UNIVERSIDAD DE LA REPÚBLICA
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ESTUDIO DE LA COMPOSICIÓN POLIFENÓLICA DE UVAS Y VINOS TINTOS DE DISTINTAS VARIEDADES *VITIS VINIFERA* CULTIVADAS EN URUGUAY

por

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RESUMEN

La sustentabilidad de la vitivinicultura del Uruguay depende de la calidad y tipicidad de sus vinos. A éstas contribuye Tannat, cultivar de buena adaptación local, características enológicas sobresalientes y de escasa distribución internacional, así como la expresión particular de otros cultivares en nuestro medio. La adecuada gestión de este potencial requiere de conocimiento que asista decisiones de manejo en el campo y la bodega. Características distintivas del vino tinto como color, astringencia y contenidos en compuestos bioactivos se deben a la presencia de polifenoles, compuestos derivados del metabolismo secundario de la vid. El objetivo de la investigación fue estudiar el efecto del cultivar y de la madurez de la uva, sobre los taninos de la baya, su distribución relativa en hollejos y semillas, y su extracción en el vino. Igualmente, profundizar en el estudio de los estilbenos, flavonoles y pigmentos de uvas producidas en el sur de Uruguay, y de los vinos tintos respectivos elaborados a escala experimental. Se trabajó con los cultivares Tannat, Syrah y Marselan durante dos vendimias, considerando uvas de diferentes grados de madurez comercial. Se caracterizó por HPLC-MS^n la composición fenólica de las bayas, así como la de los vinos y orujos. La proporción de taninos de hollejos y semillas en el vino dependió prinicipalmente del cultivar. Los vinos Marselan presentaron proporciones muy elevas de taninos de semillas mientras los Syrah y Tannat, de hollejos. La extracción de pigmentos fue selectiva, particularmente baja para los derivados *p*-cumarilados. Los contenidos de estilbenos permanecieron muy estables en el vino. El potencial de síntesis polifenólica de la vid en en sur de Uruguay fue evidente en los tres cultivares, habiéndose identificado en ellos por primera vez flavonoles acilados con ácido acético y *p*-cumárico. En Tannat un pigmento acilado con ácido ferúlico resultó característico de su perfil. Los resultados obtenidos representan una contribución a la caracterización de uvas y vinos cultivados en el país, y se espera puedan asistir decisiones de campo y bodega, así como fomentar futuras investigaciones.

Palabras clave: uvas y vinos tintos, taninos, flavonoles, estilbenos, pigmentos

STUDY OF THE POLYPHENOLIC COMPOSITION OF GRAPES AND RED WINES OF DIFFERENT *VITIS VINIFERA* VARIETIES CULTIVATED IN URUGUAY

SUMMARY

The sustainability of vitiviniculture in Uruguay depends on the quality and typicity of its wines. The wines of Tannat stand out, variety of good local adaptation with outstanding oenological characteristics and of scarce international distribution, as well as the particular expression of other cultivars in our environment. Proper management of this potential requires knowledge that assists management decisions in the field and the winery. Distinctive characteristics of red wine as color, astringency and contents in bioactive compounds are due to the presence of polyphenols, compounds derived from the secondary metabolism of the vine. The objective of the research was to study the effect of the cultivar and the maturity of the grape, on the tannins of the berry, its relative distribution in skins and seeds, and its extraction in the wine. Also, to deepen in the study of the stilbenes, flavonols and pigments of grapes produced in the south of Uruguay, and in the respective red wines elaborated on an experimental scale. We worked with Tannat, Syrah and Marselan cultivars during two harvests, considering grapes of different degrees of commercial maturity. The phenolic composition of the berries, as well as that of the wines and pomaces, was characterized by HPLC-MSn. The proportion of tannins in skins and seeds in the wine depended mainly on the cultivar. The Marselan wines presented very high proportions of seed tannins while the Syrah and Tannat, of skins. Pigment extraction was selective, particularly low for *p*-cumarylated derivatives. The contents of stilbenes remained very stable in wine. The potential of polyphenolic synthesis of the vine in southern Uruguay was evident in the three cultivars, having identified in them for the first time flavonols acylated with acetic acid and *p*-coumaric. In Tannat a pigment acylated with ferulic acid was characteristic of its profile. A contribution to the characterization of grapes and wines grown in the country ware made, and are expected to assist field and winery decisions as well as to encourage future research.

Keywords: grapes and red wines, tannins, flavonols, stilbenes, pigments

1. INTRODUCCIÓN GENERAL

1.1. MARCO TEÓRICO

La calidad del vino tinto depende de su composición. Ésta es resultado de la diversidad de sustancias presentes en las uvas que son extraídas al mosto/vino durante la maceración, de los productos de la fermentación alcohólica y otras vías metabólicas que desarrollan los microorganismos. Igualmente, de elementos que éstos ceden al vino y de las complejas transformaciones que tienen lugar durante la vinificación y conservación del vino. Para un determinado cultivar, condiciones edafoclimáticas y de manejo, la composición de la uva dependerá principalmente de su grado de madurez al momento de la cosecha (Ferrer et al., 2014, Fourment et al., 2014, González-Neves et al., 2004a). El proceso de maduración de la uva comienza a partir del envero, cuyo signo visible más característico es el cambio del color de los hollejos (piel de la uva) desde el verde distintivo del período previo (herbáceo), al característico de la variedad (Coombe y Hale, 1973). Sin embargo, los cambios que transcurren durante la maduración involucran a todas las partes de grano: pulpa, hollejos y semillas, los tejidos que contienen y los compuestos presentes en éstos (Gil et al., 2012, Pinelo et al., 2006, Kennedy y Waterhouse, 2000). A partir del envero se registra una intensa acumulación de azúcares en las bayas conforme el grano aumenta de tamaño, determinando entre otros factores, el grado alcohólico potencial del vino. Al mismo tiempo la acidez del grano, representada mayormente por los ácidos málico y tartárico, descienden significativamente de concentración por dilución, salificación y en el caso del ácido málico, además por respiración celular. La acidez total a la cosecha y su composición (tartárico, málico) tendrán una fuerte influencia en la estabilidad química y microbiológica del vino, así como en su color y gusto.

Pero al mismo tiempo que se modifican los contenidos en componentes mayoritarios de la uva con la maduración, también lo hacen los de otros compuestos como los polifenoles, que, si bien se encuentran en concentraciones menores, son determinantes de la calidad de los vinos tintos. Los polifenoles se caracterizan por

tener una estructura basada en uno o más núcleos aromáticos con más de un grupo hidroxilo (Fulcrand et al., 2006). Son metabolitos secundarios del metabolismo de la vid, a cuya reconocida importancia para los vinos, se agrega su valoración como compuestos bioactivos y moléculas de potencial uso terapéutico (Fernández-Mar et al., 2012, Rauf et al., 2018).

Los compuestos fenólicos más abundantes en las uvas tintas son los antocianos y particularmente los flavanoles (flavan-3-oles monómeros y taninos). Sin embargo, los ácidos hidroxicinámicos y familias minoritarias de polifenones como los flavonoles pueden contribuir significativamente a la calidad del vino. Por su parte los estilbenos, si bien presentes en muy bajas concentraciones, forman parte de los compuestos bioactivos del vino que mayor interés han recibido.

Los diferentes compuestos fenólicos se derivan de dos vías del metabolismo primario: la vía del acetato y la del shiquimato. En el primer caso, la acetil-CoA se transforma en malonil-CoA bajo la acción de la acetil-CoA carboxilasa. En el segundo caso, la vía del shiquimato es responsable de proporcionar los esqueletos de carbono necesarios para la producción de aminoácidos aromáticos, incluida la L-fenilalanina, que es el punto de partida de la vía de síntesis de los fenilpropanoides (Maeda et al., 2012, Tzin y Galili, 2010). Así, La biosíntesis de flavonoides comienza con la conversión de fenilalanina en *p*-cumaril CoA por la fenilalanina amonio liasa. Luego, bajo la acción de la cinamato 4-hidroxilasa, la *p*-cumaril CoA se transforma en ácido *p*-cumárico que podrá convertirse en cumaril-CoA por la 4-cumaril-CoA ligasa. A continuación, convergerán las dos vías del metabolismo primario. La reacción de la malonil-CoA y la cumaril-CoA puede ser catalizada por la estilbeno sintasa, dando lugar a la formación de estilbenos, o puede ser catalizada por la chalcona sintasa que conducirá a la formación de flavonoides (Maeda et al., 2012, Tzin y Galili 2010). En adelante, mediante la acción de diferentes enzimas, serán sintetizados los diferentes tipos de compuestos fenólicos, incluyendo flavonoles, antocianos, flavan-3-oles, así como compuestos derivados de sus estructuras primarias. Así mismo en cada uno de estos subgrupos quedarán determinados perfiles de composición que dependerán de la actividad relativa de las enzimas involucradas en las etapas finales de las síntesis de estos compuestos, que en

conjunto explican la diversidad de polifenoles presentes en las uvas (Fig. 1). Entre estas se destaca la acción de reductasas, hidroxilasas, metiltransferasas, glicosiltransferasas y aciltransferasas. La expresión de estas enzimas está regulada por diferentes factores que siguen siendo motivo de estudio. Por ejemplo, la temperatura durante la maduración afectaría particularmente la síntesis de antocianos, mientras la de flavonoles dependería principalmente de la luz (Spayd et al., 2002). Por su parte los estilbenos se encuentran a bajos niveles en forma constitutiva siendo su síntesis promovida por diferentes factores de estrés como radiación UV, incidencia de fitopatógenos, lesiones, etc (Gatto et al., 2008). Así mismo, la expresión de los diferentes genes involucrados en las rutas metabólicas mencionadas puede ser muy variable entre cultivares. Esto determina que variedades con elevado potencial de síntesis para un tipo de compuestos fenólicos, no necesariamente expresen el mismo potencial respecto a otra familia de estos compuestos.

Mientras las rutas metabólicas que conducen a la síntesis de los compuestos mencionados anteriormente se encuentran en general bien descritas, los eventos que conducen a la polimerizaión de los flavan-3-oles, así como su compartimentación en la vacuola, pared celular y otros organelos, permanecen en gran parte desconocidos (Rousserie et al., 2019).

Los oligómeros y polímeros de los flavan-3-0les son conocidos como taninos, y son los compuestos fenólicos mayoritarios de las uvas, encontrándose en los hollejos y principalmente en las semillas (Adams 2006, Kennedy et al., 2006, 2008, Downey et al., 2003). Su acumulación en ambas partes del grano parece ser independiente (Glories 1999, Downey et al., 2003) pero comienza en etapas tempranas del desarrollo de la baya, alcanzando los máximos niveles próximo a envero (Downey et al., 2003). En el transcurso de la maduración, los contenidos y proporciones de taninos en dichas estructuras se modifica, así como su extractibilidad y características estructurales, lo que a su vez tiene una fuerte determinación varietal (Cadot et al., 2012, Chira et al., 2008. Seddon y Downey 2008, Glories 1999). Estos compuestos destacan por sus propiedades de astringencia (sensación táctil) y amargor (gusto), que son dos características sobresalientes en la

definición de calidad de uvas y vinos (Chira et al., 2011). Asimismo, intervienen en la estructura al vino tinto y condicionan su capacidad de crianza (Harbertson et al., 2012, Cheynier et al., 2006, Zamora 2003). Igualmente significativa es su participación en la estabilización de los pigmentos del vino, al formar complejos moleculares con los antocianos, importantes para la permanencia del color del vino a largo plazo (Weber et al., 2013, Chira et al., 2011, Fulcrand et al., 2006). Dicha asociación también contribuiría a disminuir la astringencia de los taninos (Weber et al., 2013, Cheynier et al., 2006). El término tanino define a los compuestos fenólicos que tienen en común la capacidad de formar complejos con las proteínas y precipitarlas (Cheynier et al., 2006, McRae y Kennedy, 2011). Los flavan-3-oles se presentan en la uva como monómeros, oligómeros y polímeros. Los monómeros no precipitan las proteínas, por lo que no son considerados estrictamente taninos. El tamaño molecular de los taninos determina muchas de sus propiedades, como así también el amargor y astringencia con que son percibidos. En forma simplificada, los monómeros son percibidos más amargos que astringentes, mientras que lo inverso sería para los polímeros (Weber et al., 2013, Chira et al., 2008, Cheynier et al., 2006). Según variaciones en su estructura química los diferentes monomeros sintetizados por las uvas son : catequina, epicatequina, epicatequina-3-O-galato, galocatequina y epigalocatequina (Cheynier et al., 2006). Su naturaleza polimérica y los diferentes tipos de enlaces que pueden establecer entre sus unidades constitutivas y otros compuestos, como el ácido gálico, explican la enorme diversidad estructural en que se encuentran en las uvas, y particularmente en los vinos, donde se encuentran bajo continuas modificaciones mediante procesos de asociación, polimerización y clivaje (Cheynier et al., 2006, Fulcrand et al., 2006). En las uvas los taninos presentan diferentes características según se sinteticen en los hollejos o las semillas, las que determinan consecuencias enológicas importantes (Adams 2006, Vivas et al., 2004). La síntesis de galocatequina y epigalocatequina (flavan-3-oles tri-sustituidos, Fig. 1) no se produciría en las semillas, por lo que pueden ser utilizados como marcadores de taninos de hollejos. Por su parte los taninos de semilla tienen proporciones muy superiores a los de hollejo de epicatequina-gallato (5% vs 30% respectivamente) o bien ésta no se encuentra presente en los mismos (Boido et al.,

2011, Vivas et al., 2004), por lo que pueden ser utilizados como marcadores de taninos de semilla (Cheynier et al., 2006, Peyrot des Gachon y Kennedy, 2003). El tamaño de los taninos, generalmente expresado por su grado medio de polimerización, también difiere según su localización, siendo superior en los hollejos que en las semillas (Adams 2006, Vivas et al., 2004, Downey et al., 2003). Numerosos estudios han comprobado que las estructuras moleculares de los taninos se modifican significativamente en el transcurso de la maduración, así como sus contenidos, abundancia relativa en hollejos y semillas, asociación con otras macromoléculas y capacidad de ser extraídos durante la vinificación en tinto (Bindon et al., 2014a, 2013, 2010, Kontoudakis et al., 2011, Torchio et al., 2010, Pérez-Magariño y González-San José, 2006, Pinelo et al., 2006, Kennedy et al., 2000). Sin embargo, las bases bioquímicas de dichas transformaciones permanecen sin ser completamente conocidas, (Rousserie et al., 2019).

Se ha reportado que la uva con nivel insuficiente de madurez tiene una baja extractibilidad de taninos de hollejo, y una alta extractibilidad de taninos de semilla (Canals et al., 2005; Peyrot des Gachons y Kennedy, 2003). A su vez, en general se acepta, como lo expresa Kontoudakis et al. (2011), que las uvas inmaduras producen vinos de mayor astringencia y amargor, al ceder más fácilmente los taninos de las semillas durante la vinificación. Por lo tanto, existe una preferencia generalizada por los taninos derivados de los hollejos (Kennedy et al., 2006). En consecuencia, una tendencia en la producción de vinos de calidad es retrasar las cosechas buscando mejorar el potencial fenólico de las uvas y en particular la calidad de los taninos. Sin embargo, procurar modular mediante el nivel de madurez de la uva las propiedades sensoriales de los taninos, afectará inevitablemente al conjunto de compuestos de la uva que se sintetizan y transforman al mismo tiempo, lo que puede afectar negativamente la calidad de los vinos producidos. Investigaciones recientes (Bindon et al., 2014ab, 2013; Gil et al., 2012) muestran que los supuestos por los cuales se busca la sobremaduréz de las uvas, no serán necesariamente válidos o bien pueden ser resultado del conjunto de transformaciones que se producen en las uvas y sus consecuencias en los vinos. Fulcrand et al. (2006), Cadot et al. (2012) entre otros

señalan que poco se conoce acerca de la evolución de los fenoles y su extractibilidad durante la maduración, así como la relación con las propiedades sensoriales del vino.

Por su parte los antocianos se sintetizan en los hollejos de las uvas tintas, son extraídos al mosto durante la maceración (contacto del mosto con las partes sólidas de las uvas) y son las moléculas que le confieren el color al vino tinto joven y mediante la formación de complejos con otros compuestos, al vino de guarda (Barcia et al., 2014, Blanco-Vega et al., 2011, Alcalde-Eon et al., 2006a, Mateus et al., 2003). Los contenidos de antocianos en la uva son muy variables en función de la cepa, las condiciones ambientales durante la etapa de desarrollo y maduración, las técnicas de manejo del viñedo y el grado de madurez de la uva (Pinasseau et al., 2017, González-Neves et al., 2004a). Comienzan a sintetizarse a partir del envero, alcanzando un máximo que puede preceder al momento de madurez tecnológica (criterio de cosecha que considera la evolución de la acidez total y los azúcares en las uvas), o darse posteriormente, aspecto igualmente condicionado por la variedad de uva y las condiciones ambientales durante la maduración (Kontoudakis et al., 2011, González-Neves et al., 2010ab, Glories, 1999, Amrani y Glories, 1995, 1994). Luego sus contenidos descienden en proporciones variables, reportando algunos autores descensos de hasta 50 % en el período de una semana (Mattivi et al., 2006). Durante la vinificación en tinto, los antocianos difunden desde los hollejos al mosto desde el inicio de la maceración, dado que son moléculas hidrosolubles. Su facilidad de extracción es una característica varietal, si bien puede modificarse entre años y en función de las condiciones ambientales y el grado de madurez de la uva (Koyama et al., 2007, González-Neves et al., 2004a).

Los ácidos hidroxicinámicos *p*-cumárico, cafeico, ferúlico y sus ésteres tartáricos, son el grupo mas abundante de compuestos fenólicos no flavonoides en uvas y vinos (Ferrandino et al., 2012). Tienen una relevancia importante en la evolución del vino a través de los procesos oxidativos en los que intervienen (Fulcrand et al. 2006), su actividad como copigmentos (Bimpilas et al., 2016) y en la formación de piranoantocianos (Blanco-Vega et al., 2011, Cheynier et al., 2006).

Por su parte los flavonoles son compuestos polifenólicos localizados en los hollejos de la uva siendo extraídos al mosto durante la maceración. Ocurren en

menor concentración que los antocianos y los taninos (Cejudo-Bastante et al., 2015, Fanzone et al., 2011), sin embargo, hay un creciente interés por su estudio de acuerdo a que sus propiedades comienzan a ser más conocidas (Ferrer-Gallego et al., 2016, Castillo-Muñoz et al., 2009, Gómez-Alonso et al., 2012). Son pigmentos amarillos, pero su importancia en el vino tinto es principalmente debida a su efecto como copigmentos (Gordillo et al., 2012, Boulton 2001). Diferentes autores han reconocido la copigmentación como uno de los mecanismos más eficientes para la estabilización del cromóforo de los antocianos (Bimpilas et al., 2016, Gordillo et al., 2012, Cavalcanti et al., 2011). En los vinos tintos esto significa un incremento cuantitativo y cualitativo del color (Boulton, 2001). Sin embargo, la copigmentación es un fenómeno de escasa duración en el tiempo (Boulton, 2001) pero que promovería la asociación de antocianos con otras moleculas dando lugar a pigmentos más estables (Cheynier et al., 2006). Castillo-Muñoz et al., (2009) reportan que los flavonoles-3-O-glicósidos que pueden ser encontrados en uvas y vinos constituyen las 3 series completas de glucosidos, galactósidos y glucurónidos de las seis posibles agliconas denominadas kaempferol, quercetina, isoramnetina, miricetina, laricitrina y siringetina, así como las respectivas agliconas libres en los vinos (Fig.1). En uvas y vinos, los derivados metoxilados de los flavonoles, laricitrina, isoramnetina y siringetina, aparecen en mucha menor concentración que los derivados de quercetina, miricetina o kaempferol (Castillo-Muñoz et al., 2009, 2007, Mattivi et al., 2006).

Los estilbenos (incluyendo a su representante más conocido, el resveratrol) son reconocidas fitoalexinas, cuyo estudio ha sido motivado principalmente debido a la amplia gama de propiedades farmacológicas que se les atribuyen (Pannu y Bhatnagar 2019, Ingham 1976). Ocurre en diferentes familias de plantas, incluyendo Vitaceae (Jeandet et al., 2002), donde se sintetiza constitutivamente (Gatto et al., 2008) pero principalmente en respuesta a agentes bióticos y abióticos (Sáez et al., 2018, Vannozzi et al., 2012). La forma *trans*-resveratrol (3,5,4'-trihidroxi-trans-estilbeno) es el precursor metabólico y el núcleo estructural de diferentes estilbenoides (Sáez et al., 2018). De acuerdo con la bibliografía, los estilbenos se sintetizan constitutivamente solo en niveles muy bajos, pero se acumulan fuertemente en respuesta a una amplia gama de estrés bióticos y abióticos (Vannozzi et al., 2012).

Por lo tanto, aún en uvas sanas, se reportan en una amplia gama de concentraciones, dependiendo de la variedad de uva, la región de crecimiento, la exposición a elicitores, etc. (Belmiro et al., 2017, Vincenzi et al., 2013, Ruiz-García et al., 2012 , Gatto et al., 2008).

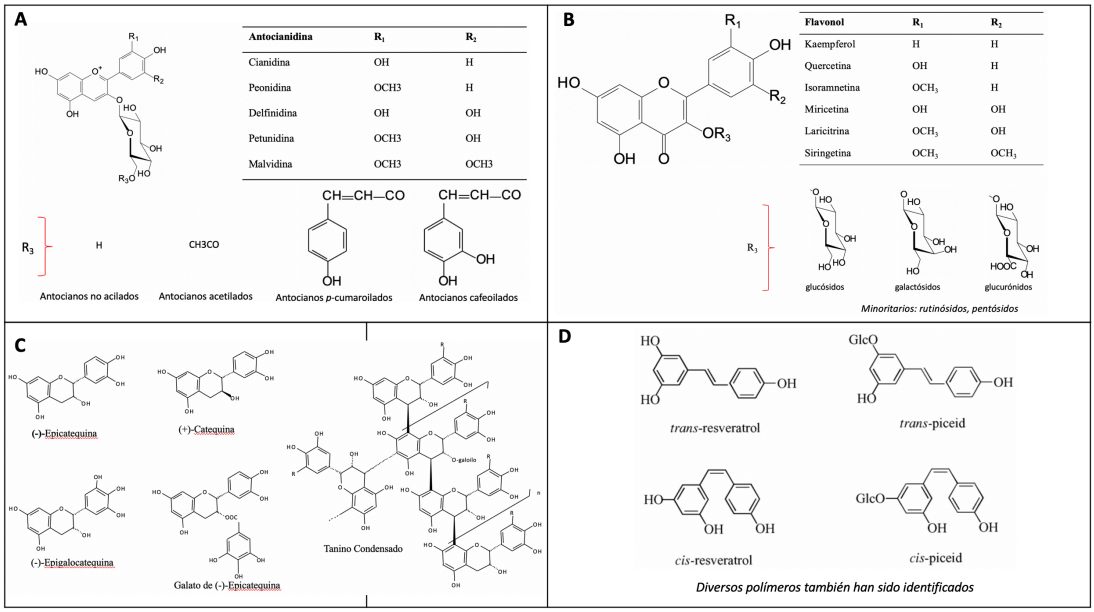

Figura 1. Principales estructuras de los compuestos fenólicos estudiados en este trabajo. A antocianos; B flavonoles; C flavanoles; D estilbenos. Glc glucosa.

En su conjunto, la literatura publicada en la actualidad resalta la importante avanzar en el conocimiento de como se determinan en el campo y la bodega los niveles y relaciones de compuestos de los cuales dependerá finalmente las características del vino.

El desconocer las causas que determinan la calidad de la uva y el vino impide tomar decisiones de manejo que se correspondan con los objetivos productivos.

En esta tesis, se hizo énfasis en el estudio de Tannat, el cultivar más representativo de la vitivinicultura nacional, reconocido por su elevada capacidad de síntesis de antocianos y taninos (González-Neves et al. 2004a, Boido et al. 2011). Al mismo tiempo, se incluyó el estudio de uvas de los cultivares Syrah y Marselan, variedades con las cuales se han producido en ensayos previos de nuestro grupo de investigación vinos con elevados contenidos fenólicos.

1.1.1. Principales características de la vitivinicultura nacional y de las variedades empleadas en las investigaciones

La viticultura nacional está representada en la actualidad por 6300 hectáreas de viñedos que produjeron en el año 2018, 105 millones de kilos de uva, de los cuales el 97% fue destinado a la elaboración de vino. El 95% de la producción total es de variedades *Vitis vinifera* y más del 80 % de uva tinta (INAVI, 2019). En el 2018, un total de 170 bodegas elaboraron 67 millones de litros de vino, de los cuales un 11% fue de vinos blancos, 34% rosados, 7% claretes y 48 % tintos (INAVI, 2019).

Tannat es la variedad más cultivada, representando en el 2018 el 27% de la producción (INAVI, 2019) siendo la cepa más representativa de la vitivinicultura del Uruguay. Esto se explica por la buena adaptación del cultivar a las condiciones productivas del Uruguay, lo que se ha presentado en diversas investigaciones que han permitido igualmente avanzar en el conocimiento de sus características varietales, comportamiento vitícola y enológico (Favre et al., 2014, González-Neves et al., 2014, 2012ab., 2010ab, 2004ab, Ferrer et al., 2011, Alcalde-Eon et al., 2006ab, Boido et al., 2006, Medina et al., 2005, etc). Tannat posee características que a la vez de distintivas le confieren buenas aptitudes enológicas, como su capacidad de generar alto grado alcohólico y bajo pH en madurez tecnológica. Pero el aspecto más sobresaliente de la variedad, es su excepcional potencial en síntesis de compuestos fenólicos como antocianos y taninos, elementos determinantes de la calidad del vino tinto (González-Neves et al., 2012a, 2004a, Ferrer et al., 2011, Alcalde-Eon et al., 2006ab, Boido et al., 2011). No obstante, a pesar del potencial de la variedad para generar vinos con alta intensidad colorante, éstos pueden presentar problemas en la estabilidad del color, lo que ha sido relacionado con su perfil en pigmentos, caracterizado por proporciones relativamente bajas de malvidina y pigmentos acilados, así como contribuciones relativamente altas de delfinidina (González-Neves et al., 2010b). Estas investigaciones igualmente han expuesto la necesidad de realizar mas estudios al respecto. Por otra parte, Tannat es una variedad de reconocido potencial en síntesis de taninos (González-Neves et al., 2012a, 2010a, 2004ab, Boido et al., 2011) que puede dar lugar a vinos de elevada astringencia (Vidal et al., 2017, 2016). Sin embargo, en ensayos previos, se han evaluado vinos

Tannat donde la astringencia no ha sido catalogada como un atributo sobresaliente, y se han analizado vinos de otras variedades con similares contenidos de taninos a Tannat, donde igualmente su astringencia no ha sido sobresaliente (datos no publicados). La necesidad de contar con mas investigaciones que permitan avanzar en el conocimiento de los factores productivos que determinan la astringencia, particularmente de los vinos Tannat, ha sido resaltada en estudios recientes (Vidal et al., 2017).

Por su parte Marselan (*V. vinifera* Garnacha x *V. vinifera* Cabernet sauvignon) significó en el 2018 un 2,3% de la producción nacional, sin embargo, en los últimos años su implantación ha crecido como demuestran los datos estadísticos de plantas de hasta 5 años de edad (INAVI, 2019) donde su porcentaje sobre el total asciende al 7%. Esto se debe al buen comportamiento enológico-productivo que ha mostrado este cultivar desde que comenzó su implantación en el país al comienzo del presente siglo, dando lugar a vinos de elevado contenido polifenólico como ha quedado de manifiesto en publicaciones nacionales (Favre et al., 2019, Neves et al., 2015). Así mismo es un cultivar con elevado porcentaje de hollejos, bajo de pulpa y que requiere de mayor investigación nacional para profundizar en el conocimiento de su compartimiento productivo y enológico.

Syrah, es una variedad que ha mostrado en Uruguay problemas productivos en años donde la pluviometría durante la maduración de la uva es abundate (desarrollo de podredumbres). Sin embargo, fuera del citado contexto, puede dar lugar a vinos con elevados contenidos fenólicos y con características distintivas del color, si bien sus pigmentos pueden presentar baja extractibilidad (González-Neves et al., 2013). En la actualidad, Syrah representa aproximadamente el 0,65% de la producción nacional (INAVI, 2019) si bien en la elaboración de vinos finos este porcentaje es claramente superior, a pesar de que no se cuente con estadísticas al respecto. Igualmente, esta variedad ha mostrado en estudios previos, características particulares, siendo una matriz interesante para el estudio de los polifenoles y su extracción al vino, como ser el relativo elevado tamaño de grano y porcentajes de pulpa de sus bayas, y la ya mencionada baja extractibilidad de sus pigmentos (González-Neves et al., 2013).

1.2. MARCO GENERAL DE LOS ESTUDIOS REALIZADOS

La investigación desarrollada durante el doctorado consideró profundizar en el conocimiento de la composición polifenólica de la uva y su relación con la calidad del vino tinto. El Grupo Disciplinario de Enología de Facultad de Agronomía (Udelar) cuenta con una larga trayectoria de investigación sobre diferentes aspectos que afectan la calidad de los vinos tintos, considerando la realidad vitivinícola del país. Entre estos se destacan los avances en el conocimiento de la composición fenólica de uvas y vinos de las principales variedades de *Vitis vinífera* destinadas a la elaboración de vinos finos en Uruguay y su relación con el manejo del viñedo y la bodega. En este contexto las investigaciones realizadas hicieron énfasis en el estudio de aquellos compuestos polifenólicos menos conocidos hasta el momento, ya sea por la dificultad que presenta su aislación y determinación analítica, como los taninos, o porque su importancia para la calidad de los vinos tintos ha cobrado mayor relevancia a partir de estudios relativamente recientes, como flavonoles y ácidos hidroxicinámicos. También se incluyó el estudio de los estilbenos, de los cuales se cuenta con escasos reportes en uvas y vinos nacionales. Igualmente se incluyó el estudio de los antocianos y pigmentos derivados de los mismos, compuestos fenólicos que han sido ampliamente descritos, pero que debido a su diversidad y complejidad de reacciones en las que intervienen, siguen siendo objetos de estudio.

De acuerdo con los objetivos de la investigación, se propuso analizar independientemente los compuestos fenólicos de los hollejos, las semillas, los vinos tintos respectivos y los residuos de hollejos y semillas apartados de la prensa luego de la vinificación (orujos). En el caso de los taninos, esto permitió realizar una valoración del reparto de los mismos en hollejos y semillas, y tras considerar los contenidos y características de éstos en el vino, estimar la magnitud derivada de cada tipología. Por su parte, el estudio de los compuestos polifenólicos retenidos en los orujos, tuvo por objetivo principal contribuir a comprender el proceso de extracción parcial y selectivo de la uva al vino que tiene lugar durante la maceración.

Las investigaciones se realizaron en los cultivares de uva *Vitis vinifera* Tannat, Syrah y Marselan, que, de acuerdo al conocimiento adquirido en ensayos previos, presentan características particulares y diferentes potencialmente favorables para alcanzar los objetivos del estudio.

En el transcurso de las investigaciones realizadas, se evaluó los efectos varietales y del grado de madurez de la uva al momento de cosecha en la síntesis, contenidos y características (perfiles de composición, extractibilidad) de taninos, pigmentos, flavonoles y estilbenos.

1.3. OBJETIVOS DEL ESTUDIO

1.3.1. Objetivos generales

Profundizar en la caracterización polifenólica de uvas y vinos producidos en Uruguay, de los cultivares Tannat, Marselan y Syrah.

Estudiar el efecto del cultivar de vid y el grado de madurez de la baya, sobre la composición polifenólica de las uvas, los vinos y los factores que afectan dicha correspondencia.

1.3.2. Objetivos específicos

1. Estudiar el efecto del cultivar y el grado de madurez de la baya en cosecha, sobre los contenidos y características de los taninos en hollejos y semillas.

2. Estudiar si el grado de madurez de la uva y el cultivar, afectan la proporción en el vino de taninos derivados de hollejos y semillas.

3. Contribuir a comprender el proceso de extracción de los pigmentos durante la vinificación en tinto considerando los contenido y perfiles de éstos en los hollejos así como en los vinos y orujos respectivos.

4. Profundizar en el conocimiento de los estilbenos y flavonoles de uvas y vinos producidos en Uruguay.

1.4. ESQUEMA GENERAL DE LA TESIS

Las investigaciones presentadas se desarrollaron a partir de las uvas y vinos elaborados en dos vendimias, 2015-2016. En estas se estudió la composición fenólica pormenorizada de las uvas (hollejos y semillas), los vinos tintos elaborados por maceración tradicional, y los residuos de vinificación (remanentes de hollejos y semillas luego de finalizadas las vinificaciones). Los viñedos involucrados se localizaron en el Sur de Uruguay, mayor zona productiva vitivinícola del país (INAVI, 2019) y se trabajó con uvas de los cultivares Tannat, Marselan y Syrah. Las vinificaciones fueron realizadas en todos los casos en la bodega experimental de Facultad de Agronomía (Udelar) al igual que los análisis espectrofotométricos. Los

análisis de composición general del vino se realizaron en el Instituto Nacional de Vitivinicultura (Las Piedras-Uruguay). Las técnicas determinaciones de compuestos fenólicos por HPLC-(ESI)-MS^n fueron realizadas durante 3 pasantías (2015-2017, de 45 días c/u) en el Instituto Regional de Investigación Científica Aplicada (IRICA), Universidad de Castilla-La Mancha (España), y en el Centro de Investigación de la Vid y el Vino de Castilla-La Mancha (España).

En la vendimia 2015 se partió de uvas Tannat de dos viñedos, en uno de los cuales se realizó la cosecha en madurez tecnológica (criterio del productor) y en una fecha posterior, representando así dos grados de madurez diferente. A su vez, se consideraron uvas Syrah y Marselan cosechadas en madurez tecnológica (según criterio del productor).

En la vendimia 2016 se trabajó igualmente con uvas de los cultivares Tannat, Syrah y Marselan, pero representados en diferentes proporciones respecto a la vendimia anterior. Mientras en Tannat se realizó una única cosecha (en uno de los viñedos considerados el año previo), en Syrah y Marselan se realizaron dos, la primera en madurez tecnológica y la segunda en una fecha posterior, obteniendo uva de grados de madurez diferente.

En el año 2017 se analizaron los vinos correspondientes a las vendimias 2015-2016, estudiando así la evolución de la composición fenólica de los mismos.

1.5. PRESENTACIÓN DE LOS CAPÍTULOS

La tesis se presenta en seis capítulos además del presente donde se exponen los antecedentes, el marco teórico incluyendo una revisión general de la literatura vinculada con la investigación, así como los objetivos que se plantearon y el diseño experimental que se propuso para conseguirlos.

El **capítulo 2** es destinado a la presentación de la investigación realizada sobre los contenidos, reparto y características de los taninos de las uvas, su correspondencia con los presentes en los vinos respectivos y el análisis de factores determinantes de dicha correspondencia. Se consideraron los trabajos realizados en

ambas vendimias, 2015 y 2016, por lo que se incluyen las tres variedades estudiadas, Tannat, Syrah y Marselan, evaluando dos niveles de madurez en cada una.

El **capítulo 3** presenta los estudios realizados en la vendimia 2015 sobre los pigmentos de las uvas, así como de los vinos y orujos respectivos. Si bien se destacan resultados relacionados al cultivar y al grado de madurez de la uva en cosecha, el énfasis estuvo en estudiar la selectividad de la extracción de los pigmentos durante la vinificación en tinto. Por lo tanto, se realizó una comparación de los contenidos y perfiles de composición del conjunto de las muestras obtenidas en la vendimia, es decir, 10 muestras de hollejos, y las correspondientes 10 muestras de vinos y orujos.

En el **capítulo 4** se incluyen los resultados obtenidos al cabo de las dos vendimias 2015 y 2016 en los contenidos y perfiles de estilbenos. Así mismo se hace un análisis de la evolución en el vino de los mismos hasta 24 meses después de las primeras determinaciones analíticas.

El **capítulo 5** presenta la investigación que surgió del estudio de los flavonoles en uvas y vinos de la vendimia 2015, donde se identificaron nuevos derivados acetilados y *p*-cumarilados de los flavonoles metoxilados laricitrina, isoramnetina y siringetina. En este capítulo también se presenta en forma detallada la metodología analítica empleada y los resultados que permitieron verificar la identidad de los nuevos compuestos reportados. La metodología empleada fue desarrollada en parte durante la investigación, pudiendo contribuir al reconocimiento de futuros metabolitos, por lo que se incluye la descripción pormenorizada de los espectros de masa que surgen de la fragmentación de la aglicona, según ésta haya sido generada por fragmentación homolítica o heterolítica del flavonol-glucósido.

Por último, en el **capítulo 6** se hace una síntesis de los principales resultados obtenidos, presentando las conclusiones y perspectivas de los estudios realizados.

2. GRAPE CULTIVAR STRONGLY AFFECTED THE RELATIVE CONTRIBUTION OF SKIN AND SEED TO WINE FLAVANOLS[1]

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2.1. ABSTRACT

The study aimed to evaluate in commercially ripe grapes of different maturity the contribution of seed and skin to the red-wine flavan-3-ols. Wines were elaborated under experimental condition by traditional maceration from *Vitis vinifera* Tannat, Marselan and Syrah grapes. Just before crashing samples of skin and seed were collected. Flavanols were isolated by SPE and analyzed through a HPLC coupled to a QqQ-MS/MS system. Seed accounted for 60 to 79% of total grape flavanol, but this had no relation with wine composition as neither did skin or total flavan-3-ol content. In Tannat and Syrah, most wine flavanols were from skin, but in Marselan from seed (up to 66%), despite this later had the most mature grapes and most skin flavanols. A Syrah wine presented the highest content of flavanols from grapes that had the lowest. Wine flavanols depended on their extractability from skin and seed, and that, mainly on grape variety.

KEYWORDS: tannins, flavan-3-ol, grape, red-wine, pyrogallol, Syrah, Tannat, Marselan

2.2. INTRODUCTION

Flavanols (flavan-3-ol monomers and their polymeric forms named tannins) are polyphenol compounds determinants of the red-wine quality because of their role in astringency, bitterness, structure and color stability [1,2] They are bioactive compounds and are the more abundant polyphenols of most red-wines [3–6].

They are present in the skin and seed of grape berries, and also in pulp, although these latter would remain not extractable during vinification [7,8] Thus, flavanols have to be extracted from grape to wine during the maceration step of winemaking.

Flavanol sensorial perception depend on their structural characteristics, and these are different in skin and seed [9]. Seeds tannins are less polymerized than those from skin and present much higher percentages of galloylation (%G). Meanwhile, just skin would present the triple-hydroxylated B ring flavanols, named prodelphinidins. Thus, in addition to the total wine flavan-3-ol content, their origin determines enological implications. Among others, the extent of galloylation has been cited to increase both, bitterness and astringency [10]. These characteristics are also affected by the flavanol mean degree of polymerization (mDP), the longer the tannin, the more astringent and less bitter it is [11]. Thus, wine flavanol composition might condition the price of wine. It has been suggested that maximization of skin tannin concentration or proportion is related to an increase in projected wine bottle price [12 13]. It is generally accepted by winemakers that the mature the grapes, the better the quality of the grape tannins, mainly because it would increase the relation skin to seed tannins. This is supported for some investigation [14–16]. Nevertheless, some authors have found the opposite result [17,18]. Thus, there is still a gap in the knowledge of how grape flavanols are affected around technological maturity (when the harvest decision has to be taken) and how it may change wine flavanols composition. Hence, this investigation aimed to contributes filling this gap throw a thoroughly study of the flavanols in grapes and the respective wines of Tannat, a grape variety cited as producing tannic wines, and in other two, a priori expected to present different characteristics, Marselan and Syrah.

2.3. MATERIAL AND METHODS

2.3.1. Experimental design

The experiments were performed from grapes of commercial vineyards located in the south of Uruguay during the years 2015 and 2016. Each vintage comprises five harvest of grapes belonged to the *Vitis vinifera* varieties Tannat, Syrah and Marselan. In 2015, one harvest of Syrah and Marselan was considered, while Tannat grapes were taken from two different vineyards, one of them, harvested on two different dates. Conversely in 2016 just one Tannat was harvested while two harvest of Syrah and Marselan could be made (Table 1). The harvests were made in the dates selected by the vineyard owner, and when a second harvest was included, it was done as later as possible, considering the vineyard sanitary condition and climatologic predictions. Thus, the grapes used in the investigations were commercially ripe grapes in all cases, in line with the objective of the research.

Each harvest was made by hand, and the clusters were carefully transported in plastic boxes (20 kg each one) to the university experimental winery. There, two batches of grapes of 70 kg each were randomly separated for vinification. Just before crushing, from each batch a sample of 100 grapes were collected, taking cluster parts from 3 to 5 berries from different parts of randomly chosen bunches. To avoid bias by size or aspect, the berries were scattered over a plain surface with a numbered grid of 5 × 5 cm squares. An order of squares was randomly chosen, and then all the grapes placed inside each square were chosen until 35 grapes were collected. Each 35-berry sample was then peeled and the seeds collected. The pulp remaining against the skins was carefully removed with the help of rounded edge blade, gently blotted with a paper towel, weighted (fresh-weight) and stored in a sample-bag at −18°C until freeze-dried. Skins were freeze-dried, weighted (dry weight) and then stored at −18 °C again in bags containing silica gel. The seeds correspondent to the 35 grapes were also conditioned, the pulp remaining against them removed, washed with distilled water, dried using absorbent paper, counted, weighted and stored in

samples-bag containing silica gel at -18 ºC. The seeds weight was also registered just after take them out from the bags, just before processing.

The general composition of the grapes belonging to each harvest were obtained from additional duplicate samples of 250 grapes each, which were analyzed within 24 h (Table 1). Then, each batch of 70 kg of grapes was vinified. Grapes were destemmed and crushed with an Alfa 60 R crusher (Italcom, Italy), and vatting was made in stainless-steel tanks (100 L capacity each). Potassium metabisulfite (50 mg SO2/100 kg of grapes) was added and dry active yeast (Saccharomyces cerevisiae ex bayanus Natuferm 804; OenoBioTech, Paris, France) was inoculated (20 g/kg of grapes). Wines were made with a classical fermentation on skins (maceration simultaneously with alcoholic fermentation) for 8 days in Marselan and Tannat, and 7 days in Syrah, one day less because of its lower anthocyanin potential, according to González-Neves et al.[19]. Along macerations, two pumpings over followed by punching the cap were carried out daily until pressing. At devatting, the fermentation was finished in all cases. The pressing was carried out with a stainless-steel manual press. Free run juices and press juices were mixed, separated from lees, stabilized by adding a dose of 50 mg/L of SO_2 and kept in glass recipients of 10-L capacity.

2.3.2. Analytical procedures and chemicals

Analytical determinations were made at the Laboratory of Instrumental Analysis placed at the Regional Institute of Applied Scientific Research (IRICA), Castilla La Mancha University, and in the Institute of the Vine and Wine of Castilla-La Mancha, Spain. All solvents were of HPLC quality, and all chemicals were analytical grade (>99%). Water was Milli-Q quality. (+)-catechin, (−)-epicatechin, (−)-epicatechin 3-gallate and (−)-gallocatechin 3-gallate were purchased from Sigma Aldrich (Tres Cantos, Madrid, Spain), while (−)-epigallocatechin and (−)-gallocatechin were acquired from Phytolab (Vestenbergsgreuth, Germany). All the standards were used for identification and quantitation by calibration curves covering the expected concentration ranges.

2.3.3. Skin and seed extraction and sample preparation

The samples of grape skin and seed were extracted each with 100 mL of a mixture 50:48.5:1.5 (v/v) of CH_3OH/ H_2O/HCOOH, using a homogenizer (Heidolph

DIAX 900) to 10000 rpm for 3 min and then centrifuging at 2500g at 5 °C for 5 min. The supernatant was separated and conserved, and the pallet was extracted again two more times. The three-supernatant obtained were mixed, the volume registered, and then stored at −18°C until analysis. Previous works made in similar conditions, confirmed that a two times extraction of the grape skin pellet yielded nearly 99% of the grape polyphenol contents able to be extracted with successive repetitions of the cited protocol [20].

2.3.4. Analysis of flavan-3-ol

The flavan-3-ols were isolated following the procedure described by [21], using SPE C18 cartridges (Waters® Sep-Pak Plus, filled with 820 mg of adsorbent). The flavan-3-ol analysis was made according to Bordiga et al.[22], using the pyrogallol-induced acid catalyzed depolymerization method. Briefly, 0.50 mL of the pyrogallol solution (100 g·L− 1 pyrogallol plus 20 g·L− 1 of ascorbic acid in 0.3 N HCl) was added to 0.25 mL of the sample in MeOH and incubating at 30 °C by 40 min. The reaction was stopped by adding 2.25 mL of sodium acetate (67 mM). The samples, before and after the depolymerization reaction, were injected (20 μL) onto an Ascentis C18 reversed phase column (150 mm × 4.6 mm with 2.7 μm of particle size), maintained at stable temperature (16 °C). The elution gradient used, and the mass spectrometer setting for identification and quantitation, were according to Lago-Vanzela et al.[23].

The analysis were performed using a HPLC Agilent 1200 series system (Agilent, Germany) equipped with DAD detector in line with an AB Sciex 3200 TRAP (Applied Biosystems) with triple quadrupole, turbo spray ionization mass spectroscopy system (ESI-MS^2). The chromatographic system was operated with an Agilent Chem Station (version B.01.03) data processing unit, and the mass spectra data was processed operating an Analyst MSD software (Applied Biosystems, version 1.5).

2.3.5. Statistical analysis

Results were subjected to ANOVA test with separation of medias through Tukey test (significant level 0.05). The program used was Infostat (2016, Professional version).

2.4. RESULTS AND DISCUSSION

2.4.1. Grape characterization

The grape basic composition at harvest may be appreciated in Table 1. In year 2015, the grapes of Tannat V1 showed a significative evolution of the primary metabolism between the two harvest dates considered, as indicate the registers of sugars, pH and acidity. Thus, Tannat V2 presented the more mature grapes while Syrah and Marselan had different ratios between acidity and sugars what could be assigned to varietal characteristics. In the vintage 2016, Syrah grapes showed an important evolution of the primary metabolism between harvest, while in Marselan, just pH and acidity changed, while sugars contents remain steady. Opposing to vintage 2015, the grapes of Tannat V2 where much less mature at harvest in 2016.

Table 1 Grape general characteristics at harvest

Year	Harvest date	Treatment	Acidity	pH	Sugars
2015	20/02	Tannat V1H1	5.63 ± 0.08 b	3.26 ± 0.02 d	221.2 ± 1.15 d
	02/03	Tannat V1H2	5.43 ± 0.09 c	3.40 ± 0.02 b	245.6 ± 2.58 b
	02/03	Tannat V2	5.55 ± 0.08 bc	3.46 ± 0.03 b	250.3 ± 1.63 a
	20/02	Marselan	6.96 ± 0.11 a	3.33 ± 0.03 c	231.9 ± 1.15 c
	12/02	Syrah	4.13 ± 0.00 d	3.64 ± 0.03 a	216.1 ± 1.15 e
2016	09/03	Tannat V2	7.42 ± 0.00 a	3.16 ± 0.03 c	207.5 ± 1.73 c
	22/02	Syrah	5.78 ± 0.04 c	3.39 ± 0.01 b	196.3 ± 2.99 d
	01/03	Syrah	4.73 ± 0.06 e	3.47 ± 0.01 a	216.0 ± 1.15 b
	03/03	Marselan	6.06 ± 0.11 b	3.36 ± 0.01 b	248.8 ± 2.99 a
	09/03	Marselan	5.28 ± 0.13 d	3.39 ± 0.01 b	252.5 ± 1.91 a

V1 and V2 corresponds to two different Tannat vineyards located in the south of Uruguay. H1 and H2 correspond to the first and second harvest made for a grape cultivar in a determined vineyard. All data are expressed as the average of 2 replicates ± standard deviation. Different letters in the same column and year indicate the existence of statistical differences ($p < 0.05$). Acidity expressed in g/L of tartaric acid

Taken together, grapes representative of different maturity degrees were used in the three grape varieties, but in all cases, were commercially ripe grapes, according with the aims of the research.

The relative proportion of skin, seed and pulp affect extraction during maceration because it changes the solid/liquid ratio [18,24] Such characterization for the grapes used in the investigation may be observed in Table 2. Both years Syrah had the heaviest berries with the highest proportion of pulp and a particular low relative contribution of seeds. Marselan presented a distinctive high proportion of skins and the smaller proportion of pulp, while Tannat had the higher relative contributions of seeds, but surpassed by those of Marselan-H2 in 2016 (Table 2).

Table 2 Grape weight and distribution in seed and skin and pulp

Year	Treatment	Berry mass (g)	% seed (m/m)	% skin (m/m)	% of pulp (m/m)
2015	Tannat V1H1	1.63 ± 0.00 c	4.5 ± 0.2 a	6.9 ± 0.1 b	88.6 ± 0.1 ab
	Tannat V1H2	1.72 ± 0.05 b	4.4 ± 0.2 a	8.5 ± 0.8 ab	87.1 ± 1.0 ab
	Tannat V2	1.50 ± 0.02 d	4.0 ± 0.7 a	7.9 ± 1.2 b	88.1 ± 2.2 ab
	Marselan	1.35 ± 0.01 e	4.0 ± 0.2 a	11.1 ± 0.2 a	84.9 ± 0.1b
	Syrah	1.83 ± 0.07 a	2.5 ± 0.2 b	6.9 ± 0.0 b	90.6 ± 0.0 a
	Tannat V2	1.42 ± 0.06 bc	4.5 ± 0.8 a	7.2 ± 0.8 c	88.3 ± 1.5 a
2016	Syrah H1	1.94 ± 0.00 a	2.8 ± 0.2 b	8.3 ± 0.0 bc	88.9 ± 0.2 a
	Syrah H2	1.80 ± 0.20 ab	2.8 ± 0.1b	8.7 ± 0.8 abc	88.5 ± 0.9 a
	Marselan H1	1.30 ± 0.02 c	4.3 ± 0.0 ab	10.5 ± 0.2 ab	85.2 ± 0.2 ab
	Marselan H2	1.47 ± 0.07 bc	4.8 ± 0.2 a	11.9 ± 1.4 a	83.3 ± 1.2 b

All data are expressed as the average of 2 replicates ± standard deviation. Different letters in the same column and year indicate the existence of statistical differences ($p < 0.05$). V1 and V2 corresponds to two different Tannat vineyards located in the south of Uruguay. H1 and H2 correspond to the first and second harvest made for a grape cultivar in a determined vineyard.

Between harvest (when two by vineyard in a year where considered), was observed that Tannat and Marselan mean berry weight increased while it decreased in Syrah. Nevertheless, the percentage of grape skin increased toward the second harvest of the three varieties considered, in agreement with other works [25].

2.4.2. Grape flavan-3-ols: contents, concentrations and distribution

Table 3 present the contents of tannins in seeds, skins and wines. The concentrations expressed in mg/g of skin or seed was presented as well since allows

to interpret results in terms of the grape flavan-3-ol accumulation potential. The Fig. 1ab shows the relative contribution of skin and seed to the total flavanol content of the grapes on a berry weight basis.

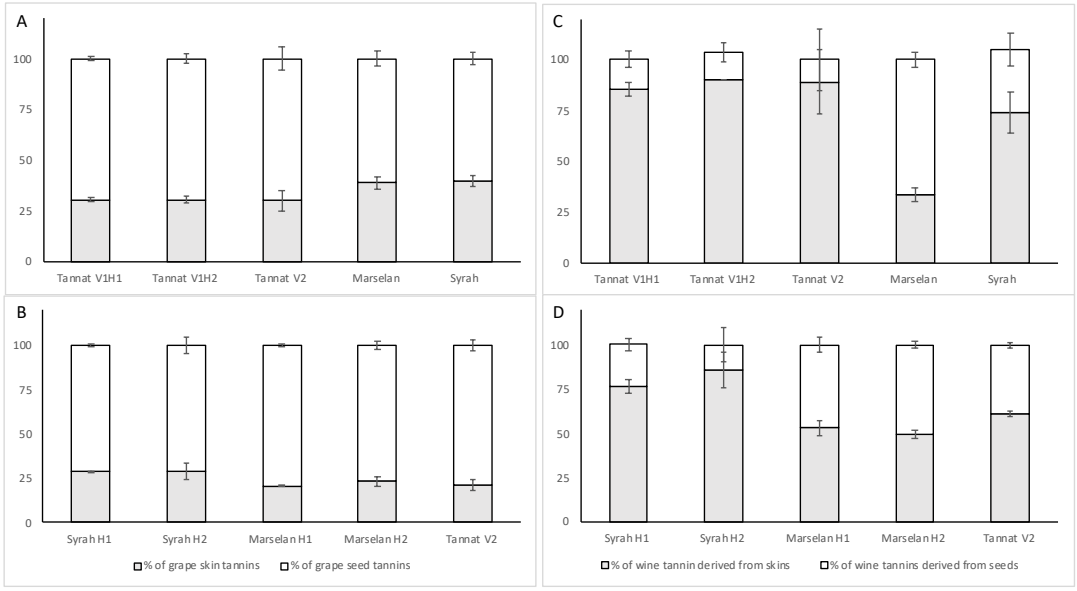

Fig. 1 Relative contribution of skin and seed to the total flavanol content of the grapes (A vintage 2015, B 2016) and estimation (based on prodelphinidin percentages comparations in skin and wines) of the relative contribution of skin and seed to the flavan-3-ol content of wines (C vintage 2015, B 2016).

Syrah showed in the average the fewest flavanol synthesis potential, in skin and seed, Marselan the highest in seeds while in the skins also presented high concentrations of these compounds. Tannat reach both years the highest concentrations of flavan-3-ol in skins, while those in seeds were in the average.

When the harvests were delayed (a second one made on the same vineyard and year), was observed that in the skins the flavan-3-ol concentrations remained steady in Marselan, while decreased in Syrah and Tannat. In the seeds, the results suggest than tannin accumulated between harvest in Tannat, while decreased in Marselan and Syrah.

Table 3 Flavanol contents and characteristics of skin, seed and wines according to treatment and vintage

Typology - Vintage	Treatment	Total F-3-ol mg/g (Sk-Sd)	Total F-3-ol mg/Kg grape	%PD	mDP	% G
Skin - 2015	Tannat 1H1	9.6 ± 0.4 a	661 ± 21 bc	25.7 ± 1.0 ab	6.60 ± 0.00 bc	4.3 ± 0.1 a
	Tannat 1H2	8.1 ± 0.2 ab	692 ± 28 b	26.0 ± 0.5 ab	6.14 ± 0.21 c	4.0 ± 0.3 a
	Tannat 2	7.8 ± 0.3 ab	612 ±59 bc	23.4 ± 2.4 b	6.60 ± 0.24 bc	4.9 ± 0.6 a
	Marselan	8.8 ± 0.7 a	968 ± 60 a	29.0 ± 0.7 a	10.76 ± 0.81 a	3.7 ± 0.1 a
	Syrah	6.8 ± 0.6 b	470 ± 69 c	25.4 ± 0.2 ab	8.32 ± 0.55 b	4.9 ± 0.4 a
Skin - 2016	Tannat V2	4.5 ± 0.4 a	338 ± 12 ab	31.9 ± 1.5 a	5.41 ± 0.04 d	6.9 ± 0.2 bc
	Marselan H1	3.7 ± 0.4 a	388 ± 51 ab	34.6 ± 0.6 a	8.55 ± 0.46 ab	6.1 ± 0.8 c
	Marselan H2	3.8 ± 0.5 a	414 ± 30 a	35.5 ± 1.7 a	9.18 ± 0.02 a	6.7 ± 0.7 c
	Syrah H1	4.2 ± 0.0 a	313 ± 1 ab	33.2 ± 1.5 a	6.89 ± 0.28 c	9.3 ± 0.3 ab
	Syrah H2	3.5 ± 0.4 a	295 ± 36 b	32.3 ± 0.6 a	7.48 ± 0.37 bc	10.0 ± 0.8 a
Seed - 2015	Tannat V1H1	33.2 ± 1.1 ab	1508 ± 127 a	0.75 ± 0.02 a	4.06 ± 0.24 a	14.1 ± 0.0 a
	Tannat V1H2	35.8 ± 0.4 ab	1584 ± 59 a	0.82 ± 0.03 a	4.09 ± 0.06 a	14.4 ± 0.1 a
	Tannat V2	36.0 ± 3.7 ab	1440 ± 221 a	0.55 ± 0.05 b	3.99 ± 0.23 a	13.8 ± 0.8 ab
	Marselan	39.1 ± 0.7 a	1535 ± 109 a	0.80 ± 0.03 a	4.17 ± 0.08 a	12.3 ± 0.2 bc
	Syrah	31.5 ± 0.5 b	713 ± 23 b	0.29 ± 0.01 c	3.73 ± 0.28 a	12.0 ± 0.3 c
Seed - 2016	Tannat V2	31.7 ± 0.7 ab	1301 ± 291 ab	0.74 ± 0.03 bc	3.26 ± 0.10 b	24.9 ± 1.7 a
	Marselan H1	36.6 ± 3.9 a	1491 ± 159 a	0.91 ± 0.11 ab	3.45 ± 0.31 ab	22.6 ± 1.2 a
	Marselan H2	31.6 ± 1.5 ab	1365 ± 91 ab	0.97 ± 0.04 a	3.91 ± 0.11 a	22.4 ± 1.0 a
	Syrah H1	29.6 ± 2.1 ab	780 ± 17 b	0.54 ± 0.02 cd	3.70 ± 0.02 ab	24.7 ± 1.6 a
	Syrah H2	27.7 ± 2.2 b	734 ± 85 b	0.41 ± 0.00 d	3.76 ± 0.14 ab	24.0 ± 0.5 a
Total grape - 2015	Tannat V1H1	42.8 ± 1.5 ab	2169 ± 148 ab			
	Tannat V1H2	44.0 ± 0.2 ab	2276 ± 32 ab			
	Tannat V2	43.8 ± 4.0 ab	2052 ± 162 b			
	Marselan	47.9 ± 0.0 a	2504 ± 49 a			
	Syrah	38.3 ± 0.1 b	1183 ± 91 c			
Total grape - 2016	Tannat V2	36.2 ± 0.3 a	1639 ± 303 ab			
	Marselan H1	40.3 ± 4.3 a	1879 ± 210 a			
	Marselan H2	35.4 ± 1.1 a	1779 ± 60 a			
	Syrah H1	33.8 ± 2.1 a	1092 ± 16 b			
	Syrah H2	31.2 ± 1.8 a	1029 ± 48 b			
Wine - 2015	Tannat V1H1	-	252 ± 14 ab	19.8 ± 0.0 a	2.98 ± 0.06 a	3.4 ± 0.1 a
	Tannat V1H2	-	268 ± 18 ab	20.3 ± 0.6 a	3.08 ± 0.14 a	2.8 ± 0.1 ab
	Tannat V2	-	270 ± 20 a	19.1 ± 1.8 a	2.86 ± 0.13 a	2.5 ± 0.1 bc
	Marselan	-	307 ± 35 a	8.4 ± 1.1 b	3.15 ± 0.17 a	3.3 ± 0.3 a
	Syrah	-	176 ± 21 b	15.5 ± 1.7 a	2.99 ± 0.13 a	2.0 ± 0.28 c
Wine - 2016	Tannat V2		*172* ± 11 b	*17.9* ± 0.6 b	*2.80* ± 0.48 a	*4.9* ± 0.7 a
	Marselan H1		*211* ± 7 ab	*14.9* ± 1.1 b	*4.06* ± 0.16 a	*5.7* ± 0.8 a
	Marselan H2		*220* ± 22 ab	*14.3* ± 0.5 b	*4.21* ± 0.95 a	*5.6* ± 1.2 a
	Syrah H1		*174* ± 17 b	*23.4* ± 0.0 a	*3.42* ± 0.11 a	*4.2* ± 0.6 a
	Syrah H2		*231* ± 3 a	*25.8* ± *2.5 a*	*3.76* ± 0.05 a	*5.7* ± 0.3 a

All data are expressed as the average of 2 replicates ± standard deviation. Different letters in the same column, typology and year, indicate the existence of statistical differences ($p < 0.05$). V1 and V2 corresponds to two different Tannat vineyards located in the south of Uruguay. H1 and H2 correspond to the first and second harvest made for a grape cultivar in a determined vineyard. % PD percentage of prodelphinidins, mDP mean degree of polymerization, %G percentage of flavanol galloylation.

Despite differences in the flavanol concentration in each typology, the relative proportion of seeds skin and pulp was determinant as well of the amounts of flavan-3-ols present in a kg of grape basis (contents), which from an enological perspective is the most important data. Hence, the differences in contents among the studied grapes were greater than those in concentrations. Thus, Marselan stood out by presenting the high contents of skin flavanols, because of its high proportions of skin and concentrations of tannins on these, while Syrah had low contents of flavan-3-ols in skin and particular in seeds because of high berry weight with high proportion of pulp, and the low percentages of seeds per berry with low concentration of flavanols in its tissues. Therefore, Marselan grapes had, in each vintage, the highest total flavanol contents, follow for those of Tannat, and Syrah presenting much lowers registers despite other variables like grape maturity.

The distribution of grape flavanols shows that they were predominantly localized in seeds (between 60 to 80 % approximately) despite other variables (Fig. 1ab). In 2015, the three Tannat grapes had a very similar flavan-3-ol distribution, characterized by higher proportions than Marselan and particularly Syrah of seed flavanols. In 2016, the proportions of seed tannins were higher than the averages observed the previous year, differences that cannot be explained by the levels of grape maturity. Nevertheless, Syrah presented once more the highest proportion of skin flavanols (without significant differences among harvests) while Tannat, but also Marselan_H1, the highest in seed. In the second harvest of Maselan grapes, a slight increase of skin flavanol proportion was observed.

From the presented results arise that the genetical factors (cultivar) had greater impact than the maturity level of the grapes defining its contents and distribution of flavan-3-ol. Nevertheless, important differences may be obtained depending on the years. The causes behind these later results were not examined in the current study and deserves ongoing research.

2.4.3. Structural characterization of skin and seed flavan-3-ols

The mean degree of polymerization (mDP) of the skin flavanols was higher than that of the seeds in agreement with literature (Table 3) [9,26]. This was not affected by the grape maturation degree, instead, a varietal effect was observed. Both years Marselan skins presented the more polymerized tannins, Tannat the least, and Syrah in between. In the seeds, the mDP was very similar in all cases with values between 3 and 4 (Table 3).

Prodelphinidins (PD) are cited to be exclusively from skins [9], but in the present investigation they were also found in seeds, even when at very low concentrations. In skins, the PD percentages were higher in year 2016 than in 2015, and both years, the highest percentages were present in Marselan. Interestingly, Marselan presented the highest proportion of PD in seeds as well.

The percentages of galloylated tannins (% G) was as expected [9,27] much higher in seed than in skin (3 to 5 more times). There were not important differences among grape varieties or due to the maturation degree of the grapes for this characteristic. Nevertheless, Syrah presented the highest registers in skins and particularly in seeds in year-2016. The % G observed in 2016 were much higher than in 2015 particularly in seeds, differences that cannot be assigned to any of the known variables affected. Future studies should try to identify factors that may affect such structural characteristic.

The relative contribution of monomers, dimers and oligomers-polymers (tannins of three and more units) to the total flavanol content of seed and skin may be observed in Fig. 2a-d. Despite the oligomer-polymer fraction is relevant in seed and skin, the formers presented more proportions of monomers and dimers and the later are almost exclusively constituted of polymers. In the cases where 2 harvest dates were evaluated, polymeric forms increased their relative importance toward the second harvest. In seeds, Tannat samples presented in all cases the highest proportion of oligomers-polymers while Syrah the least.

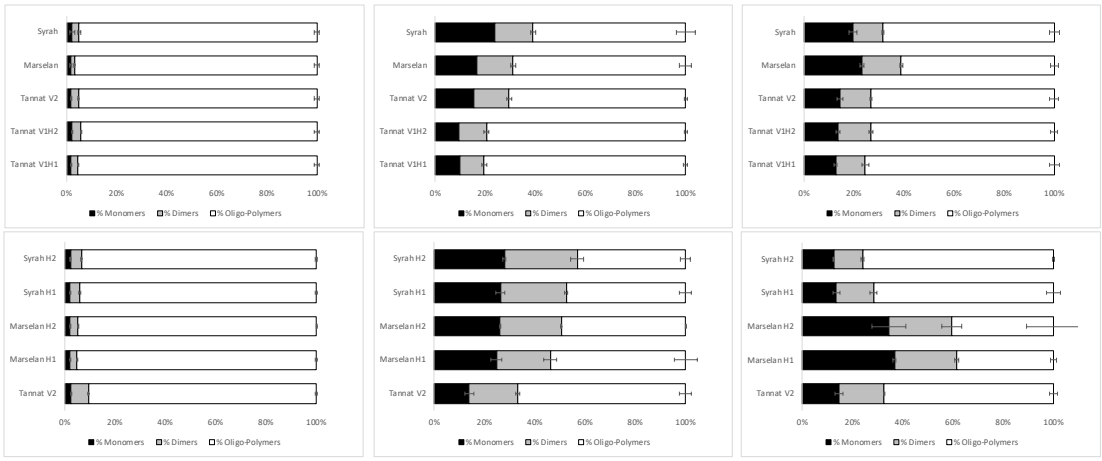

Fig. 2 Relative contribution of monomers, dimers and polymers to the total flavan-3-ol content of seed, skin and wines. **A** skin 2015; **B** skin 2016; **C** seed 2015; **D** seed 2016; **E** wine 2015; **F** wine 2016

2.4.4. Wine flavanols

The assessment in wine of the flavanol structural characteristics provided clues on how they were extracted from skin and seed (Fig. 1cd & Fig. 2ef). The comparation of prodelphinidins proportions in the grape skin and the respective wines was used to estimate the relative contribution of skins and seeds to wine tannins according to the proposed by Peyrot des Gachons & Kennedy[28]. The proportion of monomers, dimers and polymers in the wines also assisted to trace the flavanol origin since as presented previously, they are in very different proportions in skins and seeds. Differently of what has been reported by other authors, the mDP did not contribute in the current research as indicator of the flavanol origin. Similarly occurred with the %G, what could be expected since the gallic acid moiety undergo hydrolysis under wine conditions [29].

The results obtained in 2015 seemed to indicate a relationship between grape and wine flavanol contents. Thus, the grapes with more flavanols, those of Marselan, produced the wines with more flavanols, and those presenting the least, Syrah, produced the wines with the least amount of these compounds. Meanwhile, the three Tannat elaborated gave wines with similar flavan-3-ols concentrations, in between those of the Marselan and Syrah (Table 3).

The three Tannat wines elaborated presented as well very similar flavanol structure, characterized for higher proportion of prodelphinidins and low of

monomers and dimers (Table 3 & Fig. 2e). These are signs of wines containing high proportions of skin derived flavanols as indeed revealed from the estimations made considering the PD% in skins and wines (Fig. 2cd). Thus, the different maturity of these grapes at harvest, did not produced significant differences in the amount and structure of the wine flavanols.

On the other hand, in Marselan, the wine flavanols structure was clearly different to the observed in Tannat. The %PD was the lowest (despite their skins presented the highest) and the proportions of monomers and dimers were the highest. Both signs of higher proportion of seed derived flavanols, what indeed arises from the estimation made. Thus, more than 50% of Marselan wine flavanols would come from seeds (Fig. 1 cd).

Meanwhile Syrah wines showed a composition that indicate a type of flavanol extraction closer to the observed in Tannat, with a preponderance of skin derived tannins.

The assessment of 2016 results provide clarification of some result obtained the year before and revels new findings. This year, the absence of correspondence between grape and wine flavanols was evident, whether it is considered the total contents of the grapes or those in skin or seed (Table 1).

Marselan confirmed the observed the year before. Seeds made a distinctive high contribution to wine flavanols, even in very ripe grapes. At the same time, those from skin would have been little extractables, maybe because their higher mDP (Table 3). This is a major finding since expose that the grape cultivar may be determinant of wine flavanol composition, and that seed derived flavanols may be dominant already under fermentative macerations. It is generally reported that the proportion of skin and seed flavan-3-ols is mainly dependent on the maceration time, with the former being predominant in the fermentative phase, and the later becoming more important with time, being predominant in the post fermentative macerations [14,25,30,31].

Other remarkable result arises from Syrah. The concentrations of flavanols in their wines were much higher than those expected from the total contents in their grapes. Thus, Syrah_H1, produced wines with the same flavanol contents than

Tannat_V2, from grapes with similar flavonols in the skins but much lower in seeds. This shows that in 2016, the wine flavanol content of Syrah would have been even more dependent on skins than Tannat. The structural characteristics of their wines confirmed this, as Syrah once had the highest proportion of flavanols derived from skins, with the highest %PD and polymers in the wines (Fig. 1d, Fig. 2 F & Table 3).

Another main observation involving Syrah was the impressive increase of the flavanol contents in the wines derived from the second harvest grapes respect those from the first one. Such increase was of 33%, and from grapes that presented the fewest flavanol contents of the year. This indicates that albeit grape flavanol content, their extractability could be even more determinant of those in wine. In this case, that from skin. This increase of flavanol extraction with grape maturity was not observed in Marselan and Tannat. Hence, it could be also related to the grape cultivar.

Taken together, the proportion of monomers, dimers and polymers of the wine, in all cases was closer to that observed in the seeds than in the skins. This do not agree with the high %PD observed in wines in most cases. Nevertheless, It has been pointed out that the tannins more polymerized from skins, would be the less extracted during winemaking [8]. Then, the results reported here would be in line with such observations, and would reflect the preferential extraction of less polymerized flavanol fractions. Nonetheless, once extracted from grapes, flavanols undergo continuous bond making and bond braking reactions [27]. Then, a direct relation between the flavanol length of the extracted flavanol and those in wine after a period of time should not be expected.

Lastly, current investigation results showed that toward the end of the maturation process, when the harvest decision must be taken, there is not necessarily a predictable trend of the tannin evolution in the grapes or of its extraction into wines. Ongoing research would be required to further conclude in the findings reported here.

Grape cultivar may have a strong effect on the relative contribution of skin and seed to the wine flavanols, regardless grape maturity or their contents and distribution on the berries. Thus, most of the wine flavanols may be derived from seed, even in short fermentative macerations. It was verified that this is independent

of berries maturity, at least in commercially ripe grapes. Nevertheless, flavan-3-ol extractability, mainly skin one, could be strongly affected by grape maturity, thus modifying the wine flavanol contents. This later could be important at least in some cultivars, since it was observed in Syrah but not in Marselan and Tannat. In order to manage the red-wine flavanols, the grape varietal characteristics should be known and considered.

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3. SELECTIVITY OF PIGMENTS EXTRACTION FROM GRAPES AND THEIR PARTIAL RETANTION IN THE POMACE DURING RED-WINEMAKING

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Selectivity of pigments extraction from grapes and their partial retention in the pomace during red-winemaking

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ABSTRACT

The purpose of this investigation was to assess the grape pigment extraction during red-winemaking. The pigment content and profile of skin, wines and pomace of *Vitis vinifera* grapes with different pigment profiles Syrah, Marselan and Tannat was thoroughly studied. The *p*-coumaroylated anthocyanin proportions were 37% in pomace, compared to 19% in skins and just 5% in wines, highlighting their poor extraction. Di-methoxylated based anthocyanins, increased their relative contribution in pomace and wines compared to skins, indicating they were less extracted, but more stable once extracted. Remarkably, the caffeoylated anthocyanins presented significantly higher concentrations in pomace than in skins (1.9 fold on average), suggesting that their synthesis could take place during vinification. Malvidin 3-feruloyl-glucoside was found in the Tannat samples being the first evidence of an anthocyanin acylated with ferulic acid in wine. Pigment extraction was selective depending on anthocyanin B-ring substitution pattern and acylation profile.

1. Introduction

Anthocyanins are polyphenolic compounds that confer the characteristic colour of mature red grapes and are of interest for their nutraceutical properties. During the maceration step of red-winemaking, the anthocyanins are partially extracted from the grape skins to the must and wine, determining wine pigment contents and characteristics (Barcia, Pertuzatti, Gómez-Alonso, Godoy, & Hermosín-Gutiérrez, 2014; Lingua, Fabani, Wunderlin, & Baroni, 2016). Thus, the potential of pomace (the remaining of grape skins after fermentation and pressing) as a source of natural pigments and bioactive compounds depend on the extent of the extraction reached during maceration (Barcia et al., 2014).

Once extracted, anthocyanins may react with fermentation metabolites and other phenolic compounds generating a great diversity of derived pigments, some of which are much more stable than the native anthocyanins (Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2006a; Blanco-Vega, López-Bellido, Alía-Robledo, & Hermosín-Gutiérrez, 2011; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2003; Schwarz, Wabnitz, & Winterhalter, 2003). Nevertheless, anthocyanins continue to be by far the main pigments of young red wines (Fulcrand, Dueñas, Salas, & Cheynier, 2006).

In *Vitis vinifera* grapes, there are five main anthocyanins that in most varieties are also present acylated with acetic, caffeic and *p*-coumaric acid. Thus, the pigment profile of a sample may be defined considering the percentage of each of the five anthocyanin classes, but also the relative abundance of the non-acylated, acetylated, caffeoylated and *p*-coumaroylated pigments. Those characteristics are strongly determined by the grape variety and although the vinification technique may modify these to some extent, in young red-wines, the varietal fingerprint prevails. Thus, the grape pigment profile has been successfully used to discriminate grape cultivars (even among clones when associated with maturity data) and varietal young red wines (Ferrandino & Guidoni, 2009; González-Neves, Favre, Piccardo, & Gil, 2016; Ortega-Regules, Romero-Cascales, López-Roca, Ros-García, & Gómez-Plaza, 2006). It has also been taken into consideration to assess the oenological potential of grape varieties and to adapt techniques of vinification (Bosso, Guaita, Panero, Borsa, & Follis, 2009; González-Neves et al., 2004a,b; Gonzalez-San Jose, Santa-Maria, & Diez, 1990). Many works have studied the pigments content and extractability in grapes, wines

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and winemaking by-products, but just few have made a comprehensive study of such characteristics from grape to respective wines and pomace (Lingua et al., 2016).

The aim of this investigation was to assess the grape pigments extraction process from grape to wine under red-winemaking. That was carried out by a thorough comparison of the individual pigments content and pigment profiles of grape skins and their respective wines and pomace. *Vitis vinifera* L. cv. Syrah, Marselan and Tannat were used for the experiments because each one show unique characteristics. Thus, Syrah is a cultivar with a comparable proportion of both non-acylated and different acetylated and *p*-coumaroylated anthocyanins (Blanco-Vega et al., 2011), Marselan is a relatively little studied grape variety of high pigment potential presenting high proportions of di-methoxilated pigments (Alcalde-Eon, Boido, Carrau, Dellacassa, & Rivas-Gonzalo, 2006b), and Tannat is a high anthocyanin potential grape variety but with low pigment extractability, and is the most representative grape variety of Uruguay (Boido et al., 2011; Favre et al., 2014; González-Neves et al., 2004b).

2. Materials and methods

2.1. Experimental design

The experiments were performed in 2015 from commercial vineyards located in the south of Uruguay, from which ten samples of grapes and the respective red-wines and pomace were obtained to compare their pigment contents and profiles. The grapes belonged to the *Vitis vinifera* grape-varieties Tannat, Syrah and Marselan. In Tannat, grapes were taken from 2 different vineyards, and in one of them, 2 harvests were considered, which were made on different dates (Table S1). Tannat was prioritized because its exceptional anthocyanin potential (Boido et al., 2011; González-Neves et al., 2004b) was suitable for the purposes of this research. Each harvest was made by hand, and the clusters were carefully transported in plastic boxes (20 kg each one) to the university experimental winery. There, two batches of grapes of 70 kg each were randomly separated for vinification. Just before crushing, from each batch a sample of 100 grapes were collected, taking cluster parts from 3 to 5 berries from different parts of randomly chosen bunches. To avoid bias by size or aspect, the berries were scattered over a plain surface with a numbered grid of 5 × 5 cm squares. An order of squares was randomly chosen, and then all the grapes placed inside each square were chosen until 35 grapes were collected. Each 35-berry sample was then peeled, the pulp remaining against the skins carefully removed with the help of rounded edge blade, gently blotted with a paper towel, weighted (fresh-weight) and stored in a sample-bag at −18 °C until freeze-dried. Skins were freeze-dried, weighted (dry-weight) and then stored at −18 °C again in bags containing silica gel. The general characteristics and phenolic potential of the grapes belonging to each harvest were obtained from additional duplicate samples of 250 grapes each, which were analysed within 24 h (data presented in Table S1, see supplementary material). Then, each batch of 70 kg of grapes was vinified. Grapes were destemmed and crushed with an Alfa 60 R crusher (Italcom, Italy), and barrelling was made in stainless-steel tanks (100 L capacity each). Potassium metabisulfite (50 mg SO_2/100 kg of grapes) was added and dry active yeast (*Saccharomyces cerevisiae ex bayanus* Natuferm 804; OenoBioTech, Paris, France) was inoculated (20 g/kg of grapes). Wines were made with a classical fermentation on skins (maceration simultaneously with alcoholic fermentation) for 8 days in Marselan and Tannat, and 7 days in Syrah, one day less because of its lower anthocyanin potential, according to González-Neves et al. (2004a,b). Along macerations, two pumpings over followed by punching the cap were carried out daily until pressing. At devatting, the fermentation was finished in all cases. The pressing was carried out with a stainless-steel manual press. Free-run juices and press juices were mixed, separated from lees, stabilized by adding a dose of 50 mg/L of SO_2 and kept in glass recipients of 10-L capacity. After each pressing, a random sample of 35 skin pomace was taken directly from the press. The pulp, and other vegetable residues remaining against the skin pomace were carefully removed with the help of a rounded edge blade. Then, pomace were washed with distilled water and gently blotted with a paper towel, weighted (fresh-weight) and stored in a sample-bag at −18 °C until freeze-dried. Pomace were freeze-dried, weighted (dry-weight) and then stored at −18 °C again in bags containing silica gel.

2.2. Analytical procedures

2.2.1. Chemicals

Analytical determinations were made at the Laboratory of Instrumental Analysis placed at the Regional Institute of Applied Scientific Research (IRICA), Castilla La Mancha University, Spain. All solvents used were HPLC quality, and all chemicals were analytical grade (> 99%). Water was Milli-Q quality. Malvidin 3-glucoside (PhytoLab, Vestenbergsgreuth, Germany) was used as standard for quantification of anthocyanins.

2.2.2. Skin and pomace extracts

Freeze-dried skins (10 samples) and pomace (10 samples) were extracted each with 100 mL of a mixture 50:48.5:1.5 (v/v) of CH_3OH/H_2O/HCOOH, using a homogenizer (Heidolph DIAX 900) to 10000 rpm for 3 min and then centrifuging at 2500*g* at 5 °C for 5 min. The supernatant was separated and conserved, and the pallet was extracted again two more times. The three-supernatant obtained were mixed, the volume registered, and then stored at −18 °C until analysis. Previous works made in similar conditions, confirmed that a two times extraction of the grape skin pellet yielded nearly 99% of the grape polyphenol contents able to be extracted with successive repetitions of the cited protocol (Castillo-Muñoz et al., 2009).

2.2.3. Analysis of pigments by HPLC–DAD–ESI–MS^n

The pigment analysis was performed on an Agilent 1100 Series system (Agilent, Germany), equipped with a Diode Array Detector (DAD, model G1315B) and a Mass Spectrometry Detector with an electrospray ionisation (ESI-MS/MS) source (LC/MSD Trap VL, model G2445C VL), and coupled to an Agilent Chemstation (version B.04.01) data-processing station. The mass spectral data were processed with the Agilent LC/MS Trap software (version 5.3). The samples were injected (10 µL skins extracts and wine samples, 20 µL skin pomace extracts) on a reversed-phase column Ascentis Express C18 (2.1 × 150 mm; 2.7 µm particle; Sigma-Aldrich, Germany), thermostated at 40 °C. We used the chromatographic system and conditions previously described (Blanco-Vega et al., 2011) with some modification described hereafter. Briefly, the solvents were water/formic acid/acetonitrile (88.5: 8.5: 3 v/v/v, solvent A; 41.5: 8.5: 50.0, v/v/v, solvent B), and the flow rate was 0.16 mL/min. The linear gradient for solvent B was: zero min, 4%; 15 min, 30%; 20 min, 35%; 30 min, 35%; 35 min, 100%; 40 min, 100%; 45 min 4.0%. For identification, ESI-MS/MS was used employing the following parameters: positive ion mode; dry gas, N2, 11 mL/min; drying temperature, 350 °C; nebulizer, 65 psi; capillary, −2500 V; capillary exit offset, 70 V; skimmer 1, 20 V; skimmer 2, 6 V; scan range, 50–1200 *m/z*. Identification was mainly based on MS/MS data, but also on the comparison of chromatographic and spectral data (UV–vis and MS/MS) with those obtained with authentic standards. Quantification was performed using the DAD-chromatograms extracted at 520 nm and a calibration curve obtained with the standard of malvidin 3-glucoside.

2.3. Statistical data analysis

Results were subjected to ANOVA test with separation of medias through Tukey test (significant level 0.05). The variance homology was checked using the Shapiro-Wills test. The statistical analysis was carried out on 10 replicates in media comparisons of skins, wines and pomace

Table 1
Pigments identified in wines and the respective freeze-dried samples of Syrah, Marselan and Tannat grape skins and pomace: mass spectral characteristics (positive ionization mode).

Typology analysed			Wines (mg/L)	Skins (mg/kg)	Pomace (mg/kg)
Peak	Compound[a]	MS/MS (*m/z*)	MV ± SD min–max	MV ± SD min–max	MV ± SD min–max
Anthocyanins					
1	Dp-3,5-diglc	627/465/303	nd	2.2 ± 2.5 0.0–6.8	nd
2	Po-3,5-diglc	625/463/301	nd	id^{Sy}	nd
3	Dp-3-glc	465/303	8.9 ± 6.5 1.0–19	2254 ± 1190 697–4160	302 ± 256 16–620
4	Cy-3-glc	449/287	0.9 ± 0.5 0.0–1.5	291 ± 129 149–469	18 ± 19 0.0–45
5	Pt-3-glc	479/317	15 ± 9 3.9–29	2660 ± 1381 980–4715	635 ± 520 68–1384
6	Pn-3-glc	463/301	4.2 ± 0.9 2.7–5.6	1144 ± 252 773–1620	195 ± 85 95–337
7	Mv-3-glc	493/331	108 ± 31 57–143	10775 ± 2475 6531–14065	3830 ± 1755 1670–6968
8	Mv-3-pent	463/331	0.4 ± 0.1 0.2–0.6	35 ± 11 17–48	6.6 ± 6.0 0.0–17
9	Dp-3-acglc	507/303	1.5 ± 0.6 0.6–2.4	253 ± 114 75–438	38 ± 26 0.0–93
10	Cy-3-acglc	491/287	ni	id	id^{Ta}
11	Pt-3-acglc	521/317	2.6 ± 1.0 1.2–4.1	356 ± 81 153–593	97 ± 81 10–237
12	Po-6-ac-3-glc*	505/301	nd	id^{Sy}	nd
13	Po-acglc	505/301	1.6 ± 1.2 0.4–3.6	163 ± 81 92–320	43 ± 14 18–69
14	Mv-6-ac-3-glc*	535/331	nd	id	id
15	Mv-3-acglc	535/331	23 ± 9 11–40	1694 ± 234 1479–2155	843 ± 315 299–1367
16	Pt-3-cfglc	641/317	nd	15 ± 5Ta 0.0–20	18 ± 3$^{Ta;Sy}$ 0.0–23
17	Po-3-cfglc	625/301	0.2 ± 0.7 0–0.3$^{Sy;Ma}$	9.6 ± 8.9 1.9–31	15 ± 18 0.0–52
18	Mv-3-trans-cfglc	655/331	0.4 ± 0.3 0.1–0.9	75 ± 30 43–123	152 ± 27 94–186
19	Dp-3-trans-cmglc	611/303	0.8 ± 0.5 0.2–1.4	589 ± 286 224–969	273 ± 174 42–491
20	Pt-3-cis-cmglc	625/317	nd	8.6 ± 6.1 0.0–16	nd
21	Cy-3-cmglc	595/287	0.4 ± 0.1 0.2–0.5	125 ± 47 41–175	52 ± 36 0.0–111
22	Pt-3-trans-cmglc	625/317	1.1 ± 0.6 0.3–1.9	700 ± 314 250–1069	474 ± 293 71–874
23	Po-3-cis-cmglc	609/301	nd	9.7 ± 14 0.0–38	0.2 ± 0.5^{Sy} 0.0–1.7
24	Mv-3-cis-cmglc	639/331	nd	75 ± 11 63–99	21 ± 9.5 6.2–39
25	Po-3-trans-cmglc	609/301	1.2 ± 0.8 0.4–2.8	554 ± 390 250–1069	276 ± 145 118–545
26	Mv-3-trans-cuglc	639/331	7.3 ± 2.2 4.0–11	3735 ± 356 3111–4217	3478 ± 1205 1633–5341
27	Mv-3-ferglc	669/331	0.0 ± 0.0^{Ta} 0.0–0.1	24.8 ± 5.3^{Ta} 17–31	18 ± 4Ta 12–22
A-type vitisins (10-carboxy-pyranoanthocyanins)					
28	Carboxy-pydp-3-glc	533/371	0.3 ± 0.1 0.0–0.54^{Ta}	nd	nd
29	Carboxy-pypt-3-glc	547/385	nd	nd	id
30	Carboxy-pymv-3-glc (vitisin A)	561/399	3.5 ± 0.8 1.9–4.7	nd	42 ± 15 26–64
31	Carboxy-pymv-3-acglc (ac-vitisin A)	603/399	1.2 ± 0.5 0.7–1.8	nd	4.8 ± 6.2 0.0–21
32	Carboxy-pymv-3-cmglc (cm-vitisin A)	707/399	0.5 ± 0.1 0.3–0.7	ni	id
B-type vitisins (pyranoanthocyanins)					
33	Vit B Pt-3-glc	503/341	id	nd	id
34	Vit B *Pn*-3-glc	487/325	id	nd	–
35	Vit B	517/355	0.6 ± 0.3 0.3–1.0	nd	25 ± 8 13–41
36	ac-VB	559/355	0.4 ± 0.2 0.0–0.6	nd	9 ± 3 5.4–16
37	cm-VB	663/355	id	nd	9.6 ± 3.9 5–18
Flavanol–anthocyanin direct adducts					
38	Mv-3-glc-Gc	797/635/467/373	nd	9 ± 3 5.1–14	nd
39	Po-3-glc-ECat	751/589	nd	5.6 ± 1.7$^{Sy;Ma}$ 3.4–7.3	nd
40	(4–8) (-)-Ca-mv-3-glc	781/619	0.7 ± 0.2 0.5–1.0	17 ± 7 11–30	2.47 ± 3.26 0.0–7.0^{Ta}
Hydroxyphenyl-pyranoanthocyanins					
41	10-HP-pymv-3-glc	609/447	nd	nd	id
Tot	All pigments		183 ± 48 102–241	25550 ± 5372 18798–33985	10899 ± 4562 5671–18756

Peak numbers as in Fig. 1.
Data are the mean (n = 10) ± SD.
Results are expressed as malvidin 3-glucoside equivalents in mg/L of wine and mg/kg of freeze-dried grape skins or pomace
The mean and SD of compounds identified just in a particular grape variety (tagged by superscript of: **Sy**. Syrah; **Ma**. Marselan; **Ta**. Tannat) considered just the samples of the grape varieties where the compounds were identified.
* Putative identified compounds.
[a] Abbreviations: **nd**. no-detected; **id**, identified but not quantified; **dp**, delphinidin; **cy**, cyanidin; **pt**, petunidin; **pn**, peonidin; **mv**, malvidin; **pydp**, pyranodelphinidin; **pypt**, pyranopetunidin; **pymv**, pyranomalvidin; **glc**, glucoside; **pent**, pentoside; **acglc**, acetyl-glucoside; **cmglc**, p-coumaroyl-glucoside; **cfglc**, caffeoyl-glucoside; **ferglc**, feruloyl-glucoside; **HP**, hydroxyphenyl; **Cat**, catechin; **ECat**, epicatechin; **VA**, vitisin A; **VB**, vitisin B.

pigment contents and profiles. When the grape variety or grape origin (vineyard, date of harvest) was also considered, media comparisons was carried out on 2 replicates. The program used was Infostat (2016, Professional version).

3. Results and discussion

The pigments identified in skins, wines and pomace are listed in Table 1, and their place in the chromatogram showed in Fig. 1abc. As observed, some compounds were detected (MS data and correspondent pick at the 520 nm chromatogram) but not quantified due to the low concentration they presented in the samples.

As expected, Syrah, Marselan and Tannat grape skins presented each a distinctive varietal pigment profile (Tables 2 and 3). Thus, Tannat

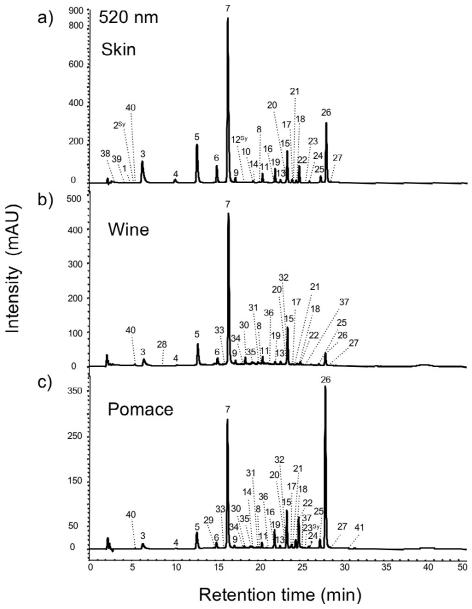

Fig. 1. HPLC-DAD Chromatograms (detection at 520 nm) of anthocyanins and anthocyanin-derived pigments of: (a) skin of Tannat; (b) 3-month Tannat red-wine; (c) pomace of Tannat. Peak numbering (see Table 1): 1–27, anthocyanins; 28–32, A-type vitisins; 33–37, B-type vitisins; 38–40, direct flavanol–anthocyanin adducts; 41, hydroxyphenyl-pyranoanthocyanin. ^{Sy} Pigment just found on Syrah. **min**, minutes.

presented in all cases the highest percentages of delphinidin, petunidin, cyanidin and non-acylated pigments, and the lowest of malvidin and peonidin. Syrah had the highest proportions of peonidin and acylated pigments, particularly of coumarates, a characteristic that has been useful to investigate wine anthocyanin acylated pigment derivatives (Blanco-Vega et al., 2011). Finally, Marselan presented the highest contributions of malvidin based pigments and an acylation pattern characterized by the lowest quotient coumarates/acetates. These results are in agreement with previous reports (Blanco-Vega et al., 2011; Alcalde-Eon et al., 2006b; Favre et al., 2014; González-Neves et al., 2016; González-Neves et al., 2007).

The five anthocyanidins presented different proportions in skins than in wines and pomace (Table 2). Malvidin based pigments had the highest contribution to pigment contents in pomace and wines while the other four anthocyanidins presented the highest percentages in the skins. This suggests that malvidin based anthocyanins, were the least extractable, but also, the more stable once extracted. Likewise, pigments based on peonidin presented in all cases higher percentages in pomace than in wine. Hence, peonidin derivatives would be less extractable than the hydroxylated anthocyanins, but less stable in wine that malvidin derivatives.

Considering what was discussed above, a canonical discriminant analysis was carried out in order to determine if the varietal origin of the sample analysed can be known by its pigment profile despite the typology it belongs to (skin, wine or pomace). The classification variable chosen was grape variety, and the relative contribution of each 5 anthocyanidins to the total pigment content the discriminant variable. The distribution of the samples in the plan defined by the two discriminant functions, is shown in Fig. S1a. The two first canonical functions accounted for 100% of the total variance (function 1 explained 89,6%). An important separation among the scores of the samples obtained by each grape-variety was observed. Thus, a perfect discrimination of all samples was obtained. This shows that the genetically controlled varietal pigment profile of the skins was transferred into wines in agreement with other works (Arapitsas, Perenzoni, Nicolini, & Mattivi, 2012; Gómez Gallego, Gómez García-Carpintero, Sánchez-Palomo, González Viñas, & Hermosín-Gutiérrez, 2013; González-Neves, Gil, Barreiro, & Favre, 2010) but also to pomace.

It was not possible to get a complete separation of the samples by grape-variety according to their acylation pigment profile, due to a greater effect of the typology analysed (data not shown). Thus, to assess if skins, wines and pomace could be associated with a distinctive pigment profile, a canonical discriminant analysis was carried out, where the classification variable was the typology (skin, wine, pomace), and the pigment acylation profile was the discriminant variable. Two canonical functions accounted for the 100% of the total variance (function 1 explained 92,9%). An important separation among the scores of the samples obtained by each grape-variety was observed (Fig. S1b), and a perfect discrimination of all samples was obtained. Thus, skins,

Table 2
Pigment profile (molar percentages), based in the relative contribution of each anthocyanidin class to the total pigment content.

Grapes	Typology	Dp	Cy	Pt	Po	Mv
Syrah	Skins	6.67 ± 0.61 cα	1.56 ± 0.08 abα	9.10 ± 0.39 bα	17.56 ± 1.00 aα	65.11 ± 0.07 bβ
Syrah	Wines	2.19 ± 0.48 cβ	0.02 ± 0.02 bγ	6.11 ± 0.64 cβ	9.37 ± 0.59 aβ	82.32 ± 1.74 bα
Syrah	Pomace	2.53 ± 0.53 bβ	0.51 ± 0.00 bβ	5.60 ± 0.61 bβ	11.70 ± 0.12 aβ	79.67 ± 1.22 bα
Marselan	Skins	8.54 ± 0.07 cα	1.00 ± 0.02 bα	8.34 ± 0.18 bα	8.36 ± 0.04 bα	73.77 ± 0.30 aβ
Marselan	Wines	1.99 ± 0.12 cβ	0.00 ± 0.00 cβ	3.99 ± 0.22 dβ	4.63 ± 0.10 bγ	89.40 ± 0.44 aα
Marselan	Pomace	1.29 ± 0.18 bγ	0.00 ± 0.00 cβ	3.04 ± 0.17 bγ	5.06 ± 0.11 bβ	90.62 ± 0.25 aα
Tannat 1	Skins	13.43 ± 0.59 bα	1.89 ± 0.03 aα	16.96 ± 0.48 aα	5.51 ± 0.62 cα	62.16 ± 0.67 bcβ
Tannat 1	Wines	6.97 ± 0.23 bβ	0.54 ± 0.16 aβ	12.12 ± 0.04 bβ	2.85 ± 0.16 cβ	77.54 ± 0.20 cα
Tannat 1	Pomace	6.25 ± 0.74 aβ	0.57 ± 0.04 bβ	12.09 ± 0.35 aβ	3.32 ± 0.25 dβ	77.67 ± 0.81 bcα
Tannat 2	Skins	14.89 ± 0.30 abα	1.91 ± 0.34 aα	17.63 ± 0.00 aα	5.36 ± 0.55 cα	60.17 ± 1.20 cdβ
Tannat 2	Wines	9.95 ± 0.35 aβ	0.87 ± 0.10 aβ	14.09 ± 0.14 aβ	2.92 ± 0.28 cβ	72.20 ± 0.69 dα
Tannat 2	Pomace	7.01 ± 1.02 aγ	0.99 ± 0.20 a$\alpha\beta$	13.90 ± 0.96 aβ	4.37 ± 0.11 c$\alpha\beta$	73.71 ± 2.28 cα
Tannat 3	Skins	16.49 ± 0.85 aα	2.05 ± 0.05 aα	18.56 ± 0.75 aα	4.94 ± 0.23 cα	57.93 ± 1.32 dβ
Tannat 3	Wines	9.78 ± 0.47 aβ	0.76 ± 0.02 aβ	14.15 ± 0.39 aβ	2.66 ± 0.11 cγ	72.66 ± 0.77 dα
Tannat 3	Pomace	7.20 ± 0.70 aβ	1.00 ± 0.13 aβ	14.16 ± 0.52 aβ	4.05 ± 0.14 cβ	73.52 ± 0.95 cα

Data are the mean (n = 2) ± SD. Different letters in the column indicate statistical significant differences between means ($p < 0.05$) according to Tukey test. Latin letters compare the % of each anthocyanidin among the types of grapes vinified for the same typology analysed. Greek letters compare the % of each anthocyanidin among the typologies analysed (skin, wine or pomace) for a certain type of grape vinified.
*Abbreviations: Tannat 1, Tannat from vineyard 1; Tannat 2, Tannat from vineyard 2; Tannat 3, Tannat from vineyard 2 harvested 10 days later; **Dp**, delphinidin; **Cy**, cyanidin; **Pt**, petunidin; **Pn**, peonidin; **Mv, malvidin.**

Table 3
Acylation profile (molar percentages) of Syrah, Marselan and Tannat according to the typology analysed: skins, wines and pomace.

Grapes	Analysis of	Ant-3-glc	Acetylated	Coumaroylated	Caffeoylated	Feruloylated	Tot. Acylated
Syrah	Skins	60.09 ± 1.98 aγ	11.10 ± 0.05 bα	28.35 ± 2.31 bα	0.47 ± 0.28 bα	–	**39.91** ± 1.98 bα
Syrah	Wines	68.96 ± 0.04 aγ	23.20 ± 0.47 aα	7.26 ± 0.23 cα	0.60 ± 0.01 bα	–	**31.05** ± 0.23 cα
Syrah	Pomace	39.22 ± 3.06 bγ	6.41 ± 0.45 cγ	51.46 ± 3.49 aα	2.93 ± 0.01 aα	–	**60.79** ± 3.06 aα
Marselan	Skins	71.03 ± 0.83 aβ	11.77 ± 0.06 cα	16.94 ± 0.88 bβ	0.27 ± 0.11 bα	–	**28.97** ± 0.83 bβ
Marselan	Wines	73.64 ± 0.40 aγ	21.05 ± 0.02 aβ	4.91 ± 0.41 cβγ	0.41 ± 0.01 bβ	–	**26.36** ± 0.40 bβ
Marselan	Pomace	50.98 ± 1.24 baβ	16.83 ± 0.50 aα	29.63 ± 1.80 aγ	2.57 ± 0.05 aα	–	**49.02** ± 1.24 aβγ
Tannat 1	Skins	73.18 ± 0.42 aaβ	9.14 ± 0.35 bβ	17.16 ± 0.14 bβ	0.45 ± 0.05 bα	0.07 ± 0.01 aα	**26.83** ± 0.52 bβγ
Tannat 1	Wines	80.80 ± 0.22 aβ	14.03 ± 0.04 aγ	5.10 ± 0.18 cβ	0.07 ± 0.01 bδ	0.03 ± 0.01 aα	**19.21** ± 0.22 cγ
Tannat 1	Pomace	47.00 ± 2.50 bβγ	10.08 ± 0.37 bβ	41.56 ± 2.52 aβ	1.26 ± 0.28 aβ	0.12 ± 0.06 aα	**53.01** ± 2.50 aaβ
Tannat 2	Skins	73.48 ± 0.95 baβ	8.39 ± 0.23 bβγ	17.88 ± 0.66 bβ	0.20 ± 0.08 bα	0.07 ± 0.01 bα	**26.53** ± 0.95 bβγ
Tannat 2	Wines	84.79 ± 0.98 aα	10.97 ± 0.61 aδ	4.18 ± 0.38 cβγ	0.05 ± 0.01 bδ	0.02 ± 0.00 caβ	**15.21** ± 0.98 cδ
Tannat 2	Pomace	58.45 ± 1.59 cα	10.15 ± 0.62 abβ	30.32 ± 2.36 aγ	0.97 ± 0.14 aβ	0.12 ± 0.01 aα	**41.56** ± 1.59 aγ
Tannat 3	Skins	75.77 ± 0.70 bα	8.11 ± 0.01 bγ	15.91 ± 0.64 bβ	0.17 ± 0.02 bα	0.06 ± 0.01 aα	**24.24** ± 0.70 bγ
Tannat 3	Wines	85.74 ± 0.59 aα	10.38 ± 0.36 aδ	3.78 ± 0.22 cγ	0.11 ± 0.00 aγ	0.01 ± 0.00 bβγ	**14.27** ± 0.59 cδ
Tannat 3	Pomace	56.62 ± 0.65 cα	9.58 ± 0.53 abβ	32.84 ± 0.06 aβγ	0.88 ± 0.06 aβ	0.09 ± 0.01 aaβ	**43.38** ± 0.65 aγ

Data are the mean (n = 2) ± SD. Different letters in the column indicate statistical significant differences between means ($p < 0.05$) according to Tukey test. Greek letters are used to compare among skins. wines or pomace among the vinified grape. Latin capital letters indicate differences among skin wine and pomace by grape type.
*Abbreviations: Tannat 1, Tannat from vineyard 1; Tannat 2, Tannat from vineyard 2; Tannat 3, Tannat from vineyard 2 harvested 10 days later.

wines and pomace, presented each a different and distinctive acylation pigment profile (see supplementary material, Table S2 & Fig. S1b). Skins could be associated with high proportions of not acylated anthocyanins, and with proportions of coumarates higher than acetates; wines with the lowest proportions of coumarates and the highest of acetates; and pomace with the highest percentages of caffeoylated and particularly of *p*-coumaroylated derivatives (Table 3 & Table S2, see supplementary material). This shows that the pigment acyl-moiety may greatly affect the pigment extractability, and once in wine, its stability. Otherwise, skins wines and pomace would have presented similar acylation pattern. The Fig. 1abc (*e.g* picks 7, 15 and 26) and Table 2 & Table S2 (supplementary material) allows changes in the relative abundance of the non-acylated, acetylate and *p*-coumaroylated anthocyanins from skins to wines and pomace to be demonstrated. The acetylated derivatives presented significant higher proportions in wines despite making similar relative contributions in skins and pomace. This suggesting that acetates would be comparatively more stable once extracted into wine than the non-acylated counterparts. In skins, the coumaroylated pigments prevailed over the acetylated in all cases, while in wines acetates represented a much higher proportion than coumarates that accounted for less of 5% in all samples, except in those of Syrah. Finally, in pomace, coumarates reached their highest pigment proportion, representing in Syrah pomace more than 50% (Table 3). Taking as reference the main grape anthocyanin, malvidin 3-glucoside, on average, 32% of it skin content remained in pomace, compared to 85% of the *p*-coumaroylated derivative. These results give evidence of the low extractability of the *p*-coumaroylated anthocyanins from skin to wine

It was noteworthy that the contents of anthocyanin-caffeoyl derivatives were significantly higher in all 10 pomace samples than in the respective skin samples (Table 3 & Table S2 see supplementary material). These were the only pigments for which such a trend was observed without exception. The peaks corresponding to the three identified caffeoyl derivatives that is petunidin-3-*O*-(6″-caffeoyl)-glucoside, peonidin-3-*O*-(6″-caffeoyl)-glucoside and malvidin 3-*O*-(6″-caffeoyl)-glucoside, were more conspicuous in the pomace than in the skin chromatogram, as their intensity increased compared to the neighbouring peaks (Fig. 2). These results were not expected as acylation is the last step of anthocyanin synthesis, an enzymatic driven process that occurred in grape during ripening (Rinaldo et al., 2015). Therefore, the increase of the caffeoyl-derivative contents in pomace could be the result of a non-enzymatically mediated reaction. It has been demonstrated that during vinification and wine storage, anthocyanins may react with hydroxycinnamic acids, but forming hydroxyphenyl-

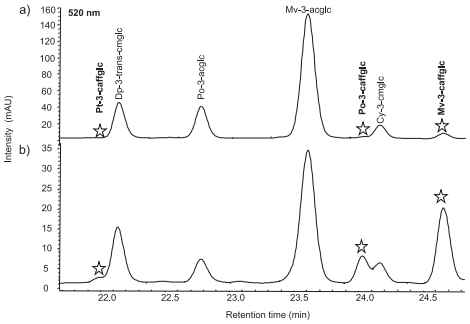

Fig. 2. Chromatographic section (DAD, detection at 520 nm) highlighting the magnitude of the caffeoylated anthocyanins relative to neighbor peaks in: (a) a skin sample; (b) a pomace sample. **Pt**, petunidin; **Dp**, delphinidin; **Po**, peonidin; **Mv**, malvidin; **Cy**, cyanidin; **acglc**, acetyl-glucoside; **cmglc**, *p*-coumaroyl-glucoside; **caffglc**, caffeoyl-glucoside; **min**, minutes.

pyranoanthocyanins (Blanco-Vega, Gómez-Alonso, & Hermosín-Gutiérrez, 2014), pigments that do not pre-exist in grapes. Alcalde-Eon et al. (2006a) speculated with the formation in wine of *p*-coumaroyl-anthocyanins, but to the best of our knowledge, synthesis of caffeoyl-anthocyanins after grape crashed onward has not been previously reported. Hydroxycinnamic acids and particularly caffeic acid, are very reactive phenols (Blanco-Vega et al., 2011; Schwarz et al., 2003). Consequently, caffeic acid as much as the caffeoylated-anthocyanins contents sharply decrease in wine in the first month after vinification (Alcalde-Eon et al., 2006a; Koyama, Goto-Yamamoto, & Hashizume, 2007). This would make the putative synthesis of caffeoyl-anthocyanins suggested here, difficult to trace, explaining the absence of previous reports on the matter. Nevertheless, the synthesis in wines of acylated anthocyanins not originally present in grapes, has been demonstrated (Alcalde-Eon et al., 2006a). These authors found that lactic acid can react in wine with the monoglucosides of the anthocyanins in an esterification reaction between the carboxyl group of the acid and the hydroxyl group in position 6″ of the sugar, originating a new compound. Future research would confirm if a process like that would explain the results here presented.

In the light of the results discussed above, *p*-coumaroylated anthocyanin synthesis during winemaking should not be discarded as an

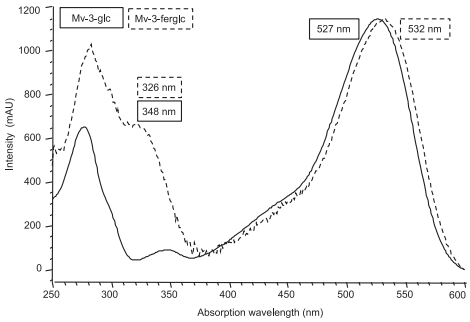

Fig. 3. UV–vis absorbance spectrum of malvidin 3-glucoside (Mv-3-glc) and malvidin 3-feruloyl-glucoside (Mv-3-ferglc).

explanation of the high percentage of *p*-coumaroylated pigments recuperated in pomace. Farther investigations should be undertaken to confirm these findings.

These results are of particular interest in grape varieties like Syrah where acylated pigments are an important proportion of their anthocyanins. Moreover, the results highlight that pomace may be an interesting source of acylated anthocyanins and malvidin based pigments, both more stables than their respective counterparts (Bakowska-Barczak, 2005; Tian et al., 2009). That is important, giving the increasing demand for food colorants from natural sources (Giusti & Wrolstad, 2003; Sigurdson, Tang, & Giusti, 2017).

Anthocyanins 3,5-diglucosides were just detected in grape skins. Unexpectedly, delphinidin 3,5-diglucoside was the main diglucoside detected, being present in all skin samples, while peonidin-3,5-diglucoside was just identified in Syrah skin samples (Table 1). Nevertheless, the main anthocyanidin, malvidin, was not detected as diglucoside in any sample, what suggests an interesting case of regioselectivity of the glucosyltransferase implied with preference towards the most hydroxylated anthocyanin, delphinidin. Interestingly, delphinidin diglucoside is the main pigment in many grape cultivars were diglucosides prevails, like in Muscadine and some hybrid grape varieties (Huang, Wang, Williams, & Pace, 2009; Rebello et al., 2013).

Direct adducts between flavan-3-ol and anthocyanin were detected in wines, but surprisingly, also in grape skins, even when at very low concentration (Table 1, Fig. 1). The synthesis of these pigments implies the reactions between the carbocations resulting from the cleavage of tannin interflavan bonds and anthocyanins (Salas et al., 2005) and is expected to occur during vinification and aging, but not in grapes. Malvidin-3-glc-gallocatechine, and peonidin-3-glc-epicatechine, were not detected in wines, while the former was also observed in pomace. Nonetheless, at lower concentration than in skins. It would be useful to study the mechanism of synthesis of these compounds in *Vitis vinifera* grapes. Others species seams to present such mechanism since tannin-anthocyanins direct-adducts have been reported in pomegranate (Sentandreu, Navarro, & Sendra 2010).

Remarkably was the presence in all Tannat skins, wines, and pomace samples of the feruloyl derivative of malvidin 3-glucoside (pick 27, Table 1, Fig. 1). The feruloyl acylation caused a bathochromical shifts in the visible absorption maxima of the anthocyanin from 527 nm to 532 nm, while the shoulder maxima change from 348 nm to 326 nm (Fig. 3). The shift at the maxima absorbance is due to the acylation of the anthocyanin, as observed in the *p*-coumaroyl derivatives, while the shift in UV part of the spectra, agrees with the presence in the molecule of the feruloyl-acyl moiety. In the MS system operated in the positive mode, this pigment gave a signal at m/z 669 corresponding to the molecular ion that farther fragmented giving at MS^2 a single ion at m/z 331, corresponding to the anthocyanidin malvidin. The loss of 338 amu may be adjudicated to a glucose moiety (162 amu) that is lost together with the ferulic acid moiety (176 amu). Under the same analytics conditions this pigment has recently been reported for the first time in grapes, in Karaerik table-grape, and further described through MS^3 experiments and the determination of their accurate mass through an online HPLC-MS-QToF system (Ayaz et al., 2017). To the best of our knowledge this is also the first time that the ferulic acid is reported acylating anthocyanins in grapes and their respective wines.

4. Conclusions

Pigment extraction during winemaking was a partial and selective process where malvidin derivatives and particularly *p*-coumaroylated anthocyanins were less extractable. Thus, pomace could be an interesting source of these pigments that are among the more stable anthocyanins. Grape varieties like Syrah with high % of *p*-coumaroylated anthocyanins, would be vinified under techniques that promote anthocyanin extraction in order to take advantage of their potential.

It is commonly accepted that the anthocyanin profile of a grape variety defines the pigment fingerprint of the respective young red wine, but as shown here, will also define that of pomace. Despite this, skin wines and pomace presented each a distinctive acylation pigment profile giving valuable information about the anthocyanin extractability during winemaking.

Our results suggest that caffeoyl-anthocyanins, and possibly the *p*-coumaroylated too, are synthetized during vinification.

An anthocyanin acylated with ferulic acid, malvidin 3-feruloyl-glucoside, was characteristic of the Tannat grape, wine and pomace pigment profile. This result also encourages to search for feruloyl-glucoside derivatives of other anthocyanidins and in more grape varieties.

The synthesis of caffeoyl-glucoside derivatives during the vinification process, important differences in anthocyanin extractability depending on their molecular structure, and the presence of a feruloyl-anthocyanin in Tannat samples, were among the main findings achieved. Results could be of scientific and technological relevance.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.10.085.

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3.8. SUPLEMENTARY MATERIAL

Table S1. General characteristics and phenolic potential of the grapes at harvest

Grape	Harvest date	Sugars (g/L)	Acidity	pH	ApH 1.0
Syrah	12/02/2015	216 ± 1.1 e	4.13 ± 0.00 a	3.64 ± 0.03 d	1404 ± 73 d
Marselan	20/02/2015	232 ± 1.1 c	6.97 ± 0.12 d	3.33 ± 0.03 b	1777 ± 73 c
Tannat 1*	2/03/2015	250 ± 1.6 a	5.56 ± 0.08 bc	3.46 ± 0.03 c	2383 ± 127 b
Tannat 2	20/02/2015	221 ± 1.1 d	5.63 ± 0.08 c	3.26 ± 0.02 a	2542 ± 41 b
Tannat 3	2/03/2015	245 ± 2.6 b	5.43 ± 0.09 b	3.40 ± 0.02 c	3172 ± 89 a

Data are the mean (n = 2) ± SD. Different letters in the column indicate statistical significant differences between means ($p < 0.05$) according to Tukey test.

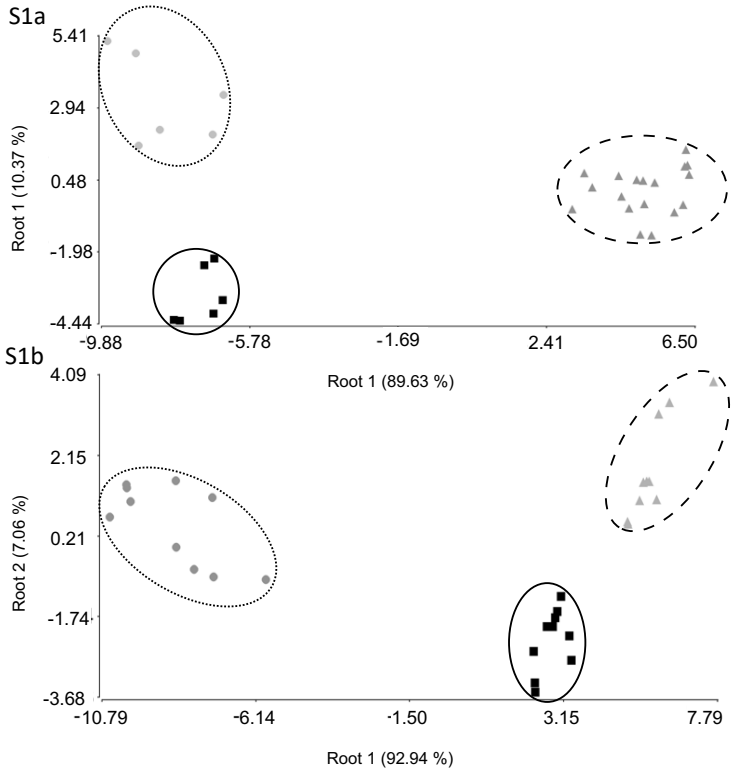
* Abbreviation: Tannat 1, Tannat from vineyard 1; Tannat 2, Tannat from vineyard 2; Tannat 3, Tannat from vineyard 2 harvested 10 days later.; Acidity was determined by titration, and is expressed as g/L of tartaric acid, sugars were determined by refractometry, pH by potentiometry. ApH 1.0 = total anthocyanin potential according to Glories and Augustin (1993) with modification of González-Neves (2004b).

Table S2. Acylation profile, expressed in molar percentages, of skin wines and pomace as mean of all the treatments considered

Typology	Non-Acylated	Acetylated	Caffeoylated	Coumaroylated	Feruloylated
Skin	70.71 ± 5.87 a	9.70 ± 1.55 b	0.31 ± 0.17 b	19.25 ± 4,92 b	0.04 ± 0.04 ab
Wines	76.11 ± 6,93 a	15.45 ± 5,29 a	0.24 ± 0.23 b	4.85 ± 1.26 c	0.01 ± 0.01 b
Pomace	50.45 ± 7.46b	10.61 ± 3.61 b	1.71 ± 0.91 a	37.16 ± 8.94 a	0.06 ± 0.06 a

Data are the mean (n = 10) ± SD. Different letters in the column indicate statistical significant differences between means ($p < 0.05$) according to Tukey test.

Figure S1. Canonical discriminant analysis of a) the grape varieties according to the molar pigment profile of the samples (skin, pomace and wine) based on the percentage of each of the five anthocyanin classes to the total pigment content. Syrah ●…..; Marselan ■ ____; Tannat ▲ --- and b) the typology analyzed according to their acylation pigment profile, wine ●…..; skin ■ ____; pomace ▲ ---



4. STILBENE PROFILES IN GRAPES AND WINES OF TANNAT, MARSELAN AND SYRAH FROM URUGUAY[2]

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4.1. ABSTRACT

This work reports the stilbene content and profile (*cis* and *trans* resveratrol, *cis* and *trans* piceid) of grapes and wines of three *Vitis vinifera* varieties, Tannat, Marselan and Syrah from Uruguay. The effect of delaying the harvests and wine storage-time in stilbene contents was evaluated aswell. Two vintages were considered, 2015 and 2016, and two harvest dates around technological maturity were evaluated, once in each cultivar. Red-winemaking was performed in duplicate under traditional maceration. After SFE, stilbenes were analysed through an HPLC-ESI-MS/MS system. Stilbene contents were between 1.6 and 7.7 mg/kg of grape berries, depending on grape cultivar, growing season, and in Syrah, on harvest date. That was the only cultivar with a very significant increase of stilbene synthesis between harvest dates, which denotes characteristics of a high stilbene producer cultivar. In wines, stilbene contents were between 0.6 and 14.0 mg/L, Syrah presenting the highest registers, Marselan the least and Tannat in between these. Marselan wines presented much lower stilbene contents than the expected from the concentrations in their grapes. Thus, extraction during winemaking can be a key factor of the wine stilbenes content. Total stilbene concentrations remained very stable during the analytical period, from wine stabilization to 24-month later.

Keywords: resveratrol, piceid, grapes, red-wine, Tannat, Syrah, Marselan

[2] Artículo en formato del Journal Foods (MDPI) https://www.mdpi.com/journal/foods

4.2. INTRODUCTION

Resveratrol is a phytoalexin with a wide range of pharmacological properties [1,2]. It occurs in different plant families including *Vitaceae* [3], where is synthetized constitutively [4] but mainly in response to biotic and abiotic agents [5,6] The *trans*-resveratrol form (3,5,4'-trihydroxy-*trans*-stilbene) is the metabolic precursor and structural core of different stilbenoids [5] such as *cis*- and *trans*- piceid [7], viniferins, pterostilbene [8,9] and piceatannol [10]. Resveratrol and their derivatives have raised attention because of their wide range of chemopreventive effect against different deceases, and its potential therapeutic use [11]. Resveratrol interfere with the ion transport and associated redox processes [12], characteristics that have been identified as the responsible of its activity against pathogens in plants and would be behind their potentiality treating human diseases [13]

The majority of studies conducted have shown that stilbenes are synthetized constitutively at only very low levels, but strongly accumulate in response to a wide range of biotic and abiotic stresses [6]. Thus, a wide range of stilbene concentrations have been reported in healthy grapes depending on grape cultivar, growing-region, exposure to elicitors, etc [4,14–16]. Therefore, it is important to have references of the synthesis potential of healthy grape-berries belonging to different cultivars in different growing regions. The stilbenes synthetized in the grape berries skin, have to be extracted into must and preserved in wine to be able to reach the consumer. Thus, in this work we report for the first time to the best of our knowledge the stilbene contents in healthy grapes of Syrah, Marselan and Tannat cultivated in the south of Uruguay, and in their respective red-wines. The stability of these compounds during wine aging, from stabilization up to 24 months later was also evaluated. Thus, covering the period of time in which most red-wines use to be consumed. The effect of delaying the harvest after technological maturity (criteria of the vintner's harvest) on the stilbene content was also considered.

4.3. MATERIAL AND METHODS

4.3.1. Experimental design

The experiments were performed from *Vitis vinifera* grape-varieties Tannat, Syrah and Marselan cultivated in commercial vineyards located in the south of Uruguay. Two vintages were considered, 2015 and 2016. When the weather conditions allowed it, a second harvest delayed from that made at technological maturity (according to the winegrowing criteria) was also made. In the year 2015, the cultivar Tannat was represented for grapes taken from 2 different vineyards, one of which was harvested on different dates. In the year 2016, just one vineyard and harvest of Tannat was considered, while two harvest at different dates were made for Syrah and Marselan (Table 1).

Table 1. General characteristics and phenolic potential of the grapes at harvest

Grape	**Harvest date**	**Sugars (g/L)**	**Acidity**	**pH**
Syrah	12/02/2015	216 ± 1.1 e	4.13 ± 0.00 a	3.64 ± 0.03 d
Marselan	20/02/2015	232 ± 1.1 c	6.97 ± 0.12 d	3.33 ± 0.03 b
Tannat 1	2/03/2015	250 ± 1.6 a	5.56 ± 0.08 bc	3.46 ± 0.03 c
Tannat 2 H1	20/02/2015	221 ± 1.1 d	5.63 ± 0.08 c	3.26 ± 0.02 a
Tannat 2 H2	2/03/2015	245 ± 2.6 b	5.43 ± 0.09 b	3.40 ± 0.02 c
Syrah H1	22/02/2016	196 ± 3.0 d	5.79 ± 0.04 c	3.39 ± 0.01 b
Syrah H2	01/03/2016	216 ± 1.1 b	4.74 ± 0.06 e	3.47 ± 0.06 a
Tannat 1	09/03/2016	208 ± 1.7 c	7.42 ± 0.00 a	3.16 ± 0.03 c
Marselan H1	03/03/2016	249 ± 3.0 a	6.05 ± 0.11 b	3.36 ± 0.01 b
Marselan H2	09/03/2016	253 ± 1.9 a	5.29 ± 0.13 d	3.39 ± 0.01 b

Data are the mean (n = 2) ± SD. Different letters in the column indicate statistically significant differences between means ($p < 0.05$) according to Tukey test.

* Abbreviation: Tannat 1, Tannat from vineyard 1; Tannat 2, Tannat from vineyard 2; Tannat 3, Tannat from vineyard 2 harvested 10 days later.; Acidity was determined by titration, and is expressed as g/L of tartaric acid, sugars were determined by refractometry, pH by potentiometry. ApH 1.0 = total anthocyanin potential according González-Neves et al. (18).

Thus, a total of five harvest were made each year. The harvest ware made by hand, and the clusters were carefully transported in plastic boxes (20 kg each one) to the university experimental winery. There, two batches of grapes of 70kg each were randomly separated from each harvest for vinification. Just before crushing, a sample

of 100 grapes were collected from each batch, taking cluster parts from 3 to 5 berries from different parts of randomly chosen bunches. To avoid bias by size or aspect, the berries were scattered over a plain surface with a numbered grid of 5 × 5 cm squares. An order of squares was randomly chosen, and then all the grapes placed inside each square were chosen until 35 grapes were collected. Each 35-berry sample was then peeled, the pulp remaining against the skins carefully removed with the help of rounded edge blade, gently blotted with a paper towel, weighted (fresh-weight) and stored in a sample-bag at −18°C until freeze-dried. Skins were freeze-dried, weighted (dry- weight) and then stored at −18 °C again in bags containing silica gel. The general characteristics and phenolic potential of the grapes belonging to each harvest were obtained from additional duplicate samples of 250 grapes each, which were analysed within 24 h (data presented in Table 1). Then, each batch of 70 kg of grapes was vinified, totaling thus, 10 vinification by year. Grapes were destemmed and crushed with an Alfa 60 R crusher (Italcom, Italy), and barrelling was made in stainless-steel tanks (100 L capacity each). Potassium metabisulfite (50 mg SO_2/100 kg of grapes) was added and dry active yeast (*Saccharomyces cerevisiae ex bayanus* Natuferm 804; OenoBioTech, Paris, France) was inoculated (20 g/kg of grapes). Wines were made with a classical fermentation on skins (maceration simultaneously with alcoholic fermentation) for 8 days in Marselan and Tannat, and 7 days in Syrah, one day less because of its lower phenolic potential according the proposed by González-Neves et al.[17,18]. Along macerations, two pumpings over followed by punching the cap were carried out daily until pressing. At devatting, the fermentation was finished in all cases. The pressing was carried out with a stainless-steel manual press. Free- run juices and press juices were mixed, separated from lees, stabilized by adding a dose of 50 mg/L of SO_2 and kept in glass recipients of 10-L.

4.3.2. Analytical procedures

4.3.2.1. Chemicals

Analytical determinations were made at the Laboratory of Instrumental Analysis placed at the Regional Institute of Applied Scientific Research (IRICA), Castilla La Mancha University, Spain, and in the Institute of Vine and Wine of

Castilla-La Mancha, Spain. All solvents used were HPLC quality, and all chemicals were analytical grade (>99%). Water was Milli-Q quality.

4.3.2.2. Skin and pomace extracts

Freeze-dried skins (10 samples each year) were extracted each with 100 mL of a mixture 50:48.5:1.5 (v/v) of CH3OH/ H2O/HCOOH, using a homogenizer (Heidolph DIAX 900) to 10000 rpm for 3 min and then centrifuging at 2500g at 5 °C for 5 min. The supernatant was separated and conserved, and the pallet was extracted again two more times. The three-supernatant obtained were mixed, the volume registered, and then stored at −18°C until analysis. Previous works made in similar conditions, confirmed that a two times extraction of the grape skin pellet yielded nearly 99% of the grape polyphenol contents able to be extracted with successive repetitions of the cited protocol [19]. The *trans*-resveratrol was purchased from Sigma Aldrich (Tres Cantos, Madrid, Spain). The *cis* isomers of resveratrol and its 3-glucosides (piceid) were generated from their respective *trans* isomers by UV irradiation (366 nm light for 5 min in quartz vials) of 25% MeOH solutions of the trans isomers.

4.3.2.3. Analysis of stilbenes by HPLC–DAD–ESI–MS^n

The analysis was achieved using a HPLC Agilent 1200 series system equipped with DAD (Agilent, Germany) and coupled to an AB Sciex 3200 TRAP (Applied Biosystems) with triple quadrupole, turbo spray ionization (electrospray assisted by a thermonebulization) mass spectroscopy system (ESI-MS/MS). The chromatographic system was managed an Agilent ChemStation (version B.01.03) data-processing unit, and the mass spectra data was processed using the Analyst MSD software (Applied Biosystems, version 1.5).

The samples were injected (20 μL) onto an Ascentis C18 reverse phase column (150 mm x 4.6 mm with 2.7 μm of particle size), with the temperature controlled at 16 °C. The solvents and gradients used for this analysis and the two MS scan types used (Enhanced MS—EMS and Multiple Reaction Monitoring— MRM) as well as all the mass transitions (*m/z*) for identification and quantitation were according to the methodology reported by Lago-Vanzela et al.[20]. Briefly, the solvents were methanol, water, formic acid (2: 97: 1 v/v/v, solvent A; 100:0:0 v/v/v,

solvent B), and the flow rate 0,30 mL/min. The gradient for solvent B was: zero min, 95%; 2 min, 95%; 25 min 70%; 40 min 45%; 50 min, 35%, 55 min, 5%; 65 min, 5%; 70 min 95%; 80 min 95%. The samples were injected at 10 µL.

4.3.3. Statistical data analysis

Results were subjected to ANOVA test with separation of medias through Tukey test (significant level 0.05). When the grape variety or grape origin (vineyard, date of harvest) was considered, media comparisons was carried out on 2 replicates. The program used was Infostat (2016, Professional version).

4.4. RESULTS AND DISCUSSION

4.4.1. Stilbenes in grape skin

Table 2 present the stilbene concentrations on a skin mass basis, what allows to analyze the synthesis capacity of the grapes, and on a grape berries mass basis, an expression more suitable for enological and practical considerations.

In the year 2015, Syrah skin stood out by presenting very high stilbene concentrations, much higher than the registered in the other grapes studied. The latter showing similar capacity of stilbene synthesis. This, despite being grapes from Marselan and Tannat, and in the latter, collected from two different vineyards and in one of them, in two different dates.

Table 2

Treatment	Total mg/kg (Sk.)	Total mg/kg (grape)	Stilbene molar profile (%)			
Year 2015			*t*-resveratrol	*c*-resveratrol	t-piceid	c-piceid
Syrah	109.4 ± 37.7 a	7.65 ± 2.99a	53.8 ± 2.7ab	3.0 ± 0.2 a	25.4 ± 6.7 a	17.8 ± 3.8 a
Marselan	29.4 ± 7.1 b	3.24 ± 0.74 ab	56.8 ± 1.1 ab	0.0 ± 0.0 b	24.5 ± 1.2 a	18.7 ± 0.2 a
Tannat 1	21.1 ± 11.7 b	1.73 ± 1.14 ab	62.5 ± 5.4 a	0.0 ± 0.0 b	28.1 ± 3.87 a	9.4 ± 1.5 b
Tannat 2 H1	23.0 ± 1.9 b	1.59 ± 0.11 b	54.6 ± 0.1 ab	0.0 ± 0.0 b	36.3 ± 0.2 a	9.1 ± 0.1 b
Tannat 2 H2	24.6 ± 5.9 b	2.11 ± 0.64 ab	47.1 ± 0.0 b	0.0 ± 0.0 b	36.9 ± 1.1 a	16.0 ± 1.6 ab
Year 2016						
Syrah H1	34.6 ± 4.3 a	2.55 ± 0.32 a	36.1 ± 4.5 a	4.2 ± 2.0 a	46.5 ± 6.9 a	13.3 ± 0.3 a
Syrah H2	61.9 ± 42.1 a	5.29 ± 3.71 a	38.5 ± 3.2 a	4.2 ± 2.2 a	45.8 ± 4.3a	11.5 ± 3.3 a
Tannat 1	35.1 ± 15.5 a	2.57 ± 0.81 a	29.2 ± 4.4 a	0.0 ± 0.0 b	63.4 ± 3.8 a	7.3 ± 0.6 a
Marselan H1	16.5 ± 2.3 a	1.73 ± 0.31 a	41.5 ± 2.2 a	0.0 ± 0.0 b	41.5 ± 2.3 a	17.1 ± 0.1 a
Marselan H2	18.9 ± 2.8 a	2.08 ± 0.41 a	40.8 ± 7.5 a	0.0 ± 0.0 b	43.7 ± 12.3 a	15.5 ± 4.8 a

Content in total stilbenes expressed in mg/kg of fresh skin (Sk.) and in mg/kg of grape berries (grape), and molar profile of the different forms of resveratrol analyzed according to the grape vinified. Tannat 1 and 2 = grapes belonging to two different nearby vineyards; H1 and H2 first and second harvest respectively.

In 2016, Tannat skins presented a similar stilbene concentration than the Syrah samples from the firs harvest, while those of Marselan registered the fewest contents despite the harvest considered. Thus, in Marselan there was not a significant evolution of the stilbene synthesis between harvest dates, matching the observed in Tannat the year before. Conversely, in Syrah these compounds accumulated at high rate between harvest presenting the skin from the second one the highest contents registered in the year. [4] found that grape genotypes that were higher resveratrol producers presented the highest synthesis of transcripts related to the enzymes involved in stilbene synthesis, thus, allowing high accumulation rates during maturation. Our results in Syrah seems in light with those findings. Anyhow, the high variability obtained between samples do not allow in all cases to obtain statistical differences. Such variability is expected as may be observed in other works [4] because this kind of compounds are synthetized constitutively at very low concentration, and factors eliciting their synthesis may inside differently, even from one berry to the other. Nevertheless, [4] identified a varietal component of variation due to grape variety as also suggest our results. These authors proposed a cultivar classification in higher stilbene producers (those presenting more than 2,3 mg per kg of grape berries of these compounds) and lower once (those with stilbene contents at

harvest between 0.2-1.8 (mg/kg). In our studies, just Syrah presented in all cases stilbene contents higher than 2,3 mg per kg of grape berries, while Marselan and Tannat, presented values higher and lower than such thresholds depending on the sampled considered.

Table 3

Wine (mg/L)	*Analysis at:	Total	*t*-resveratrol	*c*-resveratrol	*t*-piceid	*c*-piceid
Syrah	0	4.97 ± 1.75 a	2.02 ± 0.60 a	0.61 ± 0.23 a	0.84 ± 0.29 a	1.51 ± 0.64 a
	12	4.48 ± 1.13 a	1.57 ± 0.25 a	0.81 ± 0.59 a	1.05 ± 0.16 a	1.06 ± 0.13 a
	24	4.71 ± 0.26 a	1.58 ± 0.13 a	0.68 ± 0.35 a	1.32 ± 0.18 a	1.14 ± 0.04 a
Marselan	0	0.91 ± 0.18 a	0.45 ± 0.04 a	0.19 ± 0.06 a	0.04 ± 0.02 a	0.24 ± 0.07 a
	12	0.57 ± 0.17 a	0.20 ± 0.11 ab	0.34 ± 0.01 a	0.01 ± 0.01 a	0.03 ± 0.04 b
	24	0.57 ± 0.09 a	0.17 ± 0.01 b	0.40 ± 0.09 a	0.00 ± 0.00 a	0.00 ± 0.00 b
Tannat 1	0	2.04 ± 0.38 a	0.84 ± 0.06 a	0.21 ± 0.06 a	0.45 ± 0.14 a	0.55 ± 0.12 a
	12	2.30 ± 0.46 a	0.68 ± 0.11 a	0.18 ± 0.01 a	0.84 ± 0.21 a	0.60 ± 0.15 a
	24	2.77 ± 0.56 a	0.72 ± 0.13 a	0.21 ± 0.01 a	1.05 ± 0.26 a	0.79 ± 0.18 a
Tannat P H1	0	2.13 ± 0.03 b	0.80 ± 0.01 a	0.30 ± 0.06 a	0.44 ± 0.06 b	0.61 ± 0.04 a
	12	2.69 ± 0.03 a	0.78 ± 0.02 a	0.23 ± 0.00 a	0.85 ± 0.08 a	0.84 ± 0.10 a
	24	2.61 ± 0.21 ab	0.73 ± 0.03 a	0.21 ± 0.03 a	0.86 ± 0.13 a	0.81 ± 0.08 a
Tannat P H2	0	2.87 ± 0.06 a	1.07 ± 0.00 a	0.23 ± 0.07 a	0.73 ± 0.01 b	0.85 ± 0.01 a
	12	3.38 ± 0.33 a	0.92 ± 0.10 a	0.16 ± 0.01 a	1.28 ± 0.12 a	1.02 ± 0.11 a
	24	3.44 ± 0.28 a	0.87 ± 0.08 a	0.14 ± 0.01 a	1.40 ± 0.09 a	1.04 ± 0.09 a

Evolution of the stilbene content throw time in 2015 wines. Tannat 1 and 2 = grapes belonging to two different nearby vineyards; H1 and H2 first and second harvest respectively. *Time elapsed (in month) from the first analytical determinations (made at 3 months after pressing).

Table 4

Wine (mg/L)	*Analysis at:	Total	*t*-resveratrol	*c*-resveratrol	*t*-piceid	*c*-piceid
Syrah H1	0	5.82 ± 0.21 b	1.89 ± 0.15 b	1.04 ± 0.14 a	0.97 ± 0.04 b	1.93 ± 0.16 b
	12	6.57 ± 0.21 b	1.94 ± 0.04 b	1.08 ± 0.08 b	1.35 ± 0.02 b	2.20 ± 0.06 b
Syrah H2	0	12.38 ± 0.40 a	4.10 ± 0.30 a	1.66 ± 0.16 b	2.34 ± 0.08a	4.28 ± 0.18 a
	12	13.96 ± 0.52 a	4.12 ± 0.21 a	1.92 ± 0.18 a	3.28 ± 0.00 a	4.64 ± 0.13 a
Tannat 1	0	5.69 ± 0.69 b	1.87 ± 0.19 b	0.75 ± 0.01 b	1.14 ± 0.23 b	1.93 0.28 b
	12	6.23 ± 0.64 b	1.77 ± 0.31 b	0.63 ± 0.01 c	1.60 ± 0.16 b	2.23 0.18 b
Marselan H1	0	1.34 ± 0.08 c	0.49 ± 0.01 c	0.20 ± 0.03 c	0.18 ± 0.01c	0.46 ± 0.01 c
	12	1.39 ± 0.16 c	0.42 ± 0.05 c	0.21 ± 0.07 d	0.26 ± 0.00 c	0.51 ± 0.04 c
Marselan H2	0	1.73 ± 0.01 c	0.63 ± 0.01 c	0.26 ± 0.06 c	0.22 ± 0.07 c	0.62 ± 0.03 c
	12	1.76 ± 0.40 c	0.52 ± 0.14 c	0.25 ± 0.01 d	0.33 ± 0.08 c	0.67 ± 0.17 c

Evolution of the stilbene content throw time in 2016 wines. H1 and H2, first and second harvest respectively. *Time elapsed (in month) from the first analytical determinations (made at 3 months after pressing).

Nonetheless, more studies will be necessary to conclude over the behavior of these cultivars under the growing conditions of the south of Uruguay. In the context of other regional studies, the stilbene concentrations reported here are much higher than those presented by other authors [21,22]. Nevertheless, the use in some cases of different analytical procedures difficult make comparisons.

4.4.2. Stilbene profile of skin

Resveratrol exists in two isoforms, *cis* and *trans,* with the respective glucosides named *cis* and *trans* piceid [2]. As appreciated in Table 2, in samples from 2015, the free resveratrol form tended to be more important than the glucosides, while in 2016 the opposite trend was observed. Resveratrol glucosides would be preferentially expressed constitutively, while trans-resveratrol would be the inducible form [4]. Then, 2016 grapes would contain a lesser proportion of inducible stilbenes what is consistent with the lesser total concentrations mostly found such year related to 2015. [23] reported that grapes with no apparent fungal infection contained similar amounts of *trans*-resveratrol and *trans*- and *cis*-piceid, but infected grapes showed a much higher *trans*-resveratrol proportion. In our essays, all grapes considered look healthy, and even when infected berries initially would not show fungus signs, both years, 2015 and 2016, were particularly dry during the grape maturity period with very low incidence of rots in the vineyards. Then, this would be not a factor behind the differences here reported.

Another observation that could be made was that *trans* piceid was in all cases, the dominant form of the glucosides, particularly in year 2016.

In grapes, the *cis* isomer of resveratrol is usually not reported. Nevertheless, is cited that it could be only slightly detectable if so [24]. Later in wines, its presence mainly responds to the isomerization process that occurs because of factors like UV radiations [2]. But, interestingly, the *cis* isomer of resveratrol was found in all the skin samples of Syrah, in what could be a varietal characteristic.

Some authors have also reported absence of the *cis* isomer of piceid in grape varieties like Syrah [25]. Clearly in our studies it was represented by significant proportions of all stilbenes in the three cultivars considered in the experiments, including Syrah.

Important differences in the stilbene profile were detected between years in line with findings of other authors [26], while it was not possible to adjudicate other difference that the already mentioned due to grape cultivar or harvest date if the data from the two vintages are considered.

Some investigations have suggested that trans resveratrol present higher biological activity than the cis form, that because the lower steric hindrance of its substituents [27]. But a more extensive review of studies shows that each stilbene form has some featured property depending of the context on what it is assessed, while in other cases all forms present similar behavior [28].

4.4.3. Stilbene in wines

Syrah wines presented the highest stilbene contents both years. In 2016, those correspondent to Syrah second harvests, presented much higher stilbene concentration than those elaborated from the first harvested grapes, thus confirming the observed in the correspondent skin samples (Table 3 & 4).

Overall, most wines presented stilbene contents that are in the range of concentrations reported for commercial wines from different regions of South America like San Francisco valley (Pernambuco, Brazil), Rio Grande do Sul (Brasil), Argentina (Mendoza & San Juan) [14,29], or Maule region in Chile [30]. In the Syrah wines of the current investigation the concentrations found were even higher (particularly in 2016) while in Marselan tended to be lowers. This is also valid in a broader context. [31,32] cite concentrations below 4.3 mg/L of *trans*-resveratrol in wines produced in the southeast of Italy in line with most published works. Anyway, contents as high as 36 mg/L has been exceptionally reported in Mencia red wines, but that would be the result of an interaction between the cultivar and the zone of production (Galician region of Valdeorras) [33,34]. Nevertheless, it should be considered when making comparisons that the different works publish data acquired by different analytical procedures and that also may include the determination of different types of the stilbene family. Interestingly, Marselan wines presented much less stilbene contents than Syrah but also than Tannat, difference that cannot be explained for the contents found in the respective grapes. A possible explanation is that these compounds were poorly extracted from grape to wine in Marselan. In fact,

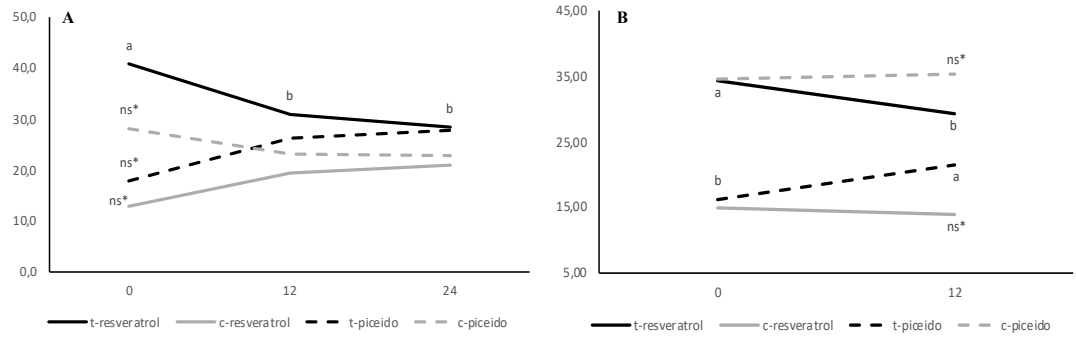
we have observed a similar behavior in other classes of polyphenols in this cultivar as well (data not shown). This result highlight that, independently of the stilbene abundance in the grapes, their extractability could be determinant of their contents in wine. Such constrain would be more or less appreciated depending on the grape-cultivar. In line with these observations, a previous work has reported very low rate of stilbene extraction during maceration [25].

Overall results expose different factors affecting red-wine resveratrol contents derived from healthy grapes, like grape variety, growing season, the easiness of extraction from grape to wine, and, at least in Syrah, the maturity of the grape berries at harvest.

Total stilbene content remained very stable over time. Indeed, there was not significant differences in their concentrations over the evaluation period in any of the wines considered (Table 3 & 4). Such stability (not found in other polyphenol classes) is interesting considering that their enological relevance, is being part of red-wine bioactive compound pool. Furthermore, the results show that the stilbene content analyzed just after wine stabilization could be representative of that reaching a potential consumer, considering that most wines are consumed young, in the analytical period covered for this investigation (until 24 months after stabilization).

While wine total stilbene content was stable over time, the relative abundance of the four forms analyzed was not (Fig. 1ab). This is expected, mainly as result of the glucoside moiety hydrolysis, and of *trans/cis* isomerization [2,35].

Fig. 1



Evolution of the mean stilbene profile of the wines over time (A) 2015 and (B) 2016 wines. Molar percentages calculated from the amounts shown in Tables 3 and 4.

The results of the wines elaborated in the 2015 vintage are those that allows to appreciate the evolution of the stilbene profile over the longer period of time, 24 months. Those from 2016 were followed just the half of time, 12 months (Fig. 1ab). Despite that the low concentrations of the minority forms difficulted in some cases to obtain statistical differences among analytical moments, a general trend was observed. The *trans*-resveratrol decreases with time concomitant an increase of the *cis* form, what is result of the isomerization that take place in wines during storage in accordance with literature [25]. Nevertheless, in the glucosylated forms, was the *trans* isomer who increased in relative abundance.

4.5. CONCLUSIONS

The stilbene content of wines depended on characteristics of grape variety like their synthesis capability, and the easiness of their extraction during winemaking. The first was clearly appreciated in Syrah, a variety that showed a much higher capability of stilbene synthesis than Marselan and Tannat, while the second one was evident in Marselan, since their wines presented much lower stilbene concentrations than the expected based on that in their grapes. Thus, it could be interesting to evaluate in Marselan maceration techniques seeking to increase phenolic extraction, with stilbene as part of the extraction efficiency indicators. Delaying the harvest time could make a great impact on grape stilbene content or not, probably depending on grape variety. Stilbene content in wine showed to be

very stable during wine storage, at least in during the time evaluated in this investigation (from wine stabilization up to 24 month later) which is also the period in which most red wines are consumed.

To the best of our knowledge, these are the first reports of resveratrol and its glucosides in grapes and wines from Uruguay.

4.6. ACKNOWLEDGEMENTS

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5. NEW ACYLATED FLAVONOLS IDENTIFIED IN *VITIS VINIFERA* GRAPES AND WINES

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New acylated flavonols identified in *Vitis vinifera* grapes and wines

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ABSTRACT

Flavonols are a class of polyphenol compounds whose importance for wine quality has increased as their structures and properties have become better understood. Here, the acetylated and *p*-coumaroylated derivatives of the flavonol 3-*O*-glucosides of isorhamnetin, laricitrin and syringetin have been identified for the first time in *Vitis vinifera* grape skins and wines. First, the MS^2 fragmentation patterns of the new flavonol derivatives showed a main signal attributable to the expected flavonol aglycone. In the *p*-coumaroylated derivatives, the signal corresponding to the intermediate loss of the phenolic acid was also observed. The structures of the aglycones were confirmed by their respective MS^3 experiments that matched with those obtained from authentic standards of the aglycones. In addition, the fragmentation signals corresponding to the aglycone radical ions generated through homolytic cleavage assisted identification, and could support future studies of flavonoid compounds by ESI-MS. Using an HPLC-ESI-Q-ToF system, the observed *m/z* values of the compounds being studied were successfully matched with the expected formula. Surprisingly, just the minority methoxylated flavonol glucosides presented acylation, suggesting a high substrate specificity of the acyltransferases implicated in their synthesis. These findings show higher diversity of grape and wine flavonols. Additional studies and isolation strategies need to be followed to further characterize these metabolites as to test their presence in other grape varieties and it wines.

1. Introduction

Flavonols are a class of flavonoid compounds derived from plant secondary metabolism. There, they act as internal regulators, antioxidants, UV screeners (Agati et al., 2013; Kaffarnik, Heller, Hertkorn, & Sandermann, 2005; Liu et al., 2014; Martinez et al., 2016), etc. In grapes, they are present in the skins, and they are extracted into must during the maceration stage of red winemaking. Compared to other flavonoid compounds such as anthocyanins and proanthocyanidins, flavonols occur in lesser concentrations (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2007; Fanzone, Zamora, Jofré, Assof, & Peña-Neira, 2011). Nevertheless, they have increasingly received consideration by the scientific community and winemakers because of their properties that are becoming known. Their main importance for red wines is due to their effect in copigmentation (Boulton, 2001; Gordillo, Rodríguez-Pulido, Escudero-Gilete, González-Miret, & Heredia, 2012), considered the most efficient mechanism for anthocyanin chromophore stabilization in nature and food systems (Cavalcanti, Santos, & Meireles, 2011; Gordillo et al., 2012). In red wines, the effects are color enhancement (quantity and quality) and stabilization since copigments act as a starting point for reactions that lead to long-term color preservation (Cheynier et al. 2006, Boulton, 2001). Flavonols may also contribute to wine astringency (Ferrer-Gallego et al., 2016) and are important bioactive compounds, with antioxidant-antiradical activity and potential actions against processes that cause different illnesses (Gómez-Alonso et al., 2012; Hilbert et al., 2015). The flavonol profile of fruits may be also used as biochemical markers to evaluate the authenticity of food products (Moussa-Ayoub, Youssef, El-Samahy, Kroh & Rohn, 2015).

In grapes, flavonols are present mainly as 3-*O*-glycosides, whereas the corresponding free aglycones can be further found in wines together with the 3-*O*-glycosides because of the acid hydrolysis that occurs during winemaking and aging. Castillo-Muñoz et al. (2009) reported that grape flavonols comprise three complete series including glucosides, galactosides and glucuronides of the six possible aglycones named kaempferol, quercetin, isorhamnetin, myricetin, laricitrin and syringetin (Fig. 1), together with the quercetin 3-*O*-rutinoside. *O*-dihexosides have also been found in hybrid grapes (De Rosso et al., 2014).

In grapes and wines, glucosides are the main flavonol derivatives, while galactosides and glucuronides are found as minor compounds,

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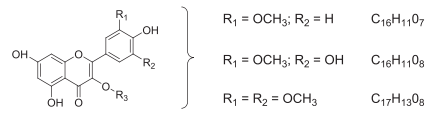

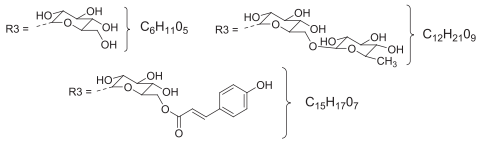

flavonol aglycone	R_1	R_2	R_3
kaempferol	H	H	H
quercetin	OH	H	H
isorhamnetin	OCH_3	H	H/acetyl /*p*-coumaroyl
myricetin	OH	OH	H
laricitrin	OCH_3	OH	H/acetyl /*p*-coumaroyl
syringetin	OCH_3	OCH_3	H/acetyl /*p*-coumaroyl

Fig. 1. Structure and molecular formula of flavonol glucosides and their aglycones.

except for quercetin-3-*O*-glucuronide that may be as abundant as the glucoside compound (Castillo-Muñoz et al., 2009). At the same time, kaempferol, and the methoxylated flavonols laricitrin, isorhamnetin and syringetin appear in much smaller concentrations than the more hydroxylated forms, myricetin and quercetin (Castillo-Muñoz et al., 2009; Castillo-Muñoz et al., 2007; Mattivi, Guzzon, & Vrhovsek, 2006; Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006).

Mass spectrometry, particularly tandem mass spectrometry and high-resolution mass spectrometry, are important tools in the structural elucidation of flavonoid derivatives (Davis & Brodbelt, 2008; Fabre, Rustan, De Hoffmann, & Quetin-Leclercq, 2001; Justesen, 2001; La Barbera et al., 2017; Panighel, De Rosso, Dalla Vedova, & Flamini, 2015; Parejo, Jáuregui, Viladomat, Bastida, & Codina, 2004).

In the case of flavonols, Castillo-Muñoz et al. (2009) have shown that using the negative ionization mode, the flavonol 3-*O*-glycosides gave stable deprotonated ions, which were only present in the MS spectra, and further underwent the loss of the 3-*O*-glycoside moiety in the ion trap, giving rise to the corresponding flavonol aglycone related ion ($[Y_0]^-$), which is the base peak in the MS^2 spectra. The latter aglycone ion is formed through heterolytic cleavage of the glycosidic bond; however, this bond can also be fragmented by homolytic cleavage, giving rise to an aglycone radical ion ($[Y_0{-}H]^{-\cdot}$) having one less unit with regards to the *m/z* value of the related aglycone ion (Davis & Brodbelt, 2008; Hvattum & Ekeberg, 2003), which can usually be observed together with the signal of the aglycone ion.

In this work, for the first time to the best of our knowledge, we present the identification of the complete series of acetylated and *p*-coumaroylated derivatives of the methoxylated flavonol 3-*O*-glucosides in grapes and their respective wines. The process used for their identification is thoroughly described, and the fact that they have been found in the methoxylated flavonols and not in the majority one is discussed.

Table 2
General composition of the studied grapes at harvest.

Grape	Harvest date	Sugars (g L^{-1})	Acidity (g L^{-1})	pH
Syrah	2/12/2015	216 ± 1.15 e	4.13 ± 0.00 a	3.64 ± 0.03 d
Marselan	2/20/2015	232 ± 1.15 c	6.97 ± 0.12 d	3.33 ± 0.03 b
Tannat V1	3/02/2015	250 ± 1.63 a	5.56 ± 0.08 bc	3.46 ± 0.03 c
Tannat V2 H1	2/20/2015	221 ± 1.15 d	5.63 ± 0.08 c	3.26 ± 0.02 a
Tannat V2 H2	3/02/2015	245 ± 2.58 b	5.43 ± 0.09 b	3.40 ± 0.02 c

Data are the mean (n = 2) ± SD. Different letters in the column indicate statistical significant differences between means ($p < .05$) according to Tukey test.
Titratable acidity expressed as g L^{-1} of tartaric acid.
[a]Abbreviation: Tannat V1, Tannat from vineyard 1; Tannat V2 H1, Tannat from vineyard 2, first harvest; Tannat V2 H2, Tannat from vineyard 2, second harvest.

2. Materials and methods

2.1. Grapes and wines

The experiments were performed in 2015 from Tannat, Marselan and Syrah *Vitis vinifera* grapes grown in southern Uruguay, and their respective wines. As shown in Table 1, five grape harvest were made, vinifying each by duplicate, comprising one of Syrah, one of Marselan, and 3 of Tannat (2 belonging to one vineyard harvested at two maturity levels, and other one from another nearby vineyard). The general composition of grapes at harvest is provided in Table 2. Analysis were carried out according to O.I·V (2015) using a refractometer Atago N1 (Atago, Tokyo, Japan) for sugars estimation and a pH meter Hanna HI8521 (Hanna instruments, Villafranca Padovana, Italy) for pH determination.

Clusters were harvested by hand and transported in plastic boxes (20 kg in one) to the college experimental winery. Just before crushing, two samples of 100 grapes each were collected taking cluster parts from 3 to 5 berries from different parts of the bunch. To avoid bias by size or aspect, each 100-berry sample was scattered over a planar surface drawn as a numbered grid of 5 × 5 cm squares. An order of squares was randomly chosen, and then, all the grapes placed inside each square were picked up until 35 grapes were collected. The grapes in each 35 grape sample were then peeled and the pulp remaining against the skins was carefully removed with the help of a rounded edge blade. The skins were gently blotted with a paper tower, weighed (fresh-weight) and stored in a sample bag at −18 °C until they were lyophilized. The skins were lyophilized, weighed (dry-weight) and then stored at −18 °C again.

Vinification were made in duplicate, employing 70 kg of grapes for each. Grapes were destemmed and crushed with an Alfa 60 R crusher (Italcom, Piazzola sul Brenta, Italy), and then, they were fermented in stainless steel tanks (100 L capacity each). Potassium metabisulfite (50 mg SO_2/100 kg of grapes) was added, and dry active yeast

Table 1
Scheme of the 20 samples analyzed over which the new flavonol compounds were identified.

Grape variety	Tannat vineyard 1	Tannat vineyard 2		Syrah	Marselan
Harvest date	03/02/2015	Harvest 1 02/20/2015	Harvest 2 03/02/2015	02/12/2015	02/20/2015
Skin samples by harvest	2	2	2	2	2
Wines made by harvest	2	2	2	2	2

(*Saccharomyces cerevisiae ex bayanus* Natuferm 804; Oenobiotech, Paris, France) was inoculated (20 g/kg of grapes). Wines were made with a classical fermentation on skins (simultaneous maceration with alcoholic fermentation) for 8 days. Two pumpings over followed by punching the cap were carried out daily until pressing. At devatting, the fermentation was finished in all cases. The pressing of the marc was carried out with a stainless steel manual press. Free-running juices and press juices were mixed, and wines were kept in glass recipients of a 10-L capacity in the cellar.

2.2. Analytical procedures

2.2.1. Skin and wine extracts for flavonol analysis

Analytical determinations were made at the Laboratory of Instrumental Analysis at the Regional Institute of Applied Scientific Research (IRICA), Castilla-La Mancha University, Spain. All solvents used were of HPLC quality, and all chemicals were of analytical grade (> 99%). Water was of Milli-Q quality. Commercial standards of flavonol 3-*O*-glucosides of quercetin, kaempferol, isorhamnetin, and syringetin (Extrasynthese, Genay, France) were used as references for identification.

Lyophilized skins were extracted with 100 mL of a mixture of 50:48.5:1.5 (*v*/v) CH_3OH/H_2O/HCOOH using a homogenizer (Heidolph DIAX 900) to 10,000 rpm for 3 min and then centrifuged at 2500*g* at 5 °C for 5 min. The supernatant was separated and conserved, and the pellet was extracted again two more times. The three obtained supernatants were mixed, the volume was registered, and then they were stored at −18 °C until analysis. Previous works in similar conditions confirmed that a grape skin pellet extracted two times allowed nearly 99% of the grape polyphenol content to be extracted with successive repetitions of the cited protocol (Castillo-Muñoz et al., 2009).

To minimize anthocyanin interference, the skin extracts were passed through SPE cartridges filled with a material that combines reverse-phase and cationic exchange phenomena (PCX Agilent Bond Elut Plexa; 6 mL capacity filled with 500 mg of adsorbent, Agilent USA) following the procedure described by Castillo-Muñoz et al. (2007). The eluate containing flavonols was dried in a rotary evaporator (35 °C) and re-solved in 1 mL of 20% methanol.

2.2.2. HPLC-DAD-ESI-MS^n analytical conditions

HPLC separation and identification of flavonols were performed on a Agilent 1100 Series system (Agilent, Waldbronn, Germany) equipped with a DAD (G1315B) and LC/MSD Trap VL (G2445C VL) ESI-MS^n system, and it was coupled to an Agilent Chem Station (version B.01.03) data-processing station. The mass spectral data were processed with the Agilent LC/MS Trap software 5.3 (version 3.3). The samples were injected (20 μL in the analysis used for the first identification and quantification of flavonols and 30 μL in the specific experiments that include MS^3) on a reversed-phase Zorbax Eclipse XDB-C18 Narrow-Bore column (2.1 mm × 150 mm; 3.5 μm particle; Agilent USA, Santa Clara-California) thermostated at 40 °C. The skin extract concentration injected into the HPLC system was of 70,4 mg/mL (mg of freeze-dried skin by mL of solution), while the wine extracts injected corresponded to a wine concentrated 3/1 for flavonol initial identification analysis, and of 6/1 for the experiments that include MS^3. The chromatographic conditions were as follows: solvent A (water/formic acid/acetonitrile, 87:10:3, v/v/v), solvent B (acetonitrile/water/formic acid, 50:40:10, v/v/v), and solvent C (methanol/water/formic acid, 90:1.5:8.5, v/v/v). The flow rate was 0.190 mL/min. The linear solvent gradient was as follows: zero min, 96% A and 4% B; 7 min, 96% A and 4% B; 38 min, 70% A, 17% B, and 13% C; 52 min, 50% A, 30% B, and 20% C; 52.5 min, 30% A, 40% B, and 30% C; 57 min, 50% B and 50% C; 58 min, 50% B and 50% C; and 65 min, 96% A and 4% B.

For identification, ESI-MS^n was used in the negative mode with the following parameters: dry gas, N_2, 8 mL/min; drying temperature, 350 °C; nebulizer, 40 psi; and scan range, 50–1200 *m/z*. The ionization and fragmentation parameters were optimized by the direct injection of the appropriate standard solutions.

2.2.3. Specific experiments up to MS^3 for Aglycone confirmation

To confirm the structures of the putative flavonol aglycones derived from the acetyl and *p*-coumaroyl 3-*O*-glucosides, specific MS^n experiments were carried out on a Tannat wine sample.

Based on the observed times in a MS^2 chromatogram obtained in an immediately previous injection of the same sample, a specific experiment was defined to search in defined time intervals for the *m/z* corresponding to the following: the flavonol 3-*O*-glucosydes of the three methoxylated-flavonols laricitrin, isorhamnetin and syringetin (MS^3 experiments); the respective acetyl glucosides and *p*-coumaroyl glucosides (MS^3 experiments); and the *m/z* corresponding to the respective aglycones (MS^2 experiments).

2.2.4. Isolation and fragmentation of the aglycone and radical ions

To independently isolate and fragment the flavonol aglycone ions generated through heterolytic cleavage of the glucosidic bond with hydrogen migration from the radical aglycone ions generated through the corresponding homolytic cleavage, specific MS^3 experiments were performed. To achieve that, the LC/MS Trap software default parameters were modified, reducing the precursor ion isolation window from ± 3 amu to ± 0.5 amu.

2.2.5. Q-ToF high-resolution mass spectrometry measurements

The analytical system used consisted of a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6545 quadrupole-time of flight (Q-TOF) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was Mass Hunter Workstation (version B.06.11). The Q-TOF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the negative ionization mode and the following parameters were set: capillary voltage, 3500 V; fragmentor, 200; gas temperature, 350 °C; drying gas, 8 L/min; nebulizer, 40 psig; sheath gas temperature, 400 °C; sheath gas flow, 12 L/min; acquisition range, 100–1000 *m/z*; and CID, linear range of 30–45. Samples were analyzed after injection (10 μL) on a Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 μm, Agilent, Santa Clara, CA) protected with a 5 mm guard column of the same material thermostated at 40 °C. The solvents system were water with 0.1% formic acid (solvent A) and acetonitrile-methanol (70:30, *v*/v) with 0.1% formic acid (solvent B). The elution gradient was (time, % of solvent A): 0 min, 95%; 1 min, 95%; 30 min, 65%; 35 min, 30%; 40 min, 20%; 45 min, 95%; and a post time of 8 min. Compounds were identified using the algorithm "Find by Formula" that evaluated the mass accuracy together with the isotopic relative abundance and isotopic separation.

3. Results and discussion

3.1. First evidence of the new flavonol derivatives

The initial identification of the new compounds was made in Tannat skin and wines, where the signals attributable to the new flavonols presented higher intensity. Then, with the information gathered in the Tannat samples, such signals could be also found in other samples of Syrah and Marselan included in the experiments, and today, in wines of the *Vitis vinifera* Cabernet Franc and Merlot from the south of Uruguay, and Tinto Fragoso, Moribel and Tempranillo from Castilla-La Mancha, Spain.

Fig. 2 shows the HPLC-DAD chromatograms (360 nm) obtained for a flavonol extract of Tannat red wine. The assignment of the chromatographic peaks was initially based on the extracted ion chromatograms (EIC) obtained at the *m/z* values of the flavonol aglycones ions ($[Y_0]^-$) of the six flavonol-3-*O*-glycosides expected to be found in grapes and wines (Castillo-Muñoz et al., 2009, 2007), that is kaempferol, quercetin,

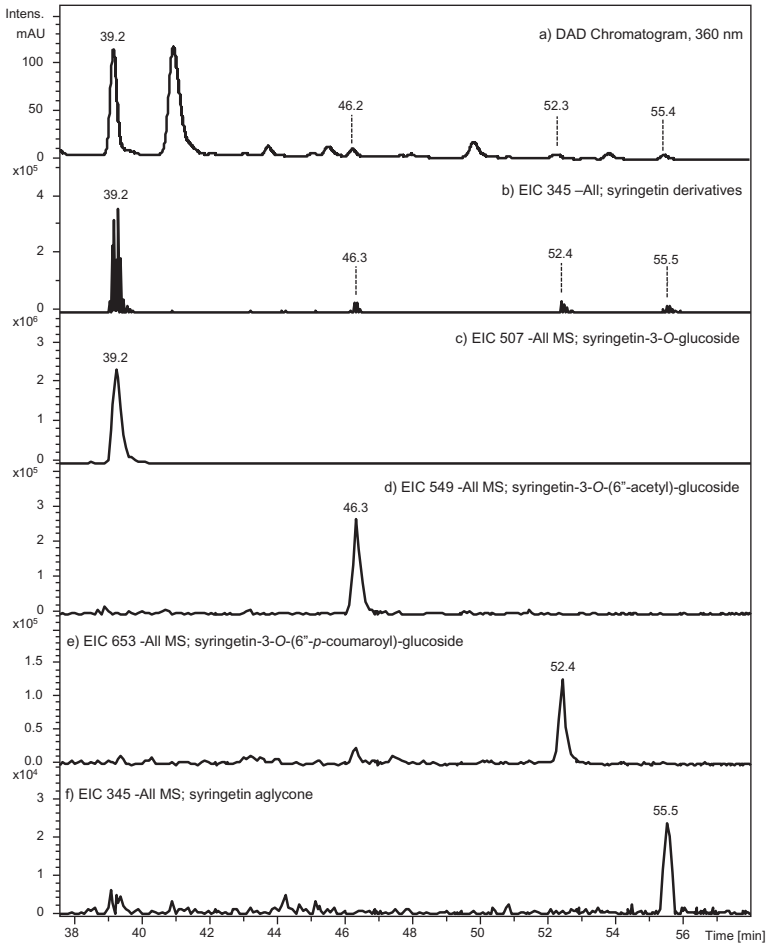

Fig. 2. Syringetin-based flavonols identified in Tannat grapes and wines: a) DAD-chromatogram at 360 nm of a sample of Tannat wine; b) extracted Ion Chromatogram (EIC) at the *m/z* value corresponding to all syringetin-based flavonols; c) EIC at the *m/z* value corresponding to syringetin-3-*O*-glucoside; d) EIC at the *m/z* value corresponding to syringetin-3-*O*-(6″-aceyl)-glucoside; e) EIC at the *m/z* value corresponding to syringetin-3-*O*-(6″-*p*-coumaroyl)-glucoside; f) EIC at the *m/z* value corresponding to free syringetin aglycone.
According to the elution time: glucoside; acetylated; p-coumaroylated and aglycone.

isorhamnetin, myricetin, laricitrin, and syringetin (Fig. 2 and supplementary data). Unexpectedly, the EIC corresponding to the three methoxylated flavonol aglycones laricitrin (*m/z* 331), isorhamnetin (*m/z* 315) and syringetin (*m/z* 345) showed two additional peaks, each which had a *m/z* that did not match any of the following expected flavonol derivatives: *m/z* 535 and 639 for laricitrin; *m/z* 519 and 623 for isorhamnetin; and *m/z* 549 and 653 for syringetin. The latter results are 204 amu and 308 amu higher than each corresponding aglycone or 42 amu and 146 amu higher than each respective flavonol glucoside, and these match the *m/z* values of an acetyl and *p*-coumaroyl moiety, respectively, two residues commonly participating in the acylation of other flavonoid-glucosides such as anthocyanins.

The DAD on-line UV/Vis spectra of the peaks assigned to the putative acetylated flavonol derivatives closely matched those of the respective flavonol-3-*O*-glucosides (Fig. 3a) because the acetic acid moiety does not add any additional chromophore absorbing in the considered wavelength range of the UV/Vis spectrum. On the contrary, the UV/Vis spectra of the putative *p*-coumaroyl flavonol derivatives closely matched the sum of the respective spectra of the flavonol 3-*O*-glucoside and that of *p*-coumaric acid. (Fig. 3b). As observed, the *p*-coumaroyl moiety adds significant radiation absorbance between 275 and 345 nm. Indeed, the screening effect against UV-B radiation has been identified as a major function of the cinnamoyl-flavonols in leaves of different plants species (Kaffarnik et al., 2005).

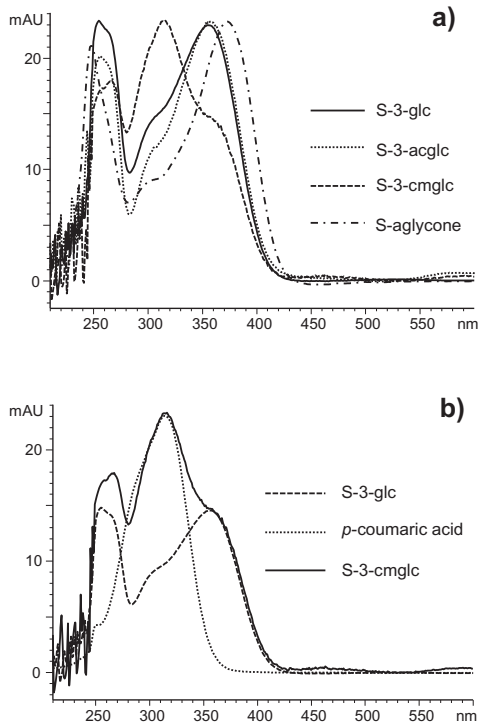

Fig. 3. UV–Vis spectra of syringetin-based flavonols identified in Tannat grapes and wines: a) comparison of different UV–Vis for all syringetin-based flavonols, including free aglycone and new acylated derivatives; b) UV/Vis spectrum of the syringetin 3-*O*-(6″-*p*-coumaroyl)-glucoside compared to those of its constituent units.

3.2. Study of the aglycone structure by MS^3 experiments

Specific MS^3 experiments were performed in order to confirm the structures of the putative flavonol aglycones derived from each tentatively identified new flavonol derivative. Thus, their fragmentation patterns were compared with those obtained from authentic standards of laricitrin, isorhamnetin and syringetin aglycones under the same experimental conditions.

3.2.1. Syringetin derivatives

The syringetin aglycone, *m/z* 345.4, eluted at 55.6 min. It fragmented, showing a main signal of *m/z* 329.9 in its MS^2 spectrum (Fig. 4a), which was assigned to the loss of a methyl group (15 amu) from the precursor deprotonated ion ($[(Y_0–H)\text{-}CH_3]^{-\cdot}$). Less favored under the operational conditions, the concurrent loss of the second methyl group of the syringetin explained the weaker signal observed at *m/z* 315.2 ($[(Y_0–H)\text{-}CH_3\text{-}CH_3]^-$). Those are the predominant fragmentations occurring in most methoxylated flavonoids under the negative ionization mode, which are indicative of the presence of an OCH_3 group in the molecule (Justesen, 2001; Panighel et al., 2015; Parejo et al., 2004). An even weaker signal at *m/z* 286.2 may be assigned to an additional loss of 29 amu, which has been described as characteristic of the fragmentation pattern of dimethoxylated flavonoids and corresponds to the loss of a $HCO^{\cdot}$ radical (Justesen, 2001).

At 39.2 min, the syringetin-3-*O*-glucoside (*m/z* 507.4) produced a MS^2 spectrum (Fig. 4b), showing a base signal corresponding to the putative aglycone radical ion ($[Y_0–H]^{-\cdot}$) obtained through homolytic cleavage (*m/z* 344.0). The presence of the aglycone ion ($[Y_0]^-$) generated through heterolytic cleavage (*m/z* 344.9) was also evident as a neighboring weaker peak. The fragmentation pathway rendered by the putatively derived aglycone for MS^3 did not mirror the one of the aglycone. Even when signals at *m/z* 329.9 and 315.2 were present, more fragments were generated, and the favored sites of cleavage changed, with *m/z* 315.9 being the base signal. This signal may be assigned to the loss of CO from the aglycone radical ion ($[(Y_0–H)\text{-}28]^{-\cdot}$), while the signal at *m/z* 301.0 may be assigned to $[(Y_0–H)\text{-}15–28]^-$, which matched the concurrent loss of a $CH_3^{\cdot}$ radical and a CO group from the aglycone. The signal observed at *m/z* 272.8 has also been cited as characteristic of polymethoxylated flavonols (Justesen, 2001). In this case, it would correspond to the loss of a $CH_3^{\cdot}$ and two CO moieties $[(Y_0–H)\text{-}15–56]^-$ from the aglycone radical ion. At *m/z* 287.2, the signal matches the loss of both $CH_3^{\cdot}$ from the syringetin aglycone and additionally a CO ($[(Y_0)\text{-}30–28])^{-\cdot}$).

The signal at *m/z* 549.5 attributable to putative syringetin 3-*O*-(6″-acetyl)-glucoside was detected at 46.3 min (Fig. 4c). In MS^2, it was rendered as the main signal at *m/z* 344.0, attributable to the syringetin aglycone radical ion, which was formed by the loss of the acetyl-glucoside moiety through homolytic cleavage. The neighboring weaker signal at *m/z* 344.9 represents the aglycone ion generated through heterolytic cleavage. The latter matches the MS^2 fragmentation pattern of syringetin 3-*O*-glucoside, with the lack of homolytic suppression derived from sugar acetylation evident, which is in contrast to the findings of Yang et al. (2014). The respective losses of 205/204 amu match the loss of an acetylated glucosyl moiety (162 amu + 42 amu). There was not evidence of the intermediate loss of the acetyl moiety, indicating that it is not favored under the operational conditions. In MS^3, the signals corresponding to *m/z* 329.9 and 315.2 were observed, as in the mass spectrum of the authentic aglycone. However, more fragments were evident, closely mirroring the fragmentation pattern already observed in the aglycone derived from the syringetin 3-*O*-glucoside.

The signal attributable to the deprotonated putative syringetin 3-*O*-(6″-*p*-coumaroyl)-glucoside (*m/z* 653.3) was obtained at 52.4 min (Fig. 4d). That compound fragmented in MS^2 rendering *m/z* 345.0 as the main signal, which is attributable to the formation of the syringetin aglycone ion through heterolytic cleavage. In this case, homolytic cleavage was clearly suppressed. An intermediate signal at *m/z* 506.8 (6.5% of the base signal) between the signal corresponding to the parental deprotonated ion (*m/z* 653.3) and the signal of the derived aglycone (*m/z* 345.0) very likely matches the loss of the *p*-coumaroyl moiety (146 amu), in contrast to that observed for *p*-coumaroylated anthocyanins that lost the entire *p*-coumaroyl-glucose residue in MS^2 spectra under positive ionization conditions (Blanco-Vega, Gómez-Alonso, & Hermosín-Gutiérrez, 2014; Mattivi et al., 2006). The further fragmentation pattern of the derived syringetin aglycone in MS^3 closely matched the pattern observed by the authentic syringetin aglycone. Thus, the loss of one of the methyl groups of syringetin was the most favored fragmentation, producing a signal at *m/z* 329.9, followed by the concurrent loss in the ion trap of the two methyl groups, which produced the signal at *m/z* 315.0.

3.2.2. Isorhamnetin derivatives

The MS and MS^2 mass spectra of the isorhamentin aglycone (53.6 min) showed the expected deprotonated ion, *m/z* 315.4 (**Fig. S1a**, see supplementary material). It fragmented giving rise to a unique fragment at *m/z* 299.8 in the MS^2 spectrum, a difference of 15 amu with respect the parent ion, which may be assigned to the loss of the $CH_3^{\cdot}$ at C-3′.

The deprotonated isorhamnetin 3-*O*-glucoside, *m/z* 477.3, was

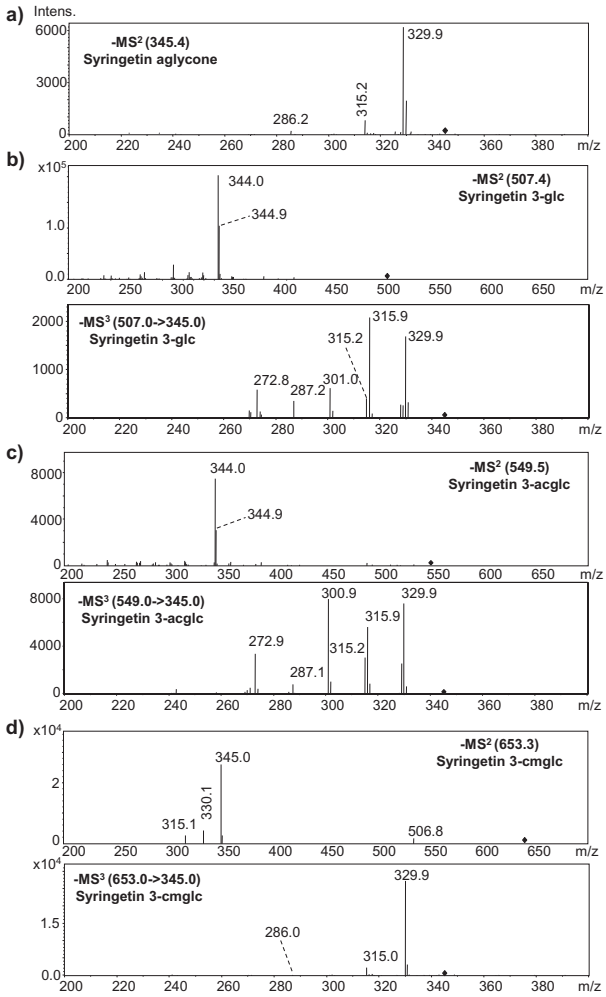

Fig. 4. MS^n experiments (isolation window of ± 3.0 amu) for identification of syringetin-based flavonols identified in Tannat grapes and wines: a) MS^2 of free syringetin aglycone; b) MS^2 and MS^3 of syringetin-3-*O*-glucoside; c) MS^2 and MS^3 of syringetin-3-*O*-(6″-acetyl)-glucoside; d) MS^2 and MS^3 of syringetin-3-*O*-(6″-*p*-coumaroyl)-glucoside.

detected at 38.4 min (**Fig. S1b**, see supplementary material). The base peak in MS^2, *m/z* 313.9, matches the aglycone radical ion ($[Y_0-H]^{-\cdot}$) formed by homolytic cleavage. The neighboring signal at *m/z* 314.9 corresponded to the expected aglycone ion ($[Y_0]^-$) formed by heterolytic cleavage. The fragmentation pattern observed in MS^3 did not mirror the one rendered by the authentic aglycone, and it was characterized by the type of losses described for syringetin glucoside and acetyl glucoside (Section 3.2.1).

The putative isorhamnetin 3-*O*-(6″-acetyl)-glucoside, *m/z* 519.3, eluted at 39.7 min (**Fig. S1c**, see supplementary material). The examination of the MS^2 spectrum showed *m/z* 314.9 as the main signal, matching the isorhamnetin aglycone ion obtained by heterolytic cleavage ($[Y_0]^-$), with a weaker neighboring signal at *m/z* 313.9, representing the aglycone radical ion ($[Y_0-H]^{-\cdot}$). The respective losses of 204/203 amu may be attributed to the concurrent loss of the acetylated glucose moiety. Differently to what was observed for the acetylated-syringetin derivative (Section 3.2.1), in this case, the flavonol acetylation modified the dominant cleavage of the *O*-glucosidic bond from mainly homolytic to mainly heterolytic, in agreement with Yang et al. (2014), but this also remarks on the difficulty of predicting the type of cleavage based on particular features of the molecular structure. For MS^3, the observed mass spectrum matched the one for the

authentic aglycone, with a major peak at *m/z* 299.8 (loss of $CH_3^{\cdot}$).

Finally, at a time of 48.7 min, the signal attributable to the putative isorhamnetin 3-*O*-(6″-*p*-coumaroyl)-glucoside with *m/z* 623.4 was detected (**Fig. S1d**, see Supplementary material). This compound produced *m/z* 314.9 as the main signal in MS^2 as well as a signal at *m/z* 313.9, which may be assigned to the putative isorhamnetin aglycone ion ($[Y_0]^-$) and radical ion ($[Y_0-H]^{-\cdot}$), respectively. The losses of 308/309 amu agree with the loss of a *p*-coumaroyl-glucoside unit (146 + 162 amu). In addition, the intermediate signal at *m/z* 477.0, attributable to the isorhamnetin-glucoside formed by the loss of the *p*-coumaroyl moiety (146 amu), was observed, with its intensity higher (47.0% of the base signal) than that found for the similar syringetin derivative. As observed above with the acetylated derivative, the glucose acylation favored the heterolytic cleavage, but in this case, the homolytic fragmentation was suppressed to a lesser extent. In MS^3, the putatively derived aglycone fragmented in the ion trap as did the authentic aglycone.

3.2.3. Laricitrin derivatives

The laricitrin aglycone ion, *m/z* 331.3, was detected at 45.5 min (**Fig. S2a**, see supplementary material). In the MS^2 spectrum, *m/z* 315.8 was the base peak, corresponding to the loss of $CH_3^{\cdot}$ at C3' ($[Y_0-CH_3]^{-\cdot}$) A much weaker signal at *m/z* 286.7 represents an additional loss of 29 amu, which may be attributable to the subsequent loss of CHO ($[Y_0-CH_3-CHO]^-$). These fragmentation patterns have been described as characteristic of monomethoxylated flavonols under electrospray ionization mass spectrometry operated in the negative mode (Justesen, 2001) and were also found by other authors (Panighel et al., 2015; Parejo et al., 2004). Two other fragments were evident at *m/z* 178.7 and *m/z* 150.7. These are commonly reported in flavonoid fragmentation studies and may be adjudicated to $[^{1,2}A]^-$ and $[^{1,3}A]^-$, respectively, derived from the flavonol C-ring cleavage (Davis & Brodbelt, 2008; Panighel et al., 2015).

At 30.8 min, the laricitrin-3-*O*-glucoside was detected (*m/z* 493.4; **Fig. S2b**, see supplementary material). It fragmented at MS^2 giving rise to *m/z* 330.9 as the main signal, corresponding to the aglycone ion following heterolytic cleavage ($[Y_0]^-$). It further fragmented in the ion trap giving rise to a MS^3 spectrum that closely matched the one obtained by the laricitrin aglycone discussed above.

The signal attributable to the laricitrin 3-*O*-(6″-acetyl)-glucoside at *m/z* 535.3 was detected at 37.5 min (**Fig. S2c**, see supplementary material). It fragmented in MS^2 with *m/z* 330.9 being the main signal, which may be assigned to the corresponding laricitrin aglycone obtained by heterolytic cleavage. Here, the signal denoting homolytic cleavage, *m/z* 329.9, was evident, which is another example where acetylation did not necessarily favor homolytic cleavage suppression. In MS^3, the derived aglycone fragmented as did the authentic one, with the characteristic $CH_3^{\cdot}$ loss (−15 amu) and the presence of the minority signals at *m/z* 150.7 and *m/z* 178.7.

The compound matching the putative laricitrin 3-*O*-(6″-p-coumaroyl)-glucoside with *m/z* 639.3 eluted at 46.5 min (**Fig. S2d**, see Supplementary material). It fragmented, rendering a *m/z* 331.0 signal in MS^2, which may be assigned to the laricitrin aglycone generated by heterolytic cleavage, as in the other laricitrin derivatives. Nevertheless, in this case, the base peak was that of *m/z* 493.1, which was assigned to the laricitrin 3-*O*-glucoside fragment derived from the intermediate loss of the *p*-coumaric acid residue (146 amu). In spite of that, in MS^3, the ion trap was able to isolate the putative aglycone and fragmented it, rendering *m/z* 315.9 as the main signal, thus matching the signal observed in the fragmentation of the authentic aglycone.

3.3. Discussion regarding the biosynthesis of the newly reported acylated flavonol glucosides

Acetic and *p*-coumaric acid are two compounds frequently participating in the acylation of plant secondary metabolites (Cunningham & Edwards, 2008; D'Auria, 2006; Kaffarnik et al., 2005; Parejo et al., 2004). Such an acylation profile is commonly present in grape and wine anthocyanins despite the hydroxylation or methoxylation substitutions pattern in their B-ring (Blanco-Vega et al., 2014; Gómez-Alonso et al., 2012; González-Neves, Gil, Barreiro, & Favre, 2010). It has also been reported for flavonols in others vegetal species (Kaffarnik et al., 2005; Kim & Jang, 2011; Onogi et al., 1993; Parejo et al., 2004; Sugiyama, Katsube, Koyama, & Itamura, 2013). However, to the best of our knowledge, the complete set of acylated flavonol glycosides we are reporting has not been previously found in grapes and wines as specific derivatives of flavonols. In agreement with our findings, the presence of isorhamnetin 3-*O*-*p*-coumaroyl-glucoside has been reported in *V. vinifera* Raboso Piave red grape skin together with a dihydrokaempferide-3-*O*-*p*-coumaroylhexoside-like flavanone and a chrysoeriol-*p*-coumaroylhexoside-like flavone (Panighel et al., 2015), while laricitrin-3-*O*-(6″-acetyl)-glucoside has been mentioned by Liang et al. (2014) and syringetin-3-*O*-(6″-acetyl)-glucoside suggested by Castillo-Muñoz et al. (2007).

In mulberries, Sugiyama et al. (2013) found that the presence of acetyl-flavonols was restricted to species that specifically contain acetyl-transferase activity. Since acylation is a final step in flavonoid synthesis (Kaffarnik et al., 2005), our findings suggest a very high specificity of the acyl-transferases involving the flavonol-glucoside substrate, which in this case would be related to the presence of methoxyl groups in the B-ring of the flavonol. This is in agreement with Kaffarnik et al. (2005), who found that hydroxycinnamoyl-transferases showed higher activity with kaempferol and isorhamnetin 3-*O*-glucosides because they have a more lipophilic B-ring than flavonols with additional hydroxyl groups. Thus, these results may also contribute to better understanding of the specificity and functionality of the acyl-transferases, which is very important as they determine new specific biological activities in their targeted molecules (Cunningham & Edwards, 2008; Yoshida, Hishida, Iida, Hosokawa, & Kawabata, 2008). Yoshida et al. (2008) indicate the importance of the caffeoyl acylation of the flavonol-glycosides in the α-glucosidase-inhibiting activity and its putative importance for diabetes treatment. The study of acyl-transferases is also of interest at the industrial level because those enzymes are used as tools to create new artificial compounds (Cunningham & Edwards, 2008; D'Auria, 2006; Rinaldo et al., 2015). Sugiyama et al. (2013) found quercetin-3-acetylglucoside just in one of the 176 cultivars of mulberries that they studied, and it was not the cultivar with the most abundant content of flavonols. Thus, further evidence should be obtained in other grape varieties to conclude if the new flavonol derivatives presented here are restricted to the Tannat grape variety, to a few varieties, or if they are widely distributed.

3.3.1. Study of the factors leading to the Homolytic cleavage of the Glucosidic bond

The homolytic cleavage of the glucosidic bond, rendering the aglycone radical ion, was the main fragmentation pathway suffered in the ion trap for the following 3 of the 9 flavonol derivatives studied here: isorhamnetin 3-*O*-glucoside, syringetin 3-*O*-glucoside and syringetin 3-*O*-(6″-acetyl)-glucoside. Otherwise, heterolytic cleavage with hydrogen migration and the formation of the aglycone ion prevailed.

Factors that would promote the homolytic cleavage have been proposed in many studies (Davis & Brodbelt, 2008; Hvattum & Ekeberg, 2003; Panighel et al., 2015; Yang et al., 2014). Hvattum and Ekeberg (2003) found a positive correlation between the number of hydroxyl substituents in the B-ring of flavonol-3-*O*-glycosides and the prevalence of homolytic cleavage. Davis and Brodbelt (2008) also observed such a trend but report an exception. We reported homolytic cleavage in methoxylated flavonols and showed that sugar acylation may be another factor determining the type of glycosidic cleavage.

Davis and Brodbelt (2008) expressed that the aglycone and saccharide structures interact determining the type of glycosidic cleavage. They showed that intramolecular interactions between the saccharide

portion of the molecule and the hydroxyl groups of the B-ring might be possible and are a deterrent of homolytic cleavage. Our results suggest that the previous theory may be generalized to extend to the interaction of the entire acyl moiety with the flavonoid B-ring structure.

In the case of isorhamnetin derivatives, just the 3-*O*-glucoside underwent homolytic cleavage. It could be expected that the acetylated and *p*-coumaroylated residues add sites of interaction and molecular length to interact with the flavonol B-ring hydroxyl group, thus hampering homolytic cleavage.

None of the laricitrin derivatives underwent homolytic cleavage as the main fragmentation pathway, which could be the result of the additional hydroxyl group of this flavonol at C5´. This could facilitate the laricitrin B-ring interaction with the non-flavonoid part of the molecule. Nevertheless, the acetyl derivative suffered radical cleavage to some extent, showing that simple associations with molecular structure cannot be made.

Finally, two of the syringetin derivatives underwent homolytic cleavage. The presence of the additional methoxyl group at C5´ may have hindered the interaction of the acyl moiety with the unique hydroxyl group of the syringetin at C3´. Nevertheless, the *p*-coumaroylated moiety underwent only heterolytic fragmentation. The extra length and sites of interaction that the *p*-coumaric acid conferred to the acyl moiety could have led to the intramolecular interaction leading to homolytic suppression.

Nevertheless, our results strongly suggest that the entire molecular geometry and composition, under the determined MS conditions, are what determine the type of cleavage that the glucosidic bound will suffer in the ion trap. This would be expected since those are also the factors affecting the molecular electronic configuration and reactivity.

3.3.2. Homolytic and heterolytic cleavages of the flavonol glucosidic bond

As mentioned above, we have found a certain degree of uncertainty regarding the identity of flavonol aglycones when they were mainly produced by homolytic cleavage because they fragmented differently than the respective authentic aglycones. In all other cases, heterolytic cleavage prevailed, and the derived aglycone ion fragmented as did the authentic one. Thus, we hypothesized that the aglycone radical ion ($[Y_0-H]^{-\cdot}$) fragmented differently than the aglycone ion ($[Y_0]^-$) but that both ions corresponded in fact to the expected flavonol aglycone.

To confirm this hypothesis, specific MS^3 experiments were performed for isorhamnetin-3-*O*-glucoside, syringetin-3-*O*-glucoside and syringetin-3-*O*-(6″-acetil)-glucoside. The first step was to modify the usual acquisition parameters of the ion trap, reducing the isolation window for the precursor ion from ± 3.0 amu to ± 0.5 amu. Thus, the second-generation precursor ions ($[Y_0]^-$) and radical ions ($[Y_0-H]^{-\cdot}$) were isolated and fragmented separately, and their single MS^3 spectra were obtained (Fig. 5). As may be observed, $[Y_0]^-$ and $[Y_0-H]^{-\cdot}$ fragmented differently, with the later producing, in all cases in MS^3, more fragments than the former. Additionally, the experiments confirmed that the fragmentation pattern generated by the derived aglycone ion in all cases matches the one generated by the correspondent aglycone, thus confirming its identity. Using the ± 3.0 amu isolation window, the radical ion and ion were jointly isolated in the ion trap for MS^2 and further fragmented together. Then, the MS^3 spectra presented the signals corresponding to the fragments generated by both ions, with an intensity defined by the relative abundances of their respective precursor ions. Thus, the overlay of both independent mass spectra resulted in one that closely matched that obtained when both ions were jointly isolated and fragmented. (Fig. 5).

The above-mentioned results suggest that the analysis of homolytic cleavage is very important to studying the identity of flavonol glycosides. When it is possible to isolate and fragment only the heterolytic-derived ion, confirmation of the aglycone is possible, but that is not suitable in generic analysis. In such cases, the knowledge of the fragmentation pathway of the radical ion could be enough to correctly identify the compounds. Likewise, if heterolytic fragmentation is remarkably suppressed, the knowledge of the general trend of the homolytic cleavage may help to not prematurely discard the presence of the compound under study in a sample. Finally, as the radical ion fragmented producing complex spectra, it is possible to obtain more structural information of the compound being studied (Feketeová, Barlow, Benton, Rochfort, & O'Hair, 2011).

4. Study of the new flavonol derivatives by Q-ToF high-resolution mass spectrometry

High-resolution mass spectrometry (HRMS) trials were performed using a HPLC-DAD-ESI-Q-ToF system to obtain the accurate masses of the analytes. As shown in Table 3, the observed *m/z* values obtained successfully matched (mass accuracy < 5 ppm) with the expected formulas of all of the compounds being studied. Interestingly, for *p*-coumaroylated derivatives of syringetin, the *cis* and *trans* isomers could be identified. The *cis* isomers eluted early and were much less abundant than their *trans* counterparts (supplementary data). Rhamnosyl-glucoside (or rutinoside) derivatives of flavonols, mainly the so-called rutin (quercetin-3-rutinoside), have been reported to occur as minor compounds in grapes and wines, and their fragmentation patterns under MS^2 conditions show a weak signal corresponding to the loss of a rhamnosyl residue (Castillo-Muñoz et al., 2007). Moreover, it has been demonstrated that flavonol-3-rutinosides quickly undergo hydrolysis under acidic wine conditions (Jeffery, Parker, & Smith, 2008). The rhamnosyl and rhamnosyl-glucose moieties have mass values of 146 amu and 308 amu under low resolution MS, similar to those for *p*-coumaroyl and *p*-coumaroyl-glucose moieties of the newly reported flavonol glucoside derivatives. Therefore, it would be possible that the initially suggested flavonol-3-(6″-*p*-coumaroyl)-glucosides could in fact be the flavonol-3-(6″-rhamnosyl)-glucosides, although we found that the suspected compounds exhibited UV–Vis spectra matching with the occurrence of a *p*-coumaroyl residue. In order to complete the correct assignment of the putative flavonol-3-(6″-*p*-coumaroyl)-glucosides, specific HRMS trials were performed using the Q-ToF equipment. Thus, every rutinoside/*p*-coumaroyl-glucoside derivative pair has the following theoretical *m/z* values and formulas for the deprotonated ion: isorhamnetin, 623.14063 ($C_{31}H_{27}O_{14}^-$) and 623.16176 ($C_{28}H_{31}O_{16}^-$), respectively, with an observed value of 623.1429; laricitrin, 639.13554 ($C_{31}H_{27}O_{15}^-$) and 639.15667 ($C_{28}H_{31}O_{17}^-$), respectively, with an observed value of 639.1381; and syringetin, 653.15119 ($C_{32}H_{29}O_{15}^-$) and 653.17232 ($C_{29}H_{33}O_{17}^-$), respectively, with an observed value of 653.1513. The latter results confirm the lack of rutinoside derivatives of isorhamnetin, laricitrin and syringetin in the studied wines and reinforce the suggested assignment made as 3-(6″-*p*-coumaroyl)-glucoside derivatives for the newly described flavonols.

5. Conclusions

In this work, we have gathered enough evidence to suggest the occurrence of acetylated and *p*-coumaroylated derivatives of the flavonol-glucosides made from the methoxylated aglycones isorhamnetin, laricitrin and syringetin in grapes and their respective wines. This evidence was mainly based on UV–Vis and MS^n spectra, including high-resolution mass spectra. A complete elucidation of the suggested assignments requires the isolation of such compounds and their structural study by nuclear magnetic resonance (NMR) spectroscopy.

The use of MS^2 experiments for the identification of the flavonol aglycones involved in these new flavonol derivatives sometimes resulted in MS^3 spectra showing more signals than those obtained for authentic aglycone standards. The reason was the breakdown of the glucosidic bond, yielding both aglycone ions ($[Y_0]^-$) and radical ions ($[Y_0-H]^{-\cdot}$) by heterolytic and homolytic cleavage, respectively, which in subsequent MS^3 experiments were isolated and fragmented simultaneously, thus resulting in a mixed spectrum of the two species. As a consequence, the predominance of heterolytic or homolytic

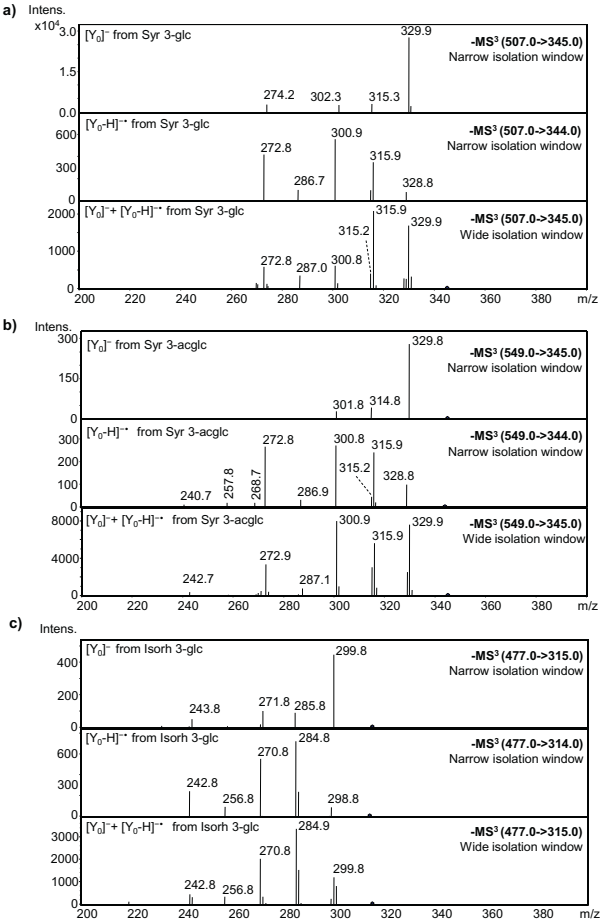

Fig. 5. MS^3 experiments, using a narrower isolation window (± 0.5 amu), for confirmation of the different fragmentation patterns showed by the aglycone ion ($[Y_0]^-$) and the aglycone radical ion ($[Y_0–H]^{-\cdot}$) formed by the heterolytic and hemolytic breakdown of the glucosidic bond, respectively: a) case of syringetin-3-*O*-glucoside; b) case of syringetin-3-*O*-(6″-acetyl)-glucoside); d) case of isorhamnetin-3-*O*-glucoside. The third MS^3 mass spectra in each case was that obtained using a wider isolation window (± 3.0 amu).

Table 3
Theoretical and Q-Tof mass spectra data of the flavonol series of methoxylated flavonol glucosides identified in samples of Tannat grapes and wines.

Flavonol	Formula	Monoisotopic mass	Theoretical m/z $[M-H]^-$	Observed *m/z* $[M-H]^-$	Mass accuracy (ppm)
Isorhamnetin 3-*O*-glucoside	$C_{22}H_{22}O_{12}$	478.11112	477.10385	477.1055	3.29
Isorhamnetin 3-*O*-(6″-acetyl)-glucoside	$C_{24}H_{24}O_{13}$	520.12169	519.11441	519.1166	2.46
Isorhamnetin 3-*O*-(6″-*p*-coumaroyl)-glucoside	$C_{31}H_{28}O_{14}$	624.14790	623.14063	623.1419	1.27
Laricitrin 3-*O*-glucoside	$C_{22}H_{22}O_{13}$	494.10604	493.09876	493.1007	3.82
Laricitrin 3-*O*-(6″-acetyl)-glucoside	$C_{24}H_{24}O_{14}$	536.11660	535.10933	535.1122	4.07
Laricitrin 3-*O*-(6″-*p*-coumaroyl)-glucoside	$C_{31}H_{28}O_{15}$	640.14281	639.13554	639.1381	2.63
Syringetin 3-*O*-glucoside	$C_{23}H_{24}O_{13}$	508.12169	507.11441	507.1143	− 0.35
Syringetin 3-*O*-(6″-acetyl)-glucoside	$C_{25}H_{26}O_{14}$	550.13225	549.12498	549.1249	− 0.29
Syringetin 3-*O*-(6″-*p*-coumaroyl)-glucoside	$C_{32}H_{30}O_{15}$	654.15846	653.15119	653.1513	0.00

fragmentation must be considered in any study dealing with flavonol glycoside identification, as in this case.

The fact that the new flavonol compounds have been identified only for the methoxylated aglycone-based flavonols, which are the minority, would mean a case of selectivity of the grape acyltransferases involved in their synthesis that needs to be investigated. Finally, it would be also interesting to search for these new acylated derivatives of flavonols in grapes and wines made from more grape varieties.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.06.019.

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6.9. SUPPLEMENTARY MATERIAL

Figure S1. MS^n spectra obtained for isorhamnetin-based flavonols identified in Tannat grapes and wines.

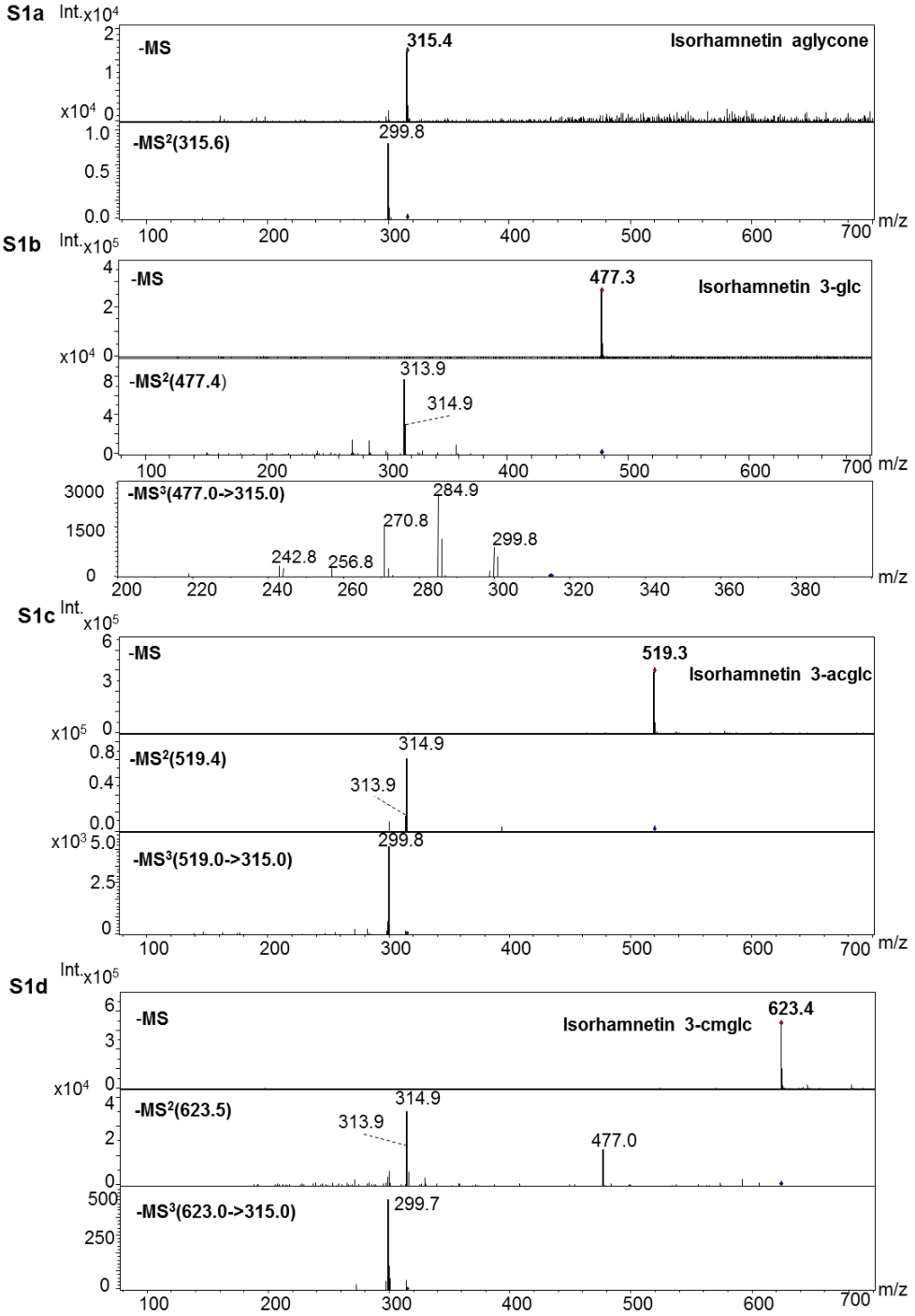
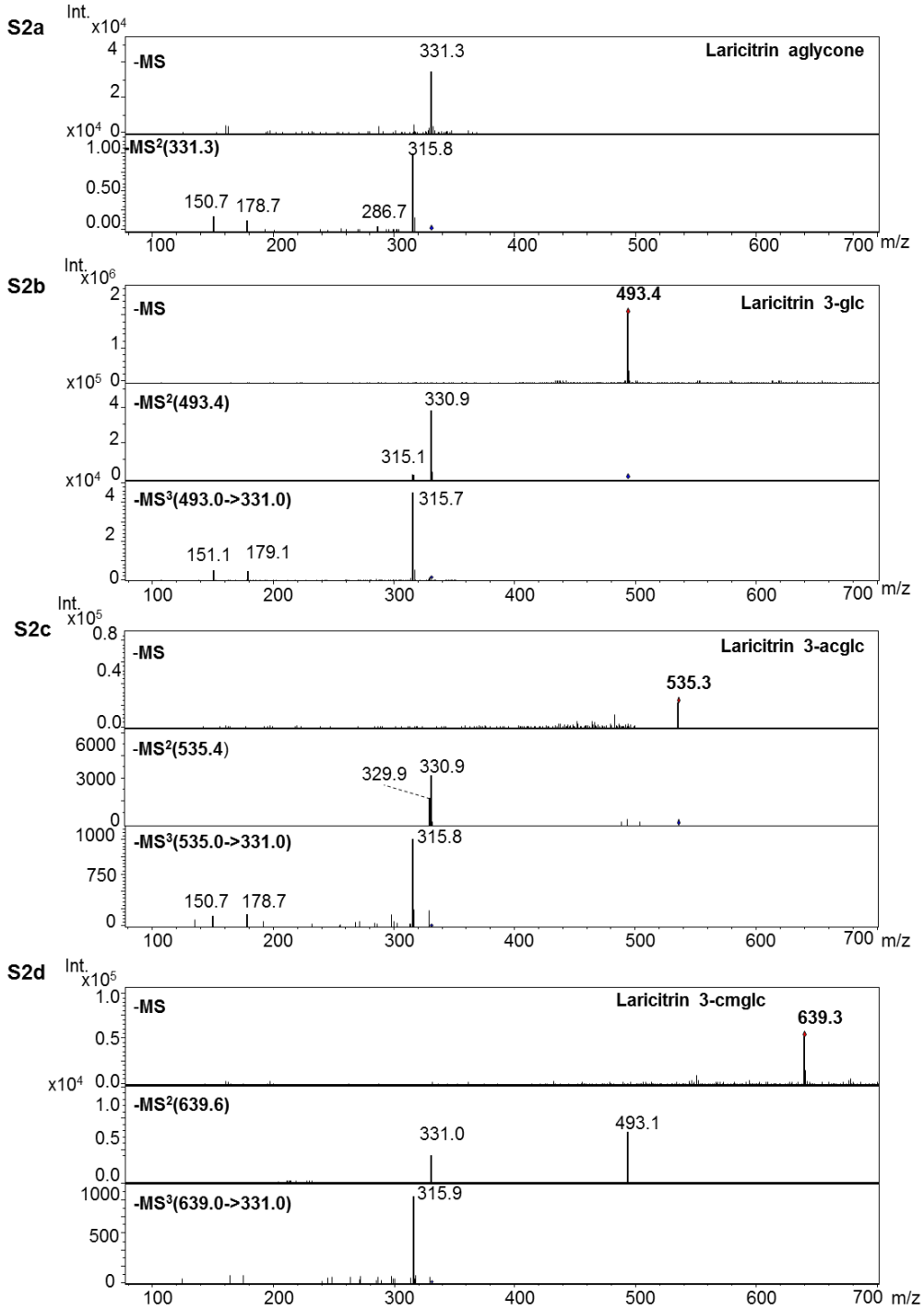


Figure S2. MS^n spectra obtained for laricitrin-based flavonols identified in Tannat grapes and wines.



6. CONCLUSIONES GENERALES Y PERSPECTIVAS

Tannat, Marselan y Syrah presentaron características diferentes que contribuyeron a los objetivos de las investigaciones realizadas. Entre otras, los elevados contenidos de pigmentos *p*-cumarilados en Syrah, se relacionaron con la dificultad de extracción de los mismos, resaltando la relevancia que esto puede tener en variedades con elevadas proporciones de este tipo de pigmentos.

El estudio conjunto de la composición de hollejos, vinos y orujos permitió identificar diferencias de extractibilidad y de estabilidad de los compuestos fenólicos relacionadas con la estructura molecular de los mismos. Al mismo tiempo permitió observar que la acilación de antocianos con ácido cafeico, y posiblemente con *p*-cumárico, también puede tener lugar durante el proceso de vinificación.

En Tannat se identificó por primera vez un antociano acilado con ácido ferúlico, que resultaría característico del perfil en pigmentos del cultivar.

Retrasar entre siete y diez días el momento de cosecha en torno a madurez tecnológica, no modificó significativamente la acumulación o reparto de taninos en las uvas. Sin embargo, en Syrah, incrementó notablemente su facilidad de extracción, particularmente desde los hollejos, mientras que en Tannat y Marselan no se detectaron diferencias cuantificables.

Las concentraciones en taninos de los vinos, así como el origen de los mismos, hollejos o semillas, no tuvo relación con los contenidos o localización de éstos en la baya. Sin embargo, su facilidad de extracción desde cada tejido habría sido la variable más importante definiendo la presencia de éstos en el vino. El cultivar sería una variable muy relevante determinando esta característica. Así, se constató que aún en uvas de elevado nivele de madurez, y en maceraciones fermentativas, la mayor parte de los taninos del vino pueden provenir de las semillas, como se observó en Marselan. Contrariamente a lo que podría esperarse, los vinos Tannat, al igual que los Syrah, se caracterizaron por la predominancia de taninos derivados de hollejos. Futuros estudios deberán incluir la determinación analítica de otros compuestos como los polisacáridos, ya que eventuales diferencias varietales podrían contribuir a explicar parte de los resultados obtenidos en este trabajo.

La relevancia de la extractibilidad de los compuestos polifenólicos quedó igualmente de manifiesto en Marselan. Esta variedad se caracterizó por presentar vinos con bajos contenidos de fenoles derivados de hollejo en relación a los presentes en las uvas respectivas. Futuras investigaciones deberían evaluar si dicha limitante está relacionada con las características estructurales de los hollejos, como sugieren los presentes estudios.

Los contenidos de estilbenos fueron particularmente elevados en Syrah, y en todas las variedades demostraron ser muy estables durante el período de guarda del vino abarcado por los estudios (24 meses). Syrah igualmente fue la única variedad donde se constató un incremento significativo de estos compuestos entre cosechas. Como la respuesta obtenida dependerá entre otras variables de la ecofisiología del viñedo en el período considerado y no es posible estandarizar dichas condiciones en viñedos bajo manejo comercial, serán necesarios más estudios para poder concluir sobre los factores que determinaron los resultados presentados.

En las tres variedades evaluadas se reportó la presencia de nuevos derivados acilados de los flavonoles metoxilados. El estudio de la especificidad de las aciltransferasas involucradas en la síntesis de estos compuestos, debería incluirse en futuros proyectos con un abordaje multidisciplinario. Así mismo, sería interesante incluir información del estado fisiológico de las plantas y del clima en eventuales estudios que busquen profundizar en las hipótesis que genera la presente investigación.

La identificación de nuevos compuestos fenólicos en uvas y vinos de Uruguay, es una prueba más del buen potencial fenólico que diferentes cultivares pueden alcanzar en las condiciones ecofisiológicas del país.

Las investigaciones realizadas generaron resultados que estuvieron de acuerdo a los objetivos propuestos, y plantean preguntas e hipótesis a evaluar en futuras investigaciones.

Conocer las características de los cultivares, de los compuestos que definen la calidad del vino y de la respuesta de éstos a diferentes manejos en el viñedo y la bodega es un insumo fundamental para tomar las mejores decisiones en cada

vendimia según los objetivos productivos. Se cree que los estudios realizados en el marco del doctorado realizan una contribución en este sentido.

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8. ANEXOS

8.1. VALORIZACIÓN DE LA TESIS

8.1.1. Artículos publicados en revistas arbitradas

Favre, G., González-Neves, G., Piccardo, D., Gómez-Alonso, S., Pérez-Navarro, J., & Hermosín-Gutiérrez, I. (2018). **New acylated flavonols identified in *Vitis vinifera* grapes and wines. Food Research International**, 112, 98-107.

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Favre, G., Hermosín-Gutiérrez, I., Piccardo, D., Gómez-Alonso, S., & González-Neves, G. (2019). **Selectivity of pigments extraction from grapes and their partial retention in the pomace during red-winemaking.** Food Chemistry, 277, 391-397.

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8.1.2. Trabajos presentados en congresos y jornadas de investigación

Favre, G., González-Neves, G., Piccardo, D., Gómez-Alonso, S., & Hermosín-Gutiérrez, I. 2016. New acylated flavonols identified in the grape skin of *Vitis vinifera* cv. **Tannat and their wines. Congreso *In Vino Analytica Scientia***. Changins.

Favre, G., González-Neves, G., Piccardo, D., Gómez-Alonso, S., & Hermosín-Gutiérrez, I. 2017. **Varietal flavonol profiles of Tannat, Marselan and Syrah *Vitis vinifera* grape skins, wines and marc, determined by an HPLC-MSn system.** Congreso HPLC. Praga.

Favre, G., González-Neves, G., Piccardo, D., Gómez-Alonso, S., & Hermosín-Gutiérrez, I. 2017. **Study of the glycosidic bond homolytic cleavage under a HPLC-ESI-MSD system, as a tool for the identification of new acylated flavonol glucosides in grapes and wines.** Congreso HPLC, Praga.

Favre, G., Hermosín-Gutiérrez, I., Piccardo, D., Gómez-Alonso, S., García-Romero, E., Mena-Morales, A., González-Neves, G. 2018. **Analysis of tannins in grapes an in the respective red wines of Tannat, Syrah and Marselan harvested at two maturity levels.** Congreso OIV, Punta del Este.

Favre, G., Hermosín-Gutiérrez, I., Piccardo, D., Gómez-Alonso, S., García-Romero, E., Mena-Morales, A., González-Neves, G. 2018. Efecto del grado de madurez de la uva sobre los contenidos en taninos de hollejos, semillas y los vinos tintos respectivos. Jornadas de Investigación Facultad de Agronomía.

Favre, G., Hermosín-Gutiérrez, I., Piccardo, D., Gómez-Alonso, S., García-Romero, E., Mena-Morales, A., González-Neves, G. 2019. **Resveratrol in grapes and wines of Tannat, Marselan and Syrah from Uruguay**. Congreso OENO/IVAS, Burdeos.

Favre, G., Hermosín-Gutiérrez, I., Piccardo, D., Gómez-Alonso, S., García-Romero, E., Mena-Morales, A., González-Neves, G. 2019. **The contribution of skin to wine flavan-3-ols could be overestimated by comparing their proportions in prodelphinidins.** Congreso OENO/IVAS, Burdeos.