

PORTOFOLIU DE LUCRĂRI ÎN DOMENIUL DE DOCTORAT VIZAT

1. Alina Margean; Mirabela Ioana Lupu; Ersilia Alexa; **Vasile Pădureanu**; Cristina Maria Canja; Ileana Cocan; Monica Negrea; Mariana-Atena Poiana (2020). An Overview of Effects Induced by Pasteurization and High-Power Ultrasound Treatment on the Quality of Red Grape Juice. *Molecules*, 25(7). Factor de impact: 4,412. Punctaj: 30,81. **Quartila JCR la anul publicării: Q2** – Chemistry, Multidisciplinary / Biochemistry & Molecular Biology. <https://doi.org/10.3390/molecules25071669>
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Article

An Overview of Effects Induced by Pasteurization and High-Power Ultrasound Treatment on the Quality of Red Grape Juice

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Abstract: In juice processing, ultrasound treatment has been tested as a potential alternative to conventional thermal methods to inactivate microorganisms and to enhance the nutritional status of juice. In this study, the impact of pasteurization and high-power ultrasound treatment on the quality of red grape juice was investigated in terms of the content of bioactive compounds such as phenolic compounds and L-ascorbic acid as well as regarding the microbiological and physicochemical properties. The grape juice was subjected to pasteurization (80 °C, 2 min) as well as to ultrasound treatment with an amplitude of 50 and 70% for 5 and 10 min. The results indicated the same level of total phenolic content for pasteurized and sonicated samples for 10 min with an amplitude of 70%, while the highest level of L-ascorbic acid was recorded for sonicated samples with an amplitude of 70% for 10 min. pH of sonicated samples decreased with amplitude and treatment time while total soluble solids and titratable acidity increased with amplitude and time. Moreover, the results indicated the usefulness of juice sonication to enhance the inactivation of microorganisms. Thus, the high-power ultrasound treatment might represent a viable technique to replace the conventional thermal treatment in grape juice processing.

Keywords: red grape juice; pasteurization; high-power ultrasound treatment; phenolic compounds; microorganisms

1. Introduction

Grapes are one of the largest fruit crops harvested around the world. However, grape juice is not consumed worldwide in large amounts because of its higher sugar content or higher acidity, but it can be mixed with apple or other juices [1–3]. The last decades were characterized by numerous studies related to the chemical constituents of grapes, especially phenolic compounds that are secondary plant metabolites with free radical scavenging activity [4,5]. The main phenols in grape belong either to the flavonoid family or the non-flavonoid one. Among the flavonoid family, anthocyanins, flavanols, flavonols and dihydroflavonols are considered to play an important role in the pigmentation and

flavor of grapes berries, while non-flavonoid phenolic compounds such as resveratrol have a role in the powerful biological activities of grape [6,7].

Many research studies have suggested that antioxidants might have positive effects on human health due to their effects against oxidative stress. Thus, it was considered that a diet based on a high daily intake of antioxidant dietary supplements from fruits and vegetables might result in a lower incidence of atherosclerosis, Alzheimer's disease, cancer, ocular disease, diabetes, rheumatoid arthritis and motor neuron disease [8].

In order to reduce microbial grow and to extend their shelf-life, fruit juices are usually processed by pasteurization. Besides the fact that this thermal procedure influences the quality of the products, it also has the disadvantage of denaturing the bioactive compounds due to ionization, hydrolysis and oxidation reactions [5,9]. Considering consumers' need for high quality, flavor and taste of fruit juices, producers are continuously looking for alternatives to the conventional thermal methods. Moreover, the consumers demand for nutritious and safe food products has resulted in increasing interest in non-thermal preservation techniques.

Recently, emerging technologies like ultrasound, microwave and pulsed electric field irradiation have been tested in the food industry to develop various effective food processing applications [10]. Ultrasounds are sound waves of frequencies higher than 16 kHz (higher than usually detected by the human ear). When transmitted through a liquid, solid or gas with elastic properties, the sound waves are moving at a speed depending on the wavelength and the type of material. When acting on a liquid medium, the fundamental effect of ultrasounds is to add to the hydrostatic pressure (P_h) an acoustic pressure (P_a) dependent on time (t), wave frequency (f) and the maximum amplitude of wave P_A . The relationship among the above factors is illustrated by the Equation (1) [10]:

$$P_a = P_A \times \sin(2 \times \pi \times f \times t) \quad (1)$$

The propagation of ultrasound waves in a medium generates physical and chemical effects which can be exploited for improving the efficiency of various food processing operations. Thus, the ultrasound technology was explored to enhance the food quality by reducing the process time, energy and increasing shelf life [11,12]. It has been reported that ultrasound energy can be used to increase extraction yields by disrupting cell tissues [12,13].

In terms of their applications, two approaches are usually used for ultrasound. Low-power ultrasound (LPU), at frequencies above 100 kHz, is a non-invasive technique and might be applied to monitor food processes and to evaluate the physico-chemical food properties, while high-power ultrasound (HPU), with frequencies between 18–100 kHz, causes physical, mechanical and chemical effects and might offer insight into the long term stability of fruit juices [14]. Most often, the power ultrasound range is used in the extraction of biologically active compounds from biological matrices, such as in the extraction of polyphenols from plant and waste food materials. Most of the commercial ultrasonic probes or cleaning baths work at low ultrasound frequencies (20–40 kHz) [15]. Low frequencies have been observed to generate large cavitation bubbles in extraction solvents which implode violently, generating high shear whiles creating microjets that ensure higher cellular degradation, increased solvent penetration and higher extraction rates [15,16]. In juice processing, HPU treatment might be used not only for controlling spoilage microorganisms, but also as an efficient and environmentally friendly treatment for enhancing the nutritional status of juices in terms of increasing the bioactive compounds [7,17]. It was reported that ultrasound was applied in extraction of plant materials because of increased sugar content, total acid content, phenolics content as well as color density of grape juice [2]. Moreover, the ultrasound treatment can influence the quality parameters of cranberry juice and nectar in terms of aromatic profile and sensory properties [18], the stabilization of cantaloupe melon juice [19] or the preservation of monomeric anthocyanins along with a significant microbial load reduction during storage [20].

Therefore, our study represents an attempt to identify alternative fruit processing techniques in order to reduce the microbial load and to improve the nutritional quality of the obtained products.

Moreover, this research reflects the possibility to replace the conventional thermal treatment with HPU treatment as a potential tool for setting up a more efficient and sustainable technology for juice processing.

In line with the above-mentioned considerations, the goal of this research was to investigate the impact of pasteurization and HPU treatment on the quality of red grape juice. In this purpose, the content of bioactive compounds such as phenolic compounds and L-ascorbic acid as well as the physicochemical attributes and the microbiological properties of pasteurized grape juice samples were compared with those of the juice samples subjected to HPU treatments at different parameters such as amplitudes of 50% (A50%) and 70% (A70%) for 5 and 10 min.

2. Results and Discussion

The effects of pasteurization and high-power ultrasound (HPU) treatments on the quality of red grape juice are discussed in terms of bioactive compounds such as total phenolic (TP) content, L-ascorbic acid (L-AsAc) or vitamin C content and the profile of polyphenolic compounds. Also, the changes in physicochemical parameters such as pH, total soluble solids (TSS) and titratable acidity (TA) as well as the microbiological quality expressed by total plate counts (TPC) and *Enterobacteriaceae* count (ENT) were investigated in response to the applied treatments. All mentioned parameters were investigated for untreated red grape juice, as a control sample (C), as well as for red grape juice samples subjected to pasteurization at 80 °C for 2 min (P), respectively to sonication with an amplitudes of 50 and 70% for 5 and 10 min, as follows: HPU (A50%, 5 min), HPU (A50%, 10 min), HPU (A70%, 5 min) and HPU (A70%, 5 min). Some authors also found that ultrasound processing parameters like amplitude and time plays a decisive role in both extraction and retaining of bioactive compounds and in determining the effects on the quality attributes [21].

To provide an overview of the effects induced by pasteurization and HPU treatments on investigated parameters, compared to untreated grape juice samples, the results obtained in this study were processed by a one-way ANOVA test. Based on the data obtained through statistical processing the significance of changes recorded in the investigated parameters in response to the applied treatments, reported with respect to the control grape juice sample can be determined. Also, the changes occurring in the investigated parameters as a result of HPU treatments were evaluated relative to pasteurization for assessing the possibility of replacing the conventional thermal treatment currently used in the grape juice processing, with HPU treatment, as a non-thermal food processing technique.

2.1. Effect of Operating Parameters of HPU Treatments on Temperature of Grape Juice Samples

The operating parameters used during the HPU treatments can impact the temperature of the grape juice samples. The average temperature values measured every 2.5 min for ultrasound- treated juice samples are shown in Table 1 while in the Figures 1 and 2 the values measured by a forward-looking infrared (FLIR) camera at the end of sonication treatment with 50 and 70% amplitude can be seen.

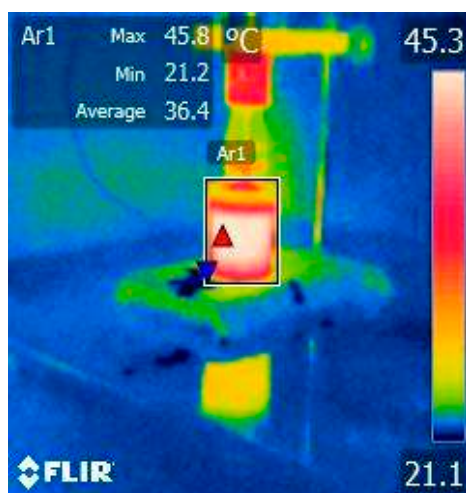
During ultrasound treatment, additional attention was paid to measure the heat generated in the samples as an effect of the sonication. To account for the heat issue and the corresponding increase of the temperature, a thermovision camera was used to ensure the temperature of the sample did not exceed 60–66 °C. Temperatures above 70 °C in particular have been shown to lead to rapid polyphenol degradation, hence the need to select efficient extraction temperatures that ensure the stability of phenolic compounds [15,22,23]. It is also important to mention that the sensitivity of a sample to temperature-induced polyphenol degradation depends on the types of polyphenol compounds available in the extract or plant matrix, and their physicochemical and biochemical characteristics, as well as solvent-sample interactions [15].

Table 1. Changes in temperature of juice samples as a result of ultrasound treatments parameters.

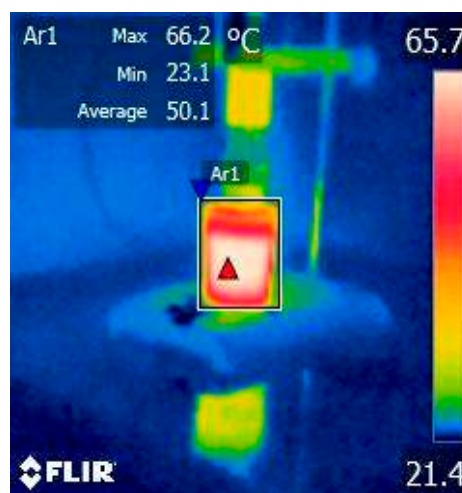
Time of Temperature Measuring (min)	T(°C)	
	HPU (A50%, 5 min)	HPU (A70%, 5 min)
0	18.9 ± 0.85	19.7 ± 0.70
2.5	25.7 ± 0.90	36.9 ± 1.00
5	36.4 ± 1.20	50.1 ± 0.70

Time of Temperature Measuring (min)	T(°C)	
	HPU (A50%, 10 min)	HPU (A70%, 10 min)
0	17.2 ± 0.85	19.9 ± 0.20
2.5	25.8 ± 0.70	34.9 ± 1.00
5	33.8 ± 1.30	51.4 ± 0.90
10	50.2 ± 1.20	66.0 ± 1.10

Results are expressed as the average value of three replicates ± standard deviation (SD).



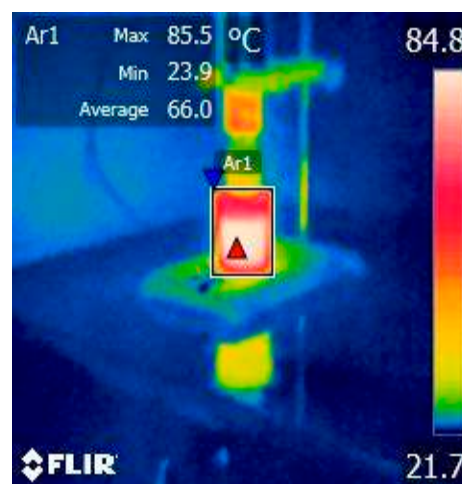
(a)



(b)

Figure 1. The temperature measured after 5 min of HPU treatment: (a) A50%; (b) A70%.

(a)



(b)

Figure 2. The temperature measured after 10 min of HPU treatment: (a) A50%; (b) A70%.

Preliminary studies related to the temperature increase mention that at a power of 100 W and a frequency of 20 kHz, a treatment over 15 min in duration resulted in a temperature in the sample of about 50 °C [21].

The results obtained by Lieu and Le revealed that temperature and time had positive effects on yield of the treatment process [2]. We observed that the pattern of the average temperatures increasing was linear for both 50 and 70% amplitudes. When the higher amplitude was applied, i.e. 70%, the average temperatures increased by 13.7 °C for the same sonication treatment of 5 min and by 15.8 °C after 10 min.

2.2. Effects of Pasteurization and HPU Treatments on Total Phenolic (TP) Content, L-ascorbic Acid (L-Asac) Content and Polyphenolic Compounds Profile

In Table 2 the total phenolic (TP) and L-ascorbic acid (L-AsAc) contents of red grape juice samples after pasteurization and HPU treatments are presented. These results reveal decreases in TP content, with respect to the control sample, in response to pasteurization and ultrasound treatments.

Table 2. Effect of pasteurization and HPU treatments on the total phenolic (TP) and L-ascorbic acid (L-AsAc) content.

Parameter	Grape Juice Sample					
	C*	P**	HPU (A50%, 5 min)	HPU (A70%, 5 min)	HPU (A50%, 10 min)	HPU (A70%, 10 min)
TP (mg GAE/100 mL)	72.86 ± 0.02 ^a	70.64 ± 0.08 ^b	50.68 ± 0.05 ^c	54.56 ± 0.07 ^d	50.71 ± 0.09 ^c	69.88 ± 0.02 ^e
L-AsAc (mg/L)	454.4 ± 0.11 ^a	340.8 ± 0.09 ^b	227.2 ± 0.04 ^c	349.8 ± 0.05 ^b	458.4 ± 0.10 ^a	568 ± 0.08 ^d

* Control sample; ** pasteurized juice sample. One-way ANOVA test was used to compare the means differences among treatments; different superscripts in the same row indicate significant differences among the treatments (Tukey's test, $p < 0.05$). Results are expressed as the average value of three replicates ± standard deviation (SD).

The content of total phenols quantified as gallic acid equivalent (GAE) in treated red grape juice samples varies from 50.68 to 70.64 mg GAE/100 mL. Another study reported a level of TP content in red grape juices in the range 744–1177 mg GAE/L [24]. TP content among sonicated samples was significantly affected by the amplitude level ($p < 0.05$) and not impacted by the treatment time ($p > 0.05$).

In Figure 3 the changes of TP content in juice samples in response to the applied treatments are depicted. It can be noted that there were losses in the TP content of the grape juice samples in response to pasteurization compared to ultrasound treatment. Thus, Figure 3a shows the losses of TP content induced by pasteurization and HPU treatment with respect to the control sample while Figure 3b reveals the losses in juice sampled subjected to HPU treatment compared to the pasteurized sample.

The first thing we could notice about the data from Figure 3a is that the pasteurization induced the lowest decreases in TP content, compared to the control sample (3.05%), while the HPU treatments (A50%, 5 min; A70%, 5 min and A50%, 10 min) led to the losses in the range 25–30%. It is worth noting that the HPU treatment (A70%, 10 min) has an effect close to that obtained through pasteurization, as regards the TP content losses (4.09%).

A closer look at Figure 3b reveals that the losses of TP content induced by HPU treatments (A50%, 5 min; A70%, 5 min and A50%, 10 min) were in the range of about 22–28%, compared to the pasteurized sample. The HPU treatment (A70%, 10 min) induced the lowest losses in TP content, about 1% compared to the pasteurized sample. Therefore, by choosing an HPU treatment for 10 min with an amplitude of 70%, the retention of total phenolic compounds in grape juice sample could be improved.

The initial content of L-ascorbic acid in untreated red grape juice sample was 454.4 mg/L. Decreases in the L-ascorbic acid content in response to pasteurization and HPU treatment applied to red grape juice were recorded, indicating that ascorbic acid is highly thermosensitive. The L-ascorbic acid content in grape juice samples decreased by 25% in response to pasteurization and HPU treatment (A50%, 5 min) and by 53% by applying HPU treatment at a higher amplitude (A70%, 5 min).

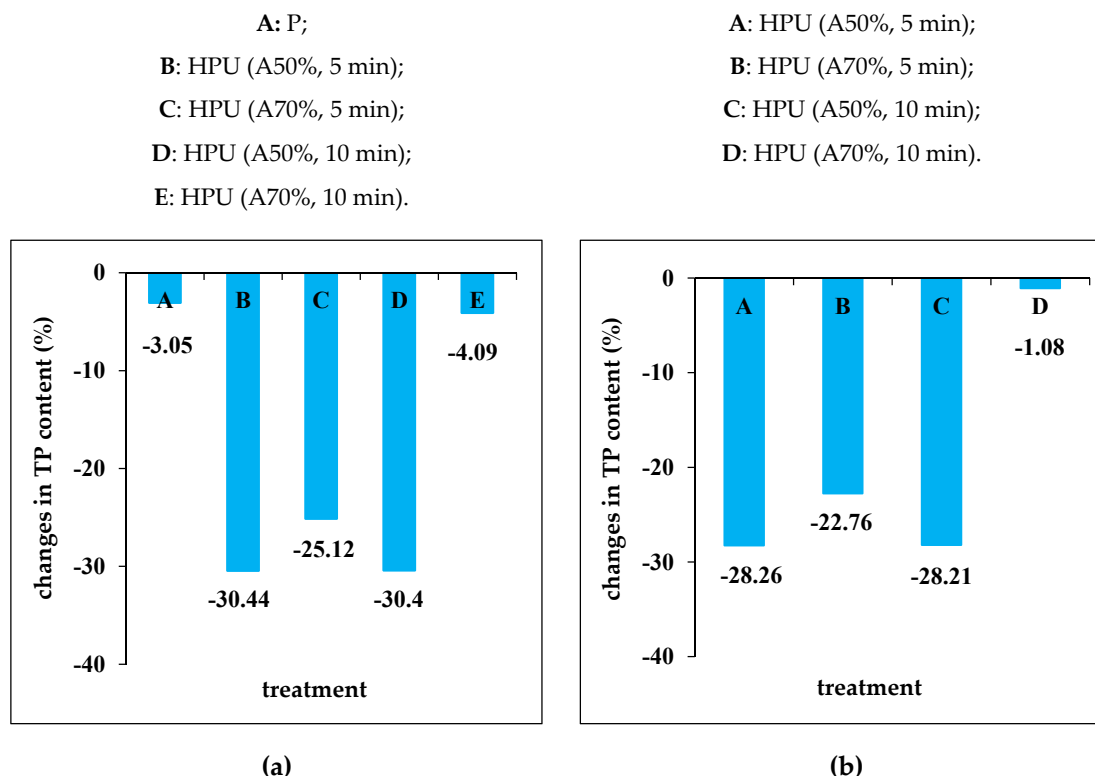


Figure 3. The changes in total phenolic (TP) content of juice samples in response to the applied treatments: (a) reported to the control sample (C); (b) reported to the pasteurized sample (P).

The degradation of L-ascorbic acid is probably attributable to the oxidation processes that occur during these treatments. The results were in agreement with other previous studies. Thus, data reported by Cao et al. [25] reveals an initial content of ascorbic acid in fresh bayberry juice of 22.82 mg/100 mL and a decrease of 25.16% in response to pasteurization. Adekunle et al. [26] also reported that the content of ascorbic acid of tomato juice decreased by 96.9 to 60.7% in response to ultrasound treatment of 24.4–61 μm for 2–10 min.

However, after HPU treatment (A70%, 10 min) an increase in L-AsAc content to 568 mg/L in comparison with the untreated grape juice was recorded. An increase of ascorbic acid was also detected by other authors at high amplitude [27–29]. An explanation could be the elimination of dissolved oxygen, essential for ascorbic acid degradation, during the cavitation produced by the sonication treatment [28] which prevents ascorbic acid breakdown.

Figure 4 provides information regarding the changes recorded in L-AsAc content in juice samples in response to the applied treatments. It can be observed that by exposing the grape juice to pasteurization, compared to ultrasound treatments, both losses and increases in the content of L-AsAc were recorded. The effect of P and HPU treatment on L-AsAc content reported to the control sample is shown in the Figure 4a. In addition, the changes in L-AsAc content recorded in juice samples subjected to HPU treatment versus the pasteurized sample are presented in Figure 4b.

Our data reveal that pasteurization and the HPU treatments (A50%, 5 min; A70%, 5 min) induced losses in L-AsAc content as follows: 25, 50 and 23.02% with respect to the control sample. Contrary to this finding, by applying HPU treatments (A50%, 10 min; A70%, 10 min) increases in L-AsAc content of 0.88, or 25%, were recorded compared to the control sample (Figure 4a). As regards the changes in L-AsAc content induced by HPU treatment with regards to the pasteurized sample, the data depicted in Figure 4b prove that only by HPU treatment (A50%, 5 min) were losses observed. By applying the other HPU treatments (A70%, 5 min; A50%, 10 min; A70%, 10 min) increases in L-AsAc content, compared to the pasteurized sample, were recorded as follows: 2.64, 34.51 and 66.67%,. These data

highlight that the HPU treatment (A70%, 10 min) was the most effective among the investigated treatments to ensure a high content of vitamin C in grape juice samples.

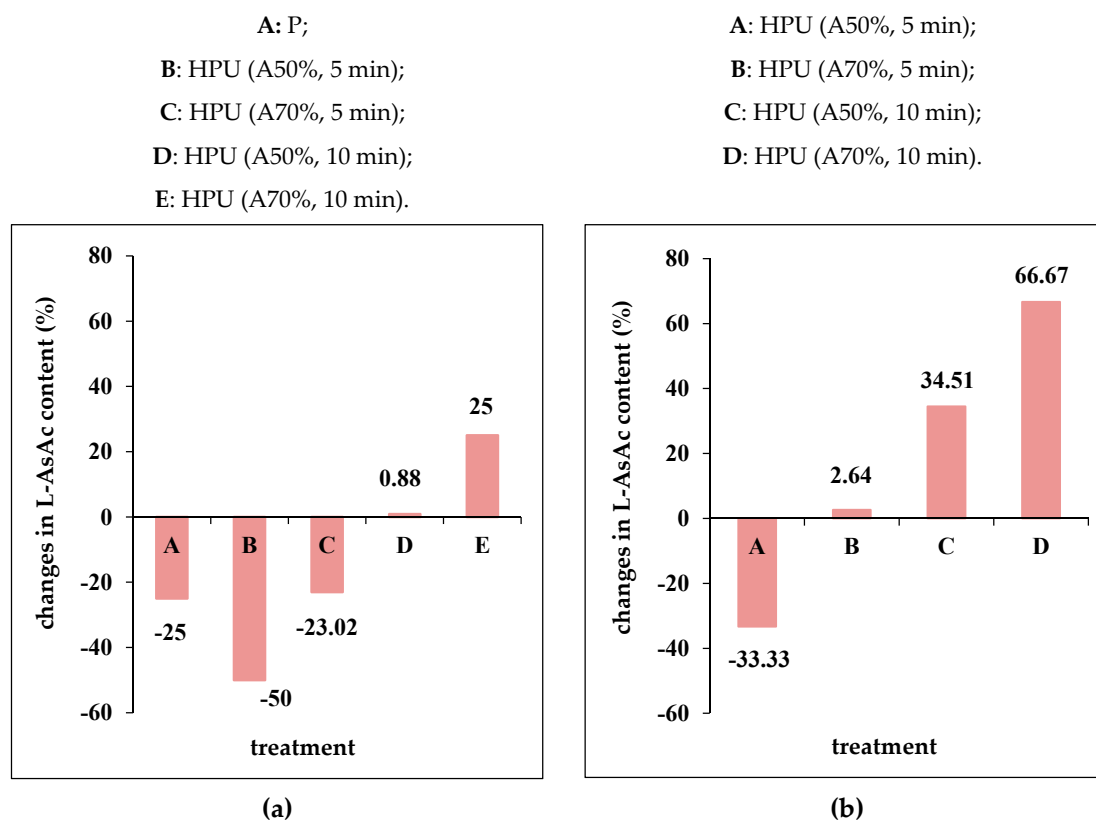


Figure 4. The changes in L-ascorbic acid (L-AsAc) content of juice samples in response to the applied treatments: (a) reported to the control sample (C); (b) reported to the pasteurized sample (P).

Polyphenols play an important role among red grape components as they determine the flavor and color of the juice [30,31] and have several beneficial properties for human health [30,32]. The evolution of individual phenolic compounds of red grape juice is shown in Table 3. Among phenolic compounds, the most known is resveratrol. Significantly higher amounts of resveratrol were observed in the grape juice samples exposed to ultrasound treatment, especially at A70%, 5 min (8.54 mg/L). Resveratrol was not detected in the juice sample subjected to pasteurization that suggests that during this thermal treatment it was destroyed. An increase of resveratrol in the ultrasound processing of grape juice was also observed by Hasan et al. [33]. A slight increase in caffeic acid has been reported in the clear apple juice treated with ultra-high pressure homogenization which is also a non-thermal technique [34] and also in a study on apple juice treatment by ultrasound at 70% amplitude and 25 kHz frequency [35]. An increase in gallic acid content from 2.15 mg/L recorded in the control sample to 2.16 mg/L in pasteurized samples and to 31.3 mg/L in a juice sample subjected to HPU treatment (A70%, 5 min) can be noted. The highest level of protocatechuic acid (5.13 mg/L), epicatechin (76.80 mg/L), *p*-coumaric acid (1.20 mg/L), rutin (63.31 mg/L), rosmarinic acid (25.93 mg/L), quercetin (5.62 mg/L) and kaempferol (4.59 mg/L) were also detected in the grape juice samples subjected to HPU treatment (A70%, 5 min). The phenolic compounds can be found both in soluble form in the vacuole or bound to the other compounds such as cellulose, hemicellulose, pectin and lignin traces of the cell wall [28]. The increase of polyphenolic compounds in sonicated juice samples might be ascribed to the increase in the extraction efficacy by ultrasounds treatment causing disruption of cell walls and ultimately the liberation of bound polyphenolic compounds [27,36]. Also, the ultrasound treatment might enhance the release of phenolic compounds from the cell walls, due to the collapse through cavitation process in the surroundings of colloidal particles found in juice [28].

Table 3. Effect of pasteurization and HPU treatments on the polyphenolic compounds profile of grape juice samples.

Polyphenolic Compounds (mg/L)	Grape Juice Sample					
	C*	P**	HPU (A50%, 5 min)	HPU (A70%, 5 min)	HPU (A50%, 10 min)	HPU (A70%, 10 min)
Gallic acid	2.15 ± 0.03 ^a	2.16 ± 0.05 ^a	15.23 ± 0.04 ^b	31.30 ± 0.07 ^c	12.82 ± 0.11 ^b	19.49 ± 0.09 ^c
Protocatechuic acid	n.d.	2.08 ± 0.35 ^a	n.d.	5.13 ± 0.05 ^b	0.79 ± 0.44 ^c	n.d.
Caffeic acid	0.93 ± 0.21 ^a	2.62 ± 0.37 ^a	1.30 ± 0.52 ^a	14.31 ± 0.06 ^{ab}	4.41 ± 0.08 ^{ab}	23.27 ± 0.04 ^{ab}
Epicatechin	3.70 ± 0.16 ^a	10.70 ± 0.33 ^a	5.45 ± 0.15 ^a	76.80 ± 0.07 ^{ab}	n.d.	35.31 ± 0.54 ^{ab}
p-cumaric acid	0.28 ± 0.03 ^a	0.1 ± 0.11 ^a	0.04 ± 0.04 ^a	1.20 ± 0.04 ^a	1.13 ± 0.05 ^a	n.d.
Ferulic acid	0.27 ± 0.01 ^a	0.32 ± 0.03 ^a	n.d.	n.d.	n.d.	0.81 ± 0.12 ^b
Rutin	2.09 ± 0.11 ^a	2.51 ± 0.06 ^a	9.61 ± 0.1 ^a	63.31 ± 0.02 ^b	n.d.	39.02 ± 0.1 ^{ab}
Rosmarinic acid	n.d.	n.d.	1.19 ± 0.06 ^a	25.93 ± 0.21 ^b	0.39 ± 0.08 ^a	4.31 ± 0.05 ^b
Resveratrol	0.21 ± 0.12 ^a	n.d.	0.65 ± 0.13 ^{ab}	8.54 ± 0.14 ^b	0.80 ± 0.06 ^{ab}	2.48 ± 0.04 ^b
Quercetin	0.38 ± 0.07 ^a	n.d.	n.d.	5.62 ± 0.02 ^b	0.31 ± 0.08 ^a	0.71 ± 0.03 ^{ab}
Kaempferol	0.83 ± 0.05 ^a	0.92 ± 0.06 ^a	n.d.	4.59 ± 0.12 ^b	n.d.	n.d.

* Control sample; ** pasteurized juice sample; n.d. - not detected. One-way ANOVA test was used to compare the means differences among treatments; different superscripts in the same row indicate significant differences among the treatments (Tukey's test, $p < 0.05$). Results are expressed as the average value of three replicates ± standard deviation (SD).

We can appreciate that the overall polyphenolic profile of the red grape juice samples is given by the contribution of a multitude of individual polyphenolic compounds. It is well known that red grapes are recognized for the significant amount of anthocyanins in their composition (300–7500 mg/kg fresh weight) and less for the content of monomeric flavanols such as epicatechin (30–175 mg/kg fresh weight) or flavonols as quercetin or kaempferol (15–40 mg/kg fresh weight) [37]. Anthocyanins, the most relevant polyphenolic compounds in red grapes, with flavonoid-like glycoside structures, show similar behavior under heating and their degradation depends on the temperature and pH [38]. At pH = 1 all the glycosidic bonds are susceptible to hydrolysis, while at a pH values in the range 2–4, specific for grape juice, aglycone-sugar bond is the most labile of the glycosidic bonds [38]. This means that under our working conditions the anthocyanins are less susceptible to destruction compared to flavonoids, which might justify the tendency of total phenolic content to increase, compared to the investigated individual polyphenolic compounds. In addition, the increase of total polyphenols content can also be attributed to hydroxycinnamic acids whose content can increase with the amplitude, respectively with the temperature.

The increase of HPU treatment time from 5 to 10 min, for a constant amplitude of 50 and 70%, leads to a significant increase in the temperature of the process, as was shown in Figures 1 and 2. The HPU treatment (A70%, 5 min) produces a heating from 23.1 up to 66.2 °C with an average value of 50.1 °C, while for HPU (A70%, 10 min) the temperature increases from 23.9 up to 85.5 °C with an average value of 66 °C. At a higher temperature, exceeding 60 °C, especially over extended periods, some phenolic compounds suffer oxidative degradation. It is also important to mention that the sensitivity of a sample to temperature-induced polyphenol degradation depends on the types of available polyphenol compounds [15]. Sharma et al. [39], highlighted that after heating, a decrease in total flavonoids was observed, which indicates that some flavonoids were probably destroyed, however, the total phenolics were increased [39]. In most fruits and vegetables, flavonoids contain C-glycoside bonds and exist as dimers and oligomers. Thermal food processing methods such as heating or boiling result in the formation of monomers by the hydrolysis of C-glycosides bonds [39]. Ross et al. [40], have shown that catechin and epicatechin content decreased with increasing heating temperature, but the content of some hydroxycinnamic acids increased.

According to the reported results it can be observed that both the ultrasound processing time and amplitude are influential factors for preserving the content of active principles. Our data reveal that HPU treatment with an amplitude of 70% for 10 min led to an increase in the TP and L-AsAc content, compared to 5 min, while HPU treatment (A70%, 5 min) might favor an increase of the levels of flavonoids such as rutin, quercetin, epicatechin and kaempferol as well as resveratrol, a natural

stilbene found in grape juice. At the same time, some hydroxycinnamic acids such as ferulic and caffeic acid are found in higher concentrations after exposing the grape juice to HPU treatment (A70%, 10 min).

2.3. Effects of Pasteurization and HPU Treatments on Ph, Total Soluble Solids (TSS) and Titratable Acidity (TA)

Physicochemical parameters such as pH, total soluble solids (TSS) and titratable acidity (TA) of untreated and pasteurized grape juice along with grape juice treated for 5 and 10 min with different ultrasound amplitudes (A50% and A70%) are shown in Table 4.

Table 4. Effects of ultrasound and pasteurization treatments on the pH, total soluble solids (TSS) and titratable acidity (TA).

Parameter	Grape Juice Sample					
	C*	P**	HPU (A50%, 5 min)	HPU (A70%, 5 min)	HPU (A50%, 10 min)	HPU (A70%, 10 min)
pH	3.62 ± 0.01 ^a	3.59 ± 0.01 ^b	3.52 ± 0.01 ^c	3.51 ± 0.01 ^c	3.50 ± 0.01 ^d	3.50 ± 0.01 ^d
TSS (°Brix)	30.18 ± 0.13 ^a	30.73 ± 0.09 ^a	26.47 ± 0.09 ^{bc}	27.73 ± 0.09 ^b	27.13 ± 0.05 ^{abc}	29.67 ± 0.09 ^{ab}
TA (g of tartaric acid /L)	3.75 ± 0.07 ^a	4.23 ± 0.08 ^b	3.79 ± 0.03 ^a	3.97 ± 0.12 ^{abc}	3.94 ± 0.16 ^{ab}	4.18 ± 0.11 ^{bc}

* Control sample; ** pasteurized juice sample. One-way ANOVA test was used to compare the means differences among treatments; different superscripts in the same row indicate significant differences among the treatments (Tukey's test, $p < 0.05$). Results are expressed as the average value of three replicates ± standard deviation (SD).

The results suggested that HPU treatment causes changes in samples as the pH decreased with increasing amplitude and treatment time. However, the pH value among sonicated samples was found to be significantly influenced by the treatment time ($p < 0.001$) and not influenced by the amplitude level ($p > 0.05$). Also, the pH values recorded for all juice samples subjected to HPU treatments were significantly different than those recorded for C and P samples ($p < 0.001$).

TSS of samples treated with different amplitudes and treatment times indicate a decrease compared with pasteurized juice. For sonicated samples, results suggested that TSS increased with amplitude and time, the value of sample sonicated with amplitude 70% for 10 min being 1.71% lower than for pasteurized juice. Although HPU treatment induced slight changes in samples, statistically the TSS content among sonicated samples was significantly influenced by amplitude level ($p < 0.001$) and not influenced by treatment time ($p > 0.05$). Also, all HPU samples were significantly different than the P sample ($p < 0.001$).

A higher value of TA was observed for pasteurized samples. For sonicated samples, results suggested that TA increased with amplitude and time, the value of sample sonicated with an amplitude of 70% for 10 min being 1.18% lower than the value recorded for the pasteurized juice sample. TA among sonicated samples was significantly influenced by both amplitude level and treatment time ($p < 0.05$).

As mentioned earlier, it is clear from the data that the different assayed combinations of ultrasound frequency and exposure time exert slight effects on the pH, TSS and TA of grape juice. Sugars are the main components of the fruit juices, being an important quality attribute and influencing consumers to accept or reject a product.

As regards the total soluble solids, around 80% are constituted by sugars [21]. The results indicated that pasteurization and sonication treatments had different effects on the total soluble solids of the grape juice. Our results related to TSS are similar to the results of sonicated sweet lime [21] and kastuni juice [41] and in contrast with the ones of sonicated orange juice and carrot [21]. However, Gao and Rupasinghe [42] obtained similar results as of both lower TSS of the sonicated sample compared with pasteurized one for an apple-carrot ratio of 60:40 and 90:10 and lower pH for an apple-carrot ratio of 90:10. Other authors found no significant changes in pH, TSS and TA due to the sonication treatment of grape juice using an ultrasonic treatment of 25 kHz at a constant temperature [27], or any changes [43] using an ultrasonic treatment of 28 kHz, respectively.

Regarding the TA, our results are not similar with the results observed by different authors in grape mash [2], apple-carrot juice [42] and grapes [44], which suggests that ultrasound treatment augmented the tartaric acid extraction. This may be attributed to the fact that we used high power ultrasound at a fixed frequency of 20 kHz.

2.4. Microbiological Quality

The application of sonication treatments has been recognized as a potential innovative technology that can meet Food and Drug Administration's (FDA's) requirements for achieving a 5-log reduction in the contaminant microorganisms (or pathogens) associated with fruit juices [45,46]. The microbiological quality of the grape juice samples was assessed on the basis of two indicators, namely the total plate count (TPC) and the *Enterobacteriaceae* count (ENT).

The total plate count (TPC) is used as a reliable indicator for the bacterial populations of a food sample. It is also called the aerobic colony count, the standard plate count, the mesophilic count or the aerobic plate count. TPC provides information about the total microbial load in a food sample. TPC quantifies the aerobic, mesophilic organisms that grow under aerobic conditions at a moderate temperature in the range 20–45 °C. The TPC includes all pathogens and non-pathogens and is used to assess the hygiene status of a food product [47]. The *Enterobacteriaceae* count (ENT) is considered by food producers as an indicator of hygiene practices, being useful to monitor the effectiveness of implemented preventive measures. The indicator microorganisms are commonly used to measure the quality of the practices used in order to ensure a proper processing of food products. The *Enterobacteriaceae* or *Escherichia coli* are used for assessing the enteric contamination of a food [48].

In Table 5 a reduction in the microbial load after pasteurization and sonication treatments of red grape juice can be observed. The control sample showed values of 4.53 log colony forming units (CFU)/mL for TPC and 1.51 log CFU/mL for ENT. The microorganisms were reduced in the pasteurized juice sample (TPC was 2.53 log CFU/mL and ENT 0.73 log CFU/mL) as well as in all samples exposed to HPU treatments. ENT and TPC of the sonicated samples were significantly influenced by the treatment time and the amplitude level ($p < 0.001$). As the data from Table 5 reveal, TPC and ENT recorded a decrease versus the control sample by increasing the amplitude level from 50 to 70% as well as by increasing the sonication time from 5 to 10 min. The lowest bacterial counts (TPC: 1.13 log CFU/mL and ENT: 0.53 log CFU/mL) were recorded after exposing the red grape juice sample to HPU treatment (A70%, 10 min). Similar results have been reported by other authors [41,45,49], revealing that the microbial load was lowered by increasing the ultrasound processing time. This indicates that the microbial cells might be resistant to sonication treatment and the cell destruction occurs only as the sonication treatment time is increased to a longer duration. Cell disruption may be caused by several factors such as combined physical and chemical mechanisms that occur during cavitation (caused by the changes in pressure), the formation of free radicals and hydrogen peroxide [49,50], leading to a thinning of microbial cell membranes, and the localized mild heating that occurs during sonication treatments [45,49].

Table 5. Impact of pasteurization and HPU treatments on the total plate count (TPC) and the *Enterobacteriaceae* count (ENT) of grape juice.

Parameter	Grape Juice Sample					
	C*	P**	HPU (A50%, 5 min)	HPU (A70%, 5 min)	HPU (A50%, 10 min)	HPU (A70%, 10 min)
TPC (log CFU/mL)	4.53 ± 0.01 ^a	2.53 ± 0.07 ^b	2.18 ± 0.03 ^{cd}	1.42 ± 0.02 ^{cd}	1.63 ± 0.02 ^c	1.13 ± 0.04 ^c
ENT (log CFU/mL)	1.51 ± 0.01 ^a	0.73 ± 0.04 ^{ab}	1.45 ± 0.05 ^{ab}	0.69 ± 0.03 ^{ab}	1.27 ± 0.02 ^{acd}	0.53 ± 0.0 ^{acd}

* Control sample; ** pasteurized juice sample. One-way ANOVA test was used to compare the means differences among treatments; different superscripts in the same row indicate significant differences among the treatments (Tukey's test, $p < 0.05$). Results are expressed as the average value of three replicates ± standard deviation (SD).

3. Materials and Methods

3.1. Grape Juice Preparation

Fresh red grapes (Merlot, from the Pietroasa Development Research Station for Viticulture and Winery, Buzau County, Romania) were split from bunches and crushed with a crusher-destemmer (Enoventa Technologies Enologiche, Piazzola sul Brenta, Italy) followed by the pressing process with a hydraulic press machine (L.U.C.M.E. Elettromeccanica, Verona, Italy). Only fruits without external injuries were used. Juice extraction and filtration were performed in a cold room at a temperature of 10 ± 1 °C. The obtained red grape juice was further subjected to pasteurization and HPU treatments. As a control sample was used the red grape juice before applying of any treatments. The control sample as well as the pasteurized and HPU treated juice samples were kept in sterilized, airtight container (bottles) and were stored at 4 °C until further analysis. All sample preparations and treatments were carried out in triplicate [45].

3.2. Pasteurization Process

Pasteurization of the grape juice samples was carried out on a hotplate with a magnetic mixer (IKA RTC Basic, Ika-Werke GmbH & Co. KG, Janke & Kunkel, Staufen, Germany) where the samples (150 mL) were placed in glass containers covered with aluminium foil over hot water bath at a temperature of 80 °C for 2 min. This temperature was chosen based on the previously reported results [18].

3.3. Ultrasound Equipment and Processing

The ultrasound treatments were conducted using a 750 W ultrasonic processor for small and medium volume applications (VCX 750, Sonics & Materials, Inc., Newtown, CT, USA) with a 1/2" (13 mm) probe used for ultrasound at a constant frequency of 20 kHz. A sample of 150 mL was placed in a glass beaker and the ultrasound probe was fixed at 45 mm depth in the juice sample. The amplitude was set at 50% (A50%) and 70% (A70%) and for each of these amplitudes, the grape juice samples were exposed to ultrasounds for 5 and 10 min. The main variables that are influencing the sonication are the intensity and the frequency of the waves. The amplitude of vibration of the ultrasonic source is proportional with the intensity of sonication that is the power dissipated per unit of surface area (W/cm^2) of the sonotrode. Thus, an augmentation of the amplitude induces an increasing of the sonochemical effects in the treated liquids. In order to identify the best extraction configuration, the amplitude may vary over a wide range [10]. Thus, depending on the amplitude and duration, four ultrasounds treatments were applied to the grape juice samples, as follows: HPU (A50%, 5 min), HPU (A50%, 10 min), HPU (A70%, 5 min) and HPU (A70%, 5 min). These combinations of amplitude and time of the applied HPU treatments were established according to the results of other previous studies, where a reduction of microbial count was obtained [28,49].

3.4. pH, Total Soluble Solids (TSS) and Titratable Acidity (TA)

The pH was measured using the electrochemical method [51] with a potentiometer (Consort C1010, Consort, Turnhout, Belgium). Titratable acidity (TA) and total soluble solids (TSS) of red grape juice samples were determined following OIV reference methods [52]. Total soluble solids (°Brix) were measured with an ABBE refractometer (ORT 1RS, KERN & SOHN GmbH, Balingen, Germany). Titratable acidity tests were performed on aliquots of 50 mL of the sample, 30 mL of boiled distilled water and 1 mL of bromothymol blue solution placed into a 250 mL beaker and titrated with standardized 0.1 mol/L NaOH (Sigma-Aldrich, Dublin, Ireland) until the same color was obtained as in the preliminary test (end-point color determination). The results were expressed as g of tartaric acid/L of juice.

3.5. L-ascorbic Acid Content (L-AsAc)

L-ascorbic acid (L-AsAc) or vitamin C determination of investigated grape juice samples was carried out by the 2,6-dichloroindophenol titrimetric method [53]. For this purpose, 10 mL of each sample was diluted with 10 mL oxalic acid 2% (Sigma-Aldrich), then, the mixture was filtered through Whatman filter paper and the clear extract was used for analysis. Further, 10 mL of the clear extract was taken and 1 mL hydrochloric acid 1N (ReAgent Chemicals, Runcorn, UK) was added. The obtained mixture was titrated with 2,6-Dichloroindophenol sodium salt (Sigma-Aldrich). A control sample titration was also performed. The results were expressed as mg/L of juice.

3.6. Determination of Total Phenolic (TP) Content and Polyphenolic Compounds Profile

The total phenolic content was determined by Folin-Ciocalteu assay [54]. For this purpose, 0.5 mL sample was treated with 1.25 mL reagent Folin-Ciocalteu (Merck, Darmstadt, Germany) diluted 1:10 with water. The sample was incubated for 5 min at room temperature and then 1 mL Na₂CO₃ 60 g/L was added. After 30 min of incubation at 50 °C the absorption of samples was measured at 750 nm using a UV-VIS spectrophotometer (Specord 205, Analytic Jena, Jena, Germany). The calibration curve was prepared using gallic acid as standard in the concentration range 5–250 µg/mL. The results were expressed in mg gallic acid equivalents (GAE)/100 mL of juice.

The polyphenolic compounds profile was determined by high performance liquid chromatography coupled with mass spectrometry (LC-MS) according to the method described by Abdel-Hameed et al. [55]. Thus, the main polyphenols from red grape juice samples were determined by LC-MS using SPD-10A UV (Shimadzu, Kyoto, Japan) and LC-MS 2010 detectors, and an EC 150/2 NUCLEODUR C18 Gravity SB 150 × 2 mm × 5 µm column (Macherey-Nagel, Düren, Germany). Chromatographic conditions were as follows: mobile phases A: water with formic acid at pH-3, B: acetonitrile with formic acid at pH-3, gradient program: 0.01–20 min 5%B, 20.01–50 min 5–40%B, 5–55 min, 40–95%B, 55–60 min 95%B. Mobile phase 0.2 mL/min, temperature 20 °C. The monitoring wavelength was 280 nm and 320. Calibration curves were performed between 20–50 µg/mL. Results were expressed in mg/L.

3.7. Microbiological Analysis

Total plate counts (TPC) and *Enterobacteriaceae* count (ENT) were carried out to evaluate the microbiological quality of the samples. The analyses were performed in triplicate according to the methodology established by the American Public Health Association. The samples were collected aseptically in a sterile vessel, immediately after processing. For sample preparation, mixing was performed using a stirrer to ensure a uniform distribution of the microorganisms in the mass product, followed by five dilutions consecutively (10^{−5}). As dilution liquid was used sterile distilled water. TPC determination was performed according to SR EN ISO 4833 [56] using Plate Count Agar (PCA) medium and the detection of *Enterobacteriaceae* was performed according to ISO 21528-1,2, [57] using VRBG medium (agar with purple red ball glucose). The PCA medium allows the growth of all aerobic germs and the other two culture media have selective character, containing inhibitory factors for the development of other microorganisms, other than *Enterobacteriaceae*, respectively yeasts and molds. After sowing of two Petri plates from each consecutive serial dilution, they were incubated for a different period of time, namely: 72 h for TPC at 30 °C and 37 °C and 24 h in the case of *Enterobacteriaceae* at 37 °C [58]. The results were expressed as log colony forming units per milliliter of juice (log CFU/mL).

3.8. Data Analysis

All values were obtained from three independent experiments and each red grape juice sample was analyzed in triplicate. All obtained results are presented as average values followed by the standard deviation (SD) of three replicates. The one-way analysis of variance (ANOVA) test was used to analyze the data and differences among means obtained in response to the applied treatments

were compared by a Tukey test with a level of significance of $p < 0.05$. The statistical data analysis was performed using the JASP (Version 0.11.1, 2019) computer software (JASP Team, University of Amsterdam, Amsterdam, The Netherlands).

4. Conclusions

The obtained results reveal that compared to pasteurization, the HPU treatment significantly improved the individual polyphenolic compounds and the L-AsAc content, without any significant effect on pH, TA and TSS. Also, important decreases in microbial counts without affecting the investigated bioactive compounds and physicochemical parameters of grape juice samples were induced by HPU treatment, compared to pasteurization. The TPC and ENT analysis results of the sonicated samples were significantly influenced by treatment time and amplitude level. The quality of red grape juice is influenced by choosing appropriate ultrasound treatment parameters. The ultrasound treatments with an amplitude of 70% were more efficient than those with A50% in terms of all investigated parameters. As for the processing time, our results reveal that while HPU treatment (A70%, 5 min) is recommended for enhancing the level of flavonoids such as rutin, quercetin, epicatechin, kaempferol as well as resveratrol, the HPU treatment (A70%, 10 min) was the most efficient in the reduction of the microbial load and obtaining the highest content of L-AsAc and TP together with a high level of some hydroxycinnamic acids as ferulic and caffeic acid. The data reported in this study suggest that HPU treatment might be used as an alternative emerging technique to successfully replace the pasteurization for improving the quality of the red grape juice. Nevertheless, we could go further in the experiments to establish the most adequate operating conditions of HPU treatment to be in agreement with national and international requirements in terms of microbial load for extending the self-life of grape juice.

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Article

Optimization of A Procedure to Improve the Extraction Rate of Biologically Active Compounds in Red Grape Must Using High-Power Ultrasound

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Abstract: The primary focus in the production of quality red wine is the extraction of grape components, which can be achieved in a variety of ways. This work investigates the extraction yield of biologically active compounds from crushed Merlot grapes, as a result of ultrasound treatment applied before maceration, and optimizes the process parameters of a laboratory scale using response surface methodology (RSM) within a central composite design (CCD) model. The two factors whose response was studied were amplitude (A) % and treatment time (t), while the dependent variables were the total phenolic compounds (TPC), monomeric anthocyanins (MA), and antioxidant activity expressed as ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. The results showed that the application of high-power ultrasound treatment to crushed grapes for a few minutes increased both the extraction rate of bioactive compounds and the antioxidant activity by a maximum of 12 times for the TPC, 14 times for the MA, 3.6 times for the FRAP value, and 18.77% for the DPPH. The optimized solution had an amplitude of 90% and a treatment time of 4 min and 24 s. The validation experiments yielded errors between—8.70% and 3.14%, confirming the proposed model. Thus, the RSM model is recommended as a tool to optimize a procedure for enhancing both the extraction rate of the bioactive compounds from grapes and the antioxidant properties of grape must. Our results demonstrate the ultimate benefits of using ultrasonic treatment on crushed grapes at the beginning of the winemaking process, as a highly effective technique for improving the extraction of high-value bioactive chemicals, with significant application potential.

Keywords: red grapes; bioactive compounds; antioxidant activity; ultrasound; extraction optimization procedure

1. Introduction

Wine is a cultural emblem, whose function has evolved over time from being a vital source of sustenance to becoming a social and cultural accompaniment to food. The production of wine grapes of 33.8 million tons and the volume of wine produced globally in 2022, which was between 257.5 and 262.3 million hectoliters of wine, with a midpoint estimate of 259.9 million hectoliters, is anticipated to be comparable to the level seen in 2021. The worldwide output level can be regarded as somewhat below average for the fourth year in a row [1].

To satisfy the varied sensory preferences of consumers, it is essential to be able to manufacture several red wine types, and wine producers are continually changing their winemaking techniques to this end. When making high-quality red wine, the extraction of grape components, which can be conducted in a variety of ways, is the major focus [2].

Due to their numerous positive health benefits, including antioxidant properties, neuroprotective action, anti-inflammation, antiaging antimicrobial activity, and lowering arterial blood pressure, phenolic compounds have attracted a lot of interest by the scientific community and the general public in recent years [3–7].

Moreover, customers place a high value on the sensory quality of red wine's color, preferring it to be a deep red. The primary pigments responsible for the young wines' vivid red color are called anthocyanins, the main polyphenolics in red grapes [8]. The initial monomeric anthocyanin profile is an element that affects the ultimate color of the wine and, additionally, might have anti-cancer and anti-glycemic properties [9].

Because of the inherent drawbacks to conventional extraction techniques, the effective extraction of these chemicals from natural sources continues to be a significant issue. The rising need for novel technical solutions that can deliver the highest extraction yields, guarantee the stability of the target compounds, and also satisfy environmental requirements, has been sparked by the growing interest in natural antioxidants [10].

Due to its eco-friendly nature, non-toxic behavior, and low energy usage, ultrasound (US) may be regarded as a sustainable, green, and innovative technology [11,12]. There are several uses for it in food technology, including analysis, preservation, and homogenization, extraction, filtering, and drying. In order to achieve these objectives, ultrasound principally employs acoustic waves, which cause mechanical and chemical reactions. These reactions are fundamentally different from those produced by conventional methods. The main goals of this technology are to speed up processing, save energy, and improve the quality, safety, and shelf life of food products [12,13]. To sum up, this technique is a green alternative for this industry, as it does not use toxic solvents. Additionally, it supports economic sustainability through its use in efficient extraction procedures [14].

Ultrasound is divided into three categories: high frequency–low power US (between 1 and 10 MHz), intermediate frequency–medium power US (between 100 kHz and 1 MHz), and low frequency–high power US (between 20 and 100 kHz). Strong physical effects are primarily produced at a US frequency of about 20 kHz by fragmentary transitory cavitation, promoting bioactive chemical release from plant matrices during cell disruption [15–17]. The effect of low frequency may be related, not only to cavitation bubble size, but also to its effect on mass transfer resistance [13]. High-power US treatment on food matrices can cause physical, chemical, and biological changes, and a variety of uses have been documented [18,19]. Numerous studies have demonstrated that high-power ultrasound is becoming more prevalent in the enological sector due to its ability to enhance a wine's organoleptic qualities, shorten processing times, and provide better microbial control in wines [20–22]. Furthermore, the International Organization of Vine and Wine has included ultrasonic treatment among the methods that can be used during winemaking [23].

Currently, adopting all the techniques designed to make color extraction and fragrance dissolution easier, throughout the winemaking process, is desired. Through the application of ultrasonic technology, the potential for the continuous extraction of qualitatively significant substances from the exocarp is explored. This is conducted by taking advantage of the phenomena caused by the transmission of ultrasound in a solid–liquid environment, such as grape must [13].

However, taking into account the fact that most research was carried out at the laboratory scale, and that the grape skins' total phenolic content varies according to the cultivar, soil type, climate, region of origin, and cultivation techniques, we consider that it is vital to optimize the factors involved in this form of extraction in order to build a workable ultrasound-assisted large-scale procedure. The extraction efficiency using high-power US is significantly influenced by a variety of factors, such as the temperature of the samples, the power of the ultrasonic device, the process frequency, the treatment's intensity, as well as the shape and size of the ultrasonic reactor [24].

Thanks to its reliable design, the statistical method known as response surface methodology (RSM) is currently widely utilized for optimization. The RSM is a collection of mathematical and statistical methods that are used to investigate the relationship between

process input variables and outputs (responses). The methodology can be used for process optimization in a variety of circumstances and is relatively affordable, practical, and effective [25].

The objective of this work was to investigate the extraction yield of biologically active compounds from crushed grapes, as a result of an ultrasonic treatment applied during the first stages of the winemaking process, before maceration. In addition, the aim was to identify the optimized process parameters in terms of amplitude (A) % and treatment time (t) at the laboratory scale, as well as to develop a procedure that improves both the extraction rate of bioactive compounds from grapes and the antioxidant properties of grape must. To this end, we first evaluated the high-value phytochemicals, such as the total phenolic compounds (TPC), which are generally associated with the organoleptic properties of finished wines and (with) their stability. We also quantified the monomeric anthocyanins (MA), which are responsible for the red color of red wines. In addition, the antioxidant activity was assessed, from all the possible biological functions performed by *V. vinifera* compounds, both the ferric reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assays are probably the most important regarding human health.

2. Materials and Methods

2.1. Grapes

The Merlot variety employed for the experimental research is the second most planted grape variety in the world, well-known for producing high-quality wines [26]. The red grapes, vintage 2019, were hand-harvested (approximately 100 kg) from a vineyard in Prahova, Romania (Pietroasa-Istria Viticulture and Winemaking Research and Development Station). The grapes were harvested at technological maturation when they reached 29.8° Brix, then quickly transported to the laboratory for processing. Only healthy, selected bunches of grapes were used for the experimental research.

2.2. Ultrasonic Equipment

The sonication of all the experimental tests was performed using a probe-type ultrasonic device (Sonics VCX-750, Sonics and Materials Inc., Newtown, CT, USA). The amplitude can be set as a percentage, between 10% and 100%. The device produces an ultrasonic power of 750 W and frequency of 20 kHz. The most typical frequencies utilized in the extraction procedures were between 20 and 100 kHz.

2.3. Optimization Design

The experimental design was developed with Design Expert® software version 13 (Stat-Ease, Inc., Minneapolis, MN, USA, 2022), to optimize the number of experiments and operating conditions.

Based on the results of the single factor experiment, an optimization design was created. For these experiments, the RSM was used within a central composite design (CCD) model. The two factors whose response was studied were amplitude (A) % and treatment time (t). The high and low levels of each variable were determined by preliminary research that took place before carrying out of these experiments. The limits of the instrument were also taken into account when choosing the ranges for all the variables. The intensity and frequency of the waves were two of the most important operating parameters that affect the performance of ultrasound technology in extracting bioactive chemicals. The results of some studies indicated that the sonochemical effects in the treated fluids increase as the amplitude increases. Moreover, the amplitude varies over a wide range, from 20% to 90% [27], to determine the ideal extraction configuration, while other studies used treatment durations ranging from 2 to 10 min [28,29].

Furthermore, according to our preliminary research, there were no significant differences in the extraction of biologically active compounds between the control sample and the ultrasound-treated samples, when ultrasound was applied at low amplitude levels

(from 30% to 50%) for a maximum treatment time of 5 min. The extraction temperature during ultrasound extraction was shown to be closely related to the extraction time, as it was found that longer extraction times resulted in higher extraction temperatures. Therefore, the extraction time selected should be within the appropriate range. However, it was found that a maximum extraction time of 5 min was necessary because high temperatures promote the oxidation and destruction of phenolic compounds.

Taking into account the aforementioned factors, the levels of the independent variables, such as percent amplitude (A) and sonication time (t), were selected at 50%, 70%, and 90% for the ultrasound time of 3, 4, and 5 min (Table 1), respectively. The dependent variables were the TPC, MA, and antioxidant activity, expressed as the FRAP and DPPH free radical scavenging activity.

Table 1. Ultrasonic extraction test treatment options.

Independent Variables				Dependent Variables
Probe Diameter (mm)	Frequency (kHz)	Amplitude (A) %	Treatment Time (t) min	
13	20	50	3	TPC
		70	4	MA
		90	5	FRAP
				DPPH

To choose the best statistical model, such as linear, quadratic, and cubic models, the experimental data were examined based on the second-degree polynomial equation (Equation (1)):

$$Y_k = \alpha_0 + \sum_{i=1}^n \alpha_i X_i + \sum_{i=1}^n \beta_i X_i^2 + \sum_{i < j}^n \alpha_{ij} X_i X_j \quad (1)$$

where Y_k is the dependent variable, α_0 , α_i , α_{ii} , and α_{ij} are the regression coefficients of the model, which are constants, and X_i and X_j are the independent variables of the model. Response surface plots were generated using the expected values from the fitted model. To illustrate the influence of the independent variables on each response, two- and three-dimensional contour plots were generated for each response.

2.4. Sample Preparation

For the laboratory tests, randomly collected grape samples (100 g) were destemmed, hand-crushed, and then treated using a probe-type ultrasonic device. The control sample (C) that was not subjected to ultrasound treatment was evaluated separately. The samples were treated in a 100 mL Pyrex glass beaker fitted with a counter-current water-cooling jacket. The water used for cooling was kept at a constant temperature of 19 °C. The positioning of the acoustic amplifier in the container was standardized at a distance of 20 mm from the bottom. At the end of the ultrasonic treatment, the samples were vacuum filtered (Nalgene Rapid-Flow Filter Units, 0.45 µm PES membrane). The filtrate was collected in a vial and stored in a refrigerator at 4 °C for further use. All extraction experiments were conducted in this manner.

2.5. Total Polyphenolic Content and Anthocyanins Determination

The total polyphenolic content (TPC) was determined by using the Folin–Ciocalteu spectrophotometric method, with gallic acid as a reference and expressed as micrograms of gallic acid equivalents per milliliter (µg GAE/mL) [30]. A 0.5 mL sample was treated with 1.25 mL of Folin–Ciocalteu reagent (Merck, Darmstadt, Germany), previously diluted 1:10 (v/v) with distilled water. After incubation for 5 min at room temperature, 1 mL Na₂CO₃ 60 g/L was added. A UV–VIS spectrophotometer (Specord 205, Analytik Jena Inc., Jena, Germany) was used to detect sample absorbance at 750 nm after 30 min of incubation at

50 °C. The calibration curve was established using gallic acid as standard, at concentrations ranging from 5 to 250 µg GAE/mL.

The monomeric anthocyanins (MA) were determined by the pH differential method [31]. Briefly, two dilutions of the same sample were prepared by adding 1 mL of wine to 14 mL of potassium chloride buffer (0.025 M, pH 1.0) and 14 mL of sodium acetate buffer (0.4 M, pH 4.5). After incubation for 15 min at room temperature, the absorbance was measured at 520 and 700 nm against deionized water. The results were expressed in milligrams of cyanidin-3-glucoside equivalents per liter (mg CGE/L). The total anthocyanin content of the samples was calculated using a molar absorption coefficient of 26,900 L/mol × cm and a molecular weight of 449.2 g/mol.

2.6. Antioxidant Activity

The antioxidant activity of the investigated samples was evaluated by FRAP and DPPH assays.

2.6.1. FRAP Assay

The protocol for the FRAP test was based on the method of Benzie and Strain [32]. For this purpose, 300 mM acetate buffer (pH = 3.6), 10 mM of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM FeCl₃ × 6H₂O solution were used as stock solutions. The working solution was prepared by mixing 100 mL acetate buffer, 10 mL TPTZ solution, and 10 mL FeCl₃ × 6H₂O solution, which was then heated to 37 °C before use. Prior to analysis, the wine samples were diluted 1:50 (v/v) with distilled water, then a 0.5 mL aliquot of diluted samples was allowed to react with 2.5 mL of working solution for 30 min at 37 °C. The absorbance of the mixture was read at 593 nm against a blank sample obtained under the same working conditions. The results were expressed in µmol of Fe²⁺ equivalents per milliliter of grape must (µmol Fe²⁺/mL).

2.6.2. DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the samples was measured following the procedure described by Kalita et al. [33], with some minor adjustments. For this purpose, 20 µL of each investigated sample was mixed in a microplate with 20 µL of distilled water, after which the resulting mixture was combined with 200 µL of a 120 mg/L DPPH radical solution prepared in 96% (v/v) ethanol. The microplate was left in the dark for 30 min, and the absorbance value (A sample) at 515 nm was measured against a blank of 96% (v/v) ethanol, using a plate reader (Tecan Sunrise™, software Magellan™, Tecan Group Ltd., Männedorf, Switzerland). The control was prepared under the same working conditions, containing 20 µL of 96% (v/v) ethanol instead of the investigated sample. The absorbance of the control (A control) was read against 96% (v/v) ethanol.

The DPPH scavenging activity (%) was calculated according to Equation (2), as follows:

$$\text{DPPH scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100 \quad (2)$$

2.7. Total Soluble Content and Acidity

The total soluble solids (TSS, ° Brix) and the titratable acidity (TA, g/L of tartaric acid) were analyzed according to the standard procedure of Council Regulation EEC 2676/90 [34].

2.8. Statistical Analysis

All the measurements were performed in triplicate and the results are presented as mean ± standard deviation (SD). The Design Expert® software version 13 (Stat-Ease, Inc., Minneapolis, USA, 2022) was used for the statistical analysis. Significant differences between the samples were assessed using a one-way ANOVA, with post-hoc analysis using the HSD Tukey test.

The Pearson correlation coefficient between the total phenolic content, monomeric anthocyanins, and antioxidant activity was calculated using JASP software, version 0.17.1 (JASP Team, Amsterdam, The Netherlands, 2023).

3. Results and Discussion

The effects of an ultrasonic pre-treatment to improve the extraction rate of polyphenols and, subsequently, to increase the antioxidant activity of several samples, with three replicates each, all from red grapes, the Merlot variety, vintage 2019, were followed in the laboratory. The experimental design provided for the randomized treatment of 13 samples with 3 repetitions each, with 5 central points, as shown in Table 2.

Table 2. Design of the central composite experimental model and results.

Std Order	Run Order	Factors		Responses			
		A (%)	B (min)	TPC ($\mu\text{g GAE/mL}$)	MA (mg CGE/L)	FRAP ($\mu\text{mol Fe}^{2+}/\text{mL}$)	DPPH Scavenging Activity (%)
2	1	90	3	1396.06 $\pm$ 5.23	223.46 $\pm$ 2.32	41.78 $\pm$ 2.25	74.5 $\pm$ 0.2
9	2	70	4	947.92 $\pm$ 3.79	167.05 $\pm$ 1.46	27.52 $\pm$ 1.21	73.3 $\pm$ 0.6
1	3	50	3	124.5 $\pm$ 1.01	20.67 $\pm$ 0.72	15.02 $\pm$ 0.83	66.8 $\pm$ 0.5
6	4	90	4	1177.85 $\pm$ 4.87	214.97 $\pm$ 2.36	42.96 $\pm$ 1.98	75.9 $\pm$ 0.6
11	5	70	4	948.05 $\pm$ 4.56	169.7 $\pm$ 1.55	28.85 $\pm$ 1.09	73.2 $\pm$ 0.3
5	6	50	4	136.4 $\pm$ 1.08	25.49 $\pm$ 0.65	16.09 $\pm$ 0.78	68.2 $\pm$ 0.2
7	7	70	3	482.31 $\pm$ 3.82	108.37 $\pm$ 1.02	27.17 $\pm$ 0.94	72.1 $\pm$ 0.5
4	8	90	5	1172.1 $\pm$ 3.75	176.29 $\pm$ 2.45	48.32 $\pm$ 2.23	77.2 $\pm$ 1.0
12	9	70	4	949.23 $\pm$ 3.98	166.6 $\pm$ 1.47	28.02 $\pm$ 0.74	73.2 $\pm$ 0.9
10	10	70	4	951.19 $\pm$ 4.56	168.15 $\pm$ 1.62	29.15 $\pm$ 1.16	73.3 $\pm$ 0.8
3	11	50	5	148.3 $\pm$ 2.04	26.22 $\pm$ 1.23	18.68 $\pm$ 0.43	71.1 $\pm$ 0.6
13	12	70	4	950.24 $\pm$ 4.21	167.24 $\pm$ 1.74	27.97 $\pm$ 0.54	73.4 $\pm$ 0.5
8	13	70	5	1126.11 $\pm$ 2.01	137.74 $\pm$ 1.03	29.97 $\pm$ 0.81	73.5 $\pm$ 0.7

Amplitude (A); time (B); total polyphenol content (TPC); monomeric anthocyanins content (MA); antioxidant activity: FRAP and DPPH. The results are expressed as the mean value of the three replicates $\pm$ the standard deviation (SD).

In terms of the content of bioactive compounds, such as the TPC and MA, as well as the antioxidant activity measured by the FRAP and DDPH assays, the effects of the high-power ultrasonic treatment on extraction kinetics were explored. All the above parameters were studied in untreated red grapes must as a control sample (C) and on the samples subjected to ultrasound treatment at 50, 70, and 90% amplitude for 3, 4, and 5 min, respectively, as follows: SM50/3 (A: 50%; t: 3 min), SM50/4 (A: 50%; t: 4 min), SM50/5 (A: 50%; t: 5 min), SM70/3 (A: 70%; t: 3 min), SM70/4 (A: 70%; t: 4 min), SM70/5 (A: 70%; t: 5 min), SM90/3 (A: 90%; t: 3 min), SM90/4 (A: 90%; t: 4 min), and SM90/5 (A: 90%; t: 5 min).

Analytical determinations were performed immediately after crushing for the control sample and immediately after treatment for the ultrasound-treated samples.

Two factors, the amplitude level and the duration of the ultrasound treatment, were found to have an effect on the parameters studied, with single or interaction effects. The ANOVA analysis of variance performed on the analytical parameters, in relation to the different conditions of amplitude and treatment time, revealed significant differences in the total polyphenolic content, the monomeric anthocyanins, and the antioxidant activity. Using the RSM and ANOVA, the best extraction parameters were statistically examined.

The process of sonication results in the development of cavitation bubbles, which have the potential to violently collapse, releasing energy and increasing the temperature and pressure. These bubbles may burst close to a solid surface or interact chemically with molecules, harming cell structures and altering the selectivity or permeability of cell membranes [20].

To monitor the corresponding temperature rise, the temperature of the sonicated samples was measured at 1 min intervals, using a thermovision camera. The mean value of the untreated sample (C), and the mean values recorded by a forward-looking infrared (FLIR) camera for each sample when the sonication treatment ended are illustrated in Figure 1.

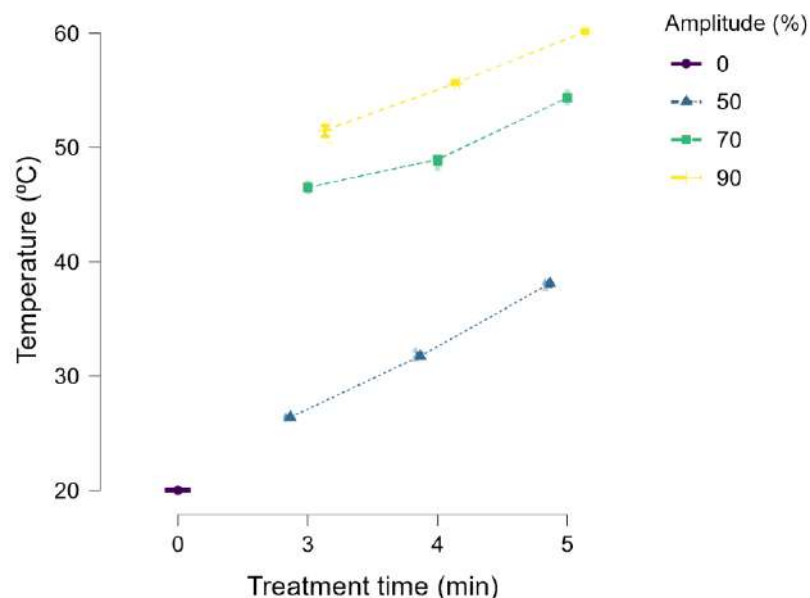


Figure 1. Changes in the temperature of the samples treated with US compared to the control sample (C).

An increase in the temperature of the samples following the ultrasound treatment was evident, compared to the reference sample, C (20 °C), correlated with the intensity and time of the treatment. The minimum and maximum average temperatures reached in the samples were 26.20 °C for SM50/3 and 60.17 °C for SM90/5, respectively. The results confirmed the fact that the increasing temperature of the must samples depends on the operating parameters used during the ultrasound treatments, as it was established that the device's real input power was transformed into heat that was released into the medium [26].

It can be observed that the increasing temperature in the sonicated samples was influenced to a higher measure by increasing the amplitude compared with the treatment time. Thereby, the temperature reached in SM70/5 (54.23 °C), treated with a lower amplitude but the maximum time (5 min), was close to that of SM90/3 (51.53 °C) treated with a higher amplitude but the minimum time (3 min).

Increased temperature often results in higher extraction yields. Temperature optimization can be performed to obtain the best yield of the target compounds without degradation, as this parameter can vary depending on the type of product [35].

It was also concluded that the intensity of the ultrasound was directly related to the amplitude of the transducer. Increasing the amplitude increased the ultrasound intensity and resulted in an increase in the sonochemical effects [36].

Using different levels of device power, intensity, frequency, amplitude, and treatment time, previous research on red grapes and wines of various types has yielded very encouraging findings on the ability of ultrasound to extract and improve the physicochemical quality of wines, as well as its ability to accelerate wine aging or control microorganisms [37–41].

In addition, other authors reported that the application of US produced interesting findings on the recovery of bioactive compounds from marc and lees, and the extraction of various aromatic compounds from wines [42–44].

Table 3 depicts the results for the physicochemical and analytical determinations of the untreated red grape must.

Table 3. Characteristics of the red grape must.

Sample	TSS (° Brix)	TA (g Tartaric Acid/L)	TPC (µg GAE/mL)	MA (mg CGE/L)	FRAP (µmol Fe ²⁺ /mL)	DPPH Scavenging Activity (%)
C	29.9 ± 0.1	3.4 ± 0.03	114.42 ± 2.67	16.06 ± 0.9	13.43 ± 1.02	65.0 ± 0.4

Untreated red grape must (C); total soluble solids (TSS); total acidity (TA); total polyphenolic content (TCP); monomeric anthocyanins content (MA); antioxidant activity: FRAP and DPPH. The results are expressed as the mean value of the three replicates ± the standard deviation (SD).

3.1. Total Polyphenolic Content

The TPC extraction yields were observed to rise with any increase in temperature. This impact might primarily be ascribed to the cavitation phenomenon, which is preferred at lower temperatures, and which makes it easier to release extractable chemicals and enhances mass movement via diffusion or by rupturing plant cell walls [23,32,45].

The TPC for sonicated samples varied from 124.5 micrograms of gallic acid equivalents per milliliter (GAE/mL) to 1396.06 µg GAE/mL, as shown in Table 2. The lowest amount of TPC was found in the SM50/3 sample (run order three) using 50% amplitude for 3 min, while the higher TPC content was found for the SM90/3 sample (run order one) using 90% amplitude for 3 min. It can be seen that there was an increase, generally proportional with the treatment conditions, of 9% to 12 times for the total polyphenol content of the sonicated samples compared with untreated sample (C). These findings indicate that amplitude is a significant factor in the extraction of total phenols.

Previous studies have shown a higher extraction yield of TPC when high-power ultrasound was applied to grapes or during winemaking [27,46]. Other authors have reported that no degradation of the polyphenols was observed after the sonication of red wine [29].

Moreover, the ultrasound treatment with an amplitude of 90% for 3 min determined the highest extraction of TPC in the sample SM90/3, while lower values were recorded when applied at the same amplitude of 90% for 4 min and 5 min, by 15.63% in the sample SM90/4 and 16.04% in the sample SM90/5, respectively. The possible explanation for this decrease might be the high temperatures reached in the immediate vicinity of the ultrasonic probe, as well as the average temperatures of 55.71 °C and 60.17 °C reached in samples SM90/4 and SM90/5, respectively, that could lead to the degradation of phenolic compounds. Other research has shown that polyphenol can be protected against thermal breakdown processes at temperatures as high as 50 degrees Celsius [47].

Despite their benefits, many phenolic compounds quickly hydrolyze and oxidize at higher temperatures, particularly when extracted for extended periods of time [48,49]. At extraction temperatures over 60 °C, Akowuah, Mariam, and Chin demonstrated that the total polyphenol content of the extracts reduced as a result of oxidative degradation [50]. The vulnerability of phenolic compounds to high temperatures was demonstrated, as early as three decades ago, by Havlikova and Mikova [51]. They demonstrated that high temperatures and prolonged extraction times enhanced the rate at which phenolic components oxidized and lowered the yield of the extracts. For instance, during maceration, the extraction of polyphenols is typically carried out between 20 and 50 °C, never over this range [52].

Furthermore, it is important to remember that the sensitivity of a sample to temperature-induced polyphenol degradation is influenced by the types of polyphenolic compounds present in the plant extract or matrix, as well as their physicochemical and biochemical properties [47].

However, additional variables such as plant species, geographical origin, or cultivars may also have an impact on the quantity of phenolic chemicals recovered using an ultrasound-assisted approach [10].

The optimal extraction parameters for the TPC were statistically investigated using the RSM and ANOVA (Table 4).

Table 4. ANOVA and coefficient table for the quadratic model. Response 1: total phenolic compounds.

Source	F-Value	p-Value	R ²	Adjusted R ²	Coefficients
Model	13.40	0.0018	0.9054	0.8378	
Intercept					921.98
A	60.33	0.0001			556.13
B	1.07	0.3361			73.94
AB	0.4989	0.5028			61.94
A ²	3.47	0.1049			−196.5
B ²	0.2193	0.6538			−49.42
Lack of fit	36229.38	<0.0001			

Amplitude (A); time (B).

As Table 4 shows, the amplitude term is significant for the enhanced extraction of polyphenols. The model F-value of 13.40 means that the model is significant. Due to the noise, there is only a 0.18% chance that such a large F-value could occur. Moreover, *p*-values less than 0.0500 indicate that the model terms are significant. In this case, amplitude is a significant model term. Values greater than 0.1000 indicate that the model terms are not significant. A high R² value (0.9054) indicates a good level of model fit. The lack of fit F-value of 36,229.38 indicates that the lack of fit is significant. There is only a 0.01% chance that such a large lack of fit F-value could occur due to noise.

The final equation in terms of the actual factors for the TPC is shown in Equation (3). The equation can be used to make predictions about the response for given levels of each factor.

$$Y_{\text{TPC}} = -5385.266 + 108.970 \times A + 686.076 \times B - 3.097 \times A \times B - 0.491 \times A^2 - 49.418 \times B^2 \quad (3)$$

where A is the amplitude and B represents the time.

The influence of the process parameters A and B on the extraction rate of the TPC in samples treated with US is shown in Figure 2.

Figure 2 clearly illustrates that the amplitude of ultrasonic waves has a greater impact on the outcomes than the time of the treatment. A higher treatment time with smaller amplitudes (50%) has a detrimental effect, and less TPC is extracted. Higher amplitude values (90%), on the other hand, greatly improve the TPC extraction yield.

The potential of ultrasonic waves to have a detrimental effect on biomass, aiding the extraction of cell contents, has been ascribed to ultrasound assisted extraction, having an improving influence on extracting phenolics from plants. Using ultrasound and different operating conditions, previous studies have shown increased yields of phenolic extracts as plant bioactive compounds, when applied to blueberry pomace, mango peels, or coffee beans [53–55].

In addition, it was observed that heat breakdown of the polyphenols may take place at high temperatures. A reduction in the extraction yield owing to temperature might be attributed to phenolic components degrading at high temperatures [56].

In the described situation, the optimal circumstances would be a higher amplitude, and a reduced duration of the treatment.

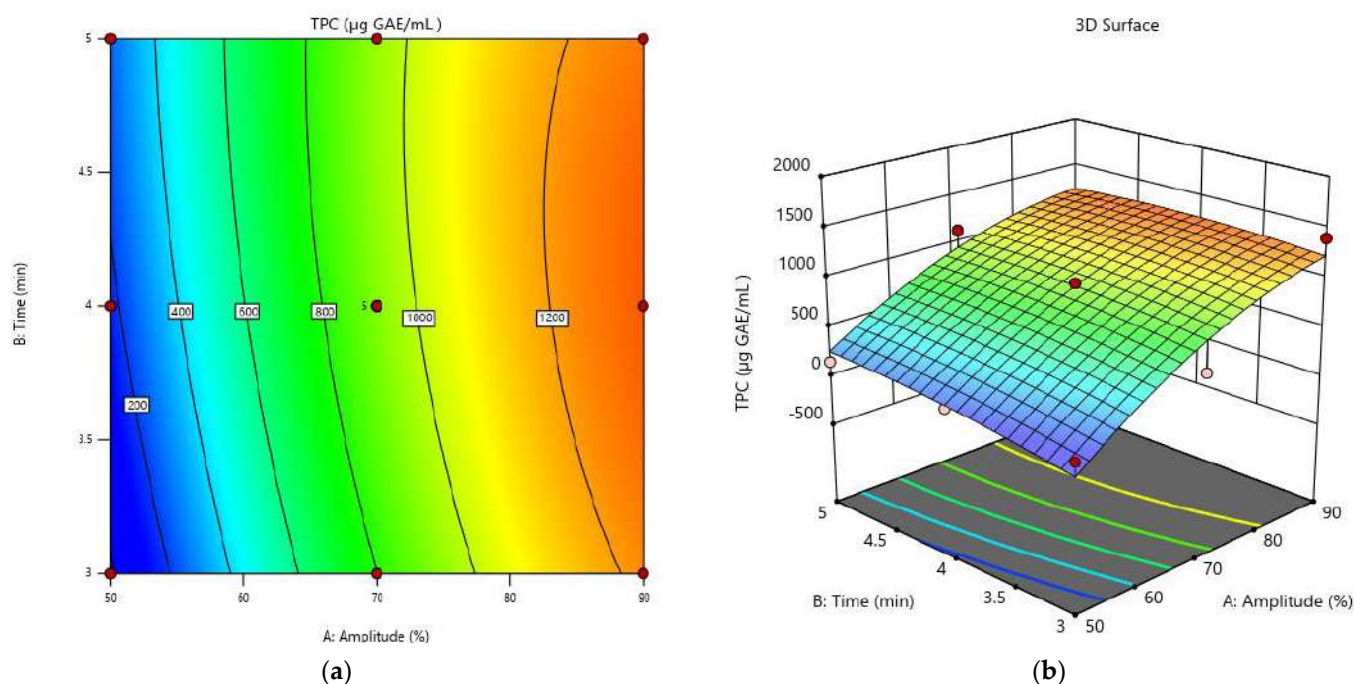


Figure 2. Influence of the process parameters on the TPC: (a) amplitude (A) and time (B) as contour graph; (b) amplitude (A) and time (B) as 3D surface.

3.2. Monomeric Anthocyanins

Anthocyanins are a major family of phenolic compounds found in red wine. The treatment tests showed a strong interaction of the cavitation phenomena with the cells of the exocarp, with an increase in the concentration of anthocyanins. Our results were consistent with others that include ultrasound-assisted extraction on the list of new technologies that have been developed with the goal of reducing extraction times and enhancing yields [57].

The MA content for the sonicated samples varied from 20.67 mg cyanidin 3-glucoside equivalents (CGE)/L to 223.46 mg CGE/L, as shown in Table 2. Similar to the TPC, the lowest amount of MA was found for the SM50/3 sample (run order three) using 50% amplitude for 3 min, while the higher MA content was found for the SM90/3 sample (run order one) using 90% amplitude for 3 min. It can be observed that there was an increase, generally proportional with the treatment conditions, of 28.7% to about 14 times for the MA of the sonicated samples compared with untreated sample (C).

Regarding the extraction of MA, US treatment with A 90% for 3 min led to the highest extraction (sample SM90/3), while slightly lower values of 3.8% (sample SM90/4) and significant lower values of 21.11% (sample SM90/5) were recorded when we used an amplitude of 90% for 4 min and 90% for 5 min, respectively. It is observed that the extraction of monomeric anthocyanins increases by a maximum value and then start to decrease, probably also due to the influence of the final temperature reached in the treated samples.

The anthocyanin content of grape juice was negatively impacted by higher amplitude levels and longer treatment times, as other authors have shown [28]. Moreover, it was observed that anthocyanins are susceptible to degradation due to a variety of variables such as pH, temperature, oxygen, water activity, and co-pigments [58].

Other researchers revealed that the temperature has a substantial impact on anthocyanin extraction, with maximal extraction occurring at roughly 30 to 35 °C. However, temperature increases enhance extraction by increasing the solubility of anthocyanins and the diffusion coefficient. However, temperatures over a particular point resulted in a reduction in anthocyanin output, e.g., at temperatures above 45 °C, anthocyanins decrease dramatically [59].

As it can be noticed, for the highest extraction of both the TPC and MA ultrasound amplitude and treatment time are the same: 90% amplitude for 3 min.

The difference in behavior between the total phenolics and anthocyanins might be explained by anthocyanins' greater vulnerability to high temperatures [56].

According to Table 5, model terms for the monomeric anthocyanins A, A², and B² are significant since their *p*-values are less than 0.0500, as shown. If the value is higher than 0.1000, the model terms are not considered relevant. An F-value this large might be caused by noise just 0.01% of the time, but the model's F-value of 44.35 suggests that the model is important. The lack of fit is implied to be significant by the lack of fit F-value of 396.80.

Table 5. ANOVA and coefficient table for the quadratic model. Response 2: monomeric anthocyanins.

Source	F-Value	<i>p</i> -Value	R ²	Adjusted R ²	Coefficients
Model	44.35	<0.0001	0.9694	0.9475	
Intercept					162.77
A	190.42	<0.0001			90.39
B	0.0972	0.7644			2.04
AB	2.70	0.1444			−13.18
A ²	9.71	0.0169			−30.08
B ²	7.97	0.0257			−27.25
Lack of fit	396.80	<0.0001			

Amplitude (A); time (B).

The adjusted R² of 0.9475 and the predicted R² of 0.8878 are in fair agreement (i.e., the difference is less than 0.2), indicating that the model makes accurate predictions. Equation (4) displays the final equation in terms of the actual factors for monomeric anthocyanins. For specific levels of each element, predictions regarding the response can be made using the equation.

$$Y_{MA} = -1134.49 + 17.683 \times A + 262.123 \times B - 0.659 \times A \times B - 0.075 \times A^2 - 27.254 \times B^2 \quad (4)$$

where A is the amplitude and B represents the time.

The effect of the process parameters A and B on the extraction rate of the MA in samples that underwent US treatment is shown in Figure 3.

According to Figure 3, which compares the effects of the treatment duration and ultrasonic wave amplitude on the extraction of the monomeric anthocyanins from red grape exocarp, the extraction yield of the MA increases with both up to a certain point before starting to decline. However, as the treatment time increases over 3 min using the ultrasound wave amplitude at the maximum level (90%), the extraction rate of the MA decreases. Excessive cavitation occurs at high amplitudes, raising the temperature around the probe, creating more hydroxyl radicals, and resulting in excessive degradation [60]. Moreover, as a smaller amplitude may not deliver enough energy to break the cell wall, the lowest extraction yields of the MA were observed in the samples where the minimum amplitude level was used (A = 50%).

Using ultrasound and different working conditions, previous studies have also shown increased yields for anthocyanin extractions from blueberry marc, mulberry wine residues, or purple sweet potatoes [61–63].

Similar to the TPC, the optimal circumstances for the MA enhanced extraction would be a higher amplitude, and a reduced duration of the treatment.

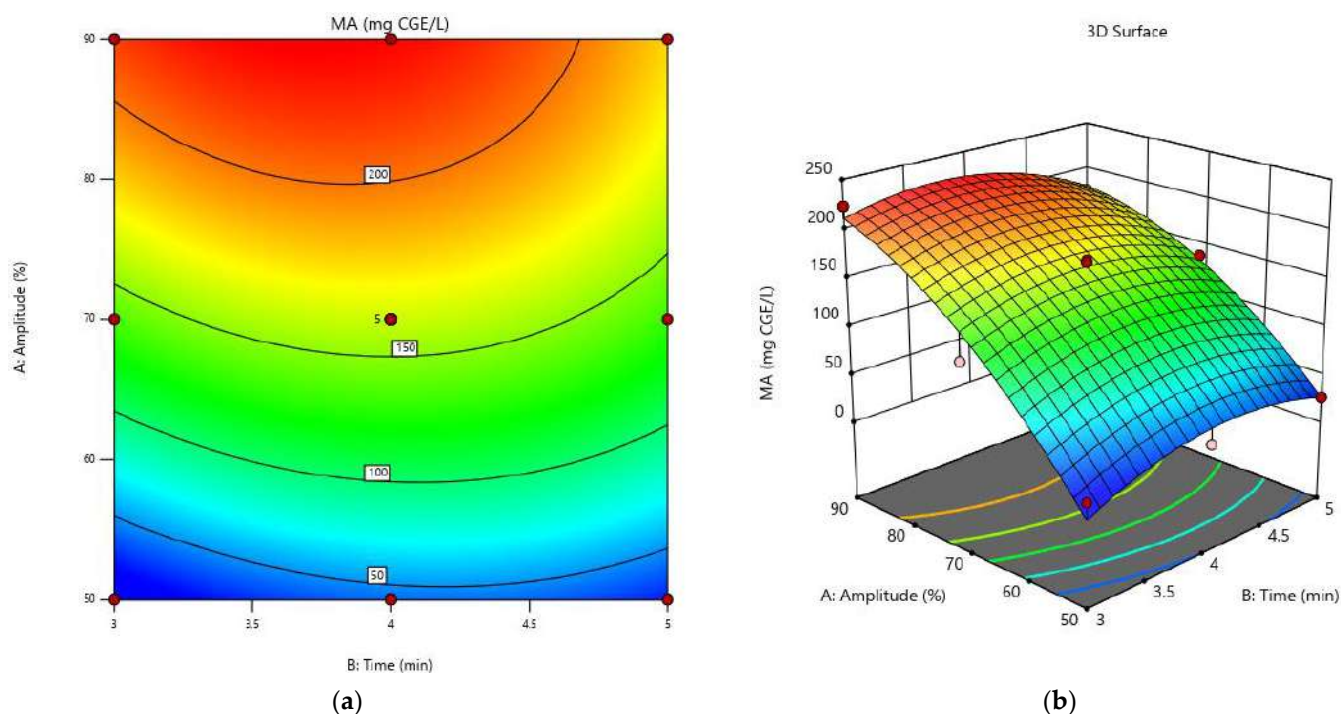


Figure 3. Influence of the process parameters on the MA: (a) amplitude (A) and time (B) as contour graph; (b) amplitude (A) and time (B) as 3D surface.

3.3. Antioxidant Activity

The antioxidant properties of the phenolic compounds from grapes have been extensively researched, including the scavenging of free radicals, the suppression of lipid oxidation, the decrease of hydroperoxide production, and so on. These properties are especially significant in terms of bioactivity. Moreover, antioxidants can stop the oxidation process by preferentially interacting with an oxidizing agent, rather than the desired target molecules or cells [64].

In addition to the FRAP assay, the DPPH scavenging activity was evaluated to further support our research findings. Both the DPPH and FRAP assays could be used to determine the antioxidant activity, as both showed high reproducibility [65].

The maximum antioxidant activity, $48.32 \mu\text{mol Fe}^{2+}/\text{mL}$ and 77.2%, as measured by the FRAP and DPPH assays, respectively, was observed in the sonicated samples of run order 8, acquired at 90% amplitude for 5 min (sample SM90/5) (Table 2). The lowest activity, $15.02 \mu\text{mol Fe}^{2+}/\text{mL}$ or 66.8% (run order 3) was observed at 50% amplitude for 3 min (sample SM50/3).

It was noted that the greatest extraction of antioxidants required the same ultrasonic amplitude (90%) as for the TPC and MA, but the maximum time was 5 min, not 3 min as for the TPC and MA. Moreover, the fact that antioxidants also include non-phenolic chemicals, which may require a higher temperature for extraction, may help to explain a slightly higher extraction temperature of 60.17°C for the maximum value of antioxidant compounds. Previous studies that claimed antioxidants required a higher temperature for extraction are consistent with this conclusion [25]. Moreover, the results might suggest that the antioxidant activity of the samples was significantly influenced by the TPC levels.

It can be seen that the antioxidant activity of the sonicated samples increases in proportion to the treatment conditions, from 11.84% to about 3.6 times as measured by the FRAP and from 2.77 to 18.77% as measured by the DPPH free radical scavenging assay, compared to the untreated sample (C).

According to the mechanism of action exhibited by antioxidant compounds, the methods used to measure antioxidant activity evaluate the ability of antioxidant species to scavenge free radicals or the ability of antioxidants to reduce an oxidant compound [65].

FRAP values quantify the ability of the antioxidant compounds contained in red grape must samples to donate electrons and, thus, reduce the oxidized species. A relevant indicator of a compound possessing potential antioxidant activity may be its reducing power [66].

It can be observed that the presence of a significant amount of phenolic compounds in red grape must, as a result of US treatment, led to high FRAP values.

The DPPH radical scavenging activity reflects the hydrogen-donating capacity of the samples, thanks to the presence of bioactive substances. When the DPPH radical scavenging activity of the samples was examined, the same conclusion was reached as in the FRAP evaluation, namely that the antioxidant potential was higher after treatment with ultrasound.

A closer examination of the results obtained from the FRAP and DPPH tests revealed that the increase in the electron-donating capacity or reducing power of the samples in response to US treatment was greater than that recorded for their free radical scavenging capacity.

The study performed by Rajurkar and Hande [67] revealed that the phenolic compounds exhibited a much higher correlation with the reducing power than with the DPPH free radical scavenging activity. Thus, it is estimated that the phenolic compounds extracted from red grapes provide antioxidant protection directly through the mechanism of reducing the oxidized intermediates in the chain reaction.

Our results suggest that the FRAP and DPPH assays could be used to distinguish the dominant mechanism, by which polyphenolic compounds from grape must act as antioxidants.

Other results showed that the antioxidant activity of phenolic compounds may have a concentration saturation limit, beyond which the activity cannot be increased by concentration. However, some results showed that there are factors other than concentration that influence the antioxidant properties of phenolic compounds, as the relationship between the phenolic compounds and the antioxidant activity was inconsistent [8,68]. Furthermore, other research showed a significant relationship between the polyphenolic compounds assessed in the various samples and the outcomes of the antioxidant activity tests [69].

As their *p*-values are less than 0.0500, Table 6 shows that the model parameters A, B, and A^2 are significant for the antioxidant activity measured by both the FRAP and DPPH assays. Values greater than 0.1000 indicate that the model terms are not decisive. The model significance is indicated by the model *F*-value of 330.16 and 58.81, respectively. The *F*-values for lack of fit, 2.40 and 102.78, respectively, indicate that the lack of fit is not significant.

Table 6. ANOVA and coefficient table for the quadratic model. Response 3: antioxidant activity.

Source	F-Value		<i>p</i> -Value		R^2		Adjusted R^2		Coefficients	
	FRAP	DPPH	FRAP	DPPH	FRAP	DPPH	FRAP	DPPH	FRAP	DPPH
Model	330.16	58.81	<0.0001	<0.0001	0.9958	0.9767	0.9928	0.9601		
Intercept									28.14	73.17
A	1587.16	246.65	<0.0001	<0.0001					13.88	3.58
B	33.68	37.65	0.0004	0.0005					2.17	1.40
AB	2.85	2.05	0.1354	0.1954					0.7200	−0.4000
A^2	12.04	6.08	0.0104	0.0431					1.78	−0.8293
B^2	2.59	0.0556	0.1515	0.8203					0.8266	−0.0793
Lack of fit	2.40	102.78	0.2085	0.0003						

Amplitude (A); time (B).

The predicted R^2 (0.9727 FRAP and 0.7829 DPPH) and the adjusted R^2 (0.9928 FRAP and 0.9601 DPPH) are in good agreement (i.e., the difference is less than 0.2), indicating that the model makes accurate predictions.

Equations (5) and (6) display the final equations in terms of the actual factors for the antioxidant activity as measured by the FRAP and DPPH assays. For specific levels of each element, the equation can be used to predict the response.

$$Y_{\text{FRAP}} = 16.030 - 0.074 \times A - 6.965 \times B + 0.036 \times A \times B + 0.004 \times A^2 + 0.826 \times B^2 \quad (5)$$

$$Y_{\text{DPPH}} = 37.996 + 0.549 \times A + 3.434 \times B - 0.020 \times A \times B - 0.002 \times A^2 - 0.079 \times B^2 \quad (6)$$

where A is the amplitude and B represents the time.

Figure 4 shows the kinetics of the antioxidant activity in samples treated with US, as well as the 3D response surfaces and contour plots.

From Figure 4, which compares the effects of the treatment time and ultrasonic wave amplitude on the antioxidant activity of the samples, it can be seen that both the FPAP and DPPH values increase with both factors, up to a maximum value of 48.32 $\mu\text{mol Fe}^{2+}/\text{mL}$ and 77.2% in sample SM90/5.

Other researchers came to the conclusion that the antioxidant activity of wines is more closely related to the type of individual phenolic compounds found in the wines than to the TPC. It has also been proposed that the antioxidant activity is mostly attributed to the flavan-3-ol fraction, rather than anthocyanins [70].

However, the grapes and wines showed a strong link between their phenolic composition and the antioxidant activity. The main substances involved in this bioactivity varied depending on the type of sample examined. The primary category, with a significant contribution to this feature, was identified as anthocyanins. Additionally, variations in the phenolic component quantities may account for the variations in the antioxidant efficacy between grape cultivars [71].

In the situation described, the optimal circumstances for antioxidant activity would be a higher amplitude and a longer duration of treatment compared to the TPC and MA.

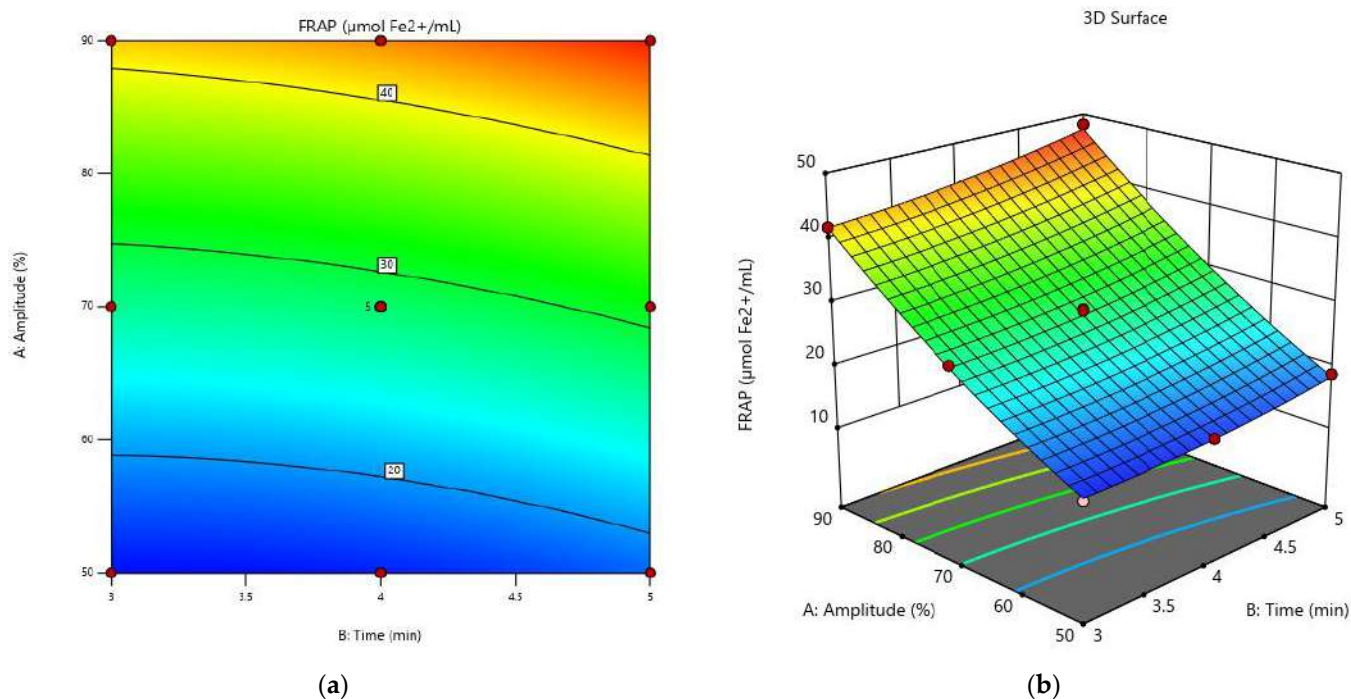


Figure 4. Cont.

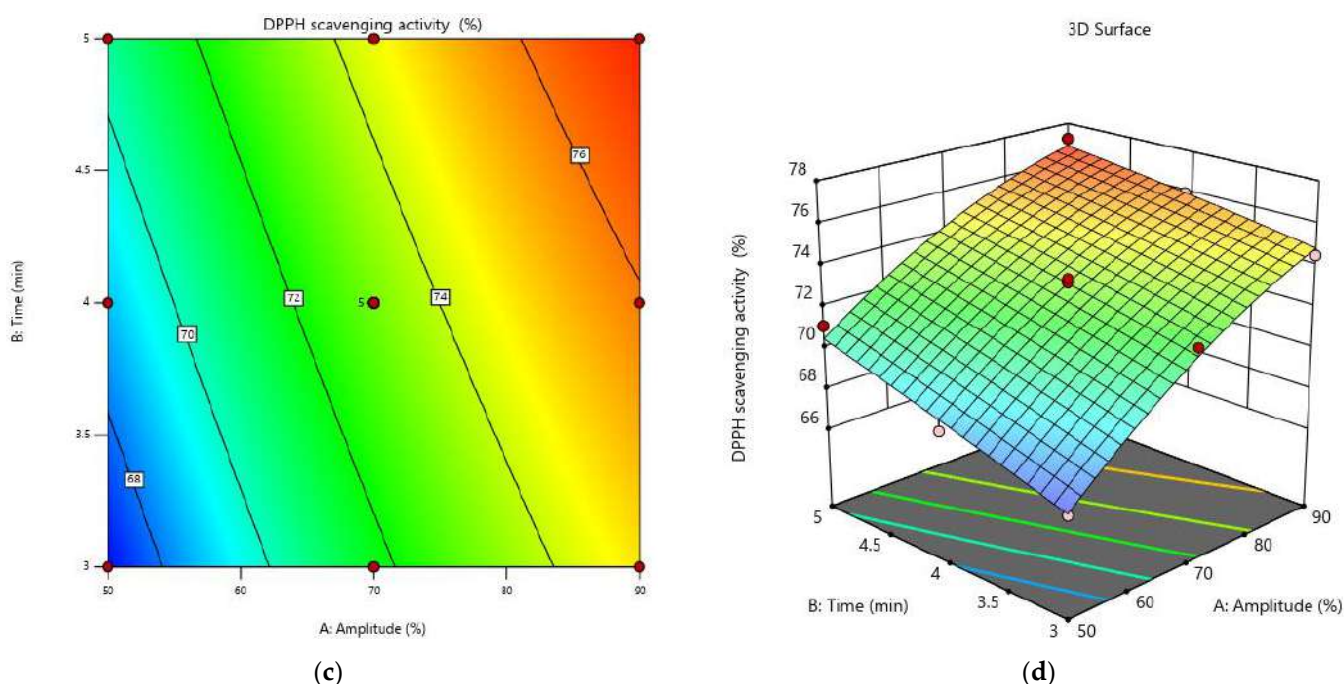


Figure 4. Influence of the process parameters on the antioxidant activity: (a) amplitude (A) and time (B) as contour graph for the FRAP values; (b) amplitude (A) and time (B) as the 3D surface for the FRAP values; (c) amplitude (A) and time (B) as the contour graph for the DPPH values; (d) amplitude (A) and time (B) as the 3D surface for the DPPH values.

3.4. Correlations between Total Phenolic Content, Monomeric Anthocyanins and Antioxidant Activity

The results of our study show that the higher the amplitude and the longer the duration of the ultrasound treatment, the higher the antioxidant activity, expressed both as the FRAP or DPPH free radical scavenging activity, as the maximum values for the antioxidant activity were found when an ultrasonic amplitude of 90% was applied for 5 min (sample SM90/5): $48.32 \mu\text{mol Fe}^{2+}/\text{mL}$ and 77.2%, respectively.

It can also be observed that the antioxidant activity of the sonicated samples, expressed by both the FRAP and DPPH tests, increased in proportion to the total polyphenol and monomeric anthocyanin content.

For the maximum values observed for the total polyphenol content ($1396.06 \mu\text{g GAE}/\text{mL}$) and the monomeric anthocyanins ($223.4 \text{ mg CGE}/\text{L}$), when an ultrasonic amplitude of 90% was applied for 3 min (sample SM90/3), the antioxidant activity was 3.11 times higher when measured with the FRAP test and 14.62% higher when measured as DPPH radical scavenging activity (%) compared to the control sample (C). These improved values confirmed the greatest influence of phenolics and anthocyanins on the antioxidant activity.

These results are in agreement with those reported by some authors who have shown a positive correlation between the TPC and the antioxidant activity evaluated by DPPH [72], or who have linked the high antiradical power of the samples to their anthocyanin concentration [73], but are in contrast to others [74,75].

The current analysis shows a very good correlation ($p < 0.01$) between the TPC and antioxidant activity as the FRAP (Pearson's $r = 0.917$) and as the DPPH radical scavenging activity (%) (Pearson's $r = 0.878$). The same, very good, Pearson correlations were found between the MA and the antioxidant activity as the FRAP (Pearson's $r = 0.928$) and as the DPPH radical scavenging activity (%) (Pearson's $r = 0.888$).

Finally, our results showed that the antioxidant activity of the sonicated samples was influenced by the TPC and MA, although the samples with the highest TPC and MA did

not always show the highest values for antioxidant activity expressed either as the FRAP or as the free radical scavenging activity, DPPH %.

3.5. Optimization of the Experimental Model for Increasing the Extraction Rate of Biologically Active Compounds and the Antioxidant Activity in Grape Must

Aiming to maximize the extraction of biologically active compounds in grape must, as well as to enhance its antioxidant activity, we investigated the optimization function of the model made with the Design Expert® software version 13, (Stat-Ease, Inc., Minneapolis, USA, 2022). For numerical optimization, any combination of one or more objectives was optimized. The goals we applied to either factors or responses were as follows: amplitude and treatment time in range; TPC, MA, FRAP, and DPPH maximized.

The optimization function of the software gave us 20 found solutions, indicating the solution with the highest value of desirability, which was 0.918 (Figure 5), namely an amplitude of 90% and a treatment time of 4 min and 24 s, as the solution selected to maximize the extraction of the TPC, MA, and antioxidant activity as the FRAP and DPPH scavenging activity.

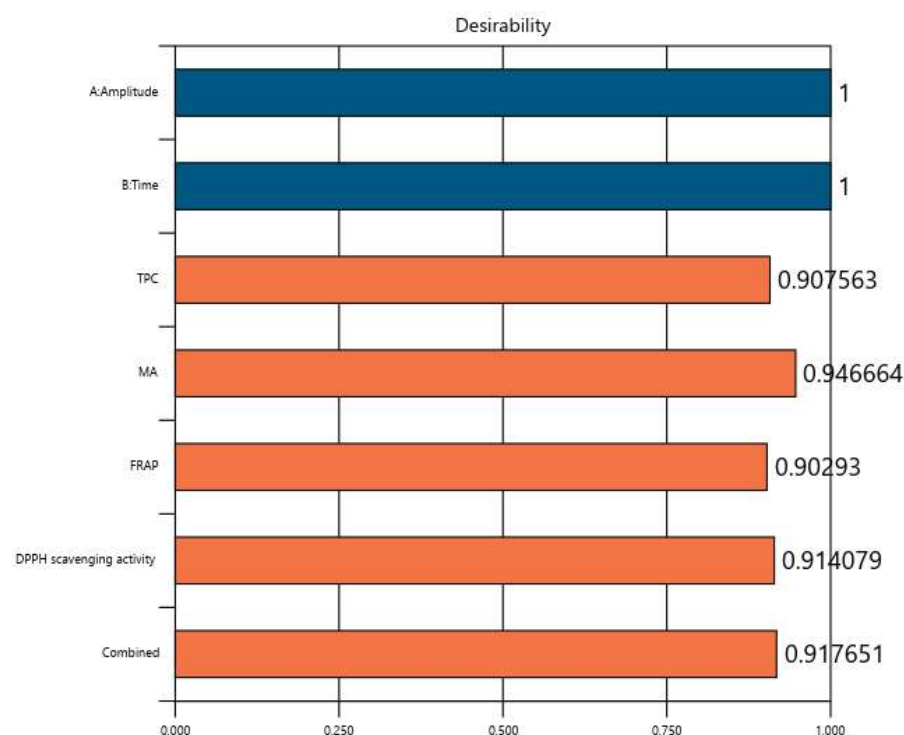


Figure 5. Graphical view of the optimal solution: the optimal factor settings are shown as red bars and the optimal response predictions are shown in blue.

To identify the factor settings that meet specified objectives, numerical optimization searches the design space using the models built during the analysis (Figure 6). Under certain conditions, the model can accurately predict the extraction rate of compounds.

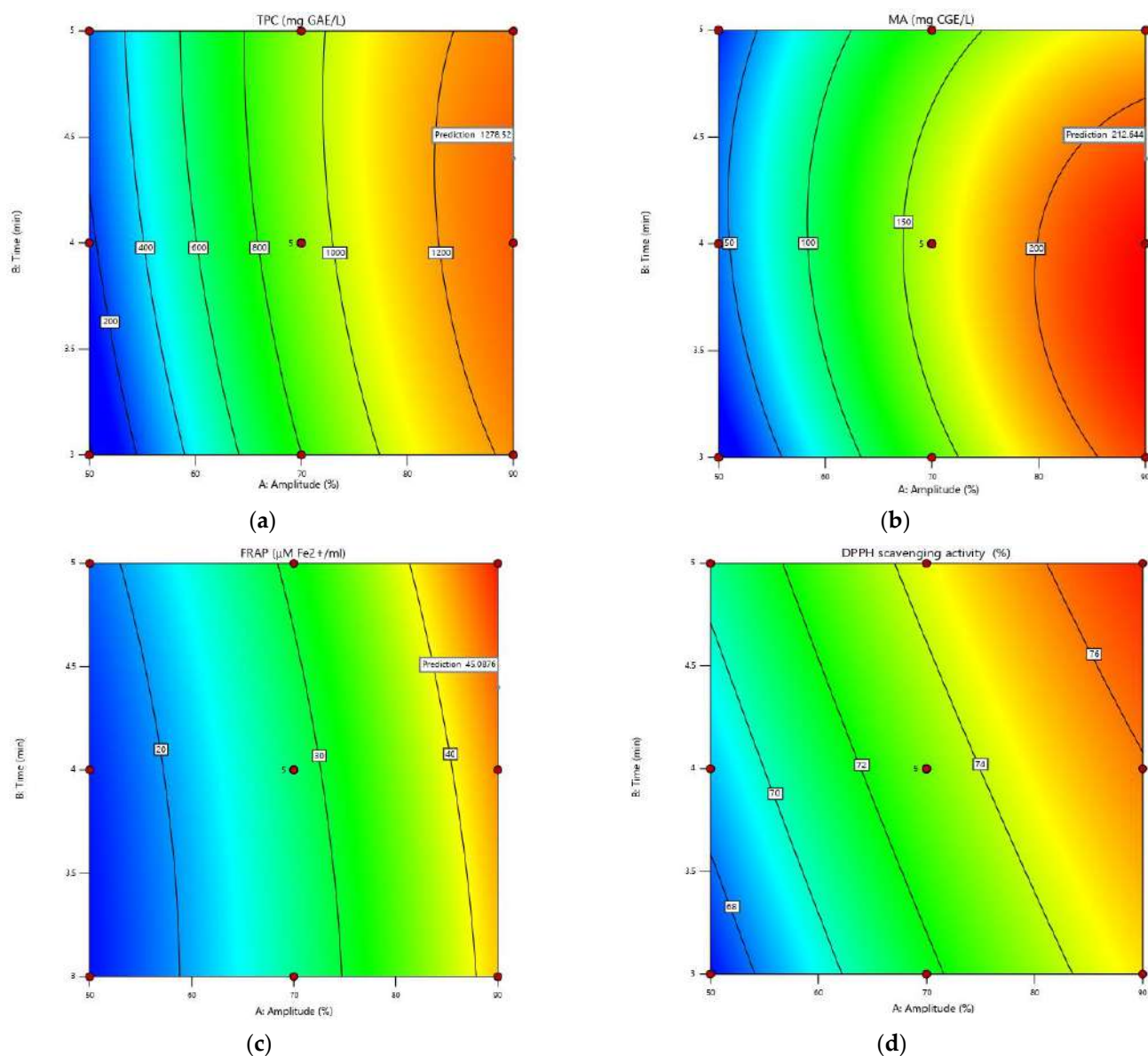


Figure 6. The numerical optimization plots for the optimal solution: (a) predicted value for the TPC; (b) predicted value for the MA; (c) predicted value for the FRAP; (d) predicted value for the DPPH.

Figure 7 shows the optimization graph for the optimal displayed solution, highlighting the point at which the response criteria is met.

3.6. Confirmation of the Model

The optimization analysis was used to guide the validation trials, which were conducted using the same methodology at the ideal selected amplitude—90% and treatment of time 4 min and 24 s. The predicted data were found to be TPC—1278.52 ($\mu\text{g GAE}/\text{mL}$), MA—212.64 (mg/L), FRAP—45.09 ($\mu\text{mol Fe}^{2+}/\text{mL}$) and DPPH—76.31%, as observed in Figures 6 and 7.

The observed response data and the predicted data from the modelled response were compared under the extraction conditions. The errors for the TPC, MA, FRAP, and DPPH were -8.70% , -0.99 , 3.14 and 0.25% , respectively, as shown in Table 7, indicating the confirmation of the model.

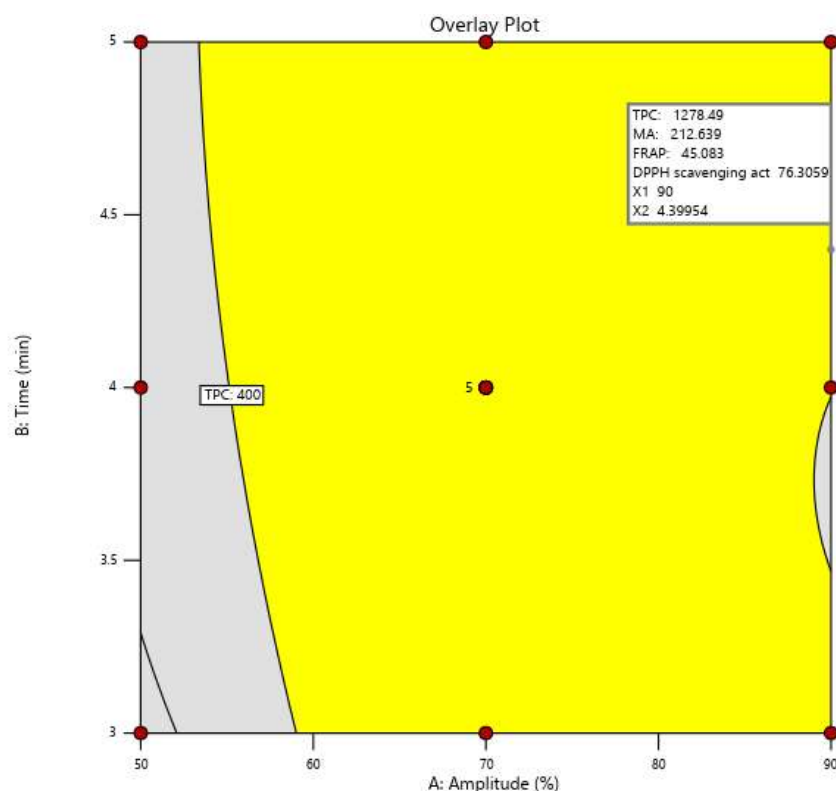


Figure 7. The overlay graph for the optimal solution: the bright yellow color defines the acceptable factor settings and grey color defines the unacceptable factor settings.

Table 7. Confirmation table of the model.

Response Data	TPC ($\mu\text{g GAE/mL}$)	MA (mg CGE/L)	FRAP ($\mu\text{mol Fe}^{2+}/\text{mL}$)	DPPH Scavenging Activity (%)
Observed	1176.16	210.56	46.55	76.5
Predicted	1278.52	212.64	45.09	76.31
Error (%)	−8.70	−0.99	3.14	0.25

Total polyphenol content (TPC); monomeric anthocyanins content (MA); antioxidant activity (FRAP).

The small differences between the predicted and observed values showed that the extraction optimization model obtained with the RSM in this study had a high degree of fit, and has the potential to be used even for large-scale extraction of phenolics and other antioxidant compounds from grapes using high-power ultrasound.

4. Conclusions

The acceleration of extraction kinetics using a physical technique can be important when the levels of physiologically active compounds in grapes are high. The use of ultrasound reduces the extraction times, while intensifying and almost maximizing the extraction yield and antioxidant activity. Overall, the use of ultrasound in winemaking opens up the possibility to optimize and better manage the vinification of red grapes. Due to the relatively short time required for ultrasound exposure, the application of ultrasound can be considered as a continuous pre-treatment for crushed red grapes prior to filling the winemaking tank. High-power ultrasound treatment of crushed grapes for a few minutes increases both the extraction rate of the bioactive compounds and the antioxidant activity by 12 times for the TPC, 14 times for the MA, 3.6 times for the FRAP and by 18.77% for the free radical scavenging activity, DPPH. However, a treatment time of 5 min and an amplitude of 90% led to a decrease in the TPC and MA, while the FRAP and DPPH values

increased with the same operating parameters. The optimal solution to develop a procedure that maximizes the TPC and MA extraction rate and the antioxidant activity during the first stage of winemaking seems to be an A of 90% and a t of 4 min and 24 s. The use of high-power ultrasound in the winemaking process for the treatment of crushed grapes has proved to be a very promising and effective technique for the extraction of high-value bioactive compounds, with great potential for commercial use. For this reason, considering the optimal solution found in this study, our further research on the impact of ultrasound treatment will include an in-depth investigation related to the content of the individual polyphenolic compounds of samples during the winemaking process, namely grape must, maceration, fermentation and bottling, with the aim of reducing the maceration period and better managing the whole process.

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Effects of Thermal and Ultrasound Treatments on L Ascorbic Acid of Grapes Juice

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Improving the nutritional quality for non-alcoholic beverages (especially the content of valuable natural antioxidants) by using modern ultrasound treatments represent a challenge for more and more food scientists. The objective of this research work was to estimate the effect of some ultrasound treatments on the total L ascorbic acid content and on several physicochemical characteristics of grape juice stored for different time periods. In order to determine this influence, the sonication parameters chosen were the amplitude (60%, 80%) and the treatment times (18, 36 min.). The samples treated by direct sonication (amplitude 80%, 36 min.) and stored 17 days had higher values of total L ascorbic acid content.

Keywords: L ascorbic acid, grapes juice, ultrasound treatments

Fruit intake is recommended as part of a healthy diet and a practical way to do so is in form of juice. When fruit juices are produced on an industrial scale, the inactivation of microorganisms and enzymes is mandatory in order to guarantee safety and stability.

Thermal processing has been quite useful for that purpose. However, excessive heating induces physical and chemical reactions that negatively affect the sensorial and nutritional properties of this kind of food [1]. The application of ultrasound-assisted extraction for plant materials and its opportunities in the food industry has proved many advantages [2-5].

Ultrasound is reported to have a minimal effect on the quality of fruit juices [6].

One of the most important micronutrient present in the fruits juice is the L ascorbic acid (vitamin C). Three main biological functions have been identified for compound: enzyme cofactor, free radical scavenger and donor/acceptor of electrons. Humans have lost ability to synthesize L ascorbic acid and depend on the diet to acquire the necessary amounts required to maintain good health. Deficiency of the vitamin C causes the disease scurvy characterized by spots on the skin, spongy gums and bleeding from mucous membranes. Is caused by deficient synthesis of collagen in which L ascorbic acid is cofactor [7-9]. Although, nowadays, scurvy is considered rare in developed nations, the vitamin C intake of significant part of the population of some of these countries may be below recommended daily acceptance (80mg per day in European Union EC) [10-12]. About 13% of the population in the USA or 1 in 7 young adults in Canada have been reported to be deficient in vitamin C with certain groups such as smokers, pregnant women and people of low socioeconomic status at a higher risk of deficiency is common. L ascorbic acid is particular important because it can reduce the chelating effect that some compound could have on iron, increasing its bioavailability [11]. Data reported by other researchers [13] revealed that the ultrasound treatments applied to different jus could inactivate microorganisms and increase antioxidants compounds. Therefore, the purpose of this study was to estimate the effect of ultrasound treatment on the level content of L ascorbic acid and on some physicochemical properties (pH, density, total soluble solids, titratable acidity, tartaric acid) of grapes juice during different storage periods.

Experimental part

Material and methods

Samples preparation

The experimental variants of juices were obtained from grape varieties Riesling (cultivated in the vineyard Dealu Mare, Romanian region and harvest at the end of September 2017). For obtain the juice, the grapes were first removed from the bunch and crushed with a crusher destemmer (Enoventa Tecnologie Enologiche, Italy). The next step was pressing the juice and solid particles with a hydraulic press machine (LU.C.M.E. Elettromeccanica, Italy). The juice was left for clearing for 4 h before starting the treatments.

Juice samples were treated by ultrasound (VCX-750, Sonics & Materials, Inc. Newtown, CT, USA) at 750 W, with constant frequency of 20 kHz at 60 and 80% amplitude for time periods of 18 or 36 min using a sample of 500 mL grape juice. This combination of amplitude and time was established according results of previous study, where a reduced of microbial count and release of antioxidant compounds was obtained [13].

Untreated juice was selected as control and pasteurized juice in a water bath at 70°C for 30 min [13], was also included to compare results. Samples were stored at 4°C and analyzed after 1, 4, 7, 10 and 17 storage days.

Determination of parameters quality, polyphenol and vitamin C content

The pH was measured with a potentiometer (Consort C1010, Consort, Belgium).

The density (g/L) was measured with a densitometer standardized at 20°C.

Total soluble solids content (°Brix) was determined using a refractometer (Brix/ ATC FG-113, Hangzhou Chinchuan Trading Co., China).

Titratable acidity was performing on aliquots of 10 mL placed into a 250 mL beaker and titration with sodium hydroxide 0.1N (Sigma-Aldrich, Dublin, Ireland). With a pipette, on a porcelain plate for titration, a drop of juice was removed and mix with two drops of indicator red phenol 0.02%. The titration is continued drop to drop, after each addition of the sodium hydroxide solution, until the indicator turns in pink-orange. The results are expressed as g tartaric acid/L. The titratable acidity (A_t) was calculated using the value of sodium hydroxide 0.1N solution volume (V), as follows:

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$$A_T = \frac{V \cdot 0.0075 \cdot 1000}{10} = 0.75 \cdot V \quad \text{g tartaric acid/L}$$

The semnifications of symbols used in these relations are:

Tartaric acid content (g/L) was determined following a colorimetric sequential technique (HI83748, Hanna Instruments).

The total phenols content was measured with an optical system of Hanna's HI 83000 series colorimeters. Photometric chemical analysis is based on the possibility to develop an absorbing compound from a specific chemical reaction between sample and reagents.

Extraction of L ascorbic acid (L-AA) was carried out by following steps (indicated by the manufacturer instructions - Megazyme Ltd., Sigma Aldrich, USA, cod product MAK074): adding 10 mL of 6% (w/v) aqueous solution of meta phosphoric acid and 40 mL 1-octanol to 10 mL sample (variants of grapes juice treated and non-treated); vortex this mixture for 1 min; adjust to pH 3.5-4; quantitatively transferring to a 20 mL volumetric flask; centrifuge at 13.684 g (14.000 rot/min.) for 5 min. (a part of the mixture was taken in 1.5 mL tubes).

Determination of the L ascorbic acid content was carried out using the supernatant and an enzymatic method (L-ascorbic acid test kit, Megazyme Ltd., Sigma Aldrich, USA, cod product MAK074) following the instructions of the manufacturer. This method is based on the color change of the absorption caused by the reduction of 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide before and after ascorbic oxides is added, the concentration of L ascorbic acid being proportional to this change. The absorbance was measured at 570 nm (Tecan Sunrise micro plate reader, software Magellan). The determination was made in 5 repetitions at different time periods of the samples storage. The content of the L-AA was expressed as mg L Ascorbic Acid per liter of juice (mg L-AA/L).

Results and discussions

All the grapes juice treated by ultrasound had higher values of L ascorbic acid content compared to the pasteurized and control samples (table 1). The juices samples treated by sonication at 80% amplitude for 36 min presented the highest value of vitamin C content (64.498 ± 4.022 mg L-AA/L). Pasteurized treatments led to a significant decrease of L ascorbic acid content (compared to the untreated control) and after 17 storage days the juice had the lowest content in this phytochemical compound (23.990 ± 6.509 mg L-AA/L).

Different researchers reported the most important mechanisms for explain the evolution and variations of vitamin C induced by the ultrasound during the storage. Maybe, the vitamin C breakdown is prevented by the elimination of dissolved oxygen (essential for ascorbic acid degradation), during the cavitations produced by the ultrasounds treatment [14]. Tiwari et al. [6] considered that the extreme physical conditions existing inside the cavitations bubbles collapsing in a micro scale and caused some chemical reactions that occur either in the same time or in isolation. Sonication cavities can be filled using water vapor and different gases dissolved in the juice, such as N_2 which may aid oxidation reactions promoted by interactions with free radicals formed during sonication [15]. This oxidation could cause that ascorbic acid converts to dehydroascorbic acid [16] explaining the variations during storage.

Significant differences regarding the vitamin C content were observed between the variants of treatment (table 1). So, the simple correlation coefficient Pearson revealed significantly higher value regarding the vitamin C content (as compared to the different treatment variants) (table 2). Our results revealed that ultrasound treatments, especially at 80% amplitude for 36 min. had a grate effect on vitamin C content during storage. Ultrasound can be considered as an alternative emerging technology for grapes juice preservation, for increase the level of vitamin C content, maintaining the antioxidant properties. However,

Table 1

TOTAL L-ASCORBIC ACID CONTENT OF THE GRAPES JUICE TREATED AND UNTREATED AFTER DIFFERENT STORAGE PERIODS
(DATA ARE MEAN OF THE VALUES $\pm$ STANDARD DEVIATION)

Variant of the treatment	Storage days				
	1	4	7	10	17
Juice untreated	43.880 $\pm$ 3.738 (fgh)*	42.425 $\pm$ 7.397 (gh)	41.455 $\pm$ 1.918 (gh)	42.182 $\pm$ 2.921 (gh)	39.272 $\pm$ 4.236 (h)
Pasteurized	25.688 $\pm$ 5.001 (i)	24.961 $\pm$ 7.357(i)	24.476 $\pm$ 5.941(i)	25.446 $\pm$ 5.584 (i)	23.990 $\pm$ 6.509 (i)
A60%,18min	52.370 $\pm$ 6.302 (de)	49.217 $\pm$ 5.259 (efg)	51.642 $\pm$ 4.896 (def)	52.855 $\pm$ 5.117 (cde)	54.553 $\pm$ 3.679 (cde)
A60%,36min	57.464 $\pm$ 4.728 (abcde)	54.796 $\pm$ 3.209 (cde)	55.038 $\pm$ 2.766 (bcde)	55.281 $\pm$ 6.220 (bcde)	57.464 $\pm$ 3.254 (abcde)
A80%,18min	55.523 $\pm$ 5.328 (bcde)	53.098 $\pm$ 6.279 (cde)	54.068 $\pm$ 6.220 (cde)	56.251 $\pm$ 3.473 (abcd)	58.677 $\pm$ 4.881 (abcd)
A80%,36min	57.949 $\pm$ 3.986 (abcd)	59.162 $\pm$ 4.666 (abcd)	61.102 $\pm$ 4.406 (abc)	63.528 $\pm$ 4.059 (ab)	64.498 $\pm$ 4.022 (a)

*Values not followed by the same letter are significantly different ($P=0.05$) according to Duncan's test.

Table 2

THE CORRELATION BETWEEN THE CONTENT OF L ASCORBIC ACID (IN GRAPES JUICE TREATED AND UNTREATED AFTER DIFFERENT DAYS) AND THE VARIANT OF TREATMENT

Variables	Statistical indicators	Days after treatments	Variant of the treatment
Vitamin C content (mg/L)	Correlation coefficient Pearson	0.042	0.722**
	Significance threshold	0.612	0.000
	N	150	150

** Correlation is significant for $p<0.01$.

N=150 (5 periods of storage x 6 variants x 5 repetitions)

Table 3

MEAN ($\pm$ SD) VALUES OF PH, TOTAL SOLUBLE SOLIDS, TOTAL ACIDITY, TARTARIC ACID AND TOTAL PHENOL CONTENT OF GRAPES JUICES (DIFFERENT VARIANTS OF TREATMENTS) DURING STORAGE

Type of analysis	Storage days	Treatments					
		Grape juice untreated (control)	Pasteurized juice	A60% 18 min	A60% 36min	A80% 18 min	A80% 36min
pH	1	3.633 $\pm$ 0.006 ^{ax}	3.593 $\pm$ 0.011 ^{bx}	3.543 $\pm$ 0.012 ^{cx}	3.536 $\pm$ 0.015 ^{cx}	3.543 $\pm$ 0.006 ^{cx}	3.507 $\pm$ 0.015 ^{dx}
	4	3.613 $\pm$ 0.006 ^{ay}	3.590 $\pm$ 0.010 ^{bx}	3.537 $\pm$ 0.006 ^{cy}	3.537 $\pm$ 0.006 ^{cx}	3.546 $\pm$ 0.006 ^{cx}	3.503 $\pm$ 0.006 ^{dx}
	7	3.600 $\pm$ 0.006 ^{ax}	3.573 $\pm$ 0.006 ^{by}	3.527 $\pm$ 0.006 ^{cy}	3.517 $\pm$ 0.006 ^{cy}	3.523 $\pm$ 0.006 ^{cy}	3.487 $\pm$ 0.006 ^{dy}
	10	3.597 $\pm$ 0.006 ^{ax}	3.563 $\pm$ 0.006 ^{by}	3.507 $\pm$ 0.006 ^{cx}	3.493 $\pm$ 0.006 ^{cx}	3.507 $\pm$ 0.006 ^{cx}	3.463 $\pm$ 0.006 ^{ex}
	17	3.583 $\pm$ 0.006 ^{aw}	3.543 $\pm$ 0.006 ^{bx}	3.493 $\pm$ 0.006 ^{cx}	3.483 $\pm$ 0.006 ^{cx}	3.487 $\pm$ 0.006 ^{cx}	3.447 $\pm$ 0.006 ^{cw}
Total soluble solids [° Brix]	1	24.37 $\pm$ 0.058 ^{ax}	24.40 $\pm$ 0.000 ^{ax}	23.73 $\pm$ 0.058 ^{bx}	23.67 $\pm$ 0.115 ^{bx}	23.33 $\pm$ 0.115 ^{bx}	23.43 $\pm$ 0.058 ^{cx}
	4	23.37 $\pm$ 0.044 ^{ay}	23.04 $\pm$ 0.574 ^{aby}	22.96 $\pm$ 0.015 ^{bcy}	22.78 $\pm$ 0.021 ^{bcy}	22.55 $\pm$ 0.044 ^{cy}	22.63 $\pm$ 0.015 ^{bcy}
	7	20.46 $\pm$ 0.557 ^{ax}	20.63 $\pm$ 0.142 ^{ax}	19.62 $\pm$ 0.047 ^{bx}	19.46 $\pm$ 0.051 ^{bx}	19.42 $\pm$ 0.026 ^{bx}	19.51 $\pm$ 0.026 ^{bx}
	10	18.70 $\pm$ 0.025 ^{aw}	17.91 $\pm$ 0.050 ^{bw}	16.61 $\pm$ 0.026 ^{cw}	16.14 $\pm$ 0.555 ^{cdw}	16.35 $\pm$ 0.042 ^{dw}	16.45 $\pm$ 0.035 ^{cdw}
	17	13.81 $\pm$ 0.015 ^{av}	12.89 $\pm$ 0.015 ^{bv}	11.46 $\pm$ 0.036 ^{cv}	11.35 $\pm$ 0.031 ^{dv}	11.13 $\pm$ 0.112 ^{ev}	11.29 $\pm$ 0.015 ^{dv}
Total acidity [g/L]	1	5.40 $\pm$ 0.100 ^{ax}	5.77 $\pm$ 0.058 ^{bx}	5.13 $\pm$ 0.153 ^{cx}	4.98 $\pm$ 0.104 ^{cx}	4.48 $\pm$ 0.029 ^{dx}	4.74 $\pm$ 0.228 ^{ex}
	4	5.07 $\pm$ 0.058 ^{ay}	5.33 $\pm$ 0.058 ^{by}	4.85 $\pm$ 0.050 ^{cy}	4.57 $\pm$ 0.061 ^{dy}	4.40 $\pm$ 0.010 ^{ey}	4.35 $\pm$ 0.025 ^{fy}
	7	4.82 $\pm$ 0.058 ^{ax}	4.95 $\pm$ 0.050 ^{bx}	4.58 $\pm$ 0.047 ^{cx}	4.41 $\pm$ 0.010 ^{dx}	4.31 $\pm$ 0.010 ^{ex}	4.17 $\pm$ 0.038 ^{fx}
	10	4.61 $\pm$ 0.015 ^{aw}	4.72 $\pm$ 0.012 ^{bw}	4.39 $\pm$ 0.015 ^{cw}	4.28 $\pm$ 0.017 ^{dw}	4.14 $\pm$ 0.047 ^{ew}	4.06 $\pm$ 0.015 ^{fw}
	17	4.26 $\pm$ 0.053 ^{bv}	4.40 $\pm$ 0.025 ^{av}	4.03 $\pm$ 0.026 ^{cv}	4.03 $\pm$ 0.042 ^{cv}	3.80 $\pm$ 0.010 ^{ev}	3.93 $\pm$ 0.032 ^{dv}
Tartaric acid [g/L]	1	3.57 $\pm$ 0.153 ^{dw}	3.68 $\pm$ 0.035 ^{cdv}	4.67 $\pm$ 0.031 ^{av}	3.79 $\pm$ 0.026 ^{cv}	4.50 $\pm$ 0.035 ^{bv}	3.81 $\pm$ 0.012 ^{cv}
	4	3.88 $\pm$ 0.025 ^{ax}	3.90 $\pm$ 0.010 ^{aw}	4.87 $\pm$ 0.015 ^{avx}	3.98 $\pm$ 0.015 ^{dw}	4.72 $\pm$ 0.006 ^{bw}	4.01 $\pm$ 0.010 ^{cw}
	7	4.10 $\pm$ 0.015 ^{ay}	4.13 $\pm$ 0.015 ^{ax}	4.99 $\pm$ 0.006 ^{axy}	4.20 $\pm$ 0.006 ^{dx}	4.91 $\pm$ 0.010 ^{bx}	4.26 $\pm$ 0.012 ^{cx}
	10	4.19 $\pm$ 0.006 ^{ay}	5.12 $\pm$ 0.006 ^{by}	5.15 $\pm$ 0.021 ^{ay}	4.36 $\pm$ 0.006 ^{ey}	5.10 $\pm$ 0.006 ^{cy}	4.42 $\pm$ 0.006 ^{dy}
	17	4.45 $\pm$ 0.020 ^{cx}	5.27 $\pm$ 0.006 ^{ax}	5.37 $\pm$ 0.021 ^{ax}	4.86 $\pm$ 0.318 ^{bx}	5.20 $\pm$ 0.010 ^{ax}	4.61 $\pm$ 0.010 ^{cx}
Total phenol content [mg GAE/L]	1	0.407 $\pm$ 0.002 ^{zv}	0.750 $\pm$ 0.003 ^{av}	0.525 $\pm$ 0.003 ^{dv}	0.578 $\pm$ 0.002 ^{cv}	0.687 $\pm$ 0.003 ^{bv}	0.511 $\pm$ 0.002 ^{ev}
	4	0.618 $\pm$ 0.002 ^{zw}	0.760 $\pm$ 0.002 ^{aw}	0.677 $\pm$ 0.003 ^{aw}	0.700 $\pm$ 0.002 ^{dw}	0.754 $\pm$ 0.002 ^{bw}	0.713 $\pm$ 0.001 ^{cw}
	7	0.783 $\pm$ 0.002 ^{ax}	0.764 $\pm$ 0.001 ^{bx}	0.765 $\pm$ 0.003 ^{ax}	0.831 $\pm$ 0.001 ^{bx}	0.890 $\pm$ 0.002 ^{ax}	0.793 $\pm$ 0.001 ^{cx}
	10	0.815 $\pm$ 0.002 ^{ay}	0.771 $\pm$ 0.002 ^{by}	1.097 $\pm$ 0.001 ^{ay}	1.124 $\pm$ 0.003 ^{dy}	1.274 $\pm$ 0.002 ^{ay}	1.143 $\pm$ 0.002 ^{by}
	17	0.951 $\pm$ 0.003 ^{ax}	0.781 $\pm$ 0.003 ^{bx}	1.233 $\pm$ 0.002 ^{dx}	1.307 $\pm$ 0.002 ^{bx}	1.592 $\pm$ 0.002 ^{ax}	1.296 $\pm$ 0.002 ^{cx}

a-f Means with the same letter in the same row are not significantly different at $p < 0.05$;

v-z Means with the same letter in the same column are not significantly at $p < 0.05$.

SD standard deviation

further studies are required to establish the most effective conditions to comply with specifications in terms of microbial load and other characteristics of juice processed by ultrasound.

Natural juices with high increase content of vitamin C could have an important impact on human health and therefore, would be of interest (for consumers, producers and policymakers) to have more information about the methods for improving the content of these valuable antioxidant using modern methods like the ultrasound treatments.

Ultrasound treatment had a significant effect on grapes juice pH after all the storage time, compared with untreated juice (table 3). Values of pH juice ranged between 3.633 ± 0.006 to 3.447 ± 0.006 . During storage, pH values and total acidity decrease in all treatment variants. Our results regarding pH and total acidity of sonicated samples are in accordance with observations of sonicated kasturi lime juice [17] and apple juice [18].

Ultrasound treatments had significantly effect on the total soluble solids (°Bx) compared with the untreated and pasteurized samples, the highest decrease was observed in case of variant 80% amplitude for 18min, after 4, 10 and 17 storage days. For all variants of treatments, this parameter decreased significantly during storage. The values of total solids content of juice (°Bx) ranged between 24.40 ± 0.000 for pasteurized variant and 11.13 ± 0.112 for variant A80%, 18min (table 3). It has been reported that sonication treatment cause a significant decrease in °Brix also of kasturi lime juice [17].

During storage, tartaric acid content increased significantly. A very strong increasement was observed after each storage days in case of samples treated by ultrasound 18 min at both amplitude (A60% and A80%).

After 10 and 17 storage days, the pasteurized juices showed a similar increase. This increase of tartaric acid content could indicate a start of fermentation none recommended in our case.

As shown in table 3, a significant increase ($p < 0.05$) was observed in total phenolic content in the entire grape juice sample sonicated at both amplitude and at both time, also at untreated grape juice. The highest increase was observed in the case of variant A80% 18 min; this increase was in range from 0.687 mg GAE/L up to 1.592 mg GAE/L. The exception is the pasteurized grape juices (in this case, the increase was not significant). The increase of the total phenolic content was found also in the apple juice [18]. The possible reason for significant increases in these phenolic phytonutrients might be attributed to the enhanced disruption of cell wall due to cavitations as a result of rapid change in pressures of the liquid by shear forces exerted during sonication which might lead to the release of some chemically bound polyphenolic phytonutrients and ultimately increased their availability in the juice. Creation of hydroxyl radicals by bubble implosion during sonication to the aromatic ring of phenolic compounds might also be a cause of their improvement in the apple juice. It has already been reported that increase in antioxidant capacity of phenolic compounds might be attributed to the addition of second hydroxyl group in different positions of these compounds chemical structure [17, 18].

Conclusions

Results of this study show a significant difference between the total vitamin C content of the grapes juices treated by different methods, minimally processed

technology (pasteurization / ultrasound used for improve the product shelf life).

Ultrasound treatments, particullary at 80% amplitude for 36 min. had a significant effect on vitamin C content during the storage (ending up with values of 64.498 ± 4.022 mg L-AA/L after 17 storage days). However, the results presented arise from working with a few of biological samples. Also, it is necessary to continue this research with extended experiments.

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INFLUENCE OF HIGH-POWER ULTRASOUND TREATMENT ON RED WINE QUALITY PARAMETERS

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Abstract

The objective of this study was to assess the impact of applying high-power ultrasonic treatment (HPU) on crushed Merlot grapes, at a laboratory scale, on the phenolic matrix of red wines, particularly anthocyanins, which are crucial for color, stability, and sensory profile. The ultrasonic treatment (US) was carried out using two amplitudes, 70% and 90%, and three treatment times, namely, 3, 4, and 5 minutes, while maceration was conducted via sequential extraction after 3, 5, and 7 days. After a bottling period of three months, there was a decrease in total polyphenol content observed compared to the content found at the end of maceration. Treatment with ultrasound caused significant variations in the optical density at 420, 520, and 620 nm, and in the content of monomeric anthocyanins. All the sonicated samples, including those extracted after three days of maceration, exhibited significantly higher color intensity values than the maximum color intensity value in the untreated samples. It is noteworthy that the change in color was a positive outcome of this treatment. The Random Forest algorithm was used to identify the most distinct variables among wines. The most significant variable was found to be the total polyphenol content, followed by antioxidant capacity and the color intensity of the wines. The algorithm grouped all the samples into 5 clusters based on three fixed factors that influenced their characteristics: amplitude, treatment time, and maceration duration. Based on these results, it can be inferred that the effects of ultrasound treatment vary significantly depending on the parameters used.

Key words: high-power ultrasound treatment, red wines, bioactive compounds, chromatic characteristics

INTRODUCTION

One of the world's earliest known alcoholic beverages is wine. Red wine can be considered a major dietary source of polyphenols due to its generally high polyphenol content (Castaldo et al., 2019). Essential substances called polyphenols give wine its color, flavor, and aroma as well as some possible health advantages (Banc et al., 2014). Red wine's phenolic content can be broadly classified into two groups: non-flavonoids, which include phenolic acids like benzoic, caffeic, and cinnamic acids and stilbenoids like resveratrol, and flavonoids, which include anthocyanins and tannins that contribute to the color and mouthfeel of the wine, flavan-3-ols (or catechins), flavonols, and their derivatives (Gutiérrez-Escobar et al., 2021). Nonetheless, flavonoids account for the majority of wine's phenolic component. Of these, the grape's stems, seeds, and skins account for up to 90% of the phenolic content found in red wine

(Nemzer et al., 2021). One-year-old red wine's polyphenol composition is composed of approximately 60–80% polymeric polyphenols, 10–15% anthocyanidins, 5–10% dimer procyanidins, 5–8% catechins, 3–6% phenolic acids, less than 1% flavonols, and less than 0.3% resveratrol, among other particular components (Buljeta et al., 2023). Red wine's phenolic components are known to provide a number of health advantages, including anti-inflammatory, cardioprotective, anticarcinogenic, neuroprotective, and gut microbiota impacts (Nardini, 2022). Many variables, including grape variety and age, pre-fermentation techniques, fermentation and aging circumstances, and technological procedures, influence the kind of polyphenols present in red wine (Luzzini et al., 2021). It is also crucial to remember that these elements may interact in intricate ways to affect how a particular wine's ultimate polyphenol profile is determined. Accordingly, the precise function and influence of polyphenols can change based

on things like the kind of polyphenols present, how long they macerate for, and the desired qualities of the finished product (Gómez-Plaza et al., 2020). Wines with good chromatic quality must have a good dispersion of the phenolic compounds from the grape to the must or must-wine. This extraction process, which is based on the disintegration of the grape skin and seed cell walls, is carried out during the maceration step to attain the release of the intended compounds into the medium. As such, the quality of the wine is influenced by the length of the maceration process (Alencar et al., 2018). Ultrasound-assisted extraction (Aadil et al., 2015; Yusoff et al., 2022), microwave-assisted extraction (Khan et al., 2022), cold plasma (Ahmadian et al., 2023; Heydari et al., 2023), supercritical fluid extraction (Molino et al., 2020), pressurized liquid extraction (Zia et al., 2022), high-voltage electric discharge (Molino et al., 2020), pulse electric field extraction (Ranjha, Kanwal, et al., 2021), microfluidization (Mukhtar et al., 2022), and enzyme-assisted extraction (Noranizan et al., 2020) are some of the advanced techniques for extracting plant bioactive compounds from foods and food-related matrices. These improved procedures are 32-36% more efficient, using roughly 15 times less energy and yielding higher-quality extracts (Sridhar et al., 2021). A technique known as high-power ultrasound (HPU) makes use of sound waves with frequency higher than 20 kilohertz (Ali et al., 2023; Hussain et al., 2023; Ranjha, Irfan, et al., 2021). The fundamental way that ultrasound affects a fluid is by increasing the hydrostatic pressure that already exists in the medium with acoustic pressure (Patist & Bates, 2011). The HPU's extraction capacity and the subsequent physico-chemical quality of the wines have been the subject of highly encouraging results in previous studies on red grapes and wines of various types (Q. A. Zhang et al., 2023).

Research has shown that ultrasound can be a valuable tool in winemaking, particularly in the extraction of polyphenols from grapes (Gómez-Plaza et al., 2020; Plaza et al., 2019). Studies have found that ultrasound treatment of grape marc can enhance the accumulation of polyphenols and anthocyanins, as well as influence the quantity of monomeric fraction of

anthocyanins (Khmelev et al., 2015). High-power ultrasounds have been shown to modify the physical characteristics of grape skin, facilitating phenolic extraction and improving wine chromatic characteristics (Pérez-Porras et al., 2021). The application of high-power ultrasounds during red wine vinification has been found to be effective in increasing the extraction of polyphenols, particularly in certain grape cultivars (Bautista-Ortin et al., 2017). These findings suggest that ultrasound can play a significant role in enhancing the quality and characteristics of wine. The International Organization of Vine and Wine decided after much investigation that this technology might be used in wineries in 2019 (OIV - International organisation of vine and wine, 2021).

However, the following concerns regarding basic research and technological implementation in industry need to be addressed. The different research groups used very different ultrasonic equipment, studied very different parameters, and obtained very different results, so they cannot be used as a standard for future research.

The objective of this study was to assess the impact of applying ultrasonic treatment (US) on crushed Merlot grapes, at a laboratory scale, on the phenolic matrix of red wines, particularly anthocyanins, which are crucial for color, stability, and sensory profile. Furthermore, to identify the most distinguishing variables among wines, we utilized the Random Forest clustering algorithm that indicates the most significant variables for grouping the samples in descending order.

MATERIALS AND METHODS

Grapes

The experimental study used about 100 kg of Merlot-varietal red grapes from the 2019 harvest. At the Pietroasa-Istrita Viticulture and Winemaking Research and Development Station in Buzau, Romania, the grapes were gathered by hand. The grapes were harvested at 29.8 °Brix, which is considered technological maturity, and were then promptly transferred to the lab for processing. For the investigation, only sound grape bunches selected at random were used.

Ultrasonic instrument

All experimental procedures were sonicated using SONIX VCX750, a probe-style ultrasonic apparatus from Sonics and Materials Inc. in Newtown, USA (SONIX VCX750, Sonics and Materials Inc., Newtown, USA). One way to express the amplitude is as a percentage, with values between 10% and 100%. The device operates at a frequency of 20 kHz and has an ultrasonic power of 750 W. The 20-100 kHz range is the most often utilized frequency range for extraction techniques.

Preparation of the sample

Samples of 1000 grams of 2019 vintage Merlot red grapes were prepared for laboratory testing. Sixty specimens, each weighing 100 grams, were randomly selected, de-stemmed, manually ground, and subjected to ultrasonic probe treatment. After sonication, we created six 1000 g samples, each containing ten 100 g specimens. The characteristics of each sample are represented by the average of the conclusions drawn from its ten instances. Ten specimens weighing 100 g each were used as the untreated control sample (C) since they had not undergone ultrasonic treatment. The specimens were evaluated independently. To treat the specimens, a 100 mL Pyrex glass beaker with a counter current water-cooling jacket was used. The cooling water was maintained at a consistent temperature of 19 °C. The acoustic amplifier was positioned 20 mm above the bottom of the container to ensure consistent treatment. The untreated sample (Control, C) and the ultrasound-treated samples underwent microvinification. After maceration, samples were sequentially extracted at 3 (D3), 5 (D5), and 7 (D7) days, pressed, and then underwent alcoholic fermentation. To ensure winemaking control, 20 g of Viniferm *Saccharomyces cerevisiae* Agrovin, Spain, fermentation yeast was added per 100 liters. The temperature was monitored daily during alcoholic fermentation, with variations ranging from 19.7 to 21.3°C. After completing alcoholic and malolactic fermentation, the samples were separated from the yeasts and clarified using 30 g of bentonite per 100 liters. Following clarification, the samples were cold-stabilized at 9°C before being bottled. All experiments were conducted in this manner.

Total polyphenolic content and anthocyanins determination

The Folin-Ciocalteu spectrophotometric method was used to calculate the total polyphenolic content (TPC), which was then expressed as micrograms of gallic acid equivalents per milliliter ($\mu\text{g GAE/mL}$) using gallic acid as a reference (Singleton et al., 1999). A 0.5 mL sample was treated with 1.25 mL of Folin-Ciocalteu reagent (Merck, Darmstadt, Germany), which had been diluted 1:10 (v/v) with distilled water. After the mixture had been incubated for five minutes at room temperature, one milliliter of Na_2CO_3 60 g/L was added. After 30 minutes of incubation at 50 °C, the sample absorbance at 750 nm was measured using a UV-VIS spectrophotometer (Specord 205, Analytik Jena Inc., Jena, Germany). The calibration curve was made using gallic acid as the standard, which was utilized in values ranging from 5 to 250 g GAE/mL.

Monomeric anthocyanins (MA) were found using the pH differential method (Lee et al., 2005). To make two dilutions of the same sample, 1 mL of wine was combined with 14 mL of potassium chloride buffer (0.025 M, pH 1.0) and 14 mL of sodium acetate buffer (0.4 M, pH 4.5). The absorbance at 520 and 700 nm was measured against deionized water after 15 minutes at room temperature. The results were expressed in milligrams (mg CGE/L) of cyanidin-3-glucoside equivalents per liter. The total anthocyanin content of the samples was calculated using a molecular weight of 449.2 g/mol and a molar absorbance coefficient of 26,900 L/mol x cm.

Ferric reducing antioxidant power (FRAP) assay

The FRAP test methodology was built upon the Benzie and Strain approach (Benzie & Strain, 1996). Two stock solutions were used: 20 mM $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ solution in 300 mM acetate buffer (pH = 3.6) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl. The working solution was prepared by mixing acetate buffer, TPTZ solution, and 10 mL of $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ solution. It was then heated to 37 °C before to use. Prior to analysis, a 0.5 mL aliquot of the diluted wine samples was allowed to react with 2.5 mL of the working

solution for 30 minutes at 37 °C. The wine samples had been diluted 1:50 (v/v) with distilled water.

Chromatic characteristics

The intensity of color (IC) and hue (N) of the samples were determined using a spectrophotometric method. The intensity of color was calculated by summing the absorptions at wavelengths 420, 520, and 620 nm with a 1 cm optical path. The hue was expressed as the ratio of absorbance at 420 nm to absorbance at 520 nm (OIV (International organisation of vine and wine), 2021).

Statistical analysis

The results, which were all measured in triplicate, are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using DESIGN-EXPERT® VERSION 13 software from Stat-Ease Inc. (Stat-Ease Inc., 2020). To assess statistically significant differences between samples, we used one-way ANOVA with post hoc analysis employing the HSD Tukey test. The Pearson correlation coefficient was computed using JASP software version 0.17.1 (JASP Team, 2023) for TPC, MA, FRAP value, IC, and N. The wines were grouped based on the parameters of ultrasound treatment using Random Forest Clustering (JASP Team, 2023). This algorithm partitions data into distinct clusters, with each observation belonging to only one group.

RESULTS AND DISCUSSIONS

Following destemming and crushing, the samples treated with ultrasound and the control sample underwent microvinification. The sequential extraction method was used to carry out the maceration operation over a period of 3, 5, and 7 days.

After fermentation and bottling, the purpose was to assess the impact of high-power ultrasound treatment parameters: amplitude (A) and treatment time (t) on wine quality parameters, specifically bioactive compounds such as TPC (total polyphenol content), MA (monomeric anthocyanin content), FRAP value, color intensity (IC), and hue (N) in the sonicated samples in comparison to the

untreated sample (C). The study analyzed the above parameters on untreated samples (C) after 3 (C3), 5 (C5), and 7 (C7) days of maceration. Additionally, the study examined samples that underwent ultrasound treatment at 70% and 90% amplitude for 3, 4, and 5 minutes after 3, 5, and 7 days of maceration. The samples were labeled as follows: SW70/3 (A: 70%; t: 3 min), SW70/4 (A: 70%; t: 4 min), SW70/5 (A: 70%; t: 5 min), SW90/3 (A: 90%; t: 3 min), SW90/4 (A: 90%; t: 4 min), and SW90/5 (A: 90%; t: 5 min). Each sample was replicated three times.

Analytical determinations performed on the samples treated with ultrasound and control samples (untreated), subjected to different durations of maceration of 3, 5, and 7 days, respectively, and microvinified and bottled for three months showed interesting results (Table 1). As these are red wines, it is important to consider the potential impact of ultrasonic treatment on the coloring matter.

The sonicated samples showed distinct chromatic features and higher concentrations of bioactive chemicals from the beginning of the maceration than the untreated samples. This difference persisted until the end of the maceration (Maier et al., 2023; Margean et al., 2020).

Effects of ultrasound treatment on wine parameters

After a bottling period of three months, both the wines made from crushed grapes subjected to ultrasonic treatment and the control wines showed a decrease in total polyphenol content compared to the content found at the end of maceration. However, the TPC values found in the sonicated samples remained higher than those of the controls, regardless of the duration of maceration (Table 1).

The total polyphenol content registered a maximum for all samples at the end of maceration and a decrease after bottling for all samples, with values ranging from 1.12% for sample SW70/3 extracted after 5 days (1169.23 $\mu\text{g GAE/mL}$ at the end of maceration) to 64.62% for sample C3 (870.32 $\mu\text{g GAE/mL}$ at the end of maceration).

High-power ultrasound applied to grapes or during winemaking has been shown in previous research to produce a greater extraction yield of

TPC (Ferraretto et al., 2011; Pérez-Porras et al., 2021). Other authors have reported no significant degradation of polyphenols after

sonication of red wine (Natolino & Celotti, 2022).

Table 1. Effects of the ultrasonic treatment on the parameters of the wine

Sample	Maceration (days)	TPC ($\mu\text{g GAE/mL}$)	MA (mg CGE/L)	FRAP ($\mu\text{M Fe}^{2+}/\text{mL}$)	IC	N
C3	3	307.91 $\pm$ 2.36	63.19 $\pm$ 1.6	39.1 $\pm$ 2.4	4.8 $\pm$ 0.3	0.56 $\pm$ 0.02
SW70/3		893.47 $\pm$ 19.6	129.51 $\pm$ 3.6	55.97 $\pm$ 0.9	16.86 $\pm$ 0.88	0.6 $\pm$ 0.03
SW70/4		876.24 $\pm$ 12.1	153.15 $\pm$ 3.9	57.70 $\pm$ 2.25	16.87 $\pm$ 1.1	0.6 $\pm$ 0.01
SW70/5		1 115.26 $\pm$ 15.4	131.07 $\pm$ 2.8	55.22 $\pm$ 1.7	16.65 $\pm$ 2.3	0.6 $\pm$ 0.01
SW90/3		946.24 $\pm$ 15.2	137.59 $\pm$ 6.5	61.57 $\pm$ 2.3	15.93 $\pm$ 1.1	0.57 $\pm$ 0.02
SW90/4		1 648.75 $\pm$ 24.9	152.04 $\pm$ 4.6	69.00 $\pm$ 2.3	17.56 $\pm$ 0.83	0.61 $\pm$ 0.08
SW90/5		1 333.94 $\pm$ 16.98	158.63 $\pm$ 2.2	68.13 $\pm$ 2.04	15.98 $\pm$ 1.47	0.54 $\pm$ 0.01
C5	5	415.76 $\pm$ 3.8	95.99 $\pm$ 3.7	54.64 $\pm$ 2.6	5.63 $\pm$ 0.76	0.57 $\pm$ 0.02
SW70/3		1 156.14 $\pm$ 11.9	138.79 $\pm$ 2.6	52.77 $\pm$ 1.6	11.6 $\pm$ 1.2	0.57 $\pm$ 0.05
SW70/4		1 094.92 $\pm$ 13.1	171.06 $\pm$ 2.5	52.07 $\pm$ 2.1	8.47 $\pm$ 1.5	0.72 $\pm$ 0.09
SW70/5		1 304.79 $\pm$ 12.4	125.51 $\pm$ 6.3	57.46 $\pm$ 2.8	14.36 $\pm$ 1.4	0.64 $\pm$ 0.02
SW90/3		1 159.05 $\pm$ 11.3	123.68 $\pm$ 5.6	57.61 $\pm$ 3.0	11.36 $\pm$ 1.2	0.67 $\pm$ 0.04
SW90/4		1 243.58 $\pm$ 10.8	126.74 $\pm$ 4.9	68.52 $\pm$ 4.9	11.49 $\pm$ 1.3	0.67 $\pm$ 0.05
SW90/5		1 605.02 $\pm$ 21.7	122.86 $\pm$ 4.8	56.06 $\pm$ 2.7	15.13 $\pm$ 0.9	0.63 $\pm$ 0.05
C7	7	439.08 $\pm$ 8.9	119.18 $\pm$ 6.1	46.65 $\pm$ 3.7	6.44 $\pm$ 1.1	0.58 $\pm$ 0.1
SW70/3		1 043.58 $\pm$ 18.4	121.63 $\pm$ 3.8	58.89 $\pm$ 2.1	13.66 $\pm$ 1.1	0.64 $\pm$ 0.07
SW70/4		1 269.82 $\pm$ 13.8	156.28 $\pm$ 4.2	61.39 $\pm$ 1.8	9.06 $\pm$ 0.8	0.65 $\pm$ 0.03
SW70/5		1 418.47 $\pm$ 21.3	121.66 $\pm$ 3.6	68.04 $\pm$ 2.9	14.76 $\pm$ 1.2	0.65 $\pm$ 0.07
SW90/3		1 261.07 $\pm$ 19.2	159.61 $\pm$ 4.1	68.84 $\pm$ 1.9	13.53 $\pm$ 1.0	0.68 $\pm$ 0.02
SW90/4		1 296.05 $\pm$ 23.1	200.36 $\pm$ 2.7	59.91 $\pm$ 1.8	11.92 $\pm$ 1.2	0.70 $\pm$ 0.04
SW90/5		1 596.28 $\pm$ 25.4	132.43 $\pm$ 2.8	61.36 $\pm$ 1.9	16.54 $\pm$ 0.7	0.67 $\pm$ 0.01

Untreated sample (C); SW70/3 (A: 70%; t: 3 min), SW70/4 (A: 70%; t: 4 min), SW70/5 (A: 70%; t: 5 min), SW90/3 (A: 90%; t: 3 min), SW90/4 (A: 90%; t: 4 min), and SW90/5 (A: 90%; t: 5 min); Total polyphenolic content (TPC); Monomeric anthocyanins content (MA); Ferric reducing antioxidant power (FRAP); Intensity of color (IC); Hue (N); The results are expressed as the mean value of the three replicates $\pm$ the standard deviation (SD).

Moreover, high-power ultrasound has been shown to have a significant impact on the phenolic structure of red wines, particularly in accelerating the aging process (Ferraretto & Celotti, 2016). It can also enhance the extraction of polyphenols from grapes during winemaking, with varying effects depending on the grape variety (Gambacorta et al., 2017). However, the specific effects on polyphenols in wine during storage are still being explored (Q. Zhang & Wang, 2017b).

As can be observed in Table 1, the treatment with HPU caused significant variations in the optical density (420, 520, and 620 nm) and in the monomeric anthocyanin content. After a period of three months of bottling, there was a significant decrease in the color intensity values of the wines for all the samples, compared to the values obtained at the end of the maceration, with the greatest decrease being recorded for the sample SW90/5 extracted after 5 days (24.85 at the end of maceration).

However, based on the data analyzed, it is evident that the ultrasonic treatment had a positive effect on the color content. All ultrasound samples, including those extracted after 3 days of maceration, recorded significantly higher color intensity values compared to the untreated samples (C7), ranging from 31.52% (SW70/4 sample, extracted after 5 days) to 172.67% (SW70/4 sample, extracted after 3 days). Other authors found that ultrasound treatment affects the evolution of color properties and major phenolic compounds in wine during storage, showing similar effects in treated and untreated wines, with quicker changes observed in treated wines (Q. Zhang & Wang, 2017a). According to additional research, it was found that the control samples that were macerated for 48 hours had the lowest color intensity. However, the differences from the other control wines were not statistically significant. Furthermore, the color intensity of the sonicated samples macerated for 48 hours was

not significantly different from the control wine that was exposed to the skin for 7 days (Pérez-Porrás et al., 2021).

The monomeric anthocyanin content was also measured for all samples immediately after maceration stage. After three months of bottling, the quantities present in the wine samples were smaller, with a decrease between 27.35% and 73.03%. The smallest decrease was observed in the case of sample SW90/4, which was extracted after 7 days (275.78 mg CGE/L at the end of maceration). This decrease in monomeric anthocyanin content is likely caused by factors such as polymerization with other phenolic compounds and oxidation reactions.

The influence of ultrasonic treatment on the monomeric anthocyanin content of wine samples is similar to its effect on their color content. The untreated samples had the highest content of monomeric anthocyanins in the C7 sample. However, the sonicated samples showed significant variations in the monomeric anthocyanin content compared to the untreated samples (Table 1). The increase ranged from 2.05% (sample SW70/3, extracted after 7 days) to 68.1% (sample SW90/4 min, extracted after 7 days). According to (Dalagnol et al., 2017), ultrasound-assisted extraction was found to accelerate the rate of anthocyanin extraction. It is worth noting that free anthocyanins are the primary source of red color in young red wines, despite the instability of monomeric anthocyanins. To stabilize these anthocyanins, one of the main methods is to condense them with tannins to generate stable anthocyanin/tannin adducts. It has been estimated that approximately 25% of the anthocyanins may undergo polymerization with flavonoid molecules towards the end of the alcoholic fermentation process. Following a year, this percentage is observed to increase to over 40% (He et al., 2012a, 2012b).

The antioxidant capacity increased for all samples after the bottling period compared to the values found at the end of maceration. The increase in antioxidant activity ranged from 3.52% for sample SW90/5, which was extracted after 7 days (59.27 $\mu\text{mol Fe}^{2+}/\text{mL}$ at the end of maceration), to 47.1% for sample SW70/4 min, which was extracted after 5 days (52.48 $\mu\text{mol Fe}^{2+}/\text{mL}$ at the end of maceration).

Nevertheless, it appears that the sonicated samples may have contained a notable quantity of phenolic compounds as a result of the US treatment, which led to elevated FRAP values.

Previous studies have indicated that the phenolic composition of wine can be influenced by the winemaking process. Moreover, a significant correlation has been found between the phenolic composition and the ferric reducing antioxidant power of samples (Lingua et al., 2016). It has also been concluded that the antioxidant capacity of wines is more closely related to the individual phenolic compounds present in the wine, rather than the total phenolic content (Banc et al., 2020).

The shade of wines after three months of bottling varies between 0.54 for sample SW90/5, extracted after 3 days, and 0.72 for sample SW70/4, extracted after 5 days (Table 1). A smaller shade indicates a larger red component compared to yellow, while high shades indicate an orange color. It is likely that anthocyanins degrade and condense with tannins, forming complexes that contribute to color stabilization.

Correlation of maceration duration, amplitude level, and sonication time

The statistical analysis of the data reveals that the investigated parameters were affected by three factors: amplitude level, ultrasonic treatment time, and maceration duration, either independently or in combination. The analysis of variance conducted on the analytical parameters for different maceration durations, amplitude, and treatment time conditions showed significant differences in the total content of polyphenols, monomeric anthocyanins, antioxidant capacity, color intensity, and hue of wines. The evolution of the analyzed parameters appears to be influenced by changes in maceration duration, percentage of amplitude, and treatment time. It is worth noting that the correlation coefficient (R) is 0.791 for CTP and 0.208 for AM. Additionally, the antioxidant activity also appears to vary, with a correlation coefficient of 0.518. The adjusted R^2 used for the three predictors: maceration duration, amplitude and treatment time shows that they can predict: 60.4% of the variation in results obtained for

CTP; 46.3% of the variation in results obtained for AM; 22.4% of the variation in FRAP results; 26.2% of the variation in results obtained for IC; and 22.3% of the variation in results obtained for hue. Previous research has also established a direct proportionality between the amplitude of the transducer and the intensity of the ultrasound. The sonochemical effects of the ultrasound were observed to increase with increasing intensity and amplitude (Mason & Lorimer, 2002). ANOVA shows that the model is significant, and the predictors introduced into the model, both alone and in the case of interaction between them, significantly influence ($p<0.05$) the total content of polyphenols, monomer anthocyanins, and FRAPs (Table 2).

Table 2. Exploring the impact of various factors on wine parameters

Factors	p-value				
	TPC	MA	FRAP	IC	N
Amplitude (%)	<0.001	<0.001	<0.001	<0.05	0.564
US treatment time (min)	<0.001	<0.001	<0.05	<0.001	<0.05
Maceration time (days)	<0.001	<0.001	<0.001	<0.001	<0.001
Maceration time *Amplitude	<0.001	<0.001	<0.001	<0.05	0.162
Maceration time *US treatment time	<0.001	<0.001	<0.001	<0.001	0.549
Amplitude *US treatment time	<0.001	<0.001	<0.001	<0.05	0.288
Maceration time *Amplitude *US treatment time	<0.001	<0.001	<0.001	0.976	0.164

Significance of each parameter (p-value); $p<0.05$ statistically significant and $p<0.001$ statistically highly significant; Total polyphenolic content (TPC); Monomeric anthocyanins content (MA); Ferric reducing antioxidant power (FRAP); Intensity of color (IC); Hue (N).

The color intensity appears to be minimally impacted by the interaction between amplitude level, treatment time, and maceration duration. However, it is worth noting that the hue of wines is significantly influenced by treatment time and duration of maceration.

Identifying the most distinguishing variables between wines by grouping them

Random Forest clustering is a hard-partitioning

algorithm that divides data into multiple clusters (groups), with each observation belonging to only one group (JASP Team, 2023). This clustering approach uses the Random Forest algorithm in an unsupervised manner, with the output variable 'y' set to NULL.

The wine dataset contains the results of analytical determinations of wines produced according to the traditional technology (control samples) and wines obtained from crushed grapes treated with ultrasound before the maceration stage. Two types of wine are represented in the 63 samples (21 samples analyzed in triplicate), with the results of 5 analytical determinations recorded for each sample. The variables recorded are TPC ($\mu\text{g GAE/mL}$), MA(mg CGE/L), FRAP ($\mu\text{M Fe}^{2+}/\text{mL}$), IC and N.

The purpose of using the Random Forest algorithm is to identify the most distinct variables among wines. Specifically, the Random Forest cluster model is optimized against its BIC value, which can be inspected in the Elbow Curve Diagram.

Table 3 and Table 4 present summary and performance statistical data, such as the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) of the model.

Table 3. Statistical data on cluster grouping

Number of clusters	Number of samples	R ²	AIC	BIC	Silhouette
5	63	0.638	162330	215910	0.220

Determination coefficient (R^2); Akaike Information Criterion (AIC); Bayesian Information Criterion (BIC); The model is optimized with respect to the BIC value.

AIC is an estimator of prediction error and provides a means for model selection. BIC is a criterion for model selection from a finite set of models. The preferred model is the one with the lowest BIC. BIC is closely related to the AIC criterion and is partly based on the probability function. When the models match, adding parameters can increase the probability, but this can lead to overlap. Both BIC and AIC introduce a penalty term for the number of parameters in the model to solve this problem. However, the penalty term is higher in BIC than in AIC. The silhouette value of the model ranges from -1 to 1, with 1 representing a perfect score. The sum of squares of each

cluster indicates the spread within the cluster, while R^2 indicates the amount of variance explained by the model (Table 4). Silhouette scores describe the degree of separation between groups.

Table 4. Grouping and sizes of clusters

Cluster	1	2	3	4	5
Dimension (number of samples)	9	11	16	14	13
Sum of squares within the group	19170	5998	36059	28188	22917
Silhouette score	0.468	0.498	0.003	0.108	0.177

Table 5 displays the Random Forest algorithm's ranking of variables in descending order of importance for grouping samples. The most significant variable is the total polyphenol content, followed by antioxidant capacity and the color intensity of the wines.

Table 5. Importance of variables in sample grouping

Parameter	Importance value
Total polyphenol content	13796
Antioxidant capacity	12996
Color intensity	12952
Monomeric anthocyanins	12628
Hue	10089

The Elbow diagram indicates the point at which adding another cluster would be unnecessary, also known as the kink in the curve. The red dot represents the minimum BIC value, which is the metric optimized by the model (Figure 2).

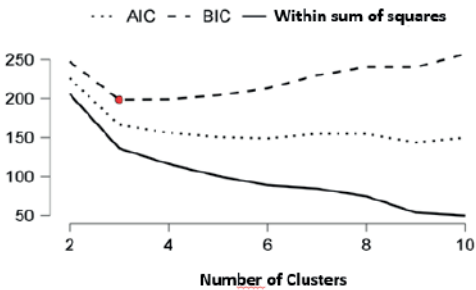


Figure 2. Elbow Diagram

Figure 3 displays the two-dimensional t-SNE graph, which provides a comprehensive overview of the various characteristics determined for the samples. However, the disadvantage is that the axes become uninterpretable. This t-SNE graph illustrates how the different clusters are grouped together. Moreover, Figure 3 displays the sample grouping into clusters based on three fixed factors that influenced their characteristics: amplitude, treatment time, and maceration duration.

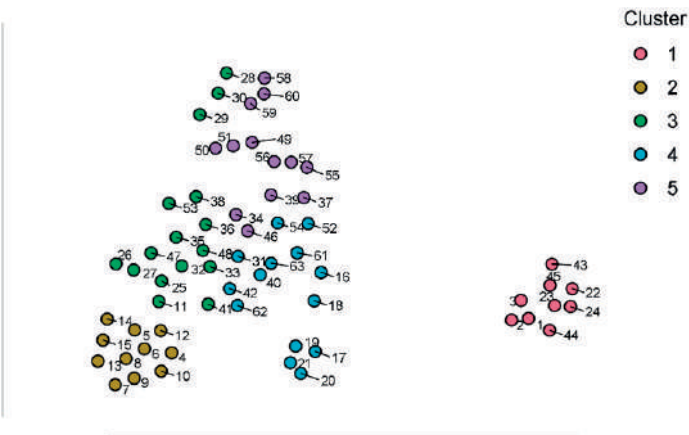


Figure 3. Two-dimensional t-SNE plot illustrating clustering of samples

Compared to all samples that underwent ultrasonic treatment, the control samples, regardless of maceration duration, recorded significantly lower values for all determined

parameters (refer to Table 1). These control samples were grouped in a separate cluster (Cluster 1).

A cluster (Cluster 2) was identified among the samples treated with ultrasound. This cluster was influenced by both the lower amplitude (70%) used during treatment, regardless of the treatment time (3, 4, or 5 minutes), and the increased amplitude (90%) used during a short treatment time (3 minutes). As a result, significantly lower values were recorded for all determined parameters compared to the other samples subjected to ultrasonic treatment. Cluster 3 confirms the positive influence of amplitude and treatment time on the properties of wines. It also shows the influence of maceration duration. The samples in Cluster 3 were extracted after 5 days, which is 2 days longer than the samples in Cluster 2. The same cluster also includes another sample that underwent maceration for 7 days but was treated with lower amplitude (70%) for only 3 minutes.

Cluster 4 and Cluster 5 group samples that underwent ultrasonic treatment with increased amplitude (90%) for 4 or 5 minutes, regardless of the duration of maceration, and additionally two samples that were treated with lower amplitude (70%) for 4 or 5 minutes, but with a maceration duration of 7 days. Clusters 4 and 5 recorded the highest values for all analyzed parameters of the wine samples obtained.

It can be inferred from these results that the effects of ultrasound treatment are subject to significant variation based on the parameters employed.

CONCLUSIONS

After a bottling period of three months, a decrease in the total polyphenol content was observed compared to the content found at the end of maceration, both in the wines made from crushed grapes treated with ultrasound and in the control samples, with the values found in the ultrasound-treated samples remaining higher than those of the control samples, regardless of the duration of maceration-fermentation of the latter. However, within each series (D3, D5 and D7), the decrease was influenced by the variation of the ultrasonic treatment parameters. The positive

effect of the ultrasonic treatment on the color content can be observed since all the ultrasonically treated samples, including those extracted after 3 days of maceration, showed significantly higher color intensity values than the maximum color intensity value of the control samples (C7), ranging from 31.52% (sample SW70/4, extracted after 5 days) to 172.67% (sample SW70/, extracted after 3 days). In conclusion, the ultrasonic treatment significantly modified the color of the wine, an effect that is very beneficial to the quality, especially for young wines, where color is one of the most important evaluation factors. The monomeric anthocyanin content of the sonicated samples showed significant variation compared to the maximum of the untreated samples, with an increase ranging from 2.05% (sample SW70/3 min, extracted after 7 days) to 68.1% (sample SW90/4 min, extracted after 7 days). The maximum anthocyanin content of the untreated samples was determined for the one extracted after 7 days. The data, subjected to statistical analysis, show that amplitude level, ultrasound treatment time and maceration duration significantly ($p < 0.05$) influence the total polyphenol content, monomeric anthocyanins and antioxidant capacity. In addition, colour intensity is not significantly influenced by the interaction between amplitude level, treatment time and maceration time, while wine hue is significantly influenced only by treatment time and maceration time. Furthermore, to identify the most distinguishing variables among wines, we utilized the Random Forest clustering algorithm. The Random Forest algorithm indicates the most significant variables for grouping the samples in descending order. Our results display that the total polyphenol content is a distinguishing feature, followed by antioxidant activity and the color intensity of the wines. All untreated samples, regardless of maceration time, consistently recorded significantly lower values for all determined parameters, in contrast to all samples exposed to ultrasound treatment, and were grouped in a separate cluster. The ultrasound-treated samples were categorized into four clusters, confirming that the effects of ultrasound treatment vary considerably depending on the parameters used (amplitude and time).

However, further research is needed to fully understand the implications of ultrasound on wine color and organoleptic characteristics, as well as the economic feasibility and scalability of implementing high-power ultrasound in winemaking processes.

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Article

Ultrasound Treatment Influence on Antioxidant Properties of Blueberry Vinegar

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Abstract: As one of the most widely used acidic condiments in the world, vinegars have demonstrated physiological functions. Due to their polyphenol content, blueberries (*Vaccinium myrtillus* L.) are a valuable source of natural flavours and antioxidants for vinegar production. Ultrasound treatment is recognized as an effective technique for improving the extraction yield of bioactive compounds from various plant materials. The aim of this research was to investigate the antioxidant properties of experimental vinegar variants obtained by an innovative manufacturing recipe using an alcoholic substrate containing blueberry juice for a rapid initiation of acetic fermentation. The substrate was subjected to ultrasound treatment at a frequency of 20 kHz and an amplitude (A) of 40%, 60%, and 80% for 3, 4, and 5 min. Under these conditions, total polyphenol content (TPC), total anthocyanins content (TAC), antioxidant activity based on ABTS and DPPH assays, as well as the sensory attributes in blueberry-vinegar formulations, were evaluated. The level of TPC and TAC and the antioxidant activity of the developed vinegar variants were optimized using response surface methodology (RSM). The obtained results revealed that ultrasound treatment resulted in increased TPC and TAC and improved antioxidant properties and sensory characteristics of blueberry vinegar. Our data revealed that the optimum values of the ultrasound treatment parameters were amplitude A: 78.50% and time t: 3.96 min. The following predicted values were determined for the main parameters: TPC: 628.01 mg GAE/L, TAC: 22.79 mg C3G/L, ABTS: 391.7 µmol/100 mL, and DPPH: 229.17 µmol/100 mL. The results of this study recommend the integration of both the use of an alcoholic substrate containing blueberry juice and the application of ultrasound treatment in vinegar production as innovative technological interventions with practical applicability for a rapid initiation of acetic fermentation and for improving the antioxidant properties of blueberry vinegar. In addition, RSM can be considered a valuable tool to optimize the ultrasound treatment's effect on the antioxidant properties of the vinegar formulations.

Keywords: blueberry vinegars; ultrasound treatment of alcoholic substrate; antioxidant activity; phenolic compounds; anthocyanins; response surface methodology



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1. Introduction

Vinegar is used as a fermented flavouring ingredient and as a special culinary constituent preservative, and also to give flavour to many food products such as salad dressings, mayonnaise, mustard, ketchup, and other food [1]. Due to its vitamins, phenolic compounds, and organic acids in its constitution, vinegar has multiple positive effects on health, especially for appetite stimulation, blood-sugar control, blood-pressure regulation, digestive, antimicrobial, body-weight management, antidiabetic, and lipid metabolism regulation [2–5].

Fruits such as grapes, apples, bananas, lemons, strawberries, rice, orange, pineapple, mulberry, blueberry, blackberry, or vegetables can be used as raw material for vinegar production all around the world [6]. Vinegars are named according to their raw material origin and uptake the properties of the corresponding raw material [7]. Berry vinegar contains numerous bioactive compounds, including phenolic components. The synergic effect of the acetic acid and these bioactive compounds could give to this product its ability to reduce the availability of carbohydrates, which is good for people with diabetes [8]. Vinegars obtained from fruit juices conserve just a fraction of these health-connected compounds [9].

According to the ‘Global Vinegar Market Report and Forecast 2021–2026’, the global vinegar market reached USD 1.32 billion in 2020. Meanwhile, the global vinegar market it is expected to grow at a Compound Annual Grow Rate (CAGR) of 1.6% between 2021 and 2026. It is expected to grow in the forecast period of 2021–2026 at a CAGR of 1.6% to attain USD 1.43 billion in 2026 [10]. The global vinegar market is led by the rising consumption of food globally. Europe is the largest vinegar market, representing nearly half of the global market. Within Europe, Italy is the leading market for vinegar as well as its major exporter [10,11].

The production of fruit vinegars as a way of making use of fruit byproducts is widely employed by the food industry since it allows them to exploit surplus and low-quality fruits without compromising the quality of the final product. The acetic nature of fruit vinegars and the high sensory impact that this acid produces on the sensorial properties of the product allow almost any type of fruit to be used for its elaboration. Every year, large amounts of fruits are produced and wasted since the excess cannot be consumed or because the fruits are considered of a second- or third-quality category [11].

In line with the Food and Agriculture Organization of the United Nations (FAO) [11], 21.6% of the fruit produced in the world is wasted, starting from the postharvest stage until its distribution. Very often, fruit is rejected simply because of its “imperfect” appearance or inadequate size, even if the fruit is edible. These actions lead to both ecological and economic problems [12]. Considering the functional properties and the perishability of these fruits, the use of such raw material in the production of high-value-added products such as vinegars could be a valuable strategy [13]. Usually, this vinegar is produced from raw materials containing sugar via sequential ethanol and acetic acid fermentations [14,15].

In the last few years, in food production, ultrasonic processing was considered a beneficial method for improving the nutritional properties of products regarding the enhancement of their biologically active compounds level, mostly the phenolic compounds [16]. It was outlined that the use of ultrasonic extraction for vegetable extracts could raise sucrose content, acidity content, phenolic compounds level, and pigmentation of liquid foods [17]. Data reported by other researchers [16,18] demonstrated the increase of the antioxidant potential of different liquid food products, such as tomato juice or cider, after the use of the ultrasound treatment. Currently, the reports of ultrasound treatment effects on the antioxidant potential of vinegars obtained from berry juices are limited, despite its potential.

Response surface methodology (RSM) can reduce workloads and the time consumption of the research work [3,19]. Therefore, RSM was used in this study, being successful in “predicting the model of various process factors in a convenient manner such as food science and engineering, chemical engineering and technology, and environment fields, etc.” [19–21].

In this study, an alcoholic substrate containing blueberry juice treated with ultrasound was used to obtain blueberry vinegar. Under these conditions, the intention was to rapidly initiate acetic fermentation. The degree of innovation of this study refers to a new recipe used for obtaining blueberry vinegar and identifying a treatment formula for this product in order to improve the polyphenols and anthocyanins content and the antioxidant properties. Consequently, the goal of this study was to optimize (using RSM) the extraction of polyphenols and anthocyanins, and the antioxidant activity of some vinegar variants obtained from an alcoholic substrate containing blueberry juices treated by ultrasound. Optimization is used in food processes, especially to minimize losses of bioactive ingredients, with RSM being preferred for this purpose. Thus, we aimed to apply the ultrasound treatment for the first time in blueberry-vinegar production by a new method and to optimize the bioactive components (TPC, TAC, DPPH, and ABTS) using RSM.

2. Results and Discussion

The effects of high-power ultrasonic treatment were explored in terms of the content of bioactive compounds such as TPC and TAC, as well as antioxidant activity measured by ABTS and DDPH assays. All the above parameters were studied on blueberry fruits, blueberry juice, juice diluted with water, and blueberry vinegar (B), as well as on the samples subjected to ultrasound treatment at 40, 60, and 80% amplitude for 3, 4, and 5 min, as follows: B403 (A = 40%; t = 3 min), B404 (A = 40%; t = 4 min), B405 (A = 40%; t = 5 min), B603 (A = 60%; t = 3 min), B604 (A = 60%; t = 4 min), B605 (A = 60%; t = 5 min), B803 (A = 80%; t = 3 min), B804 (A = 80%; t = 4 min), and B805 (A = 80%; t = 5 min).

2.1. Analysis of the Raw Material (Blueberry) and Blueberry Juices Obtained

The titratable acidity (TA), pH, and total soluble solids (TSS), expressed in °Brix, of fruit raw material, blueberry juice, and blueberry juice diluted with water are presented in Table 1.

Table 1. Titratable acidity, pH and total soluble solids of blueberry fruits, blueberry juice, and blueberry juice diluted with water.

Sample	Titrateable Acidity (%)	TSS (°Brix)	pH	TSS/TA Ratio
Blueberry	1.29 ± 0.02	12.28 ± 0.47	3.02 ± 0.14	9.44
Blueberry juice	0.94 ± 0.01	13.02 ± 0.26	3.17 ± 0.29	12.48
Juice diluted with water	0.75 ± 0.01	10.00 ± 0.00	3.3 ± 0.00	11.76

Each value represents average ± standard deviation (n = 3).

According to Saftner et al. [22], for consumption, the blueberries should have TSS values above 10%, total titratable acidity values between 0.3% and 1.3%, pH values from 2.25 to 4.25, and a TSS/TA ratio between 10 and 33. In our case, the titratable acidity varied around the value of 1.29% with a pH of 3.02, which is acceptable for fresh consumption. However, in relation to acidity and TSS/TA ratio, the value is less than 10% and, hence, their destination for the production of vinegars could be of interest for being explored. The results for pH (3.02) and titratable acidity (1.29%) indicated the fruits were quite acidic. Similar values for titratable acidity (1.58%) were reported for blueberries cultivated in Turkey [23]. Likewise, mature blueberries cultivated in Nova Scotia (Canada) were much less acidic than the blueberries used in this work [24]. High values of blueberry acidity were also reported by de Souza et al. [25] and by da Silva Fonseca et al. [13] for blueberries grown in Brazil (2.56% and 2.04%).

The results for total soluble solids content (12.28 °Brix) were similar to that reported by da Silva Fonseca et al. [13] for Brazilian blueberries and by Almenar et al. [26] who found a value of 12.67 °Brix for high bush blueberries (*V. corymbosum* L.).

Total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant potential, expressed as ABTS and DPPH assay of the berry fruits raw material are presented in Table 2.

Table 2. Total phenolic content, total anthocyanin content, and antioxidant potential of berry fruits.

Fruit Raw Material	TPC (mg GAE/100 g)	TAC (mg C3G/100 g)	ABTS ($\mu\text{mol TE/g}$)	DPPH ($\mu\text{mol TE/g}$)
Blueberry	642.37 ± 0.24^1	80.28 ± 0.82^1	38.36 ± 6.03^1	27.58 ± 0.5^1
	5246.94 ± 19.49^2	651.46 ± 6.67^2	312.38 ± 0.74^2	224.59 ± 5.94^2

¹ Values specified at fresh weight FW; ² Values specified at dry weight DW. Each value represents average $\pm$ standard deviation ($n = 3$).

Table 3 contains data on the total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant potential of the blueberry juices used in the vinegar manufacturing process.

Table 3. Total phenolic content, total anthocyanin content, and antioxidant potential of blueberry juices used in the vinegar manufacturing process.

Raw Material	TPC (mg GAE/L) (mg GAE/100 g DW)	TAC (mg C3G/L) (mg GAE/100 g DW)	ABTS ($\mu\text{mol TE}/100 \text{ mL}$) ($\mu\text{mol TE/g DW}$)	DPPH ($\mu\text{mol TE}/100 \text{ mL}$) ($\mu\text{mol TE/g DW}$)
Blueberry juice	1364.52 ± 46.4^1	195.28 ± 11.54^1	798.2 ± 18.43^3	653.9 ± 25.18^3
	1048.0 ± 3.56^2	149.98 ± 8.82^2	68.9 ± 7.40^4	50.22 ± 14.72^4
Juice diluted with water	972.18 ± 23.4^1	142.46 ± 2.80^1	600.3 ± 38.19^3	482.01 ± 28.30^3
	972.1 ± 23.4^2	142.46 ± 2.80^2	60.03 ± 3.80^4	48.27 ± 16.50^4

¹ Values expressed in mg GAE/L (FW). ² Values expressed in mg GAE/100 g DW (specified at dry weight DW).

³ Values expressed in $\mu\text{mol TE}/100 \text{ mL}$ FW. ⁴ Values expressed in $\mu\text{mol TE/g DW}$.

Similar amounts of the total phenolic compounds of the blueberry used in the present study ($642.37 \text{ mg GAE}/100 \text{ g}$) were reported by Bunea et al. [27] for the Bluecrop variety ($652.27 \text{ mg GAE}/100 \text{ g}$) and by da Silva Fonseca et al. [13] when using ethanol as an extraction solvent ($697.49 \text{ mg GAE}/100 \text{ g}$).

The results regarding the total anthocyanins tested in our study for the blueberry ($80.28 \text{ mg}/100 \text{ g}$) were similar to those found by Jacques et al. [28] ranging from 72 to $128 \text{ mg}/100 \text{ g}$ and with those obtained by da Silva Fonseca et al. [13] ($88.29 \text{ mg}/100 \text{ g}$). In the case of the anthocyanins content, the results were much less than those obtained by Bunea et al. [27] for the Romanian blueberry *V. corymbosum* L. (100.58 – $163.40 \text{ mg}/100 \text{ g}$). The content of anthocyanins depends on many factors (growing area, climatic conditions, harvest time, positioning conditions, etc.). These factors may have contributed to the observed differences in anthocyanin content.

Regarding the antioxidant activity of the blueberry tested in this study using an ABTS assay ($38.36 \mu\text{mol TE/g}$), higher values were found compared to the values reported by Bunea et al. [27] for several blueberry varieties, ranging from 24.33 to 36.46 TE mmol/g .

2.2. Vinegars Variants Obtained (Acidity, TSS)

The average values of vinegar acidity obtained in this study are presented in Figure 1 and varied between 3.597 and 4.307% after 50 days of fermentation. Generally, the acidity values were similar to the data presented by da Silva Fonseca et al. for blueberry vinegars [13]. The maximum value of acetic acid content (4.307%) was obtained for variant B804 (Figure 1).

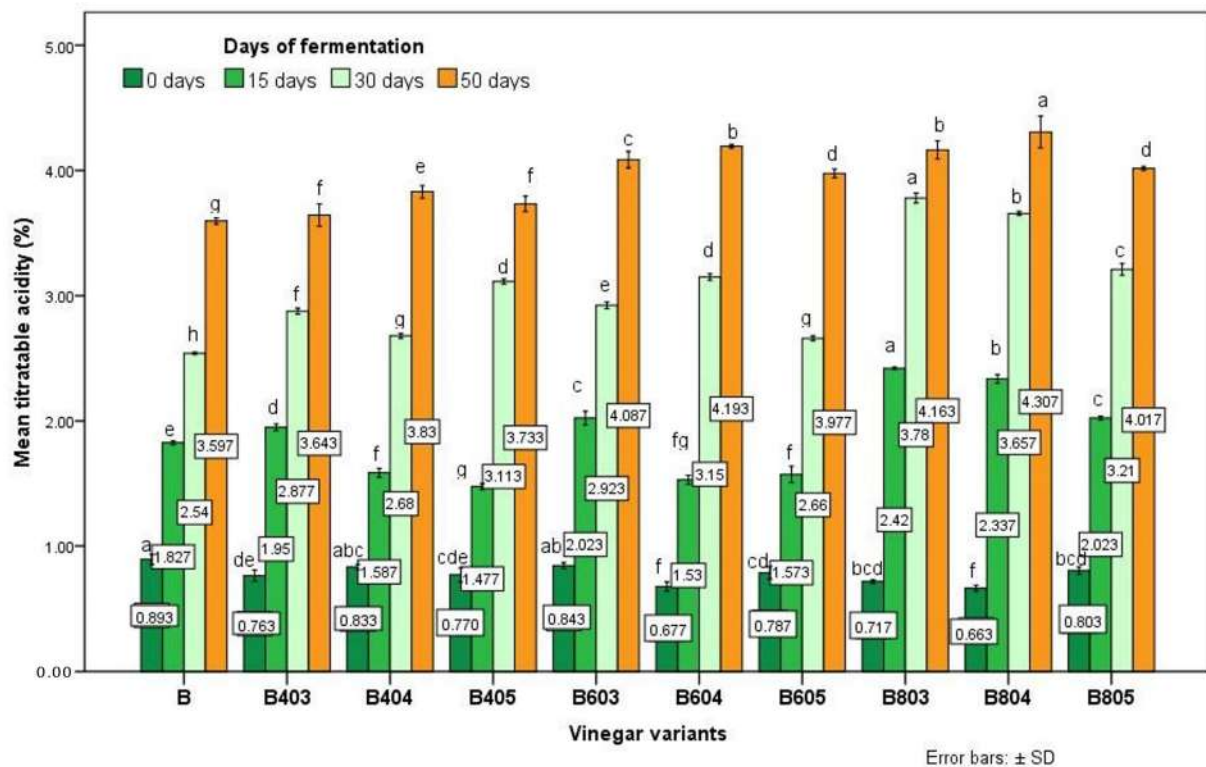


Figure 1. The average value of acidity (acetic acid %) of the blueberry variants obtained using substrates ultrasounds treated (graph of acetic acid formation in different stages of acetic fermentation). Data were expressed as the average values of 3 determinations $\pm$ standard deviation. Values with different letters differ significantly by ANOVA and Duncan's test ($p < 0.05$).

According to Tarhon M.C and to Romanian legislation, fruit vinegars must have a minimum acidity of 3.5 g/100 mL acetic acid and can present a maximum residual ethanol content of 1 g/L [29].

The vinegars obtained in this study are in accordance with legislation (Figure 1). The legislation does not establish the maximum value for the acetic acid content of the vinegar, but a vinegar with an acidity exceeding 5.5% can be rejected by consumers [13].

2.3. Total Phenolic Content and Total Antocyanins Content of Blueberry Vinegars

The average values of total polyphenol content, total anthocyanins content, and antioxidant potential, expressed as ABTS and DPPH assay, of blueberry vinegars obtained using the process described in the chapter material and methods are presented in Table 4.

Table 4. Average values of the total soluble solids, total polyphenol content, total anthocyanins content, and antioxidant potential of blueberry vinegars.

	TSS	TPC		TAC		ABTS		DPPH	
	(° Bx)	(mg GAE/L)	(mg GAE/100 g DW)	(mg C3G/L)	(mg GAE/100 g DW)	(μmol TE/100 mL)	(μmol TE/g DW)	(μmol TE/100 mL)	(μmol TE/g DW)
B	8.6	431.33 ± 24.72 ^e	501.55 ± 28.74 ^e	13.46 ± 3.22 ^d	15.65 ± 3.72 ^d	209.18 ± 42.60 ^e	24.32 ± 49.53 ^e	137.41 ± 11.35 ^e	15.98 ± 13.20 ^e
B603	8.6	441.41 ± 51.11 ^e	513.27 ± 58.75 ^e	15.25 ± 1.32 ^{cd}	17.73 ± 1.52 ^{cd}	220.63 ± 37.22 ^e	25.65 ± 42.78 ^e	146.68 ± 16.42 ^e	17.06 ± 18.87 ^e
B803	8.6	455.45 ± 38.36 ^e	529.59 ± 43.44 ^e	16.07 ± 1.96 ^{cd}	18.69 ± 2.22 ^{cd}	244.29 ± 25.87 ^e	28.41 ± 29.30 ^e	150.66 ± 11.85 ^e	17.52 ± 13.42 ^e
B403	8.5	486.77 ± 25.30 ^{de}	572.67 ± 29.42 ^{de}	16.45 ± 2.18 ^{cd}	19.35 ± 2.53 ^{cd}	260.34 ± 18.00 ^{de}	30.63 ± 20.9 ^{de}	139.99 ± 19.07 ^e	16.47 ± 22.17 ^e
B604	8.7	475.88 ± 38.39 ^e	546.99 ± 44.13 ^e	16.05 ± 2.60 ^{cd}	18.45 ± 2.99 ^{cd}	300.62 ± 34.13 ^{cd}	34.55 ± 39.23 ^{cd}	155.02 ± 13.00 ^e	17.82 ± 14.94 ^e
B805	8.7	601.84 ± 30.26 ^{ab}	691.77 ± 33.62 ^{ab}	21.08 ± 2.44 ^{ab}	24.23 ± 2.71 ^{ab}	363.69 ± 14.50 ^b	41.80 ± 16.11 ^b	217.10 ± 12.02 ^{ab}	24.95 ± 13.36 ^{ab}
B605	8.5	547.63 ± 19.22 ^{bc}	644.27 ± 22.61 ^{bc}	18.61 ± 1.50 ^{bc}	21.89 ± 1.76 ^{bc}	350.51 ± 25.26 ^{bc}	41.24 ± 29.72 ^{bc}	187.56 ± 11.07 ^{cd}	22.07 ± 13.02 ^{cd}
B804	8.83	536.53 ± 24.00 ^{cd}	607.62 ± 26.67 ^{cd}	20.74 ± 1.86 ^{ab}	23.49 ± 2.07 ^{ab}	317.31 ± 25.04 ^{bc}	35.94 ± 27.82 ^{bc}	179.96 ± 12.04 ^d	20.38 ± 13.38 ^d
B404	9.0	643.05 ± 28.05 ^a	714.50 ± 32.62 ^a	23.29 ± 1.34 ^a	25.88 ± 1.56 ^a	417.19 ± 37.53 ^a	46.35 ± 43.64 ^a	238.04 ± 14.18 ^a	26.45 ± 16.49 ^a
B405	9.0	588.15 ± 27.39 ^{abc}	653.50 ± 32.22 ^{abc}	21.15 ± 2.38 ^{ab}	23.50 ± 2.80 ^{ab}	361.03 ± 17.93 ^b	40.11 ± 21.09 ^b	204.56 ± 11.33 ^{bc}	22.73 ± 13.33 ^{bc}

Data were expressed as the average of 3 determinations $\pm$ standard deviation. Values with different letters differ significantly by ANOVA and Duncan's test ($p < 0.05$).

To understand the influence of ultrasound treatments on the biologically active compounds of blueberry vinegars and to improve the levels of these valuable substances, the response surface methodology (RSM) was used. All polynomial mathematical equations concerning the indicators of the vinegars obtained in this study are given as the results of RSM. Therefore, with the aim to optimize the process for increasing the level of TPC and TAC, the following polynomial model was used:

$$\text{TPC (mg GAE/L)} = 601.81 + 57.73 \times X_1 + 30.90 \times X_2 - 28.46 \times X_1^2 - 55.83 \times X_2^2 + 1.57 \times X_1 \times X_2 \quad (1)$$

$$\text{TAC (mg C3G/L)} = 21.08 + 2.55 \times X_1 - 0.6538 \times X_2 - 0.7289 \times X_1^2 - 1.90 \times X_2^2 - 0.1972 \times X_1 \times X_2 \quad (2)$$

where X_1 indicates the ultrasound amplitude and X_2 refers to the time of the treatment.

These models indicate the effect of amplitude and time on the TPC and TAC values in berry vinegar as a result of RSM, using the experimental design presented in Table 5. The TPC values were the highest (643.2 mg GAE/L) for the ultrasound-treated samples using an amplitude of 80% for 4 min. The lowest TPC value was found in the samples treated with an amplitude of 40% for 3 min (441.4 mg GAE/L). Regarding the TAC of the samples, the lowest value was reported at 3 min and with 40% amplitude in sample application 3 (B403) (15.31 mg C3GE/L). The highest TAC value was identified in the variant treated with 80% amplitude for 4 min (23.29 mg C3GE/L) (Table 5).

Table 5. The measured responses in the experimental design (RSM).

Sample Application (ID)	Independent Variables		Dependent Variables			
	Amplitude (X_1)	Time (X_2)	Response 1	Response 2	Response 2	Response 3
			TPC (mg GAE/L)	TAC (mg C3G/L)	ABTS ($\mu\text{mol TE}/100 \text{ mL}$)	DPPH ($\mu\text{mol TE}/100 \text{ mL}$)
B	-	-	431.33	13.46	209.18	137.41
1 (B603)	60	3	475.88	16.05	300.62	155.02
2 (B803)	80	3	536.53	20.74	317.31	179.96
3 (B403)	40	3	441.41	15.31	220.63	146.68
4 (B604)	60	4	600.19	21.01	363.43	216.94
5 (B604)	60	4	605.32	21.11	364.44	217.88
6 (B805)	80	5	588.15	21.15	361.03	204.56
7 (B605)	60	5	547.63	18.61	350.51	187.56
8 (B604)	60	4	600.21	21.05	363.32	216.44
9 (B804)	80	4	643.05	23.29	417.19	238.04
10 (B604)	60	4	603.28	21.13	363.42	217.88
11 (B404)	40	4	455.45	16.07	244.29	150.66
12 (B604)	60	4	600.19	21.12	363.84	216.37
13 (B405)	40	5	486.77	16.45	260.34	139.99

The results regarding the analysis of variance (ANOVA) for the responses in the case of TPC (mg GAE/L) and TAC (mg C3G/L) of the berry vinegar samples at different levels of amplitude and time are presented in Table 6.

For complying with the level, there was found a quadratic model ($R^2 = 0.9614$ for TPC, respectively, $R^2 = 0.9889$ for TAC). The effect of the amplitude on the TPC and TAC of the samples was statistically significant at $p < 0.001$.

The change of the TPC and TAC values, depending on the US time and amplitude is presented in Figure 2.

Table 6. Analysis of variance (ANOVA) for the responses regarding vinegars TPC and TAC.

Source	SS	DF	MS	F-Value	p-Value	Significance
Model for TPC (mg GAE/L)	52,678.71	5	10,535.74	60.73	<0.0001	**
X ₁ = A-Amplitude	26,658.15	1	26,658.15	153.67	<0.0001	**
X ₂ = B-Time	4922.79	1	4922.79	28.38	0.0001	**
AB	9.80	1	9.80	0.0565	0.8190	-
A ²	6520.57	1	6520.57	37.59	0.0005	*
B ²	16,951.25	1	16,951.25	97.72	<0.0001	**
Lack of Fit	1192.03	3	397.34	71.32	0.0006	-
Total	53,893.02	12				-
R ² = 0.9775; Adjusted R ² = 0.9614; Std. Dev. = 13.17; Average = 552.62; C.V. % = 2.38						
Model for TAC (mg C3G/L)	82.45	5	16.49	214.12	<0.0001	**
X ₁ = A-Amplitude	52.02	1	52.02	675.51	<0.0001	**
X ₂ = B-Time	3.42	1	3.42	44.40	0.0003	*
AB	0.1560	1	0.1560	2.03	0.1976	-
A ²	3.70	1	3.70	47.99	0.0002	*
B ²	25.22	1	25.22	327.42	<0.0001	**
Lack of Fit	0.5284	3	0.1761	65.72	0.0007	-
R ² = 0.9935; Adjusted R ² = 0.9889; Std. Dev. = 0.2775; Average = 19.46; C.V. % = 1.43						

SS: Sum of squares; DF: degree of freedom; MS: average squares; TPC: total polyphenols content; TAC: total anthocyanin content; * significant at $p < 0.05$ and ** significant at $p < 0.01$

In the optimization model, TPC was determined to be 628.02 mg GAE/L and TAC was found to be 22.79 mg C3G/L, after 3.96 min and 78.50% amplitude treatment (Figure 2), an increase of 45.6% was found for TPC, compared with the untreated control, and, respectively, an increase of 69.3% was identified for the TAC amount value.

Similar results regarding the positive effects of ultrasonic treatments on vinegars TPC were reported by Yikmiş et al. for apple [3] and tomato vinegars [9], by Lieu et al. [17] for grape juice, by Brezan et al. [16] for apple cider, and by Bhat et al. [30] for lime juice.

2.4. Antioxidant Activity of Berry Vinegars

With the aim of optimizing the process to increase the level of ABTS and DPPH, the following polynomial model was used:

$$\text{ABTS } (\mu\text{mol TE}/100 \text{ mL}) = 363.69 + 55.23 \times X_1 + 19.25 \times X_2 - 26.06 \times X_1^2 - 28.64 \times X_2^2 + 1.01 \times X_1 \times X_2 \quad (3)$$

$$\text{DPPH } (\mu\text{mol TE}/100 \text{ mL}) = 217.1 + 27.68 \times X_1 + 9.64 \times X_2 - 14.55 \times X_1^2 - 28.41 \times X_2^2 + 7.82 \times X_1 \times X_2 \quad (4)$$

where X_1 indicates the ultrasound amplitude and X_2 refers to the time of the treatment.

Table 7 presents the results of the ANOVA analysis for ABTS ($\mu\text{mol TE}/100 \text{ mL}$) and DPPH ($\mu\text{mol TE}/100 \text{ mL}$) values of the berry-vinegar samples. In this study, a quadratic model was used ($R^2 = 0.8607$ for ABTS and $R^2 = 0.9189$ for DPPH) and the values obtained are within the accepted limits.

The change of ABTS and DPPH values, depending on the US variables (amplitude and time) is presented in Figure 3.

At the end of optimization, ABTS was found to be 363.69 ($\mu\text{mol TE}/100 \text{ mL}$) and DPPH respectively 217.102 ($\mu\text{mol TE}/100 \text{ mL}$) for 3.96 min treatment at 78.50% amplitude (Figure 4).

The lowest DPPH ($\mu\text{mol TE}/100 \text{ mL}$) value was determined in the sample treated for 5 min with 40% amplitude, application 13 (139.1 $\mu\text{mol TE}/100 \text{ mL}$), while the highest DPPH value was detected in the sample treated with 80% amplitude for 4 min (238.9 $\mu\text{mol TE}/100 \text{ mL}$).

Factor Coding: Actual

TPC (mg GAE/L)

Design Points:

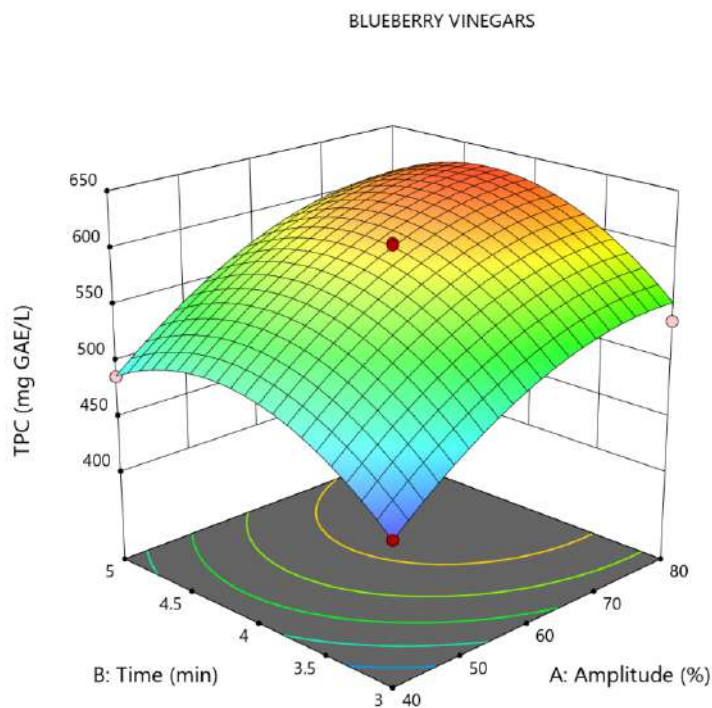
● Above Surface

○ Below Surface

441.4 643.05

X1 = A: A

X2 = B: Time



(a)

Factor Coding: Actual

TAC (mg C3G/L)

Design Points:

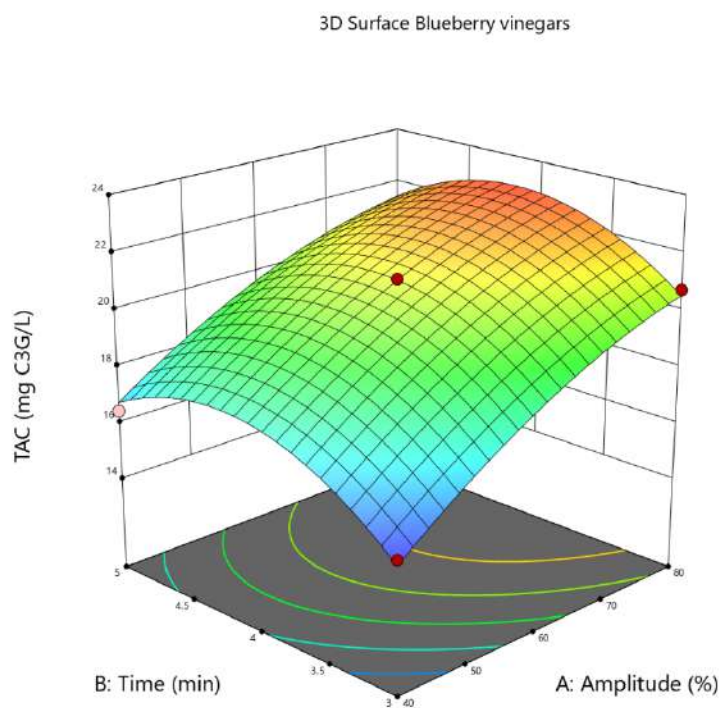
● Above Surface

○ Below Surface

15.25 23.29

X1 = A

X2 = B



(b)

Figure 2. Response surface showing the effect of time (min) and amplitude (A%) of the ultrasound treatments on bioactive compounds of the blueberry vinegars: (a) TPC (mg GAE/L); (b) TAC (mg C3G/L).

Table 7. Analysis of variance (ANOVA) of responses for antioxidant activity (evaluated by DPPH and ABTS assays) of the vinegar samples.

Source	SS	DF	MS	F-Value	p-Value	Significance
Model for ABTS * ($\mu\text{mol TE}/100\text{ mL}$)	36,608.95	5	7321.79	15.83	0.0011	*
X_1 = A-Amplitude	24,406.97	1	24,406.97	52.77	0.0002	**
X_2 = B-Time	2964.31	1	2964.31	6.41	0.0391	*
AB	4.04	1	4.04	0.0087	0.9282	-
A^2	4723.21	1	4723.21	10.21	0.0152	*
B^2	5707.83	1	5707.83	12.34	0.0098	*
Lack of Fit	3236.73	3	1078.91	5004.21	<0.0001	-
Total	39,846.54	12				-
$R^2 = 0.9187$; Adjusted $R^2 = 0.8607$; Std. Dev. = 21.15; Average = 330.03; C.V.% = 6.52						
Model for DPPH * ($\mu\text{mol TE}/100\text{ mL}$)	12,791.90	5	2558.38	28.21	0.0002	*
X_1 = A-Amplitude	6128.57	1	6128.57	67.57	<0.0001	**
X_2 = B-Time	511.31	1	511.31	5.64	0.0493	*
AB	244.77	1	244.77	2.70	0.1444	-
A^2	1592.54	1	1592.54	17.56	0.0041	*
B^2	4946.38	1	4946.38	54.53	0.0002	*
Lack of Fit	632.73	3	210.91	381.58	<0.0001	-
$R^2 = 0.9527$; Adjusted $R^2 = 0.9189$; Std. Dev. = 9.41; Average = 190.66; C.V. % = 4.94						

SS: Sum of squares; DF: freedom degree; MS: average squares; * significant at $p < 0.05$ and ** significant at $p < 0.01$.

The antioxidant activity mentioned in this study is higher than those reported by Arvaniti et al. [31] for commercial and homemade Greek vinegars (from apple cider) and similar to those specified by da Silva Fonseca et al. [13] for the blueberry and honey vinegars obtained using another fermentation process.

As stated by Wang et al. [32], ABTS and DPPH radicals have a different stereo-chemical composition and consequently confer, after interaction with antioxidants, a qualitatively different reaction for radical deactivation. In this context, more than a single assay is needed to provide the amount of information needed to estimate the antioxidant potential.

Figure 4 showed that a satisfactory antioxidant range is likely to be obtained by applying an ultrasound treatment with an amplitude of 78.50% and a period time of 3.96 min and this fact is most likely owing to the high responsiveness of phenolics to these conditions.

2.5. HPLC Analysis of Phenolic Compounds

In this study, four phenolic compounds (ellagic acid, gallic acid, ferulic acid, and chlorogenic acid) were identified in several samples chosen according to the results obtained using the model optimization. The choice of the study of the four hydroxycinnamic acids was made taking into account data from the literature that reported hydroxycinnamic acids (gallic acid and caffeic acid) as the main phenolic compounds in most types of fruit vinegars [33]. Chou et al. [34] highlighted that the number of flavonoids in vinegars is less. The authors reported that only one flavonoid compound (catechin) was detected when 21 flavonoid standards were analyzed. In persimmon vinegar, hydroxycinnamic acids were reported as major compounds [35], while in pomegranate vinegar, the protocatechuic acid ($28.88 \pm 0.02\text{ mg/L}$), followed by gallic acid, were the most abundant phenolic compounds detected [36].

The level of these antioxidants in the vinegars obtained using the US treated substrates A 80% and 60% for 4 and 5 min and the untreated substrate are presented in Table 8.

Factor Coding: Actual

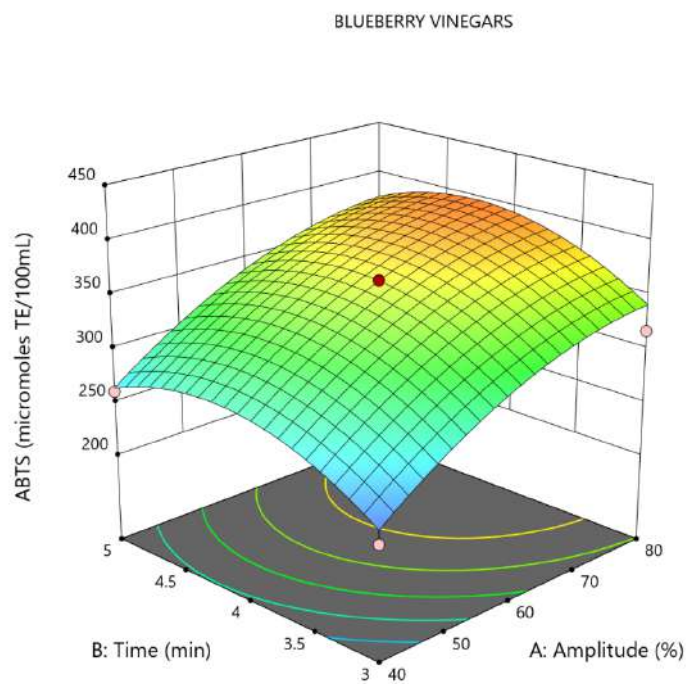
ABTS (mM TE/100mL)

Design Points:

● Above Surface
○ Below Surface
220.63 417.19

X1 = A

X2 = B



(a)

Factor Coding: Actual

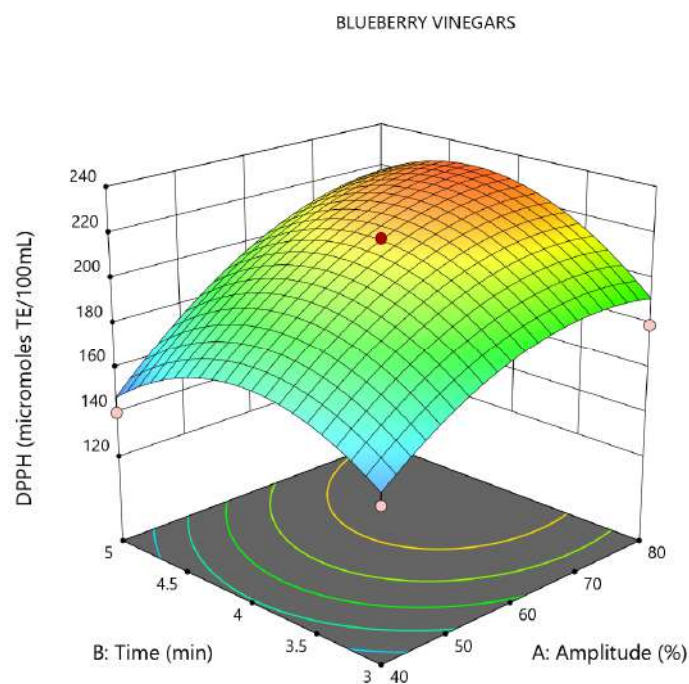
DPPH (mM TE/100mL)

Design Points:

● Above Surface
○ Below Surface
139.99 238.04

X1 = A

X2 = B



(b)

Figure 3. Response surface showing the effect of time (min) and amplitude (A%) of the ultrasound treatments on the antioxidant potential of the blueberry vinegars: (a) ABTS assay; (b) DPPH assay.

It can be observed that the level of all phenolics compounds identified by HPLC analysis was the highest in the vinegar sample B804 (ID Table 5). This vinegar sample was obtained using an amplitude of 80% for 4 min and the values of the treatment parameters were very close to those recorded after applying the optimization model (A 78.5%, 3.96 min).

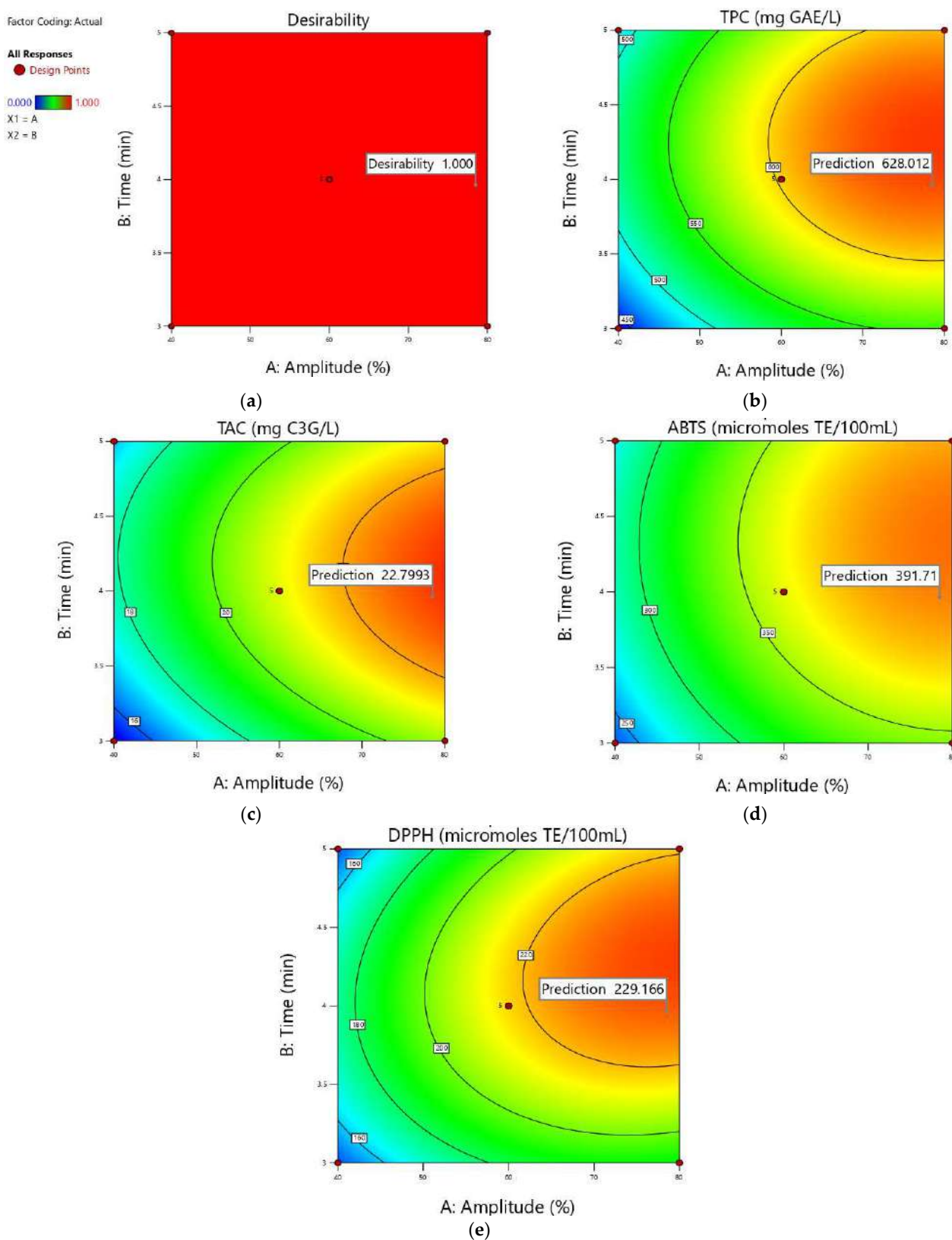


Figure 4. Contour plots showing the optimum predicted values for all the responses (parameters predicted values for amplitude 78.50% and 3.96 min time period of the US treatment): (a) desirability; (b) predicted value for TPC (612.715; 643.31); (c) predicted value for TAC (22.4769; 23.1216); (d) predicted value for ABTS (366.732; 416.689); (e) predicted value for DPPH (218.104; 240.227).

Table 8. Phenolic compounds in the vinegar samples identified by HPLC.

Sample	Ellagic Acid ($\mu\text{g}/100\text{ mL}$)	Gallic Acid ($\mu\text{g}/100\text{ mL}$)	Ferulic Acid ($\mu\text{g}/100\text{ mL}$)	Chlorogenic Acid ($\mu\text{g}/100\text{ mL}$)
B	1.781 ± 0.085^d	21.466 ± 2.72^d	1.506 ± 0.038^d	17.422 ± 1.083^b
B804	3.347 ± 0.026^a	35.22 ± 1.03^a	2.538 ± 0.116^a	18.801 ± 0.995^a
B805	3.271 ± 0.018^b	34.065 ± 0.85^b	1.967 ± 0.047^b	8.588 ± 0.084^d
B604	3.062 ± 0.104^c	28.215 ± 1.34^c	1.59 ± 0.062^c	14.238 ± 1.027^c
B605	2.353 ± 0.163^c	21.914 ± 2.78^d	1.536 ± 0.009^{cd}	8.447 ± 0.082^d

Data were expressed as the average of 3 determinations $\pm$ standard deviation. Values with different letters differ significantly by ANOVA and Duncan's test ($p < 0.05$).

2.6. Sensory Analysis

The results of sensory analysis of blueberry-vinegar samples, based on odour and taste profiles (general impression, pungent sensation, aromatic intensity, taste, and ethyl acetate odour) are presented in Figure 5. Among the judges, there were six experts (three female and three male panellists). The score of the attribute “ethyl acetate odor” is only the score of intensity.

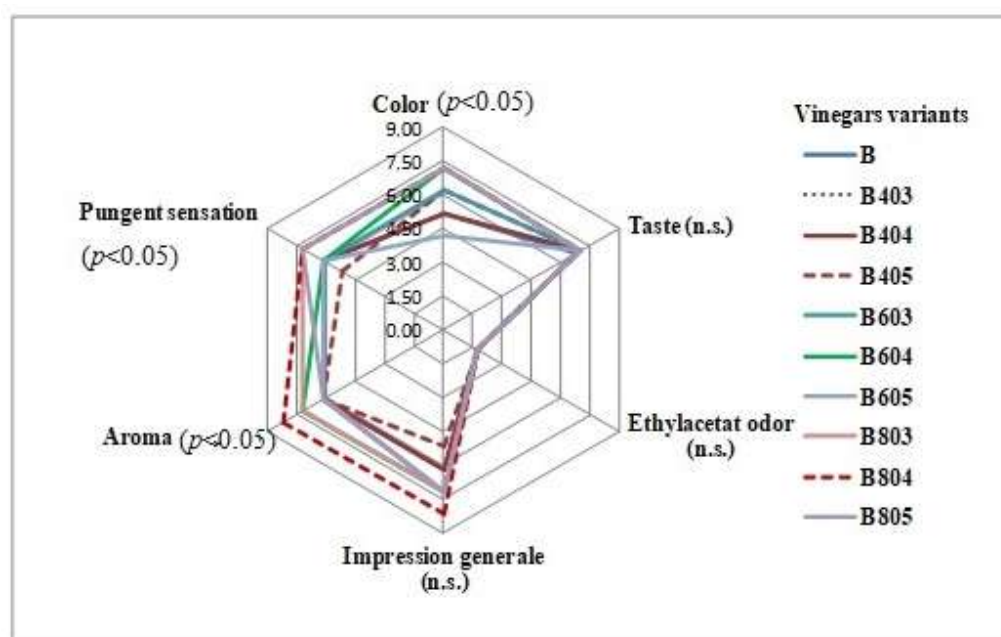


Figure 5. Results of sensory analysis for blueberry vinegars. ID of the samples were presented in Table 5. There were significant differences between samples at $p < 0.05$ and n.s., no statistical difference.

Sensory analysis of the vinegar variants evaluated revealed no abnormal odours. It was observed that there was no statistically significant difference between all samples in the case of the vinegars taste. The intensely sour taste of vinegar led to similar results, with the scores given by the tasters being close (there were no significant differences in taste).

The B804 (ID Table 5) sample was the most preferred sample regarding the evaluation of colour (8.27), aromatic intensity (8.18), pungent sensation (7.07), and general evaluation of impression (8.18) ($p < 0.05$).

The evaluation of the panellists highlights that the samples obtained using ultrasound-treated substrates were generally more liked compared with the sample control (B). Similar data were reported by Yikmuş et al. [4] for US-treated juice vinegars, products generally admired by the panellists. Other researchers reported also an improvement in the sensory

evaluations because of the ultrasound treatments used in the processing of various fruit juices [37].

3. Materials and Methods

3.1. Materials, Reagents

Folin–Ciocalteu reagent, was acquired from Sigma-Aldrich (St. Louis, MI, USA). DPPH (cat. no. D9132, purity 97%) and ABTS (cat. no. A1888, purity 98.6%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin 3 glycoside and gallic acid were acquired from Karl Roth GmbH (Karlsruhe, Germany). Hydrochloric acid, sodium acetate, sodium carbonate, and sodium hydroxide were acquired from POCH BBASIC (Gliwice, Poland). Trolox were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Wild blueberry fruits (*Vaccinium myrtillus* L., Wild), sold at a local fruit market, were used as raw materials. The berries were harvested in August from a hilly area situated in Northeast Romania 45°43'18" N 26°20'15" E (Comandau, Covasna County, Romania).

3.2. Production of Berry-Vinegar Variants

Fresh blueberries were sorted, mechanically crushed (with a beater), and the berry juice was obtained under laboratory conditions using a Bosch MES3500 (700 W) centrifugal juice extractor (Bosch GmbH, Stuttgart, Germany) following a procedure typically applied at home scale.

The clear blueberry juice obtained was used for the production of vinegar according to the technological flow chart shown in Figure 6 but the method used was not the traditional one. We have tried a new method, our intention being the faster initiation of acetic fermentation, the use of treatments leading to the accentuation of the functional valences of the finished product, and the identification of the optimal parameters of these treatments in order to obtain vinegar with improved antioxidant properties.

First, the berry juices were diluted with water in order to obtain substrates having total soluble substances of 10 °Brix. After that, these substrates were mixed with ethanol (7%, *v/v*), glucose (4%, *w/v*), and apple vinegar (10%, *v/v*) for acidification. A commercial mineral-salts blend (0.03%, *w/v*; Acetozym® (Heinrich Frings GmbH & Co. KG, Rheinbach, Germany)) was used to supplement the substrates. After the ultrasound treatments, this substrate having enough alcohol for the acetic fermentation was inoculated (in ratio 10%, *v/v*) with a culture of acetic bacteria prepared according to da Cunha et al. [1] and da Silva Fonseca [13]. A volume of 200 mL ultrasound-treated substrate was transferred to a 0.5 L capacity glass jar and inoculated with 20 mL acetic bacteria culture inoculum. The acetic bacteria culture and the inoculum were prepared according to da Cunha et al. [1]. The acetic acid bacteria were isolated from red grape (*Vitis vinifera* L.) vinegar produced in the faculty laboratory, by cultivating nonpasteurized vinegar in a GY medium (100 g/L glucose, 10 g/L yeast extract, and 100 mg/L natamycin). For obtaining the inoculum, in 500 mL Erlenmeyer flasks there were mixed the acetic bacteria culture (25 mL) with blueberry wine (150 mL), the dilution of the bacterial culture being 1:7. The flasks were incubated at 30 °C for 24 h in an orbital shaker rotating at 120 rpm for growth and cell adaptation and then added to the alcoholic substrates.

The fermentation temperature was set at 30 °C. During the process were determined the acidity (% acetic acid) and the pH values. The fermentation process was stopped when the acetic acid content was stable for a period of 7 days and until the alcohol content was between 0.5% and 1%. The acetic fermentation experiments were completed in 50 days after the inoculation. As a control (B sample), it used the untreated berry vinegar. Vinegar samples were stored at 4 °C.

3.3. Titratable Acidity, pH, and Total Soluble Solids

The pH, titratable acidity (% acetic acid), and total soluble solids values were determined for the substrates (before fermentation) and for the vinegar samples. The pH was measured using a pH meter (Consort C5020T, Consort, Turnhout, Belgium), the acidity

(% acetic acid) was determined by titration with a solution of 1N NaOH in the presence of phenolphthalein as an indicator and the total soluble solids content (TSS, °Brix) was analysed using a refractometer (OE Germany/OE Swiss/MASS) at room temperature.

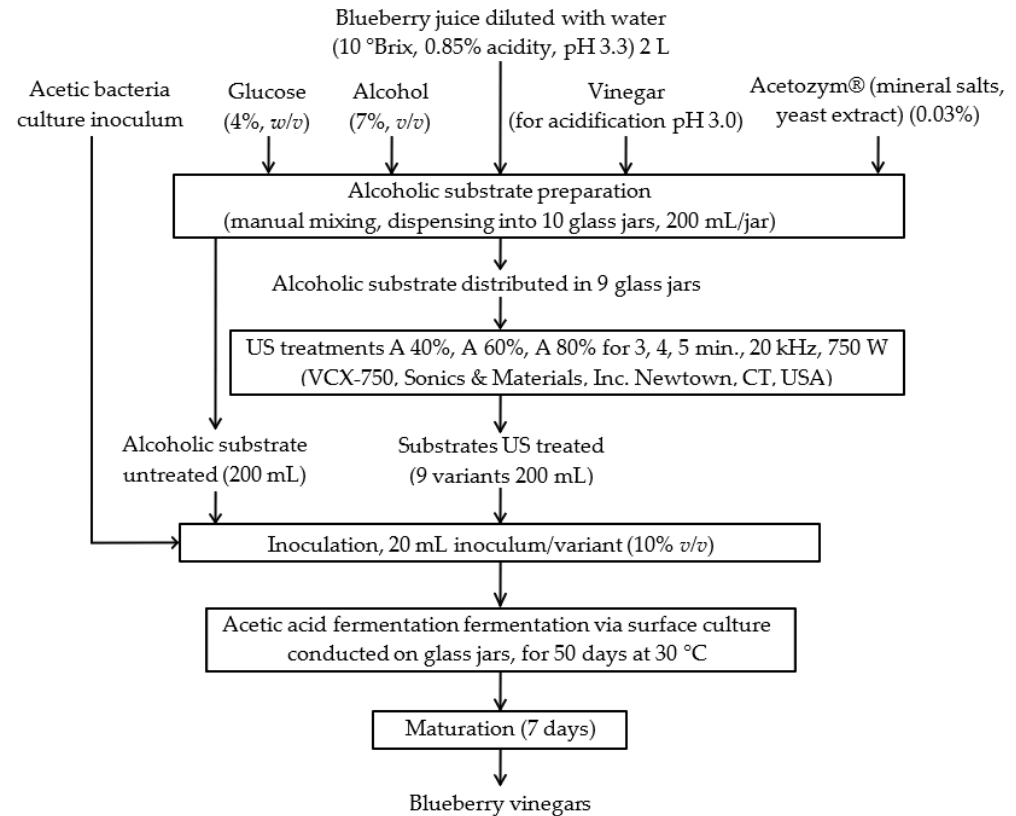


Figure 6. The method used for obtaining blueberry vinegar (US = ultrasound).

3.4. Ultrasound (US) Treatments

The substrates were ultrasonically treated (VCX-750, Sonics & Materials, Inc., Newtown, CT, USA) using a continuous frequency of 20 kHz, an amplitude A of 40%, 60%, and 80%, and a power of 750 W for periods of 3, 4, and 5 min for samples of 19 mm and 500 mL as a mixture [16].

3.5. Factorial Experimental Design

The berry vinegars' parameters were analyzed using Minitab Statistical Analysis Software (Minitab18.1.1) and response surface methodology (RSM) was employed in order to optimize the ultrasound treatment's effect on the antioxidant properties of the products. Table 5 presents the test applications (ID samples) used for optimization.

The response surface method (RSM) was used to understand the effect of ultrasound treatment of blueberry vinegar on bioactive components. Central composite design (CCD) was chosen for RSM. Two factors and 3 levels were determined in the design. There were 13 trial points for RSM optimization. Model adequacy, R^2 and corrected R^2 coefficients, lack-of-fit tests, and ANOVA results were evaluated. The independent variables were duration (X_1) and amplitude (X_2). Dependent variables were total phenolic content (TPC), total anthocyanins content (TAC), and total antioxidants (DPPH and ABTS). MINITAB statistical software (Minitab 18.1, Minitab, Inc., State College, PA, USA, 2017) was used for RSM and its graphs were developed with SigmaPlot 12.0 Statistical Analysis Software (Systat Software, Inc., San Jose, CA, USA). A polynomial was used to create model equations.

3.6. Total Phenolic Content (TPC) and Total Monomeric Anthocyanins Content (TAC)

Total phenolic content (TPC) of the samples was analyzed by the Folin–Ciocalteu method [38] using a plate reader spectrophotometer (Tecan, SunRise™, software Magellan™, Männedorf, Switzerland). The TPC was calculated as milligrams of gallic acid equivalents (GAE) per litre (L) and per 100 g dry weight (DW).

Total anthocyanins monomers were determined using the differential pH method and the absorbance was measured by the use of a spectrophotometer DR2800 type (Hach Lange, Loveland, CO, USA) [39]. The results were reported as mg cyanidin 3 glycoside (C3G) per litre of vinegar, respectively, per 100 g DW.

3.7. Antioxidant Activity

The antioxidant activity was determined by two different methods, respectively, ABTS and DPPH assays.

3.7.1. ABTS Assay

The antioxidant potential of vinegar variants in reaction with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS⁺) was determined by referring to a method related by Re et al. [40]. Trolox standards were applied to generate the calibration curve. Results were reported in μmol Trolox Equivalents per 100 mL of vinegar (μmol TE/100 mL) and per g dry weight (μmol TE/g DW).

3.7.2. DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was formulated by a method described by Kumaran et al. [41]. Absorbance was analyzed at 517 nm (spectrophotometer DR2800, Hach Lange, Loveland, USA) considering the methanol as a witness sample. The antioxidant activity of the samples against DPPH radicals was expressed as micromoles of Trolox equivalents per 100 mL of vinegar (μmol TE/100 mL) and per g dry weight (μmol TE/g DW) using a calibration curve previously created.

3.8. Polyphenols Profiles by HPLC Analysis

The polyphenolic compounds profile was determined by high-performance liquid chromatography (HPLC) according to the method described by Abdel-Hameed et al. [42]. Separations were performed using an Agilent Technologies 1200 chromatograph equipped with a UV-DAD detector, using a 250 mm $\times$ 4 mm Licrocart (Licrospher PR-18 5 μm) column (Merck, Darmstadt, Germany) operated at 30 °C. All recorded data were processed using Agilent Chem Station B.04.03 software (Agilent, Santa Clara, USA). The mobile phase consisted of water/acetic acid (97:3, v/v) (eluent A) and acetonitrile (eluent B) at the flow rate of 1 mL/min. The linear gradient profile was as follows: 97% A (0 min), 97–91% A (5 min), 91–84% A (5–15 min), 84–65% A (15–20.8 min), 65–64.5% A (20.8–36 min), 64.5–50% A (36–37 min), 50% A (37–38 min), 50–97% A (38–39 min), and 97% A (42 min). The injection volume was 20 μL .

3.9. Sensory Analysis

The overall acceptability of the vinegar samples was evaluated by considering odor and taste profiles (general impression, pungent sensation, aromatic intensity, taste, and ethyl acetate odor). Twelve female and ten male panellists participated in the vinegar samples evaluation. Each sample was dosed in glass containers 20 ± 1 g and placed at 4–6 °C until serving. For performing the analysis, a 9-point hedonic scale was used. Scale scores were the following: excellent, 9; very good, 8; good, 7; acceptable, 6; and poor, <6.

3.10. Statistical Analysis

In order to optimize the antioxidant potential of blueberry vinegar, the RSM (Minitab 18.1, Minitab, Inc.) was used. There were obtained three-dimensional graphs (Sigma

Plot 12.0 Statistical Analysis Software, Systat Software, Inc.). Samples were prepared in triplicate.

4. Conclusions

This study provides strong evidence for the integration of ultrasound treatment of alcoholic substrate containing blueberry juices as an effective step in vinegar production to increase the extraction of bioactive compounds and, consequently, to enhance the antioxidant properties of the resulting products. The added value of this study consists both in the use of a new recipe to obtain blueberry vinegar, using an alcoholic substrate containing fruit juice and in the optimization, based on the response surface methodology, of the extraction process of antioxidant compounds from blueberry. The obtained data proved that the blueberry vinegars ultrasound-treated and optimized were of superior quality in relation to the polyphenols and anthocyanins content having, at the same time, better antioxidant properties. For the optimum values of all these parameters, the better values found for US amplitude and duration were 78.50% and 3.95 min, respectively. The sensory analysis of blueberry-vinegar variants revealed that the samples obtained using ultrasound-treated substrates were generally more liked compared with the control sample. The vinegar sample obtained following ultrasound treatment at 80% amplitude for 4 min, which showed the highest TPC and TAC levels and the strongest antioxidant properties assessed by the DPPH and ABTS tests, was the most preferred sample in terms of colour, aromatic intensity, pungent sensation, and overall impression. Blueberry vinegars obtained by ultrasound treatment of the raw material are a good source of polyphenolic compounds that contribute to the daily intake of antioxidants. We plan to extend the research for a more indepth analysis of polyphenolic compounds in blueberry-vinegar variants obtained using ultrasound treatments. Our results highlight that the use of an ultrasonically treated alcoholic substrate represents innovative items with practical applicability for a rapid start of acetic fermentation and for improving the antioxidant properties of blueberry vinegar.

Limitations of this study could be due to the experimental design with only three levels of variable design (multivariate). Therefore, future approaches are considered to improve the design in order to meet the optimal condition for obtaining the best blueberry-vinegar variant with a high level of valuable bioactive compounds. In addition, any study on the antioxidant and TPC potential of blueberry vinegars should take into account the structure of the antioxidants, their contribution, raw material, technology, and the ageing process. In light of this approach, we intend to continue research on blueberry vinegars as products with a promising antioxidant potential and multiple benefits for human health.

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Original paper

Comparative analysis of alternative and standardized microbiological diagnostic methods used in food expertise

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Abstract

For the comparative analysis of the results of the alternative and the standardized reference methods used in microbiological food testing, we tested 7761 samples for the detection of foodborne pathogens (*Salmonella spp.*, *Listeria spp.*, *Campylobacter spp.*) and 5123 samples for the detection of quality indicators (Coagulase Positive Staphylococcus, *Escherichia coli*, Enterobacteriaceae, Total Aerobic Germs, Yeasts and Molds), using Mini Vidas equipment and TEMPO, respectively. Non-compliant samples resulting from food screening by alternative methods were subsequently tested by standardized reference methods to establish the correlation of the results. It has been found that fast alternative diagnostic methods used to detect food pathogens generate results equivalent to those of standardized reference methods, and rapid diagnostic alternative methods for detecting quality indicators generate results with a correlation of 98.38%. The bacterial identification achieved by comparative use of the alternative and the reference methods, namely Vitek 2 Compact and Kaufmann-White, may show differences in the obtained results, the correlation being for the *Salmonella* genus of 77.8%, and, in the case of *Listeria* genus, 100%.

Keywords

Alternative method, standardized method, pathogenic germs.

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Introduction

Conventional bacterial testing methods rely on specific media to enumerate and isolate viable bacterial cells in food. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the food sample. Traditional methods for the detection of bacteria involve the following basic steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological confirmation (PUCHIANU & al [7]).

Hence, a complete series of tests is often required before any identification can be confirmed. These conventional methods require several days to give results because they rely on the ability of the organisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates and colony counting makes these methods labor intensive. Conventional methods, generally regarded as the golden standard, often take days to complete the identification of viable pathogens. Any modification that reduces the analysis time can technically be called rapid method (MANDAL & al [4]).

Rapid and novel methods in microbiological tests provide more sensitive, precise and reproducible results compared with conventional methods (NEMATİ & al [5], VAN DER ZEE [10]).

A rapid method can be an assay that gives instant or real time results, but, on the other hand, it can also be a simple modification of a procedure that reduces the assay time. These rapid methods not only deal with the early detection and enumeration of microorganisms, but also with the characterization of isolates by use of microbiological, chemical, biochemical, biophysical, molecular biological, immunological and serological methods (MANDAL & al [4]).

The food industry is in need of more rapid methods which are sensitive for the following reasons: to provide immediate information on the possible presence of pathogen in raw material and finished products; low numbers of pathogenic bacteria are often present in complex biological environments along with many other non-pathogenic organisms; the presence of even a single pathogenic organism in the food may be a infectious dose; for monitoring of process control, cleaning and hygienic practices during manufacture; to reduce human errors and to save time and labor cost (MANDAL & al [4]).

The reviewed literature showed that rapid methods and automation in microbiology is an advanced area for studying and applying of improved methods in the early detection, and characterization of microorganisms and their products in food, pharmaceutical and cosmetic industries as well as environmental monitoring and clinical applications (NEMATİ & al [5]).

Such methods are now available for rapid detection or estimation of groups of (indicator) organisms, pathogenic micro-organisms, bacterial toxins and mycotoxins, and molds. These alternative methods can be classified by the principles on which they are based: modified conventional methods, instrumental measurement of bacterial

metabolism, bioluminescence, immunological techniques, DNA techniques and combinations of these techniques. To meet user expectations, test kits must be accurate, sensitive, specific, rapid (24 h or less), easy to use, and labor-saving. They must also offer the possibility of computerization, a low detection limit, and low investment and running costs (VAN DER ZEE & al [9], VAN DER ZEE & al [10], GUILLERMO & al [2]).

There is a continuous development of methods for the rapid and the reliable detection of food borne pathogens. Advent of biotechnology has greatly altered food testing methods. Improvements in the field of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient methods in food microbiology (MANDAL & al [4], GUILLERMO & al [2]).

To meet future challenges in an always changing environment, appropriate expertise needs to be identified and a model of specialized and continuous training is required (SHAPING THE FUTURE OF FOOD SAFETY, TOGETHER [12]).

After 48 h of incubation, the PCR method in all cases showed equal or better results than the reference cultural Food and Drug Administration, for example in the case of detection of *L. monocytogenes* (PATRINOS & al [6]). The independent validation study examined by Campden and Chorleywood Food Research Association Group (CCFRA) demonstrated that the LightCycler *L. monocytogenes* detection system shows a comparable sensitivity to reference methods (JUNGE & al [3]).

A major disadvantage of alternative and rapid methods over cultural methods is that most methods need damaging of the cells and therefore, viable cells for result confirmation and further characterization can only be obtained by repeating the analysis using standard cultural procedures. Moreover, rapid methods usually detect only one specific pathogen, while cultural methods simultaneously detect and isolate many pathogens by including several types from numerous microbiological examinations or samples, selective media in the analysis. The use of several rapid assays to do multi pathogen analyses on a food sample makes this analysis unacceptably expensive (MANDAL & al [4], BLAKE & al [1]).

It is also to be noted that in accordance with the Commission Regulation (EC) 2073/2005 of 15 November 2005 regarding microbiological criteria for food products, food business operators must be able to use other analytical methods than those reference methods, in particular faster methods as long as alternative methods give equivalent results. (COMMISSION REGULATION (EC) 2073/2005 [12]).

Materials and Methods

The purpose of the this paper was to verify the correspondence of the results of rapid alternative diagnostic methods with those of the standardized methods, by examining food samples taken during self-control programs from different processing units within Braşov County.

The samples were originally tested by alternative methods and those found with positive (non-conforming) results were then analyzed by standardized methods in order to confirm/refute the obtained results.

The exams performed by alternative methods were:

- the detection of food pathogens (*Salmonella* spp., *Listeria* spp., *Campylobacter* spp.) by using the Mini Vidas equipment;
- the enumeration of the quality indicators (*Coagulase Positive Staphylococcus*, *Escherichia coli*, Enterobacteriaceae, Total Number of Aerobic Germs, Yeasts and Molds) using the TEMPO equipment;
- the bacterial identification using the VITEK 2 COMPACT equipment.

The reference methods used to confirm the positivity cases resulted from the use of fast alternative diagnostic techniques were the standardized ones, as follows:

- the detection of food pathogens: SR EN ISO 6579-1 (*Salmonella* spp.); SR EN ISO 11290-1 (*Listeria*

spp.); SR EN ISO 10272-1 (*Campylobacter* spp.);

- the enumeration of the quality indicators: SR EN ISO 6888-2:2002/A1:2005 (*Staphylococcus* spp.); SR ISO 16649-2:2007 (*Escherichia coli*); SR EN ISO 21528-1,2 (Enterobacteriaceae); SR EN ISO 4833-1/2014 (Total Number of Germs); SR EN ISO 21527-1 (D + M);
- the Kaufmann – White method, for the bacterial identification.

The expertise was carried out within the Sanitary Veterinary and Food Safety Laboratory of Brasov, using validated and accredited methods in accordance with SR EN ISO 17025/2005, both for the reference methods and the alternative methods. The bacterial identification by the Kaufmann-White method was performed at the Institute of Hygiene and Veterinary Public Health Bucharest.

For the detection of food pathogens, we tested a number of 7761 samples using the Mini Vidas, as follows: (Table 1).

Table 1. Number of samples taken and examined by alternative techniques for detection of food pathogens

Analyzed parameters	Samples	
	No.	%
<i>Salmonella</i> spp.	4257	54.85
<i>Listeria</i> spp.	2983	38.44
<i>Campylobacter</i> spp.	521	6.71
TOTAL	7761	100.00

The Mini Vidas equipment is a compact high-performance system in the identification of highly pathogenic germs (*Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus enterotoxin*, etc.), having the immunological analysis as working principle.

Compared to the standardized methods, its use allows time savings in the preparation of culture media, glass sterilization, packaging, labeling, inoculation, plate readings, autoclaving and glassware washing, using ready-to-use reagents (Fig. 1).

The MiniVidas Automated Analyzer offers a wide range of analyzes, applications, safety and ease of use, being a automated, standardized, robust device that allows objective reading and delivering a quick result compared to classic workflows.

Matrices from which food pathogens can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens are: (Table 2).

To quote the quality indicators, we have tested 5123 samples using the TEMPO equipment, as follows: (Table 3).



Figure 1. MiniVidas Automated Analyzer

Table 2. Matrices and required times

Bacterial germs that can be identified	Matrice	Required times (hours)
<i>Salmonella</i> spp.	All food products during their validity.	48
<i>Listeria monocytogenes</i>	All food products before leaving the direct control of the unit which produced them and products marketed during their shelf life (meat and meat products, raw milk, unpasteurized milk cheeses, confectionery and pastry products, food, fish and fish products) .	72
<i>Campylobacter jejuni</i>	Chicken carcasses – surveillance period 1.04-30.09 (warm season).	72

Table 3. Number of probes sampled and examined by alternative techniques for the enumeration of quality indicators

Analyzed parameter	Number of samples	
	No.	%
<i>Staphylococ c.p.</i>	855	16.69
<i>E. coli</i>	1235	24.10
Enterobacteriaceae	943	18.41
NTG	1520	29.67
D + M	570	11.13
TOTAL	5123	100.00

The TEMPO method is a fully automated method that allows the quantitative determination of bacterial germs. It is based on traditional microbiology, based on the multiple tube method. Unlike standardized methods, it has a number of advantages, of which the most important are: sensitivity and ease of use, delivering a quick result, saving time in preparing media, preparing for glass sterilization, packaging, labeling, inoculation, plate readings, autoclaving and glassware washing, etc. (Fig. 2).

**Figure 2.** Reading station: Reading, interpreting, validation and transfer of results.

The TEMPO test consists of a card with a transfer tube and an ampule with a specific culture medium. Culture media are desiccated, sterile, ready-to-use, disposable, selective: TC (Total Coliforms); EC (*E. coli*); EB (Enterobacteriaceae); STA (*Coagulase Positive Staphylococcus*); LAB (Lactic Acid Bacteria) or non-selective: TVC (NTG); Y + M (Yeasts and Molds), identified by bar code and color code, and Stomacher TEMPO sachets.

The medium is inoculated with a dilution of the sample to be tested and transferred by the tempo filling instrument into the tempo card. The medium is homogeneously dispersed in 48 wells with three different volumes. The card is then hermetically sealed to avoid any risk of contamination during manipulations. Later, the interpretation, the validation and the transfer of the results take place in a single stage (Fig. 3).

**Figure 3.** Card with a transfer tube and an ampoule with a specific culture medium.

Example:

Tempo TVC and Tempo EC – during incubation, the microorganisms present in the card reduce the substrate from the culture medium and cause a fluorescent signal to be detected by the TEMPO reader. Depending on the number and size of the positive wells, the tempo system deducts Total Number of Germs (for the Tempo TVC test) or the *E.coli* number (for the EC test) present in the initial sample, based on the most probable number method.

Matrices from which bacterial quality indicators can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens are: (Table 4).

Table 4. Matrices from which bacterial quality indicators can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens

Bacterial germs that can be identified	Matrice	Required times (hours)
<i>E. coli</i>	Meat products, mechanically separated meat, heat-treated milk cheeses, non-dairy products.	24
<i>Staph. c.p.</i>	Raw milk cheeses subjected to lower heat treatment than pasteurization and heat-treated milk, milk powder, fish products.	24
Enterobacteriaceae	Cattle, sheep, goats, horses, pigs, pasteurized milk and pasteurized dairy products, milk powder, ice cream and dairy desserts, infant formula and food for medical purposes, egg products.	48
NTG	Milk raw material, carcasses of cattle, sheep, goats, pigs, horses, poultry, minced meat and mechanically separated.	48
D + M	Bakery products	72

Some of the non-conforming samples found during the detection of food pathogens (12 strains of *Salmonella* spp. and 9 strains of *Listeria* spp.) were reexamined with the Vitek 2 Compact and Kaufmann-White methods in order to confirm the initial results.

Vitek 2 Compact is an automatic biochemical and antibiotic confirmation system capable of selecting pathogenic and highly pathogenic organism isolates on solid media by performing biochemical tests in a very fast time, resulting in time savings in generating the results (Fig. 4).



Figure 4. Vitek 2 Compact

By using it, we save materials and reagents. Thus, the used card saves all types of reagents required for the identifications and the confirmations and it does not require any type of glassware, thermal sterilization, thermostatic or other pre-diagnostic steps.

It's an easy-to-use device with a low work-time, intuitive software and connection to the computer system. The method is fully automated, it uses different diagnosis cards to identify Gram + (GP), Gram - (GN), Anaerobic bacteria (ANC), Campylobacteria (NH), Corynebacteria (CBC), Yeast and Mold (YST) and *Bacillus* (BCL).

The working stages are the following: calibration of the apparatus by using specific kits for each methodology, inoculation of the pathological material from the previously purified microbial cultures into kit strips, determination of the bacterial optical density (OD) by means of a device called DENSIMAT (densitometer) specific to each bacteria genus, attachment of the tube with the bacterial density determined on the card specific to the method, inserting the side tube of the card into the glass tube with established bacterial optical density, inserting the card with the glass tube attached to the mobile carrier of the apparatus, closing the hatch, processing the sample identification data, assigning a sample code, requesting the genus to which the micro-organisms to be tested belong, validating requests and starting the apparatus (Fig. 5).

Example: *Salmonella* spp.



Figure 5. The main steps for identifying bacterial germs using the Vitek 2 Compact.

After a variable period of time, the equipment generates a printed report where the biochemical properties of the identified bacteria genus and the probability of its validation are indicated. Examples: *Salmonella* spp., 5-6 hours; *Listeria monocytogenes*, 6-8 hours, *Campylobacter* spp., 8-12 hours; *Escherichia coli*, 4-6 hours; *Staphylococcus* spp., 4-6 hours, Yeast and Mold, 4-6 hours.

Results and Discussion

After analyzing the 7761 samples for the detection of food pathogens and the 5123 samples for the detection of quality indicators by fast alternative diagnostic methods, the following results were obtained: (Table 5 și 6).

Table 5. Results obtained from the detection of food pathogens through fast alternative diagnostic methods

Analized parameters	Samples No.	Results			
		Noncompliant		Compliant	
		No.	%	No.	%
<i>Salmonella spp.</i>	4257	36	0.85	4221	99.15
<i>Listeria spp.</i>	2983	21	0.70	2962	99.30
<i>Campylobacter spp.</i>	521	3	0.58	518	99.42
TOTAL	7761	60	0.77	7701	99.23

Table 6. The results obtained from the examination of the quality indicators by means of rapid diagnostic alternatives

Analized parameters	Samples No.	Results			
		Noncompliant		Compliant	
		No.	%	No.	%
<i>Staphylococ c.p.</i>	855	52	6.08	803	93.93
<i>E. coli</i>	1235	67	5.43	1168	94.57
Enterobacteriaceae	943	79	8.38	864	91.62
NTG	1520	131	8.62	1389	91.38
D + M	570	41	7.19	529	92.81
TOTAL	5123	370	7.22	4753	92.78

The interpretation was carried out in accordance with the provisions of Regulation 2073/2005 laying down the microbiological safety criteria defining the acceptable process characteristics as well as microbiological safety criteria for foodproducts which set a limit beyond which a food should be considered unacceptable as being contaminated.

It was found that most non-conformities were recorded in case of Totala Number of Germs (8.62%), Enterobacteriaceae (8.38%), Yeast + Molds (7.19%), and fewest in case of *Campylobacter spp.* (0.58%), *Listeria spp.* (0.70%) and *Salmonella spp.* (0.85%).

These values express the situation of the analyzed samples from the point of view of microbiological contamination and the obtained results were the basis of the

corrective measures implemented in the processing units and of the sanctions imposed on the batches of provenance in the case of identifying the bacterial species with a toxicogenic potential. The original batches were seized until the initial results were confirmed/refused by the reference tests.

The 60 samples with non-compliant results in fast alternative tests for food pathogens were reexamined by standardized reference methods, in accordance with the provisions of Regulation 2073/2005. A correlation of the 100% obtained results was found as follows: (Table 7).

The 370 samples with non-compliant results in the rapid alternative tests for quality indicators were examined by standardized reference methods, with a correlation of 98.38% as follows: (Table 8).

Table 7. Correlation of results obtained using reference and alternative methods

Analized parameters	Non-compliant samples		Corelation %
	Alternative methods	Reference methods	
<i>Salmonella spp.</i>	36	36	100
<i>Listeria spp.</i>	21	21	100
<i>Campylobacter spp.</i>	3	3	100
TOTAL	60	60	100

Table 8. Conformity of the results obtained using the reference methods

Analized Parameters	Non-compliant samples		Corelation %
	Alternative methods	Reference methods	
<i>Staphylococ c.p.</i>	52	52	100
<i>E. coli</i>	67	67	100
Enterobacteriaceae	79	79	100
NTG	131	126	96.18
D + M	41	40	97.56
TOTAL	370	364	98.38

It means that the Mini Vidas method used for the detection of food pathogens generates equivalent results (100%) and the TEMPO method, used for the detection of quality indicators, generates results with a correlation degree of 98.38%, with those obtained using the reference methods.

However, it is worth noting that in the case of the non-correlations recorded in the quality indicators, Total Number of Germs and Y + M, where the differences were 3.82% and 2.44% respectively, the non-conforming samples insignificantly exceeded the upper limit of the admissibility. This demonstrates that for the TEMPO method, some parameters may have some mismatches

when the results record values that are at the limit of compliance / nonconformity.

As far as the bacterial identification is concerned using the Vitek 2 Compact equipment, the 12 samples of *Salmonella* spp. and the 9 samples of *Listeria* spp. were also analyzed with the Kaufmann-White method.

For both genres, a correlation of 91.68% was found, with significant differences between species *Salmonella* and *Listeria*. Thus for the *Salmonella* genus, the correlation of the results was 77.8, and in the case of the *Listeria* genus, the correlation of the results was 100%, as follows: (Table 9).

Table 9. Identified serovars of *Salmonella enterica* and *Listeria* spp.

Microorganisms	Species/Serovar	Vitek 2 Compact Method	Kaufmann-White Method	Correspondence %
<i>Salmonella enterica</i>	<i>enteritidis</i>	3	2	66.7
	<i>taksony</i>	1	1	100
	<i>infantis</i>	3	4	66.7
	<i>newport</i>	3	3	100
	<i>saintpaul</i>	2	2	100
<i>Listeria</i> spp.	<i>L. monocytogenes</i>	3	3	100
	<i>L. ivanovii</i>	3	3	100
	<i>L. innocua</i>	3	3	100

The non-conformities of the results obtained using the Vitek 2 Compact and Kaufmann-White methods were found in the case of *Salmonella enteritidis* and *Salmonella infantis*. In the case of *Listeria monocytogenes* species, the correlation of the results was 100%.

Conclusion

Following the use of alternative diagnostic methods for the examination of food samples taken from processing units, it was found that most of the non-conformities were recorded in the testing of quality indicators and significantly less in the testing of food pathogens.

The obtained results were the basis of the corrective measures implemented in the processing units and the sanctions imposed on the batches of provenance after they were confirmed / refuted by standardized reference methods, according to the provisions of Regulation 2073/2005.

Rapid alternative diagnostic methods used for the detection of food pathogens generated results equivalent to those of the standardized reference methods. In the case of fast alternative diagnostic methods for the detection of quality indicators, the results showed a correlation of 98.38% with those of the standardized reference methods.

The results obtained in bacterial identification, made using alternative and reference methods, such as Vitek 2 Compact and Kaufmann – White, may show some differences.

Competing interest

The authors declare no conflict of interest.

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THE EVALUATION OF MEAT AND MEAT PRODUCTS EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS IN ROMANIAN FOODSTUFFS

EVALUAREA EXPUNERII CĂRNII ȘI PRODUSELOR DIN CARNE LA HIDROCARBURI POLICICLICE AROMATICE ÎN PRODUSE ALIMENTARE DIN ROMÂNIA

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ABSTRACT | REZUMAT

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with a pronounced carcinogenic potential. The formation of PAH in food depends on the thermal processing methods, correlated with the chemical composition of the product, its packaging, and the structure of the coating surface. Suitable markers of the foodstuffs contamination with the potentially carcinogenic PAHs are benzo(a)pyrene and the sum of the four polycyclic aromatic hydrocarbons (PAH4): benzo(a)pyrene (C₂₀H₁₂), benzo(a)anthracene (C₁₈H₁₂), benzo(b)fluoranthene (C₂₀H₁₂) and chrysene (C₁₈H₁₂). In this survey, 206 samples of meat and smoked meat products were analyzed in order to investigate the levels of the benzo(a)pyrene and PAH4. The High Performance Liquid Chromatography (HPLC) method was used for the qualitative analysis of foodstuff samples. The values obtained were between undetectable and 3.00±1.2 µg/kg for benzo(a)pyrene, and between undetectable and 18.01±7.48 µg/kg for the PAH4.

The highest values of the potentially carcinogenic PAHs were obtained in samples of smoked pastrami, smoked pork breast and smoked sausages for benzo(a)pyrene and in samples of smoked ham, smoked pastrami and smoked pork muscles for PAH4.

Keywords: PAHs, benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, chrysene

Hidrocarburile aromatice policiclice (HAP) sunt compuși organici cu potențial carcinogen pronunțat. Formarea HAP în alimente depinde de modalitățile de prelucrare termică, corelate și cu compoziția chimică a produsului, modul în care se ambalează și structura învelișului. Markerii adecvați pentru evaluarea contaminării produselor alimentare cu HAPi potențial carcinogene sunt benzo(a)pirenului și suma celor patru hidrocarburi aromatice policiclice (HAP4): benzo(a)piren (C₂₀H₁₂), benzo(a)antracen (C₁₈H₁₂), benzo(b)fluoranten (C₂₀H₁₂) și crisen (C₁₈H₁₂). În acest studiu au fost analizate 206 probe carne și produse din carne afumate pentru determinarea nivelurilor de benzo(a)piren și PAH4. Pentru analiza calitativă a probelor de produse alimentare a fost utilizată cromatografia lichidă de înaltă performanță (HPLC). Valorile obținute au fost cuprinse între nedetectabil și 3,00±1,2 µg/kg pentru benzo(a)piren și între nedetectabil și 18,01±7,48 pentru HAP4.

Cele mai mari valori ale HAP potențial carcinogene au fost obținute în probele de pastramă afumată, piept de porc afumat și cârnați afumați pentru benzo(a)piren și în probele de șuncă afumată, pastramă afumată și mușchi afumat pentru HAP4.

Cuvinte cheie: HAP, benzo(a)piren, benzo(a)antracen, benzo(b)fluoranten, crisen

Aromatic Polycyclic Hydrocarbons (PAHs) are a class of organic compounds containing two or more combined (condensed) aromatic nuclei. They include a number of carcinogenic and genotoxic substances, of which the most important are benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(i)pyrenelene, chrysene, cyclopenta(c,d)pyrene, dibenzo(a,h)anthracene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,l)pyrene, indeno(1,2,3-cd)pyrene and 5-methylcrisene (17).

PAHs have a pronounced carcinogenic potential, which has been described over the last 200 years. In 1775, for the first time, the English physicist Percival Pott highlighted a correlation between the incidence of scrotum cancer at chimney sweepers and continuous contact with PAH-rich ash (3).

The formation of PAHs in food depends on the thermal processing methods, correlated with the chemical composition of the product, the way it is packaged and the structure of the coating. In this regard, it should be noted that most food preparation processes are performed at temperatures between 370°C and 390°C but also at temperatures higher than 400-600°C (e.g., fat frying). At temperatures between

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400°C and 1000°C, the PAHs content increase linearly, and the phenols and fatty acids reach the maximum amount at 600°C. The smoking temperature, between 20°C and 55°C, does not significantly affect the production of PAHs, and the one of 40°C allows for superior flavors than those of 20°C or 55°C (11, 12).

In roasted meat (roasting with charcoal grilling, frying) PAHs are formed during the incomplete combustion process or by the pyrolysis of the fat when oxygen and carbon are released, which ultimately lead to the formation of polycyclic compounds, which given the resulting smoke is deposited on the meat. PAHs concentration in meat roasted at a higher distance from coal is lower than in meat roasted closer to coal. In order to avoid large amounts of PAHs, grilled meat must not be in contact with the flame, the process should last longer, and the fat should be as small as possible (9, 13). Another PAH-generating process is smoking, a measure of food preservation, which has been correlated with the increased incidence of pharyngeal cancer, observed in Icelandic fishermen, large smoked fish consumers, even if they were detected in low concentrations. Accumulation of PAHs in smoked foods depends on how they are smoked, the nature of the fuel, the temperature and duration of the smoking. Wood quality plays an important role, so pyrolysis of hardwood generates smoke poorer in PAHs than softwood containing resins (19, 20). PAHs are mainly formed from carbohydrates in food, at high temperatures and in the absence of oxygen. The contaminant marker is the benzo(a)pyrene and the sum of 4 PAH (Table 1).

Depending on the number of cores contained, the hydrocarbons can be divided into:

- Light PAHs (2-4 aromatic nuclei): chrysene; benzo(a)anthracene;
- Heavy PAHs (with 5 or more aromatic nuclei): benzo(a)pyrene; benzo(a)fluoranthene; dibenzo(a)-anthracene.

Benzo(a)pyrene is a polycyclic aromatic hydrocarbon found in coal tar. Its metabolites are mutagenic

and highly carcinogenic (classified as a Carcinogenic Group 1 by IARC), as a result of incomplete combustion at temperatures between 300-600°C, following the transformation of starch, amino acids and fatty acids (8, 10). The content is expressed in micrograms / kg ($\mu\text{g} / \text{Kg} = \text{ppb}$). The carcinogenic action of benzo(a)pyrene can be achieved by contact (dermal), but especially by ingestion. The maximum permitted level laid down in Regulation (EC) 1881/2006 for benzo(a)pyrene is 2.0 $\mu\text{g}/\text{kg}$ and for the sum of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene is 12.0 $\mu\text{g}/\text{kg}$ (5).

Annually, the National Sanitary Veterinary and Food Safety Authority establishes a sampling plan for both animal and non-animal food probes in order to monitor (screening and confirmation methods) benzo(a)pyrene in food and to prevent possible risks for smoked meat and smoked meat products, in processing units and marketing units (1).

Sampling of meat and smoked meat products aims to keep contaminants at acceptable levels, within the limits of admissibility, by applying and observing good manufacturing practices. In the case of PAHs, effective consumer health protection is ensured by eliminating from consumption meat products and smoked meat products not in conformity, consumed as such or used as a blend in other food products.

The aim of this survey was the investigation of the benzo(a)pyrene and PAH4 levels in various samples of meat and smoked meat products by using the High Performance Liquid Chromatography (HPLC) method, in 25 Romanian counties and Bucharest.

MATERIALS AND METHODS

Food samples and sampling method

A number of 206 samples (Table 2) of meat and smoked meat products, from 25 counties and Bucharest, were analyzed for the detection of PAH.

All samples were taken within the official control and self-control program (1).

Table 1

Chemical formula of Aromatic Polycyclic (2, 18)

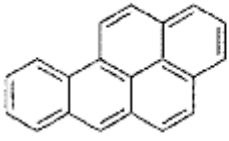
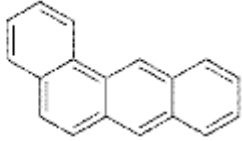
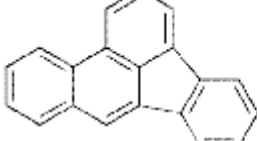
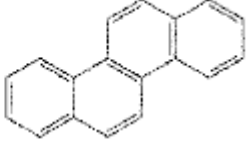
			
benzo(a)pyrene ($\text{C}_{20}\text{H}_{12}$)	benzo(a)anthracene ($\text{C}_{18}\text{H}_{12}$)	benzo(b)fluoranthene ($\text{C}_{20}\text{H}_{12}$)	chrysene ($\text{C}_{18}\text{H}_{12}$)

Table 2

Counties origin of food samples

Nr.crl.	County	Samples
1.	Alba	3
2.	Arad	3
3.	Argeş	12
4.	Botoşani	2
5.	Braşov	12
6.	Brăila	1
7.	Caraş Severin	1
8.	Covasna	20
9.	Cluj	14
10.	Dâmboviţa	2
11.	Dolj	2
12.	Harghita	3
13.	Ifov	10
14.	Maramureş	17
15.	Mureş	3
16.	Neamţ	2
17.	Olt	8
18.	Prahova	7
19.	Satu - Mare	9
20.	Sibiu	9
21.	Suceava	6
22.	Timiş	2
23.	Tulcea	9
24.	Vaslui	3
25.	Vrancea	22
26.	Bucharest city	24

The sampling was carried out in accordance with the provisions of EC Regulation 333/2007 (6). The overall sample was about 1 kg, unless this was not possible, for example, when the sample consisted of a package or a unit. The minimum number of incremental samples taken from the lot or subplot is presented in Table 3.

Table 3

Minimum number of incremental sample to be taken from batch or subplot

Lot / subplot weight or volume (in kg or liter)	Minimum number of incremental sample to be taken
< 50	3
≥ 50 and ≤ 500	5
> 500	10

If the batch or subplot was made up of separate packages or units, the number of packages or units to be sampled to form a bulk sample was established according to Table 4.

Table 4

Number of packages or units (incremental samples) to be taken to constitute the bulk sample if the lot or subplot was made up of separate packages or units

Packages or units in the lot/sublot	Number of packages or units sampled
≤ 25	At least 1 pack or 1 unit
26-100	Approximately 5%, at least 2 packages or units
> 100	Approximately 5%, maximum 10 packages or units

After sampling, each sample was placed in a clean and inert container (aluminum foil) which provides adequate protection against contamination, loss of adsorption substances on the inside of the container and damage during transport. The samples taken were sealed and coded, keeping records of each sampling.

High Performance Liquid Chromatography (HPLC)

The methods of analysis for the official control of the levels of Pb, Cd, Hg, 3-MCPD and PAHs in food-stuffs was carried out in accordance with the provisions of EC Regulation 333/2007 (6).

HPLC is a qualitative analysis method used in bio-chemistry and analytical chemistry for the separation, identification and quantification of compounds (14).

The method consists in the PAHs being extracted with the acetonitrile/acetone mixture followed by two purifications on the C18 reverse phase cartridge and on the florisil cartridge. After separation, liquid chromatography is determined, and fluorescence measured at different excitation and emission wavelengths, according to the standardized and optimized working method. In order to obtain replicable results, the ambient temperature in the laboratory was stable (≤ 20 °C), higher temperatures increase the solubility of short chain fatty acids. The detection program is presented in Table 5.

Table 5

Detection program

Component	Time min	Excitation wavelength nm	The emission wavelength nm
<i>Benzo (a)anthracene</i>	12.5	270	385
<i>Chrysene</i>	14.0		
<i>Benzo (b)fluoranthene</i>	21.8	256	446
<i>Benzo (a)pyrene</i>	31.2	292	410
<i>Dibenzo(a,h) anthracene</i>	44.1		

After the exact weighing of 2g of sample, 5 stages (0 - 4) were carried out for the analytical process followed by the actual determination (optimization of the apparatus, calibration of the calibration curve, reading, calculation and expression results).

Stage 0

The sample to be analyzed, chopped and well homogenized, was extracted with methanol and then with chloroform in two steps. The last bottom layer was collected in the same vial and then evaporated to dryness.

Stage 1

The sample to be analyzed was subjected to a three-stage Mixture 1 (acetonitrile and acetone) extraction, the supernatant (3 x 4 ml) being collected in the same vial, then evaporated to dryness. The fatty residue was thus obtained.

Stage 2

The fat residue was purified by successive extractions with the same Mixture 1 in three steps, the supernatant (3 x 2 ml) being collected in the same vial, and then purified on the C18 cartridge. Prior to switching to the cartridge, this was conditioned according to the manufacturer's operating instructions.

After passing elute, Mixture 1 was added to elute all the particles of interest. All the eluate thus obtained was evaporated to dryness. The resulting oily residue was treated with hexane and thus extracted with hexane, which was kept for 24 hours at -18 °C.

Stage 3

The hexane extract was subjected to successive extractions with Mixture 2 (hexane and dichloromethane) in three steps, the supernatant (3 x 2 ml) was collected in the same vial and then purified on the florisil cartridge. Prior to switching to the florisil cartridge, it was conditioned following the manufacturer's instructions. After passing the eluate, Mixture 2 was added to elute all the particles of interest.

Stage 4

All the eluate thus obtained was concentrated to 1 ml. It was then treated with 0.5 ml of toluene and evaporated to 50 µl, internal standard added and made up to the volume of interest (250 µl, 500 µl) with acetonitrile. Note that all of the fat residues were weighed and the evaporation to dryness was made with nitrogen in a water bath at 35°C.

The Determination

Optimizing the device

Before the determination was made, the Varian LC MS was opened, and optimization of the working parameters was done.

Drawing the standard curve

PAHs were prepared at concentrations of 1, 2, 3, 4, 5 ppb, with which the calibration curve was drawn. The curve shall be linear and pass through the origin, and the correction coefficient shall be at least 0.995.

Reading

After adjusting the device, the identification data of the samples to be analyzed was entered into the program. After the device was initialized, the samples were injected, following all the steps required by the machine's reading program. The reading was completed by the individual chromatogram for each sample read and by an analysis report, which identifies the concentration of each substance at the retention times specific to the PAHs analyzed. In the determination set was also read a fortified test at the maximum value allowed according to the legislation (5).

Calculation and Results

Before the calculation, the following aspects were pursued: the recovery percentage of the fortified sample to be in accordance with the regulations in force (16); retention times are appropriate for the analyzed PAHs; the internal standard (SI) area is the appropriate one; in the case of dilutions, take into account the dilution factor; issuing the analysis report and processing the data by correcting the value with the recovery ratio for benzo(a)pyrene and the PAH4, respectively (6).

RESULTS AND DISCUSSION

The results obtained from the laboratory examinations of meat and smoked meat products are presented in Table 6.

The recovery rate is the actual percentage of the concentration of a recovered substance during the analytical procedure. It is determined during validation if no certified reference material is available and it refers only to the benzo(a)pyrene, and not to the PAH4. For undetectable and non-quantifiable values, the recovery rate does not apply.

The obtained values were between undetectable and $3.00 \pm 1.2 \mu\text{g} / \text{kg}$ for benzo(a)pyrene in smoked pastrami and between undetectable and 18.01 ± 7.48 for PAH4 (corrected with recovery) in smoked ham, some of the maximum values being higher than the maximum permitted levels laid down in the European Regulation (5). After applying the correction index ($\pm$), finally the values were below the maximum acceptability levels.

Table 6

Results obtained from the analysis of samples of smoked meat and smoked meat products

	Matrix	Samples	Values		
			Benzo(a)pyrene $\mu\text{g/kg}$	Benzo(a)pyrene recovery %	PAH4 $\mu\text{g/kg}$
1.	Smoked bacon: traditional, frozen	4	Undetectable*	-	0.72 ± 0.31 - 2.43 ± 1.01
2.	Smoked sausages: homemade, traditional, country side, rose, from sheep and calf, Plescoi, semi-smoked extra, for grill, for grill spicy, smoked ham, bacon, garlic, for Christmas, „Carniprod”, „Stalder”, „Bătrănu Sas”, „Tătăran”, „Baroni”, „Montbellard”, „Udvarhelyi”, „Deak”, „Hajdu”.	56	Undetectable* Unquantifiable** - 2.5 ± 1.00	57.1 - 107.4	0.56 ± 0.23 - 11.08 ± 4.60
3.	Smoked pork neck	5	Undetectable* - 0.75 ± 0.30	73.6	0.45 ± 0.19 - 8.16 ± 3.39
4.	Smoked bone: pork, homemade, boned, halfboned	4	Undetectable* - Unquantifiable**	73.6	0.70 ± 0.29 - 2.31 ± 0.86
5.	Smoked sausages : from Cristur, with chicken breast	2	Undetectable* - 0.81 ± 0.32	73.6	3.43 ± 1.42
6.	Smoked pork ribs	9	Undetectable* - Unquantifiable**	85.8	0.57 ± 0.24 - 7.00 ± 2.91
7.	Smoked ribs	2	Undetectable* - 0.81 ± 0.32	73.6	3.57 ± 1.440 - 6.85 ± 2.84
8.	Smoked pork chops	4	Undetectable* - 0.87 ± 0.35	107.4	5.8932
9.	Smoked pigtales	1	1.677 ± 0.67	107.4	11.92 ± 4.95
10.	Pork jowl with pepper	1	Undetectable*	-	2.2512
11.	Smoked ham : Pădurea Neagră, with turkey breast	9	Undetectable* - 1.0490 ± 0.3429	70.7	0.25 ± 0.10 - 11.23 ± 4.66
12.	Smoked Kaizer: pork breast, extra.	9	Undetectable* - 2.584 ± 0.8448	70.7	0.47 ± 0.20 - 10.9200
13.	Smoked Leber	1	Undetectable*	-	1.76 ± 0.73
14.	Smoked pork tenderloin: file, raw smoked, smoked pork, „Azuga”, „Vilica” Delikatesa	16	Unquantifiable** - 0.97 ± 0.39	67.1 - 107.4	0.73 ± 0.30 - 13.8 ± 5.73
15.	Smoked garf bones	1	Unquantifiable**	-	1.8859
16.	Pancetta Affumicata	1	Undetectable*	-	1.62 ± 0.67
17.	Smoked pastrami: sheep, pork, Aurora, turkey breast.	17	Undetectable* Unquantifiable** - 3.00 ± 1.2	85.8 - 104.4	0.107 ± 0.04 - 15.65 ± 6.5
18.	Smoked pork legs	1	Undetectable*	-	Undetectable
19.	Hunter's smoked pork breast	5	Unquantifiable** Undetectable* - 0.82 ± 0.33	67.1	3.79 ± 1.57 - 7.26 ± 3.01
20.	Smoked pulp : pork Transylvanian	3	Undetectable* Unquantifiable** - 1.22 ± 0.49	70.7 - 95.2	0.90 ± 0.37 - 5.43 ± 2.26
21.	Smoked Salami: dry, Baranya, Deli, winter's, summer's, of Banat, Sibiu, peasant salami with ham, Selmont, Sinaia, raw smoked, Carniprod, Paprika, homemade, rustic, sax, French, Lunghetto, Brio, Victoria, București,	27	Undetectable* Unquantifiable**	69.1 - 107.4	Undetectable 0.48 ± 0.20 - 6.37 ± 2.65
22.	Smoked bacon	3	Undetectable* Unquantifiable**	67.1 - 107.4	0.48 ± 0.20 - 1.7369
23.	Pork Shoulder Bega	1	Undetectable*	-	1.4966 ± 0.6211
24.	Pork fillet specialty	1	Undetectable*	-	1.34 ± 0.56
25.	Smoked Ham: Pădurea Neagră, „Miska” from Deva, Șugatag, homemade, Transylvanian, raw	19	Undetectable* Unquantifiable** - 0.84 ± 0.34	85.8 - 99.1	2.31 ± 0.96 - 18.01 ± 7.48
26.	Smoked bacon: Transylvanian, traditional	5	Undetectable* Unquantifiable**	-	0.81 ± 0.34 - 6.58 ± 2.73
	TOTAL	206			

* Undetectable < LOD (Limit of detection, smallest measured content, from which it is possible to deduce the presence of the analyte with reasonable statistical certainty).

** Unquantifiable < LOQ (Limit of quantification, lowest content of the analyte which can be measured with reasonable statistical certainty).

In the case of the benzo(a)pyrene, the maximum value was 3.00 ± 1.2 g/kg for smoked pastrami, 2.584 ± 0.8448 for smoked pig breast, 2.5 ± 1.00 for smoked sausages, when the maximum allowable value is 2.0 μ g/kg. Values near the maximum allowable limit were also recorded in the smoked pork chest (2.584 ± 0.8448) and smoked pork loin (1.22 ± 0.49).

For the PAH₄, the maximum value was 18.01 ± 7.48 μ g/kg for smoked ham, 15.65 ± 6.5 for smoked pastrami and 13.8 ± 5.73 for smoked pork tenderloin, over the maximum admissible value of 12.0 μ g/kg. Values close to the maximum admissible limit were also recorded in smoked pigtails (11.92 ± 4.95), smoked ham (11.23 ± 4.66), smoked sausages (11.08 ± 4.60), smoked kaizer (10.92) and smoked pig head (8.16 ± 3.39).

The values obtained from other food products were lower for both parameters. Thus, it was found that, for benzo(a)pyrene, the lowest values were found in smoked leber, smoked garf bones, smoked bones, smoked bacon, smoked sausages, smoked legs, Bega backs, smoked pig fillet and some smoked salami varieties. Even if for these products and for others specified in Table 6 the obtained values are within the admissible limits, they have to be evaluated and controlled, and setting maximum levels for benzo(a)pyrene in certain foods, where the processes of smoking can cause high levels of contamination, are extremely important because they have the role of protecting public health. Various authors reported different levels of PAHs in various food products depending on their processing (charcoal, gas, oven grilling).

Farhadian *et al* (2010) found fluoranthene in all samples, with the highest concentration of total PAHs in beef satay (132 ng/g) and the lowest in oven grilled chicken (3.51 ng/g) (7). In the non-marinated meat (kebab, Kofta) the mean values of Anthracene, Fluranthene, Chrysene, Benzo(a)anthracene and Benzo(a)pyrene were 18.2 ± 11.2 ; 57 ± 28.59 ; 18.6 ± 1.69 ; 16.8 ± 7.0 and 9.2 ± 5.67 μ g / kg, respectively. The total concentration values of the existing PAHs in the examined non-marinated charcoal grilled Kebab and Kofta were 119.8 ± 54.15 and 59.2 ± 16.9 μ g/kg, respectively. Thus, marinating the meat prepared for charcoal grilling will greatly reduce the most hazardous carcinogenic PAHs compound such as benzo(a)pyrene resulting in more safe charcoal grilled meat for human consumption (4). In traditional products the PAH value is lower, with 32.46 μ g/kg benzo(a)pyrene values in smoked bacon and 15.49 μ g/kg in smoked sausage. The values are lower than twice their

value for traditionally smoked products than for commercially smoked products (15, 16).

CONCLUSION

In the case of benzo(a)pyrene, the maximum value was obtained from smoked pastrami, smoked pig chest and smoked sausages. Values close to the maximum allowable limit were recorded in smoked kaiser, smoked pork chest and smoked pulp. In the case of PAH₄, the maximum value was obtained from smoked ham, smoked pastrami and smoked tenderloins. Values close to the maximum admissible limit were also recorded in smoked pigtails, smoked ham, smoked sausages, smoked kaiser, and smoked pig neck. Values obtained from other food products were lower for both parameters. Thus, it was found that, for benzo(a)pyrene, the lowest values were found in smoked leber, smoked garf bones, smoked bones, smoked bacon, smoked sausages, smoked legs, Bega backs, smoked pig fillets and some smoked salami varieties. Even if the obtained values are within the limits of admissibility, meat and smoked meat products must be checked by laboratory tests, as smoking processes can lead to high levels of contamination with Polycyclic Aromatic Hydrocarbons, compounds with recognized carcinogenic potential.

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Article

The Influence of Different Cooking Techniques on the Biochemical, Microbiological, and Sensorial Profile of Fish-Based Catering Products

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Abstract: The present study aims to characterize five fish-based catering dishes, cooked by sous vide, by convection and microwaves in terms of their biochemical content, microbial load, and sensory analysis. The product cooked by using convection had the highest levels of MUFAs, PUFAs, and SFAs and the lowest levels of vitamin PP, riboflavin, and niacin. The sous vide maintained the highest levels of retinol, tocopherol, riboflavin, and niacin. Microwaves triggered the greatest decrease in vitamin content. All microbiological indicators exhibited levels below the acceptable limits, except for the level of fungi in the sous vide cooked product. Shelf life was estimated at 5 days for the product cooked by convection and immediately refrigerated and at 50 days for the product cooked by convection and immediately frozen. The most appreciated product from the sensory standpoint, which falls under fine dining, was the one cooked by convection and served immediately. The sous vide dish, the microwaved dish, and those refrigerated/frozen after cooking were undervalued.

Keywords: catering dishes; cook–chill; cook–freeze; cook–serve; sous vide



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1. Introduction

Nutrition is a vital issue for the sustainability of humankind. In the context of recent social and economic changes, in the aftermath of the SARS-CoV-2 pandemic, food service providers, including bars and restaurants, have diversified and intensified their catering services and widened the range of quality products that meet the needs of consumers [1–3].

Traditionally, in the hospitality industry, especially in fine dining, the taste and aesthetic criteria, such as the outer and inner aspects of the final product or the color harmony and intensity obtained using specific techniques or plating styles, have been taking precedence over other quality indicators [1]. Among other factors such as price, accessibility, or safety issues, the catering consumers' choice for such a service also depends on the type of dishes and their variety. From vegetables to meat and fish, there are so many kinds of foods with different nutritional value; nevertheless, meat and meat products play a key role in a well-balanced diet.

The nutritional quality of food supplied by the catering industry is of particular interest, as these foods may account for a significant proportion of daily food intake and even the total intake of the population [4]. Three main parameters influence the nutritional quality of food preparation: the choice of raw materials, the recipe and composition of a

meal, and the preparation process [4,5]. Considering the diverse cooking methods, it is necessary to explore the impact of such different methods on nutritional components in order to determine the differences between the calculated value and the measured value of nutritional components in catering food and to identify the correction factors for the effects of these cooking methods [4]. In catering systems, the temperature and time history during preparation and distribution, i.e., systems like cook–chill, cook–freeze, or warm–holding, require particular attention with regard to some sensitive nutrients, e.g., vitamins C and B1 and folic acid. If the main parameters and influencing factors as described are taken into account, it should not be difficult to produce food with high nutritional quality in catering.

Over the past decade, fish and seafood consumption has been increasing in the catering industry, especially in fine dining [6]. One of the reasons is that fish and seafood availability has become more diverse, ranging from wild-caught to farm-raised; moreover, their production volumes have increased significantly [7]. From a nutritional point of view, the biochemical composition of fish provides multiple much-needed substances in the human diet, such as omega-3 polyunsaturated fatty acids [8–10], especially eicosapentaenoic acid, EPA (C20:5 ω -3), docosapentaenoic acid, DPA (C22:5 ω -3), docosahexaenoic acid, and DHA (C22:6 ω -3) [11–13]. EPA, DPA, and DHA can prevent serious diseases/conditions such as high blood pressure, tumors, and brain function failure [11,12]. Omega-3 fatty acids, both n3-MUFAs (omega-3 monounsaturated fatty acids) and n3-PUFAs (omega-3 polyunsaturated fatty acids), support the functioning of the central nervous system. PUFAs are also involved in cell membranes' structural integrity and inflammatory responses [14]. Also, fish contain hydro-soluble vitamins, such as thiamine (B1) [15], riboflavin (B2) [16], niacin (B3 or PP) [15], cobalamin (B12) [17], and fat-soluble vitamins, such as retinol (A) [15], cholecalciferol (D3) [18], and tocopherol (E) [16].

To meet consumers' needs, catering companies need to diversify their techniques of processing raw materials. Food processing is usually correlated with changes in shape, sensory properties, and preservation capacity, different types of heat treatment, or the application of modern catering production systems modifying the structure of molecules, ensuring distinct physicochemical properties of the finished products and preventing nutrient losses [19]. Generally, catering production involves production systems that involve traditional cooking, namely, *cook–serve* (CS), *cook–chill* (CC), and *cook–freeze* (CF). In order to preserve the products' nutritional and sensory qualities while also ensuring food safety, sous vide technology is often employed for culinary dishes [20], including fish products [13].

Beyond the nutritional value of raw materials, for the catering industry, it is important to have a clear image of the nutritional advantages brought by different production systems and thermal treatments. The present study aims to conduct the characterization of fish-based catering products cooked using different techniques (sous vide cooking—cook–serve, convective cooking—cook–serve, microwave cooking—cook–serve, convective cooking—cook–chill, and convective cooking—cook–freeze), from a nutritive/biochemical point of view, as well as from a sensorial perspective, in a microbiological safety context. To establish the advantages of such techniques, the processing losses were analyzed, as well as vitamin retention, macronutrient values, correlated with the final product innocuity, and its sensorial profile.

2. Materials and Methods

2.1. Selected Fish Products and Applied Culinary Treatments

This study targeted five catering products derived from a fish-based dish, with each of them obtained with a different cooking technique. The differentiation consisted of both the heat treatment, where the behavior of the products and the transformations related to

convective baking (traditional method) and microwave baking (unconventional method) were analyzed, and the use of catering production systems. The behavior of the products was examined comparatively by applying not only the classic cook–serve production system but also the cook–chill and cook–freeze systems.

The fish dishes were presented as rolls, made of 42% fresh salmon fillets (*Salmo salar*) and 38% fresh carp fillets (*Cyprinus carpio*), with 13% vegetables (frozen carrots, fresh tomatoes, green parsley, and garlic), and 2% heavy cream and condiments (sunflower oil, paprika powder, salt, and white pepper). After quality control, ingredient dosing, and preliminary operations, the products were shaped into a cylindrical roll, obtained by overlapping the two types of fish with one another. The rolls were covered with breading made of finely chopped fish, mixed with brunoise chopped vegetables, heavy cream, and condiments.

The raw materials were purchased in a refrigerated state (2–4 °C), respecting the pre-set parameters of the cold chain and the company’s technical specifications. Culinary production was carried out in equipped, monitored production spaces, for which there is an integrated food quality and safety management system (QMS), certified according to ISO 9001:2015 [21] and ISO 22000:2018 [22]. The QMS ensures the safety of culinary products and superior, controlled quality (sensory, nutritional, technological, ecological, and symbolic value) [23].

The implemented hygiene programs ensured the sanitation of work surfaces, dishes, and utensils. Hygiene monitoring was carried out with a certified SystemSURE Plus device for measuring the level of surface contamination (Hygiena International, Watford, UK).

2.2. Experimental Concept and Sampling

The tested culinary product was then subjected to 5 different cooking methods, as presented in Table 1, namely: sous vide cooking—cook–serve, convective cooking—cook–serve, microwave cooking—cook–serve, convective cooking—cook–chill, and convective cooking—cook–freeze.

Table 1. Sample codes and applied cooking methods.

Applied Treatment	Sample Code	Cooking Method
None	A0	None; this sample was tested without applying any thermal treatment.
Sous vide cooking—cook–serve (SV-CS)	A1	The product was placed in a bag (Saporoso, Braşov, Romania) that was then vacuum sealed (SAM Cook, Milanówek, Poland) and cooked at 55 °C for 40 min in a sous vide cooker (Hendi, Ghimbav, Romania).
Convective cooking—cook–serve (CC-CS)	A2	In this cook–serve production system, convection baking (without steam) in an electrical oven (Rational, Luton, UK) was chosen. The product was baked at 170 °C for 25 min.
Microwave cooking—cook–serve (Mw-CS)	A3	The product was cooked in a microwave oven (Galanx, Foshan, China) for this cook–serve production system at 567 W for 15 min.
Convective cooking—cook–chill (CC-CC)	A4	In this <i>cook–chill</i> production system, convection baking (without steam) in an electrical oven (Rational, UK) was also chosen. The product was baked at 180 °C for 25 min. After cooking, the product was cooled in a controlled manner, in two stages: down to 20 °C in the first 60 min and down to 4 °C in the next 60 min, using a rapid blast chiller (Afinox, Campo San Martino, Italy).
Convective cooking—cook–freeze (CC-CF)	A5	In this <i>cook–freeze</i> production system, convection baking (without steam) in an electrical oven (Rational, UK) was also chosen. The product was baked at 180 °C for 25 min. After cooking, the product was cooled and then frozen, in a controlled manner, in three stages: down to 20 °C in the first 60 min, down to 4 °C in the following 60 min, and down to −18 °C in the last 60 min, using a rapid blast chiller (Afinox, Campo San Martino, Italy).

Sample Preparation

To limit physical, chemical, and microbiological transformations that could generate nutrient losses, the catering products were cooled down immediately to a temperature of +4 °C after thermal regeneration (which actually coincides with their consumption). Sampling was performed carefully, eliminating the possibility of contamination, and the samples were transported to the laboratory under safe and controlled conditions. The temperature during the samples' transport was maintained at +4 °C.

Sample A0 was taken on the day of processing, representing the non-thermally treated catering products, which underwent preliminary operations according to the product sheet. Samples A1, A2, and A3 were taken on the day of processing, thus representing the catering products for which the cook-serve catering production system had been used. The products were subjected to different thermal treatments, i.e., A2 was cooked in a convection oven, whereas A3 was cooked in the microwave oven. Sample A4 was taken 72 h after the processing day and stored at a temperature of +4 °C, thus representing a catering product made in the cook-chill system, thermally treated in the convection oven. The product was thermally regenerated up to a temperature of 73 °C and cooled down to +4 °C. In the physico-chemical examination, sample A4 was identical in terms of storage duration to samples A4-c, taken for the microbiological examination. Sample A5 was collected 20 days after the processing day, after storage at a temperature of −18 °C, representing catering products made in the cook-freeze system, thermally treated in the convection oven. The product was thermally regenerated to a temperature of 73 °C and cooled to +4 °C. In the physico-chemical examination, sample A5 was identical to sample A5-b, taken for the microbiological examination.

Before being subjected to nutrient content analyses, the final products were homogenized in the laboratory using a VO4006 blender (Vortex, Cesano Maderno, Italy).

2.3. Physico-Chemical Analyses

To perform the biochemical analysis, samples were taken from the homogenates corresponding to each cooking method. The following indicators were determined: dry matter content, major nutrients (carbohydrates, proteins, and lipids), total nitrogen content, non-protein nitrogen content, crude protein broken down by pepsin in hydrochloric acid, fatty acid content, and hydro-soluble (thiamine, riboflavin, and niacin) and fat-soluble (retinol, tocopherol, and F) vitamins.

The dry matter was determined through drying in a vacuum oven at 102 ± 2 °C, according to ISO 1442:2023 [24]. The result is expressed in DM% or DM (g/100 g of analyzed product).

The carbohydrate content was determined according to the specifications of AOAC 923.09 [25]. An alkaline solution of cupric salt is reduced under heat with the help of the reducing sugar from the sample to be analyzed. The excess copper sulfate is treated with potassium iodide in an acidic medium, and the released iodine is titrated with a sodium thiosulfate solution. The result is expressed in reducing sugar, %.

The lipid content was determined by means of the ether-hydrochloric extraction method in accordance with SR ISO 1443:2008 [26]. The protein substances and carbohydrates in the sample to be analyzed are hydrolyzed with hot hydrochloric acid, the insoluble part is separated via filtration, and the fat is extracted with ethyl alcohol in a Soxhlet installation.

In determining the protein content ($P = 6.25(NT - NPN)$), the Kjeldhal method was used, specifically by assessing the total nitrogen content (NT) (according to ISO 5983-1:2005 [27]), and the determination of non-protein nitrogen (NPN) was based on the method of protein precipitation with trichloroacetic acid (according to ISO 8968-4:2016 [28]), thus determining the non-protein nitrogen in the filtrate using the same method. To

evaluate the digestibility of proteins in the analyzed products, the content of crude protein decomposed with pepsin and hydrochloric acid solution was determined. The action of the enzyme (2 U/mg pepsin) on the sample heated for 48 h at 40 °C simulates the process of human protein digestion. The suspension was filtered, and the nitrogen content of the filtrate was established in accordance with the method for determining crude protein. Digestibility is expressed in %, as the ratio between the amount of digested protein and the initial amount of crude protein in the analyzed samples (in vitro digestibility (%)) = digestible protein/initial protein $\times$ 100) [29].

The fatty acid content was determined with gas chromatography, based on the separation of fatty acid methyl esters (according to ISO 12966-4:2015 [30]), using a Shimadzu GC-17A gas chromatograph equipped with a Chrompack capillary column with a length of 25 m and a diameter of 0.25 mm. The stationary phase (a polyethylene glycol derivative) was deposited inside the column as a thin film of 0.2 μm . A FID detector was used, and the mobile phase was helium of 99.9% purity. The operation parameters of the gas chromatograph were as follows: an injector and carrier temperature of 260 °C, a carrier gas flow rate of 2.0 mL/min, a 5 min plateau at the initial temperature of 70 °C, a temperature gradient of 4 °C/min, up to 235 °C, a split ratio of 1:28, and a total analysis time of 36.658 min. After the gas chromatograph reached the programmed parameters, 0.5 μL of hexane solution of fatty acid methyl esters was injected using a syringe. The retention times for short- and long-chain fatty acids had been recorded previously.

To measure the vitamin B1 content in the samples, each one was heat-treated with diluted sulfuric acid and subjected to enzymatic hydrolysis. The quantification of vitamin B1 in extracts is traditionally based on spectrofluorimetric detection (Cary-50 UV-VIS Spectrophotometer, Agilent, Santa Clara, USA) after an oxidation step to convert vitamin B1 to thiochrome. This method is similar to the AOAC Official Method 953.17 and the AACC Method 86-80. Thiamine is oxidized to thiochrome by potassium ferricyanide in a strongly alkaline solution. Thiochrome is then extracted into isobutyl alcohol, and its fluorescence is measured at 435 nm after excitation at 365 nm. Thiochrome fluorescence is directly proportional to the concentration of thiamine in the sample.

To extract vitamin B2 from the samples, hydrolysis with hydrochloric acid, enzymatic treatment, and then extraction in acetone were performed. The quantification of vitamin B2 in extracts is traditionally based on spectrofluorimetric detection (Cary-50 UV-VIS Spectrophotometer). This method is based on the automated AOAC Official Method 981.15 and is similar to the AACC Method 86-70. All methods use measurement of riboflavin's natural fluorescence under controlled pH conditions. Fluorescence is measured at 510 nm after excitation at 450 nm.

The quantification of vitamin B3 in samples is traditionally based on spectrofluorimetric detection (Cary-50 UV-VIS Spectrophotometer) and includes an initial extraction step in a 1:1 solution of sulfuric acid and water (through autoclaving at 121 °C for 45 min). This method is based on the automated AOAC Official Method 975.41 and is similar to the AACC Method 86-50A. Modifications have resulted in a safer method with equivalent responses from both niacin and niacinamide, eliminating the need for the conversion of niacinamide to niacin. Niacin and niacinamide react with cyanogen chloride created *in situ* to form an aldehyde intermediate. The subsequent addition of sulfanilic acid results in the formation of a polymethine dye absorbing light at 470 nm. The absorbance is directly proportional to the total niacin concentration.

For the determination of retinol, an RP-HPLC method was used with a UV detector ($\lambda = 325$ nm). The method, adapted from the European Commission Regulation 152/2009 [31], is based on the extraction of vitamin A in petroleum ether from the sample hydrolyzed with ethanolic potassium hydroxide solution. The solvent is removed via evaporation, and the residue is diluted in methanol and, if necessary, diluted to the required concentration. The chromatographic parameters are chosen so that there is no difference between all trans alcohol compounds of vitamin A and the cis isomers. Vitamin A was separated on a C18 reversed phase column, and an elution with the methanolic mobile phase was performed. The average peak height of several injections from the same sample solution and the average peak height of several injections of the calibration solutions were calculated.

Alpha tocopherol was analyzed using a Shimadzu VP Series liquid chromatograph with an FR-10 AXL fluorescence detector (Shimadzu, Kyoto, Japan), based on a method adapted from the European Commission Regulation 152/2009. An Alltima RP C-18 column (250 mm $\times$ 4.6 mm, 5 μ m) at 40 °C was used for chromatographic separation. The mobile phase was a mixture of methanol and acetonitrile (50:50, *v/v*) with a flow rate of 1.5 mL/min. For the detection of alpha tocopherol, a 290 nm excitation wavelength and a 325 nm emission wavelength were set on the detector.

2.4. Microbiological Analyses

The microbiological quality assessment was carried out in order to analyze the influence of the type of thermal treatment applied (sous vide, convection, and microwaves) on the main indicators. The samples prepared in the cook–chill and cook–freeze production systems were monitored throughout the minimum durability period. In the case of cook–chill products, samples were taken daily, over a 5-day period, and in the case of cook–freeze products, every 10 days, for 50 days. Before being subjected to microbiological evaluation (Table 2), the samples were ground and homogenized (Vortex, Italy).

Table 2. Microbiological indicators.

Microbiological Analyses		Analysis Methods
Quantitative Evaluation * of Microbiological Quality		
1.	Total Aerobic Microbial Count/ Aerobic Plate Count (APC)	Classic method, according to ISO 4833:2013 [32]
2.	Total Yeast and Mold Count TYMC	Classic method, according to ISO 21527-1:2008 [33]
3.	Total Coliform Count	Classic method, according to ISO 4831:2006 [34]
Quantitative evaluation * of pathogenic bacteria		
4.	<i>Staphylococcus aureus</i> count	Rapid TEMPO method (BioMerieux Industry, France).
5.	<i>Bacillus cereus</i> count	
6.	<i>Escherichia coli</i> count	
Qualitative evaluation ** of the presence/absence of pathogenic bacteria		
7.	<i>Salmonella</i> sp. detection	ISO 6579:2017 [35]
8.	<i>Listeria monocytogenes</i> detection	ISO 11290-1:2017 [36]

* With a VITEK 2 Compact system (Biomerieux, Craponne, France); ** with a Vidas Next Day rapid kit (BioMerieux, Craponne, France).

From the homogenates, samples were taken and subjected to quantitative and qualitative determinations using classical and rapid methods. The samples collected and individualized by serial number were sealed and sent immediately to the laboratory, where they were subjected to bacteriological investigations aimed at isolating, identifying, and quantifying the microorganisms, according to the standards presented in Table 2.

2.5. Sensory Analysis

The sensory characteristics of the fish dishes were evaluated by a panel of 30 assessors (60% female and 40% male, aged between 20 and 61 years) who had undergone professional training in the field of gastronomy. The panel evaluated the attributes of exterior appearance, interior appearance, consistency, odor, taste, and overall acceptability, scoring them on a linear hedonic scale of 9 points [37]. A score of 1 indicated extreme dislike, while 9 indicated maximum acceptability (extreme liking). The samples were coded to protect information on the production system and heat treatment method used so as not to influence the panelists' assessments (ISO 8589:2007 [38]). The dimensions, shape, and quantity were set to simulate a presentation mode as close as possible to the reality of serving systems in fine dining restaurants. Water was used as a palate cleanser.

The sensory tests were conducted in two stages. In the first stage, the sensory characteristics of a fish dish (A1:A5) were analyzed, influenced by the different thermal treatments (sous vide, convective baking, and microwave baking), as well as by the utilization of modern culinary production systems: cook-serve, cook-chill, and cook-freeze. In the subsequent stage, the sensory quality of products that had been enhanced by the application of specific fine dining working techniques was tested by the same panel. This process was applied to samples A1, A2, and A3, resulting in the preparation of new enhanced samples for sensory testing: OA1, OA2, and OA3.

2.6. Statistical Analysis

All experiments were conducted in triplicate, with the results expressed as the mean $\pm$ Standard Deviation (SD). The analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$) were employed to assess the differences in physicochemical and microbiological analyses. JASP Team software (2025), Version 0.19.3, was utilized for the statistical analysis [39].

The identification of patterns and relationships within complex datasets was facilitated by the implementation of a Cluster Analysis algorithm. In Random Forest clustering, data were segmented into multiple clusters, with each observation assigned to a single group [39]. The Random Forest algorithm was utilized in this clustering method, with the outcome variable 'y' set to NULL [40,41]. The frequency of observations ending up in the same leaf node is then analyzed, generating a proximity matrix that estimates the distance between observations.

3. Results and Discussions

3.1. Evaluation of the Nutrient Content of the Analyzed Fish Dishes

Thermal processing of foods can affect macronutrient content, the quality of some micronutrients, and the content of minerals in their composition. Cooking and baking can destroy some of the valuable nutrients in foods, such as PUFAs, proteins, and vitamins [15]. Table 3 offers an overview of the nutrient content of the fish dishes.

Table 3. Cooking losses and macronutrient and vitamin content.

		A0	A1	A2	A3	A4	A5
Content loss, %		-	14.25 ± 1.57 ^a	30.91 ± 2.01 ^b	28.55 ± 1.23 ^b	35.68 ± 2.32 ^c	40.25 ± 1.57 ^d
Dry substance, %		30.98 ± 0.02 ^a	31.84 ± 0.04 ^a	31.47 ± 0.38 ^a	31.40 ± 0.08 ^a	33.57 ± 0.04 ^b	33.86 ± 0.04 ^b
Proteins, %		14.32 ± 0.02 ^a	15.22 ± 0.09 ^a	16.66 ± 0.12 ^b	15.90 ± 0.16 ^a	16.93 ± 0.21 ^c	17.36 ± 0.03 ^c
Insoluble proteins, %		4.37 ± 0.01 ^a	1.53 ± 0.05 ^b	1.53 ± 0.05 ^b	1.78 ± 0.02 ^c	2.15 ± 0.04 ^c	1.58 ± 0.02 ^b
Digestibility, %		69.51 ^a	89.91	89.91 ± 0.03 ^c	89.31 ± 0.20 ^c	86.48 ± 0.11 ^b	90.67 ± 0.21 ^c
Carbohydrates, %		0.73 ± 0.02 ^a	1.10 ± 0.04 ^b	1.05 ± 0.04 ^b	1.93 ± 0.05 ^c	1.23 ± 0.05 ^b	1.25 ± 0.01 ^b
Lipids, %		15.01 ± 0.09 ^a	14.2 ± 0.08 ^b	13.64 ± 0.06 ^c	13.93 ± 0.05 ^c	14.71 ± 0.09 ^b	14.81 ± 0.01 ^b
Hydro-soluble vitamins							
Thiamine	mg	0.0478 ± 0.01 ^a	0.0347 ± 0.01 ^b	0.0256 ± 0.01 ^c	0.0289 ± 0.01 ^c	0.0183 ± 0.01 ^d	0.0159 ± 0.01 ^d
	TR%	100 ± 0.01 ^a	72.51 ± 0.01 ^b	55.32 ± 0.01 ^c	63.08 ± 0.01 ^c	41.49 ± 0.01 ^d	36.45 ± 0.01 ^d
Riboflavin	mg	0.0465 ± 0.01 ^a	0.0333 ± 0.01 ^b	0.0257 ± 0.01 ^c	0.0275 ± 0.01 ^c	0.0193 ± 0.02 ^c	0.02 ± 0.01 ^c
	TR%	100 ± 0.01 ^a	71.67 ± 0.01 ^b	55.33 ± 0.01 ^c	59.20 ± 0.01 ^c	41.57 ± 0.01 ^c	34.69 ± 0.01 ^c
Niacin	mg	5.24 ± 0.06 ^a	3.22 ± 0.04 ^b	2.05 ± 0.04 ^c	2.13 ± 0.01 ^c	1.23 ± 0.01 ^d	1.14 ± 0.05 ^d
	TR%	100 ± 0.01 ^a	61.49 ± 0.04 ^b	39.21 ± 0.04 ^c	40.74 ± 0.01 ^c	23.42 ± 0.01 ^d	21.77 ± 0.01 ^d
Fat-soluble vitamins							
Retinol	mg	0.3411 ± 0.02 ^a	0.2123 ± 0.01 ^b	0.1251 ± 0.01 ^c	0.1148 ± 0.01 ^c	0.0941 ± 0.01 ^c	0.0917 ± 0.01 ^c
	TR%	100 ± 0.01 ^a	62.24 ± 0.01 ^b	37.89 ± 0.02 ^c	34.10 ± 0.01 ^c	29.85 ± 0.01 ^c	29.40 ± 0.01 ^c
α-Tocopherol	mg	0.62 ± 0.02 ^a	0.32 ± 0.02 ^b	0.26 ± 0.03 ^c	0.22 ± 0.02 ^c	0.21 ± 0.02 ^c	0.12 ± 0.04 ^d
	TR%	100 ± 0.01 ^a	51.61 ± 0.02 ^b	43.33 ± 0.01 ^c	35.95 ± 0.01 ^c	36.65 ± 0.01 ^c	21.17 ± 0.01 ^d

Nutrient content values, dry substance, digestibility, and content loss are expressed as percentages of the total mass of each product/sample. The results are expressed as the mean value of the three replicates ± the standard deviation (SD); different letters in superscript on the same line indicate significant differences (Tukey's test, $p \leq 0.05$). TR% = true retention of vitamins after cooking treatments.

3.1.1. Evaluation of Processing Losses of the Analyzed Products

Food loss and waste, including cooking loss, are crucial factors that influence the catering sector because they can generate financial losses, depending on the selected cooking process [42]. Cooking loss is of special interest in the catering sector because of the changes in the shape, color, and texture of the product [43,44]. Cooking loss is defined as a mixture of liquids and solids removed from the fish during cooking and depends on several factors: humidity, pressure, temperature and cooking time, ventilation and the heat distribution system, muscle fiber composition, and connective tissue [45]. Cooking losses are calculated based on the weight of the fish sample before and after heat treatment, as water comes out of the product in the form of vapor and liquid [46]. %Cooking loss = $100 \times (W1 - W2)/W1$, where W1 is represented by the initial weight of the samples before cooking and W2 is the weight of the cooked samples [47].

In the case of the cook-serve production system, the highest quantitative losses were recorded for sample A2 (microwave cooking, 28.55 ± 1.23%) and sample A3 (convective cooking, 30.91 ± 2.01%), results that are not significantly different ($p > 0.05$). The lowest rate of cooking loss was observed in the sous vide system (14.25 ± 1.57%), which is represented by the liquid extracted from the product through the vacuum created in the special packaging bag. This result was found to be significantly different as compared to all of the other samples ($p < 0.05$). Through the water removed from the product, in the liquid state, significant amounts of lipids and fat-soluble compounds, soluble proteins, vitamins, minerals, etc., are lost, with the possibility of reintegrating this by-product into other culinary preparations. The two-stage cooling applied to sample A4 (CC-CC) resulted in a cooking loss of 35.68 ± 2.32%, and the three-stage cooling applied to sample A5 (CC-CF) with a temperature of −18 °C

resulted in a cooking loss of $40.25 \pm 1.57\%$. Moisture losses individualize production yields and specific raw material consumption with a direct impact on economic efficiency. Cook–chill production systems, but especially the cook–freeze ones, are consumers of both material and energy resources, so in order to obtain expected economic results, efficient cost management will have to be applied that correlates production expenses with orders and sales.

3.1.2. Evaluation of Macronutrient Content

According to the working protocol, three determinations were performed, with a coefficient of variation of reproducibility of 3.85 ($V_{Cr} \% = 3.85$) and a measurement uncertainty of 7.35 ($M\% = 7.35$). The preparation selected for evaluation in relation to different types of heat treatment or processing is a product with high protein content of approximately $14.32 \pm 0.02\%$. Starting from a protein digestibility of 69.51% in sample A0 (raw), the most useful type of heat treatment is sous vide, where the digestibility increased to 89.91%. Starting from a protein digestibility of 69.51% in sample A0 (raw), the lowest digestibility for all dishes was determined for sample A3 (Mw-CS, 86.91%), with the results being significantly different ($p < 0.05$). The results were not significantly different ($p > 0.05$) for samples A1 (SV-CS, 89.91%), A2 (CC-CS, 89.31%), and A4 (CC-CC, 90.67%). It was also found that the digestibility of sample A5 (CC-CF) was 91.74%, a result significantly different from the other samples ($p < 0.05$).

The authors of a similar study [47] concluded that thermal processes such as sous vide can induce favorable changes, such as partial unfolding or exposure of cleavage sites, in muscle proteins and improve their digestibility, whereas processes such as stewing and roasting can induce unfavorable changes, such as protein aggregation, severe oxidation, cross linking, or increased disulfide (S-S) content and decrease the susceptibility of proteins during gastrointestinal digestion. In another study [29], following treatments such as pulsed electric field, high pressure, and ultrasound, conformational and microstructural changes in proteins were found to lead to the unfolding of polypeptides and the exposure of active sites for further interactions. These changes can increase the accessibility of digestive proteases to cleavage sites. Some of these technologies can inactivate some egg proteins that are enzyme inhibitors, such as trypsin inhibitors. Sous vide is recommended to be used in order to develop new fine dining dishes with increased protein bioaccessibility.

The carbohydrate content did not undergo major changes during the treatments applied, with the observed differences being an increase of less than 1%. A0 had a sugar content of 0.73% (w/w). A1 (sous vide cooked) had a sugar content of 1.1% (w/w), A2 (convection cooked) contained 1.05% (w/w) sugars, and A3 (microwaved) contained 1.93% (w/w) sugars. The CC-CC system, A4, contained 1.23% (w/w) sugars, and the CC-CF system, A5, contained 1.25% (w/w) sugars. The raw fish dish (A0) did not have a high carbohydrate content—0.73% (w/w). Compared to the raw sample, the different values of carbohydrate content in the other samples were due to the solubility taken up by the liquid collected in the baking containers. The carbohydrate content was not significantly different for samples A1 (SV-CS, 1.1% (w/w)), A2 (CC-CS, 1.05% (w/w)), A4 (CC-CC, 1.23% (w/w)), and A5 (CC-CF, 1.25% (w/w)) ($p > 0.05$). The carbohydrate content was significantly different for sample A3 (Mw-CS, 1.90% (w/w)) ($p < 0.05$), given the rapid heating of the dish and the evaporation of water during this time.

Culinary dishes, rich in saturated and monounsaturated fats, are very resistant to heat. In the analyzed samples, the loss of lipid content ranged between 6% and 9%, depending on the applied cooking system (Table 3 shows the lipid content of all of the analyzed samples). The most protective system was the sous vide (A1) system with a 6% loss, and a 9% loss was determined for sample A3, treated with microwaves. Lipids and lipid-containing foods are particularly sensitive to microwave heating since the specific heat of lipids is low and, therefore, they are heated quickly. Microwave heating mainly favors lipid oxidation but can also cause lipolysis and polymerization. This cooking method

can have a different impact on lipid oxidation depending on the treatment conditions used (power, temperature, and time). This analysis provides an overview of the main degradation effects of microwave heating on vegetable oils and lipid-containing foods, with emphasis on the oxidation of fatty acids and cholesterol [48].

In the case of the control sample (A0), the content of SFAs (30.42%) was lower than MUFAs (34.46%) but higher than PUFAs (12.57%), which indicates high of unsaturated fatty acid content (Table 4), thus determining a high degree of risk in terms of negative transformations that it could suffer due to oxidation. The culinary techniques applied in fine dining to this category of preparations involve preliminary operations with exposure to temperature fluctuations, slicing—portioning—forming, which implies the presence of light and oxygen. These factors directly influence the stability of PUFAs and MUFAs, among other factors: pH, natural antioxidant content, the freshness of the raw material, conditions, and storage time after heat treatment (CC-CC and CC-CF).

Table 4. Fatty acid content.

Fatty Acids (%)	A0	A1	A2	A3
C6:0; Caproic	7.59 ± 0.01 ^a	2.18 ± 0.01 ^b	2.29 ± 0.01 ^b	0.42 ± 0.01 ^c
C8:0; Caprylic	0.2 ± 0.04 ^a	0.21 ± 0.01 ^a	0.22 ± 0.01 ^a	0.34 ± 0.03 ^b
C10:0; Capric	0.23 ± 0.01 ^a	0.22 ± 0.01 ^a	0.22 ± 0.01 ^a	0.33 ± 0.01 ^b
C11:0; Undecanoic	n.d.	n.d.	n.d.	n.d.
C12:0; Lauric	2.12 ± 0.04 ^a	2.24 ± 0.02 ^b	2.49 ± 0.01 ^c	3.49 ± 0.02 ^d
C13:0; Tridecanoic	n.d.	n.d.	n.d.	n.d.
C14:0; Myristic	3.18 ± 0.02 ^a	3.1 ± 0.03 ^a	3.05 ± 0.01 ^b	3.14 ± 0.02 ^a
C14:1; Myristoleic	0.16 ± 0.01	n.d.	n.d.	n.d.
C15:0; Pentadecanoic	0.22 ± 0.02 ^a	0.18 ± 0.01 ^b	0.18 ± 0.01 ^b	0.18 ± 0.01 ^b
C15:1, <i>cis</i> -10; Pentadecanoic	n.d.	0.07 ± 0.01 ^a	0.07 ± 0.01 ^a	n.d.
C16:0; Palmitic	12.5 ± 0.87 ^a	13.12 ± 0.01 ^b	14.18 ± 0.02 ^c	14.08 ± 0.02 ^c
C16:1; Palmitoleic	3.85 ± 0.09 ^a	4.08 ± 0.01 ^b	4.19 ± 0.01 ^b	4.01 ± 0.01 ^{ab}
C17:0; Heptadecenoic	0.23 ± 0.01 ^a	n.d.	n.d.	n.d.
C17:1, <i>cis</i> -10; Heptadecenoic	n.d.	n.d.	n.d.	n.d.
C18:0; Stearic	3.93 ± 0.01 ^a	4.64 ± 0.01 ^b	4.88 ± 0.02 ^b	5.48 ± 0.02 ^c
C18:1, <i>cis</i> -9; Oleic	25.65 ± 0.09 ^a	28.82 ± 0.02 ^b	30.05 ± 0.02 ^c	29.1 ± 0.09 ^b
C18:1 iso, <i>trans</i> -9; Elaidic	2.17 ± 0.01 ^a	2.18 ± 0.01 ^a	2.21 ± 0.01 ^b	2.18 ± 0.01 ^a
C18:2, <i>cis</i> -9, <i>cis</i> -12; Linoleic (n-6)	8.27 ± 0.01 ^a	9.04 ± 0.01 ^b	9.11 ± 0.01 ^c	8.7 ± 0.02 ^b
C18:3, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15; Linolenic (n-3)	2.14 ± 0.01 ^a	1.96 ± 0.01 ^a	2.02 ± 0.01 ^a	1.76 ± 0.01 ^b
C20:0; Arahidic	0.22 ± 0.01 ^a	4.1 ± 0.01 ^b	6.62 ± 0.03 ^c	4.27 ± 0.03 ^b
C20:1, <i>cis</i> -11; Eicosenoic	2.63 ± 0.01 ^a	2.86 ± 0.01 ^a	2.76 ± 0.02 ^a	2.24 ± 0.01 ^b
C20:2, <i>cis</i> -11, <i>cis</i> -14; Eicosadienoic (n-6)	2.16 ± 0.01 ^a	3.21 ± 0.01 ^b	3.07 ± 0.02 ^b	3.22 ± 0.03 ^b
S.F.A.	30.42 ± 0.77 ^a	29.99 ± 0.05 ^a	34.13 ± 0.05 ^b	31.73 ± 0.03 ^a
M.U.F.A.	34.46 ± 0.16 ^a	37.94 ± 0.03 ^b	39.21 ± 0.02 ^c	37.53 ± 0.08 ^b
P.U.F.A.	12.57 ± 0.02 ^a	14.21 ± 0.02 ^b	14.2 ± 0.03 ^b	13.68 ± 0.16 ^c
U.F.A./S.F.A	1.55 ± 0.03 ^a	1.74 ± 0.01 ^b	1.56 ± 0.01 ^a	1.61 ± 0.01 ^a
P.U.F.A./S.F.A	0.41 ± 0.01 ^a	0.47 ± 0.01 ^a	0.42 ± 0.01 ^a	0.43 ± 0.01 ^a
n-6/n-3	4.87 ± 0.01 ^a	6.25 ± 0.01 ^b	6.03 ± 0.01 ^b	6.77 ± 0.11 ^c

SFAs—saturated fatty acids; MUFAs—monounsaturated fatty acids; PUFAs—polyunsaturated fatty acids; UFAs—unsaturated fatty acids. All values are expressed as percentages of fatty acids out of the total fatty acid content. The results are expressed as the mean value of the three replicates ± the Standard Deviation (SD); different letters in superscript on the same line indicate significant differences (Tukey's test, $p \leq 0.05$).

In the control sample (A0), undecanoic (C11:0), tridecanoic (C13:0), cis 10-pentadecanoic (C15:2) and cis-10-heptadecenoic (C17:1) were not detected. Among the MUFAs, oleic acid (C18:1) represented 25.56% of the total fatty acids, and its isomer C18:1-iso (2.17%). Palmitoleic acid was determined to be in a proportion of 3.85%, and cis 11-eicosenoic acid amounted to 2.63%.

The most representative PUFAs in the A0 product were as follows: linoleic acid (8.27%), linolenic acid (2.14%), and cis-11,13-eicosadienoic acid (2.16%). The fatty acids caproic (7.59%), caprylic (0.2%), and capric (0.23%) detected in the fish dishes come from the heavy cream used as raw material, according to the product standard. These saturated, short-chain fatty acids are specific to dairy products.

Sous vide, of all of the thermal treatments applied, best protected the PUFA content, being the most balanced preparation in terms of the PUFA/SFA ratio (0.47), respectively the n-6/n3 ratio (6.25). The lack of oxygen and maintaining the vacuum inside the package during the thermal treatment contributed to maintaining the content of essential fatty acids in sample A1. From the category of ω -3 fatty acids, the analyzed samples contain only linolenic acid, determining a content between 0.19–0.61 g/portion.

3.1.3. Evaluation of Water-Soluble and Fat-Soluble Vitamin Content

Contrary to widespread opinions [49], which nevertheless led to a real “trend” in dietary nutrition—“food without fire”—most vitamins are not very sensitive to heat [50]. The vitamin content of a culinary dish can be ensured by efficiently associated raw materials but also optimized by the correct choice of culinary techniques and production systems that lead to superior vitamin retention. The heat treatment applied to culinary products is a decisive factor in preserving the native vitamin potential.

From the data analysis, it results that the most protective production system for riboflavin, used in obtaining fine dining products from fish, is the sous vide (A1 – TR = 71.67%) and microwave cooking (A3 – TR = 59.20%). Riboflavin losses are influenced by the rapid cooling/freezing treatments and storage times. After convective cooking, in sample A2, a loss of vitamin B2 content of 45% was determined, and after 72 h under refrigeration and thermal regeneration conditions, the loss is 58.5%. In the case of frozen and regenerated products, after 20 days, the loss is approximately 65.3%.

Although it is a relatively stable vitamin to thermal treatment, the literature indicates considerable losses of riboflavin during culinary preparation [51]. Thus, starting with the washing of raw materials, it was found that the first losses of riboflavin occur. By grinding raw materials, vitamin B2 losses can reach up to 7% of the existing content [52]. The type of heat treatment applied can influence riboflavin losses differently, the most unfavorable way of heat processing being frying, with losses of up to 30%. Baking at low temperatures, on the other hand, decreases the riboflavin content by very little. Riboflavin is photosensitive, so the processes that take place in light act negatively, transforming it into lumiflavin or lumichrome without vitamin activity. Significant losses occur during storage; even during short-term storage, the lack of adequate protection measures leads to its reduction [53].

Böhm et al. [54] and Kala & Prakash [55] show in similar studies on microwaved products that a possible cause of the decrease in riboflavin content in microwaved products is the fat content of the raw materials. They show that there is a direct link between the fat content and the level of riboflavin retention: the higher the fat content of the product, the lower the riboflavin retention in the microwaved product than in the conventionally cooked product, since it is a water-soluble vitamin and the high fat content of a preparation is equivalent to a low water content in the product. Sterilization, the basic method used to preserve food products, due to the harshness of the heat treatment, causes multiple reductions in vitamin content, including riboflavin. For example, in a study conducted by

Rickman et al. [56], when sterilizing canned vegetables in water, the losses of riboflavin are: in sweet corn 58.3%, mushrooms 45.6%, green beans 63.6%, and Parisian carrots 60%. These products are raw materials in the manufacturing process of catering products, raw materials that participate with a large deficit of riboflavin. Freezing is also a widely used method of food preservation, which, unlike sterilization, has the advantage of being associated with three times lower riboflavin losses. Some studies, such as the one of Awonorin et al. [57] confirm that in the case of frozen meat, the reduction in vitamin B2 content during storage is relatively small, the losses depending on the fat content and the species. When defrosting meat, in addition to the losses of major nutrients, quantities of water-soluble vitamins are also lost, losses which are much more intense when defrosting is done in water than in air thawing.

Thus, for products made in the SV system, the real retention of vitamin PP reported to the content of the non-thermally treated preparation is 61.49%, compared to the retention determined for the sample thermally treated with microwaves ($A3 - TR = 40.74\%$), respectively with the one thermally treated by convective baking ($A2 - TR = 39.21\%$). In the case of products made in the cook–chill and cook–freeze systems, the real retentions were approximately 20%.

Temperature, light, and contact with oxygen negatively influence the retention of retinol in the analyzed culinary preparations. Vitamin A undergoes oxidative decomposition under the influence of light, due to the cleavage of the double bond in the cycle and thus, the opening of the β -ionic cycle. Not only retinol in preparations is affected by processing (temperature, light) but also carotene. It undergoes an isomerization process, the trans form passing into the cis form with a reduction in vitamin activity. However, from the analysis of the results, it is found that microwave treatment is less protective of vitamin A ($A3 - TR = 30.20\%$). In the case of the sous vide system, the real retention is 62.24% and in the case of the CC-CS system it is 37.89%). In the case of products made in the cook–chill and cook–freeze systems, the real retentions were approximately 30%.

From the data analysis, significant losses of α -tocopherol were found as compared to the content of the control samples ($A0$). It was found that the most protective system is the S-V ($A1 - TR = 51.61\%$), followed by the CC-CS ($A2 - TR = 43.33\%$) and the Mw-CS ($A3 - TR = 35.95\%$). In the case of the CC-CF, also influenced by the storage duration (20 days), the real retention of α -tocopherol is approximately 20%. It was observed that the α -tocopherol content was affected by the catering processing compared to the raw sample ($A0$), with the true retentions being significantly different ($p < 0.05$) and influenced by the type of heat treatment. Thus, the most protective catering system was found to be the sous vide treatment ($A1$, $TR = 51.61\%$). No significant difference ($p > 0.05$) was observed for samples $A2$ (CC-CS, $TR = 43.33\%$), $A3$ (Mw-CS, $TR = 35.95\%$), and $A4$ (CC-CC, $TR = 36.65\%$). It was also observed that the real retention for $A5$ (CC-CF, $TR = 21.17$) was significantly different from the other heat-treated samples, being influenced by the long storage time ($p < 0.05$).

A similar study conducted on the effect of traditional and microwave heat treatment on nutrient retention in some traditional Oceanian culinary preparations (based on chicken, lamb, fish, tapioca and palusami) carried out by Kumar & Aalbersberg [58], shows a retinol retention of 91% in microwaved products and 20% in products cooked traditionally in clay pots, a niacin retention of 95% in microwaved products and 63% in traditionally heat treated products. Other studies, conducted by Gentry & Roberts [59] and Roberts et al. [60], also argue for a higher retention of micronutrients in microwaved culinary preparations compared to conventionally cooked preparations.

3.2. Microbiological Analysis

The development of microorganisms in catering products is influenced by the chemical composition of the product, the type of catering processing, the technological parameters, especially the heat treatment parameters, and also the biological relationships and relationships between different microorganisms.

All samples were analyzed in terms of innocuity by determining their microbiological load, and the results are compiled in Table 5, which provides an overall picture of the microbiological quality.

Table 5. Microbiological quality of the fish dishes.

Sample	APC (CFU/g $\pm$ SD)	TYMC (CFU/g $\pm$ SD)	Coliforms (CFU/g $\pm$ SD)	<i>E. coli</i> (CFU/g $\pm$ SD)	<i>Salmonella</i> sp. (CFU/25 g)	<i>Listeria</i> sp. (CFU/25 g)
A0	$(1.037 \pm 0.071) \times 10^4$ ^a	$(2.69 \pm 0.11) \times 10^2$ ^a	$(2.6 \pm 0.11) \times 10^1$ ^a	7 ± 0.58 ^a	nd	nd
A1	$(4.27 \pm 0.10) \times 10^3$ ^b	$(6.14 \pm 0.04) \times 10^{1b}$	$(1.67 \pm 0.09) \times 10^1$ ^b	4.67 ± 0.94 ^b	nd	nd
A2	$(4 \pm 0.01) \times 10^3$ ^b	$(6.1 \pm 0.32) \times 10^1$ ^b	$(1.1 \pm 0.11) \times 10^1$ ^b	3 ± 0.58 ^b	nd	nd
A3	$(9.83 \pm 0.76) \times 10^2$ ^c	$(4.7 \pm 0.15) \times 10^1$ ^c	3 ± 0.15 ^c	nd	nd	nd
A4	$(5.83 \pm 0.29) \times 10^3$ ^d	$(6.2 \pm 0.67) \times 10^1$ ^b	4 ± 0.8 ^c	nd	nd	nd
A4-a (day 1)	$(5.67 \pm 0.29) \times 10^3$ ^d	$(7 \pm 0.25) \times 10^1$ ^d	2 ± 0.58 ^c	nd	nd	nd
A4-b (day 2)	$(6.50 \pm 0.5) \times 10^3$ ^e	$(7 \pm 0.38) \times 10^1$ ^d	1 ± 0.58 ^c	nd	nd	nd
A4-c (day 3)	$(6.67 \pm 0.58) \times 10^3$ ^e	$(8.2 \pm 0.29) \times 10^1$ ^e	1 ± 0.58 ^c	nd	nd	nd
A4-d (day 4)	$(7 \pm 0.5) \times 10^3$ ^f	$(8.7 \pm 0.58) \times 10^1$ ^e	1 ± 0.58 ^c	nd	nd	nd
A4-e (day 5)	$(6.83 \pm 0.29) \times 10^3$ ^e	$(1.07 \pm 0.06) \times 10^2$ ^f	1 ± 0.58 ^c	nd	nd	nd
A5	$(4.97 \pm 0.15) \times 10^3$ ^b	$(2.4 \pm 0.2) \times 10^1$ ^g	3 ± 1.15 ^c	nd	nd	nd
A5-a (day 10)	$(4.63 \pm 0.12) \times 10^3$ ^b	$(2.3 \pm 0.16) \times 10^1$ ^g	2 ± 0.58 ^c	nd	nd	nd
A5-b (day 20)	$(4.5 \pm 0.01) \times 10^3$ ^b	$(2.1 \pm 0.16) \times 10^1$ ^g	3 ± 1.15 ^c	nd	nd	nd
A5-c (day 30)	$(4.17 \pm 0.29) \times 10^3$ ^b	$(2.1 \pm 0.16) \times 10^1$ ^g	2 ± 0.58 ^c	nd	nd	nd
A5-d (day 40)	$(4.5 \pm 0.01) \times 10^3$ ^b	$(2.2 \pm 0.2) \times 10^1$ ^g	1 ± 0.58 ^c	nd	nd	nd
A5-c (day 50)	$(5.13 \pm 0.23) \times 10^3$ ^d	$(2.0 \pm 0.2) \times 10^1$ ^g	1 ± 0.58 ^c	nd	nd	nd

The results are expressed as the mean value of the three replicates $\pm$ the standard deviation (SD); different letters in superscript within the same column indicate significant differences (Tukey's test, $p \leq 0.05$).

It is preferred that certain pathogens are not present in fish to avoid the use of antibiotics since the continued use of antibiotics against them can also lead to bacterial resistance to antibiotics, which may also affect human health [61]. According to EC Regulation 2073/2005 [62], the maximum accepted limits for the microbial load of heat-treated catering products are as follows: Aerobic Plate Count of 10^4 CFU/g, 10 CFU/g Total Yeasts and Molds Count, 1 CFU/25 g *S. aureus*, 1 CFU/25 g *B. cereus*, absent *E. coli* and absent in 25 g for *L. monocytogenes* and *Salmonella* sp. In the tested samples, pathogenic bacteria *B. cereus*, *S. aureus*, *Salmonella* sp., and *L. monocytogenes* were all absent.

3.2.1. Aerobic Plate Count (APC)

In the raw sample (A0), the APC was 4.02 log CFU/g, approximately 10 times lower than the maximum limit allowed by the legislation in force in Romania (5 log CFU/g, corresponding to 10^5 CFU/g). Malak et al. [63] compared the microbial load of five fish species and determined that the common carp and the grass carp had the highest values of APC (5.44 log CFU/g and 5.51 log CFU/g, respectively). Our analysis showed a lower APC, even if A0 contained fillets from two fish species and other ingredients. Their coliform counts were 1.66 log CFU and 1.93 log CFU/g, respectively, which is higher than our results.

In the sous vide cooked dish (A1), the APC decreased by 59%. Conventional thermal treatment, represented by cooking the fish dish in a convection oven, influenced the number of microorganisms in the products differently. All three cooking systems that involved convection had an average APC of 3.69 log CFU/g, still below the accepted maximum. In the cook–chill system, this level showed some fluctuations, decreasing by 0.02 log CFU/g after the first day and then increasing slightly until day four. Between day four and day five, a new decrease of another 0.02 log CFU/g was recorded. Over the 5 days of sample testing of the cook–chill system, the APC increased by a total of 0.06 log CFU/g. In the cook–freeze system, the APC level decreased by a total of 0.08 log CFU/g until day 30, after which it increased by a total of 0.09 log CFU/g. Thus, in the fish dish made in the cook–serve system, the APC decreased by 61%, in the cook–chill sample, it decreased by 44%, and in the cook–freeze sample, it decreased by 52%. Unconventional heat treatment using microwaves proved to be more effective in reducing the microbial load. Thus, the APC in sample A3 was 2.99 log CFU/g, 10.5 times lower than the non-heat-treated product and 4 times lower than the conventional heat-treated product. All APC values are presented in Figure 1 as log CFU/g.

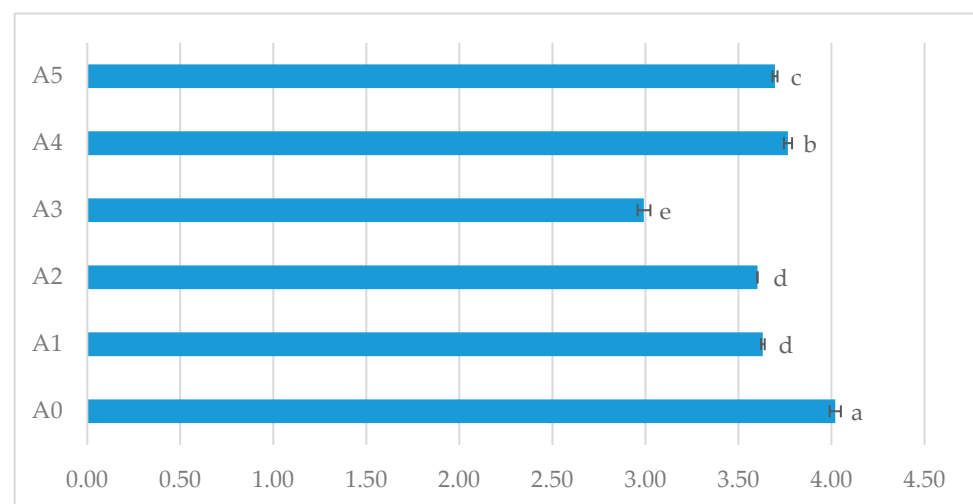


Figure 1. Total aerobic mesophilic bacterial count (log₁₀ CFU/g) in the different catering fish dishes cooked using different techniques (A0—control; A1—sous vide cook–serve; A2—convection cook–serve; A3—microwave cook–serve; A4—convection cook–chill; A5—convection cook–freeze). The results are expressed as the mean value of the three replicates $\pm$ the standard deviation (SD); different letters indicate significant differences (Tukey’s test, $p \leq 0.05$).

The production of the fish dish in the cook–chill system (CC–CC, convective oven followed by rapid cooling in two stages to the refrigeration temperature) resulted in a 45% higher level of bacterial contamination than in the product prepared in the cook–serve system. Products made in the cook–freeze system (CC–CF, convective oven followed by rapid cooling in two stages to freezing temperature) presented a 24.2% higher load compared to that of the same product made in the cook–serve system.

Other studies have reported similar APCs below the European Commission Regulation limits, such as of 4 log CFU/g (sardines [64]), 3.9 log CFU/g (red porgy [65]), 3.46 log CFU/g (bonito [66]), 3.70 log CFU/g (*Dicentrarchus labrax*, [67]), and 3.53 log CFU/g (carp fillets [68]), while some others had considerably lower levels (2.9 log CFU/g in eel [69]). Sebastia et al. [70] also analyzed four different fish species, with salmon being one of them, and obtained minimal results in the microbial loads, with the APCs under 1 log CFU/g. *E. coli*, *S. aureus*, *Salmonella* sp., *L. monocytogenes*, and *C. perfringens* were not detected in their samples. APCs that exceed the European Commission Regulation limits

have been reported by Picouet et al. [71] (4.5 log CFU/g in sous vide cooked salmon fillets), Nguvava et al. [72] (6.08 log CFU/g in sardines), and Antelm et al. [73] (9.14 log CFU/g also in sardines).

The microbiological stability of catering products was monitored during the shelf life provided by the product standard in terms of the evolution of the aerobic mesophilic microorganisms' content. The shelf life is estimated at 5 days for dishes prepared in the cook–chill system (CC-CC), stored at a temperature of 4 °C, and 50 days for dishes prepared in the cook–freeze system (CC-CF), stored at a temperature of −18 °C. Following the microbiological analysis of the sample stored for 5 (A4-e) and 50 days (A5-e), respectively, it was possible to assume and declare the shelf life of the catering products. Samples were taken and analyzed from the refrigerated products coded A4a-e every day of the shelf life.

3.2.2. Total Yeast and Mold Count (TYMC)

All samples analyzed had a total fungi count below half the APC, except for sample A3. A0, representing the raw sample, had a TYMC of 2.43 log CFU/g, which exceeds the initial fungi count that is accepted in the CE Regulation.

Two convection-cooked samples (CC-CC and CC-CS systems) had a fungal level with 0.64 log CFU/g lower than the raw sample (TYMC decreased by 77%). Over the 5 days of testing the A4 samples (cook–chill system), the total number of fungi increased by a total of 0.24 log CFU/g (a total of 73%).

The convection-cooked sample in the cook–freeze system (CC-CF) had a level of 1.05 log CFU/g lower than the raw sample (TYMC decreased by 91% in this case). Over the 50 days of testing the A5 samples, TYMC levels registered small fluctuations of 0.02–0.04 log CFU/g from one analysis to another. Overall, the TYMC count decreased by a total of 0.08 log CFU/g.

3.2.3. Coliforms Count

The initial number of coliforms in the raw sample was 1.41 log CFU/g, below the limit set by the European Commission. After the applied cooking techniques, these values decreased in the cook–serve systems, as follows: to 1.22 log CFU/g in sous vide, to 1.04 log CFU/g in convection, and to 0.48 log CFU/g in the case of microwaves. Even though the number of coliforms decreased by 36% in the case of sous vide and by 58% in the case of convection, these values still exceed the limits. In the microwaved sample, the coliforms number decreased by 88%, and this is acceptable. The samples prepared in the cook–chill and cook–freeze systems had 0.6 log CFU/g coliforms (A4) and 0.48 log CFU/g coliforms (A5). In the cook–chill system, the value decreased by half after the first day, and then coliforms were detected at a very low level until the fifth day (1 CFU/g). In the case of the cook–freeze system, the value fluctuated between 0.48 and 0.3 log CFU/g until day 30. In the determinations of days 40 and 50, the coliform level reached 1 CFU/g.

3.2.4. *E. coli* Determination

The raw sample had a 0.85 log CFU/g of *E. coli*. This level decreased by 33% to 0.67 log CFU/g after sous vide cooking, by 57% to 0.48 log CFU/g after convection, and by 100% to *nd* (not detected) in all of the other samples. All tested samples maintained a minimum content of *E. coli* below the accepted limits.

3.3. Sensorial Quality Evaluation

The sensory characteristics of fish dishes have been identified as the primary factors influencing consumer purchasing decisions. These characteristics are also considered the key determinants of the quality of such dishes. Various sensory attributes can be employed to categorize fish dishes based on these standards [63].

The fish dishes dataset (Table 6) contains the results of the sensory assessment of the fish dish samples: A1: Sous vide cooking—cook—serve (SV-CS), A2: Convective cooking—cook—serve (CC-CS), A3: Microwave cooking—cook—serve (Mw-CS), A4: Convective cooking—cook—chill (CC-CC), A5: Convective cooking—cook—freeze (CC-CF), OA1: Enhanced sample sous vide cooking—cook—serve (SV-CS), OA2: Enhanced sample convective cooking—cook—serve (CC-CS), and OA3: Enhanced sample microwave cooking—cook—serve (Mw-CS). Eight types of fish dishes were represented in the 24 samples (8 samples analyzed in triplicate), with the results of six sensory parameters recorded for each sample. The variables recorded were exterior appearance, interior appearance, consistency, odor, taste, and overall acceptability.

Table 6. Scores of sensory attributes for the fish dish samples.

Treatment	Sample	Exterior Appearance	Interior Appearance	Consistency	Odor	Taste	Overall Acceptability	Total Points
Sous vide cook—serve (SV-CS)	A1	7.73 ± 0.44 ^b	7.77 ± 0.50 ^{bc}	8.30 ± 0.69 ^a	8.37 ± 0.56 ^b	8.43 ± 0.48 ^b	8.27 ± 0.29 ^b	8.15 ± 0.31 ^b
Convective cooking cook—serve (CC-CS)	A2	8.20 ± 0.48 ^a	8.23 ± 0.76 ^a	8.30 ± 0.59 ^a	8.50 ± 0.62 ^a	8.73 ± 0.51 ^a	8.50 ± 0.61 ^a	8.41 ± 0.40 ^a
Microwave cook—serve (Mw-CS)	A3	7.40 ± 0.51 ^{cd}	7.70 ± 0.46 ^c	8.07 ± 0.51 ^b	8.07 ± 0.56 ^e	8.00 ± 0.64 ^d	8.20 ± 0.33 ^b	7.91 ± 0.45 ^c
Convective cooking cook—chill (CC-CC)	A4	7.67 ± 0.55 ^{bc}	7.47 ± 0.85 ^d	8.07 ± 0.71 ^c	8.13 ± 0.47 ^d	8.13 ± 0.35 ^c	7.87 ± 0.65 ^c	7.89 ± 0.29 ^c
Convective cooking cook—freeze (CC-CF)	A5	7.20 ± 0.65 ^d	7.77 ± 0.88 ^c	7.97 ± 0.62 ^d	7.97 ± 0.51 ^f	7.7 ± 0.67 ^d	7.87 ± 0.59 ^c	7.75 ± 0.71 ^c
Fine dining of A1	OA1	7.07 ± 0.21 ^d	7.23 ± 0.32 ^e	8.33 ± 0.50 ^a	8.37 ± 0.51 ^b	8.37 ± 0.62 ^b	8.23 ± 0.44 ^b	7.93 ± 0.63 ^c
Fine dining of A2	OA2	8.37 ± 0.34 ^a	8.27 ± 0.38 ^a	8.30 ± 0.48 ^a	8.57 ± 0.50 ^a	8.73 ± 0.56 ^a	8.57 ± 0.33 ^a	8.47 ± 0.57 ^a
Fine dining of A3	OA3	7.73 ± 0.41 ^{bc}	7.90 ± 0.46 ^b	8.07 ± 0.44 ^c	8.27 ± 0.61 ^c	8.20 ± 0.39 ^c	8.23 ± 0.62 ^b	8.07 ± 0.43 ^b

The results are expressed as the mean value of the three replicates ± the standard deviation (SD); different letters in superscript within the same column indicate significant differences (Tukey's test, $p \leq 0.05$). A nine-point hedonic scale was used, where 1: extremely unacceptable and 9: extremely acceptable. The total score was calculated as the mean of the 6 sensory parameters recorded for each sample.

Finding the most distinctive attribute among the fish meals is the aim of the Random Forest algorithm we used. The Random Forest cluster model is specifically optimized in relation to its BIC (Bayesian Information Criterion) value, which is visible in the Elbow Curve Diagram (Figure 2). The elbow diagram shows the kink in the curve or the point at which it would be unnecessary to add another cluster. The model's optimized metric, the least BIC value, is shown by the red dot.

As can be seen, the result of the cluster analysis is a set of two clusters, each one distinct from the other but largely similar to the objects or data points within them (Figure 3). The two-dimensional t-SNE (t-distributed Stochastic Neighbor Embedding) graph shown in Figure 3 provides a good summary of all of the different characteristics of the samples. The disadvantage, however, is that the axes lose their interpretability. The way in which the different clusters are grouped together is shown in this t-SNE graph. In addition, Figure 3 shows the clustering of the samples according to six fixed criteria that shaped their characteristics: external appearance, internal appearance, consistency, odor, taste, and overall acceptability.

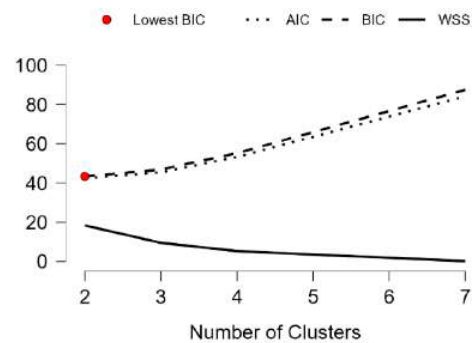


Figure 2. The determination of clusters optimized in accordance with the Bayesian Information Criterion (BIC) for the eight samples of fish dishes: A1: Sous vide cooking—cook—serve (SV-CS), A2: Convective cooking—cook—serve (CC-CS), A3: Microwave cooking—cook—serve (Mw-CS), A4: Convective cooking—cook—chill (CC-CC), A5: Convective cooking—cook—freeze (CC-CF), OA1: Enhanced sample Sous vide cooking—cook—serve (SV-CS), OA2: Enhanced sample convective cooking—cook—serve (CC-CS), and OA3: Enhanced sample microwave cooking—cook—serve (Mw-CS). Akaike Information Criterion (AIC); within sum of squares (WSS).



Figure 3. Two-dimensional t-SNE plot illustrating clustering for the eight samples of fish dishes: A1: Sous vide cooking—cook—serve (SV-CS), A2: Convective cooking—cook—serve (CC-CS), A3: Microwave cooking—cook—serve (Mw-CS), A4: Convective cooking—cook—chill (CC-CC), A5: Convective cooking—cook—freeze (CC-CF), OA1: Enhanced sample Sous vide cooking—cook—serve (SV-CS), OA2: Enhanced sample convective cooking—cook—serve (CC-CS), and OA3: Enhanced sample microwave cooking—cook—serve (Mw-CS).

As is apparent in Table 6 and Figure 3, the panelists' preferred variants are represented by sample A2, which was prepared by CC-CS, and sample 7, which was also represented by sample A2, which was improved. The two samples were grouped in a cluster (Cluster 2). Convective baking was the thermal treatment that generated the most appreciated sensory characteristics in the analyzed samples A2 (CC-CS) and OA2 (CC-CS), obtaining average scores of 8.41 and 8.47 points, respectively. All other samples, irrespective of the treatment applied, were grouped into a separate cluster (Cluster 1). It was therefore deduced that the panelists gave similar ratings for the samples in Cluster 1, as well as there being no significant differences in the scores obtained.

Additionally, the cluster analysis revealed the ranking of features in a descending order of importance for the grouping of samples. The most significant variable is the odor (index 1.383), followed by taste (index 1.343), exterior appearance (index 1.282), overall acceptability (index 1.254), interior appearance (index 1.201), and, finally, consistency (index 0.933).

It was evident that samples A1, A2, and A3 were prepared in the cook—serve system; however, they underwent different thermal treatments. The samples that were most highly regarded were those that were cooked through convection baking (A2—8.41 points), followed by those that were cooked sous vide (A1—8.15 points) and those that were cooked through microwave baking (A3—7.91 points).

Furthermore, a notable observation is that among all of the samples analyzed, those produced in the cook-serve system were the most highly regarded. The samples produced in the cook-chill and cook-freeze systems were found to be less appealing in terms of external appearance (A4—7.67 points, A5—7.2 points) and internal appearance (A4—7.47 points, A5—7.77 points), in comparison with those produced in the cook-serve system. The gradual cooling of the samples and the temporary storage at a temperature of 4 °C (A4), respectively at a temperature of −18 °C (A5), can be considered to justify slight dehydration of the products, with a direct influence on the color and external appearance of the products.

Concerning the optimization proposal, it was observed that the sous vide (SV) samples (OA1—7.93 points) did not yield a positive result and were undervalued in comparison with the initial samples (A1—8.15 points). The technical optimization proposal, in this case, failed to enhance the sensory quality, as evidenced by the contraction of the packaging resulting from the internal vacuum, which mechanically impacted the product's integrity. The internal and external appearance of the samples were influenced by the consistency of the non-thermally treated products and the mechanical actions applied during the preparation, formation, and thermal treatment. Moreover, it was observed that the samples subjected to convective baking (OA2) and microwave baking (OA3) exhibited higher scores in comparison with the initial samples (A2 and A3, respectively).

4. Conclusions

The results obtained in this study of the culinary preparation of fish made in the cook-serve, cook-chill, and cook-freeze systems, alternating with thermal treatments such as sous vide, convective baking, and microwave, are different in terms of production yield, nutritional potential, safety, and sensory value. The lowest quantitative loss was determined in the case of the fish preparation cooked under vacuum (A1, $14.25 \pm 1.57\%$), as compared to those thermally treated using convective baking and microwave, where the losses found were approximately 30%. Systems that ensure a significantly improved minimum durability by controlled cooling to refrigeration temperature (A4) and freezing (A5) resulted in quantitative losses of approximately 40%.

The open systems—convection and microwave cooking—recorded higher cooking losses than the sous vide system, largely due to moisture loss. The lowest quantitative loss was determined in the case of the sous vide dish (A1, $14.25 \pm 1.57\%$), compared to those thermally treated using convection cooking and microwaves, where the losses were found to be approximately 30%. Systems that ensure a significantly improved minimum durability by controlled cooling to refrigeration temperature (A4) and freezing (A5) resulted in quantitative losses of approximately 40%.

The macronutrient content recorded little variations between the used systems. The protein content was similar in all dishes, with an average value of 16.06%. The digestibility was also similar, with very little variation between the five cooking techniques, but it increased slightly in the cook-chill and cook-freeze systems, compared to the cook-serve (CC-CS) system. The fatty acid profile showed the highest contents of SFAs, MUFAs, and PUFAs in the convection-cooked dish. The retention of the PP vitamin was the highest in the sous vide dish and the lowest in the convective cooking. The highest amount of hydro-soluble vitamins was maintained in sous vide cooked dishes, while the convection CC-CS system caused the greatest decrease in riboflavin and niacin content. Regarding fat-soluble vitamins, namely retinol and α -tocopherol, the sous vide preserved most of their content, and microwaves caused their reduction to the lowest values. In terms of the real retention of vitamins, sous vide and convection cooking proved to be protective.

Regarding the APC, the sous vide dish had the highest value, and the microwaved dish had the lowest, but both were within the accepted limits. The number of fungi in the tested dishes was also below the limits, with the highest number in the *sous vide* dish and the lowest after microwave cooking. The coliform count was limited to very low, and the *E. coli* count was also below the limits, even reaching an absolute low of not being detected in the microwaved dish. *S. aureus*, *B. cereus*, *L. monocytogenes*, and *Salmonella* sp. were all undetected in the tested samples, which supports the fact that all of them were prepared in maximum safety and hygiene conditions. The cook–chill and cook–freeze systems offer better shelf life and rigorous control of the main indicators of safety.

Convective cooking—cook–serve (CC-CS)—was identified as the thermal treatment that produced the most appreciated sensory characteristics in the samples analyzed, as dishes OA2 and A2 obtained the highest scores. The CC-CS system was found to be the most efficient because it ensures a balance in terms of technological quality, nutritional quality, safety, and sensory quality. Through this production system, diversification in fine dining gastronomy can be ensured, thus offering versatility to the quality values of culinary products. The sous vide and microwave treatments, as well as the samples prepared in the cook–chill and cook–freeze systems, were found to be less appealing in terms of sensory attributes.

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