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Replacement of Sulfur Dioxide in White, Rosé, and Red Wines by a Blend of Tannins Extracted from Multiple Plant Materials

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Abstract: For a long time, sulfur dioxide (SO_2) has been the most universally used additive in winemaking. With its wide range of effects, ease of use, and low cost, sulfur dioxide has not yet been completely replaced by any process or substance. Since the expected trend for the near future is to keep reducing the concentration of sulfites, many investigations focus on alternative chemical, biological, or physical processes. This study aims to evaluate the chemical, antioxidant, and sensory impact of a plant-based product used as sulfur dioxide replacement (SDR) in white, rosé, and red wines produced as a result of the application of different vinification protocols. The physicochemical and sensory evaluation of the different wines produced showed that this plant-based product could be a good candidate, but appropriate winemaking treatments and optimization are needed to limit wine defects.

Keywords: winemaking; antimicrobial; microbial spoilage; antioxidant; phenolics

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1. Introduction

Today, sulfur dioxide (SO_2) is the only additive used in winemaking that combines antioxidant, antioxidative, and antimicrobial properties [1]. By combining these properties, sulfuric anhydride ensures the preservation of the quality of wines. Nonetheless, its addition to wines has come under scrutiny in recent years. Since 2005, sulfites have been included on the list of allergens [2], making it mandatory in the EU to label wines having a level of sulfites greater than 10 mg/L. This information is very helpful to those individuals sensitive to sulfites to avoid negative clinical effects [3]. Moreover, European Union legislation [4] put limits to the maximum concentrations: 150 mg/L for dry red wines, 185 mg/L for sparkling wines, 200 mg/L for dry white and rosé wines, and each level is reduced by 50 mg/L for organic wines. The permissible daily intake of sulfites, according to the World Health Organization, is 0.7 mg/kg of body weight [5]. Therefore, reducing the level of sulfites in wines remains imperative and simultaneously creates a high demand for alternatives that could replace them. Investigations involving alternative chemical, biological, or physical processes that reassemble the SO_2 effects on wine have been conducted [6–8]. In their review, Santos et al. [9] and Silva and van Wyk [10] report that different physical processes such as pulsed electric field, ultrasounds, high pressure, power ultrasound, high-pressure homogenization, filtration, low electric current and ultraviolet light have been tested in wines but not applied in wineries yet. The literature reports the experimental use of different compositions/extracts such as the extract from unripe grapes rich in phenolics [11], natural extract rich in hydroxytyrosol from olive waste [12], grapevine shoot extracts or commercial products of similar origin [13–15], phenolic extract of biological origin mainly from black radish [16], grape stem extracts [17], seed

extracts [18], commercial phenolic extracts from eucalyptus leaves and almond skins [19], powdered *Hippophae rhamnoides* L. (sea buckthorn) leaves [20], chitooligosaccharide [21], and enological tannins [22,23].

In general, wine polyphenols are reported to have inhibitory effects on the growth of lactic acid bacteria involved in enological fermentation [24]. However, sulfur dioxide qualities such as wide range of impact, ease of use, and low cost make it difficult to completely replace with other processes or substances. With its potent antibacterial properties, SO_2 offers protection against various spoilage microbes [25]. Besides its valuable effect in inhibiting microbial growth, in grapes, must, and wine, SO_2 reacts with various organic compounds to produce bound sulfur dioxide. Aldehydes, ketonic acids, sugars, uronic acids, oxidation sugar products, phenolic compounds (i.e., anthocyanins), and other substances are involved in binding reactions that happen in wines. Each of these chemicals will separate from bisulfite in its bound state, although some more easily than others. Since acetaldehyde is the most powerful electrophile in wine and reacts with phenolics and glutathione, bisulfite binding to it is important. The undesirable bruised apple fragrance which it gives to wines can be quickly counteracted by bisulfite additions [1]. SO_2 also affects the aromatic profile of wines since it is able to modulate the extent of oxidative damage caused by oxygen [26]. It interacts with some of the compounds (i.e., those having a carbonyl group) that are responsible for desirable scents (i.e., vanilla, caramel, butter, honey, potato, orange, lemon, violets, cider, and plum) in wine, which results in producing reversible non-volatile compounds that can release aromatic compounds during wine maturation and aging or under acidic conditions when levels of free SO_2 are reduced [27]. In addition, SO_2 influences color development in wines, as it bleaches and binds wine pigments reversibly, especially in monomeric anthocyanins [28].

The International Organization of Vine and Wine (OIV) and the EU have approved various physical and chemical methods to be used as alternatives to sulfur dioxide to ensure wine microbiological stability [29]. Although oenological tannins bear antioxidant protection, they are always used in combination with sulfur dioxide because they lack antimicrobial activity [16]. Recently, phenolic extracts from wine byproducts or other sources have been proposed as potential substitutes for sulfur dioxide, with encouraging results [19]. Different preparations advertised as being of natural origin are available in the market and are recommended as substitutes for SO_2 in winemaking. This study supports the great effort to substitute SO_2 in winemaking by testing a natural commercial product suggested for winemaking, referred to as a sulfur dioxide replacement (SDR).

Thus, the objective of this study is to evaluate the impact of SDR on the chemical, microbiological, and sensory characteristics of wine, in the frame of globally conducted trials with an improved formula. Taking into consideration that the SDR impact may be dependent on the winemaking process, white, rosé, and red vinification procedures were examined. The effect of SDR was evaluated on wines from four grape varieties (one international and three native Greek). Moreover, the effect of SDR on red wine produced with pre-fermentative cold maceration (CM) and whole bunch fermentation (WBF) extraction was also evaluated since these treatments result in wines richer in anthocyanins, tannins, and antioxidants in general, compared with other winemaking techniques [30,31].

2. Materials and Methods

2.1. Raw Materials

Grapes of two white varieties, Malagousia and Assyrtiko (co-vinification mixture), and two red varieties, i.e., Agiorgitiko and Cabernet Sauvignon (single-variety vinification), cultivated in the Drama PGI zone, were collected during the 2022 season. When the grapes reached their technological maturity, each grape variety was harvested by hand separately, and transferred to the Marketing Research and Development Laboratory of New Food and Beverage Products at the Department of Agricultural Biotechnology and Oenology of the Democritus University of Thrace in Drama.

The SDR product is a blend of powdered botanical hydrolysable and condensed tannins extracted from multiple plant materials (*Ananas comosus*, *Caesalpina mucronata*, *Lycium chinense*, *Mangifera indica*, *Melissa officinalis*, *Morus alba*, *Prunus domestica*, *Punica granatum*, *Rhizopus japonicus*, *Salvia rosmarinus*, *Ruta graveolens*, *Vaccinium myrtillus*) and was elaborated by the R&D team of Biolethics Europe (Laren, The Nederlands) [32]. ESTAAN® is based on a genuine blend of tannins extracted from top quality plant material. It complies with the International oenological Codex of the OIV regulation OIV-OENO 624-2022, OIV-OENO 675 A,B,C,D-2022. Its use is permitted according to EU Commission Regulation No. 2019/934 and can also be added during the vinification of organic wines according to EU Regulation No 203/2012 and Council Regulation (EC) No 834/2007 Annex VIIIa.

2.2. Chemicals and Reagents

Different analytical standards were used for the quantification of phenolics in the wine. Caffeic acid (CA), p-coumaric acid (pCA), myricetin (MYR), and quercetin (QUE) were obtained from Sigma-Aldrich (Steinheim, Germany). Rosmarinic acid (RMA), rutin (RUT), apigenin (API), kaempferol (KAE), catechin (CAT), epicatechin (EPI), quercetin-3-O-glucopyranoside (QUEGLU), and hyperoside (HYP) were purchased from Extrasynthese (Genay Cedex, France). Gallocatechin (GCAT), and dihydroquercetin (DHQ), were obtained from Carbosynth (Berkshire, United Kingdom). Protocatechuic acid (PRCA), gallic acid (GA), and luteolin (LUT) were purchased from Alfa Aesar (Karlsruhe, German). Hesperidin (HESP) was obtained from TCI (Zwijndrecht, Belgium). Resveratrol (RESV), procyanidin B1 (PRC-B1), procyanidin B2 (PRC-B2), and procyanidin B3 (PRC-B3) were purchased from Biosynth (Compton, United Kingdom). Substances used for mobile phase such as formic acid, methanol, acetonitrile, and water of LC-MS grade were from Sigma-Aldrich (Steinheim, Germany).

DPPH (2,2-diphenyl-1-picrylhydrazyl) and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas TPTZ (2,4,6-tripyridyl-s-triazine) and aluminum-chloride-6-hydrate were from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were from J&K Scientific GmbH (Pforzheim, Germany). Sodium acetate trihydrate, sodium hydroxide (HPLC grade), and Folin–Ciocalteu reagent were from Chem-Lab NV (Zedelgem, Belgium). Sodium carbonate, iron (III) chloride hexahydrate, and sodium nitrite were from Merck KGaA (Darmstadt, Germany). All the other chemicals/reagents used in this study were of analytical grade.

2.3. Winemaking Process

For each micro-vinification, two equal quantities of 25 kg of mature grapes after crushing/juice separation were used. The juice/crushed grapes were placed in temperature-controlled 30 L stainless steel fermentation vats that had previously been water-cleaned and then properly sterilized by concentrated alcohol. For each vinification, a batch of the same harvested grapes with sulfur dioxide (6 g/hL) is considered as the control, whereas the other (1 mL/L) of SDR represents the alternative. Both batches were inoculated with *Saccharomyces cerevisiae* Exelcia Terroir CH yeast (Burgundia Oenologie, Beaune, France) at 25 g/hL, followed by a nutrient (diammonium sulfite, Laffort, Bordeaux, France) addition of 20 g/hL. Sulfur dioxide or SDR product was also added to the wines after the alcoholic fermentation at the same amount as that used previously. The applied vinification protocols differ in temperatures depending on the type of wine produced. The following wines were made: white Malagousia-Assyrtiko with SO_2 (white-SO_2) and white with SDR (white-SDR), rosé Agiorgitiko with SO_2 (rose-SO_2) and rosé with SDR (rosé-SDR), red Cabernet Sauvignon wine with SO_2 (red-SO_2) and red wine with SDR (red-SDR)), red wine with the addition of SDR and application of cold maceration (CM) (red-SDR-CM) or application of whole bunch fermentation (WBF) (red-SDR-WBF), since different vinification treatments can give wines with different phenolic concentrations [33,34]. Cold maceration included the retention of grapes at low temperatures before fermentation for several days, depending

on the grape's maturation and the style of the wine produced, to enhance the extraction of compounds from the solid parts of the grapes [35]. Whole bunch fermentation consisted of using whole bunches of grapes during fermentation [31]. Fermentation was monitored by Baumé hydrometers (Dujardin Salleron) every other day. On the first and third day of alcoholic fermentation, microbiological tests were performed to detect possible microbial infection. When alcoholic fermentations in each tank were completed, the wines were pre-clarified in a new sterilized tank by fully filling it and allowed to rest for 7 days at 4 °C. After this step, wines were once again settled and bottled in 750 mL Bordeaux-styled bottles with Diam corks, manually. Chemical and sensory analyses were conducted on the finished wines. Moreover, chromatic characteristics were also analyzed after storage of bottled wines for six months at 16 °C.

2.4. Physicochemical Analysis of Must and Wine

The basic physicochemical parameters analyzed in wine are alcohol degree, relative density, free and total SO_2, total acidity, volatile acidity, and pH value. Reducing sugars were measured according to OIV–MA–AS311, relative density with an electronic hydrostatic balance, total acidity according to OIV–MA–AS313-01, pH value according to OIV–MA–AS313–15, alcohol degree by volume at 20 °C by OIV–MA–AS312–01A method, volatile acidity according to OIV–MA–AS313–02, malic acid by OIV–MA–AS313–11 method, lactic acid using an enzymatic method, and free and total SO_2 with an automatic potentiometric method.

Absorbance (A) at 280, 320, 420, 520, and 620 nm was assessed after centrifugation (10 min at 5000 rpm). Absorbance at 280, 320, and 420 nm was associated with the "total phenolic index", hydroxycinnamic acids and their derivatives, and "browning" in white wines, respectively [1,36]. The intensity (I) and hue (T) of rosé and red wines were determined by the method of Sudraud [37] and Glories [38] using the following equations:

$$\text{Intensity (I)} = \text{A420 nm} + \text{A520 nm} + \text{A620 nm},$$

$$\text{Hue (T)} = \text{A420 nm}/\text{A520 nm}$$

The yellow, red, and blue percentages of the wine colors were calculated as follows:

$$\text{Yellow: A420 (\%)} = (\text{A420}/\text{I}) \times 100; \text{ Red: A520 (\%)} = (\text{A520}/\text{I}) \times 100; \text{ Blue: A620 (\%)} = (\text{A620}/\text{I}) \times 100;$$

All the aforementioned assays were performed at least in duplicate.

2.5. Determination of Phenolics and Antioxidant Activity of Wines

The wine samples were centrifuged (5000 rpm for 15 min), and the supernatants were collected and measured. Determinations of the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant potential of wines with DPPH and ABTS assays were achieved following the methodology reported by Skendi et al. [39]. The amount of TPC and TFC was calculated based on the respective calibration curves constructed using gallic acid and catechin, respectively, whereas the antioxidant capacity was calculated using Trolox. The results are reported as milligrams of gallic acid equivalents per mL wine (mg GAE/mL), milligrams of catechin equivalents per mL wine (mg CATE/mL), and mg Trolox equivalents per mL wine (mg TE/mL), respectively. All the aforementioned assays were performed at least in triplicate.

2.6. Determination of Phenolics with LC-MS

A Shimadzu LCMS-2020 system (Shimadzu, Kyoto, Japan) using an electrospray ionization (ESI) process and a quadrupole mass analyzer, equipped with an SPD-M40 PDA (Shimadzu, Kyoto, Japan) detector, was used for the chromatographic separation and identification of phenolics. The operating conditions of the LC-PDA-MS instrument

were previously described in the modified method of Irakli et al. [40]. Phenolics present in the produced wines were injected in 10 µL volume and separated on a Poroshell 120 EC-C18 column (4.6 × 150 mm, 4 µm) operated at 35 °C. A gradient elution program was employed, using 0.1%, *v*/*v* aqueous formic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.5 mL/min, with a gradient elution program as follows: 0 min, 85% A; 0–5 min, 85–75% A; 5–10 min, 75–65% A; 10–28 min, 65–40% A; 28–35 min, 40–0% A; 35–40 min, 0–85% A. The ESI interface voltage was 4.5 kV in negative ion mode, and the curved desolvation line (CDL) voltage was 20 V. Nitrogen as a nebulizer at a flow rate of 1.5 L/min and as a drying gas at a flow rate of 15 L/min was applied. The block heater temperature and CDL temperature were maintained at 200 °C and 250 °C, respectively. A full scan mode (100–1000 *m*/*z*) was applied for the identification of peaks based on Lab Solutions LC-MS software v. 5.97.SP1 (Shimadzu, Kyoto, Japan). Quantification of compounds was achieved using the calibration curves of authentic standards, applying selective ion monitoring mode (SIM). The determination and quantification of the phenolic compounds in the wines were performed in duplicate.

2.7. Sensorial Evaluation of Wines

The sensory analysis of the wines produced from each microscale vinification was performed by 30 tasters, according to the review document on the sensory analysis of wine [41]; the protocols and the descriptive analysis test followed ISO 11035:1994 [42]. The wine tasters who participated in this study were 14 women and 16 men (mean age: 30.16 ± 13.15 years). About 26.7% of them were professional wine tasters and the rest were students of oenology who had a significant education level in the sensory evaluation of wine. The wines were blind-tasted in random order using the 100-point OIV-specific score sheet for still wines ranging from inadequate to excellent quality. No technical or analytical information regarding the wine tasted was given to the tasters. The following parameters were evaluated: appearance (limpidity and aspect other than limpidity), aroma (genuineness, positive intensity, and quality), taste (genuineness, positive intensity, harmonious persistence, and quality), and harmony/overall assessment. The total score was given by the sum of parameter scores. To comply with the research code of ethics, the sensory evaluation of wines received approval from the Research Ethics Commission of Democritus University of Thrace.

2.8. Statistical Analysis

Depending on the data to be analyzed, different statistical tests were employed using the SPSS v.25 package (SPSS Inc., Chicago, IL, USA). Differences among the samples were evaluated by *t*-test or ANOVA (Analysis of Variance) followed by the Duncan test, or the respective non-parametric Mann–Whitney U test and Kruskal–Wallis test followed by Dunn's post hoc tests. Significant differences among the wine samples were evaluated at a p-value < 0.05.

3. Results and Discussion

3.1. Effect on Physicochemical Parameters of Wines

The alcoholic fermentation in all the treatments had the same duration with a difference of +/− 1 day. The results regarding the physicochemical parameters of the wines are reported in Table 1. Chemical parameters (alcohol, reducing sugars, titratable acidity, pH value) of the SO_2 and SDR white wines were similar, while malolactic fermentation was more advanced in the SDR white wine. With regard to rosé wines, significant differences were noted, especially regarding the titratable and volatile acidity. All the SDR-containing wines had lower volatile acidity at the end of the alcoholic fermentation than the respective SO_2 wines. The only exception was the red SDR-WBF wine, which had a higher volatile acidity (1.10 g/L acetic acid) that was still below the maximum acceptable limits of EU legislation and OIV recommendations, pH value (3.89), residual sugars (6.80 g/L), and malic acid content (4.50 g/L). This is probably not directly related to the treatment but to the

fermentation scale. Absorbance at 280 nm, which is an indication of the phenolic content, was also higher in SDR than in SO_2 wines. In red wines, the two different vinification treatments (CM and WBF) gave wines with higher A280.

With regard to the monitoring of SO_2 presence in the wines, the levels of free and total sulfites in the wines are given in Table 1. On its path to its metabolic end-point in cysteine, the sulfate found in grapes is absorbed by yeast and converted to several forms, including sulfite and sulfide; therefore, the yeast may produce its own sulfur dioxide [6]. Because of yeast metabolism, alcoholic fermentation can result in comparatively high levels of SO_2. In our results, wines made without sulfite addition contained about 9.4–14.7 mg SO_2/L by the end of fermentation (free SO_2 1.7–4.7 mg/L). Most *S. cerevisiae* wine strains (80%) were found to produce less than 10 mg/L SO_2; however, other strains can release as much as 30 mg/L SO_2 [6]. The sum of free and bound sulfur dioxide is reported as total sulfur dioxide in wine. Fifteen to thirty percent of all sulfur dioxide is free sulfur dioxide. The two main forms of free sulfur dioxide are dissolved sulfur dioxide (also known as molecular SO_2), which makes up 0.5% to 9.5% of the total and is the only form with antimicrobial activity, and ionization forms of sulfite and bisulphite, which make up 90–98% [43]. Given the pH levels of the wines and the fact that molecular SO_2 content reduces when the wine pH value increases [1], it can be deduced that since the SDR wines in all cases had a significantly higher pH value than the corresponding SO_2 wine, the antimicrobial protection provided by the existing SO_2 was lower in SDR wines. This is more evident in red wines, where the wine pH value increased as follows: red-SO_2 < red-SDR < red-SDR-CM < SDR-WBF. Meanwhile, the SO_2 content showed a reverse order. However, despite the higher probability of microbial spoilage in SDR wines, phenolics in SDR wines seem to have a positive effect in controlling the development of malolactic or spoilage bacteria. Salinas Bonich et al. [44] have previously shown the inhibitory capacity of plant phenolic concentrates tested against *Oenococcus oeni* and *Brettanomyces bruxellensis*.

The most significant visual characteristic of wines is their color, which also plays an important role in the product's overall quality. During the storage of white wines, discoloration sometimes occurs [45]. The measure of absorbance in different wavelengths (280, 320, 420, 500 nm) may determine the probability of a wine showing browning or pinking discoloration in the future. Therefore, we determined in white wines the above absorbance six months after alcoholic fermentation. The evolution of white wines regarding their optical absorption at 420 and 500 nm did not differ significantly between SO_2 and SDR (Figure 1a).

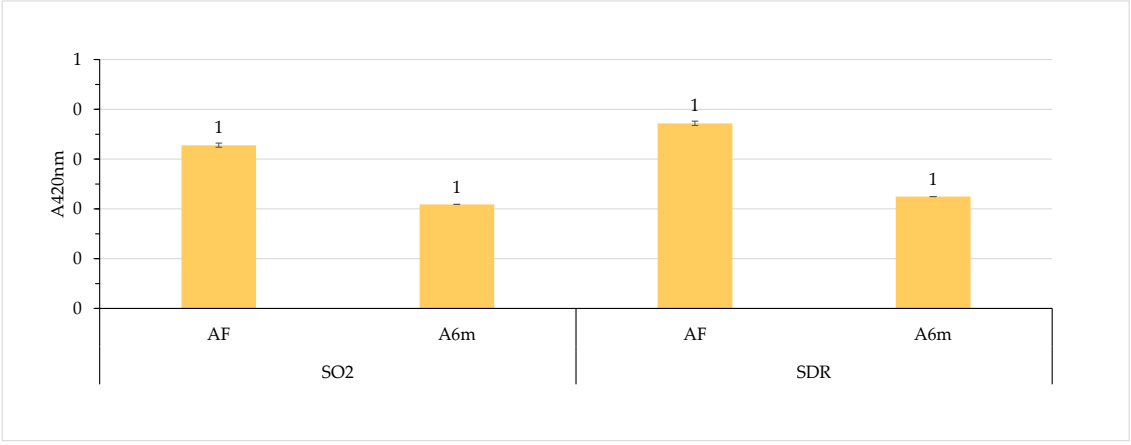
As in other wine types, the color of rosé wines has a significant influence on consumers' decisions regarding their purchase. The color of rosé wines changes inside the bottle over time [46]. Figure 1b presents the mean values of color intensity and hue as a function of time for both rosé wine samples (SO_2 and SDR, respectively).

The data evidences that the hue increased in both treatments after six months in the bottle. Specifically, the yellow hue increased for both treatments as the wine aged, with SDR wine presenting a higher increase (not significant), while the red hue and blue hue decreased (not significantly) to a similar degree for both treatments. Merrell and Harbertson [28] also reported that wines with a low SO_2 had a higher yellow hue after accelerated aging, with polymeric pigment formation most likely responsible for this change, as it also increased over time. However, there was a lack of significant differences in wine color in post-fermented wines and after six months of storage between SO_2 and SDR rosé wines. The same conclusion also arose from visual assessment during the sensory analysis.

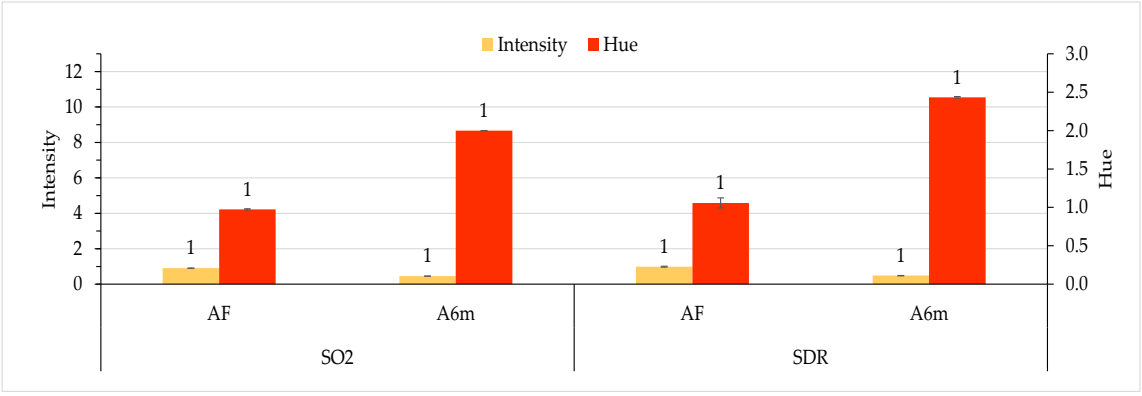
Table 1. Physicochemical characteristics * of the wines at the end of alcoholic fermentation **.

Sample	Alcohol (% vol)	pH	Titratable Acidity (g/L Tartaric Acid)	Volatile Acidity (g/L Acetic Acid)	Density (g/cm^3)	Residual Sugars (g/L)	Free SO_2	Total SO_2	Malic Acid (g/L)	Lactic Acid (g/L)	A280 nm
White-SO_2	12.00 ± 0.04^{1}	3.37 ± 0.01^{1}	6.50 ± 0.01^{2}	0.57 ± 0.00^{2}	0.993 ± 0.000^{1}	3.01 ± 0.04^{1}	17.7 ± 0.6^{2}	54.7 ± 0.6^{2}	2.51 ± 0.02^{2}	0.20 ± 0.01^{1}	7.81 ± 0.01^{1}
White-SDR	11.90 ± 0.05^{1}	3.37 ± 0.02^{1}	6.35 ± 0.01^{1}	0.40 ± 0.01^{1}	0.993 ± 0.000^{1}	3.40 ± 0.02^{2}	4.7 ± 0.6^{1}	12.3 ± 0.6^{1}	2.41 ± 0.01^{1}	0.69 ± 0.01^{2}	9.49 ± 0.01^{2}
Rose-SO2	10.20 ± 0.05^{1}	3.56 ± 0.02^{2}	7.12 ± 0.01^{1}	1.03 ± 0.01^{2}	0.993 ± 0.000^{1}	4.90 ± 0.01^{2}	11.7 ± 1.5^{2}	57.0 ± 1.0^{2}	3.21 ± 0.01^{2}	0.61 ± 0.01^{1}	13.66 ± 0.02^{1}
Rose-SDR	11.10 ± 0.05^{2}	3.50 ± 0.02^{1}	7.43 ± 0.02^{2}	0.92 ± 0.01^{1}	0.993 ± 0.000^{1}	4.61 ± 0.01^{1}	4.0 ± 1.0^{1}	13.7 ± 0.6^{1}	3.10 ± 0.01^{1}	0.70 ± 0.01^{2}	13.83 ± 0.02^{2}
Red-SO_2	12.40 ± 0.05^{3}	3.68 ± 0.01^{1}	7.01 ± 0.01^{3}	0.80 ± 0.01^{3}	0.993 ± 0.000^{1}	1.80 ± 0.02^{3}	12.3 ± 2.5^{2}	53.0 ± 1.0^{4}	3.01 ± 0.01^{3}	0.41 ± 0.01^{2}	29.50 ± 0.04^{1}
Red-SDR	13.51 ± 0.04^{4}	3.70 ± 0.01^{2}	6.59 ± 0.02^{2}	0.36 ± 0.01^{1}	0.993 ± 0.000^{1}	1.01 ± 0.01^{1}	3.0 ± 1.0^{1}	14.7 ± 0.6^{3}	2.50 ± 0.01^{2}	0.10 ± 0.01^{1}	41.30 ± 0.02^{2}
Red-SDR-CM	11.51 ± 0.04^{2}	3.72 ± 0.01^{2}	5.75 ± 0.02^{1}	0.44 ± 0.00^{2}	0.994 ± 0.000^{2}	1.20 ± 0.01^{2}	3.0 ± 1.0^{1}	11.7 ± 0.6^{2}	2.01 ± 0.01^{1}	0.70 ± 0.01^{3}	49.44 ± 0.03^{4}
Red-SDR-WBF	11.11 ± 0.07^{1}	3.89 ± 0.01^{3}	8.70 ± 0.02^{4}	1.10 ± 0.00^{4}	0.996 ± 0.000^{3}	6.80 ± 0.01^{4}	1.7 ± 0.6^{1}	9.7 ± 0.6^{1}	4.30 ± 0.01^{4}	1.20 ± 0.01^{4}	47.18 ± 0.03^{3}

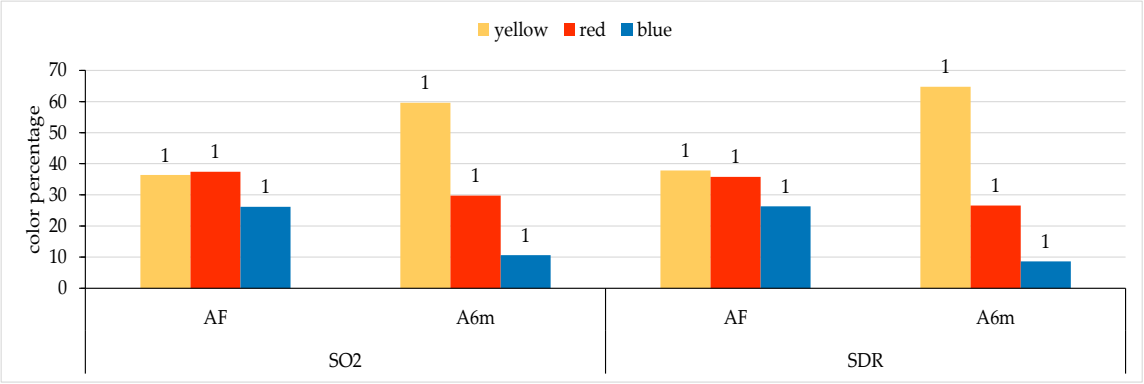
* Results represent means of triplicate values. ** Same superscript numbers in the same column for the same wine color type do not differ according to the Mann–Whitney U test ($p < 0.05$) or according to the Kruskal–Wallis test ($p < 0.05$) followed by Dunn's post hoc tests.



(**a**)



(**b**)



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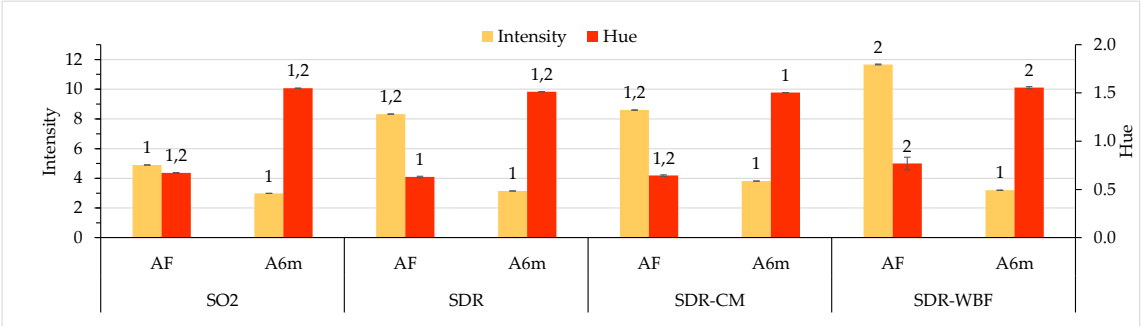
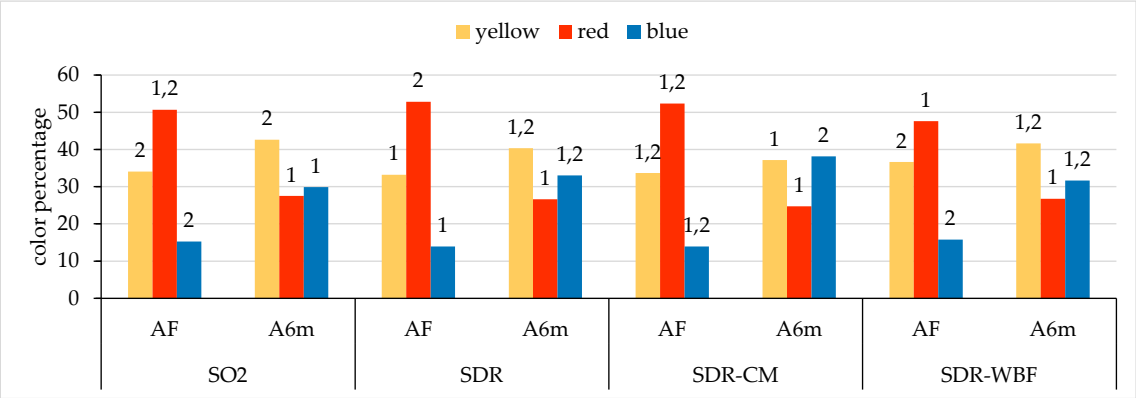


(**d**)

Figure 1. *Cont.*



(**e**)

Figure 1. Color components of wines at the end of alcoholic fermentation and six months later: (**a**) optical density values at 420 nm of the white wines. Same numbers above the bars of the same time (after fermentation, AF; after six months, A6m) of measurement do not differ according to Mann–Whitney U tests for medians ($p < 0.05$); (**b**) intensity and hue of rosé wines. (**c**) Color proportions in rosé wines. Same numbers above the bars of the same parameter and time (after fermentation, AF; after six months, A6m) of measurement do not differ according to Mann–Whitney U tests for medians ($p < 0.05$); (**d**) intensity and hue of red wines. Same numbers above the bars of the same parameter and time (after fermentation, AF; after six months, A6m) of measurement do not differ according to Kruskal–Wallis tests for medians ($p < 0.05$) followed by Dunn's post hoc tests; (**e**) color proportions in red wines. Same numbers above the bars of the same parameter and time (after fermentation, AF; after six months, A6m) of measurement do not differ according to independent sample median tests. Bars represent the means of duplicate values.

Regarding yellow, red, and blue coordinates, there are no statistically significant differences in yellow, red, or blue between SO_2 and SDR rosé wines post-fermentation and after six months of storage (Figure 1c). Both SO_2 and SDR samples subjected to six months of storage showed an increase in yellow and a decrease in red and blue. Contrary to our results, Merrell and Harbertson [28] found that the red hue of rosé wines with different SO_2 treatments increased over time (after 8 weeks of accelerated aging). This fact may be due to the difference in the period and aging method applied as well as the method for color measurement. Factors like the initial color that rosé wine has at the beginning of aging and the SO_2 concentration previously added affected its likelihood of darkening over time [28].

All studied red-SDR wines had a higher color intensity at the post-fermentative stage in comparison to red-SO_2 wine (Figure 1d), while after six months of storage, all red-SDR wines had a significantly higher hue. Other authors also noted that wines treated with SO_2 exhibited noticeably less color intensity than wines developed with alternative antioxidants produced by stem or shoot extracts [18]. This is most likely caused by SO_2's discolorizing impact on anthocyanins, which can be observed in young wines [47]. However, after six months of aging, the color intensity of red wines was comparable, indicating that the wines' color had acquired a comparable degree of oxidation.

As previously stated, the experimental wines were produced using various winemaking procedures; therefore, the variations in their color characteristics might be ascribed to both treatment and winemaking methods. As shown in Figure 1d, comparing red-SO_2 wine to red-SDR wine, the SO_2 substitution did not have a statistically significant influence on the color characteristics (intensity and hue) of the wines, neither after fermentation (AF) nor after 6 months (A6m). From the three red experimental wines produced with the application of different red winemaking protocols, only SDR-WBF wine differed significantly from red-SO_2 wine, presenting higher intensity and hue after fermentation, and higher hue after 6 months. This effect can be attributed to the winemaking technique. Incorporating stems into fermentation enhances the extraction of tannins and may also result in color loss as anthocyanins are adsorbed to the stems [48]. Additionally, the extraction of potassium

ions from the stems may alter the pH of wine [49]. As observed, SDR-WBF wine indeed had more yellow color and less red color (Figure 1e), while it also had an elevated pH value (Table 1).

Global climatic changes (hot and dry) create a gap between technological and phenolic maturity, with the latter lacking the desired quantity of anthocyanins in red wines. It was reported that adding caffeic acid and rosmarinic acid as co-pigments in wine was beneficial to obtaining a more saturated and vivid hue of wines [50]. In addition, Gordillo et al. [51] reported that an increased co-pigment concentration induced perceptible color changes toward a bluish and darkening effect that can be best correlated with hue difference (CIELAB attributes), proving the relevance of this physicochemical phenomenon on qualitative changes in anthocyanin color. Indeed, although the result is not statistically significant, the addition of SDR (rich in phenolics) resulted, after six months, in red wines with a lower yellow hue and higher blue hue compared to the SO_2-added red wines (Figure 1e). Possibly, phenolics from SDR can act as co-pigments during storage since red wines with SO_2 added immediately after fermentation have a higher yellow and blue hue than wine with SDR.

3.2. Effect on Total Phenolic and Flavonoid Content and Antioxidant Activity

The total phenolic content (TPC) and flavonoid content (TFC), as well as the antioxidant capacity of the different types of wines, are illustrated in Figure 2. It was noticed that white and rosé wines treated with SDR doubled their TPC and TFC (Figure 2a). This behavior was not noted for red wine, where there was no increase in the TPC and TFC when SDR was added (Figure 2b). Nevertheless, when treatments such as cold maceration (CM) and whole bunch fermentation (WBF) were involved, the wine resulted in significantly higher TPC. In the case of TFC, only wine with whole bunch fermentation (WBF) showed significantly higher TPC.

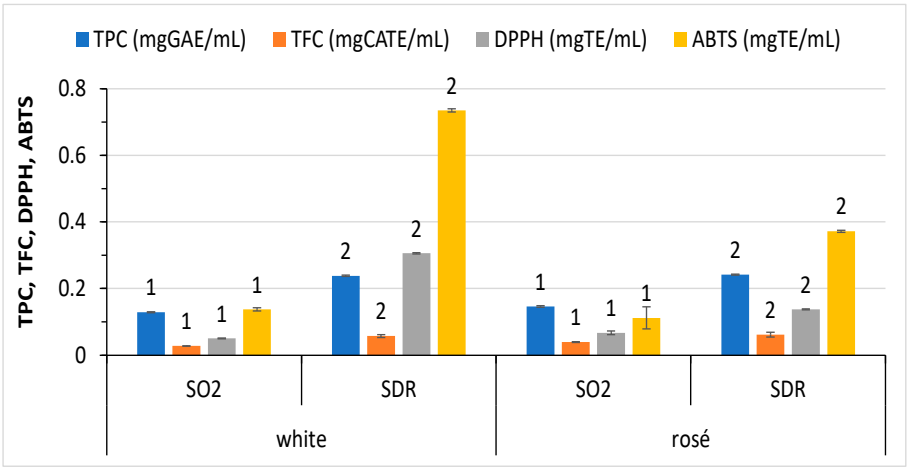
SDR addition significantly increased the antioxidant activity of the white and rosé wines by six and two times in the case of DPPH and by five and three times in the case of ABTS, respectively. Contrarily, the red wines with SDR showed significantly lower DPPH and similar ABTS values than those with SO_2. Both wines derived from cold maceration and whole bunch fermentation with SDR showed higher DPPH values compared to the control with added SO_2, yet in the case of ABTS, only wine from bunch fermentation with SDR had higher ABTS than the control with SO_2 added.

These findings indicate that SDR addition could result in white and rosé wines with higher total phenolics, total flavonoids, and antioxidant capacity, but this is not valid for red wine. The red vinification conditions are harsher than those of white or rosé vinification. This behavior could be explained by the complex formation ability of anthocyanin after reacting with other phenolic compounds [52]. The new complexes (products) formed may partially react or not with reagents used for TPC and TFC determination and may have a lower antioxidant capacity than the initial compounds that contain them. It was observed that TPC was positively correlated only with color intensity (Table 2). On the other hand, TFC did not show any significant correlation with color intensity but was positively correlated with hue. Flavonoids were positively associated with yellow and blue hues but negatively with red.

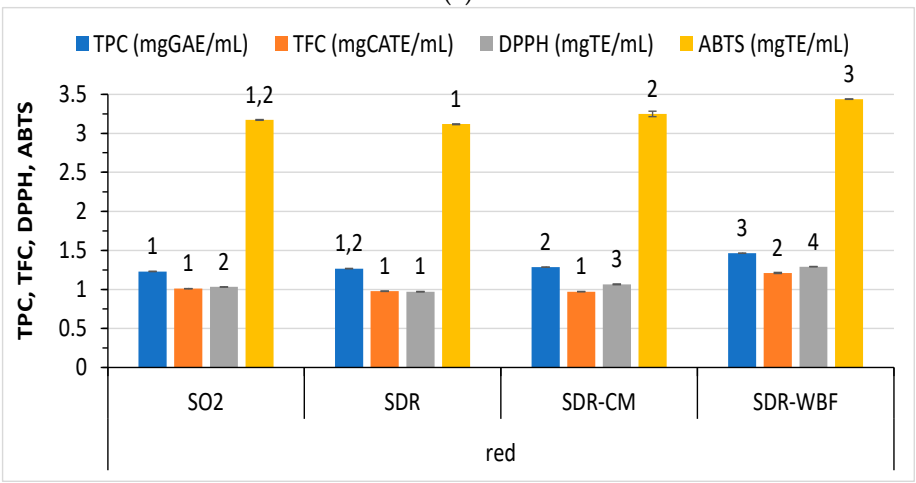
Table 2. Correlation among the phenolic content, flavonoid content, antioxidant capacity, and color parameters of red wines.

	Intensity	Hue	Yellow	Red	Blue
TPC (mgGAE/mL)	0.912 ** (0.002)				
TFC (mgCATE/mL)		0.892 ** (0.003)	0.866 ** (0.005)	−0.892 ** (0.003)	0.963 ** (0.000)
DPPH (mgTE/mL)	0.807 * (0.015)	0.731 * (0.040)	0.764 * (0.027)	−0.731 * (0.040)	
ABTS (mgTE/mL)	0.802 * (0.017)	0.714 * (0.047)	0.747 * (0.033)	−0.714 * (0.047)	

* Correlation Spearman's rho is significant at the 0.05 level (2-tailed). The significance is reported in parentheses.
** Correlation Spearman's rho is significant at the 0.01 level (2-tailed).



(**a**)



(**b**)

Figure 2. Total phenolic content (TPC), flavonoid content (TFC), and antioxidant activity (DPPH and ABTS) of (**a**) white wine (Malagousia and Assyrtiko blend) and rosé wine (Agiorgitiko) from Greek wine grape cultivars treated with SO_2 and SDR; different superscript numbers within the same response and type of wine (white, rosé) indicate significant differences ($p < 0.05$) between the means, as determined by *t*-test; (**b**) red wines made of Cabernet Sauvignon treated with SO_2 and SDR, and cold maceration (CM) and whole bunch fermentation (WBF) treated with SDR; different superscript numbers within the same response indicate significant differences ($p < 0.05$) amongst the means, as determined ANOVA followed by Duncan's multiple range test. Abbreviations: GAE, gallic acid equivalent; CATE, catechin equivalent; TE, Trolox equivalent; SDR, sulfur dioxide replacement. Bars represent the means of triplicate values.

3.3. Effect on Phenolic Profile of Wines

The phenolics in the produced wines were quantified by LC-MS and are reported in Table 3. Representative chromatograms are reported in Supplementary Figures S1 and S2. It is observed that the total amount of phenolics in white wine with added SO_2 is 11.0 μg/mL, whereas in the wine with added SDR the amount reaches 38.9 μg/mL, increasing by more than 3.5 times. The same trend is noted in rosé wines, with added SDR having 3.1 times the amount of phenolics present in SO_2-added wine (12.1 vs. 3.9 μg/mL). On the contrary, the quantity of total phenolics in red wine with added SO_2 is higher (298.1 μg/mL) than the rest of the red wines. Wines from cold maceration (183.1 μg/mL) and whole bunch fermentation (229.5 μg/mL) with added SDR seemed to contain a higher amount of phenolics compared to the red wine with added SDR (155.9 μg/mL). This behavior is similar to that observed for TPC, TFC, and antioxidant capacity.

Table 3. The major phenolic compounds quantified (μg/mL) by LC–MS in wines treated with sulfur dioxide (SO_2) and sulfur dioxide replacement (SDR).

Peak	Analyte (μg/mL)	Rt * (min)	[M − H] −	White-SO_2	White-SDR	Rosé-SO_2	Rosé-SDR	Red-SO_2	Red-SDR	Red-SDR-CM	Red-SDR-WBF
1	gallic acid	3.9	169	0.57 ± 0.09	31.73 ±0.49	0.83 ± 0.11	7.59 ± 0.63	14.93 ± 1.21	11.55 ± 1.00	12.72 ± 0.96	12.41 ± 0.86
2	gallocatechin	4.0	305	tr **	0.10 ±0.02	0.10 ± 0.01	0.10 ± 0.02	0.05 ± 0.01	1.23 ± 0.16	1.85 ± 0.21	2.65 ± 0.32
3	procyanidin B1	4.6	577	tr	0.74 ± 0.06	0.30 ± 0.03	0.21 ± 0.03	17.70 ± 2.05	9.05 ± 0.63	9.47 ± 0.57	10.30 ± 0.88
4	protocatechuic acid	5.0	153	0.22 ± 0.05	0.40 ± 0.06	0.29 ± 0.05	0.29 ± 0.06	0.24 ± 0.05	0.33 ± 0.08	0.35 ± 0.09	0.66 ± 0.11
5	procyanidin B2	5.2	577	8.60 ± 0.14	0.98 ± 0.12	tr	tr	147.12 ± 4.23	52.61 ± 2.03	62.00 ±1.34	82.40 ± 2.48
6	catechin	6.0	289	0.04 ± 0.01	1.46 ± 0.22	0.78 ± 0.13	0.82 ± 0.11	71.47 ± 2.09	55.72 ± 3.75	71.05 ± 1.83	99.63 ± 3.65
7	procyanidin B3	6.2	577	0.16 ± 0.03	tr	tr	0.16 ±0.07	23.84 ± 1.48	9.94 ± 0.76	10.17 ± 0.92	10.71 ± 0.65
8	epicatechin	7.2	289	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	12.22 ± 0.46	8.90 ± 0.53	9.27 ± 0.49	5.70 ± 0.56
9	caffeic acid	7.5	179	0.12 ± 0.01	0.29 ±0.05	0.13 ±0.03	0.15 ± 0.04	0.41 ± 0.04	0.25 ± 0.03	0.29 ±0.05	0.44 ± 0.06
10	rutin	9.1	609	0.11 ± 0.02	0.10 ± 0.02	0.06 ± 0.02	0.17 ±0.01	0.29 ± 0.02	0.35 ± 0.03	0.30 ± 0.03	0.30 ± 0.02
11	quercetin-3-O-glucopyranoside	9.6	463	0.23 ± 0.05	0.23 ± 0.03	0.23 ± 0.02	0.23 ± 0.03	0.12 ± 0.02	0.41 ± 0.05	0.41 ± 0.04	1.20 ± 0.08
12	hyperoside	9.8	463	0.01 ±0.00	0.002± 0.00	0.01 ± 0.00	0.004 ± 00	0.19 ± 0.02	0.13 ± 0.02	0.13 ± 0.03	0.65 ± 0.15
13	p-coumaric acid	9.8	163	tr	0.29 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.07 ± 0.01	0.55 ± 0.06	0.67 ± 0.05	0.15 ± 0.03
14	dihydroquercetin	10.8	303	0.07 ±0.01	0.17 ± 0.03	0.08 ± 0.03	0.16 ± 0.02	0.43 ± 0.03	0.15 ± 0.02	0.17 ± 0.01	0.13 ± 0.01
15	hesperidin	11.5	609	0.09 ± 0.01	0.08 ±0.01	tr	0.14 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.19± 0.03	0.15 ± 0.03
16	rosmarinic acid	12.1	359	- ***	0.62 ± 0.08	-	0.44 ± 0.03	-	0.31 ± 0.04	0.75 ± 0.05	0.20 ± 0.04
17	myricetin	12.5	317	tr	0.21 ± 0.02	0.21 ± 0.04	0.21 ± 0.03	2.11 ± 0.31	0.78 ± 0.05	0.96 ± 0.24	0.21 ± 0.03
18	resveratrol	13.8	227	0.16 ± 0.04	0.10 ± 0.02	tr	tr	0.16 ± 0.03	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00
19	luteolin	15.2	285	tr	0.23 ± 0.03	tr	0.19 ± 0.02	0.24 ± 0.04	0.06 ± 0.01	0.08 ± 0.01	0.11 ± 0.03
20	quercetin	15.4	301	0.08 ± 0.01	0.13 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	3.75 ± 0.42	1.26 ± 0.23	0.03 ± 00	0.03 ± 0.00
21	apigenin	17.5	269	0.03 ±0.00	0.03 ±0.01	tr	tr	0.01 ±0.00	0.03 ± 0.00	0.03 ±0.00	0.03 ± 0.00
22	kaempferol	18.3	285	tr	0.19 ± 0.05	tr	tr	1.78 ±0.12	1.25 ± 0.21	1.29 ± 0.17	0.65 ±0.10

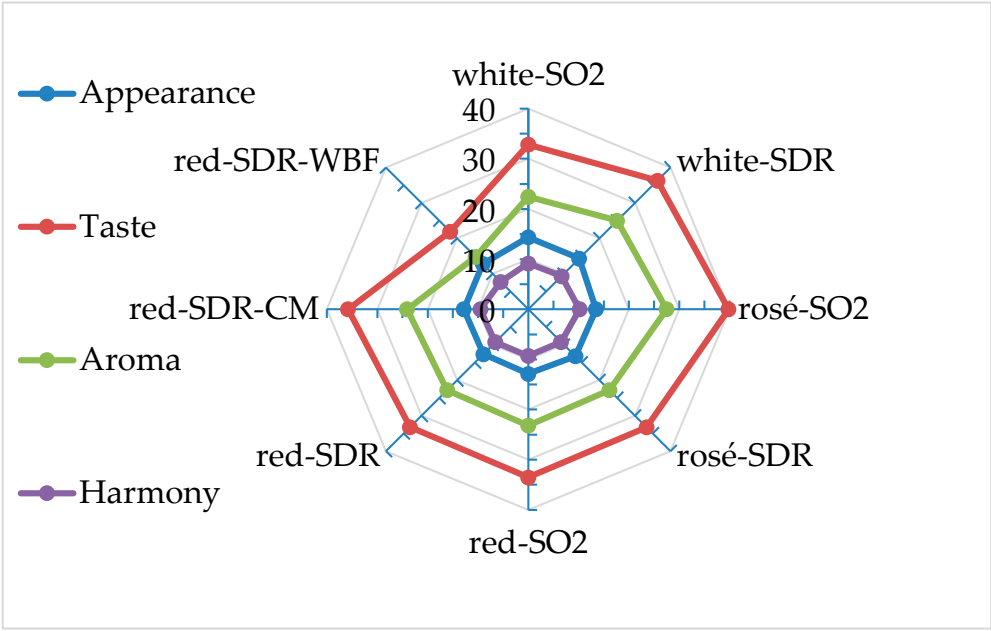
Results represent means of duplicate values ± standard deviation. * RT retention time, ** tr-trace amount, *** - not detected.

Taking a closer look at the composition of the phenolics present in the wines, one can observe that the main factor responsible for the increase in the phenolic content in the white and rosé wines with SDR is the gallic acid. The amount increases from 0.57 to 31.73 µg/mL and from 0.83 to 7.59 µg/mL for the white and rosé wines, respectively. Compounds such as gallocatechin (GCAT), kaempferol (KAE), myricetin (MYR), luteolin (LUT), p-coumaric acid (pCA), rosmarinic acid (RMA), and PRCS-B1 are not present in the white wine with SO_2 but present in the one with SDR. This fact suggests that these compounds are present in SDR. On the other hand, in red wine, the main phenolics quantified are PCB2, catechin (CAT), PCB3, PCB1, gallic acid (GA), epicatechin (EPI), quercetin (QUE), myricetin (MYR), and kaempferol (KAE), having a concentration in red wine with SO_2 higher than 1 µg/mL.

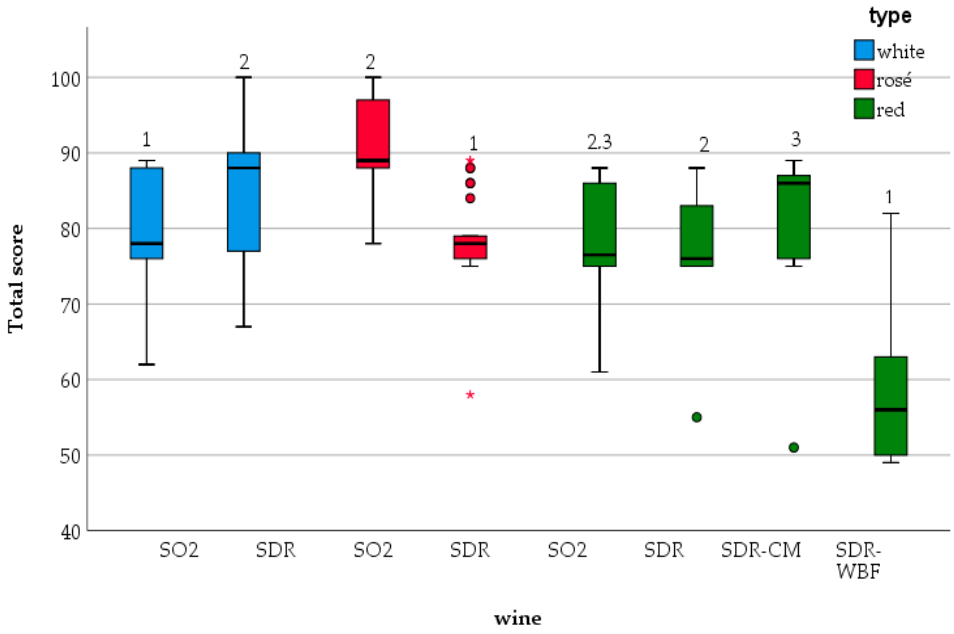
A possible explanation for the decrease in the level of phenolics in red wine is that co-pigmentation phenomena are triggered by the addition of phenolics present in SDR. Co-pigmentation leads to the creation of new compounds formed by the complexation of anthocyanin present in wine with phenolics from SDR, which in turn are not quantified by the LC-MS. Tsai, Liu, and Huang [52] reported the formation of anthocyanin–phenolic acid complexes in red wines. The decrease in catechin, gallic acid, caffeic acid, and epicatechin observed in the present study could be due to the complexation phenomena. Tsai, Liu, and Huang [52] reported ferulic acid, caffeic acid, catechin, chlorogenic acid, and gallic acid as candidates for a co-pigmentation complex with anthocyanin, whereas Zhang, Wang, Yang, Li, Niu, Shi, and Han [50] reported caffeic acid and rosmarinic acid and Darias-Martín et al. [53] reported catechin and caffeic acid as co-pigments in wine. Contrary to red wine, in white and rosé wines, the co-pigmentation phenomenon is not intense, resulting in the preservation of the phenolic compounds in their initial form.

3.4. Effect on Sensorial Attributes of Wines

The results from the sensory evaluation of the wines are shown in Figure 3. Between the two white wines, the one with SDR was preferred in terms of taste and aroma, while the other parameters had the same score (Figure 3a). Observing the results of the sensory test, we noticed that for rosé wines, the vinification using sulfuric anhydride gave a wine that was evaluated with higher scores by the tasters (appearance, aroma, and taste). Finally, the use of SDR in red wines, especially combined with pre-fermentation cold maceration, gave very good results in terms of taste and aroma compared to the rest of the red wines. On the contrary, the addition of SDR in whole bunch fermentation was rated with the lowest values by tasters for aroma and taste. According to earlier reports, WBF raises the concentration of methoxypyrazines, which may have a detrimental effect on wine quality because of the increased green features [31]. The low sensory scores may have been caused by this fact as well as the greater volatile acidity values and higher reduced sugar content (Table 1) of red SDR-WBF. According to Ribéreau-Gayon, Dubourdieu, Donèche, and Lonvaud [1], volatile acidity is difficult to detect below 0.72 g/L, but above this value, wine flavor and aroma begin to deteriorate, and at levels of 0.90 g/L and above of acetic acid, the wine develops a pronounced “harsh”, “bitter”, and “sour” aftertaste. However, Parga-Dans et al. [54] report that subjective intrinsic factors or sensory perceptions differ from objective intrinsic factors or laboratory analysis in determining wine quality. In their research, the wine’s global quality score has a significant statistical dependence on physicochemical variables, especially SO_2 levels and volatile acidity and on the sensory variables of intensity and persistence (e.g., in our case, rosé and red with SO_2 had a higher volatile acidity and total SO_2 concentration). Similarly, the literature reports that the use of alternative preparations to SO_2 causes significant changes in sensory attributes, but the changes depend on the treatment used during vinification. Santos et al. [55] demonstrated that high-pressure treatments as alternatives to SO_2 in red wines significantly altered aroma and taste perception. In addition, Fia, Menghini, Mari, Proserpio, Pagliarini, and Granchi [11] showed no significant differences between pairs of samples treated with an unripe grape extract and chitosan during oak aging.



(**a**)



(**b**)

Figure 3. Effect of SDR on the (**a**) main sensorial attributes of wines; (**b**) variation in the total evaluation score of wines. Different superscript numbers within the type of wine indicate significant differences ($p < 0.05$) amongst the means ranks, as determined by Mann–Whitney U test or Kruskal–Wallis test ($p < 0.05$) followed by Dunn's post hoc tests (with Bonferroni correction). The clustered boxplot represents the range of scores from 30 panelists. * represents outliers.

Panelists gave higher scores to the white wine made with SDR than SO_2, but the opposite was the case for rosé wines (this despite the presence of outliers). Vinification with SDR and SO_2 resulted in red wines with similar total scores. The combination of cold maceration and SDR yielded the highest score among the produced red wines, whereas whole bunch fermentation yielded the lowest score. The addition of SDR produced white and red wines with better sensorial attributes than SO_2-added wines, but not in the case of rosé wines. This, in our opinion, relates to the fact that the total evaluation score is a

complex perception, and the outliers or the extreme values deserve special attention in the evaluation of wines.

4. Conclusions

Wines (white, rosé, and red) with SDR showed similar behavior to SO_2-added wines regarding alcoholic fermentation in all of the performed tests. White-SDR and red-SDR-CM showed a better total sensory score than white and red wines with SO_2, whereas the opposite was observed in rosé ones. Red-SDR-WBF was less appreciated among red wines in terms of aroma, taste, and harmony, maybe because of its higher volatile acidity. Nevertheless, except for red-SDR-WBF, acceptable wines were produced.

SDR white and rosé wines have higher total phenolics, total flavonoids, and antioxidant capacity, while in red-SDR, the complex formation ability of anthocyanin after reacting with other phenolic compounds results in new products that may have a lower antioxidant capacity than the initial compounds that contain them. In general, using SDR was more efficient in keeping high values of TPC, TFC, and antioxidant activity than SO_2, maybe because of its plant phenolic content.

In conclusion, SDR represents a promising possibility for partial or total substitution of SO_2 by a natural product that needs further exploration. However, color evolution and sensory analyses, combined with volatile characterization and evaluation of the antioxidant capacity, should be repeated in the long term (12–18–24 months), since the results for white-SDR wine after six months of storage were very promising.

In addition, it is important to investigate the effectiveness and duration of future protection of SDR wines regarding spoilage microorganisms and potentially experiment with the partial use of both SO_2 and SDR in order to decrease the total SO_2 concentration in wines. The partial or total substitution of SO_2 by SDR should be carried out in future studies taking into consideration the vintage effect in wines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages10040110/s1, Figure S1. HPLC-PDA chromatographic profile of the phenolics determined in wine samples (black: red-SDR; pink: red-SO2). The numbers above the peaks correspond to the numbers of phenolics listed in Table 3; Figure S2. LC-MS chromatogram of selected phenolics determined in wine sample (red-SO2) in SIM mode for characteristics m/z. The numbers above the peaks correspond to numbers of phenolics listed in Table 3.

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