Study of phenolic extractability in grape seeds by means of ATR-FTIR and Raman spectroscopy.

Short title: Grape seed phenolic extractability using ATR-FTIR & Raman spectroscopy

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ABSTRACT

Near infrared hyperspectral imaging has been applied to grape seeds in order to select a representative subset of samples according to their spectral features in the 900-1700 nm range. Afterwards, selected grape seeds have been classified according to their total phenol and flavanol extractabilities. In this way, samples were sorted in three different groups identified as low, medium and high extractability levels.

In order to establish the chemical structures which can be responsible for the different extractabilities, vibrational spectroscopy has been applied to the non-extracted material after seed extractions. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) and Raman spectra of non-extracted seed material have been recorded and their main spectral features have been linked to extractabilities of flavanolic and total phenolic compounds.

The vibrational spectroscopic analysis confirms that grape seed phenolic extractability is influenced by the cell wall composition (polysaccharides, lignins, pectins) and by the degree of esterification of pectins.

KEYWORDS

Wine, grape seeds, phenolic extractability, ATR-FTIR spectroscopy, Raman spectroscopy, hyperspectral imaging.

1. INTRODUCTION

Although grape seeds represent only 0–6% of berry weight, they effect the sensory properties of wine. Depending on varieties, they contain between 20 and 55% of the phenolic compounds of the berry (Ribéreau-Gayon, Dubourdieu, Doneche, Lonvaud, Glories, Maujean, et al., 2006). Phenolic compounds are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. Flavonoids are the most numerous amongst phenolics. The generic structure of the major flavonoids comprises fifteen carbons (C6-C3-C6), with two aromatic rings (C6) connected by a heterocyclic pyran ring of three-carbon bridge (C3). Flavonoids are secondary metabolites that have important and varied roles (Crozier, Clifford, & Ashihara, 2006), including well-known health benefits. They possess ideal structural chemistry for free radical-scavenging activities, and they have been shown to be more effective antioxidants *in vitro* than vitamins E and C, on a molar basis (Rice-Evans, Miller, & Paganga, 1997).

The main subclasses of flavonoids present in grapes (*Vitis vinifera* L.) are flavonols, flavanols and anthocyanins, and they are transferred to the wine during the fermentation process. Flavonols and anthocyanins are principally localized in the skin, whereas flavanols are synthesized primarily in seeds and stems (Jackson, 2000). Flavanols constitute the most abundant phenolic family in grape seeds and play a relevant role in the sensory characteristic of red wines. They can increase the wine body, but also the sensations of astringency and bitterness (Waterhouse, 2002). Moreover, flavanols, like the main phenolic families, can increase and stabilize the wine color by means of the copigmentation phenomenon (Boulton, 2001; Gordillo, Rodríguez-Pulido, González-Miret, Quijada-Morín, Rivas-Gonzalo, García-Estévez, et al., 2015; Nogales-Bueno, Baca-Bocanegra, Jara-Palacios, Hernández-Hierro, & Heredia, 2016).

The importance of controlling the amount of phenolic compounds that may be extracted from grapes to wine lies in the fact that the phenolic content wine depends mainly on the amount of phenolic compounds released from grape to wine. It is well known that the extractabilities of phenolic compounds from the solid parts of grapes (seeds and skins) depends on the grape maturity. However, in skins, phenolic extractability increases with maturity, whereas, in seeds it decreases. (Ribéreau-Gayon, et al., 2006).

The effect of cell wall degradation on the degree of extraction of phenolic compounds from grape skins has already been widely studied. Several studies have been developed using this matrix in order to control and understand the relationship between cell wall composition and the extraction degree of phenolic compounds. An increase in the sugar content (degree of ripening) has been correlated with decreasing amounts of cell wall material, galactose, cellulose and mannose, accompanied by a decrease in the degree of methylation of pectic polysaccharides or pectins (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008). These factors could be responsible for the different extractabilities of phenolic compounds, bearing in mind that differences on thickness or density of the skin cell-wall could also play a role.

Regarding grape seeds, extractability decreases during ripening, from about 25% at the first stages to about 5% in the later (Rodríguez-Pulido, Hernández-Hierro, Nogales-Bueno, Gordillo, González-Miret, & Heredia, 2014). It might be inferred that this phenomenon could be attributed to the presence of the middle integument, which is waterproof and very hard when seeds ripen and lignify, and could prevent the extraction (Cadot, Minana-Castello, & Chevalier, 2006). On the other hand, changes in the cell wall polysaccharide structure could affect the solubility of the flavanols (Bautista-Ortín, Jiménez-Pascual, Busse-Valverde, López-Roca, Ros-García, & Gómez-Plaza, 2013). These authors found that maceration enzymes, normally used in the wine elaboration

process to degrade skin cell wall in order to increase color, can also favour the extraction of flavanols from the seeds. These oenological products easily degrade the cell walls of seeds (especially if they are unripe and show low lignification), promoting the release of seed compounds into the must-wine, increasing the wine body, but also the sensations of astringency and bitterness. It is therefore of interest to evaluate how cell wall structure changes among seed samples of different grape varieties or with different levels of phenolic compounds extractability.

Near infrared spectroscopy (NIRS) has been used in order to screen total or extractable phenolic compounds in grapes obtaining quite good results (Ferrer-Gallego, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2011; Kemps, Leon, Best, De Baerdemaeker, & De Ketelaere, 2010; Nogales-Bueno, Baca-Bocanegra, Rodríguez-Pulido, Heredia, & Hernández-Hierro, 2015; Nogales-Bueno, Hernández-Hierro, Rodríguez-Pulido, & Heredia, 2014; Rodríguez-Pulido, et al., 2014; Torchio, Río Segade, Giacosa, Gerbi, & Rolle, 2013). However, it is not possible to easily interpret the relationship between cell wall composition and phenolic compounds extractability based on near infrared (NIR) region features. The near infrared region contains absorption bands corresponding to overtones and combinations of fundamental C–H, O–H and N–H vibrations. Additionally, the presence of Fermi resonances can also increase the complexity of the NIR spectra. Therefore, many band assignments can only be tentative or unresolved which limits the usefulness of the near infrared region in structural determination. In comparison, mid infrared spectra exhibit sharp and narrow peaks essentially related to fundamental modes of vibration which can be easily assigned to chemical structures (Bokobza, 1998). Mid infrared spectroscopy has previously been used in order to relate skin cell wall composition to anthocyanin extractability (Femenia, Sánchez, Simal, & Rosselló, 1998; Hernández-Hierro, Quijada-Morín, Martínez-

Lapuente, Guadalupe, Ayestarán, Rivas-Gonzalo, et al., 2014). In these studies, mid infrared spectral features could be directly linked to the esterification of pectins, which might be responsible for the different extractabilities of the aforementioned phenolic compounds. Moreover, this spectroscopic technique has been applied to the study and quantitation of phenolic compounds extracted during winemaking (Di Egidio, Sinelli, Giovanelli, Moles, & Casiraghi, 2010; Fragoso, Acena, Guasch, Mestres, & Busto, 2011). However, in these cases, cell wall composition has not been taken into account.

Cell wall structure of grape and other fruits has been widely studied by means of vibrational spectroscopy. Vibrational spectroscopy is a subset of spectroscopy which comprises IR and Raman spectroscopy and analyses vibrations within a molecule (or material) (Byrne, Ostrowska, Nawaz, Dorney, Meade, Bonnier, et al., 2014). These two vibrational spectroscopic techniques are complementary. Whereas electric dipole transitions of IR (and UV–visible) absorption require a change of the dipole moment of the material as a result of the transition, Raman scattering requires a change in the polarizability of the bond as a result of the transition. Thus, access to molecular level information by means of two different physical processes can be achieved (Byrne, Sockalingum, & Stone, 2011).

Mid infrared spectroscopy has been employed to study cell wall structure of grapes and other fruits (Acebes, Largo-Gosens, Hernández-Altamirano, García-Calvo, Alonso-Simón, & Álvarez, 2014; Fasoli, Dell'Anna, Dal Santo, Balestrini, Sanson, Pezzotti, et al., 2016; Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015; Wilson, Smith, Kačuráková, Saunders, Wellner, & Waldron, 2000; Zietsman, Moore, Fangel, Willats, Trygg, & Vivier, 2015). However, studies which employ mid infrared spectroscopy to study the cell wall structure of grapes seeds have not been performed.

Raman spectroscopy has also been employed to study cell wall structure in fruits,

142 although, no studies have been found for grapes (Chylińska, Szymańska-Chargot, &
143 Zdunek, 2014; da Silva, Vandenabeele, Edwards, & Cappa de Oliveira, 2008; Gierlinger,
144 Keplinger, & Harrington, 2012; Szymańska-Chargot, Chylińska, Pieczywek, Rösch,
145 Schmitt, Popp, et al., 2016). Therefore, although both mid infrared and Raman
146 spectroscopy have proved to be useful and reliable techniques for the study of the cell
147 wall structure in plant and fruit tissues, they have not yet been applied to the study of
148 grape seeds.
149 In the present study, near infrared hyperspectral imaging has been applied to grape seed
150 samples in order to select a representative subset of samples according to their near
151 infrared spectral features. Afterwards, total phenol and flavanol extractabilities have been
152 obtained for these selected samples and they were sorted into three different groups,
153 identified as low, medium and high extractability levels. Next, Raman and attenuated total
154 reflectance Fourier transform infrared (ATR-FTIR) spectra have been collected for non-
155 extracted material after grape seed extractions. Finally, ATR-FTIR and Raman spectra
156 have been analyzed in order to relate their more important features to phenolic
157 extractability levels in grapes seeds. Moreover, spectral features are also related to the
158 principal sample attributes. To our knowledge, that this is the first time that ATR-FTIR
159 and Raman spectroscopy are jointly applied to grape seed non-extracted material samples
160 in order to relate their more important spectral features to their phenolic extractability
161 levels.
162
163 **2. MATERIAL AND METHODS**
164 2.1. *Samples*
165 *V. vinifera* L. cv. Syrah and Tempranillo red grape samples were collected from two
166 vineyards located in the Condado de Huelva Designation of Origin D.O. (Andalusia,

Spain). Grapes were collected on two different dates, when Tempranillo and Syrah vineyards were respectively harvested (August 7 and 11, 2014). A total of two hundred Syrah and Tempranillo grapes were collected, one hundred per variety. In order to achieve representative samples sets, single grapes were collected from the top, middle and bottom of the cluster and from the sunlight and shade side. The samples were refrigerated and immediately transported to the laboratory.

2.2. *Sample selection*

Grape seeds were removed from the whole grapes and left to dry at room temperature. Then individual near infrared hyperspectral images were recorded for grape seeds belonging to the same grape and the near infrared spectrum was saved (between 950 and 1650 nm) as described elsewhere (Rodríguez-Pulido, et al., 2014). Afterwards, sample selection was carried out as described in Nogales-Bueno, et al. (2015). Briefly, an unsupervised pattern recognition technique, principal component analysis (PCA), was used to select representative samples from the spectral data set. Eight principal components were taken into account in order to explain ninety per cent of the spectral variability of original spectral matrix. Mahalanobis distances (H) for each sample were calculated and samples were grouped according to a neighborhood H criterion ($NH \leq 0.6$). Thus, 66 groups with different spectral characteristics were created and one sample from every group was selected. These 66 selected samples were used in all the subsequent analyses. Grape seeds were weighed, immediately frozen and stored at -20 ºC until analyses were performed.

2.3. Model wine and exhaustive extractions

Two different extractions were carried out: a model wine extraction and an exhaustive extraction.

191 For model wine extraction, grape seeds were immersed in a model wine hydroalcoholic
192 solution (4 g L^{-1} tartaric acid, 12.5% (v/v) ethanol, adjusted at pH 3.6 with NaOH 0.5 M)
193 for a maceration period of 72 h. Then, supernatants were used into the subsequent
194 analyses.
195 Afterwards, grape seed samples were freeze-dried, macerated in methanol:water 75:25
196 (v/v), sonicated during 15 minutes (JP Selecta, Barcelona, Spain) and centrifuged (830×g,
197 15 minutes). These extractions were repeated twice in order to achieve an exhaustive
198 extraction of phenolic compounds. The methanolic extracts were combined and finally
199 made up to a final volume of 50 mL with methanol.
200 As a by-product of model wine and exhaustive extractions, non-extracted material (NEM)
201 was obtained from each grape seed sample. These NEM samples were freeze-dried and
202 then stored in a desiccator until further use.
203 *2.4. Total phenol and flavanol extractability determinations*
204 Total phenol and flavanol extractabilities were determined for grape seed samples. They
205 were determined for each supernatant coming from the model wine and exhaustive
206 extractions. Then, extractabilities of each sample were evaluated, respectively, as the
207 fractions of total phenols and flavanols extracted by the model wine solution with respect
208 to the exhaustive extraction. Finally, grape seed samples were sorted according to their
209 phenolic extractability levels expressed as percentages.
210 Total phenol contents were determined using the Folin–Ciocalteu method (Singleton &
211 Rossi, 1965). Two hundred microliters of exhaustive or model wine extractions were
212 mixed with 1.5 mL of sodium carbonate (20 % w/v), 500 µL of Folin reagent and made
213 up to 10 mL with ultrapure water.
214 Flavanol contents were determined following a modification of the method of Vivas,
215 Glories, Lagune, Saucier, and Augustin (1994). Ten or twenty microliters of exhaustive

or model wine extractions were mixed with 190 or 180 µL of methanol respectively and 1 mL of DMACA (4-dimethylaminocinnamaldehyde) reagent. The DMACA reagent was prepared immediately before use, containing 0.1% (w/v) DMACA in a mixture of HCl:methanol (1:10, v/v).

Both Folin and DMACA analyses were performed on an Agilent 8453 UV–visible spectrophotometer (Palo Alto, USA), equipped with diode array detection (DAD), measuring absorbance at 765 and 640 nm respectively. The extract volumes were appropriately modified for samples which needed it.

2.5. ATR-FTIR data collection

ATR-FTIR spectra were recorded with the Perkin Elmer (MA, USA) Spotlight 400N Universal Attenuated Total Reflectance (UATR) accessory of the spectrometer, which employs a 9-bounce diamond top-plate for this analysis. Spectral data were the result of 32 scans, with a spectral resolution of 1 cm^{-1} and covering the spectral range between 600 and 4000 cm^{-1}.

All samples were measured with a force gauge of 140 units. NEM from grape seed samples were powders and 6 absorption spectra were collected for each grape. A background spectrum was also recorded and automatically subtracted by the software.

2.6. Raman data collection

A Horiba Jobin-Yvon LabRAM HR800 spectrometer with an external 300 mW diode laser operating at 785 nm as source was used throughout this work. For the measurements, a ×100 objective (MPlanN, Olympus) was employed, providing a spatial resolution of ~1 µm at the sample. The confocal hole was set at 100 µm, the specified setting for confocal operation. The system was spectrally calibrated to the 520.7 cm^{-1} spectral line of silicon and the intensity response function was corrected using the Standard Reference Material (SRM) No. 2243 of the National Institute of Standards, Boulder, Colorado, USA (NIST

241 SRM 2243, 2242, 2241). The LabRAM system is a confocal spectrometer that contains
242 two interchangeable gratings (300 and 900 lines per mm respectively). In the following
243 experiments, the 300 lines per mm grating was used, giving a spectral dispersion of ~1.5
244 cm^{-1} per pixel. The detector used was a 16-bit dynamic range Peltier cooled CCD detector.
245 All spectra were recorded over the spectral range between 400 and 3500 cm^{-1} and with a
246 spectral resolution of ~0.9 to 1.6 cm^{-1}. Six spectra were collected for each NEM from
247 grape seed samples.

248 *2.7. Data analysis*

249 K-means cluster analysis of near infrared hyperspectral data was carried out in order to
250 sort grape seed samples according to their phenolic extractability levels (total phenol,
251 flavanol extractabilities). Initial cluster centers were chosen in order to maximize the
252 initial between-cluster distances. Samples were sorted into three groups, low, medium
253 and high phenolic extractability levels. K-means analysis was carried out by means of
254 Statistica v.8.0 software (StatSoft Inc., OK, USA, 2007).

255 Both ATR-FTIR and Raman raw data needed spectral pre-treatments. A multiplicative
256 scatter correction (MSC) was applied to the ATR-FTIR raw data in order to remove the
257 scattering caused mainly by particle size and compaction. Win ISI (v1.50) (Infrasoft
258 International, LLC, Port. Matilda, PA, USA, 2000) software was used for this aim.
259 Baseline correction was applied to Raman raw data. This correction was carried out using
260 MATLAB R2012b (The Mathworks, Natik, MA, USA, 2012) and following the
261 algorithm described elsewhere by Mazet, Carteret, Brie, Idier, and Humbert (2005). This
262 algorithm estimates the background of a spectrum by means of a non-quadratic cost
263 function. Afterwards, this cost function was subtracted from the raw spectra. Asymmetric
264 truncated quadratic was the cost function which gives the best results to estimate

265 background in Raman spectra of NEM from grape seed samples. Moreover, the
266 backgrounds were estimated by a 5-order polynomial and with thresholds of 0.01.
267 Peak heights were calculated for ATR-FTIR and Raman pre-treated spectra and PCA was
268 applied to both whole spectral and peak height matrices. Win ISI (v1.50) software was
269 used for PCA of whole spectral matrices, whereas PCA for peak heights matrices was
270 performed by means of Statistica v.8.0 software (StatSoft Inc., OK, USA, 2007). The
271 aforementioned peaks heights for ATR-FTIR and Raman were previously obtained from
272 spectra and pre-treated spectra respectively using MATLAB R2012b (The Mathworks,
273 Natik, MA, USA, 2012). Furthermore, univariate analysis of variance (Tukey *post hoc*
274 test) was applied to find differences in the peak heights (dependent variables) among
275 different factors (extractability levels or grape varieties). The statistically significant level
276 was considered at $\alpha = 0.05$. Statistica v.8.0 software was used to develop these analyses.

277 **3. RESULTS AND DISCUSSION**

278 *3.1. Total phenol and flavanol extractability levels*

279 K-means cluster analysis of near infrared hyperspectral data sorted grape seed samples in
280 three different groups. The analysis allocated samples into groups according to their total
281 phenols and flavanols extractability levels and, afterwards, these groups were named as
282 low, medium and high extractability levels. Table 1 shows the extractability levels of total
283 phenols and flavanols for grape seed samples and the number of samples classified in
284 each cluster by the k-means method.

285 *3.2. ATR-FTIR data*

286 Raw average spectra of NEM from grape seed samples with low, medium and high
287 extractabilities are shown in Figure 1A. MSC pre-treatment was applied to ATR-FTIR
288 spectra and average spectra were calculated for each grape seed NEM sample. Then, the
289 spectra were vector normalized and mean centered. Afterwards, a PCA was applied to

290 this spectral matrix. Overall, the spectral variability explained was 99% using 13 principal
291 components and Mahalanobis distances for each sample were calculated. Samples were
292 ranked in order of their H (Mahalanobis) distance from the mean spectrum of the entire
293 sample set and the H > 3 criterion was applied in order to look for spectral outliers. Only
294 one H-outlier was found, and it was not taken into account hereafter. Figure 1B shows
295 the scores of the NEM from grape seed samples in the space defined by the first and
296 second principal components, which described 44.31% (PC1) and 35.72% (PC2) of the
297 variability in the data. In the scores plot, the samples are represented by a color code
298 indicating the different extractability levels. It can be seen that medium extractability
299 samples overlap low and high extractability samples. In fact, if medium extractability
300 samples are removed from this plot, it is possible to find some degree of separation
301 between low and high extractability samples (Figure 1C).
302 Figure 1D shows the MSC pre-treated average spectra of NEM from grape seed samples
303 with low and high extractabilities, which are quite similar. Moreover, Figure 1D shows
304 the loadings of PC1 and PC2. PC1 and PC2 loadings show large variations in several
305 spectral regions which might influence the grape seeds phenolic extractability. For
306 example, in the scores plot, almost all high extractability samples are located in the
307 negative PC2 region (Figure 1C), accordingly, the negative features in the PC2 loading
308 (1250-1700 and 3000-3500 cm^{-1} in Figure 1D) might have significant influence on
309 phenolic compound extractability. The main features presented for grape seed NEM
310 samples are described in Table 2. These features are mainly ascribed to polysaccharides,
311 lignins, lipids, pectins and phenolic compounds (Fasoli, et al., 2016; Foo, 1981; Heredia-
312 Guerrero, Benítez, Domínguez, Bayer, Cingolani, Athanassiou, et al., 2014; Lupoi, Singh,
313 Parthasarathi, Simmons, & Henry, 2015; Szymanska-Chargot & Zdunek, 2013; Wilson,
314 et al., 2000).

315 With the aim of identifying what spectral regions have a major influence on phenolic
316 extractability, a univariate analysis of variance was carried out. Peak heights were
317 measured in MSC pre-treated grape seed NEM spectra. Thirteen peaks were taken into
318 account (Table 2). A univariate analysis of variance, a statistical method used to analyze
319 the differences among group means and their associated procedures, was performed. Peak
320 heights were used as dependent variables, whereas extractability level, shown in Table 1,
321 was used as factor or independent variable. This analysis is described in detail in Table
322 3, which shows the heights (mean and standard error) for all peaks described in Table 2.
323 Moreover, in Table 3, values are marked with letters which indicate the existence or not
324 of statistical differences between extractability levels. No significant differences were
325 found for the peak at 1743 cm^{-1}, ascribed to $(C=O)_{ester}$, and for the peaks at 2854 and 2924
326 cm^{-1}, ascribed to CH_2 stretches. Thus, they appear not to have any influence on phenolic
327 compounds extractability. Significant differences ($p < 0.05$) were found for the peaks at
328 781 and 1154 cm^{-1}, but only between medium and high extractability levels. However,
329 no significant differences were found between low and high extractability levels. These
330 two peaks are related to phenolic compounds, and therefore, this fact can show some kind
331 of differentiation between phenolic compounds in medium and high extractability
332 samples, but not between these and low extractability samples. Finally, there are
333 significant differences ($p < 0.05$) in the remaining peaks, 1020 and 3285 cm^{-1}, between
334 the three extractability levels and between low/medium levels and high level for peaks at
335 1235, 1318, 1370, 1441, 1519 and 1610 cm^{-1}. It is to be noted that almost all these peaks
336 fall within the spectral regions that principal component analysis showed to have
337 significant influence on phenolic compound extractability. Despite the obvious fact that
338 peaks related to the remaining phenolic compounds (1441 and 1519 cm^{-1}) (Foo, 1981;
339 Heredia-Guerrero, et al., 2014) influence phenolic extractability, these results confirm

that phenolic extractability is also influenced by the cell wall composition (peaks at 1020, 1235, 1318, 1370 and 3285 cm^{-1} ascribed to polysaccharides, lignins and pectins) and by the degree of esterification of pectins (1610 cm^{-1}, non-esterified pectins) (Gao, Fangel, Willats, Vivier, & Moore, 2015; Szymanska-Chargot, et al., 2013; Wilson, et al., 2000). However, this last peak is also ascribed to C=C aromatic stretching in phenolic compounds by other authors (Ricci, Olejar, Parpinello, Kilmartin, & Versari, 2015).

In addition, using grape variety as factor, a new analysis of variance was carried out and significant differences were found for peaks at 1020, 1370, 1441 and 3285 cm^{-1} among Tempranillo and Syrah samples. All these peaks also have significant influence on the extractability of phenolic compounds. Therefore, these results show that ATR-FTIR spectral features are linked to grape variety and it can be confirmed that this spectroscopic tool can be used to study the relation between grape variety and phenolic compound extractability of grape seeds.

3.3. Raman data

In order to confirm or add more information to the above findings, Raman spectra were measured for NEM from grape seeds. A baseline correction procedure was carried out for each grape seed NEM spectrum (Figure 2A) and 5 peaks were selected, those at 1269, 1442, 1609, 1655 and 2916 cm^{-1} (Table 4) (Chylińska, et al., 2014; da Silva, et al., 2008; Gierlinger & Schwanninger, 2006; Lupoi, Gjersing, & Davis, 2015; Seidler-Lozykowska, Baranska, Baranski, & Krol, 2010; Szymańska-Chargot, et al., 2016). These peaks were selected because they had a good signal to noise ratio and were present in most samples. Then, PCA was performed, both of whole spectra (data not shown) and peak heights (Lupoi, Singh, et al., 2015). The average peak heights were calculated for each sample and PCA was carried out using these data. Figure 2B shows the scores of the NEM from grape seed samples in the space defined by the first and second principal components,

365 which described 65.39% (PC1) and 23.86% (PC2) of the variability in the data. In the
366 scores plot, the samples are represented by a color code indicating the different
367 extractability levels. No trends were found, as samples are overlapped in this space, and
368 thus, PCA cannot be used to interpret the differences between phenolic extractability
369 levels in this case.
370 Afterwards, one-way univariate analyses of variance were performed. Peak heights were
371 used as dependent variables whereas grape seed variety and extractability levels (Table
372 1) were used as independent variables or factors in the different analyses. Significant
373 difference ($p < 0.05$) among grape varieties was found for the peak at 1655 cm^{-1}. This
374 peak is related to the C=C stretch in lignins and fatty acids. Moreover, significant
375 differences were found for the peaks at 1609, 1655 and 2916 cm^{-1} when extractability
376 levels were taken into account. These bands are respectively due to phenolic compounds,
377 lignins and polysaccharides (Gierlinger, et al., 2006; Lupoi, Gjersing, et al., 2015;
378 Seidler-Lozykowska, et al., 2010). The band at 1609 cm^{-1}, assigned to aromatic C=C
379 skeletal stretching, has a strong Raman activity, whereas it only weakly active in infrared
380 spectroscopy. However, the anti-symmetric stretching of COO^- only has infrared activity
381 (Sene, McCann, Wilson, & Grinter, 1994). This allows an assignment of this Raman
382 feature (1609 cm^{-1}) to phenolic compounds and not to pectins. Therefore, Raman
383 spectroscopy enables confirmation of the linkage between cell wall components and
384 phenolic extractability in grape seeds.

385 **4. CONCLUSION**

386 ATR-FTIR and Raman spectroscopy have been proven to be effective and reliable tools
387 to relate the more important spectral features to phenolic extractability levels in grape
388 seed and other sample attributes. ATR-FTIR data analysis confirmed that grape seed
389 phenolic extractability is influenced by the cell wall composition (polysaccharides,

lignins and pectins) and by the degree of esterification of pectins, despite the obvious fact that peaks related to the remaining phenolics compounds also influence on phenolic extractability. Moreover, the results obtaining from the infrared spectra have been confirmed by Raman spectroscopy. Peaks linked to cell wall composition have been shown to have significant influence on grape seed extractability.

Although FTIR spectroscopy has been applied to grape samples in several studies, to our knowledge, Raman spectroscopy had not been tested in grape seeds in order to study their cell wall structure. Raman spectroscopy has been shown to be a reliable tool for studying cell wall structure in grape seed tissue. Nonetheless, a comprehensive study should be made in order to improve spectra features, i.e., signal to noise ratio or the background produced by fluorescence.

ABBREVIATIONS USED

ATR-FTIR, attenuated total reflectance Fourier transform infrared; DAD, diode array detector; DMACA, 4-dimethylaminocinnamaldehyde; IR, infrared; H, Mahalanobis distance; MSC, multiplicative scatter correction; NEM, non-extracted material; NH, neighborhood Mahalanobis distance; NIR, near infrared; NIRS, near infrared spectroscopy; PC, principal component; PCA, principal component analysis.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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576

577 **FIGURE CAPTIONS**

578 **Figure 1**: (A) Raw average spectra of NEM from grape seed samples with low, medium
579 and high extractabilities. (B) Score plot of the first two principal components after PCA
580 performed on ATR-FTIR spectra recorded from grape seed NEM samples. The individual
581 data points have been color coded according to the results of k-means cluster analysis;
582 low, medium and high phenolic extractability. (C) Score plot without medium
583 extractability samples. (D) PC1 and PC2 loading plot and average MSC pre-treated ATR-
584 FTIR spectra for low and high phenolic extractability grape seed NEM samples.

585 **Figure 2**: (A) Raw Raman spectrum, baseline and corrected spectrum for a grape seed
586 NEM sample. (B) Score plot of the first two principal components after PCA performed
587 on peak heights of Raman spectra recorded from grape seed NEM samples. The
588 individual data points have been color coded according to the results of k-means cluster
589 analysis; low, medium and high phenolic extractability.