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INFLUENCE OF EXTRACTION CONDITIONS ON CONTENT OF BIOLOGICALLY ACTIVE SUBSTANCES FROM POMACE OF CABERNET SAUVIGNON AND RARA NEAGRA GRAPES

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Abstract. Grape pomace contains many anthocyanins, catechins, flavonoids, phenolic acids, and stilbenes, so it must be utilized. The research used grape pomace from Cabernet Sauvignon and Rara Neagra, harvested in 2023. When creating the absorption spectra, a high content of anthocyanins was observed – components that describe the most significant increases in the spectral line at wavelengths between 510 and 550 nm, specific to these phenolic compounds, confirming the participation of anthocyanins in the formation of the color of grapes as main constituents. Studying the anthocyanin content showed that most pigments that gave the red color were found in acidulated samples of Cabernet Sauvignon, with an extraction temperature of 40°C. The same trend was observed in the acidulated extract of Rara Neagra with extraction temperatures of 40°C and 55°C. When determining the total phenolic content, values ranged between 4.59-5.16 mg GAE/g DW for Cabernet Sauvignon and 4.62-5.98 mg GAE/g DW for Rara Neagra. The antioxidant activity varied in Cabernet Sauvignon between 72.06-86.4%, and in Rara Neagra between 76.79-88.64%. The lowest values were recorded in the non-acidulated samples: 72% for Cabernet Sauvignon and 76.8% for Rara Neagra. As a result, acidulated extracts were the richest in biologically active substances.

Key words: *anthocyanins, extract, phenolic substances, grape pomace.*

Rezumat. Tescovina de struguri conține mulți antocieni, catechine, flavonoide, acizi fenolici și stilbene, argumentul acestei valorificări. Spre studiu s-a folosit tescovina de struguri Cabernet Sauvignon și Rară Neagră, roada anului 2023. La realizarea spectrelor de absorbție s-a observat un conținut ridicat de antocieni – componente ce descriu cele mai semnificative creșteri ale liniei spectrale la lungimile de undă cuprinse între 510 și 550 nm, specifică acestor compuși fenolici, confirmând participarea antocienilor la formarea culorii boabelor de struguri, ca constituenți principali. Studierea conținutului de antocieni, a demonstrat că cei mai mulți pigmenți ce conferă culoare roșie s-au conținut în probele de Cabernet Sauvignon acidulate, cu temperatura de extracție 40°C. Aceeași tendință s-a observat și in cazul extractului de Rară Neagră acidulată cu temperatura de extracție 40°C și 55°C. La determinarea conținutului total de substanțe fenolice, valorile au fost cuprinse între 4,59-5,16 mg GAE/g extract la Cabernet Sauvignon și 4,62-5,98 mg GAE/g extract pentru Rară

Neagră. Activitatea antioxidantă a variat la Cabernet Sauvignon între 72,06-86,4 %, iar la Rară Neagră între 76,79-88,64%. Valorile cele mai mici s-au înregistrat la probele neacidulate: 72% Cabernet Sauvignon și 76,8% Rară Neagră. Prin urmare, extractele acidulate au fost cele mai bogate în substanțe biologic active.

Cuvinte cheie: *antocieni, extract, substanțe fenolice, tescovină.*

1. Introduction

In the production of wines, organic waste is generated, consisting of grape pomace (62%), yeast (14%), stems (12%), and sludge (12%) [1]. Grape pomace, composed of seeds and fruit skins produced during grape pressing, contains many bioactive compounds including anthocyanins, catechins, flavonoids, phenolic acids, and stilbenes [1]. This makes grape pomace a valuable source of phenolic compounds with high fiber content, suggesting its potential use as a functional food ingredient. During winemaking, some phenolic compounds end up in the wine, and a large portion is transferred to grape pomace, making it a cheap and natural source for extracting phenolic compounds and offering an alternative solution to environmental problems caused by their disposal [2].

Grape pomace has several beneficial properties. It has antioxidant activity, as the addition of grape pomace powder significantly increases the content of free bioavailable polyphenols, which may be lacking in some food products [3]. This can help in the creation of nutritious ready-to-eat products, enrich product characteristics, and significantly extend shelf life. Polyphenols in grape pomace also exhibit antimicrobial activity, potentially inhibiting the development of various bacteria. In their study, Ghendov-Mosanu et al. [4] found that grape pomace extract had a clear bactericidal activity against Gram-positive bacteria such as *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923) and against Gram-negative bacteria such as *Escherichia coli* (ATCC 25922). This effect was attributed to polyphenols, which can destabilize and modify the cytoplasmic membrane's permeability and inhibit nucleic acid synthesis in both Gram-negative and Gram-positive bacteria [4].

Moreover, recent evidence suggests that polyphenols in grape pomace can stimulate the growth of probiotic microorganisms and modify gut microbiota in terms of composition and functionality, indicating prebiotic activity [5]. The complex chemistry of grape pomace suggests that it can be used in the food, pharmaceutical, and cosmetic industries [6,7]. Recently, the anticancer properties of polyphenols in grape pomace have been demonstrated, showing a capacity to affect the proliferation of cancer cells [3]. Additionally, grape pomace has a cardioprotective effect by acting on the platelet aggregation mechanism, and its use as a seasoning can significantly reduce blood pressure and blood sugar levels [8].

Polyphenols in grape pomace also possess high antioxidant power, which prevents the formation of free radicals and blocks premature skin aging, protecting the skin from damage caused by ultraviolet rays and external weather agents such as wind, humidity, or temperature changes [9]. Today, many food additives and nutritional products derived from grape pomace, such as grape skin powder, grape seeds, and anthocyanin colorants, are available in the market. Grape pomace, a cheap by-product containing valuable products, is proposed as a food additive/ingredient for developing new products with technological and functional advantages. Including grape pomace in the food industry could be a step towards the future of food and human health, offering new functional foods and contributing to solving waste management issues in the wine industry [10,11].

The purpose of this study is to research the composition and properties of hydro-alcoholic extracts from Cabernet Sauvignon and Rara Neagra grapes harvested in 2023, obtained under different extraction conditions.

2. Materials and Methods

2.1 Raw Materials

Practical scientific studies focused on the preparation and compositional analysis of extracts from fermented grape pomace of red Cabernet Sauvignon and Rara Neagra grapes harvested in 2023 were conducted. The red Cabernet Sauvignon grapes originated from the Codru region, Milestii Mici locality, and those of Rara Neagra from the Ștefan Vodă region, Purcari commune, were technologically processed according to the classic vinification scheme within the Microvinification section of the Department of Oenology and Chemistry.

Reagents. The Folin–Ciocalteu reagent Merck (Darmstadt, Germany), gallic acid 98%, 2,2-diphenyl-1-picrylhydrazyl (DPPH) 90 %, sodium carbonate (Na_2CO_3) powder 99.5 %, ethanol (C_2H_5OH) 98%, sodium hydroxide (NaOH) solution 33% and hydrochloric acid (HCl) 37.5% were obtained from Sigma-Aldrich. All other chemicals were analytical reagent grade.

2.2. Obtaining grape pomace extracts

Samples of grape pomace, after being evacuated from the pneumatic press, were dried at a temperature of 55 ± 2°C, in oven BOV-T30C, ground with an electric mill Bosch KM13 and used to obtain the study extract by technological scheme included in Figure 1.

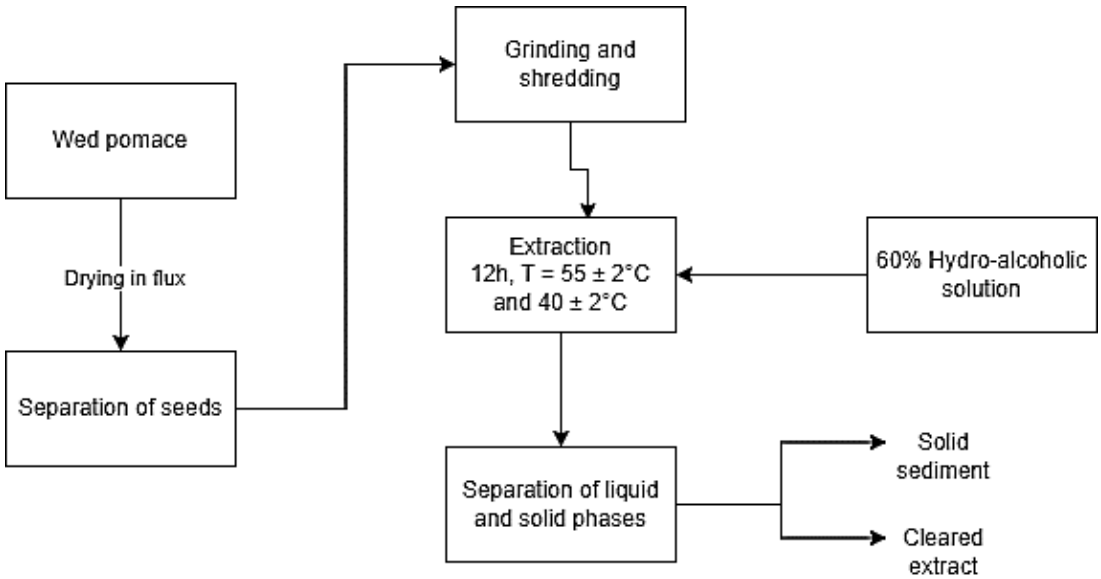

Figure 1. Technological scheme for obtaining the study extracts.

The solvent used for extraction was 60% ethyl alcohol acidified with 1 % hydrochloric acid [12] in a ratio of 1:10 (*m/v*) by volume with grape pomace powder. The experimental extraction regime included 12 hours of periodic stirring at 50 rpm at temperatures of 40±2°C and 55±2°C. After extraction, the hydro-alcoholic samples were filtered and kept in the dark in Eppendorf PCR tubes at 20 ±1°C [13].

2.3 Methods of characterizing the study extracts

Titratable acidity (AT) and pH value of the obtained extracts were established using the specialized literature [14], for the pH value having been employed the InoLab pH 7110. Dry substance content was established using ATAGO Pocket refractometer PAL-1.

To establish the value of certain chromatic indices (Ic - color intensity, color purity), and the specific profile of anthocyanins, appreciation of phenolic compounds, analysis methods present in the specialized literature were used [14], employing the T80 UV/VIS Spectrophotometer from PG Instruments Ltd.

2.4 Assessment of the chromatic index (Ic)

The experimental samples were centrifuged (2000 rpm for 15 minutes), and the absorbance was measured at 420, 520, and 620 nm, in 1 mm cuvettes. Each of these wavelengths corresponds to its light spectrum: yellow (420 nm), red (520 nm), and violet (620 nm), respectively. Based on the values obtained from the T 80 spectrophotometer, the chromatic parameters were calculated according to expressions (1) - (5):

Color intensity: $$I_c = A_{420} + A_{520} + A_{620} \quad (1)$$

Hue color: $$H_c = \frac{A_{420}}{A_{520}} \quad (2)$$

Color purity: yellow (Y), red (R) and violet (V):

$$Y = \frac{A_{420}}{I_c} * 100\% \quad (3)$$

$$R = \frac{A_{520}}{I_c} * 100\% \quad (4)$$

$$V = \frac{A_{620}}{I_c} * 100\% \quad (5)$$

where: A_{420} – absorbance at 420 nm;

A_{520} – absorbance at 520 nm;

A_{620} – absorbance at 620 nm.

2.5 Assessment of antioxidant capacity: DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals react with suitable reducing agents losing color stoichiometrically with the number of electrons consumed which was spectrophoto-metrically measured at 517 nm [15]. The study samples were centrifuged (2000 rpm for 15 minutes), adding in each glass test tube 0.1 mL of the extracts sample and 3.9 mL of DPPH (60 μM), mixing the contents, and after 30 min, measuring the absorbance at 517 nm in 10 mm cuvettes. The 50% methanol solution was used as a control. The estimated assessment of the inhibition ratio (percent) was performed using the following equation:

$$IR = \frac{A_{control} - A_{sample}}{A_{control}} * 100\ \% \quad (6)$$

where: $A_{control}$ – absorbance of control (pure DPPH sample);

A_{sample} – absorbance of sample.

2.6 Summary appreciation of phenolic compounds

In a 100 cm³ volumetric flask, add 1 cm³ of extract at a 1:5 ratio, 50 cm³ of distilled water, 5 cm³ of Folin-Ciocalteu reagent, and 20 cm³ of 20% Na_2CO_3 solution. Bring the solution to the mark with distilled water, shake thoroughly, and after 30 minutes, determine the optical absorbance at 750 nm in 10 mm cuvettes compared to distilled water. If the absorbance is not close to 0.3 UV/VIS absorbance units, repeat the determination by adjusting the dilution of the extract to achieve a value close to 0.3 units. The results were calculated

from a calibration curve using gallic acid (0–500 mg/L) and expressed in equivalents of gallic acid per 1 g of dried weight (DW) of grape marc extract (mg GAE/g DW).

2.7 Determining anthocyanin content

Involves introducing 3 cm³ of clear extract into a 25 cm³ pycnometer. Add 12.5 cm³ of rectified ethyl alcohol acidified to pH 1.2 and 3 drops of concentrated hydrochloric acid. Bring the solution to the mark with distilled water, shake vigorously, and centrifuge for 15 min at 1500 rpm. Measure the absorbance of the obtained solution at a wavelength of 520 nm in a 1 mm cuvette compared to distilled water. The anthocyanin content is calculated according to the following expression:

$$C_{ant} = 1056.7 \cdot A \quad (7)$$

where: A – absorbance of sample;

1056.7 – transfer coefficient, established for malvidin-3-glucoside isolated from grape skins.

2.8 Statistical Analysis

The experimental data were processed statistically using Microsoft Office Excel 2007 to determine the mean values along with the standard error. Using a significance level of $p < 0.05$, statistical tests such as ANOVA (analysis of variance, a statistical method used to test for significant differences between the means of experimental data groups) and PCA (principal component analysis, used for analyzing the linear dependency of varietal characteristics) were employed to analyze multiple variations according to Pearson's test [16].

3. Results and Discussions

The physical-chemical and chromatic indices were evaluated, and polyphenols and anthocyanins were quantified. In Table 1 are the physical-chemical and chromatic results of the study extracts obtained in different experimental conditions.

Table 1

Physical-chemical and chromatic indices of the study extracts

Types of extracts	pH	AT, g/L	Ic	Hc	DW, %
CS55NA	3.75 ± 0.01	5.59 ± 0.11	1.68 ± 0.01	0.04± 0.01	11.51 ± 0.05
CS55A	2.35 ± 0.01	11.55 ± 0.08	2.02 ± 0.01	0.63 ± 0.01	12.08 ± 0.05
CS40A	2.42 ± 0.01	8.04 ± 0.14	2.01 ± 0.01	0.60 ± 0.01	12.41 ± 0.05
RN55NA	4.06 ± 0.01	5.85 ± 0.16	1.33 ± 0.01	0.18 ± 0.01	11.91 ± 0.05
RN55A	2.07 ± 0.01	12.49 ± 0.21	2.50 ± 0.01	0.61 ± 0.01	10.55 ± 0.05
RN40A	3.12 ± 0.01	7.45 ± 0.08	2.07 ± 0.01	0.64 ± 0.01	12.92 ± 0.05

Note: CS55NA- Cabernet Sauvignon 55°C non-acidified, CS55A - Cabernet Sauvignon 55°C acidified, CS40A - Cabernet Sauvignon 40°C acidified, RN55NA - Rara Neagra 55°C non-acidified, RN55A - Rara Neagra 55°C acidified, RN40A - Rara Neagra 40°C acidified.

The pH value varied for Cabernet Sauvignon extracts between 2.35-3.75, and for Rara Neagra extracts between 2.07-4.06 (Table 1), which was comparable with the data commonly reported in the literature (pH 3.33–3.53) for this type of residue [17]. The titratable acidity ranged from 5.59-11.55 g /L for Cabernet Sauvignon and from 5.85-12.49 g/L for Rara Neagra. Therefore, in the case of acidified extracts and an extraction temperature of 55°C, titratable acidity showed the highest values, Table 1. These values were bigger than those in the literature [17] because of the difference in the acids used for acidulation.

The color index was 2.02 for acidified Cabernet Sauvignon at 55°C and 2.5 for acidified Rara Neagra at 55°C. In decreasing value extracts samples of Cabernet Sauvignon and Rara Neagra acidified at the extraction temperature of 40°C (Table 1).

The hue value was lowest in the non-acidified samples for Cabernet Sauvignon and Rara Neagra extracts. Acidified samples exhibited a higher hue value of 0.6-0.63 for Cabernet Sauvignon and 0.61-0.64 for Rara Neagra (Table 1). This is explained by the fact that in an acidic medium, biologically active compounds are more stable and, as a result, the color was more pronounced and more stable.

The dry substance content calculated in %, ranged between 11.51-12.41 for Cabernet Sauvignon extracts and between 10.55-12.92 for Rara Neagra (Table 1), reflecting the lower anthocyanin potential of the Rara Neagra ampelographic grape variety.

To determine the presence of essential phenolic substances in the study extracts the absorbance spectra were obtained (Figure 2). According to the spectra, there was a high content of anthocyanins - components showing significant increases in spectral lines between wave-lengths of 510 and 550 nm, specific to these phenolic compounds, confirming the involvement of anthocyanins as major constituents in grape color formation.

Experimentally (Figure 2), it was established that the experimental extraction temperature does not influence majorly the position of specific absorption peaks of phenolic compounds. However, an increase in extraction pH leads to a higher intensity of spectral lines. Absorption spectra demonstrated that extracts obtained at 55°C without acidification had curves of absorption of approximately the same shape, with slight differences in absorbance values. The maximum absorbance was detected in the Rara Neagra extract at an extraction temperature of 55°C and pH 2.07. Similar curves and approximately the same absorbance values were observed for Cabernet Sauvignon extracts at 40°C with pH 2.07, and at 55°C with pH 2.35, as well as for Rara Neagra extracts at 40°C with pH 3.12 (Figure 2).

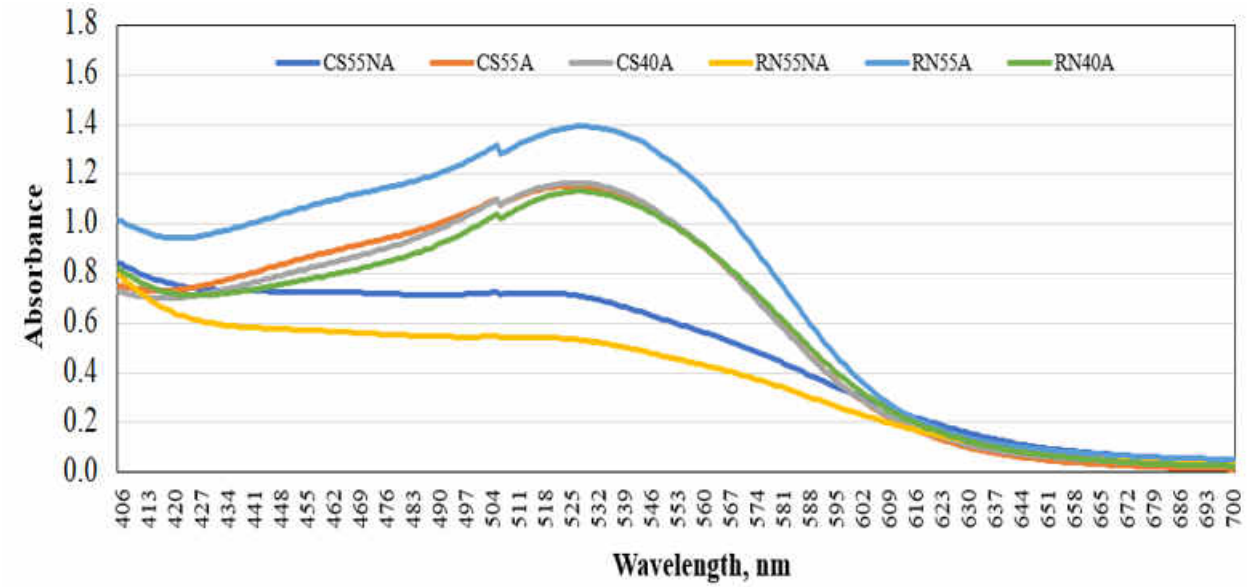

Figure 2. Study extracts' absorption spectrum (absorbance vs wavelength) obtained at the wavelength range of 406-700 nm.

CS55NA - Cabernet Sauvignon 55°C non-acidified; CS55A -Cabernet Sauvignon 55 °C acidified; CS40A - Cabernet Sauvignon 40°C acidified; RN55NA - Rara Neagra 55°C non-acidified; RN55A - Rara Neagra 55°C acidified; RN40A - Rara Neagra 40°C acidified.

The non-acidified extracts (Rara Neagra 55°C and Cabernet Sauvignon 55°C) exhibited the lowest absorbance spectra values because the colored forms of polyphenols were not in flavinic form, displaying low stability and higher susceptibility to condensation, precipitation, and polymerization reactions. Extraction conditions at 40°C resulted in similar spectra of the extracts, indicating moderate diffusion of remaining colored components in the grape pomace samples studied. Acidified extracts at 55°C showed maximal values, attributed to the higher content of constitutive phenolic compounds, as described in Figures 3 and 4.

Significant quantities of anthocyanins identified in the analyzed extracts justify the extraction of these phenolic compounds. The study of anthocyanin content, and the red pigments in the extracts, demonstrated that the highest anthocyanin content was found in acidified Cabernet Sauvignon samples at an extraction temperature of 40°C. The same trend was observed in acidified Rara Neagra extracts at both 40°C and 55°C (Figure 3), indicating increased stability of anthocyanins in acidic medium.

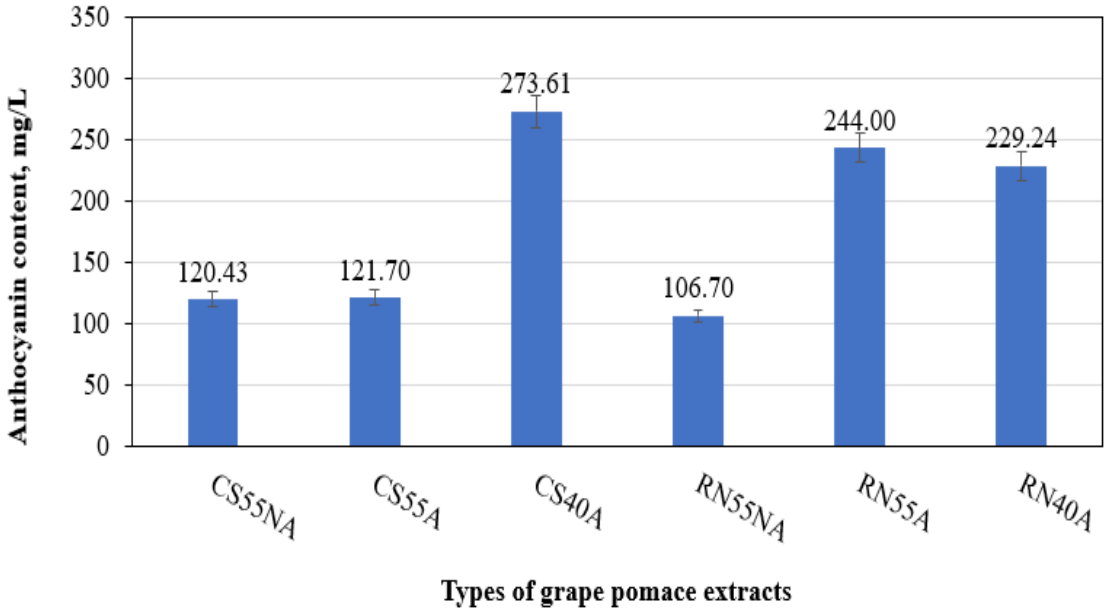

Figure 3. Anthocyanin content in the extracts of grape pomace in experimental conditions.

CS55NA - Cabernet Sauvignon 55°C non-acidified; CS55A - Cabernet Sauvignon 55°C acidified; CS40A - Cabernet Sauvignon 40°C acidified; RN55NA - Rara Neagra 55°C non-acidified; RN55A - Rara Neagra 55°C acidified; RN40A - Rara Neagra 40°C acidified.

Phenolic substances are distributed within grapes as follows (on average): 28-35% in the skin, nearly 10% in the pulp, and 60-70% in the seeds [1]. Consequently, phenolic substances are present in significant quantities in vinicultural by-products. In determining the total phenolic content in the study extracts, values varied for Cabernet Sauvignon between 4.59-5.16 mg GAE/g DW and 4.63-5.99 mg GAE/g DW for Rara Neagra (Figure 4).

In a study by Ghendov-Mosanu et al., [4], experimental values ranged from 7.51-9.52 mg GAE/g DW at 30°C and 45°C extraction temperatures, focusing on Merlot grape pomace. In comparison, the experimental results for Cabernet Sauvignon and Rara Neagra were between 3-3.5 mg GAE/g DW lower than those reported in [4]. The quantitative differences are influenced by the specific type of grape pomace and extraction conditions.

The red-colored phenolic components, which had maximum absorption at 520 nm, exhibited the highest concentration as a percentage in Cabernet Sauvignon extracts at

extraction temperatures of 40°C and 55°C, ranging from 56.50% to 57.48%. Similarly, in Rara Neagra extracts, the red components ranged between 54.98% and 55.07% (Figure 5). Yellow-colored components, with maximum absorption at 420 nm, varied between 34.94% and 44.80% in Cabernet Sauvignon extracts across all three extraction conditions, and between 35.70% and 47.83% in Rara Neagra (Figure 5).

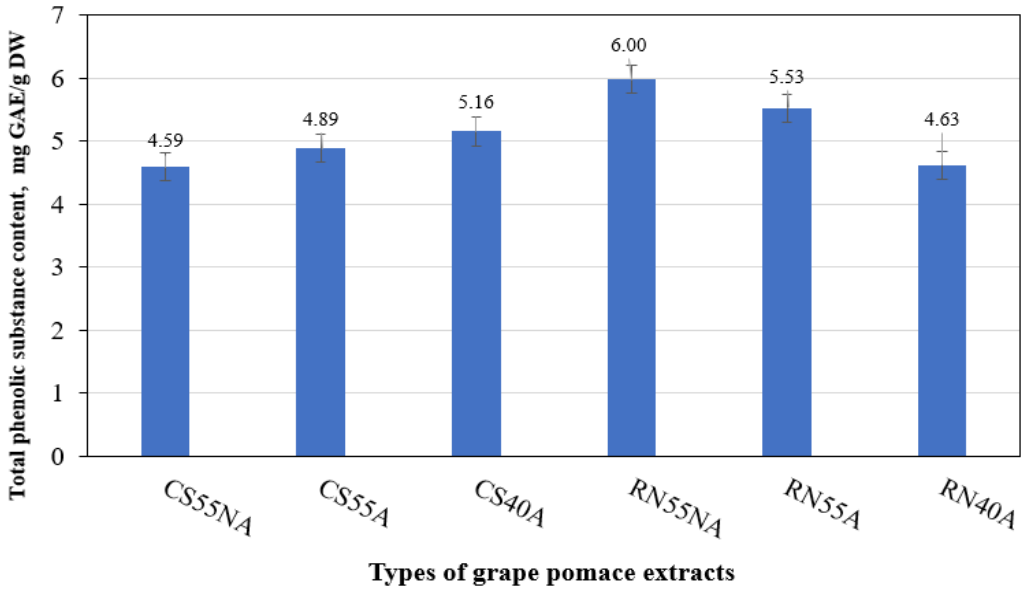

Figure 4. The total phenolic content in the study extracts.

CS55NA - Cabernet Sauvignon 55°C non-acidified; CS55A - Cabernet Sauvignon 55°C acidified; CS40A - Cabernet Sauvignon 40°C acidified; RN55NA - Rara Neagra 55°C non-acidified; RN55A - Rara Neagra 55°C acidified; RN40A - Rara Neagra 40°C acidified.

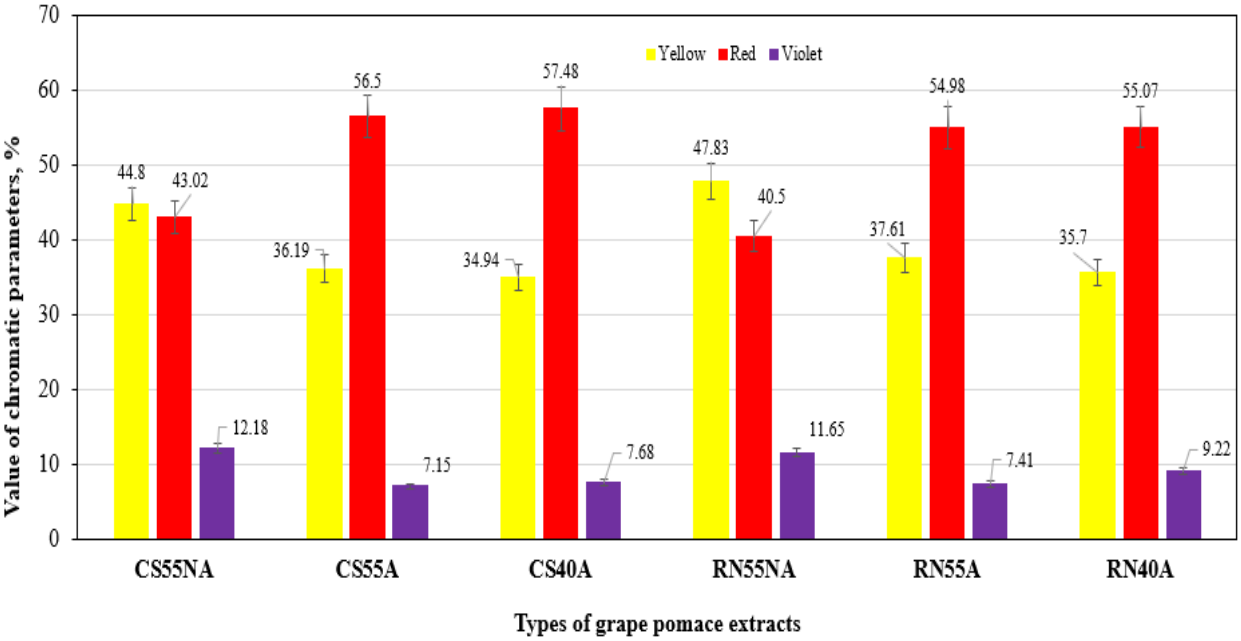

Figure 5. The chromatic parameters of study extract samples – data represented as mean values with standard deviation error lines.

CS55NA - Cabernet Sauvignon 55°C non-acidified; CS55A - Cabernet Sauvignon 55°C acidified; CS40A - Cabernet Sauvignon 40°C acidified; RN55NA - Rara Neagra 55°C non-acidified; RN55A - Rara Neagra 55°C acidified; RN40A - Rara Neagra 40°C acidified.

Violet-colored components, with maximum absorption at 620 nm, were present in lower concentrations, ranging from 7.15% to 12.18% in Cabernet Sauvignon and from 7.41% to 11.65% in Rara Neagra, a fact explained by the dominance of phenolic compounds of red color.

Grape phenolic compounds can act as powerful antioxidants by scavenging free radicals and reducing oxidative reactions. Experimental studies showed that the extracts also possessed a high inhibition ratio (capacity). This activity varied between 72.1% and 86.4% for Cabernet Sauvignon and between 76.8% and 88.6% for Rara Neagra. The lowest values were observed in the non-acidified samples: 72.1% for Cabernet Sauvignon and 76.8% for Rara Neagra (Figure 6), an effect attributed to the instability of quantified phenolic compounds.

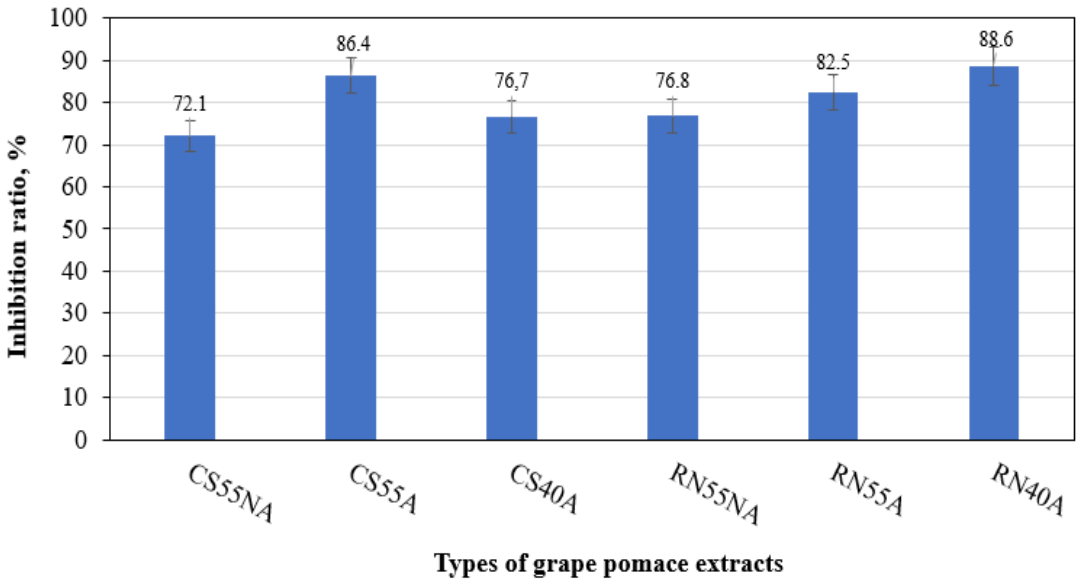

Figure 6. The inhibition ratio of the studied extracts of grape pomace in experimental conditions.

CS55NA - Cabernet Sauvignon 55°C non-acidified; CS55A - Cabernet Sauvignon 55°C acidified; CS40A - Cabernet Sauvignon 40°C acidified; RN55NA - Rara Neagra 55°C non-acidified; RN55A - Rara Neagra 55°C acidified; RN40A - Rara Neagra 40°C acidified.

Research by Ghendov-Mosanu A. [18] reported average antioxidant capacity values of 89.7% for grape pomace.

4. Conclusions

Following the research, optimal extraction conditions for biologically active substances (phenolic compounds and anthocyanins) from grape pomace of Cabernet Sauvignon and Rara Neagra have been established, it includes extraction at 55°C for 12 hours in the presence of a 60% acidified hydro-alcoholic solution. The extracts thus obtained, after concentration under vacuum, can be utilized in food technology to replace synthetic food colorants with their natural counterparts.

All studied extracts exhibited antioxidant activity and this action is correlated with their active substance's concentration.

Valorizing grape pomace is crucial for obtaining its constituent bioactive substances, which exhibit an antioxidant capacity of over 80%, a total phenolic content value of 4.9 mg GAE/g DW for Cabernet Sauvignon and higher values of 5.5 mg GAE/g DW respectively for Rara Neagra.

The results obtained demonstrate the feasibility of obtaining grape pomace extracts rich in bioactive substances without requiring technologies that would necessitate expensive equipment and chemicals. Therefore, the use of enocolorant extracts in the food industry presents an alternative that allows for the use of natural resources and the generation of inexpensive and healthy raw materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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