Supplementary Material File

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents used were of HPLC grade and purchased from Carlo Erba (Val-de-Reuil, France). Gallic acid, sodium anhydrous carbonate, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ) and Folin-Ciocalteu reagent were received from Penta (Prague, Czech Republic). *n*-Hexane was obtained from Panreac Co. (Barcelona, Spain). Hydrochloric acid, Bradford reagent, iron (III) chloride, L-ascorbic acid, β-carotene, sulfuric acid, phenol, D(+)-glucose, sodium hydroxide, and trichloroacetic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water was utilized for every experiment.

2.4.1. Fatty Acid Composition

The fatty acid content of the extracted oil was determined according to the Commission Regulation (EC) No 796/2002 (Annex XB) by producing the fatty acid methyl esters (FAMEs). FAMEs were further analyzed according to our previous study [32]. Briefly, 2 mL of *n*-hexane was added to 0.1 g of extracted oil. Following the addition of 0.2 mL of a 2 M potassium hydroxide in methanol, the mixture was vortexed for 1 min. The mixture was then left to separate the phases. A gas chromatograph (GC) equipped with a flame ionization detector (FID) was used for the analysis. The GC was an Agilent Technologies (Santa Clara, CA, USA) gas chromatograph model 7890A with an Omegawax (30 m × 320 μm × 0.25 μm) capillary column (Supelco, Bellefonte, PA, USA). Helium was the carrier gas (flow rate was 1.4 mL/min). The temperature program was: 5 min at 70 °C, then increase with a rate of 20 °C/min up to 160 °C, ramped with a rate of 4 °C/min up to 200 °C, and finally, with a rate of 5°C/min up to 240 °C. Temperatures for the injector and FID were set at 240 and 250 °C, respectively. The makeup flow of helium was 50 mL/min, the flow rate for hydrogen was 50 mL/min, and the flow rate for air was 450 mL/min. Sample injection was carried out by injecting 1 μL of the upper sample with a split ratio of 1:100. The individual peaks were identified by comparison with reference standards from FAME Mix C8–C24 (Sigma-Aldrich, St. Louis, MO, USA).

2.4.2. Determination of Total Carotenoid Content (TCC)

The determination of TCC was based on a previously reported procedure [33]. In brief, 0.01 g oil sample was diluted with 5 mL of *n*-hexane and the absorbance was measured at 450 nm using a Shimadzu UV-1700 PharmaSpec Spectrophotometer (Shimadzu, Kyoto, Japan). The total carotenoid concentration (C_{TCn}) was calculated using the subsequent equation (1):

$$C_{TCn}\ (\mu g/mL\ Oil) = \frac{A \times FD}{A^{1\%}} \times C^{1\%} \quad (1)$$

where A is the measured absorbance at 450 nm, FD is the dilution factor, $A^{1\%} = 2500$, and $C^{1\%} = 10{,}000$ μg/mL. The extraction yield in total carotenoids (Y_{TCn}), denoted as β-carotene equivalents (CtE), was subsequently determined:

$$Y_{TCn}\ (mg\ CtE/kg\ Oil) = \frac{C_{TCn} \times V}{w} \quad (2)$$

where V represents the volume of the cyclohexane phase in mL, and w denotes the amount of oil in kg.

2.5. Analyses of the Defatted Solid Residue

2.5.1. Extraction and Determination of Crude Protein Content

Initially, 1 g of the defatted blossom powder was added to 10 mL of distilled water with a pH of 12 (pH was adjusted with 1 M NaOH) and the mixture was stirred at 500 rpm for 1 h at room temperature in order to extract the proteins. The mixture was centrifuged at 3600× *g* for 5 min following the extraction, and the supernatant was collected. The solid residue was re-extracted two more times at the abovementioned conditions and the supernatants were mixed. In order to quantify the results in g/100 g, a standard curve with an equation ($y = 0.0013x + 0.0035$, $R^2 = 0.9986$) prepared using bovine serum albumin.

2.5.2. Extraction and Determination of Carbohydrate Content

1 gram of defatted sample was blended with 10 mL of distilled water and agitated at 500 rpm for an hour at 50 °C. Following a five min centrifugation, the supernatant was removed and combined with 650 μL of sulfuric acid and 130

μL of a 5% *w*/*v* phenol solution. The solution was heated at 90 °C for 5 min, then allowed cooled at room temperature for 5 min. A spectrophotometer was used to determine the absorbance of the solution at 495 nm. Using D(+)-glucose as a reference, a calibration curve (range: 0–125 mg/L, equation: y = 0.012x – 0.0505, R^2 = 0.9979) was created for quantification purposes.

2.5.3. Extraction and Determination of L-ascorbic acid Content

Initially, 5 g of finely ground defatted sample was added to a centrifuge tube along with 3 mL of a 10% *w*/*v* trichloroacetic acid solution and 27 mL of 60:40 *v*/*v* distilled water:methanol mixture. The mixture was agitated for one min and thereupon 20 mL of *n*-hexane were added into the mixture, followed by stirring for 30 min. Subsequently, the mixture underwent centrifugation for 5 min at 3600× *g*. The lower aqueous phase was retracted and centrifuged once more for 10 min at 10,000× *g*. Then 1 mL of the solution was mixed with 0.5 mL Folin-Ciocalteu reagent (10% *v*/*v*), and the mixture was incubated for 10 min. Absorbance was measured at 760 nm. Quantification was carried out with a calibration curve (0–100 mg/L, equation: y = 0.0139x + 0.0015, R^2 = 0.9994) prepared with L-ascorbic acid.

2.5.5. Extraction and Determination of TPC

The defatted blossom powder was used to extract the polyphenols. Extraction was carried out using three approaches. In the first case, 1 g of defatted blossom powder was mixed with 20 mL of 70% *v*/*v* ethanol and the sample was US in the Elmasonic P (Elma Schmidbauer GmbH, Singen, Germany) for 10 min at 45 kHz at 20 °C with a pulse program of 5 sec ON and 2 sec OFF. In the second case, 1 g of defatted blossom powder was mixed with 20 mL of 70% *v*/*v* ethanol and the sample was ST at 40 °C for 3 h at 500 rpm. For the third scenario, both techniques were utilized one after another. Following US treatment, the sample underwent an additional step of solvent, ST extraction. Subsequent to each extraction process, the mixture was centrifuged at 3600× *g* for 5 min, and the resulting supernatant was gathered for subsequent analyses.

Quantification was performed as outlined in Section 2.4.3., employing a standard calibration curve (C_{TP}, 0–100 mg/L) with gallic acid, described by the equation y = 0.0138x – 0.0044, and achieving an R^2 value of 0.9996. The extraction yield of Y_{TP} was calculated using Equation (3):

$$Y_{TP}\ (\text{mg GAE/g dw}) = \frac{C_{TP} \times V}{w} \quad (3)$$

where V represents the volume of the extraction medium (in L) and w represents the dry weight of the sample (in g).

2.5.6. Antioxidant activity by Ferric Reducing Antioxidant Power (FRAP), DPPH Radical Scavenging Activity and Hydrogen Peroxide (H_2O_2) Scavenging Activity

The determination of the FRAP activity was carried out based on our previous study [1]. Equal volumes of the prepared extracts were mixed with 0.05 mL of $FeCl_3$ solution (4 mM in 0.05 M HCl), and the mixture was then incubated for 30 min at 37 °C. Following the addition of 0.90 mL of the TPTZ solution (1 mM in 0.05 M HCl), the absorbance was measured at 620 nm after 5 min. A calibration curve (C_{AA}, 50–500 μmol/L in 0.05 M HCl) was made using L-ascorbic acid as the reference substance. The results of ferric reducing antioxidant power (P_R) were expressed as μmol of L-ascorbic acid equivalents (AAE) per g dw using Equation (S1):

$$P_R\ (\mu\text{mol AAE/g d}) = \frac{C_{AA} \times V}{w} \quad (S1)$$

where V is the volume of the extraction medium (in L) and w is the dry weight of the sample (in g).

An approach that has been used before was used to evaluate the DPPH radicals absorption activity [2]. In brief, 25 μL of the obtained extract was carefully mixed with 975 μL of 100 μM DPPH solution. The absorbance of the solution was then measured at 515 nm ($A_{515(i)}$) after mixing and after 30 min of incubation in the absence of light ($A_{515(f)}$). The antiradical activity (A_{AR}) was calculated employing Equation (S2):

$$A_{AR}\ (\mu\text{mol DPPH/g dw}) = \frac{\Delta A}{\varepsilon \times l \times C} \times Y_{TP} \quad (S2)$$

where $\Delta A = A_{515(i)} - A_{515(f)}$; ε (DPPH) = 11,126 × 10^{-6} μM^{-1} cm^{-1}; $C = C_{TP} \times 0.025$; Y_{TP} is the total phenolic yield of the extract (mg/g); and l is the path length (1 cm).

For the H_2O_2 scavenging activity, a previously described technique was used [3]. 400 μL of the extract and 600 μL of H_2O_2 solution (40 mM, made in phosphate buffer, pH 7.4) were added to an Eppendorf tube. After 10 min, the absorbance at 230 nm was measured. The capacity to scavenge the H_2O_2 was expressed as:

$$\% \text{ Scavenging of } H_2O_2 = \frac{A_o - (A - A_c)}{A_o} \times 100 \quad \text{(S3)}$$

where A_o, A_c, and A are the absorbance of the blank solution, and the extract solution in the absence of hydrogen peroxide and sample, respectively.

Anti-hydrogen peroxide activity (A_{AHP}) was determined as μmol L-ascorbic acid equivalents (AAE) per g of dw, using an L-ascorbic acid calibration curve (C_{AA}, 50–500 μmol/L in 0.05 M HCl), using the following equation:

$$A_{AHP} \text{ (μmol AAE/g dw)} = \frac{C_{AA} \times V}{w} \quad \text{(S4)}$$

where V is the volume of the extraction medium (in L) and w is the dry weight of the sample (in g).

References

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