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Bioactive Phytochemical Composition of Grape Pomace Resulted from Different White and Red Grape Cultivars

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Abstract: Grapes are rich in phenolic compounds, being important for human health with anti-inflammatory, antiatherosclerotic, antimutagenic, anticarcinogenic, antibacterial, antiviral, and antimicrobial activity. The winemaking of the grapes generates significant amounts of waste. These wastes contain bioactive compounds in their biomass that can be used as a source of food improvement or as a source of nutrition supplementation. This study looks at the content of bioactive compounds, the polyphenolic profile, and the antioxidant activity in different white and red grape pomaces. The investigation of bioactive characteristics (total polyphenols, total flavonoids, catechins, tannins, and antioxidant activity) was carried out by UV-Vis spectrophotometric methods, while the individual polyphenolic composition was investigated by target and screening UHPLC-HRMS/MS analysis. Principal components (PCA) and the heat maps analysis allows the discrimination between the grape pomace resulted from white grape cultivars (Muscat Ottonel and Tamaioasa Romaneasca) and red grape pomaces (Cabernet Sauvignon, Merlot, Feteasca Neagra, Burgund Mare, Pinot Nore), with the identification of the specific phenolic compounds for each grape pomace type.

Keywords: antioxidant activity; polyphenolic compounds; flavonoids; catechins; polyphenolic profile; grape; pomace

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1. Introduction

A particular interest for the wine industry is wine waste [1,2]. About 80% of the world's grape production is used in the wine industry. During the winemaking process, after alcoholic fermentation, about 20% of the processed grapes by weight remains as solid organic waste, namely pomace [3,4]. The principal by-products in viticulture are the grape pomace, which consists of grape stalks, seeds, and skins left after the crushing and pressing stages of wine production [5–7]. In 2021, worldwide vineyards reached a total area (including areas not yet in production) of 7,300,000 ha, which refers to the total area planted with vines for all purposes (wine and juices, table grapes, and raisins), including young vineyards which are not yet productive ("World Wine Production Outlook OIV First Estimates, International Organisation of Vine and Wine Intergovernmental Organisation," 4 November 2021). World wine production in 2021 was 260,000,000 hl, generating significant amounts of waste. It must be taken into account that, for each kg of grape that is processed, about 0.2 kg of grape pomace results, consisting of skins, stalks, and seeds. A huge amount of grape pomace is then generated, by the wine-making process itself and by the alcohol-producing industries: this by-product, or waste, has to be somehow treated and processed [8,9]. Huge quantities of waste materials are produced every year, which

are rich in nutraceutical substances and complex carbohydrates; moreover, without their recycling, this biomass represents an environmental issue due to a high concentration of polyphenolic compounds [10,11].

Grapes are rich in phenolic compounds, which makes them important for human health due to their antioxidant, anti-inflammatory, and antimicrobial activities [12,13]. There are also studies on the beneficial effects of these compounds on the heart and other organs against oxidative stress [14,15].

Grapes, grape juice, wine, and implicitly the grape pomace have been found to contain high amounts of polyphenolic compounds [16] with antioxidant properties that may also act as pro-oxidants because they may induce the production of free radicals [6,7]. Winery by-products (pomace) are rich in high-added-value compounds, mainly known as polyphenols, including phenolic acids, flavanols, and anthocyanins [17,18], which have also been identified in grapes and wine [19,20]. The quantitative and qualitative distribution of polyphenols in grape pomace may show significant differences, depending on several factors, such as grape varieties, the location of cultures, and the winemaking procedures, and even the distribution from the same cultivars may vary based on the geographic regions and vintage of the wine [21–23]. The majority of grape pomace polyphenols originate from the grape skin and seeds and consist of two main classes, flavonoids (anthocyanins, flavonols, flavan-3-ols, flavones, and chalcones) and nonflavonoids (phenolic acids, stilbenes, tannins, coumarins, and neolignans) [24], with numerous biological activities and health-promoting properties, including cardiovascular and metabolic health, cancer prevention, skin and gut health, and protection from microbial infection [12].

Byproducts resulting from the agrofood industry have received considerable attention in recent years because of the wide range of possible biotechnological applications. The complex phytochemistry of grape pomace suggests that it represents a promising source for the development of high-added-value products, such as nutraceuticals, functional foods products, skincare, and other cosmetic products [22]. Numerous food additives and nutritional products obtained from grape pomace, such as grape skin or seed extracts and powders, pomace powder, and anthocyanin colorants, are distributed in the market [25]. However, further studies are needed in order to identify the most promising source of specific bioactive phytochemicals and stimulate the implementation of sustainable strategies for the superior valorization of grape pomace at a local/regional scale. This study looks at the content of bioactive compounds, polyphenolic profile, and antioxidant activity in wine grapes, as well as their loss, by eliminating winemaking waste. The bioactive characteristics (total polyphenols, total flavonoid, and antioxidant activity) of grape pomace resulting from different white and red grape cultivars have been investigated using AnalytikJena Specord 205 UV/VIS spectrophotometer. The individual polyphenolic composition was made by screening with DAD-HPLC-MS. Principal components analysis (PCA) and heat maps analysis were used to discriminate between grape pomace resulting from white grape cultivars (Muscat Ottonel and Tamaioasa Romaneasca) and red grape cultivars (Burgund Mare, Merlot, Cabernet Sauvignon, Feteasca Neagra, and Pinot Noir). The obtained results could be helpful for the identification of the characteristic bioactive phytochemicals specific to each type of grape pomace in order to develop different technological applications.

2. Materials and Methods

2.1. Grape Sample

The grape *Vitis vinifera*, including white varieties Feteasca Regala, Riesling Italian, Sauvignon, Muscat Ottonel, and Tamaioasa Romaneasca, and red varieties Merlot, Burgund Mare, Cabernet Sauvignon, and Feteasca Neagra, from Argeș county, Ștefănești wine center (44°5′ N and 24°57′ E, 300 m above sea level, southern exhibition), was used in our studies. Grapes were harvested manually at full maturity level during the 2021 vintage. The grapes were used for winemaking, and the resulted grape pomace was further investigated. The sample musts were stored in bottles at −20 °C until the analyses.

Grape Pomace GP

The GP samples were collected in two winemaking stages: the first stage, immediately after pressing the white grapes, the fresh unfermented GP (Muscat Ottonel-MO, Tamaioasa Romaneasca-TR), and the others were supplied after 20 days of fermentation at 20 °C and must separation, and the fermented GP for red grapes (Feteasca Neagra—FN, Merlot—M, Burgund—BM, Cabernet Sauvignon—CS and Pinot Noir—PN). The samples were stored in vacuum bags at −20 °C prior to the experiments. After the end of the winemaking, the frozen grape pomace was subjected to drying in a microwave oven JW-MW-6 KW at a temperature of 45 °C. The samples of dried grape pomace were shredded with the help of SWANTEC SJ 1000 model, lab mill.

2.2. Chemicals and Reagents

All chemicals and solvents were obtained from Carl ROTH GmbH Co. (Karlsruhe, Germany) and Merck Co. (Darmstadt, Germany) and they had HPLC or analytical grade (>99%) quality. Analytical standards (gallic, abscisic, p-cumaric, cafeic, clorogenic, ferulic, elagic, vanilic, 4-hydroxibenzoic, acid 3,4-dihidroxibenzoic, t-cinamic and syringic acids, (+)-catechin, și (−)–epicatechin, rutin, naringin, hesperidin, quercetin, kaempferol, izorhamnetin, chrysin, pinocembrin, apigenin, galangin, t-resveratrol) were purchased from Sigma-Aldrich (Steinheim, Germany). Folin–Ciocalteu phenol reagent (2 N), radical scavenging assay reagents DPPH and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from obtained from Alfa Aesar (ThermoFisher GmbH Kandel, Kandel, Germany).

2.3. Extraction Procedures

In order to investigate the bioactive characteristics (total polyphenols—TP, catechins—Cat, tannins—TA, anthocianins—Ant; antioxidant activity is expressed as GAE equivalents (AA GAE) and Trolox equivalents (AA Trolox)), the extracts were prepared by adding 20 mL of ethanol 50% to 2 g of GP sample. All extracts were placed on a shaker for 48 h. Prior to each analysis, the extracts were centrifuged, and the supernatants were further used. For the determination of polyphenolic profile by UHPLC-MS/MS, the grape pomace was extracted by microwave solvent extraction (MAE) (CEM MARS 6 extractor) using water and 50% ethanol solution g/g. The ratio liquid solid (g/g) was 1:3, and a pre-set program of 5 min, pressure 13 PSI, power 480, and temperature 100 °C was applied.

2.4. Analytical Investigations

2.4.1. Quantitative UV-Vis Spectrophotometric Determinations

Spectrophotometric determinations (total polyphenols—TP, total flavonoids—TF and antioxidant capacity—AC) of the extracts were performed using an AnalytikJena Specord 205 UV/VIS spectrophotometer (Analytic Jena, Jena, Germany) equipped with 1 cm path length quartz cells.

Total polyphenols (TP) were determined by the Folin– Ciocalteu colorimetric method [26], measuring the maximum absorbance at 765 nm. In brief, 0.1 mL of extract of pomace were mixed with 5 mL of distilled water and 0.5 mL of Folin– Ciocalteu reagent. After 30 min of reaction, 1.5 mL of 20% sodium carbonate solution and 2.9 mL of distilled water were added to stop the reaction and to develop a characteristic blue color for 2 h, at room temperature and protected from light. The total polyphenols quantification was based on the standard curve generated by serial dilution of a gallic acid standard, covering the range 50–1000 mg/L of gallic acid. Values were expressed as mg gallic acid equivalents (GAE) per g of pomace, dried weight (DW), based on the formula:

$$\mathrm{TP(mgGAE/g)} = \frac{\mathrm{dilution} \times (\mathrm{DO765} - \mathrm{b})/\mathrm{a} \times \mathrm{V_e}}{\mathrm{m(g)}} \quad (1)$$

where a and b are the parameters of the calibration curve; the reading of DO765 was at wavelength 765 nm, Ve—volume of extract; and m (g)—the amount of the pomace used for the extraction.

In this study, *catechins*, monomer flavanolic units, were determined with a method based on the reaction of catechins with vanillin assay (1% alcohol solution) [27]. The vanillin test is specific to flavan-3-ols, proanthocyanins, and dihydrochalcones, which have a single bond at the 2,3-position and possess free metahydroxy groups on the B-ring, with the production of red color. For that purpose, 10 mL of extract of pomace was mixed with 10 mL HCl 11.5 N and 5 mL 1% vanillin (alcoholic solution), and after 20 min, the maximum absorbance was measured at 500 nm. A calibration curve was made using the serial dilution of catechin standard (0–0.02–0.04–0.06–0.08–0.1 mg/L), and the results were expressed in mg catechins/100 g of pomace, dried weight (DW), based on the formula

$$\mathrm{Cat}\left(\frac{\mathrm{mg}}{\mathrm{g}}\right) = \frac{\mathrm{dilution} \times (\mathrm{a} \times \mathrm{DO500} + \mathrm{b}) \times \mathrm{V_e}}{\mathrm{m(g)}} \quad (2)$$

where a and b are the parameters of the calibration curve; the reading of DO500 was at wavelength 500 nm; Ve—volume of extract; and m (g)—the amount of the pomace used for the extraction.

Tannins were determined by the leukocytanidins (LA) method based on the property of tannins to transform in a hot (100 °C) and strongly acidic (concentrated HCl) environment into cyanidin, which is red in color. The optical density at a wavelength of 550 nm is directly proportional to the concentration of tannins [28]. In detail, in two tubes, 1 mL of distilled water and 3 ml of concentrated HCl were added to 2 mL pomace extract. One of the test tubes was boiled in a water bath at 100 °C for 45 min, while the other was kept at room temperature. After 45 min, the boiled test tube was cooled with tap water, followed by the addition of 0.5 ml of 96% ethanol, in both test tubes, to stabilize the color. The optical density of the solutions in the two test tubes (boiled and unboiled) was measured at 520 nm, and the results were calculated using the formula:

$$\mathrm{Tan}\left(\frac{\mathrm{mg}}{\mathrm{g}}\right) = \frac{(15.7 \times \Delta\mathrm{OD520}) \times \mathrm{V_e}}{\mathrm{m(g)}} \quad (3)$$

where the reading of DO500 was at wavelength 520 nm; Ve—volume of extract; and m (g)—the amount of the pomace used for the extraction.

Anthocyanins. To 50 g of grape pomace, we added 85 mL of 0.1% hydrochloric acid and 15 mL of 96% ethanol; we then homogenized it and left the mixture to macerate for 1 h, at room temperature, with homogenization every 15 min. We diluted the liquid fraction at 1/20 with a 0.1% HCl solution, followed by measuring the optical density at 520 nm, in a 1 cm tube, against distilled water [29]. The quantitative results were calculated using the following formula:

$$\mathrm{Ant(mg/g)} = \frac{(\mathrm{DO520} \times 22.75 \times 20) \times \mathrm{V_e}}{\mathrm{m(g)}} \quad (4)$$

where the reading of DO520 was at wavelength 520 nm; Ve—volume of extract; and m (g)—the amount of the pomace used for the extraction.

DPPH Assay. The evaluation of the antioxidant activity was carried out by testing free radical scavenger properties. The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability by means of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), according to a the method described by Manca et al. [30]. A 100 µL sample solution of previously diluted extract was mixed with 3.90 mL of DPPH• methanolic solution (2.5×10^{-2} mg/L methanolic DPPH solution). The reaction mixtures were shaken and incubated for 45 min in the dark at room temperature. The absorbance was measured at 517 nm against methanol. Absorbance measurements were transformed into antioxidant capacity using trolox as standard, in the concentration range 6.25–100 mmol/L, and the results were expressed as mmol/L Trolox equivalents/100 g of pomace. Additionally,

the antioxidant activity was defined as the capacity necessary to reduce the initial DPPH concentration by 50% (Efficient concentration = EC50 mgGAE/L) [31], and the results were expressed as gallic acid equivalents (GAE) using a calibration curve between 0.01–1 mg GAE/L). The quantitative results were calculated using the formula:

$$AA(mg/100g) = \frac{\frac{Acontrol - Asample}{Acontrol} \times V_e}{m(g)} \times 100 \quad (5)$$

where Acontrol and Asample are the absorbances measured at 517 nm, Ve is the volume of extract, and m (g) is the amount of the pomace used for the extraction.

2.4.2. Polyphenolic Profile by UHPLC-HRMS

The quantitative analysis of individual polyphenols (phenolic acids, flavonoids, and stilbens) was performed by UHPLC–ESI/HRMS (ultra-high-performance liquid chromatography electrospray ionization tandem mass spectrometry) using a high-resolution Q Exactive mass spectrometer™ Focus Hybrid Quadrupole—OrbiTrap equipped with HESI, coupled to a high-performance liquid chromatograph UltiMate 3000 UHPLC (Thermo Fisher Scientific, Bremen, Germany). The chromatographic separation was performed on a Kinetex® C18 column (100 × 2.1 mm, 1.7 µm particle diameter) at 30 °C, under a gradient elution of two mobile phases, A (water with 0.1% formic acid) and B (methanol with 0.1% formic acid), at a flow rate between 0.3 and 0.4 mL/min, as presented in a previous paper [32]. Full scan data in negative mode covering a scan range of m/z 75–1000 were acquired at a resolving power of 70,000 FWHM at m/z 200, while variable data-independent analysis MS^2 (vDIA) was performed at the resolution of 35,000, isolation windows and scan ranges being set as follows: 75–205 m/z, 195–305 m/z, 295–405 m/z, 395–505 m/z, and 495–1000 m/z. Nitrogen was used as collision gas and auxiliary gas at a flow rate of 11 and 48 arbitrary units, respectively. The applied voltage was 2.5 kV, and the capillary temperature was 320 °C. The energy of the collision cell was set at 30 eV. The calibration was performed in the concentration range between 50 and 1750 µg/L for each of the phenolic acids and flavonoids by serial dilution with methanol from the standard mixture of concentration 10 mg/L. The data were purchased and processed using the Xcalibur software package (Version 4.1) (Thermo Fisher Scientific, Bremen, Germany). Compound Discoverer software (v. 2.1) using an untargeted metabolomics working template combined with internet database of accurate MS data, ChemSpider (www.chemspider.com, accessed on 7 October 2022) and available literature were used as a reference library to identify compounds of interest.

2.5. Data Processing

All the analyses were made in duplicate. Statistical differences between different cultivars were tested using Pearson correlation test with a 0.05 significance level. Principal component analysis (PCA) and Hierarchical Cluster 286 Analysis were performed in order to discriminate between different grape pomace varieties. All the mathematical and statistical analyses were performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and XLSTAT Add in soft version 15.5.03.3707 (Addinsoft, New York, NY, USA).

3. Results and Discussions

3.1. Bioactive Properties

The values of the TP obtained by the Folin–Ciocalteu colorimetric method ranged from 1.71 to 2.46 mg GAE/g DW, Cat ranged between 1.77 and 2.07 mg Cat/g DW, Ant ranged between 2.68 and 15.66 mg Ant/g DW, tannins ranged between 17.87 and 44.81 mg Cat/g DW, and antioxidant activity ranged from 14.55 to 75.14 mg GAE/g and between 41.66 and 85.13 µmol TE/g (Figure 1).

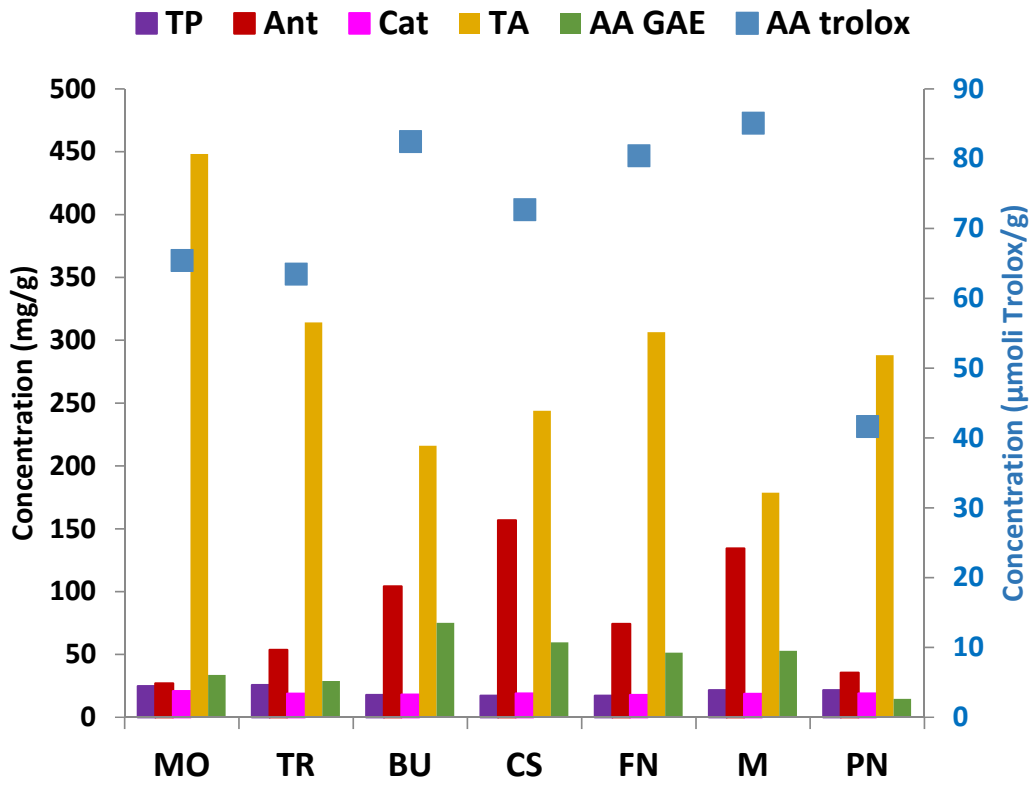
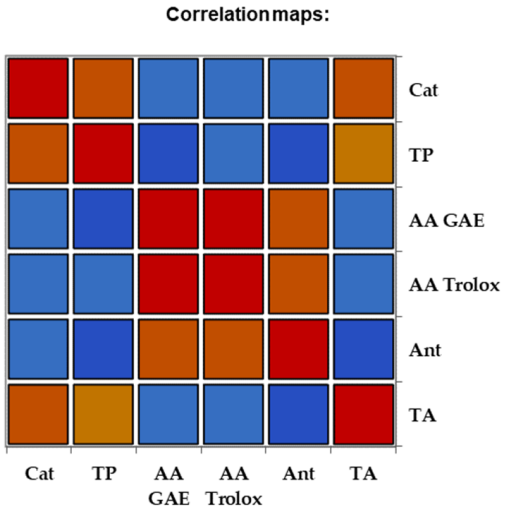


Figure 1. Bioactive properties of different grape pomace varieties (PN—Pinot Noir, M—Merlot, FN—Feteasca Neagra, CS—Cabernet Sauvignon, BU—Burgund, TR—Tamaioasa Romaneasca, MO—Muscat Ottonel).

The TP content of the investigated grape pomaces varied from 17.06 to 25.58 mg GAE/g (DW), with lower values for CS (17.06 mg GAE/g) and FN (17.07 mg GAE/g) grape pomaces and higher values for MO (24.65 mg GAE/g) and TR (25.58 mg GAE/g) grape pomaces. The obtained results are lower than the literature data for red grape cultivars Cabernet Sauvignon (55 mg GAE/g) [33], 69.83 mg GAE/g [34], 38.1 mg GAE/g [35]; Merlot (69–80 mg. GAE/g [36], Tempranillo (26.8–71.8 mg GAE/g) [33]; Malbec (196.2 mg GAE/g) [37]; Cabernet Franc (153.8 mg GAE/g) [38]; and rare Georgian red grape pomaces (27.9 mg GAE/g) [39]; and grape pomace from white grape cultivars Riesling Italian (47.94 mg GAE/g) [40], Chardonnay (58.15 mg GAE/g) [34], Viognier (99.1 mg GAE/g) and Vidal Blanc (55.5 mg/GAE/g) [38] (Table S1). A possible explanation may arise from the fact that using maceration for the extraction of the bioactive compounds from the grape pomace, an incomplete extraction could be realized. The values of the anthocyanin content of the grape pomace varied between 26.83 and 156.62 mg/g, with higher values corresponding to CS (156.62 mg/g) and M (134.22 mg/g) red grape pomaces, and lower values for grape pomaces resulted from MO (26.83 mg/g) and TR (53.53 mg GAE/g) white grapes cultivars, but also for PN red grape pomace (35.54 mg/g). The literature data show a lower amount of anthocyanin in the grape pomace of Cabernet Sauvignon (70.3 mg/g) [41], 133.79 mg/g [34], and Merlot (89.6 mg/g) [42], and higher amounts for Pinot Noir grape pomace (50.61–131.86 mg/g) [43] (Table S1).

Catechins in grape pomaces were found to be between 17.71 and 20.69 mg/g, with a higher value corresponding to MO (20.69 mg/g) white grape pomace, while tannins ranged between 178.70 and 448.15 mg/g, with high values for MO (448.15 mg/g) and TR (314.16 mg/g) white grape pomaces. The results of the antioxidant activity of all the investigated grape pomaces measured by DPPH ranged from 41.66 to 85.13 µmol Trolox/g, DW. Red grape pomace shows higher antioxidant activity, which is most likely correlated with anthocyanins content, and Merlot and Burgund Mare pomaces show higher antioxidant activities. Thus, white grape pomaces can be considered a good source of tannins, while red grape pomaces are considered a good source of anthocyanins.

The Pearson correlation analysis (Figure 2) shows moderate correlations between the bioactive properties of the investigated grape pomaces. The interpretation of correlation analysis was performed using correlation coefficients with values higher than 0.5.



Variables	Cat	TP	AA GAE	AA Trolox	Ant	TA
Cat	**1**	0.656	−0.478	−0.409	−0.452	0.741
TP	0.656	**1**	−0.702	−0.448	−0.619	0.528
AA GAE	−0.478	**−0.702**	**1**	**0.869**	**0.752**	−0.538
AA Trolox	−0.409	−0.448	**0.869**	**1**	0.653	−0.427
Ant	−0.452	−0.619	**0.752**	0.653	**1**	**−0.778**
TA	0.741	0.528	−0.538	−0.427	**−0.778**	**1**

Values in bold are different from 0 with a significance level alpha = 0.05

Figure 2. Pearson correlation between the bioactive properties of different grape pomaces (red color: positive correlations and blue color: negative corelations).

Correlation maps coefficients of determination (Pearson):

Positive correlations (red color) were obtained for TP with Cat and Ant, for AA with Ant and TA with Cat and TP, while moderate negative correlations (blue color) were observed for TP with AA, Ant and TA, for AA with TP and TA, for Ant with TP and TA and for TA with AA and Ant.

3.2. Identification of Phenolic Compounds in Grape Pomace by UHPLC-HRMS

The identification and quantification of polyphenols in grape pomace are of great interest as they make a significant contribution to its total bioactivity. A specific UHPLC-Q-Orbitrap HRMS method for rapid identification and quantification of the phenolic compounds in plant material, previously developed, optimized, and validated was, applied [44].

A total ion current (TIC) chromatogram of the Feteasca Neagra grape pomace extract in the negative ion mode, covering a scan range between 80–1000 *m/z*, is shown in Figure 3.

TIC and the extracted chromatograms of the main phenolic compounds quantified in Feteasca Neagra grape pomace (the chromatograms were extracted from TIC using a 5 ppm mass accuracy window; negative ion mode, full scan, base peak in the range 75–1000 *m/z* were illustrated in Figure S1).

A total of 26 polyphenolic compounds were simultaneously identified and quantified by comparing with reference standards, including ten phenolic acids, 13 flavonoids, stilbenes (*t*-resveratrol), plant hormone (abscisic acid), and ellagic acid, a dimeric derivative of gallic acid. The retention time, compound name, formula, *m/z* values of adduct ions, and MS/MS fragment ions in negative ESI mode, mass error, and accurate molecular mass are shown in Table 1.

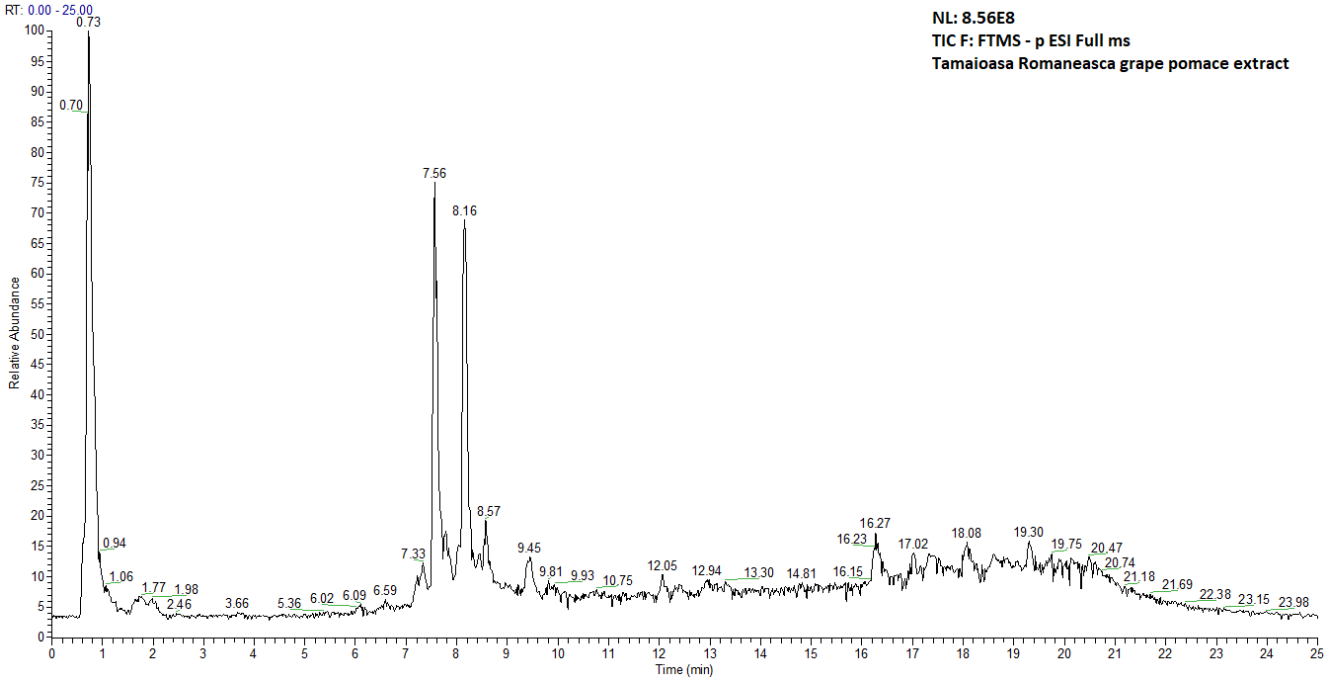


Figure 3. The obtained total ion current (TIC) chromatogram for the separation of polyphenolic compounds from Feteasca Neagra grape pomace extract by UHPLC–MS/MS detection in negative ionization mode.

Table 1. The identification of phenolic compounds in grape pomace by UHPLC-HRMS with structures confirmed by comparison with reference standards.

No	Compound	Retention Time (min)	Formula	Exact Mass	Accurate Mass $(M-H)^-$	Experimental Adduct Ion (*m*/*z*)	Mass Fragments
			Phenolic acids				
1	Gallic acid	1.98	$C_7H_6O_5$	170.0215	169.0142	169.0133	125.0231
2	3,4-Dihydroxybenzoic acid	4.26	$C_7H_6O_4$	154.0266	153.0193	153.0184	109.0281
3	4-Hydroxybenzoic acid	6.51	$C_7H_6O_3$	138.0316	137.0243	137.0233	118.9650, 96.9588, 71.0124
4	t-Ferulic acid	7.82	$C_{10}H_{10}O_4$	194.0579	193.0506	193.0499	178.0262, 134.0361
5	Chlorogenic acid	7.90	$C_{16}H_{18}O_9$	354.0950	353.0877	353.0880	191.0553
6	Caffeic acid	8.03	$C_9H_8O_4$	180.0422	179.0349	179.0343	135.044
7	Syringic acid	8.44	$C_9H_{10}O_5$	198.0528	197.0455	197.0450	182.0212, 166.9976, 153.0547, 138.0311, 123.0075
8	Cinnamic acid	8.45	$C_9H_8O_2$	148.0524	147.0451	147.0442	119.0489, 103.0387
9	p-Coumaric acid	8.72	$C_9H_8O_3$	164.0473	163.0400	163.0390	119.0489
			Flavonoids				
10	Catechin	7.57	$C_{15}H_{14}O_6$	290.0790	289.0717	289.0719	109.0282, 123.0349, 125.0232, 137.0232, 151.0390, 203.0708
11	Epi-catechin	8.14	$C_{15}H_{14}O_6$	290.0790	289.0717		
12	Rutin	9.42	$C_{27}H_{30}O_{16}$	610.1533	609.1460	609.1473	301.0352, 300.0276
13	Naringin	8.99	$C_{27}H_{32}O_{14}$	580.1791	579.1718	579.1718	363.0721
14	Hesperidin	9.33	$C_{28}H_{34}O_{15}$	610.1897	609.1824	609.1828	377.0876
15	Quercetin	10.66	$C_{15}H_{10}O_7$	302.2357	301.0354	301.0356	151.0226, 178.9977, 121.0282, 107.0125
16	Isorhamnetin	11.79	$C_{16}H_{12}O_7$	316.0582	315.0509	315.0515	300.0277
17	Kaempferol	11.59	$C_{15}H_{10}O_6$	286.0477	285.0404	285.0406	151.0389, 117.0180
18	Apigenin	11.86	$C_{15}H_{10}O_5$	270.0528	269.0455	269.0455	117.0333, 151.0027, 107.0126
19	Pinocembrin	12.58	$C_{15}H_{12}O_4$	256.0735	255.0662	255.0663	213.0551, 151.0026, 107.0125
20	Chrysin	13.41	$C_{15}H_{10}O_4$	254.0579	253.0506	253.0505	143.0491, 145.0284, 107.0125, 209.0603, 63.0226, 65.0019
21	Galangin	13.68	$C_{15}H_{10}O_5$	270.0528	269.0455	269.0455	169.0650, 143.0491
22	Pinostrobin	14.77	$C_{16}H_{14}O_4$	270.0892	269.0819	269.0822	179.0554
			Other compounds				
23	t-Resveratrol	9.55	$C_{14}H_{12}O_3$	228.0786	227.0713	227.0707	185.0813, 143.0337
24	Ellagic acid	9.69	$C_{14}H_6O_8$	302.0062	300.9989	300.9993	300.9990
25	Abscisic acid	10.08	$C_{15}H_{20}O_4$	264.1361	263.1288	263.1290	179.9803, 191.9454

Non-target UHPLC-Q-Orbitrap HRMS analysis allows one to identify other bioactive compounds and specialized metabolites that occur in grape pomace extracts, which are also responsible for antioxidant activity. Data processing analysis was carried out using Compound Discoverer software following a metabolomics working template, which includes RT alignment, background annotation, the assignment and comparison of fragmentation pattern, and molecular formula prediction, based on automated library and database search for identification purposes, including mzCloud (MS^2 fragments), Chemspider, MzVault, and Mass List Matches [45].

A total of 32 compounds were identified in the grape pomace extracts. The extracted chromatograms (using a 5 ppm mass accuracy window) of the main bioactive phytochemical compounds (polyphenols, tannins, stilbenoids) and amino acids in grape pomace extracts were presented in Figure S2. The retention times and precursor ion mass and fragment ion data of these compounds are summarized in Table 2.

Table 2. Identification of bioactive phytochemical compounds (polyphenols, tannins, stilbenoids) and amino acids in grape pomace extracts by UHPLC-Q-Exactive high-accuracy analysis of deprotonated precursors and fragment ions of specific components combined with data processing using Compound Discoverer software.

No	Compound	Retention Time [min]	Formula	Exact Mass	Accurate Mass $[M-H]^-/[M-H]^+$	Experimental Adduct Ion (*m*/*z*)	Mass Fragments
Polyphenols, tanins, stilbenoids by UHPLC-MS/MS in negative ionization mode							
1	Quercetin-3-glucoside	9.46	$C_{21}H_{20}O_{12}$	464.0954	463.0881	463.0888	301.0354, 101.0231, 300.0277
2	Kaempferol-3-glucoside	9.31	$C_{21}H_{20}O_{11}$	448.1005	447.0932	447.0938	285.0406, 151.0027, 125.0232, 174.9553
4	Isorhamnetin-3-glucoside	9.67/ 9.98	$C_{22}H_{22}O_{12}$	478.1111	477.1038	477.1042/ 477.1044	174.9553, 112.9844, 285.0406, 314.0436
5	Syringetin-3-glucoside	9.92	$C_{23}H_{24}O_{13}$	508.1217	507.1144	507.1148	289.0723, 112.9844, 174.9554, 344.0542
6	Epicatechin gallate	8.58	$C_{22}H_{18}O_{10}$	442.0899	441.0826	441.0830	169.0132, 125.0232, 289.0719
7	Epigallocatechin	7.73	$C_{15}H_{14}O_7$	306.0739	305.0666	305.0669	12.0232, 137.0233, 109.0282
8	Isohomovanillic acid	9.35	$C_9H_{10}O_4$	182.0579	181.0507	181.0499	125.0232, 146.9602
9	Suberic acid	8.92	$C_8H_{14}O_4$	174.0892	173.0820	173.0812	125.0232, 109.0282, 146.9601, 940285
10	Homoferreirin	9.42	$C_{17}H_{16}O_6$	316.0946	315.0874	315.0878	125.0960, 123.0440
11	Tricetin	10.66	$C_{15}H_{10}O_7$	302.0426	301.0354	301.0356	151.0027, 107.0126, 121.0283
12	[6]-Gingerol	13.33	$C_{17}H_{26}O_4$	294.1831	293.1759	293.1761	221.1544, 220.1465
13	Azelaic acid	9.77	$C_9H_{16}O_4$	188.1048	187.0976	187.0969	300.9992, 141.0911
14	Ursolic acid	19.40	$C_{30}H_{48}O_3$	456.3603	455.3531	455.3535	96.9587
15	Esculetin	9.15	$C_9H_6O_4$	178.0266	177.0194	177.0186	96.9588, 109.0282, 118.9651, 146.9602
16	Procyanidin dimers (B1, B2)	7.32/ 7.81/	$C_{30}H_{26}O_{12}$	578.1424	577.1351	577.1356	407.0775, 289.0720, 125.0232
17	Procyanidin dimer monogallate	8.08	$C_{37}H_{30}O_{16}$	730.1533	729.1460	729.1471	577.1359, 407.0776, 125.0232
18	Polydatin (piceid)	8.78/ 9.42	$C_{20}H_{22}O_8$	390.1314	389.1241	389.1248	227.0710, 245.0818
19	Piceatannol	8.98	$C_{14}H_{12}O_4$	244.0735	243.0662	243.0663	233.1546, 227.0347, 241.0502
20	Malvidin 3-Oglucoside	8.48	$C_{25}H_{27}O_{13}$	528.1034	527.0961	527.0965	331.0828
21	Malvidin 3-(6″-acetylglucoside)	7.99	$C_{25}H_{27}O_{13}$	535.1445	534.1372	534.1418	331.0830
22	Malvidin 3-O-p-coumaroylglucoside	10.08	$C_{32}H_{31}O_{14}$	639.1708	638.1635	638.1602	331.0830

Table 2. *Cont.*

No	Compound	Retention Time [min]	Formula	Exact Mass	Accurate Mass $[M-H]^-/[M-H]^+$	Experimental Adduct Ion (*m*/*z*)	Mass Fragments
Antochyanins and aminoacids by UHPLC-MS/MS in positive ionization mode							
23	peonidin	8.00	$C_{16}H_{13}O_6$	301.0706	301.0706	301.0712	266.9989, 283.0311
24	Peonidin-3-glucoside	8.43	$C_{22}H_{23}O_{11}$	463.1234	463.1238	463.1244	301.0716
25	Delphinidin	10.68	$C_{15}H_{11}O_7$	303.0499	303.0504	303.0504	283.0309, 266.9996, 299.0622
26	Petunidin	11.78	$C_{16}H_{13}O_7$	317.0655	317.0655	317.0663	283.0310, 266.9996, 299.0623
27	Cyanidin	7.61	$C_{15}H_{11}O_6$	287.055	287.0553	287.0557	218.3817
28	Malvidin	7.59	$C_{17}H_{15}O_7$	331.0812	331.0812	331.0800	315.0500, 287.0556
29	Tryptophan	6.55	$C_{11}H_{12}N_2O_2$	204.0898	205.0971	205.0977	118.0656, 143.0733, 115.0548
30	L-dopa	4.20	$C_9H_{11}NO_4$	197.0688	198.0761	198.0766	91.0581, 124.0397
31	Dopamine	8.65	$C_8H_{11}NO_2$	153.0789	154.0862	154.0867	91.0581, 56.9658
32	Tryptamine	7.25	$C_{10}H_{12}N_2$	160.1	161.1073	161.1077	91.0581, 56.9658

3.3. Quantitative Data of Phenolic Compounds in Grape Pomace of Different Grape Cultivars

The UPLC-HRMS/MS allows the routine determination of 26 compounds in grape pomace extracts (Table 3). The analysis was conducted in duplicate, and the results were expressed as mean values and standard deviations. Among the quantified compounds, quercetin (+)-catechin, (-)-epicatechin, gallic, and syringic acids and pinocembrin were presented in high amounts in grape pomaces, while naringin, hesperidin, apigenin, pinocembrin, chrysin, and galangin levels were found to be below quantification limits. Quercetin was found to be two- or three-fold higher in red grape pomaces compared to with white grape pomaces, higher amounts corresponding to Feteasca Neagra (1445.70 mg/100 g), Merlot (1208.27 mg/100 g) and Burgund Mare (1011.82 mg/100 g) grape pomaces, values much higher than those found in the literature for Merlot (56.65 mg/100 g [24], 31.15 mg/100 g [42]), Cabernet Sauvignon (13.98 mg/100 g [24] (Table S2). (+)-Catechin and (-)-epicatechin were quantified in higher amounts in white grape pomaces (142.080–185.68 mg/100 g for (+)-catechin and 123.94–142.83 mg/100 g for (-)-epicatechin) compared with red grape pomaces (79.24–157.54 mg/100 g for (+)-catechin and 70.90–85.87 mg/100 g for (-)-epicatechin), with higher amounts in Muscat Ottonel grape pomace. The obtained values for (+)-catechin were comparable with the literature data for pomace from red grape cultivars (Cabernet Sauvignon 150.16 mg/100 g, Merlot 122.29 mg/100 g, Isabel 94.28 mg/100 g) [24]. Literature data reported lower amounts of (-)-epicatechin in grape pomace, with values ranging between 18.24 and 44.36 mg/100 g [24]. Syringic acid from grape pomaces ranged from 18.31 to 19.80 mg/100 g in white grape pomaces and from 22.00 to 94.30 mg/100 g in red grape pomaces, with a higher amount in Merlot grape pomace. Gallic and ellagic acids were quantified in similar amounts in white and red grape pomaces with values between 6.44 and 14.02 mg/100 g for gallic acid and from 0.28 to 3.63 mg/100 g for ellagic acid. Similar amounts of syringic and gallic acids in grape pomace were reported in the literature, with gallic acid ranging from 2.52 and 36.04 mg/100 g [24,37,42,46], while syringic acid ranged from 46.9 to 173.1 mg/100 g for Cabernet Sauvignon, Merlot, and Malbec red grape pomace [37,42]. Pinostrobin was quantified in higher amounts in red grape pomaces, except Pinot Noir grape pomace (10.19–45.37 mg/100 g), in comparison with white grape pomaces (0.81–1.01 mg/100 g). Rutin was quantified in low amounts only in white grape pomaces while the literature data reported 4.20–41.43 mg/100 g of rutin in Cabernet Sauvignon, Merlot, Isabel, Malbec red grape pomace [37,38,42]. The amount of resveratrol in the studied grape pomaces ranged from 0.10 to 0.98 mg/100 g, while the literature data reported 4.02–9.00 mg resveratrol/100 g of Cabernet Sauvignon pomace [5,24], 3.02 mg/100 g. The results of the quantitative analysis are consistent with the studied literature studies (Table S2) [24,37,42,46].

Table 3. Concentration of phenolic compounds in different grape pomaces (mg/100 g DW).

Phenolic Compound	White Grape Cultivars		Red Grape Cultivars				
	Muscat Ottonel	Tamaioasa Romaneasca	Cabernet Sauvignon	Feteasca Neagra	Merlot	Burgund Mare	Pinot Noir
Gallic acid	10.71 ± 0.54	11.12 ± 0.56	9.25 ± 0.46	10.95 ± 0.55	14.07 ± 0.70	7.56 ± 0.38	6.44 ± 0.32
3,4-Dihydroxybenzoic acid	0.39 ± 0.02	0.55 ± 0.03	0.93 ± 0.05	1.55 ± 0.08	0.70 ± 0.04	0.60 ± 0.03	0.67 ± 0.03
4-Hydroxybenzoic acid	0.13 ± 0.01	0.23 ± 0.01	<0.13	0.26 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	<0.13
t-Ferulic acid	0.13 ± 0.01	0.22 ± 0.01	0.17 ± 0.01	0.23 ± 0.01	0.15 ± 0.01	<0.13	<0.13
Chlorogenic acid	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13
Caffeic acid	0.13 ± 0.01	0.19 ± 0.01	0.13 ± 0.01	0.25 ± 0.01	0.34 ± 0.02	0.19 ± 0.01	<0.13
Syringic acid	19.80 ± 0.99	18.31 ± 0.92	22.75 ± 1.14	48.43 ± 2.42	94.30 ± 4.71	22.00 ± 1.10	48.14 ± 2.41
Cinnamic acid	0.69 ± 0.03	0.73 ± 0.04	1.00 ± 0.05	3.06 ± 0.15	0.37 ± 0.02	0.73 ± 0.04	0.56 ± 0.03
p-Coumaric acid	0.22 ± 0.01	0.21 ± 0.01	0.24 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	<0.15	0.26 ± 0.01
catechin	185.68 ± 9.28	142.80 ± 7.14	126.26 ± 6.31	79.24 ± 3.96	126.56 ± 6.33	120.98 ± 6.05	157.54 ± 7.88
epi-catechin	142.83 ± 7.14	123.94 ± 6.20	76.24 ± 3.81	70.90 ± 3.55	85.87 ± 4.28	79.24 ± 3.96	83.62 ± 4.18
rutin	0.16 ± 0.01	0.32 ± 0.02	<0.14	<0.14	<0.14	<0.14	<0.14
naringin	<0.14	<0.14	<0.14	<0.14	<0.14	0.14	<0.14
hesperidin	NF	NF	<0.14	<0.14	<0.14	0.14	<0.14
quercitin	355.42 ± 17.77	672.59 ± 33.63	992.49 ± 49.62	1445.70 ± 72.28	1208.27 ± 60.41	1011.82 ± 50.59	360.90 ± 18.05
isorhamnetin	0.96 ± 0.05	1.70 ± 0.08	6.29 ± 0.31	7.32 ± 0.37	5.46 ± 0.27	8.32 ± 0.42	3.02 ± 0.15
kaemferol	0.29 ± 0.01	0.540.03	0.21 ± 0.01	0.41 ± 0.02	0.99 ± 0.05	0.79 ± 0.04	<0.10
apigenin	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
pinocembrin	<0.19	<0.19	<0.19	<0.19	<0.19	<0.19	<0.19
crysin	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18	<0.18
galangin	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13
pinostrobin	1.01 ± 0.05	0.81 ± 0.04	45.37 ± 2.27	10.19 ± 0.51	13.52 ± 0.68	38.72 ± 1.94	0.96 ± 0.05
t-resveratrol	0.35 ± 0.02	0.58 ± 0.03	0.35 ± 0.02	0.64 ± 0.03	0.35 ± 0.02	0.98 ± 0.05	<0.14
Ellagic acid	1.14 ± 0.06	0.87 ± 0.04	1.23 ± 0.06	3.63 ± 0.18	1.53 ± 0.08	1.22 ± 0.06	0.28 ± 0.01
Abscisic acid	<0.13	0.14 ± 0.01	0.14 ± 0.01	<0.13	<0.13	<0.13	0.15 ± 0.01

NF—not found.

The Pearson correlation analysis applied to the quantitative data (Figure 4) shows strong and moderate correlations between the main phenolic compounds in grape pomaces. The interpretation of correlation analysis was carried out using correlation coefficients with values higher than 0.5 (blue—negative correlations red—positive correlations).

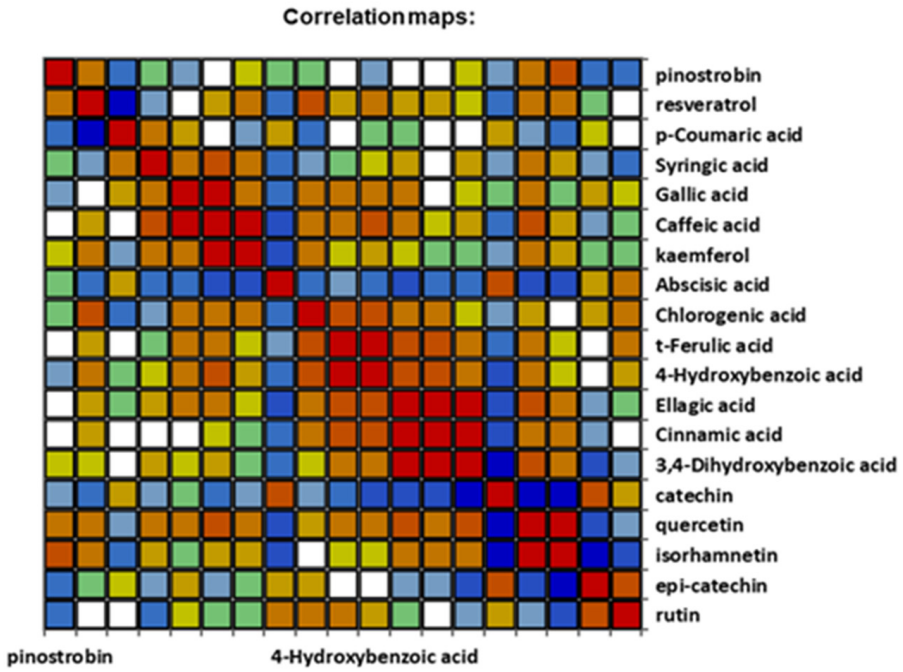


Figure 4. Pearson correlation between the main phenolic compounds of different grape pomaces (positive corelations: red color and negative corelations: blue color).

Strong correlations were obtained for t-resveratrol with p-coumaric acid, for ellagic aid with cinnamic acid, for cinnamic acid with 3,4-dihydroxybenzoic acid, and for catechin with quercetin, while the majority of the phenolic compounds shows low correlations (Table S3).

Based on the results obtained, we can state that the grape pomace obtained from the investigated grape cultivars could be considered a valuable source of bioactive polyphenols (eg. Syringic and gallic acids, (+)-catechin and (-)-epicatechin, quercetin) with numerous beneficial effects on health, including antioxidant activity, anti-cancer activity, vasodilator activity and oxidative stress management [1,47–50].

3.4. Multivariate Data Analysis

Unsupervised classification by PCA and heat map analysis were used in order to show the differentiation of the different grape pomaces based on the composition of the main phenolic compounds. The heat map profiles based of the main bioactive phytochemicals from the grape pomaces indicate a clear differentiation of red grape pomace extracts from the white grape pomace extracts, but also from PN grape pomace (Figure 5A). The grouping of Pinot Noir red grape pomace alongside white grape pomace can be explained by the late harvesting of the grapes, which cause the decrease of the phenolic compounds. Cabernet Sauvignon and Burgund Mare, but also Feteasca Neagra and Merlot grape pomaces, show similar phenolic profiles and are grouped in different clusters.

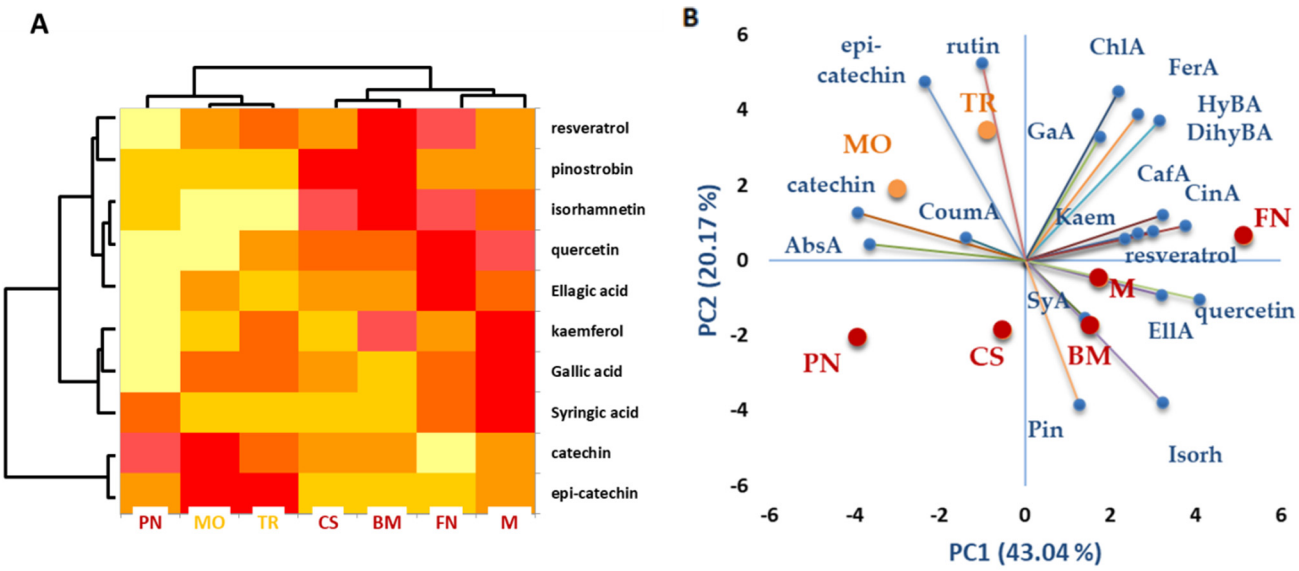


Figure 5. Differentiation of grape pomace based on the main quantified phytochemicals: Heat map (**A**) and PCA (**B**) (MO-Muscat Ottonel, TR-Tamaioasa Romaneasca, PN-Pinot Noir, CS-Cabernet Sauvignon, FN-Feteasca Neagra, M-Merlot, BM-Burgun Mare).

PCA explained 63.21% of the total variation using principal components with a higher contribution brought by PC1 (43.04%) when compared to PC2 (20.17%), (Figure 3). Along both PC1 and PC2 axes, the white grape pomaces can be differentiated from the red ones, as they are grouped in in a separate group (Figure 5B). PCA analysis also revealed the correlations among the polyphenols' compositions in different grape pomaces. Our results showed that (+)-catechin, (-)-epicatechin, rutin, and *p*-coumaric and abscisic acids are specific phenolic markers of white grape pomace, while quercetin, syringic acid, t-resveratrol, isorhamnetin, and pinostrobin are specific phenolic markers of red grape pomaces.

Statistical analysis based on the qualitative data relating to phytochemical compounds (polyphenols, tannins, and stilbenoids) and nutritional information (amino acids) identified from the HRMS/MS screening of grape pomaces of different white and red grape cultivars was performed in order to discriminate between the grape pomace category. As shown

by heat maps analysis (Figure 6A), a clear discrimination of white grape pomaces can be observed from the red ones based on the specific phytochemical compounds.

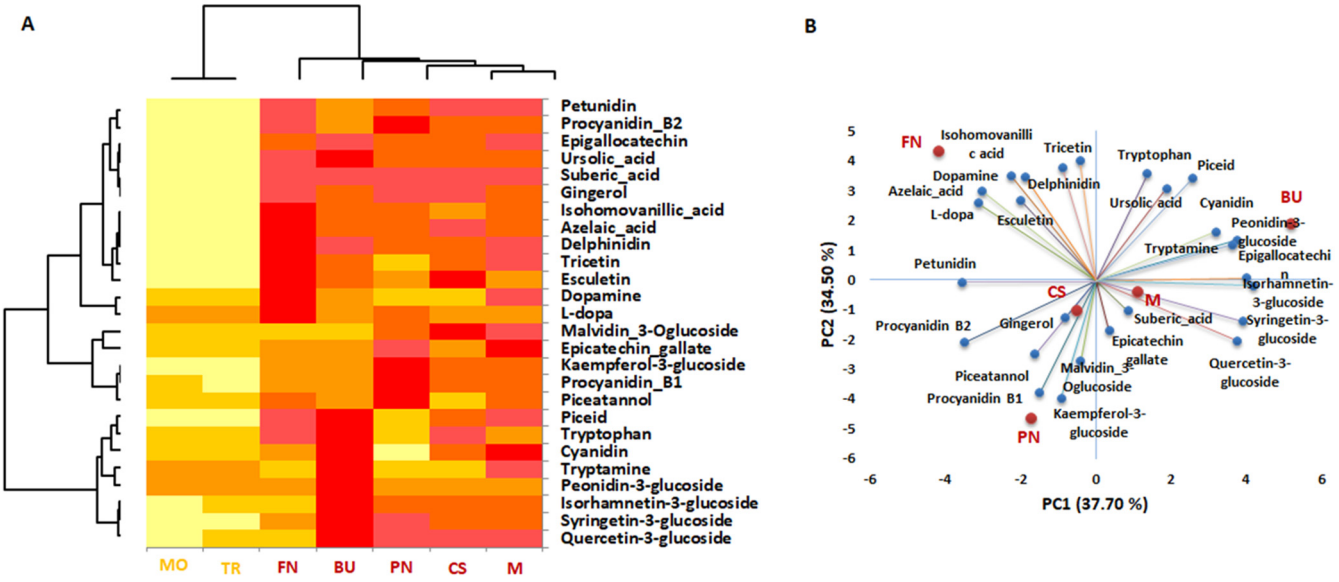


Figure 6. Differentiation of grape pomace based on the phytochemical profiles (qualitative data): Heat map (**A**) and PCA (**B**) (MO-Muscat Ottonel, TR-Tamaioasa Romaneasca, PN-Pinot Noir, CS-Cabernet Sauvignon, FN-Feteasca Neagra, M-Merlot, BM-Burgun Mare).

PCA analysis based on the phytochemical profiles of red grape pomace revealed their differentiation and correlations among the phytochemical and nutritional fingerprints of the pomace of different red grapes (Figure 6B). Thereby, nutritional compounds such as L-dopa and dopamine and its metabolite isohomovanilic acid, but also phytochemical compounds such as azaleic acid, tricetin, aesculetin, delphinidin, and petunidin, represent specific markers of Feteasca Neagra grape pomace, while procyanidins B1 and B2, piceatannol, and gingerol are representative compounds in Pinot Noir and Cabernet Sauvignon grape pomaces. Tryptofan amino acid and its metabolite tryptamine are implicated in various neuropsychiatric disorders [45], but ursolic acid, piceid, cyaniding, epigallocatechin, and isorhamnetin-3-glucoside, with antioxidant, anticarcinogenic, and anti-inflammatory properties were also characteristic of Burgund Mare grape pomace, while suberic acid, epicatechin gallate, quercetin-3-glucoside, and syringetin-3-glucoside are characteristic for Merlot grape pomace.

This study provides an integrative discussion of the correlation between the qualitative and quantitative polyphenols profiles and bioactive properties of grape pomace, also highlighting the identification of compounds that have not been commonly reported in grape pomace, but which could be involved in synergistic effects that contribute to the overall bioactive potential. Further studies are needed in order to quantify the amount of the specific bioactive compounds in grape pomace, including anthocyanins, procyanidin, stilbens, and amino acids, as well as their purification, in order to identify the potential for further biotechnological applications (food supplements and drugs).

4. Conclusions

The grape pomace represents a valuable source of high-added-value phytochemicals with important biological activities that can be used for the development of new functional foods, cosmetics, and supplements. The phytochemical bioactive composition of grape pomace varies in relation to the grape cultivar, with white grape pomaces being considered a good source of tannins, while red grape pomaces are considered a good source of anthocyanins. (+)-Catechin and (-)-epicatechin are representative of grape pomace resulting from white grape cultivars, while quercetin, syringic acid, and pinostrobin are representative

of grape pomace resulting from red grape cultivars. However, more studies are needed to improve the extraction, separation, and purification of the bioactive compounds from grape pomace in large-scale production processes, thus supporting the circular economy schemes for a sustainable and environmentally friendly management of waste resulting from wine industry.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations9120395/s1: Figure S1: TIC and the extracted chromatograms of the main phenolic compounds quantified in Feteasca Neagra grape pomace (the chromatograms were extracted from TIC using a 5 ppm mass accuracy window; negative ion mode, full scan, and base peak in the range 75–1000 *m/z*); Figure S2: TIC and the extracted chromatograms of the main phenolic compounds (A) and amino acids (B) identified in Feteasca Neagra grape pomace (the chromatograms were extracted from TIC using a 5 ppm mass accuracy window; negative ion mode, full scan, base peak in the range 75–1000 *m/z*); Table S1: Content of total phenolic compounds (TP, expressed as GAE), total anthocyanins (Ant, expressed in mg/g), tannins (expressed in mg/g), antioxidant activity (expressed in µmoli Trolox/g) in grape pomace; Table S2: Phenolic compounds in grape pomace; Table S3: Correlation matrix and Pearson coefficients of determination for individual phenolic compounds in grape pomaces.

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