Influence of Acidity and Extraction Time on the Recovery of Flavonoids from Grape Skin Pomace Optimized by Response Surface Methodology

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Grape pomace is recognized as an economical source for the recovery of a large number of biologically active compounds, such as polyphenols. Grape pomace extracts can be successfully used as raw material for functional foods production, since they are generally recognized as safe for the food industry. This study aimed to quantify the amounts of recovered flavonoids (total flavonoids, proanthocyanidins, anthocyanins, and flavonols) from grape skin pomace (Vitis vinifera L. cv. Merlot) under conventional extraction with different acidities (0.5 % – 1 % HCl) and lengths of extraction time (30–60 minutes). The grand average values in this study were as follows: (i) proanthocyanidins 12.08±0.25 mg CE g⁻¹ d.m., (ii) anthocyanins 2.17±1.02 mg Mvd-3-glc g⁻¹ d.m., (iii) flavonols 7.73±0.11 mg QE g⁻¹ d.m., and (iv) total flavonoids 36.28±0.42 mg QE g⁻¹ d.m. The amounts of studied polyphenols generally increased with acidity and length of extraction time. This relation was more evident for flavonols and total flavonoids, while less clear for other examined phenolics.

Key words: total flavonoids, proanthocyanidins, anthocyanins, flavonols, optimal extraction acidity/time, grape pomace skin extracts

Introduction

In 2012, grape production amounted to a total of 77 million tons, and 80 % of all produced grape was used for winemaking. Twenty percent of the grape in wine production accounts for by-products, such as grape pomace, totaling nearly 11 million tons of grape by-products per year (other recent sources reported pomace production of 5–14 million tons). Overproduction of grape pomace can be challenging for industrial waste management, or it can be prudently used as a valuable raw material for functional food production. The functional foods can be engineered from any sources. Hence, as expected, this type of food market has the fastest growth rate in the world, suggesting that interest for processing raw materials such as grape pomace will only increase in the future.

Grape pomace from most grape varieties (with both seeds and skins) are recognized as an economical source for the recovery of a large number of biologically active compounds (BAC), primarily polyphenols. Polyphenols such as flavonoids, phenolic acids, phenolic alcohols, stilbenes, and ligands, are the main phenolic compounds found in pomace. From this group of BAC, the most interesting polyphenols are flavonoids, as they are known to contribute to human health via their antioxidative property. For instance, literature reports that flavonoids (flavonols, isoflavones, anthocyanins, flavanols, flavones, and flavanones) in general have beneficial effects on atherosclerosis, hypertension, neurodegeneration, myocardial, and other medical conditions. Additionally, flavonoids extracted from grapes exhibit anti-inflammatory, antibacterial, antiallergic, anticancer, and antioxidative potential. The proanthocyanidins, also known as oligomeric flavonoids, have great importance in nutrition and medicine because of their antioxidant activity, which is much higher than that of monomeric phenolics. Literature reports their possible effects on reducing the risk of chronic diseases, such as cardiovascular disease and cancer. Therefore, evaluating the influences of extraction parameters on flavonoids recovered from grape pomace is essential for the nutritive and commercial value of the grape extracts.

Commonly, the objective of an extraction is to optimally recover the greatest possible amount(s) of targeted compound(s) with minimum contamination with undesirable components in the obtained ex-
tate(s). Phytochemicals such as flavonoids are commonly recovered from plants by solid-liquid extraction. Extraction of target compounds from grape pomace is mainly affected by grape variety/vintage, type of extraction/solvent, acidity, temperature, time, addition of enzymes, and particle size. Ethanol is generally recognized as safe (GRAS status), and is commonly used as organic solvent in conventional extraction of polyphenols. Increased temperature improves extraction due to better diffusion and disruption of plant cellular structures. Increased acidity induced by the use of strong acids, such as HCl, with elevated temperatures, tends to shorten the extraction time of polyphenols (e.g. flavonoids). This is due to the denaturation of grape cellular membranes, thus promoting their release from the pomace matrix. It has previously been shown that increased acidity supports extraction of phenolic acids and total phenols together with increased antioxidant capacity of extracts. Extraction time varies with regards to different plant material and particle sizes, where smaller particles of plant material tend to reduce extraction time. Due to these numerous sources of variation, it is important to quantify those influences that have an effect on the amount of extracted flavonoids in different grape pomaces. The food industry has shown increased interest for the development and evaluation of natural antioxidants. As mentioned previously, flavonoids from grape pomace are important contributors to antiradical activity in grape extracts. Hence, it is essential for health benefits and commercial value of functional foods to evaluate the major contributors of antioxidant activity.

Therefore, the objectives of this study were as follows: (i) to quantify the amounts of recovered flavonoids (total flavonoids, proanthocyanidins, anthocyanins, and flavonols) at different acidity and lengths of extraction time from red grape skin pomace; (ii) to quantify the cumulative influences of extraction time and acidity on flavonoids from objective (i); and (iii) to optimize extraction parameters with regard to the investigated flavonoid subgroups.

**Materials and methods**

**Chemicals and standards**

Ethanol, methanol, hydrochloric acid, aluminum chloride, potassium acetate, sulfuric acid, potassium chloride, sodium acetate, and ascorbic acid were of reagent grade, purchased from Gram-mol (Zagreb, Croatia). Sodium carbonate was purchased from Lach-Ner (Czech Republic). Gallic acid, quercetin, (+)-catechin hydrate and vanillin were obtained from Sigma Aldrich Chemie GmbH, Germany. Folin-Ciocalteu reagent was purchased from Kemika (Zagreb, Croatia).

**Plant materials**

This study was carried out with grape pomace by-products obtained from the Merlot red grape cultivar (Vitis vinifera L.). The samples were provided by the winemaking company Agrolaguna (Poreč, Croatia) during the 2014 harvest. The grape pomace was collected on the harvesting day after destemming, pressing, filtering of the grapes, and freeze-drying. Firstly, 500 g of grape pomace was frozen at -60 °C, then freeze-dried in a laboratory freeze-drier (CoolSafe PRO, Labogene, Denmark) under high vacuum (0.13–0.55 hPa) for 24 h with isothermal (heating) plate temperatures of 20 °C. The final water content of the dried grape pomace was 3.15 %. The freeze-dried grape pomace was stored in the dark in polyethylene bags at -18 °C until analysis. Prior to analysis, the skins and seeds were manually separated from the thawed grape pomace, and the grape skins were used for further analysis. The grape skins were then milled and pulverized into powder with a blender (Imetec Dolcevia CG1, 150W) and used for extraction. Powder particle size distribution was: d(0.9)≤ 6±1 mm; d(0.5)≤ 3±1 mm; d(0.1)≤ 2 (Malvern, Mastersizer 2000 particle size analyzer, Germany).

**Conventional extraction**

The extraction conditions (solvent polarity, addition of acid, extraction time, extraction temperature) were chosen from previously published literature. Hence, the extraction method for recovering phenolic compounds from grape skin pomace was done using 50 % aqueous ethanol (v/v) with 12 mol L⁻¹ hydrochloric acid (0, 0.5, and 1 %). The samples (0.3 g) of the grape pomace skins were weighed into Erlenmeyer flasks and extracted with 20 mL of solvent for 30, 45, and 60 min after flushing with nitrogen, in order to prevent oxidation during extraction. The extraction process was carried out under heat-reflux at 80 °C with magnetic stirrer. The extraction of each sample was done twice. The samples were then filtered through Whatman filter paper No. 40 (Whatman International Ltd., Kent, UK), and made up to 25 mL in volumetric flask with extraction solvent. Prior to analysis, the extracts were stored at -18 °C in an inert gas atmosphere.

**Determination of total flavonoids**

Total flavonoids (TF) were determined according to the method described in literature. A volume of 0.5 mL of extract was added to 1.5 mL of 96 % ethanol (v/v), 0.1 mL of 10 % aluminum chloride (m/v), 0.1 mL of 1 mol L⁻¹ potassium acetate, and 2.8 mL of distilled water. After thorough mixing, the reaction mixtures were incubated at room temperature for 30 min, followed by absorbance readings at 415 nm. The amount of 10 % aluminum...
chloride was substituted by the same amount of distilled water in blank. The calibration curve was prepared using 10 to 75 mg L$^{-1}$ of quercetin in methanol (linear regression $r^2 = 0.9971$). The concentration of TF was expressed as mg quercetin equivalents (QE) g$^{-1}$ dry matter.

**Determination of polymeric proanthocyanidins**

Polymeric proanthocyanidins (PCs) were estimated spectrophotometrically according to vanillin assay.$^{29}$ A 2.5 mL aliquot of a freshly prepared solution of 1 % vanillin (m/v) and 25 % sulfuric acid (v/v), both in 100 % methanol, were added to 1 mL of extract. The mixture was kept at 20 °C in a water bath, and after 15 min, the absorbance at 500 nm was read. The calibration curve was prepared using 50 to 400 mg L$^{-1}$ of catechin in methanol (linear regression $r^2 = 0.9961$). The concentration of PCs was expressed as mg catechin equivalents (CE) g$^{-1}$ dry matter.

**Determination of anthocyanin content**

Anthocyanin content (ACY) was measured by the pH differential method.$^{30}$ The extract was diluted to the same extent in 0.025 mol L$^{-1}$ potassium chloride (pH 1.0) and 0.4 mol L$^{-1}$ sodium acetate (pH 4.5). The mixtures were measured spectrophotometrically at both 520 and 700 nm. Anthocyanin content (mg Mvd-3-glu equivalents g$^{-1}$ dry matter) was calculated as $(A_{520, \text{pH}1.0} - A_{700, \text{pH}1.0}) - (A_{520, \text{pH}4.5} - A_{700, \text{pH}4.5}) \cdot 529 \cdot \text{dilute factor} \cdot 1000/28000$, where the molar absorptivity and molar mass of malvidin-3-glucoside (Mvd-3-glc) were 28,000 L/cm/mol and 529 g mol$^{-1}$, respectively.

**Determination of flavonols content**

Flavonols content (FL) was measured by using the method from literature.$^{31}$ The grape skin pomace extracts (0.25 mL) were mixed with 0.25 mL of 1 g L$^{-1}$ HCl in aqueous ethanol (96 %, v/v), and 4.55 mL of 2 g L$^{-1}$ 12 mol L$^{-1}$ HCl. The absorbance of the reaction mixture was then read at 360 nm. Standard curve of quercetin (100 mg L$^{-1}$) dissolved in aqueous methanol (80 %, v/v) was used to quantify HCA, and the results were expressed as mg of caffeic acid equivalents g$^{-1}$ (on a fw basis). The calibration curve was prepared using 10 to 100 mg L$^{-1}$ of quercetin in methanol (linear regression $r^2 = 0.9989$). The concentration of FL was expressed as mg quercetin equivalents (QE) g$^{-1}$ dry matter.

**Experimental methodology and statistical analysis**

As before, the experiments were designed as full factorial randomized experimental design (Table 1). Dependent variables for multivariate analysis were: amounts of proanthocyanidins, anthocyanins, flavonols, and flavonoids in mg g$^{-1}$ dry matter. Independent variables were: extraction time/min and HCl acidity/%. Descriptive statistics was used to assess basic information about the experimental dataset (e.g. to obtain sample basic metrics, check for normality of distribution). Normality and homoscedasticity was tested with Kolmogorov–Smirnov and Levene’s test, and statistical methods for analysis were chosen based on recommendations from literature.$^{33}$ Continuous variables were analyzed by multivariate analysis of variance. Pearson’s linear correlation tests were used to assess the relation between the pairs of continuous variables. Lack-of-fit tests were used to evaluate predictive power of models (i.e. modeling efficacy). The significance levels for all tests were $\alpha \leq 0.05$. Analyses were performed with IBM SPSS Statistics (v.20). The RSM optimization analysis was done with STATGRAPHICS Centurion XVII.

**Results and discussion**

**Influence of extraction parameters on concentrations of proanthocyanidins**

Recently, the recovery of phenolic compounds from food processing waste has been receiving increased attention as a potentially low-cost and valuable source of new and effective antioxidants.$^{23}$ Various novel techniques have been employed to recover phenolics from plant matrices, but from the industrial point of view, conventional solvent extraction is usually preferred due to its process simplicity, efficiency, and lower investment cost.$^{19,20,34–40}$

The type and polarity of extraction solvent play an important role in the extraction selectivity and efficiency, where ethanol and water are preferred...
solvents since they are non-toxic and environmentally friendly. In the present study, an aqueous mixture of ethanol (50 %, v/v) was chosen since 50 % aqueous solvent was found to be the optimum concentration for the extraction of phenolic compounds from grape by-products.

Table 2 lists the concentrations of proanthocyanidins, anthocyanins, flavonols, and total flavonoids in grape pomace skin extracts studied at three extraction times with or without addition of HCl. The grand average for proanthocyanidins in grape skin pomace extracts was 12.08±0.25 mg CE g⁻¹ d.m. Similar results (17.73±2.5 mg g⁻¹ d.m.) were previously reported in red grape pomaces from five grape cultivars. The differences in results were likely attributed to the use of different extraction techniques (ultrasound bath), however, the authors used somewhat similar parameters as in this study (use of 0.1 % HCl with 70 % acetone at 45 °C). It can be observed that the extraction yield of proanthocyanidins is significantly higher with addition of HCl, but remained the same in 0.5–1 % HCl interval. With regards to extraction time, it can be seen that the concentrations of proanthocyanidins did not significantly change from 30 to 60 minutes. This trend was observed for all combinations of time and percentages of HCl (Table 2). It was already shown that acidic solvent increased yields of proanthocyanidins solubilized from the grains of some sorghum varieties. Proanthocyanidins are stable at pH 4–6, and acid assists in dissociation of proanthocyanidins from the plant matrix. Comparing the contents of polymeric procyanidins from literature of 13.4 mg g⁻¹ d.m., the grape skin pomace from this study represents a potentially valuable source of phenolic compounds.

<table>
<thead>
<tr>
<th>Extraction parameters</th>
<th>Total flavonoids</th>
<th>Proanthocyanidins</th>
<th>Anthocyanins</th>
<th>Flavonols</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCl (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20.63±0.73</td>
<td>7.84±0.43</td>
<td>1.52±0.18</td>
<td>4.53±0.19</td>
</tr>
<tr>
<td>0.5</td>
<td>41.43±0.73</td>
<td>13.73±0.43</td>
<td>2.54±0.18</td>
<td>8.81±0.19</td>
</tr>
<tr>
<td>1</td>
<td>46.77±0.73</td>
<td>14.68±0.43</td>
<td>2.45±0.18</td>
<td>9.85±0.19</td>
</tr>
<tr>
<td><strong>Time (min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>29.13±0.73</td>
<td>12.19±0.43</td>
<td>2.42±0.18</td>
<td>6.86±0.19</td>
</tr>
<tr>
<td>45</td>
<td>39.09±0.73</td>
<td>11.55±0.43</td>
<td>1.96±0.18</td>
<td>7.90±0.19</td>
</tr>
<tr>
<td>60</td>
<td>40.62±0.73</td>
<td>12.51±0.43</td>
<td>2.13±0.18</td>
<td>8.43±0.19</td>
</tr>
<tr>
<td><strong>Time by HCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min, 0 %</td>
<td>18.03±1.29</td>
<td>7.25±0.83</td>
<td>1.61±0.31</td>
<td>4.39±0.22</td>
</tr>
<tr>
<td>30 min, 0.5 %</td>
<td>32.29±1.29</td>
<td>13.23±0.83</td>
<td>3.07±0.31</td>
<td>8.13±0.22</td>
</tr>
<tr>
<td>30 min, 1 %</td>
<td>37.06±1.29</td>
<td>16.1±0.83</td>
<td>2.59±0.31</td>
<td>8.05±0.22</td>
</tr>
<tr>
<td>Grand Mean</td>
<td>29.13±0.74</td>
<td>12.19±0.48</td>
<td>2.42±0.25</td>
<td>6.86±0.13</td>
</tr>
<tr>
<td>45 min, 0 %</td>
<td>19.98±0.85</td>
<td>6.84±0.61</td>
<td>1.39±0.31</td>
<td>3.66±0.18</td>
</tr>
<tr>
<td>45 min, 0.5 %</td>
<td>43.26±0.85</td>
<td>12.58±0.61</td>
<td>2.16±0.31</td>
<td>8.57±0.18</td>
</tr>
<tr>
<td>45 min, 1 %</td>
<td>54.03±0.85</td>
<td>15.22±0.61</td>
<td>2.34±0.31</td>
<td>11.48±0.18</td>
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<tr>
<td>Grand Mean</td>
<td>39.09±0.49</td>
<td>11.55±0.35</td>
<td>1.97±0.07</td>
<td>7.90±0.10</td>
</tr>
<tr>
<td>60 min, 0 %</td>
<td>23.87±1.55</td>
<td>9.43±0.76</td>
<td>1.57±0.31</td>
<td>5.54±0.51</td>
</tr>
<tr>
<td>60 min, 0.5 %</td>
<td>48.76±1.55</td>
<td>15.38±0.76</td>
<td>2.38±0.31</td>
<td>9.73±0.51</td>
</tr>
<tr>
<td>60 min, 1 %</td>
<td>49.23±1.55</td>
<td>12.71±0.76</td>
<td>2.44±0.31</td>
<td>10.01±0.51</td>
</tr>
<tr>
<td>Grand Mean</td>
<td>36.28±0.42</td>
<td>12.08±0.25</td>
<td>2.17±0.02</td>
<td>7.73±0.11</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± standard error in mg g⁻¹ dry matter of pomace
† Significant at p ≤ 0.05
‡ Not significant p > 0.05
Values with different letters are statistically different at p ≤ 0.05
Influence of extraction parameters on concentrations of anthocyanins

The average amount of anthocyanins in the grape skin extracts was 2.17±1.02 mg Mvd-3-glc g−1 d.m. (Table 2). A recent study evaluating the influence of fermentation of grape pomace has reported similar values for anthocyanins under extraction, performed with hot water at 50 °C. Further, lower concentrations were reported for grape marc from three different cultivars 0.87±0.06 mg Mvd-3-glc/g d.m., but extraction was performed with 70 % acetone (v/v) and 0.1 % HCl in an ultrasonic unit for 1 h at room temperature. Monrad et al. (2010) found two-fold higher concentrations of total anthocyanins in red grape pomace extracts (4.36 mg g−1 d.m. at 80 °C, 4.5 mg g−1 d.m. at 100 °C, and 4.11 mg g−1 d.m. at 120 °C) in comparison with our results, but under accelerated solvent extraction (ASE). Also, it is well-known that anthocyanin quantities show large variations among different grape cultivars, e.g. in aqueous grape skin pomace extracts, the concentrations of anthocyanins varied from 1.76 to 21.40 mg g−1 d.m., while in aqueous alcohol extracts concentrations varied from 45.38 to 88.44 mg g−1 d.m.54. Concerning the influence of extraction parameters, anthocyanins amounts significantly increased with addition of HCl, but remained the same for all extraction times. In addition, the cumulative influence of time and acidity was not significant, implying that extraction of anthocyanins is largely dependent on acidity (Table 2). This is likely due to the fact that anthocyanins are more stable in acidic solutions. Indeed, it was found that acidic aqueous solvents tend to be more useful for their extraction, which might be explained by the fact that the hydrolysis step helped the release of anthocyanins from plant cell walls55.

Influence of extraction parameters on concentrations of flavonoids

Regarding the total content of flavonols, previously reported data confirmed significant variations between different vintage years for fermented grape skin with averages from 2.97 to 3.10 mg g−1 48. The amount of flavonols in our samples averaged 7.73±0.11 mg QE g−1 d.m. Similarly to the anthocyanins, it was reported that the results for flavonols tend to show large variations5. For instance, almost exactly the same values (7.41±2.43 mg g−1) as our results were obtained for evaluating flavonols in commercially available grape marc skin extracts56. This process employed hot water as solute, for 1–2 h of extraction time. On the other hand, several-fold higher values were reported elsewhere (52.7±5.0 mg g−1), likely due to the application of different extraction techniques57. From all the observed polyphenols, and accordant with the favorable influence of acidity on their extraction54, flavonols showed the clearest influence of acidity on their recovery from grape skin pomace extracts (Table 2). The highest average experimental amounts of flavonols (9.85±1.55 mg g−1 d.m.) were detected if extraction time ranged between 45–60 minutes with 1 % HCl acidity (Table 2).

Influence of extraction parameters on concentrations of total flavonoids

Lastly, the average grand mean for total flavonoids was 36.28±0.42 mg QE g−1 d.m. In comparison, previous values for pomace from three red grapes ranged from 16.48 to 29.83 mg CTE g−1 51. However, extraction was carried with 40 % ethanol (v/v) as extraction solvent without addition of HCl. Makris et al. (2007) evaluated agri-food solid waste extracts of white and red grape pomace, and the content of total flavonoids was 33.45 and 50.24 mg CTE g−1, respectively (corrected for 5 % of humidity). Extraction was carried out with 0.1 % HCl in methanol/acetone/water (60/30/10, v/v/v)52. Addition of HCl resulted in increased flavonoid content, from 20.63 to 41.43 (0.5 % HCl) and 46.77 mg QE g−1 d.m. (1 % HCl). The observed results confirmed that acidity had enhanced hydrolysis of polymeric phenolic structures and release of monomeric metabolites that are easily solubilized during extraction.

For all time intervals, increased acidity resulted with increased amounts of flavonoids (Table 2). Interestingly, with longer extraction time, the amounts of proanthocyanidins and anthocyanins remained similar, while total flavonoids on average increased 36.8 % after extending extraction time from 30 minutes to 45 or 60 minutes (Table 2). Similarly, to our results, other sources have shown that higher total flavonoids concentrations were observed with longer extraction time (10 vs. 30 min) for Inula helenium53. Also, literature reports fluctuations (increase and decrease) in the flavonoid content as a function of time for aqueous ethanol extracts. It was hypothesized that increased time increased adhesion of diffused particles (flavonoid) around the cell walls of the supporting material (glass or plastic tubes) where extraction was conducted, which may hinder extraction54.

Optimization of flavonoids recovery in red grape skin pomace extracts

The quantities of all studied polyphenols were mutually strongly correlated, meaning that all of them strongly increased with each other (Table 3). To optimize the relation among proanthocyanidins (PC), anthocyanins (AC), flavonols (FL), total fla-
vonoids (TF), extraction time, and acidity, the RSM regression models (quadratic, cubic etc.) were constructed. This approach was previously published by our group\(^ {11,23,34,55-59} \). Significant models contained only statistically significant predictors (acidity, time, TF, and their various interactions). The constructed equations with RSM from experimental data were:

\[
PC = 4.70 - 0.40 \cdot t \cdot [\text{min}] + 58.95 \cdot \text{Acidity} \% + 0.61 \cdot TF - 0.51 \cdot t \cdot \text{Acidity} \% + 0.02 \cdot t \cdot TF - 1.98 \cdot \text{Acidity} \% \cdot TF - 0.02 \cdot TF^2 + 6.81 \cdot \text{Acidity} \% \\
+ 0.03 \cdot \text{Acidity} \% \cdot TF^2
\]

\[
AC = -0.87 + 6.81 \cdot \text{Acidity} \% + 0.15 \cdot TF - 0.30 \cdot \text{Acidity} \% \cdot TF - 0.002 \cdot TF^2 + 0.003 \cdot \text{Acidity} \% \cdot TF^2
\]

\[
FL = -14.31 + 1.66 \cdot t \cdot [\text{min}] + 43.30 \cdot \text{Acidity} \% - 0.80 \cdot TF - 0.05 \cdot t \cdot TF - 2.15 \cdot \text{Acidity} \% \cdot TF - 0.01 \cdot \text{Acidity} \% \cdot TF^2 + 0.0004 \cdot t \cdot [\text{min}] - 0.001 \cdot t \cdot [\text{min}] \cdot TF^2 + 0.03 \cdot \text{Acidity} \% \cdot TF^2
\]

Model fitness (i.e. model efficacy) between experimental and modeled data was evaluated with adjusted \( R^2 \), which equaled 90.3 %, 51.89 %, and 86.1 % for equations 1–3, respectively. Durbin-Watson statistic was insignificant and in the acceptable ranges from 1.4 to 2.6. Lack-of-fit tests for all models were insignificant, implying good fitness between modeled and experimental data. This also can be visually observed from Figure 1(d)-(f) for all extraction times and acidities in each group of flavonoids. Additionally, the occurrence of over-parameterization was tested with variance inflation factors that were all lower than the acceptable value (V.I.F. \( \leq 4 \)). In summary, all models were precise and with very good predictive power, and Figure 1 shows all RSM plots for all the flavonoids.

The highest amounts of PC = 16.45 mg g\(^{-1}\) d.m. can be obtained from 35.54 TF after 30.22 minutes of extraction and with addition of 0.99 % HCl. The optimal content of AC (3.74 mg g\(^{-1}\) d.m.) can be obtained from 17.91 mg g\(^{-1}\) d.m. of TF at 42.04 minutes of extraction and with acidity of 1 % HCl. In the same way, optimal amounts of FL 49.48 mg g\(^{-1}\) d.m. can be obtained from 22.80 mg g\(^{-1}\) d.m. of TF at 49.48 minutes and with 0.86 % of acidity.

Table 3 – Correlation among studied polyphenols

<table>
<thead>
<tr>
<th>Proanthocyanidins [mg CE g(^{-1}) d.m.]</th>
<th>Anthocyanins [mg Mvd-3-glc g(^{-1}) d.m.]</th>
<th>Total flavonoids [mg QE g(^{-1}) d.m.]</th>
<th>Flavonols [mg QE g(^{-1}) d.m.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proanthocyanidins [mg CE g(^{-1}) d.m.]</td>
<td>1</td>
<td>0.77( ^\dagger )</td>
<td>0.82( ^\dagger )</td>
</tr>
<tr>
<td>Anthocyanins [mg Mvd-3-glc g(^{-1}) d.m.]</td>
<td>1</td>
<td>0.56( ^\dagger )</td>
<td>0.63( ^\dagger )</td>
</tr>
<tr>
<td>Total flavonoids [mg QE g(^{-1}) d.m.]</td>
<td>1</td>
<td>0.96( ^\dagger )</td>
<td></td>
</tr>
<tr>
<td>Flavonols [mg QE g(^{-1}) d.m.]</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^\dagger \) Significant at \( p \leq 0.05 \)
Fig. 1 – Graphs showing extraction conditions for flavonoids from red grape skin pomace extracts
Equations 1–3 can be applied for an industrial extraction of proanthocyanidins, anthocyanins and flavonols by controlling acidity, time, and concentrations of total flavonoids. For instance, it is a very common industrial scenario where a facility produces anthocyanins from grape pomace for further use (e.g., colorants or additives, antioxidants, nutraceuticals etc.) but the pomace has unknown content of such compounds. The amount of all polyphenols in different grape pomaces varies greatly, due to the influence of different cultivating conditions, vintages, cultivars, winemaking, etc. Hence, it is difficult to standardize industrial processing even for extraction of the same type of compounds, let alone for different ones. On the other hand, the goal of industry is to have steady production of the highest amounts of anthocyanins under optimal conditions that are derived from the lowest expenditure of resources and with flexibility of using the same pomace for different types of flavonoids. In such a situation, it is essential to know the content of anthocyanins for provided grape pomace in order to adjust the extraction/processing parameters in the facility and to evaluate the economic value of the pomace. This data can be obtained by spectrophotometric evaluation that has to be repeated over and over again for different polyphenols such as proanthocyanidins and flavonols. Therefore, a flexible economical approach is welcomed that is able to have the least number of analytical assessments for adjustment of the production. Here is where equations 1–3 could be useful. For example, for their solutions, only one spectrophotometric evaluation is needed, and that is the amount of total flavonoids. It is reasonable to use this parameter since other mentioned polyphenols, in this instance proanthocyanidins and flavonols, are a subset of a set of total flavonoids. Thus, when pomace is delivered to the processing facility, one needs to evaluate only the amount of total flavonoids and if the goal is to produce anthocyanins, plug that data into equation 2, together with the data for acidity and extraction time. That will provide the predicted maximum yield for the aforementioned pomace. If the same pomace is going to be used for the production of proanthocyanidins or flavonols, one can use the same data already obtained for the total flavonoids, and just use it in equation 1 or 3.

**Conclusions**

The concentrations of all studied polyphenols (total flavonoids, proanthocyanidins, anthocyanins, flavonols) had increased with increasing acidity. Extraction time generally favored an increase in the studied polyphenols, but this relation was more evident for flavonols and total flavonoids, while less obvious for other examined phenolics. The flavonoid content in grape skin pomace extracts proved the interesting potential of grape pomace by-products as bioactive constituents in functional-foods production.

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