidante a niveles más bajos (Bradshaw et al., 2011).

I.4.2. Extractos naturales de subproductos vitivinícolas

El uso de compuestos fenólicos como conservantes naturales se ha convertido recientemente en un área de creciente interés (Schieber, 2017). Los compuestos fenólicos poseen actividad antimicrobiana debido a que pueden provocar cambios en la composición de los ácidos grasos y alterar la permeabilidad de la membrana celular de las bacterias (Campos et al., 2009; Rozès y Peres, 1998). También pueden modificar la estructura de la pared celular debido a la adsorción de fenol, e incluso interaccionar con enzimas celulares (Campos et al., 2003; Stivala, et al., 2017). Recientemente, se ha demostrado que la catequina posee actividad antimicrobiana por inducir estrés oxidativo mediante la producción de especies reactivas de oxígeno intracelulares, causando daño en la pared celular bacteriana (Sinsinwar y Vadivel, 2020).

Respecto a los ácidos fenólicos, los ácidos hidroxicinámicos (ácido *trans-p*-cumarico y *trans*-cafeico) ejercieron una alta inhibición del crecimiento de varias cepas de *Lactobacillus hilgardii* y *Pediococcus pentosaceus* en un medio sintético similar al vino (Stivala et al., 2017). Sin embargo, en vinificación hay que tener en cuenta que algunos compuestos fenólicos pueden actuar como activadores o inhibidores del crecimiento bacteriano dependiendo de su estructura química y concentración (García-Ruiz et al., 2008; Reguant et al., 2000; Vivas et al., 1997). Por ejemplo, el ácido gálico y otros compuestos fenólicos como las antocianinas libres activan el crecimiento celular de

Oenococcus oeni y la velocidad de degradación del ácido málico (Vivas et al., 1997). En la misma línea, Alberto et al. (2001) demostraron que el ácido gálico en bajas concentraciones (hasta 200 µg/mL) estimula el crecimiento de *Lactobacillus hilgardii*. Por el contrario, estos autores observaron que a concentraciones más altas (1000 µg/mL), el ácido gálico tiene un efecto inhibidor sobre el desarrollo bacteriano. Además, la eficacia de los compuestos fenólicos como antimicrobianos también depende de las especies microbianas. Por ejemplo, *Oenococcus oeni* es más sensible a la inactivación de los ácidos fenólicos que *Lactobacillus hilgardii* (Campos et al., 2003).

Por su parte, García-Ruiz et al. (2011), en un estudio comparativo de los efectos inhibidores de los polifenoles del vino sobre el crecimiento de las bacterias lácticas, mostraron que los flavonoles y el resveratrol poseen una actividad antimicrobiana significativa contra bacterias lácticas asociadas al proceso de vinificación como son *Oenococcus oeni*, *Pediococcus pentosaceus* y *Lactobacillus hilgardii.* En un estudio similar, Pastorkorva et al. (2013) demostraron que el resveratrol ejerce una actividad antimicrobiana significativa contra las levaduras *Dekkera bruxellensis*, *Hanseniaspora uvarum*, *S. cerevisiae*, *Zygosaccharomyces bailii, Zygosaccharomyces rouxii*, así como contra las bacterias acéticas *Acetobacter aceti*, *Acetobacter oeni* y *Acetobacter pasteurianus*.

Otro grupo de compuestos fenólicos con importantes aplicaciones durante la elaboración del vino son los taninos. Existen muchos tipos de taninos comerciales de diferentes orígenes vegetales. Entre ellos, se incluyen los taninos hidrolizables de roble o castaño y taninos condensados de semillas y hollejos de uva e incluso de otros orígenes vegetales como la tara, el quebracho y la mimosa (Versari et al., 2013). En vinificación, se puede agregar taninos con el objetivo de facilitar la clarificación de mostos y vinos, obteniéndose otros beneficios como la mejora de estructura y sensación en boca del vino (Vivas, 2001), así como una mayor intensidad y estabilidad del color de los vinos tintos (Canuti et al., 2012). Entre las diferentes funciones atribuidas a los taninos enológicos, su actividad antioxidante es una de las principales razones por las que son utilizados en vinificación, (Ricci et al., 2016). Los taninos enológicos inhiben las polifenoloxidasas, tirosinasa y lacasa, evitando el pardeamiento de los mostos (Nichols-Orians, 1991; Versari et al., 2013; Vignault et al., 2019). Además, tienen la capacidad de

consumir oxígeno protegiendo de la oxidación a los demás componentes del vino (Navarro et al., 2016; Vivas y Glories, 1996).

A partir de los subproductos generados por la industria vitivinícola, semillas, hollejos, raspones y sarmientos de vid, pueden obtenerse extractos naturales ricos en compuestos fenólicos, mediante extracción con disolventes o utilizando técnicas de extracción limpias "Green" que no emplean disolventes orgánicos. Los extractos naturales obtenidos mediante tecnologías limpias son un modo de revalorización de los subproductos respetuoso con el medio ambiente, ya que en su obtención no se generan residuos contaminantes (Alañón et al., 2017; Poveda et al., 2018). Algunos autores han mostrado que los extractos naturales obtenidos a parir de estos subproductos poseen propiedades antimicrobianas y antioxidantes debido a su riqueza en compuestos fenólicos, por lo que podrían utilizarse como conservantes en la industria alimentaria (Alañón et al., 2015; Alañón et al., 2017; Poveda et al., 2018; Raposo et al., 2016). La creciente búsqueda de alternativas naturales al SO_2, junto con el interés del sector enológico por revalorizar los subproductos generados durante la elaboración del vino, ha llevado a diferentes grupos de investigación a evaluar el potencial de estos subproductos como sustitutos del SO_2 en vinificación.

Ruiz-Moreno et al. (2015) propusieron la utilización de extracto de raspón como alternativa al SO_2 después de analizar su efecto antimicrobiano y antioxidante en vino modelo. Estos autores observaron que el extracto de raspón posee un efecto inhibidor menor que el SO_2 para *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Dekkera bruxellensis* y *Pediococcus damnosus*, mientras es más eficaz contra *Candida stellata* y *Botryotinia fuckeliana*. Posteriormente, Esparza et al. (2020) evaluaron el efecto de la sustitución parcial de SO_2 en el vino Tempranillo por un extracto de raspón de uva Mazuelo y por un extracto comercial de madera de vid (Vinetan®) y mostraron que el uso de ambos extractos como sustitutos parciales del SO_2 da lugar a vinos con buenas características organolépticas, similares o incluso mejores a los vinos controles. No obstante, estos autores observaron que los mostos con SO_2 tenían mayor actividad antioxidante y concentración de antocianos libres que los mostros con ambos tipos de extractos. Un extracto comercial rico en estilbenos (Vineatrol®) obtenido a partir de sarmientos de vid, también ha sido propuesto como alternativa al SO_2 en la elaboración

de vinos Syrah (Raposo et al., 2016 y 2018). En la misma línea de trabajo, Guitiérrez-Escobar et al. (2021) han desarrollado otro extracto comercial rico en estilbenos (ST99®), a partir de sarmientos, con una importante actividad antimicrobiana frente a *Brettanomyces bruxellensis* y *Zygossaccharomyces bailli*.

Otra industria totalmente relacionada con la industria vitivinícola es la tonelera, cuyos productos son esenciales en la elaboración del vino. Las tonelerías generan importantes volúmenes de residuos, principalmente maderas de distintas especies y orígenes cuyo contenido en compuestos fenólicos ha sido objeto de numerosos estudios (Baca-Bocanegra et al., 2018; Baca-Bocanegra et al., 2019; Alañón et al., 2011a, b). Concretamente, Alañón et al., 2011a demostraron que los ácidos fenólicos, incluidos el gálico, el protocatechico, el *p*-cumárico y el elágico, y los elagitaninos son los principales responsables de la actividad antioxidante de los extractos de maderas enológicas. En la misma línea, Pascual et al. (2017) confirmaron que los elagitaninos son muy buenos antioxidantes, con alta efectividad de consumo de oxígeno, por lo que sugirieron la posibilidad de su uso como alternativa al SO_2 en la elaboración del vino.

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II. JUSTIFICACIÓN Y OBJETIVOS

II. 1. JUSTIFICACIÓN

El dióxido de azufre (SO_2) es uno de los conservantes más utilizados en la industria vitivinícola. Sin embargo, su uso conlleva algunos inconvenientes como la producción de defectos del aroma o efectos adversos sobre la salud. Es por ello, que uno de los principales retos del sector enológico es disminuir la concentración de SO_2 utilizada en mostos y vinos. Para lograr este objetivo, se buscan alternativas adecuadas que permitan controlar los procesos oxidativos y los microorganismos que deterioran el vino.

Ante la necesidad de satisfacer la demanda de los consumidores y cumplir con una legislación cada vez más estricta sobre los conservantes alimentarios, se han propuesto nuevos conservantes y tecnologías emergentes, inofensivas para la salud y respetuosas con el medio ambiente. Entre ellas, la industria vitivinícola ha puesto en valor el uso de los subproductos generados durante el cultivo de la vid y la elaboración del vino, por ser una fuente de compuestos fenólicos con propiedades antimicrobianas y antioxidantes, como son los sarmientos, raspones, semillas y hollejos de la uva, así como los residuos procedentes de la elaboración de barricas.

A pesar de que numerosos estudios han caracterizado ampliamente los subproductos generados en la industria vitivinícola, aún son pocos los trabajos que han profundizado en el conocimiento de su potencial como alternativas al uso de SO_2, en la etapa prefermentativa, y su influencia sobre la calidad del vino. Del mismo modo sucede con productos enológicos comerciales, como son la levadura seca inactiva y el quitosano, los cuales han mostrado ejercer numerosos beneficios sobre la calidad de vino, pero su capacidad para reemplazar el SO_2 en la elaboración de vinos ha sido poco estudiada.

En base a lo expuesto, la hipótesis del presente trabajo es que los extractos procedentes de subproductos derivados de la industria vitivinícola, así como otros productos enológicos comerciales procedentes de fuentes naturales, podrían ser una alternativa al uso del SO_2 en la elaboración de vinos. Para evaluar esta posible hipótesis se realizaron diferentes vinificaciones y se analizaron los parámetros enológicos que influyen en la calidad del vino, como son los parámetros del color, composición fenólica y volátil de los vinos. Además, se determinó la actividad antioxidante de los vinos

elaborados con las posibles alternativas al SO_2, así como la resistencia a la oxidación mediante el análisis del radical 1-hidroxietilo. El análisis de este radical es uno de los principales objetivos de esta Tesis Doctoral, pues su formación durante la oxidación química del vino puede tener importantes efectos en la calidad sensorial del mismo.

Adicionalmente, y debido a las diferencias observadas en la concentración de los productos secundarios de fermentación de los vinos elaborados con quitosano, se realizó el análisis de la fracción nitrogenada de estos vinos. El objetivo era evaluar el efecto de la adición prefermentativa del quitosano sobre el perfil de aminoácidos, concentración de aminas biógenas y carbamato de etilo, y así asegurar que la adición prefermentativa de quitosano no compromete la salubridad del vino.

II. 2. OBJETIVOS

El principal objetivo de esta Tesis Doctoral fue estudiar diferentes estrategias para reemplazar el uso de SO_2 en la fase prefermentativa de la vinificación. Para ello, se elaboraron vinos con la adición de extractos acuosos liofilizados obtenidos a partir de residuos de la industria vitivinícola y otros productos comerciales, como la plata coloidal, levadura seca inactiva y el quitosano, con el fin de evaluar su influencia en la calidad del vino.

Los objetivos específicos del presente trabajo han consistido en:

I. Evaluar el potencial enológico de los extractos acuosos liofilizados de semillas de uva y raspón (*Vitis vinifera* L. cv. Tempranillo), solos o en combinación con CSC, sobre el crecimiento microbiológico, parámetros del color, composición fenólica, actividad antioxidante, composición volátil y características organolépticas del vino Airén.

II. Evaluar el potencial enológico de los extractos acuosos liofilizados de semillas de uva (*Vitis vinifera* L. cv. Tempranillo) y de roble americano (*Quercus alba*), solos o en combinación con CSC, sobre el recuento microbiológico al final de la fermentación maloláctica, composición fenólica, actividad antioxidante, composición volátil y características organolépticas del vino Cabernet Sauvignon.

III. Comparar la actividad antioxidante del quitosano, levadura seca inactiva y extractos acuosos liofilizados de raspón y sarmiento de *Vitis vinifera* L. cv. Tempranillo, como alternativas al SO_2, mediante la cuantificación del radical 1-hidroxietilo, la composición fenólica y los parámetros del color en vinos Cabernet Sauvignon.

IV. Estudiar la reacción entre el radical 1-hidroxietilo y diferentes sustancias naturales con actividad antioxidante (ácido ascórbico, quitosano, glutatión, levadura seca inactiva y extractos de semillas de uva y madera roble) mediante

resonancia paramagnética electrónica (EPR) en vino tinto y blanco. Determinar la capacidad de estos antioxidantes para prevenir la formación de acetaldehído tras la reacción de Fenton, así como su capacidad de consumo de oxígeno.

V. Como objetivo específico adicional, se decidió evaluar el efecto de la adición prefermentativa del quitosano sobre el perfil de aminoácidos, concentración de aminas biógenas y carbamato de etilo del vino Cabernet Sauvignon.

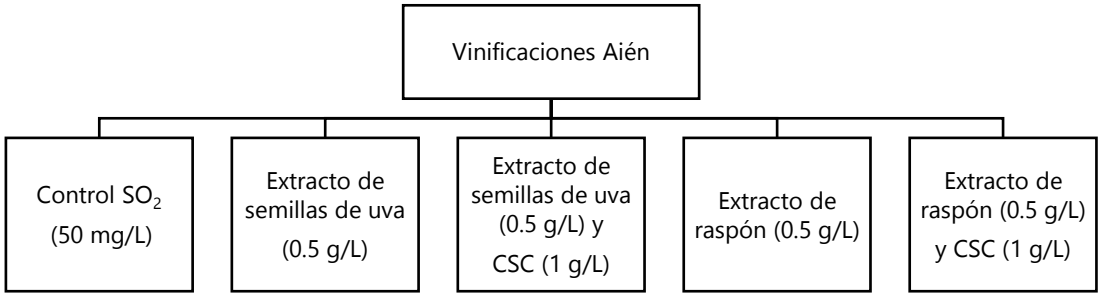
III. PLAN DE TRABAJO

El desarrollo de esta Tesis Doctoral se ha llevado a cabo según el siguiente plan de trabajo:

OBJETIVO 1.

Evaluar el potencial enológico de los extractos acuosos liofilizados de semillas de uva y raspón (*Vitis vinifera* L. cv. Tempranillo), solos o en combinación con CSC, sobre el crecimiento microbiológico, parámetros del color, composición fenólica, actividad antioxidante, composición volátil y características organolépticas del vino Airén.

VINIFICACIONES Y ALTERNATIVAS AL SO_2 EMPLEADAS EN LA FASE PREFERMENTATIVA

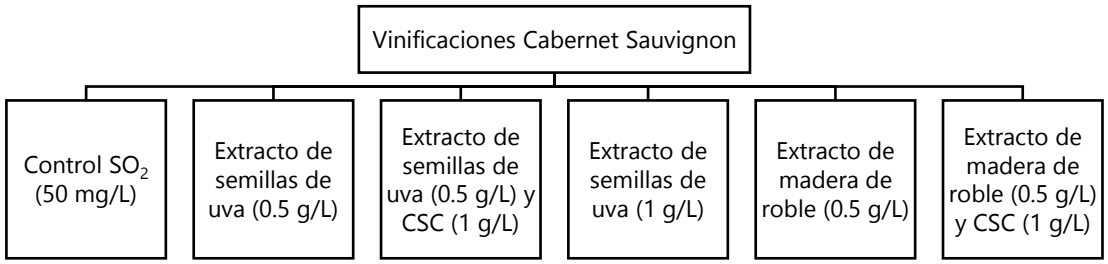


COMPUESTOS Y PARÁMETROS ENOLÓGICOS ANALIZADOS	TÉCNICAS ANALÍTICAS
Recuentos microbiológicos	Siembra en medio selectivo para levaduras, bacterias acéticas y bacterias lácticas
Parámetros enológicos convencionales: Densidad, Grado alcohólico, acidez total, acidez volátil, pH, SO_2, glucosa y fructosa, glicerol y ácidos orgánicos	Densimetría electrónica, Espectroscopia de Infrarrojo Cercano, Valoración potenciométrica, Destilación y volumetría, Paul-Rankine, HPLC-IR
Parámetros del color CIELab Contenido de polifenoles totales Actividad antioxidante	Espectroscopia UV-Vis
Composición fenólica	HPLC-DAD-ESI-MS^n
Composición volátil	GC/MS
Características organolépticas	Análisis sensorial descriptivo

OBJETIVO 2.

Evaluar el potencial enológico de los extractos acuosos liofilizados de semillas de uva (*Vitis vinifera* L. cv. Tempranillo) y de roble americano (*Quercus alba*), solos o en combinación con CSC, sobre el recuento microbiológico al final de la fermentación maloláctica, composición fenólica, actividad antioxidante, composición volátil y características organolépticas del vino Cabernet Sauvignon.

VINIFICACIONES Y ALTERNATIVAS AL SO_2 EMPLEADAS EN LA FASE PREFERMENTATIVA

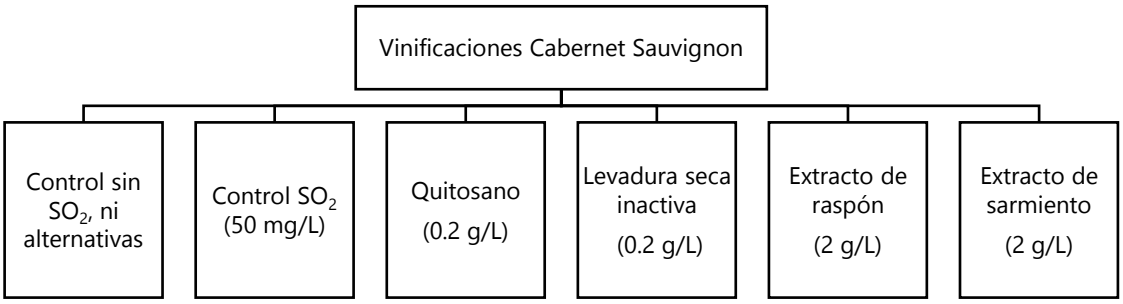


COMPUESTOS Y PARÁMETROS ENOLÓGICOS ANALIZADOS	TÉCNICAS ANALÍTICAS
Recuentos microbiológicos	Siembra en medio selectivo para levaduras, bacterias acéticas y bacterias lácticas
Parámetros enológicos convencionales: Densidad, grado alcohólico, acidez total, pH, SO_2, glucosa y fructosa, glicerol y ácidos orgánicos	Densimetría electrónica, Espectroscopia de Infrarrojo Cercano, Valoración potenciométrica, Paul-Rankine, HPLC-IR
Parámetros del color CIELab Contenido de polifenoles totales Actividad antioxidante	Espectroscopia UV-Vis
Composición fenólica	HPLC-DAD-ESI-MS^n
Composición volátil	GC/MS
Características organolépticas	Análisis sensorial descriptivo

OBJETIVO 3.

Comparar la actividad antioxidante del quitosano, levadura seca inactiva y extractos acuosos liofilizados de raspón y sarmiento de *Vitis vinifera* L. cv. Tempranillo, como alternativas al SO_2, mediante la cuantificación del radical 1-hidroxietilo, la composición fenólica y los parámetros del color en vinos Cabernet Sauvignon.

VINIFICACIONES Y ALTERNATIVAS AL SO_2 EMPLEADAS EN LA FASE PREFERMENTATIVA

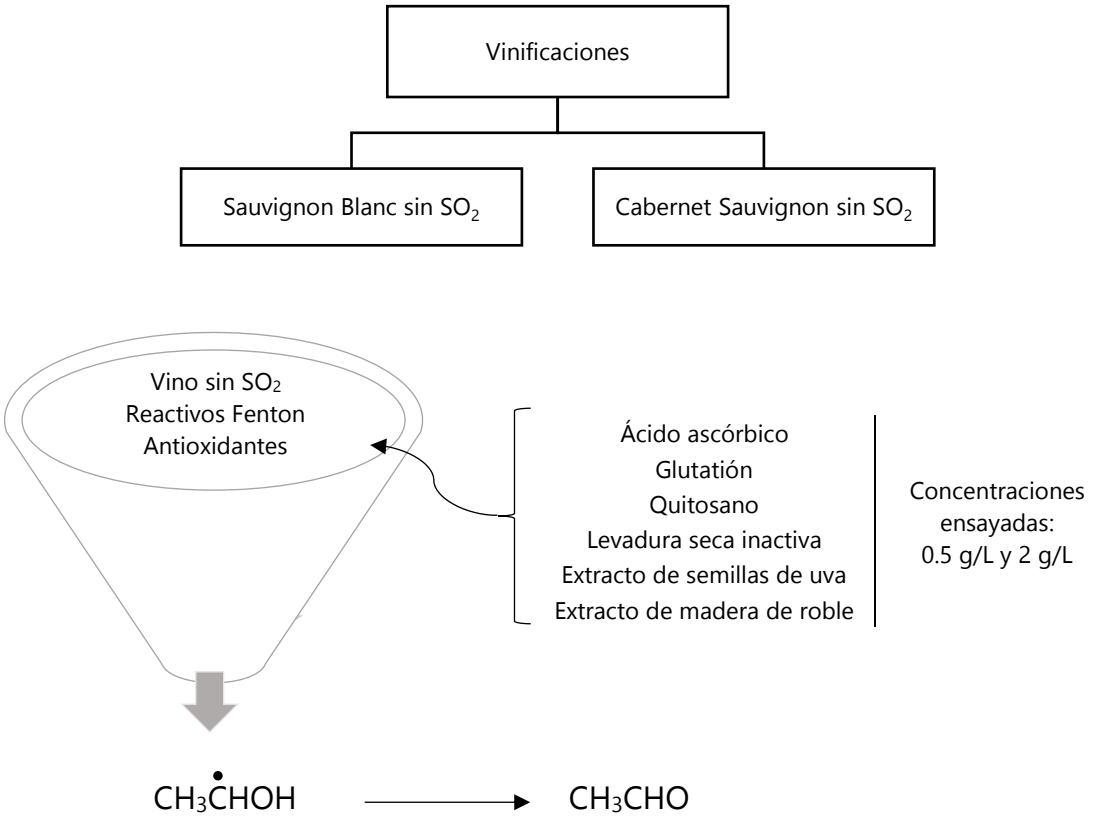


COMPUESTOS Y PARÁMETROS ENOLÓGICOS ANALIZADOS	TÉCNICAS ANALÍTICAS
Parámetros del color CIELab Contenido de polifenoles totales Actividad antioxidante	Espectroscopia UV-Vis
Composición fenólica	HPLC-DAD-ESI-MS^n
Radical 1-hidroxietilo	Resonancia paramagnética electrónica

OBJETIVO 4.

Estudiar la reacción entre el radical 1-hidroxietilo y diferentes sustancias naturales con actividad antioxidante (ácido ascórbico, quitosano, glutatión, levadura seca inactiva y extractos de semillas de uva y madera de roble) mediante resonancia paramagnética electrónica (EPR) en vino tinto y blanco. Determinar la capacidad de estos antioxidantes para prevenir la formación de acetaldehído tras la reacción de Fenton, así como su capacidad de consumo de oxígeno.

REACCIÓN DE FENTON EN VINOS CON DIFERENTES ALTERNATIVAS AL SO_2

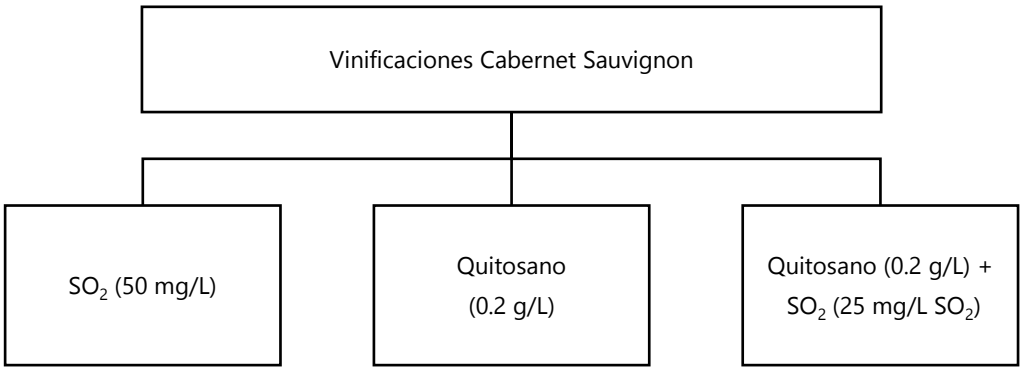


COMPUESTOS Y PARÁMETROS ENOLÓGICOS ANALIZADOS	TÉCNICAS ANALÍTICAS
Radical 1-hidroxietilo	Resonancia paramagnética electrónica
Acetaldehído	Análisis enzimático/Espectroscopia UV-Vis
Capacidad de consumo de oxígeno	Sensor óptico de fluorescencia

OBJETIVO 5.

Evaluar el efecto de la adición prefermentativa del quitosano sobre el perfil de aminoácidos, concentración de aminas biógenas y carbamato de etilo del vino Cabernet Sauvignon

VINIFICACIONES Y ALTERNATIVAS AL SO_2 EMPLEADAS EN LA FASE PREFERMENTATIVA

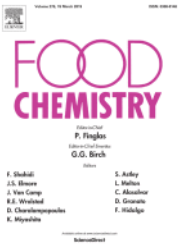


COMPUESTOS Y PARÁMETROS ENOLÓGICOS ANALIZADOS	TÉCNICAS ANALÍTICAS
Parámetros enológicos convencionales: Densidad, grado alcohólico, acidez total, pH, SO_2, glucosa y fructosa, glicerol, ácidos orgánicos y meso- y levo- butanodiol	Densimetría electrónica, Espectroscopia de Infrarrojo Cercano, Valoración potenciométrica, Paul-Rankine, HPLC-IR
Diacetilo y acetaldehído	GC-MS
Aminoácidos y aminas biógenas	HPLC-DAD
Carbamato de etilo	GC-MS

IV. RESULTADOS Y DISCUSIÓN

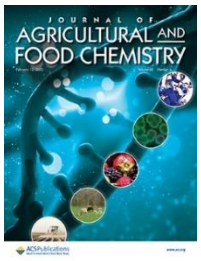
Los resultados de esta Tesis Doctoral han sido publicados en los siguientes artículos científicos:

ARTÍCULO 1: Marchante, L., Loarce, L., Izquierdo-Cañas, P. M., Alañón, M. E., García-Romero, E., Pérez-Coello, M. S., & Díaz-Maroto, M. C. (2019).
Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO_2 for white wines: Effects on chemical composition and sensorial properties.
Food Research International, 125, 108594.
https://doi.org/10.1016/j.foodres.2019.108594

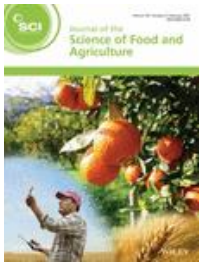


ARTÍCULO 2: Marchante, L., Izquierdo-Cañas, P. M., Gómez-Alonso, S., Alañón, M. E., García-Romero, E., Pérez-Coello, M. S., & Díaz-Maroto, M. C. (2019).
Oenological potential of extracts from winery and cooperage by-products in combination with colloidal silver as natural substitutes to sulphur dioxide.
Food Chemistry, *276*, 485–493.
https://doi.org/10.1016/j.foodchem.2018.10.072

ARTÍCULO 3: Marchante, L., Márquez, K., Contreras, D., Izquierdo-Cañas, P. M., García-Romero, E., & Díaz-Maroto, M. C. (2020).
Impact of oenological antioxidant substances on the formation of 1-hydroxyethyl radical and phenolic composition in SO_2 free red wines.
Journal of the Science of Food and Agriculture, *100*(8), 3401–3407.
https://doi.org/10.1002/jsfa.10374



ARTÍCULO 4: Marchante, L., Marquez, K., Contreras, D., Izquierdo-Cañas, P. M., García-Romero, E., & Díaz-Maroto, M. C. (2020).
Potential of different natural antioxidant substances to inhibit the 1-hydroxyethyl radical in SO_2-Free wines.
Journal of Agricultural and Food Chemistry, *68*(6), 1707–1713.
https://doi.org/10.1021/acs.jafc.9b07024



ARTÍCULO 5: Marchante, L., Mena, A., Izquierdo-Cañas, P. M., García-Romero, E., Pérez-Coello, M. S., & Díaz-Maroto, M. C. (2021).
Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO_2-free red wines.
Journal of the Science of Food and Agriculture, *101*(3), 1143–1149.
https://doi.org/10.1002/jsfa.10725

Artículo 1. Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO_2 for white wines: Effects on chemical composition and sensorial properties.

Food Research International *(2019), 125,* 108594.

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Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO_2 for white wines: Effects on chemical composition and sensorial properties

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ARTICLE INFO

Keywords:
Grape seed extract
Stem extract
Colloidal silver
Sulfur dioxide
White wine quality

ABSTRACT

The search for alternative additives to sulfur dioxide, with antioxidant and antimicrobial activity, in the production of wines is one of the current objectives of the enological industry. In the present study, aqueous extracts obtained from winery byproducts (grape seeds and stems), alone or in combination with colloidal silver complex, have been used in white vinification. The antimicrobial effect of the extracts was similar to that of sulfur dioxide, being more effective on lactic and acetic bacteria in those wines to which colloidal silver was added. The effect on the color, the phenolic compounds and the volatile fraction of the wines was evaluated, as well as their sensory profile. The use of both extracts modified the color of the wines, increasing the chromatic parameters a* and b*, indicating a browning tendency, although no other signs of oxidation were found. Wines with seed extracts contained higher amounts of flavan-3-ols, and a significant increase in some volatile compounds such as fatty acid ethyl esters and benzene compounds, which were identified in the extracts. From a sensorial point of view, the wines with stem extracts were the most similar to those elaborated with SO_2, detecting a certain bitterness in wines with seed extracts.

1. Introduction

Sulfur dioxide is one of the preservatives most used in wine industry due to its powerful antioxidant activity, antimicrobial effect and its influence on certain organoleptic characteristics such as color stability and aroma complexity. Particularly, the use of sulfur dioxide is essential for the production of white wines to avoid oxidation reactions, which entail browning of white wines and chemical composition changes in detriment of its sensorial quality (Oliveira, Ferreira, De Freitas, & Silva, 2011). However, the use of SO_2 implies some drawbacks such as the production of defects by neutralization of aromatic compounds (Ribereau-Gayon, Dubourdieu, Doneche, & Lonvaud, 2006) or even adverse health effects such as the appearance of sensitivity and development of allergic reactions (Santos, Nunes, Saraiva, & Coimbra, 2012). Therefore, one the main challenges in the oenological field is finding alternatives to sulfur dioxide for its replacement or limitation.

In the last years, several alternative methods and practices have been assayed for emulating the preservative action of SO_2 without its negative side effects. One example of them is the use of physical methods, such as high pressures or ultrasounds and pulse electric field, or alternative practices such as the use of colloidal silver complex (Garde-Cerdán et al., 2014; Guerrero & Cantos-Villar, 2015; Izquierdo-Cañas, García-Romero, Huertas-Nebreda, & Gómez-Alonso, 2012). However, despite the promising antiseptic effect of these techniques, the main drawback was the lack of antioxidant activity to avoid oxidation reactions in wines. Consequently, the addition of alternative natural substances with demonstrated antimicrobial and antioxidant properties such as vegetal extracts, lysozyme, hydroxytyrosol and oenological tannins has been recently explored (González-Rompinelli et al., 2013; Guerrero & Cantos-Villar, 2015; Raposo et al., 2016a; Raposo et al., 2016b; Salaha, Kallithraka, Marmaras, Koussissi, & Tzourou, 2008; Santos et al., 2012; Sonni, Chinnici, Natali, & Riponi,

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2011). In this sense, the use of by-products from winery as natural preservatives has been emerged in the last years. In winemaking, large quantities of by-products with antioxidant and antimicrobial properties (grape stems, grape pomace, exhausted yeast, wine lee, high loaded waste water..) are generated (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Lafka, Sinanoglou, & Lazos, 2007) whose adequate management could result in a valuable source of natural preservatives alternative to the use of SO_2.

Among them, grape pomace and grapevine-shoot have been the winery by-products more studied as plausible alternative to SO_2. Must from Pedro Ximénez dried grapes was fortified with ethanolic extract from grape pomace resulted in wines with higher content of flavanols, flavonols and procyaninids increasing the protective effect and antioxidant properties of wines (Dumitriu, Peinado, Peinado, & López de Lerma, 2015). The replacement of SO_2 by an extract from grapevine-shoot particularly rich in stilbenes, vineatrol®, as preservative in red wines was recently assessed. At bottling, wine treated with vineatrol showed higher color intensity, purity, and better score at sensory analysis than wines treated with SO_2 (Raposo et al., 2018).

On the other hand, grape seeds and stem are other by-products generated in winemaking process. The antioxidant and antimicrobial properties of ethanolic extracts from grape stems were reported (Ruiz-Moreno et al., 2015). Indeed, the chemical composition of grape stems and their potential to exert valuable biological and technological applications through their integration in distinct added-value products have been recently compiled (Barros, Gironés Vilaplana, Texeira, Baenas, & Domínguez Perles, 2015). Meanwhile, pressurised aqueous and hydroalcoholic extracts obtained from grape seeds showed major antioxidant and antimicrobial properties than those attained by other by-products like grape pomace or stems (Poveda, Loarce, Alarcón, Díaz-Maroto, & Alañón, 2018). The influence of grape seeds and stems macerations on wine composition had been evaluated in red wines (Del Llaudy, Canals, Canals, & Zamora, 2008; Pascual et al., 2016; Suriano, Alba, Tarricone, & di Gennaro, 2015). However, despite these facts, based on our knowledge the potential use of extracts from grape seeds and stems in white winemaking process as substitute of SO_2 has not been considered until now.

Therefore, the promising use of extracts from grape seeds and stems solely or in combination with colloidal silver complex as alternative to SO_2 has been evaluated in white wines. The effects of extracts addition on the antioxidant and antimicrobial activity, color, phenolic composition, volatile compounds and sensorial properties of white wines were assessed in comparison with those resulted in wines treated with SO_2.

2. Material and methods

2.1. Plant extracts obtained by pressurised water extraction

Grape seed and stem winemaking by-products from *Vitis vinifera* L. Cv. *Tempranillo* were kindly provided by Institute of Vine and Wine of Castilla-La Mancha (IVICAM, Tomelloso, Ciudad Real, Spain). Around 300 g of each fresh product were milled and stored in freezer at −18 °C until their extraction process.

Grape seeds and stems were submitted to pressurised water extraction using an accelerated solvent extractor ASE 200 (Dionex Corp. Sunnyvale, CA). Extraction method used was that optimized by Alañón et al. (2017) with some modifications. Six grams (6 g) of stems and eight (8 g) grams of seeds were introduced in steel cells of 22 mL joint two grams (2 g) of diatomaceous earth as dispersant agent. Two extractions cycles of 10 min were carried out under 120 °C of temperature and 1500 psi of pressure using water as extraction solvent. A flush volume of 60% of fresh water was used for the second cycle and one rinse was performed after each extraction to avoid any carry-over. Finally extracts were frozen −80 °C and then freeze-dried under vacuum (1.1×10^{-2} mB). Several extractions were carried out until about 12 g of lyophilizate were obtained from each sample. Homogenous lyophilized samples were stored at room temperature in a desiccator until their used. Table 1 shows the polyphenol composition of both extracts, catechins, condensed tannins and total polyphenol content expressed as mg/g of dry weight.

Table 1
Polyphenol composition of by-product extracts, catechins, condensed tannins and total polyphenol content (TPC), expressed as mg/g of dry weight.

By-product extracts	Catechins	Condensed tannins	TPC
Seeds	13.48 ± 0.06	19.44 ± 0.54	47.57 ± 2.44
Stems	7.64 ± 0.12	24.29 ± 1.63	46.79 ± 2.12

2.2. Chemicals: colloidal silver complex and SO_2

Colloidal silver complex (CSC) is a product under patent with a particle size smaller than 10 μm and insoluble in ethanol and water. An inorganic inert material was used in which surface, silver nanoparticles were deposited. SO_2 was added as potassium metabisulphite ($K_2S_2O_5$) with a purity higher than 95%.

2.3. Winemaking process

Airén grapes (*Vitis vinifera*) from 2016 vintage were destemmed, crushed and pressed. After that, must was divided in 15 batches of 4 L each one. Five different treatments applied to must were tested by triplicate which consisted of the addition of: (i) SO_2 at 50 mg/L which act as control wine, SO2; (ii) 0.5 g/L of lyophilized grape seed extract, E1; (iii) 0.5 g/L of lyophilized grape seed extract and 1 g/L of colloidal silver complex, E1Ag; (iv) 0.5 g/L of lyophilized stem extract, E2; (v) 0.5 g/L of lyophilized stem extract and 1 g/L of colloidal silver complex, E2Ag.

All must were submitted to an alcoholic fermentation at 18 °C in containers of 5 L using *Saccharomyces cerevisiae* Uvaferm VN (Lallemand Inc.) as starter culture. Fermentation evolution was monitored daily by density measurements. Wine was finally decanted when the density value was 990 g/L and simultaneously, glucose and fructose were determined at room temperature. Once fermentation finished, wines with SO_2 were adjusted to 25 mg/L free SO_2. All wines were cold stabilized at −3 °C during 30 days. Finally, prior a new adjustment of free SO_2 levels to 25 mg/L in control wines (SO2) all samples were filtered and bottled.

2.4. Physicochemical analysis and color parameters

Density, alcoholic degree, total and volatile acidity, SO_2, pH, fructose and glucose content, glycerine and organic acids (malic, lactic, citric and succinic acids) were determined by official analytical methods established in the International Organization of Vine and Wine (O.I.V., 2014).

Color parameters were obtained following the OIV method for the determination of chromatic characteristics according to CIELAB using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara CA, USA). The measuring conditions were: transmittance between 770 and 380 nm at 5 nm intervals, 1 mm cuvettes, D65 illuminant, and a 10° reference pattern observer. Results expressed were referred to 1 cm optical length (O.I.V., 2014).

2.5. Microbiological counts

Microbiological counts were carried out in four different fermentation stages: the tumultuous stage, devatting, racking and before bottle. The samples were obtained from containers in sterile bottles, under aseptic conditions.

2.5.1. Yeast

Depending on the winemaking stage, 0.1 mL of sample or 0.1 mL of serial dilution (from 10^{-1} to 10^{-5}) in sterile saline solution (0.9%) were spread onto YPD (Conda-Pronadisa, Madrid, España) supplemented with chloroanphenicol (100 mg/L). The plates were incubated under aerobic conditions at 30 °C for 2 days.

2.5.2. Lactic acid bacteria (LAB)

0.1 mL of sample and 0.1 mL of 10^{-1} dilution in sterile saline solution (0.9%) were spread onto MRS (Conda-Pronadisa, Madrid, España) plates, supplemented with cycloheximide (100 mg/L). Plates were incubated under anaerobic conditions (Gas Pack System, Oxoid Ltd., Basingstoke, United Kingdom) at 30 °C for 5 days.

2.5.3. Acetic acid bacteria

Depending on the winemaking stage, 0.1 mL of sample or 0.1 mL of 10^{-1} dilution in sterile saline solution (0.9%) were spread onto GYC (5% glucose, 1% yeast extract, 0,5% calcic carbonate, 2% agar) plates, supplemented with cycloheximide. Plates were incubated under aerobic conditions at 30 °C for 5 days.

In all cases, colonies were counted and the results were expressed as colony forming units (CFU) per milliliter of wine.

2.6. HPLC analysis of phenolic compounds

2.6.1. Flavan-3-ols and stilbenes

The flavan-3-ols and stilbenes were isolated from wine by SPE on C18 cartridges (Waters Sep-Pak Plus, 820 mg of adsorbent, Saint-Quentin En Yvelines, France) according to a previously published method (Rebello et al., 2013). Then, 30 µL of the extracts were analyzed in a HPLC Agilent 1200 series system equipped with DAD (Agilent, Waldbronn, Germany), and coupled to an AB Sciex 3200 TRAP (Applied Biosystems, Foster City, USA) with triple quadrupole, turbo spray ionization (electrospray assisted by a thermonebulization) and mass spectroscopy system (ESI-MS/MS) (Rebello et al., 2013). Chromatographic separation was achieved on an Ascentis-C18 column (4.6 × 150 mm; 2.7 µm particle; Supelco, Germany), thermostated at 16 °C and with a flow rate of 0.3 mL/min (Rebello et al., 2013).

For identification and quantification (calibration curves), the next standards were used: (+)-catechin, (−)-epicatechin and trans-resveratrol-glucoside from Sigma Aldrich (Tres Cantos, Madrid, Spain); procyanidins B1 and B2 from Extrasynthese (Genay, France). trans-Resveratrol-3-glucoside was transformed into its respective cis isomer by UV irradiation (366 nm during 5 min in quartz vials) of a 25% MeOH solution of the trans isomer.

2.6.2. Flavonols and hydroxycinnamic acids

For the analysis of flavonols and hydroxycinnamic acids, 3 mL of sample was taken to dryness in a miVac DUO concentrator operating at 35 °C (Genevac Ltd. Ipswich, UK) and resolved in 1.5 mL of a methanol/water solution (20/80). Then they were analyzed following the procedure previously developed by our research group (Castillo-Muñoz et al., 2009). An Agilent 1100 analytical HPLC system (Agilent, Germany) with a diode array detector (DAD) and a LC/MSD Trap VL electrospray ionization mass spectrometry (ESI-MSn) system, coupled to an Agilent Chem Station for data processing was used. The separation of the compounds was made in a Zorbax Eclipse XDB-C18 reversed-phase column (2.1 × 150 mm; 3.5 µm particle; Agilent, Germany) thermostated at 40 °C.

ESI-MS/MS was used for identification, while DAD-chromatograms were extracted at 360 nm and 320 nm for quantification of flavonols and hydroxycinnamic acids, respectively. Flavonol concentrations were expressed as equivalents of quercetin-3-glucoside (Extrasynthese, Genay, France) (mg/L) while hydroxycinnamic acids were expressed as equivalents of caftaric acid (Extrasynthese, Genay, France) (mg/L).

2.7. Total phenolic content and antioxidant activity

2.7.1. Total phenolic content

Total phenolic content (TPC) in wines and extracts was determined by the Folin-Ciocalteau assay. An UV–Vis spectrophotometer (Helios, Thermo Spectronic, Cambridge, UK) was used to measure the absorbance at 765 nm. Results were expressed as mg of gallic acid equivalent per litre (mg GAE/L) for wines, and mg of gallic acid equivalent per gram of dry weight (mg GAE/g) for extracts.

2.7.2. DPPH method

The method was based on the stability of 1,1-diphenyl-2-picrylhydrazyl radical (Brand-Williams, Cuvelier, & Berset, 1995). 100 µL of sample dilutions were added to 2.9 mL of a 0.06 mM methanolic DPPH radical solution (Sigma-Aldrich, Tres Cantos, Madrid, Spain). Absorbance was measured after 25 min reaction, at 515 nm in a UV–Vis spectrophotometer (Helios, Thermo Spectronic, Cambridge, UK), using methanol to set zero. Those measurements ranged between 20 and 80% of the initial absorbance of the radical DPPH were taken as reliable. Quantification was done with a calibration curve of Trolox ranging between 0.1 and 0.8 mM. Results were expressed in millimoles equivalents of Trolox per litre of wine.

2.7.3. ABTS method

The ABTS• + (radical cation) decolourisation assay (Re et al., 1999), consisted on mixture 20 µL previously diluted samples with 3 mL of working solution. The working ABTS solution was made by mixing ABTS solution (Sigma-Aldrich, Tres Cantos, Madrid, Spain) and potassium persulfate solution at 7 mM and 2.45 mM respectively which was maintained in darkness at room temperature for 12–16 h. The working ABTS solution resulted from the dilution of the previous one (1:90 v/v) which exhibited an absorbance values of 0.7 ± 0.02 at 734 nm. Ethanol was used to set the blank. Absorbance data was measured at $t = 0$ and $t = 5$ min to obtain percentage inhibition values. Quantification was made by means of a Trolox calibration curve ranged between 0.1 and 0.8 mM. Results were expressed in millimoles equivalents of Trolox per litre of wine.

2.7.4. Oxygen radical absorbance capacity method (ORAC)

The method was developed previously by Dávalos, Gomez-Cordoves, and Bartolome (2004). 96-well polystyrene microplates with black sides and clear bottom from NUNCLON ™ (Sigma-Aldrich, Tres Cantos, Madrid, Spain), were used for the essay. Samples were previously diluted and 100 µL aliquot was mixture with 1.4 mL sodium phosphate buffer. 20 µL of sodium phosphate buffer was used as a control sample and it was made a Trolox calibration curve ranged between 10 µM and 320 µM that were added into wells. Finally, 20 µL aliquot samples were added and all wells were filled with 120 µL of fluorescein solution. Microplates were incubated 15 min at 37 °C, after this time 60 µL of AAPH solution was added. Fluorescence was read every each minute during 80 min (Synergy HT de BIO-TEK). Results were expressed in millimoles equivalents of Trolox per litre of wine.

2.8. Analysis of catechins and condensed tannins of extracts

Catechins in seed and stem extracts were determinate by the method based on the reaction between catechins and DMACA 4-(dimethylamino) cinnamaldehyde (Vivas, Glories, Laguna, Saucier, & Augustin, 1994). A volume of 2.5 mL of DMACA solution at 5.7 mM was added to 0.5 mL diluted sample. For the blank 2.5 mL of methanol was used, and

the absorbance was measurement at 640 nm after 10 min. Results were expressed in mg equivalents of catechin per gram of dry weight.

For the analysis of condensed tannins, the method used was based on the tannins precipitation with the methyl-cellulose that does not absorb at 280 nm (Sarneckis et al., 2006). 150 µL of extract was mixed with 800 µL of water, 250 µL of methyl-cellulose 0.04% and 750 µL of ammonium sulphate. For the blank, the extract was replaced by water and the methyl-cellulose and the ammonium sulphate were not added. After 20 min, the samples were centrifuged and the absorbance was determined at 280 nm. The results were expressed in mg of condensed tannins per gram of dry weight.

2.9. Analysis of volatile compounds

2.9.1. Major volatile compounds

Major volatile compounds were analyzed directly by GC–MS. 100 µL of wine mixed with 100 µL of 2-pentanol-4-methyl used as an internal standard (60.35 mg/L) and 1 mL MiliQ water. Injections of 1 µL of samples were done in split mode into chromatograph Focus-ISQ (Thermo Scientic, Milan, Italy). Column used was a BP21 (60 m × 0.32 mm × 0.25 µm) FFAP phase (polietilenglicol modified with nitrotereftalic acid (TPA) (SGE, Trajan Scientific, United Kingdom). Helium was used as a carrier gas (constant flow 1.2 mL/min). The injector temperature was 195 °C and the oven temperature was programmed as follows: 32 °C during 2 min ramped at 5 °C/min to 120 °C, later it increased at 75 °C/min to 190 °C, where it was maintained during 18 min. MS operated in the electron impact mode with electron energy of 70 eV, the ion source temperature was 250 °C. Identification was carried out by comparison with commercial standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the quantification were done using calibration curves of each standard.

2.9.2. Minor volatile compounds

Volatile fraction of wines was extracted: 100 mL of wine samples skipped with 40 µL of 4-nonanol (1 g/L) as internal standard were submitted to a solid phase extraction (SPE) using 500 mg styrene divinyl benzene cartridges (Lichrolut EN Merck, KGaA, Darmstadt, Germany) previously conditioned. Subsequently, volatile fraction was eluted with 10 mL of dichloromethane. Extracts were concentrated under stream of nitrogen and stored at −20 °C prior to the chromatographic analysis.

Gas-chromatography analysis was carried out by the injection of 1 µL of previous extracts in splitless mode into a 6890 N Agilent technologies chromatographer coupled to a 5973 N Agilent technologies Mass Detector. The column used was a HP20 Carbowax type capillary column (60 m × 0.25 mm × 0.25 µm) using helium as a carrier gas with a constant flow of 1 mL/min. Injector temperature was 250 °C and oven temperature was programmed as follows: 70 °C during 5 min ramped at 1 °C/min to 95 °C holding 10 min, then 2 °C/min to 210 °C holding 40 min, up to complete the 137 min. The MS operated in electron impact mode with electron energy of 70 eV, the ion source temperature was 230 °C and the scanning was made from 45 to 550 a.m.u. (Castro-Vázquez et al., 2011).

Peaks identification was carried out comparing the mass spectrum of peaks with those from authentic standards and chromatographic libraries (WILEY and NBS75k) and based on the linear retention index (LRI) from literature. Linear retention indices were calculated using n-alkanes as external references. Quantification process was achieved by internal standard method. Calibration curves of each volatile compound were calculated by injection of commercial standards. For compounds which commercial standards were not available, the calibration curves of compounds with similar chemical structures were used (4-heptanol was quantified as 1-heptanol; 3-hexen-1-ol, acetate, (*Z*)- as ethyl hexanoate; and ethyl 9-decenoate as ethyl decanoate).

2.10. Sensory analysis

In order to evaluate wines sensory characteristics, a descriptive sensory analysis was employed; it was performed following the Sensory Profile method according to standard ISO 6564:1985 by 9 judges. Assessment took place in a standard sensory analysis chamber (ISO 8589:2007), equipped with separate booths and wine testing glasses (ISO 3591:1997) covered with a watch-glass to minimize the escape of volatile compounds.

The sensorial analysis was performed according to the ISO 11035:1994 where the identification and selection of descriptors for establishing a sensory profile by a multidimensional approach is defined. In a previous session, wines were sniffed and tasted by the assessors to carry out a product familiarization and develop a lexicon that comprehensively and accurately describes the sensorial profile of wines. The judges generated sensory terms individually. The hedonic terms were eliminated, as well as those terms provided by < 3 panellists, and synonyms or antonyms were regrouped in a single term. Once taken into account the responses of assessors and by consensus, a tasting sheet was created with 7 attributes which described the sensorial profile.

Wines were sniffed and tasted by duplicate in two sessions. The panellists used a 10 cm unstructured scale to rate the intensity of each attribute. The left extreme of the scale indicate a null intensity of the descriptor and the right extreme the maximum values.

2.11. Statistical analysis

Chemical and sensorial data were submitted to Student-Newman-Keuls test in order to identify statistical differences among different treatments applied to wines. While, major and minor volatile compound data were subjected to Principal Component Analysis (PCA). Statistical analysis were carried out using the IBM SPSS software, version 24.0 for windows statistical package.

Table 2
Oenological parameters of final Airén wines (n = 3) produced using SO_2 or by-product extracts, with and without CSC.

	SO2	E1	E1Ag	E2	E2Ag
Alcohol (%)	10.22 ^{a} ± 0.09	10.41^{a,b} ± 0.05	10.38 ^{a,b} ± 0.05	10.50 ^{b} ± 0.15	10.58 ^{b} ± 0.13
Total acidity (g/L)	3.64 ± 0.12	3.26 ± 0.33	3.27 ± 0.18	3.33 ± 0.09	3.40 ± 0.13
pH	3.64 ^{a,b} ± 0.12	3.92 ^{b} ± 0.20	3.62 ^{a,b} ± 0.18	3.72 ^{a,b} ± 0.18	3.40 ^{a} ± 0.13
Volatile acidity (g/L)	0.20 ± 0.01	0.29 ± 0.03	0.24 ± 0.01	0.24 ± 0.01	0.21 ± 0.08
Total SO_2 (mg/L)	108.67^{b} ± 2.89	nd ^{a}	nd ^{a}	nd ^{a}	nd ^{a}
Glucose and fructose (g/L)	1.60 ^{c} ± 0.28	0.41 ^{a} ± 0.12	0.62 ^{a} ± 0.05	0.38 ^{a} ± 0.35	1.17 ^{b} ± 0.06
Glycerine (g/L)	5.62 ^{d} ± 0.00	3.97 ^{a} ± 0.07	4.18 ^{b} ± 0.13	4.58 ^{c} ± 0.03	4.49 ^{c} ± 0.01

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test at $P < .05$.
SO2, control wine with SO_2; E1, wine with seed extract; E1Ag, wine with seed extract and CSC; E2, wine with stem extract; E2Ag, wine with stem extract and CSC.

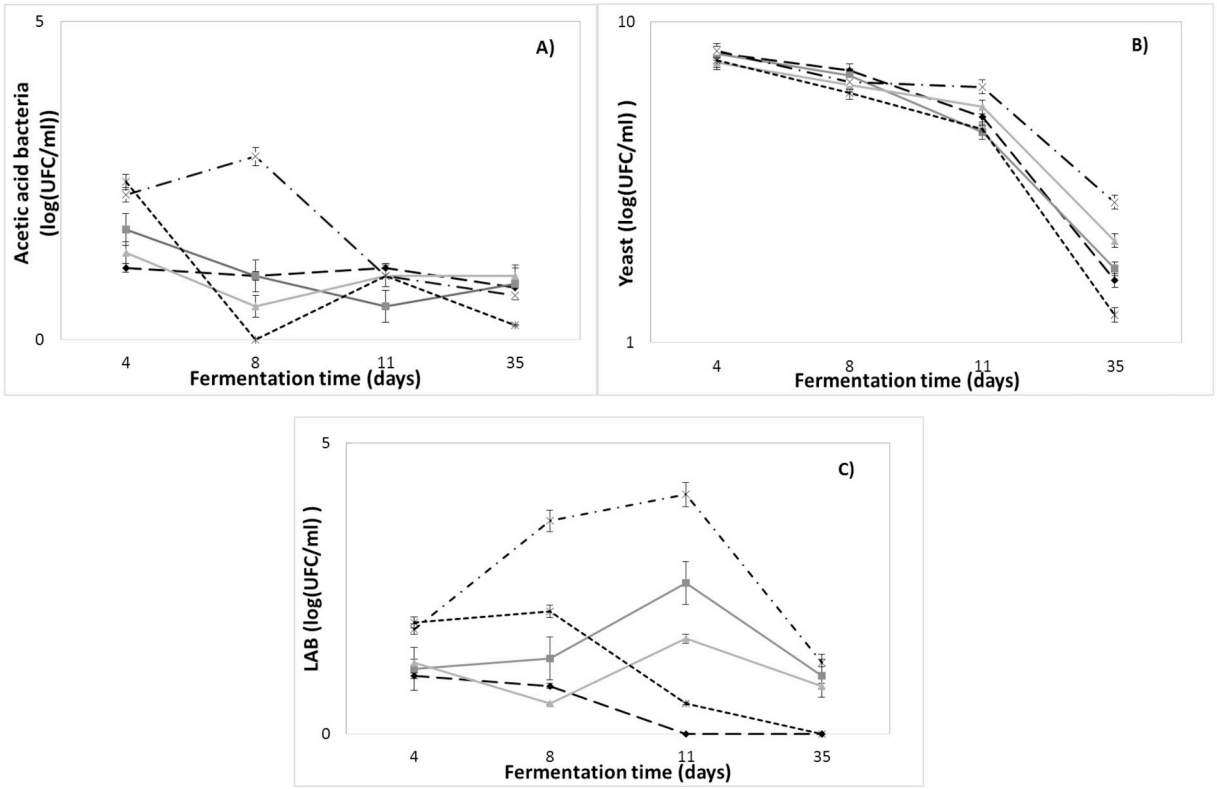

Fig. 1. Changes in microbial counts of Airén wines produced using SO_2 or winery by-product extracts, with and without CSC, during different fermentation stages. Symbols: SO2 E1 E1Ag E2 E2Ag.

3. Results and discussion

3.1. Conventional analysis

Table 2 shows the results of the physical-chemical analysis performed on the wines at the end of the alcoholic fermentation. The addition of extracts from seeds and stems and colloidal silver did not affect the content of total and volatile acidity of the wines, with values similar to those of control wine. pH values were higher in wines with extracts, although in all cases, the fermentation was developed correctly and there was a complete depletion of the sugars, with low volatile acidity values in all wines. Glycerin values were slightly lower in wines elaborated with natural extracts in absence of SO_2, probably due to the effect of SO_2 in the fermentation pathway.

3.2. Microbiological analysis

Fig. 1 shows the microbial counts of acetic bacteria, lactic acid bacteria and yeasts during alcoholic fermentation. At the beginning of the fermentation (day 4) the average counts of acetic bacteria were lower in the control wine with SO_2 (13.3 cfu/mL). In addition, the number of acetic bacteria remained practically constant in SO_2 wine until the end of fermentation. However, in the rest of the wines a decrease in the acetic bacteria counts was observed, especially in the sample with stem extract and colloidal silver (E2Ag), which was maintained until the end of the fermentation with the lowest counts (1.67 cfu/mL), although these values did not show significant differences respect to the control wine.

The lactic acid counts were also low at the start of the fermentation. The addition of seed and stem extracts produced an increase in lactic acid bacteria on days 8 and 11 of fermentation, which was more pronounced in wines with stem extract (E2). However, the addition of colloidal silver caused a decrease in the lactic acid bacteria counts at the end of the fermentation. Thus the counts were null in the sampling before bottling in wines elaborated with stem extract and colloidal silver (E2Ag). The same happened in SO_2 wines, although sulfur dioxide has less inhibitory effect on lactic acid bacteria than on acetic bacteria (Drysdale & Fleet, 1988).

Colloidal silver has already shown in previous works similar effects to SO_2 as a protector against acetic and lactic bacteria (García-Ruíz et al., 2015; Izquierdo-Cañas et al., 2018). Similarly, previous studies have shown the inhibitory effect of the stems on some strains of LAB (Ruiz-Moreno et al., 2015) and the seed and stem extracts against different pathogenic microorganisms (Poveda et al., 2018).

Table 3
Wine color parameters of final Airén wines (n = 3) produced using SO_2 or by-product extracts, with and without CSC.

	SO2	E1	E1Ag	E2	E2Ag
L *	98.668^{b} ± 0.361	93.695^{a} ± 0.569	93.062^{a} ± 2.079	95.584^{a} ± 0.689	94.487^{a} ± 1.149
a *	-0.170^{a} ± 0.120	0.828^{b} ± 0.185	0.814^{b} ± 0.370	0.512^{b} ± 0.246	0.427^{b} ± 0.245
b *	2.913^{a} ± 0.535	9.079^{b} ± 1.913	9.835^{b} ± 2.995	6.386^{b} ± 1.046	7.773^{b} ± 1.616
C *	2.921^{a} ± 0.527	9.118^{b} ± 1.916	9.869^{b} ± 3.014	6.408^{b} ± 1.061	7.786^{b} ± 1.626

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test at $P < .05$.

Respect to the yeasts, the counts were similar initially since the same amount of starter was used in all the wines. During fermentation, the yeast counts were decreased and no significant differences were observed in the final wines. These results show that the addition of extracts from seeds and stems, and colloidal silver has an effect equivalent to that of SO_2 on the yeast population of wines (Izquierdo-Cañas et al., 2012).

3.3. Wine color parameters

Table 3 lists the chromatic characteristics of the wines. The use of seed and stem extracts modified the color parameters of the wines compared to the control wines with SO_2. The wines elaborated with extracts presented lower luminosity (L *) and higher a*, b* and chroma (C*) values, and showed a darker color and a slight yellowish-brown tone. No added effect was observed due to the utilisation of colloidal silver.

These differences could be due to the effect of the use of extracts, which already had color by themselves, or to a less protective effect against the oxidation of the phenolic compounds of the must in comparison to that obtained with SO_2. It is well known the important role played by SO_2 in the reduction of quinones formed during the oxidation process to its o-diphenol form, preventing the formation of brown pigments in white wines (Singleton, 1987). An increase in the component b*, related to the browning of white wines, has been observed by other authors in SO_2 free vinifications of different white grape varieties using different SO_2 alternatives (inactive yeast strains enriched in glutathione or chitosan, hydrolysable and condensed tannins, colloidal silver or hydroxytyrosol), although these changes were not evaluated negatively from the sensory point of view (Ferrer-Gallego, Puxeu, Nart, Martín, & Andorrà, 2017; Izquierdo-Cañas et al., 2012; Raposo et al., 2016b).

3.4. Phenolic compounds

Table 4 shows the total and individual phenolic compounds of wines treated with SO_2 or with seed or stem extracts, either alone or in combination with CSC. Regarding stilbenes, resveratrol is quite sensitive to enzymatic oxidation (Mattivi & Nicolini, 1993), so greater protection against oxidation of these compounds due to SO_2 was observed (Castellari, Spinabelli, Riponi, & Amati, 1998). A smaller amount of stilbenes was observed in the wines treated with seed and stem extracts, especially in those containing CSC.

Flavan-3-ols, especially (+)-catechin, (−)-epicatechin and procyanidins, are highly correlated with the enzymatic browning of white wines (Sioumis, Kallithraka, Makris, & Kefalas, 2006). In general, the wines presented a low content of these phenolic compounds, however, the contribution due to the grape seed extracts was evident, as can be seen in Table 4, were those wines treated with seed extracts contained the highest amounts of flavan-3-ols. The important content of phenolic compounds, mainly flavan-3-ols, in grape seed and stem extracts has been evidenced by several authors (Barros et al., 2015; González-Paramás, Esteban-Ruano, Santos-Buelga, De Pascual-Teresa, & Rivas-Gonzalo, 2004; Spatafora, Barbagallo, Amico, & Tringali, 2013), although there is a great variability depending on the grape variety and extraction conditions used.

The content of total flavonols, poor substrates of polyphenoloxidase, was slightly higher in wines treated with extracts, either alone or with CSC. However, this effect was not observed in the case of hydroxycinnamic acids, where the major compound (trans-GRP) was higher in sulfur-containing control wines. GRP levels decrease in oxidation conditions (Cheynier, Trousdale, Singleton, Salgues, & Wilde, 1986) so that the protection against oxidation due to SO_2 may be more effective.

The wines treated with CSC had, in general, lower content in phenolic compounds, this effect was also observed by Izquierdo-Cañas et al., 2012. The use of colloidal silver in vinification is still under study, and there is little information on its effect on the phenolic fraction of white wines.

3.5. Total antioxidant activity

The antioxidant activity of the wines was evaluated by three different “in vitro” methods, DPPH, ABTS and ORAC. Fig. 2 shows the results obtained together with the total polyphenol content (TPI). The ORAC method is characterized by obtaining, in general, higher antioxidant activity values than those of the other two methods, because they are based on different principles (González-Centeno et al., 2012).

Table 4
Individual and total content (mg/L) of stilbenes, flavan-3-ols, flavonols, and hydroxycinnamic acids in Airén wines (n = 3) produced using SO_2 or by-product extracts, with and without CSC.

	SO2	E1	E1Ag	E2	E2Ag
Stilbenes*					
t-Resveratrol-glucoside	$10.40^{e} \pm 0.23$	$8.94^{d} \pm 0.66$	$5.55^{b} \pm 0.78$	$7.19^{c} \pm 0.92$	$2.75^{a} \pm 0.84$
c-Resveratrol-glucoside	$81.88^{d} \pm 2.15$	$66.70^{c} \pm 5.93$	$41.75^{b} \pm 5.92$	$40.09^{b} \pm 7.62$	$22.80^{a} \pm 2.75$
ΣStilbenes	$92.28^{d} \pm 2.36$	$75.65^{c} \pm 7.81$	$47.30^{b} \pm 5.32$	$47.28^{b} \pm 8.45$	$25.55^{a} \pm 3.60$
Flavan-3-ols					
Catechin	$0.19^{a} \pm 0.08$	$2.70^{b} \pm 0.45$	$2.39^{b} \pm 0.21$	$0.22^{a} \pm 0.04$	$0.23^{a} \pm 0.08$
Epicatechin	$0.47^{a} \pm 0.01$	$1.30^{c} \pm 0.20$	$1.05^{b} \pm 0.19$	$0.03^{a} \pm 0.01$	$0.02^{a} \pm 0.00$
Procyanidin B1	$0.23^{b} \pm 0.03$	$1.06^{d} \pm 0.03$	$0.61^{c} \pm 0.07$	$0.11^{a} \pm 0.04$	$0.13^{a} \pm 0.05$
Procyanidin B2	$0.28^{a} \pm 0.03$	$1.29^{c} \pm 0.03$	$0.82^{b} \pm 0.16$	$0.13^{a} \pm 0.04$	$0.15^{a} \pm 0.06$
Procyanidin Unknown	nd^{a}	$0.14^{c} \pm 0.02$	$0.10^{b} \pm 0.02$	nd^{a}	nd^{a}
Monomers glicosides	$0.86^{a} \pm 0.25$	$1.94^{b} \pm 0.15$	$1.94^{b} \pm 0.28$	$0.51^{a} \pm 0.02$	$0.69^{a} \pm 0.05$
ΣFlavan-3-ols	$2.03^{a} \pm 0.16$	$8.43^{c} \pm 0.82$	$6.91^{b} \pm 0.76$	$1.00^{a} \pm 0.11$	$1.20^{a} \pm 0.17$
Flavonols					
Quercetin 3-galactoside	$0.06^{a} \pm 0.01$	$0.07^{b,c} \pm 0.00$	$0.06^{a} \pm 0.00$	$0.07^{a,b} \pm 0.00$	$0.07^{b} \pm 0.00$
Quercetin 3-glucuronide	0.38 ± 0.00	0.51 ± 0.04	0.41 ± 0.04	0.46 ± 0.08	0.43 ± 0.08
Quercetin 3-glucoside	0.19 ± 0.01	0.24 ± 0.02	0.21 ± 0.02	0.22 ± 0.01	0.23 ± 0.03
ΣFlavonols	$0.63^{a} \pm 0.02$	$0.82^{b} \pm 0.04$	$0.68^{b,c} \pm 0.02$	$0.75^{b,c} \pm 0.09$	$0.73^{b,c} \pm 0.08$
Hydroxycinnamic acids					
trans-GRP	$19.18^{e} \pm 0.53$	$16.23^{d} \pm 0.18$	$14.21^{c} \pm 0.14$	$9.36^{a} \pm 1.27$	$13.04^{b} \pm 0.09$
cis-GRP	1.41 ± 0.02	1.44 ± 0.16	1.06 ± 0.12	1.40 ± 0.15	1.39 ± 0.23
Fertaric acid	2.83 ± 0.01	2.79 ± 0.10	2.76 ± 0.08	2.66 ± 0.09	2.81 ± 0.01
ΣHydroxycinnamic acids	$23.42^{d} \pm 0.56$	$20.45^{c} \pm 0.44$	$18.02^{b} \pm 0.09$	$13.42^{a} \pm 1.33$	$17.25^{b} \pm 0.18$

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test at $P < .05$.
* Results expressed in μg/L. nd: not detected.

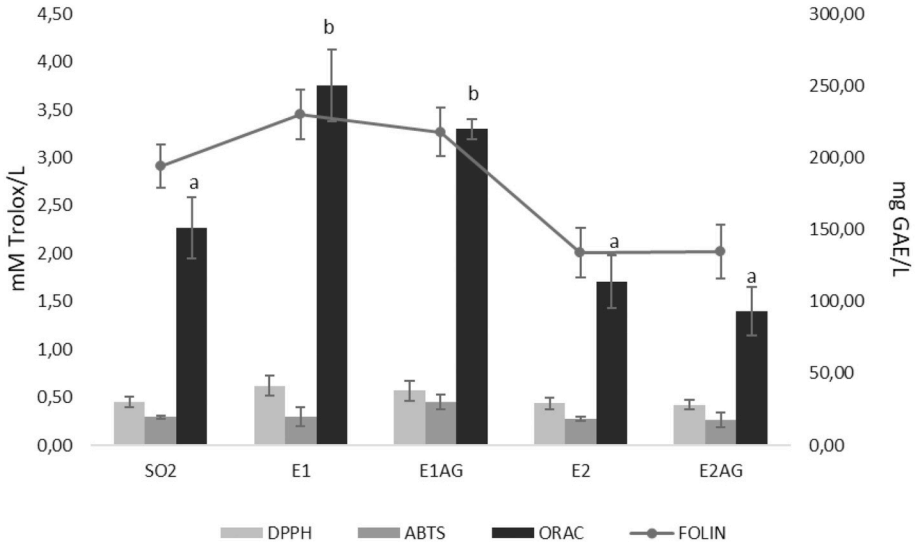

Fig. 2. Total phenolic content and antioxidant activity of final Airén wines (n = 3) produced using SO_2 or by-product extracts, with and without CSC. Different superscript letters for the same parameter denote significant differences ($p < .05$).

In general, there were no significant differences in the antioxidant activity values of the different samples, except in the wines treated with seed extract, with and without CSC (E1 and E1Ag), whose antioxidant activity (ORAC method) was significantly higher. The antioxidant activity is correlated with the content of phenolic compounds, and these wines also showed higher amounts of total polyphenols, flavan-3-ols and flavonols. In comparison with other winery by-products, several authors have shown that grape seed extracts are those that contain the highest amount of phenolic compounds and greater antioxidant activity (Marchante, Gómez Alonso, Alañón, Pérez-Coello, & Díaz-Maroto, 2018; Mattos, Tonon, Furtado, & Cabral, 2016).

These results show that the antioxidant capacity of the wines elaborated with seed and stem extracts, either alone or in combination with CSC, was similar or superior to that of the control wine elaborated with SO_2, in agreement with the results observed by other authors (Ruiz-Moreno et al., 2015).

3.6. Volatile compounds

When SO_2 substitutes are used in winemaking, the effect observed on volatile compounds varies depending on the type of additive used. The absence of SO_2 can cause the development of autochthonous yeasts in the wine, since it is demonstrated that the implantation of the starter strains is not always total. On the other hand, the lower availability of oxygen in sulfur-containing vinifications can affect the metabolism of yeasts, affecting mainly the synthesis of esters and alcohols (Garde-Cerdán & Ancín-Azpilicueta, 2007; Herraiz, Martin-Alvarez, Reglero, Herraiz, & Cabezudo, 1989). On the other hand, the use of natural extracts can incorporate specific volatile compounds to the final wine.

Tables 5 and 6 show the concentrations of the major and the minor volatile compounds of the wines, respectively. Regarding the major volatile compounds, it is worth noting the highest concentration of acetaldehyde of the SO2 wines. It has been previously described that the presence of SO_2 activates the production of acetaldehyde by yeasts (Herraiz et al., 1989) however, the addition of colloidal silver as antiseptic compound did not produce this effect (Izquierdo-Cañas et al., 2012).

The wines with a content of higher alcohols more similar to the control wine were those made with CSC, especially in the case of isoamyl alcohols, whose lowest values were only observed in wines treated with seed or stem extracts (E1 and E2) (Raposo et al., 2016b). A lower amount of isoamyl alcohols could be beneficial for the overall aroma of the wine.

Some compounds present in the grape must that can have an important role in the wine aroma can undergo oxidative processes during vinification, as terpenes and C6 compounds. SO_2 protects the must from oxidation, preventing the action of the lipoxygenase enzyme that act on the fatty acids precursors of the C6 compounds. These substances did not show significant differences between wines, so the protective effect of the seed and stem extracts, with or without CSC, on C6 compounds was equivalent to that of the SO_2.

Several authors have observed a greater quantity of alcohols in wines with SO_2, justified by a higher consumption of amino acids precursors in the presence of sulfur dioxide (Raposo et al., 2016a; Raposo et al., 2016b; Sonni et al., 2011). In our case, the total aliphatic alcohols (Table 6) did not show significant differences between the wines, although some variances in individual compounds were observed. The methionol, a compound very sensitive to oxidation, was

Table 5
Mean content and standard deviation (mg/L) of main volatile compounds in Airén wines (n = 3) produced using SO_2 or by-product extracts, with and without CSC.

	SO2	E1	E1Ag	E2	E2Ag
Acetaldehyde	51.77 ^{b} ± 2.25	3.66 ^{a} ± 0.59	4.30 ^{a} ± 0.53	4.35 ^{a} ± 0.67	3.87 ^{a} ± 0.35
Ethyl acetate	57.47 ± 2.48	64.46 ± 2.47	67.99 ± 10.95	66.94 ± 1.14	67.48 ± 4.61
2-Phenyl ethanol	10.18 ^{a} ± 0.31	9.53 ^{a} ± 0.38	12.94 ^{b} ± 2.24	9.63 ^{a} ± 0.46	10.88 ^{a} ± 0.67
Methanol	26.19 ^{b} ± 0.53	24.47 ^{a,b} ± 1.36	27.26 ^{b} ± 0.81	22.38 ^{a} ± 1.91	22.91 ^{a} ± 1.31
1-Propanol	14.38 ^{a} ± 0.57	17.42 ^{a,b} ± 0.82	28.31 ^{c} ± 3.23	19.96 ^{b} ± 1.55	29.27 ^{c} ± 1.07
Isobutanol	34.90 ± 4.35	26.42 ± 0.43	31.77 ± 5.43	31.19 ± 6.49	31.50 ± 0.41
Isoamyl alcohols	183.29 ^{b} ± 5.51	152.92 ^{a} ± 5.35	208.76 ^{b} ± 25.29	158.61 ^{a} ± 10.32	200.76 ^{b} ± 2.34

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test at $P < .05$.

Table 6
Mean content and standard deviation (μg/L) of minor volatile compounds in Airén wines (n = 3) produced using SO_2 or by-product extract, with and without CSC.

	SO2	E1	E1Ag	E2	E2Ag
Terpenes					
Linalol	5.7^{d} ± 0.2	3.9^{c} ± 0.3	3.3^{a,b} ± 0.4	3.5^{b} ± 0.1	2.9^{a} ± 0.1
β-Citronelol	3.1^{a,b} ± 0.1	4.3^{b} ± 0.3	3.6^{a,b} ± 1.0	2.6^{a} ± 0.1	3.1^{a,b} ± 0.3
Geraniol [2]	10.3^{c,d} ± 0.4	2.3^{a} ± 0.1	11.4^{d} ± 1.1	7.9^{b} ± 0.8	9.6^{c} ± 0.7
Σ Terpenes	19.2^{d} ± 0.2	10.5^{a} ± 0.4	18.4^{d} ± 1.6	13.9^{b} ± 0.8	15.6^{c} ± 0.4
C_6-Compounds					
1-Hexanol [2]	161.3 ± 12.7	170.1 ± 8.3	168.7 ± 2.7	152.1 ± 5.3	178.2 ± 14.4
3-Hexen-1-ol, (*E*)- [2]	8.0^{b,c} ± 0.9	8.8^{c} ± 0.7	6.5^{a,b} ± 0.8	7.5^{a,b,c} ± 0.2	6.1^{a} ± 0.7
3-Hexen-1-ol, (*Z*)- [2]	77.1 ± 8.6	81.0 ± 3.8	75.2 ± 3.1	70.7 ± 1.1	72.8 ± 6.8
2-Hexen-1-ol, (E)-	1.3^{a,b} ± 0.1	2.2^{b} ± 1.1	1.5^{a,b} ± 0.2	4.8^{c} ± 0.1	0.8^{a} ± 0.1
2-Hexen-1-ol, (Z)-	0.3^{a,b} ± 0.0	0.3^{a} ± 0.1	0.5^{c} ± 0.0	0.4^{a,b} ± 0.0	0.4^{b} ± 0.0
Σ C_6-Compounds	248.0 ± 22.3	262.5 ± 13.3	252.5 ± 5.9	235.5 ± 6.6	258.4 ± 22.0
Alcohols					
2-Methyl-1-propanol	251.1^{c} ± 17.0	224.7^{b,c} ± 1.5	199.3^{a,b} ± 25.6	208.6^{a,b} ± 9.9	186.4^{a} ± 8.8
1-Butanol	5.4^{c} ± 0.4	4.2^{b} ± 0.1	6.2^{d} ± 0.7	3.5^{a} ± 0.0	4.8^{b,c} ± 0.1
1-Pentanol	4.3^{a} ± 0.1	5.7^{b} ± 0.1	6.0^{b} ± 0.7	5.7^{b} ± 0.0	3.7^{a} ± 0.5
4-Heptanol	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.1
3-Methyl-1-pentanol	11.1^{a} ± 1.2	15.1^{b} ± 0.9	22.3^{c} ± 1.4	14.2^{b} ± 0.8	22.0^{c} ± 2.9
2,4-Dimethyl-3-pentanol	2.0^{b} ± 0.2	1.8^{a,b} ± 0.1	2.0^{b} ± 0.3	1.4^{a} ± 0.1	1.8^{a,b} ± 0.3
3-Ethoxy-1-propanol	0.9^{a} ± 0.1	3.8^{b} ± 0.1	6.5^{d} ± 0.3	5.7^{c} ± 0.1	7.7^{e} ± 0.1
3-Octanol [2]	0.8^{a} ± 0.1	1.2^{b} ± 0.0	1.2^{b} ± 0.1	1.1^{b} ± 0.1	1.0^{b} ± 0.1
1-Heptanol	17.6^{a} ± 0.8	41.0^{c} ± 3.9	43.4^{c} ± 3.7	42.2^{c} ± 5.5	32.4^{b} ± 4.7
2,6-Dimethyl-4-heptanol	2.5^{d} ± 0.1	1.2^{a} ± 0.1	2.3^{d} ± 0.1	1.5^{b} ± 0.1	2.0^{c} ± 0.2
2-Ethyl-1-hexanol [2]	1.3^{b} ± 0.2	0.8^{a} ± 0.0	0.7^{a} ± 0.1	1.9^{c} ± 0.3	0.6^{a} ± 0.0
1-Octanol	4.9 ± 0.5	4.5 ± 0.3	4.4 ± 0.1	4.8 ± 0.0	4.8 ± 0.5
1-Nonanol	1.1^{b} ± 0.2	1.6^{c} ± 0.1	1.3^{b} ± 0.0	1.9^{d} ± 0.2	0.8^{a} ± 0.1
2,5-Dimethyl- 3-hexanol	9.6 ± 0.9	7.6 ± 1.1	8.8 ± 1.1	8.0 ± 0.9	10.1 ± 0.9
Methionol	52.5^{c} ± 2.7	37.6^{a} ± 2.9	53.1^{c} ± 2.5	43.8^{b} ± 3.1	49.2^{b,c} ± 4.7
Σ Alcohols	365.7 ± 15.6	351.7 ± 9.1	358.4 ± 26.2	345.0 ± 15.9	327.8 ± 15.9
Acids					
Propanoic acid	0.1^{a} ± 0.0	0.1^{a} ± 0.0	0.1^{b} ± 0.0	0.1^{a} ± 0.0	0.1^{a} ± 0.0
Isobutyric acid	5.7^{b} ± 0.5	5.3^{b} ± 0.1	6.7^{c} ± 0.1	5.5^{b} ± 0.4	4.2^{a} ± 0.3
Butanoic acid [1,2]	8.4^{a} ± 0.2	9.1^{a} ± 0.8	11.4^{b} ± 1.3	9.2^{a} ± 0.6	9.0^{a} ± 0.9
2-methylbutyric acid	25.7 ± 1.5	22.4 ± 2.6	25.1 ± 0.3	22.8 ± 0.8	25.2 ± 2.1
Pentanoic acid [2]	0.4^{b} ± 0.1	0.3^{a,b} ± 0.0	0.4^{b} ± 0.0	0.3^{a,b} ± 0.0	0.3^{a} ± 0.0
Hexanoic acid [1,2]	753.6^{a} ± 34.6	875.8^{a,b} ± 56.0	898.0^{b} ± 79.6	797.0^{a,b} ± 26.5	768.4^{a,b} ± 54.2
3-Hexenoic acid, (E)-	4.9^{a} ± 0.5	5.3^{a} ± 0.4	6.0^{b} ± 0.1	4.6^{a} ± 0.2	4.6^{a} ± 0.4
Octanoic acid [1,2]	2195.1^{a,b} ± 61.6	2555.2^{a,b} ± 67.5	2701.7^{b} ± 393.5	2291.7^{a,b} ± 54.4	2093.5^{a} ± 253.6
7-Octenoic acid	7.5^{a,b} ± 2.0	7.9^{a,b} ± 1.5	8.4^{a,b} ± 1.2	5.8^{a} ± 0.2	10.5^{b} ± 0.6
Nonanoic acid [1,2]	7.5^{b} ± 2.0	2.6^{a} ± 0.2	8.9^{b} ± 0.6	2.2^{a} ± 0.3	7.5^{b} ± 0.6
Decanoic acid [1,2]	717.3^{a} ± 55.4	844.5^{b} ± 65.2	1014.8^{c} ± 11.5	660.1^{a} ± 16.6	825.8^{b} ± 7.7
Σ Acids	3726.2^{a} ± 102.8	4328.3^{a,b} ± 86.2	4681.5^{b} ± 430.8	3799.4^{a} ± 93.9	3748.9^{a} ± 299.3
Esters					
Isobutyl acetate	60.6^{c} ± 1.5	63.2^{c} ± 1.0	57.6^{b,c} ± 5.2	52.1^{b} ± 1.3	40.5^{a} ± 4.5
Ethyl butanoate [1]	87.5^{a} ± 1.4	109.4^{b} ± 2.4	115.6^{b} ± 3.8	87.5^{a} ± 10.0	88.5^{a} ± 6.7
Isoamyl acetate	920.1^{b} ± 39.8	947.8^{b} ± 29.7	1049.8^{c} ± 31.1	782.2^{a} ± 45.3	750.4^{a} ± 72.3
Ethyl hexanoate	696.6^{a} ± 9.1	804.7^{b} ± 19.9	691.5^{a} ± 53.1	681.9^{a} ± 25.3	628.2^{a} ± 73.2
Hexyl acetate	141.5^{a,b} ± 0.5	160.7^{b} ± 0.3	146.7^{a,b} ± 17.8	140.2^{a,b} ± 7.7	126.3^{a} ± 11.8
Ethyl 3-hexenoate	1.3^{a} ± 0.1	1.6^{b} ± 0.0	1.6^{b} ± 0.1	1.2^{a} ± 0.0	1.2^{a} ± 0.1
3-Hexen-1-ol, acetate, (Z)-	1.6^{a,b,c} ± 0.2	1.9^{c} ± 0.2	1.4^{a,b} ± 0.2	1.7^{b,c} ± 0.1	1.2^{a} ± 0.1
Ethyl heptanoate	0.8^{a} ± 0.0	1.3^{b} ± 0.2	1.5^{b} ± 0.1	1.5^{b} ± 0.1	1.5^{b} ± 0.2
Ethyl lactate	4.2^{a} ± 0.2	34.6^{b} ± 3.8	5.4^{a} ± 1.2	30.6^{b} ± 3.4	4.6^{a} ± 0.7
Heptyl acetate	2.0^{a,b} ± 0.4	2.3^{b} ± 0.3	3.0^{c} ± 0.1	1.7^{a} ± 0.2	1.7^{a} ± 0.0
Ethyl 2-hydroxy-3-methylbutanoate	0.7^{a} ± 0.1	2.9^{d} ± 0.1	1.2^{b} ± 0.2	2.6^{c} ± 0.2	1.0^{b} ± 0.1
Methyl 2-hydroxy-4-methylpentanoate (+ 2,3-butanediol)	12.3^{a} ± 0.3	12.5^{a} ± 1.6	17.4^{b} ± 3.5	15.2^{a,b} ± 1.4	18.0^{b} ± 0.5
Ethyl 3-hydroxybutyrate	35.8 ± 2.6	35.9 ± 2.4	36.6 ± 0.5	35.0 ± 1.5	38.1 ± 4.3
Ethyl octanoate [1,2]	1183.3^{a} ± 22.0	1811.8^{b} ± 141.2	1857.9^{b} ± 217.5	1924.8^{b} ± 189.0	1564.1^{b} ± 235.2
Isobutyl isovalerate	13.7^{b} ± 0.4	8.8^{a} ± 1.8	9.9^{a} ± 0.4	8.0^{a} ± 0.2	10.0^{a} ± 1.2
Ethyl methyl succinate	1.5^{c} ± 0.3	0.8^{a} ± 0.1	1.3^{b,c} ± 0.2	1.0^{a,b} ± 0.1	0.8^{a} ± 0.1
Ethyl decanoate [1,2]	159.0^{a} ± 12.3	400.5^{b} ± 2.1	457.7^{b,c} ± 7.1	395.4^{b} ± 50.5	503.5^{c} ± 52.7
1,3-Propanediol diacetate	3.3^{a} ± 0.1	4.4^{b} ± 0.5	4.9^{b} ± 0.2	5.0^{b} ± 0.2	4.3^{b} ± 0.4
Isoamyl octanoate	3.2^{a} ± 0.0	4.2^{b} ± 0.1	6.4^{c} ± 0.1	4.4^{b} ± 1.0	5.2^{b} ± 0.4
Diethyl succinate [1]	30.3^{a} ± 0.6	43.0^{b} ± 0.4	44.6^{b} ± 7.7	48.2^{b,c} ± 3.4	54.3^{c} ± 0.9
Ethyl 3-hydroxyhexanoate	3.3^{b} ± 0.1	3.3^{b} ± 0.3	3.5^{b} ± 0.3	2.3^{a} ± 0.0	2.4^{a} ± 0.2
Ethyl 9-decenoate	358.8^{a} ± 12.9	830.8^{c} ± 11.9	548.2^{b} ± 3.6	924.9^{d} ± 49.8	534.2^{b} ± 18.0
Allylacetate	37.4^{a} ± 1.6	42.7^{a,b} ± 2.6	54.3^{c} ± 6.9	48.1^{b,c} ± 3.2	50.9^{b,c} ± 3.9
Ethyl 2-hydroxy-4-methylpentanoate	12.6 ± 1.5	8.8 ± 0.0	9.2 ± 0.9	9.4 ± 2.6	9.4 ± 1.6
Ethyl 4-hydroxybutyrate	947.2^{b} ± 103.6	768.3^{a} ± 24.2	816.8^{a,b} ± 19.6	839.4^{a,b} ± 42.5	881.6^{a,b} ± 63.9
Diethylmalate	12.6^{b} ± 0.6	9.2^{a} ± 0.9	8.7^{a} ± 1.0	8.9^{a} ± 0.5	11.4^{b} ± 1.2
Diethylglutarate [2]	9.2^{a} ± 0.3	71.9^{b} ± 0.1	12.2^{a} ± 0.4	71.0^{b} ± 6.1	8.5^{a} ± 1.0
Σ Esters	4740.2^{a} ± 100.8	6187.6^{c} ± 206.3	5965.1^{c} ± 250.0	6124.3^{c} ± 323.6	5341.9^{b} ± 496.4
Benzenic compounds					

(*continued on next page*)

Table 6 (*continued*)

	SO2	E1	E1Ag	E2	E2Ag
Benzaldehyde	0.8^{a} ± 0.1	1.1^{b} ± 0.2	1.7^{d} ± 0.2	1.4^{c} ± 0.0	2.2^{e} ± 0.0
Guaiacol [1,2]	0.7^{a} ± 0.1	1.0^{b} ± 0.2	1.7^{c} ± 0.1	0.7^{a} ± 0.1	0.8^{a,b} ± 0.0
Benzyl alcohol [1,2]	6.0^{a} ± 0.3	13.4^{c} ± 2.8	9.5^{b} ± 0.6	19.8^{d} ± 1.2	15.1^{c} ± 1.2
4-Vinylguaiacol [1,2]	116.4^{b} ± 11.4	80.3^{a} ± 3.8	109.2^{b} ± 8.0	105.9^{b} ± 5.4	108.9^{b} ± 18.6
Syringol [1]	9.5^{b} ± 1.4	8.7^{b} ± 1.7	11.7^{c} ± 0.0	5.0^{a} ± 0.0	6.5^{a} ± 0.5
Tyrosol	3732.4^{a} ± 296.4	2897.2^{a} ± 357.0	4532.4^{b} ± 430.3	3568.0^{a} ± 87.0	3548.6^{a} ± 450.4
Vanillin [1,2]	1.8^{a} ± 0.0	4.5^{b,c} ± 0.2	4.2^{b} ± 0.3	5.1^{c} ± 0.6	5.1^{c} ± 0.4
Benzeneacetic acid	7.1^{a} ± 0.5	27.1^{b} ± 6.0	40.7^{c} ± 6.5	8.7^{a} ± 0.9	21.1^{b} ± 2.6
Acetovanillone [1]	13.8^{b} ± 1.8	8.8^{a} ± 0.9	8.8^{a} ± 0.6	11.4^{a} ± 0.9	10.2^{a} ± 0.8
p-Phenylacetophenone	29.8^{a} ± 2.6	37.1^{a} ± 0.5	61.0^{b} ± 7.1	55.3^{b} ± 5.4	57.3^{b} ± 4.4
Σ Benzenic compounds	3918.3^{a} ± 291.7	3079.2^{a} ± 359.3	4780.7^{b} ± 427.7	3781.4^{a} ± 80.0	3775.9^{a} ± 464.3
Furanic compounds					
γ-Butyrolactone [1,2]	0.1^{a} ± 0.0	0.1^{a,b} ± 0.0	0.3^{c} ± 0.0	0.2^{b} ± 0.0	0.3^{c} ± 0.0
Ethylfuroate	2.6^{a} ± 0.1	3.9^{b} ± 0.1	3.8^{b} ± 0.4	4.0^{b} ± 0.3	4.3^{b} ± 0.7
δ-Octalactone	3.5^{a} ± 0.5	7.1^{b,c} ± 1.6	8.2^{c} ± 0.6	5.7^{b} ± 0.3	5.4^{b} ± 0.1
γ-Nonalactone	1.6 ± 0.0	2.5 ± 0.8	2.5 ± 0.3	2.5 ± 0.7	2.4 ± 0.4
Pantolactone	6.5^{b} ± 0.7	4.2^{a} ± 0.4	6.6^{b} ± 1.0	5.5^{a,b} ± 0.5	5.6^{a,b} ± 0.5
γ-Undecalactone	63.0^{a,b} ± 3.1	50.4^{a} ± 8.6	75.3^{b} ± 13.2	51.9^{a} ± 1.8	62.2^{a,b} ± 4.8
Σ Furanic compounds	77.3^{a} ± 3.6	68.2^{a} ± 10.7	96.6^{b} ± 13.7	69.7^{a} ± 2.8	80.2^{a} ± 5.2

Values with different superscript letters in the same row denoted significant differences according to the Student-Newman-Keuls test at $P < .05$. Different superscript number denoted those minor volatile compounds found in the seed and stem extracts ([1] seeds; [2] stems; [1,2] seeds and stems).

found in higher amounts in SO2 wines, as has been described by other authors (Raposo et al., 2016a). While 3-ethoxy-1-propanol showed a lower concentration in SO2 wines compared to those with extracts (Sonni et al., 2011; Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2008).

The majority of the wines elaborated with extracts presented greater quantities of the major fatty acids and their ethyl esters. Several authors who have used different SO_2 substitutes in winemaking (Garde-Cerdán & Ancín-Azpilicueta, 2007; Raposo et al., 2016b; Sonni et al., 2008) have previously showed this effect. However, these differences may be due to the volatile composition of the extracts used, which can be transferred to the wine and cause a small increase in the concentration of certain compounds. Table 6 shows those compounds that were identified in the seed and stem extracts.

The increase in the concentration of fatty acids positively influences the formation of their corresponding ethyl esters. Some of the most abundant esters were ethyl hexanoate, ethyl octanoate and ethyl decanoate, which along with acetates such as isoamyl acetate and the ester derivates of hydroxy fatty acids have been shown to have an important role in the aroma of wines from neutral grape varieties. The wines elaborated with seed and stem extracts presented a total ester concentration significantly superior to the SO2 wine, so the use of these natural extracts during the vinification of Airén wines, could improve the complexity of their aroma.

Regarding the benzene compounds, tyrosol and 4-vinylguaiacol, both formed by yeasts metabolism, were the majority and presented few differences with respect to the SO2 wine. Other benzene compounds such as benzaldehyde, benzyl alcohol, vanillin or benzeneacetic acid showed slightly higher concentrations in wines with extracts.

In order to highlight the differences between the wines according to their volatile composition, a Principal Components Analysis was carried out to the major and minor volatile compound data matrix. The variance explained by the first three principal components was 70.8%. The distribution of the samples in the plane formed by the Principal Components 1 and 2 (PC1 and PC2) is shown in Fig. 3, and the most correlated compounds with both components are shown in Table 7, together with their loadings.

PC 1 separated the samples according to the presence of SO_2. The control wines were located on the left side due to their higher concentration of acetaldehyde, and lower amount of certain ethyl esters of fatty acids, and other volatile compounds present in the extracts such as vanillin and acetovanillone. The PC 2 separated the samples with colloidal silver and SO2 wine from the rest. The compounds most correlated with this component were some esters such as ethyl lactate, ethyl 2-hydroxy-3-methylbutanoate, diethylglutarate and ethyl-9-decenoate, and methionol with smaller amounts in these wines.

These results confirm that the addition of seed and stem extracts as SO_2 substitutes modify the volatile composition of the wines, and demonstrate the effect of colloidal silver complex on some groups of compounds as was shown in previous studies (Izquierdo-Cañas et al., 2012).

3.7. Sensory analysis

The negative effect that SO_2 can have on wine aroma is well known, as it can mask the fruity notes and neutralize it. Although its absence can increase the typical aromas of oxidation, especially in white wines (Ferrer-Gallego et al., 2017). In vinifications carried out with different SO_2 substitutes, an increase in the attributes related to floral and fruity aroma has been observed in wines without SO_2 (Raposo et al., 2016b; Sonni et al., 2008). However, Sonni et al., (2008), observed the opposite effect in white wines made with lysozyme and tannins with certain types of yeast.

The results obtained in the sensory evaluation of the wines are shown in Fig. 4, which includes three olfactory attributes and three gustatory attributes. All wines without SO_2 presented the “floral” attribute, independently of the extract used. The wines with stem extracts, with and without CSC, presented a sensorial profile more similar to the control wine, with higher scores for the fruity attribute, while the citrus aroma was more intense in the control wine (Izquierdo-Cañas et al., 2012).

According to other authors, the wines with seed extracts presented bitter notes (Pascual et al., 2016; Ruiz-Moreno et al., 2018), probably due to their significantly higher content of flavan-3-ols, main responsible for bitterness. Possibly for this reason the wine E1 was the worst valued by the tasters, although the differences were not statistically significant.

4. Conclusions

The use of alternative additives in winemaking requires that they demonstrate to have both antioxidant and antimicrobial activity equivalent to SO_2.

No significant differences were observed in total yeasts, lactic or

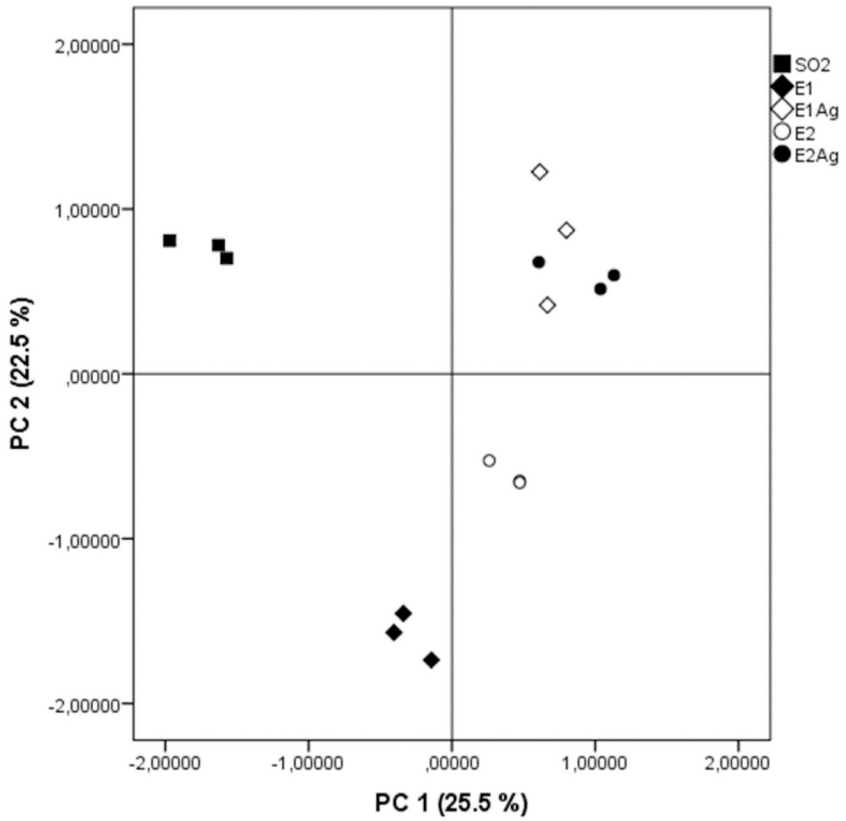

Fig. 3. Sample distribution in the space of Airén wines produced using SO_2 or by-product extracts, with and without CSC, defined by PC1 and PC2 regarding to main and minor volatile compounds.

Table 7
Results of principal component analysis applied to the significant main and minor volatile composition of Airén wines ($n = 3$) produced using SO_2 or by-product extracts, with and without CSC.

Principal component	Compound	Loadings (*)
PC1	3-Ethoxy-1-propanol	0.973
	Ethyl decanoate	0.949
	Ethyl heptanoate	0.924
	Acetovanillone	0.893
	Diethylsuccinate	0.890
	Acetaldehyde	−0.884
	Vanillin	0.859
PC2	Ethyl lactate	0.950
	Ethyl 2-hydroxy-3-methylbutanoate	0.948
	Diethylglutarate	0.925
	Methionol	0.861
	Ethyl 9-decenoate	0.826

(*) Only those volatiles with loadings > 0.800 have been included.

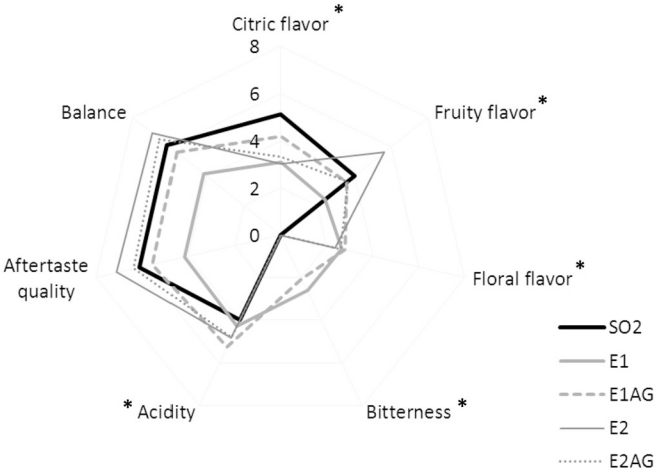

Fig. 4. Cobweb diagram of sensory scores for Airén wines produced using SO_2 or by-product extracts, with and without CSC. Descriptors with * denote significant differences ($p < .05$).

acetic bacteria counts in final wines elaborated with extracts respect to the control wine, showing an effect against these microorganisms similar to that of SO_2, being the wines with stem extract and colloidal silver those that had the lowest final counts.

The prefermentative addition of extracts influenced the phenolic composition of wines, which presented a tendency to browning and higher amounts of flavonols. Also, wines treated with seed extracts contained the highest amounts of flavan-3-ols, due to the higher content of these phenolic compounds in grape seed extracts.

Respect to volatile components, wine with extracts presented higher amounts of some compounds formed during alcoholic fermentation, mainly ethyl esters of fatty acids, and a lower concentration of acetaldehyde than SO2 wines. However, wines with colloidal silver presented similar concentrations of methionol and ethyl lactate than wines with SO_2.

From the sensory point of view, wines treated with extracts had higher scores on the fruity and floral attributes, although those with grape seed extracts were the worst evaluated presenting a slight bitterness.

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Artículo 2. Oenological potential of extracts from winery and cooperage by-products in combination with colloidal silver as natural substitutes to sulphur dioxide.

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Oenological potential of extracts from winery and cooperage by-products in combination with colloidal silver as natural substitutes to sulphur dioxide

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ABSTRACT

The aim of this study was to evaluate the oenological potential of natural extracts from winery and cooperage by-products, either alone or with a colloidal silver complex (CSC), on the quality of red wines, as possible substitutes to SO_2. Natural extracts were obtained from grape seeds and American oak wood by accelerated extraction with subcritical water. The prefermentative addition of grape seed or oak wood extracts was an useful tool to control acetic acid bacteria development, without affecting the alcoholic and malolactic fermentations. Both extracts protected the wines against oxidation, without negatively modifying their phenolic and volatile composition. They did not cause organoleptic defects in wines, which presented greater aromatic complexity and were positively evaluated by the tasters. Therefore, the use of grape seed or oak wood extracts in red vinification could be a good alternative to replace or reduce the doses of SO_2.

1. Introduction

Nowadays, there is a growing trend towards the revalorisation of the wine industry by-products, since they are an important source of bioactive compounds. Particularly, winery by-products are rich in phenolic compounds with antioxidant, anti-inflammatory, antimicrobial or bio-stimulant properties (Friedman, 2014). This phenolic composition makes them very attractive for different uses in the pharmaceutical, cosmetic, and food or agricultural industries.

On the other hand, new natural sources are being studied as an alternative to the use of SO_2 during winemaking, due to its negative effects on health (Vally, Misso, & Madan, 2009). The phenolic compounds of *Vitis vinifera* L. have been presented as a promising alternative to the use of SO_2 (García-Ruiz et al., 2012; Raposo et al., 2018). These compounds are important antioxidants, however their antimicrobial properties could be lower than those of SO_2 (Poveda, Loarce, Alarcón, Díaz-Maroto, & Alañón, 2018). In this sense, colloidal silver has been proposed as an important antiseptic agent, which could replace or reduce the doses of SO_2 in winemaking, although its lack of antioxidant capacity must be considered (Garde-Cerdán et al., 2014; Izquierdo-Cañas, García-Romero, Huertas-Nebreda, & Gómez-Alonso, 2012).

In wine industry, phenolic compounds have an important role in the production of wines, as they prevent oxidation reactions, and have an influence on wine organoleptic characteristics. Flavonoids and some phenolic acids, compounds abundant in the winery by-products, have shown to be good anthocyanin copigments, in addition to acting as oxidation substrates that partially prevent unwanted color changes (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009).

Among the grape by-products, seeds have a high antioxidant capacity (Delgado Adámez, Gamero Samino, Valdés Sánchez, & González-Gómez, 2012). The addition of seeds, from the grape pomace, during the fermentation of a red wine Syrah improved its antioxidant activity (Jara-Palacios, Hernanz, Escudero-Gilete, & Heredia, 2016). On the other hand, Cejudo-Bastante et al. (2016) showed the possibility of using an enzymatic hydrolysate of grape seeds during the fermentation of a Syrah wine to inhibit both, color and phenolic degradation. While Rivero, Gordillo, Jara-Palacios, González-Miret, and Heredia, (2017) observed a higher content of anthocyanins and procyanidins in Syrah wines fermented with the addition of grape seeds, which also showed higher quality and stability of the color.

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Other by-products related to the wine industry, are the remains of oak wood generated by the cooperages during the manufacture of the barrels. Cooperage by-products come from high quality woods; they are rich in volatile and phenolic compounds and have important antioxidant and antimicrobial capacities (Alañón, García-Ruiz, Díaz-Maroto, Pérez-Coello, & Moreno-Arribas, 2015). In previous studies, the pre-fermentative addition of oak wood chips increased the content of phenolic compounds and stabilized the color of the wines (Gordillo, Cejudo-Bastante, Rodríguez-Pulido, González-Miret, & Heredia, 2013). While the pre-fermentative addition of oenological tannins from skins, seeds and oak wood in red vinification, could improve the color, aroma and sensory properties of wines (Chen et al., 2016).

In addition, it is important to note the relevance of green extraction techniques, which do not use organic solvents, to obtain natural extracts for the food industry. Alañón, Alarcón, Marchante, Díaz-Maroto, and Pérez-Coello (2017) indicated that oak wood extracts, obtained by accelerated extraction with subcritical water, could be used in the food industry as flavouring agents and natural preservatives. While, pressurized aqueous extracts obtained from grape seeds presented greater antioxidant and antimicrobial properties than those from other winery by-products such as grape pomace or stems (Poveda et al., 2018).

Therefore, the objective of this study was to evaluate the oenological potential of natural aqueous extracts from winery and cooperage by-products, either alone or with a colloidal silver complex (CSC), utilised as alternatives to the use of SO_2, on the quality of red wines (*Vitis vinifera* L. Cv. Cabernet Sauvignon). Natural extracts were obtained from grape seeds (*Vitis vinifera* L. Cv. Tempranillo) and American oak wood (*Quercus alba*) by accelerated extraction with subcritical water.

2. Material and methods

2.1. Aqueous extracts

Aqueous extracts were obtained by accelerated solvent extraction (ASE 200, Dionex Corp. Sunnyvale, CA) with subcritical water (Alañón et al., 2017). Two wine industry by-products were used, grape seeds (*Vitis vinifera* L. Cv. Tempranillo) from the wastes of wineries, and American oak wood (*Quercus alba*) from the wastes of cooperages. Grape seeds were obtained from the Institute of Vine and Wine of Castilla-La Mancha (IVICAM, Tomelloso, Ciudad Real, Spain), and oak wood from Tonelería Magreñán (La Rioja, Spain). The samples were ground using a crusher Stephan UMC5 (Stephan Food Service Equipment GMBH). Eight grams (8 g) of seeds or 4 g of oak wood were mixed with 2 g of diatomaceous earth, used as dispersing agent to reduce the dead volume of a 22 mL stainless steel cell. Extraction conditions were as follow: temperature 120 °C, pressure 1500 psi, two extraction cycles of 10 min. To avoid possible contaminations, the extraction system was rinsed between samples. The extracts were freeze-dried under vacuum (1.1×10^{-2} mbar) for 24 h, at a condenser temperature of −53.2 °C. Several extractions were carried out until about 50 g of freeze-dried extracts were obtained from each sample. Homogenous freeze-dried extracts were kept in desiccator until their use. Both extracts were chemically characterized previously (Alañón et al., 2017; Poveda et al., 2018).

2.2. Colloidal silver complex (CSC) and SO_2

Colloidal silver complex (CSC) is a product under patent, composed by silver nanoparticles deposited on the surface of an inorganic inert material. It is a powder insoluble in ethanol and water with a particle size smaller than 10 μm. Potassium metabisulphite ($K_2S_2O_5$) with a purity higher than 95% was used as source of SO_2.

2.3. Red winemaking

Red wines from grapes *Vitis vinifera* L. Cv. Cabernet Sauvignon were elaborated during the 2016 harvest. The chemical composition of the must was: 23.9 °Brix, 3.05 g/L of total acidity, 3.98 of pH and 1.57 g/L of L-malic acid. Destemmed and crushed grapes were divided in eighteen batches (5 kg must and 1 kg skins, for each wine). A total of six treatments, by triplicate, were tested: (i) SO_2 at 50 mg/L which act as control wine, SO_2; (ii) 0.5 g/L of freeze-dried grape seed extract, SC1; (iii) 0.5 g/L of freeze-dried grape seed extract and 1.0 g/L of colloidal silver complex, SC1 + CSC; (iv) 1.0 g/L of freeze-dried grape seed extract, SC2; (v) 0.5 g/L of freeze-dried oak wood extract, QC1; (vi) 0.5 g/L of freeze-dried oak wood extract and 1.0 g/L of colloidal silver complex, QC1 + CSC. Natural extracts, alone or in combination with colloidal silver complex, or SO_2 were added as powder during the filling of the glass containers.

Alcoholic fermentation-maceration was realised in 10 L glass containers using *Saccharomyces cerevisiae* Uvaferm VN (Lallemand Inc., Zug, Switzerland) as starter culture, at 22 °C. Two punching downs were carried out every day. The evolution of the fermentation was controlled by weight loss, and it was considered finished when the weight of the containers remained stable. Then the density and glucose and fructose levels of wines were determined.

After alcoholic fermentation, wines were decanted and the malolactic fermentation was carried out at 20 °C using *Oenococcus oeni* PN4 (Lallemand Inc., Zug, Switzerland) as starter culture. The end of the malolactic fermentation was determined by measuring the malic and lactic acid content of the wines. Then, wines were decanted, cold stabilized at −5 °C, filtered and bottled. Only wines with SO_2 were adjusted to 25 mg/L of free SO_2 before bottling.

2.4. Physicochemical analysis

Conventional parameters as density, alcohol, glucose and fructose, dry extract, glycerin, total acidity, pH, acetic acid, total SO_2 and organic acids (tartaric, citric, succinic, malic and lactic acids) were determined using the official analytical methods established by the International Vine and Wine Organization (OIV, 2009).

2.5. Microbiological counts

The microbiological counts were performed at the end of the alcoholic and malolactic fermentations, before bottling. Wine samples were obtained using sterile bottles and pipettes.

2.5.1. Yeast

0.1 mL of sample was spread onto plates of YPD (Conda-Pronadisa, Madrid, España) supplemented with chloramphenicol (100 mg/L). The plates were incubated under aerobic conditions at 30 °C for 2 days.

2.5.2. Lactic acid bacteria

0.1 mL of serial dilution (10^{-2}) in sterile saline solution (0.9%) was spread onto plates of MRS (Conda-Pronadisa, Madrid, España), supplemented with cycloheximide (100 mg/L). The plates were incubated under anaerobic conditions (Gas Pack System, Oxoid Ltd., Basingstoke, United Kingdom) at 30 °C for 5 days.

2.5.3. Acetic acid bacteria

0.2 mL of sample was spread onto plates of GYC (5% glucose, 1% yeast extract, 0.5% calcic carbonate, 2% agar), supplemented with cycloheximide (100 mg/L). The plates were incubated under aerobic conditions at 30 °C for 5 days.

In all cases, colonies were counted and the results were expressed as colony forming units (CFU) per milliliter of wine (CFU/mL).

2.6. Total phenolic content (TPC) and antioxidant capacity

Total phenolic content (TPC) in wines was determined by the Folin-Ciocalteau assay. An UV–Vis spectrophotometer (Helios, Thermo Spectronic, Cambridge, UK) was used to measure the absorbance at 765 nm. Results were expressed as mg of gallic acid equivalent per liter (mg GAE/L).

The antioxidant capacity of wines was measured using three different "in vitro" methods; the DPPH assay, based on the use of 1,1-diphenyl-2-picrylhydrazyl radical as a stable radical (Brand-Williams, Cuvelier, & Berset, 1995), ABTS·+ (2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay (Re et al., 1999) and the oxygen radical absorbance capacity (ORAC) assay (Dávalos, Gomez-Cordoves, & Bartolome, 2004). Results were expressed in millimoles equivalents of Trolox per liter of wine (mM Trolox/L).

2.7. HPLC-DAD-ESI-MSn analysis

Flavan-3-ols and stilbenes were previously isolated from wines by SPE on C18 cartridges (Waters Sep-Pak Plus, 820 mg of adsorbent, Saint-Quentin En Yvelines, France), following the procedure reported by Rebello et al. (2013). 30 µL of the resulting extracts were analyzed using a HPLC Agilent 1200 series system equipped with a diode array detector (DAD, Agilent, Waldbronn, Germany) and coupled to a mass spectrometry system AB Sciex 3200 Q TRAP (Applied Biosystems, Foster City, USA) with triple quadrupole, turbo spray ionization (electrospray assisted by a thermonebulization) mass spectroscopy system (ESI-MS/MS). Chromatographic separation was achieved on an Ascentis-C18 column (4.6 × 150 mm; 2.7 µm particle; Supelco, Germany), thermostated at 16 °C and with a flow rate of 0.3 mL/min (Rebello et al., 2013).

Flavan-3-ols and stilbenes were identified and quantified according to the methodology reported by Rebello et al. (2013). The standards used for the calibration curves were: (+)-catechin, (−)-epicatechin, (+)-catechin gallate and *trans*-resveratrol-glucoside from Sigma (Tres Cantos, Madrid, Spain); (+)-gallocatechin and (−)-epigallocatechin from Phytolab (Vestenbergsgreuth, Germany); and procyanidins B1 and B2 from Extrasynthese (Genay, France). *trans*-Resveratrol-3-glucoside was transformed into its respective cis isomer using UV irradiation (366 nm during 5 min in quartz vials) with 25% MeOH solution of the *trans* isomer.

Flavonols and hydroxycinnamic acids were isolated from wines by solid phase extraction (SPE) on PCX cartridges (Bond Elut Plexa, 500 mg of adsorbent, 6 cm^3, Agilent, Waldbronn, Germany), following the procedure previously described by Castillo-Muñoz et al. (2009). Their analysis was carried out in an Agilent 1100 analytical HPLC system (Agilent, Germany) with a diode array detector (DAD) and a LC/MSD Trap VL electrospray ionization mass spectrometry (ESI-MSn) system. A Zorbax Eclipse XDB-C18 reversed-phase column (2.1 × 150 mm; 3.5 µm particle; Agilent, Germany), with a pre-column Zorbax Eclipse XDB-C8 (2.1 × 12.5 mm; 5 µm particle; Agilent, Germnay), thermostatized at 40 °C was used. The separation was achieved by using a ternary mobile phase, with an injection volume of 20 µL and a flow rate of 0.16 mL/min (Castillo-Muñoz et al., 2009).

The analysis of anthocyanins was carried out in the same chromatograph and with the same chromatographic column used for flavonols and hydroxycinnamic acids, according to the methodology previously described by Rebello et al. (2013). In this case, injection volume was 10 µL and the flow rate was 0.19 mL/min.

ESI-MS/MS was used for identification, while DAD-chromatograms were extracted at 360 nm, 320 nm and 520 nm for quantification of flavonols, hydroxycinnamic acids and anthocyanins, respectively. Flavonol concentrations were expressed as equivalents of quercetin-3-glucoside (Extrasynthese, Genay, France) (mg/L), hydroxycinnamic acids were expressed as equivalents of caftaric acid (Extrasynthese, Genay, France) (mg/L), while anthocyanin concentrations were expressed as equivalents of malvidin-3-glucoside (Phytolab, Vestenbergsgreuth, Germany) (mg/L).

2.8. Color parameters

CIELAB parameters were determined by the OIV method using a spectrophotometer (Agilent 8453 diode array, Agilent, Santa Clara CA, USA) with a home-made program for spectra treatment. The percentages of copigmentation and polymerization were determined following the method proposed by Boulton (1996).

2.9. Analysis of major volatile compounds

Major volatile compounds were analysed in a GC/MS chromatograph Focus-ISQ (Thermo Scientic, Milan, Italy). 100 µL of wine were mixed with 100 µL of 2-pentanol-4-methyl used as an internal standard (41.55 mg/L) and 1 mL of Mili-Q water. Then, 1 µL of sample was injected in split mode (1/25) into a BP-21 column (SGE) (60 m × 0.32 mm × 0.25 µm) FFAP phase (polietilenglicol modified with nitrotereftalic acid (TPA). Helium was used as carrier gas (constant flow 1.2 mL/min). The injector temperature was 195 °C and the oven temperature was programmed as follows: 32 °C during 2 min, ramped at 5 °C/min to 120 °C, and then increased 75 °C/min to 190 °C, that it was maintained during 18 min. The MS operated in the electron impact mode: electron energy 70 eV, ion source temperature 250 °C. Identification was carried by comparison with commercial standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the quantification by calibration curves of each standard.

2.10. Analysis of minor volatile compounds

Prior to their analysis, minor volatile compounds were extracted by solid-phase extraction (SPE) using 500 mg styrene divinyl benzene cartridges (Lichrolut EN Merck, KGaA, Darmstadt, Germany). 100 mL of wine and 40 µL of 4-nonanol as internal standard (1 g/L) were passed through SPE cartridges previously conditioned. One microliter (1 µL) of extract was injected in splitless mode into an Agilent 6890 GC System equipped with an Agilent 5973 inert Mass Selective Detector and a HP-20 Carbowax column (60 m × 0.25 mm × 0.25 µm). Helium was used as carrier gas (constant flow 1 mL/min). The oven temperature was programmed as follows: 70 °C during 5 min and then ramped at 2 °C/min to 210 °C. The injector temperature was 250 °C. The MS operated in the electron impact mode: electron energy 70 eV, ion source temperature 230 °C. Identification of the minor volatile compounds was performed comparing their GC retention indices and mass spectra with those provided for authentic standards from Sigma-Aldrich, and by the NBS75 K and Wiley A libraries. Linear retention indices were calculated using *n*-alkanes as external references. Semiquantitative analysis of the compounds was performed assuming that component response factors were the same as the response factor for the internal standard.

2.11. Sensory analysis

Descriptive sensory analysis was performed according to standard ISO 6564:1985 by a panel of eight expert assessors with experience in sensory analysis. Assessment took place in a standard sensory analysis chamber (ISO 8589:2007). In a first session, wines were sniffed and tasted by the assessors in order to generate the sensory terms individually. The hedonic terms and those provided by less than 3 assessors were rejected, while synonyms were regrouped in a single term. Then a tasting sheet with 21 attributes, that described the sensorial profile of the wines, was generated by consensus (ISO 11035:1994).

Wines were sniffed and tasted in three sessions and the descriptors were scored on a 10 cm unstructured scale to rate the intensity of each attribute (0: absence of a descriptor, 10: maximum intensity).

2.12. Statistical analysis

Statistical analysis was carried out by using the SPSS software, version 24.0. The Student-Newman-Keuls test was applied to identify any statistically significant differences between samples. Moreover, in order to highpoint the main contributors to the variance among wines, chemical and sensorial data separately were subjected to Principal Component Analysis (PCA).

3. Results and discussion

3.1. Physico-chemical parameters and microbiological counts

Supplementary Table S1 shows the conventional oenological parameters of wines after bottling. No significant differences were found for the density, alcohol content, residual concentration of glucose and fructose, dry extract, glycerine and total acidity, between wines. These data showed that alcoholic fermentation was not affected by any of the products added. SO_2 wines had lower acetic acid, although it is important to note that all values of acetic acid were below the legal limit for red wine.

In our results, differences in the concentration of some organic acids between wines could be observed. In general, SO_2 wines showed higher organic acid concentrations, except for acetic and tartaric acids. Previous studies have related the increase in the production of succinic acid in wines with higher concentrations of SO_2 (Shimazu & Watanabe, 1981). On the other hand, Rozès, Arola, and Bordons (2003) showed that consumption of citric acid and the production of acetic acid, by *Oenococcus oeni*, increased when phenolic compounds were added in a model wine.

Table 1 shows the microbiological counts (UFC/mL) of lactic acid bacteria, acetic bacteria and yeasts in wines before bottling. Antimicrobial activity of rich polyphenol extracts against altering microorganisms have been previously reported (Alañón et al., 2015; García-Ruiz et al., 2012). In our case, the replacement of SO_2 by seed grape or oak wood extracts, did not affect significantly to the population of acetic bacteria, while lactic bacteria and yeast were more inhibited by SO_2. Few differences were observed with the addition of colloidal silver. In previous studies, similar results over microbial population of white and red wines were obtained using colloidal silver instead of SO_2 (Garde-Cerdán et al., 2014; Izquierdo-Cañas et al., 2012).

3.2. Total phenolic content (TPC) and antioxidant capacity

Although, SO_2 helps the extraction of anthocyanins during maceration, no statistically significant differences were observed in the total polyphenol content between the different wines, probably due to the contribution of other phenolic compounds provided by natural extracts (Supplementary Fig. S1). On the other hand, the antioxidant activity of red wines is largely attributable to their high content of total polyphenols. Therefore, the results obtained by DPPH and ORAC methods did not show significant differences between wines. Previous studies reported that the addition of grape seed tannins increased the antioxidant activity, determined by the DPPH method, but only in the polyphenol-poor wines (Neves, Spranger, Zhao, Leandro, & Sun, 2010). Antioxidant activity of wines treated with grape seed extracts (SC1, SC1 + CSC, SC2) determined by ABTS method provided lower values although, no statistically differences were found. These results were in good agreement with the findings of Ghanem et al. (2017) for the increase of the antioxidant activity of wines by the addition of condensed tannins. Therefore, the use of natural extracts from wine industry by-products did not affect the total phenolic content and antioxidant capacity of the wines.

3.3. Non-anthocyanin phenolic compounds

Table 2 shows the total and individual content of flavan-3-ols, flavonols, hydroxycinnamic acids and stilbenes of wines treated with SO_2 or with natural extracts, either alone or in combination with CSC. The pre-fermentative use of oak wood extracts instead of SO_2 did not modify significantly the flavan-3-ols content of wines. The concentration of (+)-catechin, main monomeric flavan-3-ol of Cabernet Sauvignon wines (Hermosín-Gutiérrez, Lorenzo, & Espinosa, 2005) did not change in those wines with oak extract respect to that with SO_2. However, wines treated with the highest concentration of grape seed extract (SC2) presented a greater content of flavan-3-ols, mainly (+)-catechin and procyanidin B2. This fact was in consonance since (+)-catechin is regarded as the main flavan-3-ol monomer contributed to wine by seeds (Pascual et al., 2016), meanwhile procyanidin B2 is the main procyanidin dimer found in grape seeds (González-Paramás, Esteban-Ruano, Santos-Buelga, De Pascual-Teresa, & Rivas-Gonzalo, 2004). Several studies have demonstrated the high antioxidant capacity of flavan-3-ols, which was evidenced in SC2 wines (Supplementary Fig. S1). In addition, according to the results obtained by Izquierdo-Cañas et al. (2012), wines treated with CSC as antimicrobial agent exhibited lower content of flavan-3-ols, although the differences were not significant.

Different pre-fermentative treatments tested did not affect to the content of flavonols in the wines significantly, which showed the protective effect of the natural extracts against oxidation. Wines treated with oak wood extract and CSC presented a lower content of total flavonols compared to SO_2 wines and with those treated with grape seed extracts. This effect was previously observed by Izquierdo-Cañas et al. (2012) in some minor flavonol glycosides. In our particularly case, the flavonol more affected was quercetin-3-glucoside. The protective effect of natural extracts against the oxidation of flavonols was also observed for hydroxycinnamic acids.

Regarding stilbenes, it has been previously described that the use of SO_2 in the pre-fermentative phase reduces the oxidation and precipitation of resveratrol during alcoholic fermentation (Castellari, Spinabelli, Riponi, & Amati, 1998). This protective effect of stilbenes against oxidation was also observed when SO_2 was replaced by natural extracts from grape seeds and oak wood, since no significant differences were found between wines. Based on the results obtained, SO_2 did not seem to increase the extraction of flavonols, hydroxycinnamic acids and stilbenes.

3.4. Anthocyanins and chromatic characteristics

Anthocyanins are extracted from grape skins during maceration and are the main pigments responsible for the color of red wine. Table 3 shows the individual and total anthocyanin content of wines elaborated. A slight effect of SO_2 on anthocyanin extraction during

Table 1
Microbiological counts (UFC/mL) of wines treated with SO_2 and natural extracts, with or without CSC, before bottling (n = 3).

	SO_2	SC1	SC1 + CSC	SC2	QC1	QC1 + CSC
Lactic acid bacteria	$27{,}000^{a} \pm 5500$	$430{,}000^{c} \pm 45{,}000$	$420{,}000^{c} \pm 100{,}000$	$450{,}000^{c} \pm 83{,}000$	$230{,}000^{b} \pm 64{,}000$	$610{,}000^{d} \pm 45{,}000$
Acetic bacteria	$5.0^{a} \pm 0.0$	$11.7^{a} \pm 5.8$	$6.7^{a} \pm 2.9$	$10.0^{a} \pm 5.0$	$10.0^{a} \pm 0.0$	$6.7^{a} \pm 2.9$
Yeast	$380^{a} \pm 80$	$8000^{c} \pm 2500$	$3700^{b} \pm 2000$	$1300^{a} \pm 93$	$97^{a} \pm 15$	$150^{a} \pm 30$

[a,b,c,d]Different superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the test of Student–Newman–Keuls.

Table 2
Individual and total content (mg/L) of flavan-3-ols, flavonols, hydroxycinnamic acids and stilbenes in wines treated with SO_2 and natural extracts, with or without CSC (n = 3).

	SO_2	SC1	SC1 + CSC	SC2	QC1	QC1 + CSC
(+)-Catechin	$26.4^{b,c} \pm 1.4$	$22.1^{c} \pm 3.1$	$23.0^{b,c} \pm 0.5$	$36.1^{a} \pm 0.2$	$27.8^{b} \pm 0.5$	$24.0^{b,c} \pm 0.2$
(−)-Epicatechin	$19.7^{a} \pm 2.2$	$15.5^{a,b} \pm 0.6$	$13.7^{b} \pm 0.7$	$18.9^{a,b} \pm 0.8$	$16.1^{a,b} \pm 0.4$	$16.2^{a,b} \pm 2.5$
(+)-Gallocatechin	$13.4^{a} \pm 0.5$	$9.3^{a} \pm 0.2$	$9.4^{a} \pm 1.4$	$12.6^{a} \pm 1.0$	$11.0^{a} \pm 1.9$	$9.9^{a} \pm 3.4$
(−)-Epigallocatechin	$1.9^{a} \pm 0.4$	$1.7^{a} \pm 0.5$	$2.2^{a} \pm 0.9$	$2.7^{a} \pm 1.3$	$3.2^{a} \pm 0.02$	$2.6^{a} \pm 0.3$
(+)-Catechin gallate	$0.27^{a} \pm 0.06$	$0.19^{a} \pm 0.04$	$0.19^{a} \pm 0.09$	$0.26^{a} \pm 0.05$	$0.29^{a} \pm 0.03$	$0.22^{a} \pm 0.01$
Procyanidin B1	$21.2^{a} \pm 3.3$	$17.5^{a} \pm 6.1$	$18.1^{a} \pm 2.1$	$27.5^{a} \pm 2.4$	$24.2^{a} \pm 4.0$	$19.5^{a} \pm 1.0$
Procyanidin B2	$7.5^{b} \pm 1.4$	$7.0^{b} \pm 2.5$	$7.0^{b} \pm 0.8$	$12.3^{a} \pm 1.1$	$8.5^{b} \pm 0.2$	$8.3^{b} \pm 0.3$
Procyanidin Unknown 1	$21.6^{a} \pm 3.3$	$17.7^{a} \pm 6.0$	$18.4^{a} \pm 2.2$	$27.9^{a} \pm 2.4$	$24.5^{a} \pm 3.9$	$19.8^{a} \pm 1.0$
Procyanidin Unknown 2	$1.9^{a} \pm 0.4$	$1.5^{a} \pm 0.3$	$1.4^{a} \pm 0.1$	$1.8^{a} \pm 0.7$	$1.9^{a} \pm 0.2$	$1.8^{a} \pm 0.1$
Galloylated dimers	$1.3^{a} \pm 0.5$	$0.9^{a} \pm 0.3$	$0.9^{a} \pm 0.0$	$1.5^{a} \pm 0.1$	$1.4^{a} \pm 0.0$	$1.2^{a} \pm 0.1$
Monomers glicosides	$31.4^{a} \pm 3.2$	$28.7^{a} \pm 1.6$	$24.3^{a} \pm 3.3$	$31.4^{a} \pm 0.0$	$25.9^{a} \pm 2.8$	$26.6^{a} \pm 6.7$
Total Flavan-3-ols	$146.5^{a,b} \pm 12.7$	$122.0^{b} \pm 15.9$	$118.7^{b} \pm 4.5$	$172.3^{a} \pm 11.0$	$144.8^{a,b} \pm 13.0$	$130.0^{b} \pm 10.7$
Myricetin-3-glucuronide	$9.4^{a} \pm 0.1$	$11.1^{a} \pm 1.1$	$12.7^{a} \pm 0.4$	$13.1^{a} \pm 0.5$	$10.5^{a} \pm 2.2$	$9.4^{a} \pm 0.9$
Quercetin-3-galactoside	$2.3^{a,b} \pm 0.1$	$2.6^{a,b} \pm 0.0$	$2.3^{a,b} \pm 0.3$	$2.9^{a} \pm 0.0$	$2.1^{a,b} \pm 0.5$	$1.7^{b} \pm 0.3$
Quercetin-3-glucuronide	$12.4^{a} \pm 0.4$	$12.5^{a} \pm 1.4$	$13.8^{a} \pm 0.3$	$15.8^{a} \pm 1.1$	$11.1^{a} \pm 2.8$	$10.5^{a} \pm 1.8$
Quercetin-3-glucoside	$15.1^{a} \pm 0.2$	$15.6^{a} \pm 0.9$	$15.6^{a} \pm 0.7$	$16.9^{a} \pm 0.2$	$12.8^{a,b} \pm 2.6$	$10.8^{b} \pm 0.9$
Laricitrin-3-glucoside	$3.2^{b} \pm 0.1$	$4.0^{a,b} \pm 0.2$	$4.3^{a,b} \pm 0.0$	$4.8^{a} \pm 0.0$	$3.7^{a,b} \pm 0.8$	$3.4^{b} \pm 0.2$
Kaempferol-3-glucoside	$1.7^{a} \pm 0.2$	$2.1^{a} \pm 0.2$	$1.9^{a} \pm 0.2$	$2.4^{a} \pm 0.2$	$1.8^{a} \pm 0.4$	$1.5^{a} \pm 0.0$
Isorhamnetin-3-glucoside	$4.2^{a,b} \pm 0.2$	$4.8^{a,b} \pm 0.2$	$4.8^{a,b} \pm 0.3$	$5.4^{a} \pm 0.2$	$3.8^{a,b} \pm 1.3$	$3.4^{b} \pm 0.3$
Syringetin-3-glucoside	$3.6^{b} \pm 0.0$	$4.4^{a,b} \pm 0.2$	$5.2^{a} \pm 0.0$	$5.3^{a} \pm 0.1$	$4.1^{a,b} \pm 0.9$	$3.8^{a,b} \pm 0.2$
Total Flavonols	$51.9^{a,b} \pm 0.4$	$57.0^{a,b} \pm 4.2$	$60.6^{a,b} \pm 0.8$	$66.4^{a} \pm 0.9$	$50.5^{a,b} \pm 11.1$	$44.5^{b} \pm 4.6$
Caftaric acid	$56.0^{a} \pm 0.6$	$58.5^{a} \pm 0.9$	$60.0^{a} \pm 0.3$	$59.9^{a} \pm 1.5$	$57.6^{a} \pm 3.3$	$59.7^{a} \pm 2.3$
Coutaric acid	$20.2^{a} \pm 0.1$	$20.4^{a} \pm 0.2$	$21.2^{a} \pm 1.2$	$22.0^{a} \pm 0.1$	$19.5^{a} \pm 1.8$	$19.7^{a} \pm 1.7$
Fertaric acid	$9.4^{a} \pm 0.1$	$9.6^{a} \pm 1.0$	$10.8^{a} \pm 0.1$	$9.8^{a} \pm 0.7$	$10.3^{a} \pm 1.9$	$9.8^{a} \pm 0.7$
Total Hydroxycinnamic Acids	$85.6^{a} \pm 0.7$	$88.6^{a} \pm 2.2$	$92.0^{a} \pm 1.6$	$94.9^{a} \pm 2.6$	$87.3^{a} \pm 7.1$	$89.2^{a} \pm 4.6$
t-Resveratrol-glucoside*	$181.3^{a} \pm 9.2$	$158.9^{a} \pm 24.3$	$154.9^{a} \pm 14.7$	$198.0^{a} \pm 36.4$	$152.3^{a} \pm 23.6$	$157.9^{a} \pm 25.9$
c-Resveratrol-glucoside*	$448.6^{a} \pm 12.7$	$401.9^{a} \pm 28.6$	$379.4^{a} \pm 7.4$	$420.6^{a} \pm 15.9$	$424.6^{a} \pm 23.8$	$415.8^{a} \pm 29.4$
Total Stilbenes*	$629.8^{a} \pm 3.6$	$560.8^{a} \pm 4.4$	$534.3^{a} \pm 22.1$	$618.6^{a} \pm 20.6$	$576.9^{a} \pm 0.2$	$573.7^{a} \pm 55.3$

[a,b]Different superscripts in the same row mean significant differences (α = 0.05) according to the test of Student–Newman–Keuls.
* Results expressed in μg/L.

maceration was observed. However, the differences were not statistically significant neither in the total anthocyanin content nor in the concentration of the main anthocyanin of the Cabernet Sauvignon variety, malvidin-3-glucoside. Sheridan and Elias (2016) demonstrated that SO_2 did not affect the concentration of malvidin-3-glucoside in a model wine.

SO_2 wines had significantly higher luminosity values (L^*) and lower color intensity (CI) than wines with extracts (Table 4), highlighting the bleaching effect of SO_2 (He et al., 2012). SO_2 can bleach free anthocyanins by nucleophilic addition to the C4 carbon in the C ring of the flavylium cation, reducing the color of the red wines (He et al., 2012). On the other hand, by comparison with the SO_2 treatment, wines with extracts, either alone or with CSC, had significantly lower a^* and b^* values, showing a displacement of the color from the red towards the zone of the violets. Previous studies have already shown that the presence of grape seeds during fermentation increases the intensity of color and accentuates the bluish tone of wines (Pascual et al., 2016).

Anthocyanins participate in different reactions that lead to more stable derived pigments. Among these reactions, non-covalent interactions such as copigmentation (between anthocyanins and copigments) or self-association (between anthocyanins) can protect anthocyanins from degradation. In our study, the highest values for proportion of copigmented anthocyanins corresponded to SO_2 wines, coinciding with a higher content of acetaldehyde. Izquierdo-Cañas et al. (2012) also obtained higher % copigmentation in SO_2 wines compared to wines without SO_2 and with the addition of colloidal silver complex. According to Hermosín-Gutiérrez et al. (2005), a greater percentage of copigmentation was observed in wines with higher content of monomeric anthocyanins, although the differences were not statistically significant.

Table 3
Individual and total content (mg/L) of anthocyanins in wines treated with SO_2 and natural extracts, with or without CSC (n = 3).

	SO_2	SC1	SC1 + CSC	SC2	QC1	QC1 + CSC
Delphinidin 3-glc	$3.1^{a} \pm 0.4$	$2.7^{a} \pm 0.2$	$2.7^{a} \pm 0.2$	$2.9^{a} \pm 0.7$	$2.5^{a} \pm 0.4$	$2.4^{a} \pm 0.4$
Petunidin 3-glc	$8.7^{a} \pm 0.9$	$8.0^{a} \pm 0.6$	$8.2^{a} \pm 0.4$	$8.6^{a} \pm 1.4$	$7.5^{a} \pm 0.9$	$7.0^{a} \pm 1.0$
Peonidin 3-glc	$4.8^{a} \pm 0.5$	$4.0^{a} \pm 1.0$	$4.1^{a} \pm 0.4$	$4.5^{a} \pm 0.7$	$4.3^{a} \pm 0.1$	$4.1^{a} \pm 0.3$
Malvidin 3-glc	$176.0^{a} \pm 10.3$	$145.2^{a} \pm 16.4$	$172.1^{a} \pm 10.0$	$159.6^{a} \pm 16.0$	$157.6^{a} \pm 13.3$	$143.4^{a} \pm 11.3$
Delphinidin 3-acglc	$3.4^{a} \pm 0.7$	$2.9^{a} \pm 0.9$	$3.8^{a} \pm 0.9$	$3.5^{a} \pm 0.6$	$3.0^{a} \pm 0.4$	$2.5^{a} \pm 0.4$
Petunidin 3-acglc	$4.8^{a} \pm 0.4$	$4.8^{a} \pm 0.2$	$4.9^{a} \pm 0.1$	$5.2^{a} \pm 1.0$	$4.4^{a} \pm 0.5$	$3.8^{a} \pm 0.4$
Delphinidin 3-cmglc	$0.8^{a} \pm 0.1$	$0.7^{a} \pm 0.1$	$0.8^{a} \pm 0.2$	$0.8^{a} \pm 0.2$	$0.6^{a} \pm 0.1$	$0.6^{a} \pm 0.1$
Peonidin 3-acglc	$8.0^{b} \pm 0.9$	$7.2^{a,b} \pm 0.3$	$6.9^{a,b} \pm 0.3$	$7.6^{b} \pm 0.6$	$6.7^{a,b} \pm 0.5$	$5.9^{a} \pm 0.6$
Malvidin 3-acglc	$93.9^{b} \pm 5.6$	$71.4^{a} \pm 7.2$	$85.1^{a,b} \pm 4.9$	$82.4^{a,b} \pm 5.0$	$79.0^{a,b} \pm 7.3$	$70.3^{a} \pm 6.8$
Malvidin 3-cfglc	$0.7^{a} \pm 0.1$	$0.5^{a} \pm 0.0$	$0.6^{a} \pm 0.1$	$0.6^{a} \pm 0.1$	$0.6^{a} \pm 0.1$	$0.5^{a} \pm 0.1$
Petunidin 3-cmglc	$1.4^{a} \pm 0.2$	$1.1^{a} \pm 0.2$	$1.5^{a} \pm 0.1$	$1.4^{a} \pm 0.4$	$1.0^{a} \pm 0.2$	$1.0^{a} \pm 0.2$
Malvidin 3-cmglc cis	$1.3^{b} \pm 0.2$	$0.6^{a} \pm 0.1$	$0.7^{a} \pm 0.0$	$0.6^{a} \pm 0.1$	$0.6^{a} \pm 0.0$	$0.5^{a} \pm 0.1$
Peonidin 3-cmglc	$4.8^{a} \pm 0.7$	$4.0^{a} \pm 0.4$	$4.2^{a} \pm 0.5$	$4.2^{a} \pm 0.8$	$3.6^{a} \pm 0.7$	$3.4^{a} \pm 0.7$
Malvidin 3-cmgluc trans	$29.4^{a} \pm 4.5$	$22.2^{a} \pm 4.6$	$27.9^{a} \pm 3.5$	$25.6^{a} \pm 4.8$	$24.0^{a} \pm 4.4$	$22.2^{a} \pm 3.7$
Total anthocyanins	$341.0^{a} \pm 25.1$	$275.3^{a} \pm 31.9$	$323.5^{a} \pm 20.8$	$307.4^{a} \pm 32.4$	$295.5^{a} \pm 28.8$	$267.5^{a} \pm 25.7$

[a,b]Different superscripts in the same row mean significant differences (α = 0.05) according to the test of Student–Newman–Keuls.

Table 4
Color related parameters in wines treated with SO_2 and natural extracts, with or without CSC (n = 3).

	SO_2	SC1	SC1 + CSC	SC2	QC1	QC1 + CSC
L^*	$30.16^{b} \pm 3.15$	$16.84^{a} \pm 5.27$	$14.20^{a} \pm 2.88$	$14.82^{a} \pm 4.13$	$17.51^{a} \pm 4.03$	$20.03^{a} \pm 5.22$
a^*	$53.55^{b} \pm 1.20$	$45.97^{a} \pm 3.18$	$44.62^{a} \pm 2.40$	$44.77^{a} \pm 2.93$	$47.51^{a} \pm 2.88$	$48.75^{a.b} \pm 3.22$
b^*	$35.26^{a} \pm 1.53$	$26.58^{a} \pm 6.11$	$23.19^{a} \pm 3.55$	$24.35^{a} \pm 5.65$	$28.80^{a} \pm 4.90$	$30.80^{a} \pm 4.85$
C^*	$64.13^{b} \pm 0.89$	$53.19^{a.b} \pm 5.83$	$50.31^{a} \pm 3.78$	$51.05^{a} \pm 5.32$	$55.29^{a.b} \pm 4.96$	$57.70^{a.b} \pm 5.32$
H^*	$33.36^{a} \pm 1.55$	$29.75^{a} \pm 3.90$	$27.35^{a} \pm 2.25$	$28.28^{a} \pm 3.84$	$30.52^{a} \pm 2.92$	$32.15^{a} \pm 2.30$
Color Intensity (CI)	$4.88^{a} \pm 0.50$	$7.79^{a} \pm 1.74$	$8.53^{a} \pm 1.23$	$8.54^{a} \pm 1.60$	$7.84^{a} \pm 1.47$	$7.07^{a} \pm 1.35$
Tonality	$0.95^{b} \pm 0.03$	$0.87^{a.b} \pm 0.03$	$0.77^{a} \pm 0.11$	$0.86^{a.b} \pm 0.03$	$0.89^{a.b} \pm 0.00$	$0.89^{a.b} \pm 0.01$
% copigmentation	$44.45^{b} \pm 1.84$	$30.89^{a} \pm 1.13$	$35.12^{a} \pm 0.80$	$35.65^{a} \pm 2.27$	$34.64^{a} \pm 2.45$	$33.03^{a} \pm 2.25$
% polymerization	$18.92^{a} \pm 0.85$	$24.43^{b} \pm 3.28$	$26.82^{b} \pm 2.65$	$23.02^{b} \pm 1.56$	$23.74^{b} \pm 0.77$	$26.2^{b} \pm 0.36$

[a,b]Different superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the test of Student–Newman–Keuls.

The addition of polyphenols to red wine increases the polymerization reaction and, therefore, improves its color (Alcalde-Eon, García-Estévez, Puente, Rivas-Gonzalo, & Escribano-Bailon, 2014). Wines with grape seed and oak wood extracts exhibited a higher percentage of polymerization than wines treated with SO_2. Natural extracts used are rich in polyphenols, mainly flavan-3-ols and ellagitannins respectively, which would favor the formation of polymeric pigments and reduce the percentage of contribution of copigmentation to wine color, since this is considered the first step to form polymeric pigments. In addition, SO_2 can react with several components of the wine, mainly acetaldehyde, pyruvic acid and phenolic compounds, such as anthocyanins and hydroxycinnamic acids, which would reduce the rate of polymerization reactions (He et al., 2012).

According our results, the negative effect of SO_2 on the color of red wines due to anthocyanin discoloration was greater than its effect increasing the extraction of anthocyanins and protecting them from oxidation, since free SO_2 wines, despite having lower contents (although not significantly) of anthocyanins, showed higher color intensities and tonalities more appreciated by consumers in young red wines.

3.5. Volatile compounds and sensory evaluation

Supplementary Tables S2 and S3 show the major and the minor volatile compounds of the wines, respectively. Within the group of major volatiles, the main differences were observed in acetaldehyde and isobutanol, and to a lesser extent in isoamyl alcohols, while the rest of the compounds were not affected by the treatment used. Regarding to minor compounds the main differences were found in the C_6 compounds and alcohols, as well as in those volatile components given by the oak extract, such as lactones and benzenic compounds.

When principal component analysis (PCA) was applied to the volatile component concentrations presented in Supplementary Tables S2 and S3, the first two principal components (PC1 and PC2) explained nearly 58% of the variance between the wines. Fig. 1 plots the wines on the coordinate grid defined by the first two principal components.

PC1 separated the wines obtained by prefermentative addition of aqueous oak wood extracts, with or without CSC, from the rest of the samples. Compounds positively correlated with PC1 such as 2-methylpropanoic acid, diethyl glutarate, ethyl 2-hydroxy-4-methylvalerate and three C_6 alcohols, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol and (Z)-2-hexen-1-ol, showed higher concentrations in SO_2 wines and in those treated with grape seed extracts. While the substances negatively correlated with PC1 were those compounds released by the oak extracts, whose concentrations were higher in wines treated with them. These volatile compounds were two oak-lactone isomers, (E)-β-methyl-γ-octalactone and (Z)-β-methyl-γ-octalactone, eugenol, vanillin and ethyl octanoate. The extract used was obtained from American oak wood, which is characterized by its high content of oak-lactones, mainly *cis*-isomer, and eugenol, while its concentration in vanillin is similar to that of French oak (Díaz-Maroto, Sánchez-Palomo, & Pérez-Coello, 2004). Previous studies have reported higher amounts of medium-chain fatty acid ethyl esters in wines without SO_2 using lysozyme as antiseptic (Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009). In contrast, other authors showed a higher production of esters in wines treated with SO_2 in comparison with those produced using a grapevine-shoot stilbene extract as preservative (Raposo et al., 2016). These authors observed a similar behaviour for C_6-alcohols, with greater amounts of 1-hexanol and (Z)-3-hexenol in SO_2 wines.

On the other hand, PC2 separated wines treated with SO_2 from the rest of samples, and it was positively correlated with acetaldehyde, 1-butanol and isoamyl alcohols. SO_2 wines displayed higher levels of acetaldehyde, because SO_2 favours the formation of this compound by *Saccharomyces cerevisiae* (Herraiz, Martin-Alvarez, Reglero, Herraiz, & Cabezudo, 1989). This effect has been previously observed when replacing SO_2 with other antiseptic compounds such as lysozyme or colloidal silver (Izquierdo-Cañas et al., 2012; Sonni et al., 2009). In contrast, Garde-Cerdán et al. (2014) did not find significant differences on acetaldehyde content in wines made in the presence or absence of SO_2. According to Sonni et al. (2009), SO_2 also seemed to have a direct correlation with isoamyl alcohol and 1-butanol.

It is evident that SO_2 replacement with different antiseptic and antioxidant additives could alter wine aroma chemical composition; however, it should preserve or even improve organoleptic quality of wines. All wines elaborated did not present sensory defects and resulted well balanced. In order to highlight the main organoleptic attributes of wines, PCA was applied to the data obtained in the quantitative sensory analysis. Fig. 2 shows the principal component bi-plot, illustrating the simultaneous projection of wines tested and the sensorial descriptors. The first two principal components explained 78.6% of the total variance. PC1 and PC2 accounted for 45.5% and 33.1% of the variance between samples, respectively. Wines were grouped mainly into three groups according to the additive used: SO_2, grape seed extract or oak wood extract. It was worthy to note that the use of CSC, as it has been observed by other authors, hardly influenced the organoleptic profile of wines treated with natural extracts (Garde-Cerdán et al., 2014; Izquierdo-Cañas et al., 2012).

The prefermentative addition of oak wood extract instead of SO_2, either alone or with CSC, contributed to the typical flavour of oak barrel ageing, described as oak wood, vanillin and clove or spicy. These sensory notes were due to volatile compounds provided by the oak extract, as oak lactones, vanillin and eugenol (Díaz-Maroto et al., 2004), whose concentration was higher in the QC1 and QC1 + CSC wines (Supplementary Table S3). In addition, these wines presented greater body, intensity and aftertaste quality and were better valued than the rest.

On the other hand, wines treated with SO_2 and those obtained with grape seed extracts preserved the pepper flavour characteristic of Cabernet Sauvignon, and presented a more intense aromatic and gustatory profile to red fruits than wines treated with oak extract. Recently, ethyl 2-hydroxy-4-methylvalerate has been identified as a key compound of the “blackberry aroma” in Bordeaux red wine (Falcao, Lytra, Darriet, & Barbe, 2012). Wines treated with SO_2 and grape seed extracts

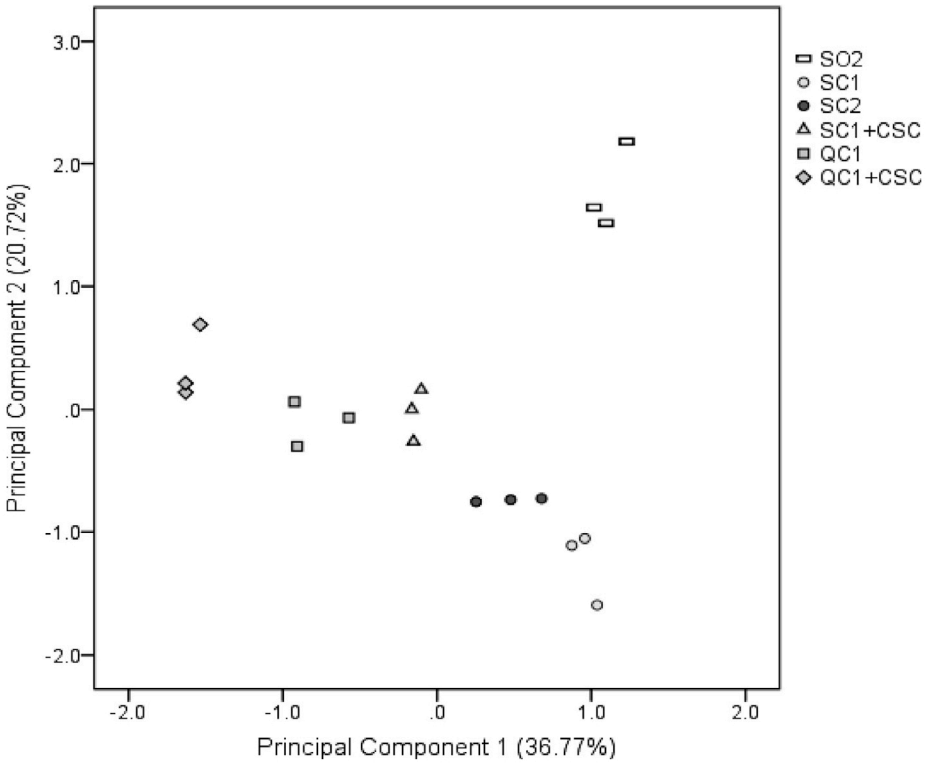

Fig. 1. Plot of wines treated with SO_2 and natural extracts, with or without colloidal silver complex, distributed in the space defining by principal components PC1 and PC2 with regard to significant volatile compounds.

(SC1, SC1 + CSC and SC2) revealed higher amounts of this volatile compound, although in all samples, their concentrations were below the perception threshold (300 μg/L in model wine solution). Therefore, its effect on the aroma of these wines would be conditioned to the existence of synergistic perceptive interactions with other compounds, such as it was revealed by Falcao et al. (2012).

Wines treated with grape seed extracts, mainly those with higher concentration of extract (SC2), were bitterer for the tasters. This fact could be related to their higher content of (+)-catechin (Table 2), since the bitterness of red wines is mainly due to the monomeric flavonoids (Noble, 1994).

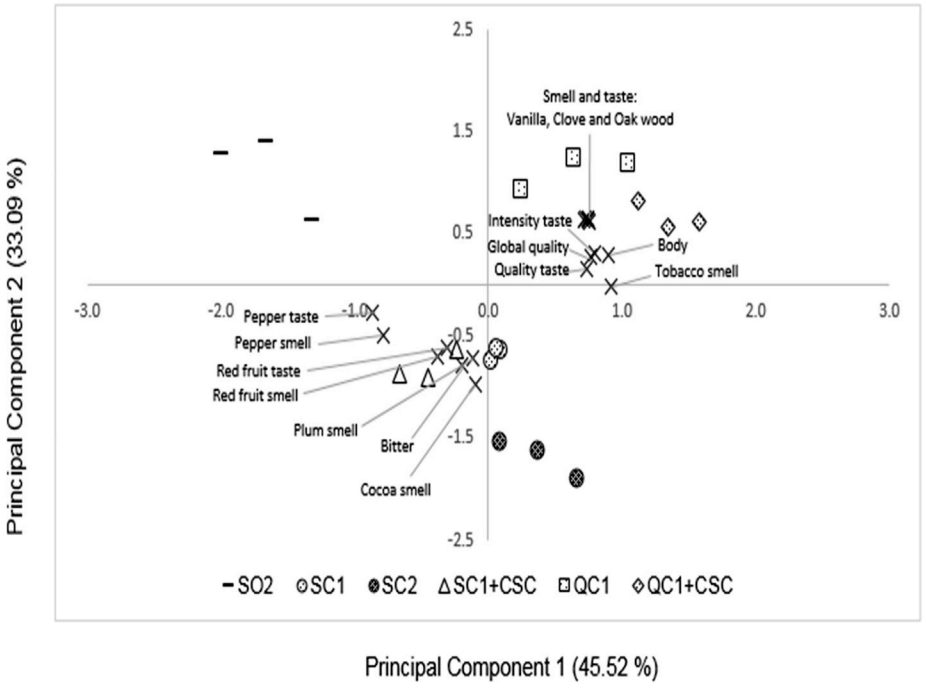

Fig. 2. Distribution in the space of wines treated with SO_2 and natural extracts, with or without colloidal silver complex, joint with their sensorial attributes of taste and smell (x).

4. Conclusions

The prefermentative addition of aqueous natural extracts obtained from wine industry by-products, either alone or in combination with CSC, instead of SO_2, did not affect to the phenolic and volatile composition of red wines negatively. Alcoholic fermentation was not altered by any of the products added. The microbiological counts (UFC/mL) of acetic bacteria in wines before bottling revealed the antimicrobial activity of natural extracts against these altering microorganisms. Both extracts protected wines against oxidation, not observing important changes in their phenolic composition.

From the sensorial point of view, grape seed and oak wood extracts did not cause organoleptic defects in wines, which presented greater aromatic complexity. Wines treated with seed extracts preserved better the varietal character of the grape variety. Meanwhile the oak extract wines presented sensory notes typical of the aged wines, which were positively evaluated by the tasters. Therefore, the use of aqueous extracts from winery and cooperage by-products in red vinification could to be a good alternative to replace or reduce the doses of SO_2.

Conflict of interest statement

The authors declare that they do not have any conflict of interest, and an informed consent was obtained from all study contributors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.10.072.

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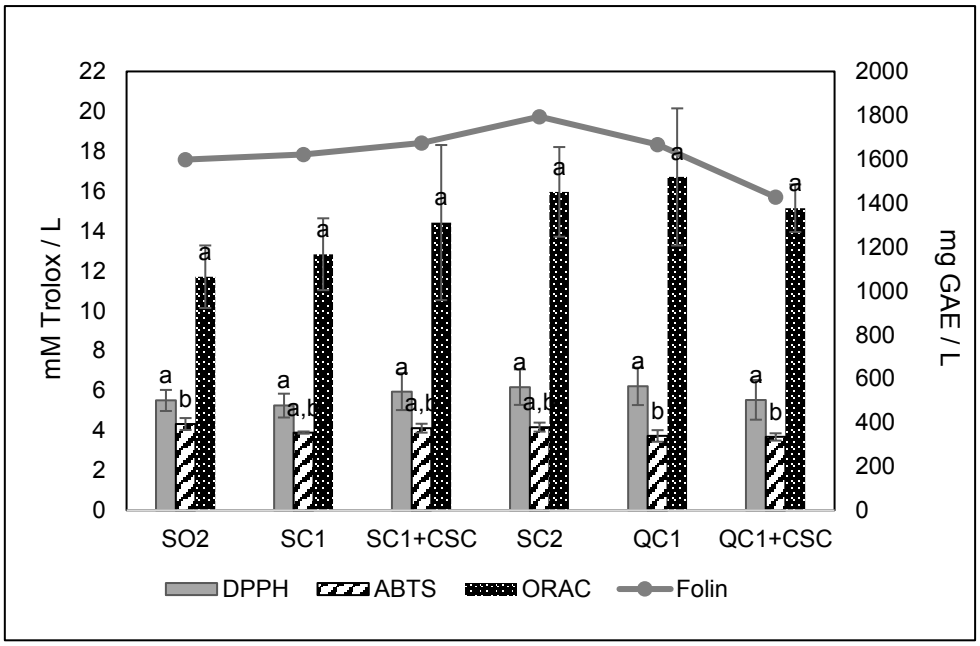

Fig. S1. Total polyphenol content and antioxidant activity of wines treated with SO_2 or natural extracts (n = 3). ^{a,b} Different superscripts in the same method of determining antioxidant activity mean significant differences (α = 0.05) according to the test of Student–Newman–Keuls.

Table S1. Physico-chemical parameters of wines treated with SO_2 and natural extracts, with or without CSC (n = 3).

	SO_2	SC1	SC1+CSC	SC2	QC1	QC1 + CSC
Density	$0.99^{a} \pm 0.00$	$0.99^{a} \pm 0.00$	$0.99^{a} \pm 0.00$	$0.99^{a} \pm 0.00$	$0.99^{a} \pm 0.00$	$0.99^{a} \pm 0.00$
Alcohol (% v/v)	$14.04^{a} \pm 0.18$	$14.16^{a} \pm 0.16$	$14.24^{a} \pm 0.39$	$14.02^{a} \pm 0.44$	$14.21^{a} \pm 0.36$	$14.38^{a} \pm 0.21$
Glucose and fructose (g/L)	$0.11^{a} \pm 0.02$	$0.08^{a} \pm 0.02$	$0.09^{a} \pm 0.01$	$0.09^{a} \pm 0.01$	$0.09^{a} \pm 0.02$	$0.10^{a} \pm 0.02$
Dry extract (g/L)	$24.81^{a} \pm 0.27$	$24.86^{a} \pm 1.18$	$23.49^{a} \pm 1.01$	$25.08^{a} \pm 1.56$	$23.18^{a} \pm 1.56$	$23.02^{a} \pm 1.87$
Glycerine (g/L)	$8.88^{a} \pm 0.30$	$8.56^{a} \pm 0.09$	$8.67^{a} \pm 0.57$	$8.56^{a} \pm 0.51$	$8.84^{a} \pm 0.46$	$8.78^{a} \pm 0.28$
Total acidity (g/L)	$3.10^{a} \pm 0.08$	$2.97^{a} \pm 0.21$	$2.70^{a} \pm 0.13$	$2.94^{a} \pm 0.17$	$3.05^{a} \pm 0.22$	$2.84^{a} \pm 0.16$
pH	$4.30^{a} \pm 0.02$	$4.37^{b} \pm 0.04$	$4.44^{c} \pm 0.01$	$4.38^{b} \pm 0.01$	$4.37^{b} \pm 0.03$	$4.35^{a,b} \pm 0.04$
Acetic acid (g/L)	$0.25^{a} \pm 0.01$	$0.34^{c} \pm 0.01$	$0.30^{b,c} \pm 0.03$	$0.28^{a,b} \pm 0.01$	$0.31^{b,c} \pm 0.02$	$0.30^{b,c} \pm 0.01$
Tartaric acid (g/L)	$1.64^{a} \pm 0.05$	$1.88^{b} \pm 0.12$	$1.80^{b} \pm 0.05$	$1.96^{b} \pm 0.11$	$1.97^{b} \pm 0.07$	$1.89^{b} \pm 0.11$
Citric acid (g/L)	$0.16^{c} \pm 0.03$	$0.01^{a} \pm 0.03$	$0.06^{a,b} \pm 0.02$	$0.09^{b} \pm 0.00$	$0.06^{a,b} \pm 0.02$	$0.04^{a,b} \pm 0.00$
Succinic acid (g/L)	$0.92^{b} \pm 0.07$	$0.81^{a.b} \pm 0.06$	$0.71^{a} \pm 0.07$	$0.78^{a,b} \pm 0.00$	$0.80^{a,b} \pm 0.08$	$0.68^{a} \pm 0.04$
Malic acid (g/L)	$0.04^{a} \pm 0.02$	$0.02^{a} \pm 0.03$	$0.01^{a} \pm 0.01$	$0.02^{a} \pm 0.01$	$0.03^{a} \pm 0.03$	$0.02^{a} \pm 0.01$
Lactic acid (g/L)	$1.66^{b} \pm 0.06$	$1.50^{a} \pm 0.04$	$1.45^{a} \pm 0.03$	$1.48^{a} \pm 0.06$	$1.49^{a} \pm 0.06$	$1.40^{a} \pm 0.03$
Total SO_2 (mg/L)	$45.33^{b} \pm 9.45$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$

^{a,b,c} Different superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the test of Student–Newman–Keuls.

Table S2. Major volatile compounds (mg/L) in wines treated with SO_2 or natural extracts, either alone or in combination with CSC (n = 3)

Compounds	SO_2	SC1	SC1+CSC	SC2	QC1	QC1+CSC
Acetaldehyde	$12.90^{b} \pm 2.15$	$7.26^{a} \pm 0.42$	$7.73^{a} \pm 0.90$	$6.99^{a} \pm 0.74$	$7.37^{a} \pm 0.68$	$8.48^{a} \pm 2.16$
Ethyl acetate	$64.61^{a} \pm 4.62$	$71.24^{a} \pm 7.83$	$77.42^{a} \pm 4.65$	$75.48^{a} \pm 3.49$	$77.12^{a} \pm 4.57$	$78.68^{a} \pm 5.39$
Methanol	$47.58^{a} \pm 5.79$	$55.54^{a} \pm 9.51$	$42.46^{a} \pm 1.90$	$47.39^{a} \pm 4.13$	$47.87^{a} \pm 5.27$	$43.76^{a} \pm 7.28$
1-Propanol	$22.16^{a} \pm 1.09$	$29.26^{b} \pm 1.63$	$23.89^{a} \pm 2.57$	$27.30^{b} \pm 1.26$	$29.01^{b} \pm 1.21$	$29.63^{b} \pm 2.77$
Isobutanol	$34.90^{a} \pm 1.15$	$50.60^{c} \pm 2.26$	$40.62^{b} \pm 5.13$	$52.16^{c} \pm 1.84$	$54.23^{c} \pm 1.87$	$38.67^{a} \pm 0.33$
Isoamyl alcohols	$386.85^{c} \pm 3.78$	$334.24^{a} \pm 20.98$	$355.76^{a.b.c} \pm 14.62$	$344.50^{a.b} \pm 12.96$	$380.44^{b.c} \pm 18.34$	$365.62^{a.b.c} \pm 17.24$

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test ($\alpha = 0.05$).

Table S3. Minor volatile compounds (µg/L) in wines treated with SO_2 or natural extracts, either alone or in combination with CSC (n = 3)

Compounds	SO_2	SC1	SC1+CSC	SC2	QC1	QC1+CSC
1-hexanol	692.8 ^{b} ± 33.9	650.2 ^{a,b} ± 18.1	626.0 ^{a,b} ± 46.7	637.1 ^{a,b} ± 44.5	645.2 ^{a,b} ± 29.8	557.6 ^{a} ± 41.8
(E)-3-hexen-1-ol	57.6 ^{c} ± 2.4	46.1 ^{b} ± 1.4	45.7 ^{b} ± 2.6	46.0 ^{b} ± 3.3	41.3 ^{b} ± 2.5	32.1 ^{a} ± 1.9
(Z)-3-hexen-1-ol	59.0 ^{d} ± 2.4	44.5 ^{c} ± 4.5	37.5 ^{a,b,c} ± 2.7	42.4 ^{a,b} ± 2.5	35.9 ^{a,b} ± 3.8	33.2 ^{a} ± 3.2
(E)-2-hexen-1-ol	29.3 ^{b} ± 6.1	23.3 ^{a,b} ± 8.0	22.4 ^{a,b} ± 6.1	23.0 ^{a,b} ± 7.8	18.6 ^{a,b} ± 4.3	12.7 ^{a} ± 3.0
(Z)-2-hexen-1-ol	14.8 ^{c} ± 3.0	12.5 ^{a,b} ± 0.9	11.5 ^{a,b,c} ± 0.8	15.2 ^{c} ± 2.3.	10.5 ^{a,b} ±1.1	8.2 ^{a} ± 0.8
Total C_6 compounds	853.6 ^{b} ± 47.5	776.6 ^{b} ± 13.1	743.1 ^{b} ± 45.0	763.6 ^{b} ± 58.3	751.4 ^{b} ± 40.6	643.8 ^{a} ± 47.6
linalool	2.3 ^{a} ± 0.7	3.1 ^{a} ± 0.7	1.8 ^{a} ± 0.1	3.1 ^{a} ± 1.5	1.9 ^{a} ± 0.1	2.0 ^{a} ± 0.4
citronellol	4.5 ^{a} ± 1.1	4.0 ^{a} ± 1.5	5.4 ^{a} ± 0.9	4.5 ^{a} ± 1.7	8.5 ^{a} ± 4.6	10.2 ^{a} ± 5.9
geraniol	13.1 ^{a} ± 0.8	12.6 ^{a} ± 0.4	17.2 ^{a} ± 5.1	13.5 ^{a} ± 2.9	12.6 ^{a} ± 1.3	16.0 ^{a} ± 3.6
geranic acid	9.2 ^{b} ± 0.7	9.7 ^{b} ± 1.0	8.9 ^{b} ± 1.3	9.3 ^{b} ± 0.8	6.6 ^{a} ± 0.8	6.4 ^{a} ± 0.6
geranial	23.7 ^{b} ± 1.8	15.6 ^{a} ± 1.5	21.7 ^{b} ± 2.0	13.9 ^{a} ± 1.2	14.2 ^{a} ± 0.9	22.2 ^{b} ± 1.4
Total terpenic compounds	52.8 ^{a} ± 2.6	44.9 ^{a} ± 4.7	55.0 ^{a} ± 9.0	44.3 ^{a} ± 5.6	43.8 ^{a} ± 5.0	56.8 ^{a} ± 10.0
3-oxo-α-ionol	64.7 ^{a} ± 8.0	65.8 ^{a} ± 12.7	89.8 ^{a} ± 8.7	73.2 ^{a} ± 10.9	85.4 ^{a} ± 13.6	89.1 ^{a} ± 18.5
β-damascenone	13.1 ^{a} ± 4.6	13.8 ^{a} ± 11.4	12.5 ^{a} ± 2.3	10.0 ^{a} ± 7.1	12.7 ^{a} ± 3.7	12.8 ^{a} ± 5.0
Total C_{13} norisoprenoids	77.8 ^{a} ± 9.3	79.6 ^{a} ± 23.5	102.3 ^{a} ± 10.9	83.2 ^{a} ± 14.5	98.0 ^{a} ± 17.0	102.0 ^{a} ± 21.7
propanoic acid	1.0 ^{a} ± 0.2	1.3 ^{a} ± 0.2	1.0 ^{a} ± 0.1	1.7 ^{a} ± 0.3	1.0 ^{a} ± 0.1	0.9 ^{a} ± 0.5
2-methylpropanoic acid	40.0 ^{d} ± 2.3	47.4 ^{e} ± 2.9	27.7 ^{c} ± 4.2	38.0 ^{d} ± 1.7	22.8 ^{b} ± 1.3	17.6 ^{a} ± 2.7
butanoic acid	56.8 ^{a,b} ± 1.3	61.9 ^{c} ± 4.3	39.0 ^{a} ± 5.5	48.3 ^{a,b} ± 5.2	50.5 ^{b} ± 4.8	47.9 ^{a} ± 4.8
3-methylbutanoic acid	331.4 ^{b} ± 0.8	322.9 ^{b} ± 4.5	250 ^{a}.8 ± 6.2	279.5 ^{a} ± 10.8	308.8 ^{b} ± 22.9	260.6 ^{a} ± 18.8
hexanoic acid*	1.5 ^{a} ± 0.1	1.4 ^{a} ± 0.0	1.5 ^{a} ± 0.0	1.4 ^{a} ± 0.0	1.4 ^{a} ± 0.0	1.5 ^{a} ± 0.0
octanoic acid*	1.5 ^{a} ± 0.1	1.4 ^{a} ± 0.2	1.7 ^{a} ± 0.2	1.3 ^{a} ± 0.2	1.5 ^{a} ± 0.1	1.7 ^{a} ± 0.1
decanoic acid	702.6 ^{a,b} ± 19.4	621.4 ^{a} ± 105.6	835.6 ^{b,c} ± 84.4	623.5 ^{a} ± 47.5	786.7 ^{b,c} ± 35.9	854.5 ^{c} ± 23.2
Total Acids*	4.1 ^{a,b} ± 0.1	3.9 ^{a,b} ± 0.3	4.4 ^{b} ± 0.3	3.7 ^{a} ± 0.2	4.0 ^{a,b} ± 0.2	4.3 ^{a,b} ± 0.2

Table S3. Continued.

Compounds	SO_2	SC1	SC1+CSC	SC2	QC1	QC1+CSC
isobutyl acetate	36.6 ^{a,b} ± 12.4	35.1 ^{a,b} ± 4.6	34.7 ^{a,b} ± 10.9	27.0 ^{a} ± 7.1	64.0 ^{c} ± 12.7	58.4 ^{b,c} ± 12.2
ethyl butanoate	158.9 ^{a} ± 21.0	172.7 ^{a} ± 20.5	166.1 ^{a} ± 21.5	166.1 ^{a} ± 21.5	160.1 ^{a} ± 21.4	133.1 ^{a} ± 22.3
isoamyl acetate	1123.8 ^{a} ± 197.9	762.2 ^{a} ± 351.6	867.1 ^{a} ± 130.3	917.3 ^{a} ± 84.8	924.5 ^{a} ± 65.8	922.0 ^{a} ± 114.7
ethyl hexanoate	392.5 ^{a} ± 60.9	373.0 ^{a} ± 8.2	414.0 ^{a} ± 25.0	354.8 ^{a} ± 43.5	409.9 ^{a} ± 26.4	406.8 ^{a} ± 37.7
hexyl acetate	20.4 ^{a} ± 0.4	18.5 ^{a} ± 3.1	14.3 ^{a} ± 5.0	19.7 ^{a} ± 1.6	17.8 ^{a} ± 1.8	15.4 ^{a} ± 1.4
ethyl 2-hydroxy-3-methylbutanoate	4.6 ^{a} ± 0.4	6.9 ^{b,c} ± 1.1	5.1 ^{a,b}± 0.3	7.2 ^{c} ± 0.8	6.1 ^{a,b,c} ± 1.0	5.7 ^{a,b,c} ± 0.4
ethyl octanoate	656.7 ^{a} ± 47.2	641.1 ^{a} ± 36.9	786.7 ^{b} ± 14.3	727.2 ^{b} ± 24.7	788.9 ^{b} ± 11.1	861.6 ^{c} ± 30.6
ethyl 3-hydroxybutanoate	68.5 ^{c} ± 4.7	64.8 ^{b,c} ± 3.7	49.4 ^{a}± 0.9	60.5 ^{b,c} ± 6.4	57.7 ^{b} ± 2.8	49.2 ^{a} ± 2.0
ethyl 2-hydroxy-4-methylvalerate	50.3 ^{b} ± 8.6	92.7 ^{c} ± 0.4	50.5 ^{b} ± 5.6	56.1 ^{b} ± 1.8	18.9 ^{a} ± 2.5	14.0 ^{a} ± 2.1
diethyl malonate	3.4 ^{a} ± 0.9	2.3 ^{a} ± 0.5	3.3 ^{a} ± 1.2	3.2 ^{a} ± 0.8	3.5 ^{a} ± 1.0	4.4 ^{a} ± 1.6
ethyl decanoate	134.4 ^{a} ± 21.2	119.5 ^{a} ± 13.8	130.5 ^{a} ± 19.5	145.1 ^{a} ± 27.2	152.1 ^{a} ± 15.2	153.8 ^{a} ± 24.6
diethyl succinate*	0.5 ^{a} ± 0.0	1.4 ^{b,c} ± 0.4	2.0 ^{d} ± 0.1	0.9 ^{b} ± 0.0	1.0 ^{b} ± 143.3	1.6 ^{c,d} ± 0.4
ethyl-9-decenoate	73.5 ^{a} ± 30.8	48.5 ^{a} ± 3.4	64.3 ^{a} ± 5.2	53.9 ^{a} ± 10.8	51.7 ^{a} ± 3.1	68.9 ^{a} ± 7.6
ethyl 4-hydroxybutanoate*	2.1 ^{a} ± 0.0	1.4 ^{a} ± 0.0	1.9 ^{a} ± 0.1	1.7 ^{a} ± 0.0	2.1 ^{a} ± 0.1	1.7 ^{a} ± 0.3
ethyl lactate	495.5 ^{a} ± 46.0	598.2 ^{a} ± 33.7	473.8 ^{a} ± 32.8	485.6 ^{a} ± 63.8	582.6 ^{a} ± 56.4	496.9 ^{a} ± 45.5
diethyl malate	17.1 ^{a} ± 2.8	16.6 ^{a} ± 3.1	11.9 ^{a} ± 3.2	13.8 ^{a} ± 7.4	12.3 ^{a} ± 1.1	14.0 ^{a} ± 0.9
diethyl glutarate	92.9 ^{b} ± 13.5	111.7 ^{b} ±17.3	87.4 ^{b} ± 8.4	96.6 ^{b} ± 6.0	22.6 ^{a} ± 4.3	21.3 ^{a} ± 2.7
ethyl 2-phenylacetate	39.5 ^{a} ± 17.3	42.3 ^{a} ± 25.1	46.2 ^{a} ± 22.1	23.2 ^{a} ± 7.1	58.5 ^{a} ± 17.3	42.9 ^{a} ± 10.2
ethyl vanillate	223.3 ^{a} ± 41.3	218.5 ^{a} ± 66.9	267.5 ^{a} ± 41.2	226.1 ^{a} ± 29.0	256.5 ^{a} ± 28.4	270.2 ^{a} ± 18.1
Total Esters*	6.2 ^{a,b} ± 2.9	6.1 ^{a} ± 0.5	7.8 ^{b} ± 0.3	6.0 ^{a,b} ± 0.0	6.7 ^{a,b} ± 0.5	6.8 ^{a,b} ± 0.7

Table S3. Continued.

Compounds	SO_2	SC1	SC1+CSC	SC2	QC1	QC1+CSC
1-butanol	35.1 ^{c} ± 2.2	23.9 ^{b} ± 0.1	20.6 ^{b,a} ± 4.7	24.9 ^{b,a} ± 0.7	21.1 ^{b} ± 0.3	17.9 ^{a} ± 0.1
1-pentanol	31.0 ^{a} ± 1.6	34.7 ^{a} ± 0.7	32.0 ^{a} ± 3.2	35.5 ^{a} ± 2.8	33.6 ^{a} ± 3.6	30.1 ^{a} ± 3.2
4-methyl-pentanol	38.4 ^{b} ±3.0	33.9 ^{a,b} ± 4.4	29.8 ^{b} ± 5.0	33.6 ^{a,b} ± 1.2	31.2 ^{a,b} ± 1.5	28.7 ^{b} ± 1.8
3-methyl-pentanol	103.6 ^{b} ± 12.5	81.6 ^{a} ± 6.3	78.7 ^{a} ± 8.7	80.8 ^{a} ± 2.2	84.1 ^{a} ± 4.2	78.3 ^{a} ± 4.7
3-ethoxy-1-propanol	3.7 ^{a} ± 1.1	7.4 ^{a} ± 1.8	5.1 ^{a} ± 1.0	8.1 ^{a} ± 4.6	4.5 ^{a} ± 0.5	4.4 ^{a} ± 0.0
4-methyl-3-penten-1-ol	3.9 ^{a} ± 0.5	4.3 ^{a} ± 1.0	4.3 ^{a} ± 0.7	7.9 ^{a} ± 6.5	3.6 ^{a} ± 0.5	3.9 ^{a} ± 0.7
heptanol	30.0 ^{a} ± 3.8	28.8 ^{a} ± 1.9	25.2 ^{a} ± 0.7	29.6 ^{a} ± 2.2	25.7 ^{a} ± 0.5	25.8 ^{a} ± 0.3
2-mercaptoethanol	6.3 ^{a} ± 1.4	9.7 ^{a,b} ± 2.3	8.9 ^{a,b} ± 1.8	13.0 ^{b} ± 3.6	8.6 ^{a,b} ± 0.9	7.1 ^{a} ± 0.5
2,3-butanodiol	40.0 ^{c} ± 2.3	8.1 ^{b} ± 0.3	3.2 ^{a} ± 1.8	3.1 ^{a} ± 0.4	3.3 ^{a} ± 1.3	1.9 ^{a} ± 0.5
octanol	17.9 ^{a} ± 0.4	19.6 ^{a} ± 0.7	19.4 ^{a} ± 1.1	17.1 ^{a} ± 3.6	20.4 ^{a} ± 1.5	21.5 ^{a} ± 1.0
3-methylthio-propanol	78.8 ^{a} ± 14.0	76.0 ^{a} ± 11.6	79.8 ^{a} ± 3.3	75.6 ^{a} ± 7.7	77.1 ^{a} ± 6.5	68.9 ^{a} ± 7.6
3-octanol	2.7 ^{a} ± 0.5	3.3 ^{a} ± 1.0	2.8 ^{a} ± 0.4	3.7 ^{a} ± 1.2	2.3 ^{a} ± 0.3	2.6 ^{a} ± 0.3
nonanol	9.4 ^{a} ± 1.4	7.9 ^{a} ± 1.4	9.5 ^{a} ± 1.6	9.9 ^{a} ± 5.2	9.8 ^{a} ± 1.9	11.5 ^{a} ± 3.4
2,5-dimethyl-4-heptanol	23.6 ^{a,b} ± 2.1	28.5 ^{b} ± 2.4	22.1 ^{a,b} ± 1.3	19.3 ^{a} ± 3.5	22.8 ^{a,b} ± 1.0	19.8 ^{a} ± 1.3
Total Alcohols	435.6 ^{a} ± 0.3	377.9 ^{b} ± 35.5	353.7 ^{b} ± 18.6	370.3 ^{b} ± 32.8	358.6 ^{b} ± 14.8	346.1 ^{b} ± 23.9
butyrolactone	26.1 ^{a} ± 3.9	30.7 ^{a} ± 0.3	28.7 ^{a} ± 5.4	33.2 ^{a} ± 5.1	24.7 ^{a} ± 3.1	31.1 ^{a} ± 2.2
(E)-β-methyl-γ-octalactone	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	36.6 ^{b} ± 2.2	38.1 ^{b} ± 0.9
(Z)-β-methyl-γ-octalactone	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	308.9 ^{b} ± 12.3	328.7 ^{b} ± 19.6
γ-nonalactone	14.2 ^{a} ± 1.8	10.6 ^{a} ± 5.4	14.6 ^{a} ± 4.7	14.0 ^{a} ±5.0	18.3 ^{a} ± 5.9	22.0 ^{a} ± 3.1
undecalactone	34.3 ^{a} ± 3.5	21.3 ^{a} ± 6.5	22.8 ^{a} ± 4.8	25.7 ^{a} ± 4.7	28.3 ^{a} ± 3.8	29.7 ^{a} ± 4.7
Total Lactones	74.5 ^{a} ± 4.9	62.6 ^{a} ± 11.1	66.1 ^{a} ± 13.4	72.9 ^{a} ± 12.8	416.8 ^{b} ± 23.6	449.5 ^{b} ± 16.1

Table S3. Continued.

Compounds	SO_2	SC1	SC1+CSC	SC2	QC1	QC1+CSC
guaiacol	133.3 ^{a} ± 28.6	140.7 ^{a} ± 11.3	154.0 ^{a} ± 29.4	161.5 ^{a} ± 16.2	165.1 ^{a} ± 17.3	126.7 ^{a} ± 10.8
benzyl alcohol	240.1 ^{a} ± 17.7	378.4 ^{a} ± 47.3	282.4 ^{a} ± 31.6	295.1 ^{a} ± 14.4	281.7 ^{a} ± 48.3	262.2 ^{a} ± 31.0
2-phenylethanol*	18.7 ^{a} ± 2.5	17.1 ^{a} ± 4.0	21.8 ^{a} ± 3.0	16.1 ^{a} ± 3.0	20.8 ^{a} ± 2.0	20.5 ^{a} ± 1.9
eugenol	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	8.2 ^{b} ± 0.7	9.7 ^{c} ± 0.6
vinyl guaiacol	279.2 ^{a} ± 35.5	303.4 ^{a} ± 4.6	277.1 ^{a} ± 84.0	260.8 ^{a} ± 32.9	289.4 ^{a} ± 57.2	252.1 ^{a} ± 33.0
syringol	718.5 ^{a} ± 81.1	748.1 ^{a} ± 69.0	847.8 ^{a} ± 214.9	654.2 ^{a} ± 76.6	820.1 ^{a} ± 52.5	652.4^{a} ± 20.6
4-hidroxybenzene ethanol*	4.7 ^{a} ± 1.9	5.0 ^{a} ± 0.4	4.7 ^{a} ± 1.0	4.1 ^{a} ± 0.9	4.8 ^{a} ± 0.6	4.6 ^{a} ± 0.8
benzoic acid	346.7 ^{a} ± 52.6	512.9 ^{a} ± 59.0	520.0 ^{a} ± 77.5	342.2 ^{a} ± 49.2	494.1^{a} ± 44.1	489.1 ^{a} ± 61.5
benzeneacetic acid	98.8 ^{a} ± 7.8	92.1 ^{a} ± 10.2	91.1 ^{a} ± 0.7	79.6 ^{a} ± 11.1	84.8 ^{a} ± 20.5	71.4 ^{a} ± 6.8
vanillin	12.6 ^{a} ± 5.7	17.4 ^{a} ± 2.9	19.8 ^{a} ± 2.6	16.8 ^{a} ± 2.7	158.8 ^{b} ± 8.6	145.5^{b} ± 14.6
acetovanillone	54.2 ^{a,b} ± 3.7	53.6 ^{a,b} ± 9.0	58.2 ^{b} ± 5.6	54.1 ^{a,b} ± 3.7	44.2 ^{a} ± 1.4	41.7 ^{a} ± 1.8
Total Benzenic compounds*	25.2 ^{a} ± 4.5	24.4 ^{a} ± 4.0	28.6 ^{a} ± 3.7	21.0 ^{a} ± 3.9	27.9 ^{a} ± 2.7	28.8 ^{a} ± 2.7

Values with different superscript in the same row denoted significant differences according to the Student-Newman-Keuls test ($\alpha = 0.05$).

*Values expressed as mg/L.

Artículo 3. Impact of oenological antioxidant substances on the formation of 1-hydroxyethyl radical and phenolic composition in SO_2 free red wines.

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Impact of oenological antioxidant substances on the formation of 1-hydroxyethyl radical and phenolic composition in SO_2 free red wines

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Abstract

BACKGROUND: Different natural substances, chitosan, inactive dry yeasts and freeze-dried aqueous extracts from two wine industry by-products (stems and shoots) were used in red winemaking as possible alternatives to SO_2. The resistance to oxidation of wines was evaluated by electron paramagnetic resonance. The phenolic composition of wines was analyzed by high-performance liquid chromatography-diode array detection/electrospray ionization mass spectrometry, antioxidant activity was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation] assays and spectrophotometric measurements of color were compared.

RESULTS: The wines elaborated with chitosan and inactive dry yeast presented greater capacity to inhibit the formation of the 1-hydroxyethyl radical compared to the wines elaborated with stem or shoot extracts. The total content of anthocyanins was higher in the wines elaborated with SO_2; however, the concentration of flavan-3-ols was higher in the wines with chitosan. In addition, the wines with chitosan and inactive dry yeast presented the highest % polymerization. Wines elaborated with stem extract had a lower concentration of flavonols and stilbenes.

CONCLUSION: Chitosan and inactive dry yeast, which are used as an alternative to SO_2 in winemaking, allow the control of the formation of 1-HER in red wines. Wines with stem and shoot extracts showed a lower resistance to oxidation.

Keywords: red wine; EPR; 1-hydroxyethyl radical; phenolic composition; antioxidant activity

INTRODUCTION

Red wines are a rich source of phenolic compounds that contribute to their sensory characteristics and have important antioxidant properties. Several studies have shown that there is a high correlation between the phenolic composition and the antioxidant activity of wine. The recommended methods to measure antioxidant activity in wines are: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and oxygen radical absorbance capacity (ORAC).[1] On the other hand, electron paramagnetic resonance (EPR) is presented as a technique that allows the direct detection of free radicals in complex systems and allows determination of the level of oxidation in wines.[2] EPR has been used successfully to investigate the effect of several components of wine on the formation or the suppression of free radicals.[3] Recently, several studies have investigated the production of free radicals in white wines by EPR and highlighted the importance of phenolic compounds in the oxidative stability of white wines.[4–6]

The dissolved oxygen in the wine can react with phenolic compounds in the presence of traces of transition metals such as iron or copper, which act as catalysts in the chemical oxidation of wines. These oxidation reactions result in the byproducts of semiquinone and benzoquinone, whereas O_2 is reduced to hydrogen peroxide.[7,8]

Hydrogen peroxide together with ferrous ion (Fe II) generates hydroxyl radicals (˙OH) capable of oxidizing any organic molecule found in wine, known as the Fenton reaction.[9] Among the free radicals produced during the oxidation of wine, 1-hydroxyethyl radical ($CH_3CH{\cdot}OH$), also known as 1-HER, a product of ethanol oxidation

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by the radical $^{\bullet}OH$, is the most abundant and could be the main agent responsible for the oxidation of other organic substances in wine.[2]

When SO_2 is added to wine, it hydrates to sulfurous acid (H_2SO_3), which dissociates in turn to form bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) ions. The antioxidant activity of HSO^{3-} has been attributed to its capacity to react with H_2O_2, thus limiting the oxidation of ethanol by the Fenton reaction.[7] HSO_3^- also performs an important role in the reduction of quinones back to phenol.[9]

As a result of the harmful effects that HSO_3^- can have on health, the oenological sector is looking for natural alternatives that can replace or reduce the dose of SO_2 used in winemaking. Chitosan, inactive dry yeast and different winery by-products have been studied as alternatives to the use of SO_2.[10–12] However, the resistance to oxidation of the red wines fermented with these products is unknown. In this sense, the present study aimed to evaluate the influence of chitosan, inactive dry yeast and two extracts from winery by-products (stem and shoot) on the formation of 1-HER, as well as phenolic composition and color parameters in red wines (*Vitis vinifera* L. Cv. Cabernet Sauvignon).

MATERIALS AND METHODS

Chitosan, inactive dry yeast and natural extracts from winery by-products

The chitosan used was BACTILESS (Lallemand, S.L., Barcelona, Spain), a biopolymer of fungal origin *Aspergillus niger*. The inactivated dry yeast used was PURE-LEES LONGEVITY (Lallemand, S.L.). It is a specific inactivated yeast with a high dissolved oxygen consumption capacity, developed by INRA (Montpellier, France).

Natural extracts from winery by-products were obtained by accelerated solvent extraction (ASE 200; Dionex Corp. Sunnyvale, CA, USA) with subcritical water.[13] Two winery by-products were used: stems and shoots (*Vitis vinifera* L. Cv. Tempranillo), obtained from the Institute of Vine and Wine of Castilla-La Mancha (IVICAM, Tomelloso, Ciudad Real, Spain). The samples were ground using a crusher Stephan UMC5 (Stephan Food Service Equipment GMBH, Hameln, Germany). Six grams of each sample were mixed with 2 g of diatomaceous earth used as dispersing agent in a 22-mL stainless steel cell. The extraction conditions comprised: temperature 120 °C, pressure 1500 psi and two static extraction cycles of 10 min. To avoid possible contamination, the extraction system was rinsed with Milli-Q water (Millpore, Bilerica, MA, USA) between samples. The extracts were freeze-dried under vacuum (1.1×10^{-2} mbar) for 24 h, at a condenser temperature of −53.2 °C. Several extractions were carried out to obtain approximately 50 g of freeze-dried extracts from each by-product. Homogenous freeze-dried extracts were kept in a desiccator until use.

Winemaking

Six types of red wines from grapes *Vitis vinifera* L. Cv. Cabernet Sauvignon were elaborated in triplicate during the 2017 harvest: control wine without SO_2 (CW), wine with SO_2 (50 mgL^{-1}; SO_2W), wine with chitosan (0.2 gL^{-1}; CHW), wine with inactive dry yeast (0.2 gL^{-1}; DYW), wine with stem extract (2 gL^{-1}; STW) and wine with shoot extract (2 gL^{-1}; SHW). The additives were added to the must before alcoholic fermentation. The dose of chitosan and inactive dry yeast were those recommended by the manufacturer. Extracts from winery by-products were added according to the bibliography and based on previous studies for other alternative substances to SO_2.[14,15]

Alcoholic fermentation was performed in 20-L containers at 22 °C using *Saccharomyces cerevisiae* Uvaferm VN (Lallemand, S.L. Barcelona, Spain) as starter culture. The evolution of the fermentation was monitored by measuring the density, and was considered finished when the density value was 0.992 gL^{-1} and the concentration of glucose and fructose was less than 1 gL^{-1} for all samples. After alcoholic fermentation of red wines, pressing was carried out for 30 min and the malolactic fermentation was performed in red wines at 20–22 °C for 10 days using *Oenococcus oeni* VP41 (Lallemand S.L.) as starter culture. The end of the malolactic fermentation was determined by measuring the malic and lactic acid content in wines. Then, the additives were removed before bottling, when the wines were decanted, cold stabilized at −3 °C for 30 days and filtered. Only wines with SO_2 were adjusted to 25 mgL^{-1} of free SO_2 before bottling.

Total phenolic content (TPC) and antioxidant activity

TPC in extracts from winery by-products was determined by the Folin–Ciocalteau assay. An UV-visible spectrophotometer (Helios, Thermo Spectronic, Cambridge, UK) was used to measure the absorbance at 765 nm. The results were expressed as mg of gallic acid equivalents (GAE) g^{-1} dry weight (DW) sample.

The antioxidant activity of the wines was measured by two methods. $ABTS^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)) radical cation decolorization assay,[16] and the DPPH assay, using the 1,1-diphenyl-2-picrylhydrazyl radical as a stable radical.[17] The results were expressed in mmol L^{-1} equivalents of Trolox L^{-1} wine.

EPR spin trapping

The resistance to oxidation of the wines was evaluated 6 months after bottling by EPR in a Bruker model EMX-micro spectrometer (Bruker Instruments, Inc., Billerica, MA, USA) with the cavity corresponding to model ER 4119HS, using α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) as spin trap (1 mol L^{-1}). EPR spectra were measured after inducing the oxidation of the wine by bubbling air (2.5 mL min^{-1}) and exposition of visible light for 6 min. The distance between reactor surface and the visible light lamp was 10 cm. Irradiation was carried out under visible radiation generated by a 400-W metal halide lamp (Powerstar HQI–E400 W/D Pro Daylight; Osram, Munich, Germany) from 400 to 700 nm. The quartz cell (AquaX-bore cell; Bruker) was immediately filled, and all readings were started 3 min later, recording the spectra for 30 min (i.e. the time interval between the different spectra was 5 min, with the accumulation of 10 scans).

The EPR microwave power was set to 2.000 mW, the modulation frequency was 100 kHz and a sweep time of 30 s was used. A sweep width of 100 G centering the field at 3510 G. The receiver gain was set to 30 dB. For the identification of the POBN/1-HER, the constants of hyperfine coupling of the samples were compared with those in the literature[2,18] and quantification was carried out cia Spin Count, a tool in Xenon software (Bruker), as described by Márquez *et al.*[4]

High-performance liquid chromatography (HPLC) analysis of phenolic compounds

The wine samples were diluted with 0.1 Eq. L^{-1} HCl before injection (1:2; v/v) for the analysis of anthocyanins. Flavonols and hydroxycinnamic acids were isolated from wine samples before injection using solid phase extraction (SPE) on PCX cartridges (Bond Elut Plexa, 500 mg of adsorbent, 6 cm^3; Agilent, Waldbronn, Germany), as described by Castillo-Muñoz *et al.* [19]

Next, anthocyanins, flavonols and hydroxycinnamic acids were analyzed in an Agilent 1100 analytical HPLC system (Agilent, Germany)

with a diode array detector (DAD) and a LC/mass selective detector Trap VL electrospray ionization mass spectrometry (ESI-MS) system coupled to an Agilent Chem Station for data processing. A reversed-phase column Ascentis Express C18 (2.1 × 150 mm, 2.7 μm particle size; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used for analysis of anthocyanins and a Zorbax Eclipse XDB-C18 reversed-phase column (2.1 × 150 mm, 3.5 μm particle size; Agilent, Germany) was used for separation of flavonols and hydroxycinnamic acids. Both columns were kept at 40 °C. Quantitation was performed using DAD-chromatograms extracted at 520, 360 and 320 nm for anthocyanins, flavonols and hydroxycinnamic acids, respectively. Their concentrations were expressed as equivalents of malvidin 3-glucoside from Phytolab (Vestenbergsgreuth, Germany), quercetin 3-glucoside from Extrasynthese (Genay, France) and caftaric acid from Extrasynthese (Genay, France), respectively.

The flavan-3-ols and stilbenes were isolated from wine by SPE on C18 cartridges (Sep-Pak Plus, 820 mg of adsorbent; Waters, Saint-Quentin En Yvelines, France). The extracts were analyzed in a HPLC Agilent 1200 series system equipped with DAD (Agilent, Germany) and coupled to an AB Sciex 3200 TRAP (Applied Biosystems, Foster City, CA, USA) with triple quadrupole, turbo spray ionization (electrospray assisted by a thermonebulization) mass spectroscopy system (ESI-MS/MS). The separation was performed in an Ascentis C18 reversed-phase column (150 × 4.6 mm, 2.7 μm particle size; Supelco, Darmstadt, Germany), with the temperature controlled at 16 °C.[20] The compounds were identified and quantified using the following: (+)-catechin, (−)-epicatechin, (+)-catechin gallate and *trans*-resveratrol-glucoside from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); (+)-gallocatechin and (−)-epigallocatechin from Phytolab; and procyanidins B1 and B2 from Extrasynthese.

Color parameters

CIELAB parameters were determined by the International Organisation of Vine and Wine method using a spectrophotometer (Agilent 8453 diode array; Agilent, Santa Clara CA, USA) with a home-made program for spectra treatment. The percentage of polymerization was determined following the method proposed by Boulton.[21]

Statistical analysis

To identify statistically significant differences between the samples, analysis of variance and Student–Newman–Keuls tests were applied to the analytical data. Statistical analysis was performed using SPSS, version 24.0 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

EPR signal identification

Spin trap POBN was used to trap the 1-HER forming a stable adduct (POBN/1-HER) which can be detected by EPR spectroscopy. The EPR spectrum of the adduct POBN/1-HER was characterized by a triplet of doublets with a hyperfine coupling constant for nitrogen (a_N) of 15.56 G and a superhyperin coupling constant for hydrogen (a_H) of 2.54 G, in accordance with the literature.[2,18]

Figure 1 shows that all of the wines produced the 1-HER induced by air bubbling and visible light exposure for 6 min. In this way, it is shown that one of the main intermediaries of the chemical oxidation of wine, mediated by radicals $^{\bullet}OH$, is the 1-HER. Therefore, the reaction between $^{\bullet}OH$ and ethanol is not totally impeded by the endogenous polyphenols of the wine, which are widely accepted antioxidants.[3]

On the other hand, variations in the intensity of the spectra were observed depending on the type of wine. The intensity of the spectra of the 1-HER in the different wines demonstrated the increasing trend: SO_2W, CHW, DYW, SHW, STW and CW. The pre-fermentation addition of natural products in winemaking, as an alternative to SO_2, influenced the subsequent formation of POBN/1-HER adduct. This difference in the intensity of the spectra could also be influenced by the different phenolic composition of the wines and their corresponding antioxidant activity, as described below.

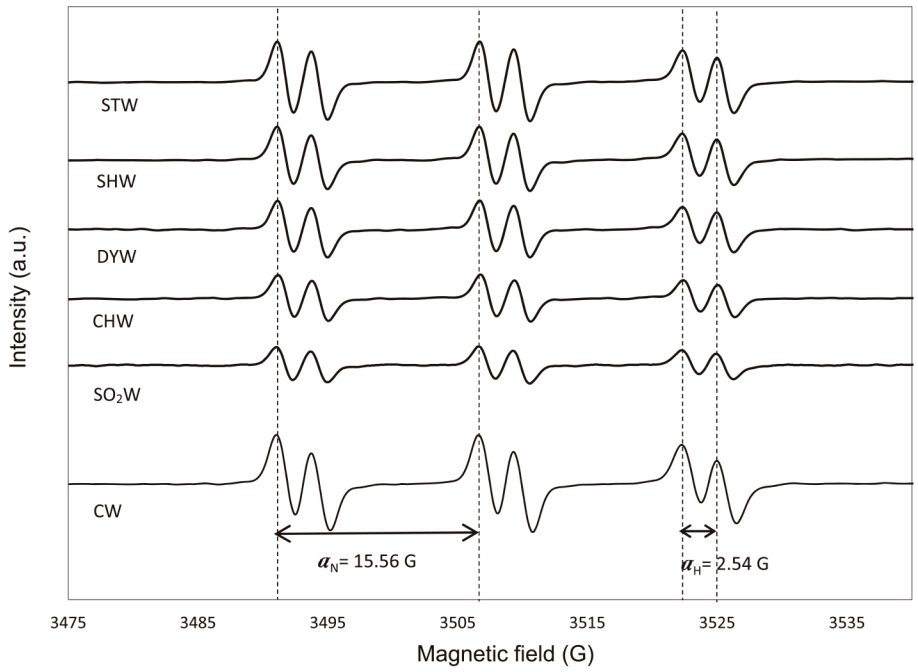

Figure 1. EPR spectrum of POBN/1-HER adducts in different wines after 30 min. CW, control wine without SO_2; SO_2W, wine with SO_2; CHW, wine with chitosan; DYW, wine with inactive dry yeast; SHW, wine with shoot extract; STW, wine with stem extract.

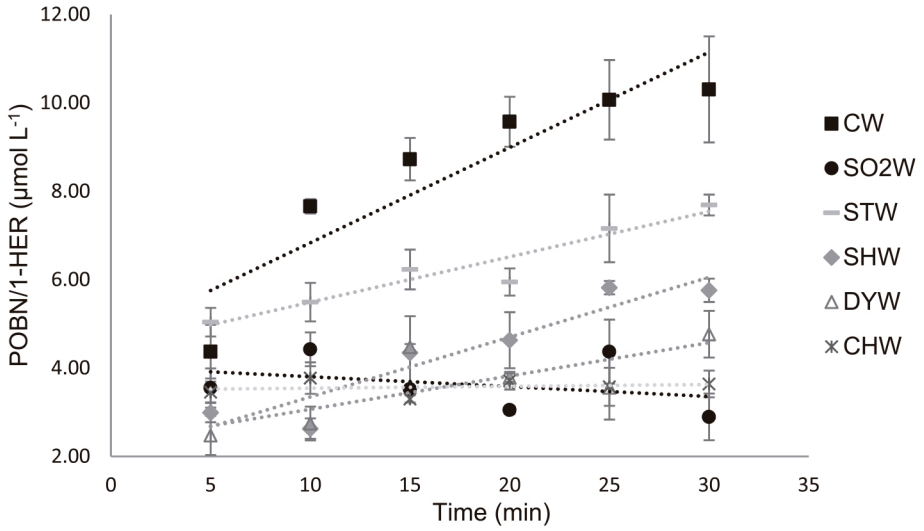

Figure 2. POBN/1-HER adduct formation kinetics in different wines for 30 min. CW, control wine without SO_2; SO_2W, wine with SO_2; CHW, wine with chitosan; DYW, wine with inactive dry yeast; SHW, wine with shoot extract; STW, wine with stem extract.

Evolution of the POBN/1-HER formation

The formation of the adduct POBN/1-HER in the different wines was recorded during 30 min after the air bubbling and exposure to visible light for 6 min (Fig. 2). The wines analyzed showed two different kinetic profiles. In wines elaborated with SO_2 or chitosan, the concentration of POBN/1-HER remained constant throughout the reaction time. In wines elaborated with SO_2, the concentration can be explained by the ability of HSO_3^- to perform as an antioxidant with respect to inhibiting the production of 1-HER, by reducing H_2O_2 to H_2O at the same time as HSO_3^- is oxidized to HSO_4^-.[2,22] Moreover, HSO_3^- can reduce quinones to their respective phenol form, increasing their antioxidant activity.[9] The wines elaborated with chitosan showed a trend of POBN/1-HER concentration similar to wines elaborated with SO_2. Chitosan can act as metal chelant and prevent the Fenton reaction by Fe (II) and/or Fe (III) removal.[23] Castro-Marín *et al.*[18] showed that chitosan added to a commercial white wine without SO_2 reduced the formation of free radicals during the oxidation of wine by its Fe (II) chelating activity.

On the other hand, the formation of POBN/1-HER in CW, DYW, STW and SHW increased over time. The biggest difference in the 1-HER increase of these wines is observed between DYW and CW. The wines elaborated with inactive dry yeast presented a lower slope, with an initial concentration of 1-HER lower compared to the rest of this group of wines. The lower initial concentration of the 1-HER can affect the kinetics of its formation. It is known that the 1-HER formed by the Fenton reaction is capable of reducing Fe (III) to Fe (II) and amplifying the Fenton reaction.[24] In addition, the lower concentration of the 1-HER in wines elaborated with inactive dry yeast may be a result of the Fe (II) chelating effect of the yeasts because these have been used successfully as biosorbents for the elimination of ions of metals,[25] which would justify the lower chemical oxidation of DYW.

1-HER quantification and comparison with antioxidant activity

Figure 3 shows the antioxidant activity values (mmol L^{-1} Trolox L^{-1}) and the concentration of the POBN/1-HER formed at 30 min

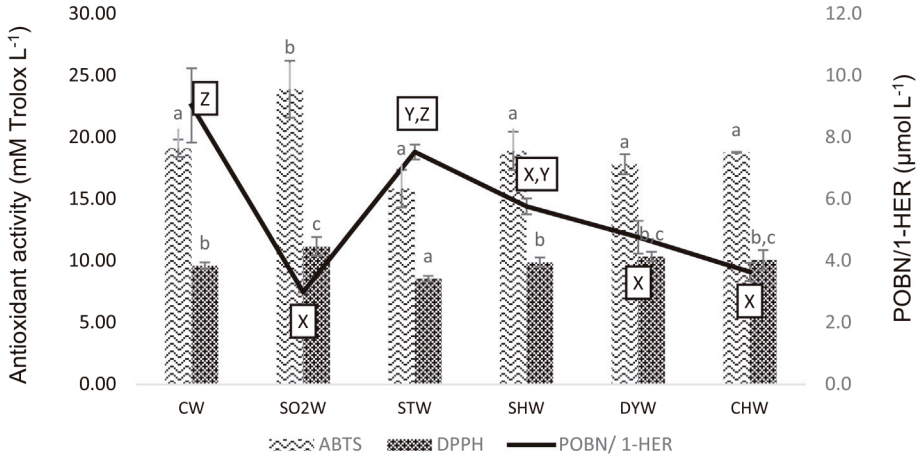

Figure 3. Antioxidant activity and POBN/1-HER adduct formation in different wines. CW, control wine without SO_2; SO_2W, wine with SO_2; CHW, wine with chitosan; DYW, wine with inactive dry yeast; SHW, wine with shoot extract; STW, wine with stem extract. Different letters appearing in the same method for determining antioxidant activity (a, b, c) and 1-hydroxyethyl radical formation (X, Y, Z) indicate significant differences ($\alpha = 0.05$) according to the Student–Newman–Keuls test.

Table 1. Individual and total content (mg L^{-1}) of anthocyanins, flavan-3-ols, flavonols, stilbenes and hydroxycinnamic acids in wines with different pre-fermentative treatments ($n = 3$)

	CW	SO_2W	STW	SHW	DYW	CHW
Delphinidin 3-glc	7.14 ± 0.86 a	12.13 ± 1.55 b	6.67 ± 0.95 a	8.56 ± 1.65 a	9.09 ± 0.22 a	9.25 ± 0.66 a
Petunidin 3-glc	11.96 ± 0.66 a	19.39 ± 3.07 b	11.07 ± 0.33 a	13.36 ± 1.79 a	14.06 ± 0.10 a	14.41 ± 1.04 a
Peonidin 3-glc	5.96 ± 0.02 a	8.06 ± 0.39 b	5.32 ± 0.15 a	6.66 ± 1.01 a	6.70 ± 0.06 a	5.86 ± 0.71 a
Malvidin 3-glc	194.10 ± 5.15 a	252.09 ± 17.50 b	174.36 ± 16.28 a	197.24 ± 3.61 a	195.91 ± 0.17 a	190.46 ± 1.71 a
Delphinidin 3-acglc	3.84 ± 0.30 a	6.91 ± 1.35 b	3.64 ± 0.13 a	4.33 ± 0.65 a	4.57 ± 0.15 a	4.63 ± 0.21 a
Petunidin 3-acglc	6.33 ± 0.40 a	8.60 ± 0.95 b	5.16 ± 0.04 a	6.04 ± 0.79 a	6.39 ± 0.08 a	6.35 ± 0.17 a
Delphinidin 3-cmglc	0.09 ± 0.02 a	0.08 ± 0.02 a	0.08 ± 0.02 a	0.11 ± 0.01 a	0.09 ± 0.02 a	0.08 ± 0.00 a
Peonidin 3-acglc	4.74 ± 0.26 a	7.17 ± 0.63 b	4.46 ± 0.16 a	5.16 ± 0.47 a	5.17 ± 0.02 a	5.00 ± 0.15 a
Malvidin 3-acglc	105.80 ± 1.71 a	148.36 ± 11.62 b	96.01 ± 6.40 a	107.57 ± 6.12 a	109.16 ± 0.62 a	105.42 ± 0.12 a
Malvidin 3-cfglc	0.78 ± 0.01 b	1.20 ± 0.02 d	0.60 ± 0.02 a	0.96 ± 0.03 c	0.86 ± 0.03 c	0.98 ± 0.03 c
Petunidin 3-cmglc	1.13 ± 0.36 a	1.82 ± 0.49 a	1.27 ± 0.03 a	1.56 ± 0.28 a	1.66 ± 0.09 a	1.61 ± 0.00 a
Malvidin 3-cmglc cis	1.19 ± 0.11 a	2.00 ± 0.35 b	1.04 ± 0.06 a	1.19 ± 0.12 a	1.22 ± 0.05 a	1.15 ± 0.03 a
Peonidin 3-cmglc	1.98 ± 0.02 a,b	2.99 ± 0.25 c	1.76 ± 0.06 a	2.01 ± 0.12 a,b	2.17 ± 0.08 b	2.08 ± 0.02 b
Malvidin 3-cmgluc trans	29.06 ± 0.50 a	40.70 ± 2.47 b	25.41 ± 2.20 a	28.53 ± 1.38 a	29.32 ± 1.17 a	29.48 ± 0.66 a
Total anthocyanins	374.11 ± 5.89 a	511.49 ± 38.35 b	336.85 ± 23.77 a	383.27 ± 15.14 a	386.38 ± 0.44 a	376.76 ± 0.39 a
(+)-Catechin	29.13 ± 1.73 a,b	29.10 ± 2.59 a,b	25.10 ± 3.66 a	27.29 ± 0.84 a	30.78 ± 0.63 a,b	33.85 ± 2.74 b
(−)-Epicatechin	12.51 ± 0.81 a	13.84 ± 1.44 a	10.54 ± 1.60 a	12.07 ± 0.54 a	13.11 ± 1.42 a	13.67 ± 2.13 a
(+)-Gallocatechin	17.37 ± 0.98 b,c	16.54 ± 0.72 a,b	14.24 ± 1.06 a	14.77 ± 0.59 a,b	19.45 ± 0.74 c	22.86 ± 2.48 d
(−)-Epigallocatechin	3.61 ± 0.52 b	4.04 ± 0.41 b	2.80 ± 0.22 a	3.01 ± 0 0.13 a	3.80 ± 0.14 b	4.21 ± 0.23 b
(+)-Catechin gallate	0.08 ± 0.01 a	0.08 ± 0.03 a	0.09 ± 0.03 a	0.08 ± 0.02 a	0.08 ± 0.02 a	0.10 ± 0.01 a
Procyanidin B1	25.75 ± 3.01 a,b	26.44 ± 2.61 a,b	22.98 ± 2.79 a	22.92 ± 1.37 a	27.30 ± 0.92 a,b	32.09 ± 4.24 b
Procyanidin B2	0.66 ± 0.03 a,b	0.74 ± 0.11 a,b	0.56 ± 0.07 a	0.61 ± 0.04 a	0.70 ± 0.04 a,b	0.83 ± 0.12 b
Procyanidin B4	2.21 ± 0.11 a	2.30 ± 0.31 a	1.90 ± 0.32 a	2.07 ± 0.15 a	2.34 ± 0.22 a	2.67 ± 0.49 a
Procyanidin (unknown 1)	3.85 ± 0.30 a,b	4.11 ± 0.44 a,b	3.19 ± 0.45 a	3.40 ± 0.01 a	4.01 ± 0.16 a,b	5.00 ± 0.92 b
Procyanidin (unknown 2)	1.64 ± 0.25 a,b	1.63 ± 0.16 a,b	1.40 ± 0.14 a	1.46 ± 0.05 a	1.66 ± 0.11 a,b	2.00 ± 0.34 b
Galloyated dimers	0.60 ± 0.07 a,b	0.56 ± 0.06 a,b	0.51 ± 0.11 a,b	0.49 ± 0.02 a	0.60 ± 0.07 a,b	0.70 ± 0.09 b
Monomers glycosides	29.64 ± 1.88 a,b,c	27.77 ± 3.50 a,b	25.64 ± 3.71 a	27.11 ± 1.77 a,b	32.69 ± 0.18 b,c	35.16 ± 2.96 c
Total flavan-3-ols	127.06 ± 9.20 a,b	127.15 ± 11.72 a,b	108.94 ± 14.06 a	115.28 ± 4.68 a,b	137.22 ± 2.21 b,c	154.19 ± 15.76 c
Myricetin-3-glucuronide	3.73 ± 0.08 b,c	4.02 ± 0.61 c	2.66 ± 0.19 a	3.23 ± 0.16 a,b	3.18 ± 0.22 a,b	3.11 ± 0.36 a,b
Myricetin-3-glucoside	23.71 ± 0.70 b	26.66 ± 1.69 c	19.99 ± 0.25 a	20.41 ± 1.32 a	21.90 ± 1.19 a,b	21.91 ± 0.39 a,b
Quercetin-3-galactoside	1.51 ± 0.05 a,b	1.73 ± 0.26 b	1.23 ± 0.09 a	1.36 ± 0.22 a,b	1.41 ± 0.12 a,b	1.55 ± 0.01 a,b
Quercetin-3-glucuronide	14.11 ± 0.17 b	16.20 ± 0.04 c	11.13 ± 0.89 a	12.81 ± 0.55 a,b	12.49 ± 0.91 a,b	13.68 ± 1.76 b
Quercetin-3-glucoside	9.45 ± 0.05 a	9.80 ± 0.98 a	7.93 ± 0.57 a	8.42 ± 1.17 a	8.86 ± 0.52 a	9.02 ± 0.04 a
Laricitrin-3-glucoside	9.14 ± 0.12 b	10.41 ± 0.78 c	7.03 ± 0.72 a	8.92 ± 0.42 b	8.83 ± 0.71 b	8.17 ± 0.37 b
Kaempferol-3-glucoside	1.21 ± 0.01 b	1.11 ± 0.02 a,b	1.03 ± 0.07 a	0.95 ± 0.14 a	1.04 ± 0.05 a	1.02 ± 0.04 a
Isorhamnetin-3-glucoside	2.53 ± 0.02 a	2.75 ± 0.45 a	2.11 ± 0.09 a	2.46 ± 0.42 a	2.56 ± 0.11 a	2.47 ± 0.00 a
Syringetin-3-glucoside	10.98 ± 0.65 b,c	11.75 ± 0.49 c	8.84 ± 1.13 a	10.36 ± 0.59 a,b,c	9.92 ± 0.24 a,b	9.73 ± 0.32 a,b
Total flavonols	76.37 ± 1.25 c	84.42 ± 4.25 d	61.96 ± 3.29 a	68.91 ± 3,87 b	70.19 ± 0.38 b,c	70.67 ± 3.20 b,c
t-Resveratrol-glucoside *	455.40 ± 9.35 d	420.24 ± 9.78 c	320.69 ± 15.09 a	361.65 ± 12.08 b	459.64 ± 21.61 d	471.17 ± 27.38 d
c-Resveratrol-glucoside *	403.47 ± 15.00 a,b	350.48 ± 4.60 a	346.77 ± 51.34 a	360.28 ± 5.99 a	427.40 ± 1.40 b	441.83 ± 35.95 b
Total stilbenes *	858.86 ± 21.95 c	770.71 ± 5.23 b	667.46 ± 57.30 a	721.94 ± 9.19 b	887.04 ± 23.01 c	913.00 ± 16.81 c
Caftaric acid	52.14 ± 1.32 a	53.92 ± 5.41 a	46.19 ± 2.86 a	47.09 ± 2.25 a	43.90 ± 3.22 a	46.16 ± 8.19 a
Coutaric acid	23.94 ± 1.00 a	24.45 ± 4.16 a	19.42 ± 1.25 a	20.73 ± 0.50 a	18.82 ± 0.69 a	20.71 ± 3.89 a
Fertaric acid	2.78 ± 0.11 a	1.92 ± 0.25 a	2.81 ± 0.37 a	1.90 ± 0.30 a	2.79 ± 0.48 a	2.74 ± 0.49 a
Total hydroxycinnamic acids	78.86 ± 2.16 a	80.28 ± 9.81 a	68.42 ± 4.13 a	69.71 ± 2.46 a	65.51 ± 2.06 a	69.61 ± 11.67 a

CW, control wine without SO_2; SO_2W, wine with SO_2; STW, wine with stem extract; SHW, wine with shoot extract; DYW, wine with inactive dry yeast; CHW, wine with chitosan.
* μg L^{-1}.
Different lowercase letters in the same row indicate significant differences ($\alpha = 0.05$) according to the Student–Newman–Keuls test.

(μmol L^{-1}) in the different types of wines. There were no significant differences in the concentration of POBN/1-HER for wines elaborated with SO_2, inactive dry yeast and chitosan. The pre-fermentative addition of chitosan or inactive dry yeast in winemaking could protect the formation of POBN/1-HER by preserving the antioxidant activity of wine according to the DPPH method. Therefore, higher values of antioxidant activity (i.e. by DPPH) could suggest lower POBN/1-HER concentrations after wine oxidation. In accordance with these results, Chinnici *et al.*[23] reported that the antioxidant activity of chitosan inhibited the oxidation of (+) -

Table 2. Color related parameters in wines with different pre-fermentative treatments ($n = 3$)

	CW	SO_2W	STW	SHW	DYW	CHW
L^*	9.24 ± 1.13 a	16.33 ± 3.07 b	13.13 ± 1.61 a	10.45 ± 0.82 a	9.75 ± 0.02 a	11.00 ± 0.89 a
a^*	40.20 ± 1.45 a	49.00 ± 3.49 c	45.09 ± 1.95 b	41.76 ± 1.05 a,b	40.79 ± 0.02 a,b	42.42 ± 1.17 a,b
b^*	15.89 ± 1.93 a	27.44 ± 4.77 b	22.43 ± 2.70 a	17.95 ± 1.40 a	16.77 ± 0.01 a	18.87 ± 1.51 a
Color intensity	13.83 ± 0.92 c	9.60 ± 1.64 a	10.99 ± 0.39 a,b	12.54 ± 0.73 b,c	13.31 ± 0.25 c	12.15 ± 0.74 b,c
% polymerization	24.09 ± 1.21 b	19.39 ± 0.89 a	23.92 ± 0.38 b	24.19 ± 0.67 b	29.37 ± 0.16 c	28.84 ± 2.35 c

CW, control wine without SO_2; SO_2W, wine with SO_2; STW, wine with stem extract; SHW, wine with shoot extract; DYW, wine with inactive dry yeast; CHW, wine with chitosan.
[a,b,c]Different lowercase letters in the same row indicate significant differences ($\alpha = 0.05$) according to the Student–Newman–Keuls test.

catechin and varietal thiols in model wine, and it was explained that such antioxidant activity could be a result of the capture of transition metals and the consequent suppression of Fenton chemistry. Also, the inactive dry yeast could have a similar effect to preserve the antioxidant activity by elimination of metal ions,[25] as previously noted.

On the other hand, wines with shoot or stem extracts showed lower values of antioxidant activity and higher concentrations of the 1-HER, and no significant differences were observed in the concentration of this radical between control wine without SO_2 and wines with stem extract. Although shoot and stem extracts have a total phenolic content of 14.82 ± 0.30 and 14.34 ± 0.18 mg GAE g^{-1} DW, respectively, they were not effective for the control of 1-HER.

Phenolic compounds and color parameters

Table 1 shows the total and individual content of anthocyanins, flavan-3-ols, flavonols, stilbenes and hydroxycinnamic acids in wines. The phenolic composition of wines is influenced both by the initial concentration of phenolic compounds in the grapes and by the different winemaking techniques used. Therefore, the pre-fermentation addition of any addition can influence the extraction of the phenolic compounds from the grapes and the different condensation and oxidation reactions during fermentation.

Anthocyanins are effective scavengers of reactive oxygen radicals and are considered to be one of the phenolic fractions responsible for the antioxidant activity of red wines.[1,26] The total content of anthocyanins was higher in the wines elaborated with SO_2 because this compound helps their extraction during maceration.[27] The rest of the wines did not present significant differences in total anthocyanins compared to the control wine without SO_2. Individually, the only differences were observed in malvidin 3-cfglc and peonidin 3-cmglc, for which the content was slightly lower in the wines elaborated with stem extract (STW). In addition, wines elaborated with stem extract had a lower concentration of flavonols, and these wines would possibly be the most susceptible to oxidation.

Another group of phenolic compounds related to the inhibition of oxygen radicals and the antioxidant activity in red wines is the flavan-3-ols.[1,28] The total flavan-3-ols content was higher in wines elaborated with chitosan (CHW), although only the (+)-gallocatechin showed significant differences with respect to the rest of the wines. The content of flavan-3-ols did not change in wines with inactive dry yeast, shoot or stem extracts (DYW, SHW or STW) with respect to the control wines, with and without SO_2 (CW and SO_2W).

Wine with chitosan, wine with inactive dry yeast and control wine without SO_2 (CHW, DYW and CW) had higher concentrations of stilbenes. The wines with shoot extract (SHW) had contents similar to SO_2 wines (SO_2W), whereas the wines with stem extract (STW) presented lower amounts of stilbenes. The difference in the concentration of stilbenes in control wines with and without SO_2 was in agreement with the findings of Kostadinovic *et al.*,[29] with the the highest concentration of stilbenes being obtained in the wines with the lowest concentration of SO_2.

Color parameters of wines treated with SO_2 and the antioxidant products tested are shown in Table 2. SO_2 wines had significantly higher luminosity values (L^*) and lower color intensity) than wines without SO_2. In this sense, SO_2 bleach free anthocyanins by nucleophilic addition to the C4 carbon in the C ring of the flavylium cation, reducing the color of the red wines.[30] SO_2 free wines had significantly lower a^* and b^* values. Therefore, the effect of SO_2 on the color of red wines due to anthocyanin discoloration was greater than its effect increasing the extraction of anthocyanins. SO_2 free wines showed a higher color intensity and more violets tonalities, although they had lower contents of anthocyanins. These results are in good agreement with the findings of Marchante *et al.*[11] with respect to using grape seed extracts and oak wood extracts as alternatives to SO_2.

Phenolic compounds can participate directly in the color stability of red wine forming polymeric pigments. These increase the wine color stability because they are more stable than monomeric anthocyanins.[31] In the present study, the wines with chitosan and inactive dry yeast (CHW and DYW) presented the highest percentage polymerization and it is well known the antioxidant activity increases with the degree of polymerization of wine.[1,32]

CONCLUSIONS

All wines analyzed produced 1-HER induced by air bubbling and exposure to visible light during 6 min, with variations being observed with respect to the intensity of the spectra according to the pre-fermentative treatment applied. The lowest concentration of the 1-HER was found in wines elaborated with SO_2, chitosan and inactive dry yeast. The wines with stem and shoot extracts showed lower resistance to oxidation. The pre-fermentative addition of chitosan or inactive dry yeast in winemaking could protect the formation of 1-HER by preserving the antioxidant activity of wine according to the DPPH method. The total content of anthocyanins was higher in SO_2 wines; however, the flavan-3-ols concentration was higher in the wines with chitosan. In addition, wines with chitosan and inactive dry yeast presented the highest % polymerization. Wines elaborated with stem extract had a lower concentration of flavonols and stilbenes. In conclusion, chitosan and inactive dry yeast could be considered

as potential natural alternatives for reducing SO_2 in red winemaking because this allowed the elaboration of red wines with a phenolic potential capable of controlling the formation of 1-HER.

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CONFLICT OF INTERESTS

The authors declare that have no conflicts of interest.

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Artículo 4. Potential of different natural antioxidant substances to inhibit the 1-hydroxyethyl radical in SO_2-Free wines.

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Potential of Different Natural Antioxidant Substances to Inhibit the 1-Hydroxyethyl Radical in SO_2-Free Wines

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ABSTRACT: The potential of different natural antioxidants to inhibit the 1-hydroxyethyl radical formation in SO_2-free wines was analyzed by electron paramagnetic resonance (EPR). Chitosan, glutathione, inactive dry yeast, oak and grape seed extracts, and ascorbic acid were tested in white and red wines. The ability of these substances to prevent the formation of acetaldehyde after the Fenton reaction and the oxygen consumption capacity were measured. Ascorbic acid was the antioxidant substance that offered higher percentages of 1-hydroxyethyl radical inhibition at 30 min of reaction. However, wines with ascorbic acid showed higher concentrations of acetaldehyde after the Fenton reaction. Grape seed extract and chitosan provided higher percentages of radical inhibition in red wine than those in white wine, in contrast to the inactive dry yeast that only produced radical inhibition in white wine. Although oak extract did not produce changes in the 1-hydroxyethyl radical, wines with that extract had lower concentrations of acetaldehyde.

KEYWORDS: *SO_2-free wines, natural antioxidants, EPR, Fenton reaction, 1-hydroxyethyl radical*

INTRODUCTION

The production of wines without SO_2 is a complex challenge for the enological industry because the free radicals produced in the oxidation reactions of the wine can cause significant deterioration according to the production of oxidizes substrates. Electron paramagnetic resonance (EPR) is a widely used technique that allows the detection of species with unpaired electrons through the use of spin traps, which are diamagnetic compounds capable of yielding long-lived radical products upon reaction with free radicals.[1] Accordingly, this technique makes it possible the direct detection of free radicals in complex systems and allows us to determine the level of oxidation in wines.[2]

The oxidation reactions in the wine begin with the activation of oxygen in the presence of transition metals, forming a hydroperoxyl radical that subsequently oxidizes the phenolic compounds, generating *o*-quinones and hydrogen peroxide. Trace transition metals, particularly iron and copper, have a fundamental role in wine oxidation, notably because they catalyze the reaction of hydrogen peroxide to $^{\bullet}OH$ by a Fenton-type reaction, being then redox-cycled by those polyphenols.[3] The $^{\bullet}OH$ radical is a very potent oxidant that can abstract a hydrogen atom from other compounds such as ethanol, tartaric acid, glycerol, and phenolic compounds, which after oxidation produces 1-hydroxyethyl radical, acetaldehyde, glyoxylic acid, glyceraldehyde, and quinones, among other oxidation products.[2,4−6]

The 1-hydroxyethyl radical resulting from the oxidation of ethanol by the $^{\bullet}OH$ radical is the most abundant radical and could be the main agent responsible for the oxidation of other organic substances in wine.[2] The different compounds that constitute the matrix of the wine and its storage conditions influence the production and destination of the 1-hydroxyethyl radical. In the presence of oxygen, the 1-hydroxyethyl radical forms 1-hydroxyethyl peroxyl, an intermediate radical that decomposes to produce acetaldehyde and a hydroperoxyl radical.[2] Another reaction route of the 1-hydroxyethyl radical is its reduction back to ethanol. Kreitman et al.[7] proposed that the formation of disulfide can occur by reducing the 1-hydroxyethyl radical back to ethanol while simultaneously forming a thiyl radical followed by dimerization. Also, Gislason et al.[8] showed that the cinnamates and the hydroxycinnamates equally inhibit the formation of oxidation products in wine exposed to the Fenton reaction (catalytic Fe(II) with hydrogen peroxide) and proposed a mechanism for the radical oxidation of cinnamic acids to the allylic alcohol via the 1-hydroxyethyl radical.

Recently, Nikolantonaki et al.[9] demonstrated that ellagitannins, provided by oak wood during wine aging, intervene in the oxidative stability of wine since they have an important effect on the protection of the 1-hydroxyethyl radical, with strong interactions with the wine matrix. On the other hand, Castro-Marín et al.[10] observed that chitosan, due to its Fe(II) chelating activity, inhibits the formation of the 1-hydroxyethyl radical in white wine. Nevertheless, the use of natural

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substances to inhibit the Fenton-type oxidation reaction remains marginal.

Since one of the main challenges in the enological field is to find alternatives of SO_2 for its replacement or limitation, the main objective of this work was to study the reaction between the 1-hydroxyethyl radical and different natural substances with potential antioxidant capacity by electron paramagnetic resonance (EPR) in red and white wine. Ascorbic acid, chitosan, glutathione, inactive dry yeast, and freeze-dried aqueous oak and grape seed extracts were tested. In addition, the ability of these potential antioxidants to prevent the formation of acetaldehyde was measured, as well as their oxygen consumption capacity.

MATERIAL AND METHODS

Natural Antioxidant Substances. Chitosan Bactiless (a natural nonallergenic biopolymer of fungal origin *Aspergillus niger*), inactivated dry yeast PURE-LEES LONGEVITY (a yeast developed to help the wine resist oxidation during storage in the cellar and aging), *Saccharomyces cerevisiae* Uvaferm VNn and *Oenococcus oeni* VP41 were acquired from Lallemand, S.L. (Switzerland). Ascorbic acid and glutathione were purchased from Sigma-Aldrich (France).

Two aqueous extracts from winery byproducts were obtained by accelerated solvent extraction (ASE 200, Dionex Corp., Sunnyvale, CA) with subcritical water.[11] The wine industry byproducts used were grape seeds (*Vitis vinifera* L. Cv. Tempranillo) from the waste of wineries and American oak wood (*Quercus alba*) from the waste of cooperages. These samples were grounded using a Stephan UMC5 crusher (Stephan Food Service Equipment GMBH). Seeds (8 g) or 4 g of oak wood was mixed with 2 g of diatomaceous earth (used as a dispersing agent to reduce the dead volume) in a 22 mL stainless steel cell. The extraction conditions used were 120 °C, 1500 psi, and two static extraction cycles of 10 min. To prevent the possible contamination, the extraction system was rinsed between samples. The extracts were freeze-dried under vacuum (1.1×10^{-2} mbar) at a condenser temperature of −53.2 °C for 24 h. These freeze-dried extracts were preserved in a desiccator until their use. These extracts were chemically characterized previously.[11]

Wine Samples. Two wine samples from grapes *V. vinifera* L. Cv. Cabernet Sauvignon and *V. vinifera* L. Cv. Sauvignon Blanc were made without SO_2 during the 2018 harvest. Alcoholic fermentations were performed in 50 L of Sauvignon Blanc must at 17 °C for 11 days, and in 100 kg of Cabernet Sauvignon grapes at 22 °C for 11 days, using *S. cerevisiae* Uvaferm VN as a starter culture in both. The evolution of the fermentation was controlled by measuring the density, and it was considered finished when the density value was 0.992 g/L; at the same time, the glucose and fructose concentration was determined. After alcoholic fermentation of red wines, pressing was carried out for 30 min and the malolactic fermentation was performed in red wines at 20−22 °C for 10 days using *O. oeni* VP41 as a starter culture. The end of the malolactic fermentation was determined by measuring the malic and lactic acid contents in wines. Finally, white and red wines were decanted, cold-stabilized at −3 °C for 30 days, filtered, and bottled.

Conventional enological parameters such as alcohol, glucose and fructose, total acidity, total and free SO_2, and pH were determined using the official analytical methods established by the International Vine and Wine Organization.[12] The total phenolic content (TPC) in wines was determined by the Folin-Ciocalteau assay. A UV−vis spectrophotometer (Agilent 8453 diode array, Agilent, Santa Clara, CA) was used to measure the absorbance at 765 nm. Results were expressed as mg of gallic acid equivalent per liter (mg GAE/L).

Fenton Reaction. The Fenton reaction was carried out as previously described by Elias and Waterhouse,[13] with some modifications. In a glass reactor (25 mL amber scintillation vial, sealed with a rubber septum) were added 2022.6 μL of wine, 300 μL of 98% EtOH (EtOH was added only in the sample called control Fenton reagents (CFR)), 100 μL of 0.8 mmol/L H_2O_2, 37.8 μL of 100 mmol/L POBN (α-(4-pyridyl-1-oxide)-*N*-tertbutylnitrone, a diamagnetic compound capable of yielding long-lived radical products upon reaction with free radicals) as a spin trap and 40 μL of 0.08 mmol/L Fe(II). Decarbonized and deoxygenated water used for the solutions and reactions was acidified to pH 3.4 with HNO_3, subsequently boiled for 10 min, and then deoxygenated with argon for another 15 min.

The natural antioxidant substances were studied at concentrations 0.5 and 2.0 g/L, in both wines. The identifier codes for each sample tested were the following: CW (control wine), SEW (wine with grape seed extract), OEW (wine with oak wood extract), CHW (wine with chitosan), GSW (wine with glutathione), DYW (wine with inactive dry yeast) and ASW (wine with ascorbic acid).

EPR Spin Trapping. The 1-hydroxyethyl radical was analyzed by EPR using a Bruker EMXmicro spectrometer with the cavity corresponding to model ER 4119HS and a quartz cell (AquaX-bore cell). EPR spectra were measured after initiating the Fenton reaction 3 min later and recording the spectra for 30 min (the time interval between the different spectra was 5 min, with accumulation of 10 scans). The instrumental conditions were as follows: center field, 3510 G; sweep width, 100 G; microwave power, 2000 mW; modulation frequency, 100 kHz; sweep time, 30 s; and receiver gain, 1000. For the identification of the 1-hydroxyethyl radical adduct, the constants of hyperfine coupling of the samples were compared with those in the literature and the quantification was carried out through Spin Count, a tool of the Xenon software program (Bruker, Germany), according to Márquez et al.[6] The capacity of different natural antioxidant substances tested to inhibit the 1-hydroxyethyl radical, in red and white wines, was expressed as % 1-hydroxyethyl radical inhibition of their respective control wines.

Acetaldehyde Analysis. Acetaldehyde concentration was enzymatically analyzed according to McCloskey et al.,[14] in a Y15 enzymatic autoanalyzer (Biosystems, Barcelona, Spain) after performing the Fenton reaction, as described above, without POBN addition. Measurements were performed at 30 min in triplicate.

Oxygen Consumption Capacity. The oxygen consumption capacity, of the natural antioxidant substances, was analyzed according to Ferreira et al.,[15] with some modifications. The wines were saturated bubbling air for 4 min, and subsequently, each antioxidant substance was added (2 g/L), followed by shaking it for 1 min for its dissolution. The dissolved oxygen was measured, initially (when the antioxidant substance was added) and after 60 min, with a Seven2Go pro meter (Mettler Toledo, Zurich, Switzerland) equipped with a DO probe. Oxygen molecules create a decrease in the fluorescence lifetime, which is proportional to their concentration. An infrared sensor located in the reader pen of the fluorimeter allows the simultaneous measurement of the sample temperature (all analyses were performed at room temperature of 20 ± 2 °C). The instrument is managed by specific software (Mettler Toledo, Zurich, Switzerland) that facilitates the immediate measurement of oxygen concentration in mg/L. The results were expressed as mg/L oxygen consumption per hour.

Statistical Analysis. To study the effect of the wine type (red or white wine) and the concentrations tested (0.5 and 2 g/L) for each natural antioxidant substance on 1-hydroxyethyl radical inhibition, a factor analysis was performed. *p*-Values less than 0.05 indicate that the factors defined by wine type, concentration of the substances tested, and the interaction between them significantly influence the 1-hydroxyethyl radical inhibition.

Analysis of variance (ANOVA) and the Student−Newman−Keuls test were applied to the analytical data of acetaldehyde and oxygen consumption capacity to identify statistically significant differences between samples. Statistical analysis was conducted using SPSS software, version 24.0 (IBM Corp, NY).[16] Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

EPR Analysis. Figure 1 illustrates the adducts formed between the POBN and 1-hydroxyethyl radical (POBN/1-hydroxyethyl radical) after the Fenton reaction in control

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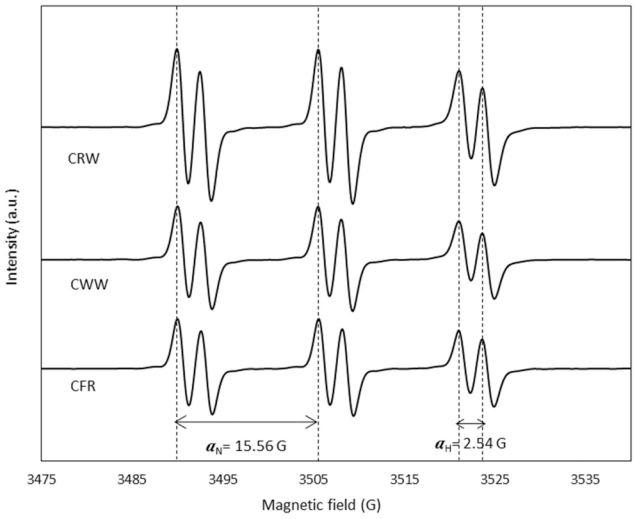

Figure 1. EPR spectrum of POBN/1-hydroxyethyl radical adducts after 30 min. CRW, control red wine without SO_2; CWW, control white wine without SO_2; and CFR, control Fenton reagents.

white and red wines for 30 min, as well as the spectrum of the Fenton reagent control. EPR spectra of the POBN/1-hydroxyethyl radical adducts were characterized by a doublet−triplet with a hyperfine coupling constant for nitrogen (a_N) of 15.56 G and a hyperfine coupling constant for hydrogen (a_H) of 2.54 G, according to the literature.[2,10] A higher intensity of the spectra in the control red wine was observed, which may be due to the higher alcohol content and phenolic compounds in red wine (Table 1), since phenolic compounds can regenerate Fe(II) from Fe(III) in the presence of oxygen,[17] thus increasing the oxidation of ethanol.

Table 1. Enological Parameters in Initial Red and White Wines[a]

	red wine	white wine
alcohol (% v/v)	13.76 ± 0.00	12.93 ± 0.07
total acidity (g tartaric acid/L)	4.97 ± 0.03	5.44 ± 0.02
pH	3.68 ± 0.01	3.28 ± 0.02
glucose and fructose (g/L)	0.31 ± 0.00	0.18 ± 0.02
free SO_2	N.D.	N.D.
total SO_2	N.D.	N.D.
total polyphenols (mg GAE/L)	1956.50 ± 26.59	185.28 ± 0.16
acetaldehyde (mg/L)	4.50 ± 0.71	8.00 ± 0.00

[a]N.D.: not detected (detection limit, 5 mg/L).

The effect of the different antioxidant substances added to SO_2-free wines on Fenton reaction inhibition in the wines for 30 min is shown in Figure 2 and Table 2. In this study, oak extract did not exert an inhibitory effect for the 1-hydroxyethyl radical in the two concentrations tested, neither in red wine nor in white wine (data not shown). Although this extract has demonstrated antioxidant activity for 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, as reported by Alañón et al.[18] While grape seed extract had an inhibitory effect on the 1-hydroxyethyl radical, there were significant differences with respect to the wine type and the concentrations of antioxidant substances tested. The inhibition by grape seed extract was greater in red wines at a concentration of 2 g/L. In addition, the wine type and extract concentration interaction had a significant effect on the % inhibition of the radical since the differences in % inhibition between red and white wines were not the same in the two concentrations tested.

Chitosan also had an inhibitory effect on the 1-hydroxyethyl radical formed by the Fenton reaction in the wines for 30 min. The Fe(II)-chelating activity of chitosan has recently been demonstrated in white wine without SO_2.[10] The addition of chitosan had significant differences in the % 1-hydroxyethyl radical inhibition both in wine type and the concentrations tested. The % inhibition of chitosan was higher in red wines in the two concentrations tested (Table 2). Then, some differential components of red wine against white wine, and some synergistic effect between the red wine matrix and chitosan, could explain this effect that should be studied more thoroughly.

In contrast to this effect, the inactive dry yeast only produced radical inhibition in white wine. The antioxidant activity of inactive dry yeast to the 1-hydroxyethyl radical in white wine may be due to its oxygen consumption capacity (as will be discussed later), thereby decreasing the Fenton reaction. The % inhibition of inactive dry yeast in white wine did not show significant differences with respect to the concentration tested.

Other natural products known for their antioxidant properties are glutathione and ascorbic acid. Glutathione can react with hydrogen peroxide and carbonyl compounds,[19] and it is known to scavenge *o*-quinone compounds efficiently in wine/juice conditions[20,21] so that glutathione could suggest efficient management of the oxidation mechanism. In this study, the effect of glutathione on the 1-hydroxyethyl radical inhibition was more noticeable at the concentration of 2 g/L, and there were no differences in the type of wine.

Figure 2 also highlights the high percentage of inhibition of the ascorbic acid in the two concentrations studied, both in white and red wines at 30 min of the Fenton reaction. It is well known that, in wine, ascorbic acid has the capacity to scavenge molecular oxygen before the oxidation of phenolic compounds. Furthermore, if the oxidation of phenolic compounds occurs, then ascorbic acid is also suggested to readily reduce the oxidized products back to the original phenolic state.[22] In line with our results, other authors have studied the interaction of ascorbic acid with the 1-hydroxyethyl radical in model cellular systems by EPR using phenyl-*N*-*t*-butylnitrone (PBN) as a spin trap.[23] These authors reported that ascorbic acid inhibited the PBN/HER signal. However, the inhibition of the PBN/HER adduct by ascorbic acid was associated with the formation of the semidehydroascorbyl radical, so they showed that the ability of ascorbic acid to reduce the PBN/HER adduct to EPR-silent product(s) might mask the quantitative detection of the 1-hydroxyethyl radical.

Formation of Acetaldehyde after the Fenton Reaction. The chemical oxidation of wine involves a series of redox reactions, where acetaldehyde appears as a secondary product of the oxidation of ethanol via the Fenton reaction.[17] Figure 3 shows the concentration of acetaldehyde after the Fenton reaction in white and red wines treated with the different antioxidant substances tested. The wines with the oak extract were the ones that presented less acetaldehyde. In a parallel experiment, without Fenton reagents, the combinations between acetaldehyde and oak extract were revealed. A decrease of acetaldehyde concentration after the addition of oak extracts of 62.5 and 90.0% in white and red wines, respectively, was observed. Furthermore, less concentration of

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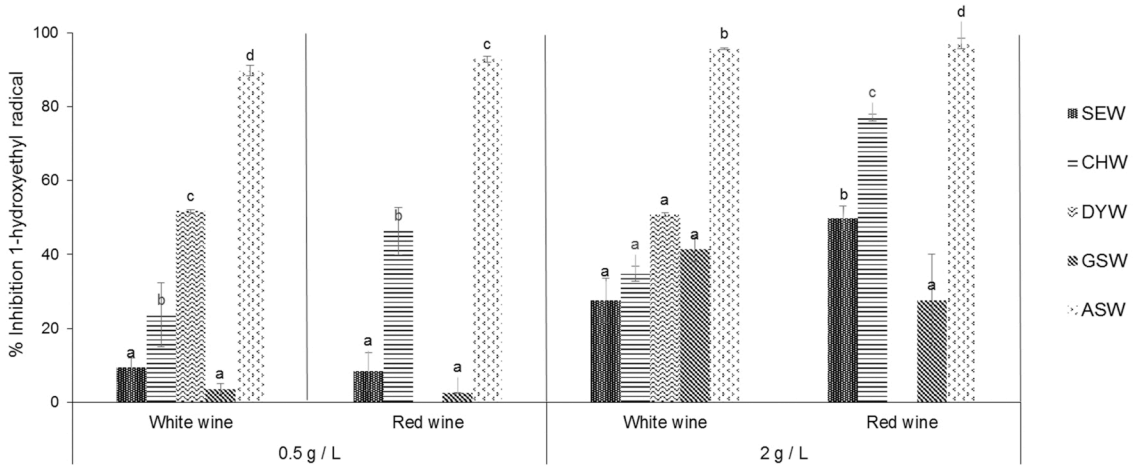

Figure 2. % Inhibition of 1-hydroxyethyl radical by different natural antioxidant substances at the concentrations tested (0.5 and 2 g/L) in red and white wines at 30 min. SEW (wine with grape seed extract), CHW (wine with chitosan), DYW (wine with inactive dry yeast), GSW (wine with glutathione), and ASW (wine with ascorbic acid). Different superscripts (a, b, c, and d) in the same group (wine type and concentration) mean significant differences (α = 0.05) according to the test of Student−Newman−Keuls.

Table 2. Degree of Statistical Significance in the Factor Analysis for Each Antioxidant Substance Added to SO_2-Free Wines to Inhibit the Fenton Reaction for 30 min[a]

factors	SEW	CHW	DYW[b]	GSW	ASW
type of wine	0.029	0.001		0.179	0.041
additive concentration	0.001	0.006	0.191	0.002	0.003
interaction factors	0.021	0.063		0.241	0.302

[a]The p-value (<0.05) indicated that the factors defined by wine type, concentrations of the natural products tested, and the interaction between them significantly influence the 1-hydroxyethyl radical inhibition. The identifier codes for each sample tested are the following: CW (control wine), SEW (wine with grape seed extract), OEW (wine with oak wood extract), CHW (wine with chitosan), GSW (wine with glutathione), DYW (wine with inactive dry yeast), and ASW (wine with ascorbic acid). [b]Only DYW showed 1-hydroxyethyl radical inhibition in white wine.

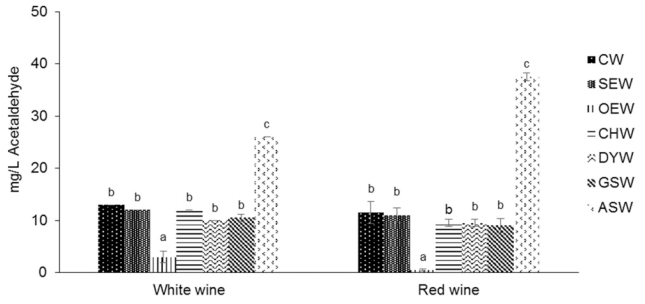

Figure 3. Acetaldehyde concentration in white and red wines with different natural antioxidant substances after the Fenton reaction. CW (control wine), SEW (wine with grape seed extract), OEW (wine with oak wood extract), CHW (wine with chitosan), GSW (wine with glutathione), DYW (wine with inactive dry yeast), and ASW (wine with ascorbic acid). Different superscripts (a, b, and c) in the same type of wine mean significant differences (α = 0.05) according to the test of Student−Newman−Keuls.

acetaldehyde was observed in red wine than that in white wine. This difference may be influenced by the condensation reaction of tannin/anthocyanin through acetaldehyde in red wine. This effect is already observed in the initial wine, where less concentration of acetaldehyde was detected in red wine (Table 1). It is also important to mention that the addition of the oak extract to wine, both red and white, had no effect on the 1-hydroxyethyl radical inhibition for 30 min, compared with that on their respective control wines (data not shown).

On the other hand, the formation of acetaldehyde after the Fenton reaction in wines treated with grape seed extract, chitosan, glutathione, and inactive dry yeast was statistically the same as their respective control wines.

The opposite effect was observed with ascorbic acid. The treatment with ascorbic acid increased the formation of acetaldehyde in red and white wines after the Fenton reaction, although a greater increase was observed in red wine. It is known that phenols can regenerate Fe(II) from Fe(III) in the presence of oxygen; Fe(II) catalyzes the oxidation reaction, and therefore, a higher concentration of acetaldehyde was formed in red wine.

Due to the high concentration of acetaldehyde in wines with ascorbic acid, it was decided to study the kinetics of % inhibition of the 1-hydroxyethyl radical by different antioxidant substances. Figure 4 shows a pro-oxidant effect in white wine with 0.5 g/L ascorbic acid at 5 min of reaction. The other antioxidant substances showed an inhibitory effect on the radical from the beginning of the reaction, keeping constant kinetics for 30 min. This pro-oxidant effect at the beginning of the reaction in wines with ascorbic acid reveals that the percentage of inhibition for the 1-hydroxyethyl radical at 30 min of reaction could be due to an oxidative effect of the radical, instead of a protective effect. Since this decrease of the radical may be due to the oxidation of the 1-hydroxyethyl radical, it thereby increases the concentration of acetaldehyde.

From a mechanistic viewpoint, the action of ascorbic acid on the 1-hydroxyethyl radical and the production of acetaldehyde, in SO_2-free wine, could be explained through the following pathways, as shown in Figure 5. On the one hand, ascorbic acid can inhibit the 1-hydroxyethyl radical by reduction to ethanol, forming the radical semidehydroascorbyl (reaction a).[23] In addition, ascorbic acid can also recycle quinones back to phenols (reaction b),[17] being available to reduce the 1-hydroxyethyl radical (reaction c). However, ascorbic acid can also increase the formation of acetaldehyde by oxidizing the 1-hydroxyethyl radical. Ascorbic acid recycles quinones back to phenols (reaction b) that, in contact with oxygen, are oxidized,

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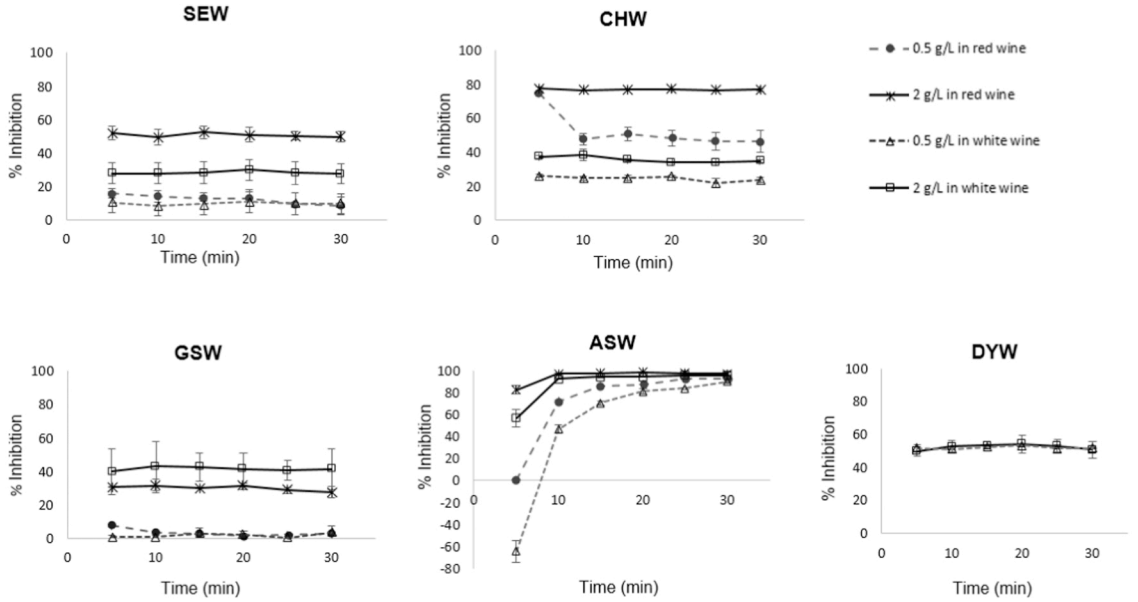

Figure 4. Kinetics of % inhibition of the 1-hydroxyethyl radical by different natural antioxidant substances at the concentrations tested (0.5 and 2 g/L) in red and white wines for 30 min. SEW (wine with grape seed extract), CHW (wine with chitosan), GSW (wine with glutathione), and DYW (wine with inactive dry yeast. * Inactive dry yeast showed 1-hydroxyethyl radical inhibition in only white wine and ASW (wine with ascorbic acid).

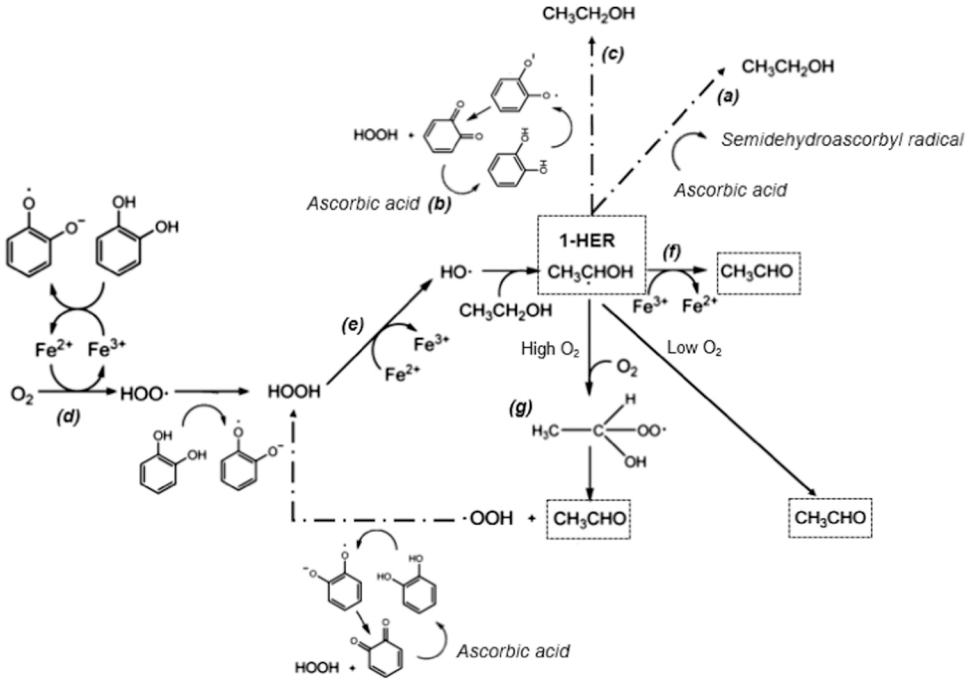

Figure 5. Scheme proposed to explain the possible pathways of the ascorbic acid for the inhibition of 1-hydroxyethyl radical and the formation of acetaldehyde. Adapted from Elias and Waterhouse.[13]

producing •OH and Fe(III) (reactions d and e). At a higher concentration of Fe(III) in the medium, the formation of acetaldehyde increases because Fe(III) catalyzes the 1-hydroxyethyl radical oxidation to acetaldehyde (reaction f). In addition, with high O_2 (reaction g), •OOH is produced, which can be reduced to H_2O_2, increasing the Fenton reaction (reaction e).

Also, ascorbic acid may act as an antioxidant by disrupting the chain propagation reactions, replacing highly reactive radicals with the monodehydroascorbate radical of lower reactivity. However, under some conditions, ascorbic acid may show pro-oxidant activity, mainly when sulfur dioxide is not present.[24] The difference between ascorbic acid effects acting as an antioxidant or a pro-oxidant, in the absence of sulfur dioxide, is related to the relative concentrations of ascorbic acid and catalytically active metal ions. Buettner and Jurkiewicz[25] proposed the idea of a cross effect, whereby ascorbic acid acts as an antioxidant at higher concentrations in relation to metal ions, while a pro-oxidant effect is observed at lower concentrations of ascorbic acid.

Oxygen Consumption Capacity of the Natural Antioxidant Substances. Figure 6 shows the oxygen

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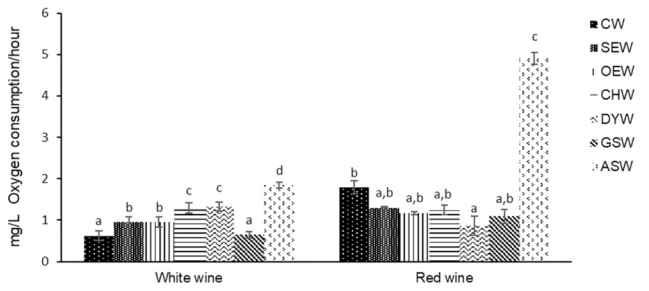

Figure 6. Oxygen consumption capacity (mg/L) per hour of different natural antioxidant substances in white and red wines. CW (control wine), SEW (wine with grape seed extract), OEW (wine with oak wood extract), CHW (wine with chitosan), GSW (wine with glutathione), DYW (wine with inactive dry yeast), and ASW (wine with ascorbic acid). Different superscripts (a, b, and c) in the same type of wine mean significant differences ($\alpha = 0.05$) according to the test of Student–Newman–Keuls.

consumption (mg/L) per hour of the antioxidant substances tested in white and red wines. Ascorbic acid was the natural substance tested with the highest oxygen consumption capacity, as reported in the literature on model wine,[26,27] highlighting the high percentage of oxygen consumed in red wine. The higher oxygen consumption in red wine compared to that in white wine is due to their different contents of phenolic compounds (Table 1) since phenols are the main substrates for oxygen in wines and the capacity of the wine to consume oxygen is related to its phenolic concentration.[28] Figure 6 also indicates that chitosan and inactive dry yeast were effective oxygen consumers in white wine. In contrast, glutathione was the least efficient; this weak effect of GSH to consume oxygen has been previously observed in model wine.[26]

On the other hand, inactive dry yeast showed the least oxygen consumption capacity in red wine. In this sense, it is known that the phenolic compounds of red wine can be linked by van der Waals bonds to the yeast cell walls, decreasing the capacity to absorb dissolved oxygen.[29] The fact that inactive dry yeast does not have the capacity to absorb dissolved oxygen in red wine can affect the formation of the 1-hydroxyethyl radical since no differences were observed for the 1-hydroxyethyl radical formed in the control red wine and that with inactive dry yeast.

The enological tannins of oak and grape seeds have the capacity to consume oxygen, as reported by several authors.[30−32] In our study, oak and grape seed extracts showed the ability to eliminate oxygen in white wine, while in red wine, no significant differences were observed with control wine.

This work shows that grape seed extract and chitosan provided higher percentages of radical inhibition in red wine than those in white wine, in contrast to the inactive dry yeast that only produced radical inhibition in white wine. However, the antioxidant activity of glutathione to inhibit the 1-hydroxyethyl radical did not depend on the type of wine. Oak extract did not produce changes in the 1-hydroxyethyl radical, and combinations between acetaldehyde and oak extract were revealed. Ascorbic acid was the only antioxidant substance tested that increased the concentration of acetaldehyde, after the Fenton reaction; therefore, the 1-hydroxyethyl radical inhibition could be masked by a pro-oxidant effect. In conclusion, the potential use of the natural substances tested to inhibit the 1-hydroxyethyl radical in SO_2-free wines is influenced by the type of wine (red or white), and their respective mechanisms of action could control or increase the formation of acetaldehyde. In this sense, other studies should be carried out in more detail to study the reactions between natural antioxidant substances and wine matrix compounds.

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Notes

The authors declare no competing financial interest.

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Artículo 5. Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO_2-free red wines.

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Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO_2-free red wines

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Abstract

BACKGROUND: Different red winemaking were carried out to evaluate the effects of the prefermentative addition of chitosan, as an alternative to the use of SO_2, on the secondary products of alcoholic fermentation, yeast available nitrogen (YAN), biogenic amines and ethyl carbamate.

RESULTS: The wines made with chitosan presented higher total acidity and higher content of tartaric and succinic acids than those made only with SO_2. The use of chitosan in winemaking resulted in wines with higher glycerol and diacetyl content without increasing the concentration of ethanol, acetic acid, acetaldehyde or butanediol. YAN was lower in wines made with chitosan, which may mean an advantage for the microbial stability of the wines. Furthermore, the use of chitosan at the beginning of alcoholic fermentation did not increase the concentration of biogenic amines or the formation of ethyl carbamate in SO_2-free red wines.

CONCLUSION: The total or partial substitution of SO_2 for chitosan at the beginning of the alcoholic fermentation gives rise to quality red wines without negatively affecting their nitrogen fraction or their very important secondary fermentation products such as acetic acid or acetaldehyde.

Keywords: SO_2 free wines; secondary fermentation products ; amino acids ; biogenic amines ; ethyl carbamate

INTRODUCTION

The nitrogen fraction in wine is complex and variable and depends on the grape variety, the agronomic and oenological techniques applied and the wine storage conditions. During the fermentation process, nitrogenous compounds are essential for yeast growth and metabolism. However, unless completely consumed, they can promote microbiological instability due to the growth of bacteria and the production of ethyl carbamate, which is a carcinogenic compound.[1,2]

Sulfur dioxide (SO_2) is the most common additive in winemaking. Its antioxidant, antioxidasic and antimicrobial capacity is necessary to give a good quality product. It is also known to influence amino acid utilization and volatile compound formation by yeast,[3-7] because it interferes with glycolysis and phosphorylation of the respiratory chain.[8] However, due to the harmful effects that SO_2 can have on health, one of the main objectives of the oenological industry is the search for natural alternatives that make it possible to reduce or replace the dose of SO_2 used in winemaking. In this sense, Chinnici *et al.*[9] emphasized the ability of chitosan to act as a surrogate for SO_2, reducing browning and protecting thiols from oxidation in model wine solutions.

Chitosan is a polysaccharide derived from chitin, widely used in recent years in the food industry due to its important antimicrobial and antioxidant activity.[9,10] Its use in winemaking was approved by the EU in 2011, mainly as a clarifier (elimination of contaminants, prevention of turbidity and oxidation, reduction

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of undesirable microorganisms, etc.).[11] Numerous studies have been carried out on chitosan and its applicability in oenology. Among them, Filipe - Ribeiro *et al.*[12] showed the efficacy of chitosan to improve the sensory profile of red wines contaminated with volatile phenols, and reported that chitosan reduces negative and bitter phenolic sensory attributes and increases positive fruit and floral notes. More recently, Castro - Marín *et al.*[13] reported the potential of using biocompatible chitosan as a healthier supplement and/or alternative to SO_2 against the oxidative deterioration of white wine due to its Fe (II) chelating activity, which inhibits the formation of 1-hydroxyethyl radicals.

These important properties make chitosan a possible alternative that can be used to reduce the use of SO_2 in winemaking. However, the literature lacks data on its effect on other components of wine of great interest, such as YAN and important by-products of fermentation. Specifically, the nitrogenous fraction of wine includes compounds related to its quality, as well as its possible harmful effects on health, such as ethyl carbamate and biogenic amines. Secondary fermentation compounds include acetaldehyde, acetic acid or glycerol, the concentration of which could vary due to the substitution of SO_2 by chitosan, affecting the quality of the wine.Thus, the objective of this work was to study the influence of the addition of chitosan as a possible alternative to the use of SO_2 at the beginning of alcoholic fermentation on the nitrogen fraction and the secondary fermentation products of red wines.

MATERIALS AND METHODS

Red winemaking and accelerated aging

Three types of red wines of *Vitis vinifera* L. Cv. Cabernet Sauvignon grapes were made in triplicate: (i) control wine with SO_2 (50 mgL $^{-1}$): SO_2 W; (ii) wine with chitosan (0.2 gL $^{-1}$): CHW; (iii) wine with chitosan (0.2 gL $^{-1}$) and SO_2 (25 mgL $^{-1}$): CH + SO_2 W. The chitosan used was Bactiless ™ (Lallemand, SL, Barcelona, Spain), a biopolymer from *Aspergillus niger* fungus .

Alcoholic fermentation was carried out in 20 L containers at 22 ° C using *Saccharomyces cerevisiae* Uvaferm VN (Lallemand) as starter culture. The evolution of the fermentation was controlled by measuring the density. It was considered finished when the density was 0.9920 g mL $^{-1}$. The glucose and fructose concentration were determined at the same time. After alcoholic fermentation, the wines were decanted and malolactic fermentation was carried out at 20-22 ° C using *Oenococcus oeni* VP41 (Lallemand) as a starter culture. The end of the malolactic fermentation was determined by measuring the content of malic and lactic acid in the wines. The wines were then decanted, cold stabilized at −3 ° C for 30 days, and filtered through 0.2 µm filters prior to bottling. Only control wines with SO_2 were adjusted to 25 mgL $^{-1}$ of free SO_2 before bottling. Once bottled, the wines were analyzed as described below.

All the wines were aged in a laboratory oven at 40 ° C for 35 days, to study the concentration of biogenic amines and ethyl carbamate after accelerated aging.

Conventional oenological parameters and secondary fermentation products

Density, pH, free and total SO_2, alcohol, total acidity, glycerol, organic acids and meso / levo-butanediol were determined by official analytical methods established by the International Organization of Vine and Wine.[14] Diacetyl and acetaldehyde were analyzed on a Focus-ISQ GC/MS chromatograph (Thermo Scientic, Milan, Italy). One hundred microliters of wine were mixed with 100 µL of 2-pentanol-4-methyl (Sigma-Aldrich Chemie, Steinheim, Germany), used as internal standard (41.55 mg L $^{-1}$) and 1 mL of Milli-Q water (Merck KGaA, Darmstadt, Germany). Then, 1 µL of sample was injected in split mode (1/25) into a BP-21 free fatty acid phase (FFAP) column (60 m × 0.32 mm × 0.25 µm) (SGE, Trajan Scientific, Milton Keynes, UK). Helium was used as carrier gas (constant flow 1.2 mL min $^{-1}$). The injector temperature was 195 ° C and the oven temperature was programmed as follows: 32 ° C for 2 min, rose to 5 ° C min $^{-1}$ at 120 ° C, and then increased to 75 ° C min $^{-1}$ at 190 ° C, which was held for 18 min. The MS operated in electron impact mode: electron energy 70 eV, ion source temperature 250 ° C. Identification was achieved by comparison with commercial standards (Sigma-Aldrich Chemie) and quantification using the calibration curves of each standard.

Liquid chromatography high resolution (HPLC) analysis of amino acids, biogenic amines and ammonium ion

The content of amino acids, biogenic amines and ammonium ion was determined simultaneously by the method described by Gómez-Alonso *et al.*[15] with some modifications. The samples were previously derivatized by mixing 1 mL of wine with 1.75 mL of 1 M borate buffer (pH = 9), 750 µL of methanol, and 30 µL of diethylethoxymethylenemalonate (DEEMM) in a screw cap test tube for 30 min in an ultrasound bath. Then, the mixture heated at 70 ° C for 2 h to allow complete degradation of excess DEEMM and reagent by-products.

Analyzes were performed on HPLC equipment with a diode array detector (Agilent, Model 1110; Agilent Technologies, Inc. Santa Clara, CA, USA). The chromatographic separation was carried out on an ACE HPLC column (5 C18-HL), particle size of 5 µm (250 mm × 2.1 mm), with the gradient shown in Table 1 (phase A: 25 mM acetate buffer , pH = 5.8 with 0.02% sodium azide; phase B: methanol; phase C: acetonitrile), and a flow rate of 0.9 mL min $^{-1}$. For detection , a photodiode array detector was used , monitored at 280 and 269 nm. Compounds were identified and quantified using the corresponding standards (Sigma-Aldrich Chemie).

Ethyl Carbamate GC/MS analysis

Ethyl carbamate from the wine samples was isolated and extracted by solid phase extraction (SPE): 1 mL of butyl carbamate

Table 1. Eluent gradient for HPLC determination of amino acids, ammonium ion, and biogenic amines

Time (min)	0	2	20	55	80	90	92
Eluent A (%)	80	80	73	50	15	15	80
Eluent B (%)	15	15	15	25	45	45	15
Eluent C (%)	5	5	12	25	40	40	5

(Sigma-Aldrich Chemie)as internal standard (400 ngL^{-1}) was added to 8 mL of wine and Milli Q -Water (Merck KGaA)was added, giving a total volume of 50 mL. This mixture was applied directly to the EXtrelut® NT20 column (Merck KGaA). After 10 min equilibration, the sample was dropped at a flow rate of approximately 2 mL min^{-1} using a vacuum system and the column was dried for 15 min to remove all the sample. Then the ethyl carbamate was eluted with 160 mL of dichloromethane. Finally, the extracts were concentrated to 200 µL on a rotary evaporator at 30 ° C and 325 mbar and stored in a freezer (-20 ° C) before their chromatographic analysis.

A total of 1.5 µL of extract was injected in splitless mode into a Focus-ISQ GC (Thermo Scientific). The column used was a BP-21 FFAP (60 m × 0.32 mm × 0.25 µm) (SGE, Trajan Scientific). The injector temperature was 200 ° C and the oven temperature was programmed as follows: 70 ° C for 3 min, ramping at 3 ° C min^{-1} to 150 ° C, which was maintained for 2 min. Then, the temperature was raised to 100 ° C min^{-1} to 195 ° C and held for 10 min. The carrier gas was helium with a flow rate of 1.2 mL min^{-1}. The MS operated in electron impact mode with electronic energy of 70 eV, the temperature of the ion source was 250 ° C and the selected ion monitoring mode (SIM) was used: 62, 74 and 89 m/z for ethyl carbamate and 62, 74 and 88 m/z for butyl carbamate. The quantitative evaluation was carried out by relative response factor. This methodology was adapted of OIV - OENO 590-2017.[16]

Statistic analysis

Statistical analyzes were performed with SPSS software, version 24.0. The Student-Newman-Keuls test was used to verify the existence of significant differences between means. Principal component analysis (PCA) was applied to highlight the main variables that contribute to the differences in nitrogen fraction between samples.

RESULTS AND DISCUSSION

Conventional oenological parameters and secondary fermentation products

Table 2 shows conventional oenological parameters and secondary fermentation products in the young red wines tested. No significant differences were found between the samples for residual glucose and fructose concentration, pH, density, and alcohol content. However, the prefermentative addition of chitosan increased the concentration of some by-products of fermentation.

Glycerol is the major by-product of fermentation. It contributes to the body and intensity of the wine flavor. Glycerol has vital physiological functions during yeast fermentation. The accumulation of glycerol allows yeast to re - adjust the osmotic gradient across the cell membrane to retain cellular functions under osmotic stress conditions.[17,18] The prefermentative addition of chitosan could induce osmotic stress on *Saccharomyces cerevisiae* and these wines contain a higher concentration of glycerol.Sun *et al.*[19] observed an increase in glycerol in *Saccharomyces cerevisiae* encapsulated in alginate – chitosan – alginate and cloncluded its action as a stress tolerance agent. Moreover, increased glycerol formation decreases the carbon source directed to ethanol synthesis in alcoholic fermentation.[20] In our study, wines with chitosan had higher glycerol concentration and lower ethanol concentration, although the differences in ethanol content were not statistically significant from the control wine with SO_2.

Some authors have tried to redirect the glycolysis flow towards glycerol; however, optimized conditions produced an excessive accumulation of acetic acid and acetaldehyde.[21-23]In our study, chitosan treatment influenced glycerol synthesis without affecting the concentration of acetic acid. Indeed, the concentration of acetaldehyde was lower in CHW and CH+SO_2W. Acetaldehyde is one of the most important carbonyl compounds in wine and can be formed by yeast during alcoholic fermentation or by other microorganisms such as acetic acid bacteria and lactic bacteria.[24,25] In this work, SO_2 W had a higher concentration of acetaldehyde. Some authors have shown that SO_2 promotes the production of acetaldehyde by *Saccharomyces cerevisiae* .[7,26] The prefermentative addition of chitosan may also influence the lower production of acetaldehyde. In this regard Yu *et al.*[27] reported that gold (Au) nanoparticles with chitosan reduced the level of acetaldehyde in wine. The fact that CH + SO_2 W also has low acetaldehyde values corroborates this second hypothesis.

The metabolites of the diacetyl-acetoinic cycle, diacetyl and meso- and levo-butanediol, were studied. Although no significant differences were obtained in the concentration of butanediol, CHW and CH + SO_2 W had a higher concentration of diacetyl and a lower concentration of malic acid. Consequently, a lower concentration of citric acid and a higher concentration of lactic acid were expected in these wines, as a result of the metabolism of lactic bacteria.[28] However, no significant differences were obtained in the concentration of these compounds with respect to the control wine. The increase in diacetyl was not therefore produced by a change in the metabolism of lactic bacteria, and was probably due to an alteration in the metabolism of *Saccharomyces cerevisiae* . The higher concentration of diacetyl in wines with chitosan could be linked to the formation of glycerol to maintain the nicotinamide adenine dinucleotide (NAD + / NADH) redox balance. As reported in the bibliography,[29-33] glycerol has a major role as an osmolyte under osmotic stress conditions and also functions as an essential redox sink in the absence of oxygen, when the reoxidation of excess cytosolic NADH is required. In line with these findings, Michnick *et al.*[34] showed that the increased utilization of NADH through glycerol formation led to a transitory accumulation of pyruvate and acetaldehyde. These compounds could then follow two pathways towards the formation of diacetyl:[35] (i) transformation of pyruvate and acetaldehyde into α -acetolactate, which can be degraded into diacetyl; (ii) condensation of acetaldehyde with acetyl coenzyme A. Thus, the prefermentative addition of chitosan could affect the NADH redox balance of the yeasts and, as a consequence, the wines elaborated with chitosan showed a higher concentration of glycerol and diacetyl.

Some differences could be observed among organic acids. Control wines with SO_2 showed a significantly lower total acidity value and smaller amounts of tartaric acid. The smaller concentration of tartaric acid in wines with SO_2 is in agreement with other authors.[36] The addition of chitosan also increased the production of succinic acid compared to control wines with SO_2. In this sense, Bach *et al.*[37] reported that the amino acid γ -aminobutyric acid (GABA) could act as a source of succinate in wine. Higher concentrations of succinic acid in wines with chitosan may therefore be due to the changes that chitosan can cause in the metabolism of amino acids, which will be discussed later.

Effect of the prefermentative addition of chitosan on the YAN content of wines

Table 3 shows the content of amino acids, ammonium ion, and the total YAN in the wines studied. The total YAN content was lower in wines elaborated with chitosan, which can mean an

Table 2. Conventional oenological parameters and secondary products of fermentation in red young wines (n = 3)

	SO_2W	CHW	$CH+SO_2W$
Glucose and fructose (gL^{-1})	$0.33^{a} \pm 0.08$	$0.17^{a} \pm 0.04$	$0.26^{a} \pm 0.15$
Density (gmL^{-1})	$0.9909^{a} \pm 0.0000$	$0.9894^{a} \pm 0.0000$	$0.9894^{a} \pm 0.0000$
pH	$3.90^{a} \pm 0.03$	$3.85^{a} \pm 0.09$	$3.86^{b} \pm 0.04$
Total SO_2 (mgL^{-1})	$61^{b} \pm 10$	nd	$19^{a} \pm 1$
Free SO_2 (mgL^{-1})	$31^{b} \pm 8$	nd	nd
Alcohol (% v/v)	$14.21^{a} \pm 0.13$	$13.85^{a} \pm 0.19$	$13.99^{a} \pm 0.19$
Total acidity (gL^{-1})	$3.71^{a} \pm 0.01$	$4.07^{b} \pm 0.02$	$3.98^{b} \pm 0.10$
Glycerol (gL^{-1})	$8.19^{a} \pm 0.06$	$8.82^{b} \pm 0.24$	$8.61^{b} \pm 0.09$
Acetic acid (gL^{-1})	$0.25^{a} \pm 0.02$	$0.29^{a} \pm 0.02$	$0.27^{a} \pm 0.02$
Tartaric acid (gL^{-1})	$1.88^{a} \pm 0.07$	$2.11^{b} \pm 0.11$	$2.10^{b} \pm 0.08$
Citric acid (gL^{-1})	$0.19^{a} \pm 0.01$	$0.18^{a} \pm 0.01$	$0.20^{a} \pm 0.03$
Succinic acid (gL^{-1})	$0.62^{a} \pm 0.01$	$0.80^{b} \pm 0.06$	$0.75^{b} \pm 0.05$
Malic acid (gL^{-1})	$0.12^{b} \pm 0.04$	$0.03^{a} \pm 0.01$	$0.06^{a} \pm 0.02$
Lactic acid (gL^{-1})	$1.01^{a} \pm 0.09$	$1.27^{a} \pm 0.16$	$1.24^{a} \pm 0.17$
meso-Butanediol (gL^{-1})	$0.12^{a} \pm 0,01$	$0.14^{a} \pm 0,01$	$0.14^{a} \pm 0,01$
levo-Butanediol (gL^{-1})	$0.49^{a} \pm 0.02$	$0.52^{a} \pm 0.03$	$0.51^{a} \pm 0.02$
Diacetyl (mgL^{-1})	$3.95^{a} \pm 0.87$	$9.20^{b} \pm 0.31$	$9.86^{b} \pm 0.48$
Acetaldehyde (mgL^{-1})	$12.87^{b} \pm 2.60$	$7.87^{a} \pm 1.92$	$8.22^{a} \pm 0.27$

[a,b]Different letters in superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the Student–Newman–Keuls test. SO_2W: wine with SO_2; CHW: wine with chitosan; $CH+SO_2W$: wine with chitosan and SO_2.
nd, not detected (detection limit: 5 mgL^{-1}).

Table 3. Concentration (mgL^{-1}) of amino acids, ammonium ion and yeast-available nitrogen in red young wines (n = 3)

	SO_2W	CHW	$CH+SO_2W$
Aspartic acid	$1.22^{c} \pm 0.09$	$0.60^{a} \pm 0.06$	$0.84^{b} \pm 0.03$
Glutamic acid + glutamine	$8.52^{c} \pm 0.20$	$4.64^{a} \pm 0.09$	$5.52^{b} \pm 0.25$
Asparagine	$13.75^{b} \pm 0.40$	$9.38^{a} \pm 0.61$	$9.87^{a} \pm 0.45$
Serine	$5.64^{c} \pm 0.13$	$4.59^{a} \pm 0.08$	$5.30^{b} \pm 0.17$
Histidine	$2.51^{a} \pm 0.12$	$2.30^{a} \pm 0.48$	$2.75^{a} \pm 0.18$
Glycine	$3.56^{b} \pm 0.23$	$2.19^{a} \pm 0.19$	$2.38^{a} \pm 0.08$
Threonine	$2.05^{b} \pm 0.04$	$0.90^{a} \pm 0.05$	$0.93^{a} \pm 0.07$
β-Alanine	$6.07^{b} \pm 0.55$	$3.74^{a} \pm 0.36$	$4.23^{a} \pm 0.84$
Arginine	$7.90^{b} \pm 0.11$	$6.76^{a} \pm 0.08$	$7.53^{b} \pm 0.40$
α-Alanine	$6.02^{c} \pm 0.12$	$3.35^{a} \pm 0.09$	$3.62^{b} \pm 0.04$
GABA	$9.55^{c} \pm 0.11$	$5.12^{b} \pm 0.16$	$4.85^{a} \pm 0.06$
Tyrosine	$43.44^{b} \pm 2.39$	$37.67^{a} \pm 0.84$	$40.74^{b} \pm 0.50$
Valine	$1.79^{b} \pm 0.10$	$0.84^{a} \pm 0.06$	$0.87^{a} \pm 0.01$
Methionine	$1.09^{a,b} \pm 0.13$	$0.98^{a} \pm 0.01$	$1.28^{b} \pm 0.15$
Cysteine	$1.28^{a} \pm 0.15$	$1.08^{a} \pm 0.07$	$0.91^{a} \pm 0.21$
Tryptophan	$0.86^{b} \pm 0.04$	$0.42^{a} \pm 0.03$	$0.80^{b} \pm 0.04$
Isoleucine	$0.89^{b} \pm 0.01$	$0.66^{a} \pm 0.04$	$0.83^{b} \pm 0.05$
Phenylalanine	$0.97^{b} \pm 0.11$	$0.70^{a} \pm 0.05$	$0.67^{a} \pm 0.08$
Leucine	$0.78^{a} \pm 0.05$	$0.87^{a} \pm 0.05$	$0.78^{a} \pm 0.10$
Ornithine	$1.96^{b} \pm 0.09$	$1.63^{a} \pm 0.07$	$2.30^{c} \pm 0.11$
Lysine	$20.48^{b} \pm 1.19$	$15.94^{a} \pm 0.17$	$15.49^{a} \pm 0.20$
Ammonium ion	$22.85^{b} \pm 0.09$	$20.67^{a} \pm 0.21$	$20.45^{a} \pm 0.17$
$\sum$ YAN	$163.19^{c} \pm 3.96$	$125.04^{a} \pm 2.34$	$132.92^{b} \pm 0.12$

[a,b,c] Different letters in superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the test of Student–Newman–Keuls. SO_2W: wine with SO_2; CHW: wine with chitosan; $CH+SO_2W$: wine with chitosan and SO_2.
YAN, yeast-available nitrogen.

advantage for the microbial stability of finished wines. It is known that amino acids are essential for the growth and metabolism of yeast. However, unless consumed completely after the fermentation process, they can promote microbiological instability due to the growth of bacteria and the production of biogenic amines and ethyl carbamate with harmful health effects.[1,2]

Principal component analysis was applied to the amino acids, ammonium ion, and total YAN content data to highlight the variables that contributed more to the differentiation of the treatments studied. The first two main components represented 86.11% of the total variance. The variables that showed the best correlations with each main component are shown in Table 4, with their correlation coefficients, and the percentages of explained and accumulated variance of each component. The distribution of the samples in the space formed by principal components 1 and 2 (PC1 and PC2) is shown in Fig. 1.

Principal component 1 separated wines elaborated with chitosan (CHW and CH + SO_2 W) from the control wines with SO_2 (SO_2 W), mainly due to the lower content of ammonium ion, GABA, threonine, valine, lysine, α -alanine, asparagine, glycine, glutamic acid and glutamine, phenylalanine, β -alanine and aspartic acid in CHW and CH + SO_2 W. The ammonium ion was the YAN source that contributed most to the differentiation. Ammonium consumption starts when two other preferred nitrogen sources, glutamate and glutamine, are exhausted.[38] In our results, the concentration of these amino acids was also lower in wines with chitosan. After ammonium, GABA was the amino acid with the highest correlation coefficient. It is known that GABA has an important role in fermentation because *Saccharomyces cerevisiae* can use it efficiently as a source of assimilable nitrogen, especially under conditions of low availability of YAN.[39] γ-Aminobutyric acid plays an important role as a source of succinate.[37,39] In our study this effect could be observed in wines treated with chitosan, which had a lower concentration of GABA and a higher concentration of succinic acid (Table 2).

Principal component 2 differentiated wines that only contained chitosan, CHW, from those with SO_2 (CH + SO_2 W and SO_2 W).

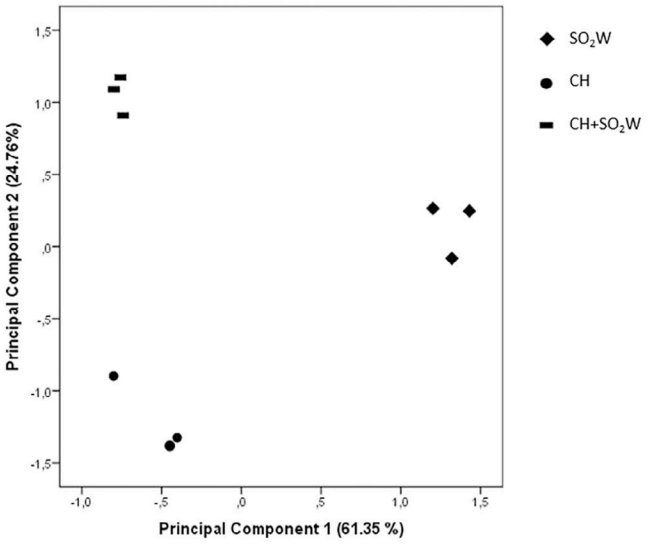

Figure 1 . Plot of wine samples in the space defined by principal components PC1 and PC2 with regard to assimilable nitrogen data. SO_2 W: wine with SO_2; CHW: wine with chitosan; CH + SO_2 W: wine with chitosan and SO_2.

CHW presented particularly lower concentrations of ornithine, tryptophan, methionine, and serine. Methionine and serine are two sulfur amino acids. It is known that SO_2 can be assimilated and used in the synthesis of sulfur-containing amino acids.[40] Moreover, Elviri *et al.*[41] showed that ionic interactions are the main drivers of the loading and release behavior of amino acids or peptides of chitosan hydrogels, and they observed that sulfur-containing amino acids exhibited greater absorption by chitosan hydrogels. In our work, the prefermentative addition of chitosan could cause the production of wines with a lower concentration of sulfur amino acids, such as serine, threonine, and methionine, with the possibility of ionic interactions existing between these amino acids and the chitosan.

Table 4. Results of principal component analysis applied to assimilable nitrogen data (n = 3)

Principal component	% Explained variance	% Cumulative variance	Compounds	Correlation coefficients
PC1	61.35	61.35	Ammonium ion	0.993
			GABA	0.992
			Threonine	0.984
			Valine	0.980
			Lysine	0.976
			α-Alanine	0.974
			Asparagine	0.971
			Glycine	0.964
			Glutamic acid + glutamine	0.942
			Phenylalanine	0.886
			β-Alanine	0.883
			Aspartic acid	0.855
PC2	24.76	86.11	Ornithine	0.931
			Tryptophan	0.824
			Methionine	0.806
			Serine	0.708

Table 5. Content of biogenic amines (mgL^{-1}) and ethyl carbamate (μgL^{-1}) in young and accelerated aged wines (n = 3)

	Young wines			Accelerated aged wines		
	SO_2W	CHW	CH+SO_2W	SO_2W	CHW	CH+SO_2W
Histamine	$0.17^{a} \pm 0.02$	$0.16^{a} \pm 0.02$	$0.14^{a} \pm 0.03$	$0.18^{a} \pm 0.01$	$0.17^{a} \pm 0.02$	$0.17^{a} \pm 0.02$
Tyramine	$15.42^{a} \pm 0.41$	$15.50^{a} \pm 0.43$	$13.96^{a} \pm 0.26$	$41.42^{b} \pm 5.19$	$39.33^{b} \pm 2.66$	$42.76^{b} \pm 10.74$
Putrescine	$13.44^{a,b} \pm 0.06$	$13.06^{a,b} \pm 0.06$	$13.57^{b} \pm 0.10$	$13.39^{a,b} \pm 0.07$	$12.96^{a} \pm 0.06$	$13.47^{a,b} \pm 0.50$
Phenylethylamine	$0.08^{a} \pm 0.00$	$0.08^{a} \pm 0.00$	$0.09^{a} \pm 0.00$	$0.08^{a} \pm 0.01$	$0.08^{a} \pm 0.00$	$0.08^{a} \pm 0.00$
Total biogenic amines	$29.11^{a} \pm 0.35$	$28.80^{a} \pm 0.47$	$27.62^{a} \pm 0.16$	$54.89^{b} \pm 5.23$	$52.54^{b} \pm 2.64$	$56.48^{b} \pm 10.41$
Ethyl carbamate	$1.73^{b} \pm 0.22$	$1.23^{a} \pm 0.29$	$1.34^{a} \pm 0.18$	$1.57^{b} \pm 0.11$	$1.14^{a} \pm 0.03$	$1.13^{a} \pm 0.08$

^{a,b}Different letters in superscripts in the same row mean significant differences ($\alpha = 0.05$) according to the Student–Newman–Keuls test. SO_2W: wine with SO_2; CHW: wine with chitosan; CH+SO_2W: wine with chitosan and SO_2.

Effect of the prefermentative addition of chitosan on the concentration of biogenic amines and ethyl carbamate, in young and accelerated aged wines

Table 5 shows the concentration of biogenic amines and ethyl carbamate in young and accelerated aged wines. Biogenic amines are mainly produced by microbial decarboxylation of some amino acids, and by amination and transamination of aldehydes and ketones.[42] Four biogenic amines were identified in our wines: histamine, tyramine, putrescine, and phenylethylamine. Among them, tyramine and putrescine were the most abundant biogenic amines detected. It is important to note that the prefermentative addition of chitosan did not affect the content of biogenic amines in young wines. However, the content of these components could increase during wine storage by the action of decarboxylase enzymes, from microorganisms naturally present in wine before filtering, that catalyze the formation of biogenic amines.[43] Accelerated aging of the wines was carried out for this reason. A significant increase in the concentration of tyramine was found in all wines after accelerated aging. In line with our results, Gerbaux and Monamy[44] reported that tyramine is one of the biogenic amines that increases most during storage.

On the other hand, another toxic substance is ethyl carbamate, which is formed through reactions of ethanol and cyanate, urea, citrulline, or other *N*-carbamyl compounds. As can be seen in Table 5, small levels of ethyl carbamate were obtained in the three types of winemaking. The prefermentative addition of chitosan also caused a lower concentration of ethyl carbamate both in young and accelerated aged wines. Chitosan could therefore be used to replace or decrease the SO_2 level without increasing - and even slightly decreasing - the concentration of ethyl carbamate in wines.

CONCLUSIONS

The total or partial replacement of SO_2 by chitosan at the beginning of alcoholic fermentation gave rise to wines with a higher glycerol, diacetyl, and succinic acid content without increasing the concentration of ethanol, acetic acid, acetaldehyde, or butanediol. Furthermore, ammonium ion, GABA, threonine, valine, lysine, α -alanine, asparagine, glycine, glutamic acid and glutamine, phenylalanine, β -alanine, and aspartic acid were the YAN sources that contributed most to differentiating wines with chitosan. The total YAN content was lower in wines elaborated with chitosan, and this was achieved without increasing the concentration of biogenic amines and ethyl carbamate. These results suggest that the prefermentative addition of chitosan is a possible alternative to the addition of SO_2 in red winemaking.

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V. CONCLUSIONES GENERALES

Las conclusiones generales obtenidas en esta Tesis Doctoral, de acuerdo con los objetivos inicialmente establecidos, son las siguientes:

I. El uso combinado del extracto de raspón y plata coloidal fue la alternativa al SO_2 que mostró los recuentos más bajos de bacterias lácticas y acéticas antes del embotellado del vino Airén. La adición prefermentativa de extractos de semillas y raspón, solos o en combinación con la plata coloidal, generó vinos con menor luminosidad y mayores valores de a*, b* y C*, mostrando un color más oscuro, con tonalidades más amarillas y un ligero pardeamiento en el color de estos vinos. Respecto a la composición fenólica, el uso de ambos extractos dio lugar a vinos con menor concentración de estilbenos respecto al uso de SO_2, especialmente los que fueron elaborados junto con CSC. La adición prefermentativa de extractos de semillas de uva aumentó la concentración de flavonoles y flavan-3-oles. En cuanto a la composición volatil, los vinos con extractos de semillas de uva y los extractos de raspón presentaron mayor contenido de ésteres etílicos de ácidos grasos y compuestos bencénicos, aportados por los extractos. Desde el punto de vista sensorial, los vinos elaborados con la adición prefermentativa de extractos fueron caracterizados por los catadores como los vinos más florales y menos cítricos que los vinos control elaborados con SO_2. Entre ellos, los vinos con extractos de semillas de uva fueron diferenciados del resto por ser más amargos, mientras que los vinos elaborados con extractos de raspón fueron los más afrutados.

II. Los recuentos microbiológicos de bacterias acéticas en vino Cabernet Sauvignon, antes del embotellado, mostraron que los extractos de semillas de uva y madera de roble parecen efectivos para controlar la población de estos microorganismos alterantes. Respecto a la composición fenólica, los vinos elaborados con extracto de semilla de uva (1g/L) contenían mayor concentración de flavan-3-oles, especialmente catequina y procianidina B2. En el perfil de flavonoles, se observó que los vinos elaborados con extracto de roble en combinación con la plata coloidal presentaron menor concentración de

quercetina-3-glucósido. Los vinos con extractos, solos o con CSC, tuvieron menores valores de a* y b* mostrando tonalidades más violáceas que los vinos elaborados con SO_2, los cuales presentaron mayor luminosidad y menor intensidad de color. Los compuestos volátiles que contribuyeron en mayor medida a la diferenciación de las muestras fueron principalmente los aportados por el extracto de roble, lactonas y compuestos bencénicos. Así, la adición prefermentativa de extracto de madera de roble en lugar del SO_2, dio vinos con aroma y sabor a madera de roble, vainilla y clavo. Además, estos vinos presentaron mayor cuerpo, intensidad, calidad de retrogusto y fueron mejor valorados por parte de los catadores. Por otro lado, los vinos elaborados con SO_2 y con extractos de semillas de uva conservaron el carácter varietal de la uva Cabernet Sauvignon, y presentaron un perfil aromático y gustativo más intenso a frutos rojos que los vinos con extractos de roble.

III. El quitosano y la levadura seca inactiva, utilizados como alternativas prefermentativas al SO_2 en vinificación, permiten controlar la formación del radical 1-hidroxietilo en vino Cabernet Sauvignon. Mientras que los vinos elaborados con extracto de raspón y sarmiento mostraron menor resistencia a la oxidación. Respecto al contenido en antocianos, el vino con SO_2 presentó mayor concentración, sin embargo, el efecto blanqueante que este ejerce sobre dichos compuestos dio lugar a vinos más claros y con menor intensidad colorante. El quitosano fue la alternativa al SO_2 que dio lugar a vinos con mayor concentración de flavan-3-oles. Mientras que los vinos elaborados con extracto de raspón fueron los vinos que mostraron menor concentración de flavonoles y estilbenos. Por lo tanto, la adición prefermentativa de quitosano, levadura seca inactiva y extractos de raspón y sarmiento, como alternativas al SO_2, influyen en la formación del radical 1-hidroxietilo, en la composición fenólica y el color de los vinos.

IV. El extracto de semillas de uva y el quitosano proporcionaron mayores porcentajes de inhibición del radical 1-hidroxietilo en el vino tinto que en el vino blanco, a diferencia de la levadura seca inactiva que solo ejerció inhibición del

radical en el vino blanco. La actividad antioxidante del glutatión para inhibir el radical 1-hidroxietilo no dependió del tipo de vino. El extracto de roble no produjo cambios en el radical 1-hidroxietilo, además los vinos con ese extracto tuvieron menor concentración de acetaldehído. El ácido ascórbico fue la única sustancia antioxidante que aumentó la concentración de acetaldehído después de la reacción de Fenton.

V. El uso de quitosano, en comparación con el SO_2, en la elaboración del vino Cabernet Sauvignon, dio como resultado vinos con mayor contenido de glicerol y diacetilo sin aumentar la concentración de etanol, ácido acético, acetaldehído o butanodiol. El nitrógeno fácilmente asimilable se encontró en menor concentración en los vinos elaborados con quitosano, lo que puede significar una ventaja para la estabilidad microbiana de los mismos. Además, el uso de quitosano no aumentó la concentración de aminas biógenas ni la formación de carbamato de etilo en los vinos. Por lo tanto, la sustitución total o parcial de SO_2 por quitosano al inicio de la fermentación alcohólica da lugar a vinos tintos de calidad sin afectar negativamente a la fracción nitrogenada.