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ORIGINAL RESEARCH ARTICLE

Valorisation of white wine lees: optimisation of subcritical water extraction of antioxidant compounds

Benjamin Poulain[1], Delphine Winstel[1], Arnaud Massot[2], Virginie Moine[2] and Claudia Nioi[1]*

[1] Université de Bordeaux, Bordeaux INP, Bordeaux Sciences Agro, INRAE, UMR OENO, 1366, ISVV, F-33140 Villenave d'Ornon, France.
[2] Biolaffort, 11 Rue Aristide Berges, 33270 Floirac.

*correspondence:
claudia.nioi@u-bordeaux.fr

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ABSTRACT

This study explored the extraction of antioxidant compounds from white wine lees using subcritical water extraction (SWE). First, SWE was compared to extraction using conventional solvents mixed with water: 50 % methanol, 50 % ethanol and 50 % acetonitrile. The antiradical activity of the extract increased to 12.8 mg TE/g of extract dry matter (DM) using conventional solvents and to 29.2 mg TE/g DM with SWE, compared to the antiradical activity of dried lees, which was 5.7 mg Trolox Equivalent/g of dry matter (mg TE/g DM). Second, SWE conditions were optimised to obtain lees extracts with a high antioxidant activity by applying a Doehlert design and response surface methodology (RSM). The extraction parameters modulated during RSM optimisation were extraction time (t, 15 – 60 min), temperature (T, 100 – 250 °C) and stirring speed (S, 100 – 1000 RPM). Antioxidant activity was assessed using DPPH and FRAP tests, as well as by measuring the Oxygen Consumption Rate (OCR) of lees extracts. Temperature was found to be the main parameter that had a significant effect on antioxidant activity. The results of the RSM showed the optimal conditions (temperature, duration and speed) for attaining maximum antioxidant activity to be: 240 °C, 15 min and 550 RPM respectively. The best-known antioxidant compounds in white wine lees, Total Polyphenol Content (TPC), Glutathione (GSH) and Total Sulfhydryl groups (TSH) were also quantified. Extracts with the highest antioxidant capacity had the highest TPC and TSH concentrations. In conclusion, this study showed (i) that subcritical water extraction is a green process that has potential as an alternative to using conventional solvents for extracting white wine lees, (ii) the chemical components involved in their antioxidant capacity, and (iii) the potential of the lees for oenological and other industrial applications.

KEYWORDS: White wine lees, subcritical water extraction, response surface methodology, Doehlert design, antioxidant

INTRODUCTION

White wine production involves various major stages, including grape pressing, settling, fermentation and aging before bottling. After winemaking, the wine lees (yeast residue from alcoholic fermentation) are a waste product, representing about 2 – 6 % of the total volume of wine produced. The composition of this sludge-like material depends on factors linked to the winemaking process (yeast strain, aging duration and state of autolysis) and is also influenced by grape variety and viticultural practices (Salgado *et al.*, 2010; Pérez-Bibbins *et al.*, 2015). The solid fraction of wine lees is mainly composed of yeast cell compounds, tartaric acid, grape residuals and inorganic matter (Dimou *et al.*, 2015; Pérez-Bibbins *et al.*, 2015). The liquid fraction mainly consists of wine rich in ethanol and organic acids (Salgado *et al.*, 2010).

Wine aging is key to ensuring organoleptic balance and thus the quality of the wine (Fornairon-Bonnefond *et al.*, 2001). It can be carried out in contact with the lees to improve the chemical stability of the wine, preventing pinking, tartaric precipitation, protein cloudiness (Fornairon-Bonnefond *et al.*, 2001), oxidative spoilage (Dubourdieu and Lavigne-Cruege, 2004) and the improvement of organoleptic properties (Marchal *et al.*, 2011). The antioxidant potential of wine lees during aging has been extensively reported (Fornairon-Bonnefond *et al.*, 2001). Certain reducing compounds are released from the lees into the wine, thus limiting the risk of oxidative spoilage during aging and after bottling (Dubourdieu and Lavigne-Cruege, 2004). Wine lees antioxidant potential seems to be partly related to glutathione (GSH), a bioactive and antioxidant tripeptide present in grape, must and wine (Lavigne *et al.*, 2007). Recently, some studies have shown that GSH is not the only compound involved in lees antioxidant potential: Bahut *et al.* (2020) proved that S- and N- containing compounds are the main contributors to the antioxidant power of wine, especially after wine ageing in contact with lees (Romanet *et al.*, 2020). Due to their ability to consume oxygen, yeast lipids, such as sterols, are also considered to be involved in the antioxidant potential of lees (Fornairon-Bonnefond and Salmon, 2003; Salmon, 2006). Mannoproteins and proteins of yeast cell walls have also been identified as a source of antioxidant compounds once they have been extracted and undergone hydrolysis (Jaehrig *et al.*, 2008).

In the last decade, several strategies have been proposed for using white wine lees: the recovery of the tartaric acid contained in the solid fraction (Salgado *et al.*, 2010; Kontogiannopoulos *et al.*, 2016; Kontogiannopoulos *et al.*, 2017), promoting the biomass as a source of nutrients for microbial media and fermentation (Rivas *et al.*, 2006; Salgado *et al.*, 2010; Dimou *et al.*, 2015; Pérez-Bibbins *et al.*, 2015), and the recovery of yeast cell polysaccharides, such as mannoproteins, for use as food additives (De Iseppi, *et al.*, 2021a; De Iseppi, *et al.*, 2021b). However, only a few studies have been carried out with the aim of recovering antioxidant compounds from white wine lees (Poulain *et al.*, 2024; Winstel *et al.*, 2024). Recently, an optimised methodology for extracting GSH from white wine lees was proposed using water (Winstel *et al.*, 2024). Thus, the literature on the valorisation of white wine lees for the extraction of antioxidant molecules is quite limited.

Based on the antioxidant potential of white lees in wine, this study aimed to propose an eco-friendly extraction strategy for obtaining antioxidant extracts from white wine lees.

Extraction is a critical primary step in the isolation, identification and use of bioactive compounds. In a context where environmental and economic sustainability is a priority, emphasis should be placed on developing green extraction processes, especially for biomass utilisation like wine lees.

Over the past 20 years, supercritical fluids, such as supercritical carbon dioxide and subcritical water, have been used in promising green processes for the extraction of bioactive compounds (Essien *et al.*, 2020; Zhang *et al.*, 2020). Subcritical water extraction is based on the use of water as a solvent in temperatures above the usual boiling point (100 °C) and below the critical point (374 °C) and under pressure high enough to keep it in the liquid state. In this subcritical state, with the increase in temperature, the water diffusivity characteristics are improved and its relative permittivity, viscosity and surface tension decreases. Under such conditions, water has properties similar to those of organic solvents and becomes a more effective solvent for medium and low polarity compounds (Kruse and Dinjus, 2007; Zhang *et al.*, 2020). The ionic product of water is also affected by temperature, and under subcritical conditions water can act as a weak acid or alkali which can promote the hydrolysis of biomass (Kruse and Dinjus, 2007; Bahari, 2010). Thus, in the context of the extraction of white wine lees, composed of a mixture of polar and apolar molecules, this process seems relevant for their valorisation. The extraction efficiency of the SWE process can be significantly influenced by the extraction conditions, including temperature, extraction time and stirring speed (Essien *et al.*, 2020; Zhang *et al.*, 2020). The response surface methodology (RSM) is a statistical modeling technique for determining the influence of variables after application of an experimental design, and is thus useful for optimising processes. In the present study, the Doehlert design was applied to optimise the SWE of white wine lees and thereby obtain lees extracts with a high antioxidant activity. The effects of extraction time, temperature and stirring speed on antioxidant activity were studied. Various complementary methods commonly used to assess the antioxidant activity of extracts (Zhong and Shahidi, 2015) were evaluated: the scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical based on the free radical scavenging mechanism (Brand-Williams *et al.*, 1995), the Ferric Reducing Antioxidant Power (FRAP) based on redox potential (Benzie and Strain, 1996), and Oxygen Consumption Rate (OCR) based on direct interaction with oxygen. Finally, the Total Phenolic Content (TPC), GSH and Total Sulfhydryl groups (TSH), the compounds most frequently described as being responsible for the antioxidant

power of lees, were quantified. Using this approach, the objective was to propose the optimal conditions for extraction by subcritical water to obtain new products with antioxidant capacity and thus valorise white wine lees.

MATERIALS AND METHODS

1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), iron (III) chloride hexa-hydrate, Copper (II) sulfate pentahydrate, sodium acetate, glacial acetic acid, gallic acid and Ellman's reagent (DTNB) were purchased from ThermoFisher Scientific (France). 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), sodium carbonate, L (+)-Tartaric acid Folin-Ciocalteu reagent, hydrochloric acid, Tris(hydroxymethyl) aminomethane (≥ 99.8 %), EDTA dihydrate sodium hydroxide and L-Glutathione reduced (GSH, ≥ 98 %) were purchased from Sigma-Aldrich (France).

2. Lees

The white wine lees comprised a mixture of several lees collected in a Bordeaux wine estate during the 2021 vintage. These were collected during first racking, 15 days after alcoholic fermentation of a sauvignon grape must (conventional winemaking with addition of sulphite) and then freeze-dried in the laboratory under the following conditions: freezing at -24 °C, vacuum setpoint at 0.050 mbar, drying at room temperature (Christ Alpha 2-4 LSCbasic, Grosseron, France). Before extraction and analysis, the lees powders were mixed and kept in a dark place at room temperature.

3. Extraction by conventional solvents

Conventional solvent extractions were performed using water at room temperature (20 °C) and mixtures of water and ethanol (EtOH/H_2O, 50:50, v/v), water and methanol (MeOH/H_2O, 50:50, v/v), water and acetonitrile (MeCN/H_2O, 50:50, v/v). Based on the preliminary results of a single-factor experiment, the solid-to-liquid ratio and extraction time were set at 1 % (2.5 g of white wine lees powder mixed with 250 mL of solvent) and 3 h respectively. At the end of the extraction, the samples were centrifuged at 8400 RPM for 15 min at 4 °C (Eppendorf 5804 R, Eppendorf, France). Then the organic solvents were removed by vacuum distillation (Rotavapor R-114, Buchi, France). Finally, the samples were frozen at -24 °C to be freeze-dried before analysis.

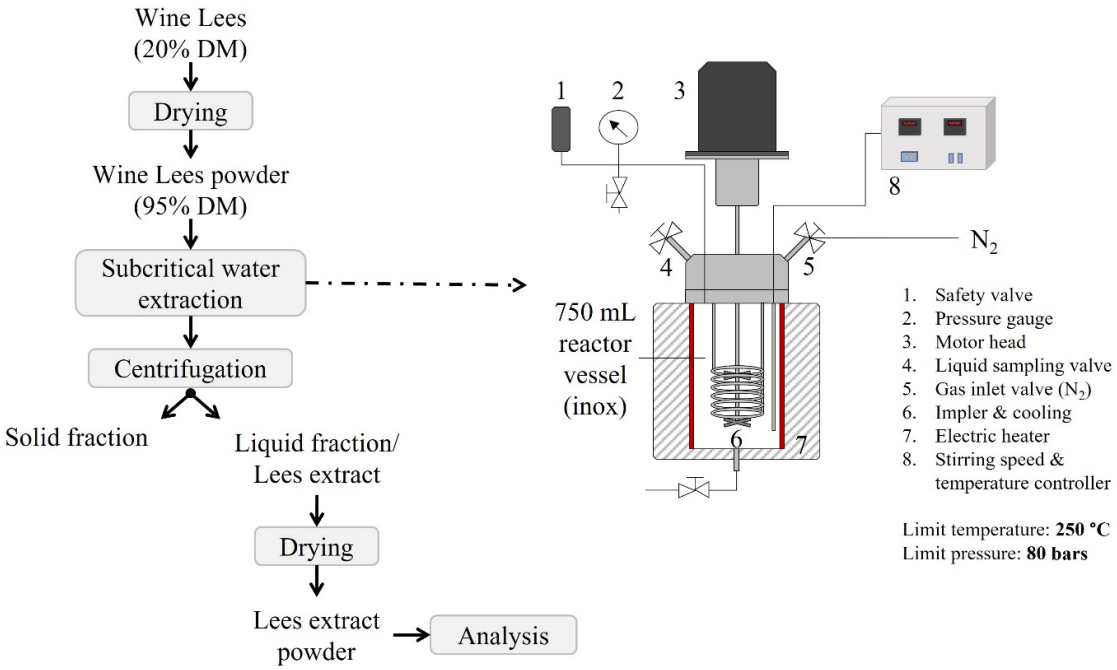


FIGURE 1. Diagram of the subcritical water extraction procedure and system used in the study (DM = Dry Matter).

4. Subcritical water extraction procedure

Under a maximum operating pressure of 80 bar and a temperature of 250 °C, SWE was conducted in a 750 mL batch-type high pressure autoclave (HPP, France) connected to a temperature and stirring controller (HPP, France), as shown in Figure 1. For all experimental runs, 2.5 g of white wine lees powder was mixed with 250 mL of water (solid-to-liquid ratio: 1 %) and the extraction was performed under a constant pressure of 40 bar with injection of nitrogen. Extraction time (t, 15 - 60 min), temperature (T, 100 - 250 °C) and stirring (S, 100 - 1000 RPM) were independent variables modulated during the extractions as defined by the RSM design. At the end of SWE, the mixture in the reactor was cooled by the internal cooler to a temperature of 60 °C before being recovered and centrifuged at 8400 RPM for 15 min at 4 °C (Eppendorf 5804 R, Eppendorf, France). The obtained liquid fraction (i.e., the lees extract), was frozen at -24 °C to be freeze-dried.

5. Determination of antioxidant activity

5.1. DPPH assay – antiradical activity

The DPPH free radical scavenging assay was carried out using the method described by Brand-Williams *et al.* (1995) with some modifications to fit in 96-well microplates (Nioi *et al.*, 2022). The reaction was carried out by mixing 15 µL of sample and 285 µL of an ethanolic solution containing DPPH• (9.0×10^{-5} M; prepared daily) into each well (four wells per sample, $n = 4$). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 515 nm using a microplate spectrophotometer UV-Vis MultiSkan Sky High (ThermoFisher Scientific, France) and all the extracts were analyzed in triplicate ($n = 12$). A calibration curve was obtained using Trolox as the standard (0-125 mg/L in EtOH/H_2O (50:50, v/v), $R^2 = 0.997$) and the results were expressed as mg of Trolox equivalents (TE) per gram of DM of extract (mg TE/g DM).

5.2. FRAP assay – Ferric Reducing antioxidant power (FRAP)

The ferric reducing power of the extracts was determined using the method described by Benzie and Strain (1996), with some modifications as reported by González-Centeno *et al.* (2012) to adapt the method to 96-well microplates. The FRAP reagent was prepared daily by mixing 1 vol of 0.01 M TPTZ solution (in 0.04 M Hcl), 1 vol of 0.02 M $FeCl•6H_2O$ aqueous solution and 10 vol of 0.3 M acetate buffer at pH 3.6. For the measurement, 15 µL of sample and 285 µL of FRAP reagent were mixed in a microplate well (four wells per sample, $n = 4$). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 593 nm and all the extracts were analyzed in triplicate ($n = 12$). A calibration curve was obtained using Trolox as the standard (0-125 mg/L in EtOH/H_2O (50:50, v/v), $R^2 = 0.999$) and the results were expressed as mg TE/g DM of extract.

5.3. OCR – Oxygen Consumption Rate (OCR)

The capacity of the lees extracts to consume oxygen was measured to evaluate their capacity to react directly with O_2. In the context of use of the extracts for oenological purposes, the extracts were dissolved at 1.0 g/L in a model wine solution composed of 12 % ethanol (v/v) and 4.0 g/L of L(+)-tartaric acid adjusted to a pH of 3.5 using 10 M NaOH. To reproduce the oxygen catalytic conditions of wine, 3.0 mg/L of iron and 0.3 mg/L of copper were added to the model wine solution in the form of iron (III) chloride hexahydrate and copper (II) sulphate pentahydrate. The model solution was then saturated with oxygen by air bubbling (8 ± 0.7 mg/L at 20 °C). A model wine solution control without added extract was also monitored. Vials with a capacity of 20 mL and integrated oxygen sensor strips (OXVIAL20, Bionef, France) were used. The vials were filled to the brim with the solution, capped under a nitrogen atmosphere and then stirred at 400 RPM on a multi-position stirring plate (Thomas Scientific, New Jersey, USA) throughout the measurements. The dissolved oxygen measurements were carried out by the luminescence technique using a FireSting-O2 (Pyroscience optical O_2 sensor, red-flash technology, Bionef, France). Dissolved oxygen measurements began after 10 min of solution equilibration and were performed by automatic measurement at 1 h intervals until full O_2 consumption was reached. The temperature was maintained at 20 ± 1 °C throughout the measurements. The dissolved O_2 over time was used to calculate the O_2 consumption rate (OCR) (expressed as mg O_2 consumed/day.g of extract) of each sample (Nioi *et al.*, 2022). Figure S1 (supplementary data) outlines the methodology for obtaining the OCR value from the oxygen consumption data.

6. Determination of Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). In a 96-well microplate, 20 µL of sample (solubilised lees extract or gallic acid standard), 80 µL of sodium carbonate (7.5 %, m/v) and 100 µL of Folin-Ciocalteu reagent (previously diluted 10-fold in demineralised water) were added to each well (four wells per sample, $n = 4$). After 30 min at 25 °C in the dark, absorbance was measured at 760 nm and determinations were performed in triplicate ($n = 12$). Gallic acid was used as the standard to obtain a calibration curve (0 to 100 mg/L in EtOH/H_2O (50:50, v/v), $R^2 = 0.999$), and the total polyphenol content was expressed as mg of gallic acid equivalent (GAE) per gram DM of extract.

7. Determination of Total Sulfhydryl groups (TSH)

The DTNB assay for determining the quantity of TSH in wine lees extracts was performed using the method described by Tietze (1969) with some modifications. A first buffer solution (referred to as TE8) was obtained by mixing a 50 mM solution of Tris with a 3 mM solution of EDTA. TE8 was adjusted to pH 8.0 with 0.1 N HCl solution and stored at 4 °C. A stock solution of DTNB was then prepared by adding DTNB to TE8 and adjusting the pH to 8.0 by adding 0.1 N NaOH solution; the solution was stored at 4 °C. For the measurements, 245 µL of TSH reagent and 5 µL of sample were put into each well of a 96-well microplate (four wells per sample, $n = 4$). After 10 min of reaction at room temperature, the absorbance at 412 nm was measured. A calibration curve was obtained using glutathione as the standard (0 to 1000 mg/L in ultrapure water with 0.1 N HCl solution, $R^2 = 0.999$), and the results were expressed as mg of glutathione equivalent (GSHE) per gram of DM of extracts. All determinations were performed in triplicate ($n = 12$).

8. Quantification of Glutathione by LC-HRMS

The quantification of glutathione in the wine lees was carried out following the protocol described by Winstel *et al.* (2024). The analyses were performed using an UHPLC appliance consisting of a Vanquish system (Thermo Fisher Scientific, Les Ulis, France) with binary pumps, an autosampler and a heated column compartment. An Atlantis Premier BEH C18 AX column (150 × 2.1 mm, 1.7 µm, Waters, Guyancourt, France) was used for the stationary phase, with water (Eluent A) and acetonitrile (Eluent B), containing both 0.1 % of formic acid as mobile phases. The flow rate was set at 350 µL/min

and the injection volume was 5 µL. The temperature of the column chamber was set at 30 °C in forced air mode. For the screening and quantitative analysis, Eluent B varied as follows: 0 min, 5 %; 3.0 min, 5 %; 6.3 min, 30 %; 6.6 min, 98 %; 8.1 min, 98 %; 8.3 min, 5 %; 10.5 min, 5 %. An Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionisation (HESI II) probe (both from Thermo Fisher Scientific, Les Ulis, France) was used. The ionisation and spectrometric parameters, optimised in positive mode, have already been described by the authors (Winstel *et al.*, 2024).

9. Doehlert Design of experiment and response surface methodology

Response surface methodology was used to assess the effects of extraction parameters and to optimise SWE. A Doehlert design with three numeric factors was chosen. The design consisted of fifteen randomised runs, including three replicates at the central point (Table 1). The independent variables used for the design of the experiments were extraction time (X_1, 15 – 60 min), temperature (X_2, 100 – 250 °C) and stirring (X_3, 100 – 1000 RPM). The investigated response variable was antioxidant activity evaluated by DPPH (Y_1, in mg TE/g DM), FRAP (Y_2, in mg TE/g DM) and OCR (Y_3, in mg O_2/day.g DM). The TPC (Y_4, in mg GAE/g DM); the extraction Yield (Y_5, in %) was also taken into account. The response variables (as a function of factors) were fitted using the following second-order polynomial function:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2$$

where Y represents the response prediction, Xi and Xj are the independent variables affecting the response, and a0, ai, aii, aij are the regression coefficients for the intercept, linear, quadratic and interaction effects.

The standard deviations of the experimental responses were determined by repeating the experiments at the center of the experimental domain (Xi = 0) three times. XLSTAT software (Version 2022.4.1, Addinsoft, France) was used to implement the experimental matrices, calculate the model coefficients, assess the significance of the regression, the validity of the model and plot the contour lines. To validate the models, the extractions were carried out in optimal conditions in triplicate and the *t*-test was employed to compare the experimental responses to those predicted by the models.

RESULTS

1. Extraction with conventional solvents

When in a subcritical state and depending on the conditions, water has similar properties to certain polar or semi-polar organic solvents and it can promote the hydrolysis of biomass (Kruse and Dinjus, 2007; Zhang *et al.*, 2020). In order to determine the potential of SWE as a green means of extraction of antioxidant compounds, the wine lees extracts obtained using conventional organic solvents and water at room temperature were compared to those obtained by water in subcritical conditions (SWE: 200 °C and 40 bar).

Figure 2 shows the results of the DDPH assay to compare the antioxidant activity of the white wine lees with that of the lees extracts obtained using water, different mixtures of organic solvents (methanol/water, ethanol/water and acetonitrile/water) and subcritical water (SW 200 °C: 15 min, 200 °C, 550 RPM and 40 bar). The antiradical activity of the lees before extraction was 5.7 ± 0.1 mg TE/g DM. The extract obtained using water at 20 °C had an antiradical activity of 4.8 ± 0.1 mg TE/g DM (no significant difference with lees before extraction). The DPPH values of the extracts using conventional organic solvents (acetonitrile, methanol and ethanol) were between 11.3 ± 0.5 and 12.8 ± 0.5 mg TE/g DM, thus more than double those obtained for lees. The antiradical activity of the SW extract was 29.2 ± 0.9 mg TE/g DM, over four times higher than the lees and more than double that of the extracts obtained using conventional organic solvents.

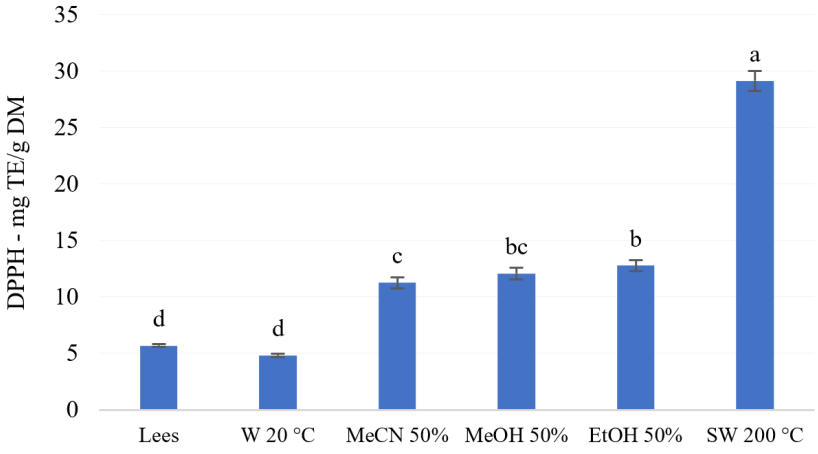


FIGURE 2. Antiradical activity by DDPH assay results of antioxidant activity of white wine lees and of extracts obtained using water at 20 °C (W 20 °C), conventional solvents EtOH 50 % (EtOH/H_2O, 50:50, v/v), MeOH 50 % (MeOH/H_2O, 50:50, v/v), MeCN 50 % (MeCN/H_2O, 50:50, v/v) during 3 h and solid- liquid ratio of 1 % and subcritical water extraction (SWE 200 °C: 15 min, 200 °C, 550 RPM, 40 bar).

These results indicate that SWE may be a suitable process for obtaining extracts with higher antioxidant capacity. In the second part of this study, the SWE conditions were optimised to maximise the antioxidant activity of the wine lees extracts by using Response Surface Methodology with Doehlert design.

2. Optimisation of subcritical water extraction

Before applying the experimental design, the impacts of the different parameters (solid/liquid ratio, pressure, temperature and time) on the extraction of the antioxidant compounds were determined in a single-factor study. The results (data not shown) were used to determine the range of the operating conditions in the experimental design. Because the solid-liquid ratio and pressure parameters did not have an impact on the antioxidant activity of the lees extracts, they were kept constant in the experimental design. Extraction time, temperature and stirring speed were integrated into the Doehlert experimental design for optimisation of the extracts' antioxidant capacity using SWE. The antioxidant activity was assessed via DPPH, FRAP, OCR and TPC (Table 1).

The antioxidant activity assessed by the DPPH method ranged between 5.82 mg TE/g DM (run 3: 37.5 min, 132 °C, 917 RPM) and 40.06 mg TE/g DM (run 7: 48.8 min, 240 °C, 550 RPM). The values obtained using the FRAP assays ranged between 9.67 mg TE/g DM (run 3: 37.5 min, 132 °C, 917 RPM) and 53.17 mg TE/g DM (run 7: 48.8 min, 240 °C, 550 RPM). The OCR in model wine solution of wine lees extracts ranged between 0.04 mg O_2/day.g DM (run 5: 26.3 min, 110 °C, 550 RPM) and 0.96 mg O_2/day.g DM (run 7: 48.8 min, 240 °C, 550 RPM). TPC varied from 16.66 mg GAE/g DM (run 3: 37.5 min, 132 °C, 917 RPM and run 13: 48.8 min, 110 °C, 550 RPM) to 54.09 mg GAE/g DM (run 12: 26.6 min, 240 °C, 550 RPM). The extraction yield, estimated from the ratio of quantity of extract recovered to quantity of wine lees used for extraction, ranged from 46.71 % (run 7: 48.8 min, 240 °C, 550 RPM) to 81.30 % (run 11: 48.8 min, 153 °C, 200 RPM).

TABLE 1. Doehlert experimental design with natural values of independent variables, experimental response values for antiradical activity (DPPH), ferric reducing power (FRAP), oxygen consumption rate (OCR), total polyphenols content (TPC), and extraction yield of wine lees extracts. LQ = Limit of quantification.

	Independent variables			Dependent variables				
	SWE conditions							
Run	X_1 (t, min)	X_2 (T, °C)	X_3 (S, RPM)	Y_1, DPPH (mg TE/g DM)	Y_2, FRAP (mg TE/g DM)	Y_3, OCR (mg O_2/day.g DM)	Y_4, TPC (mg GAE/g DM)	Y_5, Extraction yield (%)
1	60.0	175	550	14.86	19.56	0.19	28.57	74.76
2	26.3	153	200	7.32	11.67	0.05	18.93	77.63
3	37.5	132	917	5.82	9.67	0.06	16.66	63.82
4	37.5	175	550	13.88	17.87	0.13	28.99	71.79
5	26.3	110	550	6.69	12.81	*0.00*	16.79	54.36
6	37.5	218	200	32.51	42.54	0.82	49.06	60.68
7	48.8	240	550	40.06	53.17	0.96	52.10	46.71
8	26.3	197	917	26.01	33.78	0.65	38.85	68.17
9	48.8	197	917	29.36	39.11	0.75	45.25	62.86
10	15.0	175	550	10.70	14.54	0.04	24.26	78.80
11	48.8	153	200	7.72	10.95	0.05	20.08	81.30
12	26.3	240	550	39.53	51.40	0.86	54.09	52.87
13	48.8	110	550	6.32	12.80	0.15	16.66	55.84
14	37.5	175	550	12.99	16.47	0.11	26.36	77.42
15	37.5	175	550	11.92	14.80	0.12	26.18	76.89

* < LQ

Analysis of variance (ANOVA) was used to analyze the experimental results and to obtain regression models. The significance and adequacy of the models were evaluated using Fisher's test (F-test) for the model sum squares and lack-of-fit test. Determination coefficients (R^2 and adjusted R^2) provided additional information on the model fitness results (Table S1).

The significant *p-values* of the models (*p-value* < 0.05) show that the second order polynomial model gave a good approximation for DPPH, FRAP, OCR, TPC and extraction yield responses. Moreover, insignificant *p-values* > 0.05 obtained for the lack-of-fit confirm that the second-order polynomial models correctly represented the experimental data for DPPH, FRAP, TPC and extraction yield responses. However, for OCR, the *p-value* of the lack-of-fit test was 0.0019, indicating that the model did not perfectly represent the experimental data. The lack-of-fit test is a measure of the significance of the lack-of-fit error. According to Ekpenyong *et al.* (2017), the lack-of-fit error comprises error resulting from model deficiency, and pure error reflects the variability of observations that share their independent variable values. The lack-of-fit error and pure error are two components of the residuals. In this study, the model-independent variance was estimated using the replicates of experimental design's central point (run 4, 14 and 15: 37.5 min, 175 °C, 550 RPM), and the pure error was therefore also calculated using these replicates. While a significant lack-of-fit can result from noise (Kayarogannam, 2023), it can also result from the high precision of the replicates and the existence of error in other experiments leading to the underestimation of the pure error (Taheri, 2022). The central point replicates were performed on the same day, thus limiting any associated error compared to other experiments. This could explain the significant lack-of-fit for OCR model and the low *p-value* (0.05 < *p-value* < 0.10) of lack-of-fit test obtained for DPPH, FRAP, TPC and extraction yield models.

When carrying out RSM it is recommended that adjusted R^2 be used, which does not systematically increase when terms are added to the model (Myers *et al.*, 2009; Kayarogannam, 2023). The adjusted R^2 obtained for DPPH, FRAP, TPC, OCR and extraction yields models were 0.934, 0.924, 0.886, 0.865 and 0.908 respectively, indicating that the models provided a good representation of the experimental values and that they left only a low percentage of variations unexplained. The results of the ANOVA indicate that these models are able to adequately describe the five studied responses as functions of extraction time (X_1), temperature (X_2) and stirring (X_3).

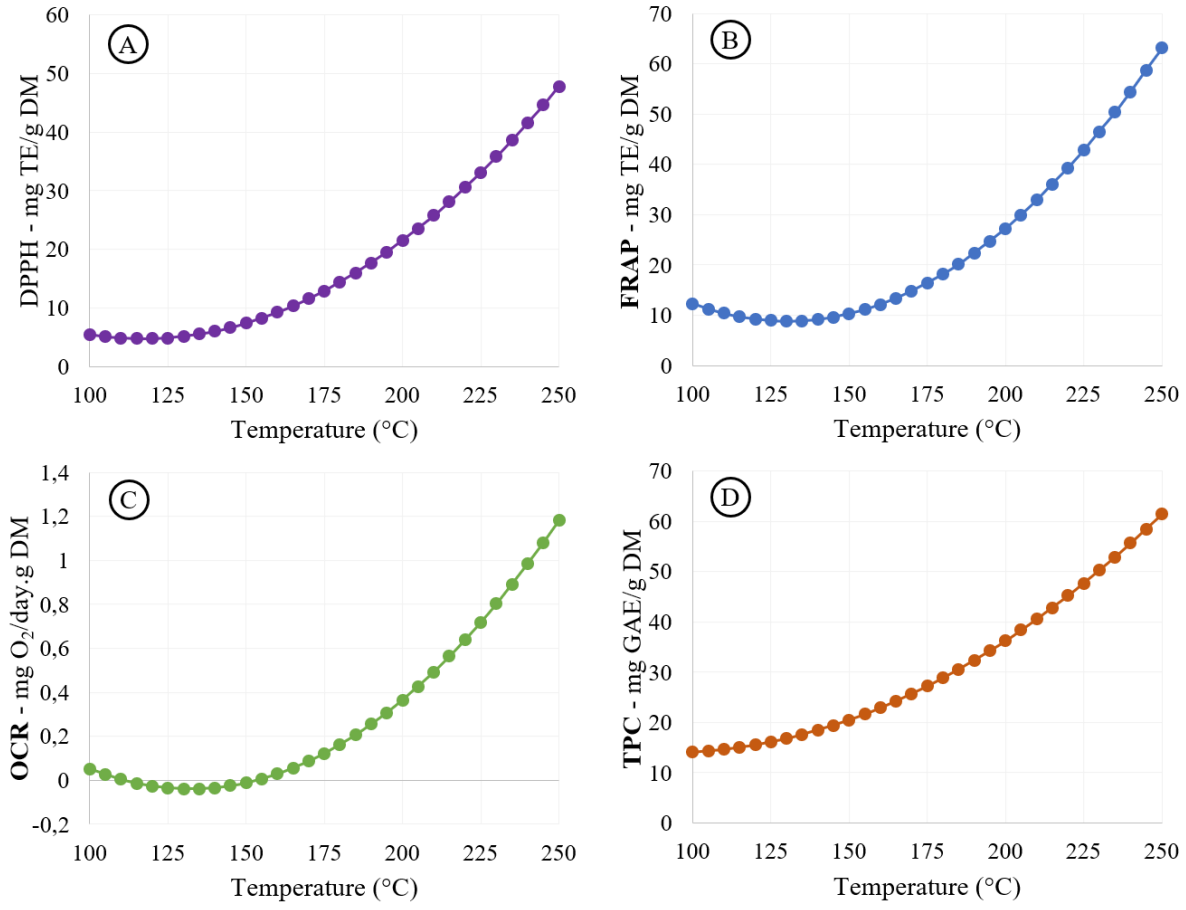


FIGURE 3. Antiradical activity by DPPH (A), ferric reducing power by FRAP (B), oxygen consumption rate (OCR) (C) and total polyphenols content (TPC) (D) models as a function of extraction temperature.

The *p-value* of the regression coefficients of the second order polynomial equations (Table S1) show that the effects of the extraction temperature on antioxidant capacity (DPPH, Y_1; FRAP, Y_2; OCR, Y_3), total polyphenol content (TPC, Y_4) and Extraction Yield (Y_5) were significant for linear (a_2) effects (*p-value* < 0.05). The quadratic effects of temperature (a_{22}) on DPPH, FRAP and OCR were significant, but on TPC (*p-value* > 0.05) they were not significant. Meanwhile, the effects of extraction time (a_1 and a_{11}), stirring (a_3 and a_{33}) and interactions (a_ia_j) were not significant for all responses (Y_1 to Y_5). The effect of the extraction temperature on extraction yield (Y_5) was significant for both linear (a_2) and quadratic effects of temperature (a_{22}). The effect of stirring was significant for linear effects (a_3) and not significant for quadratic effects (a_{33}). Finally, the effects of extraction time (a_1 and a_{11}) and interactions (a_ia_j) were not significant.

Temperature was the main parameter that influenced the antioxidant activity, total polyphenol content and extraction yield of the wine lees extracts. The models can be summarised as equations (3), (4), (5), (6) and (7) after elimination of the insignificant coefficients.

(3) $Y_1(DPPH) = 12.932 + 21.149X_2 + 13.680X_2^2$

(4) $Y_2(FRAP) = 16.380 + 25.427X_2 + 21.335X_2^2$

(5) $Y_3(OCR) = 0.122 + 0.564X_2 + 0.495X_2^2$

(6) $Y_4(TPC) = 27.201 + 23.655X_2 + 10.570X_2^2$

(7) $Y_5(ExtractionYield) = 75.363 - 4.778X_2 - 5{,}078X_3 - 31{,}007X_2^2$

Thus, using the simplified models it was possible to plot antioxidant activity and TPC as a function of extraction temperature, which was the only independent variable that had a significant effect. As shown in Figure 3A, 3B, 3C and 3D, an increase in extraction temperature led to an increase in extract antioxidant activity and total polyphenol content. Finally, the extraction yield model (Y_5) was represented in 3D response surface plots (Figure 4), showing the combined effects of temperature (°C) and stirring speed (RPM). It can be seen that the extraction yields increased with temperatures up to 175 °C then decreased with temperatures higher than 175 °C. A decrease in yield was also observed with an increase in stirring speed.

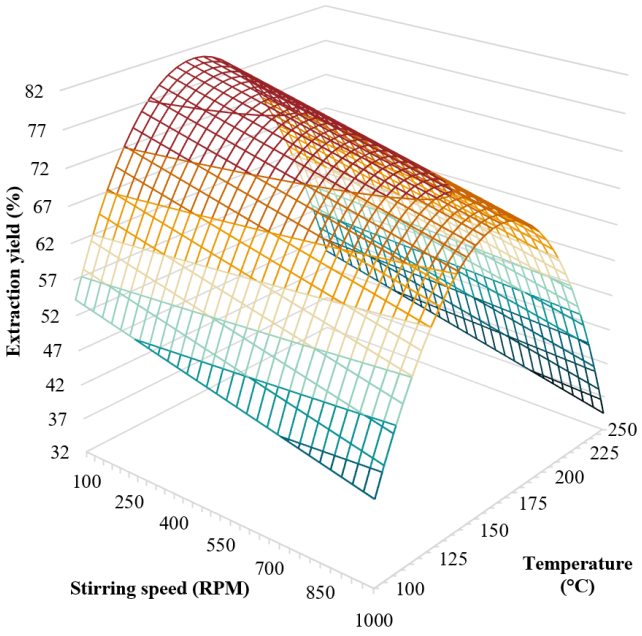


FIGURE 4. Response surface 3D plots showing combined effects of temperature (°C) and stirring speed (RPM) on extraction yield (%).

TABLE 2. Predicted and experimental values in optimum extraction conditions for all criteria: antiradical activity by DPPH, ferric reducing power by FRAP, total polyphenols content (TPC) and oxygen consumption rate (OCR).

	Extraction time (min)	Temperature (°C)	Stirring speed (RPM)	DPPH (mg TE/g DM)	FRAP (mg TE/g DM)	TPC (mg GAE/g DM)	OCR (mg O_2/day.g DM)
Predicted	15	240	550	41.53	54.44	55.64	0.98
Experimental	15	240	550	41.25 ± 0.74	55.87 ± 0.69	55.64 ± 0.48	0.98 ± 0.13
p-value				0.578	0.070	1.000	0.922

Based on these experimental responses, the predicted optimum conditions for maximum antioxidant activity when applying all analytical methods was 15 min extraction time at 240 °C and a stirring speed of 550 RPM. The experimental values obtained for the extracts via DPPH, FRAP, TPC and OCR (carried out in triplicate) were not significantly different from the predicted values (*p-value* > 0.05), demonstrating the good fit between the models and the experimental data (Table 2). Process optimisation was conducted based on the response surface methodology results and desirability function. Since the extraction time had no significant effects on the antioxidant activity of the extracts, the extraction time was limited to the shortest (15 min), thus reducing the costs associated with SWE. Finally, since the stirring speed had limited impact on the studied responses, it was decided to maintain it at an average level (550 RPM).

3. Quantification of total sulfhydryl groups (TSH) and glutathione (GSH) in lees extracts

In wines aged on lees, protection against oxidation is linked to the presence of GSH and other reducing compounds, including sulfur-containing compounds (Lavigne *et al.*, 2007; Romanet *et al.*, 2020). Here, TSH was determined in lees extracts obtained by applying the Doehlert design (Table 1). The optimisation of the extraction of these compounds was not intended, and therefore the results were not modeled using response surface methodology. However, from Figure 5 it can be established that the optimum condition is close to operating conditions of run 6: 215 °C, 30 min and 200 RPM.

Using the DTNB method, TSH was found to (Figure 5) range between 0.5 mg GSHE/g DM (run 13: 48.8 min, 110 °C, 550 RPM) and 16.0 mg GSHE/g DM (run 6: 37.5 min, 218 °C, 200 RPM). An increase in TSH in the lees extracts was observed with increasing extraction temperature (up to 218 °C); at 240 °C, a slight decrease in TSH was observed. The increase in the extraction duration from 26.3 to 48.8 min at an extraction temperature of 197 °C (runs 8 and 9) resulted in an approximately 40 % increase in TSH. Conversely, a reduction in the extraction duration from 48.8 to 15.0 min at 240 °C (runs 7, 12 and opt) also led to an increase in TSH of about 40 %.

Glutathione is the best-known antioxidant thiocompound in wine lees (Dubourdieu and Lavigne-Cruege, 2004). Here, it was measured in the lees extracts obtained by SWE under optimised conditions (Opt: 15 min; 240 °C; 550 RPM) by LC-HRMS using the method described by Winstel *et al.* (2024). In these extracts, glutathione is present in concentrations below the limits of quantification (Table S2), while thiocompounds were present at a concentration of 13.69 ± 0.30 mg GSHE/g DM.

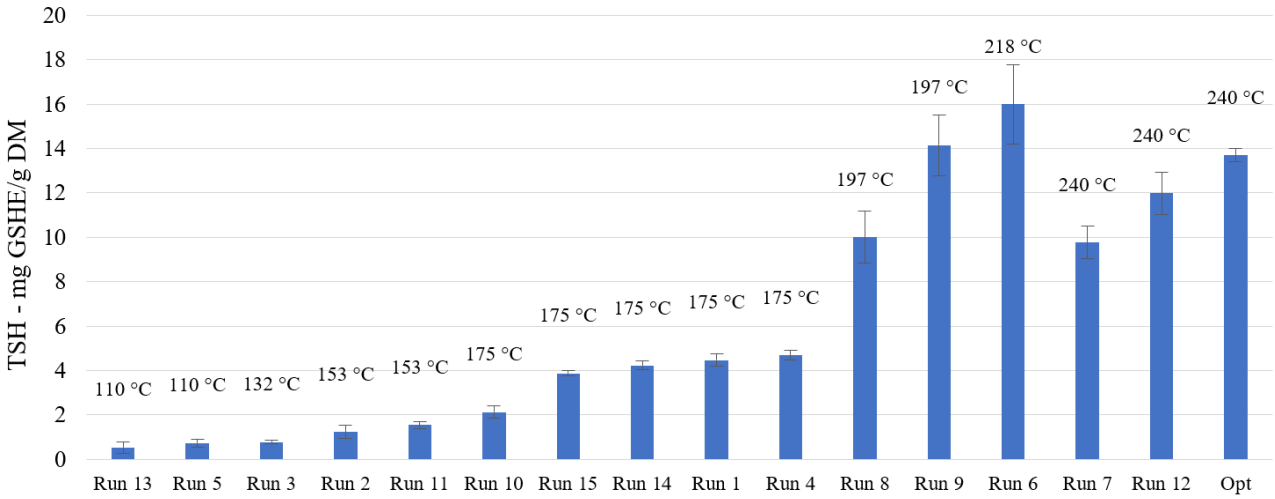


FIGURE 5. Total sulfhydryl groups (TSH) quantified by DTNB method in wine lees extract obtained using the Doehlert design (Run 1 to 15) and optimal extraction conditions (Opt: 15 min; 240 °C; 550 RPM).

DISCUSSION

When using a wine by-product to produce antioxidant extracts, extraction is the first and foremost step. In a comparison of SWE with conventional solvent extraction (Figure 2), higher antioxidant activity was found in extracts obtained via SWE than in those obtained via extraction with water at 20 °C and conventional organic solvents (EtOH 50 %, MeOH 50 %, MeCN 50 %). This is the first time that subcritical water has been proposed for use in a green and sustainable process involving white wine lees. There are many advantages in using SWE: water is an abundant, renewable, non-toxic, non-flammable and economical extraction medium (Essien *et al.*, 2020), and safe and superior extracts devoid of any traces of solvents can be obtained (Chemat *et al.*, 2012), thus ensuring the possibility of direct use of extracts in agri-food industry. Other advantages include the moderate cost of pilot installations, the easy implementation for the operators and the possibility to work in batch and continuous mode. The application of subcritical water in industry is thus of great interest. The optimisation of its operating conditions for controlled use was therefore studied.

The antioxidant activity (DPPH, FRAP and OCR) and TPC values obtained indicate that the SWE operating conditions affect the antioxidant capacity of the extracts (Table 1). The increase in extraction temperature from 110 to 240 °C resulted in an increase in the antioxidant activity of the extracts. More precisely, within this temperature range, antiradical activity (DPPH) and oxygen consumption rate (OCR) each increased 6-fold, ferric reducing power (FRAP) 4-fold, and total polyphenol content (TPC) 3-fold. The models obtained using RSM for the DPPH, FRAP, TPC and OCR assays showed similar trends (Figure 3); thus, the different methods for assessing antioxidant activity provided consistent results. The extraction duration did not have a significant impact on any of the studied responses, including extraction yield.

While such results have already been observed in studies on the optimisation of the extraction of antioxidant compounds using subcritical water for Coriandrum sativum seeds and sage (Zeković *et al.*, 2014; Pavlić *et al.*, 2016), other studies have found shorter extraction times to have an impact on antioxidants extraction from chestnut shells and soybean flour (Moras *et al.*, 2017; Pinto *et al.*, 2021). It is possible that in our experimental conditions the extraction of wine lees antioxidant compounds was already at its maximum at 15 min. In the same way, stirring speed had no impact on the antioxidant activity of the extracts, but its impact on extraction yield was significant. Increasing the stirring speed often improves mass transfer (Essien *et al.*, 2020). However, due to the small size of the lees powder particles and therefore the large contact surface during solid-liquid extraction, combined with the increased diffusivity of water in SWE (Kruse and Dinjus, 2007), the mass transfer is likely to have been maximised, limiting the impact of stirring speed. On the other hand, a decrease in yield was observed with increasing stirring speed. Some authors have shown that too high a stirring speed can cause sample particles to agglomerate on the reactor wall, leading to a reduction in the amount of obtained extract (Lin *et al.*, 2015). The extraction temperature was the main parameter to affect antioxidant activity of the wine lees extracts (DPPH, FRAP, OCR and TPC). Increasing the temperature systematically resulted in an increase in antioxidant activity and total polyphenol content. Temperature is a key parameter of SWE (Kruse and Dinjus, 2007; Zhang *et al.*, 2020). An increase in temperature gives rise to two major phenomena: a decrease in the dielectric constant or relative permittivity ($\boldsymbol{\varepsilon}_r$; from 78.5 at 25 °C to 25 at 250 °C) of water and the occurrence of hydrolysis reactions (Kruse and Dinjus, 2007). Reducing the relative permittivity improves the extraction of weakly polar and non-polar compounds (Zhang *et al.*, 2020). Numerous studies have demonstrated that reducing water polarity by increasing the extraction temperature in subcritical conditions improves the extraction of phenolic compounds from wine and cider by-products, with a positive effect on the antioxidant activity of the extracts (Bahari, 2010; Gabaston *et al.*, 2018; Yammine *et al.*, 2020). Yammine *et al.* (2020) observed an average increase of 75 % in the extraction of Total Polyphenol Content from different grape pomace when the extraction temperature was increased from 100 °C to 200 °C. This increase in extracted polyphenol reached 300 % when the temperature was increased from 100 °C to 190 °C during the extraction of stilbenes from vine by-products (Gabaston *et al.*, 2018). Thus, increasing the temperature of the SW has often been shown to increase the amount of extracted polyphenols. Associated with hydrolysis reactions, the decrease in water polarity allows for the extraction of phenolic compounds adsorbed by yeasts present in wine lees (Jara-Palacios, 2019), thus explaining the increase in polyphenol content in the extracts. It is possible that polyphenols interact with lees in a reversible way, modulating lees antioxidant capacity. The TPC extracted from wine lees therefore depends on the grape variety, geographical origin, and winemaking practices, as well as the vinification process, yeast strain, and maceration time (Delgado De La Torre *et al.*, 2013; Zhijing *et al.*, 2018). In our study, a significant correlation was observed between DPPH and TPC (Figure 6A; $R^2 = 0.985$), as well as between FRAP and TPC values (Figure 6B; $R^2 = 0.961$). These results align with numerous studies (Parikh and Patel, 2018); they indicate that the increased amount of extracted phenolic compounds enabled by the decrease in relative permittivity in SW led to an increase in the antioxidant activity of the wine lees extracts. The antioxidant capacities of the lees extracts, as determined by the DPPH assay (between 5.8 and 40.1 mg TE/g DM) and FRAP assay (between 9.7 and 53.2 mg TE/g DM), are similar to those obtained for other natural biomasses extracted using SW (Nastić *et al.*, 2018; Gan and Baroutian, 2022; Park *et al.*, 2023). They are also comparable to those obtained from extracts of white wine grape pomace; i.e., about 44.4 ± 2.8 and 113.4 ± 5.1 mg GAE/g extracts in SWE at 170 °C and 210 °C respectively (Pedras *et al.*, 2017). It is important to note that TPC and antioxidant capacity depend on the lees matrix and can vary depending on the oenological parameters (grape variety, yeasts strain and enzymatic autolysis). In our previous studies we observed this matrix effect on the GSH content of different white wine lees (Winstel *et al.*, 2024).

Because subcritical water can be used as a medium for hydrolysis, it can improve the release of peptides that have antioxidant activity, as it has been demonstrated in other studies (Powell *et al.*, 2016; Rivas-Vela *et al.*, 2021). Wine lees, linked to the presence of yeast cells, contain significant quantities of proteins (De Iseppi *et al.*, 2020). Bioactive peptides were obtained after hydrolysis of yeast proteins (Vollet Marson *et al.*, 2020). Sulfur-containing amino acids, thiopeptides, including glutathione and thioproteins from yeast biomass are known for their antioxidant activity (Dubourdieu and Lavigne-Cruege, 2004; Jaehrig *et al.*, 2008). In the present study, the concentration of TSH in the lees extracts was found to vary between 0.5 and 16.0 mg GSHE/g DM, with the most influencing parameter being temperature. The maximum total sulfhydryl content in the extracts was obtained at an extraction temperature of 218 °C (run 6) and a slight decrease was observed at the maximum extraction temperature of 240 °C (runs 7 and 12), suggesting a possible degradation of these compounds (Marcet *et al.*, 2016; Ahmed and Chun, 2018). A significant correlation between TSH and DPPH (Figure 6C; $R^2 = 0.829$) and TSH and FRAP (Figure 6D; $R^2 = 0.806$) was observed. This correlation has also been shown by other authors (Costa *et al.*, 2006; Zhou *et al.*, 2022). It is therefore possible that certain operating conditions promote the formation and/or the release of TSH with antioxidant capacity.

Glutathione was not quantifiable in extracts obtained under optimised conditions, probably as a consequence of its thermal degradation during SWE (Zhang *et al.*, 1988). The absence of glutathione in our extracts suggests the involvement of other unidentified thio-compounds in the antioxidant activity. These results indicate the presence of antioxidant compounds other than polyphenols and GSH, as already shown by Bahut *et al.* (2020) for yeast derivatives.

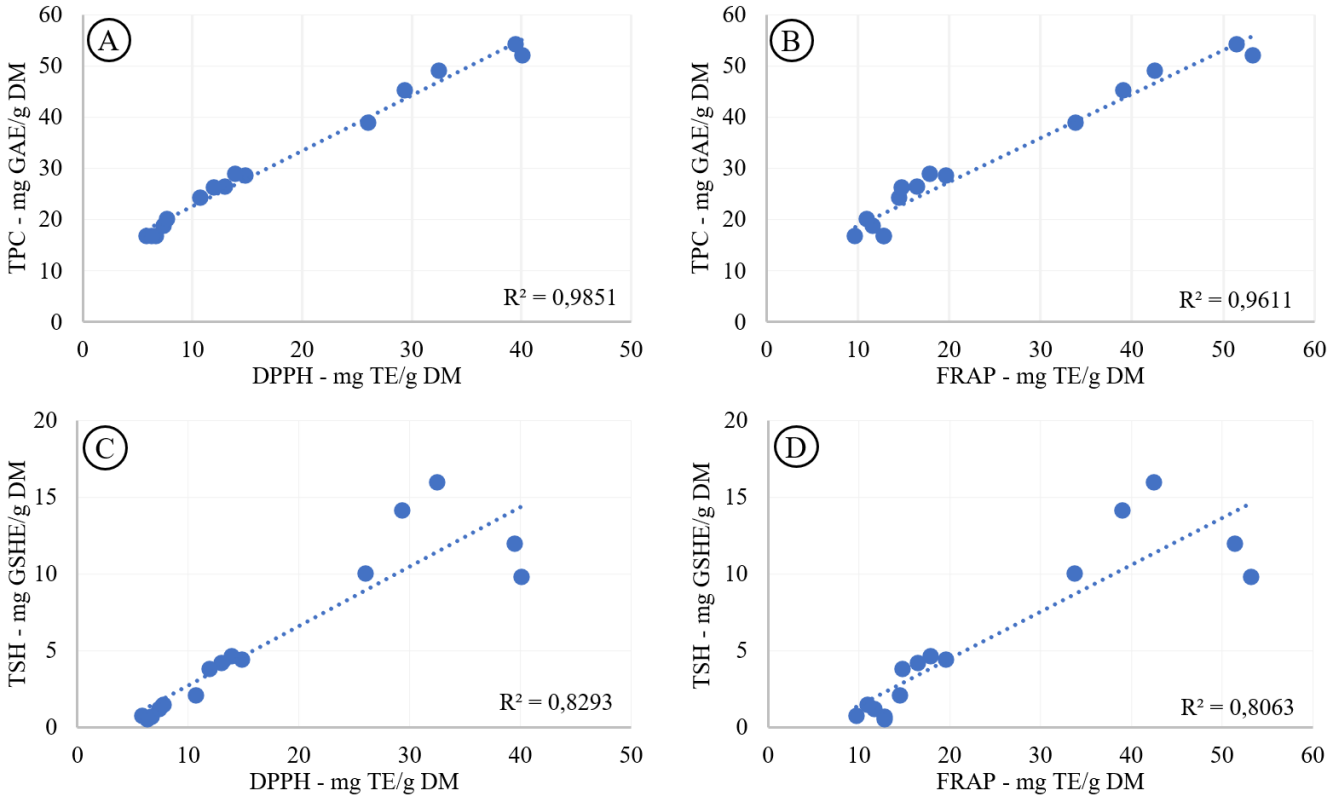


FIGURE 6. Correlation between Total Polyphenol Content (TPC) and antiradical activity (DPPH) experimental values (A); between TPC and ferric reducing power (FRAP) experimental values (B); between total sulfhydryl groups (TSH) by DTNB and DPPH experimental values (C) and between DTNB and FRAP experimental values (D).

However, the precise compounds involved in the antioxidant capacity have not yet been identified. It is also possible that the antioxidant fraction of the extracts from white wine lees differ from those of the yeast derivatives. Indeed, SWE conditions can modify the chemical composition of lees by promoting the formation of new antioxidant compounds not accessible before SW extraction.

Finally, the antioxidant activity of the lees extracts was evaluated using a method based on their OCR in model wine solution (lowest and highest OCR kinetics presented in figure S2.). This method is used to evaluate the kinetics of the oxygen consumption of oenological products like yeast derivatives, tannins and bioprotection (Pascual *et al.*, 2017; Nioi *et al.*, 2022; Windholtz *et al.*, 2023). A high OCR value is associated with a rapid reaction with oxygen. It is supposed that by reacting quickly with oxygen, the oenological product can compete with wine compounds (such as polyphenols and aromas) and prevent or limit their oxidation. Here, the values for OCR of the lees extracts in model wine solution were similar to those obtained by Nioi *et al.* (2022) for industrial yeast derivatives; these products are used during wine aging, being able to consume oxygen and thus limiting the oxidation of organoleptic compounds. In the same way, the lees extracts are a potential new product with antioxidant activity for use in the production of wine and other beverages.

All of these results demonstrate that white wine lees extracts could be useful as antioxidants in the production of food, nutraceuticals or cosmetics. In addition, lees extracts could be proposed for oenological applications during winemaking, which would be of great interest in the context of a circular economy, as this by-product can be reintegrated into the wine production chain.

CONCLUSION

By optimising subcritical water extraction using response surface methodology, the impact of extraction conditions (duration, stirring speed, and temperature) on the antioxidant activity of white wine lees extracts was assessed. Antioxidant activity was measured using DPPH, FRAP, and OCR assays. Key antioxidant compounds (TPC, GSH, and TSH) were also quantified. Temperature was found to be the most significant factor influencing antioxidant activity, with optimal conditions identified as 240 °C, 15 min, and 550 RPM. To the best of our knowledge, this is the first study to propose SWE for the valorisation of white wine lees. The study highlight that white wine lees extracts have potential applications as antioxidants in nutraceutical, pharmaceutical, or food and beverage production. Moreover, reintroducing the use of wine lees extracts in wine production to prevent oxidation could improve the shelf life and quality of wines. This would be particularly attractive to winemakers looking to enhance product stability while maintaining a clean label.

The commercial potential of lees extracts is vast, with applications across multiple industries that align with current trends in sustainability and natural product development. By tapping into this potential, companies can create innovative, marketable products while contributing to environmental goals, thereby opening up new revenue streams and enhancing their market presence.

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