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Anthocyanidins, Proanthocyanidins, and Anthocyanins Profiling in Wine Lees by Solid-Phase Extraction−Liquid Chromatography Coupled to Electrospray Ionization Tandem Mass Spectrometry with Data-Dependent Methods

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Supporting Information

ABSTRACT: A method has been developed to study the content of anthocyanidins, proanthocyanidicins, and anthocyanins in wine lees, an abundant byproduct from wineries. Detection/quantitation of the target compounds was carried out by a hyphenated system consisting of a solid-phase extraction workstation (Prospekt-2 unit) online coupled to a liquid chromatograph-triple-quadrupole tandem mass spectrometer (LC-MS/MS), where standards were used for identification/quantitation of both anthocyanidins and proanthocyanidins. Owing to the lack of anthocyanins standards, advantages from the use of data-dependent methods were taken for their identification and confirmatory analysis. Combination of the scanning methods (viz. product-ion, precursor-ion, and neutral-loss scanning) allowed identifying five different anthocyanins present in wine residues. The results thus obtained have been validated by complementary analysis of the extracts using LC-TOF/MS in high-resolution mode. Quantitation of the monitored compounds was supported on selected reaction monitoring (SRM) and calibration curves run with standards of anthocyanidins and proanthocyanidins.

KEYWORDS: *Wine lees, anthocyanins, anthocyanidins, proanthocyanidins, data-dependent methods, neutral-loss scanning, precursor-ion scanning, product-ion scanning*

INTRODUCTION

A general trend in the past decade is development of new nutraceuticals and supplemented foods based on extracts or compounds obtained from diverse natural sources as fruits or vegetables. Anthocyanins are a representative example of this trend as an onset nutraceutical industry is presently interested in this family of compounds because of their antioxidant properties and beneficial effects reported in the literature. These compounds have been featured with anticarcinogenic[1] and anti-inflammatory activity,[2] with a significant role in prevention of cardiovascular diseases and diabetes.[3] This role is essentially based on their chemoprotective, vasoprotective,[4] and free radical scavenging effects,[5] among others, most of them directly related to the flavilium ion with electron deficiency.[6] Apart from that, anthocyanins apparently are a suited option as food colorants because they are nontoxic, water soluble, and easily obtained from natural sources. All these benefits have promoted development of studies on characterization of these compounds with the aim of replacing artificial colorants, the negative side effects of which—such as attention deficit hyperactivity disorder in children[7,8] and carcinogenic activity, as is the case with Red Sudan colorants[9]—have been reported.

Most studies dealing with analysis of these natural pigments have been focused on their extraction from nonvaluable agricultural raw materials, which would involve their revalorization. Present research on raw materials from the winemaking industry is mainly targeted at quantitation of anthocyanins and anthocyanidins from grapes (skin and pomace),[5] but also research on anthocyanins encompasses wine itself,[10] where the anthocyanin content has demonstrated to be representative of each wine, thus allowing discrimination of adulterated wines.[11]

On the contrary, wine lees—the solid residue remaining at the bottom of reservoirs after wine fermentation—have received less attention. Despite their variable composition, wine lees are mainly composed by microorganisms (particularly yeasts) and, in a less proportion, by tartaric acid and inorganic matter. In red wine production, the intense red color of wine lees suggests that this agricultural waste could have an added value as source of colorants. In fact, most of the research on wine lees is focused on adsorption of anthocyanins by yeasts, being of special interest the

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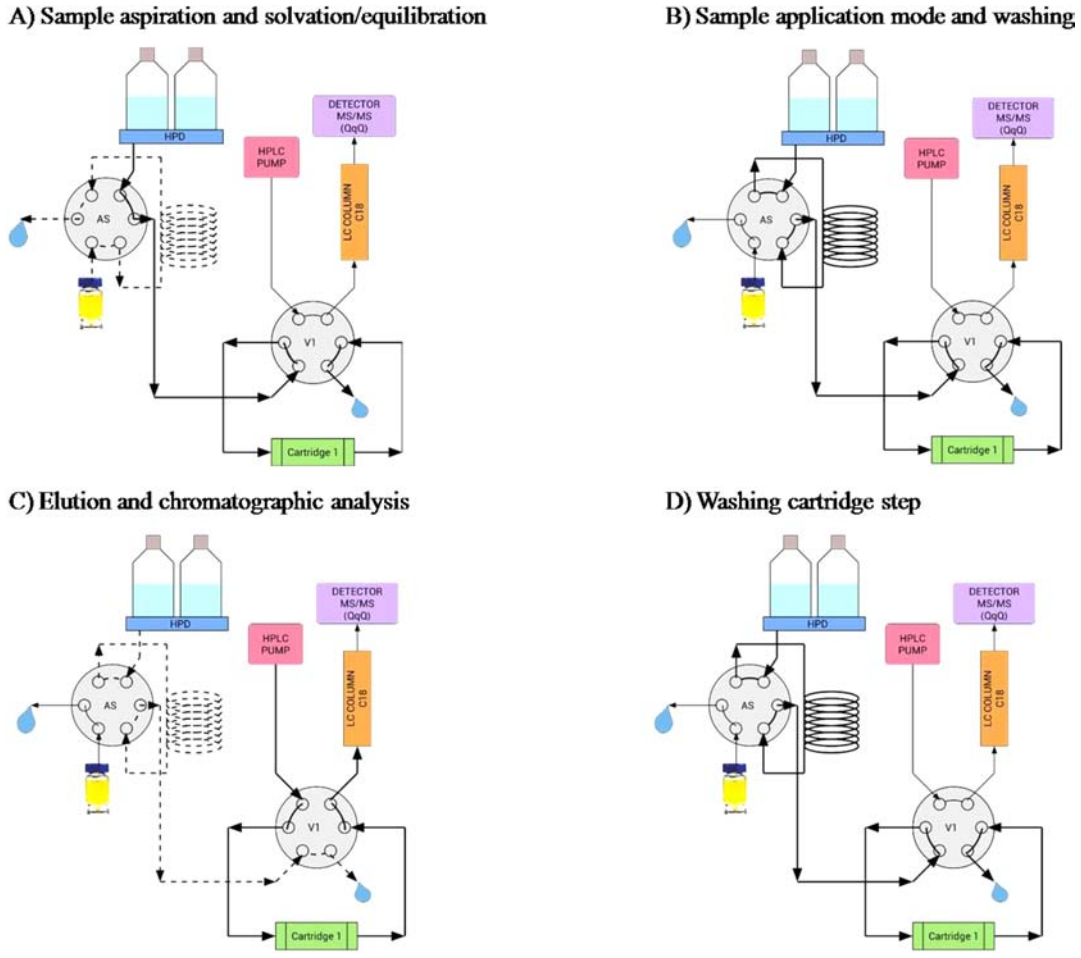

Figure 1. Scheme of the experimental approach based on SPE-LC-MS/MS. Automatic cartridge exchange (ACE), electrospray ionization module (ESI), high-pressure dispenser (HPD), and triple-quadrupole mass detector (QqQ).

studies by Mazauric et al. in 2006[12] and Morata et al. in 2003,[13] who simulated aging of a model wine over lees to prove that about one-third of the total content of free anthocyanins in wine was removed by lees after 1 week.

Methods for determination of anthocyanins are mainly based on liquid chromatography (LC) with detection by molecular absorption (usually a diode array detector—DAD), where identification is primarily based on UV−vis spectra or retention times by comparison with standards.[14] However, DAD detection is unable to discriminate between coeluted compounds, which is specially critical here taking into account the isomeric character of anthocyanins. In this sense, it is worth mentioning the research developed by Pati et al. in 2009, who developed a selective method for screening of anthocyanins and dimers in crude grape extracts with the aid of LC-DAD-MS/MS.[15]

In addition, quantitation of these compounds is a difficult task owing to the absence of commercially available standards for most of them. Two different strategies based on mass spectrometry (MS) can be used to overcome this problem. One is utilization of a high-resolution mass spectrometer such as the time-of-flight (TOF) detector, which supports identification by mass accuracy detection of precursor and product ions. Papoušková et al.[16] used a UPLC-QqTOF MS/MS configuration to study the anthocyanin profile of 68 certified red wines and stated this analytical technique as a conclusive tool for anthocyanin profiling. The second strategy takes benefits from the triple-quadrupole mass detector to confirm the presence of known compounds by application of data-dependent methods. Among these methods, product-ion scanning enables acquisition of MS2 spectra, which provide information on product ions formed by activation of a selected precursor ion. Other approaches are precursor-ion scanning and neutral-loss scanning, which are complementary tools to screen the presence of specific compounds in complex matrices.[17] Precursor-ion scanning is useful to detect all precursor ions that release known product ions, while neutral-loss scanning allows detection of compounds with a given m/z difference between precursor ions and product ions (neutral loss), which should be representative of a characteristic fragmentation. These approaches, used for screening of anthocyanins in grapes and red wine varieties, have allowed identification of compounds such as monoglucoside and diglucoside derivatives as well as other conjugates.[17,19,20] In this research, a commercial automated solid-phase extraction (SPE) system coupled online to LC-MS/MS was selected to develop a method for detection and quantitation of anthocyanins, anthocyanidins, and proanthocyanidins in wine lees and in the extracts from dried lees. To set the concentration range of the target compounds in this vinification residue, samples from eight winemakers located in different points in Spain were used. Application of data-dependent methods enabled confirming the presence of anthocyanins in the target samples. Finally, validation of identification was supported on complementary analysis by LC-TOF/MS.

MATERIALS AND METHODS

Samples. Wine lees obtained as semisolid residue decanted after alcoholic fermentation of grape juice were provided by eight different wineries, where common Spanish grape varieties were used, namely, Cune (Tempranillo), López de Heredia (Mazuelo, Tempranillo, Graciano, Garnacha), La Unión (Syrah, Tempranillo), Ramírez de la Piscina (Tempranillo), Selección de Torres (Tempranillo), Torres de Anguix (Tempranillo), Real Sitio de Ventosilla (Tempranillo, Cabernet Franc, Merlot), and Viña Hermosa (Tempranillo, Garnacha). Each sample was homogenized by agitation at room temperature for 15 min and centrifuged at 855 × *g* for 5 min to isolate the liquid fraction. The "dried solid phase" was the solid resulting from centrifugation and drying at 30 °C for 48 h in an oven, milled in a mortar, sieved to a 0.5 mm particle size, and stored at 4 °C until use. Pools of the liquid and solid phases from the different varieties of wine lees were prepared by mixing aliquots of them. These two pools were used for optimization and validation of the method.

Standards and Solvents. Cyanidin chloride (Cy), delphinidin chloride (Dp), peonidin chloride (Pn), petunidin chloride (Pt), malvidin chloride (Mv), procyanidin B1 (PB1), procyanidin B2 (PB2), and procyanidin A2 (PA2) were from Extrasynthese, (Barcelona, Spain). A standard solution of each anthocyanidin was prepared in ethanol at 1000 μg/mL (pH = 3).

Methanol, acetonitrile, and formic acid (MS grade) were from Scharlab (Barcelona, Spain). Deionized water (18 mΩ·cm) was obtained by a Milli-Q water purification system from Millipore (Billerica, MA). All chemicals were LC grade and used without further purification.

Apparatus and Instruments. A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for microwave-assisted extraction.

Shaking and centrifugation of wine lees and extracts were carried out by an MS2 minishaker (IKA, Germany) and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

The online SPE-LC-MS/MS system was configured by an automated Prospekt-2 SPE workstation from Spark Holland (Emmen, The Netherlands) coupled to an Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA) 6460 Triple Quad LC-MS detector equipped with a Jet Stream Technology electrospray ion source, also from Agilent. The SPE workstation comprises a unit for SPE cartridge exchange, an automatic cartridge exchanger, and a high-pressure syringe dispenser for SPE solvent delivery, as shown in Figure 1. The automated system was also coupled to a Midas autosampler furnished with a 200 μL sample loop. Peek tubing of 0.25 mm i.d. from VICI (Houston, TX) was used for all connections between valves. The SPE step was fully automated via the Sparklink v.2.10 software.

HySpere C8 EC cartridges (end-capped silica-based octyl phase, particle size 10 μm, 10 × 2 mm i.d.) from Spark Holland were used in the SPE step, selected after testing other types of cartridges such as CN (silica based cyanopropyl phase, particle size 7 μm), C2 (silica based ethyl phase, particle size 7 μm), C18 HD (end-capped silica based phase with a high density of octadecyl chains, particle size 7 μm), Resin GP (polymeric polydivinylbenzene phase, particle size 5−15 μm), and Resin SH (strong hydrophobic modified polystyrene−divinylbenzene phase, particle size 20−50 μm). All cartridges were 10 × 2 mm i.d.

Confirmatory analysis of the target compounds was performed by an Agilent 1200 Series LC system interfaced to an Agilent UHD Accurate-Mass LC-TOF/MS detector (Palo Alto, CA), equipped with an Agilent Technology dual electrospray ion source (dual ESI) operating in the negative and positive ionization modes.

Sample Preparation. The liquid phase of wine lees was filtered using a 20 μm pore size filter and 1:2 diluted with acidified milli-Q water (pH = 4) before analysis to avoid obstruction problems in the automated SPE system.

Concerning the solid residue of dried lees, 6.25 g was placed into the extraction vessel of the microwave-assisted digestor with 50 mL of 60:40 (v/v) ethanol−water adjusted at pH 4 with HCl. The vessel was positioned at the suited zone for irradiation with focused microwaves. Auxiliary energy was applied at 140 W irradiation power for 10 min, after which the solid residue was removed by centrifugation and the liquid fraction filtered by a 20 μm pore size filter and 1:2 diluted with acidified milli-Q water.

Analysis started by solvation of the selected SPE cartridges by 4 mL of MetOH at 5 mL/min and equilibration with 2 mL of milli-Q water acidified to pH 4 at 5 mL/min and 1 mL of acidified milli-Q water at 2 mL/min. Cartridges were then loaded with the analytical samples (200 μL) by propelling 2 mL of acidified milli-Q water at 2 mL/min and washed with 0.5 mL of acidified milli-Q water at 1.7 mL/min. Then, the analytes were eluted by the chromatographic mobile phase for 7.5 min according to the LC gradient program detailed below and finally chromatographically separated in the analytical column prior to MS detection. Rinsing of the cartridges with 4 mL of MetOH at 5 mL/min and 4 mL of milli-Q water at 5 mL/min allowed their repeated use. The sequence of automated SPE operations is listed in Table 1.

Table 1. Sequence of Operations Involved in the Online SPE Method

automated analytical protocol	flow rate (mL/min)	volume (mL)	solvent	comment
new cartridge				C8 EC
start autosampler				load sample (200 μL)
solvation	5.0	4.0	methanol	
equilibration 1	5.0	2.0	deionized water (pH 4)	
equilibration 2	2.0	1.0	deionized water (pH 4)	
sample application	2.0	2.0	deionized water (pH 4)	injected sample (200 μL)
wash cartridge	1.7	0.5	deionized water (pH 4)	
switch off valve				
elution			LC mobile phase	7.5 min
wash cartridge 1	5.0	4.0	MeOH	
wash cartridge 2	5.0	4.0	deionized water	

Separation of the analytes was performed by a Mediterranea Sea C18 analytical column (3 μm, 15 × 0.46 cm) from Teknokroma (Barcelona, Spain) using a flow rate of 0.8 mL/min. The gradient program—established by a mobile phase A (deionized water) and mobile phase B (methanol) both containing 0.5% formic acid (pH = 3)—was as follows: 0−1 min, 16% eluent B; 1−6 min, 45% eluent B, held from 6−9 min; 9−16 min, 66.4% eluent B; 16−17 min, 100% eluent B and held up to min 20. The analytical column, thermostated at 20 °C, was equilibrated by a postrun time of 8.5 min between analyses. Complete automation of the SPE and LC-MS/MS systems was accomplished by programming appropriate analysis sequences.

Detection by the triple-quad mass spectrometer used high-purity nitrogen (99.999%) as collision gas. Data were processed using the Agilent MassHunter Workstation Software (version B.05.00) for qualitative and quantitative analysis. Determination of the analytes was performed by ESI-MS/MS in selected reaction monitoring (SRM) mode. Flow and temperature of the drying gas and sheath gas (nitrogen) were 10 L/min and 325 °C, respectively. Nebulizer pressure was 40 psi, and capillary voltage was 2000 V for the positive ionization mode and 4000 V for the negative mode. Dwell time was set at 70 ms.

For LC-TOF/MS confirmatory analysis, chromatographic separation was performed using an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), kept at a temperature of 25 °C. Mobile phases in this case were water (phase A) and acetonitrile (phase B), both LC-MS/MS grade, and with 0.1% formic acid as ionization agent. The LC pump was programmed at a flow rate of 0.9 mL/min, and the following elution gradient was carried out: held from 0 to 2 min 4% eluent B, 2 to 7 min 10% eluent B, 7 to 60 min

Table 2. Optimization of the MS/MS Step for Qualitative and Quantitative Determination of Anthocyanidins and Proanthocyanidins

analyte	precursor ion (m/z)	voltage MS1 (eV)	product ion (m/z)	collision energy (eV)	quantitation SRM transition	confirmation SRM transitions	retention time (min)
proanthocyanidin B1	577.1	160	289.1	30	577.1 → 289.1	577.1 → 407.1; 124.9	6.1
proanthocyanidin B2	577.1	160	289.1	30	577.1 → 289.1	577.1 → 407.1; 124.9	6.9
proanthocyanidin A2	575.0	195	285.0	30	575.0 → 285.0	575.0 → 539.2; 449.0	10.1
delphinidin	303.1	175	229.0	35	303.1 → 229.0	303.1 → 137.1; 257.0	10.7
cyanidin	287.1	185	137.1	35	287.1 → 137.1	287.1 → 213.0; 109.1	12.6
petunidin	317.1	190	302.0	25	317.1 → 302.0	317.1 → 217.0; 203.1	13.2
peonidin	301.0	155	286.0	25	301.0 → 286.0	301.0 → 258.0; 201.1	14.5
malvidin	331.1	185	315.0	35	331.1 → 315.0	331.1 → 287.1; 242.0	15.2

Table 3. Anthocyanins Identification Using Data-Dependent Methods and Confirmation by LC-TOF/MS

				LC-TOF/MS			
compound	precursor ion[a] (m/z)	product ion[b] (m/z)	neutral loss[c] (Da)	experimental m/z	ion	exact mass	error (ppm)
peonidin-3-glucoside	463.2	301.0	162	463.1223	$[M^+]$	463.1235	2.63
peonidin-3,5-diglucoside	625.2	301.0	162 × 2	625.1765	$[M^+]$	625.1763	−0.29
petunidin-3-glucoside	479.4	317.1	162	479.1193	$[M^+]$	479.1184	−1.87
delphinidin-3-glucoside	465.0	303.1	162	465.1020	$[M^+]$	465.1028	1.51
delphinidin-3-rutinoside	611.0	303.1	146, 162	611.1598	$[M^+]$	611.1607	3.18

[a]Detected by precursor-ion scanning. [b]Detected by product-ion scanning. [c]Detected by neutral-loss scanning.

100% eluent B and held up to min 70. A post run of 4 min was included to equilibrate the column. Injection volume was 10 μL, and injector needle was rinsed between samples five times with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it. The operating conditions of the mass spectrometer were as follows: gas temperature, 325 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; and focusing voltage, 175 V. Data acquisition (2.5 Hz) in both the centroid and the profile modes was governed via the Agilent MassHunter Workstation software. The instrument was operated in auto MS/MS mode. The mass range and detection window were set at m/z 100−3000 and 100 ppm, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis (1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine or HP-921] in positive ionization mode, while ions with m/z 119.0362 (proton abstracted purine) and m/z 966.000725 (formate adduct of HP-921) were used in negative ionization mode.

Data Processing. MassHunter Workstation software (version B.05.00) Qualitative Analysis (Agilent Technologies, Santa Clara, CA) was used for processing all data obtained by LC-TOF/MS. The feature extraction algorithm took into account all ions exceeding 5000 counts with a charge state equal to or above one, and a feature had to be composed of two or more ions to be valid (e.g., two ions in the isotope cluster). Peaks with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) or entities characterized by retention time (RT), intensity in apex of chromatographic peak, and accurate mass.

RESULTS AND DISCUSSION

Optimization of the LC-MS/MS Method. On the basis of the ESI-MS/MS ionization and fragmentation patterns for the target compounds, the negative ESI mode was selected for determination of proanthocyanidins while the positive mode was selected for anthocyanidins. Both groups of compounds were separated enough in the chromatograms to require only one analysis per sample by fast polarity switching during the chromatographic run. The effect of the ionization agent on the ESI-MS/MS sensitivity and peak shape was studied by testing variable concentrations of formic and acetic acids taking into account that the stability of the target compounds requires acid pHs. Formic acid improved the sensitivity as compared to acetic acid, with 0.5% (v/v) as optimum concentration. The preferred precursor ions for proanthocyanidins were the $[M - H^-]$ forms, while anthocyanidins were mainly ionized as $[M^+]$ entities. The SRM method was developed for compounds with available standards. Three SRM transitions were selected for each compound to confirm its presence in the target samples. Table 2 lists the optimum values for each anthocyanidin or proanthocyanidin. Activation of proanthocyanidins was characterized by the fragmentation pattern associated to quinone methide (QM) cleavage of the interflavan bond, which led to different fragments depending on the structure of the given proanthocyanidin. As shows Supplementary Figure 1, Supporting Information, proanthocyanidin A is characterized by a main fragment of m/z 285.0 and a secondary fragment at m/z 290.0 as a result of QM cleavage. On the other hand, B-type proanthocyanidins led to fragments at m/z 289.0 and 271.0 as a result of QM, as shown in Supplementary Figure 2, Supporting Information.[18] Anthocyanidins, malvidin, peonidin, and petunidin shared the same fragmentation pattern by cleavage of a methyl group in position R1 (Supplementary Figure 3, Supporting Information). On the other hand, cyanidin and delphinidin were fragmented by different mechanisms. Optimum quantitation transition of cyanidin can be explained by retro-Diels−Alder reaction as described by Barnes et al.,[21] where fragments at m/z 137.1, 121.0, and 109.1 are produced. Fragmentation of delphinidin can be explained by the loss of a molecule of water and two carbonyl groups $[M - H_2O - 2CO]^+$, as reported by Montoro et al.[22]

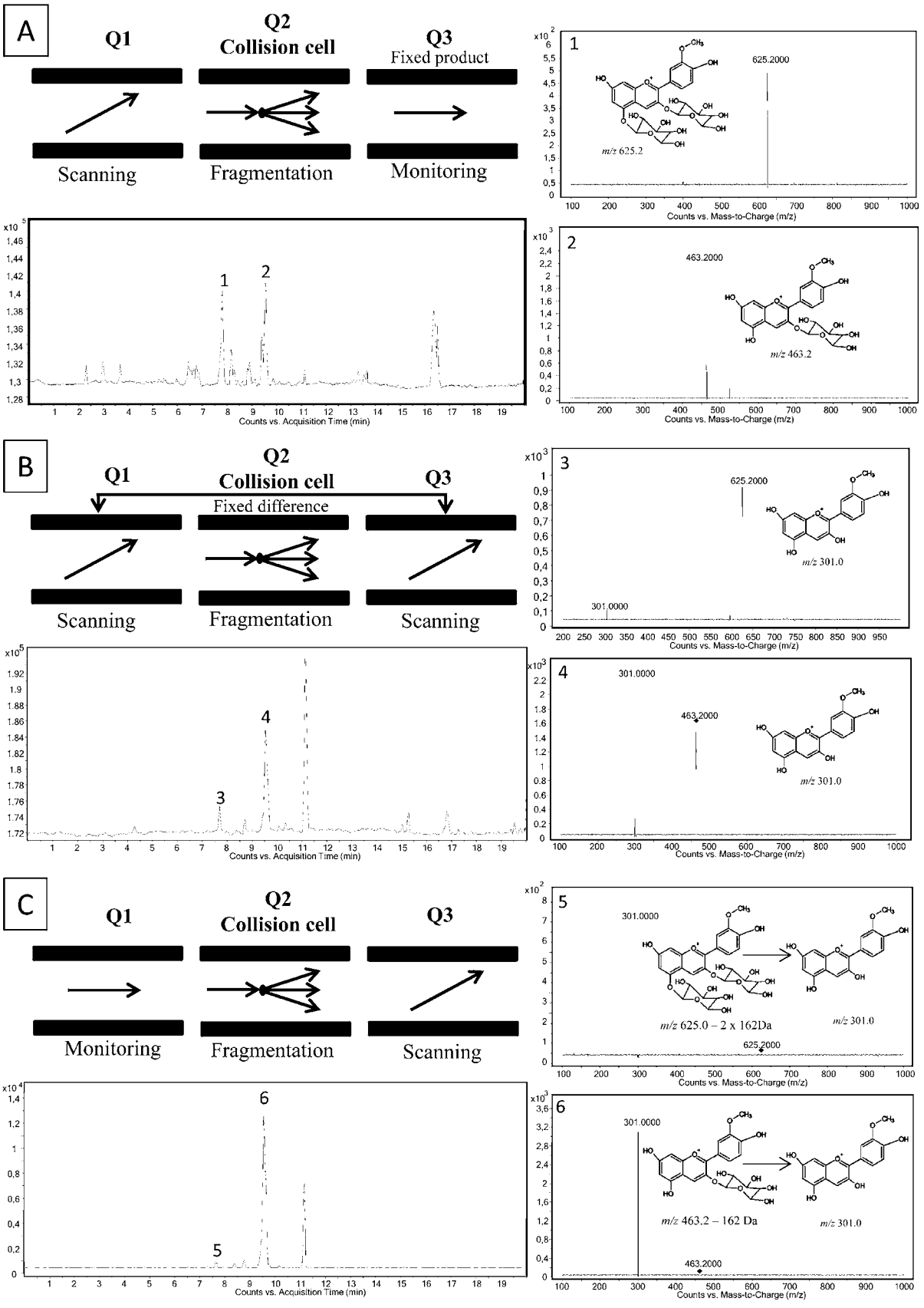

Figure 2. Data-dependent methods used for qualitative analysis. (A) Product-ion scanning for detection of peonidin-3,5-diglucoside and peonidin-3-glucoside, (B) neutral loss scanning by monitoring cleavage of the glucose moiety, and (C) precursor-ion scanning for detection of peonidin-3,5-diglucoside and peonidin-3-glucoside.

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Table 4. Optimization of the MS/MS Step for Qualitative and Quantitative Determination of Anthocyanins

analyte	precursor ion (m/z)	voltage MS1 (eV)	product ion (m/z)	collision energy (eV)	quantitation transition	retention time (min)
peonidin-3-glucoside	463.2	120	301.0	15	463.2 → 301.0	7.5
delphinidin-3-glucoside	465.0	120	303.1	25	465.0 → 303.1	8.3
petunidin-3-glucoside	479.4	120	317.1	15	479.4 → 317.1	8.9
peonidin-3,5-diglucoside	625.2	120	301.0	25	625.2 → 301.0	9.6
delphinidin-3-rutinoside	611.0	100	303.1	25	611.0 → 303.1	14.5

Data-Dependent Methods and High-Resolution Mass Spectrometry. Data-dependent methods were used to scan derivatives of the target metabolites by LC-MS/MS. For this purpose, the versatility of the triple-quad mass analyzer allowed application of three different scanning methods: precursor ion, neutral loss, and product ion. Tentative identifications carried out by data-dependent methods were confirmed by LC-TOF/MS in high-resolution mode using the same chromatographic method. The sample used in all analyses was a pool of liquid lees. Table 3 summarizes the main results obtained by combination of these methodologies. For precursor-ion scanning, the precursor ions found for the three proanthocyanidins and the six anthocyanidins were simultaneously monitored in the third quadrupole (Q3) by scanning precursor ions corresponding to derivative metabolites formed by conjugation, such as glucosides, in the first quadrupole (Q1). Scanning of the precursor ion for delphinidin (m/z 303.1) allowed detecting two ions at m/z 465.0 and 611.0 at 8.3 and 14.5 min elution time, which fit to the molecular cations of delphinidin-3-glucoside and delphinidin-3-rutinoside, respectively. Neutral-loss scanning by tuning Q1 and Q3 to monitor common neutral losses of 162 and 146 Da, corresponding to loss of glucose and rhamnose as substituents, confirmed the molecular cations of delphinidin-3-glucoside (loss of 162 Da) and delphinidin-3-rutinoside (loss of 146 Da). Third, MS/MS product-ion scanning of delphinidin-3-glucoside and delphinidin-3-rutinoside led to preferential formation of the aglycon cation (m/z 303.1, delphinidin) and a fragment at m/z 465.0 that corresponded to cleavage of the rhamnose substituent, which is indicative of the fragmentation of the glycosidic bond between glucose and rhamnose.[17] Identification of these conjugated derivatives was confirmed by high-resolution mass spectrometry by extracting ions at m/z values, as shown in Table 3. Monoisotopic masses were searched in the raw data files obtained by analysis of the samples. Search parameters were mass accuracy 4 ppm, minimum peaks height of 2500 counts on the profile and centroid spectra, and a peak spacing tolerance of 0.0025 m/z plus 7 ppm.

The same strategy was used for other compounds. Thus, glucoside derivatives of peonidin (m/z 301.0) were also detected as the scanning of its precursor ion led to detection of two ions at m/z 625.2 and 463.2, which should correspond to the cationic adducts of peonidin-3,5-diglucoside and peonidin-3-glucoside found at 7.5 and 9.6 min, respectively. Confirmation based on neutral loss scanning (162 Da) and product ion scanning enabled finding the fragment ascribed to aglycone peonidin (m/z 301.0), as shown in Figure 2. By analogy, high-resolution mass spectrometry confirmed identifications proved by data-dependent methods.

Finally, the scanning of the precursor ion of petunidin (m/z 317.1) allowed detecting the molecular cation of petunidin-3-glucoside (m/z 479.4) at 8.9 min, which was also supported on neutral loss scanning of a glucose moiety. Therefore, the product-ion scanning detected the aglycone cation of petunidin (m/z 317.1).

After identification and confirmatory analysis of conjugated derivatives in extracts from wine lees, optimization of the method for detection of these compounds by the SRM method was carried out. Voltages of the first quadrupole and collision energy applied to activate precursor ions were optimized, resulting in the final method in Table 4.

Optimization of Online SPE-LC-MS/MS. A generic protocol was used for optimization of the sorbent, where the tested SPE cartridges were automatically solvated by 4 mL of MeOH at 4 mL/min, conditioned with 2 mL of deionized water at 4 mL/min, and equilibrated first with 2 mL of MeOH and second with 2 mL of deionized water at 4 mL/min. Cartridges were then loaded with 100 μL of the pool of liquid wine lees by propelling 2 mL of deionized water at 4 mL/min. The washing step was eliminated to ensure 100% retention. Then, analytes were eluted from the SPE cartridges by the mobile phase after switching off valve 1 during the LC gradient program. Finally, the eluate components were chromatographically separated by the analytical column prior to MS detection.

The capacity of the sorbents for analyte retention (expressed as average of relative percentage of peak areas for all compounds) decreased in the sequence C8 EC (100) > C18 HD (80) > Resin SH (70) > Resin GP (65) > C2 (15) > CN (5). Best results were obtained by HySphere C8 EC cartridges, which were adopted for further testing as they provided the best overall recovery in these preliminary tests.

Response surfaces were developed to optimize the volume and flow rate for the loading solvent and washing solutions, with the best recoveries found at volumes of 0.5 mL for both and flow rates of 2.0 and 1.7 mL/min, respectively.

The effect of the type of elution solvent was also tested, obtaining the best results with acidified milli-Q water (pH 4) after checking mixtures with different percentages of acidified organic phase (i.e., 0%, 10%, 20%, 30%, and 40% MeOH). Retained analytes were eluted by pumping the LC mobile phase through the SPE cartridge to the analytical column for 7.5 min. Shorter times did not allow quantitative elution of the analytes from the cartridge, while longer elution times led to peak broadening. The efficiency of the SPE step was calculated by setting two C8 cartridges online for sequential passage of the sample through them. As shown in Supplementary Figure 4, Supporting Information, a modified configuration was used by implementation of a second valve (valve 2) in the system. Thus, if breakthrough occurs, the second cartridge should retain the fraction of compounds which was not retained in the first cartridge. Independent analysis of the eluted fraction from each cartridge should allow estimating the SPE retention efficiency, which was assessed by triplicate analysis of liquid wine lees and extracts of dried wine lees spiked at two concentrations (i.e., 1.0 and 1.5 ng/mL). The extraction efficiency was calculated as the ratio of analyte response in cartridge 1 and the sum of the response provided by both cartridges. Attending to these estimations, the extraction efficiency was above 93% in the extracts from solid lees, while in liquid lees this parameter was

Table 5. Analytical Features of the Method

analyte	sample	calibration equation	linear range (ng/mL)[a]	coefficient of regression (R^2)	limit of detection (pg/mL)	limit of detection on column (pg)	limit of quantitation (pg/mL)	limit of quantitation on column (pg)
proanthocyanidin B1	liquid wine lees	$y = 11\,2318x + 5460.80$	0.10−500	0.9804	30	3.0	100	10.0
	extract of dry lees	$y = 117\,165x + 11\,500$	0.10−800	0.9809	30	3.0	100	10.0
proanthocyanidin B2	liquid wine lees	$y = 11\,067x - 8684.50$	0.08−600	0.9963	24	2.4	80	8.0
	extract of dry lees	$y = 21\,862x + 9463.9$	0.05−900	0.9951	15	1.5	50	5.0
proanthocyanidin A2	liquid wine lees	$y = 58\,250x + 503.32$	0.08−900	0.9934	24	2.4	80	8.0
	extract of dry lees	$y = 25\,397x - 637.68$	0.08−750	0.9883	24	2.4	80	8.0
cyanidin	liquid wine lees	$y = 226\,620x - 3704.20$	0.05−900	0.9936	15	1.5	50	5.0
	extract of dry lees	$y = 221\,780x - 14\,067$	0.1−900	0.9984	30	3.0	100	10.0
peonidin	liquid wine lees	$y = 950\,387x - 18\,233$	0.05−800	0.9953	15	1.5	50	5.0
	extract of dry lees	$y = (1 \times 10^{06})x - 73\,010$	0.10−800	0.9979	30	3.0	300	30.0
petunidin	liquid wine lees	$y = 438\,493x - 4223.80$	0.10−600	0.9909	30	3.0	100	10.0
	extract of dry lees	$y = 356\,935x - 38\,870$	0.20−900	0.9931	60	6.0	200	20.0
malvidin	liquid wine lees	$y = 196\,102x + 2370.40$	0.15−750	0.9899	45	4.5	150	15.0
	extract of dry lees	$y = 370\,529x - 3383.7$	0.20−750	0.9992	60	6.0	200	20.0
delphinidin	liquid wine lees	$y = 138\,385x - 5007.70$	0.05−900	0.9999	15	1.5	50	5.0
	extract of dry lees	$y = 192\,184x - 785.22$	0.26−750	0.9998	78	7.8	260	26.0

[a]The concentration in the extracts correspond to 6.50 g of dried lees quantitatively extracted with 50 mL of extractant —60:40 (v/v) ethanol−water (see Sample Preparation).

above 97% in all types of lees. With these premises, no breakthrough was observed for any target analyte at any concentration, despite the differences in polarity between conjugated and nonconjugated compounds.

Validation of the Method. Calibration graphs ($n = 7$) made with both spiked liquid wine lees and extracts from solid lees exhibited excellent linearity for proanthocyanidins and anthocyanidins, with regression coefficients higher than 0.98 over the concentration range depending on the target analyte (Table 5). Three analytical replicates ($n = 3$) of spiked samples for each concentration level within the linear dynamic range were injected. Figure 3A shows the SRM chromatograms obtained by monitoring selected ions of the target analytes in liquid wine lees spiked with standards (1 μg/mL) subjected to the above-described online SPE-LC-MS/MS procedure. Figure 3B shows the SRM chromatograms obtained by monitoring selected ions of the target analytes in liquid wine lees from La Unión wineries.

The lower limit of detection (LLOD) and that of quantitation (LLOQ) were calculated by injecting dilution series of each analyte in both types of samples to obtain the concentrations which provided signals 3 and 10 times above the background noise, respectively. The LLODs ranged from 15 to 45 pg/mL for liquid wine lees and from 15 to 78 pg/mL for a pool of extracts of dried wine lees . The sensitivity was thus better for liquid lees, which could be ascribed to components of the solid matrix present in the extracts. Table 5 summarizes the characteristics of the method. Matrix effects could justify the differences in calibration models for certain compounds. However, this effect was not matrix dependent as it is random. This is an important aspect and explains the utilization of different calibration models.

The precision of the method was estimated by intraday and interdays studies. Both parameters were evaluated by analysis of pools of liquid lees and extracts spiked at three concentrations (0.5, 1.0, and 1.5 ng/mL) for seven consecutive days with three replicates per day. As shown in Table 6, intraday and interday variability, expressed as RSD, ranged from 0.1% to 10.9% and from 0.4% to 10.1%, respectively, depending on the target analyte.

Analysis of Anthocyanins, Anthocyanidins, and Proanthocyanidins Content in Lees. The proposed online SPE-LC-MS/MS method was used to determine the anthocyanins, anthocyanidins, and proanthocyanidins profile in liquid wine lees and extracts of dried wine lees. Table 7 shows the quantitation data obtained after interpolation of peak areas within their calibration curves for all analyzed samples. Concerning analytes with no commercial standards, it is worth mentioning that they were quantified by the aglycon form, which is the usual approach since it is supposed that the glucoside and the aglycon forms have similar biological effects.

In general terms, the concentrations of the target compounds in the extracts of dried lees were higher than in liquid lees, especially for glucoside derivatives. B type proanthocyanidins were always found at higher concentrations than those of A type, with levels between 2.0 and 31.5 ng/g in dried lees extracts and between 0.3 and 23.5 ng/mL in liquid lees. Exceptional levels were found in the case of liquid lees from López de Heredia, Ramírez de la Piscina, and Viña Hermosa, in which the

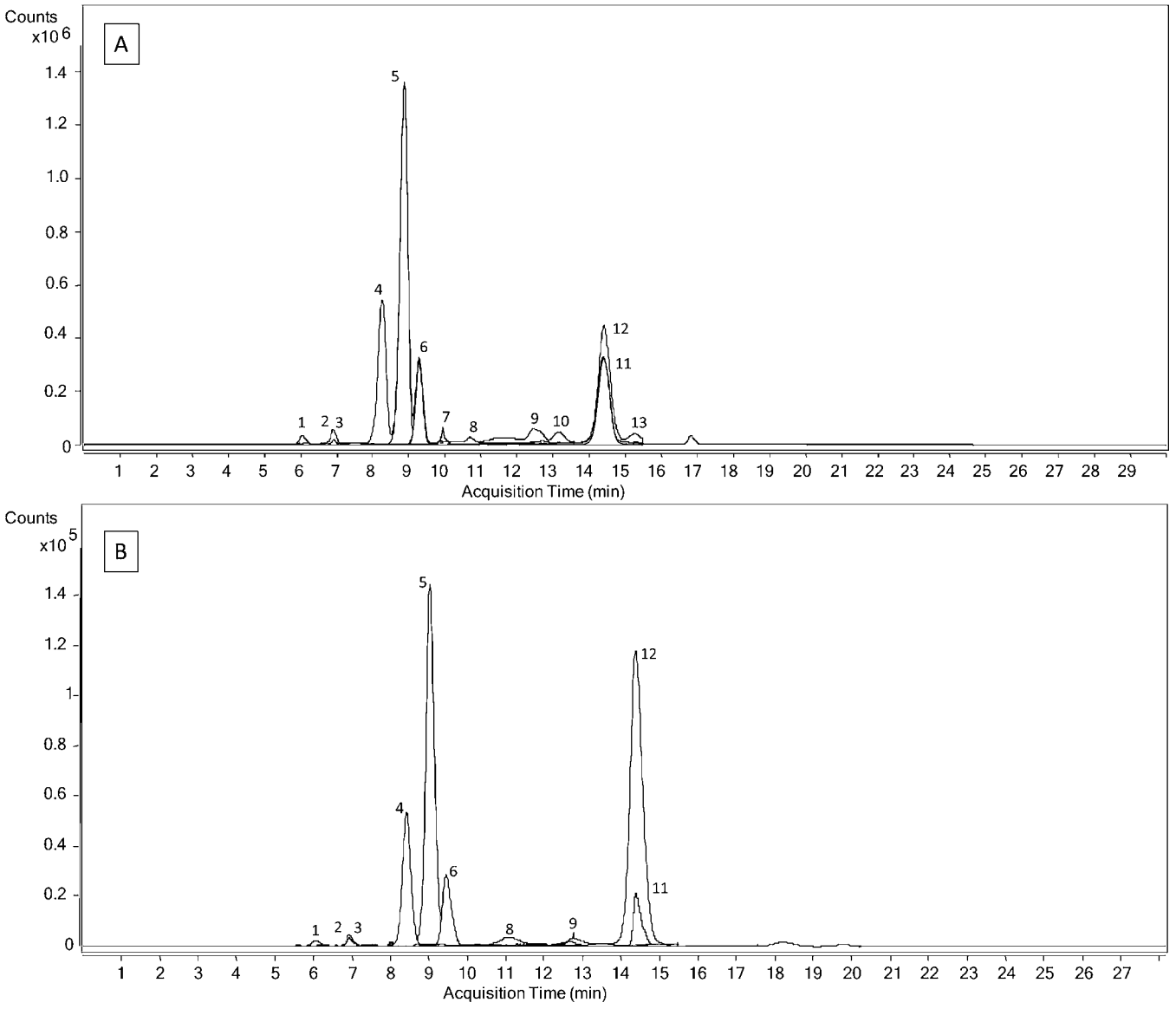

Figure 3. Selected reaction monitoring chromatogram obtained from (A) liquid wine lees from Viña Hermosa wineries spiked with standards (1 μg/mL each) and (B) extract of the solid residue from wine lees from La Unión wineries.

Table 6. Precision Study by Estimation of Intraday and Interdays Variability Expressed as Relative Standard Deviation

	liquid lees 1.5 (ng/mL)		liquid lees 1.0 (ng/mL)		liquid lees 0.5 (ng/mL)		extract 1.5 (ng/mL)		extract 1.0 (ng/mL)		extract 0.5 (ng/mL)	
analyte	intraday	interdays	intraday	interdays	intraday	interdays	intraday	interdays	intraday	interdays	intraday	interdays
proanthocyanidin B1	2.6	3.7	7.5	7.5	3.2	7.4	2.0	6.8	5.6	6.2	1.9	6.9
proanthocyanidin B2	2.5	9.8	8.9	9.9	8.0	9.9	3.6	7.4	3.7	8.8	3.6	9.9
proanthocyanidin A2	2.0	9.3	2.6	7.4	4.0	6.4	5.5	6.9	0.1	8.9	0.4	7.1
delphinidin	1.2	5.2	0.9	1.9	0.5	8.3	7.7	9.6	6.9	9.7	3.4	8.3
cyanidin	1.0	1.3	0.1	0.4	0.4	9.5	8.4	9.9	0.7	10.1	1.5	7.4
petunidin	1.1	2.4	1.4	2.4	0.1	2.5	3.2	6.7	1.9	5.3	2.3	8.5
peonidin	0.2	1.5	1.6	0.9	0.1	4.7	5.9	7.4	0.3	4.8	0.4	8.6
malvidin	3.8	4.9	6.8	7.2	8.4	8.6	4.7	5.7	2.1	6.6	1.7	8.6

concentration of proanthocyanidin B2 reached 17.5, 8.5, and 23.5 ng/mL, respectively.

Concerning anthocyanidins, the concentrations found varied between 1.06 and 77.5 ng/g in the extracts of dried lees and between 0.09 and 7.5 ng/mL in liquid lees. The highest concentrations of petunidin and malvidin were found in extracts from Selección de Torres dried lees: 71.3 and 77.5 ng/g, respectively. Detection of anthocyanidins in wine lees had previously been described by Mazauric et al. and Salmon et al., who stated that yeast lees can modify color of wines either by weak and reversible interactions between anthocyanins and yeast walls or by a periplasmic β-glucosidase enzyme.[12,22]

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Table 7. Concentration of Anthocyans Detected in Liquid Lees and Extracts from Solid Lees (expressed as ng/mL and ng/g, respectively)

analyte	sample[a]	Cune[b]	López de Heredia[b]	La Unión[b]	Ramírez de la Piscina[b]	Selección de Torres[b]	Torres de Anguix[b]	Real Sitio de Ventosilla[b]	Viña Hermosa[b]
Pro B1	A	18.4	n.d.	14.4	11.2	25.1	16.9	17.2	4.5
	B	n.d.	1.9	0.3	3.3	0.3	n.d.	0.3	3.7
Pro B2	A	28.3	n.d.	24.9	2	31.5	19.5	14.6	4.8
	B	2.3	17.5	3.1	8.5	3.9	2	5.4	23.5
Pro A2	A	1.4	3.7	1.5	4.2	1.9	1.2	0.6	1.1
	B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cyanidin	A	1.5	1.5	1.1	1.7	6	3	2.3	1.1
	B	0.1	0.1	0.1	0.1	0.5	0.5	0.3	0.1
peonidin	A	n.d.	n.d.	n.d.	n.d.	3.6	2.8	n.d.	n.d.
	B	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
petunidin	A	8	10.1	2.7	4.9	71.3	20.9	16.5	1.9
	B	n.d.	0.4	n.d.	0.3	0.4	n.d.	3.7	1.1
malvidin	A	7.7	n.d.	n.d.	1.9	77.5	25.7	21.6	n.d.
	B	0.2	0.3	n.d.	0.7	2.1	0.2	4.5	1
delphinidin	A	30.2	n.d.	n.d.	4.6	27.9	37.7	24	4.9
	B	0.1	2.4	0.09	0.2	0.2	0.1	7.5	4.6
peonidin 3-glucoside[c]	A	39.9	n.d.	n.d.	13.3	192	100	36.2	6.1
	B	0.6	3.6	0.9	0.3	0.2	0.3	3.5	0.5
peonidin 3,5-diglucoside[c]	A	11.4	n.d.	n.d.	4.8	27.9	40.3	9.3	n.d.
	B	n.d.	0.06	0.05	0.09	0.06	n.d	0.2	0.1
petunidin 3-glucoside[c]	A	421	2.1	2.2	112	3401	717	424	80.3
	B	0.9	33.4	0.6	9.8	9.9	0.9	113	85.5
delphinidin 3-glucoside[c]	A	426	n.d.	n.d.	65	4000	538	342	71.7
	B	0.4	31.8	0.3	1.2	1.7	0.8	103	61.9
delphinidin 3-rutinoside[c]	A	552	n.d.	n.d.	148	1697	690	992	39.6
	B	0.2	4.3	0.6	5.5	3.3	0.3	30.2	8.4

[a]A: Extract of dry lees. B: Liquid wine lees. [b]n.d.: not detected. [c]Concentration estimated in terms of the calibration curve of the corresponding anthocyanidin.

Variability of the results was characteristic in the case of anthocyanins. Thus, high levels of anthocyanins were found in samples from specific wineries such as Cune, Selección de Torres, Torres de Anguix, Real Sitio de Ventosilla, and Viña Hermosa. The high concentration of peonidin-3-glucoside, petunidin-3-glucoside, delphinidin-3-glucoside, and delphinidin-3-rutinoside (191.7, 3400.5, 4000.2, and 1697.4 ng/g, respectively) detected in extracts isolated from Selección de Torres as compared to other varieties should be emphasized. One other interesting result was ascribed to lees from Real Sitio de Ventosilla, in which concentrations of petunidin-3-glucoside and delphidinin conjugates were high both in liquid lees and in extracts from dried lees. The lowest concentrations of anthocyanins were found in lees from La Unión and Ramírez de la Piscina.

Application of quantitative methods enabled comparing the target analytes content of wine lees from different wineries and also the content of liquid lees and extracts isolated from dried lees. Attending to these results, the influence of the winery on the colorant content, especially that of anthocyanidins, is evident. This could be explained by the differences of wine aging protocols adopted by wineries, also influenced by the region and cultivar. This study also proved the capability of data-dependent methods combined with high-resolution MS to detect and confirm the identity of unknown compounds without use of standards.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1−4, where fragmentation pathways of proanthocyanidin A2 and B, the basic structure of anthocyanidins, and a scheme of the experimental approach based on SPE-LC-MS/MS are shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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