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The main phenolic compounds responsible for the antioxidant capacity of sweet cherry (*Prunus avium* L.) pulp

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ARTICLE INFO

Keywords:

Bioactive compounds
Multivariate analysis
Chlorogenic acids
Anthocyanins
Autoscaling

ABSTRACT

The antioxidant capacity of sweet cherry (*Prunus avium* L.) pulp extracts is strictly related to the phenolic content, starting from the fact that the higher content of phenolic compounds corresponds to the higher antioxidant indexes. This work aims to assess which compounds characterized three cultivars, namely Ferrovia, Sweetheart, and Lapins grown in Southern Italy and mainly influenced the antioxidant capacity of their extracts. HPLC-MS/MS analyses were conducted to identify and quantify 17 flavonoids and 25 hydroxycinnamates derivatives. A significant influence of cultivar was revealed from one-way MANOVA ($p < 0.05$). Furthermore, the extracts were tested for their radical scavenging activity (DPPH and ABTS assays) and reducing power using the Folin-Ciocalteu method. Lapins and Sweetheart extracts, richer in phenolic compounds, returned the highest reducing power and radical scavenging capacity. Finally, a Factorial Analysis was applied to the collected data allowing reliable correlations between phenolics and antioxidant indexes.

1. Introduction

Prunus avium L. (Rosaceae), commonly called sweet cherry, is a vigorous tree extensively distributed around the world with greater prevalence in areas with a temperate climate (Bastos et al., 2015). In Italy there is a long tradition of cultivating cherries with around 30,000 Ha of orchards, particularly located (60%) in the southeast of the country (Apulian region) producing around 32,000 tons of cherries annually (<https://rivistafrutticoltura.edagricole.it/featured/aspetti-p-roduttivi-e-di-mercato-dellattuale-cerasicoltura/>). Sweet cherry fruits are traditionally very appreciated by consumers due to their quality characteristics, such as taste, color, sweetness, sourness, and firmness; furthermore, their intake has been associated with beneficial effects (Corbo et al., 2019; Nawirska-Olszańska et al., 2017), because these fruits are very rich in nutrients (i.e., vitamins and fibers) and bioactive compounds (i.e., phenols, carotenoids, and organic acids) which have been demonstrated to prevent chronic and degenerative diseases (Lage et al., 2020; Noratto et al., 2018). Sweet cherry extracts might be a serious candidate to prevent oxidative stress-induced disorders like

intestinal inflammation disorders and neuronal cell death (Matias et al., 2016).

In particular, special interest has been focused on phenolics due to their great capacity to scavenge reactive oxygen and nitrogen species as well as the strong anti-inflammatory activity shown (Gonçalves, Bento, Silva, & Silva, 2017). Their profile has been extensively investigated using both targeted and non-targeted approaches, including nuclear magnetic resonance (NMR) and high-pressure liquid chromatography hyphenated with mass spectrometry (HPLC-MS) analyses, which highlight how phenols composition and content can significantly vary according to climatic conditions, cultivar, fruit maturity, and storage conditions (Gonçalves, Ramos, Rosado, Gallardo, & Duarte, 2019; Ballistreri, Continella, Gentile, A., Amenta, Fabroni, & Rapisarda, 2013; Longobardi et al., 2013). Anthocyanins, especially cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside, are the main flavonoids present in sweet cherries mostly responsible for the red skin and flesh color; moreover, epicatechin and quercetin-3-O-rutinoside are the principal flavanol and flavonol, respectively, identified in the pulp (Crupi, Genghi, & Antonacci, 2014; Magri et al., 2023). Finally, many chlorogenic

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<https://doi.org/10.1016/j.lwt.2023.115085>

Received 4 May 2023; Received in revised form 9 July 2023; Accepted 12 July 2023

Available online 13 July 2023

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and hydroxybenzoic acid derivatives are also present (Crupi et al., 2018). Information from the qualitative and quantitative pattern of phenolic compounds by HPLC-MS determination can be used to perform multivariate statistical analysis to find out the most important compositional differences between varieties through the metabolomic “quantitative approach” (Wishart, 2008).

To date, a panel of antioxidant assays exists based on different types of chemical reactions (namely, single electron transfer or hydrogen atom transfer assays) not all providing equivalent information. Therefore, screening biological samples for their antioxidant properties is best suited by coupling a series of assays which can give more reliable results (Huang, Ou, & Prior, 2005). Generally, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Folin-Ciocalteu are the most widespread assays for the high-throughput and cost-effective gauge of food samples, such as sweet cherries (Domínguez-Rodríguez, Marina, & Plaza, 2022; Ockun et al., 2022), due to their simplicity, rapidity, and reproducibility together with direct correlation to phenolic compounds (Bibi Sadeer, Montesano, Albrizio, Zengin, & Mahomoodally, 2020). Many reports have already demonstrated how the antioxidant capacity of cherry pulp extracts is strictly related to the phenolic content, but to the best of our knowledge, few studies deal with a possible correlation between the radical scavenging capacity and reducing power and the phenolic composition of sweet cherries pulp, without reaching a specific and validated relationship (Acero, Gradillas, Beltran, García, & Mingarro, 2019; Martini, Conte, & Tagliacuci, 2017).

Therefore, starting from the hypothesis that higher content of phenolics corresponds to the higher antioxidant indexes in sweet cherry pulp extracts, this work aims to assess which compounds characterized the three studied cultivars (namely, Ferrovia, Sweetheart, and Lapins) and mainly correlated with antioxidant indexes. Within this context, the extracts were tested for their radical scavenging power by measuring their capacity to scavenge DPPH[•] and ABTS^{•+} and their reducing power by using the Folin-Ciocalteu method. Moreover, HPLC-MS/MS analyses were carried out to identify and quantify the main polyphenol metabolites and, finally, a series of multivariate statistical analyses [i.e., one-way MANOVA and Factorial Analysis (FA)] was applied to the collected data. Finally, solutions of standard compounds at the same concentration determined in the cherries extracts were prepared and analyzed for their antioxidant capacity to validate the FA results.

2. Materials and methods

2.1. Plant materials

The experiment was conducted during the 2021 season on mature sweet cherries (*Prunus avium* L.) of Ferrovia, Sweetheart, and Lapins varieties, collected from 22 years-old trees located in Apulia (longitude 41.07° E, latitude 16.26° N, and altitude 259 m) and cultivated under organic farming. It is worth noting that organic sweet cherries were chosen because of the need for natural extracts without contaminants, which could eventually be conditioning their antioxidant capacity. The trees were trained to a *Prunus mahaleb* rootstock and planted at a spacing of 6 m × 5 m; starting from blossoming until harvest, they were irrigated through a localized system (drip irrigation) with a water supply of 16 L/h. Samples were harvested (from the first to the last decade of June) once reached technological maturity, consisting of suitable total soluble solids (TSS), measured as °Brix (TSS = 15.1 ± 1.1, 16.9 ± 1.1, and 16.5 ± 0.5 °Brix), and titratable acidity (TA), expressed as a percentage of malic acid (TA = 0.84 ± 0.11, 0.80 ± 0.06, and 0.95 ± 0.04%) for Ferrovia, Sweetheart, and Lapins, respectively.

Samples (around 1 kg) of cherries from each variety were picked on the same day, from four different branches of an individual tree and mixed; then, they were packed in plastic bags and stored in the laboratory at -80 °C until further analyses.

2.2. Chemicals

LC-MS grade water and acetonitrile, formic acid, ethanol, and methanol, were supplied from Merk Life Science S.r.l. (Milano, Italy). Cyanidin-3-O-glucoside chloride, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, (+)-catechin, (-)-epicatechin, procyanidins B₁ and B₂ were purchased from Extrasynthèse (Genay, France). Chlorogenic acid, neochlorogenic acid, cyanidin-3-O-sophoroside chloride, and quercetin-4'-O-glucoside were purchased from Phytolab (Aprilia, Italy). The stable free radical DPPH[•], 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, the Folin-Ciocalteu phenol reagent, gallic acid, and sodium carbonate were purchased from Sigma-Aldrich (Milan, Italy). ABTS^{•+} was purchased from Alfa Aesar (Karlsruhe, Germany).

2.3. Ultrasound-assisted extraction (UAE) of phenolic compounds from cherries pulp

Ultrasound extraction of flavonoids and hydroxycinnamic acid derivatives from cherries pulp was carried out accordingly to the optimized conditions determined in our previous report (Clodoveo, Crupi, & Corbo, 2022). Destoned sweet cherry berries (30 g) were freeze-dried (CHRIST Alpha 1-4LD plus, Osterode am Harz, Germany) at -42 °C under a pressure of 0.10 mbar for 72 h and ground using an IKA A11 basic homogenizer (IKA®-Werke GmbH & Co., Staufen, Germany). Powder samples (250 mg) from each cherry variety were properly sieved at 0.5 mm (Endecotts Ltd., London, UK), carefully weighed (EU-C1200, Gibertini s.r.l., Novate Milanese, Milano, Italy) into 2 mL Eppendorf tubes, and mixed with 1.5 mL of water/ethanol (1:1, v/v). UAE of phenolics was performed by an ultrasonic water bath (Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany) working in continuous mode at constant temperature (25 ± 2 °C), time (3 min), power (100 W), and frequency (37 kHz). Afterward, the extracts were centrifuged at 4000 × g for 15 min at 5 °C (EPPENDORF 5810R, Hamburg, Germany), filtered through a 0.45 µm syringe cellulose filter, and analyzed by HPLC-DAD-MS/MS. The extractions from each variety were repeated six folds.

2.4. HPLC-DAD-ESI-MS/MS analyses

Qualitative and quantitative analyses of cherry extracts were carried out through a 1290 Infinity HPLC system equipped with a binary pump, thermostatically column compartment, autosampler, and MSD triple quadrupole QQQ in a series configuration (Agilent Technologies, Santa Clara, CA). The phenolic compounds extract (3 µL) was injected onto a Luna C18 (150 × 2 mm i.d., particle size 3 µm, Phenomenex, Torrance, CA, USA) column, protected by Gemini C18 (Phenomenex, Torrance, CA, USA) 5 µm (4 × 2 mm i.d.) pre-column and maintained at 40 °C. The following binary gradient, column re-equilibration, and flow rate were the same as those reported in our previous research (Crupi et al., 2014). Diode array detection (DAD) was set in the range 190–650 nm, and absorbance was recorded at 520, 360, 320, and 280 nm. The ionization of the molecules, necessary for their identification, was acquired in positive (for anthocyanins) and negative (for the other compounds) modes adopting the same optimized parameters as previously described (Crupi et al., 2018; Crupi et al., 2014).

Retention times (RT), absorption spectra profile, MS, and MS/MS patterns were compared with those reported in other research or, eventually, of analytical standards and used for the compound identification (Crupi et al., 2018; Crupi et al., 2014); while, the quantification was made by using the calibration curves, obtained through HPLC-DAD analyses, in the concentration range 0.01–400 µg/mL of cyanidin-3-O-glucoside (R² = 0.9964) for anthocyanins, chlorogenic acid (R² = 0.9983) for hydroxycinnamic acids, quercetin-3-O-glucoside (R² = 0.9994) for flavonols, and (+)-catechin (R² = 0.9967) for flavanols.

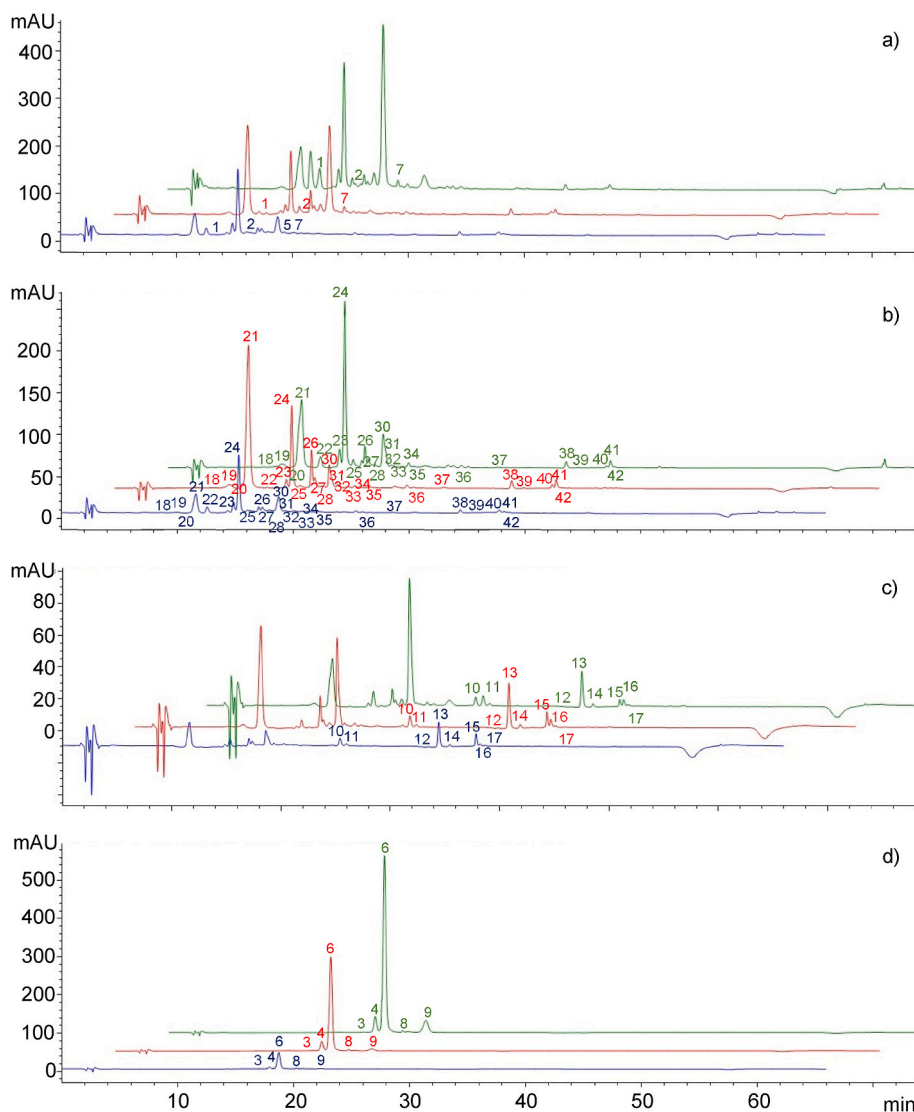


Fig. 1. HPLC-DAD chromatograms registered at a) 280, b) 320, c) 360, and d) 520 nm of Ferrovia (blue line), Lapins (red line), and Sweetheart (green line). Peaks number corresponds to that reported in [Tables 1 and 2](#) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Folin Cioalciu, DPPH, and ABTS assays

The antioxidant activity of sweet cherry extracts was assessed by the DPPH radical assay and ABTS radical cation assay according to previously reported methods ([De Leo et al., 2021](#); [Pacífico et al., 2014](#)). Briefly, for the DPPH assay an aliquot (0.35 mL) of pulp extracts at different concentrations (0.20–125 µg/mL) was mixed with 0.65 mL of a freshly prepared DPPH methanol solution (0.1 mmol/L). The reaction mixture was shaken vigorously and kept in the dark at room temperature for 30 min. Then, the mixture was recorded at 517 nm using a UV–Vis spectrophotometer (Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer, Fischer Scientific, Milan, Italy). The % of DPPH• scavenging was calculated according to the formula:

$$\text{Inhibition (\%)} = (\text{Abs}_0 - \text{Abs}_t) / \text{Abs}_0 \times 100$$

Abs₀ is the absorbance of the control DPPH solution at t = 0 min and Abs_t is the absorbance after adding the test sample at t = 30 min. Antiradical curves were obtained by plotting % DPPH• scavenging activity versus concentrations; then, IC₅₀ (µg/mL necessary for 50% reduction of the DPPH radical) was calculated and transformed into µg/g d.w.

ABTS radical cation was generated by the reaction between ABTS (7.0 mmol/L) and potassium persulfate (2.45 mmol/L). The mixture was allowed to stand in the dark at room temperature for 12–16 h. Thus, the ABTS^{•+} solution was diluted with methanol to reach an absorbance of 0.70 at 734 nm. Investigated extracts were dissolved in 1.0 mL of diluted ABTS^{•+} solution. After 15 min of incubation in the dark at room temperature, the absorbance was measured at 734 nm in reference to a blank. The results were expressed in terms of mg Trolox equivalents per g d.w. by using a calibration curve prepared at the concentrations range 0.150–0.025 mg/mL.

For the determination of reducing capacity, the Folin-Cioalciu (F–C) assay was used. In detail, 50 µL of extract was mixed with 500 µL of Folin-Cioalciu reagent and 400 µL of distilled water. After 3 min, 500 µL of 10% Na₂CO₃ was added and then the solution was left in the dark at room temperature for 1 h. Absorbance was recorded at 765 nm and, finally, results were expressed as mg of gallic acid equivalents (GAE) in g of dry weight (d.w.) cherries.

F–C and ABTS were also measured on nine standard solutions (M1 – M9) prepared in water/ethanol (1:1, v/v) and containing fixed concentrations according to the values determined in Ferrovia, Sweetheart, and Lapins extracts (expressed in µg/mL; see [Tables S1 – Supplementary](#)

Table 1HPLC-DAD-MS (ESI^{+/−}) characteristics and content of anthocyanins, flavonols, and flavan-3-ols in mature sweet cherries pulp.

Peak	Compound	RT (min)	λ_{\max} (nm)	[M] ⁺ (m/z)	[M-H] [−] (m/z)	MS/MS fragments	Ferrovia	Sweetheart	Lapins
1	Procyanidin B ₁ ^a	13.16	279		577	451, 425, 407, 289	31 ± 4 ^{b,c}	173 ± 19a	16 ± 2b
2	(+)-Catechin ^a	16.27	282		289	245, 205, 179	2.2 ± 0.2c	8.3 ± 1.2a	4.6 ± 0.7b
3	Cyanidin-3-O-sophoroside ^a	16.90	516	611		449, 287	0.09 ± 0.04c	1.12 ± 0.09a	0.64 ± 0.12b
4	Cyanidin-3-O-glucoside ^a	17.85	516	449		287	6.6 ± 1.3c	76 ± 8a	52 ± 10b
5	Procyanidin B ₂ ^a	18.20	279		577	451, 425, 407, 289	0.6 ± 0.2	n.d.	n.d.
6	Cyanidin-3-O-rutinoside	18.62	516	595		449, 287	79 ± 14c	930 ± 110a	540 ± 80b
7	(−)-Epicatechin ^a	19.95	282		289	245, 205, 179	2.9 ± 0.6b	21 ± 2a	19 ± 2a
8	Pelargonidin-3-O-rutinoside	20.20	520	579		271	1.5 ± 0.3b	4.4 ± 0.9a	3.9 ± 0.6a
9	Peonidin-3-O-rutinoside	22.20	520	609		463, 301, 286	3.3 ± 0.8c	108 ± 13a	19 ± 3b
10	Quercetin-3-O-rutinoside-7-O-glucoside	25.22	344		771	609, 463, 301	7.7 ± 0.9c	10.0 ± 1.6b	12.3 ± 1.9a
11	Kaempferol-3-O-rutinoside-7-O-glucoside	25.80	330, 266, 282sh		755	593, 447	1.9 ± 0.2b	2.3 ± 0.3a	2.6 ± 0.4a
12	Quercetin-3-O-galactosyl-rhamnoside	33.34	355		609	591, 301, 300, 257	0.39 ± 0.13b	0.81 ± 0.15a	0.86 ± 0.17a
13	Quercetin-3-O-rutinoside	34.27	354		609	301, 300, 255	23 ± 4c	37 ± 5b	50 ± 7a
14	Quercetin-3-O-glucoside ^a	35.31	354		463	301, 300	2.0 ± 0.3b	3.3 ± 0.4a	3.9 ± 0.5a
15	Kaempferol-3-O-rutinoside ^a	37.75	350		593	285, 284	9 ± 2b	4.9 ± 0.9c	13 ± 2a
16	Kaempferol-3-O-glucoside ^a	38.11	282, 330		447	327, 285, 284, 255	1.6 ± 0.3c	5.5 ± 0.7b	6.6 ± 1.1a
17	Quercetin-4'-O-glucoside ^a	38.35	282, 332		463	301, 300	0.70 ± 0.14a	0.69 ± 0.15a	0.37 ± 0.11b
Total flavonoids							170 ± 30c	1390 ± 160a	750 ± 110b

RT: retention time; λ_{\max} : absorption maximum wavelength; [M]⁺: molecular ion, only present in the anthocyanidins MS spectra; [M-H][−]: deprotonated molecule.^a Available as analytical standards.^b Mean of 6 replicates ± standard deviation expressed in mg/kg d.w.^c Different letters in each row indicate statistically significant differences between cultivars (one-way MANOVA – Tukey HSD post-hoc test, P < 0.05).

Material). Namely, M1 (Ferrovia-like), M2 (Sweetheart-like), and M3 (Lapins-like) contained procyanidin B₁, *trans*-5-*O*-caffeoyl-quinic acid, *trans*-3-*O*-caffeoyl-quinic acid, catechin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-sophoroside, epicatechin, quercetin-4'-*O*-glucoside, and kaempferol-3-*O*-glucoside; M4 (Ferrovia-like), M5 (Sweetheart-like), and M6 (Lapins-like) contained the same compounds except for procyanidin B₁, *trans*-5-*O*-caffeoyl-quinic acid, and *trans*-3-*O*-caffeoyl-quinic acid; M7 (Ferrovia-like), M8 (Sweetheart-like), and M9 (Lapins-like) contained only the procyanidin and the two chlorogenic acids.

2.6. Statistical analysis

Data from HPLC-DAD-MS/MS analysis of phenolics and antioxidant capacity assays were analyzed using the STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) software package. Specifically, after testing their normal distribution by Shapiro–Wilk's W test, one-way multivariate analysis of variance (MANOVA), followed by Tukey's HSD post hoc test was performed on the quantified phenolic compounds and antioxidant indexes to evaluate the significantly different means ($p < 0.05$). A successive Factor Analysis (FA) with orthogonal rotation of axes was carried out, including auto-scaling transformed data of phenolic compounds, DDPH, ABTS, and F–C.

3. Results and discussion

3.1. Qualitative and quantitative profile of phenolic compounds in the cherries pulp extracts

Phenolics structure and interaction with other fruit components usually influence their diffusion into the extraction solvent. Various solvents are suggested in the literature for the recovery of flavonoids and phenolic acids from berry fruits, especially methanol, ethanol, or hydroalcoholic mixtures (Crupi et al., 2014; Gonçalves et al., 2017; Watrelot & Bouska, 2022). In this study, an optimized green extraction

procedure adopting water/ethanol (1:1, v/v) as the solvent, which has allowed to obtain the maximum recovery of the main phenol compounds present in the cherry pulp, was employed (Chemat et al., 2019; Clodoveo et al., 2022).

Fig. 1 depicts the chromatograms registered at 280, 320, 360, and 520 nm of cherry pulp extracts in water/ethanol (1:1, v/v) from the three tested cultivars (Ferrovia, Sweetheart, and Lapins). The phenolic compounds identification was accomplished by matching the retention times, elution order (also confirmed using available analytical standards), UV/Vis, and MS spectra with those of our previous relevant works (Crupi et al., 2018; Crupi et al., 2014). Specifically, 17 peaks were assigned to flavonoids, including 4 flavanols (procyanidin B₁, catechin, procyanidin B₂, and epicatechin), 8 flavonols (quercetin and kaempferol derivatives), and 5 anthocyanins (cyanidin derivatives and the two rutinosides of pelargonidin and peonidin) (Fig. 1a, c, and d; Table 1). Moreover, 25 hydroxycinnamates derivatives (mainly consisting of caffeoyl and coumaroyl conjugates) were also attributed (Fig. 1b; Table 2).

From one-way MANOVA analysis coupled with the Tukey HSD posthoc test, a significant influence of cultivar was revealed ($p < 0.05$), with Sweetheart characterized by the highest amount of phenolics (Tables 1 and 2). In particular, flavanols, especially procyanidin B₁ (173 mg/kg d.w.) and (+)-catechin (8.3 mg/kg d.w.), were more concentrated in Sweetheart pulp extracts, even though procyanidin B₂ was only found in Ferrovia as already observed in our previous studies (Clodoveo et al., 2022; Crupi et al., 2014). It is worth pointing out that in recent research (Gonçalves et al., 2017), very lower values of catechin and epicatechin (procyanidins were not determined at all) were quantified in Sweetheart lyophilized cherries, probably due to different geographic origins and pedoclimatic conditions, but also extraction technique and solvent. According to previous findings in the literature (Kelebek & Selli, 2011; Serra, Duarte, Bronze, & Duarte, 2011), cyanidin-3-*O*-rutinoside was the main anthocyanin identified in all three cultivars (representing 46–72% of the total contents of anthocyanins), followed by cyanidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside. However, its content

Table 2
HPLC-DAD-MS (ESI+/-) characteristics and content of hydroxycinnamic acids derivatives in mature sweet cherries pulp.

peak	Compound	RT (min)	λ_{max} (nm)	[M-H] ⁻ (m/z)	MS/MS fragments m/z	Ferrovia	Sweetheart	Lapins
18	3-O-(4'-O-caffeoyl glycosyl)-quinic acid	8.53	294sh, 309	515	379, 353, 341, 191, 179	0.42 ± 0.05 ^{b,c}	0.85 ± 0.14b	1.09 ± 0.15a
19	cis-3-O-caffeoylquinic acid	9.80	294sh, 312	353	191, 179	0.9 ± 0.2b	3.7 ± 0.6a	3.8 ± 0.6a
20	5-O-(4'-O-caffeoyl glycosyl)-quinic acid	10.01	298sh, 315	515	379, 353, 341, 191, 179	4.5 ± 0.8b	18 ± 2a	19.5 ± 1.1a
21	trans-3-O-caffeoylquinic acid ^a	11.49	303sh, 324	353	191, 179, 135	120 ± 20c	320 ± 50b	570 ± 70a
22	Caffeic acid hexose I	13.16	290, 304sh	341	323, 281, 251, 221, 179	0.41 ± 0.04b	30 ± 4a	2.0 ± 0.2b
23	cis-3-O-coumaroylquinic acid	14.79	301	337	191, 163	24.9 ± 1.4b	39 ± 5a	19 ± 3c
24	trans-3-O-coumaroylquinic acid	15.24	302sh, 311	337	191, 163	189 ± 12b	370 ± 50a	190 ± 30b
25	Caffeic acid hexose II	15.96	292, 306sh	341	323, 281, 251, 221, 179	0.56 ± 0.10c	14.1 ± 1.4a	6.9 ± 0.9b
26	trans-5-O-caffeoylquinic acid ^a	16.98	302sh, 326	353	191, 179, 135	13 ± 3c	39 ± 5b	71 ± 9a
27	trans-4-O-caffeoylquinic acid	17.28	292, 324	353	191, 179, 173, 135	11.6 ± 1.3b	11 ± 2b	18 ± 3a
28	4-O-(4'-O-caffeoyl glycosyl)-quinic acid	17.51	–	515	497, 469, 379, 353, 341, 191, 179	2.0 ± 0.3a	0.7 ± 0.5b	2.8 ± 1.0a
29	methyl 3-O-caffeoylquinic acid	18.08	–	367	335, 193, 161, 135	0.9 ± 0.2c	2.0 ± 0.2b	3.2 ± 0.4a
30	cis-4-O-coumaroylquinic acid	18.55	–	337	191, 173, 163	6.1 ± 0.8c	78 ± 6a	49 ± 8b
31	cis-5-O-caffeoylquinic acid	18.81	295 sh, 313	353	335, 191	10.1 ± 1.9c	31 ± 4a	19 ± 3b
32	trans-4-O-coumaroylquinic acid	19.26	292, 309sh	337	191, 173, 163	4.6 ± 0.5b	5 ± 2b	8.5 ± 1.0a
33	methyl coumaroylquinic acid	20.20	292, 308sh	351	319, 163, 145, 119	1.9 ± 0.3b	1.5 ± 0.3b	3.0 ± 0.6a
34	methyl coumaroylquinic acid II	20.74	292, 308sh	351	319, 163, 145, 119	1.3 ± 0.2c	8.9 ± 1.4a	4.3 ± 0.8b
35	methyl 4-O-caffeoylquinic acid	21.17	–	367	193, 179, 161, 135	1.27 ± 0.11b	1.3 ± 0.6b	2.9 ± 0.6a
36	methyl 5-O-caffeoylquinic acid	25.32	303sh, 326	367	191, 179, 161, 135	4.8 ± 0.5b	5.7 ± 0.9b	7.4 ± 1.1a
37	methyl coumaroylquinic acid IV	28.46	303sh, 328	351	319, 163, 145, 119	2.3 ± 0.3b	2.0 ± 0.4b	3.8 ± 0.5a
38	trans,trans-3,5-di-O-caffeoylquinic acid	34.35	298, 328	515	353, 191, 179	8.8 ± 1.4c	13.9 ± 1.9b	19 ± 3a
39	cis,trans-3,5-di-O-caffeoylquinic acid	35.37	294, 308sh	515	497, 467, 447, 379, 353	0.8 ± 0.2b	1.22 ± 0.10a	1.4 ± 0.2a
40	3-O-coumaroyl-5-O-caffeoylquinic acid	37.82	294, 320sh	499	481, 431, 337, 191, 179, 163	5.0 ± 1.1b	2.8 ± 0.5c	7.0 ± 0.9a
41	3-O-caffeoyl-5-O-coumaroylquinic acid	38.17	295, 305sh	499	481, 453, 431, 353, 337, 191, 179, 163	4.4 ± 0.8c	14.4 ± 1.7b	18 ± 2a
42	3-O-coumaroyl-4-O-caffeoylquinic acid	38.71	295, 305sh	499	481, 453, 431, 353, 337, 191, 179, 163	0.7 ± 0.3b	1.0 ± 0.2b	2.1 ± 0.4a
	Total hydroxycinnamates					420 ± 50b	1020 ± 140a	1050 ± 140a

RT: retention time; λ_{max} : absorption maximum wavelength; [M-H]⁻: deprotonated molecule.

^a Available as analytical standards.

^b Mean of 6 replicates ± standard deviation expressed in mg/kg d.w.

^c Different letters in each row indicate statistically significant differences between cultivars (one-way MANOVA – Tukey HSD post-hoc test, P < 0.05).

(930 mg/kg d.w.), as well as that of the other 5 anthocyanins, was higher in Sweetheart (Table 1); our data appear in accordance with other research that reported a higher amount of this anthocyanin in Sweetheart than Lapins (Gonçalves et al., 2017; Martini et al., 2017).

Interestingly, the behavior of flavonols is less homogeneous, indeed more consistent levels of quercetin-3-O-rutinoside (50 mg/kg d.w.), which is the most abundant flavonol generally found in sweet cherries (Crupi et al., 2014), quercetin-3-O-rutinoside-7-O-glucoside (12.3 mg/kg d.w.), kaempferol-3-O-rutinoside (13 mg/kg d.w.), and kaempferol-3-O-glucoside (6.6 mg/kg d.w.) were quantified in Lapins pulp extracts; while, kaempferol-3-O-rutinoside-7-O-glucoside, quercetin-3-O-galactosyl-rhamnoside, and quercetin-3-O-glucoside were more concentrated in Lapins and Sweetheart and, finally, quercetin-4-O-glucoside were more present in Ferrovia and Sweetheart cherries (Table 1). Regarding hydroxycinnamates, in agreement with results reported by other authors (Ballistreri et al., 2013), trans-3-O-caffeoylquinic acid (neochlorogenic acid) and trans-3-O-coumaroylquinic acid represented the predominant derivatives in cherries; specifically, they were the principal phenolic acids in Lapins (570 mg/kg d.w.) and Sweetheart (370 mg/kg d.w.), respectively. Overall, coumaroylquinic and caffeoylquinic acids content was lower in the Ferrovia cultivar (Table 2). This was not a surprise, since comparing different literature reports emerges a deficit of hydroxycinnamic acids in Ferrovia concerning other cultivars (especially Lapins and Sweetheart); however, it is worth noting that the scion × rootstock combination and orchard management could play a fundamental role on the level of these compounds (Correia, Schouten, Silva, & Gonçalves,

Table 3
In vitro antioxidant indexes of sweet cherry extracts.

Antioxidant index	Ferrovia	Sweetheart	Lapins
Folin-Ciocalteu (mgGAE/g d.w.)	1.22 ± 0.16 ^{a,b}	2.4 ± 0.2a	2.1 ± 0.3a
DPPH (IC ₅₀) ^c	218 ± 14a	86 ± 14b	91 ± 2b
ABTS (mgTE/g d.w.)	1.27 ± 0.16b	2.9 ± 0.4a	2.6 ± 0.4a

^a Mean of 6 replicates ± standard deviation.

^b Different letters in each row indicate statistically significant differences between cultivars (one-way MANOVA – Tukey HSD post-hoc test, P < 0.05).

^c Concentration expressed in µg/g d.w. necessary for 50% reduction of DPPH radical.

2017).

3.2. In vitro reducing power and radical scavenging capacity

Because the analyzed cherry pulp extracts showed significant qualitative and quantitative variation regarding flavonoids (Table 1) and chlorogenic acids (Table 2) patterns, three different antioxidant assays were adopted to group more complete information for comparing the antioxidant capacity of the sweet cherry cultivars (Acero et al., 2019). Thus, F–C, DPPH, and ABTS assays were chosen because they are conventional methods for determining antioxidant properties of plant matrices and complementary estimations based on a single electron (F–C and ABTS) or hydrogen transfer (DPPH) mechanisms more related to phenolic antioxidant molecules (Bibi Sadeer et al., 2020; Opitz et al.,

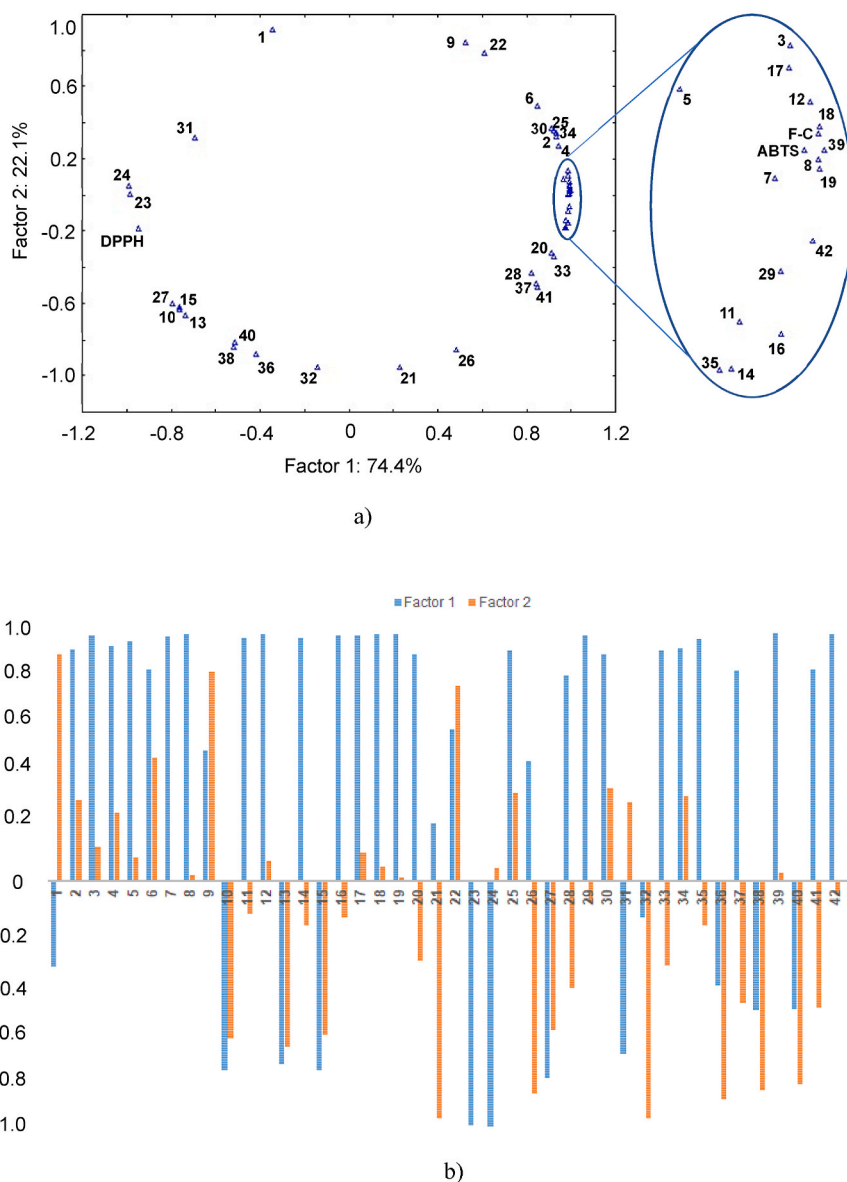


Fig. 2. Factor analysis score plot a) and factor loadings b) relative to cherry pulp phenolic compounds and F-C, ABTS, and DPPH autoscaled data. Peaks number corresponds to that reported in [Tables 1 and 2](#)

2014; Pacifico et al., 2014). The outcomes of antioxidant indexes measured on Lapins, Ferrovia, and Sweetheart extracts are listed in [Table 3](#). As expected from perusing the relevant literature (Gonçalves et al., 2017; Pacifico et al., 2014; Picariello, De Vito, Ferranti, Paolucci, & Volpe, 2016), they showed concentration dependent-manner antioxidant properties closely linked to the phenolic content; Lapins and Sweetheart extracts, which were richer in phenolic compounds, returned the highest reducing power (F-C: 2.1 and 2.4 mgGAE/g d.w., respectively) and radical scavenging capacity (DPPH: 91 and 86 $\mu\text{g/g}$ d.w.; ABTS: 2.6 and 2.9 mgTE/g d.w., respectively) ([Table 3](#)).

3.3. Relationship between phenolic compounds profile and antioxidant indexes

Positive correlations have been already reported between total phenolics in sweet cherries and antioxidant indexes (Gonçalves et al., 2021). However, as shown by the polyphenolic profile ([Tables 1 and 2](#)), in this study Sweetheart and Lapins extracts were characterized by different content of flavonoids and chlorogenic acids (i.e., the former

was richer in anthocyanins and *trans*-3-*O*-coumaroylquinic acid while the latter in flavonols and *trans*-3-*O*-caffeoylquinic acid) even though the reducing power and radical scavenging activity were the same ([Table 3](#)). Therefore, to tentatively find out which cherry pulp phenol compounds mainly influenced the antioxidant capacity, FA analysis was carried out on phenolics, DPPH, ABTS, and F-C data after their auto-scaling transformation to become equally important and allowed reliable correlation (Van den Berg, Hoefsloot, Westerhuis, Smilde, & Van der Werf, 2006).

Specifically, two factors were extracted (based on Kaiser and scree rules) to describe the maximum covariance (96.51%) of the variables in the dataset without losing too much information. Furthermore, the orthogonal axes variation (quartimax normalized) was imposed to obtain a clearer factor loadings pattern and improve the dataset structure interpretability ([Fig. 2](#)). Twenty-one compounds (among which catechin, epicatechin, cyanidin-3-*O*-glucoside, quercetin-4'-*O*-glucoside, and few caffeoyl- and coumaroylquinic acid derivatives), having high positive loadings (>0.9) on Factor 1 (accounting for 74.4% of total variance), could be considered as the main responsible of the antioxidant capacity because they were directly related to F-C and ABTS (factor

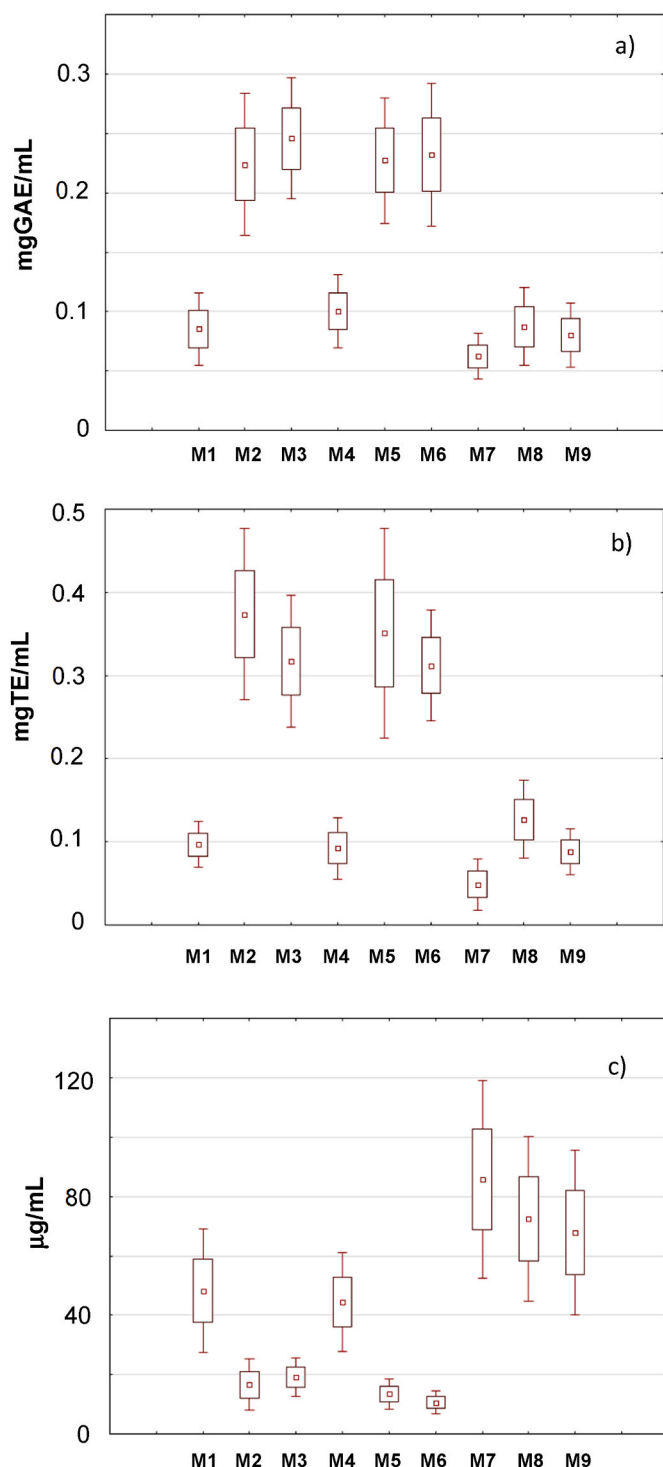


Fig. 3. Box & Whisker plots of a) F-C, b) ABTS, and c) DPPH values measured in standard mixtures (M₁-M₉) at the same concentrations of cherry pulp extracts.

loadings of 0.9955 and 0.9927, respectively) and inversely related to DPPH (factor loading of -0.9449) (Fig. 2). Evidently, it could be inferred that the selected phenolic compounds play a synergistic role in enhancing the radical scavenging activity and reducing power of the cherry extracts.

In line with our findings, previous *in vitro* studies with different sweet cherry cultivar extracts proposed how the flavonoids content, especially anthocyanin like cyanidin-3-*O*-glucoside, was strongly correlated to the

antioxidant properties (Hayaloglu & Denir, 2006; Acero et al., 2019; Ockun et al., 2022) due to their higher number of hydroxyl groups than the other compounds (Mendes, de Freitas, Baptista, & Carvalho, 2011). Conversely, hydroxycinnamic acids showed a lower relationship with antioxidant indexes (Acero et al., 2019); indeed, in our work, for instance, *trans*-3-*O*-caffeoylquinic acid, *trans*-5-*O*-caffeoylquinic acid, and *trans*-4-*O*-coumaroylquinic acid had high factor loadings on Factor 2, meaning they were not related to ABTS, F-C, and DPPH. Then, *cis*-3-*O*-coumaroylquinic acid and *trans*-3-*O*-coumaroylquinic acid had even negative loadings ($>|0.9|$) on Factor 1, meaning they were inversely related to antioxidant indexes and probably might have somewhat prooxidant effect. Finally, it is worth pointing out that procyanidin B₁ (among the identified flavanols) did not seem to influence the antioxidant capacity, having a positive loading (0.9156) on Factor 2 (Fig. 2).

At this point, to test the reliability of FA results, F-C, DPPH, and ABTS were measured and compared on 1) standard solutions obtained by mixing some of the compounds more related to the antioxidant indexes (namely, catechin, epicatechin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-sophoroside, quercetin-4'-*O*-glucoside, and kaempferol-3-*O*-glucoside) plus procyanidin B₁, *trans*-3-*O*-caffeoylquinic acid, and *trans*-5-*O*-caffeoylquinic acid, which were apparently not related, at the same concentrations found in Ferrovia (M₁), Sweetheart (M₂), and Lapins (M₃) and 2) mixtures just containing the former or the latter compounds group at the identical concentration of Ferrovia (M₄ and M₇), Sweetheart (M₅ and M₈), and Lapins (M₆ and M₉) cherry extracts, respectively. From Fig. 3, illustrating Box & Whisker plots of mean values, M₂ and M₃ showed higher reducing power and radical scavenging activity than M₁, in agreement with the statistical difference observed between Sweetheart/Lapins and Ferrovia (Table 3). Moreover, F-C, ABTS, and DPPH values of M₁, M₂, and M₃ were not statistically different from M₄, M₅, and M₆ ones, meaning that the selected anthocyanins, flavonols, and flavanols had a synergistic effect independently from the presence of procyanidin B₁, *trans*-3-*O*-caffeoylquinic acid, and *trans*-5-*O*-caffeoylquinic acid. Instead, also different concentrations of the last three compounds did not statistically change the indexes amounts measured in M₇, M₈, and M₉ solutions (Fig. 3).

Conversely to some recent works dealing with the correlation (by means of either Pearson coefficients or multivariate canonical analysis) between total phenolic compounds or total anthocyanins and antioxidant indexes in sweet cherries (Antognoni et al., 2020; Ceccarelli, Antonucci, Costa, Talento, & Ciccioritti, 2020) and eventually just speculating that the higher antioxidant activity in some varieties might depend on the presence of unique compounds (Hu et al., 2021), it is worth noting that the finding of this work not only demonstrated the starting hypothesis but also confirmed, through a validation pattern, which phenolics are more linked to antioxidant capacity providing new insights toward one of the main drawbacks of the antioxidant *in vitro* assays that is their bad correlation with bioactive compounds (Bibi Sadeer et al., 2020).

4. Conclusions

In this work, 42 phenolics, including flavonoids (i.e., anthocyanins, flavonols, and flavanols) and non-flavonoids (i.e., hydroxycinnamates), were revealed in 3 sweet cherries pulp extracts. Their contents were significantly affected by the cultivar; indeed, flavanols and anthocyanins (especially, procyanidin B₁ and cyanidin-3-*O*-rutinoside) were more concentrated in Sweetheart, while flavonols were more abundant in Lapins (i.e., quercetin-3-*O*-rutinoside) or in Ferrovia and Sweetheart (i.e., quercetin-4'-*O*-glucoside) according to the structure. Regarding hydroxycinnamates, the highest amounts were registered in Sweetheart and Lapins, even though the prevalent compounds were *trans*-3-*O*-caffeoylquinic acid and *trans*-3-*O*-coumaroylquinic acid in the former and the latter cultivar, respectively. As expected, antioxidant indexes agreed with the phenolic content, following the order Sweetheart \approx Lapins $>$

Ferrovia. However, from the FA outcome, only 21 compounds (among which catechin, epicatechin, cyanidin-3-O-glucoside, quercetin-4'-O-glucoside, and few caffeoyl and coumaroylquinic acid derivatives) seemed to play a synergistic role in influencing the antioxidant capacity of the cherry extracts; while procyanidin B₁, *trans*-3-O-caffeoylquinic acid, and *trans*-5-O-caffeoylquinic acid were not related to F-C, ABTS, and DPPH. Notably, these findings were validated by measuring the antioxidant indexes in standard solutions containing some of the aforementioned phenolic compounds at the same concentrations as the real cherry extracts.

In conclusion, this work confirmed that higher content of phenolics corresponds to higher antioxidant capacity, but also demonstrated, through a multivariate analysis validated by dedicated experiments, that just a restricted pool of compounds could influence the antioxidant capacity independently from the presence of some other phenols. However, some limitations about the applied antioxidant methods, which are generally recognized as non-specific and non-sensitive, because other species can react with the oxidant leading to an overestimation, and bear no resemblance to biological systems, cannot be hidden. On the other hand, the possible hydroxy radical formation during the ultrasound green extraction may result in the oxidation or undesirable structural changes of the recovered phenolic compounds. Therefore, further studies are in progress to evaluate these potential drawbacks.

Funding

This work was supported by Research for Innovation REFIN-POR Puglia FESR-FSE 2014/2020.

CRediT authorship contribution statement

Maria Lisa Clodoveo: Data curation, Formal analysis, Writing – original draft, Funding acquisition. **Pasquale Crupi:** Conceptualization, Validation, Writing – original draft, Methodology, Formal analysis. **Marilena Muraglia:** Investigation. **Muhammad Yasir Naem:** Resources. **Roberta Tardugno:** Writing – review & editing. **Francesco Limongelli:** Resources. **Filomena Corbo:** Project administration, Supervision.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2023.115085>.

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