

Article

From Grape to Wine: Targeted Profiling of Selected 34 Bioactive Phenolics in Negoska Variety, an Unexplored Indigenous Greek Grape Cultivar

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Abstract

Wine is a widely consumed beverage and important source of bioactive polyphenols, molecules with well-established health beneficial properties. The winemaking process produces byproducts, such as pomace, seeds and stems, which also comprise a valuable resource for sustainable valorization because of their phenolic content. The study herein concerns the exploitation of Negoska, a rare indigenous red grape variety of northern Greece cultivated in the Protected Designation of Origin (PDO) terroir of Goumenissa. In this context, we investigated various vinification fractions of Negoska, such as grapes, must, wine, pomace, seeds and stems in respect to their content in selected phenols, metabolites' content and antioxidant properties. The respective results indicated the presence of 34 non-anthocyanin phenolics, which were quantified by LC–MS/MS analysis. Principal Component Analysis (PCA) revealed a clear differentiation in the distribution between the grape fractions and the processed products. In particular, the phenolic compound pattern of Negoska wine was characterized by a high concentration of gallic (69.8 mg/L), caffeic (40.8 mg/L) and *p*-coumaric (31.1 mg/L) acids, while procyanidin B2 (24.0 mg/L) was among the most abundant compounds detected in wine. In must, procyanidin B2 was the predominant phenolic compound (9.7 mg/L). On the other hand, pomace, stems and seeds were identified as particularly rich reservoirs of various bioactive phenolics, with the molecule of procyanidin B2 representing one of the most abundant compounds in these matrices, reaching concentrations of 83.1–1015.1 mg/Kg DM. The investigated phenolic content fluctuated greatly among the samples investigated with procyanidin B2 and diosmin being the most abundant compounds in dried grapes, luteolin-4'-*O*-glucoside and diosmin in pomace, procyanidin B2, gallic acid and catechin in seeds, and procyanidin B2, epigallocatechin and luteolin-4'-*O*-glucoside were the prevailing molecules in stems. These findings constitute the first targeted characterization of selected phenolic compounds and their metabolites' profiles in Negoska grapes and the respective winemaking fractions, highlighting the potential of this indigenous cultivar and its byproducts to serve as source of valuable bioactive compounds for valorization by the food and cosmetic industries.

Keywords: Negoska variety; *Vitis vinifera*; polyphenols; antioxidants; winemaking byproducts



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1. Introduction

Wine consumption has endured for centuries and continues to enjoy widespread popularity in modern society. According to the International Organization of Vine and Wine (OIV) data, the recent (2020–2026) annual wine consumption averaged approximately 222 million hectoliters globally. In Europe, Italy, France, Germany, the United Kingdom and Spain are the five countries with the highest wine consumption level. Greece, in 2025, ranked 16th in wine consumption and 11th in wine production, highlighting its continued importance and role within the global wine sector [1]. Consequently, nowadays wine constitutes one of the most widely consumed alcoholic beverages worldwide, appreciated not only for its distinctive sensory attributes but also for its potential health-promoting properties. The possible protective effects of moderate wine consumption against the development of certain types of cancer, cardiovascular diseases and diabetes have been extensively investigated and discussed in numerous studies [2,3]. Except for alcohol and sugars, wine contains a wide range of bioactive compounds such as polyphenols [2,4] and volatile terpenes [3]. In particular, polyphenols have attracted considerable scientific interest due to their antioxidant, anti-inflammatory, anti-aging, anti-obesity, and cardioprotective properties [5–7], which are considered to contribute to many of the health benefits associated with wine consumption.

The winemaking process generates substantial amounts of diverse residues such as wine lees and wastewater as well as byproducts such as grape pomace, seeds, skins, and stems. The latter are known for their rich content of phenolic compounds and other valuable constituents, which has stimulated intense research interest in their sustainable management and the development of revalorization strategies within a circular economy framework.

Negoska is a rare indigenous grape variety of northern Greece used for red wine production. According to local wine industry sources, it is mainly cultivated on the slopes of Mount Paiko near the wine-producing town of Goumenissa, within the Protected Designation of Origin (PDO) zone. It shares a common historical background with Xinomavro, a famous Greek grape variety, since both are being referred as “Popolka”. Negoska wine is characterized by its ripe red and black fruit aroma, moderate acidity, a softer tannin content and good aging potential [8]. However, tannin perception may vary depending on winemaking conditions, grape maturity, and analytical composition. Interestingly, there are no studies in the literature concerning the comprehensive determination of the chemical composition of this important, rare cultivar. A Scopus database search conducted in February 2026 using “Negoska” as a keyword provided only two results, underlining the limited coverage of this topic in existing literature. In particular, the first source is a chapter in “The Oxford Companion to Wine” mentioning Negoska as an indigenous Greek red grape variety primarily cultivated in Central Macedonia, which exerts its viticultural characteristics, wine style and regional significance [9]. An additional source is a research report concerning the antioxidant activity and total phenolic content of Greek wines that includes the PDO Goumenissa wine as a blend consisted by 75% of Xinomavro and 25% of Negoska [10].

The present study constitutes an explorative preliminary assessment of selected phenolic compounds and the antioxidant properties of grapes, must and wine, as well as winemaking byproducts (pomace, stems, and seeds) of Negoska variety. Additionally, this study aspires to provide an overview of metabolite presence in the vinification residues and determine which bioactive molecules are transferred into the resulting wine.

2. Materials and Methods

2.1. Samples and Materials

All samples used in this study were provided by Aidarinis Winery (Goumenissa, Macedonia, Greece). They composed of grapes, must, wine, seeds, stems and pomace originated from the Negoska grapevine variety which is classified into *Vitis vinifera* species and is cultivated in the surrounding terroir. Three independent batches of each sample category (approximately 1 kg each) were supplied by the winery from three different harvesting days during the same vintage. Each batch was processed (extracted, analyzed) independently. Further details regarding the sampling details for individual vines or grape producing vineyard blocks are not available.

In particular, the grape berries were harvested at commercial maturity from a winery's vineyard during the 2024 growing period. They were manually destemmed, deseeded, freeze-dried and milled to provide an amorphous powder. The separated seeds were also dried and ground to a fine powder. The winemaking byproducts, consisting of grape pomace and stems, were collected after the vinification process, air-dried in a dark room at room temperature for approximately 40 days until constant weight and milled to powder. All powdered samples were transferred into airtight amber glass containers and stored until analysis in desiccators at room temperature and in an absence of light. For all samples, the time elapsed between grinding and sample analysis did not exceed two weeks.

All analytical determinations were performed using three independent and representative samples of each material, which were extracted and analyzed separately to ensure the reproducibility and reliability of the results.

2.2. Chemicals and Standards

Methanol, ethyl acetate and petroleum ether (40–60 °C) used for the extraction of samples were obtained as analytical purity solvents from Fisher Chemicals (Hampton, NH, USA). All chemical standards used for the determination of phenolic compounds were purchased as $\geq 95\%$ purity compounds by Sigma-Aldrich (Burlington, MA, USA), except of catechin, epigallocatechin gallate, gallic acid and isoquercetin which were provided by ExtraSynthese (Genay, France). The following solvents (LC-MS purity) were used for the LC-MS/MS determinations: Acetonitrile, methanol and water obtained from JT Baker (Radnor, PA, USA) and formic acid provided by Fisher Chemicals. The following reagents and solvents used for various determinations: Folin–Ciocalteu's reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ, $\geq 95\%$ purity), glacial acetic acid (analytical purity) were provided by Sigma-Aldrich, anhydrous sodium carbonate (99.999+% purity), magnesium sulphate, sodium chloride, and sulfuric acid (98% purity) were purchased from Chem-Lab (Zedelgem, Belgium), heptahydrate iron sulfate (99+% purity), hexahydrate ferric chloride (97% purity) and potassium hydroxide (KOH, 99.99% purity) were provided by Alfa Aesar (Heysham, UK) and vanillin (99% purity) was obtained from Acros Organics (Geel, Belgium).

2.3. Extraction Procedure

The extraction procedure was based on a protocol described by Myrtsi et al. (2021) [11]. Briefly, 50 g of the dried material (grape, pomace, stem and seed samples) were extracted with 200 mL of an acidified mixture of methanol–water (MeOH/H₂O/1.0 N HCl, 90:9.5:0.5, v/v/v). The selection of acidified methanol as extraction solvent was based on a previously validated protocol for the LC–MS/MS determination of phenolic compounds which revealed its high efficacy to act as a potent extraction solvent for a broad range of phenolic metabolites. After sonication for 10 min (35 kHz, RK 100 SH, Bandelin Sonorex Super sonicator, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) at a temperature below

35 °C, the solvent was separated by filtration and the remaining solid was extracted twice using the same procedure. The combined extracts were evaporated under reduced pressure using a Büchi Rotavapor R-210 apparatus equipped with a Büchi vacuum pump V-700 and a V-850 vacuum controller (Büchi Labortechnik AG, Flawil, Switzerland), keeping the temperature below 35 °C. The remaining residue was redissolved in 50 mL of MeOH/H₂O (1:1) and centrifuged. The supernatant was defatted with petroleum ether (3 × 30 mL), and the petroleum ether phase was discarded. The remaining aqueous phase was subsequently extracted with ethyl acetate (3 × 50 mL). The combined ethyl acetate fractions were dried over anhydrous MgSO₄, evaporated to dryness under reduced pressure, and the final residue was dissolved in methanol prior to LC–MS/MS analysis.

Wine samples were processed immediately after the bottle opening. Wine and must samples were processed in accordance with the following procedure: 5 mL of the respective sample were centrifuged for 10 min at 7500 rpm (Eppendorf Centrifuge 5430, Eppendorf SE, Hamburg, Germany), filtered through a 0.45 µm pore-size filter and subjected directly to LC–MS/MS analysis.

To avoid polyphenols' degradation, all abovementioned activities were performed in the absence of direct sunlight and at temperatures below 35 °C.

2.4. Determination of Total Tannin (TTC), Phenolic (TPC) and Flavonoid (TFC) Content

The detailed spectrophotometric methods applied for the TTC, TPC, and TFC determinations have been reported previously [11]. Specifically, all samples were placed in a 96-well microplate (Sarstedt AG & Co. KG, Nümbrecht, Germany) in triplicate, and the respective absorbance was measured in a NanoQuant, infinite M200PRO (Tecan Group Ltd., Männedorf, Switzerland) instrument. The TTC was measured at 500 nm absorption wavelength, and its value was calculated using an epicatechin standard calibration curve. The respective results are expressed as mg of epicatechin equivalents per Kg of dried material (mg EE/Kg DM) or per L (mg EE/L). The TPC was determined at 765 nm absorption wavelength, and the respective value was obtained against a gallic acid standard calibration curve. The respective results were expressed as mg of gallic acid equivalents per Kg of dried material (mg GAE/Kg DM) or per L (mg GAE/L). The TFC was measured at 415 nm absorption wavelength, and its value was calculated using a quercetin standard calibration curve. The respective results are expressed as mg of quercetin equivalents per Kg of dried material (mg QE/Kg DM) or per L (mg QE/L).

2.5. Determination of Individual Phenolic Compounds Content by LC–MS/MS Analysis

The individual phenolic compounds' presence and quantitative content was assessed using an Accela Ultra High-Performance Liquid Chromatography system coupled with a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described by Myrtsi et al. (2023) [12]. In particular, the analytes' chromatographic separation was achieved on a C18 column (150 × 2.1 mm, 3 µm, Fortis Technologies Ltd., Neston, Cheshire, UK), which was coupled with an AF C18 guard column (10 × 2.0 mm, 3 µm, Fortis Technologies Ltd., Neston, Cheshire, UK) using a mobile phase consisting of A: water with 0.1% formic acid and B: 100% acetonitrile. The Electro-Spray Ionization (ESI) technique was used for the MS/MS determination, and the determinations were performed at Selected Reaction Monitoring mode (SRM). The quantification of polyphenols was achieved by constructing a calibration curve for each analyte using six concentration levels for each standard solution, along with 2-(4-chlorophenyl)malonaldehyde as an internal standard solution, at concentration of 200 ng/mL. Calibration curves were prepared using six concentration levels covering the range of 50–800 ppb. All samples were appropriately diluted prior to analysis, when required, to ensure that the measured

concentrations were within the established calibration ranges. The respective parameters of transition, collision energy, polarity, retention time, calibration curve equation, and determination coefficient for each analyte are included as Table S1 in the Supplementary Materials section. Compound identification was performed by comparison with authentic standards, based on retention their time and the corresponding SRM transition, according to a validated analytical method previously developed by our group. Recovery and matrix effects were assessed in selected matrices (grape berries and wine). Sixteen representative compounds were selected for this assessment, covering different chemical classes. The results confirmed the applicability of the method across the evaluated matrices, although matrix-dependent effects cannot be completely excluded for all analytes. Compound identification was based on comparison with authentic reference standards analyzed under identical chromatographic and mass spectrometric conditions. Although the use of authentic standards substantially increases confidence in compound assignment, the use of a single SRM transition for quantification cannot completely exclude potential interferences from structurally related compounds in complex matrices. Therefore, confirmation of selected atypical compounds by complementary analytical approaches, such as additional SRM transitions or high-resolution MS/MS, would further strengthen their identification.

2.6. Antioxidant Properties Evaluation

The widely used antioxidant property evaluations of phenolic-rich grape and wine matrix assays FRAP (Ferric Reducing Antioxidant Power) and DPPH• (2,2-DiPhenyl-1-PicrylHydrazyl) were utilized to determine the antioxidant capacity of the investigated samples. The respective assessments were performed using a NanoQuant, infinite M200PRO (Tecan Group Ltd., Männedorf, Switzerland) instrument, according to previously reported procedures [11]. For FRAP assay the absorbance was measured at 593 nm wavelength, and the reducing capacity was determined against a FeSO₄ standard calibration curve. For DPPH assay the absorbance was measured at 515 nm wavelength and the outcome was reported against a Trolox standard calibration curve. For each sample the respective results are expressed as mmol Fe²⁺/Kg (or L) for FRAP and as Trolox equivalents (TE)/Kg (or L) for DPPH•.

2.7. Data Analysis

All experimental data have been expressed as mean values ± standard deviation. Statistical analyses were carried out using Microsoft Excel 365 and JMP® Student Edition (Edition 18). Calibration curves for the quantification of individual polyphenolic compounds were established by linear regression analysis in Microsoft Excel. Significant differences among the different grape products/byproducts (dried grapes, pomace, seeds, stems, grape must, and wine) were evaluated by a One-way Analysis of Variance (ANOVA). Subsequent post hoc comparisons of means were performed using the Tukey–Kramer Honestly Significant Difference (HSD) test at a significance level of $\alpha = 0.05$ (p -value < 0.05). ANOVA, HSD test, and Principal Component Analysis (PCA) were performed using JMP® Student Edition to assess the multivariate relationships among samples and to identify similarities and differences between the studied extracts. PCA was performed using the correlation matrix after standardization of variables. HCA was conducted using standardized variables and Ward's linkage method to evaluate similarities among samples based on their phenolic profiles.

3. Results

3.1. Total Tannin (TTC), Phenolic (TPC) and Flavonoid (TFC) Content

The determined values for total tannin (TTC), phenolic (TPC) and flavonoid (TFC) contents are presented in Figure 1 and included in Table S3 of the Supplementary Materials section. The respective results indicate that stems and seeds exhibit the highest tannin and phenolic contents, since stems were determined to contain 20.2 g of tannins (as EE/Kg DM) and 5.2 g of phenolics (as GAE/Kg DM), while seeds were found to contain 19.3 g of tannins (as EE/Kg DM) and 6.7 g of phenolics (as GAE/Kg DM). It is further notable that the Negoska wine is characterized by a high tannin content (9.3 g EE/L). It must be noted however that vanillin assay may be influenced by anthocyanin interference, particularly for red-colored matrices such as red wine and grape-derived byproducts. Therefore, the reported total tannin content values should be interpreted with caution, and further studies using more selective analytical approaches are encouraged to confirm these values. In respect to the flavonoid content, stems were identified as the richest source of flavonoids (159.1 mg QE/Kg DM), followed by pomace (78.1 mg QE/Kg DM), while the dried grapes exhibited a lower TFC value (39.4 mg QE/Kg DM) as compared to pomace. The observed difference between freeze-dried grapes and pomace can be rationalized considering that grapes were analyzed prior to vinification, whereas pomace represents the residual solid fraction remaining after the juice extraction and fermentation procedures.

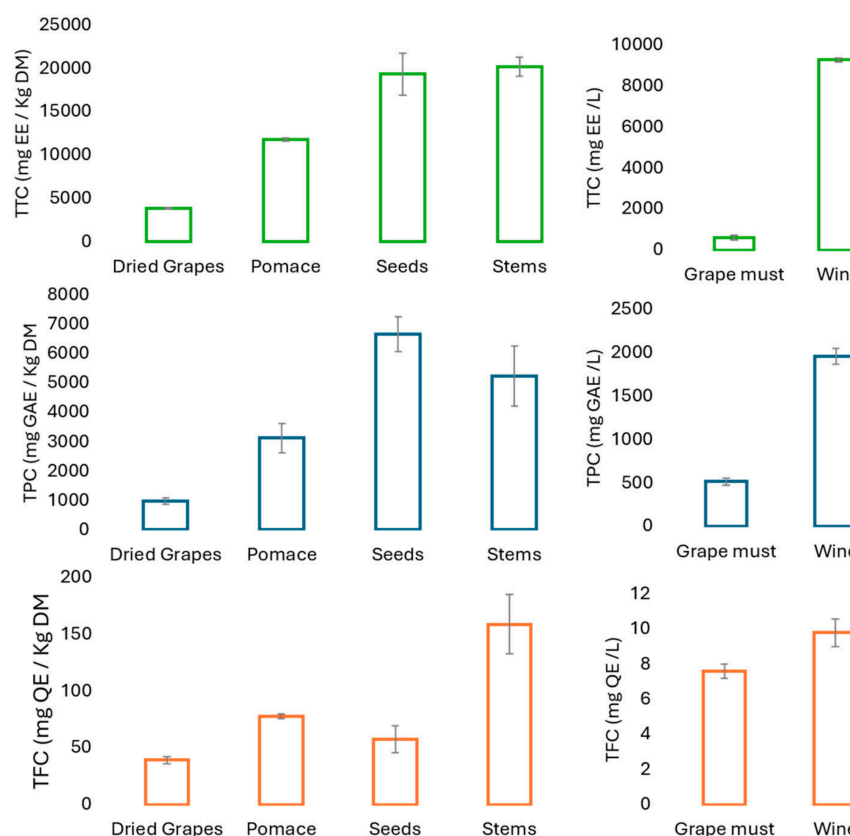


Figure 1. Total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents of samples tested. Data are presented descriptively; no statistical analysis was performed for the comparisons shown in this figure.

3.2. Individual Phenolic Compounds Content by LC–MS/MS Analysis

The quantification of the individual phenolic compounds content in the investigated samples is included in Table 1, revealing the presence of 34 bioactive phenolics. Notably, a substantial fluctuation of phenolics presence was observed among various samples, since

only the molecules of gallic acid and procyanidin B2 were detected in all samples. In addition, Negoska grape stems were determined to display a particular rich phenolic content with prevailing bioactive phenolics the molecules of epigallocatechin, luteolin-4'-O-glucoside, gallocatechin, isoquercetin and catechin.

Table 1. Quantitation of phenolic compound content in the dried grapes, pomace, seeds, stems expressed as mg/kg DM (for must and wine in mg/L).

Compounds	Dried Grapes	Pomace	Seeds	Stems	Must	Wine
	mg/Kg DM				mg/L	
Epigallocatechin	n.d. ¹	104.0 ± 3.4 ^b	n.d.	702.9 ± 31.2 ^a	4.7 ± 0.7 ^b	17.0 ± 6.4 ^a
Gallic Acid	12.6 ± 1.2 ^c	147.6 ± 12.8 ^b	248.6 ± 18.4 ^a	138.4 ± 10.2 ^b	2.9 ± 0.4 ^b	69.8 ± 11.5 ^a
Procyanidin B2	83.1 ± 16.2 ^d	284.7 ± 35.2 ^c	576.2 ± 52.2 ^b	1015.1 ± 69.3 ^a	9.7 ± 1.6 ^b	24.0 ± 8.1 ^a
Gallocatechin	n.d.	2.7 ± 1.3 ^b	Tr ²	352.3 ± 37.2 ^a	1.5 ± 0.2 ^b	3.1 ± 0.8 ^a
Chlorogenic Acid	n.d.	10.0 ± 2.3 ^a	n.d.	n.d.	0.9 ± 0.3 ^b	0.3 ± 0.1 ^a
Procyanidin B1	2.9 ± 2.0 ^b	27.5 ± 7.2 ^a	44.5 ± 16.1 ^a	30.8 ± 2.8 ^a	n.d.	0.4 ± 0.2 ^a
Protocatechuic Acid	n.d.	40.6 ± 2.9 ^b	5.7 ± 1.0 ^c	82.8 ± 3.0 ^a	tr	1.8 ± 0.4 ^b
Neochlorogenic Acid	tr	0.7 ± 0.3 ^a	tr	tr	n.d.	tr
Hydroxytyrosol	n.d.	4.4 ± 7.6	n.d.	n.d.	n.d.	18.5 ± 5.7 ^a
Catechin	18.8 ± 3.0 ^c	106.1 ± 8.5 ^b	212.7 ± 26.4 ^a	122.0 ± 10.7 ^b	0.21 ± 0.05 ^a	n.d.
Epicatechin	5.4 ± 0.2 ^b	26.5 ± 1.9 ^b	53.7 ± 15.6 ^a	6.3 ± 5.6	tr	tr
Epigallocatechin Gallate	0.6 ± 0.5 ^c	n.d.	9.7 ± 3.8 ^b	22.2 ± 3.2 ^a	n.d.	n.d.
Quercetagenin-7-O-glucoside	57.1 ± 4.6 ^a	64.1 ± 6.3 ^a	n.d.	12.6 ± 1.2 ^b	tr	4.8 ± 1.1 ^a
Caffeic Acid	n.d.	3.1 ± 0.1 ^a	n.d.	3.2 ± 0.1 ^a	n.d.	40.8 ± 8.4 ^a
Polydatin	n.d.	0.28 ± 0.01 ^b	0.25 ± 0.02 ^b	1.3 ± 0.1 ^a	n.d.	tr
Isoquercetin	31.7 ± 8.8 ^c	130.7 ± 4.5 ^b	n.d.	169.6 ± 13.3 ^a	tr	n.d.
<i>p</i> -Coumaric Acid	n.d.	5.9 ± 1.0 ^b	8.3 ± 1.4 ^a	n.d.	n.d.	31.1 ± 5.3 ^a
Taxifolin	0.3 ± 0.1 ^c	4.2 ± 0.1 ^b	1.3 ± 0.1 ^c	17.0 ± 1.7 ^a	n.d.	2.6 ± 0.5 ^a
Diosmin	82.0 ± 50.4	424.0 ± 82.0 ^a	n.d.	n.d.	n.d.	n.d.
Herperidin	17.3 ± 1.9 ^c	117.8 ± 3.3 ^a	n.d.	25.1 ± 1.6 ^b	tr	n.d.
Quercitrin	3.4 ± 0.5 ^b	5.7 ± 1.1 ^a	n.d.	n.d.	tr	n.d.
Sophoricoside	n.d.	0.41 ± 0.03 ^a	0.4 ± 0.1 ^a	n.d.	n.d.	n.d.
Luteolin-4'-O-glucoside	n.d.	450.5 ± 32.5 ^b	0.9 ± 0.2 ^c	610.6 ± 22.2 ^a	0.5 ± 0.1 ^a	0.21 ± 0.04 ^b
Phloridzin	0.3 ± 0.1 ^c	8.3 ± 0.5 ^b	9.4 ± 0.4 ^b	17.6 ± 0.9 ^a	0.12 ± 0.02 ^b	2.6 ± 0.7 ^a
Myricetin	n.d.	7.4 ± 0.4 ^a	n.d.	n.d.	n.d.	8.1 ± 1.5 ^a
<i>trans</i> -Resveratrol	n.d.	n.d.	n.d.	4.4 ± 2.9	n.d.	2.9 ± 1.3 ^a
Eriodictyol	n.d.	n.d.	n.d.	7.4 ± 0.8 ^a	n.d.	0.23 ± 0.04 ^a
Luteolin	n.d.	0.6 ± 0.1 ^a	n.d.	0.8 ± 0.2 ^a	n.d.	n.d.
Quercetin	n.d.	44.9 ± 3.3 ^a	2.2 ± 0.4 ^c	13.7 ± 0.4 ^b	n.d.	3.6 ± 0.9 ^{ac}
Calycosin-7-O-D-glycoside	tr	tr	0.5 ± 0.2 ^a	0.3 ± 0.2 ^a	n.d.	n.d.
Matairesinol	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 ± 0.2 ^a
Phloretin	n.d.	n.d.	n.d.	n.d.	n.d.	tr
Apigenin	n.d.	0.20 ± 0.03 ^b	n.d.	1.4 ± 0.1 ^a	n.d.	n.d.
Isorhamnetin	n.d.	7.4 ± 2.6 ^a	n.d.	n.d.	n.d.	0.28 ± 0.05 ^a

¹ n.d.: not detected. ² tr: traces, (between LOD and LOQ). Censored values were replaced by LOD/2 (n.d.) and LOQ/2 (tr) for statistical analysis. Different superscript letters indicate significant differences among samples within the same matrix category (solid or liquid fractions) according to a one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Statistical analyses were performed separately for solid samples expressed as mg/kg DM and liquid samples expressed as mg/L. Letters between these two groups should not be compared.

3.3. Antioxidant Properties

The antioxidant capacity of the investigated samples was assessed by applying the FRAP and DPPH• assays. The respective results are illustrated in Figure 2 and included in Table S3 of the Supplementary Materials section. Among the samples investigated, Negoska seeds and stems were determined in both assays to display the strongest antioxidant potency displaying activities measured as 76.0 and 72.0 mmol Fe²⁺/Kg DM and 5950.4 and 5501.1. mg TE/Kg DM, respectively.

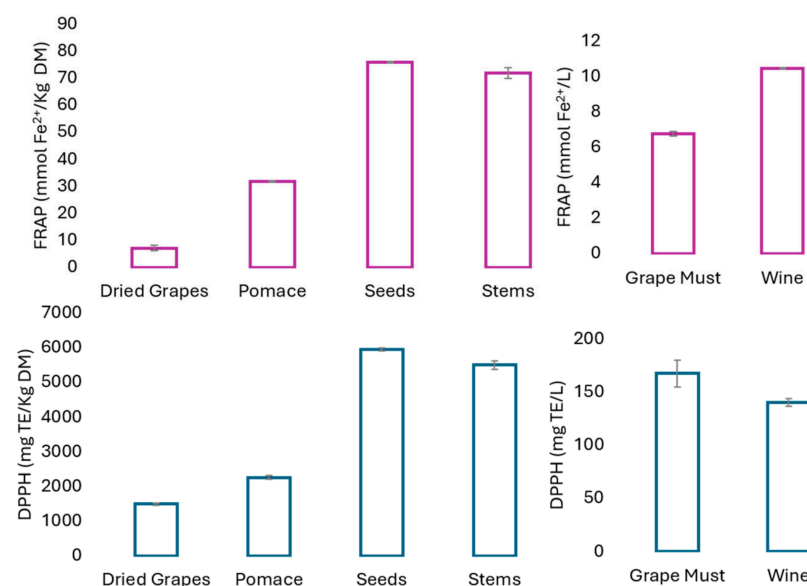


Figure 2. Results of FRAP and DPPH assay for the determination of antioxidant capacities of the investigated samples. Data are presented descriptively; no statistical analysis was performed for the comparisons shown in this figure.

3.4. Principal Component Analysis (PCA)

The Principal Component Analysis (PCA) was applied aiming to serve as an exploratory multivariate tool to visualize the similarities and differences among the investigated samples based on their phenolic compound profile. The respective results presented in Figure 3 proved capable of clearly separating the investigated samples, revealing the stem sample as strongly associated with higher levels of various flavonols with the most prominent molecules of procyanidin B2 and gallate derivatives. Similarly, pomace showed a distinct clustering based on the content of various flavonoids, such as luteolin-4'-O-glucoside, diosmin and quercetin. Nevertheless, because the implemented herein PCA refers to both solid and liquid matrices, the resulting clustering should be interpreted with caution, since the observed herein differences reflect relative compositional trends rather than absolute comparisons between the different samples.

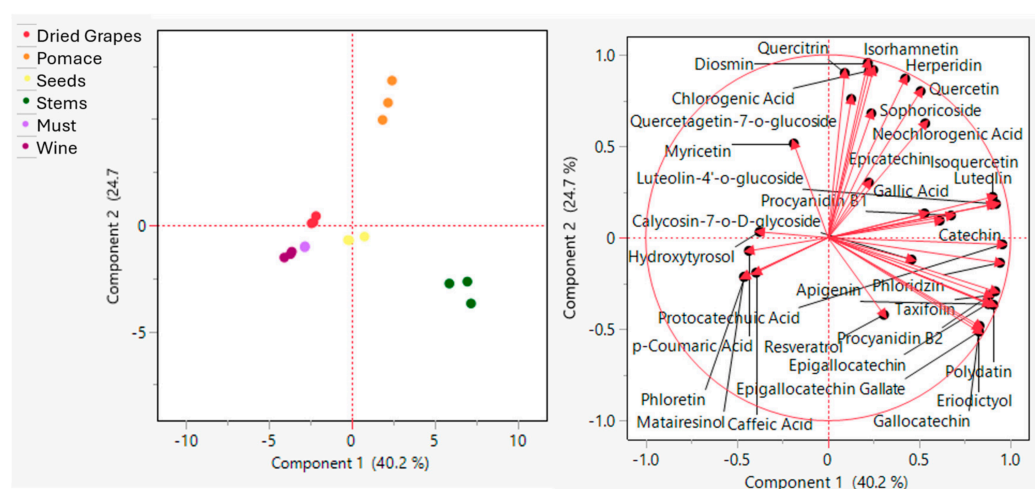


Figure 3. Principal Component Analysis (PCA) score and loading plots of the polyphenolic composition of the analyzed grape products.

4. Discussion

The increasing emphasis on sustainable resource management and circular bioeconomy strategies has stimulated a considerable research interest towards the valorization of the agro-industrial wastes and byproducts. In this context, the wine-making sector represents an intriguing case since it generates annually large quantities of pomace, seeds and stems. Although these residues were traditionally considered to be low-value byproducts, growing evidence demonstrates that they constitute important reservoirs of bioactive compounds, particularly polyphenols, which are known to possess significant antioxidant, anti-inflammatory, and other health-promoting properties. Consequently, the recovery and utilization of these compounds may reduce the environmental burdens and simultaneously create added economic value [13,14].

In the present study, the Negoska stems and seeds were determined as particularly rich sources of tannins and phenolic compounds, highlighting their potential for valorization and utilization in high-value applications (Figure 1) [15]. In this respect, Karastergiou et al. have investigated the total phenolic content (TPC) of four *Vitis vinifera* cultivars' seeds and determined a wide fluctuation ranging from 5000 to 35,000 mg GAE/kg DW [16]. In respect to the grape stems, although they are less extensively studied, as compared to grape seeds, previous studies have reported TPC values ranging from 850 to 34,000 mg/kg DM, depending on parameters such as cultivar and extraction method [17].

Considering the strong association between phenolic composition and antioxidant activity, it is evident that seed and stem samples displayed the highest antioxidant activity among the products investigated herein, in respect to their total phenolic and tannin contents. This relationship supports the widely accepted role of phenolic compounds as major contributors to the antioxidant potential of grape-derived matrices, revealing that the abundance of catechin, epicatechin, and procyanidin derivatives is related with the superior antioxidant capacity observed for these fractions and highlighting the potential of these Negoska byproducts to serve as sustainable sources of natural antioxidants for applications in food preservation, cosmetics, and pharmaceutical formulations.

The LC–MS/MS determination outcome for the individual phenolic presence in Negoska byproducts are consistent with the respective total phenolic and tannin content measurements. The results included in Table 1 and Supplementary Figure S1 revealed an increased content of flavan-3-ols (catechins) in stems, followed by seeds and pomace samples, displaying a similar trend with the respective TTC values. Although the quantified individual phenolic compounds contributed to the overall phenolic profile, they represent only a fraction of the total phenolic content determined by the Folin–Ciocalteu assay, indicating the presence of additional non-quantified phenolic constituents. The compositional analysis identified procyanidin B2 and diosmin as the most abundant compounds in dried grapes (83.1 and 82.0 mg/Kg DM respectively). Wine sample was determined to contain mostly gallic acid (69.8 mg/L) and in lesser amounts caffeic and *p*-coumaric acids (40.8 and 31.1 mg/L respectively), while must displayed the poorest phenolic content with the molecules of procyanidin B2 and epigallocatechin (9.7 and 4.7 mg/L respectively) as major components. On the other hand, the vinification residues exhibited a considerably high phenolic content with Negoska stems displaying the richest content having procyanidin B2, epigallocatechin and luteolin-4'-*O*-glucoside (1015.1, 702.9 and 610.6 mg/Kg DM respectively) as the most abundant molecules. Pomace contained the molecules of luteolin-4'-*O*-glucoside, diosmin and procyanidin B2 (450.5, 424.0 and 284.7 mg/Kg DM respectively) as the most abundant, while seeds had the molecules of procyanidin B2, gallic acid and catechin (576.2, 248.6 and 212.7 mg/Kg DM respectively) as the prevailing components.

These results are in agreement with similar literature findings determining gallic acid as a major phenolic component of wines [18] and highlighting its contribution to their

antioxidant capacity, color behavior and potential health effects [19,20]. Botta et al. 2025 have studied the gallic acid content of 24 wines and determined that its concentration varies from 0 to 6 mg/L for white wines and from 6 to 657 mg/L for red wines [21]. Negoska red wine was determined within this range (69.8 mg), while the highest content was detected for a Spanish wine produced by applying a natural winemaking process on Garnacha Tintorera and Mencía grapes. The detected large amounts of gallic acid in seeds is in agreement with Atak et al. 2021 analyses of seeds, skins and pulp obtained from 22 cultivars and hybrids determining the presence of significantly higher concentrations of gallic acid in seeds as compared to skins and pulp [19]. Beyond antioxidant activity, the presence of gallic acid is also associated with antimicrobial [22], anti-inflammatory [23] and chemopreventive properties [24], highlighting grape seeds as particularly attractive for the development of functional ingredients and nutraceutical products.

The notable feature of the Negoska variety samples investigated related to the content—in appreciable amounts—of phenolics rarely found in commercial varieties. Thus, the relatively high abundance of protocatechuic acid in Negoska pomace, stems, seeds and wine samples represents a characteristic compositional feature of cultivar and its terroir, which may serve as a potential marker of their geographical origin, since the concentration of this molecule is highly dependent on vineyard location [25].

Of special interest was the relatively high concentration of hydroxytyrosol detected in Negoska wine. Although hydroxytyrosol is a low molecular weight phenolic antioxidant, mainly associated with olives, occasionally it is reported as a constituent of wine [26,27]. González-Ramírez et al. reported hydroxytyrosol concentrations ranging from 0.12 to 2.24 mg/L in 108 commercial Spanish red wines, whereas the determined in the present study hydroxytyrosol concentration reached 18.5 mg/L [26]. Although this value exceeds the concentrations reported in that study, the comparison should be interpreted with caution because Negoska wine from only one vintage was analyzed. Based on the present data it is not possible to justify the elevated hydroxytyrosol level since neither fermentation-related parameters nor tyrosol concentrations were evaluated. Therefore, further studies involving controlled fermentation experiments and targeted analysis are required to determine whether this characteristic is associated with cultivar-specific traits, winemaking practices, or other factors. Nevertheless, considering the well-established antioxidant and cardioprotective properties of hydroxytyrosol [28,29], its presence may contribute to the overall phenolic profile of Negoska wine.

The detection of lignan matairesinol exclusively in Negoska wine contributes to its distinctive phenolic profile. The presence of lignans in wines is related to both grape variety and applied winemaking practices, such as maturation in oak barrels that contributes through the extraction of lignans from wood. Therefore, the matairesinol presence detected herein may have resulted from grape variety, fermentation process and wine aging conditions. Lignans have attracted increasing scientific interest because of their phytoestrogenic, antioxidant and potential disease-preventive properties [30,31]. Early studies have reported total lignan concentrations of 0.8–1.4 mg/L in red wines [32], while Vidlar et al. (2023) have showed that syringaresinol concentration is affected by barrel characteristics and matairesinol, pinoresinol contents are mainly influenced by grape cultivar [33]. In this respect, a 2021 study has reported very low lignan concentrations for wines fermented and stored in stainless-steel tanks, while matairesinol was among the lignans detected in higher concentrations in red wines and wines produced by applying the traditional Qvevri method [34]. It must be noted however that in general red wines contain higher concentrations of lignans as compared to white wines [32,35]. Herein, the molecule of matairesinol was detected exclusively in the Negoska wine sample (0.7 mg/L).

However, it is not possible to determine its origin since it may have resulted from the specific winemaking practice or from grape cultivar.

However, previous studies on Negoska wines have reported high total phenolic content and antioxidant activity, confirming the phenolic richness of this cultivar. Gugulyas and Mashev (2006) reported high levels of total phenolics and antioxidant activity in traditional Greek red wines, including Goumenissa wine, which is a blend of Xinomavro and Negoska [10]. Overall, the phenolic profile of Negoska is generally consistent with that reported for other Mediterranean red grape cultivars, while some quantitative differences for the presence of individual phenolic compounds were observed. Indicatively, Biniari et al. 2020 have reported TPC values for the berry skins of Mediterranean grapes ranging between 2.18 and 19.14 mg GAE/g fresh weight and 20.92–47.19 mg GAE/g fresh weight for seeds [3]. These differences may reflect cultivar characteristics, environmental conditions, and winemaking practices, highlighting the need for further comparative studies.

In this respect, the applied extraction procedure for the separation of phenolic compounds is a process highly dependent on solvent composition and extraction conditions. Although the extraction solvent acidified methanol utilized herein is widely used for the extraction and LC–MS/MS analysis of phenolic compounds, alternative solvents such as aqueous ethanol mixtures, may provide different extraction efficiencies for specific phenolic classes. Therefore, the phenolic profile comparisons among literature studies should consider the applied extraction methodology. In the present study, the selected extraction procedure was based on a previously validated protocol and was considered to be the most suitable for the targeted determination of the specific phenolic constituents in Negoska grape byproducts.

Principal Component Analysis (PCA) was applied to evaluate the variability of the phenolic profiles among the studied samples. The PCA revealed a clear separation among sample groups, in respect to their phenolic profile differences. However, because solid and liquid matrices were processed and different extraction workflows were applied, the PCA should be interpreted primarily as a comparison of phenolic composition patterns rather than an absolute quantitative equivalence among matrices. In particular, the first two principal components accounted for 64.9% of the total variance, with PC1 and PC2 summarizing 40.2% and 24.7%, respectively. The score plot revealed a clear separation of the sample groups, indicating substantial differences in their phenolic composition. PC1 was mainly associated with higher levels of flavonoids and flavan-3-ols, including quercetin, isorhamnetin, catechin, epicatechin, and procyanidins, whereas negative PC1 values were related to phenolic acids such as caffeic acid and *p*-coumaric acid, as well as hydroxytyrosol and phloretin. PC2 further discriminated the samples according to the relative abundance of chlorogenic acid, quercitrin, diosmin, and myricetin on the positive side, and gallocatechin, epigallocatechin gallate, and resveratrol on the negative side. The loading plot demonstrated strong correlations among compounds belonging to the same phenolic subclasses, suggesting common biosynthetic pathways and similar distribution patterns across the samples.

Notably, wine samples clustered in close proximity to grape must and they were clearly separated from vinification residues (pomace, stems, and seeds). This distribution indicates that the phenolic fingerprint of final wine largely originates from the liquid phase present before fermentation. At the same time, the separation of solid fractions is indicative of the substantial amounts of phenolic compounds retained within the grape matrix after vinification. This selective partitioning is particularly important from a valorization perspective, as it indicates that after wine production the winery byproducts retain significant amounts of bioactive compounds. Consequently, these materials should not be

regarded as waste but rather as valuable secondary raw materials suitable for the recovery of phenolic-rich extracts.

The clustering pattern observed in the PCA score plot was further confirmed by Hierarchical Cluster Analysis (HCA) (Supplementary Figure S2), which grouped the replicates together and revealed a clear separation among the major sample fractions. The HCA was performed using the concentrations of the quantified phenolic compounds to further investigate similarities among the analyzed Negoska fractions. Two major groups were observed. The first group included dried grapes, must, wine and seeds, whereas the second is comprised by pomace and stems. Within the first cluster, wine samples grouped more closely to grape must than with the solid byproducts, supporting the PCA findings and indicating that the phenolic profile of wine is largely derived from the liquid phase of the winemaking process. In contrast, pomace and stems formed a distinct cluster characterized by the retention of substantial amounts of phenolic constituents. These results further demonstrate that winery byproducts maintain unique phenolic fingerprints and may represent valuable sources of bioactive compounds for subsequent valorization. However, PCA and HCA were employed as exploratory tools to visualize the relationships among the analyzed matrices according to their phenolic composition. Therefore, the resulting clustering patterns should be interpreted as descriptive rather than predictive.

From an industrial perspective, the recovery of phenolic compounds from winery byproducts represent a promising strategy for the sustainable valorization of vinification residues [36]. The large quantities of grape pomace, seeds and stems generated during winemaking are rich in bioactive compounds and constitute low-cost raw materials. Thus, their valorization as natural antioxidants in functional foods, nutraceuticals, cosmetic formulations, and pharmaceutical excipients may represent a promising valorization strategy. It must be noted however that the upgrade and industrial scale adaptation of such processes depends on several factors, such as extraction efficiency, solvent selection, process scalability, solvent recovery, product purification, and economic viability. Therefore, further techno-economic assessment and process optimization are required before their large-scale industrial application.

Although the present study provides the first targeted characterization of selected phenolic compounds in Negoska grapes, wine and vinification byproducts, it does not encompass the entire phenolic metabolome. Specifically, anthocyanins, which are characteristic pigments of red grape cultivars and major contributors to wine color and antioxidant properties, were not included in the specific analytical panel. Therefore, the results should be interpreted as a targeted profile of selected phenolic compounds rather than a comprehensive polyphenolic characterization. Future investigations integrating dedicated anthocyanin analysis with untargeted metabolomics approaches would provide a more complete understanding of the phytochemical composition and bioactive potential of this indigenous cultivar.

Another serious limitation of the present study relates to the origin of investigated samples, since they have been obtained from a single vineyard, a single winery and one harvest season (2024). Although the independent sample replicates demonstrated a good reproducibility of the applied methods, they are not capable of capturing the biological variability associated with different vineyards, environmental conditions, vineyard management practices, or vintages. Consequently, the present findings should be considered as preliminary and a representation of the investigated Negoska lot. Thus, future studies involving multiple vineyards and consecutive vintages are needed to precisely evaluate the natural variability, phenolic composition and antioxidant properties of this indigenous grape cultivar.

5. Conclusions

The present study provides the first comprehensive characterization of the phenolic composition of Negoska grapes, must, wine, and winery byproducts, highlighting the richness of this indigenous Greek cultivar in bioactive phenolic compounds. The results demonstrate substantial changes in phenolic composition during vinification, with the final wine exhibiting a markedly enriched phenolic profile compared with the corresponding must. These changes are consistent with the extraction of phenolic compounds from grape skins and seeds during maceration and with biochemical transformations occurring throughout fermentation. In parallel, grape byproducts, particularly seeds and stems, were identified as rich sources of flavan-3-ols and other phenolic constituents, supporting their potential for sustainable valorization as natural antioxidants and bioactive ingredients for food, cosmetic, nutraceutical, and pharmaceutical applications. Overall, this work provides a valuable baseline for future multi-vintage and comparative studies aimed at further characterizing the Negoska cultivar and exploring its industrial potential.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/app16157689/s1>, Table S1: Transition, collision energy, polarity, and retention time (RT), equation and correlation coefficient (R^2) applied for the determination of each analyte; Table S2: Recovery and matrix effect evaluation of representative phenolic compounds in analyte-poor white wine and white grape matrices; Table S3: Total tannin (TTC), phenolic (TPC), flavonoid (TFC) contents FRAP, and DPPH values of samples tested; Figure S1: Composition of polyphenol classes in the samples. Phenolic Acids: gallic acid, chlorogenic acid, protocatechuic acid, neochlorogenic acid, caffeic acid, p-coumaric acid. Flavan-3-ols (Catechins): epigallocatechin, gallocatechin, catechin, epicatechin, epigallocatechin gallate. Proanthocyanidins: procyanidin B1, procyanidin B2. Other Flavonoids: quercetagenin-7-o-glucoside, isoquercetin, taxifolin, diosmin, herperidin, quercitrin, sophoricoside, luteolin-4'-o-glucoside, myricetin, eriodictyol, luteolin, quercetin, calycosin-7-o-D-glycoside, apigenin, isorhamnetin. Stilbenes: trans-resveratrol, polydatin. Dihydrochalcones: phloridzin, phloretin. Lignans: matairesinol. Phenylethanoids: hydroxytyrosol. Figure S2. Hierarchical clustering of samples based on polyphenol composition.

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