"Contribución de las semillas y del raspón sobre la composición, color y calidad del vino tinto; influencia del sistema de vinificación y del tratamiento del racimo"

Olga Pascual García

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Olga Pascual García

“Contribución de las semillas y del raspón sobre la composición, color y calidad del vino tinto; influencia del sistema de vinificación y del tratamiento del racimo”

Tesis Doctoral dirigida por:

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Departament de Bioquímica i Biotecnologia

Tarragona 2017

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Certifican:

Que este trabajo, titulado **"Contribución de las semillas y del raspón sobre la composición, el color y la calidad del vino tinto; influencia del sistema de vinificación y del tratamiento del racimo"**, que presenta **Olga Pascual García** para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de esta Universidad.

Tarragona, 3 de Mayo del 2017

Dr. Fernando Zamora　　　　　　　　Dr. Joan Miquel Canals Bosch

Los análisis de esta Tesis doctoral se realizaron en los laboratorios del Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili, en los laboratorios del Instituto Regional de Investigación Científica Aplicada (IRICA) de la Universidad de Castilla-La Mancha, bajo la dirección de los profesores Joan Miquel Canals Bosch y Fernando Zamora Marín, con la colaboración de los Drs. Sergio Gómez Alonso e Isidro Hermosín Gutiérrez.

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- Innovaciones en el estudio de la astringencia del vino relacionadas con los métodos de análisis, la composición y estructura de los taninos, y los polisacáridos (AGL2014-56594-C2-1-R and AGL2014-56594-C2-2-R). De 2015 a 2017.

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"Viva el buen vino, que es el gran camarada para el camino"

Pío Baroja

Dedicada a mis padres y a mi hermano,

ÍNDICE

INTRODUCCIÓN **15**

1. Los compuestos fenólicos de la uva y sus implicaciones sensoriales en el vino . **17**

1.1. No flavonoides 18

1.1.1. Ácidos fenoles y derivados 18

1.1.2. Estilbenos 20

1.1.3. Taninos hidrolizables 20

1.1.3.1. Galotaninos 21

1.1.3.2. Elagitaninos 21

1.2. Flavonoides 22

1.2.1. Flavonoles, Flavononoles y Flavonas 22

1.2.2. Antocianos 23

1.2.3. Flavan-3-oles 26

2. Distribución de los compuestos fenólicos en las diversas partes de la uva **28**

2.1. Semillas 29

2.2. Pulpa 30

2.3. Piel 30

2.4. Raspón 31

3. Evolución de los compuestos fenólicos a lo largo del proceso de madurez de la uva, vinificación y crianza del vino tinto **31**

3.1. Evolución de los compuestos fenólicos en la maduración de la uva 31

3.2. Factores que influyen en la composición fenólica durante la madurez de la uva 33

3.3. Evolución de los compuestos fenólicos durante la vinificación 35

3.4. Factores que influyen en la extracción de los compuestos fenólicos durante la vinificación 38

3.5. Evolución de los compuestos fenólicos durante la crianza 41

3.5.1. La copigmentación 41

3.5.2. Los pigmentos poliméricos 42

3.5.3. Los piranoantocianos 44

3.5.4. Evolución de las proantocianidinas 46

4. La astringencia y el sabor amargo del vino tinto **47**

4.1. La astringencia 47

4.2. El sabor amargo 49

5. Taninos enológicos: Origen, propiedades y funciones enológicas **50**

6. El oxígeno **52**

6.1. Reacciones de oxidación 53

6.1.1. Oxidaciones enzimáticas 53

6.1.2. Oxidaciones no enzimáticas 54

6.2. Métodos de medición del oxígeno disuelto 55

REFERENCIAS **56**

HIPÓTESIS Y OBJETIVOS **67**

RESULTADOS **71**

CAPÍTULO 1: Influencia de la madurez de la uva y del tratamiento pre-fermentativo del racimo de la variedad Garnacha sobre la composición y calidad del vino. **73**

CAPÍTULO 2: Influencia de las semillas y del raspón en la composición y en la astringencia del vino. **99**
CAPÍTULO 3: Velocidad de consumo de oxígeno por diferentes taninos enológicos en una solución de vino modelo. **135**

DISCUSIÓN GENERAL **157**

CONCLUSIONES GENERALES **165**

INTRODUCCIÓN

1. Los compuestos fenólicos de la uva y sus implicaciones sensoriales en el vino

Los compuestos fenólicos son responsables de algunas de las principales propiedades organolépticas del vino que determinan la calidad de los vinos tintos, tales como el color, el cuerpo, la astringencia y el sabor amargo (Kennedy, 2008). También participan en reacciones de oxidación, en la interacción con proteínas y en los procesos de crianza y envejecimiento de los vinos. Estos compuestos provienen de diversas partes del racimo y se extraen durante la fase de fermentación/maceración (Ribéreau-Gayon et al., 2006). Su concentración en el vino va a depender del tipo de uva, de su nivel de madurez fenólica y del proceso de vinificación. Los compuestos fenólicos están relacionados con los beneficios para la salud debido a sus propiedades antioxidantes, ayudando a la prevención de diversas enfermedades cardiovasculares y algunos tipos de cáncer (Soleas et al., 2002; Rodrigo et al., 2011).

Los compuestos fenólicos se caracterizan por presentar una estructura química común: un núcleo bencénico con uno o más grupos hidroxilo (-OH) (Monagas et al., 2005). Se dividen en dos grandes grupos según su estructura química, basándose en su esqueleto carbonatado: los no flavonoides y los flavonoides (**Figura 1**).

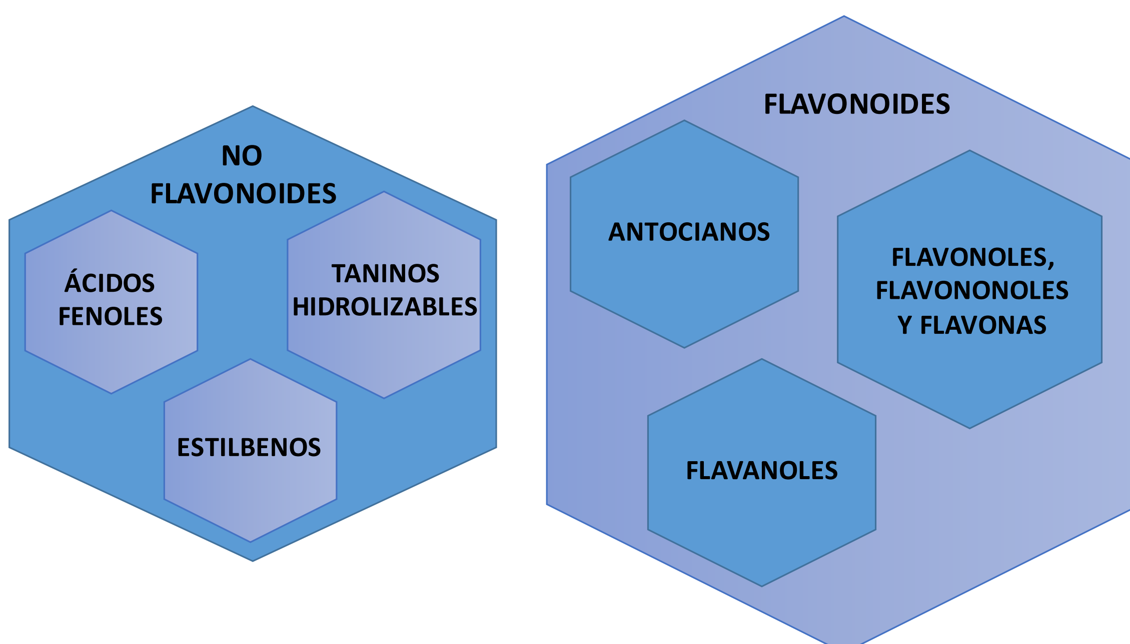


Figura 1. Clasificación de los compuestos fenólicos.

Los compuestos fenólicos no flavonoides en la uva están mayoritariamente en la pulpa, mientras que los flavonoides se localizan en las semillas, hollejos y raspones (Monagas et al., 2005) (**Figura 2**).

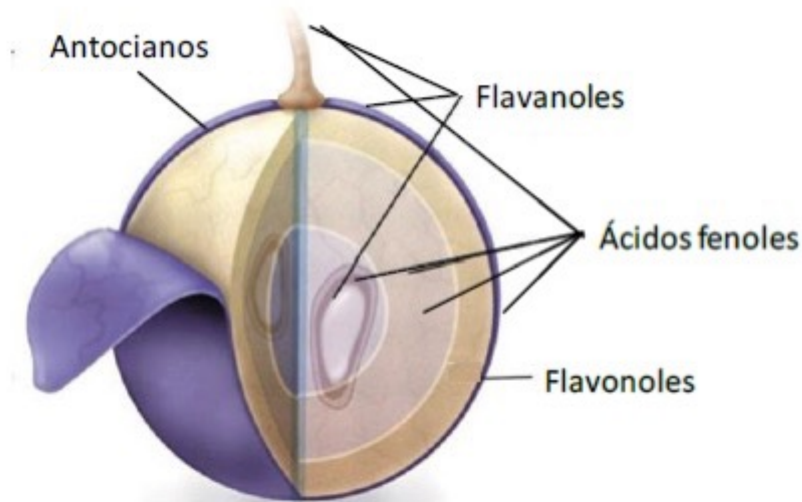


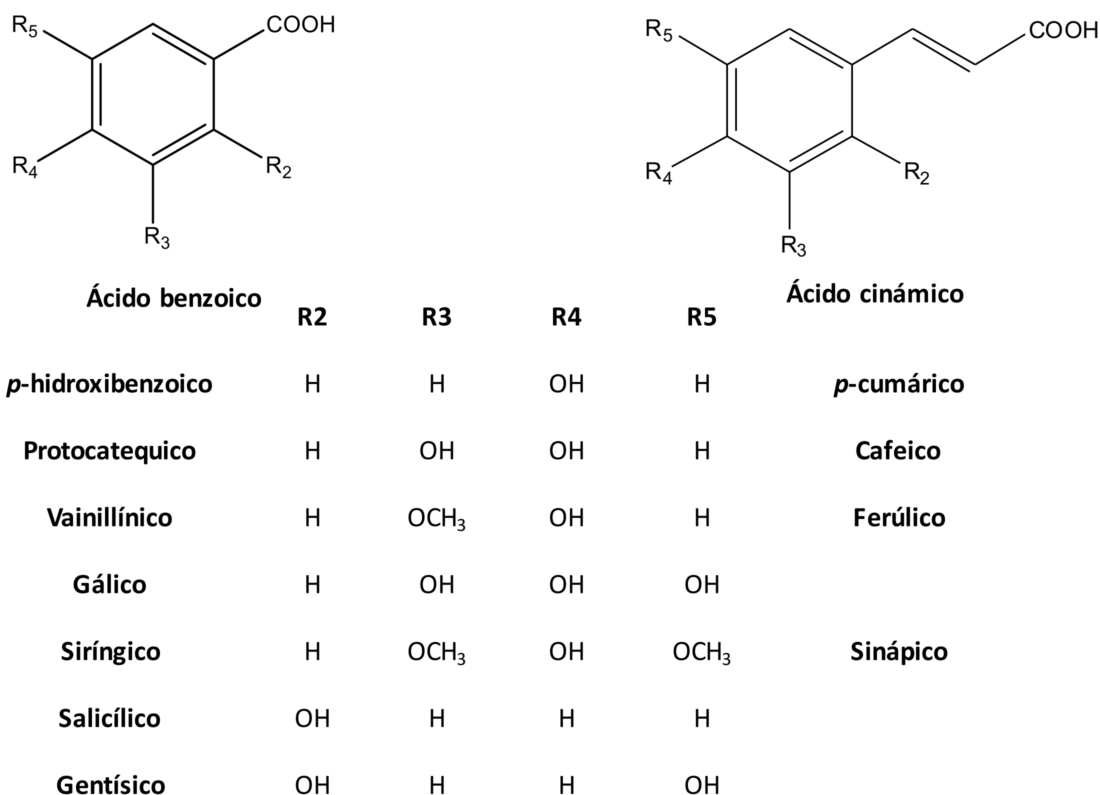
Figura 2. Distribución de los compuestos fenólicos en la uva.

1.1. No flavonoides

Los principales compuestos fenólicos no flavonoides que se encuentran en la uva y en el vino tinto son los ácidos fenoles, en su forma libre o esterificada, los estilbenos y los taninos hidrolizables.

1.1.1. Ácidos fenoles y derivados

Los ácidos fenoles son incoloros, aunque con el tiempo y oxidación dan lugar al pardeamiento de los vinos. También pueden actuar como copigmentos afectando así indirectamente al color del vino tinto. Estos ácidos participan poco en los atributos sensoriales del vino, pero bajo la acción de algún microorganismo pueden dar lugar a la aparición de fenoles volátiles, con olores desagradables (sudor de caballo, cuero, etc.) (Chatonnet et al., 1992).



Ácido benzoico	R2	R3	R4	R5	Ácido cinámico
***p*-hidroxibenzoico**	H	H	OH	H	***p*-cumárico**
Protocatequico	H	OH	OH	H	**Cafeico**
Vainillínico	H	OCH_3	OH	H	**Ferúlico**
Gálico	H	OH	OH	OH	
Siríngico	H	OCH_3	OH	OCH_3	**Sinápico**
Salicílico	OH	H	H	H	
Gentísico	OH	H	H	OH	

Figura 3.1. Estructura química de los ácidos fenoles.

Los ácidos fenoles se caracterizan por presentar un solo anillo bencénico. Se pueden dividir en dos grupos: los ácidos benzoicos (C1-C6) y los ácidos cinámicos (C3-C6) (**Figura 3.1**). Se localizan en las vacuolas de las células de la pulpa y del hollejo de la uva, siendo mayoritarios en este último. En la uva, los ácidos benzoicos se encuentran principalmente en su forma libre o como ésteres, y los ácidos cinámicos se encuentran mayoritariamente en forma esterificada con el ácido tartárico. El ácido gálico es uno de los ácidos benzoicos con mayor concentración en la uva, tanto en su forma libre como en la de éster de flavan-3-ol (galato de (-)-epicatequina). Los principales ácidos cinámicos presentes en las uvas son los ácidos caftárico, cutárico y fertárico en su forma *trans*, que es más estable (**Figura 3.2**). Aunque también se han hallado pequeñas cantidades del isómero *cis*. El ácido caftárico es el predominante (Monagas et al., 2005; Rentzsch et al., 2009). Los compuestos no flavonoides mayoritarios en la uva son los ácidos cinámicos.

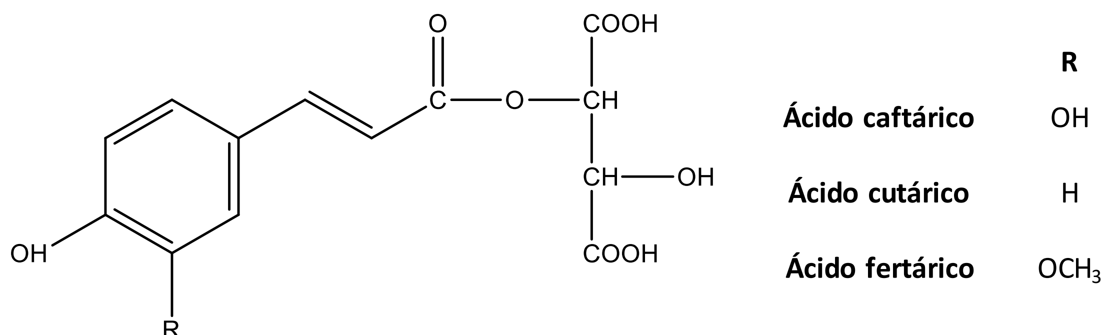
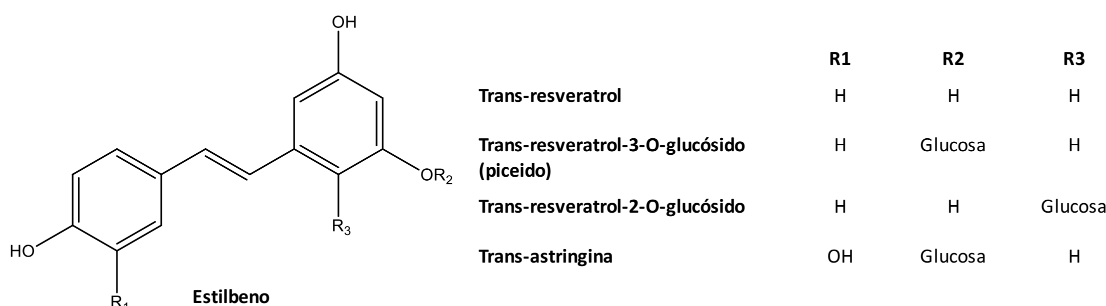


Figura 3.2. Estructura de los ácidos cinámicos esterificados.

1.1.2. Estilbenos

Los estilbenos (C6-C2-C6) se caracterizan por tener dos anillos bencénicos unidos por un doble enlace, lo que hace que tengan una alta reactividad. El *trans*-resveratrol y su derivado glucosilado (piceido) son los estilbenos mayoritarios de la uva (**Figura 4**). Estos compuestos se sintetizan en la vid como una respuesta frente a situaciones de estrés biótico y abiótico. Carecen de propiedades organolépticas, pero son beneficiosos para la salud humana, por su importancia como antioxidantes, anticancerígenos y protectores de enfermedades cardiovasculares (Athar et al., 2007; Das et al., 2010; Leifer & Barberio, 2016).



	R1	R2	R3
Trans-resveratrol	H	H	H
Trans-resveratrol-3-O-glucósido (piceido)	H	Glucosa	H
Trans-resveratrol-2-O-glucósido	H	H	Glucosa
Trans-astringina	OH	Glucosa	H

Figura 4. Estructura química de los estilbenos.

1.1.3. Taninos hidrolizables

Los taninos hidrolizables se dividen en dos grupos, los galotaninos y los elagitaninos. Estos taninos se caracterizan por tener una molécula de glucosa y por liberar ácido gálico (galotaninos) o ácido elágico (elagitaninos) por hidrolisis ácida. Los taninos hidrolizables no proceden de la uva, pero pueden estar presentes en el vino ya que son aditivos

enológicos ampliamente utilizados en vinificación (Ribéreau-Gayon et al., 2006). Asimismo, la madera de roble libera elagitaninos en los vinos de crianza, siendo mayoritarios la vescalagina y la castalagina (Fernández de Simón et al., 1999; Navarro et al., 2016).

1.1.3.1. Galotaninos

La estructura química de los galotaninos consiste en una molécula de glucosa, cuyos grupos OH están total o parcialmente esterificados con una molécula de ácido gálico (**Figura 5**). Tiene un sabor ácido y dan sensaciones muy amargas y algo astringentes (Delteil, 2000; Zamora, 2003) y no contribuyen significativamente a la estructura en boca del vino (Crespy, 2002a).

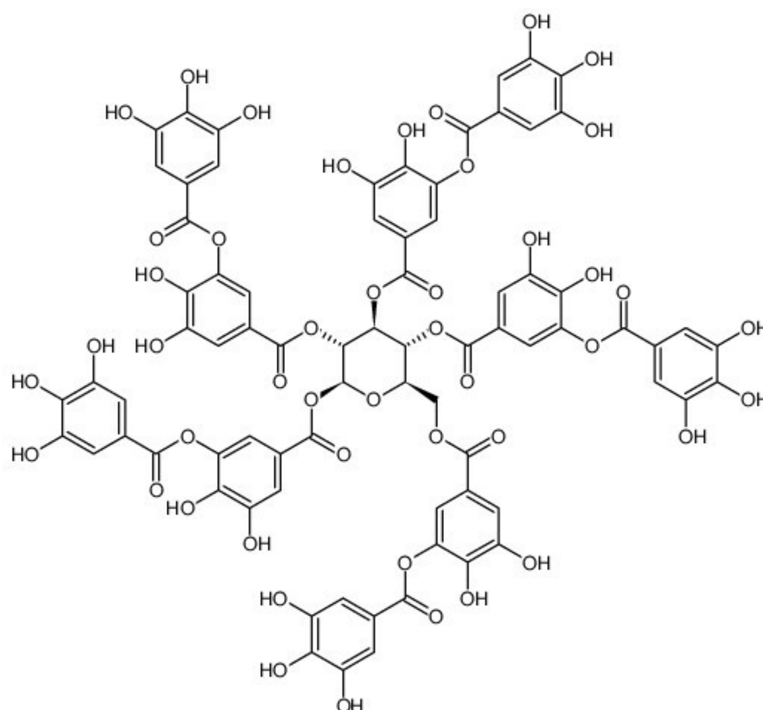


Figura 5. Estructura química de un galotanino.

1.1.3.2. Elagitaninos

Los elagitaninos presentan una estructura química compleja, son esteres del ácido hexahidroxidifénico (HHDP) y nonahidroxitrifénico (NHTP) y monosacáridos, generalmente glucosa (Takuo et al., 2009). Puede haber monómeros, oligómeros o C-glicosídos. En la **Figura 6** se muestran los principales elagitaninos presentes en la madera

de roble. En los vinos de crianza en barricas de roble, se han identificado flavano-elagitaninos (acutisimina A/B, epiacutisimina A/B), debidos a la unión de la vescalagina con una catequina y/o epicatequina (Jourdes et al., 2011). Los elagitaninos participan y/o influyen en el proceso de oxidación de los vinos tintos (Vivas, 1996; Navarro et al., 2016). Sensorialmente, estos taninos contribuyen a la sensación de astringencia y en el sabor amargo (Glabasnia & Hofmann 2006; Michel et al., 2011).

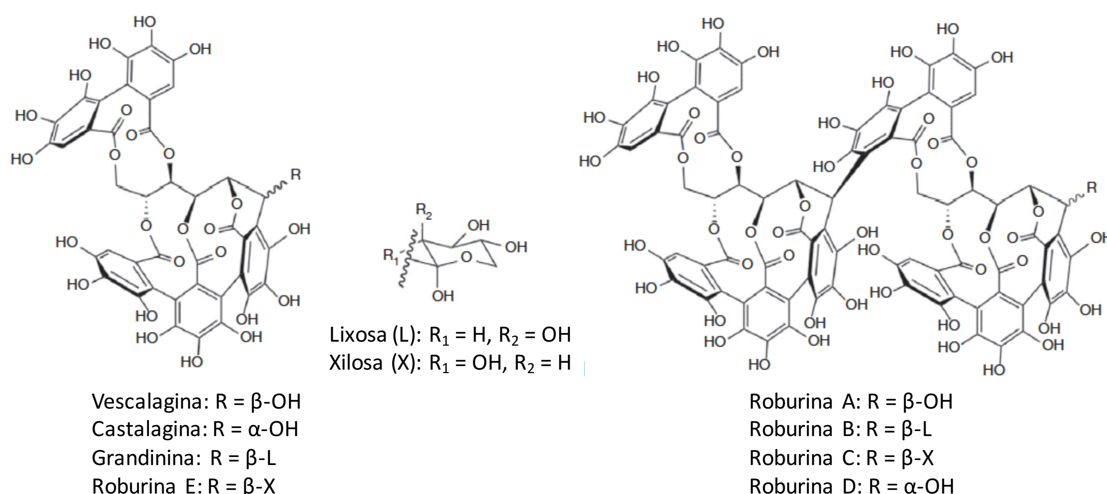


Figura 6. Estructura química de los ocho principales elagitaninos.

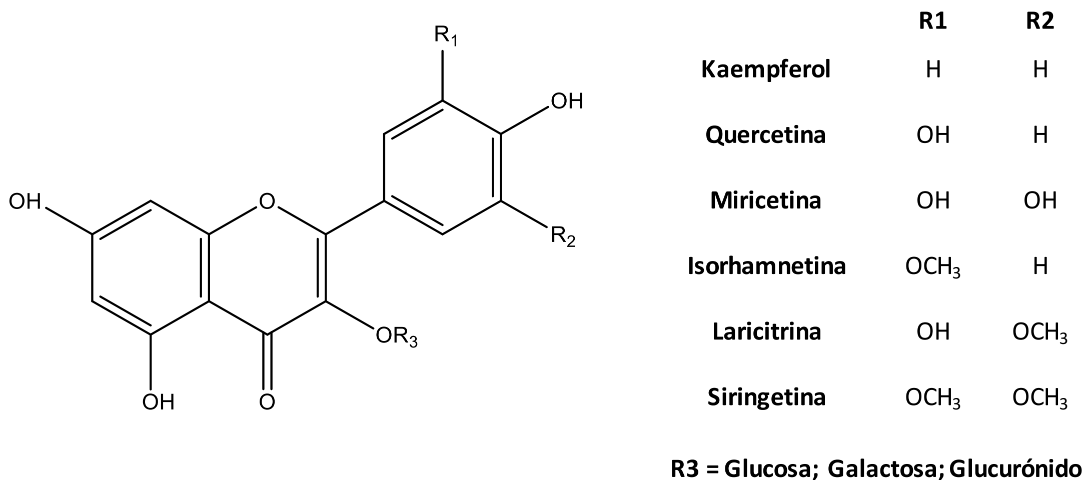
1.2. Flavonoides

Los flavonoides poseen un esqueleto de 15 átomos de carbono con estructura C6-C3-C6 formado por 3 anillos (A, B y C). Su clasificación se basa en el grado de insaturación del anillo C y de la presencia de grupos hidroxilos y metoxilos del anillo B (Fulcrand et al., 2006). Estos compuestos tienen la particularidad de que pueden actuar tanto como electrófilos como de nucleófilos, de ahí su alta reactividad en el vino (Kennedy et al., 2006). Los antocianos y flavanoles son los más abundantes en la uva y en el vino.

1.2.1. Flavonoles, Flavononoles y Flavonas

Los flavonoles son pigmentos de color amarillo, que se encuentran principalmente en los hollejos. Su concentración en la uva está en el rango de 10 a 300 mg/kg dependiendo de la variedad (Ferrandino et al. 2012). Están presentes en el vino en forma de 3-O-

glicósidos de agliconas (**Figura 7**). En los vinos tintos estos compuestos afectan al color por medio de la copigmentación (Boulton, 2001). A nivel sensorial, la quercetina aporta sensaciones de amargor y astringencia (Hufnagel & Hoffman, 2008; Sáenz-Navajas et al., 2010).



	R1	R2
Kaempferol	H	H
Quercetina	OH	H
Miricetina	OH	OH
Isorhamnetina	OCH_3	H
Laricitrina	OH	OCH_3
Siringetina	OCH_3	OCH_3

R3 = Glucosa; Galactosa; Glucurónido

Figura 7. Estructura química de los flavonoles.

Las flavonas y los flavononoles también son pigmentos amarillos. La estructura química de estos compuestos es similar a la de los flavonoles (**Figura 8**). Las principales flavonas identificadas en la uva y el vino son apigenina y luteolina, y los flavononoles son astilbina y engeletina (Monagas et al., 2005).

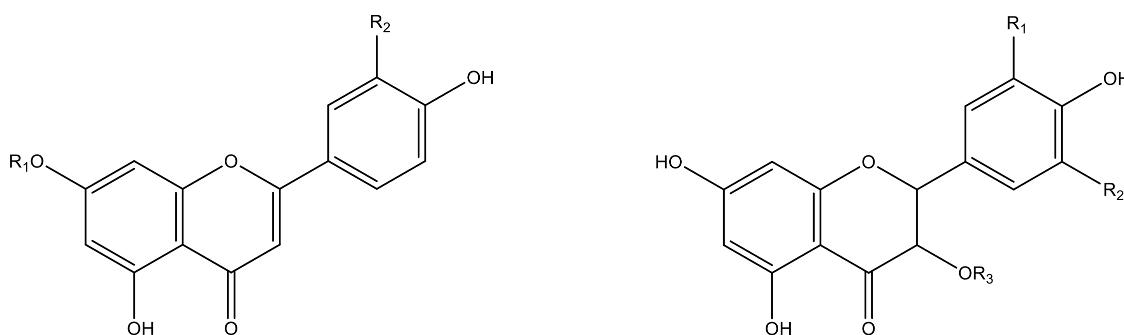


Figura 8. Estructura química de las flavonas y los flavononoles.

1.2.2. Antocianos

Los antocianos son los responsables del color rojo de las uvas y los vinos. Se localizan en la piel de la uva. También existen algunas variedades tintoreras que presentan antocianos en la pulpa (Castillo-Muñoz et al. 2009). Dependiendo de la variedad, la

concentración de antocianos en las pieles de uva tinta se encuentra entre 500 y 3000 mg/kg (Ferrandino et al., 2012). El antociano mayoritario en la mayor parte de las variedades viníferas es la malvidina-3-O-glucosido. Si bien en la uva se encuentran en forma glicosilada (antocianinas), en el vino pueden ser hidrolizadas a sus correspondientes agliconas (antocianidinas), que son mucho más fácilmente degradables.

La estructura de los antocianos, consiste en una aglicona con dos anillos aromáticos unidos a un heterociclo oxigenado. En *Vitis vinifera* se distinguen mayoritariamente cinco tipos de moléculas de antocianinas, aunque también se ha encontrado algo de pelargonina en variedades como Cabernet Sauvignon y Pinot Noir (He et al. 2010) (**Figura 9**). Los antocianos parecen carecer de propiedades gustativas a las concentraciones encontradas habitualmente en vinos tintos (0,3–1,2 g/L) (Vidal et al., 2004).

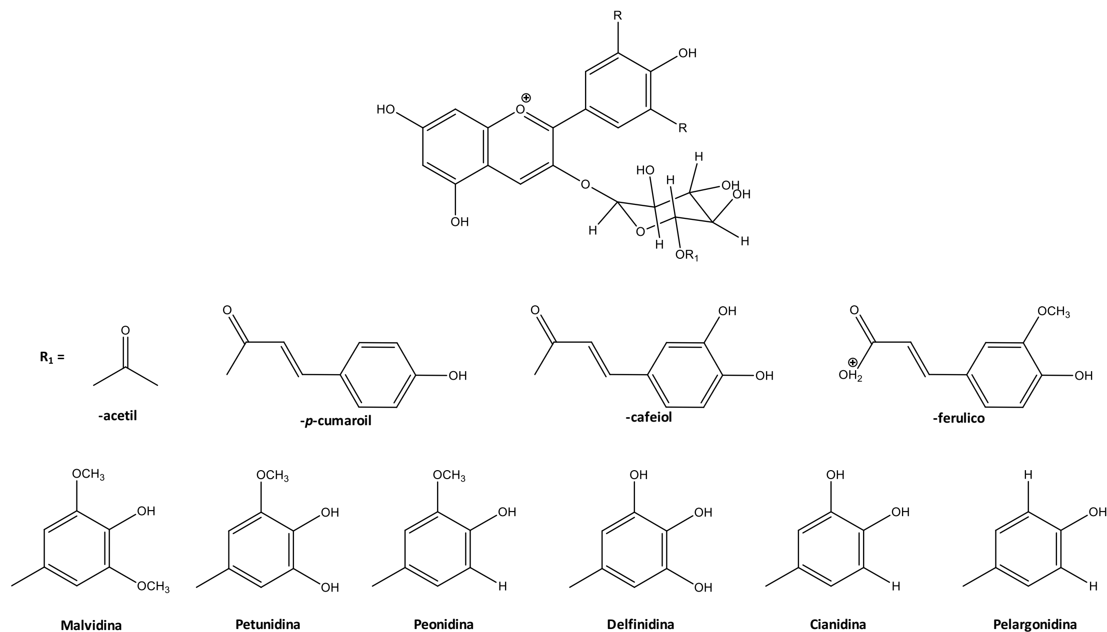


Figura 9. Estructura de los antocianos.

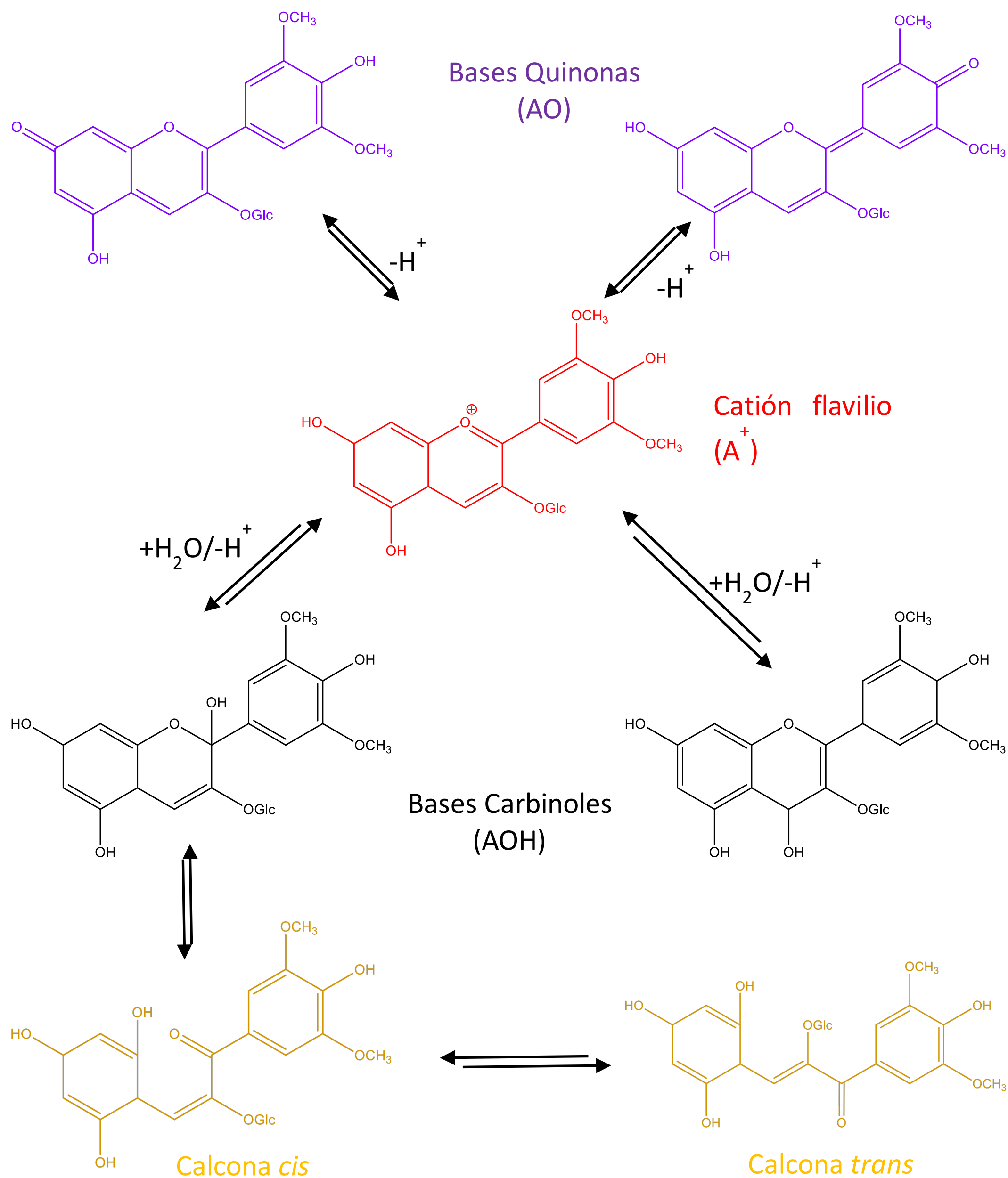


Figura 10. Cambio de color de los antocianos en función del pH.

El color de los antocianos va a depender del pH, entre otros parámetros. Estas moléculas se pueden encontrar en forma de cuatro estructuras básicas, el catión flavilio (A^+) de color rojo, la base anhidra quinoidal (AO) de color azul-violáceo, la pseudo-base carbinol o hemiacetal (AOH) incolora y la base calcona (C) de color amarillo (**Figura 10**).

A pH muy ácido el catión flavilio es la forma mayoritaria, pero a medida que el pH del medio aumenta, pasa a base quinoidal o a la pseudobase carbinol. Además, la pseudobase carbinol puede transformarse en la base calcona. Esta transformación está

favorecida por temperaturas altas y por la luz (Furtado et al., 1993). La calcona *trans* puede oxidarse a ácidos fenólicos (incoloros). Por lo que una mala conservación del vino puede dar lugar a importantes pérdidas de color.

A pH del vino (3.2-4.5), las cuatro formas coexisten siendo predominante las formas incoloras, representando entre 65-85% del total de los antocianos. Seguido de la forma catión flavilio (5-30%) y de la azul-violácea (2-5%). A pH muy ácido predomina el color rojo, en cambio cuando el pH aumenta se incrementa el incoloro (Glories, 1984) (**Figura 11**).

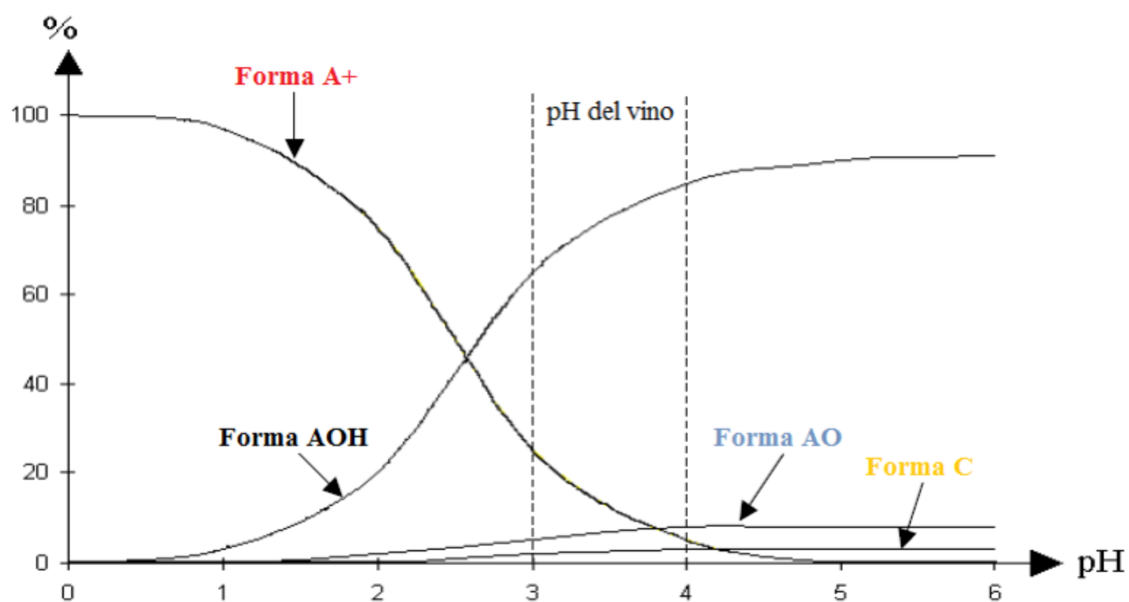
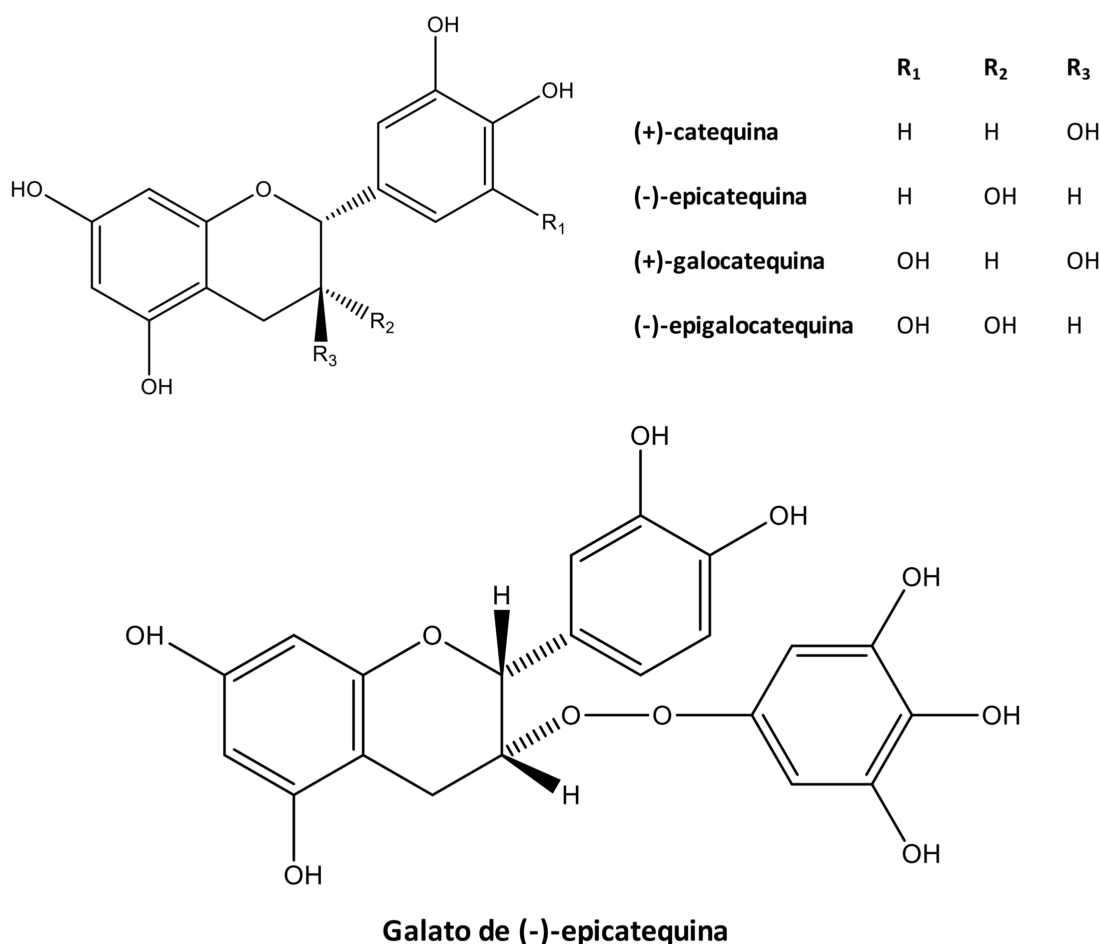


Figura 11. Evolución de la proporción de los antocianos en función del pH (Glories, 1984).

1.2.3. Flavan-3-oles

Los flavan-3-oles o flavanoles los podemos encontrar como monómeros (denominados coloquialmente como catequinas) (**Figura 12**) o como oligómeros y polímeros (proantocianidinas o taninos condensados) en las semillas, hollejos y raspones. Los flavan-3-oles influyen en la estabilidad del color, ya sea como copigmentos o por condensación con los antocianos originando nuevos pigmentos. También son responsables de la astringencia, del sabor amargo, del cuerpo y de la capacidad para envejecer del vino (Ribéreau-Gayon et al., 2006).



	R_1	R_2	R_3
(+)-catequina	H	H	OH
(-)-epicatequina	H	OH	H
(+)-galocatequina	OH	H	OH
(-)-epigalocatequina	OH	OH	H

Figura 12. Principales flavan-3-oles monómeros de la uva.

Los flavanoles monómeros con una alta concentración en la uva son la (+)-catequina, su isómero la (-)-epicatequina y el galato de (-)-epicatequina, y los que presentan una menor concentración son la (-)-epigalocatequina y la (+)-galocatequina (Terrier et al., 2009a).

Las proantocianidinas o taninos condensados poseen la propiedad de liberar antocianidinas por hidrólisis ácida a alta temperatura (Reacción de Bate-Smith; Bate-Smith, 1981). Esta es la razón por la que se denominan proantocianidinas. Según la antocianidina que liberen se clasificaran como procianidinas, prodelfinidinas, propelargoninas, profisetinidinas o prorobinetinidinas. En la uva tan sólo hay procianidinas y prodelfinidinas, pero en el vino se pueden encontrar otras proantocianidinas ya que algunos taninos enológicos procedentes de otras fuentes vegetales los contienen (Versari et al., 2013).

Las semillas contienen solo procianidinas, mientas los hollejos poseen procianidinas y prodelfinidinas (Monagas et al., 2005). Las proantocianidinas se pueden distinguir en función de la longitud de su cadena en oligómeros que se caracterizan por tener un grado medio de polimerización (mDP por la expresión inglesa *mean degree of polymerization*) entre 2-5 unidades, y polímeros que son moléculas con un mDP mayor de 5 unidades. Esto varía en función de las técnicas de detección usadas (Waterhouse et al. 2000). También dependiendo del tipo de unión interflavánica encontramos las procianidinas de tipo A y B. En las de tipo A las unidades de flavanol poseen un enlace tipo éter entre C2-C5 o C2-C7 y las de tipo B están formadas por monómeros unidos por enlaces C4-C6 o C4-C8, siendo este último más abundante (Terrier et al., 2009a). La mayoría de las procianidinas identificadas en la uva se unen por enlaces tipo B.

Los taninos de semillas tienen procianidinas altamente galoiladas (galato de (-)-epicatequina) y poseen un mDP alrededor de 10. En cambio, los taninos de piel no presentan tanta galoilación, están compuestos de procianidinas y prodelfinidinas y poseen un mDP de cerca de 30. Existe muy poca información sobre los taninos del raspón, si bien parecen estar compuestos también por procianidinas y prodelfinidinas con mDP similar al de las semillas, alrededor de 10 unidades (Monagas et al. 2005).

2. Distribución de los compuestos fenólicos en las diversas partes de la uva

Las uvas son los frutos de la vid botánicamente conocidos como bayas que se agrupan en un órgano herbáceo o leñoso conocido como el raspón o escobajo, en forma de racimo. Las bayas se unen al raspón por medio del pedicelo, por el cual se suministra a la baya de agua y nutrientes. Principalmente, las bayas están constituidas por agua, azúcares, minerales, ácidos orgánicos, compuestos nitrogenados, lípidos, compuestos volátiles y compuestos fenólicos.

Cada baya está formada por un conjunto de tejidos, denominado pericarpio, que envuelve y protege a las semillas. El pericarpio se puede dividir en exocarpio (hollejo o piel), mesocarpio (pulpa) y endocarpio (tejido que recubre las semillas). Cuando las uvas

alcanzan su madurez, los porcentajes en peso de las diversas partes de la uva respecto del racimo son aproximadamente del 0 al 6% semillas, del 75 al 85% pulpa, del 8 al 20% piel y del 3 al 7% raspón (**Figura 13**) (Ribéreau-Gayon et al., 2006).

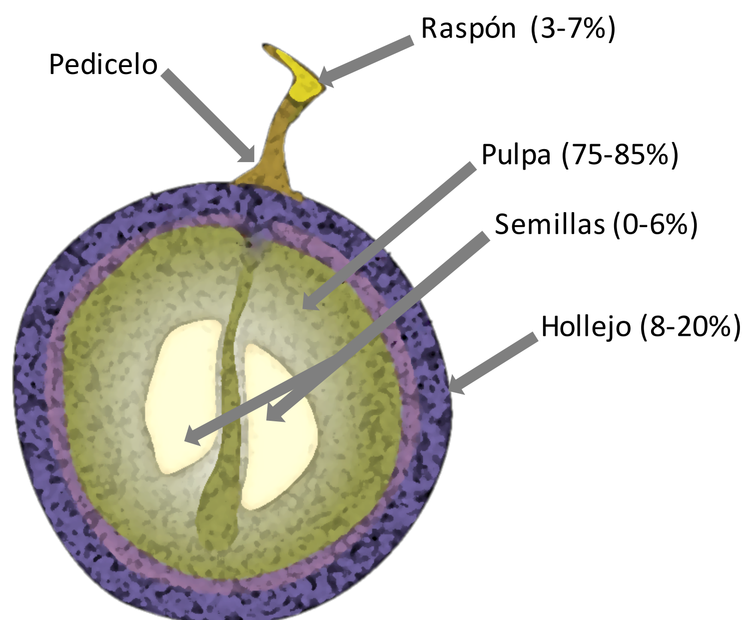


Figura 13. Estructura de una baya y el porcentaje de cada parte en función del peso fresco de un racimo de uva maduro.

2.1. Semillas

Los granos de uva pueden contener un máximo de cuatro semillas y/o también se puede dar que no contengan ninguna, una, dos o tres. Esto está relacionado con el tamaño del grano, a mayor tamaño de la baya mayor es el número de semillas que contiene (Gil et al., 2015). Las semillas contienen agua (25-45%), hidratos de carbono (35%), aceites (13-20%), compuestos nitrogenados (4-6.5%), taninos (4-6%) y minerales (2-4%). Uno de los componentes más importante de las semillas son los compuestos fenólicos, ya que, dependiendo de la variedad de uva, estos representan un 22-56% del total de los polifenoles de la baya (Ribéreau-Gayon et al., 2006). Los compuestos fenólicos mayoritarios en las semillas son las proantocianidinas (Hernández-Jiménez et al., 2009), la (+)-catequina, la (-)-epicatequina, el galato de (-)-epicatequina y el ácido gálico entre otros. Los taninos se localizan en la epidermis, el tegumento externo y el tegumento interno (Cadot et al., 2006), extrayéndose mayoritariamente en la maceración.

2.2. Pulpa

La pulpa es la parte más voluminosa de la baya. Está formada por un tejido de acumulación de sustancias. La mayor parte del tejido está ocupado por vacuolas, las cuales contienen el mosto, que es un líquido turbio, denso y ligeramente coloreado. El componente mayoritario que contiene la pulpa es el agua, representando aproximadamente un 80%. Principalmente, el mosto contiene azúcares (esencialmente glucosa y fructosa) y ácidos orgánicos (tartárico y málico, y en menor cantidad cítrico). La concentración de azúcares reductores en uvas maduras está entre 150 a 240 g/L. La acidez de un mosto puede estar entre 4 y 15 g/L expresados en ácido tartárico y su pH se encuentra entre 2.8 y 3.8. El mosto es muy rico en cationes, siendo el potasio el más abundante. También contiene compuestos nitrogenados, aminoácidos, pectinas, compuestos volátiles y polifenoles (Ribéreau-Gayon et al., 2006).

Los compuestos fenólicos que contiene la pulpa son mayoritariamente ácidos fenoles, esencialmente ácidos cinámicos esterificados con ácido tartárico. En cambio, se encuentran muy pocos polifenoles de tipo flavonoide, excepto en el caso de variedades tintoreras, en las que la pulpa contiene antocianos.

2.3. Piel

El hollejo es la parte exterior de la baya y está formado por un tejido heterogéneo. Su composición es muy similar a la del mosto, también contiene mayoritariamente agua, ácidos orgánicos, sustancias minerales, azúcares, entre otros (Ribéreau-Gayon et al., 2006). Esencialmente, la piel se caracteriza por contener cantidades significativas de productos secundarios que acumula durante la maduración, como son los compuestos fenólicos y sustancias aromáticas (Carbonell Bejerano & Martínez Zapater, 2013).

Los compuestos fenólicos que se encuentran en el hollejo son ácidos fenoles (mayoritariamente ácidos cinámicos), flavonoles, flavanoles y antocianos. Los polifenoles se sitúan especialmente en la epidermis y en la primera capa subepidérmica.

2.4. Raspón

El raspón es el esqueleto del racimo de uvas y dependiendo sobretodo de la variedad de vid tendrá una forma más o menos compacta. El tamaño definitivo del raspón se alcanza cerca del envero y durante la maduración se lignifica. En cuanto a su composición, el raspón contiene poco azúcar (<10 g/kg) y una concentración de ácidos de entre 180 y 200 meq/kg, que mayoritariamente se encuentran en forma de sales, debido a la gran cantidad de cationes presentes. Especialmente es rico en potasio y el pH de su jugo es superior a 4. También es muy rico en compuestos fenólicos, representando un 20% del total de polifenoles del racimo (Ribéreau-Gayon et al., 2006). Los compuestos fenólicos mayoritarios son los flavan-3-oles, seguidos de los ácidos fenólicos, los flavonoles (destacando la quercetina-3-glucurónido) y la astilbina (Souquet et al., 2000).

3. Evolución de los compuestos fenólicos a lo largo del proceso de madurez de la uva, vinificación y crianza del vino tinto

3.1. Evolución de los compuestos fenólicos en la maduración de la uva

El momento de cosechar la uva es un aspecto fundamental que va influir en la calidad del vino. El proceso de maduración es complejo y va a depender de muchos factores. La madurez tecnológica o madurez de la pulpa está relacionada con la concentración de azúcar, la acidez total y el pH (Nogales-Bueno et al., 2014). La uva adquiere esta madurez cuando la relación azúcar/acidez alcanza su valor máximo. La madurez fenólica o madurez de la piel es la madurez óptima de la uva para elaborar vinos tintos de alta calidad (Zamora 2003). La madurez fenólica mide el potencial de proantocianidinas y antocianos presentes en las uvas, al igual que su capacidad de extracción durante la elaboración del vino. Lo ideal sería que las uvas alcanzaran la madurez tecnológica y la fenólica simultáneamente, pero desafortunadamente la realidad acostumbra a ser muy distinta. Por lo que el momento óptimo de vendimia dependerá del vino final que se desee elaborar.

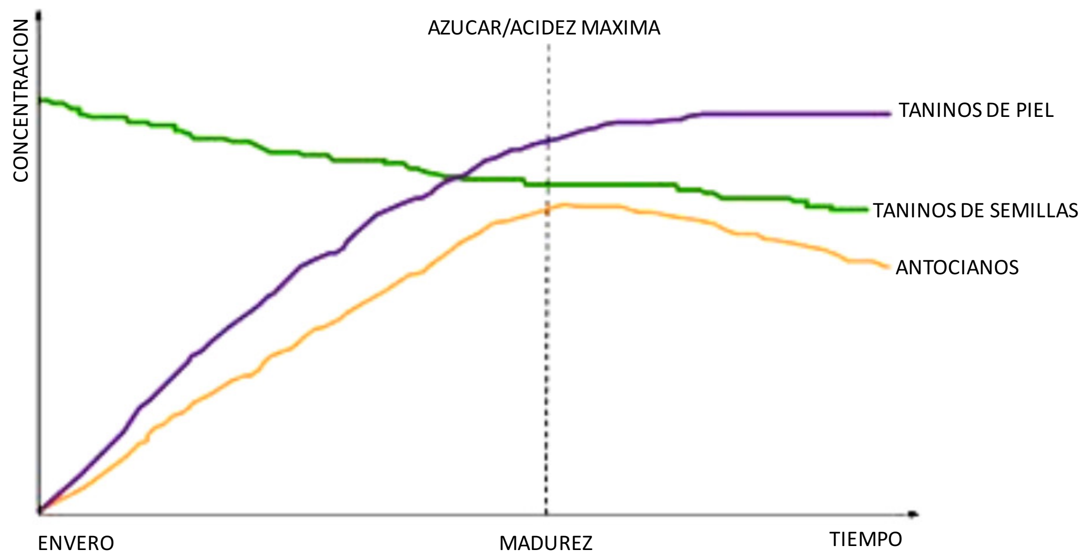


Figura 14. Evolución de los compuestos fenólicos durante la maduración (Ribéreau-Gayon et al., 2006).

La **Figura 14** muestra la evolución de los compuestos fenólicos durante la maduración de la uva. Los antocianos se acumulan en la piel aproximadamente dos semanas antes de la aparición del color en las uvas, incrementándose a lo largo de la maduración, alcanzando un máximo que generalmente coincide con la madurez tecnológica y después disminuye lentamente. La concentración de antocianos puede disminuir justo antes de la cosecha (Ryan y Revilla, 2003) y/o durante la etapa de sobremaduración (Fournand et al., 2006).

En cuanto a los taninos, su evolución es diferente dependiendo de su procedencia. Los taninos de piel se incrementan en el envero aumentando a lo largo de la maduración, aunque su máximo se alcanza pocos días después que el de los antocianos, por lo que la maduración de ambos compuestos no siempre coincide. Conforme la uva madura, la capa interna de la piel se vuelve más fina y, en presencia de etanol, la capa externa se solubiliza, lo que facilita una mayor extracción. Por el contrario, la concentración de taninos de semilla disminuye tras el envero hasta la maduración. Simultáneamente las semillas cambian de coloración de verde a amarillo y finalmente a tonalidades de color marrón grisáceo (**Figura 15**). Estos cambios de color de las semillas se pueden usar como indicador de la madurez de la uva (Quijada-Morín et al., 2016). Con la madurez, las semillas endurecen su superficie con una cutícula lignificada que dificultará la extracción

de los compuestos fenólicos en ausencia de etanol (De Freitas et al., 2000). Este cambio en la coloración de las semillas también puede deberse a fenómenos de oxidación (Kennedy et al., 2000). Durante la maduración de la uva, el contenido de proantocianidinas global aumenta, al igual que el grado medio de polimerización (Kountoudakis et al., 2011a; Gil et al., 2012).

Figura 15. Evolución del color de las semillas durante la maduración de la uva.

Los flavonoles evolucionan de una forma muy similar a los antocianos, su concentración aumenta rápidamente a partir del envero hasta alcanzar un máximo, decreciendo ligeramente en las últimas etapas de madurez. Su concentración en la uva está favorecida con una mayor radiación solar (Ristic et al., 2007).

La cantidad de ácidos cinámicos en la uva aumenta durante el periodo herbáceo alcanzando su máximo justo antes del envero, seguido de un decrecimiento en el periodo de maduración. Este descenso en la concentración de los ácidos cinámicos puede estar relacionada con su catabolismo y con su uso como sustrato para la biosíntesis de otros compuestos fenólicos (Adams, 2006).

3.2. Factores que influyen en la composición fenólica durante la madurez de la uva

La cantidad y composición de estos compuestos va a depender de una serie de factores, que se pueden agrupar en factores permanentes (el clima, el suelo, la variedad, etc.), factores variables (la temperatura, la radiación solar, etc.), factores accidentales (las heladas, infecciones, etc.) y factores modificables (la poda, el abonado, etc.). Los factores permanentes que tienen una acción constante son los que imprimen la tipicidad

de un vino. Serían por tanto el conjunto de factores que se suele englobar bajo el conocido término francés de *terroir*. La temperatura, la radiación solar y la humedad son principales factores climáticos que afectan a la maduración de la uva. Son muy variables y es casi imposible que dos añadas se parezcan.

El suelo es un factor de gran importancia y determinante, ya sea por su composición, orientación, latitud y situación. Los suelos tienen la habilidad de retener agua y nutrientes. Los suelos que tienen una alta capacidad de retención de agua provocan un retraso de la maduración de la uva, lo que conlleva elevadas concentraciones de ácidos y bajas de azúcares. En cambio, en suelos con baja capacidad de retención sucede lo contrario. En el caso de los nutrientes, una escasez de estos provoca retraso en el envero y comporta cosechas de baja producción y bayas pequeñas. Por el contrario, si el suelo es muy fértil se producen cosechas muy abundantes, pero de baja calidad. Los suelos más favorables para la viña suelen ser pedregosos con un buen drenaje, aunque los rendimientos estén limitados (Fernández-Cano & Togores, 2011).

El clima se refiere al conjunto de condiciones atmosféricas que caracterizan una región y generalmente se refiere a valores estadísticos sobre un intervalo de 30 años. En España por ejemplo hay una gran variedad de climas muy diferenciados en función de la latitud y la altitud respecto al nivel del mar. En general, la vid situada en climas cálidos produce vendimias ricas en azúcares y pobres en acidez, lo contrario sucede en climas fríos.

La temperatura que recibe la vid en el periodo de maduración de la uva tiene mucha importancia, ya que determinará la calidad y en la época de floración va incidir en la cantidad. La temperatura mínima para un buen desarrollo del racimo es alrededor de los 10°C. Las temperaturas altas provocan una mayor acumulación de azúcares y una disminución de la acidez, lo contrario sucede a temperaturas bajas. Por lo que, los excesos o extremos de temperatura perjudican el óptimo desarrollo de la uva y su maduración. No obstante, se ha descrito que los cambios pronunciados de temperatura del día y la noche que tienen lugar durante la maduración favorecen la acumulación de antocianos y taninos (Cohen et al., 2008).

La radiación solar influye en la biosíntesis de los compuestos acumulados en la uva, aumentando significativamente su concentración cuando hay mayor exposición solar (Carbonell-Bejerano et al., 2014). La cantidad de iluminación que necesita un viñedo depende de su producción, estimándose habitualmente que se necesita un metro cuadrado de superficie foliar por kilogramo de producción (Marchevsky, 2005).

La humedad es indispensable para la vida de la cepa, aunque es una planta resistente a la sequía. Durante el periodo de crecimiento herbáceo de la uva, la planta necesita bastante agua para un desarrollo normal de los racimos. En el último periodo de maduración es conveniente que sufra un cierto estrés hídrico para así lograr vendimias de calidad. Cuando hay un exceso de humedad se retrasa la maduración, da lugar a racimos con menos cantidad de polifenoles, azúcares y una mayor acidez, y los granos de uva están más aguados. También puede favorecer la aparición de enfermedades fúngicas. En casos de extrema sequía puede reducirse o pararse la actividad fisiológica de la planta.

3.3. Evolución de los compuestos fenólicos durante la vinificación

La vinificación en tintos es un proceso complejo en el cual se llevan a cabo simultáneamente dos fenómenos: la fermentación alcohólica y la maceración. Se pueden distinguir tres etapas: maceración prefermentativa, maceración fermentativa y maceración postfermentativa, como se puede ver en la **Figura 16**.

Inmediatamente después de la cosecha tiene lugar el proceso de elaboración del vino. Las uvas habitualmente se despalillan para eliminar el raspón y se estrujan para favorecer la extracción de los compuestos fenólicos de las partes solidas de la uva en el mosto, lo que facilita la maceración. Generalmente se opta por eliminar el raspón ya que este contiene un alto contenido en agua y potasio, y bajos niveles de azúcares y ácidos (Hashizume et al., 1998), y porque libera un exceso de astringencia y de sabor amargo (Llaudy et al., 2008). El mosto y las uvas estrujadas se bombean a los tanques de fermentación donde inmediatamente después se añade el dióxido de azufre. Esta

primera etapa se denomina maceración prefermentativa y tiene lugar en medio acuoso, a temperaturas moderadas y es de corta duración.

En el proceso de fermentación alcohólica que corresponde a la maceración fermentativa, las levaduras transforman el azúcar en etanol, dióxido de carbono y otros productos secundarios. Esta segunda etapa, tiene lugar en medio hidroalcohólico, a temperaturas altas (22-30°C) y su duración (5-10 días) va a depender de numerosos factores, como la especie y cepa de levadura, la concentración inicial de azúcar y los nutrientes, el nivel de aireación y la temperatura. Tanto las altas temperaturas como el etanol favorecen en la extracción de los compuestos fenólicos (Canals et al., 2005; Sacchi et al., 2005; Koyama et al., 2007; Casassa et al., 2013).

Una vez finalizada la fermentación alcohólica y hasta que se descuba, tiene lugar la maceración postfermentativa, también en medio hidroalcohólico. La duración de esta última etapa va a depender de la decisión del enólogo que a su vez dependerá del tipo de vino que desee elaborar. Los tanques se vacían mediante el sangrado del vino y los orujos se prensan, obteniendo el vino de prensa. El vino obtenido puede ser consumido como vino joven o destinado a la crianza.

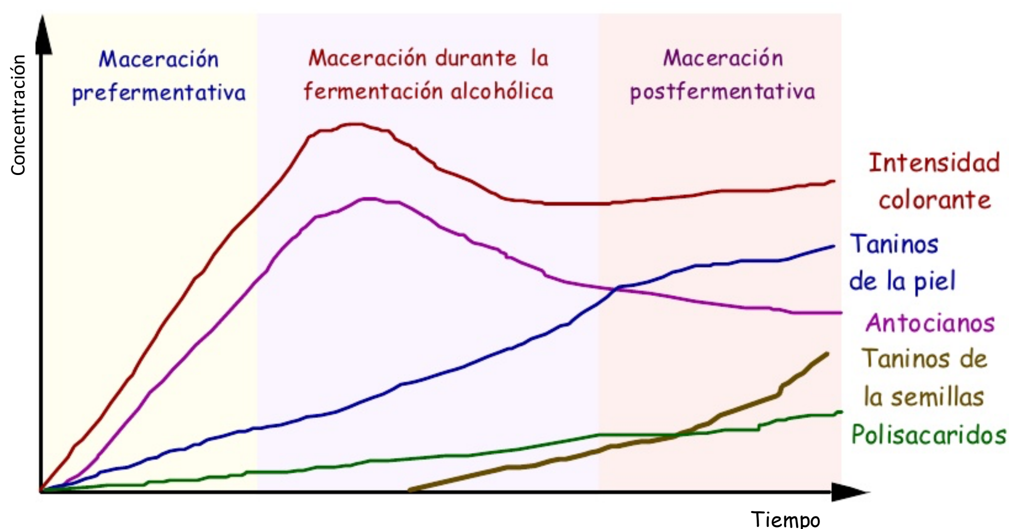


Figura 16. Cinética de extracción de los compuestos fenólicos durante la fermentación/maceración (Ribéreau-Gayon et al., 2006).

El desarrollo simultáneo de la fermentación alcohólica con la maceración incide en la extracción de polifenoles, ya que el etanol promueve la disgregación de las membranas vacuolares y de las paredes de las células de los hollejos (González-Neves, et al., 2007). En la **Figura 16** se puede observar que la cinética de extracción de los antocianos es rápida desde el principio de la maceración prefermentativa, sobre todo en la fase acuosa, prolongándose y alcanzando un máximo de extracción los primeros días de la maceración fermentativa (González-Neves et al., 2008). Luego se produce un descenso debido a fenómenos de oxidación, modificaciones estructurales y absorción de los antocianos por las paredes celulares de levaduras y por las partes solidas de la uva (Morata et al., 2003).

Los taninos de la piel se extraen a la vez que los antocianos, debido a su localización. Sin embargo, su velocidad de extracción es más lenta y va aumentar conforme aumenta la temperatura y el contenido de etanol. En el caso de los taninos de semilla, es más lenta y normalmente necesita un periodo de maceración más largo. La extracción de taninos de semilla se ve favorecida por la presencia de etanol, ya que facilita la disolución de los lípidos cuticulares (Canals et al., 2005).

De forma general, la extracción de proantocianidinas va a depender del tiempo de maceración. En los primeros días de maceración se extraen principalmente los taninos de los hollejos y conforme avanza la maceración se extraen más los procedentes de las semillas. En dicho sentido los estudios de Gil et al., (2012), de Busse-Valderde et al., (2012) y de Bautista-Ortín et al., (2016) demuestran que al aumentar el tiempo de maceración disminuye el mDP y el porcentaje de prodelfinidinas (%PD) mientras que aumenta el porcentaje de galoilación (%GAL). Dado que las proantocianidinas de la piel contienen prodelfinidinas, tienen mayor mDP y un menor %GAL que las de las semillas. Estos datos confirmarían lo anteriormente expuesto.

Los flavonoles se liberan en la fase prefermentativa y durante la fermentación. Estos compuestos se liberan en forma glicosilada, pudiéndose hidrolizar y liberar sus correspondientes agliconas. Una disminución de los flavonoles durante la etapa

postfermentativa puede estar provocada por la precipitación de las agliconas debido a su baja solubilidad (Castillo-Muñoz et al., 2007).

Los ácidos cinámicos se liberan al mosto en el momento del prensado de la uva. Durante los primeros días de maceración se van solubilizando gradualmente hasta que su concentración se estabiliza (Morel-Salmi et al., 2006).

3.4. Factores que influyen en la extracción de los compuestos fenólicos durante la vinificación

La extracción de los compuestos fenólicos es de gran interés dado su importancia en las propiedades organolépticas del vino tinto. Uno de los principales factores que condiciona su extracción es el grado de madurez de la uva. También hay que tener en cuenta las técnicas enológicas empleadas durante la vinificación para favorecer su extracción. Así como todos los sistemas que permiten un tratamiento mecánico del sombrero favorecen la extracción y disolución de los compuestos fenólicos debido a que se facilita el contacto entre las partes sólidas y el líquido. De entre los diferentes tratamientos destacan los siguientes (Zamora, 2003):

- El **remontado**: operación que consiste en bombear el mosto/vino desde el fondo del depósito para mojar el sombrero es la técnica más empleada. Esta operación es probablemente la más usada en vinificación ya que además de mejorar la extracción permite airear el mosto y deshacer las posibles estratificaciones que se originan en el tanque de vinificación. No obstante, en ocasiones el sombrero se compacta demasiado debido al empuje del dióxido de carbono. Para descompactarlo y mejorar la extracción se practican otras técnicas complementarias.

- El **bazuqueo**: operación que consiste en hundir el sombrero tanto de forma manual como mecánica. Existen sistemas automatizados que facilitan esta operación.

- La **inundación**: operación que consiste en dejar caer de golpe sobre el sombrero un gran volumen del mosto/vino, previamente extraído del tanque, para así descompactarlo.

- El ***délestage***: operación que consiste en vaciar completamente el líquido del depósito y pasado un par de horas volver a llenarlo dejando caer enérgicamente el mosto/vino sobre el sombrero depositado en el fondo de la cuba.

- La técnica del **sombrero sumergido** consiste en colocar una malla dentro del depósito para impedir que la pasta suba a la superficie y así estar en contacto continuo con el líquido. Este fenómeno debería permitir una mayor extracción de los compuestos fenólicos, pero a veces sucede lo contrario debido a que el sombrero no puede ser tratado mecánicamente al estar sumergido.

La temperatura de fermentación es un factor determinante en la disolución de los compuestos fenólicos durante la maceración. Es un factor importante que contribuye a la degradación de los tejidos del hollejo y de las semillas. Con temperaturas elevadas se ve favorecida las reacciones químicas entre los antocianos y otros compuestos, incrementando la intensidad del color y su estabilidad. Las temperaturas del orden de los 28-30°C son buenas para conseguir una buena extracción de los compuestos fenólicos. Si es mucho más alta se puede extraer una elevada concentración de taninos y los antocianos degradarse, y si es más baja la extracción es pobre (Zamora, 2003). Por ello, se han desarrollado técnicas de elaboración con el objetivo de extraer lo máximo posible, como la **termovinificación**, la **maceración postfermentativa en caliente**, la **flash-détente** y la **maceración prefermentativa por frío** entre otras.

Como ya se ha comentado, el tiempo de maceración es un factor muy determinante en la extracción de los compuestos fenólicos, ya que es el tiempo en el que las partes sólidas y el líquido están en contacto. Se aconseja adaptar el tiempo de maceración al tipo de vino que se desee elaborar, a la variedad de uva, a su estado sanitario y al nivel de madurez (Ribéreau-Gayon, et al., 2006). Una maceración larga incrementa la cantidad de proantocianidinas en el medio, pero no comporta habitualmente un

aumentó en la cantidad de antocianos. Sin embargo, se favorecen las reacciones de combinación entre las proantocianidinas y los antocianos, dando al vino mayor intensidad de color y estabilidad. Alargando la maceración también se produce un aumento de la extracción de taninos procedentes de las semillas. Hay que tener en cuenta el nivel de madurez, ya que maceraciones largas con uvas inmaduras pueden dar lugar a vinos duros, amargos y herbáceos (Zamora, 2003).

Si se quiere elaborar vinos para un consumo rápido las maceraciones son generalmente más cortas, ya que con pocos días de maceración se consigue la intensidad de color necesaria, aromas afrutados y una concentración no muy alta de taninos. Al contrario, para elaborar vinos de crianza, las maceraciones son más largas, consiguiendo así vinos con una buena estabilidad del color y estructura en boca.

En el mercado existen productos enológicos comerciales a base de taninos enológicos que permiten enriquecer el vino en dichos compuestos y también preparados con enzimas pectolíticas que aceleran la extracción de compuestos fenólicos. El uso de taninos enológicos contribuye a la mejora y estabilización del color del vino y a protegerlo de la oxidación, si bien el efecto que cause sobre el color va a depender en gran medida del tipo de tanino utilizado (Zamora, 2003). Son de gran utilidad en vendimias afectadas por *Botrytis cinerea* previniendo la acción de la lacasa (Crespy, 2002b). El incremento y estabilidad del color también se ve favorecido por el uso de enzimas pectolíticas que hidrolizan las pectinas de la pared celular de la uva. Estos preparados enzimáticos se añaden a la pasta de vendimia durante la maceración para degradar las paredes celulares acelerando así el proceso de extracción de los compuestos fenólicos procedentes tanto de las pieles como de las semillas (Bautista-Ortín et al., 2013). También afectan a la composición de los polisacáridos y oligosacáridos del vino (Ayestaran et al., 2004; Ducasse et al., 2011).

3.5. Evolución de los compuestos fenólicos durante la crianza

Desde el descube y durante toda la vida de los vinos tintos se producen una serie de modificaciones, que van a depender de la composición de cada vino y de las condiciones de conservación.

3.5.1. La copigmentación

La copigmentación es un fenómeno que consiste en la unión de las formas coloreadas de los antocianos con los copigmentos (incoloros) mediante reacciones hidrofóbicas (π-π stacking) dando lugar a la formación de estructuras tipo sándwich, con un número de capas de 2 a 10. Dentro de estas agrupaciones se origina un entorno hidrofóbico que impide que las moléculas de agua accedan al interior, previniendo la formación de las bases hemicetal y las formas de calcona (incoloras). Por lo que, un mayor porcentaje de antocianos contribuirá al color, que el correspondiente de acuerdo con el pH del vino, siempre que existan los copigmentos adecuados. La copigmentación no solo incrementa el color del vino, sino que también puede modificar su tonalidad mediante desplazamientos hipsocrómicos (desplazamiento de la longitud de onda de máxima absorción a valores más bajos; tonos más amarillentos) y batocrómicos (desplazamiento de la longitud de onda de máxima absorción a valores más altos; tonos más azulados) (Boulton, 2001).

En función de la naturaleza del copigmento implicado, la copigmentación puede subdividirse en tres categorías (Hermosín-Gutiérrez, 2007):

- **Copigmentación intermolecular**: el copigmento es una molécula de naturaleza distinta al antociano, como una molécula de flavonol.

- **Copigmentación intramolecular**: el copigmento es una porción de la propia molécula de antociano, como puede ser el caso del sustituyente cumaroilo de un antociano cumarílico.

- **Autoasociación**: en este caso el copigmento es otra molécula de antociano.

El fenómeno de copigmentación está influenciado principalmente por la concentración y estructura del pigmento y copigmentos, por factores tales como el pH, la temperatura, la fuerza iónica y la concentración de determinados solventes en el medio. Los mejores copigmentos son los flavonoles, seguidos de los ácidos hidroxicinámicos, pero en la uva y en el vino estos se encuentran en pequeñas cantidades. En cambio, los flavanoles son copigmentos menos efectivos debido a que no poseen una estructura plana, pero al ser los más abundantes en los vinos pueden llegar a tener gran relevancia en la copigmentación (Gómez-Míguez et al., 2006). Dentro de los distintos tipos de flavanoles, la epicatequina es mejor copigmento que la catequina, ya que posee una estructura prácticamente plana. En cuanto a los antocianos, a mayor grado de glicosilación y de acilación, el complejo de copigmentación será más estable (Boulton, 2001).

La copigmentación es uno de los principales mecanismos de estabilización del color en los vinos jóvenes, ya que tiene la capacidad de proteger a los antocianos de la decoloración que generan los cambios de pH y la adicción de bisulfito. Algunos autores sostienen que a medida que el vino envejece la copigmentación protege a los antocianos de la oxidación permitiendo que permanezcan estables en disolución hasta que se formen los nuevos pigmentos poliméricos mucho más estables (Berké & de Freitas, 2005; He et al., 2012). Las contribuciones de la copigmentación en el color de los vinos jóvenes son bastante altas y disminuyen gradualmente durante el envejecimiento (Hermosín-Gutiérrez et al., 2005), perdiendo importancia a partir de los nueve meses, debido a que la estructura de los pigmentos poliméricos dificulta la copigmentación.

3.5.2. Los pigmentos poliméricos

La formación de pigmentos poliméricos permite explicar la evolución y estabilización del color de los vinos tintos durante su envejecimiento. Se ha observado que los vinos añejos son más resistentes a la decoloración por la adicción de sulfitos y por cambios de pH. Asimismo, durante la crianza se produce una pérdida de intensidad colorante y un cambio de tonalidad desde matices violáceos hacia matices más anaranjados (Boido et al., 2006). Estos cambios son debidos en parte a la formación de nuevos pigmentos por unión entre antocianos y proantocianidinas (He et al., 2012).

Hay diferentes tipos de mecanismos de unión, por uniones directas o por mediación de otras moléculas. Por uniones directas hay dos tipos de pigmentos poliméricos (**Figura 17**): pigmento tipo A-T donde el C6 o el C8 del flavanol atacan a la posición electrófila C4 del antociano; pigmento tipo T-A donde el C6 o el C8 del antociano atacan a la posición C4 del flavanol, que se encuentra en forma de carbocatión resultante de la despolimerización de las proantocianidinas en medio ácido (Hermosín-Gutiérrez, 2007).

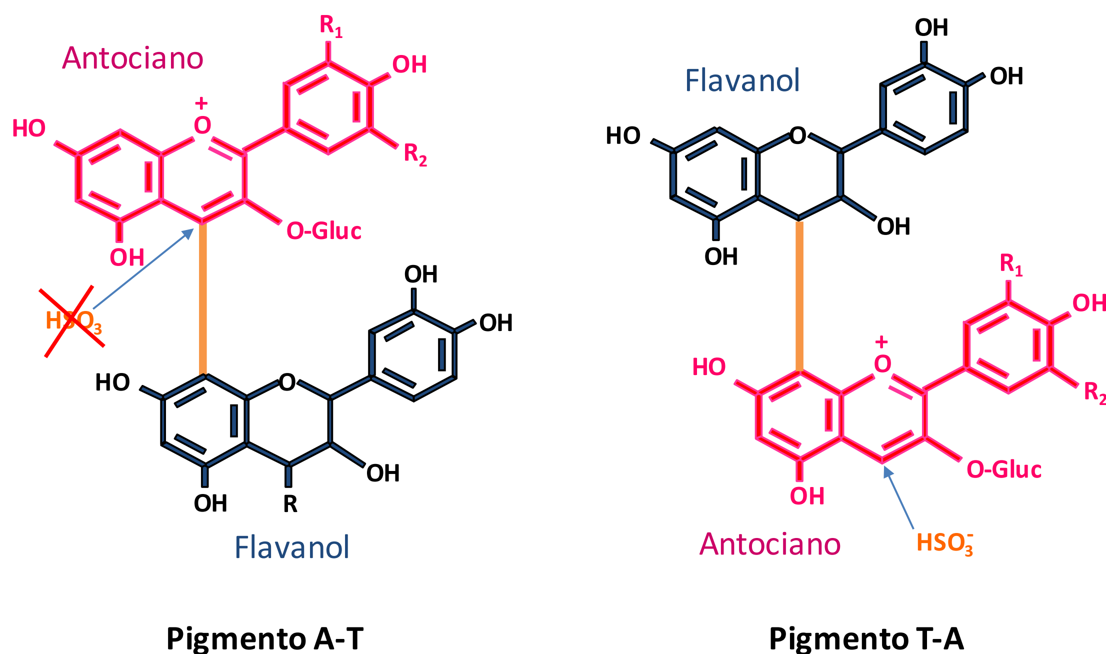


Figura 17. Estructuras de los pigmentos poliméricos resultantes de la unión entre antocianos y proantocianidinas (pigmentos tipo A-T y T-A).

Otro tipo de unión es por medio del etanal, el cual se forma gracias al metabolismo de las levaduras durante la fermentación alcohólica y también mediante la oxidación biológica o química del etanol. El etanal en medio ácido puede reaccionar con un flavanol originando un carbocatión que a su vez puede unirse a una molécula de antociano generando uniones del tipo Flavanol-etanal-Antociano de acuerdo con el mecanismo que se muestra en la **Figura 18**. En su momento se consideró que estos pigmentos eran los responsables del color de los vinos añejos (Glories, 1984). Hoy en día se sabe que no es así por dos razones. La primera es que estos pigmentos presentan una tonalidad violácea muy alejada del color de los vinos añejos. La segunda es que estos

pigmentos no son estables (Escribano-Bailón et al., 2001) y generan reorganizaciones de los compuestos fenólicos del vino que a la larga generan nuevos pigmentos.

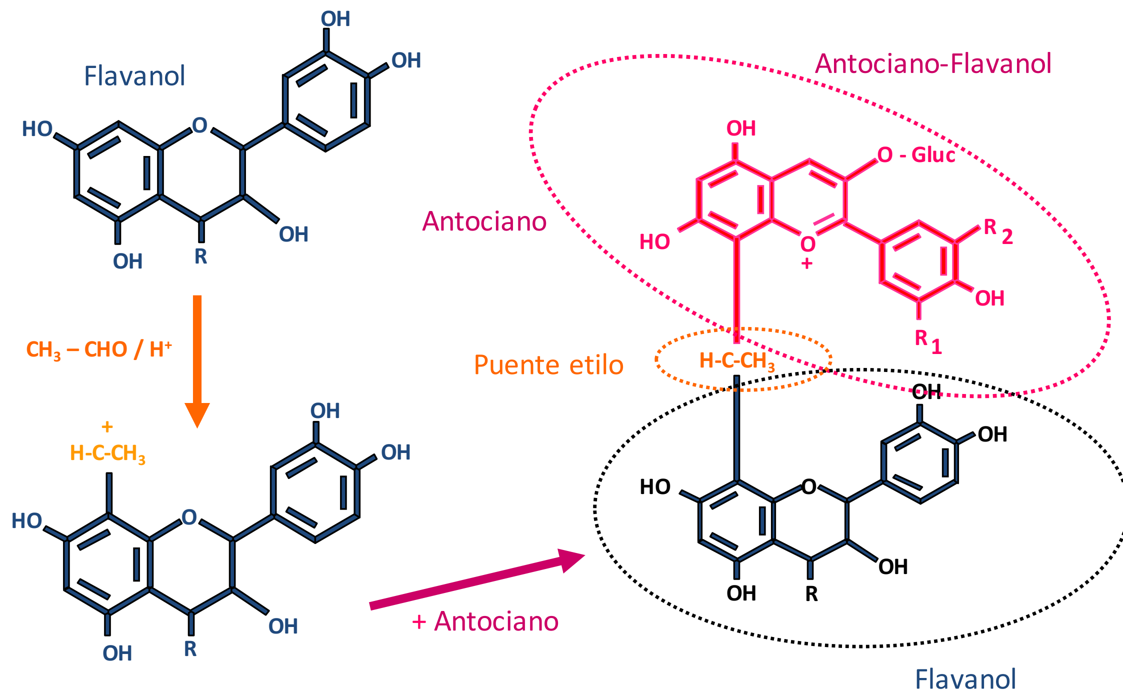


Figura 18. Mecanismo de combinación por medio del etanal.

3.5.3. Los piranoantocianos

La estructura de los piranoantocianos se caracteriza por tener un anillo pirano adicional en la posición 4 y 5 de la estructura del antociano (**Figura 19**). Los piranoantocianos se forman por la unión de los antocianos libres mediante reacciones de cicloadición con diferentes compuestos presentes en el vino que se caracterizan por tener un doble enlace polarizado. La Vitisina A (Fulcrand et al., 1998) se genera a partir del piruvato mayoritariamente durante la fermentación alcohólica, mientras que la Vitisina B (Bakker & Timberlake, 1997) se produce por la cicloadición del etanal que se origina durante la fermentación y/o durante la crianza del vino. Otros piranoantocianos se pueden originar por cicloadición con el vinilfenol generado por la descarboxilación microbiana del ácido cumárico. Finalmente, la ruptura de los puentes etilo entre antocianos y flavanoles o entre flavanoles puede originar vinil-flavanoles, los cuales pueden generar otro tipo de piranoantocianos llamados aductos vinilflavanol-antociano (Escribano-Bailón et al.,

2001). Cabe destacar que a diferencia de lo que ocurría con los otros piranoantocianos, los aductos vinilflavanol-antociano no son una sola molécula sino un amplio conjunto de pigmentos de estructura química distinta, lo que complica enormemente su identificación y cuantificación.

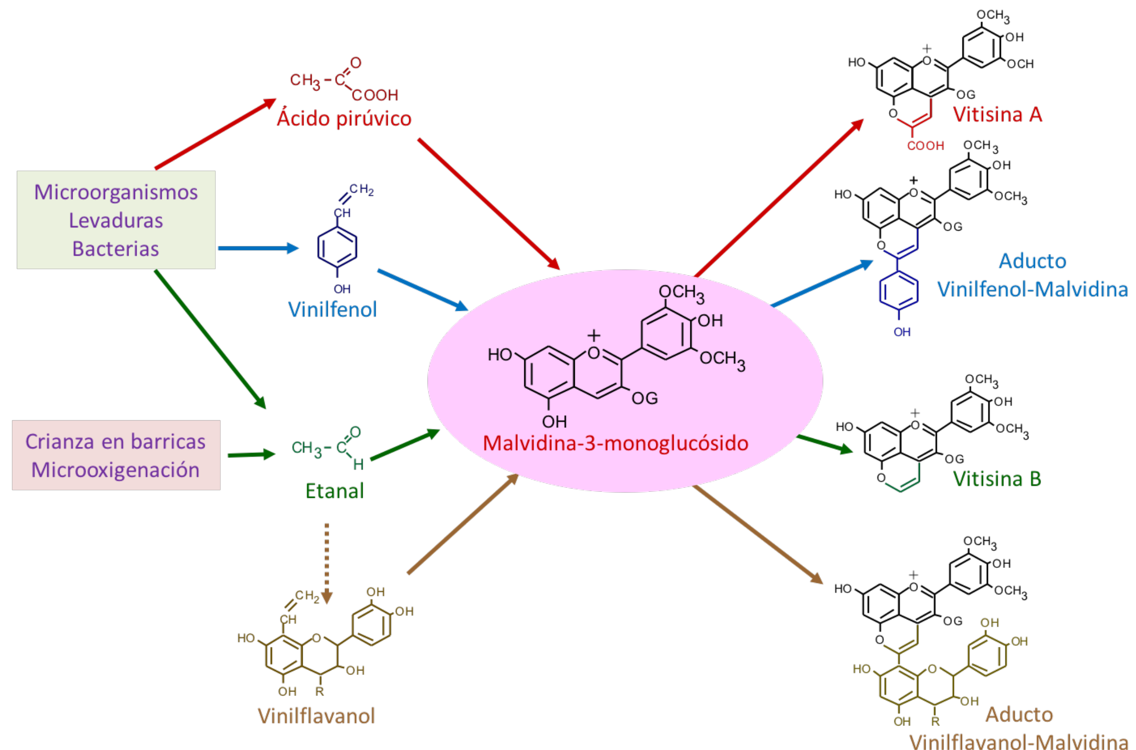


Figura 19. Posibles mecanismos de formación de los piranoantocianos (Zamora, 2004).

Durante la fermentación alcohólica se inicia la formación de estos pigmentos que sigue hasta la crianza y el envejecimiento del vino. Estos pigmentos presentan un color rojo anaranjado, son muy estables frente a la decoloración con bisulfito, a las altas temperaturas y a la degradación oxidativa. Su concentración en el vino es mucho menor que la de otros pigmentos, pero al ser muy poco sensibles a los cambios de pH, la práctica totalidad de los piranoantocianos presentes participan en el color. Con la excepción de los aductos vinilflavanol-antociano, los demás piranoantocianos tienen un tamaño similar al de los antocianos monómericos de los que proceden y se conservan disueltos en el vino, por lo que tendrán poca tendencia a precipitar durante el envejecimiento (Hermosín-Gutiérrez, 2007).

3.5.4. Evolución de las proantocianidinas

Las proantocianidinas reaccionan con los antocianos formando pigmentos poliméricos, pero también pueden reaccionar entre sí, dando lugar a proantocianidinas de mayor grado de polimerización (**Figura 20**). Los flavanoles pueden reaccionar como nucleófilos a través de los C6 y C8, debido a la ruptura de las uniones interflavánicas en medio ácido y dando lugar a un carbocatión en la posición C4. Las nuevas moléculas de proantocianidinas son de mayor tamaño y con diferente grado de polimerización, aumentando cuando los flavanoles nucleofílicos son polímeros o disminuyendo si la reacción se produce con compuestos de bajo peso molecular (Terrier et al., 2009b). Si las proantocianidinas polimerizadas alcanzan un determinado grado de polimerización estas se vuelven insolubles y pueden precipitar. Las proantocianidinas polimerizan durante la maceración y el período de crianza (Zamora, 2003).

Cuando en el medio hay presencia de oxígeno, en el mecanismo de formación de las proantocianidinas polimerizadas participa el etanal. En este caso, los centro nucleófilos del flavanol atacan al carbono carbonílico del etanal, dando lugar a un carbocatión que va a reaccionar con otro flavanol, formado un polímero unido por puentes de etilo (**Figura 20**). Otro mecanismo de polimerización, que también requieren la participación del oxígeno, es a través de la formación de semiquinonas, a partir de la oxidación de los grupos orto-difenol, formando un enlace estable mediante un mecanismo de tipo radicalario. Estas reacciones necesitan la presencia del hierro o cobre como catalizadores.

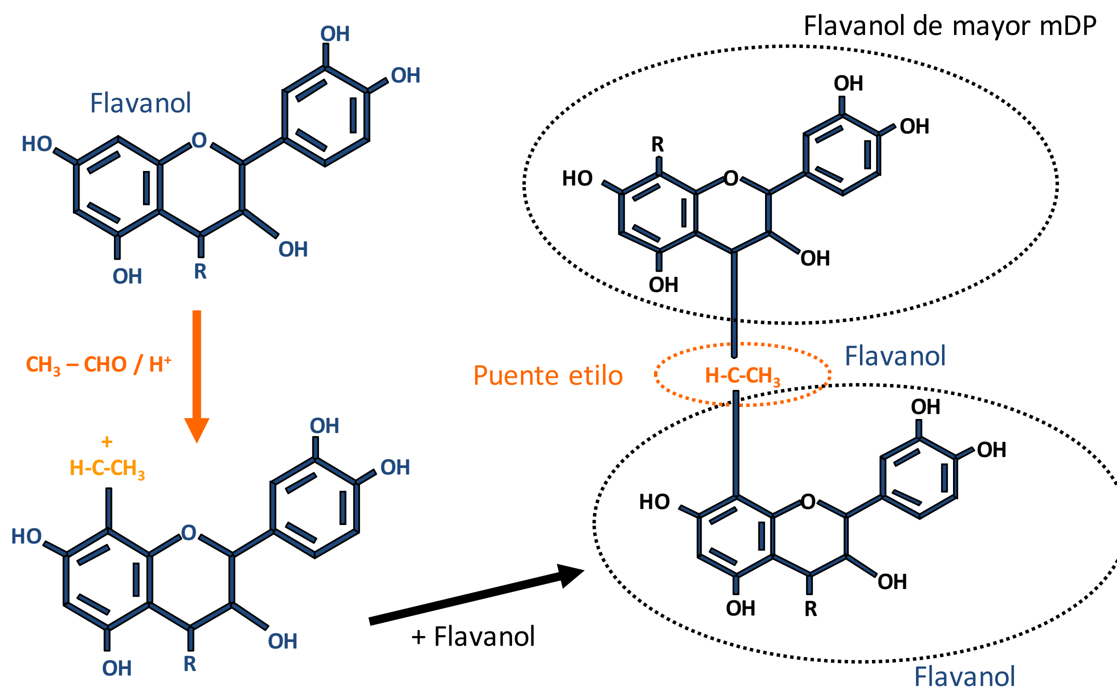


Figura 20. Polimerización de proantocianidinas por la participación del etanal.

4. La astringencia y el sabor amargo del vino tinto

4.1. La astringencia

La American Society for Testing and Materials definió la astringencia como “un complejo de sensaciones de sequedad y aspereza en el epitelio debido a la exposición a substancias como el alumbre o los taninos” (ASTM 2004). La astringencia puede estar provocada por sustancias como metales, agentes deshidratantes, minerales, ácidos orgánicos y compuestos fenólicos (Bajec & Pickering, 2008). Los principales responsables de la astringencia del vino tinto son los taninos. La astringencia es una de las propiedades más importantes que determinan la calidad de un vino tinto (Peynaud, 1987). Los vinos con un exceso de astringencia son ásperos y secos, este exceso puede ocultar otras propiedades organolépticas. Y si el nivel de astringencia es bajo, los vinos son planos y poco interesantes (Gawel, 1998). En este sentido, el gran Emile Peynaud (2013) aseguraba que “la armonía, el equilibrio y la elegancia de la astringencia son el signo distintivo de los grandes vinos tintos” o también “La astringencia en exceso en un

vino afecta a su equilibrio y a su calidad, pero, por el contrario, un vino falto de astringencia parece plano, soso y sin interés”

El 99% de la saliva es agua y el resto son sustancias inorgánicas y proteínas. Las proteínas ricas en prolina (PRPs, *Proline Rich Proteins*) tienen mayor afinidad para unirse con los taninos. Esta unión se realiza mediante puentes de hidrógeno e interacciones hidrofóbicas. El tamaño, la hidrofobicidad y la flexibilidad estructural de las proteínas influyen en la capacidad de interacción con los taninos.

La astringencia está influenciada por el nivel de madurez de la uva, la concentración y estructura de las proantocianidinas. Los vinos elaborados con uvas muy maduras darán lugar a vinos menos astringentes, ya que tienen una alta cantidad de taninos de piel y baja de semilla (Gil et al., 2012). En cambio, los elaborados con uvas verdes serán más astringentes. La sensación de astringencia aumenta con el grado de polimerización y el grado de galoilación (Vidal et al., 2003). Así, la (-)-epicatequina es más astringente que la (+)-catequina (De Freitas & Mateus, 2001). Los taninos con una mayor proporción de subunidades de epicatequina en posición de extensión aumentan la sensación de astringencia (Quijada-Morín et al., 2012).

A lo largo de la crianza y envejecimiento del vino la sensación de astringencia se va suavizando. Esto es debido a las combinaciones de antocianos y proantocianidinas. También hay que tener en cuenta la participación de los taninos hidrolizables liberados por la madera de las barricas en la sensación de astringencia (Chira & Teissedre, 2013).
Hay otros compuestos del vino que influyen en la sensación de astringencia. La percepción de astringencia disminuye conforme aumenta el pH (Rinaldi et al., 2012), los ácidos orgánicos (Fontoin et al., 2008), el grado alcohólico (Obreque-Slier et al., 2010), la concentración de polisacáridos (Gonzalez-Royo et al., 2017) y la viscosidad (Smith et al., 1996).

Para evaluar la sensación de astringencia la metodología más directa es el análisis sensorial: prueba triangular y dúo-trio; el análisis descriptivo en el que los catadores

pueden usar 53 términos que están recogidos en una rueda de aromas (Gawel et al., 2000) y el método de intensidad del tiempo (TI). La sensación de astringencia depende del tiempo. Es de las últimas sensaciones en percibirse y requiere unos segundos para desarrollarse y, va a depender de la composición y flujo de la saliva de cada individuo.

Uno de los métodos más usado en enología para medir analíticamente la astringencia es el índice de gelatina (Glories, 1984), que nos da el porcentaje de proantocianidinas capaces de reaccionar con las proteínas del vino. No obstante, al basarse en la reacción de Bate-Smith, el índice de gelatina no permite medir la astringencia generada por los taninos hidrolizables. Otras metodologías más actuales utilizan diferentes patrones proteicos como agentes de precipitación para determinar la astringencia (Llaudy et al., 2004; Mercurio & Smith, 2008). Actualmente, se ha desarrollado un método para evaluar la astringencia que reproduce la interacción proteína/polifenol en la boca. Se mide por resonancia de plasmón superficial (SPR, *Surface Plasma Resonance*), que consiste en inmovilizar las proteínas en una superficie de oro y medir su interacción con los polifenoles (Guerreiro et al., 2012).

4.2. El sabor amargo

El sabor amargo es una de las sensaciones gustativas básicas. Se detecta mediante las papilas gustativas localizadas en la parte superior de la lengua cerca de la garganta. Cada papila gustativa tiene aproximadamente 50-100 células receptoras del gusto, las cuales contienen 25 receptores acoplados a las glicoproteínas codificados por un gen de la familia TAS2Rs.

En el vino el sabor amargo es debido principalmente a los flavan-3-oles y sus polímeros (Hufnagel & Hofmann, 2008), ácidos fenólicos (Robichaud & Noble, 1990) y flavonoles (Saenz-Navajas et al., 2010).

Además, el sabor amargo también se ve influenciado por la concentración y estructura de los flavan-3-oles. La sensación de amargor aumenta a medida que disminuye la proporción de grupos galoilados y disminuye el grado de polimerización. La (-)-

epicatequina es más amarga que la (+)-catequina (Noble, 1994). El amargor va a disminuir en los vinos envejecidos.

El sabor amargo se incrementa al aumentar el grado alcohólico (Fortoin et al., 2008) y disminuye con la presencia de proteoglicanos (Vidal et al., 2004). Al aumentar la viscosidad se puede retrasar la percepción de la sensación del sabor amargo (Smith et al., 1996).

Para evaluar el sabor amargo se utiliza el análisis sensorial, al igual que en la astringencia. Sin embargo, no hay metodologías analíticas para evaluarlo en vinos, pero si en alimentos y fármacos.

5. Taninos enológicos: Origen, propiedades y funciones enológicas

Los taninos enológicos son compuestos naturales que provienen de diferentes especies vegetales. Dependiendo de su origen y composición química pueden ser taninos hidrolizables o taninos condensados (proantocianidinas).

Dentro de los taninos hidrolizables comercializados encontramos galotaninos y elagitaninos. Los taninos hidrolizables comerciales se obtienen principalmente de la corteza de diferentes variedades de árboles, los galotaninos se extraen de las nueces de gala (*Quercus infectoria* y *Rhus semillasalata*) y de la tara (*Caesalpinia spinosa*), mientras que los elagitaninos se extraen principalmente de la madera de los robles (*Quercus robur, Quercus petraea* y *Quercus alba*) y del castaño (*Castanea sativa*). La coloración que presentan es blanca cremosa para los galotaninos y marrón para los elagitaninos. Están presentes en el vino bien por contacto de este con barricas, chips o por el uso de éstos como aditivo enológico (Ribéreau-Gayon et al., 2006).

En función de su origen los taninos condensados pueden contener procianidinas, prodelfinidinas e incluso profisetinidinas y prorobinetinidinas. Las procianidinas y prodelfininas se encuentran naturalmente en los vinos tintos ya que provienen de las semillas, hollejos y raspones. Su concentración en la uva varía en función de la variedad,

las practicas vitivinícolas, el tipo de suelo y el clima (Downey et al., 2006). También pueden proceder de la adición de taninos enológicos procedentes de semillas o de pieles de uva (Parker et al., 2007). Las profisetinidinas las podemos encontrar en otras especies vegetales como el quebracho (*Aspidosperma quebracho*-blanco y *Schinopsis lorentzii*). Y las prorobinetinidinas que se encuentran en la mimosa (*Acacia dealbata*). Los taninos condensados presentan una coloración de tonos marrones.

En el mercado existe una amplia gama de estos productos. Generalmente los encontramos en forma de extracto seco, en paquetes cerrados y etiquetados. Estos taninos pueden ser relativamente puros en el caso de que hayan sido extraídos de una sola especie, mientras que si proceden de la mezcla de varias especies pueden incluir tanto taninos hidrolizables como condensados (Obreque-Slíer et al., 2009, Malacarne et al., 2016). La calidad del tanino va a depender de su origen botánico, del proceso de obtención, del tipo de solvente y del tiempo de extracción (Álvarez, 2007). La escasa información proporcionada por las casas comerciales (Obreque-Slíer et al., 2009) hace difícil saber el impacto que va a tener la adición de estos taninos en las propiedades del vino.

Hay estudios que indican que el uso de estos aditivos ha de ser cuidadoso, ya que su uso en el vino puede causar en ocasiones efectos negativos (Bautista-Ortín et al., 2005; Cliff et al., 2012; Harbertson et al.; 2012, Liu et al., 2013). La Organización Internacional de la Viña y el Vino (OIV) aprueba el uso de los taninos enológicos como coadyuvante para la estabilidad proteica en vinos y mostos (Codex OIV, 2016). Sin embargo, cada vez son más usados en la vinificación del vino tinto ya que a veces las uvas se cosechan sin haber alcanzado la madurez fenólica idónea. Esto influye en la calidad del vino que se quiere conseguir, ya que un vino de buena calidad ha de presentar una concentración de taninos óptima. Estos aditivos comerciales tienen muchos intereses de aplicación en vinificación como, inhibir la actividad lacasa (Obradovic et al., 2005), estabilizar el color de los vinos tintos (Canuti et al., 2012), dar estructura a los vinos y reducir los olores a reducción (Vivas, 2001), ejercer una actividad antioxidante protegiendo a los vinos contra la oxidación (Hagerman et al., 1998), consumo directo del oxígeno (Navarro et al. 2016), capacidad de barrido de radicales peroxilo (Magalhães et al., 2014), capacidad

para quelar el hierro (II) previniendo el daño oxidativo mediado por las reacciones de Fenton (Pérez et al., 2009), efecto en la copigmentación (Gombau et al., 2016; Neves et al., 2010) e incluso efectos bacteriostáticos (Lempereur et al., 2002).

6. El oxígeno

En 1873, Louis Pasteur dijo: "L'oxygène est le pire ennemi du vin mais c'est l'oxygène qui fait le vin et c'est par son influence qu'il vieillit" (El oxígeno es el peor enemigo del vino, pero es lo que hace el vino y es por su influencia que este envejece).

El oxígeno es un factor muy importante en el proceso de elaboración y envejecimiento del vino. Existen dos tipos de técnicas a la hora de gestionar la interacción del oxígeno con el vino que causan diferentes efectos en los vinos obtenidos. Por un lado, la tecnología de hiperreducción que es la protección total del vino frente al oxígeno. Y, por otro lado, la crianza tradicional en barricas de roble o la microoxigenación que permiten un aporte controlado y lento de pequeñas cantidades de oxígeno al vino. Dependiendo de la concentración de oxígeno disuelto, esta puede influir de una manera positiva o negativa en la composición y calidad de los vinos (Day et al., 2015). Negativamente se produce una perdida organoléptica, hay un deterioro en el color (pardeamiento) y disminuyen los aromas varietales y se desarrollan notas oxidativas (Singleton & Kramling, 1976; Blanchard et al., 2004; Ugliano et al., 2009). Y positivamente, se estabiliza el color y se reduce la astringencia y el sabor amargo (Atanasova et al., 2002; Llaudy et al., 2006; Wirth et al., 2010; Kontoudakis et al., 2011b). También va influir en el crecimiento y multiplicación de microorganismos, sobretodo dependiendo en qué momento del proceso de elaboración se aporte oxígeno. El oxígeno es necesario al principio de la fermentación alcohólica, pero se podría decir que no es aconsejable durante la fermentación maloláctica.

El 20.9% del aire atmosférico es oxígeno y se puede encontrar en mezclas gaseosas y en líquidos. La concentración máxima de oxígeno en el vino por saturación a 20°C y presión atmosférica es de 8.6 mg/L (ppm) (Singleton, 1987). La disolución del oxígeno va a depender de una serie de factores como, el tiempo de exposición, la temperatura, la

concentración de etanol y de la composición del vino (Laurie & Peña-Neira, 2012). La temperatura influye considerablemente en la saturación de oxígeno en el vino. Si la temperatura es baja la solubilidad del oxígeno aumenta y lo contrario sucede a altas temperaturas. La concentración de etanol y los extractos secos van a influir en menor medida en la solubilidad, observándose una disminución en la solubilidad del oxígeno cuando el contenido de alcohol es alto y/o en vinos con alto contenido en extracto seco.

6.1. Reacciones de oxidación

6.1.1. Oxidaciones enzimáticas

Las oxidaciones enzimáticas son muy rápidas y tienen lugar en presencia de oxígeno principalmente en la fase prefermentativa, aunque pueden aparecer en vinos insuficientemente protegidos. Estas reacciones de oxidación están catalizadas mayoritariamente por las enzimas polifenoloxidasas (PPO) sobre los compuestos fenólicos, dando lugar al pardeamiento. En las uvas sanas encontramos la enzima tirosinasa (orto-difenol-óxidoreductasa, ODOR; EC.1.14.18.1) y la lacasa (para-difenol-óxidoreductasa, PDOR; EC.1.10.3.2) que exclusivamente la encontramos en uvas afectadas por *Botrytis cinerea*. Se puede reducir el efecto de este tipo de oxidaciones usando sustancias como el glutatión, dióxido de azufre y gases inertes y controlando la temperatura (Singleton y Cilliers, 1995). La reacción de oxidación enzimática (**Figura 21**) se caracteriza y diferencia de las no enzimáticas (**Figura 22**), en que las PPO en el proceso de oxidación del *o*-difenol a *o*-quinona originan agua en vez de peróxido de hidrógeno.

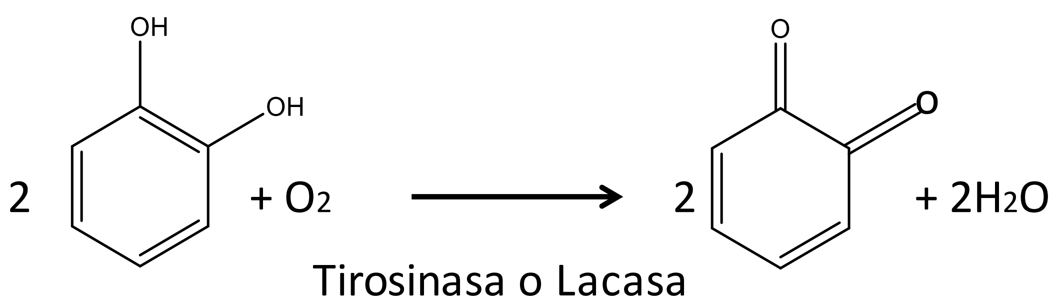


Figura 21. Reacción de oxidación enzimática.

6.1.2. Oxidaciones no enzimáticas

Las oxidaciones no enzimáticas se desarrollan más lentamente que las enzimáticas y tienen lugar en vinos ya elaborados, cuando estos no cuentan con las medidas adecuadas de protección. Generalmente, estas reacciones se consideran perjudiciales, pero en el caso de la crianza de los vinos tintos se consideran beneficiosas, ya que se producen cambios que mejoran sus características organolépticas. En el vino se encuentran sustancias que pueden reaccionar con el oxígeno, como el ácido ascórbico, el dióxido de azufre, el etanol y metales de transición como el cobre o el hierro. Los compuestos más afectados por estas reacciones son los polifenoles y en presencia de los catalizadores o en medios básicos, las reacciones son más rápidas. Sin la presencia de los metales, el oxígeno molecular no es suficientemente activo, como para oxidar de forma directa a los compuestos fenólicos del vino (Danilewicz, 2007). En la **Figura 22** se puede observar que en presencia del oxígeno y por la acción del hierro, se forma el radical hidroperóxido, que permitirá la oxidación de los *o*-difenoles a quinonas y la formación del peróxido de hidrógeno. Posteriormente, nuevamente en presencia del hierro, el peróxido de hidrógeno reaccionará dando lugar a un radical hidroxilo, capaz de oxidar sustancias como el etanol a etanal.

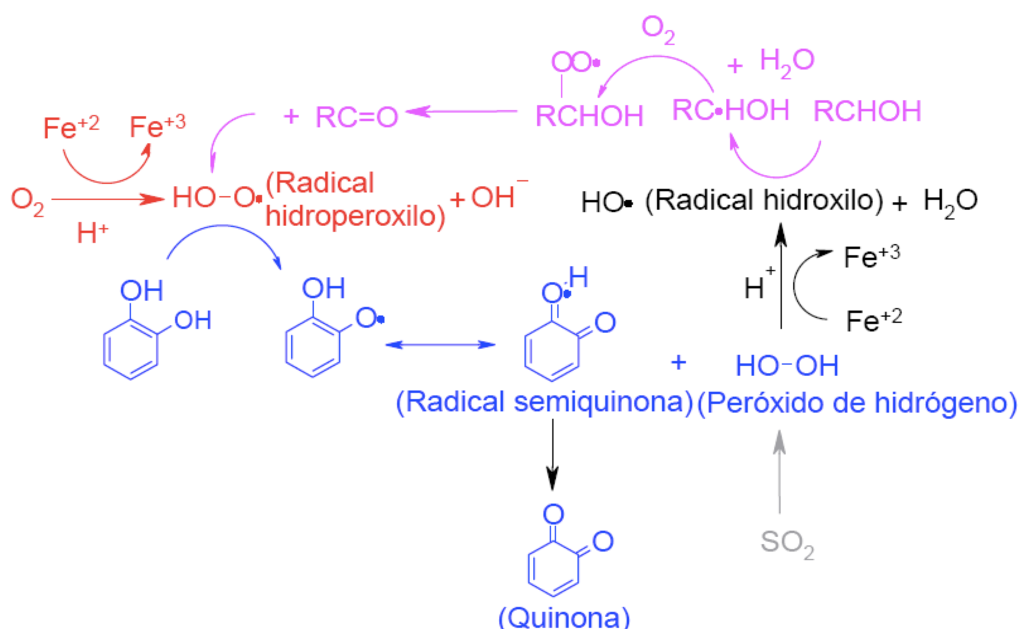


Figura 22. Reacción de oxidación no enzimática (Laurie & Peña-Neira, 2012).

6.2. Métodos de medición del oxígeno disuelto

Ribéreau-Gayon (1933) desarrollo un método colorimétrico para medir el oxígeno disuelto en vino basándose en la oxidación del bisulfato de sodio, usando como indicador el carmín índigo. Lopes et al., (2005) se apoyó en este método, desarrollando un nuevo método colorimétrico no destructivo para medir la entrada de oxígeno a través de los tapones. Otros autores han empleado el reactivo de Winkler para medir el oxígeno presente en el espacio de cabeza de las botellas tras el embotellado (Kontoudakis et al., 2008).

Tradicionalmente, en la industria se han empleados sistemas electroquímicos para medir el oxígeno disuelto basados en el electrodo de Clark, como el Orbisphere. Consisten en una célula de dos electrodos, una membrana permeable al oxígeno y un electrolito. Estos sistemas son sensibles a otras sustancias químicas y durante la medición consumen oxígeno. También necesitan que el fluido esté en movimiento para realizar la medida.

Actualmente, se ha elaborado una tecnología basada en la luminescencia, que es la propiedad de emitir luz que poseen algunos materiales (luminóforos) cuando son excitados por un estímulo, la luz. La concentración de oxígeno está relacionada con la intensidad de luz emitida por el luminóforo. Estos sistemas permiten una medición no invasiva el oxígeno disuelto en el vino en prácticamente todas las etapas del proceso de vinificación, crianza y directamente en la botella. La medición se puede llevar a cabo mediante una fibra óptica y colocando un sensor (spot) en una superficie transparente en contacto directo y continuo con el fluido o en la región del espacio de cabeza. También estos equipos nos permiten sustituir la fibra óptica por una sonda de inmersión, con la que no se necesita usar los spots, y se puede medir el oxígeno en cualquier tipo de taque. Diferentes casas comerciales fabrican estos sistemas de medición como PreSens (PreSens Precision Sensing GmbH, Alemania), Nomasense (Nomacorc SA, Bélgica), OxySense (OxySense, USA) y OpTech® O_2 - Model P (MoCon, USA). Se puede afirmar que esta metodología es actualmente la más usada para medir oxígeno en el vino.

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HIPÓTESIS Y OBJETIVOS

El principal objetivo de este trabajo fue estudiar la influencia de la composición en taninos del vino sobre su color y su calidad sensorial. Para ello, se plantearon diversas experiencias con el fin de determinar la influencia del sistema de vinificación, así como de la importancia de la contribución de las semillas y del raspón sobre la composición, color y calidad final del vino tinto. Por esta razón nos planteamos la siguiente hipótesis de trabajo:

“La composición en taninos del vino está enormemente determinada por el modo en que se lleva a cabo la vinificación que a su vez determina en gran medida su color y su calidad”

Con este propósito nos planteamos los siguientes objetivos específicos:

Objetivos:

1. **Estudiar la influencia del grado de madurez y del sistema de vinificación sobre la composición, el color y la calidad del vino tinto.**

2. **Determinar la contribución real de las semillas y del raspón sobre la composición, el color y la calidad del vino tinto.**

Objetivo adicional:

La suplementación con taninos enológicos es una operación cada vez más habitual en bodega dado que se les atribuyen múltiples efectos positivos sobre la calidad del vino sin que muchos de ellos hayan sido verificados científicamente. Por esta razón nos planteamos el siguiente objetivo adicional:

3. **Determinar la cinética de consumo de oxígeno por parte de diversos taninos enológicos para ver si su adición al vino puede ser útil para protegerlo de la oxidación.**

RESULTADOS

CAPÍTULO 1: Influencia de la madurez de la uva y del tratamiento pre-fermentativo del racimo de la variedad Garnacha sobre la composición y calidad del vino.

Uno de los factores más importantes que afectan a la composición y a la calidad del vino tinto es la madurez de la uva. La calidad de un vino está determinada por la composición fenólica, a su vez, influenciada por la madurez de la uva. Por tanto, la fecha de vendimia es un aspecto fundamental a tener en cuenta. Otro de los parámetros que influyen en la extracción de los compuestos fenólicos es las diferentes técnicas enológicas que pueden emplearse durante la vinificación.

En este capítulo se aborda el objetivo 1, en el que se planteó estudiar la influencia del grado de madurez y del sistema de vinificación sobre la composición, el color y la calidad del vino tinto.

Para ello, se realizaron diferentes tratamientos del racimo a tres niveles de madurez a escala de microvinificaciones. Paralelamente, se realizó una experiencia similar a mayor escala, en barricas, con uvas maduras.

Las analíticas de los vinos fueron realizadas en el laboratorio del grupo de Tecnología Enológica (TECNENOL) del Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili (URV, Tarragona) y en el Instituto Regional de Investigación Científica Aplicada (IRICA), Universidad de Castilla La Mancha (UCLM, Ciudad Real). **Los resultados del estudio fueron publicados en la revista *Oeno One*.**

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Influence of grape maturity and prefermentative cluster treatment of the Grenache cultivar on wine composition and quality

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Abstract

This work studied how different grape maturity levels and cluster treatments affect the color and phenolic composition of Grenache wines. Specifically, five treatments were undertaken at a microvinification scale for three maturity levels: Control (destemmed and crushed grapes), Whole Berry, Whole Cluster, Crushed Cluster and Submerged Cap. The first three treatments were also reproduced with large-scale wine fermentation in oak barrels but only with well-ripened grapes. The results indicated that the total polyphenol index (TPI), anthocyanin and proanthocyanidin concentrations, as well as the mean degree of polymerization were higher in all the treatments when the grapes were riper. Submerged Cap generated maximum color and polyphenolic extraction at the three maturity levels. Whole Berry wines were the most similar to the controls. The presence of stems (Crushed Cluster and Whole Cluster treatments) produced wines with a significantly higher pH at all maturity levels and with lower color intensity when the grapes were less ripe. The presence of stems also significantly increased the TPI in almost all cases.

Keywords: grape maturity, cluster treatment, stems, color, phenolic compounds

INTRODUCTION

The quality of red wines is highly determined by the composition of phenolic compounds. Some of their sensory attributes, such as color, body and astringency, are mainly associated with the composition of anthocyanins and proanthocyanidins (Gawel, 1998; Vidal et al., 2003). Anthocyanins are only present in grape skins of most grape cultivars, with the exception of teinturier varieties, whereas proanthocyanidins are present in skins, seeds, and stems (Ribereau-Gayon et al., 2000). Seed proanthocyanidins are made up of (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-gallate (Prieur et al., 1994), whereas skin proanthocyanidins also contain (-)-epigallocatechin and a much lower concentration of (-)-epicatechin-3-gallate (Souquet et al., 1996). Consequently, skin proanthocyanidins include procyanidins and prodelphinidins, whereas seed proanthocyanidins only consist of procyanidins. Little is known about stem proanthocyanidins, but it is thought that they are made up of the four monomers: (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-gallate, and (-)-epigallocatechin (Souquet et al., 2000; Llaudy et al., 2008). Skin proanthocyanidins have a higher mean degree of polymerization (mDP) than seed proanthocyanidins but the polymerization degree of stem proanthocyanidins is a subject of controversy (Souquet et al., 2000; Vivas et al., 2004; Llaudy et al., 2008). It has also been reported that molecular sizes, and especially the monomeric composition of proanthocyanidins, have a considerable influence on the perception of astringency. More specifically, a greater degree of polymerization and a higher percentage of galloylation cause a greater perception of astringency (Vidal et al., 2004).

It is well known that the maturity of grapes strongly influences the phenolic composition of red wines (Llaudy et al., 2008; Gil et al., 2012). Unripe grapes have lower extractability of anthocyanins and skin proanthocyanidins, but higher extractability of seed proanthocyanidins (Peyrot des Gachons and Kennedy, 2003; Canals et al., 2005). For this reason, immature grapes may produce more astringent wines because their seeds can release a greater quantity of highly galloylated proanthocyanidins (Llaudy et al., 2008). It has also been shown that stems can release highly astringent and bitter proanthocyanidins. Moreover, the presence of stems causes significant color loss and contributes to a ‘stemmy flavor’ in the wine (Boulton et al., 1995; Hashizume and

Samuta, 1997). For this reason, destemming grapes is a common procedure in red winemaking in order to avoid these negative attributes. Other arguments for removing stems are that they reduce the ethanol content and titratable acidity, increase pH and even take up valuable space in the tank (Sun and Spranger, 2005).

On the contrary, some winemakers argue that stems may occasionally have positive effects (Peynaud, 1984; Sun and Spranger, 2005). They claim that retaining stems produces wines with a higher concentration of proanthocyanidins, which helps to stabilize color and improve mouthfeel. Moreover, the presence of stems makes the cap less compact, which favors color extraction. Traditionally, stems have been used in red winemaking in such traditional regions as Châteauneuf-du-Pape (Côtes du Rhône), because their presence increased the polyphenolic content of wines and, therefore, improved their aging ability. Moreover, some winemakers in the Médoc region (Bordeaux) used to include a proportion of stems when grey rot was present, with the aim of inhibiting laccase and protecting wine color. Stems have occasionally been partially or fully used for lowtannin varieties such as Pinot Noir in traditional regions (Peynaud, 1981; Blouin, 2000). Nowadays, winemaking using the whole cluster is especially common in biodynamic/natural wine production, probably because the extra tannin contribution of stems can protect the wine against oxidation, which means that the doses of sulfur dioxide can be decreased.

Operations during winemaking can have a nonnegligible effect on color and phenolic compound extraction (Gómez-Plaza et al., 2000). Several studies have been carried out on the influence of temperature, enzymatic addition, maceration length, mechanical treatment of the cap, ethanol content, etc (Sacchi et al., 2005; Gil et al., 2013). However, to our knowledge, very little information exists about the influence of stem presence on winemaking, and wine composition and quality (Goode and Harrop, 2011). For this reason, the aim of this study was to investigate how different grape maturity levels and prefermentative cluster treatments, with or without stems, affected the color and phenolic composition of Grenache wines.

MATERIALS AND METHODS

1. Chemicals and equipment

Methanol, acetonitrile, formic acid and acetic acid of high performance liquid chromatography (HPLC) grade (>99%) and absolute ethanol and hydrochloric acid (37 %) were purchased from Panreac (Barcelona, Spain); acetaldehyde, polyvinylpoly-pyrrolidone, phloroglucinol, ascorbic acid, sodium acetate and ammonium formate were purchase from Sigma-Aldrich (Madrid, Spain); the commercial standards trans-caftaric acid (≥95%), quercetin 3-glucuronide (≥95%), caffeic acid (≥99%) and pcoumaric acids (≥99%) were purchased from Phytolab (Vestenbergsgreuth, Germany); the commercial standards malvidin 3-glucoside (≥95%), kaempferol (≥99%), quercetin dihydrate (≥99%), isorhamnetin (≥99%), myricetin (≥99%) and syringetin (≥99%), the 3-glucosides of kaempferol (≥99%), quercetin (≥99%), myricetin (≥99%), isorhamnetin (≥95%) and syringetin (≥99%) were purchased from Extrasynthese (Genay, France). Vitisin A (10-carboxy-pyranomalvidin-3-glucoside) was quantified with a previously obtained standard of ≥95% purity (Blanco-Vega et al., 2011). All spectrophotometric measurements were carried out with a Helios Alpha UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2. Grapes and wines

The experiment was carried out with a Grenache variety (Vitis vinifera L) from the AOC Montsant (Spain). About 230 kg of grapes were manually harvested at 3 maturity levels (3, 5, and 7 weeks after veraison). Five different cluster treatments and maceration techniques were performed: Control, Submerged Cap, Whole Berry (destemming without crushing), Crushed Cluster (crushing without destemming), and Whole Cluster. All microvinifications were carried out in triplicate in 25 L tanks. Around 3/5 of the grapes were carefully destemmed (Delta, Bucher-Vaslin, Chalonnes-sur-Loire, France) and the intact berries were randomly distributed in 9 batches of 15 kg. The first three batches were introduced in three tanks without any treatment (Whole Berry), whereas the other six were crushed with a manual crusher. Three of these tanks were considered as “Control” while the other three were employed for “Submerged Cap” winemaking. The remaining 2/5 of the grapes were randomly distributed in batches of 15 kg without destemming. Three of them were crushed with a manual crusher (Crushed Cluster) while

the other three were placed in the tanks intact (Whole Cluster). All the tanks were immediately sulfited (100 mg $K_2S_2O_5$/kg), inoculated with 200 mg/kg of selected yeast (EC1118, Lallemand Inc, Montreal, Canada) and maintained at a room temperature of 25 ± 1 °C. All treatments were punched-down once a day until the end of fermentation, excluding the Submerged Cap system, which was carried out according to the winemaking method described by Sampaio et al. (2007). After 2 weeks of maceration, the wines were racked into bottles (5 L plastic). All the wines were sulfited (100 mg $K_2S_2O_5$/L) and kept at 4 °C for 1 month for stabilization. Malolactic fermentation was therefore inhibited to prevent any variations caused by it. The wines were subsequently bottled and stored in a dark cellar at 15 °C until analysis.

This experiment was also performed on a larger scale (400 kg each) but only with well-ripe grapes from another vineyard and with 3 treatments only (Control, Whole Berry and Whole Cluster). This experiment was performed without replicates in opened French oak barrels (500 L) placed vertically as fermentation tanks. These wines were aged in 225 L French oak barrels for six months.

3. Standard wine analysis

The analytical methods recommended by the OIV were used to determine the ethanol content, pH and volatile acidity (Organisation Internationale de la Vigne et du Vin, 2014). The total polyphenol index (TPI) was analyzed by measuring the 280 nm absorbance of a 1:100 dilution of wine with a spectrophotometer, using a 10-mm quartz cuvette and multiplying the absorbance value by 100 as described by Ribéreau-Gayon et al. (2006). Condensed tannin concentration was estimated by precipitation with methyl-cellulose (Sarneckis et al., 2006).

4. Color parameters

Ten microliters of a 10 % (v/v) acetaldehyde solution was added to 1 mL of wine sample 20 min before color measurement to avoid sulfite interferences. The color intensity (CI) was estimated using the method described by Glories (1984). The CIELab coordinates, lightness (L*), chroma (C*), hue (h*), red-greenness (a*), and yellow-blueness (b*), were

determined according to the method used by Ayala et al. (1997) and data processing was performed with MSCV software (Ayala et al., 2001).

5. Analysis of individual low molecular mass (MM) phenolic substances in wine

The individual low MM phenolic substances in wines were prepared with solid phase extraction and analyzed with a reversed-phase HPLC diode array detector-electrospray ionization-tandem mass spectrometry system (RP-HPLC-DAD-ESI-MSn) (Blanco-Vega et al., 2011; Lago-Vanzela et al., 2013). The system comprised an Agilent 1100 Series HPLC (Agilent, Waldbronn, Germany), equipped with a DAD (G1315B) and an LC/MSD Trap VL (G2445C VL) ESI-MSn, coupled to an Agilent Chem Station (version B.01.03) data processing station. The mass spectra data were processed with the Agilent LC/MS Trap software (version 5.3). The samples (0.25 mL of wine diluted with 4.75 mL of water: formic acid, 98.5:1.5) were injected (100 µL) after filtration (0.20 µm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany) on a Ascentis Express C18 reversed-phase column (4.6 x 150 mm; 2.7 µm particle size) (Supelco, Sigma-Aldrich, Madrid, Spain), maintained at 16°C. The solvents were A [water/methanol/formic acid (89:10:1, v/v/v)] and B (methanol), and the flow rate was 0.5 mL/min. The linear gradient for solvent B was: 0 min, 1 %; 2 min, 1%; 60 min, 23 %; 75 min, 70 %; 80 min, 95 %; 90 min, 95 %; 95 min, 1 %; 100 min, 1 %. Two MS scan types were used: enhanced MS for compound identification, and multiple reaction monitoring (MRM) for quantification. The conditions for both MS scan types were ion spray voltage, −4000; ion source temperature, 450 °C; collision gas, high; curtain gas, 15; ion source gas 1, 70; ion source gas 2, 50; declustering potential, −35; entrance potential, −10; collision energy, −30; and collision cell exit potential, −3. Two injections of (+)-catechin standard solution, one at the beginning and the second at the end of every injection series, were performed to update the response factors before quantification. The analyses were carried out in duplicate. The chromatographic system was managed by an Agilent Chem Station (version B.01.03) data processing station. The mass spectral data were processed with the Analyst MDS software (Applied Biosystems, version 1.5).

6. Analysis of wine proanthocyanidins

The proanthocyanidins of the wines were extracted and analyzed by acid depolymerization in the presence of an excess of phloroglucinol (Pastor del Rio and Kennedy, 2006); the products of the reaction were separated by RP-HPLC-DAD (Kennedy and Jones et al., 2001). Proanthocyanidins were analyzed with an Agilent 1200 Series HPLC equipped with a G1362A refractive index detector (RID), a G1315D DAD, a G1311A quaternary pump, a G1316A column oven and a G1329A autosampler (Agilent Technologies, Santa Clara, CA, USA). The chromatographic system was managed by an Agilent Chem Station (version B.01.03) data processing station.

7. Sensory analysis

Sensory analyses were only performed with the wines obtained by barrel winemaking because they were considered more representative of what occurs in the wineries than micro-scale wines. Two sensory triangle tests were conducted by eleven expert tasters to compare the control wine versus the wines obtained with the Whole Berry or the Whole Cluster. In all the cases, the main objective was to determine whether the tasters were able to recognize the wine that was different. The secondary objective was to determine which wine was preferred by the panelists who had correctly identified the different wines.

8. Statistics

All the data for micro-scale wines are expressed as the arithmetic average ± standard deviation of three replicates. One-factor ANOVA tests were carried out with XLSTAT software, and multiple comparisons were performed using the Student–Newman–Keuls post-hoc test. The level of significance of sensory triangle tests was determined following Jackson’s method (Jackson, 2002).

RESULTS AND DISCUSSION

Table 1 shows the general parameters of the microscale wines. In overall terms, these results indicate clearly that the maturity level exerts a major influence on wine composition regardless of the cluster treatment and maceration procedure. As expected, the greater the maturity the higher the ethanol content, pH, TPI and tannin

concentration in all the treatments. All these data confirm that grapes underwent the correct maturation process. Volatile acidity also increased with grape maturity, probably due to the higher ethanol content.

Table 1 - Effect of grape maturity and prefermentative cluster treatment on the general parameters of micro-scale wines

Parameter	Maturity Level	Control	Submerged Cap	Whole Berry	Crushed Cluster	Whole Cluster
Ethanol (%v/v)	1	14.2 ± 0.1 A β	14.3 ± 0.1 A β	14.1 ± 0.1 A αβ	14.3 ± 0.1 A β	13.9 ± 0.1 A α
	2	16.0 ± 0.2 B αβ	16.2 ± 0.1 B β	15.8 ± 0.1 B α	15.9 ± 0.2 B αβ	15.7 ± 0.1 B α
	3	16.5 ± 0.1 C αβ	16.7 ± 0.1 C β	16.6 ± 0.0 C β	16.7 ± 0.1 C β	16.3 ± 0.1 C α
pH	1	3.17 ± 0.03 A α	3.19 ± 0.03 A α	3.20 ± 0.03 A α	3.38 ± 0.07 A β	3.28 ± 0.03 A β
	2	3.55 ± 0.06 B α	3.59 ± 0.01 B α	3.61 ± 0.03 B α	3.73 ± 0.03 B β	3.73 ± 0.01 B β
	3	3.79 ± 0.02 C α	3.79 ± 0.02 C α	3.77 ± 0.02 C α	3.89 ± 0.04 C β	3.94 ± 0.01 C β
AV (g/L)	1	0.27 ± 0.02 A α	0.28 ± 0.04 A α	0.26 ± 0.00 A α	0.29 ± 0.03 A α	0.22 ± 0.04 A α
	2	0.48 ± 0.04 B δ	0.39 ± 0.02 B γ	0.27 ± 0.02 A α	0.41 ± 0.02 B γ	0.32 ± 0.02 B β
	3	0.51 ± 0.04 B α	0.48 ± 0.02 C α	0.55 ± 0.07 B α	0.54 ± 0.04 C α	0.48 ± 0.02 C α
TPI	1	42.0 ± 2.2 A α	53.0 ± 1.9 A β	46.0 ± 0.7 A α	43.9 ± 1.6 A α	50.5 ± 3.0 A β
	2	51.7 ± 0.8 B α	55.3 ± 2.6 A α	52.6 ± 2.8 B α	54.5 ± 1.3 B α	54.9 ± 2.3 A α
	3	55.0 ± 3.1 B α	66.4 ± 2.6 B β	61.9 ± 2.1 C β	63.1 ± 0.9 C β	64.6 ± 1.1 B β
Tannins (mg/L)	1	509 ± 45 A α	637 ± 28 A γ	584 ± 10 A β	529 ± 58 A αβ	612 ± 66 A βγ
	2	566 ± 63 AB α	590 ± 52 A α	588 ± 25 A α	646 ± 55 B α	619 ± 30 A α
	3	655 ± 74 B α	839 ± 39 B β	688 ± 36 B α	811 ± 87 C β	779 ± 41 B β

Different letters indicate significant differences ($p<0.05$). Capital letters are used to compare the different maturity levels and Greek letters are used to compare the different treatments with the control (by using one-way ANOVA, and employing the Student-Newman-Keuls method for multiple comparisons). AV: Volatile acidity; TPI: Total Polyphenol Index of wines.

The different prefermentative cluster treatment of the grapes showed some interesting differences in some of the general parameters. The ethanol content was very similar in all the treatments at each maturity level. However, the ethanol content of Whole Cluster wines was slightly lower than in the other treatments, and these differences were significant in some cases. This somewhat lower ethanol content may be related to the presence of stems which can absorb ethanol and release water (Hashizume et al., 1998). It has been reported that the moisture content of stems is around 65 % (González-Centeno et al., 2010). Considering this value and that stems represent a percentage of about 4-5 % of the cluster weight, the observed decrease in ethanol content can be considered as quite logic once the osmotic equilibrium is reached. However, the Crushed Cluster wines showed similar values to the other experimental treatments although stems were also present. The influence of the presence of stems was clearer on the pH

since both treatments containing stems, Crushed Cluster and Whole Cluster, had significantly higher values in this parameter. This is probably because stems can release potassium which neutralizes the acids (Hashizume et al., 1998).

Overall Submerged Cap wines have higher TPI and tannin concentrations than Control wines, although in some of the maturity levels these differences were not significant. These results confirm that this winemaking procedure improves polyphenol extraction, as has been previously reported (Bosso et al., 2011; Ichikawa et al., 2012). Whole Berry wines also presented generally higher TPI and tannin concentration than the Control wines, although these differences were only significant in some maturity levels. These differences were in any case smaller than those observed in Submerged Cap wines. As expected, Crushed Cluster and Whole Cluster wines also had higher TPI and tannin concentrations than the Control wines, although these differences were only significant in some cases. This data confirms that stems are a source of tannins (Suriano et al., 2015).

Table 2 - Effect of prefermentative cluster treatment on the general parameters of barrel-scale wines

Parameter	Control	Whole Berry	Whole Cluster
Ethanol (%v/v)	16.6	16.5	16.1
pH	3.86	3.76	4.03
AV (g/L)	0.51	0.49	0.49
TPI	38.3	45.7	51.1
Tannins (mg/L)	300	371	474

AV: Volatile acidity; TPI: Total Polyphenol Index of wines.

Table 2 shows the general parameters of the barrelscale wines. Since no replicates were performed in that experiment, it is impossible to draw statistical differences. However, some tendencies can be confirmed in comparison with the micro-scale experiments. For example, the ethanol content of Whole Cluster wine was lower and the pH higher than in Control and Whole Berry wines in a similar way to observations in the micro-scale trials. Whole Berry and especially Whole Cluster wines also have higher TPI and tannin

concentrations than the Control wine. This data suggests that winemaking with Whole Berry favors phenolic compound extraction. This behavior was also observed in microscale assays, although the differences were smaller and not always significant. These results also confirm that the stems enrich wine in tannins, and probably also favor the extraction of phenolic compounds from skins and seed because their presence makes the cap less compact (Llaudy et al., 2008).

Table 3 - Effect of grape maturity and prefermentative cluster treatment on anthocyanins and color parameters of micro-scale wines

Parameter	Maturity Level	Control	Submerged Cap	Whole Berry	Crushed Cluster	Whole Cluster
Total Anthocyanins	1	280 ± 69 A α	388 ± 29 A βγ	291 ± 56 A α	342 ± 34 A αβ	416 ± 8 A γ
	2	322 ± 34 A α	401 ± 24 A β	388 ± 39 A αβ	434 ± 23 B β	506 ± 15 B γ
	3	365 ± 42 A α	426 ± 15 A β	361 ± 13 A α	439 ± 7 B β	497 ± 7 B γ
Non-acylated	1	250 ± 61 A α	352 ± 26 A βγ	259 ± 50 A α	308 ± 31 A αβ	373 ± 4 A γ
	2	287 ± 31 A α	360 ± 22 A β	346 ± 35 B αβ	401 ± 20 B β	464 ± 13 B γ
	3	338 ± 39 A α	394 ± 13 A β	333 ± 10 B α	411 ± 6 B β	465 ± 6 B γ
Acetylated	1	8 ± 2 A α	8 ± 1 A α	7 ± 1 A α	9 ± 1 A α	11 ± 2 A α
	2	10 ± 1 A α	10 ± 1 A α	10 ± 2 A α	9 ± 1 A α	11 ± 2 A α
	3	8 ± 1 A α	8 ± 1 A α	7 ± 1 A α	6 ± 2 A α	8 ± 2 A α
p-Coumaroylated	1	22 ± 6 A α	29 ± 3 AB αβ	25 ± 5 AB αβ	25 ± 2 A α	32 ± 2 B β
	2	25 ± 3 A α	31 ± 2 B β	32 ± 3 B β	30 ± 2 B αβ	31 ± 2 B β
	3	20 ± 2 A α	24 ± 2 A α	21 ± 2 A α	22 ± 2 A α	25 ± 3 A α
Pyranoanthocyanins	1	21 ± 4 A β	13 ± 2 A α	9 ± 2 A α	28 ± 7 B β	38 ± 16 B β
	2	32 ± 2 B γ	24 ± 2 B β	19 ± 1 B α	34 ± 7 B γ	50 ± 4 B δ
	3	27 ± 3 AB β	29 ± 3 B β	24 ± 9 B β	9 ± 1 A α	10 ± 1 A α
CI	1	6.4 ± 0.1 A β	7.6 ± 0.2 A γ	6.1 ± 0.2 A β	5.4 ± 0.3 A α	5.5 ± 0.3 A α
	2	7.8 ± 0.1 B β	9.7 ± 1.0 B γ	7.9 ± 0.3 B β	7.7 ± 0.6 B β	6.7 ± 0.3 B α
	3	10.5 ± 1.0 C αβ	13.5 ± 0.6 C γ	11.6 ± 0.4 C β	11.0 ± 1.0 C β	9.6 ± 0.4 C α
L*	1	64.4 ± 0.4 C β	59.7 ± 0.8 C α	65.6 ± 0.8 C β	68.6 ± 1.5 C γ	68.1 ± 1.5 C γ
	2	59.5 ± 0.2 B β	53.1 ± 3.6 B α	58.8 ± 1.2 B β	59.4 ± 2.1 B β	63.2 ± 1.2 B γ
	3	48.0 ± 3.0 A βγ	39.8 ± 1.4 A α	44.6 ± 1.0 A β	45.8 ± 2.7 A βγ	49.8 ± 1.4 A γ
C*	1	41.7 ± 0.8 A β	50.8 ± 1.1 A γ	41.0 ± 0.8 A β	33.7 ± 1.9 A α	38.3 ± 3.2 A β
	2	47.6 ± 0.8 B β	53.0 ± 2.4 A γ	49.5 ± 1.1 B β	42.9 ± 1.1 B α	41.1 ± 1.1 A α
	3	44.9 ± 2.6 AB αβ	50.7 ± 0.4 A γ	47.5 ± 0.9 B β	43.3 ± 2.3 B α	40.7 ± 2.3 A α
h*	1	357.3 ± 0.2 A γ	355.5 ± 0.8 A αβ	356.4 ± 0.5 A βγ	356.8 ± 0.1 A βγ	354.5 ± 0.8 A α
	2	357.7 ± 0.6 A β	358.2 ± 1.3 B β	356.2 ± 0.3 A α	358.5 ± 0.6 B β	355.7 ± 0.9 A α
	3	362.3 ± 0.7 B α	362.2 ± 0.6 C α	361.8 ± 0.4 B α	361.5 ± 0.8 C α	362.4 ± 2.1 B α

Different letters indicate significant differences ($p<0.05$). Capital letters are used to compare the different maturity levels and Greek letters are used to compare the different treatments with the control (by using one-way ANOVA, and employing the Student-Newman-Keuls method for multiple comparisons). All wine pigments (determined by RP-HPLC-ESI-MSn) are expressed as mg/L of malvidin-3-O-glucoside; Nonacylated: Summation of malvidin-3-O-glucoside, delphinidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and cyanidin-3-O-glucoside; Acetylated: Summation of malvidin-3-O-(6-acetyl)-glucoside, petunidin-3-O-(6-acetyl)-glucoside and delphinidin-3-O-(6-acetyl)-glucoside; Coumaroylated: Summation of malvidin-3-O-(6-*p*-coumaroyl)-glucoside, petunidin-3-O-(6-p-coumaroyl)-glucoside and peonidin-3- O-(6-p-coumaroyl)-glucoside ; Pyranoanthocyanins : Vitisin A; CI : Color intensity of wines ; L*: Lightness values (CIELab coordinates) ; C*: Chroma values (CIELab coordinates) ; h*: Hue values (CIELab coordinates).

Table 3 shows the influence of grape maturity and prefermentative cluster treatment on the anthocyanin concentration and color parameters of micro-scale wines. In overall terms, total anthocyanins tended to increase with maturity in all treatments, although

the differences were not always significant. This tendency was also observed in non-acylated anthocyanins but was not clear in acylated anthocyanins (acetylated and coumaroylated), maybe because the latter are minor anthocyanins in Grenache wines (Noriega and Casp, 2007). The influence of ripeness on wine color was also very clear. In specific terms, the color intensity (CI) and hue (h*) increased whereas the luminosity (L*) decreased when the grapes were riper. This data confirms that riper grapes produced wines richer in anthocyanins and with a deeper color.

The total anthocyanin concentration of Submerged Cap wine was significantly higher than in the Control wine at all maturation levels. This trend was also observed in non-acylated anthocyanins but not in acylated anthocyanins. Submerged Cap wine also had higher CI, C* and lower L* than the Control wines, although these differences were not significant in some of the maturity levels. This data confirms that this winemaking procedure improves the anthocyanin extraction.

In general terms, Crushed Cluster and Whole Cluster wines also had significant higher anthocyanin concentration than control wines. This higher anthocyanin concentration may seem surprising because stems have been reported as being able to absorb anthocyanins (Suriano et al., 2015) and their presence should consequently reduce anthocyanin concentration. By contrast, stems release tannins and other phenolic compounds that can protect anthocyanins against oxidation (Bautista-Ortín et al., 2005). Moreover, the presence of stems makes the cap less compact, which favors anthocyanin extraction (Llaudy et al., 2008). In our particular case, the presence of stems enhanced total anthocyanin concentration at almost all maturity levels. This behavior was similar for non-acylated anthocyanins but not in acylated anthocyanins. However, C* of Crushed Cluster and Whole Cluster wines tended to be lower than in Control wines, although these differences were not always significant. In the case of Whole Cluster wines, CI also tended to be lower and L* to be higher than in Control wines. However, this tendency was not observed in Crushed Cluster wines. As a whole, these results indicate that the presence of stems has a negative effect on wine color, in contrast to the higher anthocyanin concentration detected in these wines. A possible cause for this

higher anthocyanin concentration and poorer color is probably related to the higher pH observed in the wines made in the presence of stems.

In general terms, Whole Berry wine showed similar anthocyanin concentrations to Control wine. The color parameters of Whole Berry wines were also very similar to Control wines in the first harvest. However, CI and C* tended to be somewhat higher and L* to be lower in Whole Berry wines of the third harvest, when the grapes were riper.

The pigments derived from anthocyanins, pyranoanthocyanins, did not show a clear tendency according to the maturity level of the grapes or of the prefermentative cluster treatment. This result could be expected, as the main pyranoanthocyanins found in the Grenache wines, vitisin A, at this stage, namely young red wines, were produced by alcoholic fermentation yeast by-products (Blanco-Vega et al., 2011) and we used the same yeast strain for all vinifications.

Table 4 shows the anthocyanin concentration and color parameters of barrel-scale wines. Although it is not possible to draw statistical conclusions because no replicates were performed, some tendencies can be highlighted. Whole Berry wine has a higher anthocyanin concentration than Control wine. However, these data do not match with those obtained at micro-scale level, in which only small differences were found. A possible reason for this may be related to the fact that on a micro-scale level, the solubilization of anthocyanins from skins during maceration process is easier than on a barrel-scale because the punch down is more effective in small volume. This fact has probably reduced the differences. Whole Berry wine also has higher CI and C*, and lower L* and H* than the Control wine.

Table 4 - Effect of prefermentative cluster treatment on anthocyanins and color parameters of barrel-scale wines

Parameter	Control	Whole Berry	Whole Cluster
Total Anthocyanins	236	331	297
Non-acylated	225	313	280
Acetylated	3	5	4
***p*-Coumaroylated**	7	13	12
Pyranoanthocyanins	16	19	14
CI	6.8	7.8	7.1
L*	65.2	59.3	63.1
C*	32.6	40.3	32.5
h*	12.4	2.4	7.8

All wine pigments (determined by RP-HPLC-ESI-MSn) are expressed as mg/L of malvidin-3-O-glucoside; Non-acylated: Summation of malvidin-3-O-glucoside, delphinidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and cyanidin-3-O-glucoside; Acetylated: Summation of malvidin-3-O-(6-acetyl)-glucoside, petunidin-3-O-(6-acetyl)-glucoside and delphinidin-3-O-(6-acetyl)-glucoside; Coumaroylated: Summation of malvidin-3-O-(6-p-coumaroyl)-glucoside, petunidin-3-O-(6-p-coumaroyl)-glucoside and peonidin-3-O-(6-p-coumaroyl)-glucoside; Pyranoanthocyanins : Vitisin A; CI: Color intensity of wines ; L*: Lightness
values (CIELab coordinates); C*: Chroma values (CIELab coordinates); h*: Hue values (CIELab coordinates).

Whole Cluster wine also has higher anthocyanin concentrations than the Control wine, but in this case the color parameters CI, C* and L* were very similar. This higher anthocyanin concentration of Whole Cluster wine is consistent with those obtained in the micro-scale trials, which would confirm that the presence of stems favors anthocyanin extraction and/or provides protection against oxidation. The lack of differences in CI, C* and L* between Whole Cluster wine and Control wine despite the differences in anthocyanin concentration can be attributed to the higher pH of Whole Cluster wine, as mentioned in the comments on the micro-scale trials.

The hue (h*) of the control wine at barrel-scale was somewhat higher than in Whole Berry and Whole Cluster wines. This high value indicates that the color of the Control wine was more yellowish and consequently indicates a greater oxidation. Grenache is a cultivar with a great tendency to color oxidation (De Andres-De Prado et al., 2007). Winemaking in open barrel can favor a greater oxygen intake that may be the cause of the higher h*. The other winemaking conditions did not have this disadvantage, probably due to two different reasons. In the case of Whole Berry, the extraction of anthocyanins took place inside the berry at the beginning of the alcoholic fermentation, protecting the anthocyanins against oxygen. In the case of Whole Cluster, the presence of stems releases tannins and other phenolic compounds that can act as antioxidants, protecting anthocyanins from oxidation.

Table 5 - Effect of grape maturity and prefermentative cluster treatment on the compositionof the phenolic compounds of micro-scale wines

Parameter		Maturity Level	Control	Submerged Cap	Whole Berry	Crushed Cluster	Whole Cluster
Non-flavonoids	Total hydroxycinnamic acids and derivatives	1	102 ± 5 A α	167 ± 12 A β	94 ± 55 A α	82 ± 28 A α	89 ± 31 A α
		2	133 ± 1 B β	163 ± 14 A γ	136 ± 24 A βγ	85 ± 13 A α	107 ± 12 A α
		3	93 ± 32 A α	164 ± 9 A β	133 ± 19 A αβ	148 ± 10 B β	165 ± 7 B β
Flavonols	Total Flavonols	1	27 ± 5 A β	47 ± 9 A γ	23 ± 8 A αβ	16 ± 4 A α	26 ± 12 A αβ
		2	47 ± 2 B β	55 ± 10 AB β	46 ± 5 B β	30 ± 11 AB α	31 ± 5 AB α
		3	36 ± 13 AB α	64 ± 3 B β	49 ± 15 B αβ	49 ± 10 B α	43 ± 3 B α
	Aglycones	1	6 ± 2 A α	13 ± 3 A β	9 ± 1 A αβ	6 ± 2 A α	12 ± 4 A β
		2	11 ± 2 B α	14 ± 6 A α	15 ± 2 B α	13 ± 3 B α	13 ± 3 AB α
		3	14 ± 7 B α	26 ± 1 B β	20 ± 3 B α	17 ± 4 B α	17 ± 1 B α
Flavan-3-ols	Proanthocyanidins (mg/l)	1	876 ± 59 A α	749 ± 161 AB α	846 ± 59 B α	835 ± 106 AB α	1118 ± 137 B β
		2	924 ± 45 A α	882 ± 79 B α	972 ± 53 C α	955 ± 55 B α	913 ± 101 AB α
		3	776 ± 197 A α	771 ± 24 A α	731 ± 12 A α	798 ± 27 A α	794 ± 86 A α
	mDP	1	5.2 ± 0.3 A α	5.7 ± 0.4 A α	5.4 ± 0.1 A α	5.2 ± 0.3 A α	5.9 ± 0.5 A α
		2	7.0 ± 0.3 B β	6.9 ± 0.3 B β	6.6 ± 0.1 B β	6.3 ± 0.6 B αβ	6.1 ± 0.2 A α
		3	8.2 ± 0.6 C βγ	8.9 ± 0.5 C γ	7.9 ± 0.2 C β	7.8 ± 0.3 C αβ	6.7 ± 1.0 A α
	Prodelphinidins (%)	1	19.2 ± 0.3 A β	22.4 ± 0.4 A γ	19.7 ± 0.2 A β	18.2 ± 0.5 A α	21.9 ± 1.1 B γ
		2	22.2 ± 0.5 C β	23.1 ± 0.8 A β	21.5 ± 1.0 B αβ	19.9 ± 0.6 B α	20.4 ± 0.1 A α
		3	20.1 ± 0.3 B α	22.3 ± 0.3 A β	20.9 ± 0.5 B α	22.4 ± 0.4 C β	24.5 ± 1.3 C γ
	Galloylation (%)	1	6.2 ± 0.6 B β	4.8 ± 0.3 A α	5.9 ± 0.5 B β	6.2 ± 0.2 B β	5.8 ± 0.2 B β
		2	4.6 ± 0.1 A α	4.4 ± 0.1 A α	4.2 ± 0.1 A α	4.3 ± 0.3 A α	3.8 ± 1.0 A α
		3	6.9 ± 0.2 B β	7.0 ± 0.1 B β	6.8 ± 0.3 C β	6.8 ± 0.3 C β	4.4 ± 1.3 AB α

Different letters indicate significant differences ($p<0.05$). Capital letters are used to compare the different maturity levels and Greek letters are used to compare the different treatments with the control (by using one-way ANOVA, and employing the Student-Newman-Keuls method for multiple comparisons). Total amount of hydroxycinnamic acids and derivatives (determined by RP-HPLC-ESI-MSn) is expressed as mg/L of caffeic acid; Total amount of flavonols (determined by RP-HPLC-ESI-MSn) is expressed as mg/L of quercetin-3-O-glucoside; Total proanthocyanidin concentration (mg/L) was calculated by the addition of the total monomeric unit released during the phloroglucinolysis reaction; mDP, Mean degree of polymerization of wine proanthocyanidins; Prodelphinidin ratio of proanthocyanidins is expressed as a percentage; Galloylation degree of proanthocyanidins is expressed as a percentage.

Table 5 shows the influence of grape maturity and prefermentative cluster treatment on the hydroxycinnamic acid and derivative, flavonol and flavan-3-ol concentration of micro-scale wines. In general, the total hydroxycinnamic acid and derivative

concentration showed an erratic behavior throughout the maturity process. In the case of the Control wines, the total hydroxycinnamic acids and derivatives increased significantly between the first and second harvest but decreased in the third. In the case of Submerged Cap, the concentration did not change throughout ripening. Finally, in the other three prefermentative cluster treatments, the total hydroxycinnamic acids and derivatives tended to increase, although the differences were not always significant. It is therefore very difficult to draw conclusions.

In overall terms, the total flavonols and their aglycones tended to increase when the grapes were riper in all the experimental conditions, with the Submerged Cap wines being the richest in these substances. The Control and Whole Berry wines had similar levels of flavonols. By contrast, when stems were present, Whole Cluster and Crushed Cluster wines, the total flavonol concentration was significantly lower in some of the maturity levels than in Control wines.

The total proanthocyanidin concentration obtained by phloroglucinolysis is also shown in Table 5. These data are higher than those obtained by the methyl cellulose method and do not show a similar tendency than that observed for TPI or tannin concentration obtained by the methyl cellulose method. In fact, the total proanthocyanidin concentration measured by this method showed an undefined behavior throughout maturity and among the different cluster treatments. However, phloroglucinolysis provides some interesting information about the structural characteristics of proanthocyanidins, such as the mDP, the percentage of prodelphinidins and the percentage of galloylation (Kennedy and Taylor, 2003). The mDP of the proanthocyanidins clearly tended to increase with maturity in all the prefermentative cluster treatments, although in the case of Whole Cluster wine this increase was not significant. The mDP of the proanthocyanidins of all treatments was similar in wines from the first harvest and tended to be lower in wines fermented in the presence of the stems in the other two harvests, although these differences were only significant in the case of Whole Cluster wines. Finally, the percentage of prodelphinidins and galloylation did not show any clear tendency in terms of either the maturity level or the prefermentative cluster treatments.

Table 6 shows the influence of grape maturity and prefermentative cluster treatment on hydroxycinnamic acid and derivative, flavonol and flavan-3-ol concentration in barrel-scale wines. Although no replicates were performed, some conclusions can be drawn. Whole Berry wine has a higher concentration of hydroxycinnamic acids and derivatives, flavonols and proanthocyanidins than Control wine. The mDP and the percentage of prodelphinidins were also higher in Whole Berry wine than in Control wine, whereas the percentage of galloylation was similar. By contrast, Whole Cluster wine has similar levels of hydroxycinnamic acids and derivatives than the Control wine, but the total flavonol and proanthocyanidin concentrations were higher than in the Control wine. The mDP and the percentage of prodelphinidins were also higher than in the controls.

Table 6 - Effect of prefermentative cluster treatment on the composition of the phenolic compounds of barrel-scale wines

Parameter		Control	Whole Berry	Whole Cluster
Non-flavonoids	Total hydroxycinnamic acids and derivatives	79	119	72
Flavonols	Total Flavonols	15	24	22
	Aglycones	5	9	9
Flavan-3-ols	Proanthocyanidins (mg/l)	447	555	647
	mDP	5.7	6.6	6.4
	Prodelphinidins (%)	12.3	15.6	16.0
	Galloylation (%)	5.0	5.4	5.8

Total amount of hydroxycinnamic acids and derivatives (determined by RP-HPLC-ESI-MSn) is expressed as mg/L of caffeic acid; Total amount of flavonols (determined by RP-HPLC-ESI-MSn) is expressed as mg/L of quercetin-3-O-glucoside; Total proanthocyanidin concentration (mg/L) was calculated by the addition of the total monomeric unit released during the phloroglucinolysis reaction; mDP, Mean degree of polymerization of wine proanthocyanidins; Prodelphinidin ratio of proanthocyanidins is expressed as a percentage; Galloylation degree of proanthocyanidins is expressed as a percentage.

Table 7 - Sensory analysis of the wines elaborated in oak barrels using different cluster treatments

Triangular test	Positive identifications	P	Preference		
			Control	Whole berry	Whole cluster
Control vs Whole Berry	9/11	< 0.05	2	7	-
Control vs Whole Cluster	8/11	< 0.05	5	-	3

Table 7 shows the results of the sensory analysis of the various wines produced in oak barrels. The Whole Berry and Whole Cluster wines were compared with the Control wine by means of triangular tests. The results were very clear. The tasters were able to distinguish significantly between the Whole Berry wine and the Control wine (9/11). Of all the tasters who distinguished them correctly, seven preferred the Whole Berry wine, whereas the other two tasters preferred the Control wine. The tasters were also able to distinguish significantly between the Whole Cluster wine and the Control wine (8/11) and 5 of the tasters that selected the wines correctly preferred the Control wine, while the other three tasters preferred the Whole Cluster wine.

A principal component analysis was performed in order to obtain a better understanding of the influence of prefermentative cluster treatment on wine composition. Figure 1 shows the plots of varimax-rotated principal component analyses of wines from the three harvests. In the first harvest (Fig 1A), the first component explains 58.89 % of the variance, and the second accounts for 27.79 % (meaning that the aggregate variance explained by the first two components was 86.68 %). The loadings are presented as vectors, and their length and direction indicate the contribution made by both components. A clear trend can be observed in this plot, and it is possible to separate the different experimental groups. The two experimental wines produced with the presence of stems, the Crushed Cluster and Whole Cluster wines, were located on the lower side of the graph, where the vectors for pH, tannins, TPI and L* were directed. By contrast, the Control, Submerged Cap and Whole Berry wines were located in higher positions, and nearly all the points were at the top of the graph, where the vectors for CI and C* were directed.

This behavior was generally consistent in the other two levels of maturity. In the second harvest (Fig 1B), the first component accounts for 54.30 % of the variance, and the second component accounts for 21.03 % (making the aggregate variance explained by the first two components 75.33 %). This time, the Crushed Cluster and Whole Cluster wines were located on the left side of the graph, in the direction of the pH, tannins and L* vectors, whereas the other experimental wines were located on the right side, where the CI and C* vectors were again directed. Finally, in the case of the third harvest (Fig 1C), the first component accounts for 56.19 % of the variance, and the second accounts for 27.03 % (making the aggregate variance explained by the first two components 83.22 %). Once again, the experimental wines produced in the presence of stems were separated from those produced without them. In this case, Crushed Cluster and Whole Cluster wines were located towards the top of the graph, in the direction of the pH and tannins vectors. By contrast, the Control wines were located in the lower left quadrant and the other two experimental wines, Whole Berry and Submerged Cap, were located on the right side of the graph, where once again the CI and C* vectors were directed.

In general, the PCA of the three levels of maturity confirms that the presence of stems generates wines with higher tannin extraction, less color and higher pH than in wines produced without stems.

It can be concluded that grape ripening and cluster treatments have a clear effect on color and polyphenol extraction. Specifically, TPI, anthocyanin, proanthocyanidin concentrations and mDP were higher when the grapes were riper. Regarding the different treatments, there is strong evidence to suggest that Submerged Cap presents a higher polyphenol extraction than conventional winemaking. The presence of stems under Crushed Cluster and Whole Cluster conditions increases proanthocyanidin extraction. However, stems also decrease color and anthocyanin concentration, increase pH, and produce wines with poor sensory attributes. The analytical micro-scale results of Whole berry wines were very similar to the Control wine, but these presented a clearly better composition in oak winemaking, which was also preferred by the tasters.

Further studies are required on a more realistic scale, for a better understanding of how cluster treatment affects the composition and quality of red wine.

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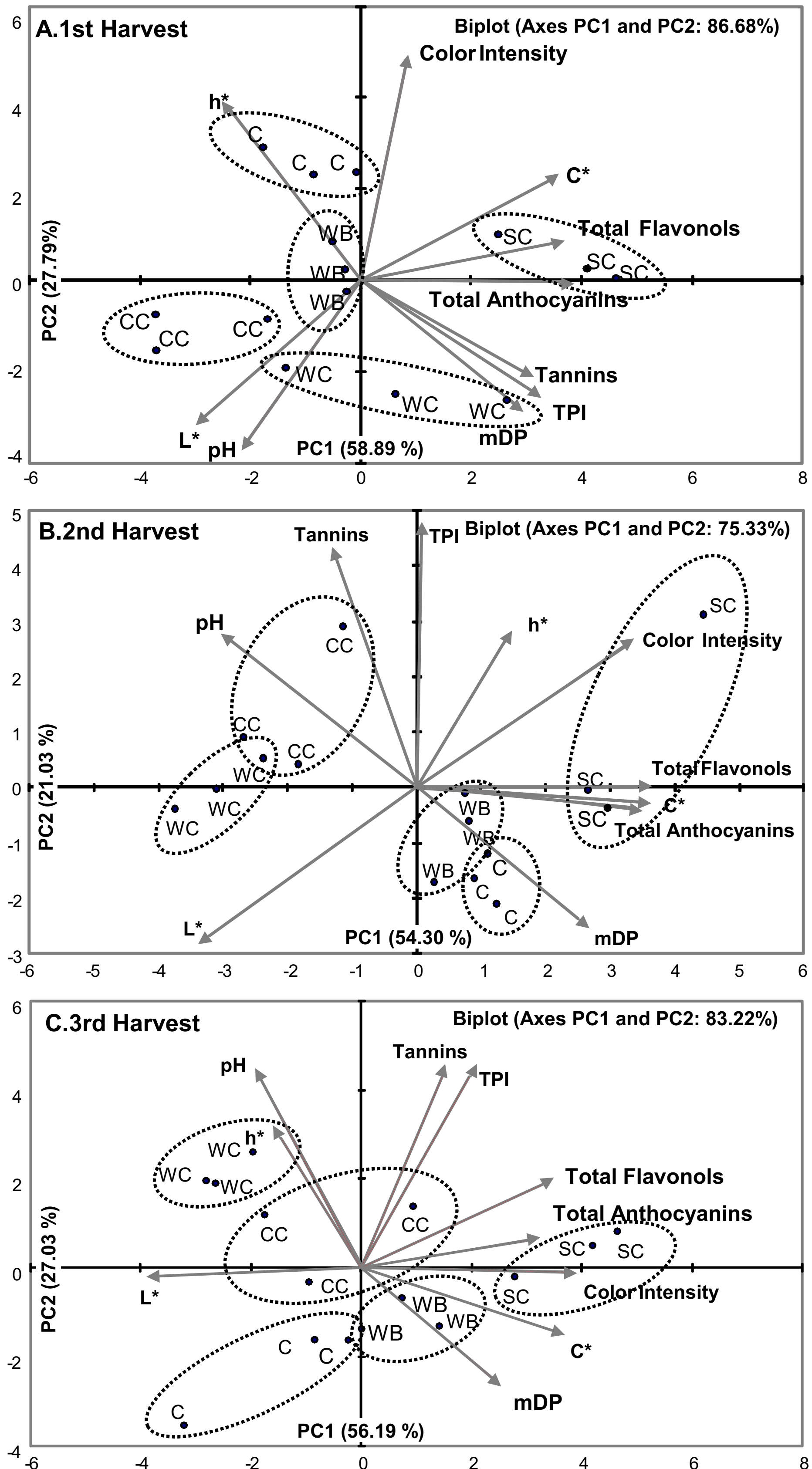

Figure 1 - Principal Component Analysis

C: Control wines; SC: Submerged Cap wines; WB: Whole Berry wines; CC: Crushed Cluster wines; WC: Whole Cluster wines

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CAPÍTULO 2: Influencia de las semillas y del raspón en la composición y en la astringencia del vino.

La composición y la calidad de los vinos tintos está influenciada por los compuestos fenólicos, sobre todo por los antocianos y las proantocianidinas. Los antocianos solo están presentes en las pieles. Mientras que las proantocianidinas se encuentran en las pieles, semillas y raspones, presentado una composición monomérica diferente dependiendo de su procedencia. En los últimos años, algunos enólogos apuestan por no despalillar, ya que en ocasiones los raspones dan al vino efectos positivos.

En este capítulo se aborda el objetivo 2, en el que se planteó determinar la contribución real de las semillas y del raspón sobre la composición, el color y la calidad del vino tinto.

Para ello, se adicionó la proporción de semillas y raspones proporcional al peso de los racimos, a un mosto decolorado y a otro que había macerado cuatro días. Estos vinos fueron analizados con el fin de dar respuesta al objetivo planteado.

Las analíticas de los vinos fueron realizadas en el laboratorio del grupo de Tecnología Enológica (TECNENOL) del Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili (URV, Tarragona) y en el Instituto Regional de Investigación Científica Aplicada (IRICA), Universidad de Castilla-La Mancha (UCLM, Ciudad Real). **Los resultados del estudio fueron publicados en la revista *Journal of Agriculture and Food Chemistry*.**

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Influence of Grape Seeds and Stems on Wine Composition and Astringency

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ABSTRACT: The aim of this paper is to study the real influence of seeds and stems on wine composition, astringency, and bitterness. A decolored grape juice and a grape juice macerated for 4 days from the same Cabernet Sauvignon grapes were fermented with or without supplementation with 100% seeds, 300% seeds, or 100% stems. Once alcoholic fermentation had finished, the wines were analyzed and tasted. The presence of seeds and stems increased the concentration of flavan-3-ol monomers with respect to the controls. However, the seeds mainly released (+)-catechin and (−)-epicatechin, whereas the stems mainly released (+)-catechin and (+)-gallocatechin. The seeds and stems also released proanthocyanidins; those from seeds have a lower mDP and a high percentage of galloylation, whereas those from stems have a higher mDP and a relatively high percentage of prodelphinidins. The presence of seeds and stems brought about a slight but significant increase in pH and lowered titratable acidity and ethanol content. The presence of seeds boosted color intensity, whereas stems had the opposite effect. Finally, both seeds and stems increased wine astringency and bitterness.

KEYWORDS: *seeds, stems, wine composition, astringency, bitterness*

INTRODUCTION

Phenolic compounds are a wide, heterogeneous family of substances responsible for a large part of the sensory attributes of red wine, such as color, mouthfeel, astringency, and bitterness.[1,2] Phenolic compounds are usually classified into two broad families: nonflavonoids, which include mainly stilbenes, hydroxycinnamic acids, and their esters; and flavonoids, which include flavonols, anthocyanins, and flavanols.[3] Stilbenes have no direct effect on the sensory attributes of wine, but they are of interest because of their health-giving properties.[2] Hydroxycinnamic acids and their esters play no direct role in wine color, but they can act as copigments.[4] It has also been described how they can contribute to wine bitterness.[5]

Flavonols are present in grape skins and are responsible for the yellow color of white grapes. Some of the flavonols are released from the skins during winemaking operations, thereby contributing to the yellow color of white wines and the yellow component of the color of red wines.[4,6] It has also been reported that flavonols can contribute to the red color of wine as copigments[4,6] and to bitterness.[7]

Anthocyanins are present only in the skins of red grapes (with the exception of teinturier varieties) and are responsible for their red-purple color. They are released into the grape juice during the maceration process, thus conferring a red color to the wine. However, wine color also depends on the wine's pH and the presence of copigmentation cofactors, mainly monomeric flavan-3-ols, flavonols, and hydroxycinnamic acids, which prevent anthocyanins from hydration and therefore enhance color intensity.[6,8]

The flavanol group includes monomeric flavan-3-ols and their polymers, also known as condensed tannins or proanthocyanidins. Proanthocyanidins contribute to long-term color stability by combining with anthocyanins.[9] They are also associated with bitterness and certain texture sensations such as body and astringency.[10] They are present in skins, seeds, and stems, although their composition varies somewhat depending on which part of the cluster they come from. Seed proanthocyanidins are made up of (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-gallate,[11] whereas skin proanthocyanidins also have (−)-epigallocatechin and a minor concentration of (−)-epicatechin-3-gallate.[12] Seed

proanthocyanidins therefore consist of only procyanidins, whereas skin proanthocyanidins include procyanidins and prodelphinidins. Little is known about the proanthocyanidins from stems, but it is assumed that they are made up of the four monomers (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-gallate, and (−)-epigallocatechin.[13,14] The mean degree of polymerization (mDP) of skin proanthocyanidins is reported to be higher than that of seeds. However, there is very little information about the mDP of stem proanthocyanidins.[13,14]

It has been reported that the molecular size and especially the monomeric composition of proanthocyanidins have a considerable infl uence on the perception of astringency. Specifically, a greater degree of polymerization and a higher percentage of galloylation lead to a greater perception of astringency.[15] It is therefore generally accepted that seed proanthocyanidins are more astringent than skin proanthocyanidins because they have a greater degree of galloylation. Stem proanthocyanidins are also generally considered to be very bitter and astringent, although to our knowledge there is no scientific evidence for this.

Operations during winemaking can greatly influence color and phenolic compound extraction.[16] Several studies have focused on the influence of temperature, enzymatic additions, the mechanical treatment of the cap, ethanol content, etc.[17−20] Nevertheless, the length of time the wine is in contact with the skins and seeds is probably the most important factor.[21,22] Gil et al.[23] showed that proanthocyanidins from skin are extracted earlier than those from seeds and that lengthening maceration therefore means that more seed proanthocyanidins are extracted. For this reason, winemakers carefully monitor maceration so as to avoid extracting an excess of seed proanthocyanidins.[3,24] A number of winemaking strategies have even been proposed that involve removing seeds from the tanks and eliminating the problems that their presence could cause.[3,24,25]

As mentioned earlier, it seems that stems can release highly astringent, bitter proanthocyanidins and other phenolic compounds. It has also been reported that the presence of stems causes significant color loss and can give rise to a “stemmy” flavor in the wine.[26,27] Hence the vast majority of wineries destem the grapes used to make red

wines in order to avoid these undesirable attributes. Other arguments for removing stems are that they reduce ethanol content and titratable acidity, increase pH and take up valuable space in the tank.[28]

However, in recent years the trend toward not destemming the grapes has become a hot topic. Some winemakers maintain that stems may occasionally have interesting eff ects[28,29] such as producing wines with a higher concentration of proanthocyanidins, which helps to stabilize color and improve mouthfeel. Furthermore, retaining the stems makes the cap less compact, which improves color extraction. Probably for these reasons some traditional wine-producing regions such as Châ teauneufdu-Pape (Cô tes du Rhô ne) do not destem because the presence of stems increases the polyphenolic content of wines, improving their aging ability. Winemakers in the Mé doc region (Bordeaux) used to include a proportion of stems when gray rot was present, with the aim of inhibiting laccase and protecting wine color. Stems have also been partly or fully used for low-tannin varieties such as Pinot noir in traditional regions such as Burgundy.[30,31] Nowadays, winemaking using the whole bunch is very common in biodynamic/ natural wine production, probably because the supplementary tannin contribution of the stems can protect the wine from oxygen, making it possible to work with lower doses of sulfur dioxide.

There are various scientific papers on the influence of seeds on wine color and composition,[24,32] but to our knowledge very little information exists with regard to how the presence of stems during winemaking influences wine color, composition, and quality.[33] Hence, the aim of this study is to explore exactly how seeds and stems contribute to the color, astringency, and phenolic composition of wine.

MATERIALS AND METHODS

Chemicals and Equipment. Methanol, acetonitrile, formic acid, and acetic acid of high-performance liquid chromatography (HPLC) grade (>99%) and absolute ethanol and hydrochloric acid (37%) were purchased from Panreac (Barcelona, Spain); enological charcoal and bentonite were purchased from Agrovin (Alcázar de San Juan, Ciudad

Real, Spain); acetaldehyde, polyvinylpolypyrrolidone, phloroglucinol, ascorbic acid, and sodium acetate were supplied by Sigma-Aldrich (Madrid, Spain); commercial standard quercetin-3-glucuronide, (−)-gallocatechin, malvidin 3-glucoside, trans-caftaric acid, caffeic, and p-coumaric acids, trans-resveratrol, and trans-piceid were purchased from Phytolab (Vestenbergsgreuth, Germany); commercial standard kaempferol, quercetin, isorhamnetin, myricetin, and syringetin, the 3-glucosides of myricetin, kaempferol, quercetin, isorhamnetin, and syringetin, PA dimers B1 and B2, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin-3-O-gallate were purchased from Extrasynthese (Genay, France). A noncommercial sample of procyanidin B4 was kindly supplied by Professor Victor de Freitas (Centro de Investigaçąõ em Química, Departamento de Química do Porto, Portugal) and used as received. The trans isomers of resveratrol and piceid (resveratrol 3-glucoside) were transformed into their respective cis isomers by UV irradiation (366 nm light for 5 min in quartz vials) of 25% MeOH solutions. Vitisin A (10-carboxy-pyranomalvidin-3-glucoside) and 10-p-monohydroxyphenyl- pyranomalvidin-3-glucoside (MHP-pymv-3-glc) were quantified with previously obtained standards.[34] All spectrophotometric measurements were carried out using a Helios Alpha UV−vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Grapes. Grapes from Vitis vinifera cv. Cabernet Sauvignon (clone 15) grafted onto 110 Richter rootstock (planted in 1992) were obtained from the experimental vineyard of the Enology Faculty of the Rovira i Virgili University in Constantí (AOC Tarragona; 41°8′54.17″N and 1°11′53.89″ E) during the 2013 vintage. The vineyard is 87 m above sea level, and groundwater is located at a depth of around 4 m. The vines were trained on a vertical trellis system and arranged in rows 2.80 m apart, with 1.20 m spacing within each row. They were pruned following a double Cordon de Royat system, with 16 buds per vine remaining after winter pruning. The grapes were harvested manually on September 22 when they were considered to be sufficiently ripe (around 13.5% potential ethanol content and 6.5 g tartaric acid/L titratable acidity).

Cluster Characterization. Ten clusters were randomly selected and weighed. They were then manually destemmed. All of the berries and stems were weighed to determine the

weight of 100 berries as well as the proportion of stems and grapes. After that, the seeds of 100 berries were manually extracted and weighed, and the result was used to calculate the percentage of seeds with respect to the cluster weight. This operation was done by triplicate. The results obtained were 4.29 ± 0.57% (w/w) of stems and 4.53 ± 0.22% (w/w) of seeds with respect to the weight of the whole cluster. These data were used to calculate the necessary weight of stems or seeds to be added to the grape juice fermentations.

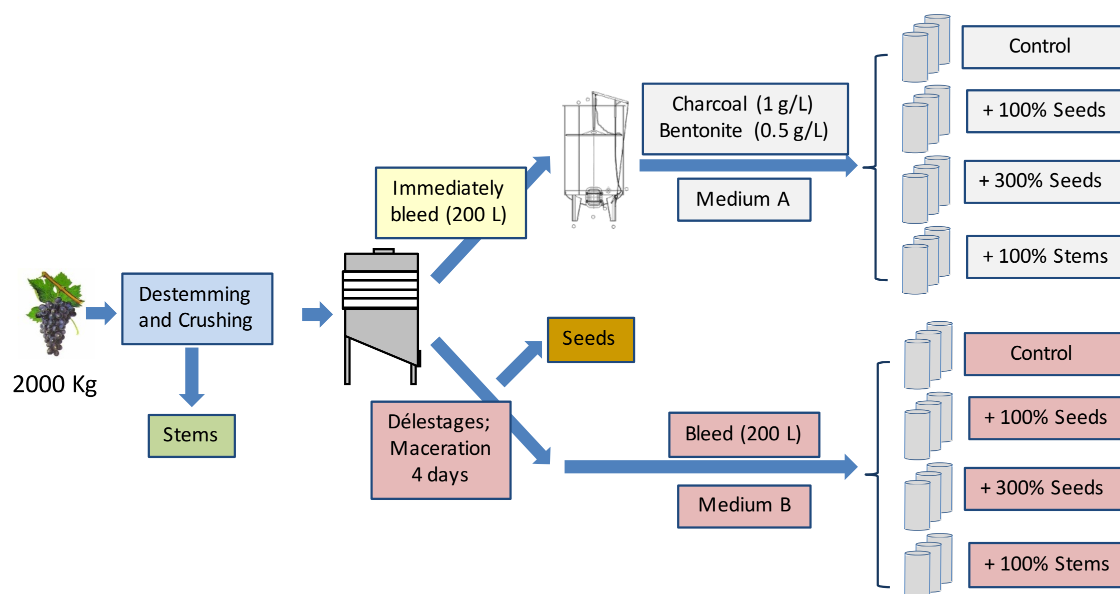

Figure 1. Experimental design.

Winemaking Conditions. Figure 1 shows a schematic diagram of the experimental design. Around 2000 kg of grapes was carefully destemmed, crushed (Delta V2, Bucher Vaslin SA, Chalonnes sur Loire, France), sulfited (100 mg K2S2O5/L), and pumped to a selfemptying, 2500 L capacity stainless steel tank. Around 5 kg of stems was kept at 4°C. Immediately after the tank was filled, 150 L of grape juice was bled off and pumped to a variable-volume 200 L tank in a refrigerated chamber (4°C) and immediately settled with charcoal (1 g/L) and bentonite (0.5 g/L) to eliminate all phenolic compounds. The aim of this discoloration was to be completely sure that all polyphenols present in the obtained wine were specifically released by an only source of polyphenols (seeds or stems). The clean must was racked to another variable-volume 200 L tank and kept for 4 days at a low temperature (4°C) to prevent fermentation.

Meanwhile, the pomace of the self-emptying stainless steel tank was inoculated with 200 mg/kg of selected yeast (EC1118, Lallemand Inc., Montreal, QC, Canada). The temperature of the tank was 23 °C at the beginning and controlled to remain below 28°C throughout the fermentation process. Once alcoholic fermentation began, one rack and return (*deléstage*) per day was carried out to extract enough seeds for the experiment. Briefly, the liquid in the tank was completely extracted by gravity and pumped into another tank. Once this operation was done, all of the liquid was returned to the tank so as to disaggregate the cap, thereby making many seeds fall to the bottom. In the following *deléstage*, these seeds were removed from the tank with the liquid in such a way that the seeds could be separated. The recovered seeds were immediately washed with cold water, dried with a hot-air dryer, and stored at 4 °C. After three *deléstages* around 20 kg of seeds had been recovered, which means that around 22% of the total seeds was extracted, bearing in mind the data obtained in the cluster characterization.

The grape juice bled off at the beginning (medium A) was distributed among 12 small tanks (8 L). Three tanks were designated "control", another three tanks were given the original proportion of seeds in the bunch (100% seeds), another three were given 3 times this value (300% seeds), and finally the last three were given the original proportion of stems (100% stems). Calculating the weight of the seeds and stems to be added was done by considering the grape juice density (1.097 g/L) and also that the theoretical percentage of grape juice was 80% of the weight of the whole cluster. Consequently, 497 g of seeds (5.66% w/w) was added to the 100% seed tanks, 1491 g of seeds (16.98% w/w) to the 300% seed tanks, and finally 471 g of stems (5.36% w/w) to the 100% stem tanks. All tanks were immediately inoculated with 200 mg/kg selected yeast (EC1118, Lallemand Inc.).

On day 4 of fermentation, the self-emptying stainless steel tank was bled again (200 L) and the liquid (medium B) distributed among 12 small tanks (8 L), following the same experimental approach described above. Specifically, three tanks were designated "control", whereas the other tanks were given an added proportion of seeds (100% seeds), 3 times the proportion of seeds (300% seeds), or a proportion of stems (100%

stems). These tanks were not inoculated because the liquid was already fermenting with the same selected yeast.

The microscale vinifications were checked daily by measuring the temperature and density of the juice. All tanks were kept at a room temperature of 27 ± 1°C. After 2 weeks of maceration, the wines from the tanks were racked. All wines were sulfited (100 mg K2S2O5/L) and kept at 4°C for 1 month for tartaric stabilization. Malolactic fermentation was therefore inhibited so as to prevent its causing any variations. The wines were finally bottled and stored in a dark cellar at 15°C until analysis. The analysis of the wines commenced about 2 months after bottling.

Standard Wine Analysis. The analytical methods recommended by the OIV were used to determine ethanol content, pH, glucose+fructose, and titratable acidity.[35] The total polyphenol index (TPI) was analyzed by measuring the 280 nm absorbance of a 1:100 dilutionof wine with a spectrophotometer, using a 10 mm quartz cuvette and multiplying the absorbance value by 100, as described by Ribéreau-Gayon et al.[36] The condensed tannin concentration was estimated by precipitation with methylcellulose.[37]

Color Parameters. Ten microliters of 10% (v/v) acetaldehyde solution was added to a 1 mL sample of wine 20 min before color measurement to avoid sulfite interferences. Color intensity (CI) was estimated using the method described by Glories.[38] The CIELAB coordinates, lightness (L*), chroma (C*), hue (h*), red−greenness (a*), and yellow−blueness (b*), were determined following the method used by Ayala et al.,[39] and data processing was performed with MSCV software.[40]

Spectrophotometric Analysis of Anthocyanins and Related Parameters. The concentration of anthocyanins was determined by spectrophotometry.[41] The ionization index (which indicates the proportion of red-colored anthocyanins at wine pH) and the PVPP index (which indicates the proportion of anthocyanins combined with proanthocyanidins) were determined in line with Glories.[38] The copigmentation index was measured in accordance with Boulton.[6]

Astringency Index. The astringency index was estimated using the method described by Llaudy et al.[42]

Analysis of Individual Low-Molecular-Mass Phenolic Substances in Wine. Monomeric anthocyanins and pyranoanthocyanins were analyzed by direct injection of wine diluted samples (0.5 mL of wine diluted with 1.0 mL of 0.1 N HCl). With the exception of monomeric flavan-3-ols, the rest of the non-anthocyanin individual low-molecular-mass phenolic substances in wines were analyzed after removal of interfering anthocyanins by solid-phase extraction.[43] All of the latter compounds were analyzed by the reversed-phase HPLC−diode array detector−electrospray ionization−tandem mass spectrometry system (RP-HPLC-DAD-ESI-MSn) following previously described methods.[34,43] This comprised an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a DAD (G1315B) and an LC/MSD Trap VL (G2445C VL) ESI-MSn, coupled to an Agilent Chem Station (version B.01.03) data processing station. The mass spectral data were processed using Agilent LC/MS Trap software (version 5.3). The monomeric flavan-3-ols were analyzed by RPHPLC-DAD-ESI-MSn after dilution of the wine samples (0.25 mL of wine diluted with 4.75 mL of water/formic acid, 98.5:1.5). The diluted samples were filtered (0.20 µm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) and then injected (100 µL) on an Ascentis Express C18 reversed-phase column (4.6 Å~ 150 mm; 2.7 µm particle size) (Supelco, Sigma-Aldrich, Madrid, Spain) maintained at 16 °C. The solvents were A [water/methanol/formic acid (89:10:1, v/v/v)] and B (methanol), and the flow rate 0.5 mL/min. The linear gradient for solvent B was as follows: 0 min, 1%; 2 min, 1%; 60 min, 23%; 75 min, 70%; 80 min, 95%; 90 min, 95%; 95 min, 1%; 100 min, 1%. Two MS scan types were used: enhanced MS for compound identification and multiple reaction monitoring (MRM) for quantification. The conditions for both MS scan types were as follows: ion spray voltage, −4000; ion source temperature, 450 °C; collision gas, high; curtain gas, 15; ion source gas 1, 70; ion source gas 2, 50; declustering potential, −35; entrance potential, −10; collision energy, −30; and collision cell exit potential, −3. The selected mass transitions (m/z pairs) for the MRM scan and quantification were as follows: (+)-catechin and (−)- epicatechin, 289−245; procyanidins B1, B2, and B4, 577−425 and 577−407; (−)-epigallocatechin and (−)-gallocatechin, 305−221 and 305−219; and (−)-epicatechin-3- gallate, 441−289. The

compounds were quantified with external calibration curves of (+)-catechin, with the previously obtained response factors for each of the other compounds, and expressed as mg/L. Two injections of (+)-catechin standard solution, one at the beginning and one at the end of every injection series, were performed to update the response factors before quantification. The analyses were carried out in duplicate. Flavan-3-ols (monomers and B-type procyanidins) were separated, identified, and quantified on an Agilent 1200 series HPLC system equipped with DAD and coupled to an AB Sciex 3200 Q TRAP (Applied Biosystems) and ESI-MSn. The chromatographic system was managed by an Agilent Chem Station (version B.01.03) data processing station. The mass spectral data were processed using Analyst MDS software (Applied Biosystems, version 1.5).

Analysis of Wine Proanthocyanidins. The proanthocyanidins of the wines were extracted and analyzed by acid depolymerization in the presence of an excess of phloroglucinol;[44] the products of the reaction were separated by RP-HPLC-DAD.45 The proanthocyanidins were analyzed with an Agilent 1200 series HPLC equipped with a G1362A refractive index detector (RID), a G1315D DAD, a G1311A quaternary pump, a G1316A column oven, and a G1329A autosampler (Agilent Technologies, Santa Clara, CA, USA). The chromatographic system was managed by an Agilent Chem Station (version B.01.03) data processing station. The number of terminal subunits was considered to be the difference between the total monomers measured in normal conditions (with phoroglucinol) and thus obtained when the analysis was performed without phloroglucinol addition. The number of extension subunits was considered as the addition of all the phloroglucinol adducts. The mean degree of polymerization (mDP) was calculated by adding terminal and extension subunits (in moles) and dividing by the terminal subunits. The percentage of prodelphinidins was calculated as the quotient between total (−)-epigallocatechin units and total monomeric units expressed as a percentage. The percentage of galloylation was calculated as the quotient between total (−)-epicatechin-3-gallate units and total monomeric units expressed as a percentage.

Sensory Analysis. All of the sensory analyses were performed in the tasting room of the Faculty of Enology in Tarragona (Rovira I Virgili University), which was designed according to UNE-EN ISO 8589:2010.[46] Triplicates were blended for simplifying the

sensory analysis. The samples were tasted by a group of eight expert enologists from our university. Two training sessions in tasting were carried out with a red wine enriched with 0−2 g/L of seed commercial tannin (Protan pepin, AEB, Brescia, Italy) before the criteria were standardized by the panelists. Taster performance and the agreement between panelists were checked using Panelcheck GNU software.[47] The panelists wore dark glasses so that they would not be influenced by the color of the samples.[48] Two trials were carried out: one for the wines obtained with the decolored grape juice bled off at the beginning (medium A) and one for the wines obtained with the liquid bled off 4 days later (medium B). A time of 1 h was allowed to elapse between the two trials to avoid fatigue. The tasters had to score in a structured scale from 1 to 5 the intensity of the astringency and the bitterness of the different samples, which were served anonymously according to a Latin squares of Williams design to avoid range and carry-over effect.

Statistics. All of the chemical and physical data for the samples are expressed as the arithmetic average ± standard deviation of three replicates. Sensory data are expressed as the arithmetic average ± standard deviation of the scores of the eight tasters. One-factor univariate analysis of variance (ANOVA) was carried out with SPSS software (SPSS Inc., Chicago, IL, USA), and multiple comparison between samples was performed by using the least-squares differences (LSD) post hoc test.

RESULTS AND DISCUSSION

Effect on Wine Parameters. Figure 2 shows the general parameters of the wines corresponding to the grape juice obtained by bleeding-off immediately after the tank was filled (medium A). The final concentration of glucose+fructose was <0.5 g/L in all samples, confirming that all alcoholic fermentations were completed. As expected, the titratable acidity of the wine made with the presence of stems was significantly lower and the pH significantly higher than in the control. This result is only natural because it has been reported that stems can release potassium that causes the precipitation of a fraction of tartaric acid in the form of potassium hydrogen tartrate.[49] This trend was also observed when seeds were present, although the differences were significant only for

pH and not for titratable acidity. These data therefore suggest that seeds might also release potassium or other cations that can neutralize the acids.

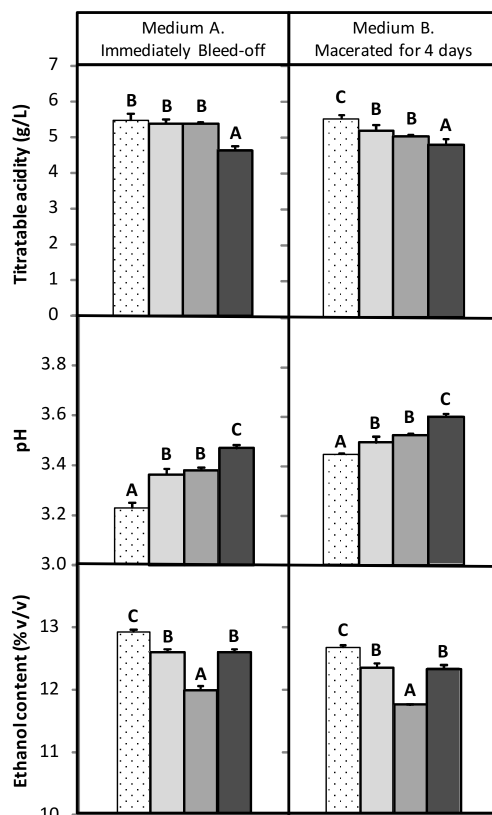

Figure 2. General parameters: dotted bars, control; light gray bars, 100% seeds; medium gray bars, 300% seeds; black bars, 100% stems. Results are expressed as the mean ± standard deviation of three replicates. Different letters indicate a statistical difference ($p < 0.05$) between samples.

The ethanol content of the wine produced in the presence of stems was significantly lower than in the control. This result may be easily explained by the fact that it has been reported that stems can absorb ethanol and release water.[49] Indeed, the moisture content of stems is reported to be around 65%.[50] Considering this value and the percentage of stems added (5.37%), which was the original percentage (w/w) of stems in the bunch, the theoretical amount of water provided by the stems was 3.49%, which should reduce the original ethanol content from 12.93 to 12.49% once osmotic equilibrium is reached. This theoretical reduction matches quite well with the ethanol content obtained in the experimental sample (12.61%).

The ethanol content of the wines produced with the addition of seeds was also signifycantly lower than in the control. This result was quite surprising but reasonable bearing in mind that seeds also contain water. It has been reported that the moisture of seeds is around 35%.[51] This value is not negligible, and indeed seeds need to be dried to facilitate the industrial process of oil extraction.[52] Under our experimental conditions, two different supplementations of seeds were carried out. In the 100% seed wines, the original proportion of seeds in the bunch (5.66%) was added, whereas 3 times this proportion (16.99%) was added to the 300% seed wines. Considering the reported moisture of seeds and the percentage of seeds added, the theoretical percentages of water contained in the seeds were 1.98 and 5.95%, respectively, which should reduce the ethanol content to 12.68 and 12.20%, respectively. Once again, these theoretical data match reasonably well with the ethanol contents obtained in the experimental samples (12.61 and 12.00%).

Figure 2 also shows the general parameters of the wines corresponding to those obtained using the liquid bled off after 4 days of maceration (medium B). The titratable acidity, pH, and ethanol content follow the same trend as in the wines obtained from the grape juice that was bled off immediately. Briefly, the titratable acidity and ethanol content decreased significantly through the presence of stems and seeds, whereas the pH did the opposite. In the particular case of reduced ethanol content, the calculation of the theoretical decrease brought about by the moisture from the stems and seeds also matches quite well with the experimental results. In addition, all of the wines obtained from the liquid bled off after 4 days of maceration had an ethanol content significantly lower (an average of $0.25 \pm 0.01\%$) than those obtained from the grape juice that was bled off immediately. Taking into account the aforementioned moisture content of the seeds and the fact that skins also contain water (around 54%),[53] we can hypothesize that this decrease was due to the water content from the solid parts of the bunch.

It is generally considered that the lower yield in ethanol content in red compared to white winemaking is because the higher fermentation temperature and the aeration induced by racking encourage ethanol evaporation. In view of these results it seems that

moisture from the solid parts of the bunch plays a non-negligible role that must be taken into account.

Table 1. Proanthocyanidins and Related Parameters[a]

CONDITION	PARAMETER	Control		+ 100 % of Seeds		+ 300 % of Seeds		+ 100 % of Stems	
Medium A. Immediately Bleed-off	TPI	7.7 ± 0.3	A	32.7 ± 0.1	C	71.8 ± 1.9	D	19.5 ± 0.7	B
	Astringency Index (mg of tannic acid/L)	95 ± 4	A	196 ± 8	C	340 ± 9	D	146 ± 6	B
	Tannins (mg/L)	168 ± 57	A	679 ± 46	C	1496 ± 167	D	475 ± 30	B
	Proanthocyanidins (mg/L)	n.d.		753 ± 24	B	1509 ± 61	C	368 ± 20	A
	mDP	n.d.		3.38 ± 0.05	A	3.45 ± 0.18	A	5.79 ± 0.21	B
	%Pd	n.d.		3.02 ± 0.53	A	2.61 ± 0.04	A	18.35 ± 0.94	B
	%Gal	n.d.		5.65 ± 0.24	B	7.86 ± 0.29	C	4.88 ± 0.22	A
Medium B. Macerated for 4 days	TPI	42.0 ± 1.0	A	63.0 ± 1.5	C	78.2 ± 3.4	D	48.2 ± 1.4	B
	Astringency Index (mg of tannic acid/L)	373 ± 10	A	571 ± 10	C	700 ± 9	D	455 ± 18	B
	Tannins (mg/L)	647 ± 37	A	1177 ± 54	C	1765 ± 213	D	747 ± 33	B
	Proanthocyanidins (mg/L)	403 ± 16	A	1262 ± 72	C	1960 ± 145	D	778 ± 149	B
	mDP	6.79 ± 0.27	C	5.01 ± 0.25	B	4.50 ± 0.17	A	6.80 ± 0.16	C
	%Pd	32.57 ± 0.30	D	17.38 ± 0.21	B	12.38 ± 0.27	A	26.71 ± 0.68	C
	%Gal	2.92 ± 0.29	A	3.83 ± 0.03	B	4.56 ± 0.03	C	2.79 ± 0.06	A

[a] Results are expressed as the mean ± standard deviation of three replicates. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples.

Effect on Wine Proanthocyanidins and Related Parameters. Table 1 shows the proanthocyanidin concentration and related parameters of the different wines. It includes two different measurements for tannins: first, the tannin concentration as measured by the methylcellulose method[37] and, second, the more specific analysis of proanthocyanidins by phloroglucinolysis,[45] which also provides information about the mDP and the percentages of prodelphinidins and galloylation. The tannin concentration measured by the methylcellulose method and the total proanthocyanidins measured by phloroglucinolisis showed a similar general trend, although some small differences were detected.

The total phenolic index (TPI), the astringency index, and the tannin and proanthocyanidin concentrations of the control wine obtained with medium A were very low and even undetectable in the case of proanthocyanidins. These data can be considered very logical because medium A was not macerated and was decolorized with charcoal and bentonite. The presence of seeds significantly increased all of these parameters, confirming that seeds are a good source of proanthocyanidins. In addition, the relationships between the percentage of added seeds and TPI, tannin, and proanthocya-nidin concentrations are nearly linear (linear regression coefficients r^2 of 0.9959, 0.9967, and 0.9647, respectively), which makes it possible to establish that, in this case, 1 g of seeds releases an average of 7.73 mg of tannins and 8.57 mg of proanthocyanidins and increases TPI by an average of 0.32 absorbance unit. These values were calculated using the slopes of the linear regression lines. Similarly, the astringency index also increased due to the presence of seeds, following a behavior almost parallel to that of tannins and proanthocyanidins. The mDP of the proanthocyanidins released by the seeds was lower (around 3.4) than the usual values found in Cabernet Sauvignon wines, confi rming that the size of seed proanthocyanidins is relatively small.[54] The percentage of prodelphinidins was also very low in both wines made with the addition of seeds. Because prodelphinidins are present only in skin proanthocyanidins,[12] this low percentage, which was the same regardless of the amount of seeds added, may be due to remaining traces of proanthocyanidin from the skins. In contrast, the percentage of galloylation was relatively high. Because seed proanthocyanidins are highly galloylated,[11] this result is quite logical as seeds were the only source of proanthocyanidins in these wines. Similar results were obtained by Rinaldi et al.[55]

The presence of stems also significantly increased the TPI, astringency index, tannins, and proanthocyanidins, but to a lesser degree than seeds. In this case 1 g of stems released an average of 5.72 mg of tannin and an average of 6.86 mg of proanthocyanidins and increased the TPI by an average of 0.22 absorbance unit. These values were calculated as the quotient between the increase of each parameter induced by the presence of stems and the weight of the added of stems. The mDP and the percentage of prodelphinidins observed were significantly higher and the percentage of

galloylation significantly lower than in the case of the seed wines. These data strongly suggest that stem proanthocyanidins have a greater mDP and a lower galloylation percentage than seed proanthocyanidins and, most especially, that they contain a noticeable percentage of prodelphinidins.

Table 1 also shows the proanthocyanidin concentration and related parameters of the wines obtained with medium B. As expected, the 4 days of maceration mean that the TPI, the astringency index, and the tannin and proanthocyanidin concentrations of the medium B control wine were much higher than those of the medium A control. The mDP and the percentages of prodelphinidins and galloylation of proanthocyanidins of the medium B control are also shown in Table 1. In general, these values are in agreement with data described for red wines.[24,56] It should be noted that the mDP and the percentage of prodelphinidins of the medium A wines supplemented with seeds were lower and the percentage of galloylation higher than in the medium B control wine. These differences are simply because the medium B control was macerated for 4 days and consequently contains proanthocyanidins from skins and seeds, whereas the only source of proanthocyanidins in medium A was seeds.

Supplementation with seeds in medium B significantly increased the TPI, astringency index, and tannin and proanthocyanidin concentrations in a similar way to medium A, again confirming that seeds are a source of proanthocyanidins. Similar results have been reported by Kovac et al.[32] by adding supplementary quantities of seeds during fermentation and by Mateus et al.[57] by adding grape seed tannins to Porto wines. Moreover, the addition of seeds brought about a significant decrease in the mDP and the percentage of prodelphinidins and a significant increase in the percentage of galloylation. These data simply indicate that the proportion of seed proanthocyanidins increased as their supplementation increased, because seed proanthocyanidins have no prodelphinidins and their mDP and percentage of galloylation are higher than those of skin proanthocyanidins. Similar results were reported by Canals et al.[24] In contrast, it has been shown that seed removal originates wines with lower proanthocyanidin concentration and higher mDP and higher percentage of prodelphinins.[24,58]

The addition of stems also increased the TPI, astringency index, and tannin and proanthocyanidin concentrations, although the eff ect, as with medium A, was lower than in the case of seeds. Here the supplementation with stems did not change the mDP or the percentage of galloylation and caused only a slight but significant decrease in the percentage of prodelphinidins. These results suggest that the proanthocyanidins released by stems are more similar to those from skins than to those from seeds.

Table 2. Flavan-3-ol Monomers and Dimers[a]

CONDITION	PARAMETER	Control	+ 100 % of Seeds	+ 300 % of Seeds	+ 100 % of Stems
Medium A. Immediately Bleed-off	Catechin	5.2 ± 0.7 **A**	96.5 ± 21.9 **C**	233.0 ± 14.9 **D**	44.9 ± 6.9 **B**
	Epicatechin	1.6 ± 0.2 **A**	72.6 ± 19.7 **C**	172.5 ± 17.1 **D**	5.8 ± 1.1 **B**
	Gallocatechin	3.8 ± 0.2 **A**	3.4 ± 0.8 **A**	6.6 ± 0.8 **B**	42.3 ± 2.9 **C**
	Epigallocatechin	0.6 ± 0.1 **A**	1.5 ± 0.4 **B**	3.9 ± 0.4 **C**	6.5 ± 1.1 **D**
	Total Monomers	11.2 ± 0.8 **A**	173.9 ± 42.8 **C**	416.0 ± 33.1 **D**	99.6 ± 11.4 **B**
	Total Dimers	6.9 ± 0.7 **A**	229.2 ± 96.8 **C**	711.7 ± 51.3 **D**	67.8 ± 8.8 **B**
Medium B. Macerated for 4 days	Catechin	50.5 ± 2.9 **A**	129.4 ± 6.6 **C**	158.5 ± 14.7 **D**	83.3 ± 9.0 **B**
	Epicatechin	18.4 ± 1.2 **A**	87.0 ± 4.1 **B**	117.3 ± 13.4 **C**	20.1 ± 1.4 **A**
	Gallocatechin	37.0 ± 1.5 **B**	34.6 ± 1.1 **AB**	31.5 ± 3.9 **A**	67.7 ± 12.0 **C**
	Epigallocatechin	7.0 ± 0.2 **B**	6.8 ± 0.3 **AB**	6.3 ± 0.3 **A**	11.7 ± 1.4 **C**
	Total Monomers	113.0 ± 5.6 **A**	257.8 ± 11.6 **C**	311.6 ± 31.0 **D**	182.8 ± 23.4 **B**
	Total Dimers	79.6 ± 5.1 **A**	319.8 ± 15.3 **C**	439.2 ± 76.7 **D**	134.1 ± 13.3 **B**

[a] Results are expressed as the mean ± standard deviation of three replicates. All data are expressed as mg/L. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples.

Effect on Wine Flavan-3-ols Monomers and Dimers. Table 2 shows the flavan-3-ol monomers and dimers of the different wines. As in the case of tannins, the concentrations of flavan-3-ol monomers and dimers in the control wine obtained from medium A were very low. The presence of seeds also significantly increased the concentration of flavan-3-ol monomers and dimers, and this was almost proportional to the amount of seeds added (linear regression coefficients r^2 of 0.9940 for monomers and 0.9999 for dimers), making it possible to estimate that 1 g of seeds released 23.5

mg of flavan-3-ol monomers and 41.7 mg of dimers. The flavan-3-ol monomers released by the seeds were mainly (+)-catechin (55%) and (−)-epicatechin (42%), whereas (+)-gallocatechin and (−)-epigallocatechin were present only in very low proportions (<2%). This small presence of (+)-gallocatechin and (−)-epigallocatechin is probably due to the presence of a slight proportion of skins with the seeds because these compounds are not present in seeds. These results are in agreement with those reported by Monagas et al.,[59] who have found that Cabernet Sauvignon seeds have around 53% of (+)-catechin and 46% of (−)-epicatechin, the other flavan-3-ol monomers being in negligible proportion. Other authors have found that (+)-catechin is the most abundant flavan-3-ol monomer in seeds from Malbec.[60] However, in other cultivars, such as Tempranillo or Graciano, (−)-epicatechin seems to be in higher proportion than (+)-catechin.[59]

The stems also released flavan-3-ol monomers and dimers in medium A, but in a relatively lower proportion than in the case of seeds. Specifically, 1 g of stems released 16.5 mg of flavan-3-ol monomers and 11.4 mg of dimers. Indeed, the proportions of the different monomers released by the stems were completely different from the proportions released by the seeds. In this case the main monomers were (+)-catechin (45%) and (+)-gallocatechin (43%), whereas (−)-epicatechin and (−)-epigallocatechin were present in minor proportions (6 and 7%, respectively).

The concentration of flavan-3-ol monomers and dimers in the medium B control was around 10 times higher than in the medium A control because the maceration continued for 4 days. Similarly to what happened with medium A, supplementation with seeds also significantly increased the total concentrations of monomers and dimers. This increase was similar to that observed in medium A when 100% seeds was added, but it was lower when a higher proportion of seeds was added (300%). A possible explanation for this lower increase in monomers and dimers in medium B may be related to the presence of anthocyanins, which can combine with flavan-3-ols to form new pigments.[6,61] The addition of seeds clearly changed the proportions of the different monomers. Specifically, (+)-catechin increased from 45 to 51% and (−)-epicatechin from 16 to 36%, whereas (+)-gallocatechin decreased from 33 to 12% and (−)-epigallocatechin from 6 to

2%. These results can be easily explained by the fact that the seeds released mainly (+)-catechin and (−)-epicatechin into medium A.

Table 3. Hydroxycinammic Acid Derivatives[a]

CONDITION	PARAMETER	Control	+ 100 % of Seeds	+ 300 % of Seeds	+ 100 % of Stems
Medium A. Immediately Bleed-off	t-Caftaric	9.65 ± 0.10 **B**	9.53 ± 0.17 **B**	5.37 ± 2.04 **A**	12.53 ± 0.30 **C**
	t-Coutaric	0.22 ± 0.01 **A**	0.22 ± 0.00 **A**	0.18 ± 0.05 **A**	0.34 ± 0.01 **B**
	c-Coutaric	0.11 ± 0.03 **A**	0.14 ± 0.02 **A**	0.11 ± 0.03 **A**	0.19 ± 0.02 **B**
	Caffeic	1.06 ± 0.05 **A**	1.05 ± 0.08 **A**	0.71 ± 0.32 **A**	0.94 ± 0.08 **A**
	t-Fertaric	0.25 ± 0.02 **A**	0.25 ± 0.02 **A**	0.18 ± 0.05 **A**	0.26 ± 0.02 **A**
	Total	11.28 ± 0.16 **B**	11.19 ± 0.08 **B**	6.54 ± 2.47 **A**	14.27 ± 0.35 **C**
Medium B. Macerated for 4 days	t-Caftaric	18.38 ± 0.51 **A**	18.90 ± 0.29 **A**	17.48 ± 2.35 **A**	20.24 ± 1.45 **A**
	t-Coutaric	1.03 ± 0.04 **A**	1.00 ± 0.02 **A**	0.92 ± 0.16 **A**	1.03 ± 0.03 **A**
	c-Coutaric	0.62 ± 0.08 **A**	0.75 ± 0.06 **A**	0.61 ± 0.14 **A**	0.72 ± 0.16 **A**
	Caffeic	2.12 ± 0.11 **A**	2.20 ± 0.04 **A**	2.08 ± 0.24 **A**	2.20 ± 0.24 **A**
	t-Fertaric	0.45 ± 0.03 **A**	0.54 ± 0.06 **A**	0.48 ± 0.07 **A**	0.50 ± 0.03 **A**
	Total	22.60 ± 0.68 **A**	23.39 ± 0.38 **A**	21.58 ± 2.88 **A**	24.69 ± 1.84 **A**

[a] Results are expressed as mean ± standard deviation of three replicates. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples. Total amount is expressed as mg/L of caftaric acid.

Effect on Wine Hydroxycinnamic Acids and Derived Tartaric Esters. Table 3 shows the concentration of hydroxycinnamic acids and derived tartaric esters. In general, the total concentration in the medium A control was around half the amount found in the medium B control, probably because this medium was decolored with charcoal and bentonite. In both media trans-caftaric acid was clearly the most abundant compound (>80%), and this result is in agreement with previous work.[62] No differences were found in any of the different hydroxycinnamic acids and derived tartaric esters in the two media when 100% seeds was added, and neither were there any differences when 300% seeds was added to medium B. This suggests that seeds are not a source of these compounds. However, when 300% seeds were added to medium A, the concentration

of trans-caftaric acid decreased significantly, which may indicate that seeds can absorb this compound.

The addition of stems slightly but significantly increased the concentration of *trans*-caftaric, *trans*-coutaric, and *cis*-coutaric acids in medium A. Although these differences were not significant in medium B, this still suggests that stems might release discrete amounts of these compounds.

Table 4. Flavonols[a]

CONDITION	PARAMETER	Control		+ 100 % of Seeds		+ 300 % of Seeds		+ 100 % of Stems	
Medium A. Immediately Bleed-off	**Total heterosides**	**0.03 ± 0.02**	**A**	**0.27 ± 0.14**	**C**	**0.54 ± 0.12**	**D**	**0.08 ± 0.04**	**B**
	Total Aglycones	**n.d.**		**0.45 ± 0.07**	**A**	**3.43 ± 0.45**	**B**	**n.d.**	
	Total Flavonols	**0.03 ± 0.02**	**A**	**0.71 ± 0.17**	**C**	**3.98 ± 0.31**	**D**	**0.08 ± 0.02**	**B**
Medium B. Macerated for 4 days	Myricetin-3-Glu	1.01 ± 0.02	**B**	1.19 ± 0.11	**B**	0.89 ± 0.17	**B**	0.67 ± 0.03	**A**
	Myricetin-3-Gal	0.15 ± 0.02	**A**	0.62 ± 0.03	**B**	n.d.		0.16 ± 0.04	**A**
	Myricetin-3-Glc	0.32 ± 0.04	**A**	2.06 ± 0.19	**C**	n.d.		0.54 ± 0.04	**B**
	Quercetin-3-Gal	0.05 ± 0.02	**A**	0.36 ± 0.05	**B**	0.18 ± 0.13	**AB**	0.10 ± 0.03	**A**
	Quercetin-3-Glu	4.69 ± 0.41	**A**	7.40 ± 0.37	**B**	4.77 ± 0.71	**A**	3.98 ± 0.29	**A**
	Laricitrin-3-Glc	2.38 ± 0.33	**AB**	4.60 ± 1.42	**B**	3.55 ± 1.05	**AB**	1.77 ± 0.27	**A**
	Syringetin-3-Glc	4.18 ± 0.28	**A**	4.06 ± 0.03	**A**	3.66 ± 0.51	**A**	3.75 ± 0.15	**A**
	Total heterosides	**12.78 ± 1.12**	**A**	**20.28 ± 2.20**	**B**	**13.06 ± 2.56**	**A**	**10.97 ± 0.85**	**A**
	Myrincetin	10.64 ± 0.30	**B**	8.96 ± 1.67	**B**	5.54 ± 0.55	**A**	4.35 ± 0.65	**A**
	Quercetin	1.93 ± 0.34	**B**	14.22 ± 0.96	**D**	3.61 ± 2.89	**C**	0.49 ± 0.34	**A**
	Laricitrin	2.21 ± 0.01	**C**	1.49 ± 0.02	**B**	1.24 ± 0.17	**A**	0.98 ± 0.11	**A**
	Kaempferol	0.02 ± 0.00	**A**	0.34 ± 0.05	**B**	0.04 ± 0.02	**A**	0.02 ± 0.00	**A**
	Isorhamnetin	0.18 ± 0.04	**B**	1.37 ± 0.11	**C**	0.36 ± 0.16	**B**	0.04 ± 0.01	**A**
	Syringetin	0.22 ± 0.06	**A**	0.17 ± 0.01	**A**	0.24 ± 0.10	**A**	0.25 ± 0.17	**A**
	Total Aglycones	**15.19 ± 0.77**	**B**	**26.54 ± 2.81**	**C**	**11.03 ± 3.25**	**B**	**6.14 ± 1.28**	**A**
	Total Flavonols	**27.96 ± 0.85**	**B**	**46.82 ± 1.91**	**C**	**24.10 ± 3.75**	**B**	**17.11 ± 0.20**	**A**

[a] Results are expressed as the mean ± standard deviation of three replicates. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples. Total amount is expressed as mg/L of quercentin-3-O-glucoside. Abbreviations used for glycosylation correspond to glucuronide (Glu), galactoside (Gal), and glucoside (Glc).

Effect on Wine Flavonols. Table 4 shows the concentration of flavonols in the different wines. The presence of flavonols in medium A was very low in all cases. In fact, most of the individual flavonols were not detected, and for that reason only total heterosides, total aglycones, and total flavonols are shown. In general, supplementation with 100% and more so with 300% seeds slightly but significantly increased the concentration of

some flavonols, especially quercetin aglycone (data not shown). Furthermore, because seeds are not reported to be a source of flavonols, this effect may be associated with a slight contamination of the seeds with small fragments of skin during the separation procedure from grape pomace.

In contrast, the flavonol levels in the medium B samples are much higher because this medium was macerated with skins for 4 days. However, the supplementation of seeds had a surprising effect. When 100% seeds was added, the total flavonol concentration almost doubled, whereas when 300% seeds was added no significant changes were observed with respect to the control. The observed increase was mainly due to quercetin aglycone, which rises from 1.93 to 14.22 mg/L, and, to a lesser extent, quercetin-3-glucuronide, laricitrin-3-glucoside, myricetin- 3-glucoside, and isorhamnetin aglycone. Quercetin aglycone represents 65% of the augmentation of total flavonols and was also the one that increased most through the addition of seeds to medium A. A small proportion of this increase may be associated with a slight contamination by small fragments of skin, as previously explained for the results observed in medium A. However, such a drastic increase cannot be explained by the presence of seeds alone because this part of the grape is not a source of flavonols. Therefore, another cause must be responsible. One possible hypothesis may be an increase in the solubility of flavonols by association with the phenolic compounds released by the seeds. Seeds release flavan-3-ols (Tables 1 and 2), which can form stable associations with flavonols that favor their dissolution, especially in the case of aglycones that are poorly soluble.[63] A proportion of the quercetin heterosides released from skins can be hydrolyzed during alcoholic fermentation,[63,64] and a great proportion of quercetin aglycone can precipitate. The presence of flavan-3-ols released by seeds can therefore favor the solubility of quercetin aglycone, justifying the data observed when 100% seeds was added. However, when 300% of seeds was added, the levels of flavonols were similar to controls. These data suggest that stems can also absorb flavonols, so that when 300% of seeds was added, the suppressive effect due to the absorption of flavonols surpasses the additive effect due to the increase of the solubility by the higher presence of flavan-3-ols.

It is interesting to note that the levels of quercetin in young red wines can range from 20 to 50 mg/L, whereas it is difficult to get >5 mg/L dissolved into model wine solutions. This unexpected higher solubility of quercetin in red wine has been explained on the basis of its involvement in copigmentation interactions with anthocyanins.[6] According to this hypothesis, it is possible that the flavan-3-ols released by the seeds when the addition was 100% boosted the solubility of the flavonols, especially quercetin aglycone, by forming associations similar to copigmentation complexes. Indeed, flavan-3-ols also participate as a copigment of anthocyanins in red wines. Moreover, the kind of interaction behind the phenomenon of copigmentation, namely, the formation of associations between phenolic compounds, has been suggested to affect not only the phenolic compounds involved in copigmentation but also any kind of phenolic compounds in red wine. In fact, this general interaction between phenolic compounds has been suggested to be why the reconstituted absorbance of red wines at 280 nm measured after a 20-fold dilution is lower than that measured directly without dilution.[6]

The supplementation of medium B with stems results in a significant decrease in total flavonols that was mainly due to aglycones. This suggests that stems absorb flavonols.

Effect on Wine Anthocyanins and Related Parameters. Anthocyanins and related parameters are shown in Table 5. No data are shown for anthocyanins for the wines from the grape juice that was bled off immediately because the treatment with charcoal and bentonite completely eliminated these pigments. The presence of seeds significantly decreased the total anthocyanin concentration measured by spectrophotometry and increased the PVPP index, and these changes were especially pronounced in the case of 300% seeds. The PVPP index measures the percentage of anthocyanins combined with proanthocyanidins.[38] Because the seeds released large amounts of flavan-3-ols, it is quite normal that the presence of seeds would encourage the production of polymerized pigments. Simultaneously, the contribution of anthocyanins to wine color (ionization index) increased and the percentage of color due to the copigmentation process (copigmentation index) decreased with the addition of 100% and, especially, 300% seeds. Both changes were probably due to the increase in the combination of anthocyanins and proanthocyanidins as suggested by the results of

the PVPP index. If a proportion of anthocyanins was combined with proanthocyanidins, the final concentration of free anthocyanins would be lower, and consequently the percentage of color due to the copigmentation of free anthocyanins with other copigments should also be reduced.

Table 5. Anthocyanins and Related Parameters of Wines from Medium B[a]

PARAMETER		Control	+ 100 % of Seeds	+ 300 % of Seeds	+ 100 % of Stems
Anthocyanins and related parameters by SP	Total Anthocyanins (mg/L)	474.5 ± 18.4 **C**	427.6 ± 21.2 **B**	264.3 ± 12.4 **A**	426.4 ± 13.1 **B**
	PVPP Index (%)	19.3 ± 0.9 **A**	41.8 ± 3.9 **C**	56.7 ± 1.7 **D**	21.6 ± 1.0 **B**
	Copigmentation Index (%)	42.7 ± 1.6 **C**	31.9 ± 1.1 **B**	19.7 ± 3.2 **A**	44.7 ± 1.6 **C**
	Ionization Index (%)	11.2 ± 1.5 **B**	24.4 ± 1.6 **C**	46.5 ± 2.8 **D**	8.9 ± 0.9 **A**
Anthocyanins by HPLC (mg/L)	Anthocyanidin-3-monoglycosides	98.1 ± 4.0 **C**	61.2 ± 3.2 **B**	28.7 ± 0.8 **A**	92.2 ± 2.9 **C**
	Acetylated anthocyanins	27.2 ± 1.3 **C**	18.0 ± 1.0 **B**	7.2 ± 0.3 **A**	27.0 ± 1.5 **C**
	p-Coumaroyl anthocyanins	11.6 ± 0.8 **D**	6.2 ± 0.6 **B**	2.7 ± 0.2 **A**	8.9 ± 0.8 **C**
	Total free anthocyanins	136.8 ± 6.2 **C**	85.4 ± 4.7 **B**	38.5 ± 8.6 **A**	128.9 ± 8.4 **C**
	Total pyranoanthocyanins	33.1 ± 1.1 **B**	23.9 ± 1.1 **A**	28.3 ± 7.9 **AB**	25.3 ± 3.7 **A**

[a] Results are expressed as the mean ± standard deviation of three replicates. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples.

The presence of stems also significantly decreased the total anthocyanin concentration measured by spectrophotometry in a similar proportion to 100% seeds. However, the changes observed in the different indices, although statistically significant, were rather slight and did not follow the same trend as in the case of seeds. Specifically, the PVPP and copigmentation index slightly increased, whereas the ionization index slightly decreased. These results suggest that the presence of stems is not as effective as the presence of seeds when it comes to encouraging the formation of polymerized pigments.

Table 5 also shows the anthocyanins as measured by HPLC-DAD. The total anthocyanin concentrations determined by HPLC-DAD were lower than those measured by spectrophotometry. This is only to be expected because spectrophotometric analysis

includes the contribution from other pigments in the measurement and therefore overestimates the total anthocyanin concentration, whereas the HPLC-DAD methods detect only free anthocyanins.[65] However, the general trend was similar to that observed by spectrophotometry. Briefly, the total anthocyanin concentration decreases in the presence of seeds, and this can be explained by the formation of polymerized pigments with the flavan-3ols they release. In general, this trend was observed for anthocyanidin-3-monoglucosides, acetylated anthocyanins, *p*-coumaroyl anthocyanins, and pyranoanthocyanins. Anthocyanins measured by HPLCDAD also tended to decrease in the presence of stems, but the differences were significant only in the case of *p*-coumaroyl anthocyanins and pyranoanthocyanins.

Effect on Wine Color. The color intensity (CI) and Cielab coordinates of the different samples are shown in Table 6. The presence of seeds increased the CI, chroma (C^*), hue (h^*), green−red component (a^*), and yellow−blue component (b^*), but it decreased the lightness (L^*) of the wines obtained from the grape juice bled off immediately (medium A). Stems also increased CI, C^*, h^*, and b^* and decreased L^*, but had no significant effects on a^*. In any case these changes in color were due to the phenolic compounds released by seeds and stems, which provided brownish hues to the samples and can have some influence on the final hue of the wine in real conditions.

More interesting are the data for the color of the samples obtained with the liquid bled off after 4 days of maceration (medium B). The presence of seeds significantly increased CI and decreased L^*, and this effect was more marked in the 300% than in the 100% seed wine. h^* and b^* also increased, but in this case no differences between the 300 and 100% seed wines were detected. Finally, C^* and a^* did not change. These results indicate that the color of the wines obtained in the presence of seeds was deeper than that of the control. In general, this is in line with results obtained in previous studies.[24,32]

Looking at the different color components, the percentages of the yellow ($A_{420\ \%)}$) and red ($A_{520\ \%)}$) components tended to decrease, whereas the percentage of the blue component ($A_{620\ \%)}$) increased significantly in the presence of seeds. Consequently, the presence of seeds not only increases the color intensity but also accentuates its bluish hue. There

are different possibilities for explaining these changes. First, the higher pH of the seed wines favors the displacement of the anthocyanin equilibrium toward the formation of the blue quinoidal base.[8] The higher pH must also displace the red form of anthocyanins toward the colorless form (hemiketal), thereby decreasing the red component of the color.[8] However, the higher presence of phenolic compounds released from the seeds favors copigmentation, which brings about hyperchromic and bathochromic eff ects,[4] and this could compensate for the loss of color and displace the wavelength of maximal absorptivity toward higher values. Finally, the presence of flavan-3-ols released from the seeds also favors the formation of polymerized pigments that contribute in a higher proportion to wine color.[66] The effect of seed supplementation on the PVPP and ionization index confirms this. Consequently, it can be asserted that the presence of seeds enhances wine color because it favors the formation of polymerized pigments through the combination of anthocyanins and the flavan-3-ols released by them.

In contrast, CI, C^*, a^*, and b^* were significantly lower and L^* was significantly higher when the stems were present. This indicates that the presence of stems leads to wines with a poorer color. Moreover, the yellow ($A_{420\ \%)}$ and blue ($A_{620\ \%)}$ components of the color increased significantly, whereas the red component ($A_{520\ \%)}$ decreased significantly. As happened in the case of seeds, stems also bring about an increase in pH that was even higher. As with seeds, this higher pH must favor the formation of the blue quinoidal base and the colorless hemiketal,[8] thereby causing a decrease in color intensity and an increase in bluish hues. Stems naturally also release other phenolic compounds that favor copigmentation and, in particular, flavan-3-ols, which could combine with anthocyanins to form new pigments. However, in this case the stems released lower amounts of flavan-3-ols and other phenolic compounds, and thus these phenomena seem to be insufficient to compensate for the loss of color that came about because the stems absorbed some anthocyanins and increased the wine’ s pH.

Table 6. Color Parameters[a]

CONDITION	PARAMETER	Control		+ 100 % of Seeds		+ 300 % of Seeds		+ 100 % of Stems	
Medium A. Immediately Bleed-off	Color Intensity (CI)	0.31 ± 0.01	**A**	0.85 ± 0.08	**C**	1.69 ± 0.04	**D**	0.50 ± 0.03	**B**
	Lightness (L*)	98.3 ± 0.1	**D**	95.4 ± 0.6	**B**	91.1 ± 0.3	**A**	97.6 ± 0.2	**C**
	Chroma (C*)	2.4 ± 0.1	**A**	5.7 ± 0.2	**C**	10.6 ± 0.2	**D**	3.9 ± 0.2	**B**
	Hue (h*)	38.4 ± 3.0	**A**	41.3 ± 2.7	**A**	39.4 ± 0.9	**A**	64.1 ± 3.2	**B**
	Green−Red Component (a*)	1.9 ± 0.1	**A**	4.3 ± 0.3	**B**	8.2 ± 0.1	**C**	1.7 ± 0.1	**A**
	Blue−Yellow Component (b*)	1.5 ± 0.0	**A**	3.7 ± 0.2	**B**	6.7 ± 0.2	**C**	3.5 ± 0.3	**B**
Medium B. Macerated for 4 days	Color Intensity (CI)	9.7 ± 0.3	**B**	12.1 ± 0.2	**C**	14.4 ± 0.4	**D**	8.1 ± 0.3	**A**
	• A_{420}%	31.5 ± 0.5	**B**	31.6 ± 0.1	**B**	30.9 ± 0.1	**A**	33.2 ± 0.4	**C**
	• A_{520}%	59.6 ± 0.4	**C**	57.4 ± 0.3	**B**	55.4 ± 0.1	**A**	56.9 ± 0.4	**B**
	• A_{620}%	8.9 ± 0.1	**A**	11.0 ± 0.3	**C**	13.7 ± 0.1	**D**	9.9 ± 0.1	**B**
	Lightness (L*)	55.6 ± 0.9	**C**	47.7 ± 0.7	**B**	40.8 ± 0.6	**A**	60.2 ± 0.9	**D**
	Chroma (C*)	52.0 ± 0.6	**B**	53.0 ± 0.5	**B**	52.5 ± 0.5	**B**	45.2 ± 0.6	**A**
	Hue (h*)	7.7 ± 1.4	**A**	9.4 ± 0.3	**B**	9.6 ± 0.7	**B**	7.1 ± 0.3	**A**
	Green−Red Component (a*)	51.5 ± 0.6	**B**	52.3 ± 0.2	**B**	51.9 ± 0.3	**B**	44.9 ± 0.5	**A**
	Blue−Yellow Component (b*)	6.3 ± 1.0	**B**	8.8 ± 0.2	**C**	8.8 ± 0.7	**C**	4.9 ± 1.3	**A**

[a] Results are expressed as the mean ± standard deviation of three replicates. Different letters in a row indicate a statistical difference ($p < 0.05$) between samples. A420% = percentage of yellow component; A520% = percentage of red component; A620% = percentage of blue component.

Effect on Wine Astringency and Bitterness. Figure 3 shows the sensory appreciation of the astringency and bitterness of the different wines. Astringency and bitterness scored significantly higher in the samples supplemented with seeds in both media than in their corresponding controls. Moreover, the score for both sensory attributes tended to be higher when 300% rather than 100% seeds was added, although in this case no significant differences were observed. The lack of significance may be because the perception of astringency and bitterness varies among individuals and, because it is an accumulative sense, its objective assessment is very complicated.[67] In any case these results are in concordance with those reported by Cliff et al.[68] using a model of a red wine enriched with grape seed tannins.

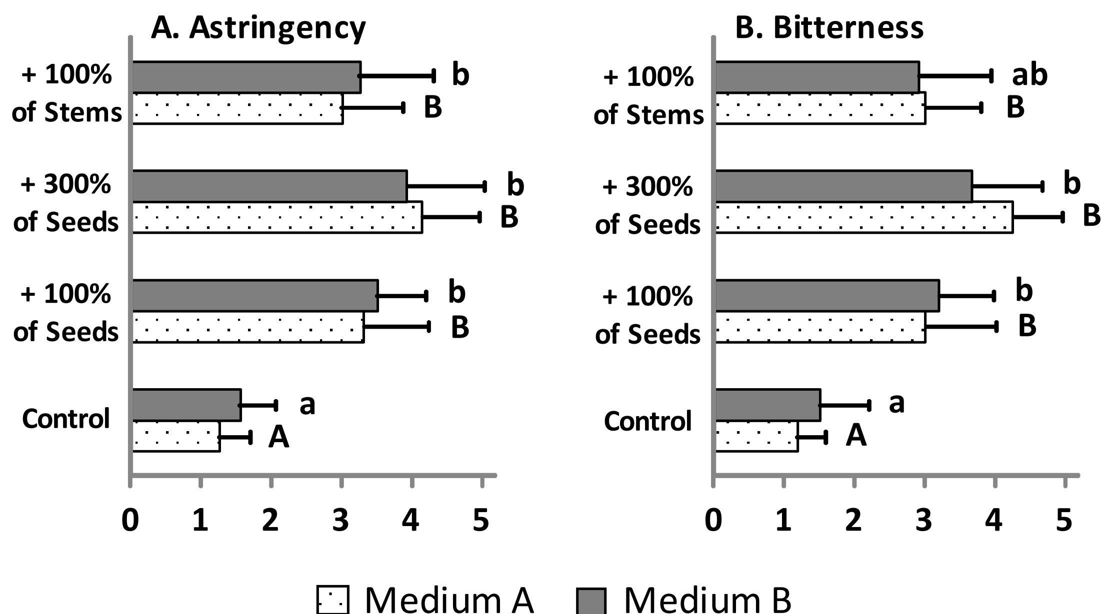

Figure 3. Sensory appreciation of astringency and bitterness. Results are expressed as the mean ± standard deviation of eight tasters. Different letters indicate a statistical difference ($p < 0.05$) between samples, capital letters for medium A and lower case letters for medium B.

The presence of stems also significantly increased astringency in both media in a similar proportion to 100% added seeds. Bitterness tended to increase in the presence of stems, but in this case the difference was not significant.

In summary, seeds and stems are major sources of phenolic compounds that condition the final composition of the wine. Specifically, seeds release significant amounts of flavan-3-ol monomers, mainly (+)-catechin and (−)-epicatechin, as well as proanthocyanidins with a relatively low mDP and a high percentage of galloylation. The presence of seeds also significantly increases the color of red wine because it boosts the formation of polymerized pigments through the combination of flavan-3-ol monomers and proanthocyanidins with anthocyanidins. Seeds also increase astringency and bitterness and generate a slight but significant decrease in ethanol content, probably through the release of potassium and water. However, stems also release flavan-3-ol monomers and proanthocyanidins, but their composition was quite different from the case of seeds. Specifically, the flavan-3-ol monomers released by stems were mainly (+)-catechin and (+)-gallocatechin, and the proanthocyanidins released had a higher mDP than those from seeds and a high percentage of prodelphinidins. Stems also

significantly increased the pH and decreased the titratable acidity and ethanol content, probably through the solubilization of potassium and water. In contrast with what happened with seeds, the presence of stems impoverishes the color of the red wine, probably because stems absorb anthocyanidins and/or polymerized pigments and also because they increased the wine' s pH. Moreover, the higher concentrations of flavan-3-ol monomers and proanthocyanidins released from stems do not have the positive effect on wine color that was observed when seeds were added. Finally, the presence of stems also increases the wine' s bitterness and astringency.

Consequently, the natural presence of seeds produces wines with a higher color intensity and tannin concentration, which can be appropriate for red wines for long aging. Seed extraction is naturally also conditioned by other factors, such as temperature, ethanol content, ripeness, and maceration length. However, the industrial elimination of seeds could be recommended in some cases to avoid an excess of astringency and bitterness in wines for early consumption. In contrast, stems have no positive effects on wine color and provide astringency and bitterness, and these are reasons why destemming should be systematically applied in red wine-making. However, further studies are needed to better understand how the ripeness of seeds and stems influence red wine color and composition and also to verify how these parameters evolve during aging.

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CAPÍTULO 3: Velocidad de consumo de oxígeno por diferentes taninos enológicos en una solución de vino modelo.

Actualmente, el uso de taninos enológicos es una operación muy frecuente en las bodegas. Se les atribuye diferentes funciones y efectos sobre las propiedades de los vinos. Una de las mayores razones por las que se usan es la de prevenir al mosto y al vino de la oxidación, debido que los taninos enológicos poseen una capacidad antioxidante. El aporte de oxígeno al vino tinto da lugar a una serie de transformaciones que permiten estabilizar el color, suavizar la astringencia y el sabor amargo.

En este capítulo se aborda el objetivo 3, en el que nos planteamos determinar la cinética de consumo de oxígeno por parte de diversos taninos enológicos para ver si su adición al vino puede ser útil para protegerlo de la oxidación. Para realizar este estudio se utilizaron cinco tipos de tanino enológico y se usó un sensor de oxígeno no invasivo.

Las analíticas de los vinos fueron realizadas en el laboratorio del grupo de Tecnología Enológica (TECNENOL) del Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili (URV, Tarragona). **Los resultados del estudio fueron publicados en la revista *Food Chemistry*.**

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Oxygen consumption rates by different oenological tannins in a model wine solution

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ABSTRACT

The kinetics of oxygen consumption by different oenological tannins were measured in a model wine solution using the non-invasive method based on luminiscence. The results indicate that the oxygen consumption rate follows second-order kinetics depending on tannin and oxygen concentrations. They also confirm that the oxygen consumption rate is influenced by temperature in accordance with Arrhenius law. The indications are that ellagitannins are the fastest oxygen consumers of the different oenological tannins, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins. This methodology can therefore be proposed as an index for determining the effectiveness of different commercial tannins in protecting wines against oxidation.

Keyword: Tannins, oxygen consumption rate, antioxidant capacity.

1. Introduction

Nowadays the use of oenological tannins is common practice in winemaking. However, their use is only authorized by the International Organization of Vine and Wine (OIV) to facilitate the clarification of wines and musts (International Oenological Codex, 2016). Nevertheless, it is unquestionable that they are also currently used for many other purposes. Indeed, the literature has attributed several other characteristics to oenological tannins, such as antioxidant activity (protection of wines against oxidation) (Ricci et al., 2016), direct consumption of dissolved oxygen (Navarro et al., 2016), ability to scavenge peroxyl radicals (Magalhães, Ramos, Reis & Segundo, 2014), ability to chelate iron (II), prevention of oxidative damage mediated by Fenton-based reactions (Pérez, Wei & Guo, 2009), antioxidasic activity (anti-laccase activity) (Obradovic, Schulz & Oatey, 2005), improvement of wine structure and mouthfeel (Vivas, 2001), color improvement and stabilization of red wines (Canuti, Puccioni, Giovani, Salmi, Rosi & Bertuccioli 2012), copigmentation effect (Neves, Spranger, Zhao, Leandro & Sun, 2010), direct formation of new pigments (Versari, du Toit & Parpinello, 2013), elimination of reduction odours (Vivas, 2001) and even bacteriostatic effects (Lempereur, Blayteyron, Labarbe, Saucier, Klebek & Glories, 2002).

Obviously, to all these functions must be added the interactions between oenological tannins and proteins and the resulting ability to prevent protein haze (Ribéreau-Gayon, Glories, Maujean & Dubourdieu, 2006), help protein fining avoid gelatin over-fining (Mierczynska-Vasilev & Smith, 2015) and the effects on wine astringency and bitterness (Obreque-Slier, Peña-Neira & Lopez-Solis, 2012).

Although oenological tannins are commonly employed to these ends, there are questions about them that need to be clarified. Many commercial types of tannin of different plant origins and chemical compositions are available (Malacarne, Nardin, Bertoldi, Nicolini & Larcher, 2016; Obreque-Slíer, Pena-Neira, Lopez-Solis, Ramirez-Escudero & Zamora-Marin, 2009). The so-called oenological tannins on the market include ellagitannins from oak or chestnut, gallotannins from oak galls, and condensed tannins from grape seeds and skins and even other plant origins such as tara, quebracho and mimosa (Versari et al., 2013).

The chemical structure of ellagitannins consists of an open-chain glucose esterified at positions 4 and 6 by a hexahydroxydiphenoyl unit (HHDP) and a nonahydroxyterphenoyl unit (NHTP) esterified at positions 2, 3 and 5 with a C-glycosidic bond between the carbon of the glucose and position 2 of the trihydroxyphenoyl unit (Quideau et al., 2004; Takuo, Takashi, Tsutomu & Hideyuki, 2009). Several different ellagitannins have been described but castalagin and vescalagin are the most abundant in oak wood, accounting for between 40% and 60% of the total by weight (Fernández de Simón, Cadahía, Conde & García-Vallejo, 1999).

Gallotannins are formed by the esterification of gallic acid with the hydroxyl group of a polyol carbohydrate such as glucose (Hagerman, 1998). Gallotannin extracts, which are also commercially known as tannic acid, are mixtures of polygalloyl glucoses or polygalloyl quinic acid esters with a number of galloyl moieties per molecule ranging from 2 to 12 depending on the plant source used to extract the tannic acid (Sylla, Pouységu, Da Costa, Deffieux, Monti & Quideau, 2015).

The composition of condensed tannins depends on their plant origin. Thus grape-seed condensed tannins are procyanidins with a lower mean degree of polymerization (mDP) and a high level of galloylation (Santos-Buelga, Francis-Aricha & Escribano-Bailón, 1995), whereas grape-skin condensed tannins are a mixture of procyanidins and prodelphinidins with a higher mDP and a lower level of galloylation (Souquet, Cheynier, Brossaud & Moutounet, 1996). Condensed tannins from other plant sources also have different composition. Those from quebracho, for instance, are profisetinidins and not procyanidins because their acidic cleavage originates fisetinidin and not cyanidin (Celzard et al., 2015), while those from mimosa are prorobinetinidins (Celzard et al., 2015) because their acidic cleavage originates robinetinidin. Condensed tannins as a whole are called proanthocyanidins.

Of the different functions attributed to oenological tannins, their antioxidant capability is probably one of the main reasons why they are widely used in winemaking for preventing grape juice and wine oxidation. It is traditionally accepted that oenological tannins inhibit polyphenol oxidases, tyrosinase (EC 1.14.18.1) and laccase (EC 1.10.3.2),

thus protecting wines against browning (Nichols-Orians, 1991; Versari et al. 2013), and that they directly consume oxygen, thereby protecting the other wine components from oxidation (Navarro et al., 2016; Vivas & Glories, 1996).

There are a number of references to the antioxidant properties of commercial tannins (Neves et al., 2010; Laghi, Parpinello, Del Rio, Calani, Mattioli & Versari et al., 2010; Magalhães et al., 2014) using different antioxidant assays (CUPRAC, DPPH, FRAP, ORAC, Folin-Ciocalteu, …). However, all these methods are indirect methods and none of them really measure the direct oxygen consumption. Moreover, the different antioxidant assays produce different and sometimes contradictory results (Magalhães et al., 2014). For these reasons, the aim of this paper is therefore to measure the kinetics of oxygen consumption by different commercial tannins in order to determine their real antioxidant capacities to protect wine against oxygen.

2. Materials and methods

2.1. Chemicals and equipment

All samples and standards were handled without exposure to light. ABTS: 2,2'azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97%, manganese dioxide, AAPH: 2,2-azobis(2-methylpropionamidine) dihydrochloride, gallic acid, copper (II) sulfate pentahydrate, iron (III) chloride hexahydrate, Folin-Ciocalteu reagent and fluorescein sodium salt were purchased from Sigma® (St. Louis, MO, USA); DPPH: 2,2-diphenyl-1-picrylhydrazyl from Extrasynthèse (Genay, France); L(+)-tartaric acid, sodium hydroxide, potassium metabisulfite, ascorbic acid, ethanol and methanol HPLC grade were purchased from Panreac Química (Barcelona, Spain). Water was ultrapure Milli-Q quality (Millipore, Bedford, MA, USA).

2.2. Oenological tannins

Five different tannins were used in this study. Quebracho tannin (Tanin SR) from Enotecnia Cataluña S.L. (Vilafranca del Pènedes, Barcelona, Spain); seed tannin (Protan pépin), ellagitannin (Ellagitan Chêne) and gallotannin (Galovin) from AEB Iberica S.A.

(Castellbisbal, Barcelona, Spain); and skin tannin (Tan Sutil) from Agrovin, (Alcazar de San Juan, Ciudad Real, Spain).

2.3. Experimental design

The experimental design previously described by Navarro et al. (2016) was used for oxygen consumption measurements. A model wine solution composed of ethanol (12% v/v) and tartaric acid (4 g/L) adjusted at pH = 3.5 with sodium hydroxide was used. This solution was enriched with 3 mg of Iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L in the form of copper (II) sulfate pentahydrate. We have worked with this model wine solution and not with real wine because the naturally occurring phenolic compounds would compete with oenological tannins in oxygen consumption making it impossible to determine the kinetic constants. The different doses of oenological tannins, potassium metabisulfite or ascorbic acid were placed in clear glass bottles into which a pill had previously been inserted (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203-01_PSt3-0828-01, Regensburg, Germany) for the non-invasive measurement of dissolved oxygen by luminescence (Nomasense TM O2 Trace Oxigen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium). The bottles were completely filled with the model wine solution and closed immediately after with a crown cap and bidule so as to minimize the volume of headspace. The bottles were then gently shaken to dissolve the different antioxidants: ascorbic acid, sulfur dioxide (added as potassium metabisulfite) or the different oenological tannins. Oxygen (Diéval, Vidal, & Aagaard, 2011) was measured periodically to determine the oxygen consumption rate. All the experiments were performed in triplicate.

2.4. Estimation of the influence of sulfur dioxide, ascorbic acid and oenological tannin concentrations on the oxygen consumption rate

In this assay the oxygen concentration was fixed at saturation level, whereas the tannin concentration was variable and the temperature maintained at 20 ± 1 ºC. Three doses of the different oenological tannins (2, 4 and 6 g/L) were assayed. These doses were higher than those usually applied to musts and wines in order to minimize the measurement time required for the complete consumption of oxygen. Three doses (50, 100 and 150 mg/L) of sulfur dioxide (E220) and ascorbic acid (E300) were also studied to compare the oxygen

consumption rate (OCR) of the different tannins with the OCR of the antioxidant additives most used in winemaking. The model wine solution was saturated with oxygen (8.0 mg/L) by bubbling with air for 10 min and then used immediately for the experiment. All assays were performed in triplicate, taking control bottles with the oxygen-saturated model wine solution without any addition as the control reference.

2.5. Estimation of the influence of temperature on the OCR

In this assay the oenological tannins (4 g/L) and the oxygen concentrations (saturation level; 8.0 mg/L) were fixed and the temperature was variable (8 ± 1, 20 ± 1 and 30 ± 1 ºC). All assays were performed in triplicate, taking control bottles with the oxygen-saturated model wine solution without any addition as the control reference.

2.6. Estimation of the influence of oxygen concentration on the OCR

In this assay the oenological tannin concentration was fixed (4 g/L), whereas the oxygen concentration was variable (2.5, 4.0 and 8.0 mg/L) and the temperature maintained at 20 ± 1 ºC. The model wine solution was initially saturated with oxygen by bubbling with air for 10 minutes and then used directly for the trials with 8 mg/L. The rest of the saturated solution was then bubbled with nitrogen to eliminate the excess oxygen until the desired value was reached (2.5 or 4.0 mg/L). All assays were performed in triplicate, taking control bottles with the different oxygen concentrations without any addition as the control reference.

2.7. Antioxidant capacity

The antioxidant capacity of the different oenological tannins was measured using various methods: total concentration of phenolic substances by means of the Folin-Ciocalteu index (TPC, OIV, 2009), $ABTS^+$ and DPPH (Vallverdú-Queralt, Regueiro, Rinaldi de Alvarenga, Torrado & Lamuela-Raventos, 2014) and ORAC assays (Suarez, Romero, Ramo, Macìa & Motilva, 2009).

2.8. Statistics

All the chemical and physical data for the samples are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance tests ($p < 0.05$)

were carried out with SPSS software (IBM, Barcelona, Spain), and multiple comparisons were performed using the Student–Newman–Keuls post-hoc test.

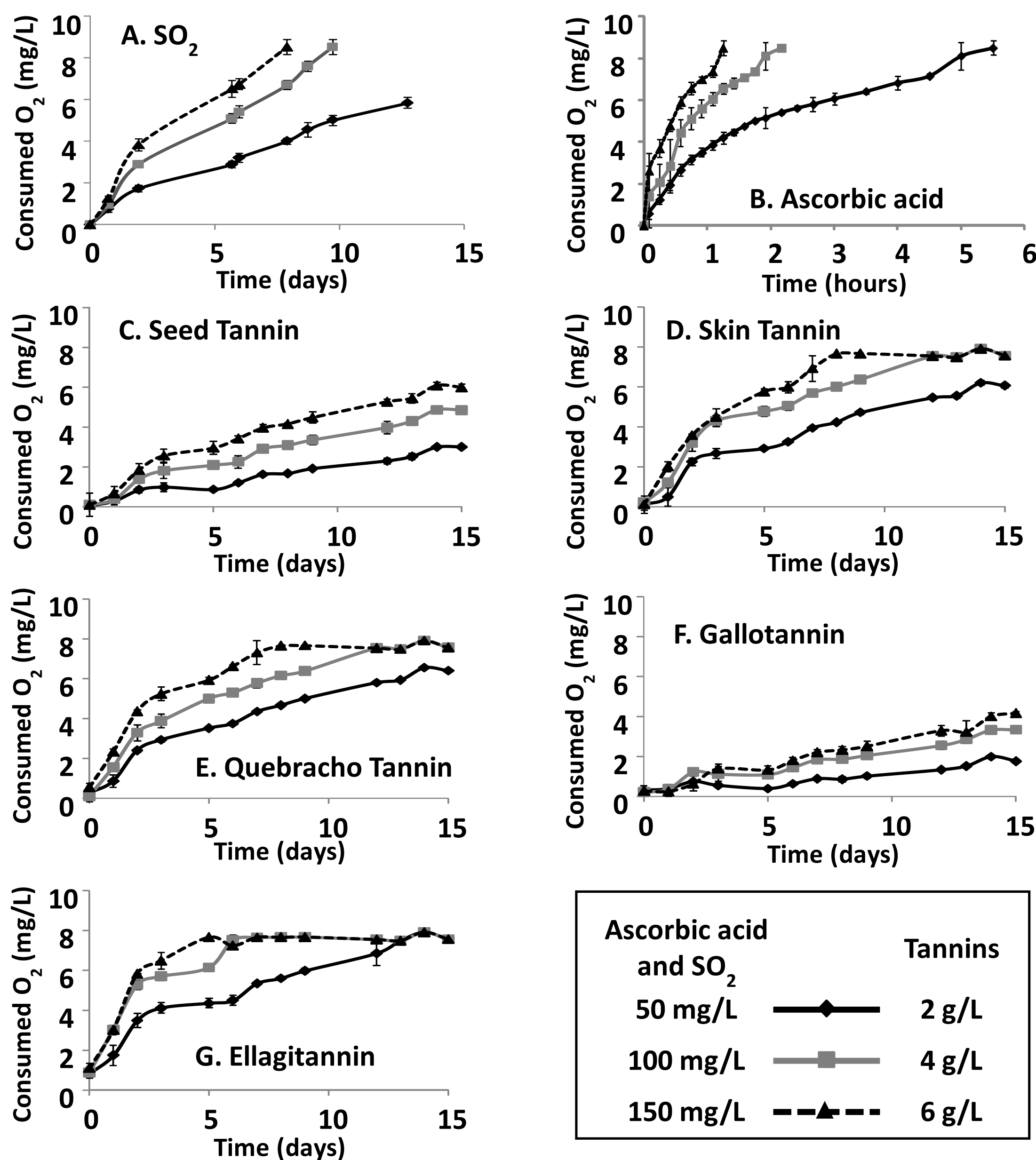


Fig. 1. Influence of sulfur dioxide, ascorbic acid and different oenological tannin concentrations on oxygen consumption in an oxygen-saturated model wine solution at 20ºC.

3. Results and discussion

Fig. 1 shows the oxygen consumption kinetics of ascorbic acid, sulfur dioxide and different oenological tannins at different concentrations in an oxygen-saturated model wine solution at 20 ºC. The oxygen consumption of the control model wine solution (without addition of any antioxidant) was very low and can be considered negligible (data not shown). In contrast, the supplementation with ascorbic acid, sulfur dioxide and all the oenological tannins resulted in an oxygen consumption that was clearly influenced by the nature and dosage of the added antioxidant. Ascorbic acid was clearly the most efficient antioxidant (Fig. 1B) because complete consumption of all oxygen took place in a matter of hours, whereas all the other antioxidants needed days to obtain the same effect. A comparison of the oxygen consumption kinetics of sulfur dioxide and the different oenological tannins seems to indicate the existence of differences in antioxidant effectiveness among all these antioxidant additives. However, the quantitative comparison of the oxygen consumption kinetics of all these compounds is not evident from looking at these graphics. A kinetic modelization is therefore necessary in order to better quantify the antioxidant effectiveness of the various additives.

Different mathematical regression approaches were tried (lineal, logarithmic, exponential …), but none of them provided satisfactory results (data not shown). After considering other possibilities, an acceptable modelization was obtained when the inverse of consumed oxygen was plotted versus the inverse of time. Fig. 2 shows the results obtained for sulfur dioxide (Fig. 2A), ascorbic acid (Fig. 2B) and seed tannins (Fig. 2C). It can be seen that satisfactory lineal regression coefficients were obtained and relatively low Mean Square Errors (MSE), thus confirming that this mathematical model works quite well. Similar results were obtained for the other oenological tannins (Supplementary Fig. 1), but they are not shown in this figure so as not to overcomplicate the graphic. Their OCRs are shown below.

According to this modelization, the following equation can be established: $1/[O_2] = A/t + B$. This equation describes the relationship between the consumed oxygen versus time and is shown in Fig. 2D, which also shows how the consumed oxygen can be cleared up,

how the first derivative is obtained, and finally how the OCR at time zero ($OCRt_0$) can be determined. These correspond to the inverse of the slope of the initial equation.

Using this procedure it is possible to determine the relationship between the $OCRt_0$ and the antioxidant concentration for all the antioxidants studied. Fig. 2E, F and G show the representation of the $OCRt_0$ versus the concentration of sulfur dioxide, ascorbic acid and seed tannin respectively. These graphics clearly indicate that the $OCRt_0$ is lineally dependent on the antioxidant concentration, considering that the linear regression coefficients are reasonably good in all three examples (> 0.9700) and the MSE relatively low (< 0.02). Moreover, the slope of the straight lines obtained provides the real OCR in function of the antioxidant concentration. Similar behaviors were obtained for the other oenological tannins (Supplementary Fig. 2), enabling calculation of the OCR of each antioxidant expressed as mg of oxygen per day and per gram of antioxidant.

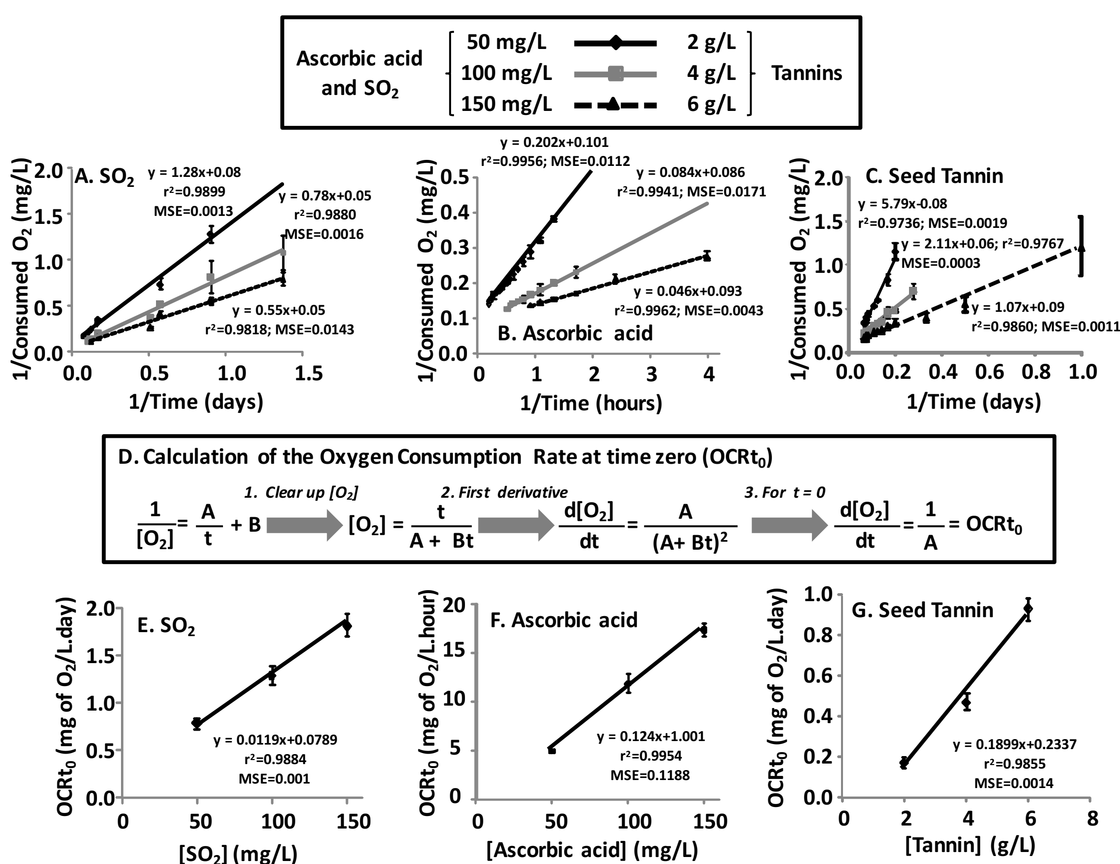


Fig. 2. Influence of sulfur dioxide, ascorbic acid and seed tannin concentrations on the oxygen consumption rate (OCR) in an oxygen-saturated model wine solution at 20 ºC.

Table 1 shows the antioxidant capacity of the different oenological tannins assessed by different analytical methods (TPC, DPPH, ABTS and ORAC) and also the OCR determined as described above. The aim of this comparison was to test whether or not the real OCR matches the most usual antioxidant capacity tests. Table 1 also shows the OCR of sulfur dioxide and ascorbic acid in order to compare the effectiveness of the different oenological tannins with the two most frequently used wine antioxidants.

In general the data confirm that the different antioxidant capacity tests obtained different and contradictory results. Briefly, the TPC test gave gallotannin the highest value followed by, in decreasing order, skin tannin, quebracho tannin, seed tannin and ellagitannin. The DPPH test also gave gallotannin the highest value but placed ellagitannin in second position followed by skin tannin, quebracho tannin and seed tannin. The results obtained with ABTS were quite similar to the DPPH test, the only difference being the position of quebracho tannin in last place just behind seed tannin. Finally, the ORAC test determined that the tannin with the highest antioxidant capacity was skin tannin followed by, in descending order, gallotannin, quebracho tannin, seed tannin and ellagitannin. Magalhães et al. (2014) obtained similar results using different antioxidant capacity tests on various oenological tannins, concluding that each antioxidant assay yields different information of a complementary nature. However, none of these assays provides useful information about the ability of each individual oenological tannin to protect wines against oxidation. In contrast, the method developed for OCR measurement determines the direct consumption of oxygen by the different oenological tannins in a model wine solution which provides a direct reference value for estimating the real capacity of the different oenological tannins to protect wines against oxidation. Moreover, the OCR obtained for the different oenological tannins can be compared with those of the most frequently used antioxidants in wine, such as sulfur dioxide and ascorbic acid. However, to obtain a real comparison the OCR needs to refer to the usual dose of each of the antioxidants. In this regard we have considered 0.05 g/L for sulfur dioxide, 0.1 mg/L for ascorbic acid and the maximum authorized dose of 0.4 g/L for all oenological tannins. Using all these data, the percentage relative antioxidant capacity (RAC) of sulfur dioxide was calculated (Table 1). The RAC expresses the relative effectiveness as oxygen consumers of ascorbic acid and each of the oenological tannins compared to sulfur

dioxide. The results indicate that ascorbic acid consumes oxygen 500 times faster than sulfur dioxide, confirming its reported high effectivity (Gibson, 2006). However, it must be taken into account that ascorbic acid generates hydrogen peroxide after consuming oxygen and its use in wine may therefore cause subsequent oxidations (Oliveira, Ferreira, De Freitas & Silva, 2011), which can affect the sensory quality of the wine. The RAC of different oenological tannins is also shown in Table 1. It can be seen that ellagitannins have a similar effectivity for oxygen consumption to sulfur dioxide, but that it is even higher (122%). This confirms that ellagitannins are very good antioxidants and suggests the possibility of their use as a complement (or even an alternative) for reducing (or even eliminating) the need for sulfur dioxide to protect wines against oxidation. Other tannins such as quebracho tannins and skin tannins have acceptable effectiveness (38% and 27% respectively). Finally, seed tannins (13%) and especially gallotannins (4%) showed lower values, indicating poor effectiveness in protecting wine against oxygen. It seems somewhat surprising to see the low RAC of gallotannins for oxygen, bearing in mind that their antioxidant capacity obtained via all the other procedures was the highest. Moreover, gallotannins are widely employed to prevent enzymatic browning in grape juice (Caillet, 2015; Crespy, 2002). A possible explanation for this could be that gallotannins can act as inhibitors of polyphenol oxidases (tyrosinase and laccase) (Nichols-Orians, 1991; Obradovic, 2006; Sugimoto et al., 2009), and therefore their protecting effect against oxidation would relate more to their polyphenol oxidase inhibitory effect than to their direct reaction with oxygen.

Table 1. Antioxidant capacity of oenological tannins assessed by different analytical methods compared with the oxygen consumption rate (OCR) in a model wine solution.

Type of tannin	TPC (mg gallic acid/g)		DPPH (mmol Trolox/g)		ABTS (mmol Trolox/g)		ORAC (mmol Trolox/g)		OCR (mg O2/day.g)		RAC (%)
Sulfur dioxide	-		-		-		-		**11.90 ± 0.63**	**E**	**100**
Ascorbic acid	-		-		-		-		**2,976 ± 625**	**F**	**50,000**
Seed tannin	**385 ± 17**	**B**	**4.91 ± 0.11**	**A**	**9.17 ± 0.48**	**A**	**2.26 ± 0.23**	**AB**	**0.19 ± 0.05**	**B**	**13**
Skin tannin	**484 ± 70**	**C**	**5.89 ± 0.44**	**B**	**9.41 ± 0.54**	**A**	**3.78 ± 0.26**	**C**	**0.40 ± 0.10**	**C**	**27**
Quebracho tannin	**434 ± 11**	**C**	**5.65 ± 0.48**	**B**	**8.92 ± 0.68**	**A**	**2.92 ± 0.74**	**B**	**0.57 ± 0.15**	**C**	**38**
Gallotannin	**780 ± 32**	**D**	**13.27 ± 0.73**	**D**	**16.00 ± 0.33**	**C**	**3.61 ± 0.25**	**C**	**0.06 ± 0.05**	**A**	**4.2**
Ellagitannin	**266 ± 7**	**A**	**7.10 ± 0.65**	**C**	**11.02 ± 0.49**	**B**	**1.86 ± 0.18**	**A**	**1.81 ± 0.15**	**D**	**122**

All data is expressed as the arithmetic mean of three replicates with standard deviation. Different letters indicate statistically significant differences. TPC: total concentration of phenolic substances; DPPH: antiradical activity determined using 2,2-Diphenyl-1-picrylhydrazyl; ABTS: antiradical activity determined using 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); ORAC: oxygen radical absorbance capacity; OCR: Oxygen consumption rate; RAC: Relative Antioxidant Capacity referring to sulfur dioxide.

Fig. 3 shows the influence of temperature on oxygen consumption by the different oenological tannins. As expected, the higher the temperature, the quicker the oxygen consumption. These data were used to determine the OCR for all the tannins at the different temperatures in accordance with what was described above. Considering the concentration of tannin used (0.4 g/L) and the OCR obtained, the kinetic constants (K) were calculated following the equation OCR = K • [Tannin]. The Arrhenius plot (Ln K versus 1/Temperature) was then graphed using these data. Fig. 3F shows the results obtained, which indicate that the OCRs of the different oenological tannins match reasonably well with the dependence of temperature described by Arrhenius law (Arrhenius, 1889).

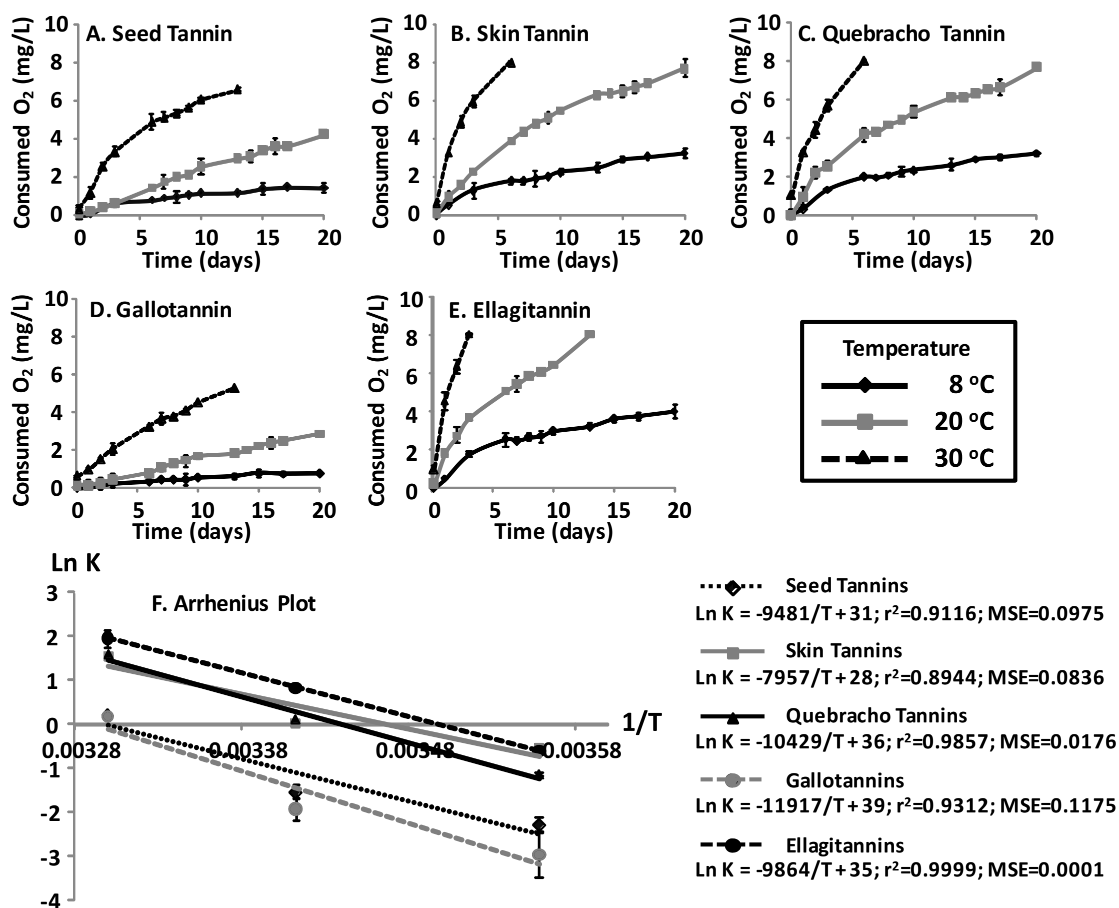


Fig. 3. Influence of temperature on oxygen consumption by different oenological tannins in an oxygen-saturated model wine solution.

Fig. 4 shows the influence of oxygen concentration on oxygen consumption by the different oenological tannins. Tannin concentration was fixed at 0.4 g/L in all the cases. As expected, the higher the oxygen concentration, the faster the oxygen consumption. Since OCR is clearly influenced by tannin and oxygen concentration, it seems that oxygen consumption by all the oenological tannins is a second-order reaction from a kinetic point of view. In short, the oxygen consumption kinetics by oenological tannins can be described using the equation OCR = *K* • [Tannin] • [O_2]. Taking all the reported data into account it was possible to calculate the kinetic constant (*K*) for all the oenological tannins studied. This was done by including all the calculated OCRs on a graph according to the product of the tannin and oxygen concentrations. The slope of the linear regression obtained should therefore be the kinetic constant (*K*). Table 2 shows these kinetic constants expressed as two different units (L.g of tannin^{-1} day^{-1} and L.µg of tannin^{-1} s^{-1}). These data clearly indicate

that ellagitannins are the fastest oxygen consumers of the various oenological tannins, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins.

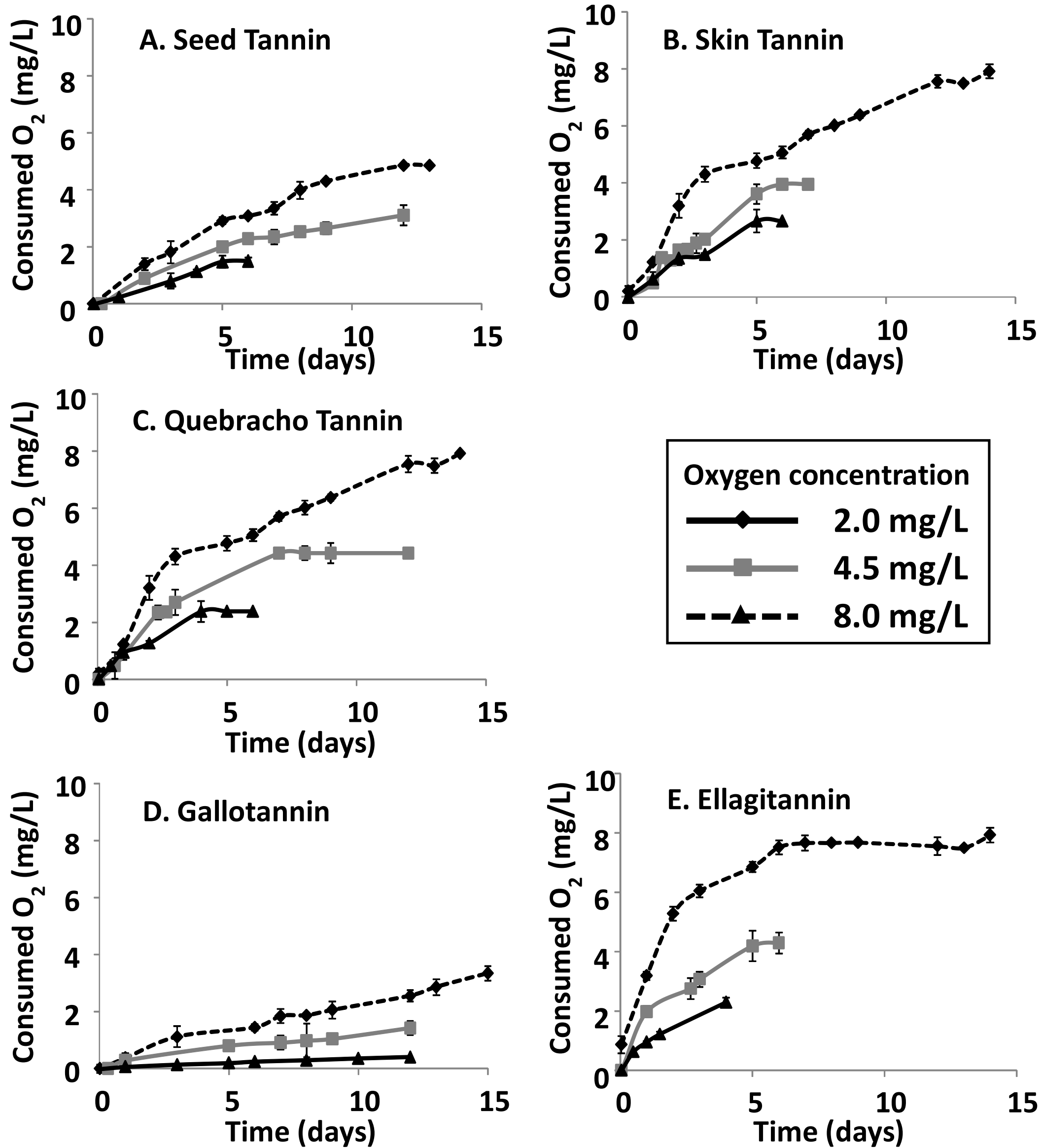


Fig. 4. Influence of oxygen concentration on oxygen consumption by different oenological tannins in a model wine solution at 20 ºC.

Table 2. Reaction rate constant (OCR=K•[Tannin]•[O_2]) for the different oenological tannins.

Type of tannin	Reaction Rate Constant (K)		Significativity ($p < 0.05$)
	(L.g of tannin^{-1}.day^{-1})	(l.µg of tannin^{-1}.s^{-1})	
Seed Tannins	0.0234 ± 0.0021	6.46 ± 0.58	**B**
Skin Tannins	0.0397 ± 0.0039	10.95 ± 1.07	**C**
Quebracho Tannins	0.0572 ± 0.0047	15.78 ± 1.30	**D**
Gallotannins	0.0068 ± 0.0009	1.88 ± 0.25	**A**
Ellagitannins	0.2063 ± 0.0151	56.93 ± 4.18	**E**

All data is expressed as the arithmetic mean ± standard deviation. Different letters indicate statistically significant differences.

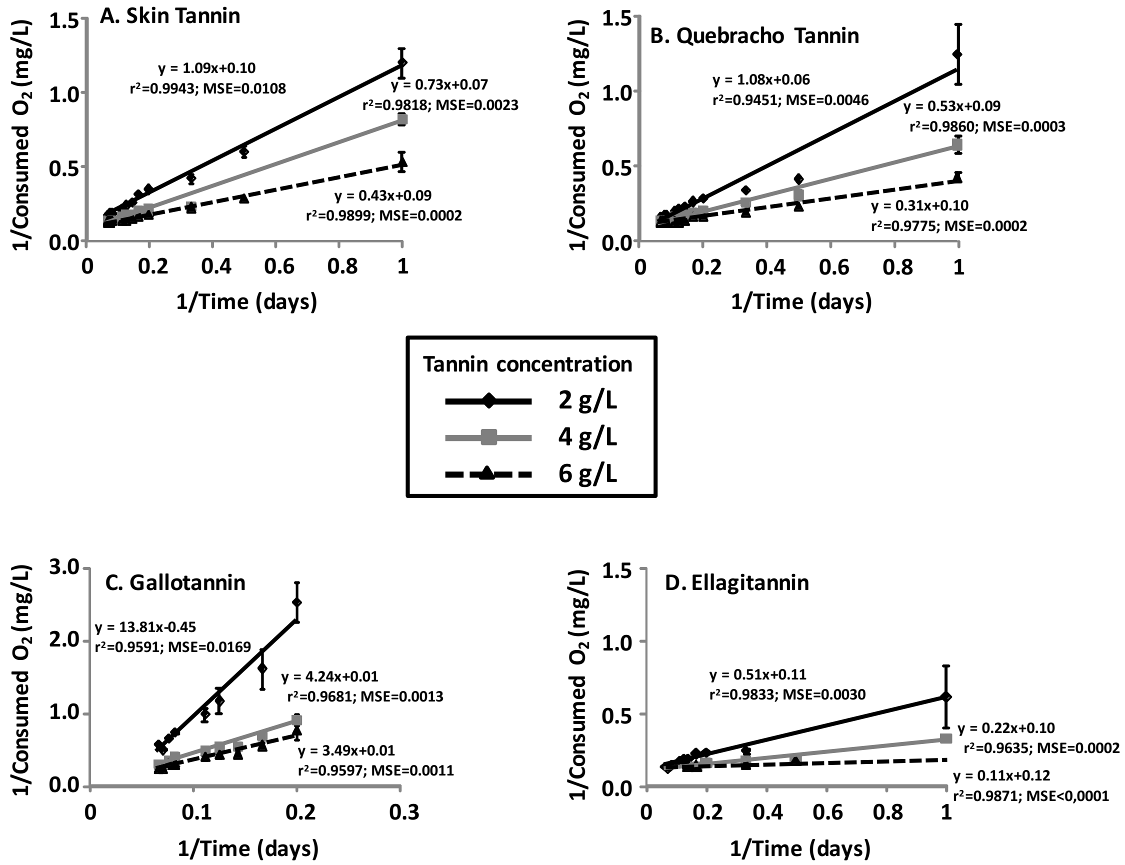
4. Conclusions

The measurement of the oxygen consumption rate (OCR) of different oenological tannins in a model wine solution using different concentrations of tannins and oxygen confirms that oxygen is consumed with a second-order kinetic in accordance with the equation (OCR = K • [Tannin] • [O_2]). These results also confirm that the OCR is influenced by temperature in line with Arrhenius law. Of the various oenological tannins, ellagitannins are the fastest oxygen consumers, followed in decreasing order by quebracho tannins, skin tannins, seed tannins and finally gallotannins. This methodology seems to be suitable for evaluating the oxygen consumption kinetics of the different oenological tannins and can therefore be proposed as an index to classify oenological tannins in terms of their effectiveness to consume oxygen.

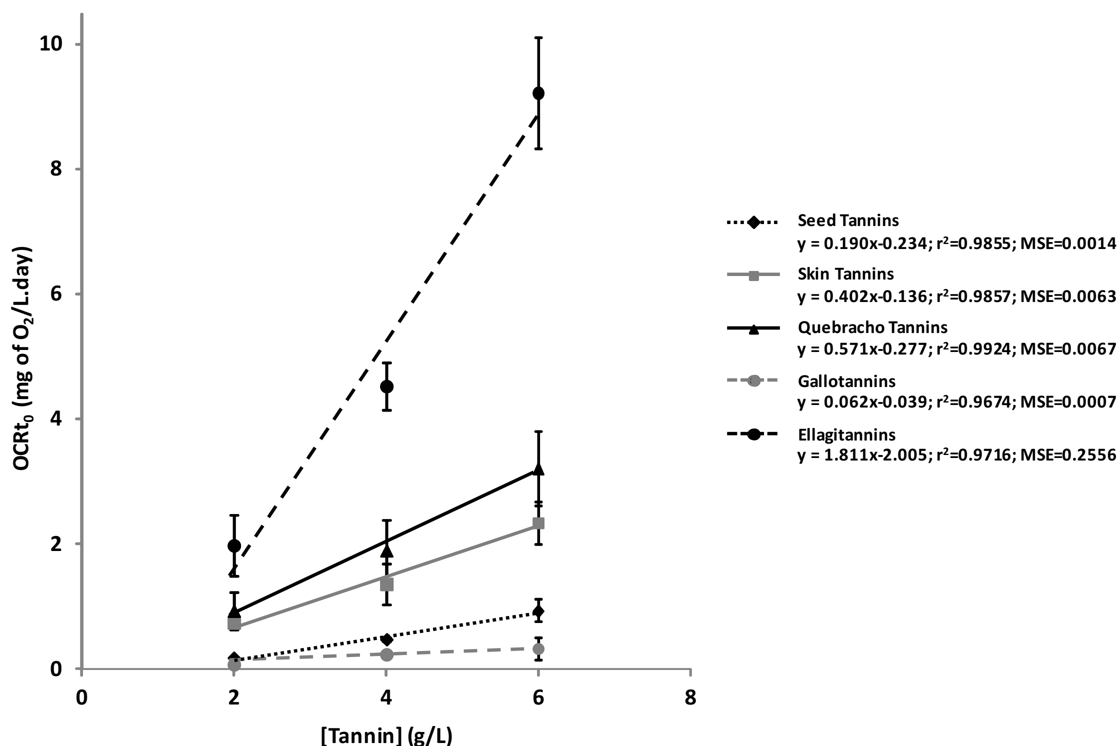
Acknowledgements

We would like to thank CICYT (Projects AGL2014-56594-C2-1-R and AGL2014-56594-C2-2-R) and the Spanish *Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente* for their financial support. We are also grateful to the OIV for the scholarship granted to Jordi Gombau.

Appendix A. Supplementary data



Supplementary Fig. 1. Modelization of oxygen consumption by different oenological tannins in an oxygen saturated model wine solution at 20 ºC.



Supplementary Fig. 2. Oxygen consumption rate of the different oenological tannins.

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DISCUSIÓN GENERAL

El grupo de Tecnología Enológica (TECNENOL) del Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili, en el que se ha desarrollado esta tesis, lleva años investigando sobre la composición química y la calidad de los vinos tintos, con el objetivo de proporcionar información tanto a nivel académico como a nivel práctico para profesionales del sector del vino.

La madurez de la uva, así como el procedimiento empleado para procesarla y vinificarla son algunos de los factores más importantes que determinan la composición química y la calidad del vino tinto. Estos factores influyen directamente sobre la composición fenólica del vino, especialmente sobre la concentración de antocianos y proantocianidinas. En el capítulo 1 de la presente Tesis se aborda el estudio de como la madurez de uvas de la variedad Garnacha Tinta afecta a la composición, el color y la calidad del vino. Asimismo, en este capítulo se estudia la influencia del tratamiento del racimo y el sistema de vinificación. Esta experiencia se realizó mediante microvinificaciones (15 kg) de las uvas a tres niveles de madurez y aplicando cinco tratamientos distintos: racimos despalillados y estrujados (Control), racimos despalillados y estrujados con sombrero sumergido (Submerged Cap), racimos despalillados sin estrujar (Whole Berry), racimos enteros estrujados sin despalillar (Crushed Cluster) y racimos enteros sin estrujar ni despalillar (Whole Cluster). Además, para el nivel de mayor madurez se realizaron vinificaciones a una escala superior (400 kg) únicamente con tres de los tratamientos: racimos despalillados y estrujados (Control), racimos despalillados sin estrujar (Whole Berry) y racimos enteros sin estrujar ni despalillar (Whole Cluster). Es necesario señalar que en la bibliografía existen diversos estudios científicos que tratan sobre algunos de estos factores por separado, pero, a nuestro conocimiento, éste es el primer trabajo que los aborda de forma tan completa y conjunta.

En términos generales los resultados demuestran claramente que la madurez de la uva y los tratamientos del racimo ejercen un efecto muy claro sobre el color y la extracción de compuestos fenólicos en general. Específicamente, el índice de polifenoles totales (IPT), las concentraciones de antocianos y proantocianidinas, así como el grado medio de polimerización eran superiores cuando mayor era el nivel de madurez de la uva.

En cuanto a los diferentes tratamientos del racimo, se ha podido comprobar que la aplicación del sombrero sumergido (Submerged Cap) comporta una mayor extracción de compuestos fenólicos que la vinificación convencional (Control). Por otra parte, la presencia de los raspones en general originó vinos con una menor graduación alcohólica y un pH superior a los vinos obtenidos con uvas despalilladas. Estas diferencias son muy probablemente debidas a que los raspones contienen un alto contenido en agua y potasio que son liberados al vino. La presencia de los raspones también generó vinos con una menor intensidad del color y una concentración superior de antocianos, muy probablemente debido a que los raspones aumentan significativamente el pH del vino, lo que disminuye la proporción de catión flavilio.

En contra de lo que era de esperar, los vinos elaborados mediante microvinificaciones en presencia de los raspones presentaban en general una concentración en proantocianidinas similar a los de controles. Sin embargo, la presencia de los raspones en la experiencia a nivel de bodega (400 kg) dio lugar a un vino con concentración de proantocianidinas claramente mayor que en el vino control. Además, el porcentaje de prodelfinidinas era también superior en el vino obtenido en presencia del raspón. Finalmente, el vino obtenido en presencia de los raspones fue el peor valorado por el panel de cata debido a un exceso de astringencia y sabor amargo. Se puede concluir, por tanto, que la presencia de los raspones debe considerarse como negativa ya que aumenta el pH, disminuye la intensidad del color y disminuye su calidad sensorial.

La práctica habitual de la vinificación en tinto suele eliminar los raspones de los racimos para evitar el aporte de excesiva astringencia y sabor amargo. Sin embargo, algunas zonas de tradición vitivinícola han usado y continúan usando el raspón en la vinificación en tinto. Tal es el caso de Châteauneuf-du-Pape (Côtes du Rhône) donde se atribuye a la presencia del raspón una mayor longevidad de los vinos o de algunos elaboradores del Medoc (Bordeaux) que en los años con presencia de *Botrytis cinerea* acostumbran a incluir un porcentaje del escobajo ya que según dicen protege el color del vino de la acción de la lacasa. Por otra parte, en los últimos años algunos elaboradores de vinos tintos de nuestro país han recuperado la tradición de elaborar sus vinos con los racimos enteros sin despalillar. Estos elaboradores argumentan que la presencia de los raspones

favorece la extracción y origina vinos con más cuerpo. La razón se atribuye a que los raspones facilitan la extracción debido a que hacen que el sombrero sea más esponjoso y también a que liberan taninos. De hecho, el empleo de los raspones se está convirtiendo en una práctica habitual en la vinificación de vinos orgánicos/biodinámicos/naturales. No obstante, existe muy poca información científica sobre la verdadera influencia del raspón sobre la composición y calidad del vino. Los resultados obtenidos en el capítulo 2 confirmaban que los raspones son una importante fuente de proantocianidinas y que su presencia influye de forma significativa sobre el grado alcohólico, el pH, la composición en compuestos fenólicos, el color y la calidad sensorial del vino. Por esta razón decidimos diseñar un nuevo experimento que permitiese obtener más información sobre su verdadera influencia. Asimismo, se decidió profundizar en la contribución de las semillas y compararla de este modo con la de los raspones.

Para tal fin, se planteó un experimento con uvas de la variedad Cabernet Sauvignon vendimiadas a su nivel adecuado de madurez. Los racimos fueron caracterizados para determinar el porcentaje en peso de semillas y raspón para así poder reproducir después las condiciones que habría en su vinificación a escala de bodega. Los raspones eran separados tras el despalillado y las semillas eran recuperadas, de forma más laboriosa, mediante délestages. Se realizaron dos grupos de microvinificaciones. El primero (medio A) se realizó con el mosto extraído inmediatamente después de finalizar el llenado del depósito de vinificación a escala de bodega. Este mosto fue decolorado con carbón y bentonita y se repartió en diferentes tanques que eran vinificados tal cual (Control), añadiéndoles el 100 % de las semillas, añadiéndoles el 300 % de las semillas, o añadiéndoles el 100 % del raspón. De forma paralela, este diseño experimental se repitió utilizando el mosto del depósito tras 4 días de maceración (medio B). Así se disponía de dos matrices, una un mosto en cuya única fuente de compuestos fenólicos era la de la parte del racimo que se le había añadido (medio A), y la otra un mosto en el que ya había un aporte significativo de antocianos y proantocianidinas (medio B).

Los resultados obtenidos en este segundo capítulo confirman los obtenidos en el primero ya que demuestran que la presencia del raspón disminuye el grado alcohólico

y aumenta el pH, lo que incide negativamente en el color del vino. También se confirma que los raspones liberan una importante cantidad de proantocianidinas las cuales presentan un grado medio de polimerización (mDP) superior al de las semillas, pero inferior al de las pieles. Asimismo, se confirma que los raspones aportan también un porcentaje importante de prodelfinidinas.

Por su parte, al añadir semillas se pudo comprobar que el grado alcohólico también disminuía. Este resultado resultó algo sorprendente, pero es muy lógico ya las semillas contienen alrededor de un 35 % de humedad y durante la fermentación alcohólica se restablece el equilibrio osmótico entre el interior de las semillas y el vino. Se suele considerar que el menor rendimiento de transformación de los azúcares en etanol de la vinificación del vino tinto respecto de la del blanco es debido a que las temperaturas más altas y la aireación favorecen su evaporación. A la vista de nuestros resultados y considerando que las pieles también contienen una proporción importante de agua se puede afirmar que parte del menor rendimiento que se observa en la vinificación en tinto sería debido al agua que aportarían las partes solidas de la uva.

Por otra parte, las semillas aportan importantes cantidades de proantocianidinas al vino, lo que genera un aumento significativo del color. Este incremento de la intensidad colorante no parece estar relacionado con el fenómeno de la copigmentación ya que el índice de copigmentación disminuye al añadir semillas. Sí que se observa un incremento del índice de PVPP y una disminución de los antocianos determinados por HPLC, lo que apuntaría a que la liberación por parte de las semillas de proantocianidinas ha favorecido la formación de combinaciones antociano-flavanol, las cuales tendrían una mayor contribución al color del vino.

Finalmente, tanto semillas como raspones liberan al vino importantes cantidades de flavan-3-oles monómeros. Así las semillas liberan mayoritariamente flavan-3-oles monómeros no galoilados ((+)-catequina y (-)-epicatequina), mientras que los raspones liberan una proporción muy alta de galato de (+)-catequina. Este mayor aporte de flavan-3-ol monómeros galoilados podría ser la causa del sabor amargo que los raspones proporcionan al vino.

Durante los últimos años, el empleo de tanino enológico en las bodegas se ha convertido en una práctica muy habitual. A los taninos se les atribuyen diversas funcionalidades entre la que destaca su posible efecto protector de la oxidación. No obstante, no existen evidencias científicas de que así sea y por esta razón se decidió abordar el estudio de la velocidad de consumó de oxígeno de diferentes tipos de tanino enológico (tanino de semillas, tanino de piel, tanino de quebracho, galotanino y elagitanino) para verificar si realmente estos aditivos enológicos pueden proteger al vino de la oxidación.

Los resultados confirmaron que todos los taninos enológicos consumen oxígeno en una solución sintética similar al vino. En este estudio también se incluyeron el ácido ascórbico y el dióxido de azufre a fin de comparar la posible eficacia de los diferentes taninos enológicos con la de los aditivos antioxidantes más utilizados en enología.

El análisis de la cinética de consumo de oxígeno por parte de los diferentes taninos enológicos mostró que la velocidad de consumo de oxígeno depende de la naturaleza del tanino, así como de la concentración de los dos substratos de la reacción (tanino y oxígeno). La modelización matemática de los resultados permitió determinar la velocidad de consumo de oxígeno (OCR-Oxygen Consumption Rate) de los diferentes taninos y compararla con la del ácido ascórbico y el dióxido de azufre. También permitió verificar que se trata de una cinética de segundo orden y determinar la constante cinética para cada tanino. Asimismo, se verificó que la OCR depende de la temperatura en consonancia con la Ley de Arrhenius.

También se comparó la OCR de los diferentes taninos enológicos con la capacidad antioxidante obtenida mediante los métodos más usuales (TPC, DPPH, ABTS y ORAC). Los resultados muestran que las diferentes metodologías para la determinación de la capacidad antioxidante originan resultados diferentes e incluso contradictorios ya que según el procedimiento cambia cual es el tanino de mayor capacidad antioxidante. La razón de estas diferencias es debida a que cada uno de estos métodos se basa en una reacción diferente que no necesariamente tiene que indicar la velocidad de consumo de oxígeno por cada tanino. Por el contrario, el método desarrollado en el presente trabajo mide directamente el consumo de oxígeno y por consiguiente parece mucho más

adecuado para determinar la capacidad real de los diferentes taninos enológicos para proteger al vino de la oxidación.

En este sentido, el tanino con mayor OCR fue el elagitanino, seguido en orden descendente por el tanino de quebracho, el tanino de piel, el tanino de semilla y el galotanino. Por otra parte, al comparar la velocidad real de consumo de oxígeno por parte de las dosis habituales de los taninos con la del ácido ascórbico y el dióxido de azufre se puede afirmar lo siguiente. El ácido ascórbico consume el oxígeno unas 500 veces más rápido que el dióxido de azufre por lo que se demuestra su gran utilidad para eliminar el oxígeno del mosto o del vino. No obstante, debe tenerse en cuenta que la oxidación del ácido ascórbico genera peróxido de hidrógeno y que por tanto necesita ser utilizado de forma conjunta con el dióxido de azufre para evitar la oxidación. Por su parte, el elagitanino parece el tanino más eficaz para proteger al vino de la oxidación ya que presenta una velocidad de consumo de oxígeno similar a la del dióxido de azufre. Estos datos confirman que los elagitaninos son muy buenos antioxidantes y sugieren su posible uso como complemento (o incluso alternativa) para reducir las dosis de dióxido de azufre en la elaboración del vino.

Finalmente, la metodología desarrollada parece muy adecuada para evaluar la OCR de los diferentes taninos enológicos y por tanto proponemos su uso como índice de clasificación de los diferentes taninos comerciales en términos de su efectividad para consumir oxígeno. Esta metodología ha sido presentada en la última reunión de la Subcomisión de Específicos dependiente de la Comisión de Enología de la OIV en París (Abril 2017).

Se puede concluir por tanto que, de acuerdo con los resultados presentados en esta memoria, se ha confirmado la hipótesis inicialmente planteada, ya que se ha podido comprobar la gran influencia de la madurez de la uva, de los sistemas de vinificación y del tratamiento previo del racimo sobre la composición, el color y la calidad del vino tinto.

CONCLUSIONES GENERALES

1. El grado de madurez de la uva, así como el tratamiento previo del racimo y el sistema de vinificación ejercen una influencia muy significativa sobre la composición y calidad de los vinos tintos. De forma más específica se puede afirmar que:

    1.1. Los vinos obtenidos a partir de uvas más maduras presentan, como era de esperar, un mayor grado alcohólico y pH. Asimismo, la mayor madurez de la uva comporta una mayor concentración de compuestos fenólicos en general, y de antocianos en particular. El grado medio de polimerización de las proantocianidinas fue significativamente mayor en los vinos obtenidos de las uvas más maduras.

    1.2. La presencia del raspón ya sea mediante la vinificación del racimo entero sin estrujar o bien estrujado, generó diferencias significativas en la composición y en el color del vino. Por regla general, los vinos elaborados con el raspón presentaban un menor grado alcohólico, un mayor pH, una mayor concentración en compuestos fenólicos y una menor intensidad del color que los vinos elaborados de forma convencional. Asimismo, los vinos elaborados con el raspón a escala de bodega eran peor valorados sensorialmente.

    1.3. La vinificación mediante sombrero sumergido generó vinos con una mayor concentración en compuestos fenólicos y un color más intenso que la vinificación convencional a nivel de las microvinificaciones. A mayor escala, la vinificación de la uva despalillada entera (sin estrujar) fue la que presentaba mejor color y mayor concentración en antocianos. También, fue la mejor valorada sensorialmente.

2. La presencia de semillas o de raspón como única fuente de compuestos fenólicos del vino generó un incremento significativo de los compuestos fenólicos totales, de las proantocianidinas y de la astringencia. El análisis de las proantocianidinas liberadas por el raspón muestra que presentan un grado medio de polimerización superior al de las semillas, pero inferior al de las pieles. Además, las proantocianidinas liberadas por el raspón presentan un porcentaje de galoilación algo inferior al de las semillas, y un porcentaje bastante alto de unidades (-)-epigalocatequina. Por lo tanto, los

taninos condensados liberados por el raspón son una mezcla de procianidinas y prodelfininidinas.

3. Las semillas liberan mayoritariamente (+)-catequina y (-)-epicatequina como principales flavan-3-oles monómeros mientras el que raspón libera además galatos de (-)-epicatequina y especialmente de (+)-catequina. Esta mayor presencia de galatos de flavan-3-oles monómeros podría ser la causa del sabor amargo que se atribuye a los vinos elaborados sin despalillar.

4. Tanto la presencia de las semillas como del raspón disminuye la concentración de antocianos probablemente debido a dos fenómenos. Por una parte, la presencia de flavanoles favorece la síntesis de combinaciones antociano-flavanol tal y como muestra el aumento del índice de PVPP, y por otra parte de que los antocianos podrían ser absorbidos por semillas o raspones.

5. La presencia de las semillas incrementa el color de los vinos. Este aumento no parece estar relacionado con los fenómenos de copigmentación ya que el índice de copigmentación disminuye con la presencia de semillas. Por consiguiente, el color más intenso debe estar relacionado con la aparición de nuevos pigmentos con mayor contribución al color. En cambio, la presencia del raspón no disminuye la intensidad del color, muy probablemente por los fenómenos de absorción de antocianos antes comentados, pero sobre todo por el importante aumento del pH que su presencia genera.

6. Todos los taninos enológicos presentan la capacidad de consumir oxígeno en una solución sintética similar al vino.

7. El análisis de la cinética de consumo de oxígeno por los diferentes taninos enológicos mostro una cinética de segundo orden acorde con la ecuación: OCR = K • [Tanino] • [Oxígeno]. Asimismo, la cinética de consumo de oxígeno mostró una dependencia de la temperatura acorde con la ley de Arrhenius.

8. La velocidad de consumo de oxígeno (Oxygen Consumption Rate – OCR) es mayor en los elagitaninos, seguida en orden decreciente por los taninos de quebracho, los taninos de piel, los taninos de semilla y los galotaninos. La comparación de la OCR con la de los dos antioxidantes más utilizados en vinificación mostró que los elagitaninos presentan una OCR similar a la del dióxido de azufre a las dosis habituales de ambos aditivos. Estos resultados sugieren que los elagitaninos podrían ser útiles para proteger al vino de la oxidación y disminuir las dosis de dióxido de azufre a utilizar. El ácido ascórbico presentó una OCR mucho mayor.