Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MS and LC-ESI-FTICR-MS

Ismael Ivan Rockenbach a,b,*, Elvira Jungfer a, Christina Ritter a, Beatrix Santiago-Schübel c, Björn Thiele d, Roseane Fett d, Rudolf Galensa a

a Department of Nutrition and Food Sciences – Food Chemistry, University of Bonn, Endenicher Allee 11-13, 53115 Bonn, Germany
b Department of Food Science and Technology, Agricultural Sciences Centre, Federal University of Santa Catarina – UFSC, Admar Gonzaga 1346, 88034-000, Itacorubi, Florianópolis, SC, Brazil
c ZCH/BioSpec, Forschungszentrum Jülich GmbH, Leo-Brandt-Str., 52425 Jülich, Germany
d Institute of Bio- and Geosciences (IBG), IBG-2: Plant Sciences/BioSpec, Research Centre Jülich, 52425 Jülich, Germany

Article info
doi:10.1016/j.foodres.2012.07.001

1. Introduction

Grape pomace, an industrial by-product from the winemaking process, has been recognized as an important and inexpensive source of polyphenols. The grape pomace polyphenols are mainly anthocyanins, flavonols, flavanols, phenolic acids and resveratrol. Several studies reported antioxidant activity of the grape pomace extracts, suggesting the winery by-products for production of natural antioxidants (Boussetta et al., 2011; Rockenbach, Gonzalez, et al., 2011; Rockenbach, Rodrigues, et al., 2011). The seeds constitute a considerable proportion of the grape pomace, amounting to 38–52% on a dry matter basis (Ghafoor, Choi, Jeon, & Jo, 2009). The composition of grape seeds is basically (w/w) 40% fiber, 16% essential oil, 11% protein, 7% complex phenolic compounds like tannins, and other substances like sugars and minerals (Campos, Leimann, Pedrosa, & Ferreira, 2008). In grape seeds, procyanidins represent in general the major part of the total polyphenol extract, and their extreme complexity is the result of the large number of different compounds with very similar structures. Procyanidins are, in fact, composed of flavan-3-ols units, (+)-catechin and (−)-epicatechin, linked together through C4–C6 and C4–C8 interflavanoid bonds, and various gallate esters (De Freitas, Glories, Bourgeois, & Vitry, 1998; Nawaz, Shi, Mittal, & Kakuda, 2006). The flavan-3-ol compounds possess two chiral centers, located at the C2 and the C3 position of the C-ring. Due to the biosynthetic pathway of the flavonoids in the plant, predominantly (+)-catechin and (−)-epicatechin are formed through epimerization from (+)-catechin and (−)-epicatechin in hot aqueous solution or alkaline medium (Kofink, Papagiannopoulos, & Galensa, 2007a). Considering food processes, for example, roasted cocoa beans and cocoa products contain the atypical flavan-3-ol (−)-catechin as a result of the high temperatures applied during the cocoa bean roasting process and particularly due to the alkalization of the cocoa powder (Kofink, Papagiannopoulos, & Galensa, 2007b; Ritter, Zimmermann, & Galensa, 2010). In seeds of grape pomace from the winemaking process, as far as we know, no publication regarding the possible presence of enantiomers of catechin and epicatechin as a result of the fermentation process is available in the literature.

Reversed-phase high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS)
are the usually employed techniques for analysis of phenolic compounds. The former enables the distinction of the various classes of phenolic compounds on the basis of their characteristic UV-visible spectrum, whereas the latter gives access to intact molecular ions and fragment ions from which both the molecular weight of the compound and information on its structural features can be established (Fulcrand et al., 2008; Sun, Leandro, De Freitas, & Spranger, 2006). Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized molecules to separate one from another. This requires first that the various molecular species of interest have been charged (often by loss or addition of protons) and transferred into the gas phase, and that they are then separated as a function of their m/z values. These two steps are achieved by the mass spectrometer source and analyser, respectively. Analysers that have been used to analyse phenolic compounds are quadrupole (Q), magnetic sector (B), ion-trap (IT), time-of-flight (TOF), and Fourier-transform ion cyclotron resonance (FTICR) that differ, among other factors, by the available mass range and resolution. FTICR provides the highest mass resolution and most accurate mass determination, making it theoretically possible to assign molecular formula unambiguously for smaller oligomers (Fulcrand et al., 2008; Hofmann, Wirtz, Santiago-Schübel, Disko, & Pohl, 2010). These high specifications mean that FTICR is ideal for analysing complex mixtures, as demonstrated by prior electrospore ionization (ESI) FTICR-MS analysis of black tea samples containing thousands of chemically distinct constituents (Kuhnert, 2010).

In this study, pressurized liquid extraction (PLE), solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) coupled to ion trap mass spectrometry (MS) were applied to determine the profile of galloylated and non-galloylated flavan-3-ols in seeds of pomace from different grape varieties. Capillary electrophoresis (CE) was applied to investigate the possible presence of enantiomers of catechin and epicatechin, concerning the possibility of epimerization or racemization of the rare flavan-3-ol enantiomers during the fermentation process of winemaking. Finally, LC-ESI-FTICR-MS was applied to elucidate the extension of the presence of isomers and other flavan-3-ol compounds in the grape seeds analyzed.

2. Materials and methods

2.1. Pomace samples

Three 2 kg samples of each red grape pomace were obtained from the Experimental Research Station plantation located in Santa Catarina state, Brazil. The varieties, all widely grown in Brazil for wine production, were as follows: ‘Primitivo’, ‘Sangiovese’, ‘Pinot Noir’, ‘Negro Amaro’, ‘Cabernet Sauvignon’ (Vitis vinifera), and ‘Isabel’ (Vitis labrusca). The samples studied were by-products of the winemaking process, obtained after fermentation. The seeds were manually separated and stored at —80 °C before analysis. After lyophilization, grape seeds were ground with a ball mill (MM2000, Retsch, Haan, Germany) under cooling with liquid nitrogen to obtain a fine powder.

2.2. Chemicals and reagents

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, MA). HPLC-MS and extraction solvents were obtained from J. T. Baker (Griesheim, Germany). Gallic acid (≥99%) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). (2-Hydroxypropyl)-γ-cyclodextrin and standards of (+)-catechin, (-)-catechin and (-)-epicatechin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standards of procyanidin B1 and procyanidin B2 were obtained from Extrasynthese (Genay, France). Sodium tetraborate was obtained from KMF Laborchemie Handels GmbH (Lohmar, Germany) and sodium hydroxide, dimethylsulfoxide and boric acid were obtained from Merck (Darmstadt, Germany).

2.3. Extraction of polyphenols from solid samples

Pressurized liquid extraction (PLE) was carried out using an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany). One gram of diatomaceous earth (Hydromatrix HM-N, Upsala, Sweden) at the bottom and 2 g of the sample material mixed with 2 g of diatomaceous earth on the top were packed into 11 ml stainless steel extraction cells after insertion of two cellulose filters (Schleicher & Schuell GmbH, Dasseln, Germany). The PLE parameters were as follows: solvent acetone plus water, 70 + 30 (v + v) (Rockenbach, da Silva, Rodrigues, Kuskoski, & Fett, 2008), temperature 25 °C, time 10 min, two cycles, 100% flush volume. The extracts were submitted to solid-phase extraction (SPE) before analysis.

2.4. Solid-phase extraction (SPE)

SPE was performed using a Gillson ASPEC XLI system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany) according to a method described previously (Friedrich, Eberhardt, & Galensa, 2000; Papagiannopoulos et al., 2002). Polyamide SPE cartridges (1 g of polyamide, 6 ml cartridge, Macherey-Nagel, Düren, Germany) were conditioned with 10 ml distilled water for 10 min and washed with 5 ml water. The extract was diluted with water to contain 2% (v + v) of the organic solvent prior to loading onto the cartridge. The cartridge was washed with 15 ml water to remove matrix interferences, and the phenolic compounds bound to the polyamide were eluted with dimethylsulfoxide plus formic acid, 95:9:1 (v + v). While eluting, the initial 1.5 ml was discarded because it did not contain compounds of interest, and the next 3 ml was collected, containing all of the compounds. These elution samples were then subjected to analysis.

2.5. Chiral capillary electrophoretic analysis

Catechin and epicatechin standards were diluted in dimethylsulfoxide (DMSO) at a concentration of 0.1 mg/ml. The background electrolyte (BGE: 0.2 mol/l) was prepared by dissolving appropriate amounts of boric acid and anhydrous sodium tetraborate adjusted to pH 8.5 by using NaOH (Kofink et al., 2007a). The chiral selector was solubilized in the running buffer to a final concentration of 15 μmol/L. Before use the buffer solution was filtered through a 0.45 μm regenerated cellulose syringe filter (JVA, Meerbusch, Germany).

Chiral separations were carried out on a Beckman P/ACE MDQ capillary electrophoretic system (Beckman Instruments, Fullerton, CA, USA) equipped with a photodiode array detection system as described by Kofink et al. (2007a). Electropherograms were stored at 210 nm. Data were processed on an IBM personal computer with Beckman 32 Karat software version 7.0. The identification of the analytes was achieved comparing the migration time and the spectra of the flavan-3-ols. Uncoated fused-silica capillaries of 375 μm o.d. × 75 μm i.d. were obtained from Beckman and cut to 70 cm (effective length 60 cm). The capillary was first washed by 20 psi pressure with 0.25 mol/l NaOH for 20 min and finally with purified water for 10 min; between each run with 0.25 mol/l NaOH for 3 min, with purified water for 2 min, and equilibrated with the running buffer for 5 min. The capillary temperature was set to 18 °C and the applied voltage was 18 kV. Samples were injected hydrodynamically (0.3 psi) for 3 s.

2.6. Identification of galloylated and non-galloylated flavan-3-ols by HPLC-DAD-MS^n

The liquid chromatograph was a Dionex summit system consisting of a Degasys DG-1310 degasser (Uniflows, Tokyo, Japan), a Ultimate 3000 pump, an ASI-100 T automated sample injector, an STH-585 column oven, and an Ultimate 3000 diode array detector equipped with a capillary cell (Dionex, Germering, Germany). The system was controlled.
using the Chromleven software package version 6.80 SR9 Build 2673 (Dionex). The analytical column (Aqua 3 μm C18, 150 mm, 2 mm i.d.; both Phenomenex, Aschaffenburg, Germany) and kept at 35 °C. 1% (v/v) acetic acid in high-purity water (mobile phase A) and 1% (v/v) acetic acid in acetonitrile (mobile phase B) were used as solvents with a flow rate of 300 μl/min. A gradient elution program was used starting at 0% B with a linear gradient to 8% B after 60 min, to 15% B after 70 min, to 40% B after 130 min and to 100% B after 131 min. The column was washed with 100% B for 9 min and reequilibrated for 9 min with the initial conditions. For analysis, 10 μl of each sample was injected. An LCQ classic ion-trap mass spectrometer (MS) equipped with an electrospray interface (ESI) and a metal needle kit was coupled to the HPLC and controlled with the Xcalibur software version 2.0 (Thermo Finnigan, Egelsbach, Germany). A flow of 100 μl/min methanol delivered by a System Gold programmable solvent module 116 (Beckman, Unterschleissheim, Germany) was added through a T-union before the HPLC eluent entered the ion source to enhance ionization of very polar compounds. The settings for the MS were as follows: source voltage, 4.5 kV (negative mode); sheath gas flow, 60; auxiliary gas flow, 60; capillary voltage, −45 V; capillary temperature, 300 °C; first octapole offset, +3.0 V; second octapole lens voltage, +22.0 V; second octapole offset, +7.0 V; ion trap DC offset, +10.2 V.

The identification of phenolic compounds was performed with authentic standard in the case of gallic acid, catechin, epicatechin and procyanidin B1 and B2. All other compounds were tentatively identified by combining UV spectra and MS/MS fragmentation data. Additionally, compound assignment was supported by comparison with data from the literature when available.

2.7. LC-ESI-FTICR-MS analysis

HPLC analysis was carried out with an Agilent HPLC system (1200 series, Santa Clara, CA, USA) equipped with a diode array detector. Polyphenols were separated with the same analytical column described before. The mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). In comparison with the mobile phase applied in HPLC-DAD-MSn analysis, acetic acid was replaced by formic acid to avoid the formation of acetal adducts in FTICR-MS. The same gradient as in HPLC-DAD-MSn analysis was at a flow rate of 300 μl/min. The injection volume was 10 μl for each sample. The column temperature was 35 °C.

Mass spectrometry was performed using a hybrid linear ion trap FTICR mass spectrometer LTQ-FT Ultra (Thermo Fisher Scientific, Bremen, Germany) equipped with a 7 T supra-conducting magnet. The electrospray ionization (ESI) source was operated in the negative mode with an ion spray voltage of 3.3 kV. Nitrogen was employed as sheath gas (40 arb). The transfer capillary temperature was set to 300 °C. Voltages for capillary and tube lens were set to −137 and −218 V, respectively. Mass spectra were recorded in full scan from 200 to 2000 Da and from 150 to 1500 Da with a resolution of 100,000 at m/z 400. The automatic gain control was set to 5E5 for the FTMS full scan. Spectra were recorded in the profile mode. Mass measurements are based on the “monoisotopic” ion (i.e., the species in which all carbons are 12C, all oxygens are 16O, etc.). All data were processed using the Xcalibur software version 2.0.

3. Results and discussion

3.1. Chiral capillary electrophoretic analysis

From an analytical point of view, compounds as catechin and epicatechin can be detected and quantified using well known and relatively simple methods. However, most methods employed cannot identify the presence of enantiomers of these monomers. The enantiomers of an analyte exhibit the same charge density, thus, their electrophoretic mobilities are the same and they cannot be separated. Therefore, it is important to add a chiral selector to the background electrolyte which is able to interact with the analyte. The chiral selector forms temporary diastereomeric associates with each enantiomer (Kofink et al., 2007a). Fig. 1 shows the overlay of electropherograms of the chiral separation of (+)/(−)-catechin and (−)-epicatechin standards and Pinot Noir grape seed extract using (2-hydroxypropyl)-γ-cyclodextrin as a chiral selector. (+)-Catechin and (−)-epicatechin were identified in all grape seed samples. (−)-Catechin and (+)-epicatechin were not detected. These results indicate that these enantiomers are not formed under the fermentation conditions of winemaking.

3.2. HPLC-DAD-MSn analysis of galloylated and non-galloylated flavan-3-ols

Table 1 shows the phenolic compounds identified in the seeds of different grape varieties. Fig. 2 shows the HPLC-DAD chromatogram of Pinot Noir grape seed extract recorded at 280 nm. Gallic acid was identified in all grape seed samples. The identification of gallic acid was confirmed by the same retention time and MS data of the pure standard which gave [M – H]+ ions at m/z 169 that dissociated to form ions at m/z 125 via loss of CO2. Gallic acid has been previously identified in grape pomace of the V. vinifera varieties Merlot and Cabernet Sauvignon and V. labrusca varieties Bordeaux and Isabel (Rockenbach, Rodrigues, et al., 2011). Catechin and epicatechin ([M – H]+ at m/z 289) were also identified with authentic standards and showed characteristic MS2 fragments at m/z 245 (loss of CO2), 205, and 203 (cleavage of the A-ring of flavan-3-ol) (Sandhu & Gu, 2010). As confirmed by the chiral capillary electrophoretic analysis, these compounds correspond to (+)-catechin and (−)-epicatechin enantiomers.

The MS3 spectrum of [M – H]+ ions at m/z 441 produced ions at m/z 289 and 169 corresponding to the deprotonated ions of (epi)catechin and gallic acid, respectively. The major fragment MS4 was at m/z 245, indicating decarboxylation of (epi)catechin, and minor fragments at m/z 205 and 203 suggesting the characteristic fragmentation pattern of (epi)catechin. On the basis of mass spectral data and previously published data (Sandhu & Gu, 2010), this compound was tentatively identified as (epi)catechin monogalolate.

Singly charged negative ions with a [M – H]+ at m/z 577 corresponded to the different procyanidin dimers. A procyanidin dimer consists of an extension unit and a terminal unit. The heterocyclic ring of the flavan-3-ol units fragments through retro-Diels-Alder (RDA) and heterocyclic ring fission (HRF) mechanisms. Both fragmentation pathways could take place on the extension unit or the terminal unit. However, fragmentation on the extension unit gives rise to fragment ions with a larger π–π conjugated system; thus, it is more energetically favorable (Gu et al., 2003). In the present study, procyanidins B1 (epicatechin-(4β→8)-catechin) and B2 (epicatechin-(4α→8)-epicatechin) were identified with authentic standards. The RDA fragmentation of the dimers produced mostly [M – H]–152– product ions at m/z 425. The m/z of 407 [M – H]–152–18– results from water elimination of m/z 425, most likely from the 3-OH (Gu et al., 2003). The [M – H]–126– fragment ions at m/z 451 result from the elimination of the phloroglucinol molecule (A-ring) (Hayasaka, Waters, Cheynier, Herderich, & Vidal, 2003), [M – H]–126–18– ions at m/z 559 result from water elimination of m/z 577, and [M – H]–288– ions at m/z 289 result from the loss of an (epi)catechin molecule (Sandhu & Gu, 2010).

[M – H]+ ions at m/z 605 were presumably assigned to (epi)catechin-ethyl dimers, condensed products of (epi)catechin with acetaldehyde, corresponding to two (epi)catechin units linked by an ethyl-bridge (Fig. 3) (Saucier, Guerra, Planet, Laguerre, & Glories, 1997). As described by He, Pan, Shi, and Duan (2008), acetaldehyde is the most abundant aldehyde present in wine and would arise from different sources such as a fermentation intermediary product, from oxidation of ethanol, or from the addition of wine spirit to the must in order to stop the fermentation.
In acidic media such as wine, after the protonation of acetaldehyde, the flavanols can undergo electrophilic substitution by acetaldehyde on the nucleophilic C6 or C8 positions of their A-ring, giving rise to the formation of condensation products. The presence of these compounds in the samples analyzed in this study can be expected since the seeds were separated from pomace collected after fermentation. As product ion, signal at m/z

![Fig. 1. Overlay of electropherograms of the chiral separation of (+)/(−)-catechin and (−)-epicatechin standards and Pinot Noir grape seed extract using (2-hydroxypropyl)-γ-cyclodextrin as a chiral selector.](image)

**Table 1** Negative-ion ESI-MS fragmentation pattern of phenolic compounds in seeds of pomace from red grape varieties (*Vitis vinifera* and *Vitis labrusca*).

<table>
<thead>
<tr>
<th>m/z</th>
<th>Compound (peak numbers)</th>
<th>Fragments (m/z)</th>
<th>Varieties&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PN</td>
</tr>
<tr>
<td>169</td>
<td>Gallic acid (1)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [169]: 125</td>
<td>X</td>
</tr>
<tr>
<td>289</td>
<td>(epi)Catechin (7, 22)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [289]: 245, 205, 203</td>
<td>X</td>
</tr>
<tr>
<td>441</td>
<td>(epi)Catechin monogallate (41)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [441]: 289, 169</td>
<td>X</td>
</tr>
<tr>
<td>577</td>
<td>Procyanidin dimer (3–6, 13, 20, 21, 34)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [577]: 425, 407, 451, 289, 559</td>
<td>X</td>
</tr>
<tr>
<td>605</td>
<td>(epi)Catechin-ethyl dimer (40, 50, 58, 61–65, 67–70)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [605]: 315, 289, 453</td>
<td>X</td>
</tr>
<tr>
<td>655</td>
<td>Unknown compound (12)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [655]: 407, 425, 529, 365, 449</td>
<td>X</td>
</tr>
<tr>
<td>729</td>
<td>Procyanidin dimer monogallate (26, 28, 29, 33, 49, 57)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [729]: 407, 451, 425, 441, 603, 289</td>
<td>X</td>
</tr>
<tr>
<td>865</td>
<td>Procyanidin trimer (2, 8, 14, 15, 17–19, 30–32, 37, 38, 45–47)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [865]: 695, 577, 575, 407, 543, 739, 713, 449, 587, 451, 407</td>
<td>X</td>
</tr>
<tr>
<td>879</td>
<td>Procyanidin dimer digallate (A-type) (9)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [879]: 709, 727, 457, 439</td>
<td>X</td>
</tr>
<tr>
<td>881</td>
<td>Procyanidin dimer digallate (B-type) (44, 60)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [881]: 729, 559, 407, 711, 577</td>
<td>X</td>
</tr>
<tr>
<td>893</td>
<td>(epi)Catechin-ethyl trimer (42, 56, 59, 66)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [893]: 603, 577, 451, 433, 407, 425, 315, 741</td>
<td>X</td>
</tr>
<tr>
<td>1017</td>
<td>Procyanidin trimer monogallate (11, 23, 24, 27, 35, 39, 48, 52–54)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [1017]: 729, 865, 847, 677, 891, 695, 577, 575, 577, 727, 739, 559, 771, 407</td>
<td>X</td>
</tr>
<tr>
<td>1153</td>
<td>Procyanidin tetramer (10, 16, 25, 36, 43)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [1153]: 865, 983, 575, 695, 739, 1027, 863, 577, 1001, 449</td>
<td>X</td>
</tr>
<tr>
<td>1169</td>
<td>Procyanidin trimer digallate (51, 55)</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt; [1169]: 1017: 729, 881, 729, 847, 999, 891, 577</td>
<td>X</td>
</tr>
</tbody>
</table>

Gallic acid, catechin, epicatechin and procyanidins B1 and B2 were identified with authentic standard; all other compounds were tentatively identified.

<sup>a</sup> PN = Pinot Noir, NA = Negro Amaro, CS = Cabernet Sauvignon, P = Primitivo, I = Isabel, S = Sangiovese.
315 would correspond to a vinyl-catechin adduct. Saucier et al. (1997) also found a high relative abundance of vinyl-catechin residues \((m/z 315)\) in their mass spectrometry analyses and attributed them to the instability of the ethyl-bridged catechin oligomers. Furthermore, another fragment at \(m/z 453\) was also observed, which presumably corresponded with a RDA fragmentation of the dimer (Saucier et al., 1997).

The singly charged negative ions at \(m/z 655\) detected in Cabernet Sauvignon grape seeds were not identified. However, the data of MS\(^n\) fragmentation \((m/z 407, 425)\) indicated that it is a pseudo-molecular ion of an oligomeric procyanidin.

\([M−H]^-\) ions at \(m/z 729\) generated MS\(^2\) \(\text{([M−H−152]^-})\) fragment ions at \(m/z 577\) corresponding to the loss of a galloyl group, \(m/z 441\) \(\text{([M−H−288]^-})\) corresponding to the loss of an (epi)catechin molecule, and \(m/z 603\) \(\text{([M−H−126]^-})\) corresponding to the elimination of a phloroglucinol molecule (A-ring). Ions at \(m/z 407, 559, 425\) and \(451\) suggest the characteristic fragmentation pattern of the procyanidin dimers described above.

\([M−H]^-\) ions at \(m/z 865\) were assigned to procyanidin trimers, known components of grape seeds (Tsang et al., 2005). Tsang et al. (2005) also found MS\(^2\) fragment ions at \(m/z 713, 695, 577,\) and 407. According to these authors, the fragment ions at \(m/z 713\), a loss of 152 Da, come from RDA fission of the heterocyclic ring system. The base ions at \(m/z 695,\) a loss of 170 Da, correspond to the RDA fission with an additional loss of water. The ions at \(m/z 577,\) a loss of 288 Da, correspond to the interflavanyl bond cleavage. Ions at \(m/z 451\) and \(m/z 407\) suggest the characteristic fragmentation pattern of the procyanidin dimers described above.

\([M−H]^-\) ions at \(m/z 879\) detected in the seeds of the varieties Pinot Noir, Negro Amaro, Cabernet Sauvignon and Primitivo suggest the structure of an A-type procyanidin dimer digallate. Passos et al. (2007) described for the first time the occurrence of A-type galloylated procyanidins in grape seeds. In accordance with these authors, the major fragment ions for an A-type procyanidin dimer digallate were observed at \(m/z 709\), resultant from a loss of a gallic acid unit \((-170\ \text{Da})\). Ions at \(m/z 727\) correspond to a loss of a gallic acid residue \((-152\ \text{Da})\). An additional loss of a gallic acid residue/unit from ions at \(m/z 709\) results in fragments at \(m/z 557/539\). Furthermore, ions at \(m/z 439\) \((-440\ \text{Da})\), a loss of a galloylated monomer from \([M−H]^-\) result from the opening of the \((C4−C8)−(C2−C7)\) ring of the A-type procyanidin dimer digallate.

\([M−H]^-\) ions at \(m/z 881\) suggest two possible structures, either the digallate ester of a procyanidin dimer or a trimer in which one of the flavanol units is (epi)gallocatechin. The masses of these compounds differ by only 0.04 Da, however, the structures were readily distinguished by collision-induced dissociation. The prominent product ion at \(m/z 729\) was formed by the loss of a neutral fragment of 152 Da from the parent ion due to the loss of either a galloyl group or the flavanol B-ring together plus portions of the C-ring from a RDA reaction. In addition, collision-induced dissociation caused successive expulsions of one or two additional 152 Da fragments.

**Fig. 2.** HPLC-DAD chromatogram of Pinot Noir grape seed extract recorded at 280 nm. The numbered peaks are denoted in Table 1.

**Fig. 3.** Elemental structure of possible dimers and trimers resulting from (epi)catechin-acetaldehyde condensation.
yielding ions at m/z 577 and 407 (425 – H2O). Collision-induced of the m/z 729 ion (MS3 analysis) further demonstrated losses of 152 Da fragments yielding ions at m/z 577 and 559 (577 – H2O). Interflavan bond cleavage occurred as well producing ions at 289 and 441 Da corresponding, respectively, to (epi)catechin and the gallate ester of one of these flavanols. The dimer digallate structure is expected to fragment with multiple losses of 152 Da fragments both from losses of galloyl groups and RDA reactions as mentioned above. On the other hand, the alternative trimer structure mentioned is not expected to undergo more than one 152 Da loss. In addition, interflavan bond cleavage within the trimer would not produce an ion at m/z 441 seen in the MS3 spectrum (Agarwal et al., 2007). These data, therefore, are consistent only with a procyanidin dimer digallate structure.

[M − H]− ions at m/z 893 were presumably assigned to (epi)catechin-ethyl trimers, condensed products of (epi)catechin with acetaldehyde, corresponding to three (epi)catechin units with one ethyl-bridge (Fig. 3). As fragment ions, vinyl-catechin adducts with a signal at m/z 315 (as observed in (epi)catechin-ethyl dimer fragmentation, m/z 605), ions resulted from RDA fragmentation of trimers, and characteristic fragmentation pattern ions of procyanidin dimers were observed. To our knowledge, this is the first time that (epi)catechin-ethyl trimers have been shown in winery by-products. Thus, the presence of (epi)catechin-ethyl trimers in the seeds of grape pomace constitutes a new element to take into account in their structural analysis.

[S − H]− ions at m/z 1017 were presumably assigned to procyanidin trimer monogallates. According to Valls, Millán, Martí, Borràs, and Arola (2009), the characteristic fragmentation of these compounds produces ions at m/z 891, 847, 729, 695, 603, 559, 451, 407 and 289.

Singly charged negative ions at m/z 1153 were tentatively identified as pseudo-molecular ions of procyanidin tetramers. MS2 fragmentation of these ions resulted in major signals at m/z 865, 983, 575, 863 and 1027. [M − H − 126]− ions at m/z 1027 corresponded to the loss of the phloroglucinol molecule (C6H6O3, A-ring). [M − H − 288]− ions at m/z 865 and [M − H − 578]− ions at m/z 575 resulted from the loss of the upper unit of flavanol and the loss of the two lower flavanol units, respectively (Sun et al., 2006).

3.3. LC-ESI-FTICR-MS analysis

High-resolution mass spectrometry revealed the presence in the grape seeds of several isomers and other flavan-3-ol compounds not detected by HPLC-DAD-MSn. Fig. 4 shows the negative-ion ESI-FTICR mass spectrum of each of the six grape seed extracts. Detailed information is presented hereafer for the variety Cabernet Sauvignon (Table 2). For this grape variety, elemental compositions were assigned to 251 different flavan-3-ol compounds, including isomers of 28 different molecular classes. The maximal tolerated range of deviation was 2 ppm. Range of deviation (ppm) was calculated as follows: \[ \frac{|m/z \text{ (measured)} - m/z \text{ (calculated)}|}{m/z \text{ (measured)}} \times 1,000,000 \]. Elemental compositions were assigned to flavan-3-ol procyanidins up to heptamers (compounds with six and seven monomeric units were detected as doubly charged ions). Additionally, elemental compositions were also assigned to mono and digalloylated flavan-3-ols, in both cases up to tetramers. A-type procyanidins were detected for all corresponding B-type galloylated flavan-3-ols and for non-galloylated compounds up to pentamers, including compounds with two A-type bonds in its structure in the case of non-galloylated procyanidin trimers. Furthermore, elemental compositions were assigned to condensed products of catechin with acetaldehyde.
An elemental composition of $C_{30}H_{24}O_{12}$ was assigned to eight different isomers of B-type procyanidin dimers ($m/z$ 575.1195) detected in the Cabernet Sauvignon grape seeds, probably corresponding to the typical isomers B1–B8. In addition to the B-type procyanidin dimers, seven isomers of A-type procyanidin dimers ($m/z$ 575.1195) were also detected, corresponding to an elemental composition of $C_{45}H_{36}O_{18}$.

Accurate masses of ten isomers of B-type procyanidin trimers ($m/z$ 865.1985), nine isomers of procyanidin trimers with one A-type bond in their structures ($m/z$ 863.1829), and ten isomers of procyanidin trimers with two A-type bonds in their structures ($m/z$ 861.1672) were detected, corresponding to elemental compositions of $C_{47}H_{42}O_{18}$, $C_{44}H_{32}O_{20}$, and $C_{43}H_{34}O_{18}$, respectively.

In the case of procyanidin tetramers, thirteen isomers of B-type structures ($m/z$ 1153.2619) and eight isomers of A-type structures ($m/z$ 1151.2463) corresponding to elemental compositions of, respectively, $C_{44}H_{34}O_{20}$ and $C_{45}H_{36}O_{22}$ were detected.

An elemental composition of $C_{23}H_{22}O_{14}$ was assigned to eleven different isomers of B-type procyanidin pentamers ($m/z$ 1441.2523), while the elemental composition of $C_{23}H_{22}O_{14}$ was assigned to seven isomers of A-type procyanidin pentamers ($m/z$ 1439.3097).

Hexamers and heptamers of B-type procyanidins were detected as doubly charged ions. An elemental composition of $C_{32}H_{30}O_{12}$ was assigned to two isomers of procyanidin hexamers ($m/z$ 1153.2619) and eight isomers of A-type structures ($m/z$ 1151.2463) corresponding to elemental compositions of, respectively, $C_{29}H_{30}O_{14}$ and $C_{30}H_{32}O_{16}$.

An elemental composition of $C_{30}H_{24}O_{12}$ was assigned to fourteen different isomers of procyanidin dimer monogallates, $C_{22}H_{22}O_{14}$ ($m/z$ 1015.1938; eight isomers of procyanidin trimer monogallates) and $C_{27}H_{38}O_{18}$ ($m/z$ 1303.2572; thirteen isomers of procyanidin tetramer monogallates) were also detected. Furthermore, the presence of two galloyl groups was observed in B-type structures with elemental compositions of $C_{44}H_{34}O_{20}$ ($m/z$ 881.1571; two isomers of procyanidin dimer digallates), $C_{45}H_{36}O_{22}$ ($m/z$ 1169.2205; ten isomers of procyanidin trimer digallates), and $C_{52}H_{40}O_{22}$ ($m/z$ 1457.2838; twelve isomers of procyanidin tetramer digallates). A-type structures with two galloyl groups corresponding to elemental compositions of $C_{43}H_{34}O_{18}$ ($m/z$ 1167.2048) and $C_{42}H_{32}O_{14}$ ($m/z$ 1455.2682) were also detected.

Finally, elemental compositions were assigned to condensed products of (epi)catechin with acetaldehyde. Ten isomers of (epi)catechin-ethyl dimers ($m/z$ 605.1664) corresponding to an elemental composition of $C_{23}H_{22}O_{12}$, twenty six isomers of (epi)catechin-ethyl trimers ($m/z$ 893.2298) corresponding to an elemental composition of $C_{27}H_{38}O_{18}$, and thirteen isomers of (epi)catechin-ethyl tetramers ($m/z$ 1181.2932) were detected.

4. Conclusions

Results indicate that seeds obtained from the pomace of red grape vinification contain a great variety of flavan-3-ols, including both galloylated and non-galloylated compounds. Oligomers up to seven monomeric units were detected. The presence in winery by-products of (epi)catechin-ethyl trimers and tetraters, besides the presence of the correspondent dimers was described for the first time. It was also possible to show that the fermentation process of winemaking did not give rise to (−)-catechin and (+)-epicatechin enantiomers. Only (−)-catechin and (−)-epicatechin were detected, which allows to presume that the condensed flavan-3-ol compounds in grape seeds are...
formed only by these enantiomers. LC-ESI-FTICR-MS turns out to be an appropriate method to analyze the complex phenolic composition of grape seeds, enabling the assignment of elemental composition for most of the detected compounds. The data obtained will support the exploitation of the grape seeds as a potential source of natural antioxidants.

Acknowledgments

This work was supported by CNPq (National Council for Scientific and Technological Development — Brazil). The authors are grateful to EPAGRI — Videira (Agricultural Research Governmental Company of Santa Catarina state) for help in obtaining samples from industrial producers.

References

Sandhu, A. K., & Gu, L. (2010). Antioxidant capacity, phenolic content, and profiling of phenolic compounds in the seeds, skin, and pulp of Vitis rotundifolia (Muscadine grapes) as determined by HPLC-DAD-ESI-MS”. Journal of Agricultural and Food Chemistry, 58, 4681–4692.