Journal of the Serbian Chemical Society
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J. Serb. Chem. Soc. 85 (4) S191–S193 (2020) *Supplementary material*

SUPPLEMENTARY MATERIAL TO

Serbian aromatized wine "Bermet": Electrochemical, chemiluminescent and spectrophotometric determination of antioxidant activity

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J. Serb. Chem. Soc. 85 (4) (2020) 517–529

EXPERIMENTAL

Chemicals. Folin–Ciocalteu reagent, hydrogen peroxide, ammonium peroxodisulphate, sodium carbonate, sodium acetate trihydrate, acetic acid, hydrochloric acid, iron(III) chloride hexahydrate, and iron(II) sulphate heptahydrate were of analytical grade and supplied by Merck (Darmstadt**,** Germany)**.** DPPH (2.2-diphenyl-1-picrylhydrazyl) was supplied by Fluka (Buchs, Switzerland) and methanol (HPLC grade) was purchased from J. T. Baker (Deventer, Netherlands). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), TPTZ (2,4,6-tripyridyl-*S*-triazine), ABTS (2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) as well as the gallic acid (GA) were obtained from Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany).

Determination of total phenols by FC assay. Total phenol content (*TPC*) was determined spectrophotometrically according to a modified Singleton *et al.*[1] method with Folin–Ciocalteu's reagent. Briefly, 0.5 mL of the sample was added into a 50 mL volumetric flask containing 2.5 mL of Folin–Ciocalteu's reagent, 30 mL of distilled water and 7.5 mL of 20 % Na_2CO_3, and filled up to the mark with distilled water. Absorbance was measured after two hours at 765 nm, against a blank sample. Gallic acid was used as the standard and the results are expressed as mg L^{-1} of gallic acid equivalents (GAE). All measurements were performed in triplicate.

Determination of AO capacity by DC polarographic HPMC assay. DC polarographic assay was used according to a previously reported procedure.[2] The current-potential (*i–E*) curves were recorded using PAR (Princeton Applied Research) polarographic analyzer, model 174A, equipped with a *X–Y* recorder (Houston Omnigraphic 2000). A dropping mercury electrode (DME) with a programmed dropping time of 1 s and a mercury flow of 1.22×10^{-3} g s^{-1} was used as the working electrode, saturated calomel electrode (SCE) as the reference and a

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Pt-foil as the auxiliary electrode. Clark Lubb's (CL) buffer (pH 9.8) was prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl, and 40.8 mL of 0.2 M NaOH. Starting H_2O_2 concentration of 5 mM was obtained by adding 100 μL of 1.00 M H_2O_2 into 19.9 mL of buffer in an electrolytic cell. Samples were gradually added into the electrolytic cell with the buffered H_2O_2 solution. Before recording each *i–E* curve, a stream of pure nitrogen was passed through the cell solution, for 2 min before the first recording and for 30 s after the addition of each aliquot. Inert atmosphere was maintained by passing nitrogen above the cell solution. The initial potentials were 0.10 V, and potential scan rate was 10 mV s^{-1}. DME current oscillations were filtered with a low pass filter positioned at 3 s. Decrease of the anodic current of HPMC (*I*) was recorded, starting from the initial value (I_0), obtained by recording 5 mM H_2O_2 solution, to which the investigated samples were added, leading to a decrease in *I*. All experiments were performed in triplicate, at room temperature.

Determination of hydroxyl free radical-scavenging activity (SA_{HFR}*).* Chemiluminescence (CL) was measured according to Parejo *et al.*[24] using a Jenway 6200 fluorimeter (Jenway Ltd, Gransmore, Essex, UK), keeping the lamp off and using only the photomultiplier of the apparatus. One millilitre of borate buffer solution (0.05 M, pH 9.0), containing $CoCl_2 \cdot H_2O$ (2 mg mL^{-1}) and EDTA (10 mg mL^{-1}) was vortexed for 15 s with 0.1 mL luminol (100 mg/mL in a buffer solution) in a test tube. 0.025 mL of diluted wine (1:100 volume ratio with 50 % ethanol in deionised water) was transferred into another test tube with 0.05 mL of a H_2O_2 aqueous solution (5×10^{-3} M). The luminol-buffer solution was added into the diluted sample and vortexed for 30 s. Immediately afterwards, the *CL* intensity plateau was recorded in a 1 cm path length glass cuvette. The I_0/I ratio was calculated for each wine (decrease in light intensity in the absence of wine /light intensity after sample addition). The I_0/I ratio for five different dilutions of quercetin in 50 % ethanol was plotted against concentration (μM). I_0/I ratio was extrapolated onto the calibration curve to obtain the equivalents of quercetin.

Determination of free radical scavenging ability using DPPH radical. Antioxidant capacity was determined using the DPPH radical scavenging assay described by Brand–Williams, Cuvelier & Berset,[21] with some modifications. Briefly, 100 μL of the wine was added to 1.9 mL of 0.094 mM 2.2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. Free radical scavenging capacity was evaluated by measuring the absorbance at 517 nm after 30 min. Antioxidant capacity was expressed as mmol L^{-1} Trolox equivalents, using the calibration curve of Trolox (0–1000 μM), a water-soluble vitamin E analogue. All assays were performed in triplicate.

Determination of free radical scavenging ability using ABTS radical cation. The Trolox equivalent antioxidant capacity (*TEAC*) was also measured using ABTS radical cation decolorization assay.[22] Stock solutions of ABTS (7 mM) and potassium peroxodisulphate (140 mM) in water were prepared and mixed to a final concentration of 2.45 mM potassium peroxodisulphate. The mixture was left to react overnight (12–16 h) in the dark, at room temperature. On the day of the analysis, the ABTS radical solution was diluted with ethanol to the absorbance of 0.70±0.02 at 734 nm. All measurements were performed as follows: 20 μL of the wine was added to 2.0 mL of the ABTS radical solution and the absorbance readings were taken after exactly 6 min against the appropriate reagent blank prepared with 20 μL of ethanol instead of the sample. These results, obtained from triplicate analyses, were expressed as Trolox equivalents derived from a calibration curve constructed using standard solutions (100–1000 μM).

Determination of ferric reducing/antioxidant power (FRAP) assay. The ferric reducing/antioxidant power (*FRAP*) assay was carried out according to a standard procedure by Benzie & Strain.[23] FRAP reagent was prepared by mixing acetic buffer, TPTZ and $FeCl_3\times6H_2O$ (20

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mM water solution) at a ratio of 10:1:1. Briefly, 50 μL of wine was added to 950 μL of *FRAP* reagent. After 4 min, the absorbance of blue coloration was measured against a blank sample. All measurements were performed in triplicate. Aqueous solutions of $FeSO_4 \times 7H_2O$ (100––1000 μM) were used for the calibration and the results are expressed as mmol L^{-1} Fe(II).

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