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PROPERTIES OF WINEMAKING BY-PRODUCTS OF *FETEASCA NEAGRA* GRAPE SEEDS

Angela Gurev [a*], Veronica Dragancea [a], Alexei Baerle [a], Natalia Netreba [a], Olga Boestean [a], Svetlana Haritonov [a], Boris Gaina [b]

[a] *Technical University of Moldova, 7, Studentilor str., Chisinau MD-2012, Republic of Moldova*
[b] *Academy of Sciences of Moldova, 1, Stefan cel Mare si Sfant blvd., Chisinau MD-2001, Republic of Moldova*
[*] *e-mail: angela.gurev@chim.utm.md; phone: (+373 69) 60 70 90*

Abstract. The aim of this study was to perform a quantitative and qualitative assessment of the biologically active compounds in winemaking by-products. The properties of the lipophilic and hydrophilic extracts from the seeds recovered from fermented pomace of the local grapes - *Feteasca Neagra*, grown in three vineyards, in the 2020 season, were studied. The physicochemical indicators of the seeds and the oil quality indicators were determined. The content of carotenoids and polyphenols in the lipophilic extracts was evaluated by spectrophotometric methods. The difference between the total content of polyphenols and flavonoids in the hydrophilic extracts from ground grape seeds (I) and degreased ground grape seeds (II) was registered. Some phenolic and flavanol constituents were identified and quantified using reversed-phase (C_{18}) gradient-elution HPLC/PDA. The Trolox equivalent antioxidant capacity assay proved the increased antioxidant activity of the hydrophilic extracts, with the highest DPPH• scavenging effect of almost 91.70 and 93.81%, an equivalent of 281.66 and 288.27 µM/L Trolox. It was concluded that the seeds recovered from *Feteasca Neagra* by-products are a rich source of functional compounds, with significant antioxidant properties.

Keywords: antioxidant, flavonoid, grape seed, polyphenol, waste.

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Introduction

The winemaking industry in the Republic Moldova has one of the most important roles in the national economy. The National Office of Vine and Wine reported that in 2019 the wine industry provided 16% of the agro-industrial complex, generating 3% of the country's Gross Domestic Product. Officially, in 2019, the total area of vineyards in the Republic Moldova was of 124.000 hectares, with 199 registered wineries, of which 69 with their own vineyards [1]. Agro-industrial grape residues - stems, pomace with seeds and liquid filtrate, may exceed 14.5% of the total grape volume [2,3]. Recent research has shown a growing interest in winemaking by-products, which are not being managed anymore as waste, but as a source of functional compounds [4]. The literature shows that grape seeds contain proteins, lipids, carbohydrates, minerals, fibre, and a wide variety of biologically active substances, such as vitamins, carotenoids, sterols, tocopherols, and polyphenols [2-5]. Moreover, grape seeds resulting from the winemaking process, are an accessible source of unsaturated fats and unsaturated fatty acids, the content of which varies between 8% and 20%, depending on the grape variety, cultivation conditions, and the type of extraction procedure [6,7]. Seeds contain about 62% of the total content of grape polyphenols, some of the most important bioactive substances [8,9]. The most common identified polyphenols are flavonoids, including the gallic acid, flavan-3-ol, catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin-3-ol gallate, dimers and trimers of procyanidin, polymers of procyanidin, stilbenes (resveratrol), *etc.* [7,8].

Several studies have shown that the content of biologically active substances in grapes, depends on several factors, such as cultivation conditions (soil, water, light, temperature, *etc.*), ripeness, and genotype, which is also the most important factor [10,11]. From the same grape variety can be produced wines with different tastes, depending on the conditions and place of cultivation as well as the processing method [12]. The quantitative and qualitative content of the biologically active substances, recovered from

winemaking by-products, from the same grape variety, is considerably affected by the method used to obtain them (cold or hot pressing, type of extraction method, type of used solvents, temperature regimes, degree of sample grinding, seed moisture, *etc.*) [6,13-15]. Thus, the cultivation conditions, the grape processing method, and the secondary metabolite extraction method used, influences the total content of biologically active substances in the lipophilic and hydrophilic extracts that were obtained after fermentation, from the same grape variety.

Bioactive compounds, recovered from winemaking by-products, have antioxidant, antibacterial [16,17], anti-inflammatory, anticancer [18,19], cardiovascular [20], and hepatic protective properties [21], which can be used in various fields, such as food industries, pharmaceuticals, cosmetics, animal farming, agriculture, *etc.* Winemaking by-products have also caught the interest of food industry researchers. The by-products are considered to be a valuable source of phytonutrients that can be incorporated into various products, thus increasing the nutritional value and turning them into functional foods, with health benefits [2-4,22].

The main aim of this study is to identify the quantitative and qualitative composition of the lipophilic and hydrophilic extracts from the winemaking by-products of the local grapes - *Feteasca Neagra*, grown in three vineyards, as well as to study the antioxidant activity of the extracts. Further, to evaluate the difference between the composition of the extracts and to suggest possible field of applicability of the recovered functional compounds.

Experimental

Materials

The 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) (≥95%) 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (purity≥97%), were provided by Alpha Aesar (USA). Aluminium chloride hexahydrate (≥98%) and the standard compounds: *β*-carotene (≥95%), gallic acid (GA) (≥97%), catechin, epicatechin (≥98%), rutin (≥94%) and quercetin (≥95%), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu phenol reagent (2.1N) was purchased from Chem-Lab NV (Belgium). Ethanol, *n*-hexane, methanol, sodium carbonate, diethyl ether, acetonitrile, chloroform, acetic acid, potassium iodide, sodium thiosulphate, sodium hydroxide, and potassium hydroxide were purchased from Chemapol (Czech Republic). All reagents used in this study were of analytical or chromatographic grade.

Methods

Sample preparation

The grape seeds were separated from *Feteasca Neagra* grape pomace, grown in the season of 2020, in the vineyards of Hincesti (FNH), Nisporeni (FNN), and Speia (FNS). The grapes were manually collected, at a distance of 1 day, and were further processed and fermented for 7 days in stainless steel containers, at a temperature of 25-26ºC, under identical conditions, in the Laboratory of Micro-Vinification of the Department of Oenology and Chemistry of the Technical University of Moldova. The pomace was obtained by pressing and stored at a temperature of -20ºC.

Obtaining lipophilic and hydrophilic extracts

The grape seeds, previously washed with distilled water, dried at 60ºC for an hour, and ground (100 g), were extracted with *n*-hexane (1:3, v/v) for 48 hours, at room temperature. The mixture was filtered; the extraction procedure was repeated twice. The degreased seed meal was used as written below. The filtrates were combined and the solvent was removed by simple distillation, at a temperature of 60ºC. The oil obtained in three repetitions was dried in the oven for 2 hours at 60ºC, then treated with anhydrous sodium sulphate. The refractive indices were measured at a temperature of 20ºC, using an ABBE Refractometer (ISOLAB Laborgeräte, Germany); the relative density (kg/L) was determined by the pycnometer method, at 20ºC [23]; the mass fractions of moisture and volatile substances (%) were calculated by air oven method [23]. To determine the saponification values (mg KOH/1 g oil), a sample of oil was refluxed with potassium hydroxide alcoholic solution and the excess was further titrated with standard hydrochloric acid [23]. The acidity index (mg KOH/1 g oil) was determined by directly titrating the oil in a diethyl ether - alcoholic medium, against standard potassium hydroxide solution [24]

The hydrophilic extracts were obtained from grape seeds (I) separated from pomace and dried for 60 min, at a temperature of 60ºC and, from degreased seeds (II), (left after extracting the oil with *n*-hexane). Each variant (I, II) was obtained in three replicates. The ground I and II seed samples, weighted with a three-decimal precision (0.500 g), were placed in test tubes with lids, together with 25 mL of solvent ethanol: water (60:40, v/v). They were used for extraction by *ultrasound-assisted method*

(ISOLAB Laborgeräte GmbH, Germany), for 30 min, at a temperature of 40ºC (30 kHz and 300 W).

Extraction of the polyphenols from oil

The extraction of polyphenols from oil was conducted according to the methods described in the literature [25]. A sample of oil weighed on an analytical balance (1.25 g), was dissolved in 2.5 mL of *n*-hexane, while the hydrophilic compounds were extracted with 1.5 mL of a 60% aqueous methyl alcohol solution by vortex stirring. The lipophilic and hydrophilic phases were separated by centrifugation (3500 rpm for 10 min). The procedure was repeated twice. Both methanolic extracts were combined and then analysed.

Evaluation of physicochemical indicators of the seeds

Grape seeds were separated from pomace, washed with distilled water, and dried. The samples were brought to a constant mass, at 105ºC. The content of dry matter was determined in the drying oven SPJ SLN 53 SMART (POL-EKO APARATURA, Poland) according to the known methods [26]. To determine the ash content, the samples were calcinated at a temperature of 550ºC in a furnace (Omron E5CC, Lithuania) [27]. To calculate the mass fractions of the lipids, weighed samples of ground seeds were placed in three repetitions in a Soxhlet extractor SER 148 (VELP Scientifica, Italy) and extracted for 3 hours by continuous reflux with *n*-hexane in a ratio of 1:10 (sample: solvent m/v).

UV-Vis spectroscopic analysis of grape seed oil

Spectroscopic analysis of the grapeseed oil samples was performed on a DR5000 spectrophotometer (HACH- Lange GmbH, USA-Germany). The concentration of the samples was of 2% of oil in hexane. Carotenoid content was determined as per the calibration graph of standard *β*-carotene solutions in *n*-hexane and expressed as mg *β*-carotene equivalent per 100 g of sample (mg *β*CE/100 g). For the construction of the calibration curve, *β*-carotene solutions were prepared with a concentration range of 40 to 0.32 mg/L; the absorbances were recorded at a wavelength of 450 nm.

Quantification of total polyphenols

The total content of polyphenols (TPC) was determined using the DR5000 spectrophotometer (HACH- Lange GmbH, USA-Germany), by the method described in the literature [28], using the Folin-Ciocalteu Phenol reagent [29]. The polyphenol content was determined according to the calibration curve of the gallic acid standard, expressed as mg of GAE/g of seeds. The calibration curve equation (0-500 mg/L, R^2= 0.9977) was used to determine the TPC.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined by the spectrophotometric method, using the same spectrophotometer, according to the method from the literature [28] with aluminium chloride. The absorbance reading for the samples was performed at a 430 nm wavelength. The TFC was determined according to the quercetin (0-160 mg/L, R^2= 0.9972) and rutin (0-160 mg/L, R^2= 0.9991) calibration curve. The results were expressed in milligrams equivalent of quercetin per gram of seeds (mg QE/g) or milligrams equivalent of rutin (mg RE/g).

Reversed-phase HPLC by gradient elution

The Provinence-i LC-2030C 3D Plus Chromatograph (Shimadzu, Japan), with integrated Photodiode Array Detector (PDA) was used. Extracts were filtered through 0.22 μm PES-filters and chromatographed through C_{18} type column "Phenomenex" (150 mm x 4.6 mm x 4 μm x 80 A) length at an oven temperature of 25ºC and flow rate of 0.5 mL/min. Phases: Water (Phase A) and Acetonitrile (Phase B), both containing Acetic Acid 0.1% (v). Gradient program for Phase B: 2 min – 5%, 18 min – 40%, 20-24 min – 90%, 25 min – 5%.

Determination of DPPH• free radical scavenging activity

The antioxidant activities were determined using the reaction of DPPH• with the antioxidants contained in the samples, by the method described in the literature [30], using the DR5000 spectrophotometer (HACH- Lange GmbH, U.S.A.-Germany). Absorbances were read at a 515 nm wavelength. Results are interpreted using the Trolox (Thermo Fisher Scientific, USA) calibration curve (500-3.95 μmol Trolox/L). To calculate the DPPH• scavenging effect, Eq.(1) was used.

$$DPPH\ (\%\ inhibition) = \frac{(A_0 - A_1)}{A_0} \cdot 100\% \qquad (1)$$

where, A_0 - the absorbance of control, nm;

A_1 - the absorbance of standard, nm.

Statistical processing of experimental data

Three parallel measurements were conducted to exclude the results with accidental errors and those with high levels of uncertainty [31]. The statistical analysis was performed with

the IBM SPSS Statistics 23 and Microsoft Excel 2010 programs, with a significance level of 95% (q<0.05). Errors ΔX were calculated as 2σ after 3 replications.

Results and discussion

Physicochemical indicators of the seeds from Feteasca Neagra

For the sample of the grape seeds of *Feteasca Neagra*, were determined the physicochemical properties and indicators. The mechanical composition of the seeds separated from the fermented pomace of *Feteasca Neagra* grapes, grown in three vineyards in the centre of Moldova, has insignificant differences. A quantity of 100 g of grape seeds collected in Hincesti comprise 58 more seeds than those collected in Speia and 196 more than in Nisporeni (Table 1).

Table 1

The mechanical composition of the seeds separated from Feteasca Neagra grape pomace.

Composition	*FNN**	*FNH**	*FNS**
Seeds (g)	*100*	*100*	*100*
Seeds (pcs.)	*3822±11*	*4018±15*	*3960±8*

**Feteasca Neagra grown on the vineyards from Hincesti (FNH), from Nisporeni (FNN), from Speia (FNS). Results are expressed as average ±2σ (n= 3).*

Analysis of the physicochemical indicators revealed that the seeds from Hincesti have a higher content of oil and dry matter. The ratio of the mass fraction of the oil to the mass of the dried seeds is by 11.87% lower in the seeds from Speia grapes, compared to Hincesti, and by 8.03% lower, compared to Nisporeni (Table 2).

Properties of Feteasca Neagra seed lipophilic extracts

The ground seeds of FNN, FNH, FNS (previously washed and dried at 60ºC for an hour) were extracted with an organic solvent (*n*-hexane), at room temperature. The oil obtained using a cold organic solvent, has an almost 20% lower yield compared with the Soxhlet method. The oil samples have the following organoleptic characteristics: relatively transparent; a pleasant taste of oil; lack of foreign smell and taste. The use of an organic solvent is not a suitable method for obtaining edible oils. Contrariwise, cold pressing is considered to be a harmless method of obtaining vegetable oils as it preserves their functional components [32,33]. The conducted research has shown that hexane, at low temperatures, solubilizes well the fat-soluble components of plant matter: chlorophylls, chlorophyll derivatives, carotenes, tocopherols, sterols, *etc.* This method could be used in analysing the composition of grapeseed oil.

The physicochemical properties and quality indices of the oil samples (Table 3), extracted with *n*-hexane, at room temperature, from *Feteasca Neagra* grape seeds were determined. The acidity indices of the oil samples are low, with values between 0.51±0.05 and 0.56±0.08 mg KOH/1 g oil. The peroxide values are low, and could not be determined by the classical titrimetric method. The analysed oil samples have the content of water and of volatile substances within the range of 0.073–0.081%. The low values of peroxide, acidity, and other indices are due to the extraction method used.

Table 2

Physicochemical indicators of the seeds from *Feteasca Neagra* grape pomace.

Seed samples	*Moisture, %DW*	*Content of dry matter, %DW*	*Ash content, %DW*	*Mass fraction of oil in 100 g of dried seeds, %*
FNN	9.67±0.12	90.34±0.16	2.41±0.11	10.10±1.53
FNH	6.53±0.01	93.46±0.01	2.37±0.05	10.53±1.12
FNS	10.13±0.04	89.87±0.04	2.44±0.05	9.30±0.05

DW: dry weight; results are expressed as average ±2σ (n= 3).

Table 3

Quality indices of the *Feteasca Neagra* grapeseed oil, extracted with *n*-hexane at room temperature.

Oil sample	*Refractive index*	*Relative density*	*Acidity index*	*Saponification index*	*Moisture and volatile substances, %*
FNN	1.4763±0.0002	0.921±0.011	0.56±0.08	189.7±0.2	0.078±0.002
FNH	1.4764±0.0002	0.921±0.011	0.53±0.02	188.3±0.4	0.073±0.002
FNS	1.4760±0.0001	0.922±0.010	0.51±0.05	189.5±0.6	0.081±0.002

Results are expressed as average ±2σ (n= 3).

The UV-Vis absorption spectra for the FNN, FNH, FNS oil samples were recorded, with an essential difference in the 300–700 nm range. The absorbance values of the oil obtained from *Feteasca Neagra* grape seeds, cultivated in Hincesti, show a higher content of pheophytins (derived from chlorophylls, after the loss of magnesium ions), as per the absorbance values recorded in the 600–620, 650–700 nm intervals (Figure 1) [34].

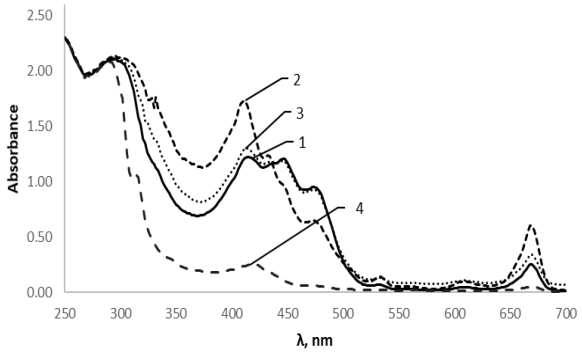

Figure 1. UV-Vis spectra for grapeseed oil samples: from FNN - *1*; from FNH – *2*; from FNS – *3*; unrefined from the supermarket -*4*.

According to characteristic absorption bands at 400-540 nm, FNN and FNS grapeseed oils have an increased content of carotenoids. The concentration of carotenoids was calculated using the calibration curve constructed on standard β-carotene solutions. The FNH seed oil has a carotenoid content of 38.71±0.44 mg βCE/100 g oil. FNN and FNS oils have a high content of carotenoids, 49.71±0.43 and 48.53±0.47 mg βCE/100 g oil, respectively. These values fall within the total carotenoid content range of 33.85–59.85 mg βCE/100 g oil, determined for the cold-pressed grape oil by Brazilian researchers [35]. A similar result was recorded for palm oil: 32.7–45.8 mg βCE/100 g of crude palm oil [36]. However, other researchers reported a much lower content of carotenoids in grape seed oil [13].

In the UV-Vis spectra of the unrefined grapeseed oil, local produce, the absorbances for carotenoids and chlorophyl derivatives are missing. Due to the high content of chlorophyll derivatives, the FNH oil is of olive-green colour. The oils from grapes from Nisporeni and Speia are yellow, due to the increased content of carotenoids. Therefore, the colour difference of the 3 oil samples is due to the content of antioxidants. The grapeseed oil P has the lightest colour, probably as a result of thermal treatment and subsequent purification.

The total polyphenol content

Polyphenols are mono- or polycyclic compounds with several hydroxyl functional groups (which may also contain carboxyl and carbonyl groups) attached to aromatic rings. Due to their structure, polyphenols have reducing properties. They form complex compounds with the Folin-Ciocalteu reagent, which absorb in the visible range of the spectrum at 750 nm [25,28]. The components of the oil (phenolic compounds, catechins, epicatechins, oligomeric procyanidins, *etc.*) were extracted in the hydrophilic phase of the aqueous methyl alcohol solution; it was further spectrophotometrically analysed. The grape seed oils have a reduced content of phenolic compounds (Figure 2), with values between *approx*. 0.005 and 0.004%; the highest content being in the lipophilic extract from FNN, of 4.89±0.05 mg GAE/100 g of sample. These values are comparable to those from literature, where the total amount of polyphenols in cold-pressed grape seed oil varies from 48 to 153 mg GAE/kg of oil [6]. Other studies have established that the mass part of the phenolic compounds in cold-pressed grape oil constitutes 0.013–0.019% of the total mass of polyphenols embedded in grape seeds; with a reduced TPC of 2.9 mg GAE/kg oil, and a content of catechins and epicatechins of 1.3 mg/kg of oil [7].

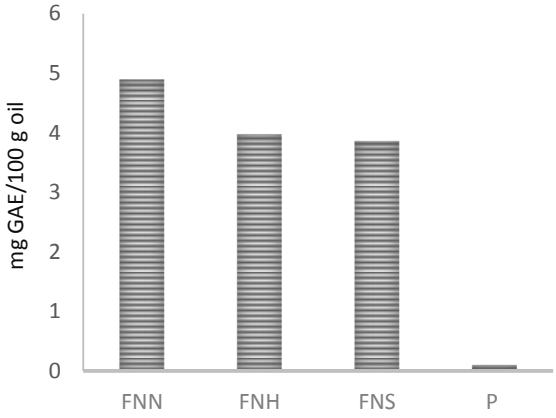

Figure 2. TPC in grapeseed oil. P -unrefined oil from the supermarket. (Results are expressed as average ±2σ (n= 3)).

It can be concluded that the lipophilic extracts from the *Feteasca Neagra* grape seeds, obtained at low temperatures and using an organic solvent, contain a higher quality and quantity of functional compounds, if compared with grapeseed oil P from the market. However, TPC in grapeseed oils is much lower than the TPC in crude olive oils from the market, which has an average value of 25 mg GAE/100 g of sample,

or 0.025%. The olive oil, extracted with organic solvents (chloroform-methanol), has a content of polyphenols of up to 57.4 mg GAE/100 g of oil, according to the bibliography [25].

Properties of hydrophilic extracts from Feteasca Neagra seeds

The TPC was determined for the hydrophilic extracts I (obtained from ground grape seeds) and II **(**obtained from ground and degreased seeds, after extracting the oil). According to the results that can be seen in Figure 3, the content of total soluble polyphenols is by 19.26–28.30% higher in all of the extracts II than in the seed extracts I. Hence, after removing the lipids from the seed, the hydrophilic compounds are better solubilized by the polar solvent. The highest TPC, of approximately 4.02%, was registered in extracts FNN II. It can be seen in Figure 3 that in extracts FNN I and II, the TPC of 32.28±2.03 and 40.15±0.16 mg GAE/g seeds respectively, is twice higher than in extracts FNH and FNS.

Several studies state that this difference is due to the influence that various factors can have on the content of polyphenols in grapes and grape seeds, such as humidity, soil fertility, and nitrogen content in the soil, methods of cultivation, climatic conditions and the ripeness of the grapes.

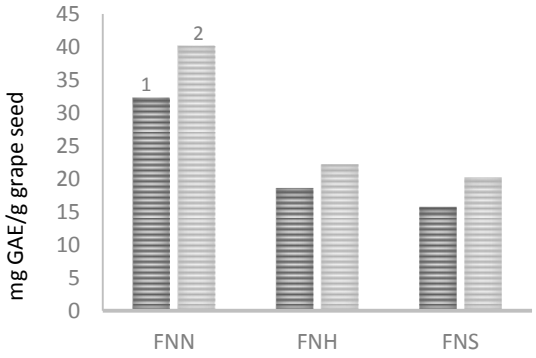

Figure 3. TPC in grape seed extracts I (*1*) and in grape seed extracts II (*2*).
(Results are expressed as average ±*2σ* (*n*= *3*)).

Another index of grape ripeness, besides the sugar content, is the phenolic ripeness, or changes in the tannin content of the grapes. Grapes harvested form vineyards planted on moderately fertile soil, with a longer sunlight exposure, will have a higher content of polyphenols. Sun rays activate the cluster formation and the synthesis of the secondary metabolites, such as quercetin, which is a good indicator of the amount of sunlight to which the grapes have been exposed [37,38]. It should be mentioned that the TPC values for FNN, FNH, and FNS seeds fall within the data gathered from literature (regarding the content of extractable polyphenols in the seeds of different grape varieties), which is between 22.47 and 72.01 mg GAE/g of dry sample [17], and between 34.63 and 71.24 mg GAE/g of fresh seeds [39].

Gallic acid derivatives and flavonoids, among which flavan-3-ols ((+)-catechin and (-)-epicatechin) and flavonol (quercetin), account for a big part from the total phenolic compounds contained in grape seeds [40], Figure 4.

The TFC in the extracts I and II of *Feteasca Neagra* seeds was determined by the spectrophotometric method, according to the calibration curves recorded for different concentrations of quercetin and rutin. The method is based on the formation of yellow aluminium compounds (III), which absorb in the range of 404-430 nm. Chelate formation is due to the C-3′, C-4′ hydroxyl groups from ring B and C-3 from ring C of flavan-3-ols and flavon-3-ols (Figure 4), hence quercetin, catechin or rutin solutions can be used as reference substances.

Extract I, from dried at 60ºC for an hour, and ground grape seeds, has a higher content of soluble flavonoids (Table 4). In all of the I and II extracts, a higher portion of flavonoids is in form of glycosides; the concentration was calculated in relation to rutin, which is a disaccharide (*α*-L-ramnopyranosyl-(1→6)-*β*-D-glucopyranoside of quercetin).

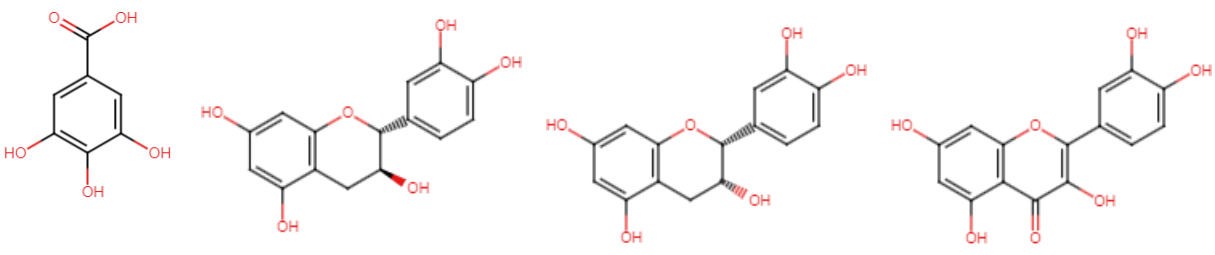

Figure 4. Common polyphenols found in grape seeds.

A reason for this could be that the functional compounds of the seeds are mostly stored as glycosides, which are also a resource for the growth of the plant. The values calculated in quercetin equivalents for all samples match the data obtained by a group of researchers, who determined by the colorimetric method, with $AlCl_3$, that the TFC in 30 grape varieties varies between 1.130 and 3.957 mg QE/g of fresh seeds, depending on the variety [39].

According to the results shown in Table 4, all type I extracts have a higher TFC. TPC, on the contrary, is higher in extracts type II of degreased seeds (Figure 3). This difference is a consequence of the obtaining method that is used: type II extracts were obtained from grape seeds that were washed with distilled water, dried and extracted with *n*-hexane. As a result, the protective layer of tannins (soluble proanthocyanidins) was removed from the surface of the seeds. According to the results (Table 4), *Feteasca Neagra* seeds I, cultivated on the vineyards of Nisporeni, have a higher content of flavonoids, with the following total concentration: 2.74±0.09 mg QE/g of seeds (dried at 60ºC, 60 min) however, the concentration expressed in mg RE/g is lower compared to FNH and FNS samples.

The difference between the content of biologically active substances in the FNN, FNH, FNS seeds separated from fermented pomace is admissible within the same grape variety. The phenomenon can be explained by the cultivation conditions [10,11,41] such as altitude, fertility, the physicochemical properties of the soil in the micro zone of the Western Suburbs of Codri, where the Nisporeni wine area is located [42], as well as the viticultural practices [43]. The rest of the technological techniques used (harvesting, processing, grape fermentation, separation of the seeds from the pomace frozen at -20ºC, obtaining of the hydrophilic and lipophilic extracts) were done in the same conditions.

Reversed-phase HPLC assay results

To identify the phenolic components in the hydrophilic extracts I and II, their retention time and spectral characteristics were compared to those of the standards: gallic acid (GA), (+)-catechin, and (-)-epicatechin. In addition to the peaks of the available standards, chromatograms showed the presence of several compounds with UV-Vis (PDA) spectra, characteristic for GA-derivates and flavanols A, B, and C, which are yet unidentified compounds, presumably glucosides of Catechin and/or of Epicatechin (Figure 5).

Table 4

TFC concentration in seed extracts of *Feteasca Neagra* grapes.

Sample	*TFC*			
	Extracts I		*Extracts II*	
	mg QE/g	*mg RE/g*	*mg QE/g*	*RE/g*
FNN	2.74±0.09	5.44±0.21	1.93±0.08	4.03±0.05
FNH	2.62±0.15	6.99±0.16	1.76±0.05	4.77±0.12
FNS	2.44±0.11	6.65±0.30	1.75±0.03	4.70±0.08

Results are expressed as average ±2σ (n= 3).

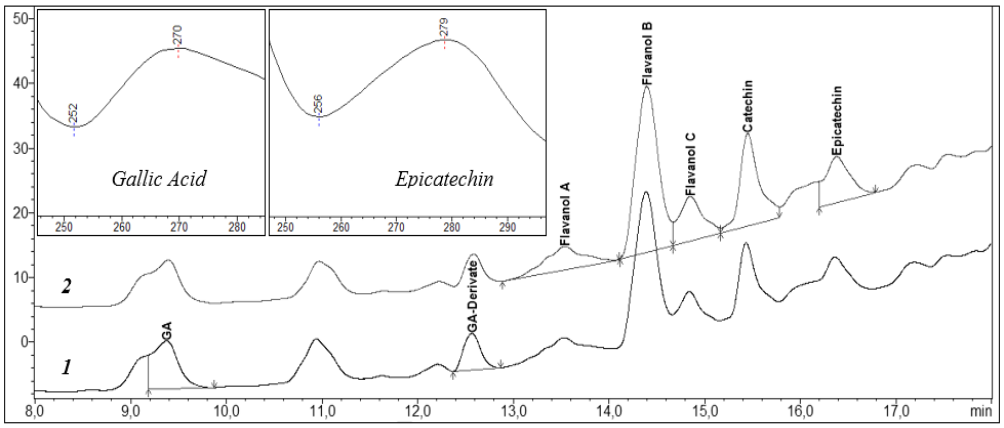

Figure 5. ***Feteasca Neagra* grape seeds polyphenols-rich extract chromatograms: (at 271 nm - *1*; at 279 nm – *2*) and UV-Vis spectra of confirmed compounds - gallic acid (GA) and epicatechin.**

The HPLC measurement results are consistent with the results recorded by spectrophotometric methods. The highest content of bioactive substances was detected in FNN extracts I and II. Extracts I from all of the seed samples have a higher content of catechin monomers, hence, a total content of flavanols is higher than in extracts II, from degreased seeds (Table 5).

The values of the total flavanols content in extracts I is within the 490.2 and 699.2 mg/100 g of seed range, on average, by 11% more than in extracts II. The content of gallic acid and gallates is higher in extracts II. Also confirmed by the spectrophotometric analysis (Figure 3), degreasing the seeds favours the release of these polar compounds from the grape seeds. The total content of soluble phenols determined by HPLC is lower than the one determined by the spectrophotometric method. Some non-phenolic substances, organic acids, and saccharides, may interact with the Folin-Ciocalteu reagent, thus TPC may be overestimated. At the same time, the total content of flavanols determined by the HPLC method (Table 5) is close to the TFC values recorded by the spectrophotometric method, with $AlCl_3$ (Table 4). The difference between the TPC values obtained by the Folin-Ciocalteu method and by HPLC chromatography was also recorded by Greek researchers [44], thus TPC dosed colorimetrically in 9 grape varieties ranged from 143 to 2228 mg GAE/100 g seeds, while by the HPLC method, values between 55 and 964 mg/100 g seeds were obtained. This source mentions a GA content (measured by the HPLC method) between 1.15 and 17.9 mg/100 g; a content of catechins between 36.4 and 454 mg/100 g, and a content of epicatechins between 17.5 and 249 mg/100 g of seeds. Another study, performed on 7 grape varieties, showed a content of gallic acid between 44.01 and 221.41 mg/100 g, and a content of catechins between 56.42 and 480.50 mg/100 g of fresh grape seeds [45].

Determination of DPPH• Free Radical Scavenging Activity of the grape seeds

The DPPH method implies the spectrophotometric measurement of the changes in the concentration of the DPPH• radical, arising from its reaction with an antioxidant. A freshly prepared methanolic DPPH• solution is purple. The colour fades in the presence of antioxidants. The antioxidant molecules can neutralize the free DPPH• radical, turning it into a colourless reaction product (DPPH-H), reducing so the absorbance of the solution. Consequently, a higher content of antioxidants leads to a more colourless solution. The Trolox equivalent antioxidant capacity assay (TEAC) of the oil samples and the hydrophilic FNN, FNH, FNS extracts was assessed (Table 6).

Table 5

The phenolic compounds identified and quantified using HPLC analysis in *Feteasca Neagra* grape seed extracts (mg/100 g seeds).

Samples	*Extracts I*					
	Catechin	*Epicatechin*	*ΣFlavanols*	*Gallic acid*	*Gallic acid derivatives*	*ΣGallates*
FNN	80.51±0.91	49.43±0.56	699.2±7.9	18.26±0.21	14.31±0.17	32.58±0.37
FNH	44.49±0.50	46.45±0.52	525.9±5.9	17.68±0.20	13.78±0.16	31.46±0.36
FNS	45.56±0.51	32.14±0.36	490.2±5.5	16.92±0.19	13.30±0.15	30.22±0.34
	Extracts II					
	Catechin	*Epicatechin*	*ΣFlavanols*	*Gallic acid*	*Gallic acid derivatives*	*ΣGallates*
FNN	67.33±0.76	60.26±0.68	585.6±6.6	21.09±0.24	15.30±0.18	36.39±0.41
FNH	49.77±0.56	51.95±0.59	479.9±5.6	20.09±0.23	14.58±0.17	34.66±0.39
FNS	40.15±0.45	33.71±0.38	457.5±5.2	21.26±0.25	14.60±0.17	36.13±0.41

Results are expressed as average ±2σ (n= 3).

Table 6

Antioxidant activity of extracts of *Feteasca Neagra* seeds.

Sample	*The Trolox equivalent antioxidant capacity assay*					
	Oil		*Extracts I*		*Extracts II*	
	% of inhibition DPPH•	*Concentration, μmol Trolox/L*	*% of inhibition DPPH•*	*Concentration, μmol Trolox/L*	*% of inhibition DPPH•*	*Concentration, μmol Trolox /L*
FNN	26.11	0.08±0.02	93.81	288.27±0.22	91.70	281.66±0.36
FNH	25.04	0.07±0.02	93.28	286.62±0.18	91.44	280.84±0.34
FNS	25.33	0.07±0.01	93.54	287.45±0.27	92.16	283.11±0.27

All results are reported as the mean of three replicates ±2σ.

To determine the antioxidant activity of the oil samples, extracts were obtained from 1.25 g oil with a 3 mL aqueous methanol solution of 60%. The antioxidant activity was determined for the hydrophilic extracts with the 1:50 (m/v) seed/solvent ratio.

Both our research and the bibliographic data [46,47] prove that fermentation, the only significant process that occurs before the pomace is obtained, does not induce considerable chemical changes and does not affect the antioxidant properties of the grape seeds. It has been established that the hydrophilic and lipophilic extracts of *Feteasca Neagra* grape seeds have an increased antioxidant activity, with the strongest free radical scavenging activity being detected in the hydrophilic extracts I and II, with a DPPH• scavenging effect of almost 91.70 and 93.81%, an equivalent of 281.66 and 288.27 µmol Trolox/L (1408 and 1441 µmol Trolox/100 g of seeds). The antioxidant activity of the extracts depends on the concentration of polyphenols as well as on the concentration of other substances with antioxidant properties (carotenoids, tocopherols, *etc.*). The data recorded by us fall within the range of the results obtained by a group of researchers who studied the difference between the quantitative and qualitative content of grape seeds from nine clones '*Karaerik*' (Turkish table grape cultivar), harvested from different vineyards. The researchers determined that the antioxidant potential of the above-mentioned grape seeds ranged from 1510 to 1918 µmol Trolox/100 g of fresh seeds [48].

The research results have confirmed that grape seeds have a higher TPC and antioxidant potential than most fruits [49]. The seeds recovered from winemaking by-products are rich sources of antioxidants; further research should be conducted in order to identify new areas of applicability and the value the winemaking by-products could bring to the pharmaceutical and food industry.

Conclusions

The conducted research showed that grape seeds from wine by-products are a cheap and accessible source of phytonutrients and biologically active substances. Thus, the properties of the lipophilic and hydrophilic extracts of seeds separated from fermented pomace of *Feteasca Neagra* grapes, cultivated in vineyards from Nisporeni (FNN), Hincesti (FNH), and Speia (Anenii-Noi region) (FNS), in the season of 2020, were elucidated.

According to the UV-Vis absorbance values, *Feteasca Neagra* grape seed oil has an increased content of chlorophyll derivatives, and the concentration of carotenoids varies from 38.71 to 49.71 mg *β*CE/100 g oil. The content of total soluble polyphenols (TPC), in all of the oil samples, is a reduced one, FNN having the highest TPC of 0.005%.

It was determined that TPC for the FNN hydrophilic extracts (32.28 and 40.15 mg GAE/g seeds) is almost twice higher than the TPC of FNH and FNS. Studies have shown that degreasing the seeds leads to better solubilization of the polar compounds, which leads to a 28.30% increase in TPC. Nevertheless, washing and extracting the seeds with *n*-hexane has as a consequence the removal of the protective layer of soluble proanthocyanidins from the surface of the seeds, leading to a reduced content of total soluble flavonoids (TFC) in the extracts.

The HPLC measurement results are consistent with the results recorded by spectrophotometric methods; hydrophilic extracts from ground seeds have a higher TFC, with values between 490.20 and 699.20 mg/100 g of seeds, which is on average by 11% higher than that of extracts from degreased ground grape seeds.

It has been established that the hydrophilic and lipophilic extracts of *Feteasca Neagra* grape seeds have an increased antioxidant activity. The strongest free radical scavenging activity was detected in the hydrophilic extracts, with a DPPH• scavenging effect of almost 91.70 and 93.81%, an equivalent of 1408 and 1441 µmol Trolox /100 g of seeds.

The seeds recovered from winemaking by-products retain their antioxidant potential and are an accessible source of phytonutrients and biologically active compounds, which can be recovered and used by the pharmaceutical industry for the production of supplements or as natural additives for functional foods.

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