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Manoproteínas y enzimas en la extracción y estabilidad del color de vinos tintos de tempranillo
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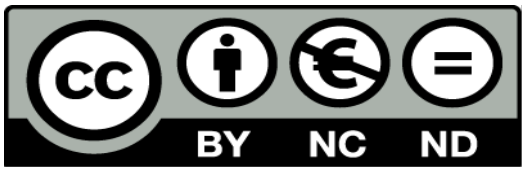

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MANOPROTEÍNAS Y ENZIMAS EN LA EXTRACCIÓN Y ESTABILIDAD DEL COLOR DE VINOS TINTOS DE TEMPRANILLO

TESIS DOCTORAL ZENAIDA GUADALUPE MÍNGUEZ

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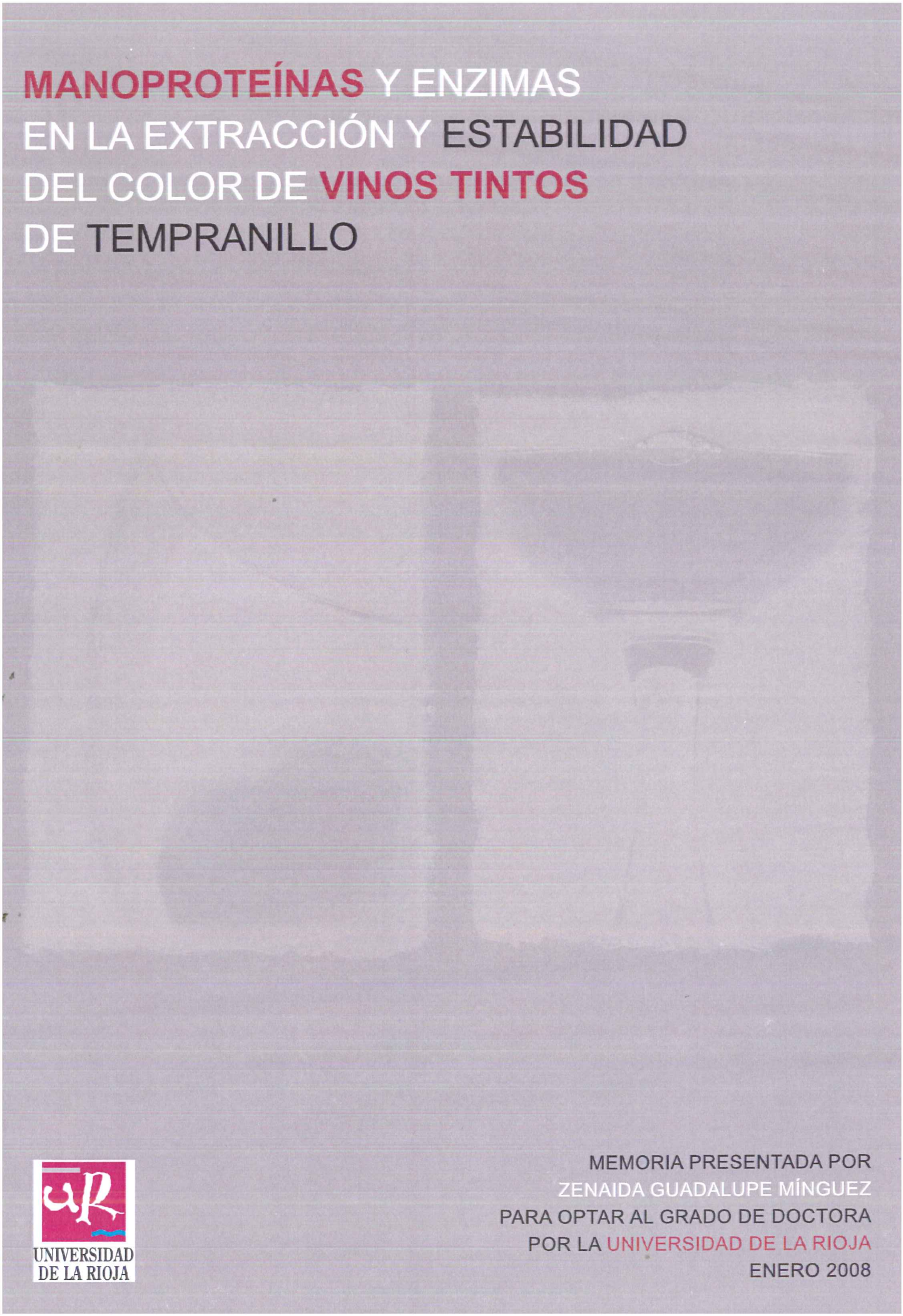

MEMORIA PRESENTADA POR
ZENAIDA GUADALUPE MÍNGUEZ
PARA OPTAR AL GRADO DE DOCTORA
POR LA UNIVERSIDAD DE LA RIOJA

ENERO 2008

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BELÉN AYESTARÁN ITURBE, profesora Titular del Área de Tecnología de los Alimentos de la Universidad de La Rioja,

CERTIFICA:

Que la memoria titulada *"Manoproteínas y enzimas en la extracción y estabilidad del color de vinos tintos de Tempranillo"*, que presenta ZENAIDA GUADALUPE MÍNGUEZ, ha sido realizada en el Departamento de Agricultura y Alimentación de la Universidad de La Rioja, bajo mi dirección, y reúne las condiciones exigidas para optar al grado de Doctor.

Logroño, 18 de Enero de 2008

Fdo: Belén Ayestarán Iturbe

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A Julia y a Diego;

a Mari Carmen, mi madre.

A la memoria de mi padre.

Índice

Presentación

Esta memoria de Tesis Doctoral se presenta en forma de compendio de publicaciones científicas siguiendo la normativa de la Universidad de La Rioja aprobada por Consejo de Gobierno el 22 de julio de 2005.

El objetivo principal de la tesis es estudiar el efecto de las manoproteínas y de las enzimas de maceración en la extracción y estabilidad del color durante la vinificación y crianza de vinos tintos de variedad Tempranillo, así como su influencia en la composición de los polisacáridos y los compuestos fenólicos. Para ello se validó en primer lugar un método de análisis que permitiera analizar las distintas familias de polisacáridos tanto en los mostos como en los vinos (*artículo 1*), así como una metodología que permitiera identificar y cuantificar de forma sencilla el mayor número posible de compuestos fenólicos (*artículo 2*). Ambos métodos se aplicaron en las muestras tomadas durante la vinificación y la crianza de los vinos. Así, el *artículo 3* analiza el perfil y el contenido de los distintos polisacáridos durante la maceración-fermentación de los vinos y su evolución durante la fermentación maloláctica, la crianza y el envejecimiento de los mismos, y el *artículo 4* estudia la evolución de las principales familias de compuestos fenólicos durante estas etapas, así como su relación con la composición del color de los vinos. En el *artículo 5* se evalúa el efecto global de las manoproteínas y las enzimas de maceración en la extracción del color y la estabilidad coloidal durante la vinificación y crianza de los vinos, así como su influencia en las propiedades sensoriales de los vinos obtenidos. Finalmente, los *artículos 6 y 7* analizan de forma más detallada el efecto de la adición de manoproteínas comerciales y de levaduras súper-productoras de manoproteínas en la composición de polisacáridos y polifenoles durante la vinificación y crianza de los vinos, así como influencia en la estabilidad del color.

Con el objetivo de facilitar la lectura de esta memoria de tesis se incluye en primer lugar un apartado denominado *Justificación y objetivos* donde se centra la temática de la tesis y se describen los objetivos de la misma. A continuación la *Introducción* recoge los estudios más relevantes que han sido realizados en relación a la temática de la tesis. En el capítulo de *Metodología de la investigación* se describe brevemente la metodología y el plan de trabajo desarrollado para llevar a cabo los objetivos planteados en la tesis. Se definen así una serie de objetivos parciales cuyos resultados han dado lugar a las publicaciones científicas que forman esta memoria. Dichas publicaciones se adjuntan en

el capítulo de *Resultados y discusión*, donde también se incluye un resumen de cada artículo y de los resultados más relevantes obtenidos. Finalmente se incluye un capítulo con las conclusiones obtenidas.

Justificación y Objetivos

La tendencia actual del consumo de vinos tintos de calidad con alto contenido polifenólico y estancia en barrica, hace necesaria la implantación de prácticas enológicas que posibiliten la elaboración de vinos de elevado color y que a su vez faciliten la estabilización del mismo. El éxito de dichas técnicas residirá en el equilibrio en la extracción y estabilización de los compuestos fenólicos, buscando estrategias que permitan obtener vinos de elevado color y cuerpo, pero que no sean excesivamente astringentes.

El presente trabajo plantea el empleo de distintos tratamientos que mejoren la extracción y estabilización de la materia colorante durante el proceso de vinificación de vinos tintos de variedad Tempranillo. La alternativa que se propone para aumentar la extracción de la materia colorante de los hollejos es el empleo de enzimas de maceración comerciales, y para incrementar la estabilidad de la materia extraída, el enriquecimiento del medio con coloides protectores como las manoproteínas.

Las enzimas de maceración aumentan la extracción, entre otros compuestos, de los compuestos fenólicos, conocidos por su papel en el color de los vinos tintos pero también en la sensación tánica, cuerpo y astringencia de los mismos. Los preparados comerciales de enzimas de maceración actúan debilitando las paredes celulares del hollejo y de la pulpa de la baya, facilitando así la difusión sucesiva de antocianos libres, copigmentos y taninos en la fase alcohólica durante la fermentación. De esta forma, desde el inicio de la fermentación-maceración se favorece la extracción de materia colorante y la formación de complejos de copigmentación y de pigmentos poliméricos, responsables ambos del color estable de los vinos tintos.

La inestabilidad de la materia colorante durante la vinificación y el envejecimiento de los vinos tintos se debe fundamentalmente a la precipitación y destrucción de pigmentos estables, con liberación de moléculas libres menos coloreadas. Una posible alternativa para disminuir estas pérdidas de color es la utilización de coloides protectores, que adsorberían la materia colorante inestable, evitando su agregación y posterior precipitación. Diferentes estudios señalan que ciertos polisacáridos, en su rol de coloides protectores, pueden unirse a los compuestos fenólicos, reduciendo su reactividad y aumentando su estabilidad coloidal. El hecho de que las manoproteínas de levadura limitan la quiebra proteica y la cristalización tartárica es conocido desde hace

tiempo, y recientemente se ha demostrado que manoproteínas aisladas de un vino tinto son capaces de evitar la agregación de los taninos en un medio sintético. Así, se puede intuir que las manoproteínas de levadura podrían jugar también un papel importante en la estabilización del color extraído durante la maceración-fermentación de los vinos tintos. De hecho, y fundamentalmente debido a su potencial como moduladores de las características organolépticas de los vinos tintos, todas las empresas suministradoras de productos enológicos comercializan uno o varios productos a base de manoproteínas. Hoy por hoy son numerosas las bodegas que utilizan estos productos, y hay quien incluso postula que podrían constituir una alternativa para la gestión de las lías, siempre muy delicada. No obstante, uno de los principales problemas relacionados con la aplicación práctica de derivados de levadura en bodegas es, a menudo, la falta de información científica sobre sus efectos. De hecho, aunque la acción de las manoproteínas sobre las propiedades gustativas de los vinos tintos parece clara, no existen resultados concluyentes en cuanto a su relación con el color.

Por las razones anteriormente expuestas, la presente tesis propone los siguientes tratamientos biotecnológicos para incrementar la extracción y estabilización del color de vinos tintos: a) adición de enzimas de maceración comerciales en la etapa prefermentativa, b) adición directa de manoproteínas purificadas industrialmente en la etapa prefermentativa, y c) inoculación de los mostos con levaduras súper productoras de manoproteínas. Así, nos propusimos evaluar la efectividad de los tratamientos propuestos sobre la extracción de coloides protectores (polisacáridos), y analizar el efecto de dichos tratamientos en la extracción y estabilidad del color durante la maceración-fermentación de vinos tintos de Tempranillo y su evolución en la conservación del vino.

Concretamente los objetivos que se plantearon fueron:

1. Analizar el efecto de los tratamientos propuestos en la extracción y evolución de los polisacáridos durante la vinificación y crianza de los vinos.
2. Analizar el efecto de los tratamientos propuestos en la extracción del color y en su evolución durante la vinificación y crianza de los vinos.
3. Analizar el efecto de los tratamientos propuestos en la extracción y en la evolución de los compuestos fenólicos durante la vinificación y crianza de los vinos.
4. Analizar el efecto de los tratamientos propuestos en la calidad organoléptica de los vinos obtenidos.

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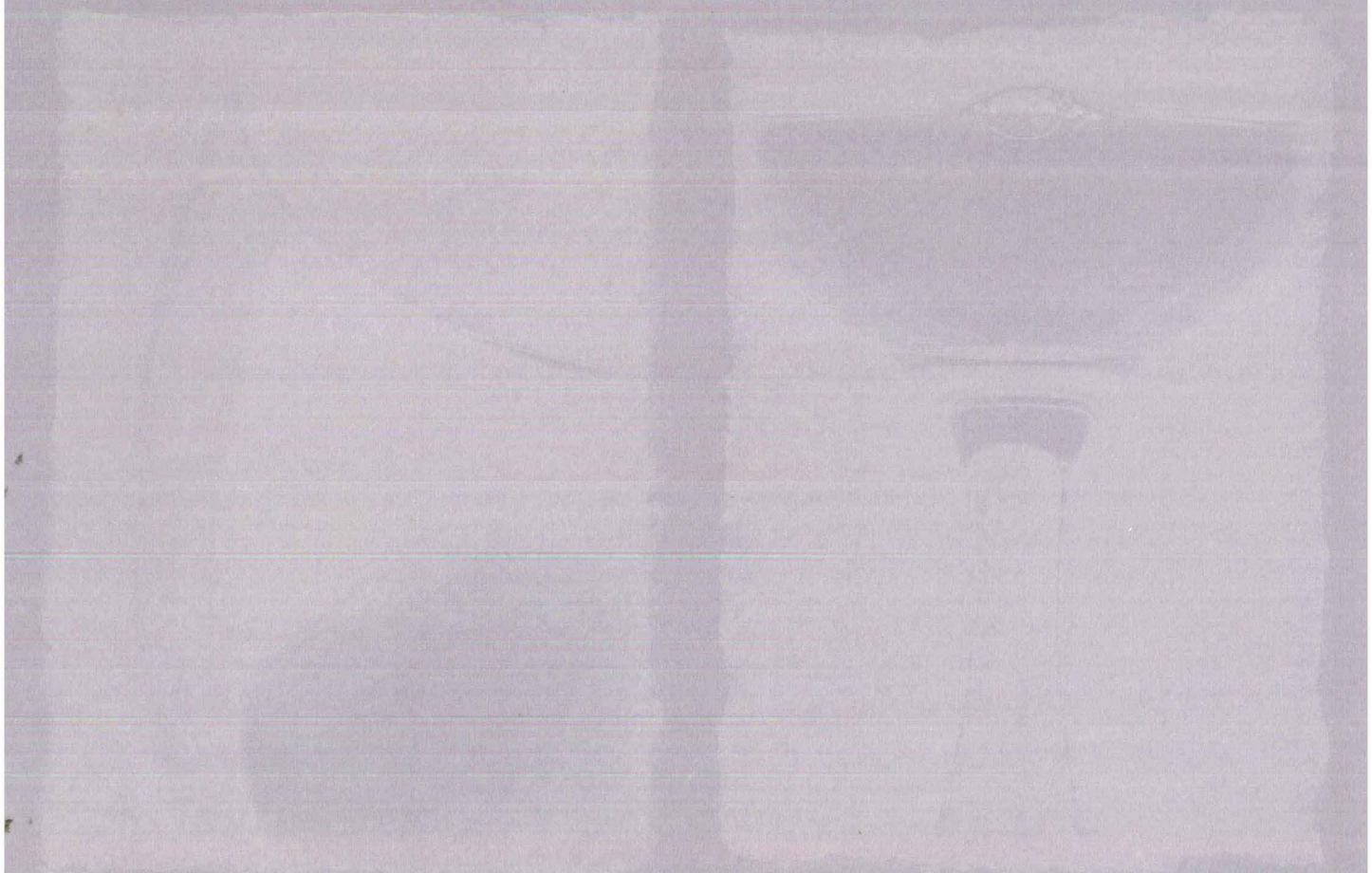


INTRODUCCIÓN

1.1. LOS COMPUESTOS FENÓLICOS DEL VINO TINTO

En la uva existen una cantidad importante de compuestos que contienen grupos fenoles altamente reactivos. Estos compuestos son extraídos durante la maceración y pasan al vino, donde participan en sus características organolépticas, determinando el color los vinos tintos, contribuyendo al flavor (olor, aroma, sabor, astringencia, etc.), y modulando además el retrogusto, la persistencia o el cuerpo.

Desde un punto de vista químico los compuestos fenólicos constan de un anillo bencénico que contiene uno o varios grupos hidroxilo. Según su estructura química se distinguen dos grupos: fenoles flavonoides, con un esqueleto del tipo 2-fenil benzopirona, y fenoles no flavonoides, que son básicamente ácidos fenólicos (Figura 1).

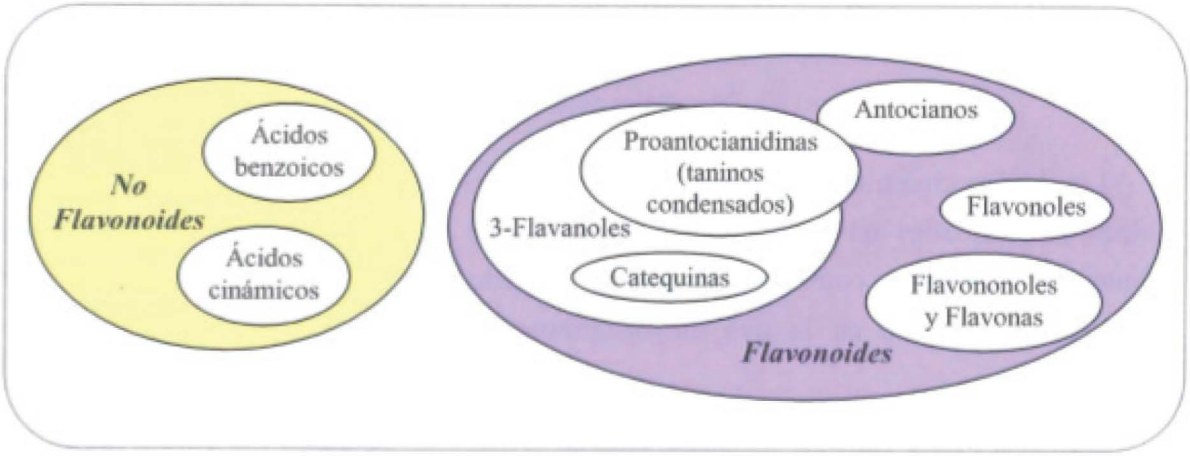


Figura 1. Clasificación de los compuestos fenólicos

La uva contiene compuestos no flavonoides en la pulpa, piel, semillas y raspón, mientras que los compuestos flavonoides se localizan preferentemente en la piel, semillas y raspón (Figura 2). De todos ellos, los flavonoides son los compuestos más importantes tanto en cantidad como en calidad, siendo los compuestos responsables del color, estructura y capacidad de crianza de los vinos tintos.

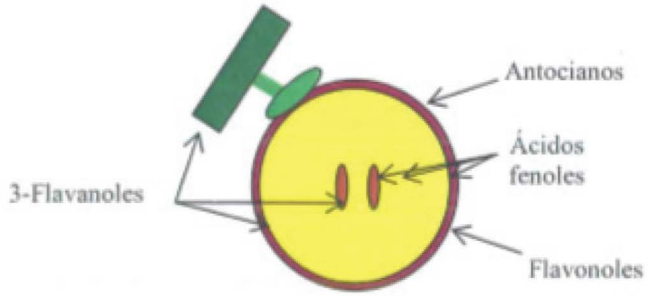
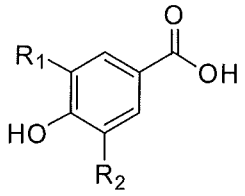


Figura 2. Localización de los compuestos fenólicos en la uva

Los ***Fenoles no Flavanoides*** son básicamente ***Ácidos fenólicos*** de dos tipos, los ácidos benzoicos, y los ácidos cinámicos.

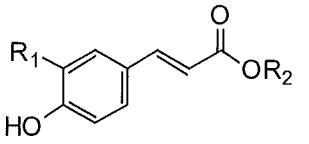
Los ácidos benzoicos se encuentran en el vino en muy baja concentración (1), encontrándose mayoritariamente en forma de ácido gálico, siríngico y p-hidroxibenzoico (Figura 3). El ácido gálico y su dímero, ácido elágico, forman parte de los taninos hidrolizables (galotaninos y elagitaninos), que son cedidos por la madera cuando los vinos son criados en barrica.



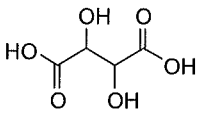
Ácidos benzoicos	R_1	R_2
ácido gálico	OH	OH
ácido p-hidroxibenzoico	H	H
ácido siríngico	OCH_3	OCH_3

Figura 3. Principales ácidos benzoicos del vino

Los ácidos cinámicos son los principales fenoles de la pulpa y se encuentran en los vinos en cantidades más importantes que rondan los 200 mg/L (1). Se pueden encontrar tanto en forma libre como esterificados con ácido tartárico y otros componentes del vino, encontrándose en la uva mayoritariamente bajo formas de ésteres tartáricos (Figura 4).



Esterificación R_2



ácido tartárico

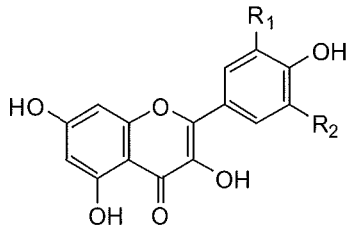
Ácidos cinámicos	R_1	Ésteres tartáricos
ácido cafeico	OH	ácido caftárico
ácido p-cumárico	H	ácido cutárico
ácido ferúlico	OCH_3	ácido fertárico

Figura 4. Ácidos cinámicos de la uva y vino

El ácido cafeico y su éster tartárico son los principales sustratos de la oxidación enzimática de los mostos; los ácidos cafeico y cumárico se encuentran muchas veces acilando los sustituyentes glucósidos de los antocianos. Estos compuestos no afectan directamente al color del vino tinto, aunque sí participan en la estabilización del color mediante fenómenos de copigmentación. Desde el punto de vista gustativo no parecen tener impacto directo sobre el sabor del vino tinto, aunque su degradación da lugar a fenoles volátiles que pueden provocar defectos a nivel olfativo (2).

Los principales ***Fenoles Flavonoides*** del vino tinto incluyen los flavonoles, los antocianos y los flavanoles. Esta última familia incluye a los taninos condensados o proantocianidinas.

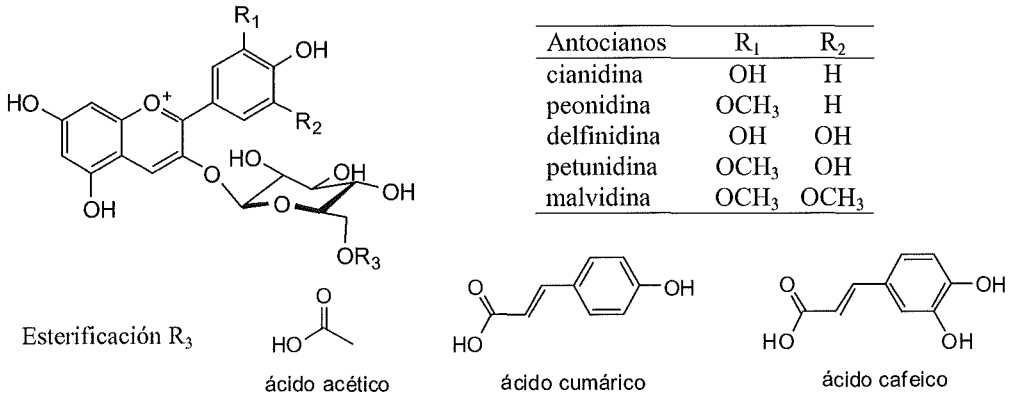
Los ***Flavonoles*** son sustancias amarillas que se encuentran en la piel de la uva bajo forma heterosídica (3). Los más comunes son la quercetina, el kaempferol, la miricetina y la isoramnetina (Figura 5), y los glucósidos más comunes son la glucosa, la galactosa y el ácido glucurónico (4). Su contenido varía de 10 a 100 mg/Kg de bayas (5) y son fácilmente extraíbles en la vinificación, aunque no son muy solubles en agua y es preciso que exista algo de alcohol como solvente. Son amargos, tienen un poder de copigmentación fuerte (6, 7), y pueden estar involucrados en las reacciones de polimerización de los fenoles (8).



Flavonoles	R_1	R_2
kaempferol	H	H
quercetina	OH	H
miricetina	OH	OH
isoramnetina	OCH_3	H

Figura 5. Principales flavonoles del vino

Los ***Antocianos*** o ***Antocianinas*** (del griego *anthos* flor y *kianos* azul) representan una parte importante tanto cuantitativa como cualitativa de los flavonoides de las uvas tintas y, en consecuencia, de los vinos resultantes. Metabolitos secundarios de las plantas, se almacenan en las células de las vacuolas del hollejo de las uvas tintas durante su maduración, siendo los compuestos responsables del color rojo azulado de su piel. Durante el proceso de maceración pasan al vino, otorgándole su color.



Antocianos	R_1	R_2
cianidina	OH	H
peonidina	OCH_3	H
delfinidina	OH	OH
petunidina	OCH_3	OH
malvidina	OCH_3	OCH_3

Figura 6. Estructura química de los antocianos monoglucósidos de la uva

Su estructura corresponde a heterósidos formados por la combinación de un aglicón (antocianidina) y de un azúcar, normalmente la glucosa. Asimismo la glucosa puede estar acilada, siendo importantes en *Vitis vinífera* los derivados acetilados y los p-cumarilados (Figura 6). En las variedades de *Vitis vinífera* se distinguen la malvidina, la petunidina, la delfinidina, la peonidina y la cianidina, de las cuales la malvidina se

encuentra en mayor proporción, por lo que será la principal responsable del color del vino tinto. En los vinos jóvenes los antocianos se encuentran en su forma libre aglicona, siendo estas formas bastante más inestables que sus correspondientes heterósidos.

Los ***Flavanoles*** constituyen una familia compleja dentro de los compuestos fenólicos. Se encuentran en elevadas concentraciones en la uva y juegan un papel muy importante en las características sensoriales de los vinos tintos. Los flavanoles están presentes en la uva en estado de monómeros y en formas más o menos polimerizadas, denominadas taninos condensados, procianidinas o proantocianidinas. Los principales flavanoles monómeros de la uva son la catequina y sus formas isoméricas (Figura 7), aunque la mayor parte de flavanoles están en forma de polímeros. Todos los productos de condensación a partir de dos unidades monoméricas son considerados como procianidinas o taninos, término que hace referencia a su capacidad para precipitar proteínas.

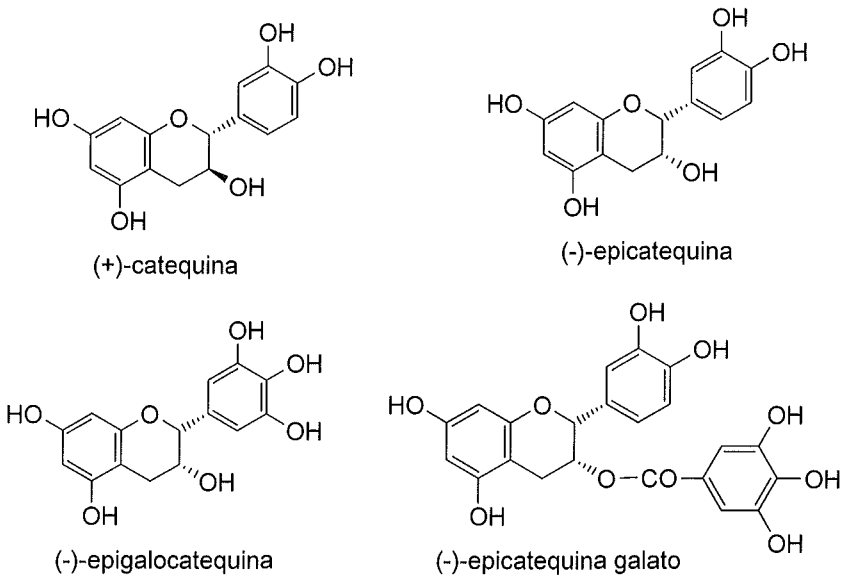


Figura 7. Principales flavanoles monómeros de la uva

El término tanino engloba una gran cantidad de compuestos fenólicos que pertenecen fundamentalmente a dos familias: la de las proantocianidinas, que proceden directamente de la uva, y la de los taninos hidrolizables, que provienen de la madera de roble y se encuentran únicamente en los vinos envejecidos en barrica (9).

Las proantocianidinas (Figura 8) son los flavonoides cuantitativamente más importantes en el vino tinto. Se distinguen las procianidinas, derivadas de la catequina y epicatequina, y las prodelfinidinas, derivadas de galocatequina y epigalocatequina, aunque comúnmente a ambas se les conoce como procianidinas o proantocianidinas. Estas moléculas pueden presentar un número muy elevado de unidades ya que los grados de polimerización medios son de 11 en las semillas y de 30 en los hollejos, si bien el grado de polimerización medio del vino se sitúa alrededor de 7 (2).

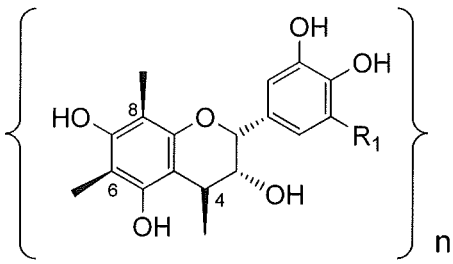


Figura 8. Estructura de las proantocianidinas

Las procianidinas son responsables del sabor amargo y la astringencia de los vinos tintos, de su estructura y cuerpo, pero también de la componente amarilla del color y, mediante su combinación con los antocianos, del color estable de los vinos envejecidos (2, 10).

Durante el proceso de vinificación y crianza del vino tinto, tienen lugar reacciones de polimerización de las proantocianidinas que a nivel sensorial producen variaciones en la astringencia y contribuyen a la estabilidad del color. Por otra parte, y a través de sus grupos hidroxilo, las proantocianidinas son capaces de formar complejos con otras moléculas como sales inorgánicas, polisacáridos y péptidos, lo cual también modifica sus propiedades organolépticas.

La ***Astringencia*** es probablemente uno de los atributos sensoriales más importantes de los vinos tintos. La sensación de astringencia que percibimos al degustar un vino tinto es el resultado la interacción de las proantocianidinas del vino con un grupo de proteínas salivales, produciéndose sensaciones de sequedad, aspereza y rugosidad en boca. Las catequinas son amargas y ligeramente astringentes pero no se clasifican como taninos porque no precipitan a las proteínas.

La percepción de astringencia está condicionada por las interacciones tanino-proteína, que dependen en gran medida de la naturaleza de la proteína así como de la composición monomérica y del tamaño de las proantocianidinas (11, 12). De hecho, son las proteínas ricas en prolina, como las proteínas salivales y las gelatinas, las que parecen reaccionar mejor con los taninos (11). Por otro lado, cuanto mayor es el grado de polimerización y el porcentaje de unidades galoiladas de los taninos, mayor es la sensación de astringencia (13, 14). Así se explicaría la elevada astringencia de los taninos de las semillas, que poseen mayor número de unidades galoiladas que los taninos del hollejo (2).

1.2. EL COLOR DEL VINO TINTO

El color es la carta de presentación de un vino tinto, no sólo porque es la primera e inmediata imagen, sino también porque es indicador de otros aspectos como su edad, estado de conservación, estructura, cuerpo o sabor en boca. Es la característica más inmediata que reconoce el consumidor y representa el elemento que, muchas veces, define la aceptación del vino a degustar.

El color del vino tinto, así como una parte importante de las características que definen su calidad, viene determinado por su composición fenólica. Los antocianos y sus pigmentos derivados (reacciones de copigmentación, polimerización y cicloadición) son los compuestos directamente responsables del color de los vinos tintos. En el caso de vinos jóvenes, el color dependerá básicamente de su composición en antocianos copigmentados. Los pigmentos que proceden de reacciones de polimerización y/o cicloadición, formados durante el proceso de vinificación, parecen ser los responsables de la coloración de vinos maduros y de crianza.

Así, el color de un vino tinto dependerá del grado de extracción de los antocianos y otros compuestos fenólicos de la uva al vino, pero también de las transformaciones que éstos sufran durante el proceso de vinificación y envejecimiento (Figura 9).

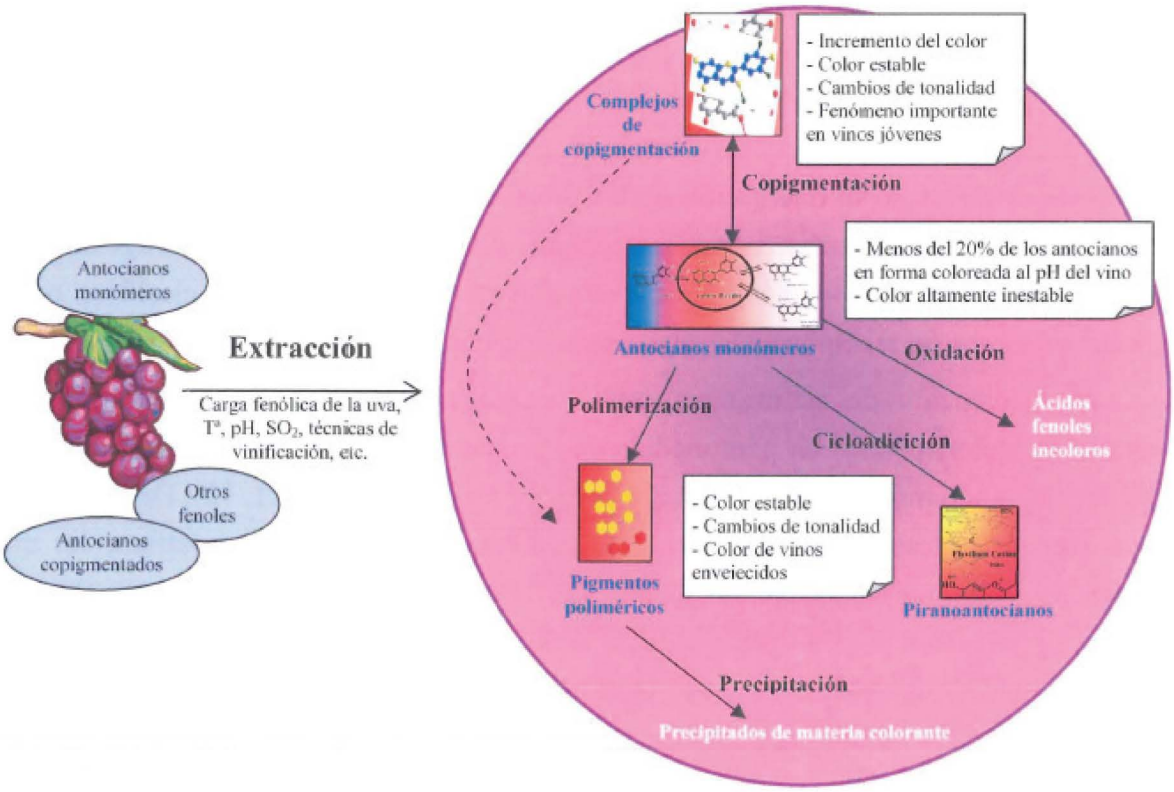


Figura 9. Fenómenos implicados en la extracción y evolución del color durante la vinificación y el envejecimiento del vino tinto

➢ *Extracción de los compuestos fenólicos*

Los cambios en los compuestos fenólicos que se producen durante la vinificación comienzan con una extracción selectiva de los mismos de la uva al vino durante el estrujado y posterior maceración. En el momento en que comienza el proceso de vinificación, los antocianos son extraídos desde los hollejos por ruptura de las células y las vacuolas, pasando rápidamente al mosto. De las células de las pieles se extraen además flavonoles tales como quercetina, kaempferol o miricetina, y de las de la pulpa, ácidos fenólicos, en especial ácidos hidroxicinámicos. Un proceso similar se aprecia en los taninos presentes en las vacuolas tánicas aunque requieren una concentración mayor de alcohol para su solubilización. Todos estos compuestos fenólicos contribuirán, como se verá más adelante, a la estabilidad del color de los vinos tintos.

El grado de extracción de los compuestos fenólicos definirá en primer término la extracción de compuestos coloreados durante la vinificación y su posterior evolución, así como la astringencia o cuerpo del vino y su capacidad para envejecer. En este sentido, son numerosos y muy diversos los ensayos y técnicas de vinificación encaminados a incrementar el porcentaje de extracción de compuestos fenólicos durante la vinificación (Tabla 1). Su objetivo es buscar la mayor extracción posible de los compuestos fenólicos deseables y evitar la cesión de otros indeseables, aunque lamentablemente esto no es fácil de lograr porque mayoritariamente producen un incremento en todo tipo de compuestos.

Tabla 1. Técnicas enológicas empleadas para aumentar el grado de extracción de compuestos fenólicos en vinos tintos (2, 15, 16, 17)

Prefermentativas	Fermentativas
– Disminución del pH del mosto – Aumento de la cantidad de SO_2 añadido – Variación de la temperatura previa a la fermentación: criomaceración, flash-expansión, maceración prefermentativa en frío, termovinificación – Adición de enzimas pectolíticas – Sangrado parcial, doble pasta	– Duración y temperatura de maceración – Tratamiento mecánico del sombrero: bazuqueo, remontado, inundación, sombreo sumergido, fermentadores rotatorios, delestage, fermentadores Ganimede, etc. – Variación de la temperatura posterior a la fermentación: maceración final en caliente – *Mezcla con primeras* fracciones de vino prensa de alta calidad

➢ Origen del color de los vinos tintos

Los compuestos primarios responsables del color del vino son los antocianos monómeros.

El color de los antocianos varia en función de su estado de equilibrio, dependiente del pH, y su combinación con otras moléculas, pudiendo presentar coloraciones rojas, malvas, amarillas o incoloras (Figura 10). Así, a pH muy ácido, la forma mayoritaria es el catión flavilio que presenta coloración roja. A medida que el pH aumenta, el catión flavilio se transforma en una base quinona de color azulado y en la forma carbinol que es incolora. Ambas reacciones ocurren simultáneamente de acuerdo con sus constantes de equilibrio. Por otra parte, a temperaturas elevadas, la forma carbinol puede transformarse en chalcona, cuya oxidación da lugar a ácidos fenoles incoloros. A diferencia del resto de reacciones, que son reversibles, la oxidación de las trans-chalconas implica una pérdida irreversible del color del vino, por lo que las temperaturas de conservación de los vinos tintos serán determinantes en términos de estabilidad de color (2). La presencia de anhídrido sulfuroso en los vinos tintos produce también una fuerte decoloración de los antocianos, que, mediante una reacción reversible, supone también una pérdida temporal de la intensidad de color.

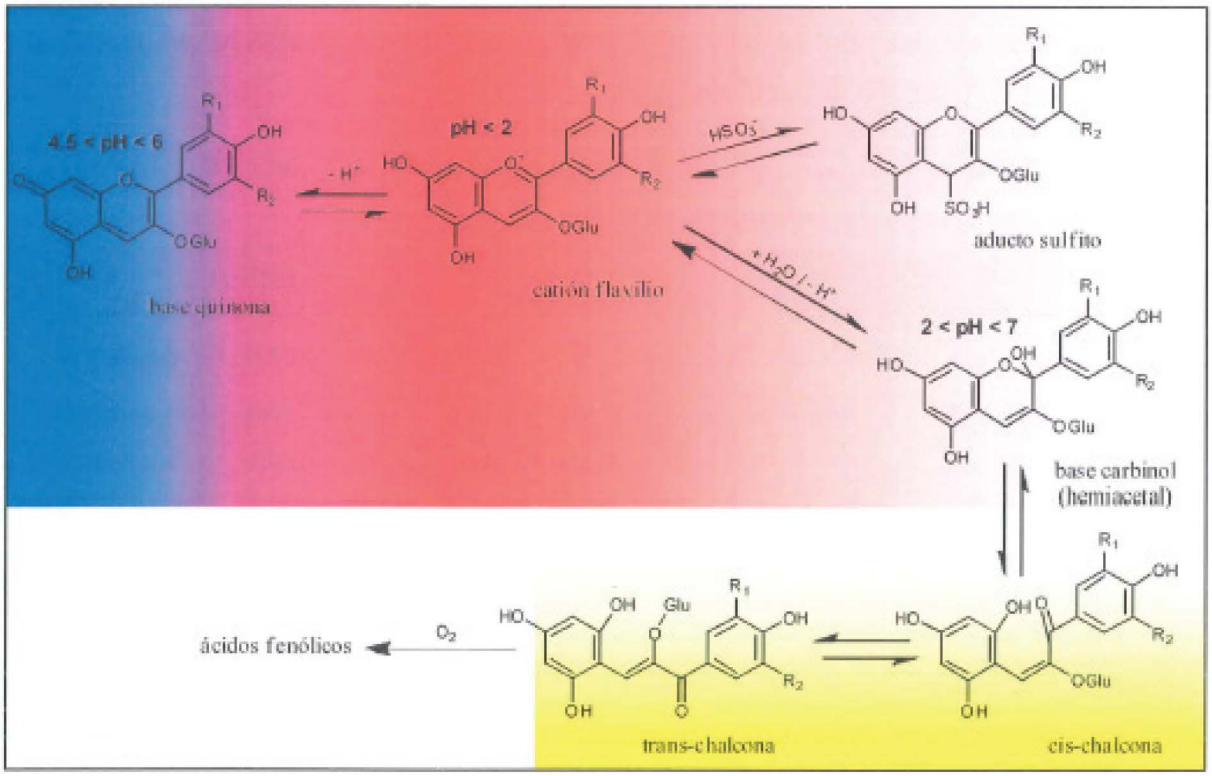


Figura 10. Equilibrio y coloración de los antocianos en el vino tinto

En resumen, a los valores de pH del vino tinto (3,5-4,1), existe un equilibrio entre las formas rojas, incoloras y azules de los antocianos, contribuyendo al color del vino sólo entre un 20 y un 30% de los antocianos. Dicho de otra manera, entre un 70 y 80% de los antocianos que podrían aportar color al vino, presentarían formas incoloras, a no ser que tenga lugar algún mecanismo que los estabilice. En efecto, la estabilidad de los antocianos no sólo depende del pH y la temperatura, sino también de la concentración y estructura de los mismos y de la presencia de otras moléculas con las que combinarse. Básicamente hay tres fenómenos que modifican este equilibrio y estabilizan el color: la copigmentación, las reacciones de polimerización y las reacciones de formación de nuevos pigmentos.

➢ *La Copigmentación*

El fenómeno de copigmentación se considera uno de los principales mecanismos de estabilización del color del vino tinto.

La copigmentación es un proceso que implica la asociación preferencial y no covalente entre las formas coloreadas de los antocianos y otros compuestos, normalmente no coloreados, denominados copigmentos o cofactores de copigmentación. Desde el punto de vista molecular, el fenómeno consiste en la formación de un complejo en el que un antociano con estructura plana se asocia con un copigmento, también de estructura plana, dando lugar a estructuras en forma de sándwich con un número de capas variable entre 2 y 10 (5). Su unión se mantiene por enlaces de baja energía, estableciéndose unas débiles interacciones entre los anillos aromáticos de las moléculas implicadas (Figura 11), y su estabilidad se debe a la presencia de glucosa en el antociano y a su disposición espacial hacia el exterior.

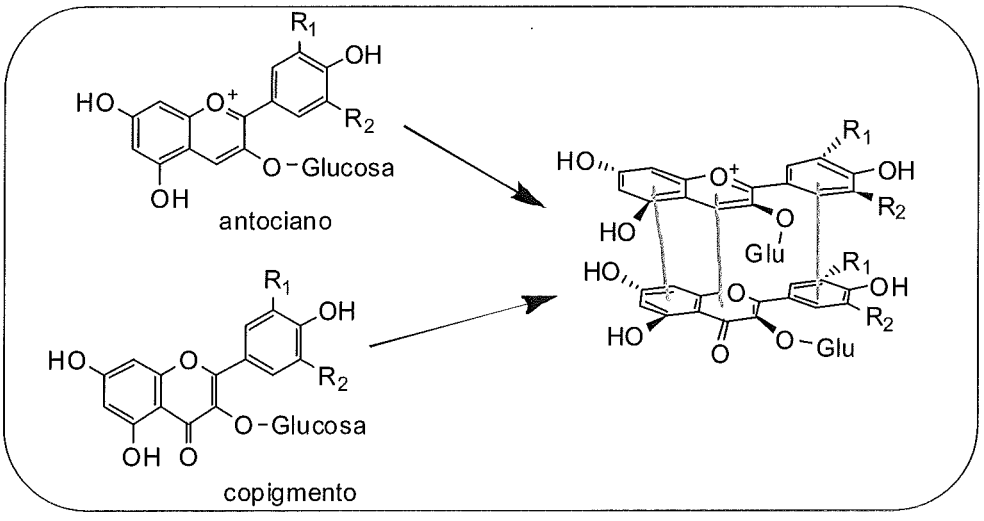


Figura 11. Formación de un complejo de copigmentación entre un antociano y un copigmento

Las agrupaciones tipo sándwich generan un entorno hidrofóbico que impide el acceso de las moléculas de agua a los antocianos. De esta forma se reduce la formación de bases hidratadas incoloras (carbinol), y se desplaza el equilibrio hacia la formación de estructuras coloreadas que no se decoloran con la presencia de bisulfito (Figura 12). Por lo tanto, un porcentaje mayor de antocianos del que correspondería de acuerdo con el pH del vino, contribuirá al color, siempre y cuando en el medio existan los copigmentos adecuados.

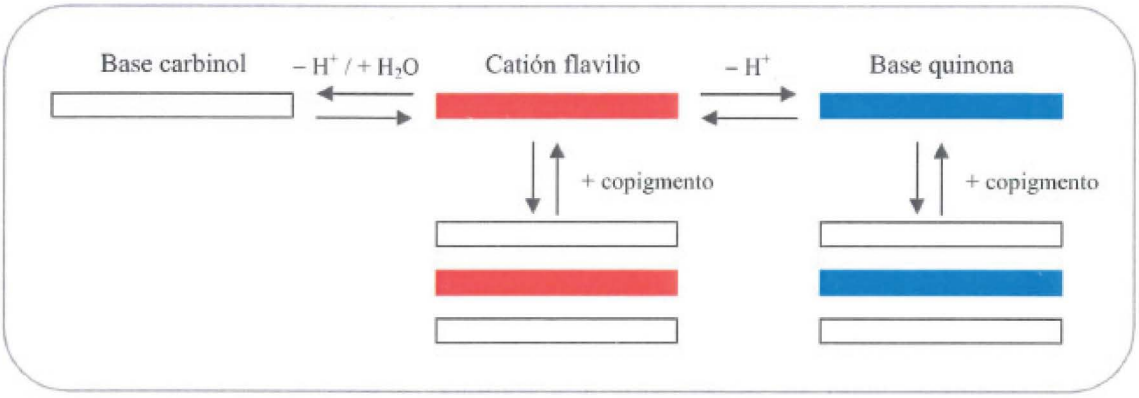


Figura 12. Principio del proceso de copigmentación

La formación del complejo de copigmentación no sólo incrementa el color del vino (efecto hipercrómico) sino que también puede modificar su tonalidad hacia tonos más azulados (efecto batocrómico) (18). Ambos efectos se suelen manifestar simultáneamente en los vinos tintos, sobre todo en los jóvenes, en diverso grado, según su composición fenólica y las relaciones molares pigmento/copigmento (19). De esta forma se explica la estabilidad del color en los vinos tintos jóvenes y su coloración púrpura.

Como copigmentos pueden actuar sustancias muy distintas, como polisacáridos, ácidos orgánicos, nucleótidos, aminoácidos y sobre todo flavanoides (20). La copigmentación se subdivide en tres categorías según la naturaleza del copigmento implicado:

a) *Copigmentación intermolecular*: el copigmento es una molécula distinta al antociano monómero, como una molécula de flavanol (7, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32).

b) *Copigmentación intramolecular*: el copigmento es una porción de la propia molécula de antociano, como el sustituyente cumaroilo de un antociano cumarílico (33, 34, 35).

c) *Autoasociación*: el copigmento es otra molécula de antociano monómero (36, 37, 38).

La estructura y la concentración tanto del antociano como del copigmento son esenciales para describir los procesos de copigmentación (6). El antociano formará estructuras más estables a mayor grado de glicosidación y de acilación (39). Los mejores copigmentos son los flavonoles (7, 22, 23, 24) pero se encuentran en pequeñas cantidades en las uvas y en los vinos. Otros copigmentos efectivos de naturaleza fenólica encontrados en los vinos son los ácidos hidroxicinámicos (7, 26, 28, 29) pero también se encuentran en pequeñas cantidades. Sin embargo, los compuestos fenólicos más abundantes en los vinos tintos, los flavanoles monómeros, son copigmentos menos efectivos (34) excepto la (-)-epicatequina, que por su estructura prácticamente plana forma copigmentos con los antocianos con gran facilidad (31).

Los complejos de copigmentación se forman ya en las bayas tintas durante la maduración, contribuyendo en gran medida a su color. A pesar de que parece que estas combinaciones se destruyen cuando la uva se airea en el estrujado (5), se forman de nuevo de forma fácil y rápida durante la maceración-fermentación. De hecho, los complejos de copigmentación se forman con mucha facilidad pero también se disocian fácilmente (17). La estabilidad de los copigmentos dependerá en gran medida de la temperatura, la fuerza iónica, el pH y de la presencia de determinados solventes en el medio (20). Así, una acidez suficiente con un nivel óptimo de pH de 3,3 facilitará la formación de copigmentos (5), pero la presencia de oxígeno y las temperaturas elevadas producirán la disociación de los complejos de copigmentación (5, 39). Además, el efecto de la copigmentación disminuirá a medida que vaya acumulándose etanol en el mosto/vino y disminuya la concentración de agua durante la fermentación (40).

En los vinos jóvenes la copigmentación envuelve prácticamente a la mitad de los antocianos (6, 31), protegiéndolos de la adición de bisulfito y los cambios de pH. En las etapas posteriores de crianza y envejecimiento de los vinos, los fenómenos de copigmentación se consideran prácticamente despreciables debido fundamentalmente a la transformación de los antocianos monómeros libres, necesarios para formar los complejos de copigmentación, en pigmentos poliméricos. Así, hay estudios que señalan que los complejos de copigmentación desaparecen totalmente después de 9 meses de embotellado (7, 31). No obstante, otros estudios concluyen que la copigmentación es un fenómeno aun relevante después de la crianza de los vinos en barrica (41), e incluso hay autores que observan valores de copigmentación del 20% en vinos obtenidos después de dos años de envejecimiento en botella (42). Diversos autores postulan además que la copigmentación es un paso previo a la formación de pigmentos poliméricos más estables ya que podría facilitar la unión covalente entre antocianos y flavanoles (25, 43, 44). De este modo los complejos de copigmentación constituirían un *reservorio* de antocianos, permitiendo que los antocianos extraídos permanezcan estables en disolución hasta que se formen los pigmentos poliméricos.

Resulta obvio entonces que una forma de incrementar tanto la intensidad del color del vino como su estabilidad consistiría en aumentar la copigmentación. Desde el ámbito de la enología, la estrategia consistiría en aumentar las relaciones molares de pigmento/copigmento. En este sentido se ha observado que la adición de copigmentos puros en la etapa prefermentativa (rutina, ácido cafeico o ácido cumárico) permite obtener vinos con mayor intensidad de color y color más estable (7). No obstante, esta posibilidad plantea no sólo problemas legales debido a la prohibición de su uso, sino fundamentalmente problemas de viabilidad económica. Así, sería interesante centrarse en otras estrategias que faciliten las reacciones de copigmentación en los vinos jóvenes y consigan que sus efectos sean más notables y duraderos. En este sentido parece importante conocer de forma exhaustiva no sólo los fenómenos que intervienen en la formación de los complejos de copigmentación, analizados fundamentalmente en medios sintéticos, sino también su estabilidad y evolución durante la vinificación y envejecimiento de los vinos.

➢ ***Formación de Pigmentos Poliméricos***

La crianza y el envejecimiento de los vinos tintos se caracterizan principalmente por una evolución armoniosa de los compuestos fenólicos que contienen, afectando en primer lugar a su color. Durante su envejecimiento, los vinos tintos van perdiendo intensidad en su color rojo, y la tonalidad va cambiando, pasando desde el rojo cereza vivo de los vinos jóvenes, hasta el rojo teja e incluso anaranjado de los vinos muy viejos. Por otro lado, mientras que los vinos jóvenes se decoloran en gran medida por el bisulfito, los vinos envejecidos apenas pierden color con este reactivo, y además, se ven menos afectados por los cambios de pH. Por estos motivos, se dice que el color del vino tinto se estabiliza durante el envejecimiento.

La evolución y estabilización del color de los vinos tintos durante su envejecimiento han sido clásicamente atribuidas a la formación de pigmentos poliméricos, por reacción entre los antocianos monómeros y los compuestos fenólicos mayoritarios de los vinos tintos, los taninos. La combinación de los antocianos con los taninos supondrá también una importante mejora del *gusto del vino*, aumentando las sensaciones de redondez y volumen en boca, y disminuyendo la astringencia y el amargor de los vinos recién elaborados.

En principio son posibles dos tipos de pigmentos poliméricos (Figura 13), uno en los que la molécula de antociano ocupa la posición inicial del polímero (pigmento tipo A-T), y otro en que los que el antociano es la parte final (pigmento tipo T-A). Se han descrito diferentes mecanismos de unión, bien de forma directa (Figura 13a y 13b), o mediada por acetaldehído (Figura 13c), que actúa de nexo de unión entre los antocianos

y los taninos y acelera así la formación de los pigmentos poliméricos. Estas combinaciones entre antocianos y taninos presentan coloraciones similares a las de los antocianos, presentando también un equilibrio entre las diferentes formas en función del pH. No obstante, las constantes de equilibrio son diferentes, lo que se traduce en que a un mismo pH, exista una mayor proporción de formas coloreadas (2).

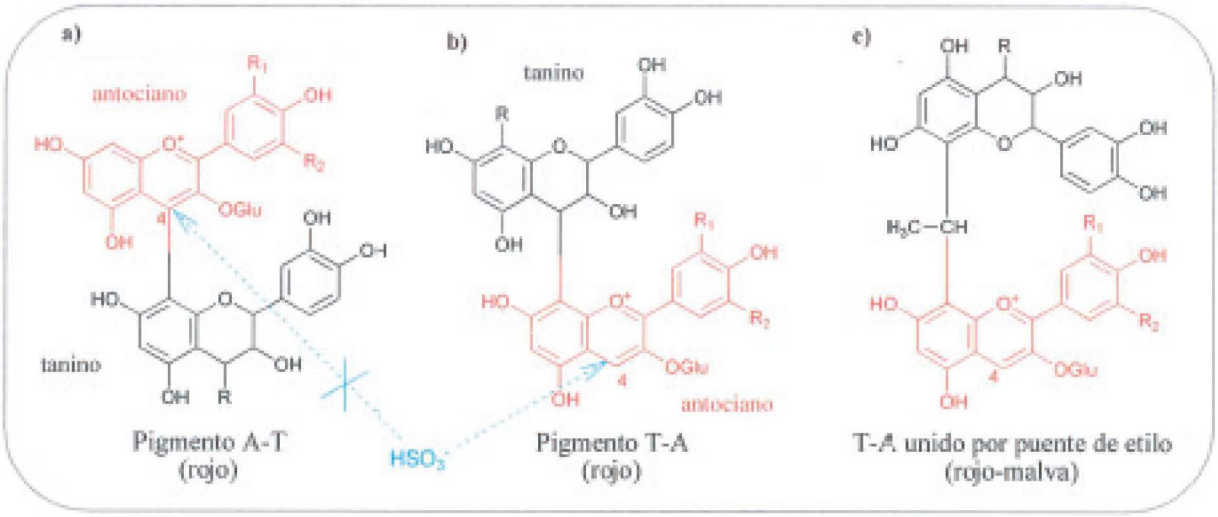


Figura 13. Estructuras de los pigmentos poliméricos resultantes de la unión entre los antocianos y los taninos

La formación de pigmentos poliméricos explica en gran medida todos los cambios de coloración que sufren los vinos tintos durante su crianza y envejecimiento. Así, la formación de estos compuestos permite explicar la pérdida de intensidad colorante del vino, ya que el mayor grado de polimerización implica también mayor grado de insolubilidad y, por tanto, de precipitación, formándose los depósitos de materia colorante observados en los vinos envejecidos. Como se ha explicado anteriormente, la polimerización de antocianos tiene además una relación directa con los fenómenos de copigmentación y, por lo tanto, con los cambios de tonalidad del vino. La desaparición de antocianos monoméricos libres para formar pigmentos poliméricos supone una disminución de las reacciones de copigmentación, produciéndose un descenso importante del color debido a la copigmentación en favor de un aumento del color debido a los antocianos poliméricos. Como consecuencia, se pierde el efecto hipercrómico y batocrómico asociado a la copigmentación, provocando una disminución de la intensidad colorante y una desaparición de las tonalidades púrpuras de los vinos tintos jóvenes. Por último, la formación de este tipo de compuestos también explica la estabilización de color. Tanto la decoloración por bisulfito como los cambios de color asociados a la variación de pH necesitan tener libre la posición C-4 del anillo pirano del antociano, que es por donde éste reacciona con el anión bisulfito o el anión hidroxilo del agua. En los pigmentos A-T está posición esta bloqueada por la unión

entre la molécula de antociano y el tanino, por lo que resultan estables; en cambio, los pigmentos T-A tienen esta posición libre y se pueden decolorar (Figura 13).

La formación de pigmentos poliméricos mediante puente de etilo compite en cierta forma con la polimerización natural de los taninos mediada por etanal, y con la formación de otras estructuras poliméricas también mediadas por este compuesto. El etanal o acetaldehído es un producto natural que se genera en los vinos tanto en la fermentación por el metabolismo de las levaduras, como en etapas posteriores de crianza por oxidación química del etanol (45), por lo que, aunque su disponibilidad no sea muy grande por la presencia de SO_2, existe en cantidad suficiente para participar en tales reacciones. Este compuesto, a través de puentes de etilo, sirve de puente de unión entre muchos polifenoles, bien entre compuestos de la misma familia o entre compuestos de familias distintas. La condensación entre unidades de antocianos no tiene lugar con la misma facilidad que con los flavonoles pero origina compuestos de color púrpura mucho más resistentes a la decoloración por hidratación y SO_2 que los antocianos libres (45). La condensación entre unidades de flavanoles tiene lugar fácilmente pero los polímeros formados son incoloros y se despolimerizan fácilmente, dando lugar a unidades reactivas de flavanol-etilo (vinilflavanol) que pueden volver a condensarse con los taninos o reaccionar con los antocianos (46). En el caso de que exista una gran disponibilidad de etanal, la polimerización proseguirá dando lugar a polímeros insolubles que precipitarán. Una aireación excesiva del vino producirá por tanto una pérdida de color importante ya que provocará por un lado, la oxidación directa de los antocianos, y, por otro, su polimerización excesiva y precipitación. Asimismo, las pérdidas de antocianos que se producen durante la fermentación alcohólica podrían ser atribuidas a la presencia de etanal como intermediario del metabolismo de las levaduras.

El que en un vino predominen un tipo u otro de las reacciones descritas anteriormente depende de su composición original. Si el vino de partida contiene una concentración de antocianos mucho mayor que la de flavanoles y proantocianidinas, la reacción que predominará será la degradación de antocianos por oxidación. Por el contrario, si la concentración de antocianos es mucho menor que la de flavanoles, la reacción que predominará será la de su polimerización, lo que se traducirá en un aumento de la coloración amarilla a lo largo de la crianza. Evidentemente si el vino contiene concentraciones de antocianos y flavanoles equivalentes, todas las reacciones serán igual de probables y la crianza conducirá a un vino de coloración estable y de características organolépticas muy equilibradas.

➢ *Formación de Piranoantocianos*

Recientemente se han identificado en el vino nuevos pigmentos derivados de los antocianos y conocidos con el nombre de piranoantocianos o vitisinas.

Los piranoantocianos se originan por reacciones de cicloadición de los antocianos con compuestos de bajo peso molecular, principalmente metabolitos producidos por las levaduras como el acetaldehído, ácido pirúvico o vinilfenol, formándose un nuevo anillo de pirano adicional. También se han identificado incorporaciones de otras moléculas como ácidos hidroxicinámicos u otras moléculas más voluminosas como los vinilflavanoles y sus derivados. Todas estas moléculas contienen un doble enlace polarizado, necesario para la reacción de cicloadición (Figura 14).

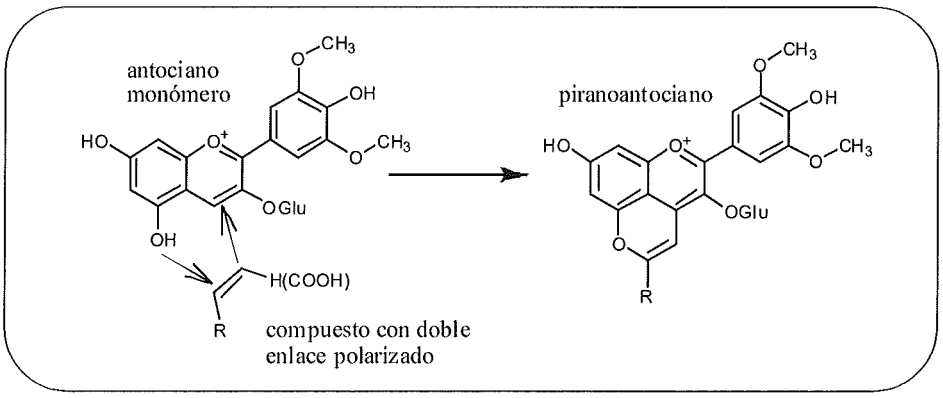


Figura 14. Mecanismo de formación de los piranoantocianos

Los piranoantocianos son pigmentos no decolorables por el bisulfito (44, 47, 48, 49) y bastante estables frente al pH (47, 50, 51), la degradación oxidativa (52) e incluso la temperatura (47), ya que también tienen bloqueada la posición 4 del antociano en el anillo pirano. A diferencia de los antocianos monómeros de los que derivan, estos pigmentos son de color rojo anaranjado, la misma tonalidad que suelen presentar los vinos envejecidos, y poseen unos cromóforos más débiles (53), otorgando al vino menos intensidad colorante. Estos compuestos no están presentes en la uva, sino que se forman durante la fermentación alcohólica y en las etapas posteriores de vinificación (50, 52).

La importancia real de la contribución de los piranoantocianos al color del vino tinto es todavía objeto de estudio. En principio, su concentración en el vino es mucho menor que la de otros pigmentos, pero dado que son poco sensibles al pH, la práctica totalidad de estos compuestos participa en el color del mismo, mientras que sólo una pequeña parte de otros pigmentos lo hará. Al contrario que los pigmentos poliméricos, que normalmente se encuentran en un estado coloidal en el vino y tienen tendencia a

precipitar, los piranoantocianos son moléculas de un tamaño fijo y similar a la de los antocianos monómeros de los que derivan y se mantienen disueltos en el vino, por lo que tendrán poca tendencia a perderse en los precipitados de materia colorante que se forman en los vinos envejecidos, o a quedarse retenidos en las superficies filtrantes por las que se pasan los vinos antes de su embotellado.

La Tabla 2 muestra un resumen de los principales piranoantocianos detectados en los vinos tintos y las moléculas implicadas en su formación.

Tabla 2. Principales piranoantocianos encontrados en los vinos tintos

Piranoantociano	Moléculas que lo forman
Vitisinas	→ malvidina 3-monoglucósido + ácido pirúvico (Vitisina A) (54), acetaldehído (Vitisina B) (48), vinilflavanol (50), vinilfenol (55) y otros (ácido a-cetoglutárico, ácido glioxílico, acetoina, acetona, diacetilo, formaldehído, benzaldehído, etc.) (56)
Pinotinas	→ antocianos + ácidos hidroxicinámicos y 4-vinilfenoles (57, 58, 59)
Portisinas	→ vitisina A + vinilflavanol (60) y vinilfenol (61)
Oaklinas	→ antocianos y/o catequinas + aldehídos cinámicos (62, 63)

1.3. POLISACÁRIDOS DE LA UVA Y DEL VINO

Los polisacáridos son uno de los principales grupos de macromoléculas presentes en los mostos y vinos. Conocidos desde hace tiempo por su efecto sobre la estabilidad coloidal de los vinos, son capaces de interaccionar con las partículas coloidales presentes en los mostos y vinos, reduciendo su reactividad y limitando su agregación y floculación. Así, estos compuestos juegan un papel fundamental tanto en las distintas etapas del proceso de vinificación como en las características organolépticas del producto final, aunque no todos los polisacáridos muestran el mismo comportamiento con respecto a los vinos, y su efecto concreto dependerá tanto de su tamaño como de su origen.

Los polisacáridos del vino son liberados durante el prensado y en el curso de la vinificación, y proceden tanto de las paredes celulares de la propia uva como de las levaduras y otros microorganismos que actúan durante el proceso de elaboración. Así, se originan familias de polisacáridos muy diversas tanto en su composición como en su estructura. La Figura 15 muestra una clasificación de los polisacáridos según su origen, si bien tanto su clasificación como su terminología (pectinas, gomas, sustancias pécticas ácidas, sustancias pécticas neutras, etc.) es confusa pues varía según los autores.

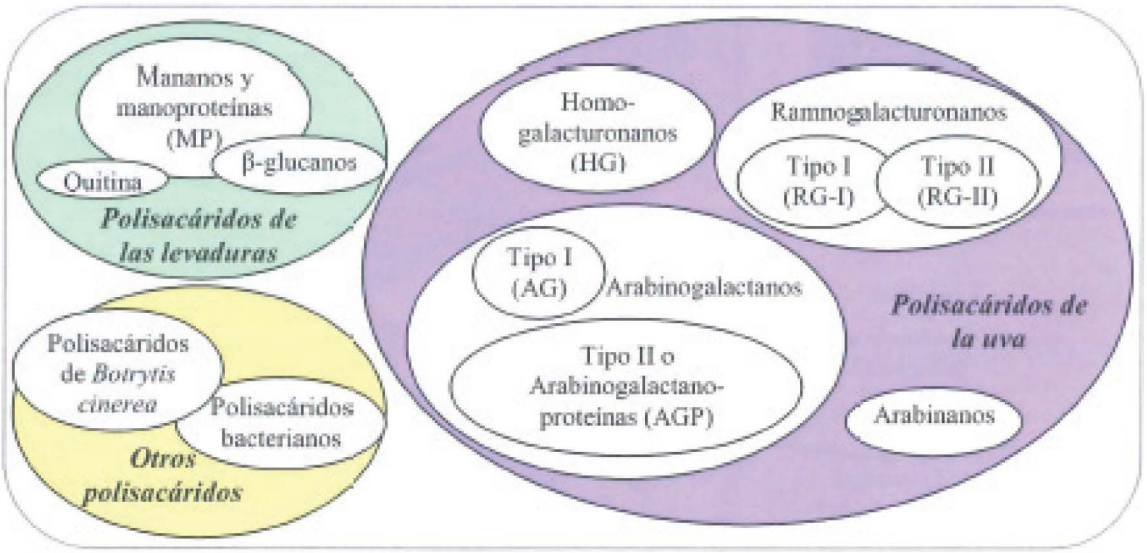


Figura 15. Clasificación de los principales polisacáridos del vino según su origen

Desde el punto de vista enológico los polisacáridos procedentes de la uva y de las levaduras son los más importantes tanto cuantitativa como cualitativamente. Los polisacáridos producidos por *Botrytis cinerea,* glucanos y botritizina, cobran relevancia cuando se produce una infección por dicho hongo, provocando clarificaciones y filtraciones particularmente difíciles. Los polisacáridos bacterianos están presentes en el vino en muy baja concentración.

➢ Polisacáridos procedentes de la uva

Los polisacáridos procedentes de la uva son el resultado de la degradación y de la solubilización de una parte de las sustancias pécticas contenidas en la pared de las células del hollejo y de la pulpa de la baya de uva. Debido a su origen muchos autores denominan a estos polisacáridos con el término de *sustancias pécticas*, aunque la división tradicional de las sustancias pécticas en gomas y pectinas puede conducir a error y ha quedado en desuso. Una terminología más reciente clasifica sencillamente a los polisacáridos de la uva en *sustancias pécticas neutras* y *sustancias pécticas ácidas* según contengan o no ácido galacturónico en su molécula. Así, los homogalacturonanos y ramnogalacturanonanos pertenecen al grupo de las sustancias pécticas ácidas, mientras que los arabinanos, galactanos o arabinogalactanos se engloban dentro de las sustancias pécticas neutras.

La Figura 16 muestra el corte de una baya y un esquema de la pared celular vegetal tipo I que rodea a las células de los tejidos vegetales de la pulpa y del hollejo de las bayas.

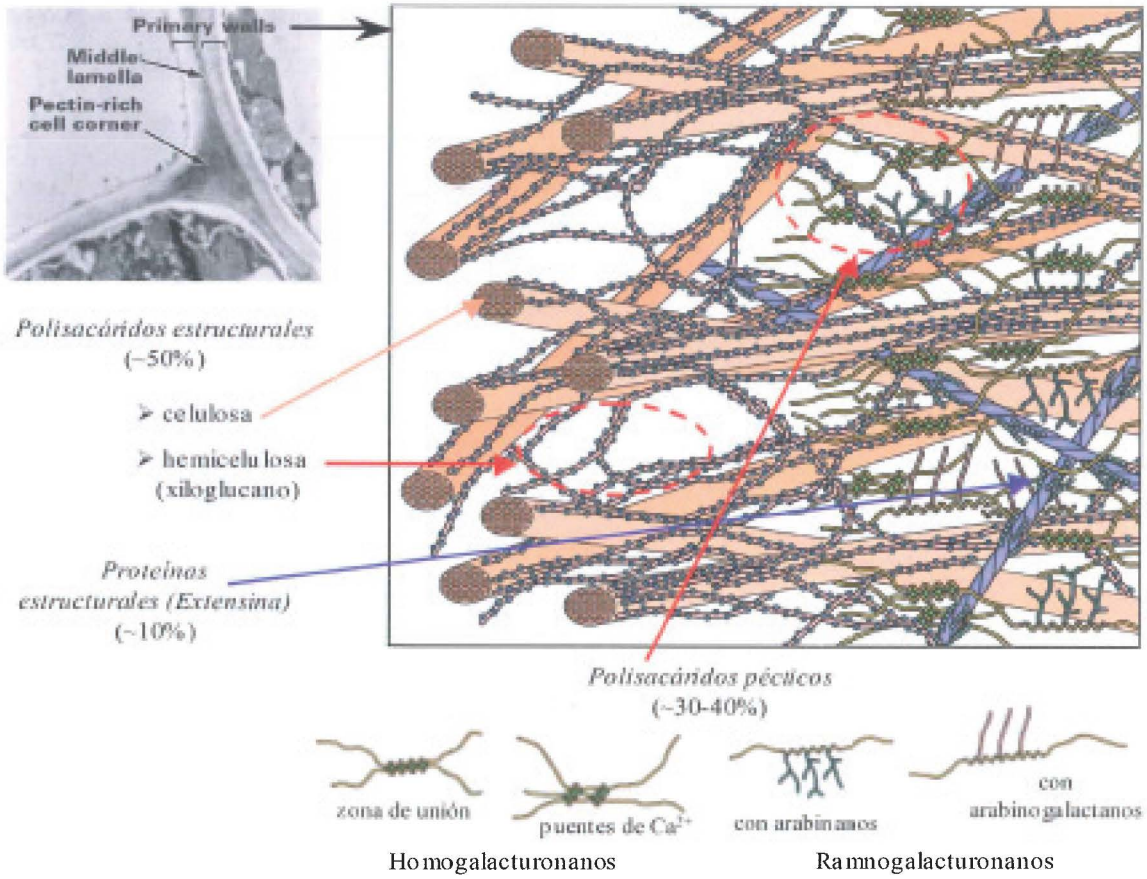


Figura 16. Esquema de la pared celular tipo I de las células vegetales

Esquema obtenido de Cárpita y Gibeaut (64). Se indica el porcentaje en peso de cada componente según estudios realizados en las paredes celulares de la uva (65)

Las paredes celulares de las bayas están formadas por una red de microfibrillas de celulosa entrelazadas por una red de xiloglucanos, embebidos en una matriz compleja de polisacáridos pécticos y glucoproteínas estructurales. La matriz péctica, que constituye aproximadamente un tercio de la pared celular (65), está formada por una mezcla compleja de homogalacturonanos (HG) y ramnogalacturonanos tipo I (RG-I) y tipo II (RG-II). El RG-I presenta a su vez ramificaciones laterales de arabinanos y arabinogalactanos (Figura 17). Estudios recientes señalan que la molécula de RG-I puede estar unida covalentemente a la red de xiloglucanos que componen las paredes celulares de la uva (66).

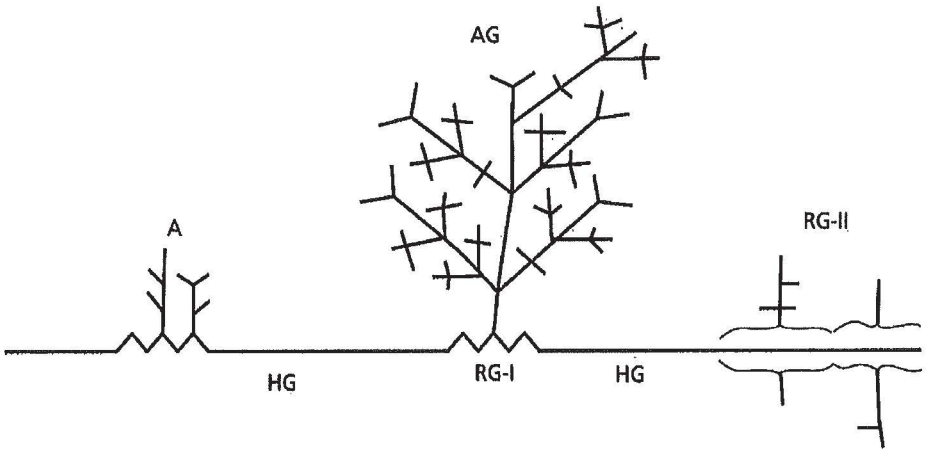


Figura 17. Modelo estructural de las sustancias pécticas ácidas de la uva
(Modelo obtenido de Doco y col. (67). A: arabinanos, HG: homogalacturonanos, RG-I: ramnogalacturonano I, RG-II: ramnogalacturonano II, AG: arabinogalactanos tipo I y tipo II)

Los ***Homogalacturonanos*** (HG) están formados por largas cadenas lineales de ácido galacturónico unidos por enlaces α-(1→4), que pueden estar esterificados con metanol y/o ácido acético (Figura 18). Las uniones osídicas α-(1→4) en la cadena de HG conducen a una estructura secundaria en hélice abierta, en la cual cada espiral está constituida por tres unidades de ácido galacturónico. El grado de metilación, que confiere a la pectina sus propiedades estructurales y funcionales, es elevado, del orden del 70 al 80% (5).

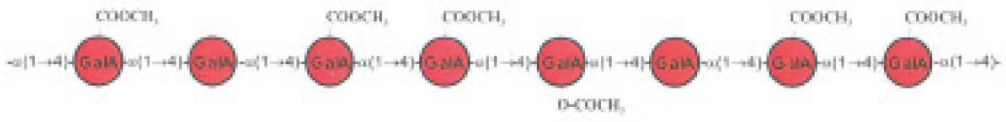


Figura 18. Estructura del Homogalacturonano (HG)
(GalA: ácido galacturónico)

Los homogalacturonanos son los polisacáridos pécticos más abundantes en las uvas, constituyendo el 80% de los polisacáridos pécticos presentes en las paredes celulares de la pulpa y los hollejos (16, 68).

Entre las cadenas lineales de los homogalacturonanos se insertan aleatoriamente estructuras de ***Ramnogalacturonano tipo I*** (RG-I), en las cuales unidades de ramnosa alternan con unidades de ácido galacturónico (Figura 19). La inserción de estos compuestos hace girar el eje de la hélice de HG en ángulo recto, formando un codo péctico. La cadena principal del RG-I lleva además cadenas laterales de polisacáridos neutros unidas al carbono 4 de la ramnosa (Figura 19), constituyendo estructuras altamente ramificadas. Así, las largas cadenas lineales de HG constituyen la *región lisa* de la pectina, mientras que las zonas ocupadas por el RG-I constituyen las *zonas erizadas* (Figura 17).

Los RG-I aislados de la uva presentan cadenas laterales constituidas principalmente por arabinanos y arabinogalactanos tipo II (68). Los RG-I aislados de los vinos, con un peso molecular entre 45.000 y 50.000 Dalton, contienen fundamentalmente arabinogalactanos tipo I y tipo II y sólo una pequeña proporción de arabinanos (66).

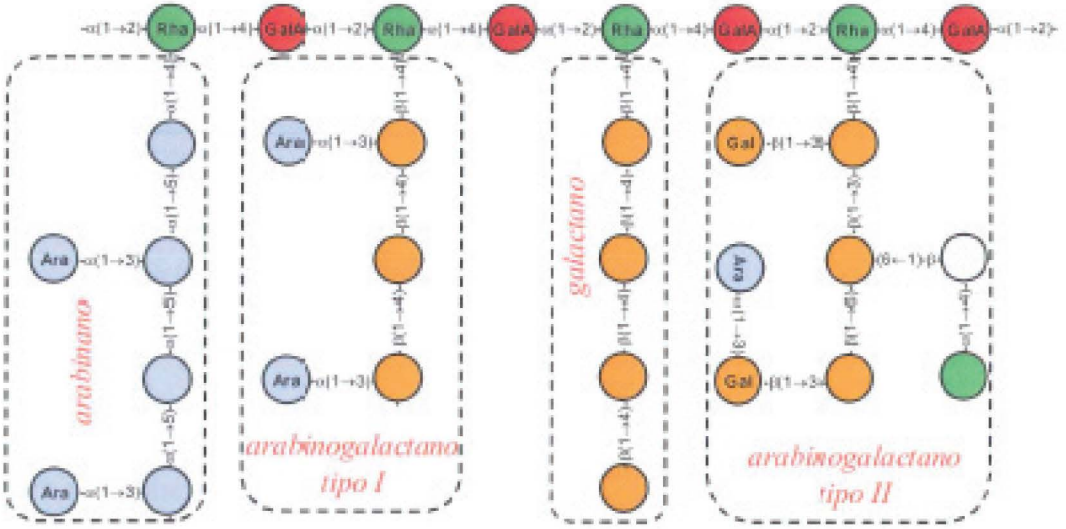


Figura 19. Estructura del Ramnogalacturonano tipo I (RG-I)
(GalA: ácido galacturónico, Rha: ramnosa, Ara: arabinosa, Gal: galactosa, GlcA: ácido glucurónico)

Los *arabinanos* son pequeños polímeros de aproximadamente 6.000 Dalton formados por una cadena lineal de L-arabinofuranosas unidas por enlaces α-(1→5), con un 30 a 40% de los residuos sustituidos con arabinofuranosas terminales en posición del carbono 3 (Figura 19). Las cadenas laterales de (1→4) β-D-galactano con residuos terminales de arabinosa en posición *O*-3 constituyen el *arabinogalactano tipo I* (Figura 19). Estos compuestos son muy abundantes en frutos como la manzana, encontrándose en menores cantidades en la uva (5). Los *arabinogalactanos tipo II* o *arabinogalactano-proteínas* poseen una estructura más compleja que se verá más adelante.

El ramnogalacturonano tipo I constituye aproximadamente el 15% de los polisacáridos pécticos de la uva (68).

El ***Ramnogalacturonano tipo II*** (RG-II) es un polisacárido péctico de estructura muy compleja y con una masa molecular muy pequeña, de aproximadamente 5.400 Dalton. Su estructura se encuentra muy conservada en las paredes celulares de todos los vegetales superiores. Está constituido por una cadena principal bastante corta de unidades de ácido galacturónico unidas por enlaces α-(1→4), unida a su vez a cuatro cadenas laterales de oligosacáridos que contienen arabinosa, ramnosa, fucosa, galactosa, ácidos galacturónico y glucurónico y también diferentes *azúcares raros* como 2-*O*-metil-fucosa, 2-*O*-metil-xilosa, apiosa, ácido acérico o 3-carboxi-5-deoxi-L-xilosa, Dha o ácido 3-deoxi-D-*liso*-2-heptulosónico, y Kdo o ácido 2-ceto-3-deoxi-D-mano-octulosónico (Figura 20). Dichos azúcares son exclusivos de la molécula de RG-II y se utilizan para su identificación y cuantificación (66, 69, 70).

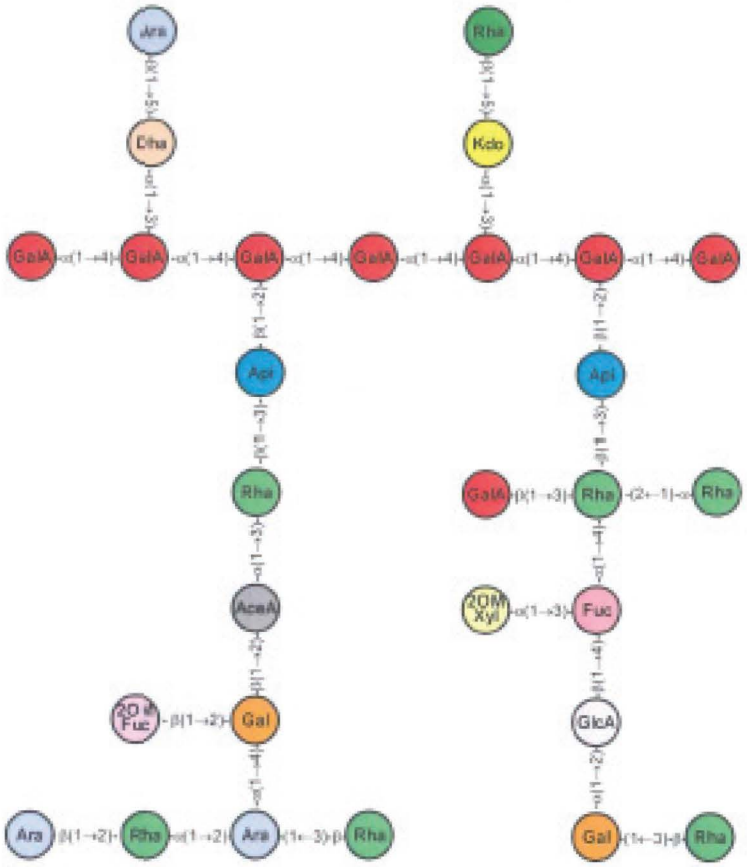


Figura 20. Estructura del Ramnogalacturonano tipo II (RG-II)

(GalA: ácido galacturónico, Rha: ramnosa, Ara: arabinosa, Gal: galactosa, GlcA: ácido glucurónico, Fuc: fucosa, AceA: ácido acérico, 2OMFuc: 2-*O*-metil-fucosa, 2OMXyl: 2-*O*-metil-xilosa, Api: apiosa, Dha: ácido 3-deoxi-D-*lixo*-2-heptulosónico, Kdo: ácido 2-ceto-3-deoxi-D-mano-octulosónico)

El RG-II, que representa menos del 5% de los polisacáridos de las paredes celulares de las uvas, suele encontrarse en la uva, en los mostos y en los vinos en forma de dímeros unidos por diésteres de ácido bórico (71, 72, 73, 74). Sin embargo, estudios recientes han detectado la forma monomérica del RG-II tanto en los mostos como en los vinos (66, 68, 75).

Los ***Arabinogalactanos tipo II*** son en realidad ***Arabinogalactano-proteínas*** (AGP). Son una clase de glucoproteínas que se encuentran formando parte del RG-I en las paredes celulares (Figura 19), aunque se localizan fundamentalmente en forma soluble en la membrana plasmática y en la matriz extracelular de las células (65), siendo su concentración mayor en la pulpa de la uva que en el hollejo (68).

La unidad núcleo del carbohidrato del AGP consiste en un esqueleto de β-D-galactano enlazado en (1→3), del cual se ramifican cadenas cortas de β-D-galactano en enlace (1→6). Estas cadenas están normalmente fuertemente saturadas con residuos de arabinosa y también con otros azúcares como xilosa, ramnosa, fucosa o ácido glucurónico (Figura 21). La estructura formada tienen aspecto de matorral debido a sus numerosas ramificaciones y forma por lo tanto la *zona erizada* de las sustancias pécticas (Figura 17). Desde un punto de vista estructural, los AGP aislados de las zonas erizadas de las sustancias pécticas ácidas están más ramificados y son más cortos que aquellos de las sustancias pécticas neutras (5).

Los AGP típicos aislados de los vinos, con una masa molecular entre 50.000 y 260.000 Dalton, contienen menos de un 5% de proteínas, y de sus azúcares, sólo entre un 3 y un 10% tienen carácter ácido (66, 69).

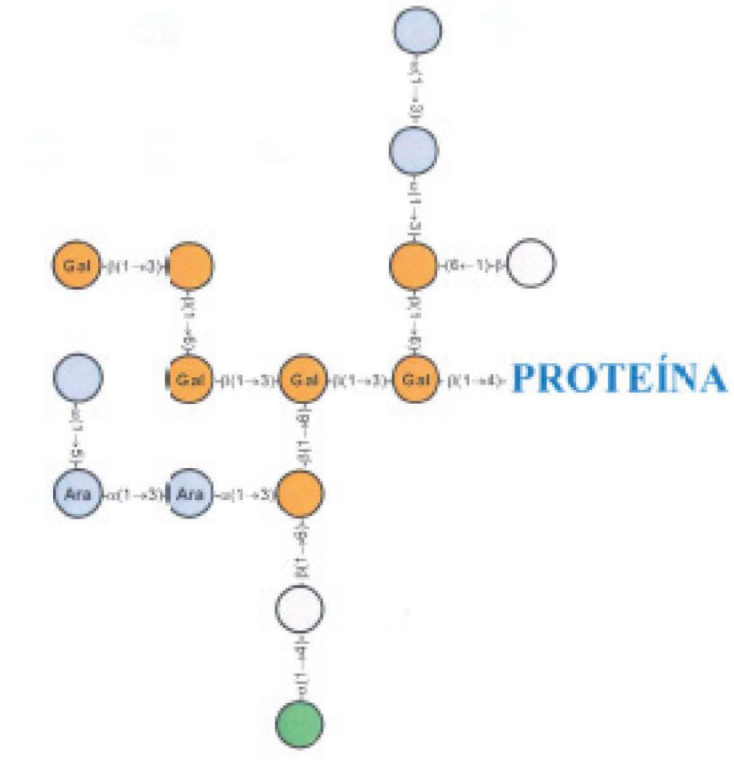


Figura 21. Estructura del Arabinogalactano tipo II o Arabinogalactano-proteína (AGP)
(Gal: galactosa, Ara: arabinosa, GlcA: ácido glucurónico, Rha: ramnosa)

Del mosto al vino las sustancias pécticas de la uva sufren modificaciones profundas debido a la acción de las pectinasas naturales de la uva o de las enzimas industriales exógenas agregadas durante la vinificación. Así, los homogalacturonanos, que son los compuestos mayoritarios en la uva (16, 68), se encuentran en cantidades importantes en los mostos (76), pero no se encuentran en los vinos (75, 77) pues son particularmente sensibles a su hidrólisis por las enzimas pectolíticas (68). Lo mismo ocurre con el ramnogalacturonano tipo I pero no así con el ramnogalacturonano tipo II. El RG-I, que constituye el polisacárido péctico más abundante en las uvas después de los HG (66), se encuentra en el vino en cantidades prácticamente despreciables (77), indicando una baja solubilidad o una degradación importante por las enzimas pectolíticas durante la vinificación. El RG-II es sin embargo muy resistente a la acción de las enzimas pectolíticas debido a las numerosas uniones osídicas raras que contiene, encontrándose por lo tanto intacto en los mostos y en los vinos (72). Así, aunque su concentración en la uva es tres veces menor que la del RG-I, se encuentra en cantidades importantes en los vinos, representado aproximadamente el 20% de los polisacáridos solubles del vino tinto (66, 78). Los AGP se encuentran en las paredes celulares vegetales en forma soluble (68), y por lo tanto son fácilmente extraíbles durante el prensado (76), representando el polisacárido más abundante de los vinos, con unos valores que alcanzan el 40% (66, 69).

- ***Polisacáridos procedentes de las levaduras***

La levaduras son la segunda fuente mayor de los polisacáridos del vino. Los polisacáridos procedentes de las levaduras están situados en su pared celular, que representa hasta un 25% de su peso seco (79). La composición concreta de la pared parece ser específica de la cepa de levadura y está compuesta por dos capas, una pared externa de carácter elástico formada por manoproteínas y β-1,6 glucanos, y una pared interna de carácter rígido formada por una red tridimensional de β-1,3 glucanos y quitina (Figura 22). Así, el 90% de la envoltura celular de las levaduras está constituida por polisacáridos, siendo el resto proteínas y lípidos. La pared celular se halla separada de la membrana plasmática por el llamado espacio periplasmático.

Los ***Glucanos*** representan el 50% del peso seco de la pared celular de las levaduras. Están compuestos por unidades de D-glucosa con uniones β-(1→3) y β-(1→6), siendo mayoritarios los β-1,3 glucanos (Figura 22). Estos últimos, con un peso molecular comprendido entre 25.000 y 270.00 Dalton (80), forman una red amorfa tridimensional que modula la rigidez y elasticidad de la pared celular y da forma a la levadura.

La ***Quitina***, componente minoritario de la pared celular, es un polímero lineal de N-acetil-glucosamina que se encuentra unido covalentemente a la malla de β-1,3 glucanos.

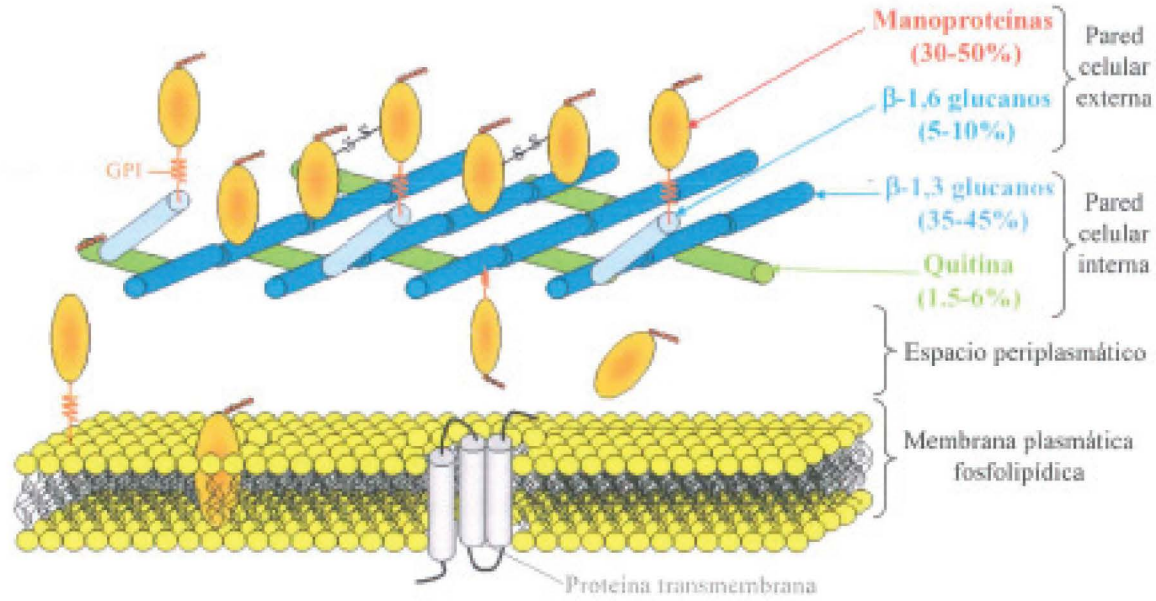


Figura 22. Esquema de la envoltura celular de las levaduras

Esquema obtenido de Molina y col. (81). Se indica el porcentaje en peso de cada componente según datos obtenidos por Klis y col. (80)

Las ***Manoproteínas*** de levadura son los compuestos más interesantes desde el punto de vista enológico. Representan del 30 al 50% de la pared celular (80) y están unidas covalentemente a la malla de β-1,3 glucanos, bien de forma directa, y fundamentalmente de forma indirecta a través de los β-1,6 glucanos (80). La pared celular de las levaduras contiene también una pequeña proporción de manoproteínas unidas de forma no covalente; son las denominadas *manoproteínas de excreción,* que se sitúan en la superficie celular, concretamente en el espacio periplasmático, al igual que muchas enzimas glicolíticas de levaduras (82).

Desde el punto de vista químico, las manoproteínas son glicoproteínas, normalmente con alto grado de glicosidación (80-90%), compuestas mayoritariamente por manosa (>90%) y glucosa.

Se pueden distinguir en el vino dos tipos de manoproteínas:

a) manoproteínas secretadas durante la fase de crecimiento exponencial de las levaduras y que se acumulan durante la fermentación (83, 84), y

b) manoproteínas liberadas por la autolisis celular de las levaduras durante la crianza del vino sobre lías (85).

La estructura molecular general de las manoproteínas exocelulares de levadura es similar a la de las manoproteínas localizadas en la pared. Poseen una estructura tridimensional basada en un núcleo proteico con dos tipos de cadenas glicánicas: cadenas cortas de manosa unidas a la parte proteica a nivel de residuos de serina o treonina, y cadenas largas polimanosídicas ramificadas con cadenas laterales de manosa que se enlazan a la parte peptídica por intermediación de una N-acetil-glucosamina unida a un residuo de asparragina (Figura 23). En el género *Saccharomyces*, la fracción glicánica de las manoproteínas posee también residuos glicosilados ácidos (86).

Las manoproteínas presentan tamaños moleculares muy variables, desde 5.000 hasta más de 800.000 Dalton (87), y su carga eléctrica varia según el pH del medio. En el rango de pH del vino las manoproteínas están cargadas negativamente, pudiendo establecer interacciones electrostáticas e iónicas con otros componentes del vino (88).

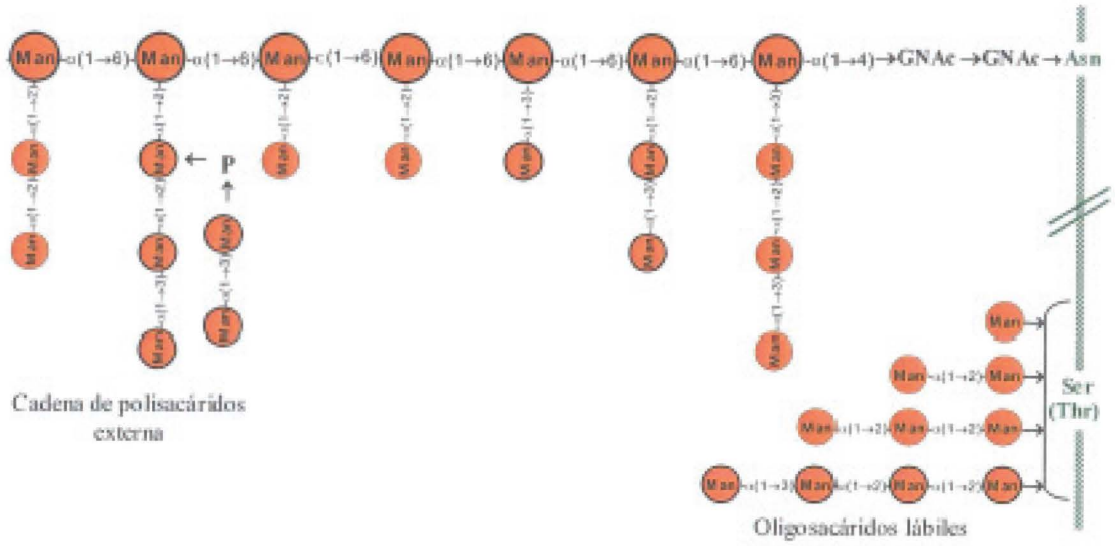


Figura 23. Estructura de las manoproteínas exocelulares de levadura (83, 87, 89)
(Man: manosa, P: fosfato, GNAc: N-acetil-glucosamina, Asn: asparragina, Ser: serina, Thr: treonina)

La cantidad de manoproteínas liberadas por la levadura durante la fermentación depende de la cepa de levadura utilizada (90, 91), de la temperatura de fermentación (67) y del nivel de macromoléculas del mosto (intensidad de la clarificación) (92, 93). La levadura libera más polisacáridos a medida que la temperatura se eleva, el medio se agita y se prolonga la conservación con la biomasa. La mayor parte de manoproteínas liberadas durante la fermentación se excreta, pues no se usa para formar paredes celulares.

Las manoproteínas liberadas en el curso de la autolisis se deben al efecto de la degradación enzimática de la pared de las células de levaduras muertas. Así, la hidrólisis de los glucanos por la acción de enzimas parietales β-1,3 glucanasas conduce

a una liberación de las manoproteínas de alto peso molecular ancladas sobre el β-glucano de las paredes celulares. Dichas manoproteínas pueden a su vez ser hidrolizadas por proteasas y α-manosidasas, liberandose pequeños polipéptidos al vino. Todas estas actividades enzimáticas son importantes durante la fermentación alcohólica y se mantienen durante varios meses después de la muerte de la célula (67). De este modo, el enriquecimiento de los vinos tintos en coloides procedentes de la autolisis de levaduras ocurre esencialmente durante la maceración postfermentativa, donde la temperatura aun es elevada (30-35 ºC). Tras este periodo la liberación de manoproteínas es muy limitada ya que la mayoría de las lías se separan del vino en el momento del descube. En el caso de la vinificación sobre lías, los polisacáridos de levaduras son también liberados durante la crianza sobre lías aunque el fenómeno de liberación es más lento porque la temperatura de conservación es baja (12-16 ºC).

Las manoproteínas de levadura constituyen la segunda familia de polisacáridos más abundante de los vinos tintos, llegando a constituir más del 30% de los polisacáridos totales solubles (66, 75).

➢ *Interés enológico de los polisacáridos de la uva y del vino*

Como se ha comentado anteriormente, los polisacáridos presentes en los mostos y en los vinos juegan un papel decisivo en las distintas etapas de vinificación y en las propiedades sensoriales de los vinos. De hecho, la influencia de los polisacáridos en dichos procesos ha sido objeto de numerosos estudios, realizados tanto en medios sintéticos como en situaciones reales de vinificación.

El rol de los coloides glucídicos sobre la estabilidad de los vinos ha sido muy estudiado. Numerosos trabajos analizan el efecto protector de los polisacáridos sobre las precipitaciones tartáricas y proteicas (77, 94, 95, 96, 97) así como su influencia en los procesos de filtración y clarificación (67, 98). Otros estudios atribuyen a los polisacáridos un papel importante en la modulación de las percepciones aromáticas de los vinos a través de la estabilización de ciertos aromas (99, 100, 101). Estudios más recientes señalan además que ciertos polisacáridos son capaces de interaccionar con los compuestos fenólicos y reducir su reactividad, consiguiendo un efecto estabilizador en el color de los vinos tintos y una modificación de sus propiedades gustativas (102, 103).

No todos los polisacáridos muestran el mismo rol en el proceso de elaboración de los vinos. De hecho, la influencia de los polisacáridos en las distintas etapas de vinificación dependerá de su concentración en los mostos y vinos pero fundamentalmente de su estructura y composición. Además, el efecto de dichos compuestos en las características sensoriales de los vinos también variará según el tipo

de polisacárido implicado. En particular, los arabinogalactanos tienen más influencia en los procesos de filtración de los vinos que las manoproteínas procedentes de las levaduras (67), que son sin embargo más eficaces contra la quiebra proteica (67). Los ramnogalacturonanos I y II y algunas manoproteínas son inhibidores de la cristalización de las sales tartáricas en los vinos (96), fenómeno sobre el cual los AGP no tienen efecto alguno (67). Además, las manoproteínas excretadas por las levaduras durante la fermentación alcohólica no poseen las mismas propiedades que las procedentes de la autolisis, siendo estas últimas las que poseen efectos protectores frente a los enturbiamientos proteicos y tartáricos (5). En relación a su interacción con los compuestos fenólicos de los vinos, los RG-II dímeros favorecen la autoagregación de los taninos en medio sintético mientras que las manoproteínas la limitan y los RG-II monómeros y AGP neutros no muestran ningún efecto (102), lo que se traduce en diferentes efectos sobre las propiedades gustativas de los vinos (103). Además, las manoproteínas de levadura están implicadas en la estabilización de ciertos aromas (100, 101) y hay autores que también las relacionan con la estabilización del color en los vinos tintos (91, 104, 105). Finalmente, la presencia de ciertos polisacáridos en los vinos podría tener efectos beneficiosos para la salud al eliminar compuestos tóxicos para el organismo. En particular, los dímeros de RG-II pueden formar complejos de coordinación con los iones plomo, de tal forma que la mayoría del plomo dosificado en los vinos se encuentra bajo la forma de complejos estables con el RG-II (77). La red de β-glucanos de las levaduras es capaz de asociarse a las micotoxinas del vino y eliminarlas (106), y diversos estudios señalan el papel de las manoproteínas en la eliminación de la ocratoxina A en mostos y vinos (107).

Aunque es obvio que cada polisacárido presenta propiedades biotecnológicas interesantes, son las manoproteínas de levadura las que despiertan mayor interés enológico. Se trata de compuestos más estudiados y conocidos que los polisacáridos procedentes de la uva, tanto en su estructura y composición como en sus funciones y aplicaciones tecnológicas. Además, son compuestos fáciles de producir y hace ya tiempo que se comercializan bajo diversas fórmulas.

➢ ***Manoproteínas. Interés enológico y aplicaciones tecnológicas***

Las manoproteínas de levadura juegan un papel muy importante en el proceso de elaboración de los vinos tintos y en sus propiedades sensoriales. Según parece las manoproteínas actúan de la siguiente manera:

- Activan la fermentación maloláctica

Diversos estudios señalan que las manoproteínas parietales de levadura estimulan el crecimiento de las bacterias responsables de la fermentación maloláctica en los vinos (90, 92, 108). Así, las manoproteínas adsorberían ciertos ácidos grasos que resultan tóxicos para dichas bacterias e inhiben su crecimiento (109). La eliminación del medio de estos ácidos sintetizados por *Saccharomyces cerevisiae* supondría por tanto una activación de la fermentación maloláctica.

- Mejoran la estabilidad tartárica

El efecto de las manoproteínas de levadura en la estabilidad térmica de los vinos blancos fue la primera propiedad enológica descrita para dichas moléculas. Es de sobra conocido que tras unos meses de crianza del vino blanco sobre lías, el tartrato se vuelve estable y no requiere de la estabilización por frío. Esto es debido a las manoproteínas, que inhiben la formación de cristales de bitartrato potásico al disminuir su temperatura de cristalización (110, 111). Así, la adición de manoproteínas retarda en más de 30 meses la aparición de cristales en vinos tintos, blancos y rosados almacenados a – 4ºC, mostrando un efecto más duradero que el ácido metatartárico usado habitualmente para su estabilización (111).

- Mejoran la estabilidad proteica

La crianza de vinos blancos sobre lías supone también una mejora considerable en términos de estabilidad proteica, hecho de nuevo atribuido a la presencia de las manoproteínas cedidas por las lías (85). De hecho, se ha observado que algunas manoproteínas tienen una influencia notable sobre la estabilidad proteica de vinos blancos y rosados (85). No está claro el mecanismo implicado en favorecer la estabilidad proteica, pero hay trabajos que establecen que las manoproteínas no evitan la precipitación de proteínas en el vino, pero sí que disminuyen el tamaño de las partículas responsables de la turbidez (94), siendo además las manoproteínas liberadas durante la fermentación las que mayor efecto protector tienen (112). La presencia y/o la adición de manoproteínas hace que sea necesaria una menor adición de bentonita para la estabilización de los vinos (67), consiguiendo de esta manera una menor pérdida de moléculas aromáticas y otros coloides del vino.

Por las razones expuestas anteriormente, los productos a base de manoproteínas han sido autorizados recientemente por la OIV, pero sólo para la estabilización tartárica o proteica de los vinos (Resolución Oeno 4/2001, Oeno 15/2005, Reglamento CE 2165/2005) (113).

- Participan en la formación del velo en la crianza en flor

Ciertas manoproteínas parecen estar implicadas en la formación del velo durante la crianza de los vinos en flor (114), típica en la elaboración de los vinos de Jerez.

- Mejoran las características espumantes

Recientemente se ha visto que el empleo de un coadyuvante liberador de manoproteínas en la elaboración de un cava y un Champagne mejoró sustancialmente sus características espumantes (115).

- Mejoran la persistencia aromática

Otro efecto descrito para las manoproteínas es el de retener aromas e incrementar por tanto la persistencia aromática de los vinos (100). Así, se ha observado que una clarificación y estabilización excesiva del vino, cuando la eliminación de macromoléculas alcanza el 30%, ejerce una influencia negativa en las propiedades sensoriales del mismo, produciéndose una pérdida aromática importante (116). Parece ser que la estabilización del aroma del vino por las manoproteínas depende del carácter hidrofóbico de los compuestos aromáticos, siendo el componente proteico de la fracción de manoproteína de gran importancia para la estabilización global del aroma (117).

- Disminuyen la astringencia de los vinos tintos y aumentan su untuosidad y cuerpo

Diversos estudios han mostrado que las manoproteínas de levadura pueden unirse con los compuestos fenólicos y controlar su estabilidad coloidal. Así, se ha observado que las manoproteínas pueden interaccionar con los taninos de los vinos tintos y disminuir su reactividad, lo que se traduce en una disminución de la astringencia de los vinos (91). Estos trabajos señalan además que dicho efecto parece depender de la cepa de levadura implicada, así como del momento de su liberación, siendo las manoproteínas liberadas durante la fermentación alcohólica más efectivas que aquellas procedentes de la autolisis (91).

En la Figura 24 se muestra el mecanismo propuesto para explicar la razón por la cual las manoproteínas actúan disminuyendo la astringencia de los vinos (118). Básicamente se postula que los taninos, en ausencia de interacciones con las manoproteínas y/o los polisacáridos, presentan una gran reactividad por las proteínas de la saliva. Por el contrario, en presencia de interacciones con manoproteínas y/o polisacáridos, se

agrupan formando monoestructuras estables, que al no poder reaccionar con las proteínas de la saliva, no generarían sensación de astringencia. Según este modelo, en las asociaciones entre taninos y polisacáridos estarían implicadas interacciones hidrofóbicas, no específicas y de tipo Van der Waals.

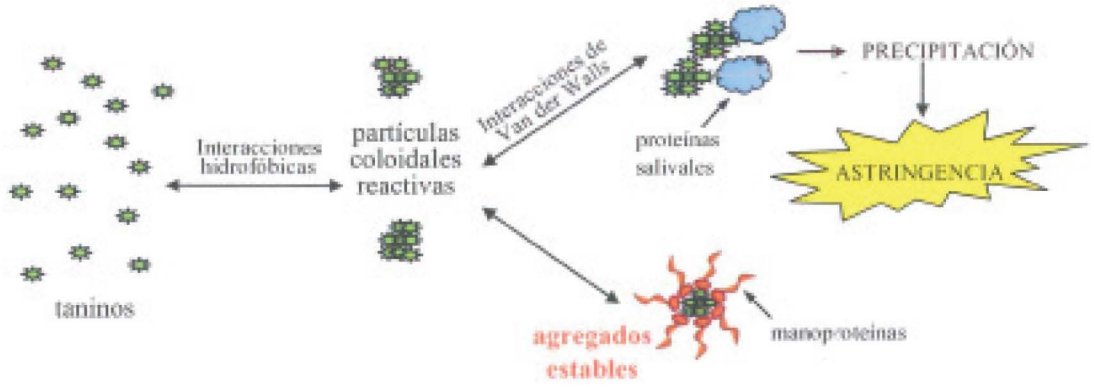


Figura 24. Modelo de la acción de las manoproteínas sobre los taninos del vino (118)

Trabajos recientes analizan el efecto de las manoproteínas, los RG-II y los AGP en disoluciones modelo que contienen taninos de semillas, concluyendo que sólo las manoproteínas tienen habilidad para prevenir la agregación de los taninos (102). Los mecanismos implicados en el efecto protector de las manoproteínas no están claros pero se proponen dos posibles hipótesis: una primera basada en la asociación molecular entre polisacáridos y polifenoles compitiendo con la agregación de los taninos, y una segunda basada en la adsorción de los polisacáridos sobre las partículas coloidales formadas por los taninos, previniendo el incremento de tamaño de la partícula, siendo esta segunda hipótesis más probable ya que observaron que los polisacáridos limitaban el crecimiento de las partículas de tanino y no su formación. La figura 25 muestra un esquema de este posible mecanismo.

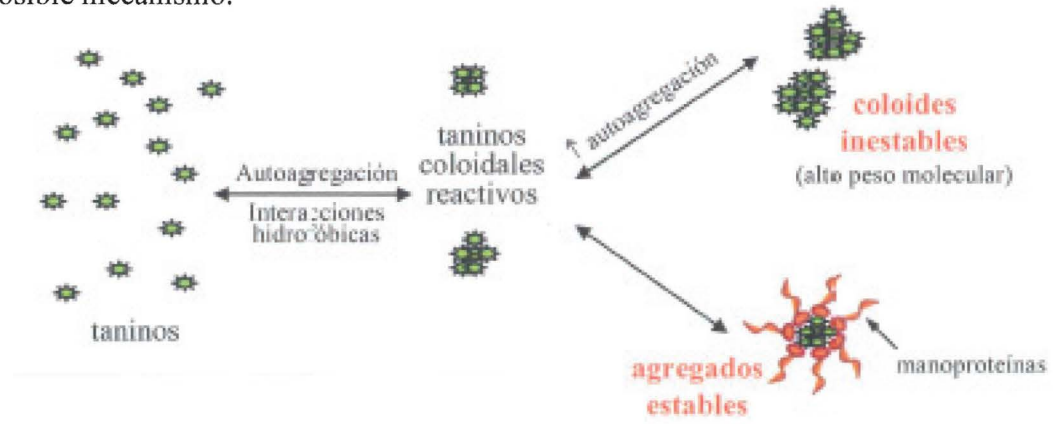


Figura 25. Esquema de la interacción tanino-manoproteína en medio sintético (102)

La estabilización de las partículas de tanino por las manoproteínas parece además ser dependiente del tipo de manoproteína implicada así como de la concentración de etanol y de la fuerza iónica del medio. De hecho, sólo las fracciones de manoproteína de bajo peso molecular (50.000-60.000 Dalton) muestran este efecto protector en las condiciones estándares del vino (12% de etanol, 2 g/L de ácido tartárico, pH 3,4), mientras que las de alto peso molecular no muestran efecto alguno (119). Estos autores sugieren además que el mecanismo implicado en la interacción tanino-manoproteína es de tipo estérico y no electrostático.

En resumen, y sea cual sea el mecanismo implicado, la formación de complejos manoproteína-tanino disminuye la reactividad de los taninos, impidiendo de esta manera su polimerización y precipitación con las proteínas salivales, lo que se traduce en una disminución de la astringencia de los vinos tintos y un aumento de su redondez, untuosidad y volumen en boca (101, 103, 118).

- *Mejoran la estabilidad colorante de los vinos tintos*

De todas las propiedades atribuidas a las manoproteínas, es quizás su influencia en el color del vino el aspecto menos conocido. Todas las casas comerciales distribuidoras de productos a base de manoproteínas atribuyen a dichos compuestos un efecto beneficioso en la estabilización del color de los vinos tintos. De hecho, las manoproteínas son tratadas en la bibliografía como *moléculas estabilizadoras del color,* aunque en la mayoría de los casos no se presentan evidencias que avalen dichas afirmaciones.

Escot y col. (91) analizaron el efecto de la adición de manoproteínas comerciales y de levaduras súper-productoras de manoproteínas en el color de vinos tintos de Pinot Noir y no observaron ninguna diferencia con los controles en ninguno de los parámetros de color analizados (intensidad colorante, tonalidad, componentes del color, limpidez y saturación). No obstante sí que observaron un aumento del índice de PVPP en algunos de los vinos elaborados con adición de manoproteínas, lo que indicaría un posible aumento de los antocianos poliméricos, y por tanto del color estable. La estabilización del color se produciría por medio de interacciones manoproteína-antociano polimérico, con un mecanismo similar al que se produce en la estabilización manoproteína-tanino. Así, la formación de los complejos manoproteína-pigmento polimérico disminuiría la reactividad de los pigmentos poliméricos, impidiendo de esta manera que aumente su polimerización y precipiten, y evitando así la pérdida de materia colorante inestable (Figura 26).

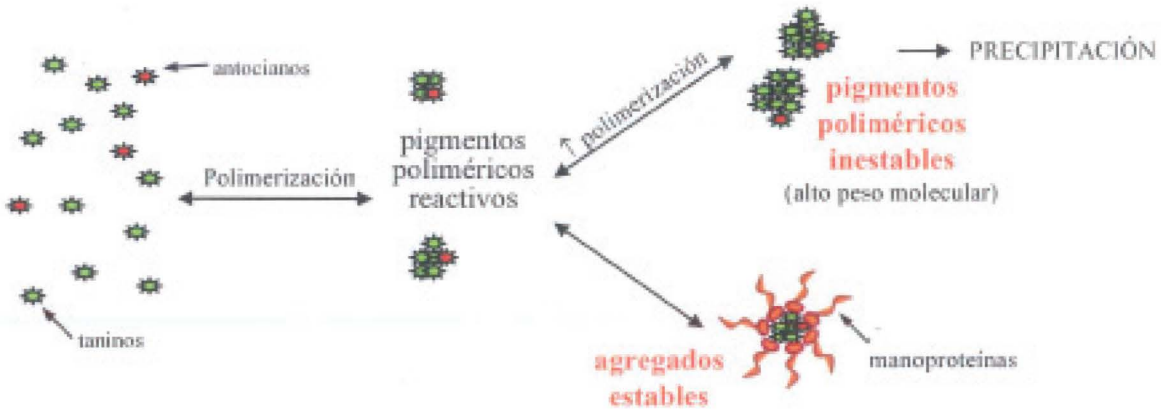


Figura 26. Modelo de actuación de las manoproteínas sobre el color estable del vino

A pesar de esta hipótesis alentadora, es importante tener en cuenta que no hay estudios que encuentren una relación directa entre las manoproteínas y el color del vino tinto, por lo que serían necesarios más ensayos para establecer si existe relación entre las manoproteínas de levadura y el color de los vinos.

De todo lo expuesto anteriormente se deduce que las manoproteínas pueden ejercer múltiples efectos positivos en la elaboración del vino tinto. No obstante, es su efecto sobre las características sensoriales de los vinos tintos lo que está fomentando su uso en las empresas vitivinícolas. Hoy por hoy, todas las empresas suministradoras de productos enológicos comercializan uno o varios productos a base de manoproteínas. El objetivo es claro. Se trata de obtener un producto que al adicionarlo al vino permita obtener todas las ventajas antes señaladas. Hay quien incluso postula que estos productos podrían constituir una alternativa a la tradicional crianza sobre lías.

La crianza del vino blanco sobre lías es una técnica bastante común en muchas regiones vinícolas. Recientemente se ha comenzado a realizar la crianza de los vinos tintos sobre lías buscando mejorar sus atributos sensoriales y estabilidad fisico-química. La crianza sobre lías origina la liberación de manoproteínas procedentes de las envolturas celulares de las levaduras muertas, y es precisamente el enriquecimiento del vino en manoproteínas y polisacáridos, la principal razón de los cambios que se producen. Así, la conservación del vino tinto en presencia de lías finas modifica el equilibrio coloidal y la composición química del vino, repercutiendo positivamente en el aroma, estructura gustativa y estabilidad del color del vino tinto (120, 121). No obstante, la principal limitación radica en que la crianza del vino sobre lías es una práctica complicada y trabajosa, que implica una inmovilización grande de los stocks y una importante dedicación de los recursos de la bodega. Asimismo, entraña cierto riesgo de reducción (104, 122) y de aparición de desviaciones de índole microbiológico (122,

123). De este modo, su sustitución por el empleo de fórmulas a base de manoproteínas resulta una alternativa tecnológica ciertamente interesante.

En la actualidad existen distintos métodos de extracción de las manoproteínas de la pared de las levaduras, siendo los más habituales la extracción enzimática y la extracción física por tratamientos térmicos. En general se encuentran disponibles pocas preparaciones comerciales a base de manoproteínas purificadas, y normalmente se emplean derivados no purificados de levadura, como extractos y autolisados.

Hoy en día, la principal limitación en el empleo de las manoproteínas es de tipo normativo. En Estados Unidos se pueden utilizar únicamente como nutrientes para las levaduras, en Sudáfrica no están permitidas, y en Australia y Nueva Zelanda se está actualmente regulando su empleo. Respecto a España, la Unión Europea ha autorizado recientemente su uso para garantizar la estabilización tartárica y proteica de los vinos (113), y todavía se espera una directiva que especifique sus modalidades de uso. En este sentido, la utilización de levaduras seleccionadas súper-productoras de manoproteínas constituiría una alternativa interesante al empleo de los preparados purificados industriales, consiguiendo además una reducción en el coste de elaboración de los vinos. El empleo de levaduras secas activas supondría un coste de 0,9 euros por hectolitro de vino producido, mientras que la adición de preparados comerciales de manoproteínas purificadas quintuplicaría este valor (según el precio medio de mercado). Además, las levaduras comerciales actuarían como agentes naturales de fermentación, liberando al medio distintos metabolitos que también modularán las propiedades sensoriales del vino obtenido.

1.4. ENZIMAS DE MACERACIÓN

Las enzimas pectolíticas utilizadas durante la maceración en la vinificación en tinto facilitan la liberación del contenido celular de la baya de uva. El objetivo de este tratamiento es la obtención de vinos con más color, más ricos en compuestos fenólicos y más fáciles de clarificar y filtrar.

Aunque todas las enzimas son producidas por el mismo microorganismo (*Aspergillus niger*), las preparaciones de pectinasas disponibles actualmente en el mercado enológico son muy distintas. Tanto la concentración en pectinasas de dichas preparaciones como la naturaleza de su actividad dependen de la cepa de *Aspergillus*, de las condiciones utilizadas para su producción y de la naturaleza del sustrato fermentado. La distinta naturaleza de las pectinasas presentes en estas preparaciones y su nivel de actividad condicionan la eficacia de los productos. En la mayoría de los casos la actividad enzimática de las preparaciones comerciales viene expresada en unidades propias de cada fabricante, utilizándose nomenclaturas globales que no hacen distinción entre las distintas pectinasas. Además, los preparados enológicos contienen generalmente otras actividades secundarias que refuerzan la acción de las pectinasas en sentido estricto. De hecho, las enzimas pectolíticas de maceración son cócteles enzimáticos con diferentes actividades específicas sobre la pared celular de las uvas.

Entre las pectinasas propiamente dichas se distinguen las actividades poligalacturonasa, pectina-esterasa y pectina-liasa, que degradan exclusivamente las cadenas de homogalacturonano de las sustancias pécticas de la pared celular. Entre las actividades secundarias se distinguen esterasas, ramnogalacturonasas, celulasas, hemicelulasas, proteasas y glicosidasas, que actúan sobre moléculas muy diversas de la pared celular, consiguiendo así una mayor ruptura de dicha estructura. Todas estas enzimas poseen actividades complementarias, necesitándose al menos 6 actividades enzimáticas distintas para degradar las cadenas de pectina (Figura 27).

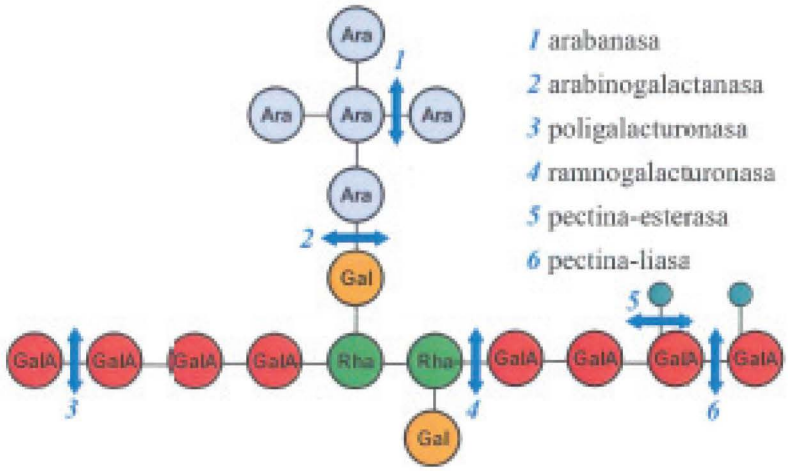


Figura 27. Actuación de las enzimas con actividad pectolítica (124)

Los preparados comerciales de enzimas de maceración actúan debilitando las paredes celulares del hollejo y de la pulpa sin llegar a destruirlas, favoreciendo así la liberación de los compuestos atrapados en dicha estructura. Los taninos mejor valorados desde el punto de vista sensorial son los taninos que se encuentran dentro de la pared celular vegetal, por lo que es importante degradar las celulosas y hemicelulosas para poder extraerlos. Los polifenoles y aromas varietales son liberados durante el prensado y la maceración debido a la acción de las actividades pectolíticas endógenas de la uva, facilitándose su extracción por las acciones del sulfuroso y las acciones mecánicas de remontados y bazuqueos.

El empleo de enzimas de maceración comerciales provoca modificaciones aún más profundas sobre las estructuras celulares del racimo que las producidas por las enzimas pectolíticas endógenas. Tras la ruptura de la baya, la primera acción de las enzimas es disgregar la pulpa adherida al hollejo y facilitar el acceso a su cara interna, consiguiendo así una mejor extracción y difusión de sus componentes y un mayor rendimiento de mosto. Sucesivamente su acción se desenvuelve en la pared celular del hollejo y de la membrana vacuolar, donde facilitan la extracción de polisacáridos pécticos (homogalacturonanos, ramnogalacturonanos, arabinogalactanos) y no pécticos (celulosas y hemicelulosas), antocianos solubles en el jugo vacuolar, precursores aromáticos y distintos taninos, taninos ligados a polisacáridos, taninos ligados a proteínas de la membrana vacuolar y taninos parietales (Figura 28).

Figura 28. Efecto de las enzimas de maceración en la extracción de los distintos compuestos fenólicos durante la maceración-fermentación (125)

Las enzimas de maceración, al modificar el estado físico de la estructura del hollejo, facilitan la extracción sucesiva de todos estos compuestos en la fase alcohólica durante la fermentación, que sin enzimas se extraerían en menor cantidad y en estadios más avanzados en presencia de etanol. Conforme avanza la fermentación, la concentración de compuestos fenólicos bloquea la acción de las enzimas, y la extracción que se produce durante estas fases es debida al efecto del etanol.

En resumen, las enzimas de maceración aceleran y mejoran la extracción de todo tipo de compuestos fenólicos, ayudando especialmente a la liberación de taninos durante las primeras fases de maceración. Desde el punto de vista del color, el empleo de enzimas de maceración comerciales produciría un incremento del contenido de los antocianos libres desde el inicio de la fermentación-maceración, pero también de taninos y otros copigmentos, favoreciendo así los fenómenos de copigmentación y polimerización de antocianos, y por tanto, la estabilización del color extraído. En este sentido, diversos trabajos analizan el efecto de distintos preparados enzimáticos comerciales en la extracción y estabilización del color de los vinos tintos, obteniéndose resultados contradictorios (126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137). En algunos casos el empleo de enzimas produce una mejora considerable en la extracción y estabilidad del color del vino, mientras que en otros no se detectan diferencias significativas entre los vinos tratados y los controles, y hay incluso trabajos que encuentran una disminución de los antocianos y del color del vino cuando emplean enzimas de maceración comerciales. Esta amplia variabilidad de resultados puede ser atribuida a las diferentes actividades enzimáticas de las preparaciones comerciales utilizadas, y principalmente a la presencia de glucosidasas o fenol-esterasas. De hecho, los resultados negativos obtenidos con la adición de enzimas comerciales han sido fundamentalmente atribuidos a la presencia de estas dos actividades enzimáticas en los preparados comerciales (127, 128, 129).

Las actividades de las enzimas de maceración en los preparados comerciales se ven también influenciadas por distintos parámetros durante el proceso de elaboración de los vinos. Así, la actividad de las enzimas es menor cuanto menor es el pH y la temperatura, obteniéndose su máxima actividad con valores de pH por encima de 4 y temperaturas de 28-30 ºC (125). El incremento del etanol durante la fermentación o de los niveles de polifenoles interfiere también con la acción de las enzimas, reduciendo significativamente su actividad (125).

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2

METODOLOGÍA DE LA INVESTIGACIÓN

2.1. METODOLOGÍA Y PLAN DE TRABAJO

Para llevar a cabo los objetivos planteados en la tesis la metodología de la investigación se dividió en dos fases.

Se realizó primero una vinificación tradicional en tinto de *Vitis Vinífera var. Tempranillo* aplicando los distintos tratamientos objeto de estudio. La vendimia se realizó en el término de Autol, D.O.Ca. Rioja, y la vinificación se llevó a cabo en la Bodega Experimental de la Universidad de La Rioja.

Las vinificaciones se realizaron en seis etapas: a) tratamiento mecánico y sulfitado de la vendimia, b) adición de los tratamientos propuestos, c) maceración-fermentación alcohólica, d) fermentación maloláctica, e) crianza en barrica de roble, f) crianza en botella. La toma de muestras se realizó al inicio de la maceración prefermentativa, durante la fermentación alcohólica (0, 30, 60 y 99% de azúcares consumidos), al inicio y final de la fermentación maloláctica, al inicio y final de la crianza en barrica de roble, y tras dos años de envejecimiento en botella. El detalle de desarrollo del diseño experimental de vinificación en tinto se muestra en la Figura 1.

Todos los productos enológicos utilizados fueron suministrados por Lallemand (Lallemand-Inc., Montreal, Canadá). La cepa de levadura *Saccharomyces cerevisiae* BM45 fue seleccionada por ser una cepa súper-productora de manoproteínas, mientras que la cepa *Saccharomyces cerevisiae* RC212 se escogió por ser una cepa neutra desde el punto de vista coloidal (1). Las enzimas de maceración (Lallyzme EXV) se seleccionaron por presentar alta actividad poligalacturonasa, pectina-liasa y pectina-esterasa, así como un elevado nivel de otras actividades secundarias como proteasa ácida, ramnogalacturonasa, y celulasa/hemicelulasa, siendo especialmente recomendadas para la obtención de vinos tintos de alta calidad (2). El preparado de manoproteínas comerciales utilizado se seleccionó por presentar una fracción bastante purificada de dicho polisacárido[a].

Cada ensayo se realizó en triplicado para garantizar la fiabilidad y consistencia de los resultados.

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[a] Información suministrada por la casa comercial y corroborada en nuestro laboratorio (ver artículo tercero de la presente tesis).

En la segunda fase de la tesis se abordó el estudió analítico de las muestras tomadas con el fin de conocer el efecto de los tratamientos biotecnológicos realizados. Concretamente se plantearon los siguientes objetivos:

1. Seleccionar y validar un método de análisis que permitiera la identificación y cuantificación de las distintas familias de polisacáridos de la uva y de las levaduras tanto en mostos como en vinos.
2. Desarrollar y validar un método de análisis sencillo que permitiera identificar y cuantificar el mayor número posible de compuestos fenólicos tanto en mostos como en vinos.
3. Aplicar la metodología desarrollada para conocer la evolución en el perfil y el contenido de los diferentes tipos de polisacáridos durante las distintas etapas de vinificación y crianza analizadas.
4. Aplicar la metodología desarrollada para conocer la evolución en el perfil y el contenido de los diferentes tipos de compuestos fenólicos durante las distintas etapas de vinificación y crianza estudiadas, así como su posible relación con la evolución en la extracción y estabilidad del color.
5. Evaluar el efecto de los tratamientos propuestos en la extracción del color y estabilidad coloidal durante la vinificación y crianza de los vinos, así como en las propiedades sensoriales de los vinos obtenidos.
6. Analizar el efecto de la adición de manoproteínas comerciales en la composición del color, de los polisacáridos y polifenoles durante la vinificación y crianza de los vinos.
7. Analizar el efecto de las levaduras súper-productoras de manoproteínas en la composición del color, de los polisacáridos y polifenoles durante la vinificación y crianza de los vinos.

Los resultados obtenidos en cada uno de los siete objetivos planteados han dado lugar a la elaboración de publicaciones científicas independientes, estando cuatro de los trabajos ya publicados en revistas científicas de alto índice de impacto, y otros tres enviados para su publicación. Dichas publicaciones se presentan en el capítulo de *Resultados y discusión,* donde también se incluye un resumen de cada trabajo y de los resultados más relevantes obtenidos.

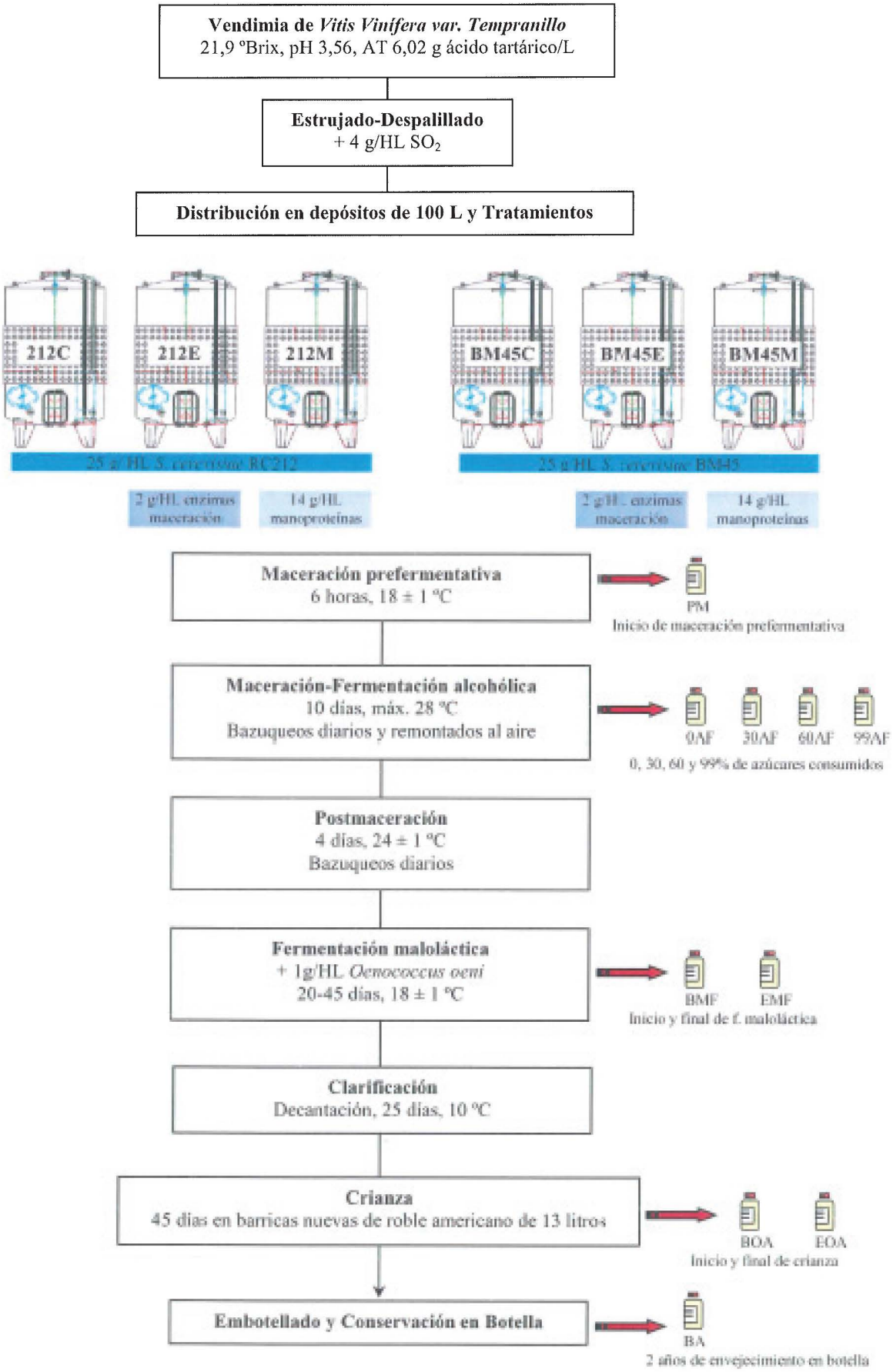

Figura 1. Esquema de Vinificación y Toma de muestras

2.2. TÉCNICAS ANALÍTICAS

Las técnicas analíticas empleadas en esta tesis ha sido:

1. Análisis de parámetros enológicos generales

Durante la vinificación se realizaron controles y análisis de densidad, temperatura, pH, acidez total, acidez volátil, sulfuroso libre y total, azúcares reductores y grado alcohólico siguiendo los métodos oficiales de la OIV (3). El ácido málico se determinó mediante métodos enzimáticos de acuerdo a los métodos oficiales de análisis de la AOAC (4).

2. Análisis microbiológicos

El seguimiento de la implantación de las cepas de levadura y bacteria inoculadas se realizó mediante técnicas de Biología Molecular, PCR y ECP (5), en muestras tomadas al final de la fermentación alcohólica y maloláctica. Dichos análisis se llevaron a cabo en el *Laboratorio de Identificación Genética de Microorganismos* (Sigmo Vertou, Francia).

3. Cuantificación de polisacáridos totales

La cuantificación de polisacáridos totales solubles y no solubles se realizó mediante los métodos colorimétricos del fenol-sulfúrico y *o*-hidroxidifenilo siguiendo la metodología descrita por Segarra y col. (6) aunque se incluyó alguna pequeña modificación (7).

4. Análisis de la materia colorante

Las características cromáticas de las muestras se midieron mediante técnicas espectrofotométricas según la metodología recomendada por la OIV (3). A partir de estos datos se calcularon las distintas expresiones de color: intensidad colorante, matiz y tonalidad. Las coordenadas de CIELAB se calcularon según el método de Ayala y col (8). El análisis de la estabilidad de la materia colorante se realizó siguiendo el método propuesto por Levengood y Boulton (9), que permite medir la aportación de los distintos componentes al color total del vino: color debido a los antocianos monómeros, color debido a la copigmentación y color estable frente al bisulfito. Este último es atribuido en la bibliografía al color debido a los antocianos poliméricos, aunque esto no es del todo correcto ya que incluye compuestos que no son poliméricos, como los piranoantocianos, y además no todos los antocianos poliméricos son estables frente al bisulfito (pigmentos poliméricos tipo T-A).

5. Cuantificación de las distintas familias de polisacáridos

El análisis de las diferentes familias de polisacáridos se realizó en distintas etapas utilizando técnicas de cromatografía de exclusión molecular y cromatografía de gases-FID y gases-masas, según la metodología validada en la tesis (10) y que se describirá más adelante (ver apartado 3.1. del capítulo de *Resultados y discusión*). El seguimiento de la distribución de pesos moleculares de los polisacáridos solubles e insolubles se realizó mediante técnicas de cromatografía líquida de alta resolución (11).

6. Cuantificación de compuestos fenólicos

La cuantificación de la composición antociánica, de ácidos hidroxicinámicos, flavonoles monómeros, flavanoles y taninos, se realizó mediante la metodología desarrollada en la tesis (12). Dicha metodología, que se describe en el apartado 3.2. del capítulo de *Resultados y discusión*, incluye técnicas de cromatografía de permeación en gel a baja presión (GPC), cromatografía líquida de alta resolución con detector de fila de diodos y detector de masas, electroforesis capilar y técnicas espectrofotométricas.

7. Análisis organoléptico de los vinos

Los vinos antes y después de la crianza fueron analizados sensorialmente por un panel de catadores expertos, evaluándose en sus tres fases: visual (color), aromática (fracción volátil) y gustativa (equilibrios en boca). Los catadores seleccionaron 12 atributos para la fase olfativa y 10 para la gustativa, eligiendo una escala de puntuación del 1 al 10. Los resultados obtenidos se evaluaron mediante un análisis de componentes principales (7).

Todos los parámetros anteriormente descritos se analizaron por triplicado en todas las muestras tomadas a lo largo de la vinificación y crianza. Los valores obtenidos fueron sometidos, variable por variable, a un análisis de varianza (ANOVA o Kruscal-Wallis). Las diferencias se expresaron con un nivel de confianza mayor del 95%.

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3

3.1. SELECCIÓN Y VALIDACIÓN DE UN MÉTODO DE ANÁLISIS PARA LA CUANTIFICACIÓN DE DIFERENTES FAMILIAS DE POLISACÁRIDOS EN MOSTOS Y VINOS

3.2. DESARROLLO Y VALIDACIÓN DE UN MÉTODO DE ANÁLISIS PARA LA DETERMINACIÓN DE POLIFENOLES EN MOSTOS Y VINOS

3.3. EVOLUCIÓN DEL CONTENIDO Y DEL PERFIL DE POLISACÁRIDOS DURANTE LA VINIFICACIÓN Y EL ENVEJECIMIENTO DE VINOS TINTOS

3.4. EVOLUCIÓN DE LA COMOSICIÓN DEL COLOR Y DEL CONTENIDO DE POLIFENOLES DURANTE LA VINIFICACIÓN Y EL ENVEJECIMIENTO DE VINOS TINTOS

3.5. EFECTO DE MONOPROTEÍNAS Y ENZIMAS DE MACERACIÓN EN LA ESTABILIDAD COLOIDAL Y LA EXTRACCIÓN DEL COLOR DE VINOS TINTOS

3.6. EFECTO DE LA ADICION DE MANOPROTEÍNAS COMERCIALES EN LA COMPOSICIÓN DE DE POLISACÁRIDOS, POLIFENOLES Y COLOR DE VINOS TINTOS

3.7. EFECTO DEL EMPLEO DE LEVADURAS SUPERPRODUCTORAS DE MANOPROTEÍNAS EN LA COMPOSICIÓN DE POLISACÁRIDOS, POLIFENOLES Y COLOR DE VINOS TINTOS

RESULTADOS Y DISCUSIÓN

3.1

SELECCIÓN Y VALIDACIÓN DE UN MÉTODO DE ANÁLISIS PARA LA CUANTIFICACIÓN DE DIFERENTES FAMILIAS DE POLISACÁRIDOS EN MOSTOS Y VINOS

Quantification of major grape polysaccharides (Tempranillo v.) released by maceration enzymes during the fermentation process VINOS

Artículo publicado en:

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Resumen

Este artículo aborda 2 objetivos fundamentales:

1. Seleccionar y validar un método de análisis que permita la identificación y cuantificación de las diferentes familias de polisacáridos presentes en mostos y vinos.
2. Aplicar el método anterior para evaluar el efecto de las enzimas de maceración comerciales en la extracción de los distintos polisacáridos durante la maceración-fermentación de los vinos tintos de Tempranillo elaborados para la presente memoria.

Para cumplir el primer objetivo se realizó una revisión bibliográfica detallada sobre los métodos analíticos existentes para la identificación y cuantificación de los polisacáridos; se seleccionaron los procedimientos más adecuados en relación a nuestro objetivo y se analizaron en detalle.

Se propuso un método que permite la identificación y cuantificación, tanto en mostos como en vinos, de las distintas familias de polisacáridos de la uva, arabinogalactanos y arabinogalactano-proteínas (AG), homogalacturonanos (GU) y ramnogalacturonanos tipo II (RG-II), así como de los polisacáridos procedentes de las levaduras, mananos y manoproteínas (M).

El método analítico se desarrolló en cuatro etapas:

1. Separación de los polisacáridos del resto de macromoléculas del mosto y vino por precipitación con etanol-ácido previa concentración de la muestra, siendo ésta última fundamental para poder recuperar y cuantificar los polisacáridos de bajo peso molecular, como los GU y RG-II.
2. Fraccionamiento de las distintas familias de polisacáridos por cromatografía líquida de exclusión molecular de alta resolución, consiguiendo así una separación de los polisacáridos en distintas fracciones según su tamaño molecular.
3. Identificación y cuantificación de los residuos glicosídicos de cada fracción por cromatografía de gases con detector de llama (GC-FID) y cromatografía de gases con detector de masas (GC-MS) de sus trimetilsilil-ester *O*-metil glicósidos (TMS) previa metanolisis ácida y derivatización de la muestra. El método de derivatización seleccionado permite la cuantificación de monosacáridos tanto neutros como ácidos.
4. Estimación de las distintas familias de polisacáridos a partir de su composición individual de residuos glicosilados según datos publicados en la bibliografía.

El método propuesto presentó unas características analíticas satisfactorias en términos de sensibilidad, repetibilidad, reproducibilidad y límites de detección y cuantificación, así como en su recuperación.

En relación al efecto de las enzimas de maceración sobre la extracción de los polisacáridos, se observó que los vinos tratados con enzimas de maceración comerciales presentaron concentraciones considerablemente más altas de arabinogalactano-proteínas y ramnogalacturonanos-II que los vinos sin tratamiento enzimático, mientras que no se produjeron diferencias significativas en cuanto a los mananos y manoproteínas. Este hecho indicó que las enzimas comerciales de maceración hidrolizaron los polisacáridos pécticos de la uva durante la maceración-fermentación, pero no actuaron sobre los polisacáridos parietales de las levaduras. Los resultados obtenidos indicaron además que, en ambas vinificaciones, una parte importante de los polisacáridos extraídos precipitaron durante la etapa de maceración-fermentación, representando los polisacáridos insolubles más de un 28% de los polisacáridos totales (solubles + insolubles) al final de dicha etapa.

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Quantification of major grape polysaccharides (*Tempranillo* v.) released by maceration enzymes during the fermentation process

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Abstract

The influence of commercial enzymes on wine polysaccharide content was studied. Tempranillo wines were made using commercial maceration enzyme preparations along with controls. The analytical method for the quantification of wine polysaccharides was carried out by a multistep procedure. Wine-soluble polysaccharides were isolated by wine concentration polysaccharides precipitation with an acid–alcohol medium and separation of each polysaccharide family by high resolution size-exclusion chromatography on a Superdex-75 HR column. The glycosyl-residue compositions of the fractions obtained were determined by gas chromatography with flame ionisation and mass spectrometry of their trimethylsilyl-ester *O*-methyl glycosides after acidic methanolysis and derivatization. The content of each fraction was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides. The analytical method proposed had good sensitivity, repeatability, reproducibility and accuracy. Soluble polysaccharides in wine were essentially composed of grape cell wall polysaccharides: arabinogalactans and arabinogalactan-proteins (38–41%), and rhamnogalacturonans-II (38–46%). Yeast mannans and mannoproteins were also present but in smaller proportions (14–19%). Wines treated with commercial enzymes had larger concentrations of arabinogalactans, arabinogalactan-proteins and rhamnogalacturonans-II than control wines, but the content of mannans and mannoproteins was similar in both wines. This indicated that the commercial enzymes hydrolysed grape pectic polysaccharides during the maceration–fermentation stage but had no influence on yeast parietal polysaccharides.
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Keywords: Wine; Polysaccharides; Maceration enzymes; Polysaccharide analysis

1. Introduction

Polysaccharides are one of the main groups of wine macromolecules. They are considered as "protective colloids", liable to prevent or limit aggregation, flocculation and thereby haze formation and tartrate salts crystallisation [1,2]. Wine polysaccharides have also been described for their detrimental role in filterability [3–5], their influence on the fermentation flora [6–8] and their interaction with aromatic compounds [9,10]. These compounds also contribute to the organoleptic properties of wines, as they stabilise flavour, colour and foam [11].

Wine polysaccharides originate from both grape primary cell walls (pectic polysaccharides) and yeast cell walls (mannoproteins and mannans) [12]. Hence, this origin diversity leads to polysaccharide families that are different in composition and structure. Two criteria widely used for polysaccharide families discrimination are acidity and protein content [10]. Neutral pectic substances mainly comprise type II arabinogalactans (AGs) and arabinogalactan-proteins (AGPs), which represent more than 40% of total red wine polysaccharides [13,14]. Their common structural feature is a (1 → 3)-β-D-galactan backbone with (1 → 6) linked β-D-galactan side chains highly substituted by arabinofuranosyl residues. Typical AGPs commonly contain less than 10% protein [14]. Other neutral polysaccharides are weakly branched (1 → 5)-α-L-arabinans [15] and type I arabinogalactans that are (1 → 4)-β-D-galactans substituted in position 6 by arabinofuranosyl residues. Acidic pectic polysaccharides, characterised by a high proportion of galacturonic acid, involve homogalacturonans and rhamnogalacturonans. Rhamnogalacturonans-II (RG-IIs), which represent about 20% of soluble polysaccharides in red wine, are (1 → 4)-α-D-galacturonans branched with four

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different side chains containing primarily rhamnopyranose, arabinofuranose and galactopyranose [16,17].

Mannans and mannoproteins are produced by yeast, such as *S. cerevisiae*, during alcoholic fermentation [13,18]. Pectic polysaccharides arise from native cell wall pectines of grape berry after degradation by pectinases during grape maturation and during the first steps of their processing to wine [19]. Commercial enzymes have been traditionally used in wine technology in order to produce even higher modifications in grape polysaccharides than those produced by endogenous pectinases. Ducruet et al. [20] observed that the addition of commercial enzymes to musts produced an increase in the amount of total acid (49%) and neutral polysaccharides (5%), but they did not study this effect in the different polysaccharide families.

The identification and structural characterisation of each polysaccharide family require its previous isolation from total wine macromolecules, followed by ion-exchange, size-exclusion and affinity chromatographies [3,16,21,22]. The methods most commonly used for extraction are precipitation with ethanol, dialysis and ultrafiltration [23]. Several methods for the direct quantification of polysaccharides in wines have been proposed. Most of these are based on the precipitation of total wine colloids, followed by colorimetric assays [3,18,21,23–26] or by the determination of peak areas in size-exclusion chromatography [27–29]. However, these global methods do not allow the identification of the different families of polysaccharides present in wines. Because of their diversity and chemical complexity, the identification and quantification of wine polysaccharide families requires a series of complex analytical procedures. However, reliable quantification may be achieved, from the concentration of individual monosaccharides that are characteristic of well-defined wine polysaccharides. Many chromatographic methods have been proposed for the identification and quantification of carbohydrates [30]. Due to their high sensitivity combined with an ability to achieve efficient separation of complex mixtures, gas chromatography (GC) and GC-mass spectrometry (GC-MS) have gained general acceptance [31]. The neutral glycosyl-residue compositions of plant polysaccharides are typically determined, after acid hydrolysis, by GC and GC-MS analysis of their aditol acetate derivatives [32] but the acidic glycosyl-residue composition cannot be determined directly by this procedure.

In the present study, published references about all these methodologies were revised. The most appropriate steps of the revised methods, to the best of our knowledge, were studied in detail. The analytical method for the quantification of wine polysaccharides was carried out in a multistep procedure. This method was applied to quantify the major family grape polysaccharides released by commercial enzymes during the maceration–fermentation of the Tempranillo grape. The properties of the proposed method were studied in detail and a validation study was carried out.

2. Experimental

2.1. Reagents and samples

All reagents were of analytical grade unless otherwise stated. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose and Kdo (3-deoxy octulosonic acid) were supplied by Sigma (St. Louis, MO), and D-galacturonic acid, D-glucuronic acid and myo-inositol from Fluka via (Sigma). D-apiose was obtained from Omicrom (South Bend, IN).

Ethanol (96%, v/v) and acetyl chloride were supplied by Scharlab (Barcelona, Spain), hydrochloric acid 37% by Carlo Erba (Rodano, Milan, Italy), dried methanol Merck (Darmstadt, Germany) and trimethylsilylation reagent (TriSil®) by Pierce (Rockford, MA). HPLC-grade ammonium formiate supplied by Fluka and Milli-Q water (Millipore, Molsheim, France) were used. A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain). All the solutions were filtered through a 0.45 μm filter before use in liquid chromatography (LC).

Wine samples were produced from *Vitis Vinifera Tempranillo* grapes of the qualified origin denomination Rioja (D.O.Ca Rioja). The yeast *S. cerevisiae* RC 212 and the maceration enzymes were purchased from Lalvin (Lallemand Inc., Montreal, Canada).

2.2. Vinification and sample collection

Six experimental vinifications were carried out using stainless steel tanks of 100 l. Destemmed-crushed grapes were homogenised and distributed into the tanks, 30 mg l^{-1} SO_2 was added and yeast *S. cerevisiae* RC 212 inoculated. After 1 h, 0.02 g l^{-1} maceration enzymes were added to three of the tanks and the musts were mixed thoroughly. These enzymes showed standard activities >4000 uPG g^{-1} (polygalacturonase units), 1000 uPE g^{-1} (pectin-esterase units) and 120 uPL g^{-1} (pectin-liase units). Vinifications without enzymes were considered as control treatments.

In the prefermentation stage, the initial measurements of pH, g tartaric acid/100 ml and g l^{-1} reducing sugars, were 3.5, 0.6 and 230, respectively. The fermentation–maceration process was carried out at a maximum temperature of 28 ± 2 °C and went on for 10 days. *S. cerevisiae* RC 212 was implanted in all the vinifications and this was corroborated by molecular biological techniques (PCR and EPC).

Wine samples were taken at the end of maceration–fermentation, when 99% of sugars had been consumed, and were collected from both enzyme-treated tanks (EXV wine) and control tanks (control wine). Sample bottles were filled completely to minimise oxygen contact and immediately frozen at −18 °C. All samples were analysed for titratable activity, pH, percent of alcohol, total phenols and reducing sugars prior to freezing.

2.3. Preparation of wine polysaccharides

Samples were homogenised and 400 ml was taken with a peristaltic pump and centrifuged (9500 × *g*, 20 min, 4 °C) using a RC-5B Sorvall refrigerated centrifuge (Du Pont, BH, Germany).

The insoluble pellets were recovered and precipitated with 50 ml of cold 96% ethanol containing 0.3 M HCl. After 18 h at 24 °C, samples were centrifuged (9000 × *g*, 20 min, 4 °C) and the pellets obtained washed in ethanol 96% several times (until the supernatant was colourless) to remove interfering materials. The residues obtained (fraction A) were freeze-dried using a Virtis freeze drying (New York, NY).

The supernatants were first concentrated five times under reduced pressure at 34 °C. Total soluble polysaccharides were then precipitated by adding of four volumes of cold ethanol containing 0.3 M HCl, and kept for 18 h at 4 °C. Thereafter, the samples were centrifuged (9000 × *g*, 20 min, 4 °C), the supernatants discarded and the pellets washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried (fraction B).

2.4. Fractionation of soluble polysaccharides by high resolution size-exclusion chromatography

In order to separate the different polysaccharide families, the soluble fractions B were submitted to high resolution size-exclusion chromatography (HRSEC). Four mg of freeze-dried fraction B were dissolved in 1 ml of ultrapure water and centrifuged (4000 × *g*, 5 min, 4 °C) to remove the insoluble material prior to analysis.

HRSEC was performed using an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with one G1310A HPLC pump, an on-line G1379A degasser, a G1362 refractive index detector, and a Windows 2000 Hewlett-Packard computer, and furnished with a Superdex-75 HR column (1.3 cm × 30 cm, Pharmacia, Sweden). Samples were injected using a manual injector (Rheodyne, CA) and collected in a Gilson fraction collector (Middletown, WI).

The mobile phase used was 30 mM ammonium formiate, pH 5.8; the flow rate and the injection volume were 0.6 ml min^{-1} and 500 μl, respectively. Chromatographic separation was carried out at room temperature.

The peaks obtained were collected in different fractions according to their elution times: fraction B1 (12–17 min), fraction B2 (18–22 min) and fraction B3 (25–30 min). The isolated fractions were freeze-dried, redissolved in water, and freeze-dried again four times to remove the ammonium salt. Each sample was injected at least 20 times in order to obtain enough freeze-dried quantities for further analysis.

The molecular weight distribution of the different fractions was determined by calibration of the Superdex-75 HR column with a pullulan calibration kit. Chromatographic separation of the pullulan standards was performed under the same conditions described above.

2.5. Identification and quantification of polysaccharides by GC and GC-MS

The carbohydrate composition of the fractions (fractions A, B and B1–B3) was determined by GC with flame ionisation detector and GC-MS of their trimethylsilyl-ester *O*-methyl glycolsyl-residues (TMS) obtained after acidic methanolysis and derivatization of these fractions. This derivatization procedure allows the identification of both neutral and acidic monosaccharides.

The polysaccharide contents of fractions B1, B2 and B3 were estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

2.5.1. Sample preparation: acidic methanolysis and derivatization

Polysaccharide fractions were treated with the methanolysis reagent MeOH 0.5 M HCl in order to hydrolyse neutral and acidic monosaccharides to their corresponding methyl glycosides. The methanolysis reagent was prepared by adding acetyl chloride (140 μl) to 4 ml of dried methanol. Freeze-dried samples (0.5–1 mg) and 1 mg of inositol (internal standard) were hydrolysed with 0.5 ml of the methanolysis reagent. The reaction was conducted under reduced pressure at 80 °C for 16 h. Thereafter, the excess of reagent was removed using a stream of nitrogen gas.

The conversion of the methyl glycosides to their trimethylsilyl (TMS) derivatives was performed using the TriSil® reagent. An excess of TriSil® reagent (0.3 ml) was added to the dried material. The reaction was carried out at 80 °C for 20 min and the reagents again removed with a nitrogen stream. The derivatized residues were then extracted with 1 ml of hexane, evaporated to dryness with a nitrogen stream, and mixed again with 40 μl of hexane. GC-FID and GC-MS were performed with 2 μl of these solutions. All analyses were performed in triplicate.

Different quantities of standard carbohydrates (0.1–5 mg) were also converted to their corresponding TMS derivatives and analysed by GC and GC-MS in order to obtain patterns for identification and standard calibration graphs. The procedure followed with the standards was the same as with the fraction samples but the derivatized residues were extracted with 2 ml of hexane, and 1 μl was used for GC and GC-MS analysis.

2.5.2. Gas chromatography

The GC system consisted of an HP5890 Series II gas chromatograph (Hewlett-Packard, USA) coupled to a FID. The GC system was equipped with a capillary split/splitless inlet and a fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Teknokroma). The carrier gas was helium at a flow rate of 1.3 ml min^{-1}. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector and the FID were operated at 250 °C. The chromatograph was operated with temperature programming (120–145 °C

at 1 °C min^{-1}, 145–180 °C at 0.9 °C min^{-1} and 180–230 °C at 50 °C min^{-1}).

2.5.3. Gas chromatography-mass spectrometry

GC-MS was performed with a Hewlett-Packard HP-G1800B GCD coupled to a mass detector operated in the electron ionisation (EI) mode under the control of a GCD Plus Chemstation Agilent, G 2070. Chromatographic separation was performed under the same conditions described above but the flow rate was 1 ml min^{-1}. EI mass spectra were obtained over the range *m/z* 50–450 every 2.8 s in the total ion-monitoring mode using a source of temperature 230 °C, a quadrupole temperature of 136 °C, and a ionisation voltage of 70 eV.

3. Results and discussion

3.1. Preparation of wine polysaccharides

Soluble polysaccharides were isolated from total wine macromolecules by precipitation with an acid–alcohol medium. In order to select the best conditions for this precipitation from those proposed by other authors [3,6,9,21,23–26,33–35], several experiments were done in order to optimise the precipitation time and temperature.

Aliquots (5 ml) of centrifuged wine sample were precipitated with four volumes of ethanol–acid for different times (6, 10, 14, 18, 22 and 26 h) and at different temperatures (4 °C and ambient temperature). The precipitates were washed with ethanol, as described in the above method, freeze-dried and weighed. Table 1 shows the means and standard deviations of the quantities obtained. As can be observed in this table, the quantity of polysaccharide precipitate increased with the time of precipitation up to 18 h and after this time the weight was constant. Larger amounts of precipitate were obtained at 4 °C in comparison with ambient temperature. Thus, 4 °C and 18 h were chosen as precipitation conditions.

In order to isolate the polysaccharides present in the insoluble pellets (fraction A), these fractions were also precipitated with ethanol–acid. Although this precipitation is not normally done by other authors when treating with insoluble fractions [35], non-precipitated pellets contain a very large quantity of insoluble wine macromolecules, such proteins, and tannins, which could cause interferences when measuring carbohydrate content. It was observed that the weight of non-precipitated pellets was almost double than that of the precipitated ones, but the amount of total polysaccharides, measured by the colorimetric methods of phenol and *o*-hydroxydiphenyl [23], was higher in the latter.

The supenatants (fraction B) obtained after removal of insoluble pellets were concentrated prior to the addition of cold ethanol–acid. This was necessary to ensure the quantitative precipitation of all soluble polysaccharides since some polysaccharide families (rhamnogalacturonans and homogalacturonans) are precipitated only partially in non-concentrated wines [35]. Several authors have analysed polysaccharide families in concentrated wine samples [3,14,35,36] and the concentrations used differed from one author to another. Similar studies made in musts focus on non-concentrated samples [12,37]. As there were different criteria for the concentration rate, and since no previous studies of the influence of sample concentration on polysaccharide precipitation were found it was considered interesting to analyse this in detail. Must and wine samples were centrifuged, the supernatants concentrated different times, precipitated, and submitted to HRSEC analysis. The chromatograms obtained are shown in Figs. 1 and 2.

Must samples were concentrated zero, two and three times. It was impossible to concentrate them more than three times because the polysaccharides began to caramelise in the media and became non-miscible with the precipitation reagent. Wine samples were also concentrated different times (0-, 2-, 4-, 5-, 7- and 10-fold).

In both musts and wines, the quantity of polysaccharide precipitate obtained after the precipitation procedure increased with the sample concentration rate (Table 2), indicating that part of the polysaccharides did not precipitate in less concentrated samples, probably due to their high solubility.

Non-concentrated musts showed only one peak in the HRSE chromatogram whereas two-fold concentrated samples also showed a second peak, and three peaks were obtained in must samples concentrated three times (Fig. 1). Thus, it could be concluded that it was necessary to concentrate must samples three times in order to obtain the three peaks and avoid loosing some polysaccharide families, as RG-IIs and homogalacturonans mainly elute in these fractions [35]. In wines, non-concentrated and two-fold

Table 1
Polysaccharide precipitate concentration[a] (mg l^{-1}) found in non-concentrated wine samples precipitated for various times at two temperatures

Precipitation temperature	Precipitation time (h)					
	6	10	14	18	22	26
Ambient	448 ± 32	490 ± 46	658 ± 41	756 ± 25	728 ± 62	745 ± 51
4 °C	470 ± 28	554 ± 56	742 ± 48	848 ± 54	890 ± 62	863 ± 62

[a] Mean ± S.D. ($n = 6$).

Table 2
Polysaccharide precipitate concentration[a] ($mg\,l^{-1}$) found in wine and must samples concentrated a different number of times

Sample	Times concentrated							
	0	2	3	4	5	6	7	10
Must	215 ± 18	368 ± 42	874 ± 61	–	–	–	–	–
Wine	423 ± 35	435 ± 56	–	761 ± 58	896 ± 62	–	991 ± 71	1278 ± 123

Precipitation carried out at 4 °C for 18 h.
[a] Mean ± S.D. ($n = 6$).

concentrated samples showed only one and two peaks, respectively, whereas the rest of the concentrated samples showed the three fractions needed for the quantification of all polysaccharide families (Fig. 2). These three peaks showed higher resolution in the samples concentrated five times and, therefore, and also in order to minimise the concentration time, this concentration was chosen for wine samples.

The repeatability of the polysaccharide precipitation method was also analysed in both wines and musts. Eight wine samples and eight must samples were centrifuged and the supernatants concentrated and precipitated. The residues were washed with ethanol, freeze-dried and weighed. Repeatability was expressed as the coefficient of variation of the precipitate quantities obtained and was 3.02% for wine samples and 2.89% for must samples. These values showed the precision of the method under the conditions selected.

3.2. Fractionation of soluble polysaccharides by HRSEC

HRSEC was carried out using a Superdex-75 HR column. This pre-packed column, with a molecular weight range from 3000 to 75,000 Da, was used for the fractionation of wine-soluble polysaccharides. In the wine samples

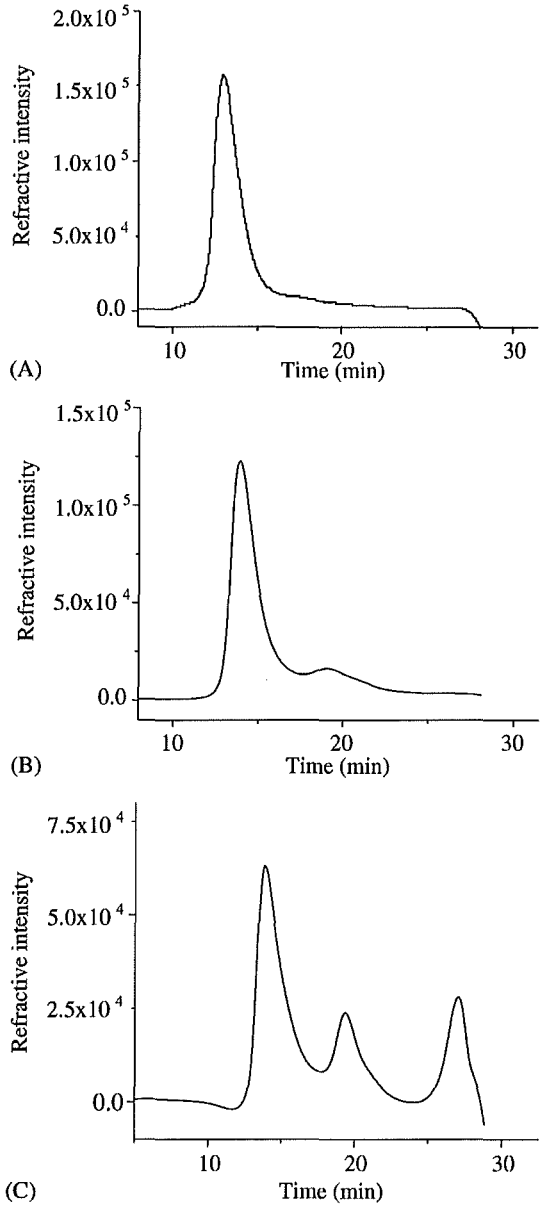

Fig. 1. HRSEC profiles of soluble polysaccharides in must samples concentrated a different number of times. (A) Non-concentrated must sample. (B) Must sample concentrated two-fold. (C) Must sample concentrated three-fold.

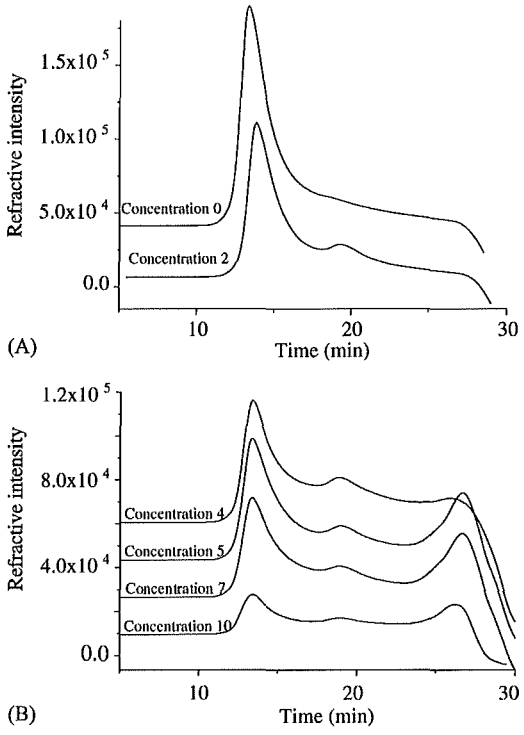

Fig. 2. HRSEC profiles of soluble polysaccharides in wine samples concentrated a different number of times. (A) Wine samples concentrated zero- and two-fold. (B) Wine samples concentrated 4-, 5-, 7- and 10-fold.

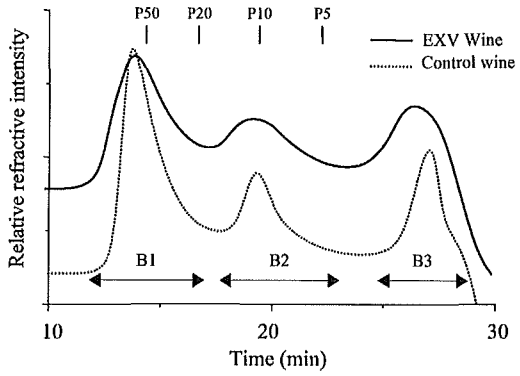

Fig. 3. Molecular weight distribution of fractions B1, B2 and B3 obtained by HRSEC on a Superdex-75 HR column. Elution times of pullulan standards (P5 → P50) are also shown.

analysed, the HRSEC fractionation allowed the separation of soluble polysaccharides into three different fractions: B1, B2 and B3. The molecular weight distribution of these fractions and the refractometric profiles are shown in Fig. 3. The population eluting between 11 and 17 min (fraction B1) corresponded to molecules with a molecular weight > P20 (22,800 Da). According to previously published data, these molecules correspond to arabinogalactans, arabinogalactan-proteins, mannans and mannoproteins [19,35]. A second population (fraction B2), with an average molecular weight between P20 and P5 (22,800 and 6000 Da), eluted between 17 and 24 min. This population correspond to a complex mixture of mainly RG-II dimers (average molecular weight ~10,000 Da) [19,35]. The third population (fraction B3), with a weight < P5 (6000 Da), eluted between 26 and 32 min and could be attributed to oligosaccharides and low molecular weight fragments of very large macromolecules.

The reproducibility and repeatability of the HRSEC fractionation (Table 3) was assessed by analysing the results obtained with each sample; 20 injections per sample were carried out. The peak area repeatability, expressed as the coefficient of variation of each sample, was <5% in all samples. The coefficient of variation of the retention time was <0.6% in all cases. The peak area reproducibility, expressed as the mean of the coefficients of variation of all the measures, was 3 ± 1%. This value was 0.2 ± 0.1% for the retention time.

Table 3
Reproducibility of relative concentrations and retention times of the fractions obtained by HRSEC on a Superdex-75 HR column

Sample	Fraction	Relative concentration[a] (%)	Retention time[b] (min)
Control wine	B1	50.1 ± 0.6	13.85 ± 0.07
	B2	27.5 ± 0.9	19.40 ± 0.03
	B3	22.4 ± 0.8	27.09 ± 0.06
EXV wine	B1	43.7 ± 0.6	14.02 ± 0.04
	B2	29.5 ± 0.6	19.43 ± 0.02
	B3	26.9 ± 0.5	26.98 ± 0.05

[a] Calculated on the basis of total recovered carbohydrates. Mean±S.D. ($n = 20$).
[b] Mean ± S.D. ($n = 20$).

3.3. Identification and quantification of glycosyl residues by GC and GC-MS

The monosaccharide composition of all the fractions (A, B, B1, B2 and B3) was first determined by GC and GC-MS of their TMS residues. In order to be able to identify and quantify the monosaccharides in the gas chromatograms (Fig. 4), calibration graphs of sugar standards were required. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, Kdo, D-galacturonic acid, D-glucuronic acid and D-apiose were used as monosaccharide standards, myo-inositol was used as internal standard. The equation, slope and intercept standard deviations, the correlation coefficients (r) and the limits of detection (LD) and quantification (LQ), for the carbohydrate standards are shown in Table 4. In addition, a recovery study of each standard was carried out (Table 4). The correlation coefficients obtained from the linear calibration graphs were all ≥ 0.992 ($P < 0.001$). These curve were therefore, were considered to be linear for the range of amounts studied (0–5 mg). The LDs and LQs showed an acceptable sensitivity; all the values obtained for the monosaccharides present in the fractions were above these limits. The recovery results showed the precision of the calibration curves.

GC-MS was used to identify those monosaccharides for which no commercial standards were available: 2-*O*-methyl fucose, aceric acid and Dha (3-deoxy-D-*lyxo*-heptulosaric acid). The identification of the peaks in the chromatogram was based on their GC retention times and MS fragmentation patterns reported in [38]. These carbohydrates were quantified using the 2-*O*-methyl xylose calibration curve.

3.4. Validation of the proposed method

The applicability of the method was checked by analysing real wine samples. Repeatability was evaluated by analysing of 10 wine aliquots under normal operating conditions. Wine samples were centrifuged and the supernatants concentrated and precipitated. The residues obtained were freeze-dried, methylated, derivatizated and submitted to GC and GC-MS analysis. The amount of D-galactose, D-glucose and D-mannose, major wine carbohydrates, was quantified in each aliquot and the repeatability expressed as the coefficient of variation ($n = 10$). The values obtained were 2.4% for galactose, 3.8% for glucose and 3.2% for mannose. The reproducibility of the method was calculated as the mean of the coefficients of variation of each of the samples analysed, from three replicate measurements. The same sugars described above were determined and the values obtained were 2.0 ± 0.6% for galactose, 4.2 ± 1.2% for glucose and 3.8 ± 0.9% for mannose. In addition, a recovery study of galactose was carried out in order to assess the accuracy

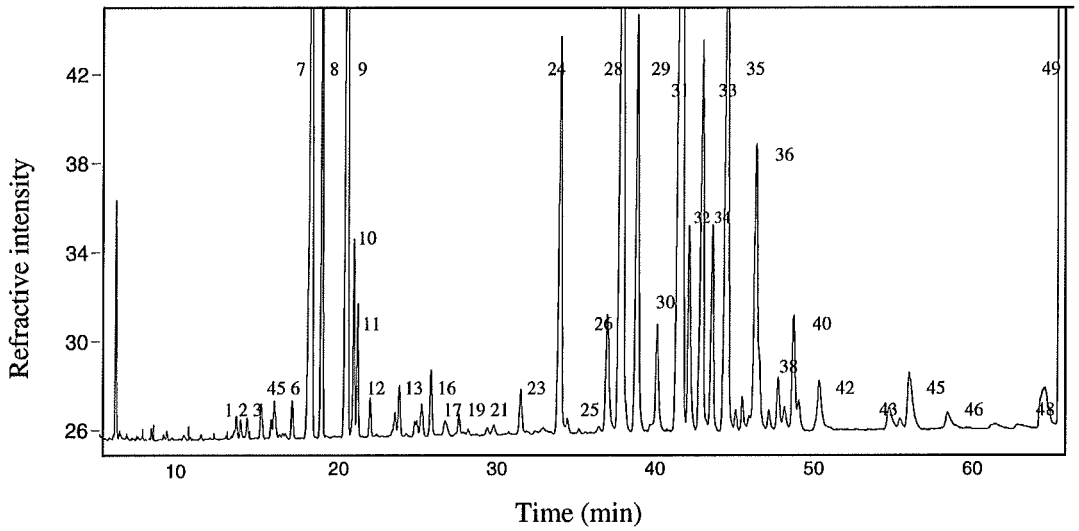

Fig. 4. GC-FID profiles of the glycosyl residues of red wine polysaccharides (fraction B1 of the control wine). Aceric acid (peaks 1, 2), 2-*O*-Me-fucose (peak 3), 2-*O*-Me-xylose (peaks 4, 5), apiose (peaks 6–8), arabinose (peaks 7, 8, 11, 15), rhamnose (peaks 9–11), fucose (peaks 10, 12, 13), xylose (peaks 14, 16, 21, 25), galacturonic acid (peaks 17, 24, 26, 33, 34, 39, 41), glucuronic acid (peaks 22, 37, 38, 42), Dha (peaks 27, 30, 32), mannose (peaks 28, 30), galactose (peaks 29, 31, 32, 35), Kdo (peaks 34, 45, 47, 48), glucose (peaks 36, 40, 44, 46), inositol (peak 49).

of the method. Ten wine samples were analysed to give the amount of galactose present and analysed again after the addition of two different quantities (1 and 2 mg) of this monosaccharide. The recovery obtained was 99 ± 4%.

3.5. Analysis of insoluble fraction A and soluble fraction B

The carbohydrate compositions of fractions A and B are shown in Table 5.

The carbohydrate content of insoluble fractions A was quite similar in both wines analysed. Glucose, a constituent of condensed polyphenolic compounds and microbial cell walls [35,39], was the main sugar detected in fractions A, representing >42% of total insoluble polysaccharides. Mannose, the main component of yeast mannans and mannoproteins [40–42], was also found in large amounts. The other sugars detected were galactose, arabinose and rhamnose, the glycosyl residues found in arabinogalactans [14]. Galacturonic acid, the main component of homogalacturonans and rhamnogalacturonans [10], was also present in small amounts. The insoluble polysaccharides present in fractions A represented quite an important amount of total wine polysaccharides, 44% for the control wine and 29% for the wine treated with enzymes (EXV wine). Thus, the precipitation of polysaccharides is an important phenomenon during the fermentation–maceration stage. This polysaccharide insolubilisation, due to the effect of ethanol, affects mainly mannans, mannoproteins, arabinogalactans and arabinogalactan-proteins.

Table 4
Monosaccharide standard calibration parameters and validation results for GC-FID

Sugar	Equation[a]	S.D.$_{slope}$ (n = 10)	S.D.$_{intercept}$ (n = 10)	LD (μg)	LQ (μg)	r^{b}	Recovery (%) (n = 5)
Fucose	$A = 0.496C - 0.004$	0.016	0.010	<1	5	0.997	103 ± 4
Rhamnose	$A = 0.642C - 0.009$	0.032	0.012	<1	<1	0.994	103 ± 7
2-*O*-Me Xyl[c]	$A = 0.294C - 0.002$	0.006	0.003	<1	7	0.998	101 ± 2
Arabinose	$A = 0.449C - 0.006$	0.035	0.004	<1	4	0.992	98 ± 5
Galactose	$A = 0.401C - 0.022$	0.010	0.025	<1	<1	0.999	101 ± 4
Xylose	$A = 0.4616C - 0.0003$	0.017	0.001	3	10		99 ± 5
Glucose	$A = 0.857C - 0.089$	0.007	0.020	14	21	0.999	99 ± 2
Mannose	$A = 0.828C - 0.009$	0.071	0.002	<1	1	0.999	100 ± 2
Kdo[c]	$A = 0.0812C - 0.0009$	0.004	0.002	2	9	0.997	97 ± 5
GalA[c]	$A = 0.250C - 0.003$	0.001	0.004	<1	<1	0.999	100 ± 2
GlcA[c]	$A = 0.282C - 0.003$	0.017	0.010	<1	7	0.999	100 ± 1
Apiose	$A = 0.224C - 0.005$	0.011	0.007	2	9	0.992	97 ± 5

[a] A and C denote the peak area and amount in mg, respectively.
[b] Linear correlation coefficient for 0–5 mg (n = 8).
[c] 2-*O*-Me Xyl, 2-*O*-methyl xylose; Kdo, 3-deoxy octulosonic acid; GalA, galacturonic acid; GlcA, glucuronic acid.

Table 5
Carbohydrate composition ($mg\,l^{-1}$) of fractions A and B determined by GC and GC-MS of their TMS derivatives

Sugars	Control wine		EXV wine	
	Fraction A	Fraction B	Fraction A	Fraction B
2-*O*-Me Fuc[a]	–[b]	16 ± 1	–	37 ± 1
Rhamnose	39.9 ± 0.2	50.7 ± 0.3	40.0 ± 0.1	87.5 ± 0.3
Fucose	–	16.5 ± 0.3	–	39.2 ± 0.4
2-*O*-Me Xyl[a]	–	16 ± 1	–	38 ± 1
Arabinose	42.5 ± 0.3	134 ± 1	47 ± 1	168 ± 1
Xylose	–	3.9 ± 0.2	–	7.8 ± 0.2
Apiose	–	38.8 ± 0.2	–	8.9 ± 0.2
Mannose	151 ± 1	134 ± 1	119 ± 1	194 ± 2
Galactose	152 ± 1	193 ± 1	157 ± 1	369 ± 1
Glucose	358 ± 2	119 ± 1	304 ± 2	270 ± 1
GalA[a]	52.6 ± 0.1	64 ± 1	48 ± 1	117 ± 2
GlcA[a]	–	23.1 ± 0.1	–	54 ± 1
Kdo[a]	–	110 ± 1	–	231 ± 2
Dha[a]	–	59.6 ± 0.4	–	125 ± 1
AceA[a]	–	38.8 ± 0.2	–	8.9 ± 0.2
Total	796 ± 2	1017 ± 3	715 ± 3	1755 ± 4

[a] 2-*O*-Me Fuc, 2-*O*-methyl fucose; 2-*O*-Me Xyl, 2-*O*-methyl xylose; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; AceA, aceric acid.
[b] $<1\,mg\,l^{-1}$.

The soluble fractions B contained all the sugars that form wine polysaccharides. Large quantities of mannose, arabinose, galactose, rhamnose, glucuronic and galacturonic acid were found and several rare sugars, such as apiose, fucose, 2-*O*-methyl L-fucose, 2-*O*-methyl D-xylose, aceric acid (3-*c*-carboxy-5-deoxy-L-xylose), Kdo (3-deoxy octulosonic acid), and Dha (3-deoxy-D-*lyxo*-heptusolaric acid) were also quantified. These rare sugars are known as markers of the RG-II molecule [16]. The presence of all these glycosyl residues confirmed the predominance of mannans, mannoproteins, arabinogalactans, rhamnogalacturonans and RG-II molecules in red wines [43]. The presence of glucose in fractions B could be attributed to microbial polysaccharides and condensed anthocyanins [35]. The presence of xylosyl residues indicated that traces of hemicelluloses (arabinoxylans or xyloglucans) might also be solubilised from grape cell walls [35].

The quantity of total soluble monosaccharides of fractions B was higher in the wine treated with enzymes (EXV wine) than in the control one, indicating that the commercial enzymes had greater effects on wine carbohydrates than the endogenous ones.

3.6. Estimation of polysaccharide concentrations

Wine fractions B were submitted to HRSEC, and three different fractions were obtained: B1, B2 and B3. The monosaccharide composition of these fractions (Table 6) was determined by GC and GC-MS of their TMS residues as described previously. The polysaccharide content of each fraction (Table 7) was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

High molecular weight polysaccharides, collected in fraction B1, were composed mainly of mannose, arabinose, galactose, rhamnose and glucuronic acid (Table 6), confirming the predominance of mannans and mannoproteins (M) and arabinogalactans and arabinogalactan-proteins (AGs) among wine polysaccharides. Arabinogalactans (AGs) are mainly composed of galactose and arabinose and minor amounts of rhamnose and glucuronic acid [14]. The molecular ratios of these residues depend on type and the hydrolysis rate of the molecule [14,22,44]. AGs were therefore estimated from the sum of galactosyl, arabinosyl, rhamnosyl and glucuronosyl residues. All the mannose content was attributed to yeast mannans and mannoproteins (M).

The composition of fraction B2 was more complex and all the rare diagnostic sugars of the RG-II molecule were detected (Table 6), confirming the predominance of this polysaccharide. These characteristic sugars included apiose, 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, aceric acid (3-*c*-carboxy-5-deoxy-L-xylose), Kdo (3-deoxy octulosonic acid), and Dha (3-deoxy-D-*lyxo*-heptusolaric acid). However, the molar ratios of arabinosyl, rhamnosyl and galactosyl residues were greater than expected for a purified RG-II molecule [17,45], and mannose and glucuronic acid were also present in these fractions (Table 6), indicating the presence of low molecular weight arabinogalactans, mannans and mannoproteins. The RG-II content was calculated from the sum of its diagnostic sugars, which represent approximately 25% of the RG-II molecule [35]. For one residue of 2-*O*-methyl fucose, RG-II contains 5 rhamnosyl, 3 arabinosyl, 2 galactosyl and 10 galacturonosyl residues [17,45]. Taking into account these molar ratios, it was possible to estimate their respective amounts in the RG-II. The remaining part was attributed to the

Table 6
Carbohydrate composition (mg l^{-1}) of fractions B1, B2 and B3 obtained by HRSEC on a Superdex-75 HR column and determined by GC and GC-MS of their TMS derivatives

	Control wine fractions			EXV wine fractions		
	B1	B2	B3	B1	B2	B3
2-*O*-Me Fuc[a]	–[b]	6.0 ± 0.1	4.6 ± 0.1	–	10.6 ± 0.1	4.9 ± 0.1
Rhamnose	18.9 ± 0.4	20.8 ± 0.2	8.9 ± 0.1	17.2 ± 0.3	32.6 ± 0.2	11.1 ± 0.1
Fucose	6.0 ± 0.1	5.7 ± 0.1	4.4 ± 0.1	6.9 ± 0.2	9.9 ± 0.1	4.8 ± 0.1
2-*O*-Me Xyl[a]	–	6.2 ± 0.1	4.8 ± 0.1	–	11.3 ± 0.1	4.8 ± 0.1
Arabinose	37.4 ± 0.8	40.0 ± 0.4	13.5 ± 0.2	124 ± 1	65.9 ± 0.5	17.1 ± 0.1
Xylose	2.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	1.5 ± 0.1
Apiose	–	14.2 ± 0.1	9.5 ± 0.1	–	24.4 ± 0.2	10.0 ± 0.1
Mannose	106.5 ± 2.2	18.1 ± 0.2	16.9 ± 0.2	114 ± 3	36.2 ± 0.3	21.4 ± 0.3
Galactose	141.6 ± 2.9	50.9 ± 0.5	34.9 ± 0.4	142 ± 3	89.6 ± 0.6	42.6 ± 0.3
Glucose	4.6 ± 0.8	30.1 ± 0.3	41.6 ± 0.5	5 ± 1	49.0 ± 0.4	49.7 ± 0.4
GalA[a]	18.0 ± 0.4	27.2 ± 0.2	14.4 ± 0.2	15.0 ± 0.4	46.7 ± 0.3	20.9 ± 0.2
GlcA[a]	12.8 ± 0.3	8.5 ± 0.1	5.3 ± 0.1	13.4 ± 0.3	13.5 ± 0.1	5.9 ± 0.1
Kdo[a]	–	16.8 ± 0.2	10.5 ± 0.1	–	36.2 ± 0.3	11.2 ± 0.1
Dha[a]	–	9.1 ± 0.1	5.7 ± 0.1	–	19.5 ± 0.2	6.1 ± 0.1
AceA[a]	–	14.2 ± 0.1	10.3 ± 0.1	–	24.0 ± 0.2	9.7 ± 0.1

[a] 2-*O*-Me Fuc, 2-*O*-methyl fucose; 2-*O*-Me Xyl, 2-*O*-methyl xylose; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; AceA, aceric acid.
[b] <1 mg l^{-1}.

presence of AGs in the case of rhamnose, arabinose and galactose.

Fraction B3 contained all the sugars known to participate in the composition of wine polysaccharides but they were present only in small amounts (Table 6). The presence of these carbohydrates was attributed to low molecular weight arabinogalactans and mannans and oligomers of homogalacturonans and rhamnogalacturonans. Homo- and rhamnogalacturonans oligomers (GU) were estimated from the galacturonic acid content [10]. All the diagnostic sugars of RG-II were found although the presence of RG-II in this fraction has never been reported [35] and it has a higher molecular weight than the molecules eluting in this fraction (see Section 3.2). In fact, it is possible that the presence of these rare sugars was due to low molecular weight fragments of the RG-II rather than to the entire molecule. Thus, RG-II was not quantified in this fraction.

The polysaccharide content (AGs, mannans and mannoproteins, RG-IIs and galacturonans) of wine samples was deduced from the sums of the respective polysaccharides present in fractions B1, B2 and B3 (Fig. 5). The comparison of the polysaccharide contents of both wines allowed an evaluation of the action of the commercial enzymes added to the EXV wine. In both wines, the polysaccharides consisted mainly of arabinogalactans, arabinogalactan-proteins, mannans, mannoproteins and rhamnogalacturonans-II (Fig. 5). AGs represented about 40% of total soluble polysaccharides in both samples, which is in good agreement with previous observations [13,14]. However, the RG-II level found (38% in the control wine and 46% in the EXV wine) was high in comparison with previous studies [16,17], although these studies dealt with Carignan noir red wines, which present a different polysaccharide quantity than Tempranillo wines. Mannoproteins and mannans were about 19% and GUs represented only a small percent (2%).

The content of mannans and mannoproteins was very similar in both wines with values in the range of 141 (control

Table 7
Polysaccharide concentration (mg l^{-1}) of Wine fractions B1, B2 and B3

Wines	Fractions	AG[a]	M[b]	RG-II[c]	GU[d]
Control wine	B1	211	106	–	–
	B2	76	18	289	–
	B3	21	17	–	14
EXV wine	B1	297	114	–	–
	B2	120	36	544	–
	B3	28	21	–	21

[a] AG, arabinogalactans and arabinogalactan-proteins.
[b] M, mannans and mannoproteins.
[c] RG-II, rhamnogalacturonans-II.
[d] GU, oligomers of homo- and rhamnogalacturonans.

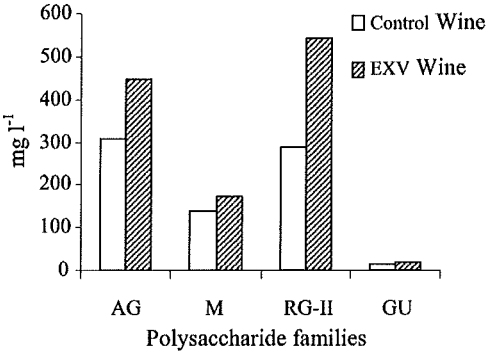

Fig. 5. Total concentration of AG, M, RG-II and GU in wines. AG, arabinogalactans and arabinogalactan-proteins; M, mannáns and mannoproteins; RG-II, rhamnogalacturonans-II; GU, oligomers of homo- and rhamnogalacturonans.

wine) to 171 mg l^{-1} (EXV wine). The same commercial yeast strain was used to produce of both wines, which excluded any variability based on the nature of the strain. A completely different behaviour was observed with AGs and RG-IIs. The content of AGs was much higher in the wine treated with enzymes than in the control, and the amount of RG-II was almost double in the EXV wine. These findings indicated that solubilisation of arabinogalactans, arabinogalactan-proteins and RG-IIs had occured during the maceration–fermentation process of EXV wine due to the action of the commercial enzymes added. These enzymes hydrolysed the polysaccharides from grape berry cell walls but did not affect yeast cell wall polysaccharides.

4. Conclusions

Wine polysaccharides play an important role in wine technology, either for their sensory characteristics, their implications during fermentation or their detrimental role in filtration. Enzymic treatments represent powerful tools to control these phenomena since they may alter wine polysaccharide composition. In this study, the influence of commercial enzymes on wine polysaccharide content was analysed. Tempranillo wines were made using maceration commercial enzyme preparations together with controls.

The analytical method for the quantification of wine polysaccharides was carried out by a multistep procedure: concentration of wine, precipitation of polysaccharides by the addition of an acid-ethanol medium, fractionation of polysaccharide families by HRSEC on a Superdex-75 HR column, and determination of carbohydrate compositions of the fractions by GC-FID and GC-MS of their trimethylsilyl-ester *O*-methyl glucosides (TMS) after acidic methanolysis and derivatization. The polysaccharide content of each fraction was estimated from the concentration of individual glycosyl residues that are characteristic of well-defined wine polysaccharides.

This study indicated that wine and must samples needed to be concentrated (tree times for musts and five times for wines) before precipitation in order to ensure the quantitative precipitation of all polysaccharide families. The proposed method presented a good sensitivity, reproducibility and accuracy.

Soluble polysaccharides in wine consisted essentially of grape cell wall polysaccharides: arabinogalactans and arabinogalactan-proteins (38–41%), and rhamnogalacturonans-II (38–46%). Yeast mannans and mannoproteins were also present but in smaller amount (14–19%). In comparison with the controls, wines treated with commercial enzymes presented higher concentrations of arabinogalactans, arabinogalactan-proteins (445 mg l^{-1} versus 308 mg l^{-1}) and rhamnogalacturonans-II proteins (544 mg l^{-1} versus 289 mg l^{-1}). The content of mannans and mannoproteins was similar in both wines. These findings indicated that grape pectic polysaccharides were hydrolysed and solubilised during the maceration–fermentation due to the action of the commercial enzymes added. However, these enzymes had no influence in yeast parietal polysaccharides.

The precipitation of wine polysaccharides was observed as an important phenomenon occurring during the maceration–fermentation process, as the polysaccharide content present in the insoluble pellets accounted for a relatively significant proportion of total wine polysaccharides (29–44%).

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3.2

DESARROLLO Y VALIDACIÓN DE UN MÉTODO DE ANÁLISIS PARA LA DETERMINACIÓN DE POLIFENOLES EN MOSTOS Y VINOS

Analysis of polimeric phenolics in red wines using different techniques combined with gel penneation chromatography fractionation

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Resumen

En este artículo se desarrolla un método de análisis que permite cuantificar distintas familias de compuestos fenólicos tanto en mostos como en vinos tintos.

El método propuesto consta de cuatro etapas:

1. Fraccionamiento de los polifenoles mediante cromatografía de permeación en gel (GPC) utilizando distintos solventes. Se obtienen así dos fracciones: F1, que contiene los compuestos fenólicos monómeros y antocianos dímeros y poliméricos, y F2, que contiene las proantocianidinas de naturaleza polimérica.
2. Identificación y cuantificación de los polifenoles monómeros en F1 por cromatografía líquida de alta resolución con detector de fila de diodos (HPLC-DAD) y detector de masas (HPLC-MS), determinándose de forma simultánea el contenido de distintas formas de antocianos monómeros, ácidos hidroxicinámicos, flavonoles y flavan-3-oles monómeros.
3. Análisis de los pigmentos poliméricos en F1 mediante electroforesis capilar de zona (CZE).
4. Cuantificación de las proantocianidinas en F2 con una variación del método de la vainillina.

La etapa inicial de fraccionamiento por GPC permitió eliminar los compuestos que podrían causar interferencias en los análisis posteriores, produciéndose una mejora sustancial en los distintos métodos de análisis. Así, se mejoró el análisis de los antocianos poliméricos por CZE, tanto en términos de respuesta, como de repetibilidad y reproducibilidad, consiguiéndose una separación de los antocianos poliméricos del vino en siete picos diferenciados. La etapa previa de fraccionamiento eliminó además los antocianos y flavanoles monómeros que interfieren en la medida de la vainillina, obteniéndose de este modo una determinación más precisa y fiable del contenido de las proantocianidinas en los vinos.

Se realizó además un estudio de validación de todas las etapas del método así como una validación global del mismo, obteniéndose resultados satisfactorios en todos los casos en términos de reproducibilidad, repetibilidad y recuperación.

Por último, se propuso una variación del método anterior con el objetivo de cuantificar las proantociadinas astringentes, ofreciéndose una alternativa a los índices generales utilizados habitualmente como el *índice de gelatina*. La aplicación de dicho método mostró una buena correlación entre el contenido de proantocianidinas astringentes medidas en distintos vinos y sus puntuaciones sensoriales.

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Analysis of polymeric phenolics in red wines using different techniques combined with gel permeation chromatography fractionation

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Abstract

A multiple-step analytical method was developed to improve the analysis of polymeric phenolics in red wines. With a common initial step based on the fractionation of wine phenolics by gel permeation chromatography (GPC), different analytical techniques were used: high-performance liquid chromatography–diode array detection (HPLC–DAD), HPLC–mass spectrometry (MS), capillary zone electrophoresis (CZE) and spectrophotometry. This method proved to be valid for analyzing different families of phenolic compounds, such as monomeric phenolics and their derivatives, polymeric pigments and proanthocyanidins. The analytical characteristics of fractionation by GPC were studied and the method was fully validated, yielding satisfactory statistical results. GPC fractionation substantially improved the analysis of polymeric pigments by CZE, in terms of response, repeatability and reproducibility. It also represented an improvement in the traditional vanillin assay used for proanthocyanidin (PA) quantification. Astringent proanthocyanidins were also analyzed using a simple combined method that allowed these compounds, for which only general indexes were available, to be quantified.
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Keywords: Red wine; Anthocyanins; Polymeric pigments; Proanthocyanidins; Astringent proanthocyanidins; GPC/HPLC/DAD/CZE

1. Introduction

Polyphenolic compounds are widely known for their role in the organoleptic properties of wines and are extremely important for determining the final quality of the product, especially in red wines. The quality of these organoleptic characteristics of red wines will depend not only on the quantity of phenolic pigments, but also on their type, composition and distribution.

Two important families of polyphenolic compounds present in grapes are known to influence final wine quality: proanthocyanidins (condensed tannins with a polymerization degree over 5) and anthocyanins. Proanthocyanidins (PAs) are important for providing wine with bitterness and astringency. Polymeric anthocyanins are known to be responsible for the stable colour of red wines. The colour of these types of pigments is more stable to pH increases than that of monomeric anthocyanins; these pigments are also less sensitive to oxidation and to bleaching by sulphur dioxide than monomeric anthocyanins [1–3].

Colour changes in red wine from the initial red–purple to a more brick hue, occurring continuously during winemaking and ageing, are supposedly due to irreversible mechanisms and prompt the formation of new and stable pigments, with either high or low molecular weights [4–10]. The pigments in aged red wine appear to be primarily large polymeric compounds and are formed due to reactions of direct or indirect condensation between anthocyanins and proanthocyanidins [11].

Grape proanthocyanidins are responsible for some major wine organoleptic properties, as well as for the physiological effects associated with its consumption [12]. The mechanism of astringency perception is commonly ascribed to interactions of proanthocyanidins with salivary proteins. This property is known to vary both with PA structure and degree of polymerization, the larger PA molecules being the most important in the astringency sensation [13]. Kallithraka et al. [14], in an attempt to compare taste-panel results with analytical data, suggested that perceived astringency could be closely correlated to the amount of flavanols not precipitated by salivary proteins.

Many chromatographic methods have been developed for analyzing grape and wine phenolics. High-performance liquid chromatography (HPLC) techniques with photodiode array or mass spectrometry (MS) detection are widely used for the separation and quantitative determination of individual monomeric and oligomeric flavonoids from red wines. However, these

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techniques are limited in their ability to analyze high molecular weight polyphenols. The analysis of polymeric pigments is relatively complex at present; their complex structure and low quantity makes their detection and separation difficult. Many methods have been used so far for the fractionation of polymers from red wine. Most of these methods are based on the separation of red wine pigments by gel permeation chromatography (GPC), solid-phase extraction (SPE) on C_{18} cartridges or more recent techniques such as countercurrent chromatography. These methods have been mainly developed to fractionate, isolate and identify new anthocyanin-derived pigments [3–5,7–9,15,16]. However, there have only been a few attempts to separate and quantify more complex polymeric pigments [11,17–19]. Recently, a method based on capillary zone electrophoresis (CZE) proved to be efficient enough to separate red wine polymeric pigments [20].

Whereas only a few studies of polymeric pigments from red wine have been published, many publications deal with proanthocyanidins from grape or grape skin. Different chromatographic techniques (HPLC–DAD, HPLC–EIS–MS) have been applied in the analysis of individual PAs, dimers, trimers and oligomers [21]; however, polymers of higher molecular weight cannot be resolved by these techniques. The methods used to estimate these polymers differ in terms of basic principles and specificity, and none of them can be considered totally satisfactory [21]. Traditionally, wine astringency is estimated using the gelatin index method proposed by Glories [22,23]. This method, based on the reaction between proanthocyanidins and gelatin, seems to be appropriate for estimating astringency; however, it only provides an index and not an absolute quantity.

The aim of this paper is to describe a versatile method that allows different families of polyphenols in red wines to be analyzed and quantified. Given the importance of polymeric phenolics in wine quality and the difficulties encountered in analyzing them, this method has been mainly developed for the analysis of these polymeric compounds. For this reason, a combination of techniques is proposed, all of them with a common initial step, namely the fractionation of red wine phenolics by GPC in order to separate monomeric and polymeric compounds. Thereafter, different analytical techniques were used: (a) identification and quantification of monomeric phenolic compounds of red wines by HPLC–DAD; (b) analysis of polymeric pigments by CZE; and (c) quantification of proanthocyanidins by reaction with vanillin. The interactions between polymeric phenolics and gelatin were also analyzed. Taking into account these results, a method for quantifying astringent proanthocyanidins is proposed. The properties of the described method were also studied and a validation study was performed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. All chromatographic solvents were of HPLC grade. Malvidin-3-glucoside, peonidin-3-glucoside, ferulic acid, syringic acid, caffeic acid, *p*-coumaric acid, catechin, epicatechin, myricetin, quercetin, isorhamnetin, kaempferol and rutin were purchased from Extrasynthèse (Lyon, France), and gallic acid from Sigma (St. Louis, MO, USA). Formic acid and acetonitrile supplied by Sigma and MilliQ (Darmstadt, Germany) ultrapure water were used. Acetone was obtained from Riedel-deHäen (Sigma), and pure methanol, ethanol and disodium tetraborate from Merck. Trifluoroacetic acid and toluene-α-thiol (benzyl mercaptan) were supplied by Fluka (Sigma), phosphoric acid, hydrochloric acid, and sulfuric acid by Carlo Erba (Rodano, Italy), sodium hydroxide by Prolabo (France), and tartaric acid by Sigma. Vanillin was obtained from Aldrich (Sigma) and gelatin 80–100 blooms from Panreac (Montcada i Reixac, Barcelona, Spain). All the solutions were filtered through a 0.45 μm filter and sonicated for 15 min before use in HPLC or CZE.

The samples used were red wine from the Qualified Origin Denomination Rioja (D.O.Ca. Rioja). The wines were selected at several stages of vinification and maturation, presenting different organoleptic characteristics.

2.2. Fractionation of wine phenolics by GPC

TSK Toyopearl gel HW-50F (Tosohaas, Montgomery-ville, PA, USA) was suspended in the mobile phase and, after swelling, it was packed in a Millipore (Bedford, MA, USA) Vantage L column (120 mm × 12 mm i.d.) at atmospheric pressure. Gel permeation chromatography data were analyzed by connecting the column to a diode array detector (Agilent, G1315B). Two milliliters (2 ml) of wine were directly applied to the column and flow rate was regulated at 1 ml min^{-1} using a peristaltic pump. A first fraction (F1) was eluted with 60 ml of ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v). A second fraction (F2) was recovered by elution with 50 ml of acetone/water (60:40, v/v). The two fractions collected were taken to dryness under vacuum. All the wines were fractionated three times.

2.3. HPLC–DAD analysis

HPLC–DAD was performed in an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with a G1313A injector, a G1311A HPLC quaternary pump, an online G1379A degasser, a G1316A oven, a G1315B photodiode array detector, and an Agilent Chemstation software. The column was a reversed-phase Kromasil 100-C18 (5 μm packing, 200 mm × 46 mm i.d.) protected with a guard column of the same material (Teknokroma, Barcelona, Spain). Phenolic compounds were eluted under the following conditions: 1 ml min^{-1} flow rate; oven 30 °C; solvent A: formic acid/water (2:98, v/v); solvent B: acetonitrile/water/formic acid (80:18:2, v/v/v); gradients: isocratic 2% B in 3 min, from 2 to 10% B in 2 min, from 10 to 15% B in 10 min, from 15 to 30% B in 10 min, from 30 to 50% B in 10 min, from 50 to 60% B in 5 min, from 60 to 90% B in 5 min, followed by washing and reconditioning of the column.

Fractions were dissolved in 2 ml of solvent A and 50 μl was directly chromatographed. UV–vis spectra were recorded every second from 250 to 600 nm, with a bandwidth of 1.2 nm. The chromatograms were acquired at 515 nm for anthocyanins, 365 nm for flavonols, 310 nm for phenolic acids and 280 nm for flavan-3-ols. The different compounds were identified on the basis of their UV–vis spectra, retention times and by comparison with commercial standards. Unknown peaks were identified by mass spectrometry. The calibration curves were obtained by injecting different concentrations of standards: malvidin-3-glucoside for anthocyanins and anthocyanin derivatives, caffeic acid for phenolic acids, quercetin for flavonols, and catechin for flavan-3-ols. The range of the linear calibration curves ($r^2 > 0.99$ in all the cases) was from 0.01 (limit of detection) to 1 mg l^{-1} for the lower concentration compounds and from 1.0 to 100 mg l^{-1} for the higher concentration compounds. Unknown concentrations were determined from the linear regression equations. Each measurement was run in triplicate.

2.4. HPLC–MS analysis

MS analysis was performed by coupling the Agilent 1100 liquid chromatograph described above to a MS detector (Hewlett-Packard, Palo Alto, CA, USA). The mass spectrometer was equipped with an electrospray ionization source and a quadrupole mass analyzer, which were controlled by the MS Agilent 1100 software. Chromatographic separation was performed under the same conditions described above. The flow was split into a ratio of 35:100 between the HPLC detector and the MS detector in order to introduce the optimal flow-rate (35 μl min^{-1}) into the electrospray ionization interface. The eluted compounds were mixed with nitrogen at a 30 l min^{-1} flow-rate and 225 °C in the electrospray ionization interface. The mass spectrometer was operated in the positive ion mode for anthocyanins (*m/z* 100–600) and in the negative ion mode for flavonols (*m/z* 100–600), phenolic acids (*m/z* 200–600) and flavan-3-ols (*m/z* 200–600). Cone voltage was a linear function of the relative function of the relative molecular mass, starting at 40 V for *m/z* 100 and ending at 80 V for *m/z* 600, nebulizer pressure was 80 psi, and capillary voltage, 4000 V. The compounds were chemically ionized by proton transfer, the ions generated were introduced into the mass spectrometer and the abundance of selected *m/z* corresponding to $(M\text{-}H)^+/(M\text{-}H)^-$ ions of compounds was recorded.

2.5. Thiolysis conditions

Fractions F1 and F2 were dissolved in 2 ml of pure methanol and introduced in a glass vial with an equal volume of thiolytic reagent (toluene-α-thiol 5% in methanol containing 0.2 M HCl). After sealing, the mixture was shaken and heated at 90 °C for 2 min. The solutions were then analyzed by HPLC–DAD under the same conditions described above. Quantification of each terminal and extension unit was based on peak areas at 280 nm [24]. Each mixture was analyzed in triplicate.

2.6. CZE analysis

Capillary zone electrophoresis was performed using an Agilent CE instrument (Waldbronn, German) equipped with a standard cassette containing an uncoated fused-silica capillary and diode array detector. Sodium tetraborate buffer solutions (50 mM) of pH 9.4 with 10% methanol (v/v) content, and 56 cm (effective length) capillary were used to separate anthocyanins and polymeric anthocyanins. The remaining CZE conditions were those described by Sáenz López et al. [20].

Wine fractions were dissolved in 500 μl of synthetic wine (12% (v/v) ethanol in aqueous solution containing 6 g l^{-1} tartaric acid, pH 3.5). Thereafter, the samples were centrifuged (5000 rpm, 5 min, room temperature) using a 5804 Eppendorf centrifuge, and directly injected in the CZE system. Wine samples were also prepared in order to compare their response with that of the fractions. These wine samples were concentrated four times prior analysis in order to reach the same degree of concentration than wine fractions.

Electrophoregrams were recorded at 280, 420, 520 and 599 nm, and the spectrum from 200 to 600 nm was also collected for each peak. The polymeric anthocyanins were detected at 280 nm and 520 nm, and anthocyanins at 599 nm because they were present as a blue quinoidal base at pH 9.4. The identification of monomeric anthocyanin and pyranoanthocyanin peaks was based on the migration times of these compounds in the electrophoregrams and on [25,26]. All the analyses were performed in triplicate.

2.7. Determination of the total proanthocyanidin content by the vanillin assay

The vanillin assay was performed according to the method described by Sun et al. [27] but with few modifications. Fraction F2 was dissolved in a suitable quantity of methanol in order to have a final absorbance within the linear range of the standard curve; for the general case, 7.5 ml of methanol was added. One milliliter (1 ml) of this solution was placed in a vial and 2.5 ml of 1% (w/v) vanillin in methanol and 2.5 ml of sulphuric acid/methanol (10:90, v/v) were added. The absorbance of the coloured adducts formed between vanillin (4-hydroxy-3-methoxybenzaldehyde) and proanthocyanidins was measured at 500 nm in 1 cm-cuvettes. The reaction was performed at room temperature and left until the maximum absorbance value at 500 nm was reached, which occurred at around 15 min of reaction time.

The spectrophotometric measurements were performed on a Cary 300 Scan UV–vis spectrophotometer (Varian Inc., Madrid, Spain). First, a blank was made to eliminate the absorbance of residual pigments present in F2. The blank was prepared in the same way described for the sample, but adding methanol instead of vanillin. When measuring the samples, reference solutions (adding methanol instead of the sample) were used for each sample. Quantification of proanthocyanidins was performed by means of a standard curve prepared with different concentrations of catechin. In this case, the reactions were performed at 30 °C

and the maximum absorbance value at 500 nm was reached at 18 min. Samples were analyzed in triplicate.

2.8. Determination of the total astringent proanthocyanidin content

The method proposed for the quantification of astringent proanthocyanidins is a combination of two methods, the vanillin assay and the assay based on the ability of proanthocyanidins to precipitate with gelatin.

Ten milliliters of a solution of gelatin (35 g l^{-1}) was added to 50 ml of wine. The wines, with and without gelatin (control wine), were shaken and kept at 4 °C for 72 h. Thereafter, the samples were centrifuged and the supernatants were collected and fractionated by GPC. Two milliliters of the supernatants was directly applied onto the TSK Toyopearl gel HW-50F column (120 mm × 12 mm i.d.). The fractionation was carried out in the same conditions described in Section 2.2. Fractions F2 from both wines were then analyzed by the vanillin assay as described in Section 2.7. The quantity of astringent proanthocyanidins was calculated as the difference found between the control wine and that with gelatin addition.

2.9. Determination of the tannin content

In order to determine the gelatin index in wine samples, these were precipitated with gelatin as described above. Precipitated proanthocyanidins were determined and calculated by comparing the tannin content obtained before and after precipitation, as described in [28].

2.10. Study of validation: repeatability, reproducibility and recovery

Repeatability was checked by analyzing six samples of the same wine under normal operating conditions. Wines were fractionated by GPC and the resulting fractions submitted to HPLC–DAD and CZE (fractions F1) and to the vanillin assay (fractions F2). The amount of different families of monomeric phenolics was quantified in each sample by HPLC–DAD and the content of total PAs was determined by the vanillin assay. In CZE, the resulting peak areas were quantified. Moreover, the content of astringent PAs was also determined in the six wines as described in Section 2.8. Repeatability results were expressed as the coefficient of variation obtained for the six measurements.

Reproducibility was assessed using five different wines at different stages of vinification, except for the quantification of total PAs in fractions F2, for which 60 samples of wines and musts were analyzed (unpublished results). Reproducibility was expressed as the mean value of the coefficients of variation obtained for the different wines from three replicate measurements.

GPC recovery was studied using five aliquots of the same wine fractionated by GPC and monomeric phenolics were quantified by HPLC. The same aliquots were directly injected in HPLC, without previous fractionation, and the same phenolics were quantified. The recovery was calculated by comparing the amounts for each compound in the samples with and without fractionation. To study proanthocyanidin recovery in the proposed method (fractionation + vanillin reaction), 20 ml of wine was fractionated in a bigger Millipore Vantage L column (120 mm × 320 mm) to obtain a suitable quantity of proanthocyanidin extract. Using the vanillin assay, an aliquot was analyzed to estimate the PA richness in the extract. Two different quantities of the lyophilized extract were added to a wine (equivalent to 68 and 150 mg l^{-1} of PAs). The original wine and the two enriched samples were fractionated and the proanthocyanidin content was determined in fractions F2 by the vanillin assay. This protocol was repeated three times and the recovery expressed as the mean of the six recoveries calculated.

3. Results and discussion

3.1. Analysis of fractions F1 and F2 obtained by GPC

Red wine was submitted to GPC in order to separate the phenolic compounds and avoid interferences in further analysis. Fig. 1 shows the chromatogram recorded at 515 nm and obtained during the GPC fractionation of red wine. It can be observed that most wine pigments were eluted in fraction F1, although a small quantity was also collected in fraction F2. Several authors [24,13] employing the same chromatographic conditions as in the present study, report that fraction F1 is mainly composed of monomeric phenols (flavan-3-ols, anthocyanins, flavonols and phenolic acids) and dimeric flavan-3-ols, while fraction F2 contains the oligomeric and the polymeric material (proanthocyanidins). Remy et al. [24] observed that polymeric pigments and oligomeric proanthocyanidins are distributed between the two fractions F1 and F2.

In order to confirm the findings reported in the bibliography, both fractions obtained after GPC fractionation underwent different analysis. A thiolysis reaction was performed in order to obtain information about the mean degree of polymerization (mDP) of the compounds present in both fractions. The depolymerization of the possible polymeric pigments and tannins by the thiolysis assay enabled us to calculate the mDP. This value, calculated as the ratio between the total number of units and the number of terminal units, was estimated as 1.5 for fraction F1.

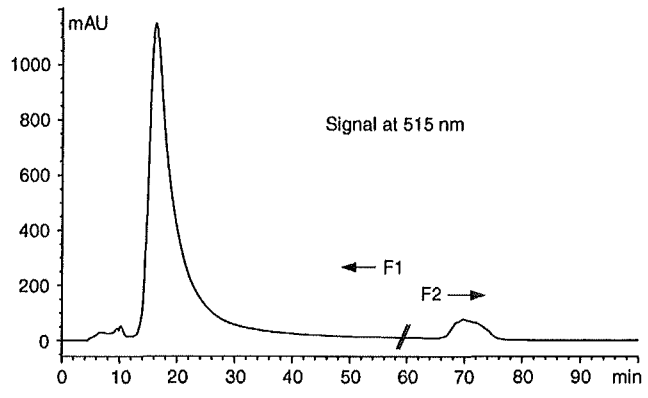

Fig. 1. Chromatogram of the GPC fractionation of a wine sample using a Toyopearl HW-50F column. See text for GPC conditions.

Table 1
Validation results[a] obtained for GPC analysis (see text for conditions and calculations)

	Monomeric anthocyanins	Phenolic acids	Flavonols	Flavan-3-ols
Repeatability (%)	4.9 ± 0.1	4.9 ± 0.1	4.7 ± 0.4	4.9 ± 0.1
Reproducibility (%)	4.9 ± 0.6	4.5 ± 0.2	4.8 ± 0.6	4.7 ± 0.5
Recovery (%)	101 ± 1	99 ± 4	96 ± 5	92 ± 7

[a] Expressed as a mean for each phenolic family (mean ± SD).

This suggested that most of the polyphenols present in F1 were monomers and dimers. Comparatively, the mDP of fraction F2 was estimated as 20, confirming the presence of proanthocyanidins.

Fraction F2 was also analyzed by HPLC–DAD and a minimum response (below the quantification limit) was observed for monomeric anthocyanins at 515 nm. This suggested that the low signal visualized at 515 nm when fractionating F2 (Fig. 1), may be due to the presence of this small amount of residual monomeric anthocyanins. However, the presence of polymeric pigments could not be ruled out. This possibility will be discussed later in Section 3.3., when analyzing polymeric pigments by CZE.

3.2. Analysis of pigments in fraction F1 by HPLC–DAD and HPLC–MS

Fraction F1 was submitted to HPLC–DAD and HPLC–MS analysis in order to identify and quantify the monomeric pigments present in it. Eleven monomeric anthocyanins were identified: delphinidin-3-*O*-glucoside; cyanidin-3-*O*-glucoside; petunidin-3-*O*-glucoside; peonidin-3-*O*-glucoside; malvidin-3-*O*-glucoside; delphinidin-3-glucosylacetate; petunidin-3-glucosylacetate; malvidin-3-glucosylacetate; delphinidin-3-glucosylcoumarate; petunidin-3-glucosylcoumarate; and malvidin-3-glucosylcoumarate. Different phenolic acids, flavan-3-ols, and flavonols were also identified and quantified in this fraction. Among the phenolic acids, gallic acid, *c*-caftaric acid, *t*-caftaric acid, *c*-coutaric acid, *t*-coutaric acid, caffeic acid and *p*-coumaric acid were identified; among the flavonols, rutin, myricetin-3-glucoside, kaempferol, myricetin, quercetin and isorhamnetin-3-glucoside; and among flavan-3-ols, catechin, epicatechin and epigallocatechin.

In order to validate the methods used, GPC fractionation and HPLC analysis, repeatability, reproducibility and recovery were assessed (see Section 2.10.) and expressed as a mean for each phenolic family (Table 1). All these results showed that the method proposed had good reliability and accuracy. These analytical characteristics are well established for the HPLC technique but not for fractionation by GPC on a Toyopearl column, which is widely used.

3.3. Analysis of pigments in fractions F1and F2 by CZE

Monomeric phenolics were successfully identified by HPLC–DAD and HPLC–MS; however, the polymeric pigments could not be visualized by these techniques. In order to be able to study this group of compounds, capillary zone electrophoresis was chosen. This technique allows the separation of many classes of compounds based on the electrophoretic migration of charged analytes. Recently, CZE has shown to be efficient enough to separate red wine polymeric pigments from monomeric anthocyanins and anthocyanin derivatives, and showed higher separation efficiency than HPLC and reduced analysis time and solvent consumption [20]. Moreover, pigmented polymers were properly separated in different peaks, whereas with other techniques these compounds eluted as diffuse humps or as a single peak [17,19,29].

Firstly, fractions F1 and F2 obtained after GPC fractionation were analyzed by CZE in order to determine where polymeric pigments were contained. Fig. 2A shows the electrophoregram recorded at 599, 520 and 280 nm obtained for fraction F1; Fig. 2B shows the electrophoregram recorded at 520, 420 and 280 nm obtained for fraction F2. In F1, two different zones of peaks were observed: a first group of peaks with migration times around 15 min (zone I, peaks 1–9); and a second one, with migration times around 24 min (zone II, peaks a–g). However, in F2 only a diffuse and big hump was observed, with a maximum migration time around 30 min (zone III), just after the second zone visualized in fraction F1.

Peaks 1–9 in zone I (Fig. 2A) were identified as reported in previous papers [25], corresponding to monomeric and dimeric anthocyanins. This finding demonstrated the presence of these compounds in F1, and was consistent with our observations in HPLC–DAD. Peaks a–g of zone II (Fig. 2A) corresponded to polymeric anthocyanins as described by Sáenz-López et al. [20], who observed that the global CZE response of these polymeric pigments was linearly correlated with the spectrophotometric determination of polymeric pigments (96%) and age index (92%). The migration times of these peaks were longer than those of peaks in zone I, confirming their higher charge/size ratios. Moreover, they absorbed at 520 nm, thus confirming their pigment nature. Finally, zone III in F2 (Fig. 2B) was attributed to the absorption of PAs. Compounds in this zone migrated more slowly than those of F1, which confirmed their higher charge/size ratio. These molecules, with an mDP of 20 (see Section 3.1.), are known to be in the F2 fraction [30]. Besides, its spectra, with a maximum absorbance at 200 and 280 nm and very low absorbance in the visible region, coincided with that of a mixture of monomeric and oligomeric apple PAs [31]. The low absorbance seen at 520 and 420 nm may be attributed to residual polymeric anthocyanins eluting in fraction F2.

After confirming the presence of monomeric anthocyanins in F1 and demonstrating that most of the polymeric pigments were

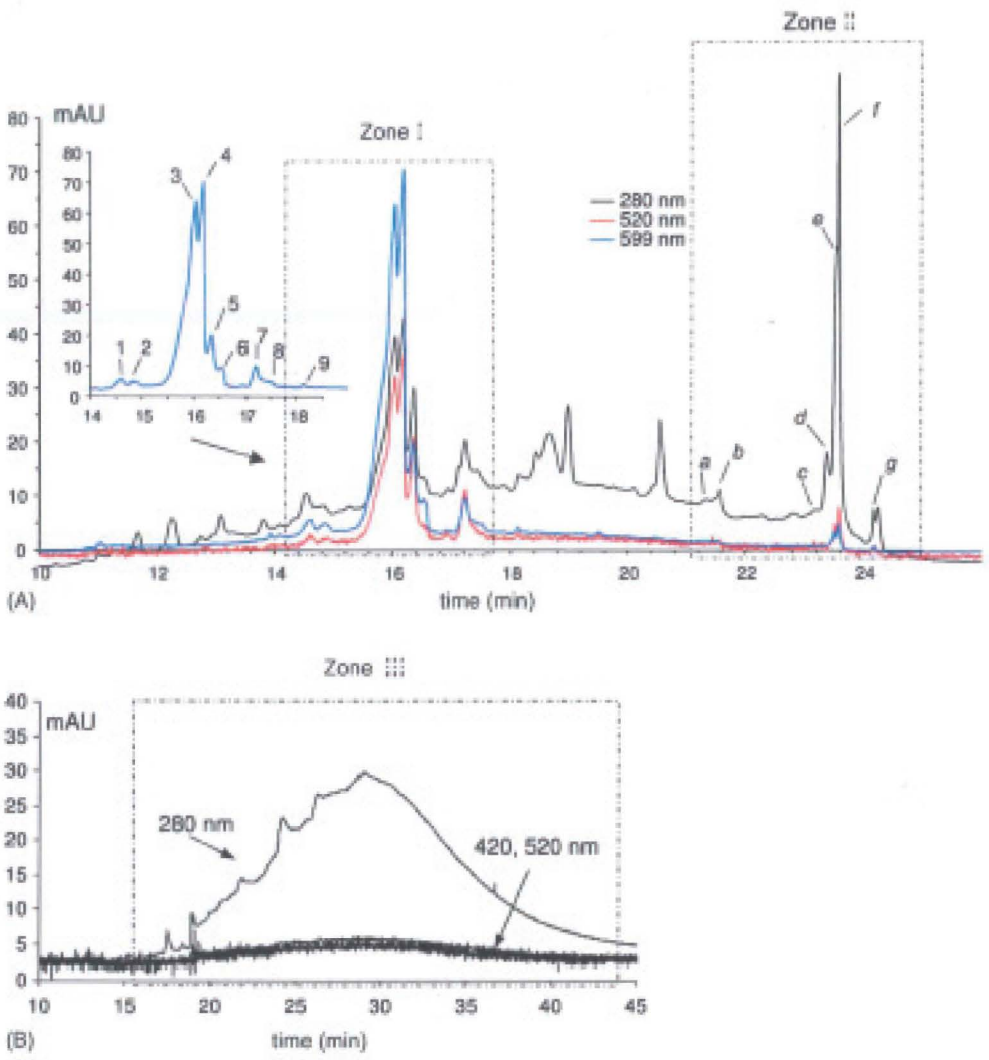

Fig. 2. Electrophoregrams of fraction F1 (A) and fraction F2 (B) from the same wine. See text for GPC and CZE conditions. Peaks: (1) malvidin-3-*O*-(6-coumaroyl)-glucoside; (2) malvidin-3-*O*-(6-acetyl)-glucoside; (3) malvidin-3-*O*-glucoside; (4) peonidin-3-*O*-glucoside; (5) malvidin-3-*O*-glucoside catechin dimer; (6) (4) malvidin-3-*O*-glucoside and pyruvic acid derivative; (7) petunidin-3-*O*-glucoside; (8) delphinidin-3-*O*-glucoside; (9) cyanidin-3-*O*-glucoside; (a–g) unidentified polymeric pigments.

contained in F1, the methods proposed, both GPC fractionation and CZE, were validated (see Section 2.10). The results obtained were also compared with those obtained from direct wine analysis in order to determine whether the fractionation step was worthwhile. Fig. 3 shows the electrophoregrams recorded at 520 and 280 nm for wine fraction F1 (Fig. 3A) and for the same wine without fractionation (Fig. 3B).

When analyzing the wines submitted to GPC fractionation, the repeatability values obtained were 2.99% for monomeric anthocyanins (zone I) and 2.68% for polymeric pigments

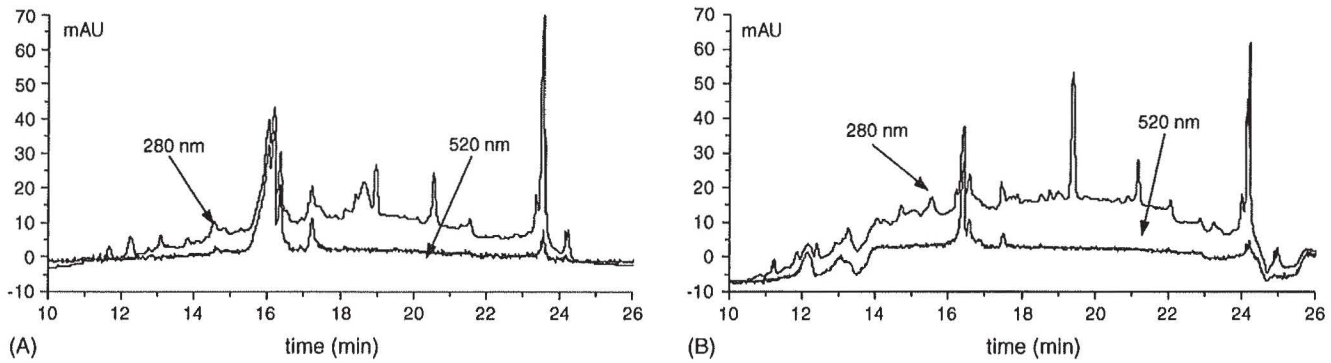

Fig. 3. Electrophoregrams for wine fraction F1 (A) and for the same wine without fractionation (B). See text for GPC and CZE conditions.

(zone II). The values of reproducibility were 2.2 ± 0.6% for monomeric anthocyanins and 2.7 ± 0.6% for polymeric pigments. These results showed a good reliability of the combined methods; however, the same parameters assessed in wine samples without previous fractionation revealed poorer analytical characteristics. In this case, the values obtained for repeatability and reproducibility were, respectively 16.2 and 14.6 ± 6.6% for monomeric anthocyanins, and 9.2 and 9.1 ± 10.8% for polymeric pigments. Additionally, the CZE response obtained was considerably higher (four to five times) in F1 fractions than in wines, and the baseline remained flatter and more stable. All these improvements could be explained by the fact that GPC fractionation eliminates interfering compounds, such as proanthocyanidins. Therefore, GPC fractionation is recommended prior to analysis by CZE.

3.4. Analysis of proanthocyanidins in fraction F2

As stated above, proanthocyanidins (polymers of flavan-3-ols) were contained in fraction F2 obtained after GPC fractionation. Several spectrophotometric methods have been developed for their quantification [21]. Of these, the vanillin reaction is an interesting procedure due to its simplicity and its specificity for flavan-3-ols, both monomers and polymers [32]. The formation of the coloured adduct is relatively slow, so the absorbance must be kinetically monitored and the value of the maximum absorbance taken as the signal value. Besides, autocondensation of vanillin in excess generates coloured compounds that cause interference. This was taken into account through subtraction of the absorption of a reference cuvette in which the sample was substituted by methanol.

When this procedure was performed directly in red wines without prior fractionation, several problems arose, giving poor values of reliability and accuracy. This was due to the interference of monomeric flavan-3-ols and the presence of large amounts of pigments absorbing at 500 nm. GPC fractionation allowed the elimination of all these interfering compounds, which remained in fraction F1. Thus, the vanillin assay was performed in fraction F2, which contains practically all the PAs.

According to Sun et al. [27], catechin can be used as a calibration standard for proanthocyanidins with a small error when the suitable concentration of acid (sulfuric acid 3.6 N) is selected. Hence, there is no need to isolate the proanthocyanidins to be used as standards, thus simplifying the analysis. Besides, the interferences of monomeric catechin itself, which is found in wine, monomeric flavan-3-ols, and wine pigments absorbing at 500 nm, are circumvented by the preliminary fractionation step. However, a blank had to be performed to eliminate the interference of the residual wine pigments present in F2, as discussed in Section 3.1.

The analytical parameters of the calibration curve are shown in Table 2. The values of the limit of detection (LD) and quantification (LQ) showed an acceptable sensitivity. A validation study of the proposed method (fractionation + vanillin reaction) was carried out as described in Section 2.10. The repeatability, reproducibility and recovery values obtained, 3.5, 1.90 ± 1.99, and 101 ± 6.4%, respectively, showed good reliability and accuracy.

Table 2
DL-Catechin standard calibration curve for vanillin assay (analytical parameters)

Equation[a]	$A = 0.002386 \times C - 0.0019$ ($r^{2[b]} = 0.994$)
SD_{slope} ($n = 7$)	0.0001
$SD_{intercept}$ ($n = 7$)	0.0079
LD (mg l^{-1})	1.66
LQ (mg l^{-1})	5.55
Repeatability[c] ($n = 6$)	2.5%
Recovery[d]	101.4 ± 0.2%

[a] A in absorbance units and C in mg l^{-1}.
[b] Linear correlation coefficient for the range 0–122 mg l^{-1} ($n = 7$).
[c] Expressed as the variation coefficient of 6 measurements of the same sample.
[d] Calculated by adding two different quantities of catechin (40 and 60 mg l^{-1}) to a previously analyzed fraction F2.

3.5. Gelatin precipitation of polymeric phenolics

Large polymeric pigments are said to precipitate in the presence of proteins like bovine serum albumin or gelatin, whereas smaller polymeric pigments remain in solution [33]. The addition of gelatin to wine samples before fractionation and further analysis by CZE of fraction F1 revealed a substantial decrease in polymeric anthocyanin content. An overall 65% decrease was measured by CZE for the seven main peaks (a–g) present in the electrophoregram (Fig. 2A). In contrast, the decrease in monomeric pigments was about five times lower (10% for malvidin-3-glucoside, according to HPLC quantification before and after gelatin precipitation). This suggested that most of the polymeric pigments present in wines were large macromolecules, confirming the findings reported in literature [33]. It also revealed a negative ionic nature in polymeric pigments, as gelatin itself is a positive-charged molecule. This supported the idea that large polymeric pigments, which should be the most important polymeric pigments in wines, are tannins containing a covalently bonded anthocyanidin moiety [33].

3.6. Analysis of astringent PAs. gelatin precipitation and vanillin assay

Through a combination of both proanthocyanidin analysis and precipitation with gelatin, a method was developed to measure the content of astringent PAs (the astringent PAs being defined by their ability for gelatin precipitation). This method permitted direct quantification of the precipitated PAs, not just an index in which the concentration of astringent PAs is only implicitly taken into account. Hence, the absolute value of astringent PA content should be a better estimate of wine astringency, if compared to the classical gelatin index.

Different types of wines from D.O.Ca.Rioja were chosen following a sensorial guidance and trying to cover a large range of astringent PA concentrations (Table 3). Wine astringency was assessed by six trained tasters and punctuated from 1 to 5. Wine 1 was a 2003 vintage young wine; Wine 2 was a 2000 vintage *Crianza* wine that was subjected to mannoprotein treatment. Wines

Table 3
Analysis of total PA concentration (TPA) and astringent PA concentration (APA) of different wine samples (comparison with the Gelatin Index)

Wine	TPA[a]	APA[a]	R[b]	GI[c]	SAP[d]
1	465 ± 15	437 ± 15	93.9	63 ± 2	3
2	238 ± 7	146 ± 14	61.1	35 ± 8	1
3	2411 ± 46	2377 ± 11	98.6	82 ± 1	5
4	428 ± 15	403 ± 19	94.4	41 ± 9	3
5	278 ± 12	271 ± 9	97.2	69 ± 8	2

[a] Expressed in mg l^{-1} (mean ± SD).
[b] Ratio APA/TPA expressed in % (mean ± SD).
[c] Gelatin Index (mean ± SD).
[d] Sensorial astringency punctuations (1–5) evaluated by six trained tasters.

3 and 4 were 2003 vintage young wines termed as "astringent" (3) and "non astringent" (4) by the tasters. Finally, Wine 5 was a very aged wine (1982 vintage), where PAs (and other compounds) had precipitated to a large extent during bottle storage.

From the results presented in Table 3, it can be observed that more than 90% of the PAs present in the wines analyzed had an "astringent" character. Wine 2 was an exception, probably due to the mannoprotein effect, which is supposed to diminish wine astringency. It was also observed that the absolute content of astringent PAs was well correlated with the sensorial astringency punctuations. However, there was a weak correlation between the content of astringent PAs and the gelatin index. This index is also based on the precipitation of PAs by gelatin. In this case, the amount of precipitated PAs is determined by the difference in tannin content before and after gelatin addition, and the index is expressed as a percentage of total tannin content. Tannin content measurement is based on the depolymerization of PAs in a hot acidic medium, and colorimetric determination of the anthocyanidins released. This assay has many drawbacks: the yield of this reaction is low due to the formation of polymeric byproducts, and depends on the structure of the PAs present in the sample [21]. For this reason, the gelatin index may be considered as a poor estimate of astringency. This index should be correlated to the ratio of astringent PAs against the total PA content. The poor correlation observed was an indication of the shortcomings of the gelatin index for measuring astringency.

To validate the method proposed, repeatability and reproducibility were assessed, being 3.4 and 4.5 ± 1.9%, respectively.

4. Conclusions

A multiple-step analytical method was developed to improve the analysis of polymeric phenolics. This method, based on the fractionation of wine phenolics by GPC on a TSK Toyopearl gel HW-50F column, was valid for analyzing different families of phenolic compounds. Monomeric flavonoids, dimeric anthocyanins and polymeric pigments were contained in fraction F1, while proanthocyanidins eluted in fraction F2. GPC fractionation enabled the elimination of interfering compounds, and thus the later analyses were substantially improved.

Monomeric flavonoids (anthocyanins, phenolic acids, flavonols and flavan-3-ols) were identified and quantified by HPLC–DAD and HPLC–MS. The methods used – both GPC fractionation and HPLC analysis – enabled high recovery of monomeric compounds in wine and yielded satisfactory repeatability and reproducibility values. The CZE technique was chosen for analyzing polymeric pigments. These compounds were properly separated in seven peaks, with longer migration times than the monomeric and dimeric anthocyanins. GPC fractionation offered a considerable improvement in response, repeatability and reproducibility in wine pigments analysis by CZE. Thus, GPC fractionation is recommended prior to analysis by CZE. GPC fractionation also improved the quantification of proanthocyanidins by the traditional vanillin assay.

Polymeric anthocyanidins were mostly precipitated with gelatin, confirming their overall negative charge. A combined method for quantifying astringent proanthocyanidins was proposed as an alternative to the general indexes available.

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3.3

EVOLUCIÓN DEL CONTENIDO Y DEL PERFIL DE POLISACÁRIDOS DURANTE LA VINIFICACIÓN Y EL ENVEJECIMIENTO DE VINOS TINTOS

Polysacharid Profile and Content during the Vinification and Aging of Tempranillo Red Wines

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Resumen

Este artículo analiza la evolución de los polisacáridos totales solubles y de las principales familias de polisacáridos durante la vinificación y el envejecimiento de vinos de Tempranillo, incluyendo las etapas de maceración-fermentación, fermentación maloláctica, crianza en barrica y envejecimiento en botella. En particular, se analizaron los cambios que se producen en los arabinogalactano-proteínas (AGP), ramnogalacturonanos tipo II monómeros (mRG-II) y dímeros (dRG-II), y oligómeros de homo y ramnogalacturonanos (GL) procedentes de la uva, y en las manoproteínas (MP) procedentes de las levaduras. Se realizó además un estudio más detallado diferenciando entre distintas formas de estos compuestos según su tamaño molecular, y un análisis de los polisacáridos insolubles y su evolución.

A diferencia de lo descrito por otros autores, nuestros resultados indicaron que los polisacáridos estructurales de bajo peso molecular, fragmentos derivados de celulosas y hemicelulosas, constituían los azúcares mayoritarios en los mostos, siendo éstos relativamente pobres en sustancias pécticas. Los primeros estadios de maceración-fermentación produjeron sin embargo una precipitación de la mayor parte de estos compuestos, mientras que se iba produciendo una extracción paulatina de las sustancias pécticas de la uva y una liberación de las manoproteínas de levadura. Los AGP se solubilizaron más rápidamente que los RG-II, que necesitaron más tiempo de maceración para su extracción, y la liberación máxima de las manoproteínas coincidió con la fase de crecimiento exponencial de las levaduras. La postmaceración llevada a cabo para incrementar el color del vino produjo una disminución importante en todos los polisacáridos pécticos de uva, especialmente en los AGP, que continuaron disminuyendo de forma acusada durante la fermentación maloláctica. Por el contrario, el contenido de manoproteínas siguió aumentando progresivamente durante estas etapas, indicando que continuaba su liberación al medio debido a la autolisis de las levaduras. Así, la liberación de manoproteínas durante estas etapas contrarrestó las perdidas que se producían debido a su precipitación. El envejecimiento de los vinos en barrica y en botella no supuso ningún cambio significativo en el contenido de las distintas familias de polisacáridos.

La diferente evolución en la familias de polisacáridos durante la vinificación produjo una modificación significativa en el patrón de polisacáridos observado en los mostos. De hecho, la presencia de polisacáridos estructurales en los vinos fue prácticamente despreciable. Así, los AGP fueron los polisacáridos mayoritarios (50%) en los vinos jóvenes, seguidos de las MP (30%) y los dRG-II (15%), obteniéndose valores muy similares a aquellos encontrados en otros vinos tintos por otros autores.

Por el contrario, las manoproteínas de levadura predominaron en los vinos envejecidos (45%), seguidos de cerca por los AGP (37%) y los dRG-II (15%). Los GL fueron detectados en los vinos en cantidades inferiores a 15 mg/L y, aunque también en bajas concentraciones, se detectó la forma monomérica del RG-II, cuya presencia en los mostos y vinos es actualmente tema de debate.

Un análisis más detallado reveló que tanto los arabinogalactano-proteínas como las manoproteínas presentes el los mostos y vinos eran mayoritariamente compuestos de alto peso molecular (50–400 kD), mientras que las formas de menor tamaño molecular, con un peso molecular medio de aproximadamente 12 kD, representaron menos de un 30%.

Por último, el análisis de los polisacáridos insolubles reveló una precipitación importante de los polisacáridos durante la vinificación, fundamentalmente en la maceración-fermentación pero también en la fermentación maloláctica. Los polisacáridos insolubles representaron más del 30% de los polisacáridos totales (solubles + insolubles) al final de la maceración-fermentación, confirmando nuestras observaciones previas. Se observó además que la precipitación afectó fundamentalmente a los AGP y MP de alto peso molecular, siendo prácticamente despreciable en el resto de polisacáridos.

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Polysaccharide Profile and Content during the Vinification and Aging of Tempranillo Red Wines

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Passing from must to wine produced a loss of low-molecular-weight grape structural glucosyl polysaccharides, and an important gain in yeast mannoproteins (MP) and grape-derived arabinogalactan proteins (AGP), and rhamnogalacturonans-II (RG-II). AGP were more easily extracted than RG-II, and small quantities of RG-II monomers and galacturonans were detected. Postmaceration produced a reduction in all grape polysaccharide families, particularly acute in AGP. The reduction of polysaccharides during malolactic fermentation only affected grape AGP, and MP were continuously liberated during the entire vinification process. Wine oak and bottle aging was associated with a relative stability of the polysaccharide families. AGP were thus the majority polysaccharides in young wines but, contrary to what may be thought, structural glucosyl oligosaccharides dominated in musts and MP in aged wines. Precipitation of polysaccharides was noticeable during vinification, and it mainly affected high-molecular-weight AGP and MP. Hydrolytic phenomena affected the balance of wine polysaccharides during late maceration-fermentation.

KEYWORDS: Red wine; winemaking; grape and yeast polysaccharides; arabinogalactan-proteins; homogalacturonans; rhamnogalacturonans II; mannoproteins.

INTRODUCTION

Must and wine polysaccharide analysis is of great interest because they have an important influence on several stages of the winemaking process such as must racking, fermentation, wine filtration, and wine stabilization (*1–5*). Moreover, these compounds affect the organoleptic properties of red wines (*6–9*), which are very important for the final quality of the product.

Two criteria widely used for the discrimination of polysaccharide families are acidity and protein content. Grape neutral pectic substances mainly comprise type II arabinogalactans or arabinogalactan–proteins (AGP). These compounds, which represent more than 40% of total red wine polysaccharides (*10, 11*), consist of a core structure of (1 → 3)-β-D-galactopyranose chains with (1 → 6) linked β-D-galactan side chains highly substituted by arabinofuranosyl residues (*10*). Grape acidic pectic polysaccharides, characterized by a high proportion of galacturonic acid, involve homogalacturonans (GL), rhamnogalacturonans I (RG-I), and rhamnogalacturonans II (RG-II). From the structural point of view, RG-II is the most remarkable of these polysaccharides due to its highly conserved structure. Type II rhamnogalacturonans are (1 → 4)-α-D-galacturonans branched with four different side chains containing some rare sugars that allow their identification and quantification (*11–13*). RG-II is usually found in cell walls and fruit juices in the form of dimers cross-linked by lead-diol esters (*14, 15*). Mannoproteins (MP) produced by yeasts are the second most abundant family of polysaccharides in wine (*11*). These polymers, with highly variable sizes, are almost pure mannans with a variable protein content (*11, 16*) and can be released by yeast in the early stages of fermentation or later on during aging on lees (*17*).

Not all polysaccharides show the same behavior with respect to wines; their influence on wine processing will depend not only on the quantity of polysaccharidic compounds but also on their structure, composition, and distribution. Some authors have even identified the importance of the type of polysaccharide on such wine characteristics. In particular, it has been shown that AGP have greater influence on the filtration procedures than MP (*18*), which are more efficient at reducing protein haze in white wines (*3, 19, 20*). RG-I and -II inhibit hydrogen tartrate crystallization, (*1*) whereas AGP do not affect this phenomenon (*18*). Among the MP classes present in wine, some have been found to act as protective factors with regard to tartaric acid precipitation (*1, 3*). Besides, it has also been shown that RG-II is responsible for borate complexation to the extent that most lead present in cell walls and wine would be bound to RG-II dimers (*2, 21*). There are also important differences with regard to the quality of the organoleptic characteristics of red wines. It has recently shown that wine RG-II dimer favors the self-aggregation of grape seed proanthocyanidins in winelike solutions, whereas wine MP and acidic AGP tend to inhibit tannin aggregation (*7*) and therefore have a different influence on wine astringency and fullness (*8*). Interactions between aroma compounds and MP have also been described (*6, 9*).

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Given the importance of must and wine polysaccharides, an understanding of their content and release kinetics is essential. It is widely known that pectic polysaccharides are liberated from grape skins and pulp during grape maturation and during the first steps of winemaking and that parietal mannoproteins are released in the wine during and after alcoholic fermentation. Several studies, mainly in white wines, have been performed in order to analyze the evolution of total polysaccharides during the winemaking process, and previous studies have included the evolution of concrete polysaccharide families during bottle storage of red wines (*22*). However, little is known about the behavior of the different types of polysaccharides during the winemaking process. Hence, the aim of this paper is to analyze the changes occurring on must and wine polysaccharide families during the different stages of red wine processing, including maceration-fermentation and postmaceration, malolactic fermentation, and oak aging and bottle aging.

MATERIALS AND METHODS

Vinification and Sample Collection. Mature Tempranillo grapes were harvested from Autol, La Rioja, Spain, at 21.9 Brix, pH 3.56, and 6.02 g tartaric acid/L. Three experimental vinifications were carried out in the wine cellar of the University of La Rioja, and wines were prepared by traditional wine technology. Grapes were destemmed, crushed, and fermented into 100 L stainless steel tanks. The prefermentation process went on for 6 h at 18 ± 1 °C; the fermentation-maceration process was carried out at a maximum temperature of 28 ± 2 °C and lasted 10 days. Postfermentative maceration went on for 4 days at 24 ± 1 °C, and wines were run off. Wines were then inoculated with a commercial preparation of *Oenococcus oeni* (1g/HL) to induce malolactic fermentation, carried out at 18.5 ± 1 °C. After 20 days of malolactic fermentation, all the wines were racked and clarified by settling for 25 days at 10 °C. Wine aging was performed in new 13 L American oak barrels, which have a larger area/volume than traditional 225 L barrels. For this reason, and on the basis of organoleptic analysis, the oak aging process went out for only 45 days. Wines were then bottled and stored at 4 °C.

Samples were taken at the beginning of the maceration-fermentation (0AF), during the maceration-fermentation (25–30% of sugars consumed, 55–60% of sugars consumed, and 99% of sugars consumed, namely, 30AF, 60AF, and 99F, respectively), and at the beginning and end of malolactic fermentation (BMF, EMF). Sample bottles were filled completely to minimize oxygen contact and immediately frozen at −18 °C. Samples were also analyzed at the beginning and end of wine oak aging (BOA, EOA) and after two years of wine bottle aging (BA).

Isolation of Must and Wine Polysaccharides. Samples were homogenized, and 400 mL was taken with a peristaltic pump and centrifuged. The insoluble pellets were recovered and precipitated with 5–10 mL of cold 96% ethanol containing 0.3 M HCl (*23*). After 18 h at 22 °C, the samples were centrifuged and the pellets obtained were washed in ethanol 96% several times and freeze–dried (*23*). The residues obtained (fractions I) contained the insoluble polysaccharides. The supernatants were first concentrated (five times for wines and three times for musts) under reduced pressure at 34 °C and were then precipitated by adding four volumes of cold ethanol containing 0.3 M HCl and kept for 18 h at 4 °C (*23*). Thereafter, the samples were centrifuged, the supernatants were discarded, and the pellets were washed with 96% ethanol. The precipitates were finally dissolved in ultrapure water and freeze–dried. The freeze–dried precipitates obtained (fractions S) contained the soluble polysaccharides.

Monosaccharide composition in fractions S and I was determined by gas–liquid chromatography as described bellow. Protein concentration was determined using the procedure described by Lowry et al. (*24*) with bovine serum albumin as standard.

Molecular Weight Distribution of Must and Wine Polysaccharides. To obtain the molecular weight distribution of must and wine polysaccharides, the soluble fractions S and the insoluble fractions I were subjected to high-resolution size-exclusion chromatography (HRSEC) on two serial Shodex OHpack KB-803 and KB-805 columns (30 × 0.8 cm, Showa Denko, Japan) equilibrated at 1 mL/min in 0.1 M $LiNO_3$. Chromatographic separation was carried out at room temperature and calibration was performed with narrow pullulan molecular weight standards (P-5, M_w = 5900 D; P-10, M_w = 11 800 D; P-20, M_w = 22 800 D; P-50, M_w = 47 300 D; P-100, M_w = 112 000 D; P-200, M_w = 212 000 D; P-400, M_w = 404 000 D).

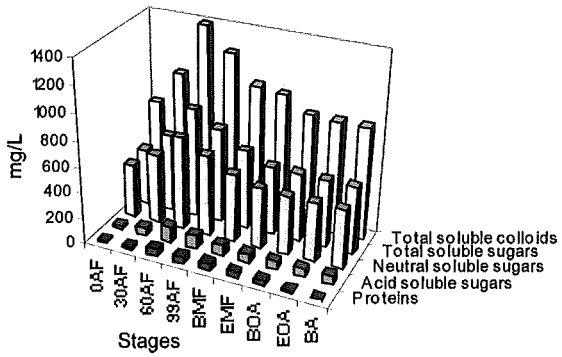

Figure 1. Analysis of soluble fractions S. Evolution of total soluble colloids, total, neutral, and acid soluble sugars, and proteins, during vinification and aging. See text for conditions and calculations.

Fractionation of Must and Wine Soluble Polysaccharides by HRSEC. To separate the different polysaccharide families, the soluble fractions S were subjected to high-resolution size-exclusion chromatography on a Superdex-75 HR column equilibrated at 0.6 mL/min in 30 mM ammonium formiate, pH 5.8 (*23*). The peaks obtained were collected in different fractions (S1, S2, and S3) according to their elution times. The isolated fractions were freeze–dried, redissolved in water, and freeze–dried again several times to remove the ammonium salt (*23*).

Identification and Quantification of Must and Wine Polysaccharides by GC and GC-MS. The carbohydrate composition of the insoluble fractions I and soluble fractions S, S1, S2, and S3 was determined by GC with flame ionization detector and GC-MS of their trimethylsilyl-ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization as previously described (*23*). Total soluble sugars were calculated in fractions S as the sum of all individual sugars, and neutral and acid soluble sugars were calculated as the sum of neutral and acid sugars, respectively. Total insoluble sugars were calculated in fractions I as the sum of all individual sugars. Polysaccharide families were quantified in fractions S1, S2, and S3 from the concentration of individual glycosyl residues characteristic of well-defined wine polysaccharides (*22, 23*).

Statistical Procedures. Vinifications and analysis were performed in triplicate. Significant differences between samples were analyzed with the SPSS 12.0 program for Microsoft Windows (SPSS Inc., Chicago, IL). Monosaccharide and polysaccharide content values were analyzed by a one-way analysis of variance (ANOVA) with repeated measurements to test the effect of the vinification stage, if the data adhered to assumptions of normality. If these assumptions were not adhered to, a Kruskal–Wallis test was used. In this paper, whenever we refer to differences between samples, we are referring to significant differences with at least $p < 0.05$.

RESULTS AND DISCUSSION

Analysis of Soluble Fractions S. Evolution of total soluble colloids, total soluble sugars, and their constituents, i.e., neutral and acid sugars, and proteins, in the fractions S during the vinification and aging are shown in **Figure 1**. Total soluble sugars accounted for about 60% of total soluble colloids in all the stages analyzed; the rest were attributed to other compounds such as salts, proteins, or phenolics. In all the stages, neutral soluble sugar content was considerably higher than that of acid soluble sugars, which represented only between 6 and 15% of total soluble sugars. The values obtained were quite similar to those obtained by our workgroup when a colorimetric method

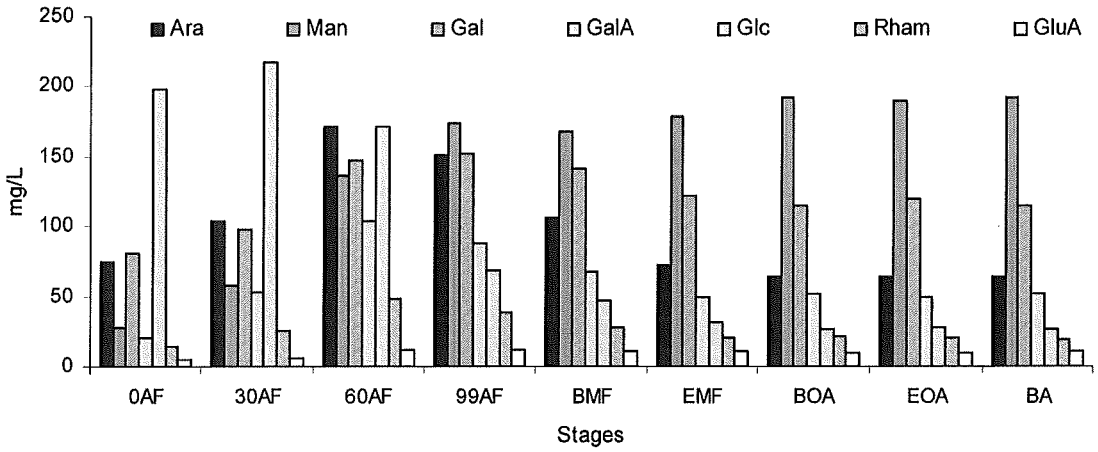

Figure 2. Evolution of major glycosyl residues in the soluble fractions S during vinification and aging. Ara, arabinose; Man, mannose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; Rham, rhamnose; GluA, glucuronic acid. See text for conditions and calculations.

was used for quantification instead of capillary GC (*25*). This finding confirmed that both methods were reliable for measuring must and wine carbohydrates, although the latter was chosen in the present study because it gave information on individual glycosyl composition.

Red winemaking increased the concentration of total soluble colloids and sugars; maceration-fermentation was the main process affecting this content (**Figure 1**). The concentration of total soluble sugars increased progressively by 90% between 0 and 4 days (60AF), reaching more than 800 mg/L, but decreased substantially at the end of the maceration-fermentation and during postmaceration and malolactic fermentation, indicating that during these periods the precipitation rate of polysaccharidic compounds was higher than their solubilization. Proteins, which accounted for less than 6% of total colloids, increased from 17 to 60 mg/L during early maceration-fermentation and then stabilized until malolactic fermentation. During oak and bottle aging, soluble sugar content was maintained, reaching values usually found in other red varieties. However, the protein content was drastically reduced during oak aging (~65%), which might be due to the well-known phenomenon of formation of wine tannin–protein insoluble complexes during this period.

Important differences were observed in glycosyl residue patterns of soluble fractions between wine and must samples (**Figure 2**). Glucose was the most prevalent sugar detected in must samples, representing more than 40% of total soluble sugars. Although its origin in must is not clear in the bibliography, it has been shown that glucose is the prevalent sugar in both the skin and pulp cell walls of grape berries (*26*) because it is the main component of major structural polysaccharides from grape cell walls such as cellulose and hemicellulosic xyloglucans, arabinoglucans and mannans. The large amount of glucose in musts would therefore be attributed to the partial solubilization of these components and to the solubilization of complexes between them and pectic polysaccharides, which have been reported to occur in cell walls from both grape pulp and skin tissues (*27*). Xylose was found to be the most prevalent among the minor sugars detected in musts (data not shown), confirming the presence of hemicellulosic xyloglucans and arabinoxylans. Other sugars detected in musts were arabinose, galactose, and rhamnose, the glycosyl residues found in AGP, and mannose, the main component of MP.

Soluble sugar content and profile changed as the maceration-fermentation process went on (**Figure 2**). The content of glucose slightly increased in the early maceration-fermentation, but it was significantly reduced later, reaching final values of less than 50 mg/L at the end of maceration. However, the other sugars detected in musts behaved in the opposite manner, and their concentrations increased significantly during maceration-fermentation to more than double at the end. The greatest increase was observed in the case of galacturonic acid and mannose, whose concentrations increased 4- and 6-fold, respectively. Mannose, galactose, and arabinose were thus the most prevalent sugars in wines at the end of alcoholic fermentation (24, 21, and 20%, respectively), followed by galacturonic acid and glucose (12 and 9%, respectively). During postmaceration and malolactic fermentation, a significant change was once again observed in the sugar profile because there was a significant decrease in all the glycosyl residues except for mannose. Thus, wines after these stages were mainly composed of mannose (33%), followed by galactose (23%) and arabinose (14%), whose molar ratio arabinose/galactose decreased from 1 to 0.6. No noteworthy changes were observed in sugar composition during wine oak and bottle aging.

Analysis of Insoluble Fractions I. The carbohydrate composition of the insoluble fractions I was also studied (**Table 1**) in order to determine the extent of precipitation occurring during the winemaking process and the type of polysaccharides precipitating.

The amount of sugars in the insoluble fractions was substantially high during maceration-fermentation, mainly during the early stages, when total insoluble sugars represented more than 30% of total sugars, i.e., the sum of sugars of soluble and insoluble fractions. Among the sugars present in the insoluble fractions, and as in the case of soluble sugars, acid residues represented only a small percentage. Glucose was the main sugar detected during maceration-fermentation, representing more than 90% in the early stages. This finding confirmed that an important amount of grape structural glucosyl polysaccharides were extracted immediately after grape crushing (>500 mg/L of glucose in 0AF of fractions S and I), although their solubilization was limited, and more than 60% of these compounds were unstable and precipitated, being detected in the insoluble fractions. Mannose, arabinose, galactose, and galacturonic acid were also detected in insoluble fractions during maceration-fermentation, indicating a precipitation of other polysaccharide families such as MP, AGP, and galacturonans. During malolactic fermentation, mannose was the main sugar detected, followed closely by glucose, which seemed to indicate that the precipitation of polysaccharides during this period mainly affected MP or other microorganism cell wall polysaccharides. The insoluble fractions of wines after malolactic fermentation contained all

Table 1. Carbohydrate Composition (mg/L) of Insoluble Fractions I of Must and Wine Samples Determined by GC and GC-MS of their TMS Derivatives[a]

	vinification stages								
sugars	0AF	30AF	60AF	99AF	BMF	EMF	BOA	EOA	BA
aceric acid	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
2-*O*-M Fuc[c]	1.51 ± 0.03	1.87 ± 0.04	1.31 ± 0.03	2.38 ± 0.05	*b*	*b*	*b*	*b*	*b*
2-*O*-M Xyl[c]	1.03 ± 0.04	1.05 ± 0.04	0.92 ± 0.04	1.19 ± 0.05	0.28 ± 0.02	0.31 ± 0.01	*b*	*b*	*b*
apiose	0.45 ± 0.02	0.27 ± 0.01	0.16 ± 0.01	0.25 ± 0.01	0.66 ± 0.03	0.73 ± 0.03		*b*	*b*
arabinose	6.1 ± 0.2	4.2 ± 0.1	3.86 ± 0.12	3.07 ± 0.09	4.5 ± 0.1	5.0 ± 0.2	5.6 ± 0.2	1.07 ± 0.03	1.01 ± 0.03
rhamnose	0.82 ± 0.07	1.3 ± 0.1	1.36 ± 0.11	1.04 ± 0.08	1.02 ± 0.08	1.14 ± 0.09	0.18 ± 0.01	0.24 ± 0.02	0.23 ± 0.02
fucose	0.13 ± 0.01	0.12 ± 0.01	0.18 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	*b*	*b*	*b*
xylose	0.51 ± 0.03	0.58 ± 0.03	0.45 ± 0.03	0.40 ± 0.02	0.22 ± 0.01	0.24 ± 0.01	*b*	*b*	*b*
mannose	8.1 ± 0.2	32.2 ± 0.9	48.3 ± 1.3	31.0 ± 0.9	42.2 ± 2.0	47.1 ± 3.3	1.14 ± 0.03	1.47 ± 0.04	1.39 ± 0.04
Dha[c]	1.12 ± 0.04	*b*	*b*	0.77 ± 0.03	0.27 ± 0.01	0.30 ± 0.03	*b*	*b*	*b*
galactose	4.5 ± 0.2	4.45 ± 0.21	3.8 ± 0.2	3.7 ± 0.2	5.2 ± 0.5	5.8 ± 0.3	0.53 ± 0.03	1.45 ± 0.07	1.37 ± 0.06
GalA[c]	3.1 ± 0.2	5.2 ± 0.4	4.2 ± 0.3	4.0 ± 0.3	2.8 ± 0.2	3.1 ± 0.2	0.44 ± 0.03	0.53 ± 0.04	0.50 ± 0.03
glucose	319.8 ± 7.3	249.9 ± 5.7	136.4 ± 3.1	78.8 ± 1.8	32.6 ± 2.7	36.3 ± 2.9	1.64 ± 0.04	1.08 ± 0.02	1.02 ± 0.02
GlcA[c]	0.98 ± 0.06	0.85 ± 0.05	0.93 ± 0.05	0.86 ± 0.05	1.39 ± 0.08	1.55 ± 0.09	0.68 ± 0.04	0.24 ± 0.01	0.23 ± 0.01
Kdo[c]	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
total[d]	348 ± 7	302 ± 6	202 ± 3	128 ± 2	91.2 ± 3.4	102 ± 4.4	10.3 ± 0.2	6.3 ± 0.1	5.93 ± 0.09

[a] Average values of 3 replicates (mean ± SD). [b] <0.1 mg/L. [c] 2-*O*-M Fuc, 2-*O*-methyl fucose; 2-*O*-M Xyl, 2-*O*-methyl xylose; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid. [d] Calculated as the sum of individual sugars (mg/L).

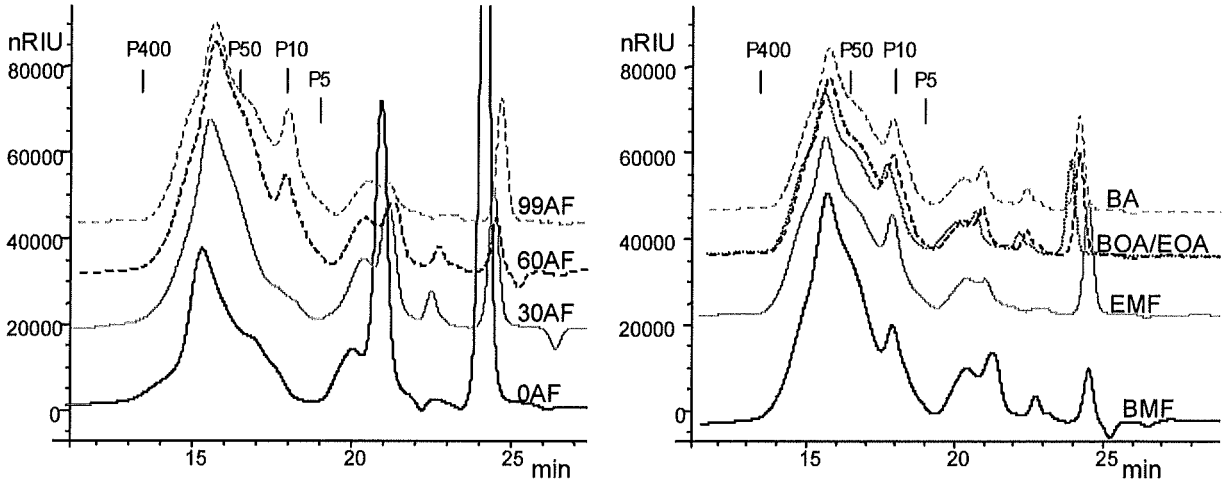

Figure 3. Molecular weight distribution of soluble fractions S by HRSEC on Shodex columns. **(a)** Evolution during maceration-fermentation, and **(b)** evolution during malolactic fermentation and oak and bottle aging. Elution times of pullulan standards (P5 → P400) are also shown.

the sugars known to participate in the composition of AGP, MP, and GL. However, the insolubilization of polysaccharides was a minor phenomenon in these stages because the sugars present in the insoluble fractions represented less than 3% of total wine sugars.

Analysis of Molecular Weight Distribution of Soluble and Insoluble Polysaccharides. HRSEC on Shodex columns of soluble and insoluble fractions S and I from must and wine samples allowed us to follow the qualitative changes in the molecular weight distribution of both soluble and insoluble polysaccharides.

In 0AF must samples, the distribution of soluble polysaccharides was characterized by the presence of three major populations, eluting at approximately 15.4, 20.9, and 24.1 min (**Figure 3a**). The population eluting at 15.4 min corresponded to molecules with molecular weight between P50 (47.3 kD) and P400 (404 kD), and it was mainly attributed to the presence of arabinogalactans or arabinogalactan–proteins because previous studies had shown that the apparent molecular weight of AGP isolated from wine ranged from 48 to 262 kD (*10, 11*). The other two populations, with molecular weights below P5 (5.9 kD), were attributed to oligosaccharides and low-molecular-weight fragments of larger macromolecules. Passing from must to wine was characterized by a progressive increase in the area of the first population, which was thought to be due not only to an enrichment of AGP but also to a progressive appearance of yeast mannoproteins, with highly variable sizes ranging from 5 to 800 kD (*17*). A signal eluting at 17.4 min was observed in samples taken in advanced fermentation (60AF). This population, not clearly defined in must samples, corresponded to molecules with an average molecular weight of P10 (11.8 kD). According to previously published data, these molecules corresponded to rhamnogalacturonan type II dimers (RG-IId), with an average molecular weight of 10–12 kD (*12, 28*), and to low-molecular-weight AGP and MP (*22, 23*). As the vinification process went on, changes in the areas of the signals could be observed (**Figure 3b**), indicating that transformations in the polysaccharide quantities were occurring. During oak and bottle aging, chromatograms were almost superimposable, showing no evolution during this period.

The molecular weight distribution of insoluble polysaccharides (fractions I) was also different depending on the vinification stage (**Figure 4**). From 0AF to 60AF, the distribution of insoluble polysaccharides was characterized by the presence of several signals eluting after 18 min (<P5). Contrary to what was thought earlier, this fact seemed to indicate that the insoluble polysaccharides in musts were in fact low-molecular-weight oligosaccharides. At the end of maceration-fermentation, the

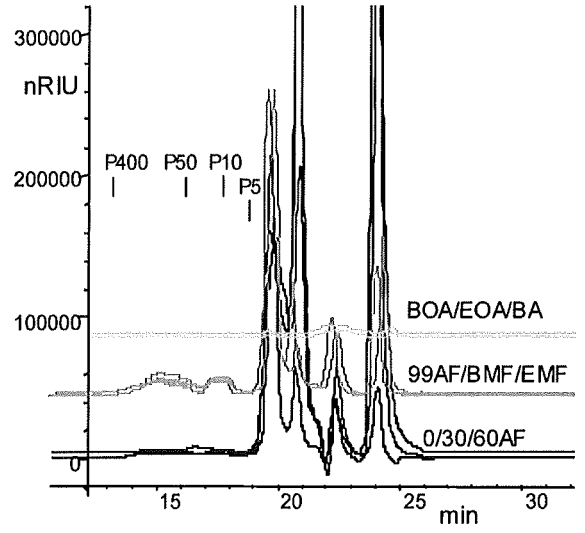

Figure 4. Molecular weight distribution of insoluble fractions I by HRSEC on Shodex columns. Elution times of pullulan standards (P5 → P400) are also shown.

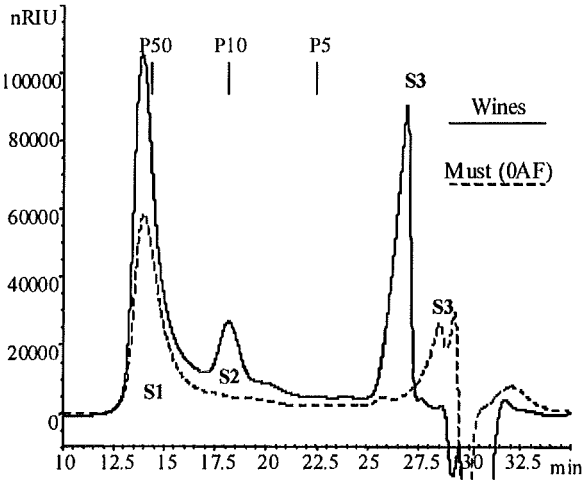

Figure 5. Molecular weight distribution of fractions S1, S2, and S3 by HRSEC on a Superdex 75-HR column. Elution times of pullulan standards (P5 → P50) are also shown.

low-molecular-weight peaks had reduced significantly, and a population of molecules ranging from P50 (47.3 kD) to P400 (404 kD) was clearly observed. The analysis of chromatograms of the insoluble fractions during postmaceration and malolactic fermentation revealed similar patterns, and insolubilization of molecules ranging from P50 to P400 was also observed. As expected, no significant signals were seen in HRSEC profiles of insoluble fractions during oak and bottle aging.

Fractionation of Must and Wine Soluble Polysaccharides by High-Resolution Size-Exclusion Chromatography. Soluble fractions S were injected on a Superdex 75-HR column in order to separate the different polysaccharide families. This prepacked column, with a molecular weight range from 3 to 75 kD, enabled the separation of soluble polysaccharides into different fractions. Chromatograms revealed a fractionation of compounds into three peaks, S1, S2, and S3, similar to that previously described (*23*), except for 0AF must, which showed only two peaks, S1 and S3 (**Figure 5**). The results obtained revealed a fractionation of compounds similar to that obtained with the Shodex columns.

Sugar Composition of Fractions S1, S2, and S3. Glycosyl residue composition of must and wine fractions S1, S2, and S3 obtained after HRSEC fractionation is shown in **Tables 2**, **3**, and **4**, respectively. It was remarkable that in all the cases important differences were again observed between must samples (0AF and 30AF) and wine samples, the 60AF sample appearing in a middle position between musts and wines.

Sugars in fractions S1 of wines accounted for more than 50% of total soluble sugars, i.e., sugars obtained by direct injection of fractions S in GC (**Figure 1**), while sugars in the second fraction represented about 30% and sugars in S3 were less than 20%. However, sugars of fractions S1, S2, and S3 of 0AF and 30AF must samples represented less than 30%, 10% and 8%, respectively, of total soluble sugars. Hence, sugars obtained after HRSEC fractionation represented around 100% of total soluble sugars in wine samples, but they were only 39–43% in must samples, mainly due to the glucose content, which was less than 15% in must fractions S1, S2, and S3 when compared with that obtained by direct injection of fractions S. This fact indicated that the majority of soluble polysaccharides in musts were basically low-molecular-weight oligosaccharides (<3 kD) because they eluted after 30 min and were not included in the fractionation range of the Superdex 75-HR column. In contrast to the descriptions contained in the bibliography (*29*, *30*), major must polysaccharides were in fact fragments of cellulose and hemicellulose, which would be easily extracted from grape cell walls during grape maturation and crushing and during early maceration-fermentation. However, they would be highly unstable and would precipitate, and it became apparent that must insoluble fractions, mainly composed of glucose (**Table 1**), showed only low-molecular-weight populations (**Figure 4**). Therefore, passing from must to wine produced a precipitation of extracted low-molecular-weight grape structural glucosyl polysaccharides, and an important enrichment of larger-sized polysaccharides, collected in fractions S1 and S2.

Wine fractions S1 mainly comprised arabinose, galactose, and mannose (**Table 2**), confirming the predominance of AGP and MP among wine polysaccharides. The composition of fractions S2 was more complex, and all the rare diagnostic sugars of the RG-II molecule were detected (**Table 3**), confirming the presence of this polysaccharide. However, as previously observed (*22*, *23*), the molar ratios of rhamnosyl, arabinosyl, galactosyl, and glucuronosyl residues were greater than expected for a purified RG-II molecule, and mannose was also present in this fraction, indicating the presence of low-molecular-weight AGP and MP. Fractions S3 contained all the sugars known to participate in the composition of wine polysaccharides, but they were present only in small amounts (**Table 4**). Galacturonic acid, galactose, arabinose, rhamnose, glucose, and mannose largely dominated this fraction and were attributed to the presence of homo- and rhamnogalacturonans oligomers (GL) and low-molecular-weight fragments of AGP, MP, and other glycosylated compounds. Unlike musts, the small proportion of glucose in wine samples was mainly attributed to the presence of condensed anthocyanins or microbial polysaccharides. As previously observed by our work group (*23*), rare sugars were also detected in S3 fractions except for must samples. These residues were attributed to RG-II monomers (mRG-II) on the basis of the molecular weight of the eluted fraction. The presence of the monomeric form of the RG-II in wines is still poorly understood as RG-II has been traditionally described as being mainly dimeric in cell walls (*31*), in fruit juices obtained by liquefaction (*15*), and in wines (*28*). However, RG-II monomer has been recently detected in polysaccharides solubilized from grape pulp tissue and also in red wines (*11*, *26*). Anyway, we cannot rule out the hypothesis that monomeric RG-II could be

Table 2. Carbohydrate Composition (mg/L) of Must and Wine Fractions S1 Obtained by HRSEC on a Superdex-75 HR Column and Determined by GC and GC-MS of their TMS Derivatives[a]

	vinification stages								
sugars	0AF	30AF	60AF	99AF	BMF	EMF	BOA	EOA	BA
arabinose	21.8 ± 1.1	28.5 ± 1.2	76.0 ± 2.9	77.9 ± 5.4	58.5 ± 1.4	42.4 ± 0.8	33.4 ± 0.4	28.5 ± 0.3	27.4 ± 0.4
rhamnose	6.0 ± 0.2	8.8 ± 0.1	13.2 ± 0.1	7.63 ± 0.02	5.9 ± 0.1	3.5 ± 0.3	3.0 ± 0.1	2.6 ± 0.2	2.3 ± 0.2
fucose	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	*b*	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.18 ± 0.01
xylose	0.81 ± 0.03	0.93 ± 0.02	1.4 ± 0.1	0.93 ± 0.01	1.74 ± 0.07	1.07 ± 0.05	1.19 ± 0.06	0.84 ± 0.03	0.92 ± 0.02
mannose	17.0 ± 0.9	30.0 ± 1.3	99.4 ± 1.0	122 ± 2	138 ± 2	146 ± 1	147 ± 4	138 ± 3	142 ± 3
galactose	61.6 ± 5.0	63.8 ± 4.1	123 ± 2	101 ± 3	102 ± 2	85.2 ± 1.0	76.1 ± 2.8	68.7 ± 1.7	71.2 ± 1.3
GalA[c]	7.4 ± 0.2	12.0 ± 1.0	15.8 ± 0.9	8.2 ± 0.2	4.9 ± 0.3	2.3 ± 0.2	1.24 ± 0.06	1.12 ± 0.08	1.32 ± 0.06
glucose	10.3 ± 0.3	7.9 ± 0.5	12.2 ± 0.8	9.3 ± 0.2	10.7 ± 0.4	9.8 ± 0.9	7.9 ± 0.2	13.2 ± 0.6	11.9 ± 0.5
GlcA[c]	3.2 ± 0.1	3.40 ± 0.05	7.3 ± 0.2	6.6 ± 0.7	6.3 ± 0.2	5.1 ± 0.1	5.15 ± 0.07	4.2 ± 0.1	3.9 ± 0.1

[a] Average values of 3 replicates (mean ± SD). [b] <0.1 mg/L. [c] GalA, galacturonic acid; GlcA, glucuronic acid.

Table 3. Carbohydrate Composition (mg/L) of Must and Wine Fractions S2 Obtained by HRSEC on a Superdex-75 HR Column and Determined by GC and GC-MS of their TMS Derivatives[a]

	vinification stages								
sugars	0AF	30AF	60AF	99AF	BMF	EMF	BOA	EOA	BA
aceric acid	*b*	0.54 ± 0.01	1.49 ± 0.03	3.4 ± 0.2	3.15 ± 0.06	3.40 ± 0.06	3.28 ± 0.04	3.17 ± 0.02	3.0 ± 0.2
2-*O*-M Fuc[c]	b	0.45 ± 0.01	1.38 ± 0.07	2.71 ± 0.04	2.57 ± 0.09	2.5 ± 0.1	2.2 ± 0.1	2.60 ± 0.06	2.81 ± 0.06
2-*O*-M Xyl[c]	*b*	0.53 ± 0.01	1.29 ± 0.01	3.09 ± 0.01	2.89 ± 0.05	3.2 ± 0.2	2.74 ± 0.06	2.8 ± 0.2	3.0 ± 0.2
apiose	*b*	0.52 ± 0.01	1.13 ± 0.01	3.23 ± 0.08	3.22 ± 0.05	3.36 ± 0.03	2.79 ± 0.09	2.3 ± 0.05	2.0 ± 0.1
arabinose	1.81 ± 0.08	9.9 ± 0.4	30.0 ± 1.2	47.3 ± 3.3	33.8 ± 0.7	23.6 ± 0.4	18.2 ± 0.2	20.1 ± 0.2	19.0 ± 1.3
rhamnose	0.63 ± 0.03	8.16 ± 0.06	18.5 ± 0.1	18.0 ± 0.05	13.6 ± 0.2	11.2 ± 0.8	9.5 ± 0.2	10.7 ± 0.8	9.8 ± 0.8
fucose	*b*	0.30 ± 0.01	0.89 ± 0.02	1.5 ± 0.1	1.40 ± 0.05	1.47 ± 0.02	1.22 ± 0.01	1.45 ± 0.02	0.8 ± 0.02
xylose	*b*	0.31 ± 0.01	0.65 ± 0.05	0.69 ± 0.01	0.82 ± 0.03	5.3 ± 0.3	0.73 ± 0.04	0.69 ± 0.02	0.71 ± 0.05
mannose	1.40 ± 0.09	8.2 ± 0.4	25.0 ± 0.3	31.2 ± 0.5	42.5 ± 0.6	47.0 ± 0.4	54.9 ± 1.4	51.7 ± 1.2	55.2 ± 4.3
Dha[c]	*b*	0.79 ± 0.05	3.1 ± 0.04	3.7 ± 0.1	2.16 ± 0.03	0.99 ± 0.01	3.1 ± 0.1	2.58 ± 0.05	1.5 ± 0.1
galactose	3.29 ± 0.10	17.4 ± 1.5	36.8 ± 2.2	38.1 ± 1.0	40.4 ± 2.3	40.6 ± 4.1	38.6 ± 1.9	40.1 ± 2.8	43.1 ± 2.6
GalA[c]	1.16 ± 0.03	13.6 ± 0.6	32.9 ± 2.0	38.4 ± 0.9	31.9 ± 1.1	30.7 ± 2.7	23.9 ± 0.7	26.6 ± 0.9	24.1 ± 0.8
glucose	1.56 ± 0.05	4.52 ± 0.06	7.2 ± 0.2	6.1 ± 0.6	7.0 ± 0.2	5.5 ± 0.1	5.90 ± 0.08	5.1 ± 0.2	5.0 ± 0.3
GlcA[c]	0.19 ± 0.01	1.16 ± 0.02	2.8 ± 0.2	4.13 ± 0.06	4.0 ± 0.1	4.2 ± 0.2	3.9 ± 0.2	4.1 ± 0.1	4.6 ± 0.1
Kdo[c]	*b*	0.54 ± 0.02	1.47 ± 0.01	3.36 ± 0.09	3.11 ± 0.05	3.36 ± 0.03	3.2 ± 0.1	3.13 ± 0.06	3.0 ± 0.3

[a] Average values of 3 replicates (mean ± SD). [b] <0.1 mg/L. [c] 2-*O*-M Fuc, 2-*O*-methyl fucose; 2-*O*-M Xyl, 2-*O*-methyl xylose; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid.

Table 4. Carbohydrate Composition (mg/L) of Must and Wine Fractions S3 Obtained by HRSEC on a Superdex-75 HR Column and Determined by GC and GC-MS of their TMS Derivatives[a]

	vinification stages								
sugars	0AF	30AF	60AF	99AF	BMF	EMF	BOA	EOA	BA
aceric acid	*b*	0.12 ± 0.01	0.25 ± 0.02	0.43 ± 0.01	3.15 ± 0.06	0.36 ± 0.01	0.38 ± 0.02	0.31 ± 0.01	0.28 ± 0.02
2-*O*-M Fuc[c]	*b*	0.09 ± 0.01	0.21 ± 0.01	0.41 ± 0.02	2.57 ± 0.09	0.38 ± 0.01	0.26 ± 0.01	0.31 ± 0.01	0.42 ± 0.02
2-*O*-M Xyl[c]	*b*	0.08 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	2.89 ± 0.05	0.10 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
apiose	*b*	0.09 ± 0.01	0.28 ± 0.01	0.21 ± 0.01	3.22 ± 0.05	0.10 ± 0.01	0.20 ± 0.01	0.11 ± 0.01	0.95 ± 0.03
arabinose	4.05 ± 0.08	7.2 ± 0.2	7.8 ± 0.4	15.4 ± 0.3	33.8 ± 0.7	10.5 ± 0.4	7.9 ± 0.3	7.3 ± 0.2	7.0 ± 0.2
rhamnose	0.45 ± 0.03	0.77 ± 0.02	1.9 ± 0.1	4.09 ± 0.04	13.6 ± 0.2	2.4 ± 0.1	2.9 ± 0.3	2.5 ± 0.1	2.80 ± 0.03
fucose	*b*	0.12 ± 0.01	0.25 ± 0.02	0.94 ± 0.08	1.40 ± 0.05	0.36 ± 0.02	0.18 ± 0.01	0.37 ± 0.01	0.45 ± 0.04
xylose	0.44 ± 0.03	0.33 ± 0.02	0.68 ± 0.05	1.77 ± 0.01	0.82 ± 0.03	0.8 ± 0.1	0.80 ± 0.04	0.77 ± 0.04	0.63 ± 0.15
mannose	3.03 ± 0.09	3.58 ± 0.04	5.8 ± 0.3	25.7 ± 0.5	42.5 ± 0.6	14.8 ± 0.4	17.1 ± 1.4	18.9 ± 1.4	19.60 ± 0.02
Dha[c]	*b*	0.57 ± 0.05	0.27 ± 0.01	1.03 ± 0.08	2.16 ± 0.03	0.47 ± 0.01	0.47 ± 0.02	0.68 ± 0.02	0.42 ± 0.12
galactose	3.9 ± 0.1	5.8 ± 0.2	6.0 ± 0.1	20.4 ± 1.0	40.4 ± 2.3	15.3 ± 0.4	20.9 ± 0.2	19.2 ± 0.5	18.2 ± 0.6
GalA[c]	2.50 ± 0.03	3.0 ± 0.2	10.0 ± 0.4	14.4 ± 0.6	31.9 ± 1.1	9.3 ± 0.2	8.38 ± 0.01	6.0 ± 0.2	6.8 ± 0.13
glucose	18.0 ± 0.5	12.40 ± 0.06	10.7 ± 0.2	66.1 ± 0.8	7.0 ± 0.2	34.3 ± 0.1	24.8 ± 0.1	29.2 ± 1.5	30.20 ± 0.08
GlcA[c]	0.71 ± 0.01	0.37 ± 0.02	0.56 ± 0.02	1.74 ± 0.05	4.0 ± 0.1	0.77 ± 0.05	1.14 ± 0.06	0.97 ± 0.01	1.02 ± 0.09
Kdo[c]	*b*	0.12 ± 0.01	0.25 ± 0.01	0.43 ± 0.02	3.11 ± 0.05	0.36 ± 0.03	0.38 ± 0.03	0.31 ± 0.02	0.51 ± 0.02

[a] Average values of 3 replicates (mean ± SD). [b] <0.1 mg/L. [c] 2-*O*-M Fuc, 2-*O*-methyl fucose; 2-*O*-M Xyl, 2-*O*-methyl xylose; Dha, 3-deoxy-D-*lyxo*-heptulosaric acid; GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy octulosonic acid.

generated under the conditions used for precipitation and separation procedures even though the shift from dimeric to monomeric state requires very low pH values (*14*).

Evolution of Must and Wine-Soluble Polysaccharide Families during Vinification and Aging. The evolution of major must and wine polysaccharides was monitored during winemaking and aging (**Figure 6**), and the results obtained showed good agreement with the observations described in the previous sections. Wine and must AGP and MP were calculated from the sum of high-molecular-weight AGP and MP from fractions S1 and smaller AGP and MP from fractions S2.

The rate of extraction and solubilization of grape and yeast polysaccharides differed depending on the polysaccharide family in question. AGP, localized in soluble form within grape cell walls (*26*), were easily extracted by endogenous enzymes during grape maturation, crushing, and the early maceration-fermenta-

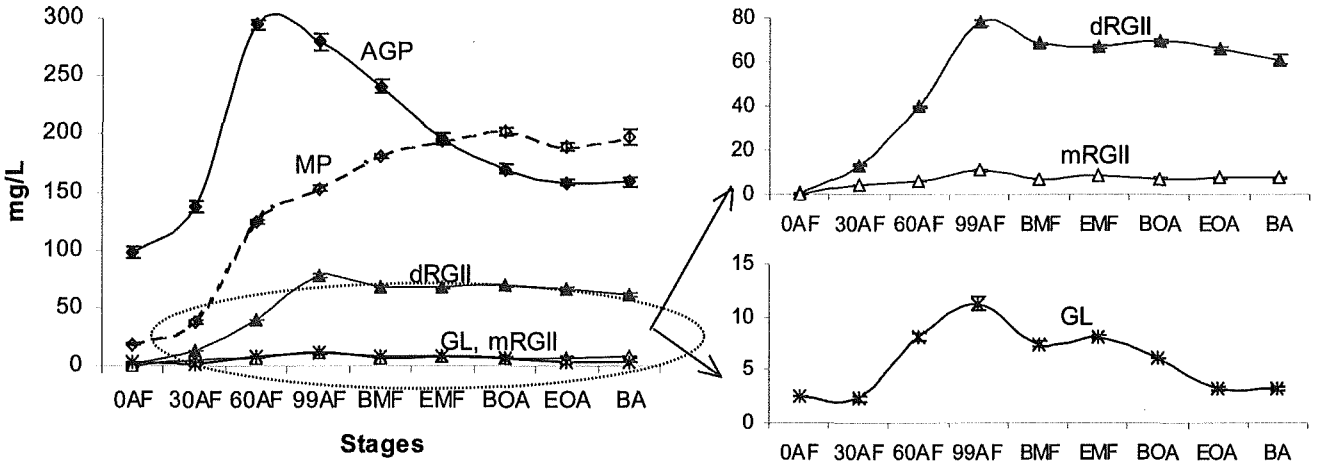

Figure 6. Evolution of major polysaccharide families in must and wine samples during vinification and aging. AGP, arabinogalactan–proteins; MP, mannans and mannoproteins; dRG-II, rhamnogalacturonan-II dimers; mRG-II, rhamnogalacturonan-II monomers; GL, oligomers of homo and rhamnogalacturonans. See text for conditions and calculations.

tion, increasing around 200% between 0AF and 60AF. Unlike AGP, RG-II were resistant to the endogenous pectolytic enzymes of grape berries and seemed not to be affected by crushing of berries. The extraction of RG-II dimers was as well almost negligible between 0AF and 30AF but increased 500% between 30AF and 99AF, indicating that dRG-II were more tightly bound to the cell wall matrix of grape cell walls, needing maceration time to solubilize. As regards the other grape polysaccharides, RG-II monomers and GL displayed similar behavior to RG-II dimers although they were present in very low quantities, mRG-II content being more than 6-fold lower than dRG-II content. The liberation of yeast mannoproteins was progressive during maceration-fermentation, with a higher extraction rate between 30 and 60AF, coinciding with the yeast exponential phase of growth. The reduction in sugar content previously observed at the end of maceration-fermentation only affected AGP molecules; the other polysaccharides were highly extracted during this period.

As a result, 0AF and 30AF must samples were largely dominated by AGP, although, as mentioned previously, low-molecular-weight glucosyl polysaccharides were the most prevalent molecules in these samples. The content of RG-II was almost negligible in musts and only traces of GL could be quantified, which was somewhat unexpected because it is the main pectic polysaccharide occurring in grapes (*27*) and it has also been detected in high amounts in blanc musts (*30*). AGP were also the majority polysaccharides in young wines after maceration-fermentation, followed by MP, dRG-II, and GL. These compounds represented about 50%, 30%, 15% and 2%, respectively, of total quantified soluble polysaccharides, in quite similar proportions to those described for other red varieties (*11*).

Postmaceration performed to enhance wine color did not yield any additional polysaccharide increase; on the contrary, it prompted a considerable reduction in AGP, dRG-II, mRG-II, and GL. Mannoproteins were the only molecules liberated during this period, resulting in an overall decrease of 8% in total polysaccharide content. MP increased 20%, possibly sufficient to improve the organoleptic qualities of wines after postmaceration. As described previously, malolactic fermentation also induced a reduction in total sugar content. However, contrary to what was thought, it only affected AGP molecules and the content of the other macromolecules remained stable, and MP even rose slightly during this period. Probably due to yeast cell wall fragmentation, the liberation of yeast mannoproteins was still high after alcoholic fermentation, thus compensating for their partial precipitation (**Table 1**).

As expected, the content of major polysaccharide families remained stable during oak and bottle wine aging, coinciding with what was observed in previous studies for other red wine varieties (*22*). In contrast to young wines, and due to the changes described previously, AGP were no longer the majority polysaccharides after malolactic fermentation and MP were the most prevalent polysaccharides in aged wines, where AGP, MP, and RG-II represented 37, 45, and 15%, respectively, of total quantified polysaccharides.

The evolution of arabinogalactan–proteins and mannoproteins was analyzed in detail and they were classified according to their molecular size (**Figure 7**). We noted that these were the highest molecular weight AGP and MP, the most prevalent both in wine and must samples, while smaller molecules, collected in fractions S2, represented less than 30% in wine samples and even less in musts (< 10%). Therefore, AGP and MP from grape and yeast cell walls were basically high-molecular-weight molecules and their solubilization rate was similar to the smaller compounds except for two aspects. On the one hand, the smaller AGP and MP were first liberated while the increase in larger molecules began as from 30AF. On the other hand, the precipitation of AGP and MP observed during late maceration-fermentation, postmaceration, and malolactic fermentation (see previous sections) had a greater extent on the bigger AGP and MP. A substantial increase in AGP and MP fragments between 60 and 99AF indicated as well an enzymatic degradation of both AGP and MP during this period. However, during postmaceration and malolactic fermentation, precipitation was probably the major phenomenon influencing the polysaccharidic balance. Precipitation during these stages may be a consequence of the formation of unstable complexes between polysaccharides and other wine polyphenolic compounds (*25*), although in the case of MP, it was fully compensated by their continuous liberation.

As regards the sugar composition of the different polysaccharide families, the molar ratio of arabinose to galactose for predominant high-molecular-weight AGP was between 0.4 and 1, and this ratio changed with the vinification process (**Figure 8**). AGP with lower arabinose/galactose molar ratios were extracted first, while AGP with higher ratios were extracted in the later stages of maceration-fermentation. After this period, high-molecular-weight AGP showed similar arabinose/galactose ratios to those described in the bibliography for AGP isolated

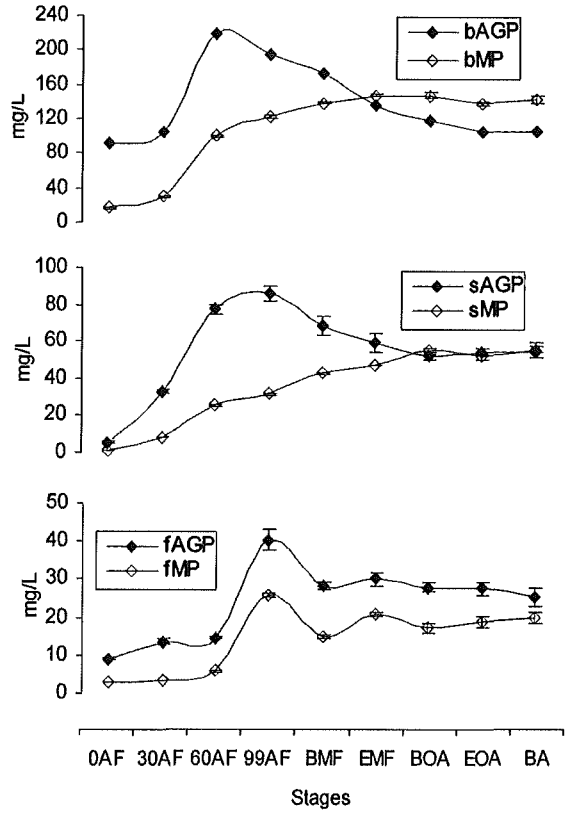

Figure 7. Concentration of arabinogalactan–proteins (AGP) and mannoproteins (MP) during vinification and aging. bAGP, bMP, high-molecular-weight AGP and MP collected in fractions S1, sAGP, sMP, low-molecular-weight AGP and MP collected in fractions S2, and fAGP, fMP, polysaccharide fragments of AGP and MP collected in fractions S3. See text for conditions and calculations.

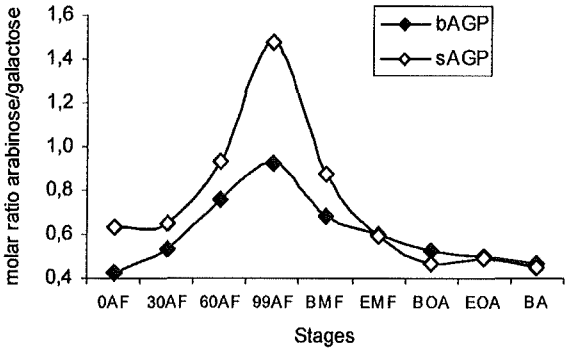

Figure 8. Molar ratio of arabinose to galactose from high-molecular-weight AGP (bAGP) and low-molecular-weight AGP (sAGP) during vinification and aging. See text for conditions and calculations.

from red wines obtained after fermentation of other red grape varieties (*10, 11*). The AGP with the highest arabinose/galactose ratios seemed to be the most affected by precipitation, as this ratio was substantially reduced during postmaceration and malolactic fermentation. The arabinose/galactose molar ratio of smaller AGP showed a similar evolution, although it was substantially higher for smaller AGP extracted after maceration-fermentation. The molar ratio of both RG-II dimers and monomers found coincided with results published previously for the purification of RG-II fractions (*11, 12*).

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NOTE ADDED AFTER ASAP PUBLICATION

The original posting of November 15, 2007, contained minor errors in the second paragraph of the Introduction and in ref *30*. This has been corrected with the posting of November 27, 2007.

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3.4

EVOLUCIÓN DE LA COMPOSICIÓN DEL COLOR Y DEL CONTENIDO DE POLIFENOLES DURANTE LA VINIFICACIÓN Y EL ENVEJECIMIENTO DE VINOS TINTOS

Changes in the color composition and phenolic content of red wines from *Vitis vinifera* Tempranillo during vinification and aging

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Resumen

Este artículo analiza la evolución de las principales familias de polifenoles durante la vinificación y el envejecimiento de vinos de Tempranillo, incluyendo las etapas de maceración-fermentación, fermentación maloláctica, crianza en barrica y envejecimiento en botella. En particular, se analizaron los cambios que se producen en las antocianinas monómeras, los flavan-3-oles monómeros, los ácidos hidroxicinámicos y las proantocianidinas. Asimismo, se realizó un estudio de la evolución en la composición del color del vino y su posible relación con los cambios en los compuestos polifenólicos.

Para determinar la composición del color del vino se midió la aportación de los distintos componentes al color total del vino: color debido a los antocianos monómeros (MAC), color debido a la copigmentación (CC) y color estable frente al bisulfito (BSC). Este último es atribuido en la bibliografía al color debido a los antocianos poliméricos, y, aunque como se ha explicado en el *Capítulo 2*, esto no es del todo correcto, dicho parámetro es muy útil en términos de medida de estabilidad de color.

El paso de mosto a vino produjo un aumento progresivo de los flavan-3oles, antocianos monómeros y ácidos hidroxicinámicos, aunque la cinética de extracción varió según la familia analizada. Así, los diferentes ácidos hidroxicinámicos, flavan-3-oles monómeros y antocianos glucosilados y sus derivados cumarílicos alcanzaron su máxima concentración al final de la fermentación alcohólica, mientras que los antocianos acetilados y las proantocianidinas alcanzaron su máximo con la postmaceración, indicando una óptima extracción de estos compuestos a altas concentraciones de etanol. Con excepción de los ácidos hidroxicinámicos, la fermentación maloláctica produjo una pérdida considerable en todos los compuestos fenólicos analizados, que no sufrieron variaciones durante la corta crianza del vino en barrica. Por el contrario, el envejecimiento del vino en botella sí que produjo una reducción significativa en los polifenoles monómeros, tanto en las diferentes formas de antocianos como de flavan-3-oles monómeros y ácidos hidroxicinámicos.

Como era de esperar, la evolución de los distintos compuestos fenólicos durante la vinificación y el envejecimiento de los vinos produjo cambios significativos en la evolución del color total de los vinos y en su composición. El color rojo de los mostos se atribuyó únicamente a la presencia de antocianos en su forma monomérica ya que los valores de color debidos a sus formas poliméricas o copigmentadas resultaron despreciables.

Al igual que se observó en los compuestos fenólicos, la etapa que más afectó a la evolución del color del vino fue la fase extractiva de maceración-fermentación. La extracción de los compuestos fenólicos durante esta etapa supuso un aumento sustancial en el color del vino, tanto en el color debido a los antocianos monómeros (MAC) como en el color estable debido a la copigmentación (CC) y a la polimerización de los antocianos (BSC), alcanzando todos estos componentes su máximo valor al final de dicha etapa. La disminución del contenido de antocianos durante la fermentación maloláctica produjo una pérdida sustancial en el color del vino a pesar del fuerte aumento en el color estable frente al bisulfito (BSC). Ninguno de los parámetros de color analizados sufrió modificaciones durante la crianza del vino en barrica, mientras que el color debido a los antocianos monómeros disminuyó significativamente durante su estancia en botella a favor de un incremento del BSC, de tal forma que el color del vino se mantuvo estable. Así, la participación de los antocianos y flavan-3-oles monómeros en diferentes reacciones de formación de pigmentos estables explicaba la disminución observada en dichos compuestos.

Durante todo el periodo estudiado, el color debido a los antocianos monómeros, de naturaleza altamente inestable, fue superior al color estable debido a la copigmentación y a la polimerización de antocianos (CC + BSC), aunque este último aumentó significativamente a lo largo de la vinificación y el envejecimiento. Así, la formación de color estable se produjo fundamentalmente en dos etapas diferenciadas: la maceración-fermentación y el envejecimiento en botella. El aumento de color estable que se produjo en la fermentación alcohólica fue debido fundamentalmente a la copigmentación, mientras que la postmaceración produjo un incremento del color estable en sus dos componentes, BSC y CC. En los vinos jóvenes la copigmentación afectó al 30% de los antocianos, sin despreciar la contribución al color de los antocianos poliméricos, que superó el 10%. El aumento de color estable durante la crianza en botella de los vinos fue debido únicamente a la formación de pigmentos poliméricos, cuya contribución al color de vino se incrementó hasta valores del 35%. Sin embargo, y a pesar de que hay estudios que señalan que los complejos de copigmentación desaparecen totalmente después de 9 meses de embotellado, el color debido a la copigmentación fue aún relevante al final de este periodo, contribuyendo en más de un 10% al color total del vino.

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Changes in the color composition and phenolic content of red wines from *Vitis Vinífera* Tempranillo during vinification and aging

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ABSTRACT

Changes in phenolics and color composition during vinification and aging of Tempranillo wines were studied. Different phenolics have different diffusion into the must, and with the exception of proanthocyanidins and acetyl-glucoside anthocyanins, with a maximum concentration at the end of postmaceration, the rest of the unacylated and coumarated anthocyanins, monomeric flavan-3ols, and hydroxycinnamic acids reached their maximum at the end of alcoholic fermentation. This resulted in a significant increase in both wine color and stable color, mainly due the formation of copigmentation complexes, although polymeric pigment formation was also important. Malolactic fermentation produced a significant decrease in the flavonoid content while non-flavonoids were maintained, prompting a considerable loss in wine color, despite the dramatic increase in bisulfite-stable color. Wine oak aging did not produce any significant change in the studied parameters, while bottle aging reduced the level of monomeric anthocyanins and flavan-3ols, and hydroxycinnamic acids. However, wine color remained stable due to a significant increase in the stable color. Polymerization reactions of anthocyanins prevailed over pigment degradation reactions, and copigmentation was still relevant after two years of bottle aging.

Keywords

Color composition, anthocyanins, proanthocyanidins, monomeric flavan-3ols, hydroxycinnamic acids, winemaking, aging, evolution.

1. INTRODUCTION

Red grape phenolics play a number of important roles in viticulture, including UV protection, disease resistance, pollination, color and defense against predation in plants [1]. They are also essential for the quality of wine as they are involved in haze formation and hue, and are responsible for the color, taste and antioxidant capacity of wines [2, 3].

Grape and wine phenolics belong to two main groups: flavonoid and nonflavonoid compounds. Flavonoids, located in grape skins, seeds and stems, include anthocyanins, flavan-3-ol monomers, oligomeric and polymeric proanthocyanidins, flavonols, flavanonols and flavones. Nonflavonoids, which derive primarily from the pulp and skins of the grape berries, includes phenolic acids and resveratrol and its derivatives. Flavonoids are the most important family among grape polyphenols, both in quantity and quality. Anthocyanins and derived pigments are directly responsible for the color of red wines and grapes, while monomeric flavanols and proanthocyanidins are mainly responsible for the astringency, bitterness and structure of wines [4]. Flavonols also contribute to bitterness and affect red wine color [5, 6], and certain phenolic acids participate in copigmentation [6].

Wine phenolic composition is conditioned by the grape used and by the winemaking processes that determine their extraction into the must and their further stability in wine. Grape phenolics depend on the variety and other factors that affect berry development such as soil, geographical location and weather conditions [7]. Fruit ripeness, ethanol content [8], perhaps berry size [9], and maceration conditions influence the extraction of phenolic compounds from the grape, and in particular the relative proportion of anthocyanins and flavanols diffusing into the wine [10]. Numerous reactions of phenolic compounds occur in the course of winemaking and aging, producing a huge variety of colorless products and pigments.

Changes in polyphenolic composition are mainly due to the participation of grape phenolics in haze formation [2] and in numerous copigmentation, cycloaddition, polymerization, and oxidation reactions [11]. These enzymatic and non-enzymatic reactions start just after grape crushing and continue throughout fermentation and aging [12], contributing to important changes in wine sensory properties [13, 14, 15]. Thus, the decrease in astringency as wine ages is ascribed to reactions of proanthocyanidins. Similarly, the color change from the red-blue of young red wines to brick-red in aged wines results from reactions of grape anthocyanins. In particular, the stable color in red wines is associated with anthocyanin copigmentation and polymerization, and other derived anthocyanin reactions resulting in more stable pigments.

The occurrence of such reactions in wine will depend on many factors [13] but mainly on the polyphenolic concentrations during the different winemaking stages. The extraction of anthocyanins and proanthocyanidins during alcoholic fermentation is generally acknowledged [16, 17, 18, 19, 20], and in-depth research into changes in anthocyanin composition during maturity and bottle aging has also been carried out [21, 22, 23, 24]. However, few studies include a global evolution of color and polyphenolic composition from the obtainment of must to aging in bottles, after passing through maturation in barrels. This research aims to study the evolution anthocyanins, hydroxycinnamic acids and flavanols during maceration-fermentation, malolactic fermentation, maturity and aging in bottles, and its relation with color composition and stability.

2. MATERIALS AND METHODS

2.1. Reagents and samples

All chemicals used were of analytical reagent grade. All chromatographic solvents were of HPLC grade. Malvidin-3-glucoside, peonidin-3-glucoside, caffeic acid, *trans-p*-coumaric acid, (+)catechin and (-)epicatechin, were purchased from Extrasynthèse (Lyon, France), and vanillin from Sigma (St. Louis, MO, USA).

Formic acid, acetonitrile and trifluoroacetic acid supplied by Sigma and MilliQ ultrapure water (Millipore, Molsheim, France) were used. Acetone was obtained from Riedel-deHäen (Sigma), and pure methanol and acetaldehyde were purchased from Merck (Darmstadt, Germany). Ethanol 96% v/v, sulfuric acid, sodium metabisulphite and tartaric acid were supplied by Scharlab (Barcelona, Spain), and sodium hydroxide and hydrochloric acid 37% were obtained from Carlo Erba (Rodano, MI, Italy).

Wine samples were produced from *Vitis vinífera Tempranillo* grapes of the qualified origin denomination Rioja (D.O.Ca. Rioja). *Saccharomyces cerevisiae* var. *cerevisiae* RC212 was a commercial yeast from Lallemand (Lallemand Inc., Montreal, Canada). The bacterial strain *Oenococcus oeni* was also purchased from Lallemand.

2.2. Vinification and sample collection

Tempranillo grapes were sourced from Autol, La Rioja, Spain, and harvested at 21.9 ºBrix, pH 3.56 and 6.02 g tartaric acid/L. Experimental vinifications were carried out in the wine cellar of the University of La Rioja and wines were prepared by traditional wine technology. Grapes were destemmed and crushed and distributed into three 100 L stainless steel tanks. The prefermentation process lasted for six hours at 18 ± 1 ºC and the experimental scaled fermentations were performed with a yeast inoculum of 25 g/Hl *S. cerevisiae* RC212. The fermentation-maceration process was carried out at a

maximum temperature of 28±2°C and lasted 10 days. Postfermentative maceration lasted for four days at 24±1°C and wines were run off. The cap was punched down twice a day until it remained submerged during the entire maceration period. Wines were inoculated with a commercial preparation of *Oenococcus oeni* (1g/HL) to induce malolactic fermentation, carried out at 18.5±1°C. After 20 days of malolactic fermentation, all the wines were racked and clarified by settling for 25 days at 10°C. Wine aging was performed at 13°C and 80-85% relative humidity in new 13 L American oak barrels, which have a larger area/volume than the traditional 225 L barrels. For this reason, and based on the organoleptic analysis, the oak aging process was only performed for 45 days. After this, the wines were filtered through SEITZ K250 filters (2.5-3.0 μm) (Sert Schenk Filter System GmB, Bad Krevnach, Germany) and finally bottled and stored for 24 months at 14°C and 80-85% relative humidity.

Samples were taken during alcoholic maceration-fermentation (beginning, 25-30%, 55-60%, and 99% of sugars consumed: namely 0AF, 30AF, 60AF, and 99AF, respectively), and at the beginning and end of malolactic fermentation (BMF, EMF). Samples were also taken at the beginning and end of wine oak aging (BOA, EOA), and after 24 months of wine bottle aging (BA).

2.3. Determination of usual enological parameters

L-Malic acid was determined by an enzymatic method in accordance with AOAC official analysis methods [25]. Conventional enological parameters (density, ethanol concentration, pH values, and reducing sugars, titratable and volatile acidities) were determined at wine pH according to official OIV practices [26].

2.4. Color components and total polyphenol index values

Spectrophotometric measurements were performed on a Cary 300 Scan UV-vis spectrophotometer (Varian Inc., Madrid, Spain) using 2-mm and 10-mm path length quartz cells. All the samples were analyzed in triplicate and all absorbance values corrected to 10-mm path length.

Wine color (WC), monomeric anthocyanins color (MAC), copigmentation color (CC), and bisulfite-stable color (BSC) were determined using the method proposed by Levengood and Boulton [27]. The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine with synthetic wine (12% alcohol, 5 g/L of tartaric acid in water, pH 3.6).

2.5. Fractionation of phenolics by GPC

Must and wine samples were directly fractionated by GPC on a Toyopearl gel HP-50F column (Tosohaas, Montgomery-Ville, PA, USA) using the method described by

Guadalupe et al. [28]. A first fraction (F1) was eluted with ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v), and a second fraction (F2) was recovered by elution with acetone/water (60:40, v/v). The two fractions collected were taken to dryness under vacuum, and then the first fraction was analyzed by HPLC-DAD-MS, and the second one was used to determine the proanthocyanidin content using the vanillin assay. All samples were fractionated two times.

2.6. HPLC-DAD determination of monomeric phenolic compounds in Fraction F1

F1 fractions were subjected to HPLC-DAD on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with a G1313A injector, a G1311A HPLC quaternary pump, an on-line G1379A degasser, a G1316A oven, a G1315B photodiode array detector, and Agilent Chemstation software. A Kromasil 100-C18 reverse phase column (5 μm packing, 200 x 46 mm i.d.) protected with a guard column of the same material (Teknokroma, Barcelona, Spain) thermostatted at 30°C was used.

The HPLC-DAD conditions had been previously used with satisfactory results in our laboratory to analyze fraction F1 [28]. The solvents used were: (A) formic acid/water (2:98, v/v), and (B) acetonitrile/water/formic acid (80:18:2, v/v/v), establishing the following gradient: isocratic 2% B in 10 min, from 2 to 10% B in 2 min, from 10 to 15% B in 10 min, from 15 to 30% B in 10 min, from 30 to 50% B in 20 min, from 50 to 60% B in 5 min, from 60 to 90% B in 5 min, at a flow rate of 1 ml min^{-1}. Spectra were recorded from 250 nm to 600 nm.

Quantification was carried out by peak area measurements at 515 nm for anthocyanins, 310 nm for phenolic acids and 280 nm for flavan-3ols. Since most of the individual phenolic compounds are not commercially available as reference standards, anthocyanin content was expressed as malvidin-3-glucoside, phenolic acid content as caffeic acid and flavan-3ols content as (+)catechin by an external standard calibration curve. Individual phenolic compounds were identified by HPLC-MS [28]. Each measurement was run in triplicate.

Anthocyanin glucosides (A-Glu) were calculated as the sum of delphinidin (Df), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin-3-glucosides (Mv); acetyl-glucoside anthocyanins (A-Ac) as the sum of delphinidin (Dfac), cyanidin (Cyac), petunidin (Ptac) and malvidin-3-(6-acetyl)-glucosides (Mvac); coumaryl-glucoside anthocyanins (A-Cm) included delphinidin (Dfcm), petunidin (Ptcm), and malvidin-3-(6-*p*-coumaryl)-glucosides (Mvcm). The sum of A-Glu, A-Ac and A-Cm was referred to as total monomeric anthocyanins (TMA). Total hydroxycinnamic acids (TCin) were calculated as the sum of *trans*-caftaric (*trans*-caffeoyl-tartaric acid), *cis*-caftaric (*cis*-caffeoyl-tartaric acid), *trans*-coutaric (*trans*-*p*-coumaryl-tartaric acid), *cis*-coutaric (*cis*-

p-coumaryl-tartaric acid), caffeic and *trans-p*-coumaric acid. The monomeric flavan-3ols (M-Flava) included (+)catechin, (-)epicatechin and (-)epigalocatechin.

2.7. Determination of Total Proanthocyanidin Content by the Vanillin Assay in Fraction F2

Total proanthocyanidins (PA) were quantified in F2 fractions by means of the vanillin assay using the method described by Sun et al. [29], but with slight modifications described by Guadalupe et al. [28]. Total Flavan-3ols (T-Flava) corresponded to the sum of flavan-3-ols monomers (M-Flava) and proanthocyanidins (PA).

2.8. Statistical procedures

Phenolic composition and color measurement data corresponding to the samples taken in the different vinification stages were analyzed using Student's t-test (SPSS version 12.0, SPSS Inc) to search for significant differences. In this paper, differences between samples always refer to significant differences with at least $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Conventional enological analysis in wines

Values of pH, titratable acidity, ethanol concentration and volatile acidity were in the ranges usually found in Tempranillo variety wines (Table 1). The pH, titratable acidity, and volatile acidity values obtained confirmed the absence of microbial alterations. Wine pH increased 0.2 units at the end of malolactic fermentation and titratable acidity decreased by 29%. Furthermore, malolactic fermentation prompted a decrease in both the total polyphenol index (TPI) and color intensity, while hue increased 0.1 units. The analyses performed on the wine indicated proper storage of wines during oak and bottle aging. Although wood polyphenols are extracted during wine aging, TPI values obtained before and after wine aging were not significantly different, indicating that polyphenol extraction was minimal during short aging. As expected, oak and bottle aging was accompanied by a decrease in color intensity and an increase in hue, while the TPI value of the wine increased slightly after two years of bottle aging.

3.2. Evolution of must and wine color composition during vinification and aging

The evolution of wine red color (WC), monomeric anthocyanins color (MAC), copigmentation color (CC), and bisulfite-stable color (BSC) was analyzed during maceration-fermentation, postmaceration, malolactic fermentation, oak aging and bottle

aging (Figure 1). Bisulfite-stable compounds are usually termed *polymeric pigments*, which is erroneous since it includes compounds that are not polymeric in nature (i.e. pyranoanthocyanins) and not all anthocyanin-derived polymers are colored or bisulfite-stable pigments (i.e. the so-called T-A polymeric pigments). However, the practice of measuring bisulfite-stable color continues to be very useful [30].

Red winemaking increased the value of WC, MAC, CC and BSC, and maceration was the main process affecting these values. Throughout the period studied, the value of monomeric anthocyanin color (MAC) was considerable higher than CC and BSC, contributing more than 50% to wine color. However, the ratio of stable bisulfite and copigmentation color (BSC + CC) to unstable anthocyanin color (MAC) changed with the winemaking process (Figure 2).

The light red color of must samples was simply due to monomeric anthocyanins, indicating that the copigmentation complexes present in the grapes [31] were broken during crushing, probably due to air contact. MAC, BSC, and CC values increased significantly in the samples during alcoholic fermentation, indicating a high extraction of grape anthocyanins as well as an important transformation of these compounds into sable copigmentation complexes and polymeric pigments. These reactions were seen to be greater with higher ethanol concentrations, and CC increased more than 6-fold between 30AF and 99AF, while BSC increased 9-fold. As expected, the postmaceration carried out to enhance wine color increased the WC value, and it was seen to be due to an increase in the color-stabilizing components while the MAC remained stable. At the end of this period, the contribution of copigmentation color to wine color was almost 30%, coinciding with Boulton's results [32], who reported that the contribution of copigmented anthocyanins to young red wine color ranged from 30 to 50%. Copigments in young wines are substantially important for wine color since they increase color intensity and transform the color of wine red to purple. Moreover, they may also be seen as a storage form of anthocyanin, allowing more anthocyanin to be removed from the skins and stabilized in solution until the polymeric pigments are formed. In this regard, and although it has been reported that polymeric pigments form during wine maturity, we found that the amount of anthocyanin-derived polymeric pigments started to become significant at the end of maceration-fermentation, contributing in around 10% to young wine red color.

Malolactic fermentation resulted in a significant decrease in the content of wine color (16.7%), due to a decrease in both CC and MAC. However, bisulfite-stable color increased dramatically by 71% in the wine, coinciding with previous observations reported by our group [33]. It is widely known that malolactic fermentation produces important losses of color due to the precipitation of unstable colloidal material [34], and

it also produced the dissociation of the copigmentation complexes, probably due to the pH shift. However, new and stable pigments were also formed during this period. These new pigments are the source of stable color in aged red wines because they are more resistant to bisulfite bleaching and their color is not pH-dependent like free anthocyanins.

During short-term barrel aging, the MAC, BSC and CC values did not change significantly. Bottle aging induced an important loss of MAC and a slight decrease of CC, but this was offset by the significant increase in bisulfite-stable color (58%), in such a way that wine color remained stable. This fact indicated that reactions of anthocyanins with proanthocyanidins and other phenolic compounds prevailed over pigment degradation reactions, and thus the contribution of bisulfite-stable color to WC was higher than 30%. Although copigmentation phenomena have been described as negligible during wine maturity, and some authors describe that copigments disappear completely after 9 months of bottle aging [6, 35], our results confirmed the presence of copigmented anthocyanins in the bottle-aged wine, contributing more than 10% to wine color. Our workgroup had previously detected the presence of these compounds in important amounts in oak aged wines [33].

All the changes described above resulted in a continuous increase in the stable/instable color ratio, from less than 0.2 in the must samples to almost 1 at the end of bottle aging, with three main stages of stable color formation: middle/end maceration-fermentation, postmaceration and bottle aging (Figure 2).

3.3. Evolution of must and wine phenolics during vinification and aging

Figure 3 shows the concentration of total phenolic compounds (TP), total monomeric anthocyanins (TMA), total hydroxycinnamic acids (TCin), and total flavan-3ols (T-Flava) during vinification and aging. Total polyphenols were calculated by adding the amounts TMA, TCin and T-Flava. Monomeric anthocyanins were analyzed because they are directly responsible for red wine color, although both hydroxycinnamic acids and flavan-3ols are associated with the formation of stable color. Hydroxycinnamic acids and monomeric flavan-3ols are involved in the copigmentation reactions, the former having been described as highly effective copigments [36, 37]. The so-called polymeric pigments are formed by the reaction between anthocyanins and polymeric flavan-3ols.

Winemaking increased total polyphenolic content, and maceration-fermentation was again the main process affecting this content. TP increased 9-fold between day 0 (0AF) and day 14 (end of postfermentative maceration), the moment when the wine achieved the maximum TP concentration, since polyphenol extraction continued while pomace

contact existed. As in the case of wine color, malolactic fermentation resulted in a substantial decrease in TP (29%). During short-term barrel aging, wine phenolics remained stable but the TP value decreased during the two years of bottle aging (10%).

As regards the results for individual polyphenolic families, important differences were observed between the must and wine samples (Figure 3). Monomeric anthocyanins were the predominant polyphenols in the must aqueous phase, representing more than 77% of total polyphenols, followed some way behind by the flavan-3ols (16.5%) and hydroxycinnamic acids (5.7%). Alcoholic fermentation produced a 5-fold increase in the concentration of monomeric anthocyanins (TMA), while the colorless phenolics TCin and T-Flava increased by more than 9-fold and 19-fold respectively, the T-Flava/TMA ratio increasing from 0.2 in the musts to 0.8 at the end of fermentation. Both anthocyanins and hydroxycinnamic acids reached their maximum extraction at the end of this stage, while flavan-3ol extraction continued during postmaceration favored by the high temperature and ethanol content. As a result, the T-Flava/TMA ratio increased to 1.1, T-Flava representing more than 49% of total phenolics, followed by monomeric anthocyanins (44%) and hydroxycinnamic acids. Evolution of anthocyanins resulted from the equilibrium between their rate of extraction and their rate of disappearance, mainly attributed to their conversion to other molecular species, either through chemical reactions or colloidal associations, rather than their precipitation or absorption phenomena. As a result, a gradual increase in stable color, both in copigmented and polymeric anthocyanins, was observed during maceration (Figure 1).

The decrease in monomeric anthocyanin content during malolactic fermentation was 1.6 times greater than that of total flavan-3ols (39.4% vs. 24.2%), while hydroxycinnamic acid concentration was maintained. The considerable reduction in monomeric anthocyanins increased the T-Flava/TMA ratio to 1.4 and coincided with the important loss in wine color (Figure 1).

During bottle aging, the content of T-Flava was maintained while anthocyanin and hydroxycinnamic acid content was considerably reduced, 34% and 19% respectively, with the subsequent increase in the T-Flava/TMA ratio (>50%). This reduction was attributed to both the precipitation of instable colorant material and the transformation of monomeric anthocyanins into polymeric pigments, corroborating the results obtained when analyzing wine color composition (Figure 1). The fact that the total flavan-3ols remained nearly constant during this stage did not mean that the monomeric flavan-3ols and proanthocyanidins did not change quantitatively and qualitatively (see section 3.4).

3.4. Evolution of individual phenolics during vinification and aging

The evolution of the mean concentration of individual anthocyanins, individual monomeric flavan-3ols and proanthocyanidins, and individual hydroxycinnamic acids during the stages of the vinification process is shown in Figures 4, 5, and 6, respectively.

The extraction of grape skin anthocyanins and monomeric flavan-3ols and skin-pulp hydroxycinnamic acids rapidly increased due to the diffusion of anthocyanin glucosides (Figure 4), (-)epigalocatechin (Figure 5), and *trans*-hydroxycinnamate derivatives (Figure 6) into the must. However, the polymeric flavan-3ols displayed limited solubility into the aqueous medium (Figure 5). As a result, anthocyanins were the predominant molecules in 0AF must samples; unacylated anthocyanin glucosides (A-Glu) accounted for 89% of total monomeric anthocyanins while coumaroylated forms (A-Cm) and acetylated forms (A-Ac) accounted for 8.2% and 2.8%, respectively (Table 2). As expected in a *Vitis vinifera* variety, malvidin-3-glucoside was the major anthocyanin, accounting for 72% of total anthocyanins, and its derivates were also the main anthocyanins in the acetylated and coumaroylated forms (Figure 4). Monomeric flavan-3ols represented 73% of total flavanols (Table 2), and (-)epigallocatechin, present only in skin [38], represented 34% of total monomeric flavan-3ols in must samples (Figure 5). *Trans*-caftaric acid was found to be the main hydroxycinnamic acid, accounting for 42% of the latter (Figure 6).

The relative proportion of the different anthocyanins, hydroxycinnamic acids and flavan-3ols significantly changed with the progression of the maceration-fermentation (Table 2). Thus, the proportion of unacylated anthocyanins and *trans*-caftaric acid decreased while the proportion of acylated anthocyanins and *trans*-coutaric acid increased. This different behavior was attributed to the different diffusion of the glucosides and hydroxycinnamate derivatives but also to the characteristic profile of the Tempranillo variety. The results obtained at the end of the fermentation stage showed that the content of malvidin-3-(6-coumaroyl)-glucoside was 3-fold higher than the content of malvidin-3-(6-acetyl)-glucosides, and that the amounts of *trans*-caftaric acid and *trans*-coutaric acid were comparable (Figures 4 and 6), in accordance with the Tempranillo variety, characterized by a higher proportion of anthocyanin coumarate forms than acetylated forms, and similar quantities of *trans*-caftaric and *trans*-coutaric acid [39].

As regards the different anthocyanic forms, we found that the proportion of peonidin (Pn) and cyanidin (Cy) decreased from 0AF to 30AF while the proportion of delphinidin (Df), petunidin (Pt) and malvidin (Mv) increased (Table 2). Although it has been widely described that the initial diffusion of the bi-substituted anthocyanins (Pn

and Cy) is higher than that of the tri-substituted anthocyanins (Df, Pt and Mv), our results coincide with those of other authors for other grape varieties [40]. As maceration-fermentation progressed, the relative proportions of the unacylated bi-substituted anthocyanins and malvidin glucoside decreased, and the proportions of Df and Pt increased (Table 2). Thus, the quantity of Df and Pt increased more than 10-fold at the end of fermentation although malvidin glucoside was still the prevalent anthocyanin (Figure 4). In relation to the flavan-3ols, both monomeric and polymeric forms significantly increased during maceration-fermentation with a greater increase in the polymeric forms (Figure 5); as a result the proportion of polymeric proanthocyanidins more than doubled the monomeric flavan-3ols at the end of this period (Table 2). This fact was attributed to the progressive extraction of the seed tannins, which need higher ethanol content to be solubilized than the skin tannins [41].

The prolongation of alcoholic maceration favored the extraction and solubility of seed tannins and minority acetylated anthocyanins [42] but not majority anthocyanins. The extraction of major anthocyanins (A-Glu and A-Cm) was probably not sufficient to compensate for their loss due to the formation of new polymeric and copigmented anthocyanins (Figure 1). Postmaceration also produced a decrease in the concentration of *trans*-caftaric and *trans*-coutaric acid, which did not coincide with the increase observed in the corresponding free forms (Figure 6), and it was explained by their roll as copigments. An additional source of caffeic acid and *trans-p*-coumaric acid may also be the hydrolysis of cinnamoyl-glucoside anthocyanins [21].

The proanthocyanidins and A-Glu were the main phenolics in young wines after the postmaceration period, followed by monomeric flavan-3ols, A-Cm, hydroxycinnamic acids and A-Ac. These compounds represented about 42.7%, 24.1%, 15.9%, 7.2%, 6.8% and 3.3% of the total quantified soluble phenols, respectively. Malvidin-3-glucoside was again the main anthocyanin in wines and its derivates were also the main anthocyanins in the acetylated and coumaroylated forms after the postmaceration period.

Malolactic fermentation prompted a significant decrease in the content of the different anthocyanin glucosides, proanthocyanidins and *trans*-hydroxycinnamate derivatives, 39.6% for A-Glu, 44.6% for A-Ac, 34.4% for A-Cm, 38.7% for PA, and 4.8% for *trans*-hydroxycinnamates. At the same time, monomeric flavan-3ol and free acid values were maintained or even slightly increased (Figures 4, 5 and 6). The lower values observed at the end of malolactic fermentation for monomeric anthocyanins, color intensity (Table 1) and wine color (Figure 1) were in good agreement with bibliography [33, 43]. However, it is important to take into account that the stable/instable color ratio remained almost constant (Figure 2) and the anthocyanin

pattern did not change (Figure 4). García-Beneytez et al. [44] state that the anthocyanin profile remains stable because the polymerization reactions of the different anthocyanins with the proanthocyanidin molecules occur at the same rate, but other authors have reported otherwise [45]. The tannin-anthocyanin combination and the precipitation of unstable colloids tannin-tannin, tannin-polysaccharides and tannin-proteins would explain the reduction in proanthocyanidins and monomeric anthocyanins. At the same time, the partial disappearance of tartaric esters with caffeic acid and *trans-p*-coumaric acid was detected, with an increase in the corresponding free acids. Other authors have reported the complete disappearance of *trans*-caftaric and *trans-p*-coutaric acid in Tempranillo wine during this phase [46]. However, the amount of caffeic acid obtained (8.4 mg/l) was similar to that in most varietal red wines [47].

In contrast to what occurred with short-term barrel aging, long-term aging in bottles caused a considerable decrease of anthocyanins and hydroxycinnamic acids (Figure 4 and 6). The anthocyanins least resistant to disappearance were the glycosilated anthocyanins, showing a 37% decrease, followed some way behind by coumarate derivatives (27%) and acetates (10%). However, the anthocyanin pattern remained stable from postmaceration to aging (Figure 4). The hydroxycinnamic compounds underwent dramatic changes. The forms of combined *trans-p*-coumaric and caffeic acid decreased by around 28% after two years of bottle aging while free acids increased. It was also observed that the amounts of *trans*-caftaric and *trans*-coutaric acid were comparable, which together with the higher proportion of coumarated anthocyanin forms detected than acetate forms, confirmed that the varietal characteristics of the Tempranillo wine were maintained after two years of aging.

Total flavan-3ols remained stable during aging, but in-depth analysis revealed an increase in proanthocyanidins (6.7%) at the same time as a decrease in monomeric flavan-3ols (9.4%). Epigalocatechin content was reduced during aging and, as previously reported by other authors [19, 23, 48], epicatechin was also reduced (Figure 5). These changes were the result of complex mutual polymerization-depolymerization processes accompanied by combination with the different anthocyanins to give more stable pigments [49].

Contrary to what was observed in the young wine after postmaceration, the polymeric flavan-3ols were the most prevalent compounds in the aged wines (41.3%), followed some way behind by monomeric flavan-3ols (24%) and A-Glu (21.8%), and lastly hydroxycinnamates (7.3%), A-Cm (3.1%), and A-Ac (2.3%).

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TABLES

Table 1. Conventional enological parameters[a] of wines

Wine stage[b]	% v/v[c]	pH	TA[d]	VA[e]	TPI[f]	CI[g]	Hue[h]
BMF	12.6±0.2	3.77±0.007	5.2±0.1	0.23±0.05	40±0.7	6.79±0.03	0.53
EMF	12.5±0.2	3.96±0.03	3.69±0.04	0.28±0.06	35±0.5	5.80±0.02	0.60
BOA	12.5±0.2	3.97±0.02	3.71±0.05	0.29±0.05	35.5±0.6	5.82±0.01	0.59
EOA	12.6±0.2	3.98±0.01	3.98±0.05	0.33±0.5	36.3±0.4	5.008±0.004	0.62
BA	12.5±0.2	3.98±0.01	3.95±0.05	0.36±0.5	37±0.5	5.01±0.01	0.75

[a] Mean ± S.D. (n=3).

[b] Wine stage: BMF, beginning of malolactic fermentation; EMF, end of malolactic fermentation; BOA, beginning of oak aging; EOA, end of oak aging; BA, end of bottle aging.

[c] mL of ethanol for 100 mL of wines at 20°C.

[d] Titratable Acidity as g of tartaric acid per liter.

[e] Volatile Acidity as g of acetic acid per liter.

[f] Total polyphenol Index as absorbance units (AU).

[g] Color Intensity as sum of absorbances at 420, 520, and 620 nm.

[h] $UA_{420\ nm}/UA_{520\ nm}$.

Table 2. Evolution of the percentage of different anthocyanic forms, hydroxycinnamic acid forms, and monomeric and polymeric flavan-3ols in maceration-fermentation and postmaceration

Stage[a]	Anthocyanic forms								Hydroxycinnamic acid forms						Flavan-3ols	
	Mv	Pt	Df	Pn	Cy	A-Glu	A-Ac	A-Cm	t-caft[b]	c-caft[c]	caffeic	t-cout[d]	c-cout[e]	coumaric	M-Flava	PAs
0AF	80.8±1	7.9±0.4	6.5±0.3	3.9±0.3	0.9±0.03	89.1±0.6	2.8±0.2	8.2±0.4	42.5±1	9.4±0.1	1.6±0.3	31.5±0.5	13.4±0.5	1.6±0.05	72.8±0.5	27.2±0.2
30AF	81.2±0.2	8.8±0.2	6.8±0.4	2.7±0.2	0.6±0.1	85.1±0.4	4.2±0.4	10.7±0.5	28.8±0.9	7.7±0.3	6.7±0.2	32.6±0.4	11.6±0.3	12.6±0-3	46.5±0.4	53.5±0.4
60AF	78.7±0.4	10.0±0.3	8.1±0.1	2.7±0.3	0.5±0.06	80.9±0.3	4.7±0.3	14.4±0.5	35.2±0.4	6.1±0.2	4.3±0.1	34.0±0.6	12.2±0.4	8.3±0.2	43.8±0.3	56.2±0.1
99AF	75.9±0.5	11.9±0.2	9.0±0.2	2.7±0.3	0.5±0.04	80.3±0.3	6.2±0.1	13.6±0.6	36.2±0.5	4.0±0.3	4.0±0.3	38.7±0.5	9.1±0.3	8.0±0.5	39.7±0.2	60.3±0.2
BMF	77.3±0.3	11.7±0.1	8.2±0.3	2.3±0.1	0.4±0.06	83.2±0.4	7.0±0.2	9.7±0.6	34.0±0.3	4.9±0.1	4.6±0.2	36.4±0.3	11.7±0.2	8.3±0.2	28.0±0.2	72.0±0.4

[a] Vinification stage: 0AF, 30AF, 60AF, 99AF: alcoholic fermentation (0, 30, 60, 99% of sugars consumed respectively); BMF: beginning of malolactic fermentation

[b]: *trans*-caftaric acid

[c]: *cis*-caftaric acid

[d]: *trans*-coutaric acid

[e]: *cis*-coutaric acid

FIGURES

Figure 1

Wine color (WC), Monomeric anthocyanins color (MAC), Copigmentation color (CC), and (c) Bisulfite-stable color (BSC) during vinification and aging. See text for conditions and calculations.

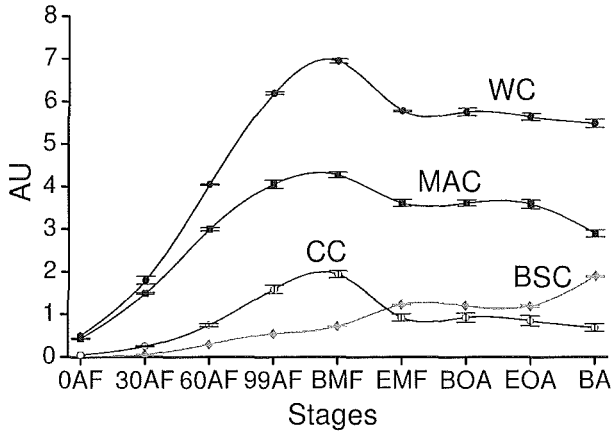


Figure 2

Ratio of Stable color (bisulfite-stable color + copigmentation color) to monomeric anthocyanins color (MAC).

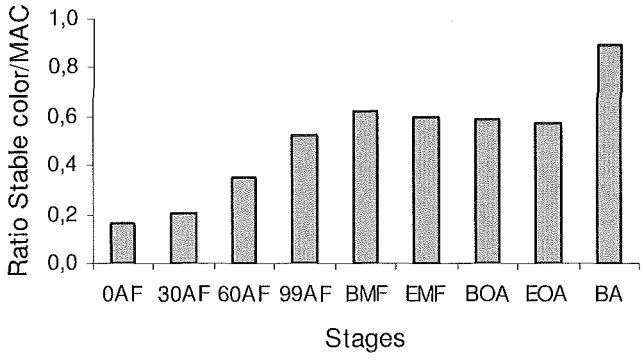


Figure 3

Concentration of Total polyphenols (TP), Total monomeric anthocyanins (TMA), Total flavan-3ols (T-Flava) and Total hydroxycinnamic acids (TCin) during vinification and aging. See text for conditions and calculations.

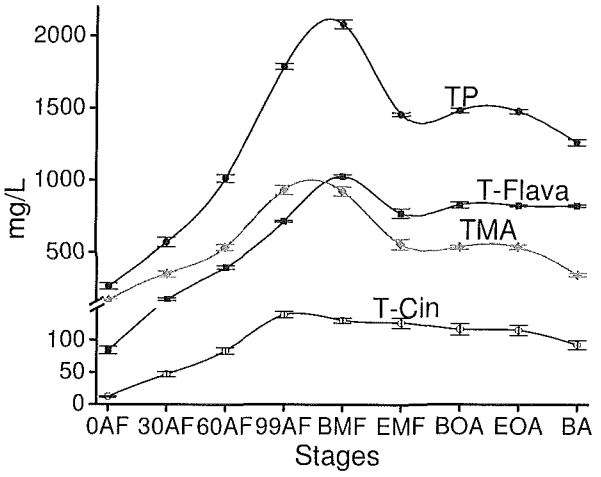


Figure 4

Concentration of monomeric anthocyanins during vinification and aging: Anthocyanin glucosides, Acetyl-glucoside anthocyanins (A-Ac), and Coumaryl-glucoside anthocyanins (A-Cm). See text for conditions and calculations.

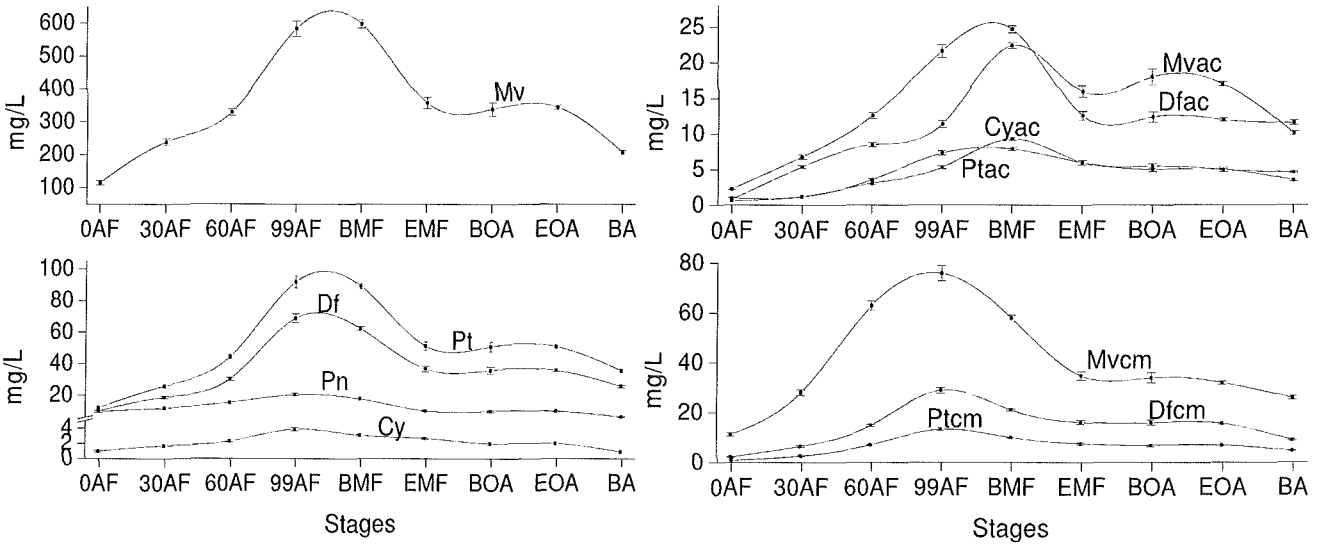


Figure 5

Concentration of Total proanthocyanidins (PA), Total monomeric flavan-3ols (M-Flava), and monomeric epigalocatechin, epicatechin and catechin during vinification and aging. See text for conditions and calculations

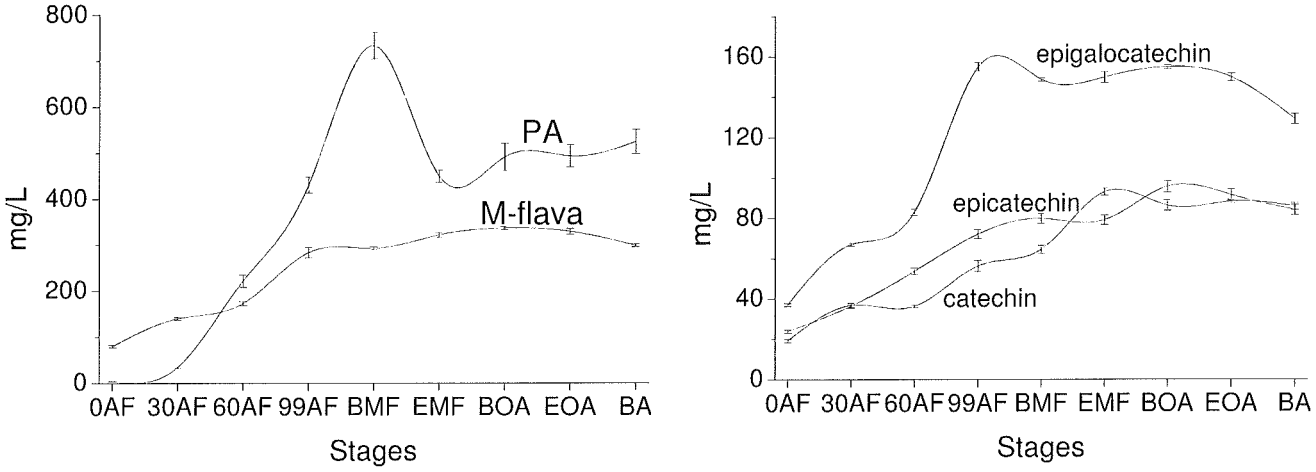
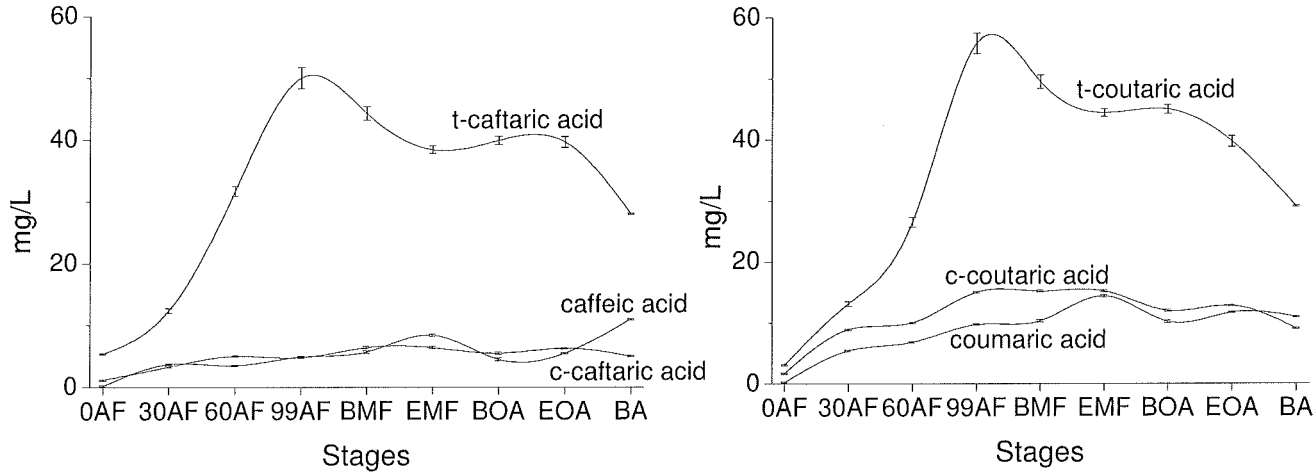


Figure 6

Concentration of Hydroxycinnamic acids during vinification and aging. See text for conditions and calculations.



3.5

EFECTO DE MONOPROTEÍNAS Y ENZIMAS DE MACERACIÓN EN LA ESTABILIDAD COLOIDAL Y LA EXTRACCIÓN DEL COLOR DE VINOS TINTOS

Maceration Enzymes and Mannoproteins:

A Possible Strategy to Increase Colloidal Stability and Color Extraction in Red Wines

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Resumen

Este artículo evalúa la efectividad de los tratamientos biotecnológicos planteados en esta tesis para mejorar la extracción y estabilización de la materia colorante durante el proceso de vinificación de vinos tintos de variedad Tempranillo. Concretamente los tratamientos que se propusieron fueron: a) adición de enzimas de maceración comerciales en la etapa prefermentativa, b) adición directa de manoproteínas purificadas industrialmente en la etapa prefermentativa, y c) inoculación de los mostos con levaduras superproductoras de manoproteínas. Así, este trabajo analiza la efectividad de estos tratamientos sobre la extracción de coloides protectores (polisacáridos), y su efecto en la extracción del color y en el contenido de polifenoles totales, así como en la calidad organoléptica de los vinos obtenidos.

Los tres tratamientos biotecnológicos propuestos en la tesis incrementaron el contenido de polisacáridos totales solubles durante la fermentación alcohólica. El tratamiento cuantitativamente más efectivo fue la adición de preparados de manoproteínas comerciales, que supuso un incremento de un 19 a un 23% en los polisacáridos totales, seguido de la utilización de levaduras súper-productoras y de enzimas de maceración, que presentaron una efectividad similar, con un incremento de un 10 a un 14%. A diferencia de los tratamientos con manoproteínas, que sólo incrementaron la concentración de los polisacáridos neutros, el empleo de enzimas de maceración produjo un incremento tanto en los polisacáridos neutros como en los ácidos, mostrando incluso mayor efecto en estos últimos. Así, las enzimas de maceración hidrolizaron los polisacáridos pécticos procedentes de la uva, hecho que ya habíamos observado anteriormente, pero el resto de tratamientos no parecía tener ningún efecto sobre los mismos. Desde el final de la fermentación alcohólica se produjo una precipitación parcial de los polisacáridos extraídos, siendo más acusada en las muestras tratadas, por lo que las diferencias encontradas disminuyeron en todos los casos en los vinos acabados.

La adición de enzimas de maceración comerciales favoreció la extracción de color en los vinos así como su estabilización. Así, los vinos elaborados con enzimas presentaron los valores más altos de color total, intensidad de color y color estable frente al bisulfito, siendo además los que obtuvieron las puntuaciones más altas de color en el análisis sensorial. La acción hidrolítica del las enzimas produjo también un incremento del índice de polifenoles totales, a menudo utilizado como medida indirecta de las proantocianidinas. De hecho, estos vinos presentaron mayor tanicidad, astringencia y longitud en boca que el resto.

Contrariamente a lo esperado, los vinos elaborados mediante el empleo de manoproteínas industriales o de levaduras súper-productoras de manoproteínas presentaron valores más bajos de polifenoles totales que sus respectivos testigos desde el inicio de la fermentación maloláctica. Estos resultados indicaban que las manoproteínas no mantenían en dispersión coloidal a los polifenoles, hecho que contradice a la bibliografía, que describe a las manoproteínas como coloides protectores y como inhibidoras de la auto-agregación de los taninos. Por otro lado, el empleo de manoproteínas industriales no incrementó ni estabilizó el color de los vinos; por el contrario, estos vinos presentaron los valores más bajos de intensidad de color y de color total. Sin embargo, el efecto de las manoproteínas liberadas sobre el color no fue tan claro, no pudiendo obtenerse resultados concluyentes. Respecto a la evaluación sensorial de los vinos, la adición de manoproteínas comerciales en la etapa prefermentativa modificó claramente la estructura aromática y gustativa de los mismos. La adición de manoproteínas incrementó la intensidad aromática de los vinos, que se caracterizaron por un predominio de los aromas a madera y ahumados. En la fase gustativa, estos vinos se describieron como los más grasos en boca y los más cálidos. Como en el caso del color, la utilización de distintas cepas de levadura no supuso diferencias destacables en el análisis sensorial de los vinos.

Teniendo en cuenta los resultados obtenidos en este trabajo, se decidió estudiar con más detalle el efecto de los purificados de manoproteínas industriales y de las levaduras súper-productoras en el contenido y la evolución de los polisacáridos y los compuestos fenólicos.

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Maceration Enzymes and Mannoproteins: A Possible Strategy To Increase Colloidal Stability and Color Extraction in Red Wines

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Different strategies were adopted to achieve increases in color stability in Tempranillo wines: (i) addition of maceration enzymes directly to the must, (ii) addition of commercial mannoproteins to the must, and (iii) inoculation of must with yeast overexpressed of mannoproteins. The addition of enzymes favored color extraction, and the wines obtained presented higher values of wine color, color intensity, bisulfite-stable color, and visually enhanced color intensity. The enzyme hydrolytic activity produced an increase in the acid polysaccharide content and polyphenol index and yielded to wines with more astringency, tannin, and length. Added mannoproteins had clearer effects on the analyzed parameters than yeast. Contrary to what may be thought, mannoproteins did not maintain the extracted polyphenols in colloidal dispersion and neither ensured color stability. These compounds clearly modified the gustative structure of the wines, enhancing the sweetness and roundness.

KEYWORDS: Red wine; maceration enzymes; mannoproteins; polysaccharides; polyphenol index; wine color; bisulfite-stable color; copigmentation color; sensory analysis

INTRODUCTION

The color of wines, especially that of red wines, is one of the most important properties used for the commercial evaluation and exerts a considerable influence on the marketability of the products. The color in young red wine is due to phenolics, especially anthocyanins, which are unstable molecules and highly susceptible to degradation (*1*). The color stability of anthocyanins is influenced by diverse factors (*2*–*4*).

The reactions that lead to the formation of stable pigments in wines have been mentioned by several authors (*5*–*8*). The reaction between anthocyanins and tannins is known as "polymeric pigments", which are primarily responsible for stable wine color and the main pigments in the aged wines (*9*). Copigmentation, the formation of complexes between anthocyanins themselves (self-association) or with other colorless cofactors, is another phenomenon that stabilizes color in young red wines (*10*–*15*). These interactions result in an increase in absorbance intensity (hyperchromism) and a positive shift in the visible wavelength (bathochromism) (*16*).

Color stability is directly related to wine quality, so the application of enological techniques that improve this factor is of major interest. The enrichment of the medium with polysaccharides could be a way to increase color stability. These compounds, described as protective colloids, are able to interact with tannins and anthocyanins in wines and reduce their reactivities and also lead to an increase in color stability (*17*–*19*). Recently mannoproteins have been shown to play a role as protective colloids by limiting self-aggregation of tannins (*20*), increasing the roundness and body of red wines (*21*, *22*) and reducing astringency and bitterness (*22*, *23*).

The addition of commercial macerating enzymes directly to the must is a common practice usually employed to facilitate the extraction of phenolic compounds during the vinification process (*24*–*26*). The enzyme preparations used contain pectolytic activities (polygalacturonase and pectin-lyase) in addition to hemicellulases, cellulases, and occasionally glycosidase activities. These preparations degrade the structural polysaccharides of grape cells and facilitate the liberation and solubilization of the phenolics bound to the cells of the skin, flesh, and seeds. Thus, the content of polyphenols would be increased since the first stages of the alcoholic fermentation, compensating the partial destruction of copigmentation anthocyanins due to the ethanol effect and temperature increase occurring during fermentation (*27*, *28*). Besides, the polymerization of tannins with anthocyanins would be favored, thereby avoiding their degradation and loss and, hence, color instability during storage of wine (*29*).

Different enological treatments are proposed in the present study in order to improve color extraction and stability during the winemaking process. The strategy adopted to achieve increases in color is the addition of maceration enzymes directly to the must and to increase the stability of the extracted color, the enrichment of the medium with protective colloids like mannoproteins. In order to do so, both yeast strains overexpressed of mannoproteins and industrial mannoproteins will be

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Table 1. Treatments Applied in the Study[a]

treatment	*S. cerevisiae* strain[b]	industrial man addition[c]	macerating enzyme addition[c]
212C: control 212	RC212	no	no
212E: 212 + enz	RC212	no	yes
212M: 212 + man	RC212	yes	no
BM45C: control BM45	BM45	no	no
BM45E: BM45 + enz	BM45	no	yes
BM45M: BM45 + man	BM45	yes	no

[a] Vinifications with each treatment were done in triplicate. [b] *S. cerevisiae* strain used for fermentation. RC212: underexpresser of polysaccharides. BM45: overexpresser of polysaccharides. [c] Addition in the prefermentative stage.

used. This paper reports the results of the strategies adopted during the vinification and aging of the Tempranillo red wines in the colloid content and color extraction and stability, as well as in the organoleptic qualities of the final wines. To the best of our knowledge, no reports of the effect of mannoproteins on wine color stability have been published.

MATERIALS AND METHODS

Reagents and Samples. All reagents were analytical grade unless otherwise stated. D-(+)-Galactose, D-(+)-glucose, and D-(+)-mannose were supplied by Sigma (St. Louis, MO), and myo-inositol and D-α-galacturonic acid were supplied by Fluka via Sigma.

Ethanol 96% v/v, acetyl chloride, sulfuric acid, sodium metabisulphite, tartaric acid, and phenol reagent were supplied by Scharlab (Barcelona, Spain), sodium hydroxide and hydrochloric acid 37% were supplied by Carlo Erba (Rodano, MI, Italy), acetaldehyde, dried methanol, and *o*-hydroxydiphenyl (biphenyl-2-ol) were supplied by Merk (Darmstadt, Germany), sodium tetraborate was supplied by Sigma, and the trimethylsilylation reagent (TriSil) was supplied by Pierce (Rockford, IL). Hexane supplied by Scharlab and Milli-Q water (Millipore, Molsheim, France) were used.

Wine samples were produced from *Vitis vinifera* Tempranillo grapes of the qualified origin denomination Rioja (D.O.Ca. Rioja). *Saccharomyces cerevisiae* var. *cerevisiae* RC212 and *Saccharomyces cerevisiae* var. *cerevisiae* BM45 were commercial yeast from Lallemand (Lallemad-Inc., Montreal, Canada). Industrial mannoproteins (Optired), maceration enzymes (Lallzyme EXV), and the bacterial strain *Oenococcus oeni* were also purchased from Lallemand.

Vinification and Sample Collection. Tempranillo grapes were sourced from Autol, La Rioja, Spain and harvested at 21.9 °Brix, pH 3.56 and 6.02 g tartaric acid/L of titratable acidity. Experimental vinifications were carried out in the experimental wine cellar of the University of La Rioja using stainless steel tanks of 100 L. Destemmed−crushed grapes were homogenized and distributed into the tanks, and 4 g/HL SO_2 was added. The different treatments were then applied, and three vinifications were made for each treatment (**Table 1**).

The first three treatments were inoculated with 25 g/HL *S. cerevisiae* yeast strain RC212 previously rehydrated. This yeast strain is a commercial active dry wine yeast selected by our laboratory because it is a strain low-expressed of polysaccharides (*17*). The rest of the deposits were inoculated with 25 g/HL *S. cerevisiae* yeast strain BM45 previously rehydrated, selected because it is a strain overexpressed of mannoproteins (*17*). Thereafter, 13.5 g/HL of industrial mannoproteins was added to tanks 212M and BM45M, and 2 g/HL of macerating enzymes was added to tanks 212E and BM45E. These enzymes, added as a liquid formulation to grapes, showed standard activities higher than 4000 uPG/g (polygalacturonase units), 1000 uPE/g (pectin-esterase units), and 120 uPL/g (pectin-lyase units) and other secondary activities such as acid protease, galacturonase, and cellulase/hemicellulase.

The prefermentation process went on for 6 h at 18 ± 1 °C; the fermentation−maceration process was carried out at a maximum temperature of 28 ± 2 °C and went on for 10 days. The postfermentative maceration went on for 4 days at 24 ± 1 °C. The cap was punched down during the maceration−fermentation and postfermentative maceration. Pumping-over extractions were also done during active fermentation. Wines were then runoff and inoculated with a commercial preparation of *O. oeni* (1 g/HL) to induce the malolactic fermentation, carried out at 18.5 ± 1 °C. After malolactic fermentation, all the wines were racked, corrected to 3.5 g/HL of free SO_2, and clarified by settling for 25 days at 10 °C. Wine aging was performed in new American oak barrels of 13 L, which are of higher area/volume than the traditional ones of 225 L. Due to this, and on the basis of the organoleptic analysis, the oak aging process went out for only 45 days. After this, wines were bottled and stored at 4 °C until analysis.

Samples were taken at the beginning of the prefermentative maceration (PM), during the alcoholic maceration−fermentation (beginning, 25−30% of sugars consumed, and 55−60% of sugars consumed, namely, 0AF, 30AF and 60AF, respectively), and at the beginning of the malolactic fermentation (BMF). Sample bottles were filled completely to minimize oxygen contact and immediately frozen at −18 °C. Samples were also taken at the beginning and end of wine aging (BA, EA).

Determination of Usual Enological Parameters. L-Malic acid was determined by an enzymatic method according to AOAC official methods of analysis (*30*). Conventional enological parameters (density, ethanol concentration, pH values, reducing sugars, titratable and volatile acidities) were determined according to official OIV practices (*31*). Color intensity (CI) was calculated as the sum of absorbances at 420, 520, and 620 nm, and the hue of wine was calculated as the ratio of absorbance at 420 and absorbance at 520 nm. These values were determined at wine pH (*31*). All these measurements were done in triplicate prior to freezing or cooling.

Microbiological Analysis. To ascertain the dominance of the inoculated strains, isolates were selected at random and subjected to molecular biology techniques (PCR and EPC) as described by Ness et al. (*32*). Samples were taken at the middle and final stages of alcoholic and malolactic fermentation. Analyses were carried out in the Laboratory of Microorganism Genetic Identification (Sigmo Vertou, France).

Exocellular Polysaccharide Production in a Synthetic Medium. After rehydration yeast were added to a synthetic medium without polysaccharides as described by Escot et al. (*17*) in order to obtain the polysaccharides released by the strains employed. Yeast was grown with stirring at 28 °C.

Isolation of Polysaccharides. Polysaccharides were isolated from the synthetic medium at the end of alcoholic fermentation. Samples were homogenized, and 5 mL was taken and centrifuged (9500*g*, 20 min, 4 °C). Polysaccharides were precipitated by addition of four volumes of cold ethanol containing 0.3 M HCl and kept 18−20 h at 4 °C (*33*). Thereafter, the samples were centrifuged (9000*g*, 20 min, 4 °C), the supernatants were discarded, and the pellets were washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried.

For the isolation of must and wine polysaccharides, samples were homogenized, and 50 mL was taken with a peristaltic pump and centrifuged (9500*g*, 20 min, 4 °C). The supernatants were then concentrated (five times for wines and three times for musts) under reduced pressure at 34 °C. Polysaccharides were precipitated by addition of four volumes of cold ethanol containing 0.3 M HCl and kept 18−20 h at 4 °C (*33*). Thereafter, the samples were centrifuged (9000*g*, 20 min, 4 °C), the supernatants were discarded, and the pellets were washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried.

Polysaccharide concentration was determined by a colorimetric method, and monosaccharide composition was determined by gas liquid chromatography as further described. Protein concentration was determined by the procedure of Lowry et al. (*34*).

Quantification of Polysaccharide Concentration. Soluble polysaccharides were first isolated as described previously and dissolved in water. The *o*-hydroxydiphenyl and the phenol methods were followed as described by Segarra et al. (*35*), but instead of 5% (w/v) phenol reagent, a solution of 3% was used. This concentration was found to be optimal to determine the 100% of the neutral polysaccharides (NPS) present in the sample but only 50% of the acid polysaccharides (APS), which were determined by the *o*-hydroxydiphenyl method. NPS concentration was calculated using a galactose calibration curve (r = 0.999 for the range 0−100 mg/L), whereas a galacturonic acid

calibration curve (r = 0.999 for the range 0−100 mg/L) was used for APS. All the analyses were conducted in triplicate on samples after polysaccharide isolation.

Determination of Monosaccharide Composition by GC. Sugars were determined in the polysaccharide extracts isolated from the synthetic medium by gas−liquid chromatography of their trimethylsilyl-ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization (*33*). The composition of the commercial mannoproteins used was also determined by this procedure. All analyses were performed in triplicate.

The GC system consisted of an HP5890 Series II gas chromatograph (Hewlett-Packard, U.S.A.) coupled to a FID. The GC system was equipped with a capillary split/splitless inlet and a fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Tecnokroma). The carrier gas was helium at a flow rate of 1.3 mL/min. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector and the FID were operated at 250 °C. The chromatograph was operated with temperature programming (120−145 °C at 1 °C/min, 145−180 at 0.9 °C/min, and 180−230 at 50 °C/min).

Color Composition and Total Polyphenol Index Measurements. The spectrophotometric measurements were performed on a Cary 300 Scan UV−vis spectrophotometer (Varian Inc., Madrid, Spain) using 2 mm and 10 mm path length quartz cells. All the samples were analyzed in triplicate, and all absorbance values were corrected to 10 mm path length.

Wine red color (WC), copigmentation color (CC), and bisulfite-stable color (BSC) were determined by the method proposed by Levengood and Boulton (*36*). Wine samples were adjusted to pH 3.6 to eliminate color differences due to pH and ultra centrifuged at 20 000*g* for 10 min. Wine color (WC) was measured by the addition of 20 μL of 10% acetaldehyde to a 2 mL of wine sample. The sample was kept for 45 min and measured at 520 nm in a 2 mm path length cell. Bisulfite-stable color (BSC), the result of the addition of 160 μL of a 5% SO_2 solution to 2 mL of wine sample, was measured at 520 nm after 50 min in a 2 mm path length cell. Copigmentation was measured by comparing diluted and undiluted samples. Samples were diluted 20-fold with synthetic wine (12% alcohol, 5 g/L of tartaric acid in water, pH 3.6) and measured at 520 nm after 20 min in a 10 mm path length cell ($A_{diluted}$). The CC is the difference between the WC value and the $A_{diluted}$ value.

The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine (1/20, v/v). The diluted samples used to measure the value of $A_{diluted}$ were also used to determine this parameter. Spectrophotometric measurements were made using 10 mm path length cells, and absorbance values were maintained along the time of reaction.

Sensory Analysis. Aged wines were analyzed by a panel of expert tasters for sensory profiling. Wines were judged on three levels: visual (color), olfactory (volatile fraction), and gustatory (taste and mouth-feel sensations).

A panel of six tasters (three males and three females), wine professionals from the D.O.Ca. Rioja, was convened. All wine tasters had participated on previous aroma and mouth-feel sensory descriptive panels and had regularly participated in quality scoring Tempranillo wine sensory panels. The wines were presented at 18 °C in coded standard wine-tasting glasses according to standard 3591 (ISO 3591, 1997). Assessment took place in a standard sensory analysis chamber (ISO 8589, 1998) equipped with separate booths. Wines were presented in two stages. In a first session, the panelists were asked to describe the olfactory and gustative attributes in their own words. Descriptive terms and their definitions were debated among the assessors, and a common consensus vocabulary was then compiled and discussed further with panelists. Tasters selected 12 attributes for the olfactory phase and 10 for the gustative, which were agreed upon as best for describing the sensory characteristics of the wines. All the generated terms were usual wine-tasting terms for describing red wines. In following sessions, assessors used the consensus vocabulary, scoring the intensity of each attribute on an interval scale with 10 levels of intensity (0 = no aroma or no taste; 1 = weak aroma or weak taste; 5 = intense aroma or intense taste; 9 = strong aroma or strong taste; intermediate values did not bear description). The color was also judged, and blue-red color was rated according to its intensity on an anchored scale with four levels of intensity (0 = no blue-red color; 1 = weak blue-red color; 2 = intense blue-red color; 3 = extremely strong blue-red color). Wine samples were assessed in triplicate.

Statistical Procedures. All the treatments were vinificated in triplicate, and all the analysis were performed in triplicate. Significant differences between samples were performed with the SPSS 12.0 program for Microsoft Windows (SPSS Inc., Chicago, IL). The values of color composition, TPI, and polysaccharide content were analyzed by a two-way analysis of variance (ANOVA) with repeated measurements in one factor, to test the effects of the treatments and vinification stage (repeated measurements), if the data met assumptions of normality. If this assumption was not satisfied, a Kruskal−Wallis test was used for each factor. The Excel program for Microsoft Windows was used, when necessary, to calculate the P value and the contrast statistical value by means of the conventional hypothesis tests. In the present paper, always we refer to differences between samples, either with different time or treatment; we will be referring to significant differences with at least $p < 0.05$.

Sensory data were subjected to ANOVA using the SPSS 12.0 program to determine reproducibility of attribute scores. Separate principal component analyses (PCA) were carried out on the mean ratings for olfactory and for gustatory attributes. The PCA was conducted using the covariance matrix with no rotation (XLSTAT 2007 program for Microsoft Windows). Average configuration plot dimensions were interpreted taking into account the descriptors used by each of the assessors, which were most highly correlated with each dimension. Dimension 1 explained 45% (aroma) and 56% (mouth), and dimension 2 explained 28% (aroma) and 31% (mouth) of the original variance. In this study, there were no significant differences in the scores given by the tasters for each attribute ($p < 0.05$), indicating that all the panelists used all attributes reproducibly.

RESULTS AND DISCUSSION

Microvinifications and Microbiological Analysis. Eighteen microvinifications were carried out as detailed previously. All alcoholic fermentations finished within 10 days, yielding final reducing sugar contents of less than 2 g/L. No differences in the fermentative progress were detected. Genetic analysis showed that the yeast strains *S. cerevisiae BM45 and RC212* were successfully implanted in all the vinifications (data not shown).

The bacterial strain *O. oeni* was also successfully implanted in all the malolactic fermentations (data not shown). This fermentation was finished when the L-malic acid concentration was below 0.1 g/L, and the required time was different depending on the yeast strain inoculated in the alcoholic fermentation. Thus, the malolactic fermentation went on for 20 days in 212 wines, whereas it took longer in BM45 wines, between 18 and 30 days more. This fact does not agree with bibliography as liberated mannoproteins are described as stimulators of malolactic fermentation (*22*).

Conventional Enological Analysis in Wines. Values of pH, titratable acidity, ethanol concentration, and volatile acidity were in the range usually found in wines of Tempranillo variety (**Table 2**). The values obtained for pH, titratable acidity, and volatile acidity confirmed the absence of microbial alterations. Analyses performed on the different wines were in accordance with a good conservation of wines during oak aging. As expected, the aging was accompanied by a decrease in color intensity and an increase in hue (**Table 2**).

The ethanol production was similar in all tests (12.3−12.7% v/v) regardless of enzyme addition, showing that the amount of enzymes added did not affect the cell wall stability. On the other hand, titratable and volatile acidity and pH were not modified by grape enzyme treatment. These results confirmed the results of Lao et al. (*37*), who report that the use of pectic enzymes does not modify these parameters. Mannoproteins and

Table 2. Conventional Enological Parameters of Wines[a]

stage[b]	wine	% v/v[c]	pH	TA[d]	VA[e]	CI[f]	hue[g]
BMF	212C	12.6 ± 0.2	3.79 ± 0.007	5.2 ± 0.1	0.23 ± 0.05	6.79 ± 0.03	0.53
	212E	12.5 ± 0.3	3.75 ± 0.002	5.36 ± 0.05	0.19 ± 0.07	7.316 ± 0.002	0.49
	212M	12.6 ± 0.3	3.81 ± 0.01	5.6 ± 0.3	0.2 ± 0.1	6.099 ± 0.005	0.59
	BM45C	12.4 ± 0.2	3.74 ± 0.01	5.59 ± 0.05	0.22 ± 0.06	6.96 ± 0.01	0.58
	BM45E	12.6 ± 0.2	3.76 ± 0.007	5.87 ± 0.03	0.18 ± 0.05	7.491 ± 0.008	0.50
	BM45M	12.4 ± 0.2	3.81 ± 0.02	5.7 ± 0.1	0.2 ± 0.03	6.556 ± 0.001	
							0.54
BA	212C	12.5 ± 0.2	3.97 ± 0.02	3.71 ± 0.05	0.29 ± 0.05	5.82 ± 0.01	0.59
	212E	12.5 ± 0.3	3.91 ± 0.02	3.94 ± 0.03	0.30 ± 0.08	6.195 ± 0.02	0.50
	212M	12.5 ± 0.3	3.94 ± 0.007	3.86 ± 0.05	0.31 ± 0.02	5.478 ± 0.004	0.60
	BM45C	12.5 ± 0.2	3.92 ± 0.007	4.03 ± 0.03	0.37 ± 0.08	5.679 ± 0.005	0.57
	BM45E	12.7 ± 0.2	3.96 ± 0.007	3.96 ± 0.08	0.4 ± 0.02	6.453 ± 0.006	0.49
	BM45M	12.3 ± 0.2	3.93 ± 0.002	4.24 ± 0.05	0.35 ± 0.03	5.061 ± 0.003	
							0.53
EA	212C	12.6 ± 0.2	3.98 ± 0.01	3.98 ± 0.05	0.33 ± 0.5	5.008 ± 0.004	0.62
	212E	12.3 ± 0.3	3.91 ± 0.01	3.9 ± 0.05	0.34 ± 0.01	5.756 ± 0.003	0.55
	212M	12.7 ± 0.3	3.95 ± 0.01	3.79 ± 0.05	0.34 ± 0.03	4.891 ± 0.005	0.68
	BM45C	12.6 ± 0.2	3.94 ± 0.005	3.92 ± 0.03	0.39 ± 0.08	5.372 ± 0.001	0.60
	BM45E	12.4 ± 0.2	3.98 ± 0.01	4.22 ± 0.03	0.42 ± 0.06	5.732 ± 0.009	0.55
	BM45M	12.6 ± 0.2	3.93 ± 0.01	3.77 ± 0.08	0.42 ± 0.08	4.991 ± 0.002	0.58

[a] Mean ± SD (n = 3). [b] Vinification stage: BMF, beginning of the malolactic fermentation; BA, beginning of the oak aging; EA, end of the oak aging. [c] Milliliters of ethanol for 100 mL of wines at 20 °C. [d] Titratable acidity as g of tartaric acid per L. [e] Volatile acidity as g of acetic acid per L. [f] Color intensity as sum of absorbances at 420, 520, and 620 nm. [g] A_{420nm}/A_{520nm}.

yeast neither modified the ethanol concentration nor pH in the wines. Wine pH increased 0.1−0.2 units at the end of the malolactic fermentation in all the cases, and titratable acidity decreased by 25−31%. After malolactic fermentation, BM45 wines presented higher values of volatile acidity than 212 wines, which coincided with the longer duration of the malolactic fermentation.

The addition of enzymes increased color intensity values after both fermentations. Parley et al. (*38*) also observed that Pinot Noir wines produced with macerating enzymes presented higher color intensities at the end of the alcoholic fermentation. Enzyme-treated wines had the highest values of color intensity at the end of the aging process. However, the addition of mannoproteins had the opposite effect, and the color intensity of wines 212M and BM45M was significantly lower than in the rest of wines at the end of the aging process.

Evolution of the Polysaccharide and Polyphenol Content and Color Composition during the Different Vinification Stages. Polysaccharides were analyzed during the maceration−fermentation, postmaceration, malolactic fermentation, and oak aging. **Figures 1** and **2** show the concentration of NPS and APS in all the musts and wines studied. The ANOVA analysis verified that the polysaccharide content depended significantly on the time when the sample was taken and enological treatment applied. In all the cases, the NPS content was higher than that of APS, the APS being only a small percentage of total polysaccharides (4−18%), in agreement with what was observed by other authors (*35*). The values found ranged from 421 to 1026 mg/L for NPS and from 31 to 139 mg/L for APS. These values were in the range described in other studies for red varieties (*33*, *39*).

Red winemaking increased the polysaccharide content, the maceration−fermentation being the main process affecting this content (**Figures 1** and **2**). Concentration of total polysaccharides (NPS + APS) increased progressively by 91−142% between zero time and 4 days (60AF) reaching 871−1117 mg/L. NPS were doubled during this period, and APS, which were liberated later (between 30 and 60AF), were tripled or quadrupled. The evolution of polysaccharides could be interpreted, in addition to the liberation of yeast mannoproteins during fermentation,

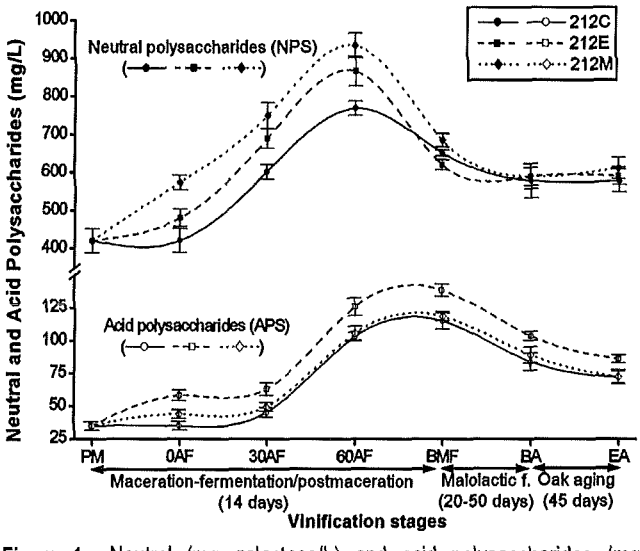

Figure 1. Neutral (mg galactose/L) and acid polysaccharides (mg galacturonic acid/L) of the samples vinificated with the yeast strain *S. cerevisiae* RC212. See text for conditions and calculations.

as the progressive release of carbohydrate polymers from skin and pulp cell walls. This interpretation is well sustained by the fact that in white wines (no pomace contact during fermentation), the fermentation process decreases the polysaccharide content (*35*, *37*). In all the cases, the content of NPS decreased substantially (15−28%) at the end of the maceration−fermentation process and during postmaceration, while the APS content remained constant or slightly increased. This different behavior, previously observed by our workgroup (*33*), could be due to the fact that APS are smaller molecules than NPS and their precipitation due to the ethanol formed during the fermentation process (*18*, *33*) is later than in the case of NPS. Both APS and NPS decreased again during the malolactic fermentation. After this stage, wine stopped giving off CO_2 and the temperature changed from 18.5 ± 1 to 15 ± 1 °C. Both effects would produce the precipitation of all the unstable material that was suspended while the temperature was high enough and there were convection currents formed by the fermentative processes. During the oak aging, the polysaccharide values were maintained

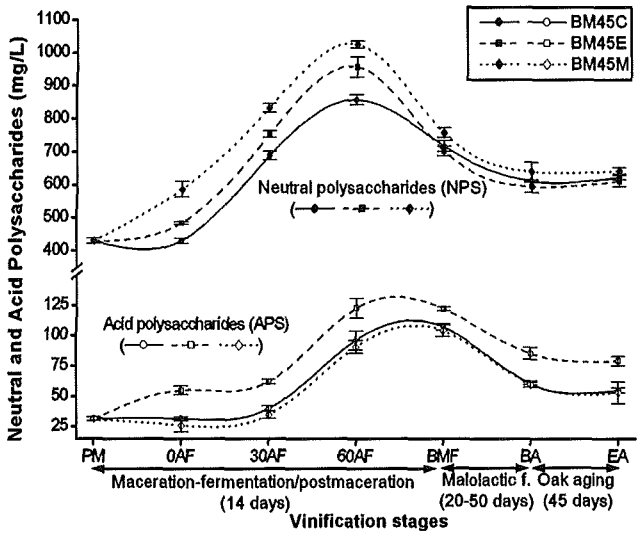

Figure 2. Neutral (mg galactose/L) and acid polysaccharides (mg galacturonic acid/L) of the samples vinificated with the yeast strain *S. cerevisiae* BM45. See text for conditions and calculations.

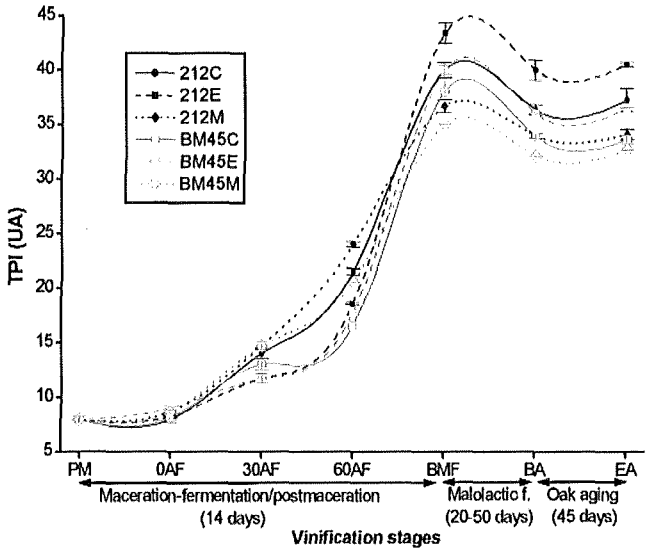

Figure 3. Total polyphenol index (TPI) of the must and wine samples.

as they would be low molecular weight molecules and, thus, maintained in solution.

Figure 3 shows the evolution of the TPI of the samples during the different vinification stages. **Figure 4a–c** shows, respectively, the evolution of WC, CC, and BSC, of the samples during the different stages. Bisulfite-stable compounds are usually termed "polymeric pigments", which is erroneous as they include compounds that are not polymeric in nature (i.e., pyranoanthocyanins), and not all anthocyanin-derived polymers are colored or bisulfite-stable pigments (i.e., the so-called T-A polymeric pigments). However, the practice of measuring BSC continues to be very useful (*40*). As in the case of polysaccharides, red winemaking increased the value of TPI, WC, CC, and BSC, the maceration being the main process affecting these values. The values of TPI, WC, BSC, and CC increased significantly in all the samples during maceration–fermentation and postmaceration, being more accused in WC (1890–2408%) and CC (1245–1918%) than in TPI and BSC (305–405% and 600–966%, respectively). Spranger et al. (*41*) observed that the evolution of the TPI during these stages is positively correlated with the content of tannins or condensed proanthocyanidins, which are major wine polymeric compounds. As in

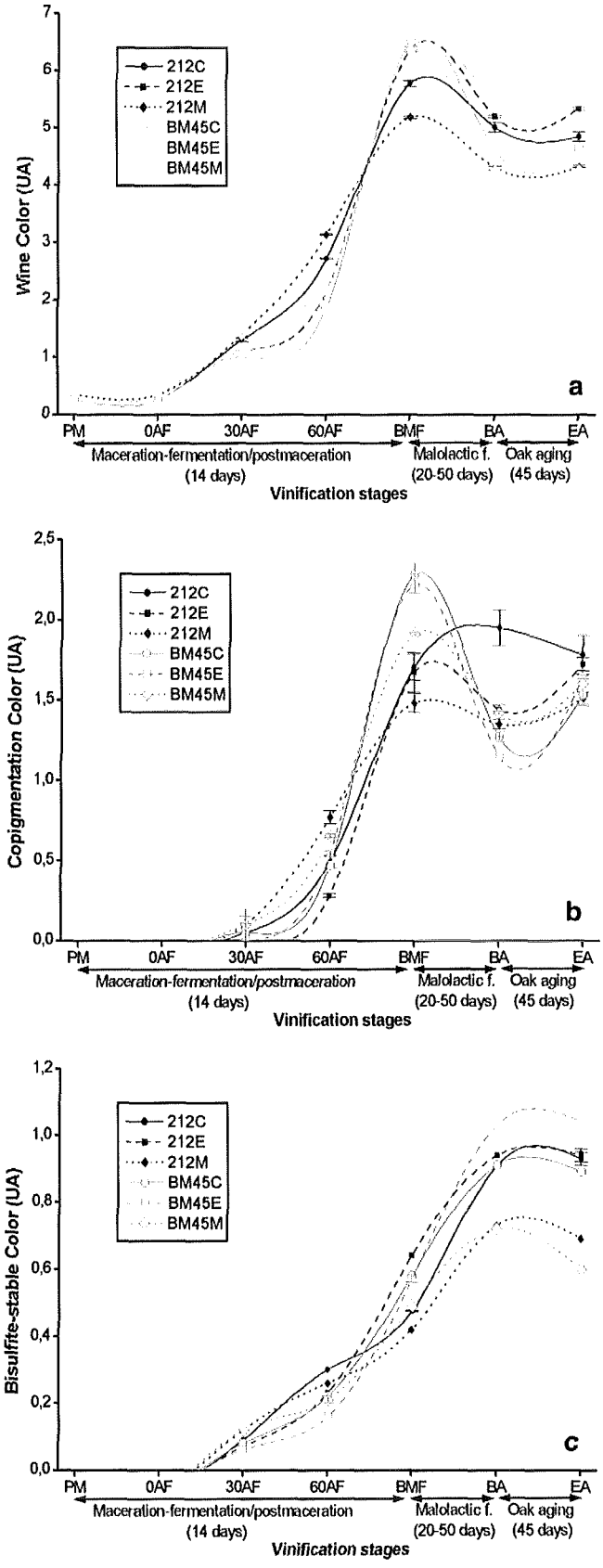

Figure 4. (a) Wine color (WC), (b) copigmentation color (CC), and (c) bisulfite-stable color (BSC) of the must and wine samples. See text for conditions and calculations.

the case of polysaccharides, the malolactic fermentation resulted in a considerable decrease in the content of total polyphenols (7.5–15%), WC (13–33.2%), and CC (5.8–48.2%), except in

the wine 212C, with an increase of 14%. However, the BSC increased significantly by 57% and 91.6% in all the wines. It is widely known that both malolactic fermentation and wine aging produce important loss of color due to the precipitation of the unstable colloidal material (*42*). However, new and stable pigments are also formed during these periods, with either high or low molecular weights. These new pigments are described to be resistant, not only to bisulfite addition, but also to the formation of the hemiketal through pH changes, and resistant to oxidation (*43*). Although polyphenols from the wood are extracted during wine aging, TPI values obtained before and after wine aging were not significantly different, indicating that polyphenol extraction was minimum during the short aging carried out. The values of WC and BSC were also maintained in this period, but CC increased by 11.8−42.6% in all the wines except for 212C, which had a decrease of 8.7%. Other authors point out that copigmentation in older red wines is negligible as this phenomenon has only been demonstrated to occur with monomeric anthocyanins (*40*). This fact would explain the decrease in CC occurring in the control wine 212C; the increase in this parameter in the rest of the wines could be due to the enological treatments applied.

Effect of Macerating Enzymes on the Polysaccharide and Polyphenol Content and Color Composition. Polysaccharide concentration was different in enzyme-treated samples and in controls, both in 212 and BM45 (**Figures 1** and **2**). From the beginning of the maceration−fermentation to 60% of sugars consumed (60AF), wines treated with commercial enzymes (212E and BM45E) had larger concentrations of NPS (10−14%) and APS (32−74%) than control wines. These findings indicated that solubilization of polysaccharides had occurred during the maceration−fermentation process due to the action of the commercial enzymes added. This solubilization affected to a greater extent to APS and therefore parietal polysaccharides, such as homogalacturonans and rhamnogalacturonans (RGs). Commercial enzymes have been traditionally used in wine technology in order to produce higher modifications in grape compounds than those produced by endogenous pectinases. As in the present study, Ducruet et al. (*44*) observed that the addition of commercial enzymes to must produced an increase in the amount of total acid (49%) and NPS (5%). Ayestarán et al. (*33*) also observed that after the maceration−fermentation process, wines treated with enzymes had larger concentrations of soluble monosaccharides, arabinogalactans, arabinogalactan proteins, and RG-IIs than control wines. However, very few reports on the evolution of these polysaccharides after the maceration−fermentation are available. As can be observed in **Figures 1** and **2**, most of the NPS liberated by macerating enzymes during the first stages of maceration−fermentation precipitated at the end of this period and during postmaceration (27−28%). Therefore, the polysaccharide content was similar in both control and enzyme-treated wines at the beginning of the malolactic fermentation. This precipitation phenomenon did not occur in the case of APS, and the differences were maintained, the enzyme-treated wines being the ones with the highest concentration of APS during all the vinification process and aging.

Commercial macerating enzymes enhance the degradation of structural polysaccharides of grape skin cell walls during the maceration−fermentation process, thus enhancing the extraction of grape phenolics (*24*, *25*). Therefore, the TPI was higher in enzyme-treated wines than in controls at the end of maceration−fermentation, 7.8% in 212E and 5% in BM45E (**Figure 3**). The value of WC was also higher in the 212E wine in comparison with the control one (10.4%) (**Figure 4a**). Enzyme-treated wines presented also the highest value of color intensity and WC both before and after wine aging (**Figure 4a** and **Table 2**). The BSC was also higher in BM45E and 212E wines before aging, but this difference was not maintained in 212E wines after wine aging (**Figure 4c**).

Table 3. Composition of Mannoproteins Produced by Lallemand and Macromolecules Released in Synthetic Medium by Yeast at the End of the Alcoholic Fermentation

	mannoproteins from Lallemand	yeast strain	
		RC212	BM45
proteins (%) (nitrogen)[a]	2.1	10.9	11.8
polysaccharides (%)[b]	72.8	81.1	78.1
mannose (%)[c]	90.6	71	55
glucose (%)[c]	9.4	29	45

[a] Lowry method. [b] Colorimetric method. [c] Capillary GC.

Effect of Industrial Mannoproteins on the Polysaccharide and Polyphenol Content and Color Composition. Commercial mannoproteins were added to samples in the prefermentative stage. The commercial preparation of mannoproteins consisted of 73% polysaccharides and 2% proteins (**Table 3**). Mannose was the main sugar (91%) with glucose far behind (9%). The remaining 25% of the preparation was probably represented by other cellular components isolated during the extraction procedure.

From the beginning of the alcoholic fermentation, mannoprotein-added samples (212M and BM45M) had higher values of NPS than their respective controls (144−168 mg/L), but the content of APS was obviously similar (**Figures 1** and **2**). This fact indicated that the mannoproteins added in the prefermentative stage were maintained soluble during this period and did not precipitate. However, these differences were minimized due to the polysaccharide decrease occurring at the end of the maceration−fermentation, which was more pronounced in the samples 212M and BM45M, which reached the highest values of total polysaccharides during this period. Therefore, the polysaccharide values tended to be similar at the beginning of the malolactic fermentation in all the wines although there were still significant differences between the samples with mannoprotein addition and the controls.

Mannoprotein-added wines presented the lowest values of the TPI at the end of the maceration process (**Figure 3**), which could be a consequence of a higher extent of polyphenol precipitation due to the formation of extra mannoprotein−polyphenol colloidal complexes. These results do not agree with what is reported in bibliography, where polysaccharides are described to function as protective colloids that slow or prevent self-aggregation of tannins in synthetic media (*20*, *45*). However, the complex polysaccharide−polyphenol could be unstable and precipitate as described by Siebert et al. (*46*, *47*), who observed that certain polysaccharides interact with gliadin and catechin and precipitate. During malolactic fermentation, polyphenolic compounds precipitated in all the samples, being more important in the control wines (10% in 212C and 15% in BM45C) than in 212M and BM45M wines (~7.5%). These data suggested that the precipitation of the complex "added mannoprotein−polyphenol" occurred mainly at the end of maceration−fermentation and during postmaceration, coinciding with the higher precipitation of the NPS. Wines produced with commercial mannoproteins presented the lowest levels of TPI at the end of wine aging.

In regard to color, samples 212M and BM45M showed lower values of WC, CC, and BSC than controls at the end of

maceration–fermentation (**Figure 4a–c**). WC and BSC differences increased during malolactic fermentation and aging, and these wines presented the lowest levels of these parameters both at the beginning and end of aging. Moreover, wines produced with mannoprotein addition had the lowest values of color intensity (**Table 2**). These results question the strategy of using polysaccharides as "color protection molecules", a hypothesis mentioned in general bibliography and research papers but not properly contrasted and studied. However, it is important to highlight that wine polysaccharides are very different both in origin and composition and thus in physical and chemical properties; the results of the present paper are referred to the effect of the mannoproteins previously described (**Table 3**).

Effect of Yeast Mannoproteins on the Polysaccharide and Polyphenol Content and Color Composition. Vinifications were also affected by the yeast strain selected, RC212 or BM45. Both strains differed in their capacity to secrete polysaccharides in the growth synthetic medium. The results showed that the yeast strain BM45 released twice the polysaccharides released by the strain RC212 at the end of the alcoholic fermentation (118 vs 62 mg/L). The analysis of macromolecules released by both strains revealed that they were polysaccharides (~80%) and some protein (~11%) (**Table 3**). Of the sugars present in the polysaccharidic fraction, mannose was the prevalent one followed, at some distance, by glucose (**Table 3**). This composition confirmed not only the parietal nature of the polysaccharidic fraction but also that the polysaccharides released into the medium were essentially mannoproteins with a low proteic fraction. The distribution of neutral sugars was different for both strains. For RC212, mannose was the main component and glucose represented only 29%. In macromolecules from strain BM45, mannose and glucose were present in the same proportion. The greater proportion of glucose in BM45 macromolecules has also been observed by other authors (*17*) and may be due to a different composition of its cell wall.

Polysaccharide content was higher in BM45 than in 212 samples (**Figures 1** and **2**). In both cases, polysaccharides were continuously released from yeast during the first stages of maceration–fermentation, coinciding with the yeast exponential phase of growth. From the beginning of the winemaking process (30AF), the amount of NPS was significantly higher (10–14%) in the samples vinificated with the overexpresser yeast strain in comparison with their respective controls. This difference was attributed to the mannoproteins released by the overexpresser yeast strain and was quite similar to that found by other authors when analyzing the same yeast strain (*17*). Although these differences were reduced at the end of maceration–fermentation, there were still significant differences between BM45 and 212 samples at the beginning of malolactic fermentation. In this stage, wines produced with the strain BM45 had higher levels of NPS than those produced with the strain RC212 (10–11% more). The NPS content of wines BM45 and 212 was not significantly different after malolactic fermentation, indicating that the precipitation of these compounds was greater in wines made with the BM45 strain. In relation with APS, the yeast strain also influenced on their release but with a contrary effect. The content of APS was always equal or even higher in the wines made with the RC212 strain.

As in the case of industrial mannoproteins, the use of a strain overexpresser of mannoproteins produced wines with lower values of the TPI than 212 wines at the end of maceration–fermentation (**Figure 3**). This fact confirmed the observation previously done about the precipitation of the complex mannoprotein–polyphenol, although in this case the yeast mannoprotein is the one forming the unstable complex and not the commercial one. The precipitation of polyphenols occurring during malolactic fermentation was more accused in BM45C and BM45E wines (15% and 11%, respectively) than in 212C and 212E wines (10 and 8%, respectively) and 212M and BM45M (~7.5%). These data suggested that the precipitation of the complex yeast mannoprotein–polyphenol began at the end of maceration–fermentation and postmaceration but went on in a marked way during malolactic fermentation, coinciding again with the higher precipitation of NPS occurring in the BM45 wines. Therefore, the precipitation of the unstable complex yeast mannoprotein–polyphenol occurred later than in the case of "industrial mannoprotein–polyphenol". This different behavior observed with both mannoproteins could be due not only to their different liberation moment but also to their different structure composition and thus reactivity (**Table 3**). During wine aging, BM45 wines presented lower values of TPIs than 212 wines, and among them, the one with enzyme addition had the highest value of this parameter during all the vinification stages (**Figure 3**). This indicated that the higher content of phenolics in wines due to the enzymatic treatment counteracted the effect of the precipitation of the complex yeast mannoprotein–polyphenol.

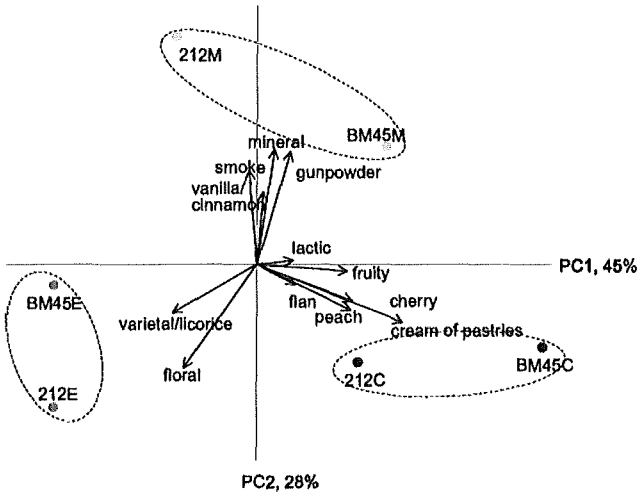

Figure 5. Biplot of principal components I and II of the olfactory attributes of the aged wines.

In relation to color, wines BM45 presented higher values of WC than 212 at the end of maceration–fermentation (13% in BM45C and 29.8% in BM45M) (**Figure 4a**), and the value of the copigmentation color (CC) was also higher (34%, 22.5%, and 24.7% in BM45C, BM45M, and BM45E) (**Figure 4b**). Note the different behavior of wines made with addition of industrial mannoproteins, which had lower values of WC, CC, and BSC than their respective controls. At the end of aging there were not clear differences in color composition parameters between wines produced with each of the strains.

Sensory Analysis of the Wines. Sensory evaluations of the aged wines were performed to verify the differences observed between wines on the organoleptic perception.

In the visual phase, enzyme-treated wines showed enhanced color intensity, obtaining mean punctuations between 2 and 3. Wines with mannoproteins, both commercial and from the yeast, had a color intensity similar to controls and even weaker, with mean punctuations close to 1.5. **Figures 5** and **6** provide a graphic representation of the relationship of the wines as determined by their olfactory and gustatory perceptions. Wines were properly located in the vectorial dimension defined by the first two factors, which accounted for 73% of the total variance

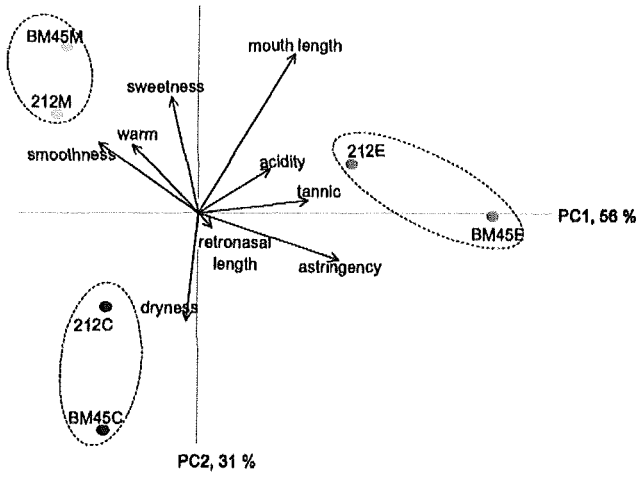

Figure 6. Biplot of principal components I and II of the gustatory attributes of the aged wines.

in the olfactory PCA space and for 87% in the gustatory PCA space (**Figures 5** and **6**, respectively). As can be observed, the technology applied in the wine making process (enzyme or industrial mannoprotein addition) was the most significant effect, being much more evident than the differences found with the different yeast strains. In the olfactory phase (**Figure 5**), control wines were highly related with the sweet and fruity descriptors. Enzyme-treated wines had high floral and varietal characters, with licorice aromas, which are characteristic of the Tempranillo variety (*48*). Oak aging and smoke and mineral aromas were predominant in the wines made with addition of industrial mannoproteins. This could be due to the fact that mannoproteins have been found to interact and reduce the volatility of fruity, floral, and green aromas (i.e., ethyl hexanoate, β-ionone, and hexanol) at the concentrations that these macromolecules occur in wine (*49*), which would cause an enhancement in the perception of the rest of the aromas. In the mouth (**Figure 6**), wines with mannoprotein addition obtained the highest scores in sweet perception and roundness sensation. The enzyme addition had a strong effect on the mouth-feel and length, enhancing acidity, tannin, astringency, and length. Control wines were described as quite dry.

All the data obtained in the wine tasting were in good agreement with the analytical data found in the present paper. The enzymatic treatment produced the wines with the strongest visual color intensity, tannin, and astringency, coinciding with the highest values of color intensity, WC, and TPI. The use of industrial mannoproteins produced a considerable decrease in the TPI, related with the roundness and low astringency of these wines, and did not maintain the stable color, which also was in good agreement with the sensory data.

Taking into account these findings, the combination of both industrial mannoproteins and maceration enzymes could be considered to be of potential application in red wine maceration process in order to improve the sensory characteristics of the wines. However, a more detailed analytical study should be performed in order to confirm and better understand the differences observed between treatments. Further investigations will examine the effect of these treatments on wine polysaccharide and polyphenolic composition.

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EFECTO DE LA ADICIÓN DE MANOPROTEÍNAS COMERCIALES EN LA COMPOSICIÓN DE DE POLISACÁRIDOS, POLIFENOLES Y COLOR DE VINOS TINTOS

Effect of Comercial Mannoprotein Addition on Polysaccharide, Polyphenolic and Color Composition in Red Wines:

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Resumen

La adición directa de manoproteínas purificadas industrialmente en la etapa prefermentativa de la vinificación en tinto se planteó como posible tratamiento biotecnológico para mejorar la estabilización de la materia colorante extraída durante el proceso de vinificación. Contrariamente a lo que esperábamos, dicho tratamiento no parecía estabilizar el color de los vinos ni mantener a los polifenoles extraídos en disolución (ver *apartado 3.6.*), por lo que decidimos realizar un estudio más detallado de dichos efectos. Por ello, este artículo analiza la influencia de la adición de manoproteínas comerciales en el contenido y la evolución de las diferentes familias de polisacáridos y de compuestos fenólicos durante el proceso de vinificación de vinos tintos de variedad Tempranillo, así como su repercusión en el color estable.

Los resultados obtenidos del análisis de las diferentes familias de polisacáridos indicaron que más del 75% de las manoproteínas adicionadas se mantuvieron solubles y no precipitaron tras su adición en la etapa prefermentativa. Así, la adición de estos preparados no modificó el contenido de manoproteínas de bajo peso molecular, pero incrementó en más de 100 mg/L el contenido de manoproteínas de alto peso molecular (bMP) respecto a las muestras control, indicando además que las manoproteínas adicionadas eran compuestos de elevado tamaño molecular (50-400 kD). A pesar de que la maceración-fermentación produjo la precipitación del 60% de las manoproteínas adicionadas, los vinos tratados, tanto los jóvenes como los envejecidos, presentaron un contenido significativamente más elevado de bMP. Como era de esperar, la adición de manoproteínas no produjo ninguna modificación en el contenido y la evolución de los polisacáridos pécticos procedentes de la uva: arabinogalactanos, ramnogalacturonanos tipo II y homogalacturonanos. En ambas vinificaciones, la evolución de dichos polisacáridos durante la vinificación y el envejecimiento de los vinos fue similar a la observada por nuestro grupo en estudios anteriores (ver *apartado 3.3.*).

Los resultados obtenidos del análisis de polifenoles indicaron que la adición de manoproteínas no afectaba al contenido de los diferentes polifenoles monómeros, antocianos, flavan 3-oles o ácidos hidroxicinámicos. Por el contrario, dicho tratamiento sí que tuvo una gran influencia en el contenido de proantocianidinas así como en el color estable de los vinos.

En relación a los fenoles monómeros, la única diferencia observada fue que las muestras elaboradas con manoproteínas presentaron un ligero retraso en la extracción de las diferentes formas de antocianos monómeros y ácidos hidroxicinámicos durante la maceración-fermentación. Asimismo, dichas muestras presentaron una reducción del contenido de los trans-hidroxicinamatos durante la fermentación maloláctica debido a la hidrólisis de sus ésteres tartáricos.

En cuanto a los polifenoles poliméricos, la vinificación con manoproteínas industriales produjo un descenso acusado en el contenido de taninos. De hecho, los vinos controles presentaron un contenido de proantocianidinas 1,5 veces superior al de los vinos elaborados con manoproteínas desde el final de la maceración-fermentación, y estas diferencias se mantuvieron durante todas las etapas posteriores. Este hecho, que confirmaba lo observado mediante la medida del índice de polifenoles totales, cuestionaba lo expuesto en la bibliografía, donde las manoproteínas se describen como coloides protectores estabilizadores de los taninos e inhibidores de su auto-agregación. Contrariamente a esta teoría, nuestros resultados indicaban que la presencia de manoproteínas en el medio desestabilizaba a las partículas de tanino, provocando su precipitación y dando lugar a una reducción sustancial de su contenido. Se plantearon dos posibles hipótesis para explicar este mecanismo. Por un lado, la combinación manoproteína-tanino formaría estructuras de alto peso molecular que serían inestables y precipitarían. Por otro, las manoproteínas podrían actuar como agentes floculantes en vez de estabilizadores, interaccionando con diferentes partículas de tanino y produciendo su precipitación. Así, e independientemente del mecanismo implicado, la co-agregación entre las manoproteínas y las partículas de tanino, explicaría el mayor grado de precipitación de bMP observado en las muestras con manoproteínas al final de la maceración-fermentación.

De forma análoga a lo observado en el caso de los taninos, y también contradiciendo lo descrito en la bibliografía, el empleo de manoproteínas produjo una desestabilización y precipitación de los pigmentos responsables de color estable de los vinos. De hecho, las manoproteínas adicionadas, no sólo no incrementaron el color estable de los vinos, sino que lo redujeron considerablemente, explicando los valores más bajos de intensidad de color y de color total en las muestras elaboradas con manoproteínas. Por el contrario, las manoproteínas no afectaron al color debido a los antocianos monómeros, hecho en concordancia con lo observado en el análisis de los antocianos monómeros, donde no se observó ninguna modificación en el contenido de dichos compuestos. Asimismo, tampoco se observó ninguna diferencia significativa entre los vinos controles y los elaborados con manoproteínas en relación a sus coordenadas de Cielab.

Según nuestra información, este es el primer trabajo en el que se analiza el efecto de las manoproteínas sobre el contenido de taninos en un ensayo real de vinificación y no en un medio sintético, donde se han obtenido resultados distintos a los de nuestro estudio, pero también diferentes entre ellos según el tipo de manoproteína utilizada, su concentración y la composición del medio sintético (pH y fuerza iónica).

Así, nuestro estudio se realiza en la propia matriz de los mostos y los vinos, teniendo en cuenta las condiciones reales de vinificación, y analizando los taninos reales procedentes de las semillas y la piel de la uva, y no agregados sintéticos añadidos. Por otro lado, tampoco hemos encontrado estudios que analicen el efecto protector de las manoproteínas sobre el color estable de los vinos, propiedad que se les atribuye constantemente pero sobre la que no existen evidencias científicas.

Effect of Commercial Mannoprotein Addition on Polysaccharide, Polyphenolic and Color Composition in Red Wines. Part I

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ABSTRACT

Commercially available mannoprotein preparations were tested in Tempranillo winemaking to determine their influence on polysaccharide, polyphenolic and color composition. No differences were found in the content of grape arabinogalactans, homogalacturonans and rhamnogalacturonans-II, but mannoprotein-treated samples showed considerable higher values of high-molecular-weight mannoproteins from the beginning of the alcoholic fermentation, these differences diminishing with the vinification progression. Although mannoprotein addition did not modify the content and composition of neither monomeric anthocyanins nor other monomeric phenolics, it had a strong influence on wine tannin content and stable color. Regarding to polymeric proanthocyanidins, yeast mannoproteins did not act as stabilizers colloids but quite the contrary, leading to an important precipitation of these compounds and thus to an overall reduction in the tannin concentration and polyphenol index. Mannoproteins did not show either any protective effect against wine stable color. On the contrary, mannoprotein-treated wines showed the lowest values of stable color although monomeric anthocyanin color was not affected.

KEYWORDS: Tempranillo red wine; winemaking; mannoproteins; polysaccharides; monomeric polyphenols; proanthocyanidins; colloidal stability.

INTRODUCTION

Wine technology is in continuous evolution in order to be able to satisfy the current consumer preferences. The growing trend toward the consumption of red wines aged in new barrels and with highly phenolic content involves the use of enological practices that produces wines with greater body, and better mouthfeel and overall stability. Natural yeast mannoproteins, initially used for the chemical stabilization of white wines, have recently attracted the attention of enologists for the winemaking of red wines, not only for their well-known effect on wine stability but also for their positive influence over a number of technological and quality properties of red wines. In fact, yeast cell wall mannoproteins play a very important role in the overall vinification process, and most of their technological functions have been widely described: (a) inhibition of tartrate salt crystallization (1, 2, 3), (b) reduction of protein haze (4, 5, 6, 7, 8), (c) stimulation of malolactic fermentation (9, 10, 11), (d) wine enrichment during autolysis of less (12, 13), (e) interaction with *flor* wines (14, 15), (f) yeast flocculation and autolysis in sparkling wines (16), and (g) adsorption of toxic ochratoxin A (17, 18, 19, 20).

Even more interesting, yeast mannoproteins have been described for their positive effect over diverse sensorial properties of red wines. Initially described for their interaction with aromatic compounds (21, 22, 23, 24), recent studies relate yeast mannoproteins with other wine sensory properties, including color stabilization (25, 26, 27, 28), reduction of astringency (25, 29, 30), and increased body and mouthfeel (23, 25 29, 30). These properties, especially those of red wines, are substantially important for the final wine quality, and in fact, it is due to these effects that numerous wine industries are introducing mannoprotein-based products in different stages of the red winemaking. The use of these products may even be seen as an alternative to the traditional wine aging on lees. It is well known that the advantages of wine maturation on lees are due to the process of lysis of the dead yeast cells in the fine lees, and mainly to the mannoproteins released in this process. However, this is a very complex and slow process which may require months or years, can create microbiological and organoleptic risks, and involve an important immobilization of the wine cellar resources.

The positive effect of yeast mannoproteins on the sensorial quality of red wines does not only attract the wine industry, and researches in enology are obviously showing a growing interest to better understand the effect of these polysaccharides in such sensory properties. In this sense, some studies have been developed in model systems in order to explain the interactions between mannoproteins and polyphenols (31, 32). To get closer to the real winemaking process, we explored different winemaking techniques to

increase the concentration of this polysaccharide and studied the effects produced on the wines obtained (30). The following techniques were explored: (a) the addition of exogenous commercial mannoproteins directly to musts, (b) the use of selected active yeast which produce high levels of mannoproteins during alcoholic fermentation. Exogenous mannoproteins clearly modified the gustative and aromatic structure of wines and seemed to have clearer effects on the analyzed parameters than yeast (30). Contrary to what was described in bibliography (25, 31, 32), we found that neither the addition of commercial mannoproteins nor the use of selected yeast maintained the extracted polyphenols in colloidal dispersion, and neither seemed to ensure color stability. Taking into account these unexpected findings, and on the basis that they were obtained from the sensory evaluation of the wines and general oenological parameters, we thought that a more detailed analytical study should be performed in order to confirm such observations.

Therefore, the aim of this first paper is to study the effect of the use of commercial mannoproteins in the red winemaking on the content and profile of wine polysaccharide and polyphenolic families as well as on the wine color. A second paper (Part II) analyzes the effect of yeasts overexpressers of mannoproteins.

MATERIALS AND METHODS

Reagents and Samples

All chemicals used were of analytical reagent grade. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, Kdo (3-deoxy octulosonic acid), vainillin and gallic acid were supplied by Sigma (St. Louis, MO), and D-galacturonic, D-glucuronic acid and myo-inositol were supplied by Fluka via (Sigma). D-apiose was obtained from Omicrom (South Bend, IN), and malvidin-3-glucoside, peonidin-3-glucoside, ferulic acid, syringic acid, caffeic acid, *p*-coumaric acid, catechin, epicatechin, myricetin, quercetin, isorhamnetin, kaempferol and rutin were purchased from Extrasynthèse (Lyon, France). Ethanol, formic acid, acetonitrile, acetone, acetyl chloride, sulfuric acid, sodium metabisulphite, and tartaric acid were supplied by Scharlab (Barcelona, Spain), sodium hydroxide, hydrochloric acid, and phosphoric acid were supplied by Carlo Erba (Rodano, Milan, Italy), dried methanol, disodium tetraborate, and acetaldehyde were supplied by Merck (Darmstadt, Germany), and the trimethylsilylation reagent (TriSil) was obtained from Pierce (Rockford, MA). HPLC-grade ammonium formiate, and trifluoroacetic acid supplied by Fluka, and Milli-Q water (Millipore, Molsheim, France) were used. A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain).

Wine samples were produced using *Vitis Vinífera Tempranillo* grapes from the qualified origin denomination Rioja (D.O.Ca. Rioja). Commercial mannoprotein preparation and the bacterial strain *Oenococcus oeni* were purchased from Lallemand (Lallemand-Inc., Montreal, Canada).

Vinification and Sample Collection

Mature Tempranillo grapes were harvested from Autol, La Rioja, Spain, at 21.9 °Brix, pH 3.56 and 6.02 g tartaric acid/L. Experimental vinifications were carried out in the wine cellar of the University of La Rioja and wines were prepared using traditional wine technology. Around 1000 Kg of grapes were destemmed, crushed, distributed into 100 L stainless steel tanks, and inoculated with 25 g/HL *S. cerevisiae* yeast strain RC212. Thereafter, 13.5 g/HL of industrial mannoproteins were added to half of the tanks (212M) while the rest remained as control vinifications (212). The prefermentation process lasted for 6 h at 18 ± 1°C; the fermentation–maceration process was performed at a maximum temperature of 28 ± 2°C and went on for 10 days. Postfermentative maceration went on for 4 days at 24 ± 1°C and wines were run off. Wines were then inoculated with a commercial preparation of *Oenococcus oeni* (1g/HL) to induce malolactic fermentation, carried out at 18.5 ± 1°C. After malolactic fermentation, all the wines were racked and clarified by settling for 25 days at 10°C. Wine aging was performed in new 13-litre American oak barrels, which are of higher area/volume than the traditional 225-litre barrels. For this reason, and based on the organoleptic analysis, the oak aging process went out for only 45 days. Wines were then bottled and stored at 4°C.

Samples were taken at the beginning of maceration–fermentation (0AF), during maceration–fermentation (25-30% of sugars consumed, 55-60% of sugars consumed, and 99% of sugars consumed, namely 30AF, 60AF, and 99F, respectively), and at the beginning and end of malolactic fermentation (BMF, EMF). Sample bottles were filled completely to minimize oxygen contact and immediately frozen at –18°C. Samples were also analyzed at the beginning and end of wine oak aging (BA, EA).

Isolation of Soluble Polysaccharides

Samples were homogenized, and 400 mL was taken and centrifuged. The supernatants were concentrated under reduced pressure as previously described (33), and polysaccharides were then precipitated by adding four volumes of cold ethanol/acid and kept for 18h at 4°C. Thereafter, the samples were centrifuged, the supernatants discarded, and the pellets were washed several times with 96% ethanol. The precipitates

were finally dissolved in ultrapure water and freeze-dried. The freeze-dried precipitates obtained (fractions S) contained the soluble polysaccharides.

Fractionation of Polysaccharide Families by HRSEC

In order to separate the different polysaccharide families, the soluble fractions S were subjected to high resolution size-exclusion chromatography on a Superdex-75 HR (1.3 x 30 cm) column (Pharmacia, Sweden) equilibrated at 0.6 mL/min in 30 mM ammonium formiate, pH 5.8. Chromatographic separation was carried out at room temperature on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) as previously described (33). Calibration was performed with narrow pullulan molecular weight standards (P-5, Mw = 5900 D; P-10, Mw = 11800 D; P-20, Mw = 22800 D; P-50, Mw = 47300 D; P-100, Mw = 112000 D; P-200, Mw = 212000 D; P-400, Mw = 404000 D). The peaks obtained were collected in different fractions (S1, S2 and S3) according to their elution times. The isolated fractions were freeze-dried, redissolved in water, and freeze-dried again four times to remove the ammonium salt.

Estimation of Polysaccharide Concentrations by GC and GC-MS

The carbohydrate composition of the fractions (fractions S, S1, S2 and S3) was determined by gas chromatography with flame ionization detector and GC-MS of their trimethylsilyl-ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization. GC was performed with a Hewlett-Packard HP5890 chromatograph using a fused-silica capillary column (30 m x 0.25 mm x 0.25 µm, Teknokroma, Barcelona, Spain) with helium as carrier gas and the rest of conditions previously described (33). Total sugars were calculated from the sum of all individual sugars from fractions S. Neutral and acid sugars were calculated as the sum of neutral and acid sugars, respectively, from fractions S. The content of each polysaccharide family in fractions S1, S2 and S3 was estimated from the concentration of individual glycosyl residues characteristic of well-defined wine polysaccharides (33, 34).

Fractionation of Phenolics by GPC

Samples were directly fractionated by gel permeation chromatography on a Toyopearl gel HP-50F (Tosohaas, Montgomery-ville, PA, USA) as described by Guadalupe et al. (35). A first fraction (F1) was eluted with ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v), and a second fraction (F2) was recovered by elution with acetone/water (60:40, v/v). Both fractions were taken to dryness under vacuum.

Determination of Monomeric Phenolic Compounds by HPLC-DAD

Fractions F1 were subjected to HPLC-DAD on a Kromasil 100-C18 reverse phase column (5 μm packing, 200 x 46 mm i.d.) protected with a guard column of the same material (Teknokroma, Barcelona, Spain). Chromatographic separation and quantification of monomeric phenolics was carried out on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) as previously described (35).

Anthocyanin glucosides (A-Glu) were calculated as the sum of delphinidin, cyanidin, petunidin, peonidin and malvidin-3-glucosides; acetyl-glucoside anthocyanins (A-Ac) as the sum of delphinidin, cyanidin, petunidin and malvidin-3-(6-acetyl)-glucosides; coumaryl-glucoside anthocyanins (A-Cm) included delphinidin, petunidin, and malvidin-3-(6-*p*-coumaryl)-glucosides. The sum of A-Glu, A-Ac and A-Cm was referred to as total monomeric anthocyanins (TMA). Total hydroxycinnamic acids (TCin) were calculated as the sum of *trans*-caftaric (*trans*-caffeoyl-tartaric acid), *cis*-caftaric (*cis*-caffeoyl-tartaric acid), *trans*-coutaric (*trans-p*-coumaryl-tartaric acid), *cis*-coutaric (*cis-p*-coumaryl-tartaric acid), caffeic and *trans-p*-coumaric acid. The monomeric flavan-3ols (M-Flava) included (+)catechin, (-)epicatechin and (-) epigalocatechin.

Determination of Total Proanthocyanidin Content

Total proanthocyanidins (PAs) were quantified in F2 fractions by the vanillin assay according to the method described by Sun et al. (36) but with few modifications (35). The spectrophotometric measurements were performed on a Cary 300 Scan UV-vis spectrophotometer (Varian Inc., Madrid, Spain).

Determination of Color and Total Polyphenol Index

Wine color, monomeric anthocyanin color, copigmentation color, and bisulfite-stable color were determined by the method proposed by Levengood and Boulton (37). The CIE tristimulus values (*X*, *Y*, *Z*) and CIELAB rectangular (*L**, *a** and *b**) parameters (illuminant D65 and 10° observer conditions) were determined according to Ayala et al. (38). Color intensity was calculated as the sum of absorbances at 420, 520, and 620 nm, and visual color was analyzed by sensory analysis of the wines as previously described (30). The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine with synthetic wine (12% alcohol, 5 g/l of tartaric acid in water, pH 3.6).

Statistical Procedures

Analyses were performed in triplicate. Significant differences between samples were analyzed using the SPSS 12.0 program for Microsoft Windows (SPSS Inc., Chicago, IL). The values of polysaccharide and polyphenolic content, and color composition were analyzed by a two-way analysis of variance (ANOVA) with repeated measurements in one factor (vinification stage) to test the effect of mannoprotein addition. If the data did not meet normality assumptions, a Kruskal–Wallis test was used. In this paper, differences between samples always refer to significant differences with at least $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of the Commercial Mannoproteins Used

A commercial preparation rich in mannoproteins was purchased from Lallemand and characterized with regard to glycosyl-residue composition. This commercial preparation was actually a yeast derivative product obtained by a specific refining process which leads to a high level of reactive yeast cell wall polysaccharides, mainly mannoproteins (information supplied by the manufacturer). The moment of addition and the dose used were followed as recommended by the manufacturer.

The glycosyl-residue analysis revealed a composition of 73% polysaccharides and 2% proteins, mannose being the main sugar (91%) with glucose far behind (9%), which confirmed the prevalence of parietal mannoproteins. The remaining 25% of the preparation was probably represented by other cellular components isolated during the extraction procedure.

Effect of Commercial Mannoprotein Addition on the Polysaccharide Composition during Vinification and Oak Aging

Mannoprotein treatment increased the amount of total sugars from the moment they were added, and mannoprotein-treated musts presented 20% more total soluble sugars than the controls (Figure 1). These differences were maintained during the extractive period of the maceration–fermentation, i.e., from 0AF to 60AF, where treated samples contained around 100 mg/L more total soluble sugars than their respective controls. The late maceration–fermentation and the postmaceration supposed a considerable reduction of more than 25% in the amount of total soluble sugars in both vinifications, being more accused in the mannoprotein-added samples. Thus, the difference in the sugar content previously observed was considerably reduced, to the point that both vinifications

presented similar values of total soluble sugars during the later stages of malolactic fermentation and oak aging. These facts, previously observed by our workgroup by using different methods of analysis (30), seemed to indicate that the mannoproteins added in the prefermentative stage were maintained in solution after their addition but they were also more affected by precipitation as the winemaking went on. As expected, there were not significant differences in the content of acid sugars between both vinifications.

Must and wine polysaccharides were fractionated by HRSEC in order to analyze the concrete polysaccharide families and confirm that the differences observed in the sugar content between both vinifications were actually due to the added mannoproteins. Soluble polysaccharides from both vinifications were directly injected on a Superdex 75-HR column, with a molecular weight range from 3 to 75 kD, showing quite similar chromatographic profiles (data not shown). Wine samples revealed a fractionation of compounds into three peaks, S1, S2 and S3, similar to that previously described (33), while must refractometric profiles showed only two of these peaks, S1 and S3. The glycosyl residue composition of the fractions was determined by GC and GC-MS, and the polysaccharide composition was estimated from the concentration of these individual glycosyl residues which form wine polysaccharides structurally identified. Higher molecular weight polysaccharides, eluting in fraction S1, corresponded to molecules with a molecular weight higher than 50 kD, and, according to their glycosyl composition and to previously published data, it corresponded to a complex mixture of high-molecular-weight arabinogalactans and arabinogalactan-proteins (bAGP) from grape berries and high-molecular-weight mannoproteins (bMP) from yeasts (39). Polysaccharides with an average molecular weight of 12 kD, fractionated in the second fraction (S2), mainly corresponded to grape rhamnogalacturonan-II dimers (dRG-II), but also to low-molecular-weight arabinogalactan-proteins and mannoproteins (sAGP and sMP, respectively). Fraction S3 displayed a molecular weight of less than 6 kD and it was mainly dominated by homo and rhamnogalacturonans oligomers (HG). However, glycosyl residues characteristics of AGP, MP and RG-II polysaccharides were also detected. The firsts were attributed to fragments of larger AGP and MP and were not quantified in this study, and the presence of rare sugars was attributed to monomeric RG-II (mRG-II).

Figure 2 shows the concentration of sample polysaccharide families during the winemaking and oak aging. It all the cases, the polysaccharide evolution pattern was quite similar to that obtained in previous studies by our workgroup (39). When comparing the control vinification with the mannoprotein-treated one, no significant differences could be observed in the content of grape polysaccharides, neither in the

case of high-molecular-weight compounds such as bAGP or dRG-II, nor in the case of smaller AGP, mRG-II or GL. Regarding the content of yeast mannoproteins, there was obviously a great difference between both vinifications. Mannoprotein-treated musts presented around 100 mg/L more high-molecular-weight mannoproteins (bMP) than their respective controls but no significant differences were obtained for the smaller molecules (sMP), indicating that more than 75% of the added mannoproteins remained in solution after their addition and that they were big size compounds. These bMP showed a molecular weight between 50 and 400 kD, with an average size of around 150 kD. The difference in the mannoprotein content between both musts was maintained during the maceration–fermentation, and although it was substantially diminished later on, there were still significant differences between the wines after malolactic fermentation and oak aging, the treated-wines showing 40-50 mg/L more bMP than the controls. Our results confirmed that an important precipitation of the added mannoproteins occurred during postmaceration, but it was partial, affecting to 60% of the added mannoproteins.

The results showed that control wines, both young and aged ones, were essentially composed of grape cell wall AGP, followed by yeast MP and dRG-II, showing similar proportions than those described in bibliography (40). However, mannoprotein-treated wines had larger concentrations of MP and these compounds were thus in quite similar proportions in wines than AGP polysaccharides. The content of mRG-II and HG was less than 2% of the total polysaccharide families in both control and treated wines.

Effect of Commercial Mannoprotein Addition on the Monomeric Phenolics during Vinification and Oak Aging

Figure 3 shows the content of total monomeric anthocyanins (TMA), hydroxycinnamic acids (TCin) and monomeric flavan-3ols (M-Flava) on the samples taking during winemaking and wine oak aging. Control and mannoprotein-treated samples presented comparable values of these phenolics throughout the vinification process except for the values of TMA and TCin during late maceration–fermentation. Thus, mannoprotein-treated samples showed an slight delay in the extraction of pomace anthocyanins and hydroxycinnamic acids between 60AF and 99AF, coinciding with the period of the maximum concentration of high-molecular-weight mannoproteins (Figure 2). To establish any relationship between the presence of big MP and the slower diffusion rate of anthocyanins or hydroxycinnamic acids was dismissed, because during postmaceration the 212M wine still presented higher amounts of these colloids and a higher extraction rate than the control. As a result, the differences observed during the maceration–fermentation fully disappeared after this stage. The identical TMA and

TCin contents observed for both wines during the later stages of malolactic fermentation and oak aging confirmed that high-molecular-weight mannoproteins had no influence on the evolution of these compounds.

As expected, we did not observe either any significant difference in the different forms of the monomeric anthocyanins between controls and 212M wines after the postmaceration period (Figure 4a). Surprisingly, the slower TMA extraction observed for MP samples at the end of the alcoholic fermentation was seen to be caused by a slower extraction in all the anthocyanic forms, the majority unacylated forms and the minority coumarated and acetylated forms (Figure 4a). A more detailed analysis revealed that it was the malvidin-3-glucoside together with their respective coumarated and acetylated forms the compounds responsible for these differences (data not shown).

With regard to monomeric flavan-3ols, we did not find any difference in the extraction and evolution of (+)catechin, (-)epicatechin, and (-)epigalocatechin between control and 212M samples (Figure 4b). However, hydroxycinnamic acids showed quite different behavior between both vinifications (Figure 4c). As in the case of anthocyanins, samples with MP addition showed a slower extraction of hydroxycinnamic acids during late maceration–fermentation but the biggest differences between both vinifications occurred during malolactic fermentation. In the case of MP wines, this stage produced a substantial decrease in the sterified hydroxinnamic acids (SCin) in favor of an increase in their respective free acids (FCin), which was seen to be due to a reduction in the *trans*-hydroxycinnamate derivatives, specially of *trans*-caftaric and *trans*-coutaric acid, because the *cis* forms remained stable (data not shown). Some authors have observed more or less intense changes in the *trans*-hydroxycinnamates during malolactic fermentation (41) and aging in oak barrels (42) due to hydrolysis of the tartaric esters. However, we just observed this hydrolysis phenomenon in the MP-enriched wine.

Effect of Commercial Mannoprotein Addition on the Proanthocyanidins during Vinification and Oak Aging

Contrary to what was observed with monomeric phenolics, the amount of polymeric proanthocyanidins was significantly affected by the addition of commercial mannoproteins (Figure 5). Thus, mannoprotein-added samples presented significantly lower proanthocyanidin contents from the end of the maceration–fermentation. These differences were maintained throughout the malolactic fermentation and oak aging, where the PA content of the controls was around 1.5 times higher than that of the treated wines.

In year 2000, Saucier et al. (25) proposed a model that could possibly explain the polysaccharide–tannin interactions. Basically, it suggests that wine proanthocyanidins alone are highly reactive towards salivary proteins with the subsequent increase in wine astringency. However, the combination tannin–mannoprotein would produce stable structures which are not reactive towards proteins, explaining why wine tannins are less astringent in the presence of mannoproteins (26). In addition, more recent studies have shown that adding polysaccharides to a model tannin suspension have a strong impact on tannin particle size evolution (31). Thus, it was observed that mannoproteins did not prevent initial tannin aggregation but they strongly inhibited it at wine concentrations. A more detailed studied suggested that the tannin–mannoprotein combination could be steric rather than electrostatic (32).

As in bibliography, we found that mannoproteins had a strong impact on tannin colloids and thus in wine astringency. This effect would imply a combination/adsorption of mannoproteins and tannins, confirming the widely accepted hypothesis proposed by Saucier. However, and contrary to what is described, our results suggested that mannoproteins did not have any protective effect towards tannins but quite the contrary. In order to be able to explain it, two hypothesis could be proposed. On the one hand, at the studied concentrations, the combination tannin–mannoproteins could result in high molecular weight structures that would be instable and precipitate, leading to a decrease in the total PA content. On the other hand, mannoproteins could act as flocculating polymers instead of stabilizers. Flocculation is usually explained by bridging, the same mannoprotein molecules would bind to different tannins, leading to the formation of bigger aggregates and further precipitation. Although studies in a synthetic media seems to indicate the opposite (31, 32), it must be noted that authors have obtained different results depending on the conditions of the medium, such as ethanol content and ionic strength, and the concentration and type of the studied polysaccharide. Thus, only the smallest mannoproteins, with average molecular size of 50-60 kD, limited tannin aggregation under standard wine conditions (pH 3.4, 12% ethanol, 2 g/L tartaric acid), while bigger mannoproteins did not show any effect (32), and other wine polysaccharides enhanced it (31) To the best of our knowledge, our study is the first one that analyze the protective role of mannoproteins towards tannins in a real vinification situation, with the real matrix of the wine and with well-dissolved seed and skin grape tannins rather than purified aggregates. A previous study carried out in Pinot Noir wines, by means of ethanol and gelatin indexes, suggested that yeast mannoproteins can combine with tannins and lead to less astringent tannins (26), but they did not analyze the effect produced on the tannin content.

In conclusion, and regardless of the implied mechanism, the addition of mannoproteins reduced the proanthocyanidin content, which resulted in wines with decreased astringency and tannicity, and enhanced sweetness and roundness (30). The lower PA content observed for the 212M wines was also in good correlation with their lower values in the total polyphenol index (Figure 5), which it is often used as an indirect measure of proanthocyanidins.

Effect of Commercial Mannoprotein Addition on Wine Color

For some time now there have been speculations that mannoproteins might well bind with other phenolic compounds besides proanthocyanidins and by so doing, stabilize the color of the red wines. Although no studies have been conducted on the interactions mannoproteins–wine pigments, it is hypothesized that mannoproteins would be adsorbed by colloidal molecules of anthocyanin–tannin, copigmented anthocyanins, etc., covering completely the surface of these unstable colloids and therefore avoiding their degradation and precipitation (43).

On the basis of this hypothesis, the protective colloid role of mannoproteins towards instable colorant material precipitation was studied. Figure 6 shows the evolution of wine color (WC), monomeric anthocyanin color (MAC) and stable color (SC) in the samples throughout the period studied, the latter being considered as the sum of copigmented and bisulfite-stable color.

As previously described by our workgroup (30), young wines produced with mannoprotein addition presented considerable lower values of wine color at the end of the maceration–fermentation, and these differences were maintained during malolactic fermentation and aging. It was observed that wine color differences were due to the stable color component, and no significant differences were found between both vinifications in the monomeric anthocyanin color throughout the period studied, which confirmed that mannoproteins had no effect on the monomeric anthocyanins (see previous section on monomeric phenolics). On the contrary, mannoprotein-treated wines showed lower values of stable color than controls at the end of the maceration–fermentation and these differences become even more evident during malolactic fermentation. Thus, 212M wines presented less than 20% of stable color than control wines both before and after wine oak aging, which resulted in wines with lower values of color intensity (Table 1) and visually weaker color intensity (30). Control and 212M wines did not show any remarkable difference in the CIELAB parameters a^* (from green to red), b^* (from blue to yellow) and L^* (lightness) at the end of oak aging (Table 1).

On the basis of our results, the stabilizing effect of mannoproteins towards color was also called into question. As in the case of tannins, our observations pointed out that mannoproteins did not have any protective role towards instable colorant material but they would act as colloidal-destabilization or precipitation species. Thus, and taking into account that the stable color is of the uppermost importance in enology, this effect should be further investigated.

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TABLES

Table 1. Color attributes in wines after oak aging

Wine	CI	Hue	*a**	*b**	*L**
212	5.008±0.004	0.62	26.8	0.90	72.2
212M	4.831±0.005	0.68	25.6	0.94	69.4

CI (color intensity as sum of absorbances at 420, 520 and 620)

Hue (A_{420}/ A_{520})

a* (from green to red), b* (from blue to yellow) and L* (lightness)

FIGURES

Figure 1. Effect of commercial mannoprotein addition on the evolution of total and acid sugars during vinification and oak aging. See text for conditions and calculations.

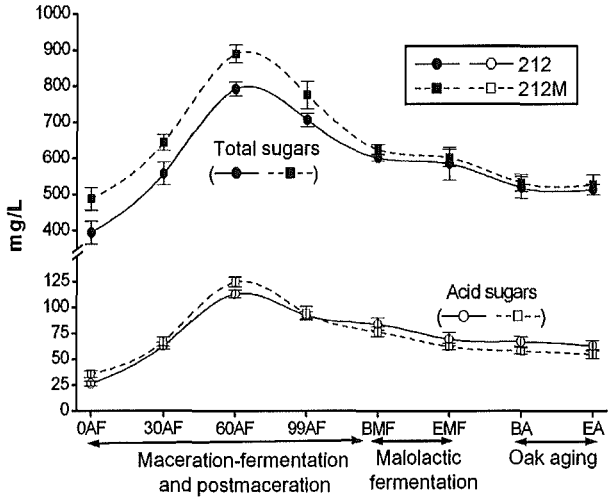


Figure 2. Effect of commercial mannoprotein addition on the evolution of major polysaccharide families during vinification and oak aging. bAGP, high-molecular-weight arabinogalactan-proteins; sAGP, low-molecular-weight arabinogalactan-proteins; bMP, high-molecular-weight mannoproteins; sMP, low-molecular-weight mannoproteins; dRG-II, rhamnogalacturonan-II dimers; mRG-II, rhamnogalacturonan-II monomers; GL, oligomers of homo- and rhamnogalacturonans. See text for conditions and calculations.

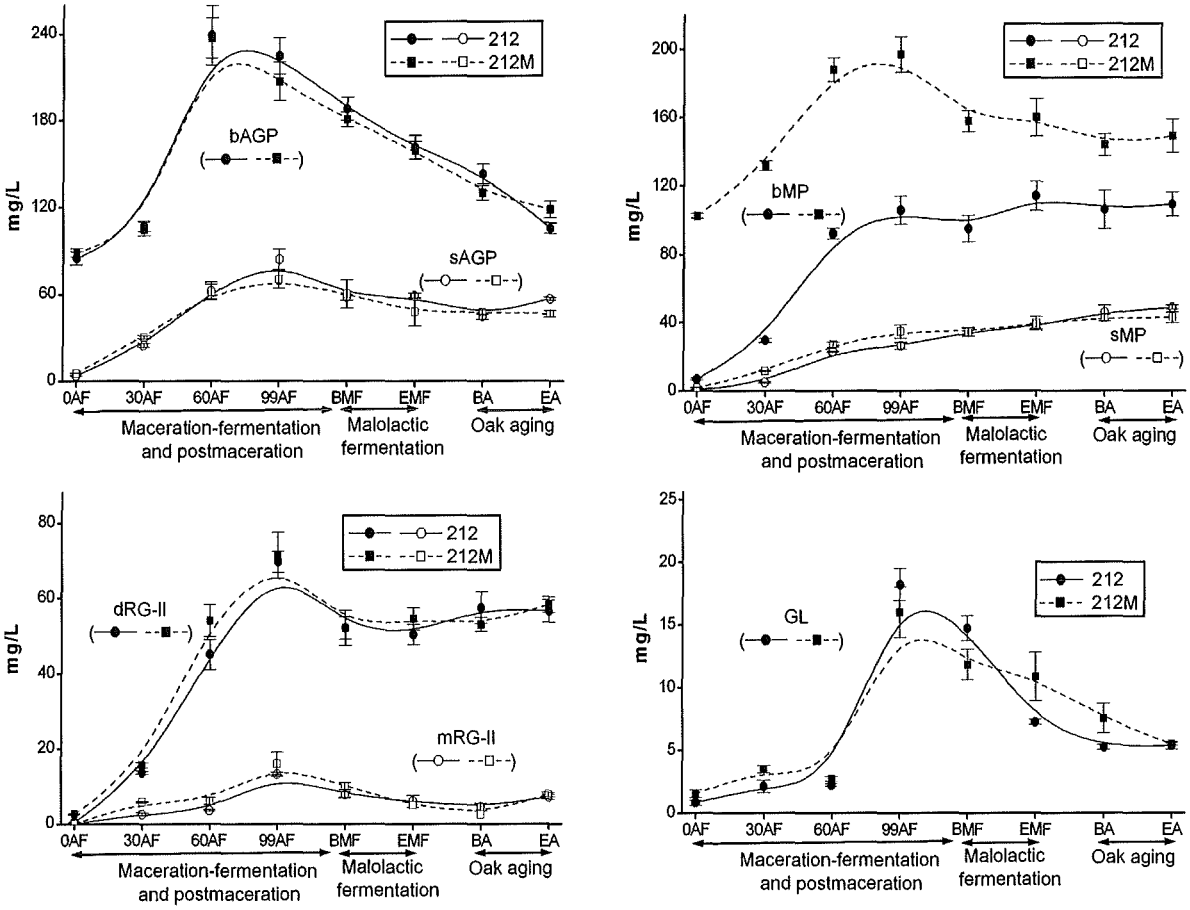


Figure 3. Effect of commercial mannoprotein addition on the evolution of total monomeric anthocyanins (TMA), total monomeric flavan-3ols (M-Flava), and total hydroxycinnamic acids (TCin) during vinification and oak aging. See text for conditions and calculations.

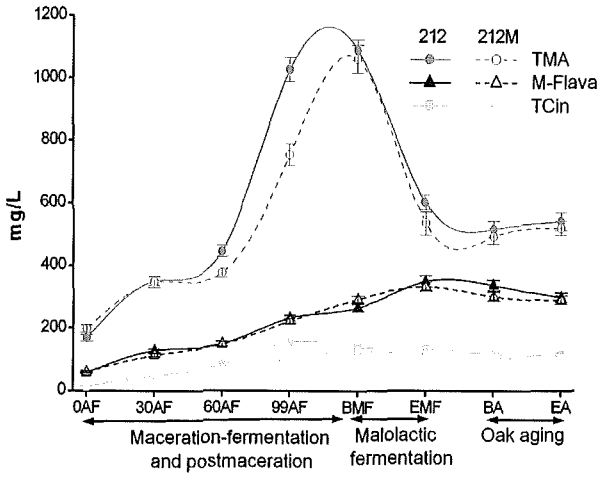


Figure 4. Effect of commercial mannoprotein addition on the evolution of: a) anthocyanin glucosides (A-Glu), coumaryl-glucoside anthocyanins (A-Cm), and acetyl-glucoside anthocyanins (A-Ac); b) monomeric flavan-3ols; c) sterified hydroxycinnamic acids (SCin) and free hydroxycinnamic acids (FCin). See text for conditions and calculations.

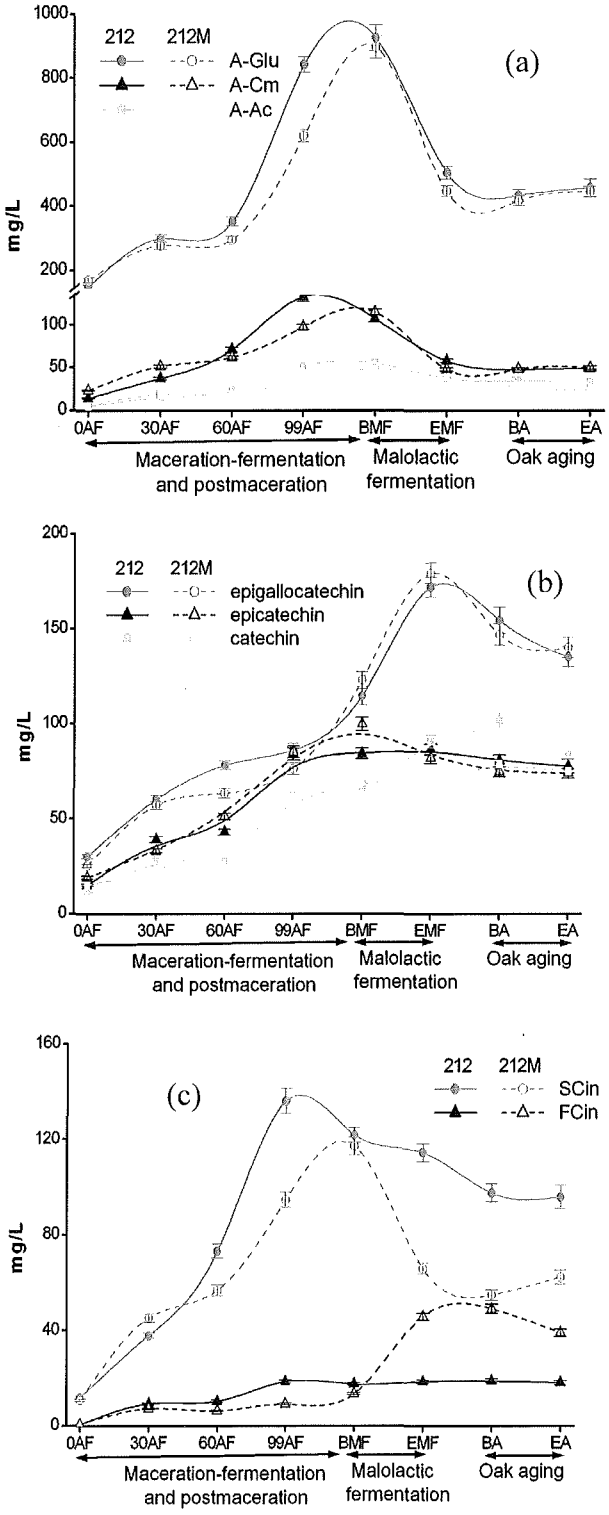


Figure 5. Effect of commercial mannoprotein addition on the evolution of total proanthocyanidins (PAs) and total polyphenol index (TPI) during vinification and oak aging. See text for conditions and calculations.

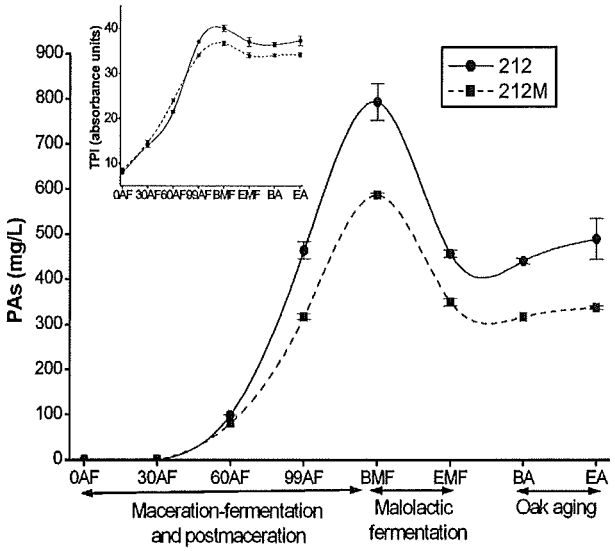
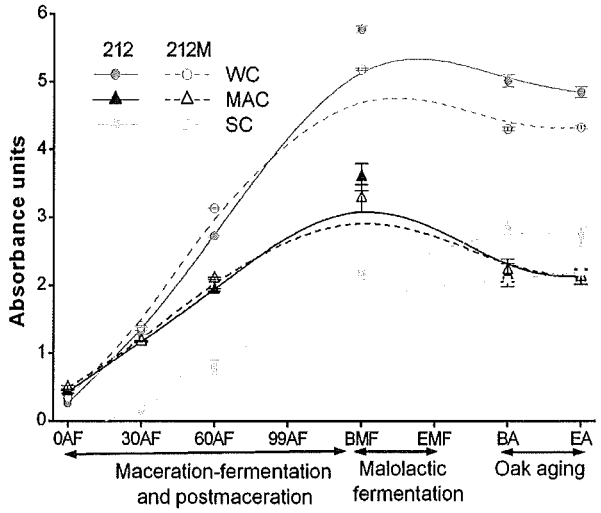


Figure 6. Effect of commercial mannoprotein addition on the evolution of wine color (WC), monomeric anthocyanin color (MAC) and stable color (SC) during vinification and oak aging. See text for conditions and calculations.



3.7

EFECTO DEL EMPLEO DE LEVADURAS SUPERPRODUCTORAS DE MANOPROTEÍNAS EN LA COMPOSICIÓN DE POLISACÁRIDOS, POLIFENOLES Y COLOR DE VINOS TINTOS

Effect of Yeast Overexpresser of Mannoproteins on the Polysaccharide, Polyphenolic and Color Composition in Red Wines. Part II

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Resumen

La utilización de coloides protectores como las manoproteínas se planteó como posible tratamiento biotecnológico para mejorar la estabilización de la materia colorante durante el proceso de vinificación de vinos tintos de variedad Tempranillo. Concretamente se propusieron dos tratamientos: a) adición directa de manoproteínas purificadas industrialmente en la etapa prefermentativa, y b) inoculación de los mostos con levaduras superproductoras de manoproteínas. En la parte I de este artículo (ver artículo presentado en el *apartado 3.6*) se analizó el efecto de la adición de manoproteínas industriales en la composición de polisacáridos, polifenoles y color durante el proceso de elaboración de los vinos, obteniéndose unos resultados un tanto sorprendentes.

Este artículo analiza el efecto de las manoproteínas liberadas por cepas de levaduras súper-productoras en el contenido y la evolución de las diferentes familias de polisacáridos y de compuestos fenólicos durante el proceso de vinificación de vinos tintos de variedad Tempranillo, así como su repercusión en el color estable. Además, se evalúa el efecto de ambos tratamientos combinados: levadura súper-productora + adición de manoproteínas comerciales.

El uso de cepas súper productoras favoreció la liberación de manoproteínas de alto peso molecular (bMP) en comparación con las cepas no productoras de manoproteínas. A pesar de que la evolución de estos polisacáridos fue diferente en las vinificaciones tratadas con manoproteínas comerciales y en las no tratadas, el contenido final de bMP en los vinos fue similar en todas las muestras con manoproteínas independientemente de su origen. Como se esperaba, la fermentación del mosto con la levadura súper productora no afectó al contenido y evolución de los polisacáridos de la pared celular de la uva (AGP, RG-II y GL).

Entre los vinos elaborados por las dos cepas de levaduras no se observaron diferencias en el color debido a los antocianos monómeros ni en su contenido. Tampoco se observaron diferencias en el contenido de ácidos hidroxicinámicos y flavan-3oles monómeros. Estos resultados confirmaron que las manoproteínas, independientemente de su origen, no influyen en el contenido de los polifenoles monómeros estudiados.

Los resultados obtenidos en el análisis de las proantocianidinas confirmaron lo observado con las manoproteínas industriales. Así, la presencia en el medio de manoproteínas de alto peso molecular liberadas por la cepa súper productora produjo una disminución sustancial en el contenido de proantocianidinas desde la maceración-fermentación.

Los vinos obtenidos con la cepa súper-productora presentaron concentraciones más bajas en el contenido de proantocianidinas y de polifenoles totales que sus respectivos testigo, confirmando el efecto desestabilizador de las manoproteínas sobre los taninos. Por el contrario, y a diferencia de lo observado con el uso de manoproteínas comerciales, la utilización de levaduras superproductoras de manoproteínas no tuvo un efecto desestabilizador claro sobre el color del vino.

Los resultados obtenidos del análisis de las vinificaciones realizadas con el tratamiento combinado de levaduras súper-productoras y adición de manoproteínas comerciales confirmó que:

1. El efecto de las manoproteínas comerciales sobre los taninos y el color estable de los vinos fue independiente del tipo de cepa de levadura empleada.
2. El efecto de la cepa súper-productora de manoproteínas sobre los taninos fue independiente de la adición de manoproteínas en la etapa prefermentativa.
3. La efectividad del tratamiento combinado sobre el contenido de manoproteínas de alto peso molecular fue similar al obtenido empleando cada tratamiento por separado.
4. El efecto del tratamiento combinado sobre los taninos y el color estable no fue aditivo.

A partir de los resultados obtenidos en estos trabajos, y teniendo en cuenta que el color y su estabilidad es de suma importancia en la elaboración de los vinos tintos, sería interesante realizar más ensayos para dilucidar el efecto de las manoproteínas sobre el color estable del vino, así como el mecanismo implicado en la precipitación del agregado manoproteína-tanino.

Effect of a Yeast Overexpresser of Mannoproteins on Polysaccharide, Polyphenolic and Color Composition in Red Wines. Part II

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ABSTRACT

The use of yeast overexpressers of mannoproteins produced an increase in high-molecular-weight mannoproteins in wines similar to that obtained when purified mannoproteins were added. The results confirmed that mannoproteins have a precipitation effect on wine tannins, independently of their origin. As with commercial mannoproteins, the released mannoproteins did not influence the different monomeric polyphenols or monomeric anthocyanin color. In contrast, no stable color loss was observed caused by industrial purified mannoproteins. The combination of adding commercial mannoproteins and using overexpresser yeast strains on the parameters studied did not have an additive effect. Future studies must clarify the colloidal mechanism involved in the precipitation of the mannoprotein-tannin complex, as well as the effect of these polysaccharides on stable color in wines.

KEYWORDS: Tempranillo red wine; yeast overexpresser of mannoproteins; polysaccharides; monomeric polyphenols; proanthocyanidins; colloidal stability.

INTRODUCTION

Mannoproteins are glycoproteins, often highly glycosylated, with carbohydrate fractions consisting mainly of mannose (> 90%) and glucose. These polysaccharides, which can account for up to 50% of the cell wall dry mass of *Saccharomyces cerevisiae*, are located in the outermost layer of the cell wall, where they are connected to a matrix amorphous β-1,3 glucan by covalently bonds (1). Two classes of mannoproteins have been described in wines. The first group is released by the action of a β-1,3 glucanase upon the wall during aging in the presence of nonmultiplying cells (autolysis); the second group is released by yeast when actively growing during fermentation. These last compounds, with highly variable sizes from 5 to more than 800 kDa (2), are similar to those from yeast cell walls, except for a lower protein content. The amount of mannoproteins released by yeast depends on the specific yeast strain (3), as well as the winemaking and maturation conditions (4), and these molecules become the second polysaccharide family in red wines (5).

Commercial formulates of mannoproteins have been used from a long time as technological adjuvants in winemaking, mainly of white wines, due to their positive effect in the overall vinification process (6, 7). More and more, enologists are being attracted to the use of mannoproteins for the winemaking of red wines, and not for their well-known effect on wine stability but for their positive influence on the sensorial quality of wines. Thus, numerous industries of enological products have launched of several mannoprotein-based products for production and sale, which include yeast extracts and yeast autolysates, purified polysaccharide preparations from yeast cell walls, and selected yeast strains that produce large quantities of parietal polysaccharides. So far, only a few scientific studies have reported the use of these products in winemaking (8, 9, 10), and their advantages and effects on wine composition are mainly claimed by their producers themselves.

Based on these considerations, we aimed to analyze the effect of these commercial products on the winemaking of the Tempranillo red wine. In Part I of this study, the effect of the addition of exogenous mannoproteins directly to the must on the wine polysaccharide and polyphenolic composition was studied, and it was found that it clearly reduced the content of polymeric proanthocyanidins but it did not affect to the content of monomeric polyphenols. This paper studies the effect of vinification with a selected active yeast, which produces mannoproteins abundantly in the coarse of alcoholic fermentation, and the results obtained with both alternatives will be then compared. If both showed similar behavior in terms of their effects and their

effectiveness, selected yeast would be, in principle, more easily handled in enological practice than exogenous mannoprotein by-products.

Nowadays, the main problem relating to the use of mannoproteins as enological adjuvants is a legislative one. The Organisation Internationale de la Vigne et du Vin (OIV) and the European Council Regulation (EC) permit the addition of mannoproteins from yeast walls to wines, but only to improve tartaric and/or protein stabilization (Resolution Oeno 4/2001, Oeno 15/2005, EU Regulation 2165/2005). The USA regulations allow for yeast cell walls to be used but only as yeast nutrients, not for stabilization. Neither South Africa nor Switzerland permit their use, and the situation in Australia and New Zealand is now under review. Another reason for using yeast may simply be economic because it would reduce production costs when compared with other yeast by-products. The cost of using a selected dry yeast would be about 0.007 euros per liter of wine while the use of highly purified mannoprotein-based products would cost three times more (average market price). Besides, the role of the selected yeast would not be limited to increasing mannoprotein content; they would act as natural fermentation agents in winemaking, producing other secondary metabolites which would interact with other wine compounds and modify the composition and organoleptic qualities of wine in different ways.

MATERIALS AND METHODS

Reagents and Samples

All chemicals used were of analytical reagent grade. L-Fucose, L-rhamnose, 2-*O*-methyl-D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, Kdo (3-deoxy octulosonic acid), vainillin and gallic acid were supplied by Sigma (St. Louis, MO), and D-galacturonic, D-glucuronic acid and myo-inositol were supplied by Fluka via (Sigma). D-apiose was obtained from Omicrom (South Bend, IN), and malvidin-3-glucoside, peonidin-3-glucoside, caffeic acid, *p*-coumaric acid, catechin and epicatechin were purchased from Extrasynthèse (Lyon, France). Ethanol, formic acid, acetonitrile, acetone, acetyl chloride, sulfuric acid, sodium metabisulphite, and tartaric acid were supplied by Scharlab (Barcelona, Spain), sodium hydroxide, hydrochloric acid, and phosphoric acid were supplied by Carlo Erba (Rodano, Milan, Italy), dried methanol, disodium tetraborate, and acetaldehyde were supplied by Merck (Darmstadt, Germany), and the trimethylsilylation reagent (TriSil) was obtained from Pierce (Rockford, MA). HPLC-grade ammonium formiate, and trifluoroacetic acid supplied by Fluka, and Milli-Q water (Millipore, Molsheim, France) were used. A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain).

Wine samples were produced using *Vitis Vinífera Tempranillo* grapes from the qualified origin denomination Rioja (D.O.Ca. Rioja). *Saccharomyces cerevisiae* var. *cerevisiae* RC212 and *Saccharomyces cerevisiae* var. *cerevisiae* BM45 were commercial active dry yeast from Lallemand (Lallemand-Inc., Montreal, Canada), commercial mannoprotein preparation (OptiRED) and the bacterial strain *Oenococcus oeni* was also purchased from Lallemand.

Vinification and Sample Collection

Mature Tempranillo grapes were harvested from Autol, La Rioja, Spain, at 21.9 °Brix, pH 3.56 and 6.02 g tartaric acid/L. Experimental vinifications were carried out in the wine cellar of the University of La Rioja and wines were prepared using traditional wine technology. Grapes were destemmed, crushed, and distributed into nine 100 L stainless steel tanks. Three of the deposits were inoculated with 25 g/HL *S. cerevisiae* yeast strain BM45 previously rehydrated (BM45 samples), which was selected due to its high liberation of mannoproteins (10); other three deposits were inoculated with *S. cerevisiae* yeast strain RC212 previously rehydrated and were considered as control vinifications. The remaining tanks were inoculated with the yeast strain BM45 and thereafter 13.5 g/HL of exogenous commercial mannoproteins were added (BM45M samples). On the one hand, this vinification allowed us to confirm if the results obtained with each yeast strain (BM45 y RC212) was independent of the addition of commercial mannoproteins in the prefermentative stage. On the other hand, we could confirm if the addition of industrial mannoproteins produced the same effects with independently of the yeast strain employed. In addition, we checked the combined effect of both treatments. The prefermentation process lasted for 6 h at 18 ± 1 °C; the fermentation–maceration process was performed at a maximum temperature of 28 ± 2 °C and went on for 10 days. Postfermentative maceration went on for 4 days at 24 ± 1 °C and wines were run off. Wines were then inoculated with a commercial preparation of *Oenococcus oeni* (1g/HL) to induce malolactic fermentation, carried out at 18.5 ± 1 °C. After malolactic fermentation, all the wines were racked and clarified by settling for 25 days at 10 °C. Wine aging was performed in new 13 L American oak barrels, which are of higher area/volume than the traditional 225 L barrels. For this reason, and based on of the organoleptic analysis, the oak aging process went out for only 45 days. Wines were then bottled and stored at 4 °C.

Samples were taken at the beginning of maceration–fermentation (0AF), during maceration–fermentation (25-30% of sugars consumed, 55-60% of sugars consumed, and 99% of sugars consumed, namely, 30AF, 60AF, and 99F, respectively), and at the beginning and end of malolactic fermentation (BMF, EMF). Sample bottles were filled

121 completely to minimize oxygen contact and immediately frozen at –18 °C. Samples
122 were also analyzed at the beginning and end of wine oak aging (BA, EA).

123 ***Isolation of Must and Wine Polysaccharides***

124 Samples were homogenized, and 400 mL were taken and centrifuged. The
125 supernatants were first concentrated under reduced pressure as previously described
126 (11). Polysaccharides were then precipitated by adding four volumes of cold
127 ethanol/acid and kept for 18h at 4 °C. Thereafter, the samples were centrifuged, the
128 supernatants discarded, and the pellets washed several times with 96% ethanol. The
129 precipitates were finally dissolved in ultrapure water and freeze-dried. The freeze-dried
130 precipitates obtained (S fractions) contained soluble polysaccharides.

131 ***Fractionation of Must and Wine Polysaccharide Families by HRSEC***

132 In order to separate the different polysaccharide families, the soluble S fractions
133 were subjected to high resolution size-exclusion chromatography on a Superdex-75 HR
134 (1.3 x 30 cm) column (Pharmacia, Sweden) equilibrated at 0.6 mL/min in 30 mM
135 ammonium formiate, pH 5.8. Chromatographic separation was carried out at room
136 temperature on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany)
137 as previously described (11). Calibration was performed with narrow pullulan molecular
138 weight standards (P-5, Mw = 5900 D; P-10, Mw = 11800 D; P-20, Mw = 22800 D; P-
139 50, Mw = 47300 D; P-100, Mw = 112000 D; P-200, Mw = 212000 D; P-400, Mw =
140 404000 D). The peaks obtained were collected in different fractions (S1, S2 and S3)
141 according to their elution times. The isolated fractions were freeze-dried, redissolved in
142 water, and freeze-dried again four times to remove ammonium salt.

143 ***Estimation of Polysaccharide Concentrations by GC and GC-MS***

144 The carbohydrate composition of the fractions (fractions S1, S2 and S3) was
145 determined by GC with flame ionization detector and GC-MS of their trimethylsilyl-
146 ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization.
147 GC was performed with a Hewlett-Packard HP5890 chromatograph using a fused-silica
148 capillary column (30 m x 0.25 mm x 0.25 µm, Teknokroma, Barcelona, Spain) with
149 helium as carrier gas and the rest of conditions previously described (11). The content
150 of each polysaccharide family in fractions S1, S2 and S3 was estimated from the
151 concentration of individual glycosyl residues characteristic of well-defined wine
152 polysaccharides (11, 12).

Determination of Color and Total Polyphenol Index

Wine color (WC), monomeric anthocyanin color (MAC), copigmentation color (CC), and bisulfite-stable color (BSC) were determined by the method proposed by Levengood and Boulton (13). El stable color (SC) was calculated as the sum of CC and BSC. The CIE tristimulus values (*X*, *Y*, *Z*) and CIELAB rectangular (*L**, *a** and *b**) parameters (illuminant D65 and 10° observer conditions) were determined according to Ayala et al. (14). Color intensity was calculated as the sum of absorbances at 420, 520, and 620 nm, and visual color was analyzed by sensory analysis of the wines as previously described (10). The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine with synthetic wine (12 % alcohol, 5 g/l of tartaric acid in water, pH 3.6).

Fractionation of Must and Wine Phenolics by GPC

Samples were directly fractionated by GPC on a Toyopearl gel HP-50F (Tosohaas, Montgomery-ville, PA, USA) as described by Guadalupe et al. (15). A first fraction (F1) was eluted with ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v), and a second fraction (F2) was recovered by elution with acetone/water (60:40, v/v). Both fractions were taken to dryness under vacuum.

Determination of Must and Wine Monomeric Phenolic Compounds by HPLC-DAD

Fractions F1 were subjected to HPLC-DAD on a Kromasil 100-C18 reverse phase (5 μm packing, 200 mm x 46 mm i.d.) column protected with a guard column of the same material (Teknokroma, Barcelona, Spain). Chromatographic separation and quantification of monomeric phenolics was carried out on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) as previously described (15).

Anthocyanin glucosides (A-Glu) were calculated as the sum of delphinidin (Df), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin-3-glucosides (Mv); acetyl-glucoside anthocyanins (A-Ac) as the sum of delphinidin (Dfac), cyanidin (Cyac), petunidin (Ptac) and malvidin-3-(6-acetyl)-glucosides (Mvac); coumaryl-glucoside anthocyanins (A-Cm) included delphinidin (Dfcm), petunidin (Ptcm), and malvidin-3-(6-*p*-coumaryl)-glucosides (Mvcm). The sum of A-Glu, A-Ac and A-Cm was referred to as total monomeric anthocyanins (TMA). Total hydroxycinnamic acids (TCin) were calculated as the sum of *trans*-caftaric (*trans*-caffeoyl-tartaric acid), *cis*-caftaric (*cis*-caffeoyl-tartaric acid), *trans*-coutaric (*trans*-*p*-coumaryl-tartaric acid), *cis*-coutaric (*cis*-*p*-coumaryl-tartaric acid), caffeic and *trans*-*p*-coumaric acid. The monomeric flavan-3ols (M-Flava) included (+)catechin, (-)epicatechin and (-)epigalocatechin.

Determination of Total Proanthocyanidin Content in Must and Wine Samples

Total proanthocyanidins (PAs) were quantified in F2 fractions by the vanillin assay according to the method described by Sun et al. (16) but with few modifications (15). The spectrophotometric measurements were performed on a 300 Scan UV-vis spectrophotometer (Varian Inc., Madrid, Spain).

Statistical Procedures

Vinifications and analyses were performed at least in triplicate. Significant differences between samples were analyzed using the SPSS 12.0 program for Microsoft Windows (SPSS Inc., Chicago, IL). The polysaccharide and polyphenolic content values were analyzed by a two-way analysis of variance (ANOVA) with repeated measurements in one factor (vinification stage) to test the effect of mannoprotein. If the data did not meet normality assumptions, a Kruskal–Wallis test was used. In this paper, differences between samples always refer, significant differences with at least $p < 0.05$.

RESULTS AND DISCUSION

Characterization of the commercial active dry yeast used

It has been show that the amount of mannoproteins released by yeast is dependent upon the specific strain used (3, 8), and that mannoproteins released during fermentation itself are more reactive than those released during yeast autolysis (8). Based on these considerations, an active dry yeast was selected that had been shown to release inherently higher levels of mannoproteins in the course of alcoholic fermentation (8 and the musts were inoculated with the dose recommended by the manufacturer. Both the overexpresser yeast selected (*S. cerevisiae* BM45) and the commercial yeast selected for the controls (*S. cerevisiae* RC212) were grown in a synthetic medium in order to obtain and further analyze the polysaccharides released by both strains (10). The analysis revealed that the macromolecules released by both strains were mainly polysaccharides (~80%) and some proteins (~11%), and that the BM45 yeast strain released more macromolecules than the RC212 strain in a synthetic medium and in must during fermentation (10). In both cases, mannose was found to be the prevalent sugar followed by glucose, which confirmed that the polysaccharides released into the medium were essentially mannoproteins with a low proteic fraction. However, the distribution of both sugars was different for both strains. For RC212, mannose was the main component and glucose represented only 29%, while they were present in relatively similar proportions in macromolecules from the strain BM45 (10). The

greater proportion of glucose in BM45 polysaccharides has also been observed by other authors (8) and may be due to different composition of its cell wall.

Effect of the yeast overexpresser of mannoproteins on the polysaccharide composition of the wine during vinification and aging

Figure 1 shows the evolution of major polysaccharide BM45 and BM45M samples during winemaking and oak aging. The evolution of the polysaccharides in the samples obtained with yeast strain RC212 are shown in Part I. The effect of the overexpresser yeast on the must and wine polysaccharides was the same independently of the presence or absence of purified mannoproteins.

As expected, fermenting with the overexpresser yeast did not produce any significant difference in the content and evolution of grape cell wall AGP, bAGP or sAGP, or monomeric or dimeric RG-II and GL. However, the use of the BM45 strain enhanced the release of mannoproteins in comparison with the RC212 strain. No significant differences were observed in the amount of sMP but significant differences were observed in bMP, indicating that the selected overexpresser yeast released an extra amount of large mannoproteins; this is important because yeast mannoproteins have been found to act differently regarding to colloidal stabilization depending on their molecular size (17). Although the maximum difference in the amount of bMP observed between the two strains was the same, independently of the addition of commercial mannoproteins, this occurred during a different vinification stage. Thus, in the samples without added mannoproteins this occurred at the end of post-maceration, while in the samples with added mannoproteins the maximum different was observed earlier (end of alcoholic fermentation). This difference is due to the fact that more bMP was precipitated than released in the treated samples. Obviously, the higher content of mannoproteins favored bMP precipitation in the treated samples. This precipitation of bMP was progressive and continued during aging, producing similar contents of bMP in the 212M and BM45M wines at the end of aging. Logically, not adding mannoproteins caused the difference in bMP content between both strains to be maintained during malolactic fermentation and aging. The result was that at the end of aging, the bMP content of the BM45 wine was similar to that of the BM45M and 212M wines, and obviously higher than that of the 212 wine. This was the main polysaccharide in the BM45, BM45M and 212M wines, followed by AGP, RG-II and GL. These compounds accounted for approximately 53%, 40%, 15%, and 2%, respectively, of total quantified soluble polysaccharides, the proportion of MP was considerably higher than that described in the bibliography for red varieties (5, 11, 12).

Effect of the yeast overexpresser of mannoproteins on monomeric phenolics during vinification and aging

The differences observed in the total content of monomeric anthocyanins (TMA), hydroxycinnamic acids (T-Cin) and monomeric flavan-3ols (T-Flava) between the vinifications performed with the overexpresser strain diminished during ageing (Figure 2). At the end of ageing, no differences were observed in the content of the different monomeric polyphenols between the wines obtained with the two yeast strain selected BM45 and RC212 (Part I). These results confirmed that the mannoproteins did not influence the content of monomeric anthocyanins, hydroxycinnamic acids and monomeric flavan-3ols of the aged wine.

Effect of the yeast overexpresser of mannoproteins on polymeric proanthocyanidin content during vinification and aging

The BM45 samples displayed a significantly lower content of proanthocyanidins (PAs) as from the end of maceration-fermentation (Figure 3). Theses differences were maintained throughout malolactic fermentation and oak aging, when the PA content of the 212 wine was around 1.7 times higher than in the BM45 wines (Part I). Also, in the BM45M samples PA content diminished with respect to that in the 212M samples (Part I), but this decrease in PAs was lower than that observed in the samples not treated with commercial mannoproteins (1.3 vs 1.7). These results confirmed that the bMP from the *S. cerevisae* BM45 strain had the same effect on the proanthocyanidins as that observed with the added mannoproteins (see Part I). Other studies performed in synthetic wine describe the mannoproteins released by the BM45 strain, with a mannose/glucose ratio close to the unit, as the most efficient for tannin stabilization (18). Our results in wine showed the opposite. Moreover, the bMP released by the BM45 strain did not stabilize or prevent the precipitation of the released mannoprotein-tannin colloid complex. The scarce protector effect of the bMP released by the BM45 strain on the tannin prompted a significant reduction in PAs at the end of aging of the BM45 and BM45M wines. These wines also presented the lowest IPT values IPT (Figure 3).

Effect of the yeast overexpresser of mannoproteins on wine color

Figure 4 shows wine color (WC), color due to monomeric anthocyanins (MAC) and stable color (SC) of the BM45 samples. As with the 212 samples, the differences observed in WC between the BM45 and BM45M samples after post-maceration continued until the end of aging. However, no differences were observed in WC between the 212 and BM45 wines at the end of aging nor between the 212M and BM45M wines. The difference in WC that existed between these wines was not due to

290 the type of yeast strain used but to the addition of industrial mannoproteins. In terms of
291 color composition, significant differences were observed between oak-aged wines
292 produced with the two selected yeast strains (Figure 4 and Part I). The Stable Color
293 (SC) of the BM45 wine was slightly lower than that of the 212 wine, and was similar in
294 the BM45M and 212M wines, albeit with lower values. These results indicated that
295 mannoprotein release induced by the overexpresser strain produced a slight loss in SC.
296 Previous studies by our group reported that the released mannoproteins did not have
297 noteworthy effects on polymeric anthocyanins (10).

298 At the end of aging, color due to monomeric anthocyanins (CMA) was similar in the
299 212 and BM45 wines, and in the BM45M and 212M wines. These results indicated that
300 the mannoproteins released by the overexpresser strain did not affect monomeric
301 anthocyanin color.

302 Aged wines fermented with the yeast overexpresser of mannoproteins presented
303 higher coloring intensity values than the 212 wines (Table 1). In terms of the CIELAB
304 parameters, the 212 and BM45 wines did not show any significant difference in the a^*
305 and L^* values, which were around 26 and 70, respectively (Table 1). However,
306 fermentation with the BM45 yeast strain produced wines with significantly higher
307 values of the b^* coordinate (from blue to yellow), these values being 2.8 in BM45-aged
308 wines and 0.92 in 212 wines.

309 ***Overall differences between the use of exogenous commercial mannoproteins and a***
310 ***selected yeast overexpresser of mannoproteins***

311 As indicated in Part I, the addition of commercial mannoproteins prompted a
312 decrease in proanthocyanidins and stable color, but this treatment had not effect on
313 either the monomeric polyphenols or on color due to monomeric anthocyanins.

314 This study examined the influence of the addition of commercial mannoproteins
315 using a different yeast strain (BM45 vs BM45M). The results obtained in the wines
316 (Figure 1-4) indicated that the addition of commercial mannoproteins had the same
317 effect on the proanthocyanins and stable color regardless of the type of yeast strain used
318 to prepare them. Moreover, the addition of mannoproteins did not affect either
319 monomeric polyphenol content or color due to monomeric anthocyanins in either the
320 wines prepared with the BM45 strain or those obtained with the RC212 strain.

321 If we compare the results obtained with the industrial mannoproteins and those of
322 the mannoproteins released by the BM45 strain, it was observed that, although the
323 evolution of bMP changed during winemaking, the final content of these compounds in
324 the wines was similar in all the samples with the added mannoproteins, regardless of

their origin. Surprisingly, the BM45M samples did not display higher mannoprotein contents than the other samples. Therefore, treatment using commercial mannoproteins combined with an overexpresser yeast strain did not have an additive effect.

The use of overexpresser yeasts produced similar decreases in proanthocyanidins as those observed when mannoproteins were added. The addition of commercial mannoproteins to the must inoculated with overexpresser yeast strains did not cause a greater decrease in proanthocyanidins in the wines and therefore did not have an additive effect. In turn, no differences were observed in terms of the effect of the overexpresser yeast strains and commercial mannoproteins on the different monomeric polyphenols or on color due to monomeric anthocyanins (CMA). Nor did combined treatment in the BM45M samples have an additive effect on the different monomeric polyphenols and CMA. The main difference between both mannoproteins was their contribution to the stable color of the wines. Unlike the commercial mannoproteins, the released mannoproteins did not have a clearly destabilizing effect on wine color. Therefore, and bearing in mind their role as fermentation agents and their lower cost, it would seem to be more advisable to use overexpresser mannoproteins than add industrial purified mannoproteins during the pre-fermentation stage.

The effect of both mannoproteins on the PAs was clear but the colloidal mechanism involved in the precipitation of the mannoprotein-tannin complex is unknown. Therefore, further studies must be performed to identify the mechanism responsible for this. The effect of these polysaccharides on the stable color of the wines must also be clarified.

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TABLES

Table 1. Color attributes in wines after oak aging

Wine	CI	Hue	*a**	*b**	*L**
BM45	5.372±0.001	0.60	26.7	2.8	72
BM45M	4.991±0.002	0.58	26.1	2.84	69.4

CI (color intensity as sum of absorbances at 420, 520 and 620)

Hue (A_{420}/ A_{520})

a* (from green to red), b* (from blue to yellow) and L* (lightness)

FIGURES

Figure 1. Effect of the use of a yeast overexpresser of mannoproteins on the evolution of major polysaccharide families during vinification and oak aging. bAGP, high-molecular-weight arabinogalactan-proteins; sAGP, low-molecular-weight arabinogalactan-proteins; bMP, high-molecular-weight mannoproteins; sMP, low-molecular-weight mannoproteins; dRG-II, rhamnogalacturonan-II dimmers; mRG-II, rhamnogalacturonan-II monomers; GL, oligomers of homo- and rhamnogalacturonans. See text for conditions and calculations en Parte I.

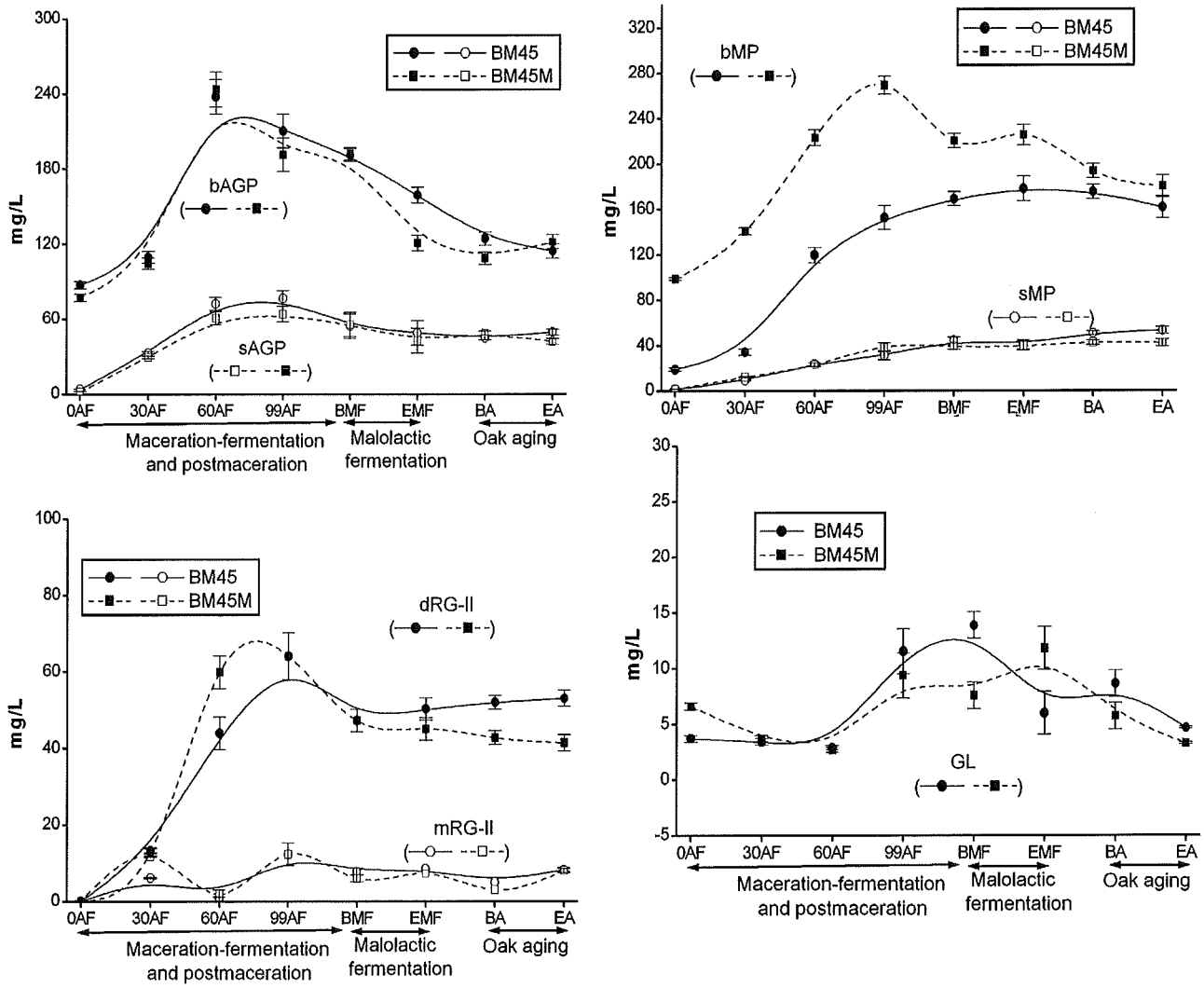


Figure 2. Effect of the use of a yeast overexpresser of mannoproteins on the evolution of total monomeric anthocyanins (TMA), total monomeric flavan-3ols (M-Flava), and total hydroxycinnamic acids (TCin) during vinification and oak aging. See text for conditions and calculations.

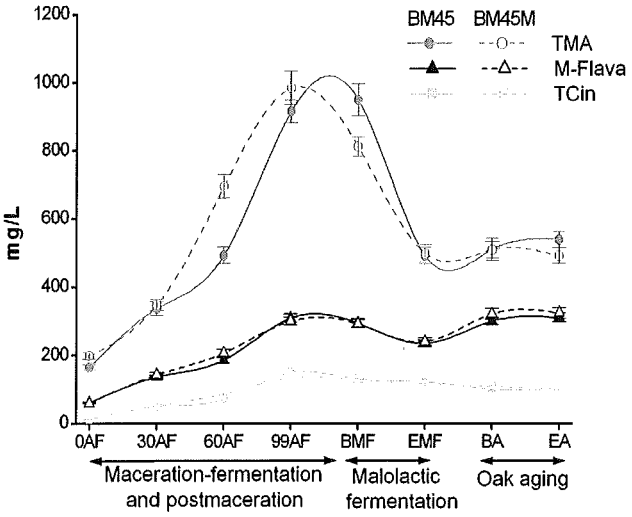


Figure 3. Effect of the use of a yeast overexpresser of mannoproteins on the evolution of total proanthocyanidins (PAs) and total polyphenol index (TPI) during vinification and oak aging. See text for conditions and calculations.

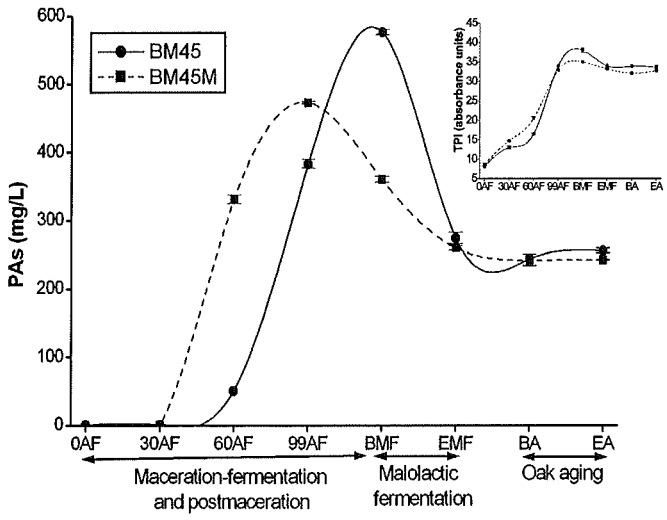
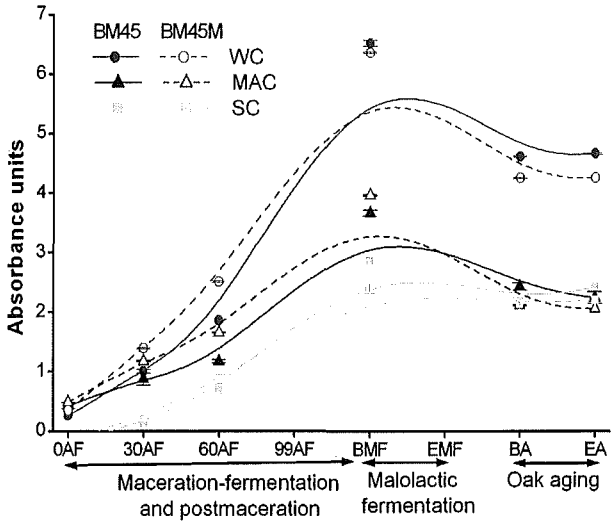


Figure 4. Effect of the use of a yeast overexpresser of mannoproteins on the evolution of wine color (WC), monomeric anthocyanin color (MAC) and stable color (SC) during vinification and oak aging. See text for conditions and calculations



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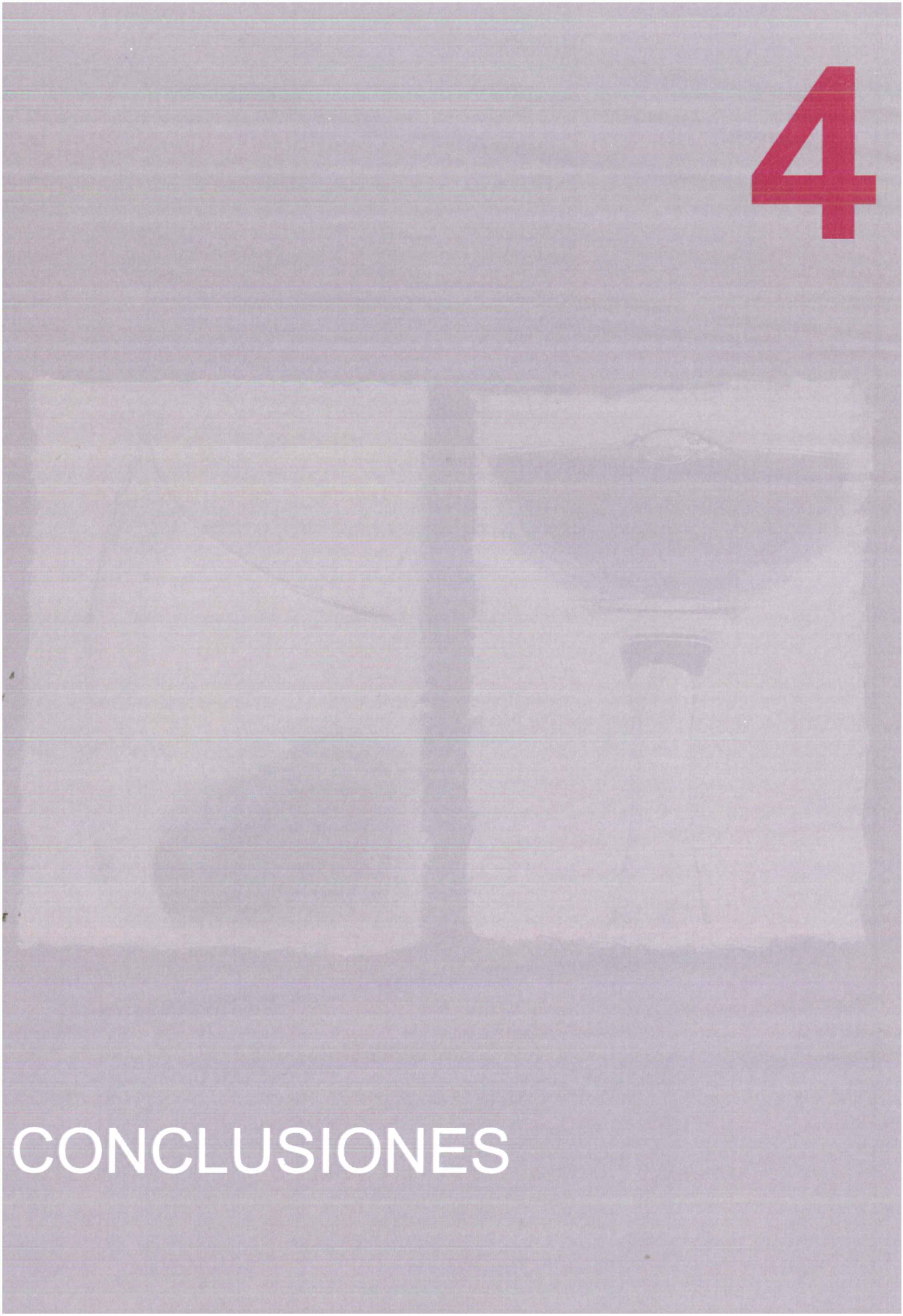


CONCLUSIONES

Las conclusiones generales de esta memoria pueden resumirse en los siguientes puntos:

1. En este trabajo se ha validado un método de análisis que permite la cuantificación de las distintas familias de polisacáridos de la uva y de las levaduras en muestras de mostos y vinos.

2. En este trabajo se ha desarrollado y validado un método de análisis que permite determinar de forma simultánea el contenido de antocianos monómeros, ácidos hidroxicinámicos, flavonoles monómeros y flavanoles, en muestras de mostos y vinos, y produce una mejora sustancial en la cuantificación de las proantocianidinas por el método de la vainillina y en el análisis de pigmentos poliméricos por electroforesis capilar de zona.

3. La vinificación en tinto de Tempranillo produce cambios sustanciales en el perfil y el contenido de las diferentes familias de polisacáridos.

   Los polisacáridos estructurales de bajo peso molecular derivados de celulosas y hemicelulosas son los polisacáridos mayoritarios en los mostos, mientras que los arabinogalactano-proteínas (AGP) predominan en los vinos jóvenes y las manoproteínas (MP) en los vinos envejecidos.

   La maceración-fermentación produce una precipitación de la mayor parte de los polisacáridos estructurales y un enriquecimiento en los polisacáridos de la uva y de las levaduras. Los AGP se extraen más fácilmente que los ramnogalacturonanos tipo II (RG-II).

   La postmaceración produce una disminución en todos los polisacáridos pécticos de uva, especialmente en los AGP, que también precipitan de forma acusada durante la fermentación maloláctica. Por el contrario, las manoproteínas se liberan de forma continua debido a la autolisis de las levaduras.

   La mayor parte de los AGP y MP presentes en los mostos y en los vinos son compuestos de alto peso molecular y se detecta además la forma monomérica del RG-II.

   La precipitación de AGP y MP de alto peso molecular es relevante durante la vinificación, fundamentalmente durante la maceración-fermentación.

   El envejecimiento de los vinos en barrica y en botella no produce ningún cambio significativo en el contenido de las distintas familias de polisacáridos.

4. La vinificación en tinto de Tempranillo produce cambios sustanciales en el contenido de las diferentes familias de polifenoles y por tanto en la estabilidad del color del vino.

   Existen dos etapas diferenciadas de formación de color estable: la maceración-fermentación y el envejecimiento en botella.

   El aumento del color estable durante la maceración-fermentación se debe fundamentalmente a fenómenos de copigmentación mientras que el aumento de color estable durante la estancia del vino en botella es debido únicamente a la formación de pigmentos poliméricos.

   La copigmentación afecta al 30% de los antocianos en los vinos jóvenes, donde ya se detecta la presencia de pigmentos poliméricos, que contribuyen al color del vino en más de un 10%.

   El 35% de los antocianos se encuentra en forma de pigmentos estables en los vinos envejecidos en botella, donde aún se detecta más de un 10% del color debido a la copigmentación.

5. Los tres tratamientos biotecnológicos propuestos en esta tesis incrementan el contenido de polisacáridos totales solubles durante la vinificación.

   El tratamiento cuantitativamente más efectivo para aumentar la concentración de polisacáridos totales es la adición de preparados de manoproteínas comerciales, seguido de la utilización de levaduras superproductoras y enzimas de maceración.

   Las enzimas de maceración comerciales liberan al medio mayor cantidad de polisacáridos de la uva que las enzimas endógenas, pero no actúan sobre las paredes celulares de las levaduras.

   El empleo de manoproteínas industriales o de levaduras superproductoras de manoproteínas supone un incremento en la cantidad de manoproteínas de alto peso molecular pero no afecta al resto de polisacáridos.

   La precipitación de polisacáridos durante la vinificación es más acusada en las muestras elaboradas con los tratamientos biotecnológico, por lo que las diferencias observadas disminuyen en los vinos acabados.

6. El uso de enzimas de maceración comerciales incrementa tanto el color extraído como el color estable de los vinos, así como el contenido de polifenoles totales, dando lugar a vinos con más capa de color, astringencia y tanicidad.

7. Tanto la vinificación con manoproteínas industriales como con levaduras superproductoras de manoproteínas producen una disminución acusada en el contenido de taninos.

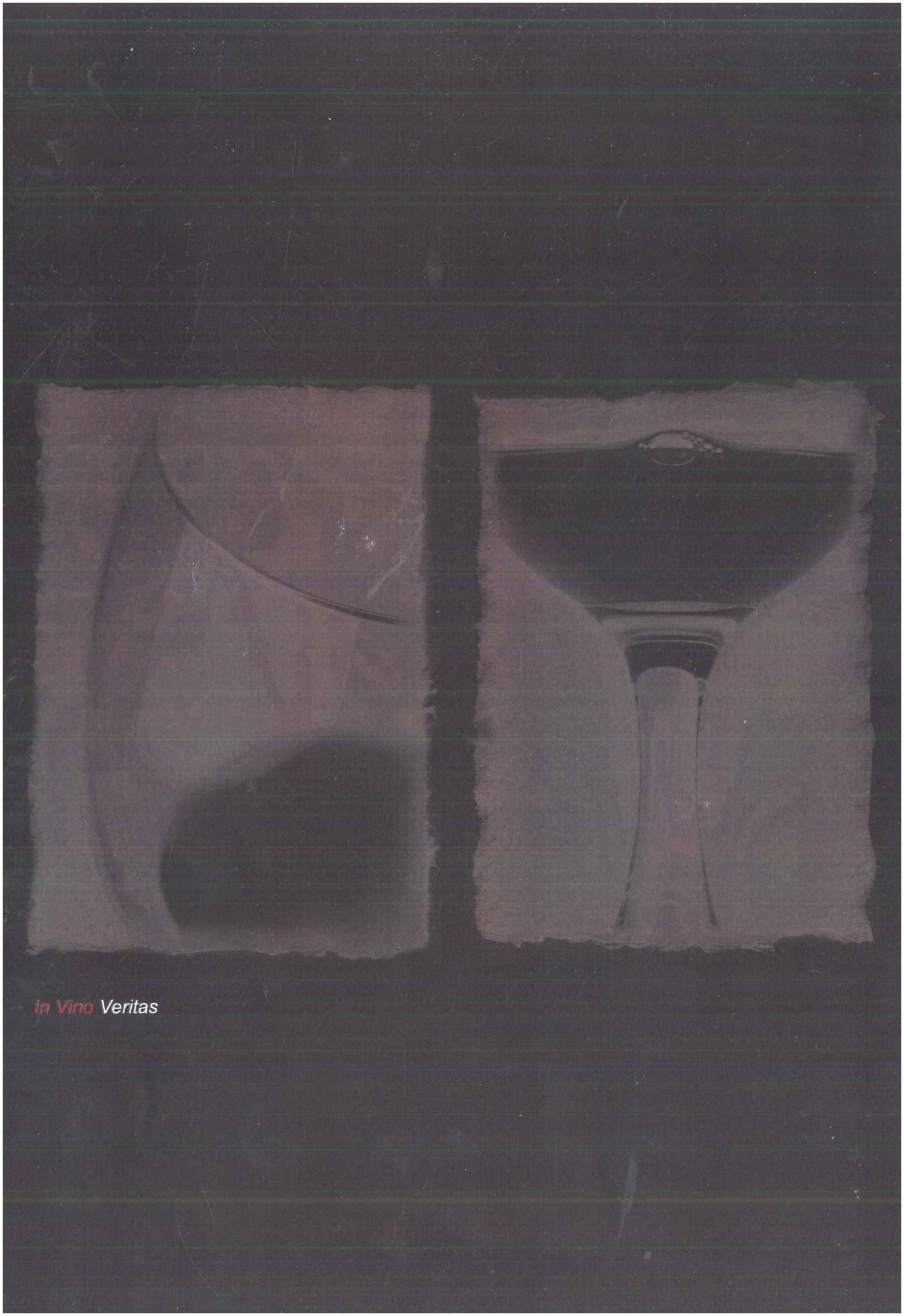
8. El empleo de manoproteínas industriales provoca una desestabilización y precipitación de los pigmentos responsables del color estable de los vinos tintos, pero no se observa este efecto con las manoproteínas liberadas por las cepas superproductoras.

9. Las manoproteínas no afectan al contenido de los diferentes polifenoles monómeros ni al color debido a los antocianos monómeros.

10. Los resultados obtenidos en esta tesis cuestionan el efecto protector de las manoproteínas sobre los taninos y el color estable de los vinos tintos.

    La combinación manoproteína-tanino forma un co-agregado inestable que precipita.

    Futuros estudios serán necesarios para verificar el efecto de las manoproteínas sobre el color estable de los vinos.


In Vino Veritas