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Evaluation of grape stems and grape stem extracts for sulfur dioxide replacement during grape wine production

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A R T I C L E I N F O

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A B S T R A C T

Sulfur dioxide (SO_2), the main preservative in wine, may affect the sensory properties of the wines, as well as cause allergic reactions and headaches in sensitive people. The aim of this work was to evaluate the replacement of SO_2 in Tempranillo wines with Mazuelo grape stem products. Five Tempranillo red wines were elaborated: positive control (60 mg/L SO_2); negative control with no preservatives; Mazuelo extract (200 mg/L); Mazuelo extract combined with SO_2 (100 mg/L + 20 mg/L); and Mazuelo stem (400 mg/L). The oenological parameters, antioxidant capacity, total phenolic (TP), total flavonoids (TF) and total anthocyanins (TA) contents were determined. Additionally, individual phenols were analyzed by HPLC-DAD-FLD. The spectrophotometric analyses showed that the wines had similar antioxidant capacities and concentrations of TP and TF. However, TA was more affected by the lack of SO_2 as anthocyanins presented higher concentrations in positive control samples. The concentrations of individual phenols followed a similar path in all samples. Wines containing sulfites were more similar than the other treatments. However, these similarities were not reflected on the sensory analysis performed, as triangle test did not show differences between the wine with extract addition and the positive control wine. Therefore, Mazuelo stem extract could be a possible strategy for SO_2 replacement. Nevertheless, further studies are necessary to confirm the potential of grape stem extracts as wine preservative.

1. Introduction

Sulfur dioxide (SO_2) is the main preservative in wine as it has multiple positive effects on the winemaking process (antiseptic, antioxidant, antioxidasic, solubilizing activity). However, SO_2 can cause allergic reactions in sensitive individuals. Moreover, wine with SO_2 can cause mild symptoms, like headaches, in consumers with liver enzymatic deficiency or alterations in the metabolism of B1 vitamin (Giacosa et al., 2018). In the last decade, wine producers strive to replace or reduce SO_2 in this beverage to strengthen wine image as a natural product and to avoid health issues in sensitive individuals. According to European Union (EU) legislation, wine producers have to indicate in wine labels the phrase "contains sulfites" if the amount of this additive is higher than 10 mg/L (European Union, 2003). Moreover, the World Health Organization (WHO) established a daily ingestion limit of 0.7 mg/kg of body weight (WHO, 1998). The search for alternative additives to SO_2, with antioxidant and antimicrobial activity, in the production of wines is challenging. Most of SO_2 substitutes have antimicrobial and/or antioxidant effects, although, to our knowledge, there is no preservative as effective as SO_2 in wine production. Concerning antimicrobial activity, the main compounds permitted by the EU and active against Gram-positive bacteria or yeast growth are dimethyl dicarbonate, lysozyme and sorbic acid (Ancín-Azpilicueta et al., 2016; Lisanti et al., 2019). In this regard, a grapevine cane extract with 99% content of stilbenes also showed

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promising properties against wine-related spoilage microorganisms in a wine matrix (Gutiérrez-Escobar et al., 2021). Other compounds such as chitosan or colloidal silver complexes (Izquierdo-Cañas et al., 2012; Petrova et al., 2016), or even purely physical methods such as pulsed light or high hydrostatic pressure (Christofi et al., 2020; Escott et al., 2021) have also proven their efficacy as antimicrobial agents in winemaking. However, many of the wines thus obtained have shown (or could show, if studied) clear signs of oxidation, mainly due to the absence of antioxidant capacity of these methods or additives. Therefore, even if these methods allow reducing the necessary levels of SO_2 in wines, they only serve as complementary to the use of SO_2, and no as substitute for it.

There are different researches to determine the antioxidant activity in wine of several natural products. Raposo et al. (2016) added hydroxytyrosol to red wines before bottling and observed that after 6 months of bottle storage, wines with this phenol were more oxidized than wines with SO_2. Glutathione has also been proposed for SO_2 replacement in wines due to its antioxidant activity, although it can lead to the degradation of malvidin-3-glucoside in red wines as a consequence of oxidation reactions (Gambuti et al., 2017), thus limiting its potential use in red winemaking.

In the last years, plant extracts from different wastes of agri-food and winemaking industries have received attention due to its importance in different fields, including nutraceuticals, functional foods, food additives and cosmetics (Jiménez-Moreno et al., 2020; Galanakis, 2021; Galanakis et al., 2018). These extracts are rich in phenolic compounds with recognized antioxidant and antimicrobial action, and therefore, they rise as promising candidates to replace SO_2 in winemaking. In this regard, some authors have obtained white wines with a sensory evaluation similar to that of wines made with SO_2, replacing this preservative with extracts obtained from winery byproducts such as grape seeds and stems (Marchante et al., 2019b). The current worldwide grape production is in the millions of tons, generating important amounts of byproducts with bioactive compounds showing significant antioxidant activity, like grape stems (Gouvinhas et al., 2019). Therefore, it could be very interesting to search for different applications of this byproduct in food, cosmetic and pharmacological industries. In this sense, several research works have shown that the grape stem's components can prevent degenerative diseases (cancer, cardiovascular and neurodegenerative diseases) (Quero et al., 2021; Tsao, 2010), and also have antimicrobial and anti-inflammatory properties (Salehi et al., 2019).

Therefore, the aim of this work was to study the physicochemical properties of Tempranillo (*Vitis vinifera* L. cv. Tempranillo) red wines in which sulfur dioxide was replaced by different stem products from Mazuelo grape (*Vitis vinifera* L. cv. Carignan). In this case, the results were compared with wines made with a combination of extracts and SO_2, a positive control wine with only SO_2, and a negative control sample free of preservatives.

2. Materials and methods

2.1. Winery byproduct extracts preparation and chemical characterization

Grape stem extracts from Mazuelo variety (*Vitis vinifera* L. cv. Carignan) obtained using GRAS (Generally Recognized as Safe) solvents were used. The grapes were harvested in 2018 in Navarra (north of Spain) in the optimum stage of ripeness for winemaking. Grapes were separated from the stems, and the latter were oven dried at 25 °C, ground and sieved ($\varphi < 0.3$ mm). The extraction process consisted in maceration of the grape stem powder in 50% (v/v) ethanol/water (1:100, w/v ratio) at 40 °C for 24 h in an orbital shaker at 250 rpm. Next, the samples were centrifuged (5000 RCF), filtered in paper filter and freeze-dried. The phenolic characterization protocol of the grape stem extract can be found in a previous work (Esparza et al., 2021). In addition, grape stem biomass and the resulting extract were chemically characterized using the National Renewable Energy Laboratory (NREL) official protocols, and included ethanol extractives (NREL/TP-510-42619), structural carbohydrates (namely cellulose and hemicellulose), Klason and acid soluble lignin (NREL/TP-510-42618) and ash content (NREL/TP-510-42622). Fat content was determined according to the official AOAC method (nº 920.39). Total proteins content was performed using a Kjeldahl distiller (Kjeltec 8400 Analyzer, FOSS, Hilleroed, Denmark) by quantification of nitrogen after samples digestion and estimated by N conversion factor of 6.25. Moisture was determined gravimetrically using a moisture analyzer (MAC 50/1/NH, RADWAG, Radom, Poland).

2.2. Winemaking

The wines were made in the facilities of the Navarra Viticulture and Oenological Research Station (EVENA). The winemaking process began by destemming and crushing the 2019 vintage Tempranillo grapes (*Vitis vinifera* L. cv. Tempranillo). Then, the mixture of must, seeds and skins was transferred to 50 L stainless steel tanks. The different treatments applied were: positive control (PC) with 60 mg/L of SO_2; negative control (NC) free of preservatives; Mazuelo extract (ME) with 200 mg/L of this extract; Mazuelo extract mixed with SO_2 (MM) with an initial addition of 100 mg/L of the extract (reduced doses of SO_2 were added later, in advanced winemaking stages); and ground Mazuelo stem (MS) with 400 mg/L of stem powder (raw material). The SO_2 was added in the form of potassium metabisulfite solution to reach the aforementioned concentrations. Next, all samples were inoculated with commercial *Saccharomyces cerevisae* var. *bayanus* yeasts (Oenoferm® Be-Red, Erbslöh, Geisenheim, Germany). The fermenting must was pumped over periodically to help the maceration. Alcoholic fermentation took place at controlled temperatures varying from 25 to 27 °C. When the density was close to 992 kg/L, the alcoholic fermentation ended and wines were racked to 25 L stainless steel tanks. The pomace was pressed, and the resulting wine was mixed with the free run wine. The malolactic fermentation started by inoculating commercial lactic acid bacteria (PN4 MBR *Oenococcus oeni*, Lallemand, Aurillac, France). After the malolactic fermentation, wines were racked to 16 L plastic bottles and the SO_2 content was corrected in the positive control wines (to 60 mg/L) and in the wines with a combination of extract and sulfur dioxide (20 mg/L SO_2). Nothing was added to the other samples during winemaking. After a month, another racking took place to remove settled lees. Before bottling, the concentrations of SO_2 were corrected again to the same concentrations. The wines were bottled in 750 mL Antique Green glass bottles and stored at cellar conditions (18–20 °C) during a year. In order to analyze the evolution of the different wine parameters throughout the winemaking process, samples were taken from the tanks before alcoholic fermentation (M), when 50% of the sugars in the must were consumed (50%AF), after the alcoholic fermentation (AF), after the malolactic fermentation (MLF), and after one year of bottle aging under cellar conditions (YB). The winemaking process of the different treatments under study were made in duplicate in separate tanks. These samples were frozen and kept at −20 °C until the analysis.

2.3. Antioxidant capacity determination and oenological parameters of wines

For the ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, a 7 mM solution ABTS in ethanol with 2.45 mM potassium persulfate was prepared. Then, this mixture reacted for 16 h at room temperature in darkness. Next, the solution was diluted in methanol until reaching an absorbance of 0.70 ± 0.02 at 734 nm. The concentration of the Trolox calibration curve ranged from 0.05 to 2.11 mM, and two quality control standards at intermediary concentrations were prepared. Musts were diluted with methanol to 1:7 and wine samples were diluted 1:15. For the analyses, 30 μL of each sample were mixed with 2.97 mL of $ABTS^{\bullet+}$ solution, and after 30 min in darkness, the

absorbance was read at 734 nm. For the DPPH assay, a DPPH (2,2-diphenyl-1-pycrilhydracyl) solution of 0.09 mM was prepared, and the absorbance was adjusted to 0.9 ± 0.05 at 517 nm. Trolox calibration standard solutions ranging from 0.05 to 0.62 mM were prepared along with two quality control standards at intermediary concentrations. For the DPPH antioxidant capacity determination, 150 μL of samples were mixed with 2.85 mL of reagent, and the cuvettes were kept in darkness for 30 min. The absorbance was read at 517 nm. The dilutions applied to the samples were the same as for the ABTS method. Finally, for the FRAP method performance, a solution was prepared by mixing a 300 mM acetate buffer, a 2,4,6-tris(2-pyridyl)-s-triazine 9.99 mM solution and a $FeCl_3 \cdot 6H_2O$ 20 mM solution, in 10:1:1 proportion. Next, 2.85 mL of this solution were mixed with 150 μL of sample and the absorbance was read at 595 nm after 30 min in darkness. The sample dilutions with methanol were 1:15 for musts and 1:25 for wine samples. The Trolox calibration curve ranged from 0.05 to 1.18 mM, and two quality control standards at intermediary concentrations were also prepared. All spectrophotometric analyses were performed in a 7315 Jenway UV-Vis spectrophotometer (Staffordshire, UK). In all cases, the samples were analyzed in triplicate, and the results were presented as mmol of Trolox equivalent/L.

General parameters of the wines were measured before bottling following the OIV (International Organization for Vine and Wine) compendium of international methods of wine and must analysis in a laboratory accredited by ENAC (UNE-EN ISO/IEC 17025:2017).

2.4. Spectrophotometric determination of total anthocyanins, total phenolics and total flavonoids content

The total monomeric anthocyanins concentration (TA) was determined according to AOAC method: buffer solutions at pH 1 (0.025 M potassium chloride buffer) and at pH 4.5 (0.4 M sodium acetate buffer) were made and used to dilute the samples to the appropriate concentrations (musts 1:7, wines 1:20). The absorbance was read at 520 and 700 nm for each pH. The results were expressed as mg of malvidin-3-glucoside/L.

For the total phenolic content determination (TP), the samples were previously diluted with methanol to concentrations of 1:2 in must, and 1:4 in the rest of samples. The calibration curve was prepared using gallic acid as standard with concentrations ranging from 0.038 to 0.956 g/L, and control standards were also prepared at different concentrations. For the analyses, aliquots of 0.1 mL of sample or standard solution were transferred to a 15 mL tube and mixed with 0.5 mL of Folin reagent, 7.9 mL of deionized water and 1.5 mL of Na_2CO_3 (20%, w/v). Samples were left in darkness for 2 h and, then, the absorbance of each sample was read at 765 nm. All the samples were analyzed in triplicate and the results were expressed in g of gallic acid/L.

Finally, for the determination of total flavonoid content (TF), a solution of 2% $AlCl_3$ in 5% acetic acid was prepared. Quercetin was used as standard for the calibration curve, with concentrations ranging between 3 and 25 mg/L, and control standards were prepared at three different concentrations. Must samples were not diluted and the wines were diluted 1:7 with methanol. For the analyses, 0.75 mL of sample or standard were combined with 0.75 mL of reagent. The mixture was left in darkness for 30 min before reading the absorbance at 420 nm. All the samples were analyzed in triplicate and the results were expressed as mg of quercetin/L.

2.5. Identification and quantification of phenolic composition by HPLC-DAD-FLD

The identification and quantification of the phenolic compounds was performed with a high-performance liquid chromatograph (HPLC) equipped with two 510 pumps, a 717 Plus autosampler, a 996-photodiode array detector and a 474-scanning fluorescence detector (Waters Div., Milford, MA, USA). The column used was a Zorbax Eclipse Plus C18 reversed-phase (250 × 4.6 mm, particle size 5 μm, Agilent, Santa Clara, CA, USA). Samples were centrifuged and, then, 3 mL of sample were freeze-dried in a Cryodos-50 freeze-drier (Telstar, Barcelona, Spain) and reconstituted with 0.6 mL of methanol:water (50:50 v/v) with 1% HCl. Wine samples were also injected in their original concentration to quantify anthocyanins and caftaric acid. The mobile phases were deionized water with 2% acetic acid (HPLC grade) (phase A), and methanol (HPLC grade) with 2% acetic acid (phase B). The linear gradient scheme was as follows: [time (min); phase A (%)] [0; 95]; [0.5; 95]; [10; 90]; [25: 80]; [35; 70]; [50; 50]; [60; 30]; [65; 10]; [70; 5]; [75; 95]. The column temperature was 30 °C, the injection volume was 40 μL and the flow rate was 1 mL/min. The HPLC quality solvents were from Scharlab (Barcelona, Spain) and PanReac AppliChem (Barcelona, Spain). The identification of the phenolic compounds was performed by the double coincidence of the UV-Vis spectrum at the characteristic wavelength of each compound, and the retention time of its corresponding standard. (+)-Catechin, (−)-epicatechin and procyanidin A2 were identified and quantified by fluorescence with excitation and emission wavelengths of 290 nm and 315 nm, respectively. For quantitation, a calibration curve was prepared weighing from 50 μg to 200 μg of each standard, according to the concentrations previously estimated in the samples. All the anthocyanins were quantified as malvidin-3-glucoside. Quercetin-3-glucuronide was quantified as quercetin-3-glucoside. All the samples were analyzed in triplicate. (−)-Epicatechin, malvidin, malvidin-3-glucoside and procyanidin A2 were from Extrasynthese (Genay, France), while the other standards were from Sigma-Aldrich (Madrid, Spain). All the standards injected in the identification step are compiled in Table S1 of the Supplementary Material.

2.6. Triangle sensory analysis

In order to determine sensory differences between the wines after one year of storage in bottle under cellar conditions a triangle test was performed. The sensory analysis was conducted according to the method described by the ISO 4120:2021 standard (European Committee for Standardization, 2021). The wines were tasted by 11 expert panelists belonging to EVENA (Navarra Viticulture and Oenological Research Station) staff, in a standard sensory analysis laboratory (ISO 8589:2010) at their facilities. The assessment took place in separate booths with controlled temperature (20–22 °C) and light conditions.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviation. When the concentration of a certain compound was below the limit of quantification (LOQ) of the technique and less than 25% of data were 'not detected', a left-censored data completion model was applied (Helsel, 2011). Later, a principal component analysis (PCA) was applied with the aim of clustering the variables under study in the wines according to their evolution in the winemaking process. Data analyses were performed with the packages implemented in the R.4.1.1 software (R Core Team, 2013).

The triangle sensory analysis results were examined applying the ISO 4120:2021 norm considering a significance level (α) of 0.01 (European Committee for Standardization, 2021).

3. Results and discussion

3.1. Extracts characterization and oenological parameters of the wines

The chemical composition of dry stem powder and extract was determined. The general composition of the stem is summarized in Table S2 in Supplementary Material. The fresh stems had 70% moisture, but after drying it was 8%, which is important to maintain and preserve the raw material (humidity lower than 10%). The major fraction of the grape stems was lignin, representing 32.84%, and their polysaccharides composition was 47.7%, with a predominance of the cellulose fraction,

with 31.34% of glucose content. Other constituents' contents such as protein, ash and fat represented 5.72%, 1.85% and 0.45% of stems dry weight, respectively. The general chemical composition of grape stems based on the bibliographic data was reported in Blackford et al. (2021) including cellulose (19.6–37.9%), hemicellulose (9.8–35.3%), lignin (12.8–47.3%), protein (4.9–11.2%), and ash (3.9–11.2%). Our values were in line with those previously reported. Furthermore, it should be noted that the comparison of published data is difficult, as the chemical composition can be affected by different factors such as grape variety, ripeness, harvesting and storage processes, as well as the characterization methodologies used.

Regarding the stem extract composition (Table S2 in Supplementary Material), lignin was also the most representative fraction with 30.62%, followed by cellulose (19.68%). Hemicellulose and fat were not detected in the extracts. Ash content was 3.14% and proteins 2.67%. In a previous study (Esparza et al., 2021), we reported the phytochemical characterization of the same Mazuelo stem extract compared to different varieties of grape stem extracts (Garnacha, Mazuelo, Tempranillo, Chardonnay and Cabernet Sauvignon). Mazuelo stem extract had the highest antioxidant capacity, total phenolic and total flavonoids contents among all the grape stems analyzed. The antioxidant capacity obtained for this extract was 1.51 ± 0.17, 0.80 ± 0.09; and 0.81 ± 0.08 mmol of Trolox equivalent/g of extract by ABTS, DPPH and FRAP assays, respectively. The total polyphenol content was 172 ± 6 mg of gallic acid equivalent/g of extract and the total flavonoid concentration was 2.8 ± 0.1 mg of quercetin equivalent/g of extract. Moreover, 11 phenolic compounds were identified (gallic, ellagic and caftaric acids, catechin, procyanidin B1, quercetin, quercetin-3-derivative, *trans*-resveratrol, ε-viniferin, malvidin-3-glucoside and an unknown anthocyanin) by HPLC-DAD. The most abundant compounds were catechin (3.5 ± 0.3 mg/g of extract) and procyanidin B1 (2.5 ± 0.2 mg/g of extract), while the lowest concentration was found for ellagic acid (54 ± 5 μg/g of extract).

The oenological parameters for all the experimental wines were within a normal range of concentration, and there were not discrepant results among the treatments. This indicates that the SO_2 replacement did not negatively impact these parameters. These results are presented in Table 1.

Table 1
Oenological parameters of the experimental wines at the time of bottling.

Analysis	NC	PC	ME	MM	MS
Alcoholic Content (% v/v)	15 ± 0	15 ± 0	15 ± 0	15 ± 0	15 ± 0
Titratable acid (g/L)[a]	4.4 ± 0.2	4.3 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	4.0 ± 0.1
pH	4.2 ± 0.2	4.3 ± 0.1	4.4 ± 0.0	4.5 ± 0.0	4.4 ± 0.0
L-Malic acid (g/L)	<QL (0.2)	<QL (0.2)	<QL (0.2)	<QL (0.2)	<QL (0.2)
Total reducing sugars (g/L)	<QL (1.0)	1.0	<QL (1.0)	<QL (1.0)	<QL (1.0)
Volatile acidity (g/L)[b]	0.58 ± 0.06	0.53 ± 0.01	0.72 ± 0.03	0.68 ± 0.04	0.68 ± 0.01
Free SO_2 (mg/L)	<QL (7.0)	20.5 ± 4.9	<QL (7.0)	<QL (7.0)	<QL (7.0)
Total SO_2 (mg/L)	<QL (15.0)	33 ± 3	<QL (15.0)	<QL (15.0)	<QL (15.0)
Total Phenolic Index (Abs/cm)	46 ± 5	55 ± 4	50 ± 1	51 ± 1	49 ± 3
Optical density 420 nm (Abs/cm)	3.6 ± 0.4	3.5 ± 0.1	4.1 ± 0.2	4.2 ± 0.2	4.0 ± 0.2
Optical density 520 nm (Abs/cm)	4.2 ± 0.8	4.0 ± 0.3	4.2 ± 0.2	4.3 ± 0.1	4.2 ± 0.2
Optical density 620 nm (Abs/cm)	1.2 ± 0.2	1.2 ± 0.0	1.4 ± 0.1	1.5 ± 0.0	1.4 ± 0.1
Calcium (mg/L)	53 ± 12	59 ± 6	62 ± 4	63 ± 3	60 ± 3
Potassium (mg/L)	1429 ± 335	1779 ± 366	2110 ± 31	2182 ± 73	1759 ± 122
Magnesium (mg/L)	92 ± 6	92 ± 4	100 ± 0	103 ± 0	95 ± 1

NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder.
QL: Quantifiable Level.
[a] Expressed as tartaric acid.
[b] Expressed as acetic acid.

3.2. Wine spectrophotometric characterization

The must and wine antioxidant activity are presented in Table 2. Wines and must are complex matrixes that owe their antioxidant properties to a combination of bioactive compounds, mainly phenolic compounds. Therefore, to quantify their activity accurately it is advisable to apply methods with different mechanisms of action (Marchante et al., 2019b). The antioxidant capacity determination of the samples was performed applying three methods with different action mechanisms: radical scavenging methods (ABTS and DPPH assays) and reducing antioxidant power method (FRAP). The antioxidant capacity of the musts was low probably because the phenolic compounds, present in grape skins and seeds, take time to be extracted by the must. The antioxidant capacity was higher in wine after alcoholic fermentation (AF) than halfway through fermentation (50% AF) in all cases. Thereafter, the antioxidant capacity of all wines remained constant or slightly decreased, probably due to chemical modifications of the phenolic compounds, more specifically oxidation reactions (Garrido and Borges, 2013). In general, positive control samples showed the highest values of antioxidant capacity at the end of the study, independently of the method used. This could be due to the solubilizing effect of SO_2, as it helps the diffusion of phenolic compounds from the skins to the must (Giacosa et al., 2018; Jackson, 2000a). However, Garaguso and Nardini (2015) found no significant differences between the different samples, when comparing the antioxidant activities of commercial SO_2-free organic wines with conventional ones. Esparza et al. (2020) also studied the reduction of SO_2 in wines with grape stem extract and a commercial extract from vine wood and found no differences in the antioxidant

Table 2
Antioxidant activity (mmol Trolox equivalents/L) Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines with different preservative treatments determined by different methods of analysis.

Assay	Sample	NC	PC	ME	MM	MS
ABTS	M	2.6 ± 0.4	3.1 ± 0.2	3.1 ± 0.3	3.0 ± 0.2	2.7 ± 0.3
	50%AF	12.8 ± 0.7	14.6 ± 0.6	13.9 ± 0.8	15.4 ± 0.7	14.0 ± 0.7
	AF	15.6 ± 0.9	17.4 ± 1.2	15.9 ± 0.7	17.0 ± 1.2	16.9 ± 0.8
	MLF	14.7 ± 1.3	16.2 ± 1.4	14.1 ± 0.4	15.5 ± 0.6	15.1 ± 0.6
	YB	13.5 ± 2.3	16.9 ± 0.5	14.4 ± 0.8	15.2 ± 0.4	14.6 ± 1.0
DPPH	M	1.1 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.3 ± 0.0	1.2 ± 0.1
	50%AF	5.7 ± 0.2	6.2 ± 0.6	5.9 ± 0.1	6.4 ± 0.4	6.0 ± 0.4
	AF	6.8 ± 0.3	7.7 ± 0.1	7.2 ± 0.3	7.6 ± 0.5	7.2 ± 0.2
	MLF	7.3 ± 0.3	8.0 ± 0.2	7.4 ± 0.3	7.7 ± 0.2	7.8 ± 0.3
	YB	6.7 ± 0.9	8.5 ± 0.1	7.4 ± 0.3	7.9 ± 0.2	7.4 ± 0.4
FRAP	M	1.7 ± 0.2	2.6 ± 0.4	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
	50%AF	8.7 ± 1.1	10.3 ± 0.5	9.3 ± 0.2	9.6 ± 0.6	8.7 ± 0.3
	AF	10.5 ± 0.2	12.6 ± 1.1	10.3 ± 0.6	11.7 ± 0.5	10.6 ± 0.4
	MLF	10.1 ± 0.9	11.5 ± 0.5	9.6 ± 0.4	10.2 ± 0.4	9.3 ± 1.0
	YB	9.2 ± 1.4	12.1 ± 1.0	9.8 ± 0.5	10.5 ± 0.2	9.9 ± 0.7

NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. M: must; 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

capacity between their experimental treatments. However, Christofi et al. (2020) found that wines subjected to high hydrostatic pressures with SO_2 concentration values lower than 60 mg/L showed reduced

values of total flavanols, anthocyanins and antioxidant activity after six months of storage compared to untreated samples. Table 3 shows the evolution throughout the winemaking process of total anthocyanins (TA), total flavonoids (TF) and total phenolic (TP) content. TA presented a higher extraction degree in positive control than in the rest of the samples. The maximum TA was reached at mid alcoholic fermentation in all the samples and subsequently decreased, probably due to their adsorption by suspended solids, polymerization with tannins, precipitation or chemical degradation (Ribérreau-Gayon et al., 2006). This reduction was less pronounced in positive control samples than in the other samples. Flavonoids were especially extracted during the first half of the alcoholic fermentation, probably because these compounds are mainly found in grape skins and are usually extracted by the aqueous phase (Boulton et al., 1999; Ribérreau-Gayon et al., 2006). Subsequently, TF showed a slight decrease in all the samples analyzed. Finally, TP increased until the end of alcoholic fermentation and, thereafter, hardly changed in the samples under study. The observed decrease in TF and TA, without observing hardly any impact on TP, could be due to the copigmentation phenomenon, in which flavonoids and anthocyanins can combine, changing their structures and becoming new phenolic compounds (Qian et al., 2017). After one year in bottle, the wines with added SO_2 (PC and MM) showed the highest concentrations of total anthocyanins, total flavonoids and total phenolic content (Table 3).

Fig. 1 shows the PCA of individuals and of variables obtained from the spectrophotometric data of all the samples analyzed. The winemaking process established the discrimination of the treatments in the PCA. As can be seen in Fig. 1A, musts (M) were positioned on the left because these samples showed low concentrations of all components analyzed (TA, TF, TP) and, therefore, also presented a low antioxidant capacity. The subsequent stages of the winemaking process were positioned clockwise. Wines at mid alcoholic fermentation (50%AF) presented the highest content, mainly of anthocyanins, but also of flavonoids, and this determined the position of these samples in the PCA graph. As winemaking progressed, TA and TF decreased, which determined the position of the wines after malolactic fermentation and bottle aging. On the other hand, all the treatments were clustered in the center of the PCA, but it was noticeable that the wine containing a mixture of SO_2 and extract was close to the positive control wine. Contrarily, the wines without SO_2 were more similar among themselves. Therefore, PCA could be an important tool to differentiate between wines made with or without added SO_2 by applying wine spectrophotometric analyses, which are very simple and fast. The PCA plot of variables showed the low correlation that exist between antioxidant capacity and total anthocyanin and flavonol content of the wines, while the total phenolic content was significantly correlated with antioxidant capacity (Fig. 1B). In a previous study of sulfur dioxide substitution in red wine conducted by Salaha et al. (2008), the antioxidant capacity of wines was strongly correlated with phenolic compounds, but also with flavonoids and anthocyanins. This discrepancy with regard to correlation of TA and TF with antioxidant capacity in both research works could be attributed to the different analytical methods used for quantification, as they used a chromatographic method for anthocyanin analysis, and a different spectrophotometric method for flavonoid determination than the one used in the present work. So, these results point out the need to complement the spectrophotometric results with chromatographic analyses of individual phenolic compounds, including flavonoids and anthocyanins.

Table 3
Evolution of total anthocyanins, total flavonoids, and phenolic content in Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines with different preservative treatments.

Assay		NC	PC	ME	MM	MS
Total Anthocyanins (mg malvidin-3-glucoside/L)	M	39 ± 11	77 ± 7	42 ± 0	44 ± 2	33 ± 4
	50% AF	337 ± 11	368 ± 19	381 ± 11	397 ± 30	381 ± 8
	AF	313 ± 38	366 ± 10	310 ± 7	349 ± 6	314 ± 8
	MLF	244 ± 14	272 ± 16	228 ± 9	244 ± 6	235 ± 13
	YB	107 ± 9	192 ± 15	113 ± 2	136 ± 3	112 ± 5
Total Flavonoids (mg of quercetin/L)	M	16 ± 1	15 ± 1	17 ± 1	17 ± 1	13 ± 2
	50% AF	90 ± 9	96 ± 5	96 ± 4	109 ± 8	101 ± 8
	AF	91 ± 7	104 ± 14	87 ± 10	97 ± 6	91 ± 7
	MLF	70 ± 4	79 ± 4	67 ± 2	74 ± 2	71 ± 2
	YB	54 ± 9	73 ± 6	60 ± 1	66 ± 2	59 ± 3
Total Phenolic Content (g gallic acid/L)	M	0.38 ± 0.03	0.46 ± 0.03	0.43 ± 0.01	0.41 ± 0.01	0.39 ± 0.02
	50% AF	1.52 ± 0.08	1.67 ± 0.07	1.57 ± 0.06	1.70 ± 0.09	1.59 ± 0.10
	AF	1.68 ± 0.06	1.79 ± 0.07	1.78 ± 0.05	1.95 ± 0.12	1.76 ± 0.06
	MLF	1.63 ± 0.15	1.86 ± 0.05	1.54 ± 0.01	1.59 ± 0.05	1.57 ± 0.08
	YB	1.37 ± 0.18	1.78 ± 0.10	1.50 ± 0.06	1.53 ± 0.07	1.45 ± 0.09

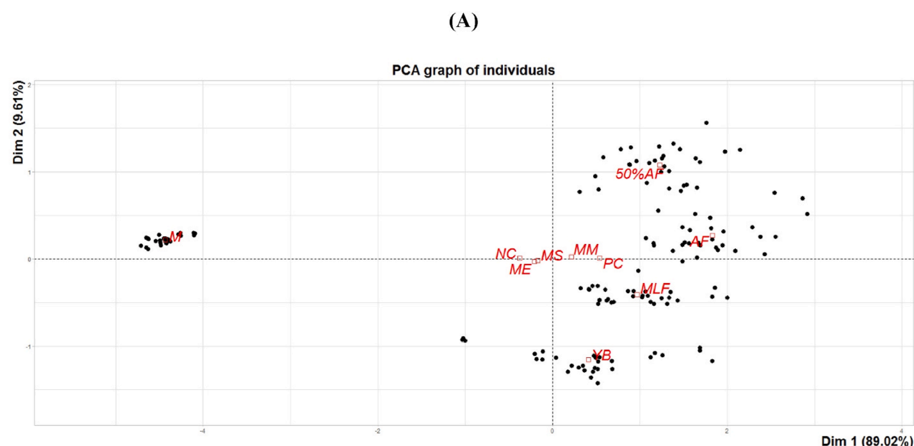
NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. M: must; 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

3.3. Wine HPLC-DAD-FLD characterization

Fig. 2 shows the evolution of the concentration of the anthocyanins identified in the wines. It was possible to identify malvidin, malvidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-glucoside. In addition, four unknown anthocyanins were identified and quantified by their spectra at 523 nm. The evolution of the monomeric anthocyanins during the winemaking process was similar to that observed in TA. Malvidin-3-glucoside was the major anthocyanin found in all the samples analyzed, which agrees with results from previous works (Marchante et al., 2019a; Salaha et al., 2008). Maximum extraction, in general, occurred at mid alcoholic fermentation with a subsequent decrease, due to polymerization, complexation with other phenolics, reaction with other compounds, precipitation and even destruction of the molecules (Ivanova et al., 2011). Besides the aforementioned reasons for their decrease, anthocyanins can also degrade into phenolic acids such as protocatechuic acid, which is the main metabolite of cyanidin-3-glucoside (Song et al., 2020). This could explain the disappearance of cyanidin-3-glucoside and anthocyanin 2 observed in the first stages of winemaking in all the wines. Another reason for the observed decrease in the concentration of anthocyanins could be that the extraction rate of these compounds was slower than their loss rate (Guadalupe and Ayestarán, 2008). However, although the decrease in the monomeric anthocyanins could be expected, their concentration in positive control wines after one year in bottle were, at least, 39% higher than those found in the rest of wines. This may be due to the chemical structure of anthocyanins, since they have a 2-phenyl-benzylpyrilium structure, which makes them susceptible to oxidation by hydrogen peroxide (H_2O_2), a substance naturally formed during the aging process (Gambuti et al., 2017; Zhao et al., 2022). The action of SO_2 as a H_2O_2 scavenger (Christofi et al., 2020; Gambuti et al., 2017) could explain why positive control wine had a higher anthocyanin content at the end of storage (see Table S3 in Supplementary Material).

Fig. 3 presents the evolution of the concentration of the phenolic acids identified in the samples under study. In the initial musts, only caftaric acid and chlorogenic acid were found, both derivatives of caffeic acid. Chlorogenic acid showed a progressive increase in concentration throughout the winemaking process in all the samples. All the wines

(A)



(B)

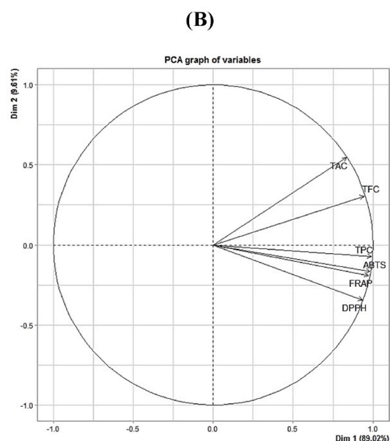


Fig. 1. Principal components analysis graphs of individuals (A) and of variables (B) of the Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines with different preservative treatments. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. M: must; 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled. TAC: total anthocyanins content; TFC: total flavonoid content; TPC: total phenolic content.

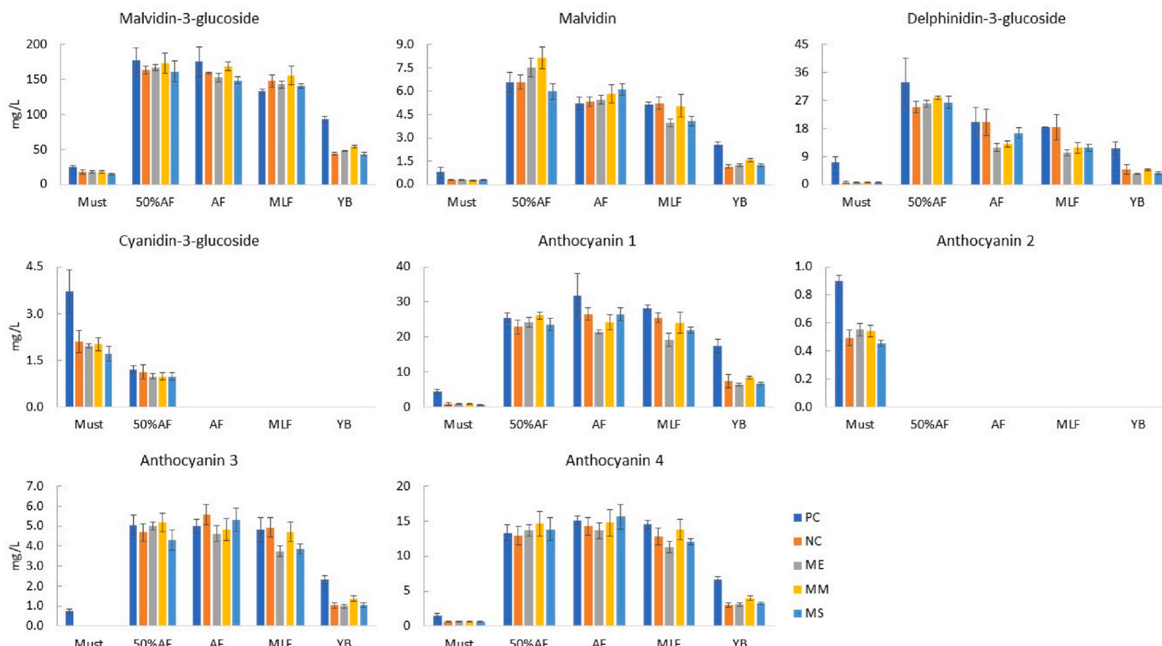


Fig. 2. Anthocyanins located and quantified in Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines organized by treatment and winemaking stage. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

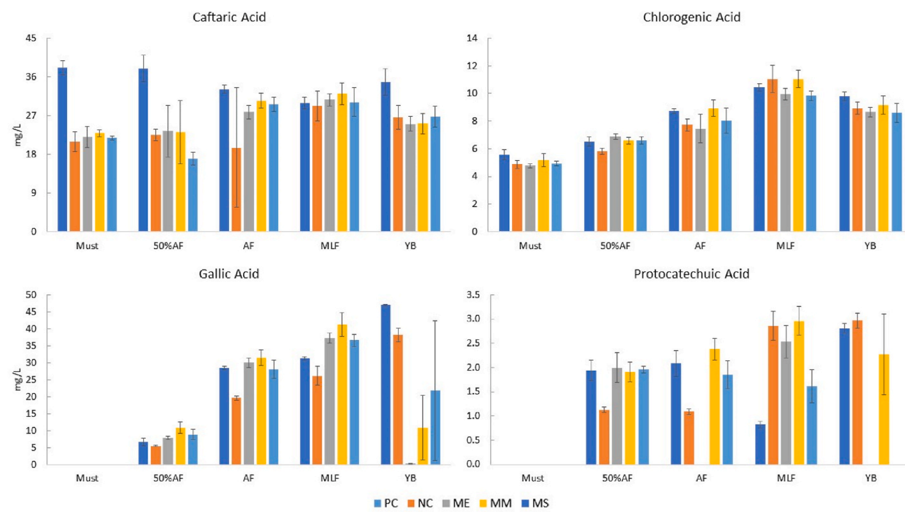


Fig. 3. Graphs presenting the concentration evolution of the acids in Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines through time. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

showed similar contents of this phenolic acid during winemaking. However, caftaric acid was found in higher concentration in the positive control samples during the initial winemaking stages, although at the end of the vinification process these values were very close to those of the rest of wine samples. Subsequently, during the bottle aging of the wines, the concentration of both acids remained constant or decreased slightly. The concentration of the hydroxybenzoic acids (gallic and protocatechuic acid) evolved irregularly throughout the winemaking process, especially in the wines obtained with addition of stem extract or powder. Gallic acid concentration increased during winemaking in all the samples, but during bottle aging this increase was only maintained in both control wines. Raposo et al. (2018) also found an increase in gallic acid content after the storage of wines treated with a commercial extract rich in stilbenes comparing with control wines (with SO_2). The release of this hydroxybenzoic acid from its galloylated precursor could explain this behavior. Gallic acid is also known to complex with anthocyanins and form more stable pigments, so, its reduction and variability may be an indicator of the existence of this type of reactions (Qian et al., 2017). The behavior of protocatechuic acid in the different wines was highly variable. This phenolic acid can be generated from various phenolic compounds, such as anthocyanins and quercetins (Song et al., 2020; Wang et al., 2019). Moreover, this transformation can occur both naturally and catalyzed by lactic acid bacteria, which would explain the increase in protocatechuic acid observed in some wine samples during malolactic fermentation (Song et al., 2020; Zhao et al., 2021). On the other hand, the oxidation reactions that can occur between these phenols and some metals and/or enzymes, as well as their polymerization, may also contribute to the variable behavior observed in the concentration of both hydroxybenzoic acids (Li et al., 2008; Zhang et al., 2018).

The evolution of the flavan-3-ols and derivates found in the wines is shown in Fig. 4. The concentration of these compounds increased mainly in the second half of the alcoholic fermentation in all the samples. Later, during bottle aging, the content of flavan-3-ols and derivates decreased in all the wines analyzed. The maximum values were observed at the end of alcoholic or malolactic fermentation, since these compounds take longer to extract than flavonols (Jackson, 2000b). Flavan-3-ols are found mainly in grape seeds and, there, they are protected by a lipid layer that slowly dissolves as the alcohol level increases, favoring the extraction of these compounds as the primary fermentation progresses (Ivanova et al., 2011). The decrease observed after one year of bottle

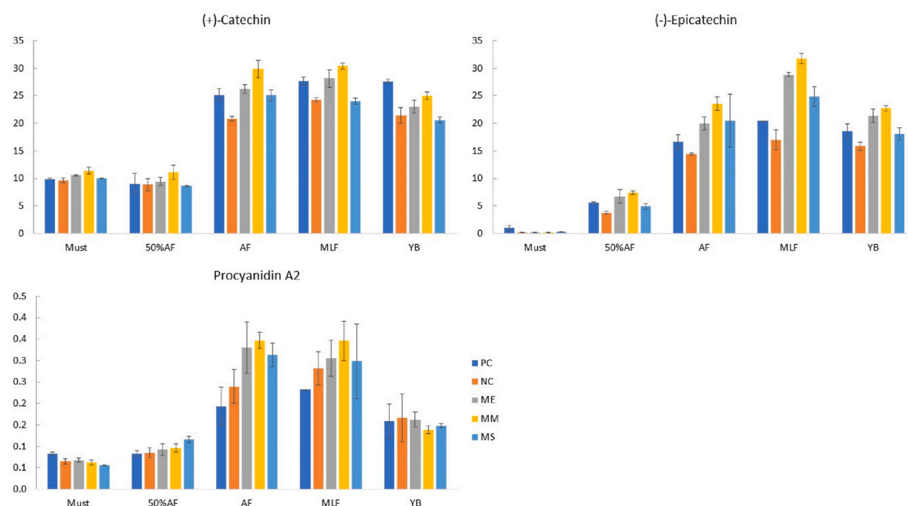


Fig. 4. Graphs presenting the concentration evolution of flavan-3-ols and derivates in Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines through time. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

aging could be explained by the binding and precipitation of these phenolic compounds along with proteins, remaining yeast cells and other wine components (González-Rompinelli et al., 2013; Jackson, 2000b). It should be noted that the samples containing stem extract or powder showed the highest concentrations of (−)-epicatechin at the end of the period under study, probably due to the hydrolysis of tannins present in the added grape stem products (Molino et al., 2020). These results are in agreement with Marchante et al. (2019b), who also found that when using grape stem and seed extracts as SO_2 substitutes, the flavan-3-ols content increased compared to wines with SO_2. When other substances without tannins in their composition, such as colloidal silver, have been used to replace SO_2, no statistical differences have been found in catechin concentration (Izquierdo-Cañas et al., 2012).

Fig. 5 displays the evolution of the concentration of quercetin and its derivatives in the analyzed samples. Quercetin content mainly increased during the second half of alcoholic fermentation in all samples, while its derivatives were more significantly extracted in the first half of this fermentation. The maximum concentration of quercetin-3-O-glucoside was reached halfway through alcoholic fermentation in all the samples, and subsequently decreased progressively so that after one year of bottle aging, this flavonoid was not detected in any of the wines. Even though the 3-O-glucoside forms of flavonols are the most abundant in grapes, their concentration can decrease due to acid hydrolysis, thus increasing the aglycones concentration (Favre et al., 2018), which is consistent with what was observed in this work. Regarding glucuronide flavonols, they usually have low concentrations in grapes, with the exception of quercetin-3-O-glucuronide (Castillo-Muñoz et al., 2009; Favre et al., 2018). Quercetin-3-O-glucuronide showed similar concentrations from mid-alcoholic fermentation onwards, so it seems less prone to degradation than quercetin or quercetin-3-glucoside. These results agree with those of Castillo-Muñoz et al. (2009), who found that quercetin-3-O-glucoside was more susceptible to acid hydrolysis than its 3-O-glucuronide counterpart. On the other hand, as mentioned previously, the reaction of flavonols with anthocyanins to form stable polymeric pigments could also be responsible for the observed decrease in the concentration of these compounds.

Fig. 6 shows the PCA graphs of individuals (A) and of variables (B), which summarizes the results obtained by HPLC analyses. Data for protocatechuic acid, anthocyanin 2 and cyanidin-3-glucoside were not considered for PCA because more than 25% of these data were 'not detected'. The initial must stage was again placed on the left side of the graph but, in this case, the other winemaking stages were arranged counterclockwise. Again, the positive control wine and the wine with a combination of extract and SO_2 were found in close positions on the right side of the graph, due to their higher contents of anthocyanins (especially the positive control) and other phenolic compounds such as flavan-3-ols or caftaric acid. Conversely, the wines obtained with no addition of SO_2 showed a lower phenolic content and, hence, their position on the left side of the graph. These results would indicate that grape stem extract could be a possible agent to replace SO_2 in wines but only partially, because this preservative has an important role as a solubilizing agent and as a protector against the chemical degradation of the previously extracted phenolic compounds.

Finally, the triangle tests performed to assess the sensory differences among the wines did not show clear discrepancies among the control wines and the wines free or with a reduced content of SO_2 (Table S4 of Supplementary Material). This would indicate that the physicochemical differences detected among the wines were not enough to alter their sensory profile and, furthermore, it could be stated that the grape stem extract did not negatively impact the sensory properties of the wine.

4. Conclusions

In this work, the influence on the phenolic composition of Tempranillo red wines of the partial or total substitution of SO_2 by different products from Mazuelo grape stems was studied. At bottling, the oenological parameters were not negatively affected by the SO_2 substitution. The spectrophotometric analyses evidenced that the final concentrations of total phenols and flavonoids were not very different among the treatments studied. However, the total anthocyanin content was lower in the wines obtained without adding SO_2. The PCAs obtained from analytical data showed that the samples containing SO_2 were more similar to each other than to the rest of the samples. However, the physicochemical differences detected among the wines did not translate into sensory perceptible differences. Thus, the partial or total substitution of SO_2 by grape stem extracts did not produce clear differences in the sensory quality of the wines, despite producing wines with lower phenolic content than the wines obtained with the addition of this preservative. Therefore, it seems viable to use these extracts as substitutes for SO_2, which would offer the double advantage of reducing the risks associated with the consumption of this additive, and of contributing to the circular economy, allowing the valorization of the byproducts generated in the cellar itself. However, before their implementation in wineries, additional studies should be carried out to demonstrate the preservative action of these extracts in wines subjected to some kind of stress or stored for longer periods. This will help adjust

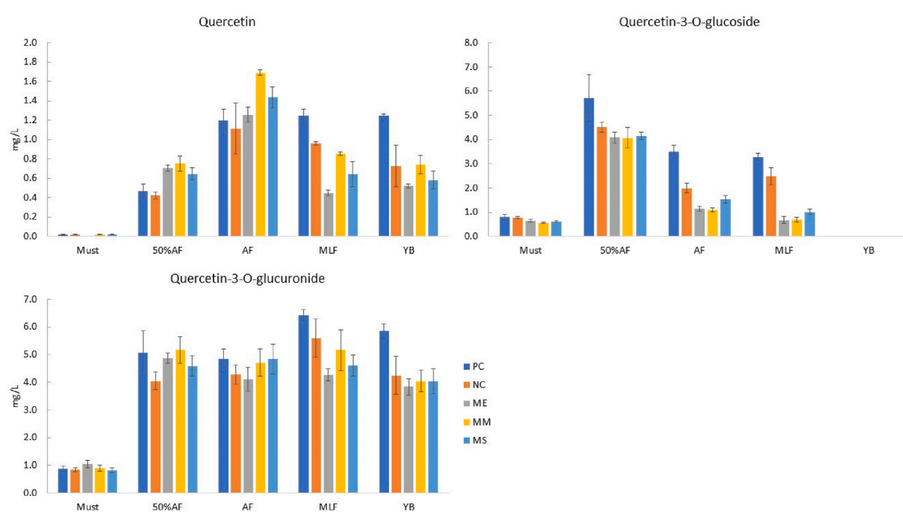


Fig. 5. Graphs presenting the concentration evolution of the quercetins in Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines through time. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled.

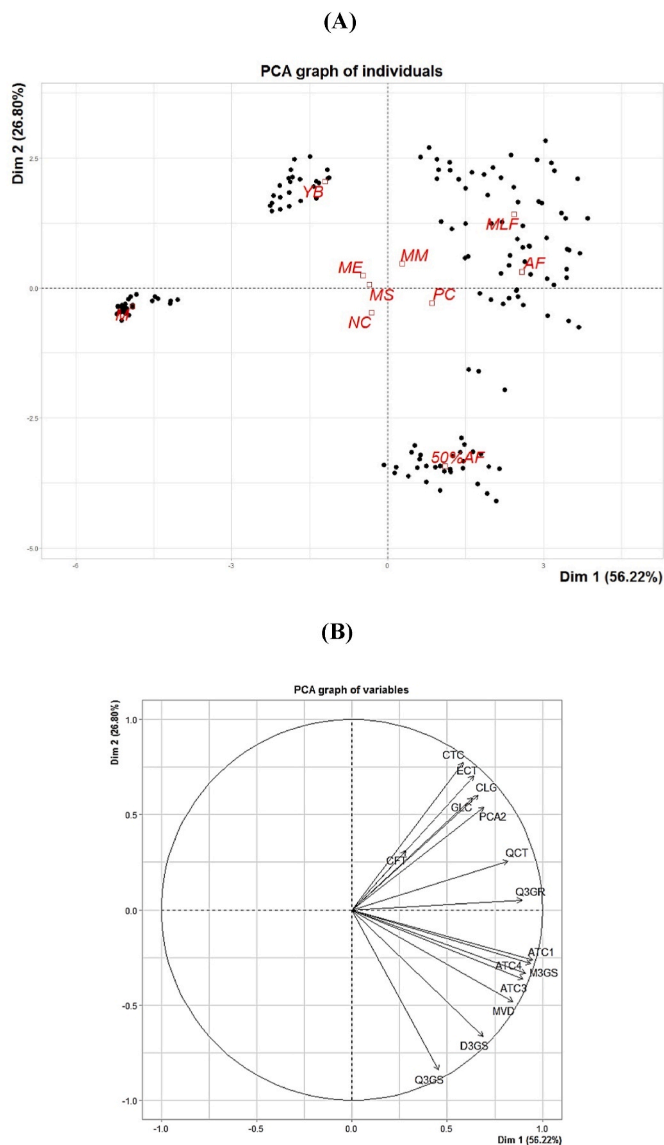


Fig. 6. Principal components graphics of individuals (A) and variables (B) of the Tempranillo (*Vitis vinifera* L. cv. Tempranillo) musts and wines with different preservative treatments. NC: negative control; PC: positive control; ME: stem extract; MM: stem extract mixed with SO_2; MS: stem powder. 50%AF: mid alcoholic fermentation; AF: end of alcoholic fermentation; MLF: end of malolactic fermentation; YB: one year bottled. CTC: (+)-catechin; ECT: (−)-epicatechin; CFT: caftaric acid; GLC: galic acid; CLG: chlorogenic acid; QCT: quercetin; Q3GS: quercetin-3-glucoside; Q3GR: quercetin-3-glucuronide; PCA2: Procyanidin A2; MVD: malvidin chloride; M3GS: malvidin-3-glucoside; D3GS: delphinidin-3-glucoside; ATC1: anthocyanin 1; ATC3: Anthocyanin 3; ATC4: Anthocyanin 4.

the most suitable dose of extract and establish the half-life of these innovative SO_2-free wines.

CRediT authorship contribution statement

Danielle P. Nogueira: Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Nerea Jiménez-Moreno:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. **Esparza Irene:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing, Funding acquisition. **Jose Antonio Moler:** Formal analysis, Writing – original draft, Writing – review & editing. **Pedro Ferreira-Santos:** Formal analysis, (extract characterization), Writing – original draft. **Ana Sagües:** experimental (winemaking processes in EVENA facilities). **José António Teixeira:** Writing – original draft. **Carmen Ancín-Azpilicueta:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100453.

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