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Impact of Deep Eutectic Solvents on Extraction of Polyphenols from Grape Seeds and Skin

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Featured Application: The interest for green and sustainable utilization of industry by-products, such as grape pomace, is increasing rapidly, and is in line with the recognition of the necessity to protect the environment. Our investigation, mainly focused on natural deep eutectic solvents (NADES), could contribute to further exploitation of these solvents for extraction of biologically active compounds from different plants. Finally, the present study provides profound understanding of antioxidant activity and polyphenol composition of grape seeds and skin, highlighting the quality of some insufficiently investigated Serbian autochthonous varieties and suggesting their broaden use.

Abstract: In the past few years, research efforts have focused on plant exploitation for deriving some valuable compounds. Extraction has been usually performed using petrochemical and volatile organic solvents, but nowadays, increased recognition of environmental pollution has prompted the utilization of green solvents as alternatives. Therefore, the aim of the present study was to exploit deep eutectic solvents (DES) (choline chloride: citric acid and choline chloride: glucose) as solvents for extracting valuable phenolic antioxidants from grapes. Investigation was conducted on ten grape varieties, observing seeds and skin as different matrix. Total polyphenol content (TPC) was determined by *Folin-Ciocalteu* spectrophotometric microassay. Antioxidant activity was investigated using four different tests and results were combined in a unique Antioxidant Composite Index (ACI) to reveal comprehensive information about this biological activity. Polyphenol compounds were identified and quantified with the aim of HPLC-diode array detector (DAD). Overall results support that DES (particularly choline chloride: citric acid) were comparable to conventional solvent, and in most cases even outperformed acidified aqueous ethanol (concerning extraction efficiency and antioxidant activity). Regardless of varietal distinctions, grape seeds have higher antioxidant capacity compared to grape skin, and such findings are according to their phenol compound concentrations.

Keywords: grape seed; grape skin; green extraction; deep eutectic solvents; antioxidant activity; polyphenols

1. Introduction

Grape (*Vitis Vinifera* L. ssp sativa) is generally cultivated in moderate-warm climate zones, and it is of worldwide interest for nutritional and medical purposes [1]. This fruit, as one of the main natural dietary sources of polyphenols, is associated with numerous health benefits. It is important

to emphasize that phenolic composition is affected by the grape variety, degree of ripeness, as well as agronomic and environmental conditions [2]. Moreover, there is a significant difference between parts of fruits (seeds, skin, and stems) considering not only the number of phenolic compounds, but also their concentrations [3]. There are three main classes of polyphenols abounding in grapes: phenolic acids, flavonoids, and stilbenes, each with their own family members. Numerous studies suggest that polyphenols work in more than one way, expressing antioxidant activity and influencing cell communications that affect important biological processes. In particular, grape polyphenols could contribute to a healthy heart by promoting the relaxation of blood vessels to help maintain healthy blood flow and function [4]. Extensive information is available on grape antioxidant activity, concerning the prevention of the degenerative pathophysiological state developed in healthy adults [5].

Approximately 75 million tons of grapes are produced every year worldwide, whereby almost 80% of this quantity is utilized in wine production [6]. Progressively increasing amounts of wine waste represents a serious environmental pollution problem [7,8]. Amid wine generation, most of the valuable health promoting compounds from grape berries are extricated into juice or wine, but a noteworthy sum remains caught within the pomace (skin and seeds). An increasing body of evidence has recently appeared that the revalorization of wine by-products is conceivable. Grape pomace, such as value-added products containing plenty of active compounds, have been proposed for enrichment of food, pharmaceutic, or even cosmetic items [9].

Extraction of valuable and health-promoting constituents was usually performed using petrochemical and volatile organic solvents. Most of these solvents are of serious concern, since they are flammable, low biodegradable, toxic, and volatile. Along with the growing recognition of the human impact on the environment, green extraction has become an optimal and sustainable way for raw material utilization [10]. Nowadays, several types of solvents, including natural deep eutectic solvents (NADES), have been suggested as an alternative for conventional solvents. NADES are prepared by mixing quaternary ammonium salts (e.g., choline chloride) and naturally derived hydrogen bond donors (e.g., sugars, alcohols, amines) [11]. Commonly, NADES components are non-toxic, cheap, and readily available [12]. Due to their unique physicochemical properties, such as adjustable viscosity, wide polarity range, and high solubilization strength for a broad range of compounds variety, NADES have a great potential for different health related purposes [13]. They were confirmed as excellent solvents for sustainable and environmentally friendly extraction. Choline chloride based natural deep eutectic solvents were demonstrated to have high stabilizing ability for phenolic compounds, which can be correlated with the strong hydrogen bonding interactions between solutes and solvent molecules [14]. Besides the extraction, they have been used in enzyme stability and enzymatic reactions [15–17]. Furthermore, the literature indicates that natural deep eutectic solvents could be designed with specific biological activity. They were reported to play an important role in enhancing antioxidative activities of plant extracts by possessing this activity itself [12].

Serbia has a long viticulture tradition (it is one of the oldest grapevine growing areas in Europe) due to its favorable geological position. Concerning the rapid growth of the wine industry, there was a need to perform comprehensive research on biologically active compounds of the grape that would include different varieties cultivated in this area. To the best of our knowledge, most of the studies explored the composition and bioactivity of seeds and/or skins obtained from international varieties [18–20]. Investigations of typical Serbian grapes have been undertaken using only a few autochthonous varieties [21–24]. Keeping in mind all of the mentioned above, the main aim of the present study was to determine total phenolic content and antioxidant capacity of grape seed and skin extracts obtained from ten grape varieties (both international and autochthonous varieties were included). Moreover, the present study was designed as an opportunity to broaden previous investigations of extraction efficiency of NADES [10,25,26] Thus, the complementary aim was to exploit deep eutectic solvents (choline chloride: glucose and choline chloride: citric acid) as solvents for extracting valuable phenolic compounds from the grape. Overall results showed that acid based NADES outperformed acidified aqueous ethanol, concerning both seeds and skin. There were no

significant differences between widely used international and autochthonous varieties. Additionally, seeds and skin obtained from some Serbian traditional grapes were outstanding with markedly high antioxidant potential.

2. Materials and Methods

2.1. Standards and Reagents

Ethanol, Trolox (97%), TPTZ (i.e., 2, 4, 6-tris(2-pyridyl)-s-triazine), DPPH (i.e., 2, 2′-diphenyl-1-picrylhydrazyl), ABTS (i.e., 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), choline chloride, glucose, citric acid. Analytical standards of quercetin 3-*O*-glucoside, (+)-catechin, (−)-epicatechin, protocatechuic acid, and gallic acid were obtained from Sigma (St. Luis, MO, USA). Hydrochloric acid, pyrogallol, *Folin-Ciocalteu* reagent (FC), potassium hydroxide, sodium carbonate, ferric chloride hexahydrate, and potassium peroxydisulfate were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of NADES

Choline chloride (ChCl) was dried in the vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA) for 24 h at 60 °C. NADES solutions with 30% (*v/v*) of water were prepared by mixing water, ChCl and hydrogen bond donor (citric acid (Cit) or glucose (Glc)) at the respective molar ratio (ChCit = 2:1 and ChGlc = 1:1). The mixture was stirred in the sealed flask for 2 h at 50 °C until a homogeneous transparent uncolored liquid was formed.

2.3. Sample Preparation

Ten grape varieties (Table 1) were harvested in autumn 2017 at technological maturity. Immediately after collecting berries, seeds and skins were manually separated. Seeds were washed with cold, distilled water and air dried at room temperature until moisture content was below 10% (approximately three weeks). Crushed seeds were continuously extracted with chloroform for six hours at 70 °C in order to eliminate lipids from the samples. Skins were lyophilized and stored under nitrogen up to subsequent analysis. All samples were grinded into a powder form (BOSCH domestic coffee mill). Extraction was conducted by mixing samples with solvent of interest (70% acidified aqueous ethanol ChCit and ChGlc). The solid-liquid ratio was 1:10. Polyphenol-rich fraction was extracted from grape skins and defatted seeds using an ultrasound bath (FALC, Treviglio, Italy). The extraction parameters were: time 30 min, temperature 50 °C, and frequency 59 kHz [25,27]. Afterwards, mixtures were centrifuged (Janetzki T32 C, Wallhausen, Germany) for 15 min at 5000× *g* and supernatants were decanted to further analysis.

Table 1. Characteristics of grape varieties.

Grape Variety	Origin	Epoch of Maturation	Locality
'Chardonnay' (white)	i	II	3 Morave region- Trstenik
'Bagrina' (white)	a	III	Negotinska Krajina region- Negotin
'Župljanka' (white)	a	III	3 Morave region- Trstenik
'Gamay' (red)	i	II	3 Morave region- Trstenik
'Začinak' (red)	a	III	Negotinska Krajina region- Negotin
'Black Tamjanika' (red)	a	II	Negotinska Krajina region- Negotin
'Merlot' (red)	i	III	3 Morave region- Trstenik
'Prokupac' (red)	a	III/IV	3 Morave region- Trstenik
'Frankovka' (red)	i	II	3 Morave region- Trstenik
'Cabernet Sauvignon' (red)	i	III	3 Morave region- Trstenik

Abbreviations: i—international; a—autochthonous.

2.4. Determination of Total Polyphenol Content (TPC)

Total polyphenol content of extracts was determined using the rapid microtiter plate *Folin-Ciocalteu* method [28]. Briefly, 10 μL of diluted samples and serial standard solutions (gallic acid) were loaded on a 96-well microtiter plate (MTP). The repeated volumes of *Folin-Ciocalteu* reagent diluted 10 times (100 μL), and 1 M Na_2CO_3 (80 μL) were transferred to wells. After an hour of incubation at room temperature in the dark, the absorbance of blue coloration was measured at 630 nm against a blank sample on an MTP reader (BIOTEK, USA, ELx800 Absorbance Microplate Reader). The results were expressed as mg Gallic Acid Equivalents (GAE) per gram of dry weight (mg GAE g^{-1} DW). Calibration curve (y = 0.0028x + 0.0087), prepared for the working solutions of Gallic Acid in the concentration range of 10 to 80 mg L^{-1}, showed good linearity (r^2 = 0.9983). Limit of detection was 1.341 mg L^{-1}, and limit of quantification was 4.470 mg L^{-1}. Spectrophotometric analyses were done in triplicate.

2.5. Antioxidant Activity Evaluation

2.5.1. Ferric Ion Reducing Antioxidant Power (FRAP) Microassay

This test was performed according to Bolanos et al. with some modifications [29]. FRAP working solution was prepared by mixing 300 mM acetate buffer (pH = 3.6), 10 mM TPTZ solution (i.e., 2, 4, 6-tripyridyl-s-triazine in 40 mM HCl) and 20 mM $FeCl_3 \times 6H_2O$ at volume ratio 10:1:1. Diluted samples and Trolox solutions (20 μL) were added together with FRAP working solution (280 μL) in 96-well microplate in triplicate. Reaction mixtures were incubated at 37 °C for 30 min in dark conditions. The absorbance was measured at 630 nm. Antioxidant activity was calculated from calibration curve (y = 1.2368x + 0.0592) with the range 0.1–1 mmol Trolox L^{-1} and with linearity (r^2 = 0.9947). Limit of detection (LOD) was 6 μmol L^{-1}, while limit of quantification (LOQ) was 20 μmol L^{-1}. Results were expressed as mM Trolox Equivalents (TE) per gram of dry weight (mM TE g^{-1} DW).

2.5.2. Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Microassay

CUPRAC assay was done as briefly described Zengin et al. [30]. Diluted extracts and Trolox solutions (67 μL) were pipetted in 96-well microplate in triplicate. Antioxidant capacity was evaluated after 30 min of reaction at room temperature between extracts and 61 μL 0.01 M $CuCl_2$, 61 μL 7.5×10^3 M neocuproine in ethanol and 61 μL ammonium acetate buffer (1 M, pH = 7). Absorbance readings were made against a reagent blank at 450 nm. Calibration curve (y = 1.4453x + 0.0467), calculated using a range 0.05–0.6 mmol Trolox L^{-1}, showed good linearity (r^2 = 0.9968). Limit of detection was 11 μmol L^{-1}, and limit of quantification was 37 μmol L^{-1}. Results were expressed as mM Trolox Equivalents (TE) per gram of dry weight (mM TE g^{-1} DW).

2.5.3. 2, 2'-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Microassay

Antioxidant activity was investigated using DPPH microassay as it was described by Melendez et al. with some modifications [31]. Diluted samples and standard solutions (7 μL) were allowed to react with 193 μL of the DPPH radical solution (1.86×10^{-4} mol L^{-1} DPPH in ethanol, prepared ex tempore) in 96-well microplate in triplicate. The mixtures were shaken and after incubation (1 h at room temperature); the absorbance was measured at 490 nm on an MTP reader. Trolox was used as a standard (range 0.2–1 mmol L^{-1}) for obtaining calibration curve (y = 62.691x − 0.112) with good linearity (r^2 = 0.993). LOD was 0.1 μmol L^{-1}, and LOQ was 0.4 μmol L^{-1}. Results were expressed as mM Trolox Equivalents (TE) per gram of dry weight (mM TE g^{-1} DW).

2.5.4. Trolox Equivalent Antioxidant Capacity (TEAC) Microassay

This method was based on ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) radical decolorization adjusted to a microplate reader [32]. Stock solutions of ABTS (14 mM) and potassium peroxodisulfate (4.9 mM) in phosphate buffer (pH 7.4) were prepared and mixed in equal volumes.

The mixture was left at room temperature in the dark (1216 h) to allow free radical generation. On the day of analysis, the ABTS radical solution was diluted with phosphate buffer in order to achieve an absorbance of 0.7 ± 0.02 at 734 nm (approximately 1:80, *v/v*). The method was performed in the following way: diluted samples and Trolox solutions (20 μL) were added to 280 μL of the ABTS radical solution in wells of MTP in triplicate. Trolox solutions in the concentration range 0.02–0.15 mmol L^{-1} were used for calibration curve (y = 283.25x + 0.2318), with good linearity (r^2 = 0.998). LOD and LOQ were determined (0.1 and 0.5 μmol L^{-1}, respectively). After exactly 6 min, the absorbance readings were taken at 630 nm. Results were expressed as mM Trolox Equivalents (TE) per gram of dry weight (mM TE g^{-1} DW).

2.5.5. Antioxidant Composite Index (ACI)

ACI values were calculated in order to provide a comprehensive information about antioxidant activity of extracts. Combination of the results obtained from all performed tests (FRAP, CUPRAC, DPPH and ABTS) enabled the ACI calculation. An index value of 100 is assigned to the best score for each method and then an index score for all other samples was calculated following the equation:

$$\text{Antioxidant Index Score} = \frac{\text{sample score}}{\text{best score}} * 100 \tag{1}$$

The average of all four tests for each grape variety was then taken as antioxidant potency composite index [33].

2.6. HPLC Analysis

All samples were filtered through 0.22 μm polytetrafluoroethylene (PTFE) filter before the injection. HPLC analyses were performed on the Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA) equipped with a diode array detector (DAD) according to Panić et al., with small modifications [26]. Polyphenols were separated on a Phenomenex C18 column (Kinetex 150 mm × 4.6 mm, 2.6 μm, 100 Å) using the mobile phase, consisted of 2% HCOOH in H_2O (solvent A) and methanol (solvent B) at a flow rate of 0.8 mL/min. Gradient conditions were as follows: 3–8% B linear 0–13 min, 8% B isocratic 13–18 min, 8–10% B linear 18–20 min, 10% B isocratic 20–45 min, 10–100% B linear 45–50 min, 100% B isocratic 50–54 min, 100–3% B linear 54–55 min, 3% B isocratic 55–60 min. The wavelength used for the quantitative determination of catechins and phenolic acids was 280 nm, while flavonol derivatives were detected and quantified at 360 nm. Individual phenolic compounds were identified by comparing their spectral data and retention times with those of the authentic external standards. Calibration curve of external standard was used for their quantification. The calibration curves of standards were made by diluting the stock standards with methanol. Parameters of linear regression, LOD and LOQ for phenolic compounds by HPLC analysis are presented in Table 2.

Table 2. Parameters of linear regression, LOD and LOQ for phenolic compounds by HPLC analysis.

Compound	Concentration Range (mg L^{-1})	Regression Equation	r^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Gallic acid	0.22–60.60	34.008 x − 2.593	0.9996	0.198	0.696
Protocatechuic acid	0.24–490	34.025x + 15.663	0.9999	0.025	0.085
(+)-Catechin	0.43–56.67	7.832 x + 1.208	0.9999	0.191	0.637
(−)-Epicatechin	0.25–41.00	8.111 x − 1.309	0.9999	0.077	0.256
Quercetin 3-*O*-glucoside	0.19–22.40	35.138 x − 3.079	0.9998	0.069	0.231

Abbreviations: LOD: limit of detection; LOQ: limit of quantification.

2.7. Statistical Analysis

The statistical analysis was performed using the software SPSS (Version 20, Chicago, IL, USA). Homogeneity of variance was checked with Levene's test. Independent sample t- test was applied to

evaluate differences between international and autochthonous grapes. Analysis of variance (ANOVA) was used to identify differences between varieties. Tukey's post-hoc test was used for multiple comparisons between groups. The relationship among all results was described by the Pearson's correlation coefficient. *p* value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Total Polyphenol Content (TPC)

Total polyphenol content of grape seeds and skin was investigated in order to emphasize previous disclosures about the importance of profitable and sustainable utilization of wine by-products, considering appreciable amounts of biologically active compounds remained caught within the grape pomace [34,35]. The present study was conducted on seeds and skin from ten different grape varieties, among which, five of them represent Serbian autochthonous varieties ('Bagrina', 'Župljanka', 'Začinak', 'Black Tamjanika', and 'Prokupac'). With intention to compare conventional and natural deep eutectic solvents, investigation of total phenolic content was performed using three different solvents: acidified aqueous ethanol (AcEtOH), and green solvents ChCit, and ChGlc. Natural deep eutectic solvents are designer solvents, and there are more than 10^8 different possible combinations. Commonly, it is recommended to try several typical combinations, which considerably differ in physiochemical properties in order to select a suitable NADES. In our study, choline chloride-based NADES containing citric acid or glucose were chosen based on former reports and their physicochemical properties. For instance, ChCit is characterized with low pH value and with polarity similar to polarity of conventional solvents widely used for polyphenol extraction. Conversely, pH of ChGlc is almost neutral. High stability of phenolic compounds in sugar based NADES, as well as in ChCit, was previously published [10,14]. Furthermore, different water content in NADES should be optimized in order to reduce viscosity that can cause problem with mass transfer and pumping if the NADES are applied in industrial level [36]. In many cases, the addition of water between 20 30% (w/w) reduced viscosity of NADES and beneficially influences extraction yield of both polar and non-polar compounds. According to our experience and previous optimized works with polyphenol extraction, 30% of water was added to the NADES ChCit and ChGlc [25].

Choosing the right NADES for polyphenol extraction is a major part of the extraction process optimization, since it directly influences pH, polarity, and viscosity of a solvent, and as such, directly influences the extraction efficiency. However, NADES choice is followed by selection of the extraction method, which also significantly contributes to the extraction efficiency. In our case, ultrasound assisted extraction (UAE) was chosen since extraction yield could be improved with cavitation phenomena resulting cell leakage and better mass transfer. Additionally, process parameters to achieve the maximum extraction efficiency within NADES should be adjusted. This means optimization of a temperature, extraction time, and power of ultrasound. According to our experience, and previously published and optimized ultrasound assisted extractions of polyphenols from the grape within NADES, extraction was carried out 30 min on 50 °C [25].

TPC values of grape seed extracts obtained from acidified aqueous ethanol ranged from 70.86 to 146.69 mg GAE per gram of dry weight (Figure 1). The highest total phenolic content was extracted from 'Gamay' and 'Prokupac' seeds, while lowest TPC appeared in seeds obtained from 'Merlot' and 'Cabernet Sauvignon' varieties. Our results were similar to those reported for the same varieties [18,22] and slightly lower than seed data obtained for Mediterranean grape pomace by Ky et al. [37]. despite of the similarity of the varieties included in our and in the study conducted by Pantelic et al., minor observed differences are presumably present due to seasonal variations of weather, agronomical practices, and vineyard management [22]. Nevertheless, mentioned study also reported the highest total polyphenol content in seeds obtained from Serbian autochthonous variety 'Prokupac'. Regarding NADES, TPC values of grape seed extracts ranged from 92.36 to 182.60 and 56.17 to 156.17 mg GAE per gram of dry weight for ChCit and ChGlc extracts, respectively (Figure 1). ChCit had higher extraction

efficiency than ChGlc in almost all varieties (with the exception of seeds of two red grape varieties: 'Merlot' and 'Prokupac'). Interestingly, seeds from three Serbian autochthonous varieties, 'Prokupac', 'Black Tamjanika' and 'Župljanka', stood out with pronounced high TPC content in both NADES.
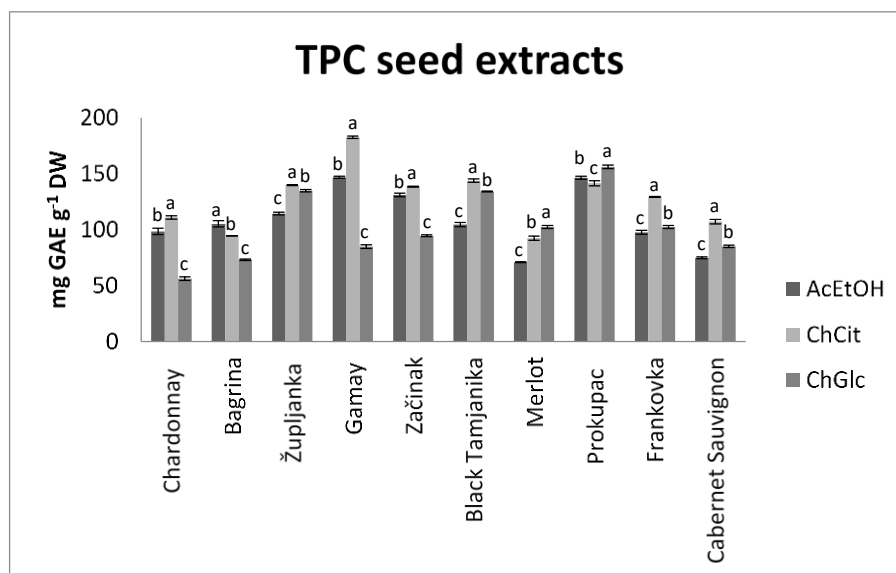

Figure 1. Total polyphenol content of seed extracts obtained from ten grape varieties. Data are expressed as mg GAE g^{-1} DW for each variety, means ± SD, *n* = 3. Different lower case letters (a–c) indicate significant differences among solvents ($p \leq 0.05$) based on post hoc Tukey's test. Abbreviations: TPC—total polyphenol content; GAE—gallic acid equivalents; DW—dry weight; SD—standard deviation; AcEtOH—acidified aqueous ethanol; ChCit—choline chloride: citric acid; ChGlc—choline chloride: glucose.

By analyzing skin extracts (Figure 2) obtained using acidified aqueous ethanol, 'Merlot', 'Cabernet Sauvignon', and 'Začinak' were outstanding with the highest TPC (16.21, 16.14, and 15.00 mg GAE g^{-1} of DW, respectively). Concerning only white varieties, 'Župljanka' (9.41 mg GAE g^{-1} of DW) had the highest total phenolic content. The results of TPC in skin extracts revealed in the present study were in good agreement with literature [18,23]. When it comes to NADES skin extracts (Figure 2), ChCit outperformed ChGlc, with some exceptions. ChCit and acidified aqueous ethanol showed similar potential considering solvent extractability; therefore, the same varieties ('Cabernet Sauvignon', 'Merlot', and 'Začinak') had the highest total phenolic content in both extracts. ChGlc distinguished those, but also 'Frankovka', as grape varieties, which skin is abundant in phenolic compounds. The present study confirmed the observations of Cvjetko-Bubalo et al., who screened five different DES and proposed acid based NADES as the most promising for extraction of grape skin polyphenols [25].

In general, TPC values varied significantly among varieties (Figures 1 and 2). In addition, it is clearly observed that total phenolics of grape skin extracts were several times lower than those determined for grape seed extracts. These findings were in accordance with previous reported data for the *Vitis vinifera* species [18]. In addition, TPC values determined for red skin were slightly higher in comparison with white skin extracts.

Extracting solvent affected total polyphenol content of both seeds and skins (Figures 1 and 2). It is known that physicochemical properties play an important role in solvents selectivity towards some particular compounds. More polar solvent is expected to have a higher yield of polar molecules in comparison with non-polar ones. Thus, differences in extraction efficiency between ChCit and ChGlc could be present due to distinction in their polarity (acid based NADES are more polar). Solvent acidity is another important feature found to affect extraction selectivity. For example, anthocyanins are flavonoid phenolic compounds whose chemical form and stability depend on pH value. Namely,

they are prevalent in the flavylium cation form, which is stable at low pH. With the increased pH value, they undergo structural transformation into forms prone to further degradation due to their instability [25,38]. Therefore, extraction of these polyphenols is enhanced along with the growing acidity of a solvent. This could be a possible explanation for lower TPC values found in most ChGlc extracts, considering almost neutral pH of this solvent (pH values of AcEtOH and ChCit are both less than 7). ChCit showed equal or even higher extraction efficiency than referent solvent, acidified aqueous ethanol, observing both, seeds and skin. Extraordinary extraction capacity of ChCit extracts confirmed previous findings about high extraction ability of acid based NADES for some phenolic compounds [25,38–40]. Moreover, acidified aqueous ethanol and ChCit extracts were significantly correlated (Pearson's coefficient of 0.792 and 0.950; $p < 0.01$, for seeds and skin, respectively); due to similar acidity and polarity of the solvents.
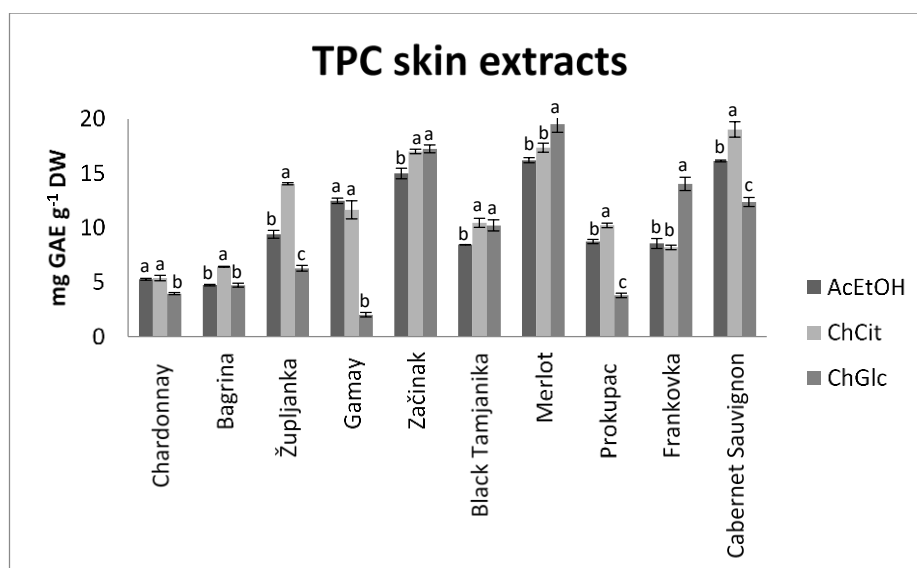

Figure 2. Total polyphenol content of skin extracts obtained from ten grape varieties. Data are expressed as mg GAE g^{-1} DW for each variety, means ± SD, $n = 3$. Different lower case letters (a–c) indicate significant differences among solvents ($p \leq 0.05$) based on post hoc Tukey's test. Abbreviations: TPC—total polyphenol content; GAE—gallic acid equivalents; DW—dry weight; SD—standard deviation; AcEtOH—acidified aqueous ethanol; ChCit—choline chloride: citric acid; ChGlc—choline chloride: glucose.

3.2. Antioxidant Activity

Dietary antioxidants, widely spread in fruits and vegetables, have received considerable scientific attention and investigation of their bioactivity became the main goal of many conducted studies. Regardless of the large number of validated antioxidant in in vitro tests, there is still a problem concerning the differences in chemical principles and mechanisms of the most common methods. In order to provide comprehensive information about total antioxidant capacity, and to distinguish the dominant mechanism of antioxidant activity, it was suggested to perform several tests pointing different aspects of antioxidant properties [41]. Therefore, four antioxidant methods were conducted—FRAP, CUPRAC, DPPH, and ABTS (Table S1: Antioxidant activity of grape seed extracts, Table S2: Antioxidant activity of grape skin extracts).

FRAP is a typical electron transfer method based on the capability of antioxidants to reduce Fe^{3+} tripyridyl triazine complex (colorless) to Fe^{2+} tripyridyl triazine (blue color) in acidic medium. CUPRAC assay measures the ability of test compounds to reduce Cu^{2+} to Cu^{+} in aqueous-ethanolic medium (pH 7.0) in the presence of neocuproine. DPPH assay, the oldest indirect method for evaluating antioxidant capacity, measures the reduction of stable organic DPPH radical in presence of antioxidants

to the yellow colored 2, 2-diphenyl-1-picrylhydrazyn. Another commonly used radical scavenging test is ABTS/TEAC assay, based on the ability of test compounds to reduce ABTS radical [41].

Taking into account many limitations and lacks of each test applied for evaluating antioxidant activity, results obtained from them were used for calculating unique antioxidant composite index (ACI). ACI is a simple, widely used mathematical calculation that enables deeper insight into antioxidant activity, since it combines several tests based on different chemical mechanisms.

ACI values calculated for grape seed extracts are presented in Figure 3. Regarding conventional solvents, the highest antioxidant activity was determined for seeds obtained from 'Gamay' variety. Extraction with ChCit also revealed 'Gamay' as the most potent, followed by Serbian autochthonous varieties, such as 'Black Tamjanika' and 'Prokupac'. In the case of ChGlc, the highest ACI value was calculated for seed extracts of 'Prokupac' variety. When it comes to grape skin (Figure 4), ACI calculation revealed apparently red skin prominence in antioxidant actions. 'Merlot', 'Začinak', and 'Cabernet Sauvignon' were characterized with the highest antioxidant capacity. Observed differences among varieties, regarding both seeds and skin, represent a confirmation of many research studies conducted so far [20,42].
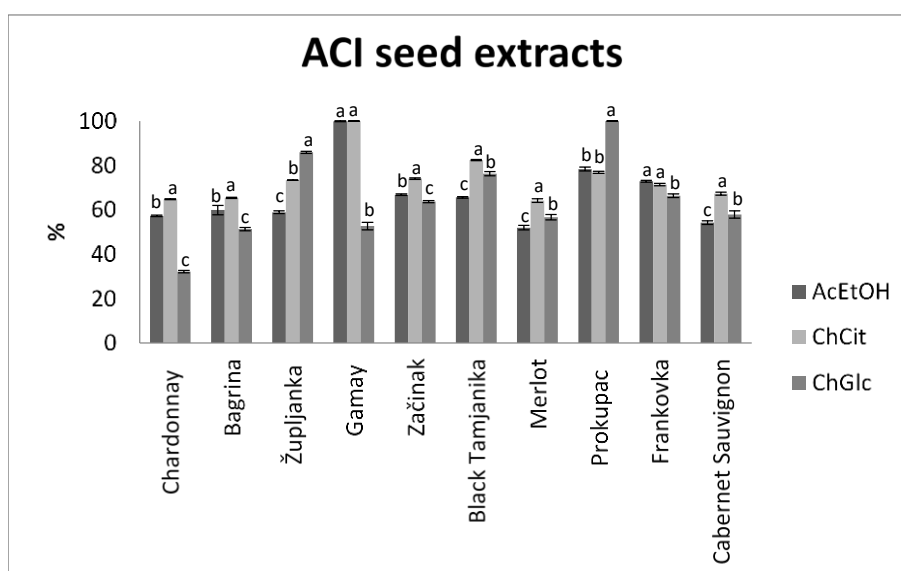

Figure 3. Antioxidant composite index- grape seed extracts.Data are expressed as % for each variety, means ± SD, *n* = 3. Different lower case letters (a–c) indicate significant differences among solvents (*p* ≤ 0.05) based on post hoc Tukey's test. Abbreviations: ACI—Antioxidant Composite Index; SD—standard deviation; AcEtOH—acidified aqueous ethanol; ChCit—choline chloride: citric acid; ChGlc—choline chloride: glucose.

ACI values for both, seeds and skin (Figures 3 and 4) were significantly correlated with TPC of corresponding extracts (0.798 ≤ r ≤ 0.967; *p* < 0.01). Therefore, such strong relationship confirmed the previous disclosures about the significant contribution of polyphenol compounds to the antioxidant activity. Nevertheless, prominent antioxidant activity of ChCit extracts was revealed. Higher antioxidant activity of these extracts in comparison with ChGlc and acidified aqueous ethanol extracts could be explained by the antioxidant activity of ChCit itself, or by antioxidant activity of NADES forming compound, citric acid. This naturally occurring organic acid possess antioxidant and anti-inflammatory properties. Furthermore, antitumor activity of this compound has been reported [43]. Citric acid is widely used food additive, approved and closely regulated as acidulant, pH regulator, flavoring agent, preservative and antioxidant synergist in soft drinks, baked nutrients, jam, candy, jelly sweet, marmalade, and tinned vegetable [44].
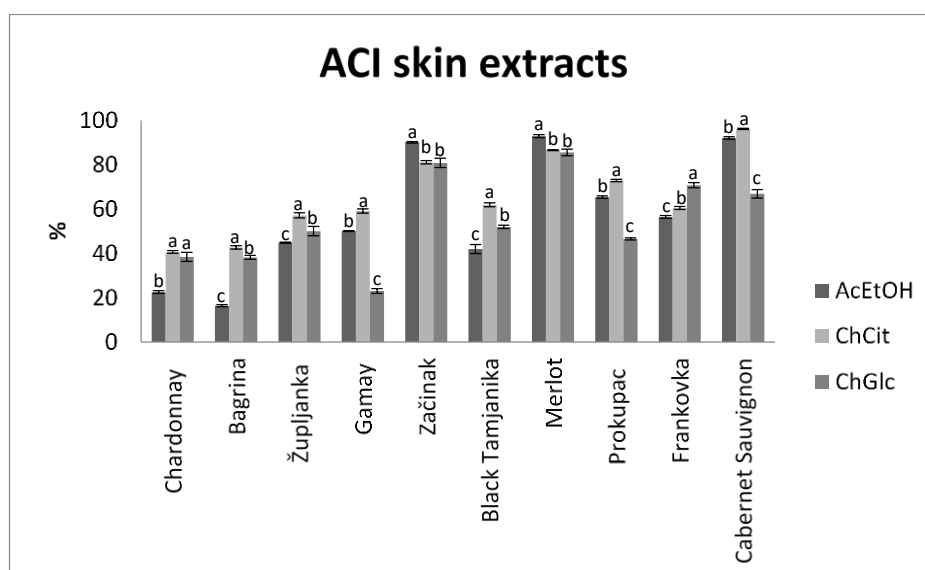
Figure 4. Antioxidant composite index- grape skin extracts. Data are expressed as % for each variety, means ± SD, *n* = 3. Different lower case letters (a–c) indicate significant differences among solvents ($p \leq 0.05$) based on post hoc Tukey's test. Abbreviations: ACI—Antioxidant Composite Index; SD—standard deviation; AcEtOH—acidified aqueous ethanol; ChCit—choline chloride: citric acid; ChGlc—choline chloride: glucose.

3.3. Phenolic Composition

Structural and physicochemical features of a solvent have a strong influence on selectivity towards biologically active compounds. In general, considering solvent extractability, the highest content of polyphenols from both, seeds and skin, was obtained with ChCit. Thus, further HPLC analysis was done to precisely characterize the phenolic profile of such ChCit extracts. Moreover, acidified aqueous ethanol extracts were also screened in order to compare NADES versus conventional solvent in terms of extraction efficiency for some of the main grape polyphenols (gallic acid and protocatechuic acid within the phenolic acids group, (+)-catechin and (−)-epicatechin as flavan-3-ols representatives, quercetin 3-*O*-glucoside like flavonol main member).

The sum of identified polyphenols by HPLC varied noticeably among varieties, for both seed and skin extracts. Polyphenol compositions evaluated by HPLC-DAD analysis of grape seed extracts are presented in Table 3. Flavan-3-ols were predominantly present, which is in accordance with previously published data [22,45]. (+)-Catechin was the most abundant followed by (−)-epicatechin. Gallic acid and protocatechuic acid were present in smaller quantities. When it comes to grape skin (Table 4), distribution of phenolic compounds was significantly different amid varieties, which is in accordance with literature data [46]. Nevertheless, grape skin peaks could be assigned to the four different compounds: (+)-catechin, (−)-epicatechin, protocatechuic acid, and quercetin 3-*O*-glucoside.

Regardless of the similar polarity and acidity of extraction solvents, differences between acidified aqueous ethanol and ChCit selectivity were observed. Conventional solvent, almost without the exception, had a higher affinity than NADES for (+)-catechin, (−)-epicatechin, and gallic acid in seeds. The highest concentrations of (+)-catechin were determined in the seeds of 'Župljanka' (15.587 and 10.197 mg g^{-1} DW, for acidified aqueous ethanol and ChCit, respectively), followed by 'Začinak' and 'Prokupac'. 'Prokupac' was the variety with the highest content of (−)-epicatechin, regardless the solvent. The abundance of dominant flavan-3-ols was in agreement with Ky et al. [34] and Bakkalbaşı et al. [47]. Gallic acid was predominantly present hydroxybenzoic acids. Interestingly, there was no difference between seeds obtained from white and red varieties regarding gallic acid content, although such observation was previously reported [21,48]. Among all varieties, red grape 'Prokupac'

showed significantly higher amounts of gallic acid (2.450 and 1.850 mg g^{-1} DW, for acidified aqueous ethanol and ChCit seed extracts, respectively).

Table 3. Phenolic composition of grape seed extracts.

Grape Variety	Solvent	Gallic Acid (mg g^{-1} DW)	Protocatechuic Acid (mg g^{-1} DW)	(+)-Catechin (mg g^{-1} DW)	(−)-Epicatechin (mg g^{-1} DW)	Total (mg g^{-1} DW)
'Chardonnay'	AcEtOH	0.947 [e]		3.661 [d]	1.737 [d]	6.345 [d]
	ChCit	0.885 [e]	0.482 [b]	3.347 [d]	1.466 [d]	6.181 [d]
'Bagrina'	AcEtOH	1.204 [d]	0.158 [e]	4.355 [d]	3.605 [c]	9.322 [c]
	ChCit	1.142 [d]	0.249 [d]	3.045 [d]	1.626 [d]	6.062 [d]
'Župljanka'	AcEtOH	1.546 [c]	0.569 [a]	15.587 [a]	4.391 [b]	22.093 [a]
	ChCit	1.219 [d]	0.605 [a]	10.197 [b]	2.808 [d]	14.829 [b]
'Gamay'	AcEtOH	0.689 [f]	0.101 [e]	2.911 [d]	2.371 [d]	6.073 [d]
	ChCit	1.096 [d]	0.291 [c]	3.998 [d]	1.785 [d]	7.169 [d]
'Začinak'	AcEtOH	1.230 [d]	0.115 [e]	6.884 [c]	2.189 [d]	10.419 [c]
	ChCit	1.167 [d]	0.214 [e]	4.883 [d]	0.948 [e]	7.213 [d]
'Black Tamjanika'	AcEtOH	1.691 [c]	0.228 [e]	4.937 [d]	3.550 [c]	10.406 [c]
	ChCit	1.519 [c]	0.444 [b]	4.248 [d]	2.292 [d]	8.504 [d]
'Merlot'	AcEtOH	0.992 [e]	0.140 [e]	3.799 [d]	2.356 [d]	7.287 [d]
	ChCit	0.940 [e]	0.276 [d]	3.020 [d]	1.275 [e]	5.510 [d]
'Prokupac'	AcEtOH	2.450 [a]	0.219 [e]	6.709 [c]	6.269 [a]	15.647 [b]
	ChCit	1.850 [b]	0.383 [c]	5.338 [d]	2.999 [d]	10.570 [c]
'Frankovka'	AcEtOH	1.908 [b]	0.522 [b]	5.224 [d]	5.140 [b]	12.794 [c]
	ChCit	1.315 [d]	0.508 [b]	3.281 [d]	2.705 [d]	7.810 [d]
'Cabernet Sauvignon'	AcEtOH	0.786 [f]	0.135 [e]	3.960 [d]	1.797 [e]	6.679 [d]
	ChCit	0.745 [f]	0.262 [d]	3.177 [d]	0.970 [e]	5.154 [d]

Values represent mean of three replicates. Standard deviation was < 5%. Different lower case letters (a–f) indicate significant differences among solvents and varieties ($p \leq 0.05$) based on post hoc Tukey's test. Abbreviations: AcEtOH—acidified aqueous ethanol, ChCit—choline chloride: citric acid; DW—dry weight.

Table 4. Phenolic composition of grape skin extracts.

Grape Variety	Solvent	Protocatechuic Acid (mg g^{-1} DW)	(+)-Catechin (mg g^{-1} DW)	(−)-Epicatechin (mg g^{-1} DW)	Quercetin 3-O-Glucoside (mg g^{-1} DW)	Total (mg g^{-1} DW)
'Chardonnay'	AcEtOH		0.240 [b]			0.240 [e]
	ChCit	0.562 [b]	0.245 [b]			0.807 [e]
'Bagrina'	AcEtOH	0.139 [c]	0.227 [b]			0.366 [e]
	ChCit	0.293 [c]	0.294 [b]			0.587 [e]
'Župljanka'	AcEtOH		0.277 [b]			0.277 [e]
	ChCit	0.147 [c]	0.275 [b]	0.035 [d]		0.457 [e]
'Gamay'	AcEtOH			0.429 [c]	0.115 [e]	0.544 [e]
	ChCit	0.330 [c]	0.146 [d]	0.689 [c]		1.165 [d]
'Začinak'	AcEtOH		0.095 [e]	2.479 [b]	0.312 [d]	2.886 [c]
	ChCit	1.663 [a]	0.191 [c]	2.768 [b]	0.274 [d]	4.896 [b]
'Black Tamjanika'	AcEtOH				0.348 [d]	0.348 [e]
	ChCit	0.275 [c]	0.061 [e]	0.032 [d]		0.369 [e]
'Merlot'	AcEtOH		0.060 [e]	2.066 [b]		2.127 [c]
	ChCit	0.085 [c]	0.265 [b]	4.654 [a]	0.334 [d]	5.338 [b]
'Prokupac'	AcEtOH		0.061 [e]	0.068 [d]	0.187 [e]	0.316 [e]
	ChCit	0.166 [c]	0.252 [b]	0.222 [c]	0.130 [e]	0.770 [e]
'Frankovka'	AcEtOH		0.085 [e]	0.106 [c]	0.420 [c]	0.611 [e]
	ChCit	0.065 [c]	0.307 [a]	0.329 [c]		0.701 [e]
'Cabernet Sauvignon'	AcEtOH		0.017 [e]	2.612 [b]	0.569 [b]	3.198 [c]
	ChCit	0.098 [c]	0.180 [c]	5.219 [a]	0.739 [a]	6.237 [a]

Values represent mean of three replicates. Standard deviation was < 5%. Different lower case letters (a–e) indicate significant differences among solvents and varieties ($p \leq 0.05$) based on post hoc Tukey's test. Abbreviations: AcEtOH—acidified aqueous ethanol, ChCit—choline chloride: citric acid; DW—dry weight.

Interestingly, ChCit skin extracts contained higher amounts of (+)-catechin and (−)-epicatechin in comparison with acidified aqueous ethanol extracts. (−)-Epicatechin was dominantly present in red grape skin and the highest concentrations of this compound were determined in 'Cabernet Sauvignon' (2.612 and 5.219 mg g^{-1} DW, for acidified aqueous ethanol and ChCit extracts, respectively). Among white varieties, isomer (+)-catechin was the most abundant.

ChCit also showed better extraction efficiency for protocatechuic acid irrespective of investigated matrix. Concerning seeds, 'Župljanka' was characterized with the highest concentration of protocatechuic acid (0.569 and 0.605 mg g^{-1} DW, for acidified aqueous ethanol and ChCit, respectively). When it comes to skin, acidified aqueous ethanol demonstrated extremely low extraction efficiency towards protocatechuic acid. Concentrations of protocatechuic acid in ChCit skin extracts varied from 0.065 to 1.663 mg g^{-1} DW.

The concentration of quercetin 3-*O*-glucoside, found in skin extracts obtained from red grape varieties, tended to be higher in acidified aqueous ethanol compared to NADES, with some exceptions. The highest concentration of this compound was observed in skin of 'Cabernet Sauvignon' (0.569 and 0.739 mg g^{-1} DW, for acidified aqueous ethanol and ChCit, respectively).

In general, 'Župljanka' seeds distinguished with the highest content of total phenolics evaluated applying HPLC, regardless the solvent. Such domination of 'Župljanka' among the varieties with the highest TPC content determined by *Folin-Ciocalteu* method, such as 'Gamay', 'Prokupac', and 'Black Tamjanika' is due to remarkably higher (+)-catechin concentrations in 'Župljanka' seeds. Thus, it is expected that mentioned varieties have higher concentrations of some unquantified polyphenol compounds in seeds. When it comes to grape skin, highly pigmented red varieties, such as 'Cabernet Sauvignon', 'Začinak', and 'Merlot' could be emphasized as cultivars extremely rich in phenolic compounds.

Finally, chromatographic analysis of obtained extracts revealed some interesting observations. Namely, acidified aqueous ethanol was more efficient in extracting phenolics from grape seeds, while ChCit have proved more selective towards target polyphenol compounds in grape skin. Considering the domination of ChCit in TPC determination, such lack of consistency in results obtained from HPLC analyses could be explained by the presence of some phenolic compounds that we were not able to quantify. For example, flavanols derivatives and polymers such as: (−)-epicatechin gallate, epigallocatechin gallate, and proanthocyanidins have been identified in both seeds and skin extracts, although seeds contained significantly higher amounts. Perhaps the quantification of these compounds would shed a new light on a phenolic composition of grape seeds, since it was previously reported that acid based NADES possess an excellent extraction performance for catechins [49].

The food industries add value both by reducing waste disposal and by transforming by-products into new value food items. Based on obtained results in this study, we would like to suggest utilization of grape pomace as a good source of biologically active compounds. Recovery of polyphenols from grape waste appears to be a new strategy in commercialized applications especially using ecological and sustainable extraction.

4. Conclusions

Considering a diverse array of biologically active compounds present in grape, valorization of grape pomace by extracting potent antioxidants represents a great challenge. This study investigated antioxidant potential and polyphenol composition of seeds and skin obtained from some widely grown grapes with an emphasis on Serbian old autochthonous varieties. International grapes did not surpass autochthonous ones concerning parameters of interest. Moreover, some Serbian varieties distinguished as potent sources of polyphenols. For example, high polyphenol concentrations were determined in 'Prokupac' and 'Black Tamjanika' seeds. Furthermore, variety 'Župljanka' was outstanding with significantly higher amounts of catechins in seeds. When it comes to skin, noteworthy TPC values were determined for 'Začinak' variety. Therefore, broaden exploitation of seeds and skin from Serbian traditional varieties is strongly recommended.

Additionally, because of a growing awareness about environmental pollution, there is a need to minimize and even eliminate the use of hazardous chemicals in extraction processes. Within this research, it was shown that conventional solvents could be replaced with natural deep eutectic solvents, yielding the same or better polyphenol content. NADES were reported as safe for human consumption, and they could be used in the industry without difficult and expensive downstream purification steps.

Since ChCit exhibited high extraction efficiency towards polyphenols from grape seeds and skin, the present work strongly emphasizes its utilization for green and sustainable extraction of biologically active compounds from wine industry pomace.

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