Functional Tannins in Grape Pomace Flours of Feteasca Neagra and Italian Riesling

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The aim of this study was to analyze the content of functional tannins in grape pomace skin, seed, and mixed flour, from Feteasca Neagra and Italian Riesling varieties. Results showed that all compounds were present in the highest concentration in grape pomace seed flours and the lowest in skin flours. The concentration of total tannins and total flavan-3-ol monomers in all flour samples ranged from 37.34 to 72.28 mg epicatechin/g dry weight and 0.57 to 3.13 mg/g dry weight, respectively. Among monomers, (+)-catechin was found in the highest concentration, except in Italian Riesling seed flours, where (-)-epicatechin was predominant. The mDP of proanthocyanidins in seed flour (7.15 to 8.07) is lower than that of skin flours (10.83 to 18.43) while the opposite was observed for %G. As a result, all Feteasca Neagra and Italian Riesling grape pomace flours, especially seed flour, could be used as sources of functional tannins in alimentary or pharmaceutical industry.

Keywords: grape pomace flour, tannins, mDP, flavan-3-ol monomers, %G

The management of the wastes from wine making industry involves both economical and ecological issues, with grape pomaces representing an environmental risk due to the huge quantities that need to be disposed of (5 to 9 million t/y around the globe), possessing a high pollution load which can have a negative impact on flora and fauna [1, 2]. Pomace represents around 25% of the raw processed material and is considered a waste from winemaking industry resulted after pressing the grapes to release the juice. This sub-product is composed mainly of seeds and skins. Grape pomace is also known as a source rich in polyphenols, due to a relatively low extraction of these compounds during the winemaking process. Furthermore, polyphenols are bioactive compounds which have powerful antioxidant properties responsible for their capacity to reduce oxidative stress in cells [3]. Many beneficial health effects have been reported in terms of inhibiting cardiovascular diseases [4] and some types of cancer [5, 6], an exhibition of hepatoprotective effects [7] and protection against neurodegenerative diseases [8].

The most abundant and functional polyphenols being present in both red and white grape pomaces are flavan-3-ol monomers, (+)-catechin and (-)-epicatechin, and their oligomers and polymers also called condensed tannins or proanthocyanidins [9]. The structure of these compounds differs depending on the fraction of the grape, through their mean degree of polymerization (mDP) and their percentage of galloylation (%G) [10].

In the past few years, Romanian research concentrated more on polyphenols from fresh grapes or wines [11, 12] and too little on the concentration of compounds that remain in grape pomace with the perspective of using it as raw material in other industries. Thus, this study was triggered by the lack of scientific literature on the chemical composition of grape pomaces obtained from Romanian varieties or from any varieties grown within the Romanian borders, with respect to polyphenols' beneficial health properties and the exploitation potential of grape by-products. The aim of this research was to evaluate different tannins present in grape pomaces processed in the form of 6 different flours obtained from Feteasca Neagra and Italian Riesling, grown in Pancota, Minis-Maderat vineyard, Romania. Since grape pomace in Romania is barely used as fertilizer; this study was also conducted with respect to possible use in alimentary industry, in order to obtain new functional products or to be used as a source of antioxidant in pharmaceutical industries.

Experimental part

Materials and methods

Biological material

The grape pomace of two *Vitis vinifera* cultivars was used to conduct the experiment: one black Romanian variety, Feteasca Neagra and one white variety, Italian Riesling. Both cultivars were grown in Pancota as part of the Minis-Maderat vineyard (46°05' and 46°35', latitude and 21°15' and 22°00', longitude), in Western Romania. All grapes were harvested at technological maturity, in 2015. The technological process of obtaining the grape pomace flours was shown in figure 1.

Moisture analysis

The moisture was analyzed by the modified method from Gul et al. [13]. The percentage of moisture was obtained based on the difference in weight of the flour samples, before and after drying to constant weight at 100°C.

Preparation of ethanolic extracts for tannins' extraction

The extracts of grape pomace flour were prepared by weighing 5 g of each type of flour and adding 50 mL of alcoholic solution 50% and 1 mL of sodium metabisulfite 5.25%. All the extracts were then placed in a water bath at 50°C for one week and manually stirred each day. Prior to each analysis, the extracts were centrifuged and the supernatants were further used.

Spectrophotometric determination of total tannins

This method was slightly modified from Sarneckis et al. [14]. Aqueous (-)-epicatechin solutions (0, 20, 40, 60, 80,

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100 mg/L epicatechin) were used to establish a calibration curve for reporting total tannins content. Samples were prepared with and without the addition of methyl cellulose in a medium saturated in ammonium sulfate. The absorbance was read at 280 nm (Helios Alpha UV-Vis spectrophotometer 124 - Thermo Fisher Scientific Inc., Waltham, MA, USA) and the difference between the total polyphenols and the quantities of tannins precipitated by methyl cellulose gave the total tannin concentration. All the results were expressed in mg of epicatechin/g DW.

HPLC-DAD determination of proanthocyanidins and flavan-3-ol monomers

The HPLC analyses were performed using an Agilent 1200 liquid chromatograph equipped with a G1315D diode array detector (DAD), a G1311A quaternary pump, a G1316A column oven, and a G1329A autosampler (Agilent Technologies, Santa Clara, CA, USA). Proanthocyanidins' concentration, monomeric composition, the %G, and the mDP were analyzed by phloroglucinolisis according to the procedure reported by Kennedy and Jones [15]. Proanthocyanidins were depolymerized by the addition of phloroglucinol in acid medium. In order to quantify the flavan-3-ol monomers in grape flour, this assay was also carried out without the addition of phloroglucinol and the retention times were compared with those of pure compounds. The number of terminal sub-units was considered to be the difference between the total monomers measured in normal conditions (with phloroglucinol) and the total measured without phloroglucinol addition. The number of extension subunits was considered to be the sum of all the phloroglucinol adducts. The ratio of the sum of galloylated flavanols to the sum of all flavanols represents the %G. The mDP was calculated by adding terminal and extension subunits (in moles) and dividing by the terminal subunits. The total proanthocyanidins concentration was considered to be the sum of all terminal and extension sub-units.The whole method, the used equipment, the elution conditions, flow rate and composition of the mobile phases were previously described by Gil et al. [16].

Statistical analysis

Data are expressed as the arithmetic average of three replicates (n = 3). One-way ANOVA and Tukey's HSD post-hoc test were carried out using SPPS 20 software, IBM Corporation, Armonk, New York, USA.

Results and discussions

Moisture

The remaining moisture in grape pomace flours under natural drying and after grinding is presented in figure 1. The flour with the lowest percentage of water content was the skin flour from Feteasca Neagra (7.63%), while the skin flour from Italian Riesling variety had the highest water content (10.13%). It is known that the degradation of tissue causes an increase in the rate of water evaporation in intracellular spaces [17]. Since skin degradation naturally occurs during maceration processes [18] and Feteasca Neagra grapes have undergone this process, it can be fairly assumed that the differences in water content between the skin flours of the two varieties are rooted in these factors. Among flours obtained from Feteasca Neagra statistical differences ($p < 0.05$) in term of moisture were determined only between skin (7.63%) and seed flour (8.22%) and between seed and mixed flours (8.06%). In the case of Italian Riesling, all flours are statistically significant when compared to each other. Moreover, Italian

Fig. 1 Technological process of obtaining the grape pomace flours from Feteasca Neagra and Italian Riesling

Riesling mixed flour water content (9.48%) was significantly different from the same form of Feteasca Neagra flour (8.06%). The same thing was observed in the case of the two skin flours. No differences were observed between seed flours. By naturally drying the whole pomace at room temperature, other studies showed that moisture content ranged from 5.05 to 7.03% [19].

Total tannins

Results about total tannins are presented in table 1.When samples were compared within the same variety, in both cases, grape seed flours presented the highest tannins concentration (from 71.52 to 72.28 mg epicatechin/g DW in Feteasca Neagra and in Italian Riesling), while the lowest was determined in grape skin flours, finding which is consistent with some other research on grape marcs of red varieties [20]. Tannins in mixed flours ranged from 54.69 to 62.98 mg epicatechin/g DW, in between the values registered for the other two types of flour. Between Feteasca Neagra seed flours and mixed flours, statical differences were observed, but the same situation was not encountered in Italian Riesling.

Table 1

TOTAL TANNINS, PROANTHOCYANIDINS, mDP, AND %G IN GRAPE POMACE FLOURS

Type of flour / Parameter	Feteasca Neagra skin flour	Feteasca Neagra seed flour	Feteasca Neagra mixed flour	Italian Riesling skin flour	Italian Riesling seed flour	Italian Riesling mixed flour
Total tannins (mg epicatechin/g DW)	44.00 ± 3.35 ab	72.28 ± 3.60 d	54.69 ± 6.21 bc	37.34 ± 6.07 a	71.52 ± 7.70 d	62.98 ± 6.17 cd
Proanthocyanidins (mg/g DW)	4.57 ± 1.12 a	12.35 ± 0.64 bc	9.50 ± 2.39 b	3.94 ± 0.13 a	15.25 ± 1.61 c	10.71 ± 1.69 b
mDP	10.83 ± 1.27 bc	7.15 ± 2.15 a	9.07 ± 0.34 abc	18.43 ± 1.26 d	8.07 ± 0.10 ab	10.96 ± 3.98 c
%G	14.05 ± 2.03 a	21.47 ± 3.38 b	17.92 ± 4.00 ab	18.58 ± 0.51 ab	22.11 ± 1.73 b	19.78 ± 1.21 ab

Values are expressed as the average ± standard deviation (n = 3). Different letters within the same row indicate significant differences (Tukey HSD test; $p < 0.05$) among the analyzed samples of flour. DW - Dry weight; mDP – mean degree of polymerization; %G – percentage of galloylation

It seems that the quantity of total tannins in our seed flour samples is higher than that reported by others in defatted seeds of Raboso Piave (41.72 mg catechin/g) [21]. Other studies have shown a higher tannins' concentration in both seeds and skins from the red grape pomace of numerous varieties, than that present in our seed flours [20]. The same trend can be observed in the case of mixed flour samples in terms of tannins concentration when compared with other values obtained in whole pomaces (189 mg cyanidin equivalents/g DW) [22]. These differences, as showed in the case of other compounds along this study, are related to variety, climate differences, analysis methods and winemaking techniques.

Proanthocyanidins

The proanthocyanidins (table 1) were present in all our flour samples, but the variety did not influence their concentration, since no statistical differences were observed between the same types of flour from Feteasca Neagra and Italian Riesling, respectively. The richest flour in proanthocyanidins was the one obtained from the seeds of the white pomace (15.25 mg/g DW). Between the seed and skin flours of the red variety, the latter was already expected to contain a lower amount of proanthocyanidins. Though, both seeds and skins were macerated the same period of time it is known that the diffusion of these compounds from skins into the must is a lot faster than their extraction from seeds, which requires a higher temperature and alcohol concentration [23]. The same trend was observed between the skin and seed flours of Italian Riesling. De Freitas and Glories [24] showed that white grapes contain a lot more proanthocyanidins in seeds than in the skins and also it can be stated that the only loss in proanthocyanidins caused by pressing the berries is only from skins and not from seeds. Some other concentrations of proanthocyanidins in grape by-products were previously reported in the literature in the whole pomace [25] and in the skins and seeds [26].

Mean degree of polymerization (mDP) and percentage of galloylation (%G) of proanthocyanidins

The mDP and the %G of the proanthocyanidins are reported in table 1. Thus, the highest mDP of proanthocyanidins in our samples was found in Italian Riesling skin flour (18.43) statistically different from the identical flour form of Feteasca Neagra (10.83). Within and between both varieties, skin flours had a higher mDP than seed flours. Mixed flours had an mDP situated in between that of skin and seed flours. Other studies also state that grape seeds have a lower mDP than skins and that they can vary according to the studied variety, vintage, and region [25].

Concerning the amount of gallates, the highest % was determined in Italian Riesling seed flour (22.11%), followed closely by the same flour form of Feteasca Neagra (21.47%). A big difference was observed between the %G in Feteasca Neagra skin (14.05%) and seed flours (21.47%). It is already known that grape seeds contain a higher amount of gallates compared to grape skins [27]. The value of %G was also higher in the seed flour of the white variety compared to the skin flour of Feteasca Neagra.

Multiple studies have shown that galloylation and polymerization are important structural factors of polyphenols that influence their scavenger capacity and antitumoral properties. Lizarraga et al. [28] concluded that polyphenolic extracts that contain polymers and compounds that have a higher degree of galloylation are the most suitable to be used as anti-proliferative agents.

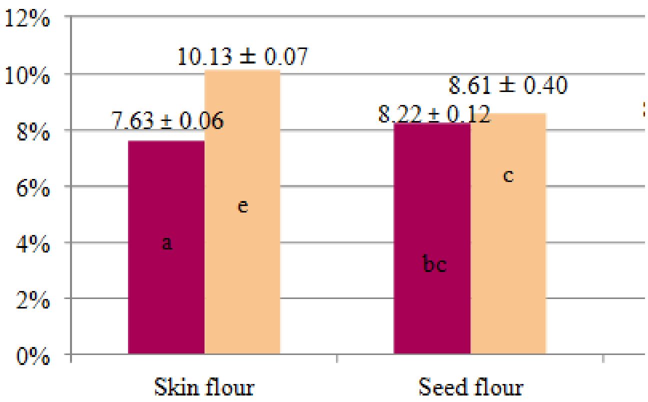

Fig. 2. Moisture (%) in grape pomace flours of Feteasca Neagra and Italian Riesling Values are expressed as the average ± standard deviation (n = 3). Different letters in the columns indicate significant differences (Tukey HSD test; $p < 0.05$) among the analyzed samples of flour

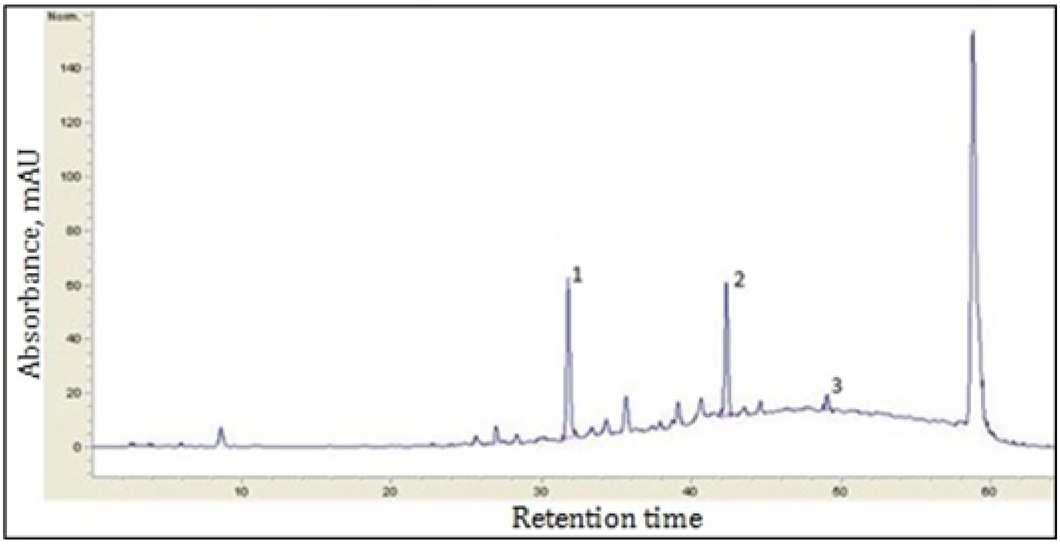

Fig. 3. HPLC chromatogram of flavan-3-ol monomers in Feteasca Neagra seed flour 1- (+)-catechin; 2- (-)-epicatechin; 3- epicatechin-3-gallate Reversed-phase HPLC analysis was carried out with an Agilent Zorbax eclipse XDB C18,4.6 mm × 250 mm, 5 μm column. The injection volume: 30 μL. Solvent A - 1% aqueous acetic acid and Solvent B- methanol. Flow rate of 1 mL/min. The elution conditions were 1.0 mL/min. Elution was performed with a gradient starting at 5% B for 10 min, a linear gradient from 5-20% B in 20 min, and a linear gradient from 20-40% B in 25 min. The column was then washed with 90% B for 10 min and re-equilibrated with 5% B for 5 min before the next injection. The monomers (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-O-gallate were identified by comparing their retention times with those of the pure compounds

The latter parameter also seems to play a much more important role in this matter than the mDP. It has been proved that polymeric proanthocyanidins are not well absorbed by the gut, but it seems that for them to exhibit their health benefits this is not necessary [29].

Total and individual flavan-3-ol monomers

Total and individual flavan-3-ol monomers were determined and their concentration is presented in table 2. A HPLC chromatogram of the flavan-3-ol monomers was also presented in figures 3 and it was representative for all the studied samples. Concerning the sum of flavan-3-ol monomers, no notable differences were observed between the same flour forms of Feteasca Neagra and Italian Riesling with one exception. Thus, Italian Riesling seed flour had a higher concentration of monomers (3.13 mg/g DW), compared to the same flour form of Feteasca Neagra (2.40 mg/g DW). Skin flours were characterized by the lowest amount of monomers. Other studies also reported lower amounts of monomers in the grape pomace skins compared to grape pomace seeds independent of the studied variety [20, 30]. The values of monomers in both mixed flours (1.34 mg/g DW - Feteasca Neagra; 1.60 mg/g DW – Italian Riesling) are significantly different from those of other types of flour. Our results regarding the concentration of total flavan-3-ols monomers in skins and seeds of Italian Riesling grown in Pancota are strikingly higher than those reported for the same variety (14 mg/kg fresh grape) cultivated in the region of Castilla-La Mancha, Spain [30]. As it happens with the concentration of monomers in Feteasca Neagra skin and seed flours, our study shows lower values than those found in grape marc skin (1.86 - 7.71 mg/g DW) and seeds (5.01 -13.84 mg/g DW) of some French varieties [20].

Furthermore, the individual quantification of each of the three flavan-3-ol monomers revealed a particular composition among the various analyzed samples. Significant differences ($p < 0.05$) in the concentration of (+)-catechin were determined between all flours obtained from the same cultivar. Hence, seed flours were proved to have a four to fivefold higher amount of (+)-catechin (1.24 mg/g DW Feteasca Neagra; 1.22 mg/g DW Italian Riesling), compared to the skin or mixed flours. Moreover, mixed flours were richer in (+)-catechin than skin flours. As to (-)-epicatechin, its concentration ranged from 0.20 to 1.67 mg/g DW, with the highest value determined in Italian Riesling seed flour. The latter was significantly different from the same type of flour obtained from Feteasca Neagra (0.99 mg/g DW), while all other significances in the case of (-)-epicatechin followed the same trend as those registered in the above differences in the content of (+)-catechin. Epicatechin-3-gallate was uniformly distributed among the majority of the flour samples. There were no statistical differences in terms of epicatechin-3-gallate concentration, with one exception. Thus, Italian Riesling seed flour registered an epicatechin-3-gallate concentration (0.24 mg/g DW) around twofold higher compared to Feteasca Neagra skin flours (0.10 mg/g DW). Guendez et al. [31] discovered that not all white seed varieties contain epicatechin-3-gallate, while in the seeds of some other white varieties a higher amount of this compound was determined compared to the seeds of red varieties examined. The concentration of individual monomers we found in Feteasca Neagra and Italian Riesling seed flour is comparable to the quantities determined by the above-mentioned authors, in the seed extract of some Hellenic native and international varieties [(+)-catechin: 183 mg/100g of seeds - Merlot, 186 mg/100g of seeds-Negoska; (-)-epicatechin: 97.8 mg/100g of seeds - Mavrofdani, 172 mg/100g of seeds Agiorgitiko; epicatechin-3-gallate: 27.9 mg/100g of seeds - Cabernet Sauvignon, 18.6 mg/100g of seeds -Grenache Rouge]. Mixed flours from our analyzed varieties contained lower amounts of (+)-catechin and (-)-epicatechin, but a higher concentration of epicatechin-3-gallate, compared to Muscadine whole pomace [32]. Our skin flour samples were richer in flavan-3-ol monomers than the skin of finger pressed white [(+)-catechin: 9.5 – 23 mg/kg fresh grape; (-)-epicatechin: 2.6 - 8.3 mg/kg fresh grape] and red grapes [(+)-catechin: 8.5 -25 mg/kg fresh grapes; (-)-epicatechin: 6.2=13 mg/kg fresh grapes] varieties analyzed by Montealegre et al. [30]. However, they contained a lower amount of these compounds when

Table 2
TOTAL AND INDIVIDUAL FLAVAN-3-OL MONOMERS IN GRAPE POMACE FLOURS

Type of flour / Parameter	Feteasca Neagra skin flour	Feteasca Neagra seed flour	Feteasca Neagra mixed flour	Italian Riesling skin flour	Italian Riesling seed flour	Italian Riesling mixed flour
∑ monomers (mg/g DW)	0.57 ± 0.11 a	2.40 ± 0.26 C	1.34 ± 0.17 b	0.64 ± 0.14 a	3.13 ± 0.27 d	1.60 ± 0.04 b
(+)-catechin (mg/g DW)	0.27 ± 0.02 a	1.24 ± 0.13 c	0.65 ± 0.09 b	0.28 ± 0.09 a	1.22 ± 0.05 c	0.75 ± 0.01 b
(-)-epicatechin (mg/g DW)	0.20 ± 0.01 a	0.99 ± 0.11 c	0.53 ± 0.07 b	0.21 ± 0.04 a	1.67 ± 0.18 d	0.67 ± 0.03 b
Epicatechin-3-gallate (mg/g DW)	0.10 ± 0.08 a	0.17 ± 0.00 ab	0.16 ± 0.00 ab	0.15 ± 0.00 ab	0.24 ± 0.04 b	0.18 ± 0.01 ab

Values are expressed as the average ± standard deviation (n = 3). Different letters within the same row indicate significant differences (Tukey HSD test; $p < 0.05$) among the analyzed samples of flour. DW - Dry weight

compared to skin by-products of some French varieties, extracted with a hydroalcoholic solution [20]. In general, among monomers, (+)-catechin was found in the highest concentration, except in Italian Riesling seed flours, where (-)-epicatechin was predominant.

Conclusions

Grape pomace flours from both varieties could be considered a good source of functional tannins, in obtaining new functional products for alimentary industry, or to be used in pharmaceutical industry. In general, the same form of flour from Feteasca neagra and Italian Riesling had similar tannins' concentration, except for total flavan-3-ol monomers and (-)-epicatechin which were determined in a higher concentration in Italian Riesling seed flours. The seed flours are more suitable as tannins sources than skin flours based on compounds concentration. Epicatechin-3-gallate had a rather similar concentration in all types of flour. In general, among monomers, (+)-catechin was found in the highest concentration, except in Italian Riesling seed flours, where (-)-epicatechin was predominant.

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