GRAPE VARIETY AND EXTRACTION METHODS EFFECT ON PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF WHITE GRAPES POMACE

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This study assessed the phenolics and antioxidant activities of two local white grape pomace extracts (i.e., Feteasca alba and Tamaioasa Romaneasca). The influence of the extraction procedure (i.e., maceration and ultrasonication) on the total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity (AA) of extracts (pomace, stems, skin, and seeds) was investigated as well. The results revealed that grape pomaces from two white varieties showed high and various values for TPC and AA, depending on the extraction procedure i.e., maceration and ultrasonication. On the other hand, statistical analysis revealed that TPC and TFC were positively correlated with the grape variety ($p < 0.01$). The experimental values are consistent with the predicted values.

Keywords: white grape by-products; phenolics; antioxidant activity – IC_{50}; statistical analysis

1. Introduction

Currently, grape by-products are used for various purposes in agriculture, cosmetics, pharmaceuticals, biorefining, animal feed, and fortified/functional foods. Grape pomace and wine yeast are the main wastes of interest in foods [1, 2]. Grape pomace is the waste resulting from the pressing process of red and white grapes, with the final goal being to obtain high-quality wine. Grape pomace is usually composed of stalks/clusters, skins, and seeds/kernels, representing approximately 25% of the total grapes weight used in the winemaking process [3,4]. Grape pomace consists of two fractions: pomace without seeds (residual pulp, skin, and bunches) and pomace with seeds. Both fractions are rich in bioactive compounds such as phenolic compounds (flavonoids, phenolic acids, and stilbenes) [1,3,5]. The most abundant phenolics in grape pomace are represented by the anthocyanins concentrated in the skin, respectively the flavonols present especially in the seeds (56-65% of the total), as reported Garćia-Lomillo et al. (2017). In addition, grape bunches or stalks constitute a

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residue that can be used as a source of astringent compounds, mainly proanthocyanins [6]. Several studies have reported that grape pomace contains high amounts of resveratrol and polyphenols such as flavanols (myricetin, quercetin, and kaempferol), flavan-3-ols (catechin and epicatechin), cinnamic acids (p-coumaric acid), and benzoic acids (syringic, gallic, and protocatechuic, 4-hydroxybenzoic) [1, 7-8]. The presence of relevant phenolic compounds in grape pomace components (i.e., seed, skin and pulp) are investigated in various studies [9-28]. Recent studies revealed that bioactive compounds of grape pomace are beneficial to human health, particularly in protecting against chronic diseases [29-33]. Polyphenols, the major compounds in grape pomace, are known to have anti-inflammatory, anti-obesity, antihyperlipidemic, cardioprotective, and cancer-prevention effects, as well as improved insulin sensitivity and glucose homeostasis improvement [1, 34]. Moreover, in the case of polyphenolic compounds, it was found that they have antioxidant properties, but they can also act as prooxidants, as they can induce free radicals [7, 35-37]. Several authors highlighted that the phenolic compounds, as prooxidants, may reduce the evolution of atherosclerosis [9, 36, 38]. The comparative evaluation of the antioxidant activity of the unfermented and fermented pomace indicated a significant positive correlation between the antioxidant activity and the concentration of tannins, total content of anthocyanins, flavan-3-ol monomers, and stilbenes [39]. In addition, grape pomace can be reused to obtain add-value products by increasing the fiber content and antioxidant compounds [40-43]. In food-added value production, the antioxidant capacity of pomace extract can be a valuable alternative for substituting synthetic antioxidants [44].

The hydroxyl groups from phenolics as free radical scavengers [38, 45] or hydrogen atom donors [46, 47] are related to antioxidant activity (AA). Based on this concept several characterization methods of AA such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays were developed [38]. These assays analyze antioxidant activity by hydrogen atom donation (ABTS and DPPH) or electron transfer (FRAP) [48-50]. The Folin–Ciocalteu method for assessing total polyphenol content (TPC) is the reference test for measuring polyphenols in plants and biological samples [46, 51]. Consequently, the phenolic content was determined by spectrophotometric evaluation of this reaction [52, 53].

White grape by-products are examined less in terms of bioactive compounds and antioxidant properties, despite the high demand for food that exhibits good health and prevents diseases caused by oxidative stress. The current study highlighted that grape stem, a serious pollutant in the environment, could be valorized to extract phenolic compounds for use as bioactive and/or functional ingredients. The objectives of this study were: (i) to investigate the composition of grape by-products in terms of phenolic and flavonoid content and antioxidant activities, regarding grape variety, extraction procedure, and grape anatomical parts; (ii) to evaluate the correlation between the phenolic content, AA, grape variety, anatomical part, and extraction procedure. In this respect, the established correlation could be utilized to optimize the

extraction of phenolics from grape by-products and for further utilization as functional ingredients. In addition, the findings from this study will be useful for winemaking producers and producers of food ingredients, based on the correlations established by the statistical approach.

2. Materials and Methods

2.1 Site description

The vineyards where the grape samples were collected (i.e., Feteasca Alba and Tamaioasa Romaneasca) are in the middle of the Dealu Mare region (Fig. 1), known for the quality of grapes and wine production. These vineyards are situated on the hills around the Carpathian Mountains and benefit from different types of calcareous soil [54-56].

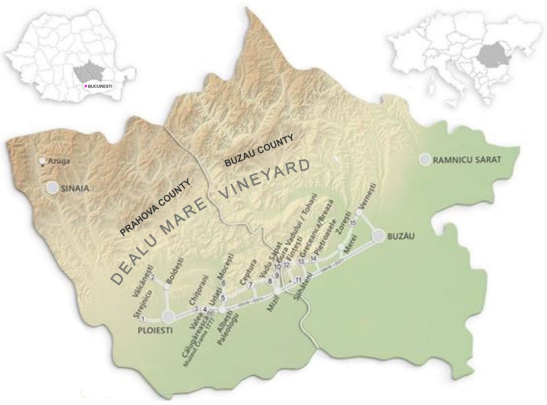

Fig. 1. Dealu Mare vineyard according to REVINO Gourmet 2023 [57]

The first stage of the investigation of the phytochemical profile of the extracts from the pomace of white grapes (Feteasca Alba and Tamaioasa Romaneasca) considered the following aspects: organic culture; climate; geographical characteristics; (5) type of soil (black-brown clay-calcareous) and calcareous subsoil [58].

2.2 Materials and reagents

Grape samples of two white indigenous varieties (Feteasca Alba and Tamaioasa Romaneasca) were harvested from the abovementioned Romanian vineyards (Fig. 1). Representative portions of each sample were used for further analysis. Morphological characteristics of local white grape (*Vitis Vinifera* L.) cultivars [59] are provided according to data presented by authors in previous study [60]. All reagents (Merck, KGaA, Germany, and Sigma-Aldrich, Saint Louis, USA) were of high purity (analytical grade), and the liquid samples were filtered before use to avoid accidental contamination of the samples (including ultrapure water) or possible interference in chemical investigations.

2.3 Sample preparation

The main stages of obtaining hydroalcoholic extracts from pomace, bunches (separated from pomace), and skins and seeds for the two varieties of white grapes are summarized in Fig. 2 and Table 1.

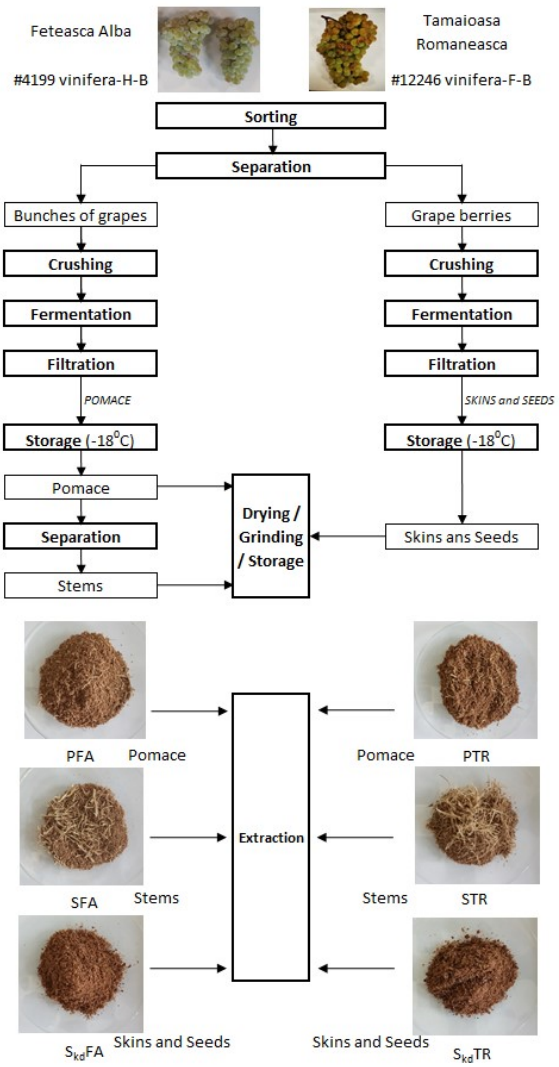

Fig. 2. Flow chart for obtaining hydroalcoholic extracts from pomace bunches and skins and seeds, from white grape varieties - Feteasca Alba, Tamaioasa Romaneasca

The pomace samples (P - "pomace"), bunches (S - "stems") and skins and seeds (S_{kd} - "skins and seeds") were dried in an oven for 48 h at 35°C. The resulting dry plant material, subsequently ground and used for extraction, had an average moisture content in the range of 74.90-76.75% (PFA); 72.55-74.03% (PTR); 75.28-75.61% (SFA); 74.78-76.30% (STR); 79.42-77.88% (S_{kd}FA); 74.94-75.84% (S_{kd}TR). After drying, the

plant material was ground (electric grinder/2 intervals of 10 s) and stored at cold (-18°C).

2.3.1 Extraction by ultrasonication

Extraction by ultrasonication was carried out according to the procedure described by Ivanova et al. (2011), with slight modifications to optimize the use of raw materials and reagents [62]. Ultrasound-assisted extraction was performed by using a USC500TH ultrasound bath (45 kHz / 2°C accuracy / resolution 1 min). The optimal parameters of the process were: dry material extraction ratio: extraction solvent (g:cm^3) = 0.5:10; solvent used for extraction: ethanol 50% (mixture of absolute ethanol: double-distilled water = 1:1, v/v) acidified (0.1%, v/v) with concentrated hydrochloric acid (HCl).

Table 1

Investigated white grape parts (hydroalcoholic extracts) and sample code

Grape variety	Grape parts	Sample code*	Grape variety	Grape parts	Sample code*
Feteasca Alba	Pomace	PFA-M	Tamaioasa Romaneasca	Pomace	PTR-M
	Stems	SFA-M		Stems	STR-M
	Skins and seeds	S_{kd}FA-M		Skins and seeds	S_{kd}TR-M
	Pomace	PFA-U		Pomace	PTR-U
	Stems	SFA-U		Stems	STR-U
	Skins and seeds	S_{kd}FA-U		Skins and seeds	S_{kd}TR-U

**M – maceration, U – ultrasonication.*

On the other hand, the hydroalcoholic extracts obtained by ultrasonication was achieved taking into account several steps such as (a) 0.5 g of dried plant material was placed directly into 15 mL vials over which was added 10 mL of extraction solvent (worked in triplicate); (b) second, the extraction is performed by ultrasounds, in an ultrasonic bath equipped with an ampoule holder, at ordinary temperature for 20 minutes (the temperature at the beginning and at the end of the process were noted) with intermittent agitation (vortex) every 5 minutes; (c) the third step revealed that the supernatant was separated by centrifugation (10 minutes/5000 rpm); (d) the collection of the supernatant was made by filtration (PRAT DUMAS France/Whatman equivalence 42, thickness 290 mm, micrometric retention 7-9 mm) in a 25 mL volumetric flask; (e) next, was add 10 mL of solvent over the precipitate in the ampoule, and the extraction process was resumed, followed by separation; the supernatant was separated and combined with the supernatant from the first extraction phase; (f) at the end, the 25 mL volumetric flask was brought to the mark with double-distilled water, then was stored in the refrigerator (4°C) until the moment of analysis for the determination of phytochemical parameters.

2.3.2 Extraction by maceration

Extraction by maceration was achieved in two phases, following the same steps of the abovementioned ultrasound extraction procedure, pooling the supernatant fractions collected in each phase. The only exception in terms of the working stages was that the extraction by maceration was carried out at room temperature for 24 hours in the dark under slight continuous agitation.

2.3.3 Phytochemical analyses

Total polyphenol content (TPC) from grape pomace samples was determined by Folin-Ciocalteu method [63] slightly adapted [33, 63, 60]. Analytic grade Folin-Ciocalteu (Merck KGaA, Germany) consists of a mixture of sodium tungstate ($Na_2WO_4 \bullet 2H_2O$) and sodium molybdate ($Na_2MoO_4 \bullet 2H_2O$) in water, and hydrochloric acid (HCl) and phosphoric acid (H_3PO_4) were added. The chemical process, occurring at a basic pH, is based on molybdenum reduction from +6 (yellow) to +4 (blue) after polyphenols oxidation in the samples, quantified colorimetrically at 765 nm. Gallic acid (Carl Roth GmbH and Co. KG, Germany) was used as a standard in order to obtain calibration curve. TPC was expressed as milligrams of gallic acid equivalents (GAE) per mL of pomace extract and then reported to dry weight (mg GAE/g).

Total flavonoid content (TFC) was assessed using a colorimetric assay with aluminum chloride ($AlCl_3$), according to several studies [33, 60, 64-68]. As standard was used Quercetin, QE (Sigma-Aldrich, Germany). TFC was expressed as µg QE/mL of grape part (pomace, stems, skins, and seeds) extract, finally reported as dry weight (mg QE/g).

The *antioxidant activity* (AA) of grape extracts was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [69], which is a widely used spectrophotometric assay [60, 70]. The method was described by several authors [71-74]. Regarding investigated extracts, the authors [60] have described a method that was applied at seven concentrations, and gallic acid was used as a standard to obtain a reference value for the antioxidant amount necessary to decrease the initial DPPH• concentration by 50% (IC50, µg/mL). AA of the grape extracts was indicated as µg GAE/mL.

All experiments were performed in triplicate, and the means ± standard deviations (SD) were reported [60].

2.3.4 Statistical analysis

Data are reported as the mean ± standard deviation (SD) of three replicates. The Student's t-test ($p = 0.05$) was applied to determine the significant differences among the samples in terms of TPC, TFC, and AA, depending on the grape variety, grape anatomic part, and extraction procedure applied. Differences between samples were considered significant when the p-value was < 0.05. Significant differences between means were determined using One-way Analysis of Variance (ANOVA), with TPC, TFC, and AA as dependent variables and grape variety, anatomic part, and extraction procedure, respectively, as fixed factors. Following the identification of significant differences, multiple comparisons between the experimental groups (twelve hydroalcoholic extracts) were performed using Tukey's test with $p = 0.05$. Data were investigated by SPSS (version 26.0, SPSS, USA). Principal Component Analysis (PCA) and hierarchical clustering were applied to the obtained data.

3. Results and Discussion

The processing of grapes results in a large amount of waste (i.e., grape pomace, grape seeds, wine lees), which still contains various ingredients of grapes, especially

phenolic compounds, with beneficial properties for nutrition [74]. Although the phenolic content and antioxidant capacity of grapes and grape products (i.e., wine/juice) have been extensively investigated, information on the phenolic profile and antioxidant activity of by-products obtained from two Romanian white varieties (Feteasca Alba and Tamaioasa Romaneasca) has been reported for the first time. For grape pomace, ethanol has been used as an extractive solvent owing to its numerous features, such as its natural presence in wines, safety, and environmentally friendly behavior [75]. As mentioned above, hydroalcoholic extracts were obtained for all experiments. The TPC values of the analyzed hydroalcoholic extracts obtained from the grape by-products are shown int Table 2:

Table 2

Phytochemical parameters (hydroalcoholic extracts) of grape pomace obtained from Feteasca Alba and Tamaioasa Romaneasca varieties.

Sample code	TPC*	TFC**	AA (IC_{50})***
PFA-M	9.58 ± 0.58^{c}	2.36 ± 0.06^{i}	16.46 ± 0.24^{n}
PFA-U	7.55 ± 0.38^{d}	1.91 ± 0.25^{i}	16.61 ± 0.38^{n}
SFA-M	7.18 ± 0.51^{d}	1.75 ± 0.12^{ij}	16.51 ± 0.33^{n}
SFA-U	5.72 ± 0.80^{de}	1.49 ± 0.13^{j}	17.01 ± 0.37^{m}
S_{kd}FA-M	6.01 ± 0.52^{de}	1.54 ± 0.12^{j}	16.69 ± 0.19^{np}
S_{kd}FA-U	5.24 ± 0.53^{de}	1.38 ± 0.07^{j}	16.97 ± 0.39^{m}
PTR-M	15.04 ± 0.72^{a}	3.25 ± 0.32^{g}	16.26 ± 0.05^{o}
PTR-U	15.32 ± 1.93^{a}	3.53 ± 0.40^{g}	16.19 ± 0.11^{o}
STR-M	4.88 ± 0.23^{e}	1.21 ± 0.05^{jk}	17.10 ± 0.25^{l}
STR-U	3.32 ± 0.34^{f}	1.15 ± 0.07^{k}	17.21 ± 0.20^{l}
S_{kd}TR-M	11.79 ± 1.61^{b}	2.69 ± 0.52^{h}	16.28 ± 0.13^{o}
S_{kd}TR-U	12.53 ± 0.87^{b}	3.09 ± 0.24^{h}	16.27 ± 0.26^{o}

**as mg GAE/g DW; **as mg QE/g DW; ***as μg GAE/mL. The results are presented as mean ± SD (triplicate). The values followed by different superscripts within the same column are significantly different according to Tukey's test ($p < 0.05$) among all the three factors analyzed (grape variety, anatomic part, and extraction method respectively).*

They ranged between 15.32 mg GAE/g DW (pomace of Tamaioasa Romaneasca, extracted by ultrasonication), and 3.32 mg GAE/g DW (stem of Tamaioasa Romaneasca, extracted by ultrasonication). The Tamaioasa Romaneasca variety, compared with Feteasca Alba, provided extracts with high amounts of TPC and TFC when pomace, skin, and seeds were used as raw materials, regardless of the extraction procedure applied. In contrast, extracts from the stem of Feteasca Alba had higher TPC and TFC values than the ones of Tamaioasa Romaneasca.

The maceration procedure seemed to be more efficient than ultrasonication when extraction of bioactive compounds (total phenolics and flavonoids) is discussed in the case of Feteasca Alba, regardless of the anatomic part. However, ultrasonication led to higher amounts of phenolics compared to the maceration of Tamaioasa Romaneasca extracts obtained from pomace, skin, and seeds. Maceration was also more efficient (by a factor of 1.5) for the extraction of phenolics from the stems of Tamaioasa Romaneasca. Interestingly, the total polyphenol content extracted from the stems of Feteasca Alba and the pomace of Feteasca Alba was 26% higher in the case of the maceration process compared with ultrasonication. Maceration led to an

increased amount of flavonoids from Feteasca Alba by-products with 24% (pomace), 17% (stem), and 12% (skin and seeds), respectively, compared to ultrasonication. It can be concluded that, ultrasound-assisted extraction (40 minutes) favors the faster release of extractable species in the solvent, maceration, through the longer contact time (24 hours), allows establishing the optimal balance between the concentration of extractable species from the plant matrix and the extraction solvent respectively. On the other hand, in maceration process was performed 24 hours at dark, to avoid degradation of extractable species, and thus were obtained the improved parameters. These data could be useful for analytical issues in agreement with the limited information provided in the literature. Thus, although grape stems are serious pollutants owing to their high organic material content that is biodegraded in soil and water, it was emphasized that they contain phytochemicals with important biological properties [76]. The TPC of grape stem extracts derived from three native Greek vine varieties ranged between 264.795 and 374.765 mg gallic acid equivalent/g dried weight of the extract. Concerning the total flavonoid content, the same varieties exhibited values between 12.630 and 17.478 mg catechin equivalent/g dried weight of extract [76]. The antioxidant properties of the stem extracts expressed as IC_{50} value (the concentration that induced the scavenging of the DPPH radical at 50%) ranged between 10.88 μg of extract/mL (*Mavrodaphne* variety, exhibiting strong antioxidant activity) and 14.87 μg of extract/mL (*Rhoditis* variety). Grape stem extracts are important antioxidant agents and an underestimated source of chemical compounds with beneficial roles in human health.

Although several data have been reported for TPC, TFC, and AA values of grapes grown worldwide, it is difficult to compare them because of the different methods applied and units of measure used. In addition, there is abundant literature on red grapes and red grape by-products and is relatively scarce when the TPC, TFC, and AA of white grapes and their anatomic parts are searched. It has been reported that the polyphenol content was lower in white-skinned cultivars than in black- and red-skinned cultivars [77]. Grape pomace is an excellent and affordable source of polyphenolic compounds [75] and a potential source of natural antioxidant agents [42, 78-80]. The pomace extracts obtained from two white grape varieties by Xu Y et al., 2015 had TPC and TFC values of 55.5 mg GAE/g and 32.8 mg CE/g for Vidal Blanc and 99.1 mg GAE/g and 75.0 mg CE/g for Viognier respectively. Vidal Blanc exhibited a DPPH scavenging capacity of 7.71μmol/g, whereas Viognier was 3.54μmol/g. The extracts examined in the present study exhibited scavenging activities ranging between 16.19 and 17.21 μg GAE/mL, with the order of STR-U > STR-M > SFA-U >S_{kd}FA-U >S_{kd}FA-M > PFA-U > SFA-M > PFA-M >S_{kd}TR-M >S_{kd}TR-U > PTR-M > PTR-U. The results obtained in this study are consistent with those reported by Tkacz et al. (2019) for white grapes [81]. The minimum value was 1380.29 mg/100g dm for cv. Hibernal, while the maximum value within the group of white grapes analyzed was 2886.16 mg/100 g dm for cv. Freiminer. Seeds, an integral part of grape marc, were found to contain total polyphenolic compounds exceeding at least five times that in white grape skins. The concentrations of polyphenolic compounds in seeds were

reported as follows: 11253.73 mg/100 g dm for the Seyval Blanc cultivar and 24041.90 mg/100 g dm for the Freiminer variety respectively [81]. Grape variety, maturity, genetic diversity, viticulture practices, soil characteristics, environmental stress, and vine health status have been reported to influence the phenolic profile of grapes [82]. Studying the influence of grape variety on phenolic profiles is essential [82]. The total phenolic content of 11 grape cultivars from China ranged between (1.296 and 10.525) mg GAE/g FW in skins, which was higher than that in pulps (0.189–1.134 mg GAE/g FW) of the same grapes. The DPPH radical scavenging capacity was reported to be highest in the pulp of the Beni Fuji variety (EC_{50}=14.1 μg/mL) and in the skin of the Muscat Kyoho variety (EC_{50}=11.7 μg/mL) [82]. The differences in the AA of different grapes may be very large [78]. The dissimilarities in the AA of grape varieties can be attributed to differences in cultivars and conditions, such as climate, environmental factors, soil conditions, and irrigation [79]. The white grape pomace obtained in the present study showed an important phenolic content, indicating that significant amounts of these bioactive compounds with antioxidant activity (which should be preserved/used in a specific matrix) could be recovered from these winemaking byproducts for food industries, feed, or pharmaceuticals. The inconsistency between our data and those reported by others might be a consequence of geographical location and varietal differences.

Statistical analysis of data

The T-test was applied to check if there were significant differences between the analyzed groups (i.e., extracts obtained from three kinds of anatomic parts of grapes, two grape varieties, and by applying two extraction procedures) in terms of their TPC, TFC, and AA content. According to the results of the T-test, all three dependent variables were registered differences ($p < 0.05$) labeled by anatomic part, namely pomace vs. stem and skin and seeds vs. stem. The groups consisting of TPC, TFC, and AA of the extracts were not different for the two grouping variables: extraction procedure (maceration/ultrasonication) and anatomical part (pomace/skin and seeds). There were no statistically significant differences in the antioxidant activities between the extracts of cv. Feteasca Alba and cv. Tamaioasa Romaneasca, which is in line with the data reported by Tkacz et al. (2019) [81], refers to the antioxidant potential determined in the flesh of white (four varieties) and red (three varieties) grapes. The differences between the average values of AA and TPC, depending on each of the independent factors used in the analysis, are shown in Fig. 3.

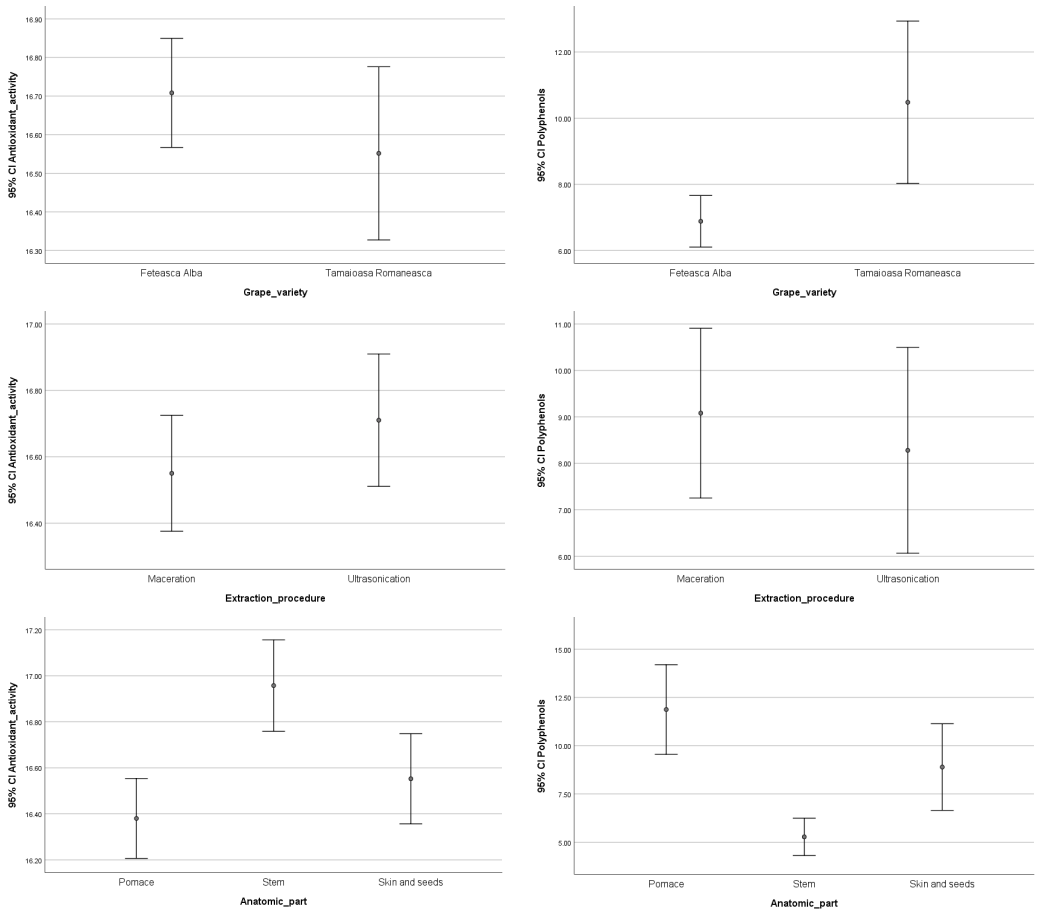

Fig. 3. Differences between average values of AA and TPC depending on factors: a) grape variety, b) extraction procedure and c) anatomic part

Interestingly, the error bars associated with the groups representing the average values of AA were in full agreement with the results of the t-test, indicating that there were significant differences between groups in relation to each independent variable analyzed. The AA of the extracts obtained from cv. Tamaioasa Romaneasca registered the highest variability in the values. In contrast, the variability of data, influenced by grape variety and extraction procedure, was smaller in the set of values of AA related to extracts obtained from the pomace, stem, skin, and seeds. It can be assumed that the lack of overlapping error bars was due to the natural differences between samples that contributed to the data variability. Screening of the composition of extracts, namely, the categories of polyphenols, can be explained in this sense. The phenolic contents of the extracts obtained from cv. Tamaioasa Romaneasca, respectively, by ultrasonication has been distinguished by large variability of data, meaning that the interaction between the independent parameters and their impact on each dependent variable must be analyzed when optimizing and eventually standardizing the content of grape byproduct extracts.

A one-way ANOVA consolidated the results of the *t*-test. Thus, there was no significant variation ($p > 0.05$) between extracts obtained by maceration and ultrasonication in terms of TPC, TFC, and AA. In addition, there was no significant variation ($p > 0.05$) between the hydroalcoholic extracts obtained from the two white grape varieties concerning AA, but there was a significant variation ($p < 0.05$) between the same extracts regarding TPC and TFC. A significant variation ($p < 0.05$) between extracts obtained from different anatomical parts of grapes in terms of TPC, TFC, and AA was emphasized through a one-way ANOVA analysis. Two-factor ANOVA was used to evaluate the influence of two independent variables from the total set of three on each dependent variable (TPC, TFC, and AA); the results are reported in Table 3. TPC, TFC, and AA of the analyzed extracts were influenced by the interaction between grape variety and extraction procedure, grape variety, and the anatomic part of the grapes.

Table 3

The interaction of the independent variables and their influence on TPC, TFC and AA, according to Two – Factor ANOVA analysis

Source / Type of interaction	TPC	TFC	AA
grape variety * extraction procedure	√	√	√
grape variety * anatomic part	√	√	√
anatomic part * extraction procedure	—	—	—

√ there are principal effects ($p < 0.05$) and also interaction ($p < 0.05$); there are significant effects of each source ($p < 0.05$) but no interaction ($p > 0.05$)

The cumulative effects of the anatomic part of the grapes and the extraction procedure on the TPC and AA of the hydroalcoholic extracts obtained from Feteasca Alba and Tamaioasa Romaneasca are shown in Fig. 4.

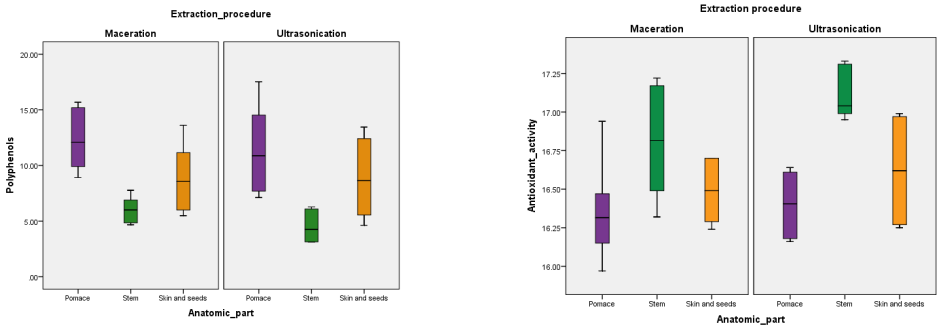

Fig. 4. The influence of extraction procedure and anatomic part of grapes on the phenolics content (a) and AA (b) and of the hydroalcoholic extracts obtained from white grape varieties

The pomace subjected to ultrasonication led to high variability in TPC values, whereas in the case of maceration, a high variability of AA values was obtained. The closed average values of TPC were determined by maceration and ultrasonication of the skin and seeds. In contrast, pomace and stem maceration seemed to be more efficient. Smaller average AA values (expressed as IC_{50}) were determined for the extracts obtained by maceration, regardless of the type of grape by-product. To highlight the association between dependent and independent variables, the assessment

of the degree of association between variables was carried out using Pearson correlation (Table 4). Correlation analysis revealed that the level of antioxidant activity of the white grape extracts was dependent on the total phenolic content. An indirect correlation was established because, in our study, AA was expressed as IC_{50}, so a lower IC_{50} value corresponded to a higher antioxidant activity. TPC and TFC exhibited a significant positive correlation with a significant level of 95%. These compounds were uncorrelated with either the extraction procedure or the anatomic part of the grapes, meaning that a correlation other than the linear one could be established between the analyzed dependent and independent parameters. A significant negative correlation between TPC, TFC, and antioxidant activity was observed. In agreement with our results, Elfalleh et al. (212) [83] found negative correlations between TPC, TFC, and DPPH scavenging capacity in pomegranate juice. The special capacity of each phenolic compound to scavenge different free radicals is due to its distinctive structure, namely the number of hydroxyl groups and side chains of benzoic acid [84]. Liu et al. (2018) [78] reported, based on the high correlation established between TEAC (Trolox equivalent antioxidant capacities) and TPC in pulps of 30 grape varieties, that phenolic compounds could be the main components responsible for scavenging free radicals.

Table 4

Pearson correlation coefficient values and type of association for the analyzed parameters

Association	Pearson coefficient / type of association	Type of association	Level of significance
Total phenolics * AA	0.829 / strong	indirect	p = 0,01
Total flavonoids * AA	0.819 / strong	indirect	p = 0,01
Total phenolics * Total flavonoids	0.984 / strong	direct proportional	p = 0,01
Total phenolics * Grape variety	0.451 / moderate	direct proportional	p = 0,01
Total flavonoids * Grape variety	0.449 / moderate	direct proportional	p = 0,01

A strong linear positive correlation between TPC and antioxidant activity has also been previously reported by Tournour et al. (2015) [75] for pomace extracts obtained from Portuguese grape cultivars, and by Shiraishi et al. (2018) [77] for white-skinned table grape cultivars commercially grown in Japan. It should be noted that the antioxidant activity was expressed as mmol TE (Trolox equivalent)/g dry residue in the first case and as mmol/L TE in the second case.

A moderate association between the phenolic content of the extracts and the grape varieties analyzed was determined (Table 5). According to Li et al. (2019) [82], the phenolic compounds in grapes strongly depend on the grape variety. The grape varieties investigated by these authors were mainly red and pink. The relationship between all the analyzed parameters is shown in Fig. 5. The pomace of the Tamaioasa Romaneasca variety was characterized by the highest amount of phenolics and flavonoids, correlated with the smallest AA values (expressed as IC_{50}), which displayed a positive and significant effect on all these parameters compared with maceration. In contrast, although in the pomace from Feteasca Alba, the TPC, TFC, and AA values were higher than those of the other two Feteasca Alba by-products, maceration was indicated as the extraction procedure. If the anatomical part is analyzed in the context

and aim of the study, it can be observed that there are significant differences between the values of variables of interest in pomace, skin, and seeds. As a result, increased attention is recommended to producers when choosing suitable byproducts. Choosing a suitable method for the extraction of bioactive compounds is compulsory and should be made in agreement with the grape variety. The statement previously mentioned, referring to the stem as a source of phenolic compounds, especially in cv. Feteasca Alba is underlined in the graphical representation. Maceration is recommended as an adequate treatment for extraction, leading to average AA values.

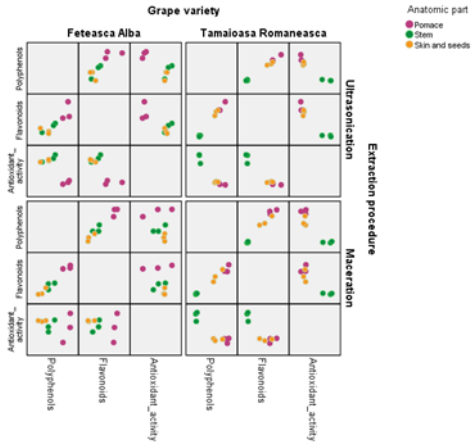

Fig. 5. The relationship between TPC, TFC, and AA of the hydroalcoholic extracts, the grape variety, the anatomic part of byproducts, and extraction procedure

Multiple linear regressions were applied to highlight the relationship between AA as a variable of interest and the other variables that affected AA, as described above. Prediction of AA with high R-squared values can help both academics and industry avoid wasting time and effort. In the case of the parameters taken in the analysis at the individual level, the predicted values of AA for a good value of R^2 = 0.75, can be calculated as follows:

Pred (Antioxidant activity) = 17.135 – 0.047 × total polyphenol content – 0.203 × total flavonoid content – 0.043 × atomic part + 0.163 × grape variety + 0.114 × extraction procedure

Grape variety = 1 / Feteasca Alba; 2 / Tamaioasa Romaneasca

Anatomical part = 1 / pomace; 2 / stem; 3 / skin and seeds

Extraction procedure = 1 / maceration; 2 / ultrasonication

The R^2 value of 0.75 underlines that other factors, not introduced in the analysis, influence the predicted antioxidant values. To increase the R2 value, a combination of parameters was considered based on their abovementioned relationship. R2 increased to 0.84 (Fig. 6), the equation for predicted values of AA being the following:

Pred (Antioxidant activity) = 11.711 + 0.433* Total polyphenol content – 0.658* Total flavonoid content + 0.729*Anatomic part + 3.051*Grape variety + 1.024*Extraction procedure – 0.307* Total polyphenol content *Grape variety +

0.546* Total flavonoid content *Grape variety – 0.568*Grape variety*Extraction procedure - 0.434*Grape variety * Anatomic part

Principal Component Analysis (PCA) was used to analyze the data for TPC, TFC, and AA of the 12 hydroalcoholic extracts obtained from the by-products of two grape varieties by applying two different methods of extraction (maceration and ultrasonication). Only one component was extracted (Fig. 7), and the loading values of the samples ranged from 0.875 (P_TR_U) to 0.998 (P_FA_M).

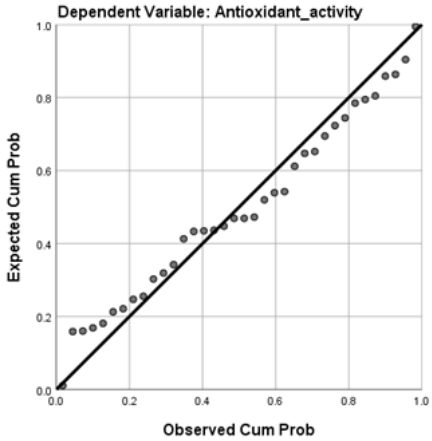

Fig. 6. The Normal P-P Plot of Regression Standardized Residual

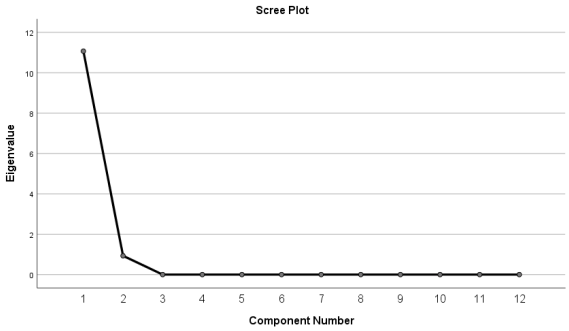

Fig. 7. The SPSS output of Scree Plot for data related to the 12 hydroalcoholic extracts

Except for the extracts obtained from the pomace of Tamaioasa Romaneasca, both by maceration and ultrasonication, the loading values of the extracted components in the component matrix were higher than 0.96, indicating a high contribution of these variables to Principal Component 1 (the only one extracted). PCA was also used to analyze the data for the dependent (TPC, TFC, and AA) and independent (grape variety, anatomic part, and extraction procedure) factors. Relevant factor loadings (0.55) were considered for each component (Table 5 and Fig. 8). Three PCs were extracted, accounting for 85.29% of the total variance. PC1 explained a high percentage of the total variance (51.25%), being formed by TPC, TFC, and grape variety in its positive part and by AA in the negative part. The structure of PC1 emphasizes the relationship between these parameters, showing that the phenolic content and antioxidant activities of grape by-products varied among the grape varieties investigated. PC2 was highly positively associated with the extraction procedure, while PC3 was highly positively associated with the anatomic part of the grapes. The interpretation of the first component loadings suggests that high TPC and TFC values contribute to a smaller AA (IC_{50} values) of the extracts. This indicator of free radical scavenging (to whom they are attributed to numerous health effects) showed a high factor loading. Among the three independent factors analyzed, grape variety was the main factor affecting the antioxidant activity of hydroalcoholic extracts. It is tentatively suggested that the extraction procedure of phenolics (maceration/ultrasonication), located in the positive part of PC2 and showing a high factor loading, had a higher influence on AA than the

anatomic part of the grapes. The type of by-products (i.e., anatomic part, located in the positive part of PC3) affects the AA of the hydroalcoholic extracts concerning the grape variety.

Table 5

Factor loadings (Varimax normalized) using principal component extraction

Factor	Eigenvalue	Cumulative variance (%)	Anatomic part	Grape variety	Extraction procedure	Polyphenols	Flavonoids	Antioxidant activity
Factor 1	3.07	51.25	-0.248	**0.607**	-0.084	**0.970**	**0.968**	**-0.841**
Factor 2	1.04	68.63	-0.102	0.247	**0.951**	-0.044	0.024	0.274
Factor 3	1.00	85.29	**0.861**	0.491	-0.052	-0.131	-0.123	0.157

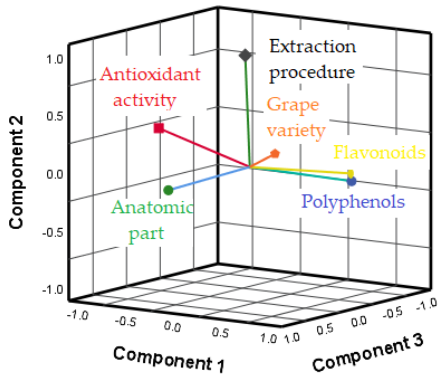

Fig. 8. 3D plot of loadings of the extracted components using Principal Component Analysis

Hierarchical Cluster Analysis was applied to both the experimental data (TPC, TFC, and AA of the hydroalcoholic extracts of the white grape by-products) and the values of descriptors (grape variety, anatomic part of grapes, and extraction procedure). This analysis aimed to identify the hydroalcoholic extracts obtained from different anatomical parts of the two Romanian white grape by-products by maceration/ultrasonication, which are similar to each other but at the same time different from the extracts of the other groups in terms of the phytochemical attributes (content of bioactive compounds and AA). The Ward method was used for clustering variables of type intervals by applying the Squared Euclidean distance.

The Ward linkage method is well known to be effective, and cluster membership is assessed by calculating the total sum of the squares of deviations from the average of the respective cluster. Hierarchical Cluster Analysis was statistically significant at a significant threshold of 5%. The dendrogram associated with this analysis is presented in Fig. 9.

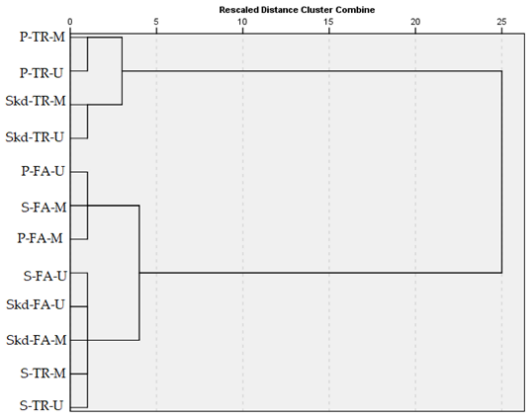

Fig. 9. Dendrogram of variables of interest (using Ward linkage

The affiliation of each grape by-product extract to a cluster in the first stage of clustering was obtained as follows: Cluster 1: P-TR-M and PTR-U (two extracts); Cluster 2: S_{kd}-TR-M and S_{kd}TR-U (two extracts); Cluster 3: PFA-U, SFA-M, and PFA-M (tree extracts); Cluster 4: SFA-U, S_{kd}FA-U, S_{kd}FA-M, STR-M, STR-U, and PFA-M (five extracts). In the second stage of clusterization, two clusters were produced: one by aggregating the previously mentioned clusters 1 and 2 (with a total of four extracts) and the other by aggregating the previously mentioned clusters 3 and 4 (resulting in a total of eight extracts).

The analysis of the hydroalcoholic extracts classification based on their phytochemical composition, AA, and three descriptors (grape variety, type of grape by-product, and extraction procedure) as variables led to an optimum number of two classes. These extracts clearly demarcated the extracts characterized by the highest TPC and TFC, respectively, and the smallest AA (expressed as IC50) values, meaning extracts from pomace, skin, and seeds of Tamaioasa Romaneasca (regardless of the extraction method), from all the other extracts. Furthermore, both clusters aggregated in the third stage of clusterization, including all the extracts obtained from grape by-products, regardless of grape variety and extraction procedure.

4. Conclusions

The valorization of food by-products is a fast-growing trend aimed at sustaining the circular economy and reaching some of the targets set by the UN Sustainable Development Goals. The modern approach to winemaking is focused on the recovery of valuable compounds from grape pomace to close the food loop and provide the ingredients requested by food and pharmaceuticals. White grape by-products are examined less for their bioactive compounds and putative antioxidant properties, despite the high demand for food that exhibits good health and prevents diseases caused by oxidative stress. In this article, the authors propose to describe and analyze in terms of phytochemical parameters; for the first time, new autochthonous grape varieties, very old, with a valuable phytochemical profile, varieties that were only included in the international catalog, but which had not been characterized (neither grapes nor

pomace). Therefore, this first study regarding the antioxidant activity, total polyphenol content, and total flavonoid content of grape pomace extracts, corroborated with statistical analyses, represents a novelty for the characterization of these new Romanian grape varieties, and the authors hope that there will be interested readers. Significant differences were found between the grape by-products from the two Romanian-grown grape varieties concerning the concentrations of total phenolics, total flavonoids, and antioxidant activity. According to the abovementioned experiment, this study revealed that the TPC, TFC, and AA decreased as follows: pomace, skin, and seeds of Tamaioasa Romaneasca extracted by ultrasonication>pomace, skin, and seeds of Feteasca Alba extracted by maceration > stem of Feteasca Alba extracted by maceration > stem of Tamaioasa Romaneasca extracted by maceration. As such, the efficient recovery of phytochemicals from grape by-products represents a contribution to a sustainable and circular economy, as well as a cost-effective source of potentially valuable food ingredients. These results provide new insights into the characterization of winemaking residues, helping to use bioactive compounds in different areas of human health. Moreover, the relationship between the parameters managing the complex process of polyphenol and flavonoid extraction was analyzed. Further research should focus on increasing the number of grape varieties studied, optimizing the extraction procedures, and analyzing the phenolic constituents. These comprehensive efforts will help reduce pollution, adding value to raw materials, and meeting consumers' needs for healthy food, at least based on the recognized antioxidant and antimicrobial properties of polyphenols.

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