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Article

Comparison of Content in Phenolic Compounds and Antioxidant Capacity in Grains of White, Red, and Black Sorghum Varieties Grown in the Mediterranean Area

Paola Pontieri,*,□ Giacomo Pepe,□ Pietro Campiglia, Fabrizio Merciai, Manuela Giovanna Basilicata, Dmitriy Smolensky, Matteo Calcagnile, Jacopo Troisi, Roberta Romano, Fabio Del Giudice, Mariarosaria Aletta, Marco Guida, Pietro Alifano, and Luigi Del Giudice

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ABSTRACT: Sorghum (*Sorghum bicolor* L.) is a gluten-free cereal with many varieties containing greater amounts of bioactive compounds than other grains. In this work, the phenolic composition and antioxidant capacity were compared in three sorghum varieties grown in the Mediterranean, consisting of different pericarp colors: white, red, and black. The qualitative and quantitative profiles of acidified aqueous methanol extracts of whole grain sorghum flours were elucidated through ultrahigh performance liquid chromatography (UHPLC) coupled to photodiode array (PDA) and ion trap time-of-flight (IT-TOF) mass spectrometry. The investigated sorghum varieties contained polyamines, such as spermidines, and polyphenolic compounds belonging to different classes: hydroxycinnamic acids, flavanols, flavones, and flavanones. Pyrano-flavanone-flavanols were detected only in the black sorghum. Precolumn and postcolumn 2,2′-diphenyl-1-picrylhydrazyl (DPPH)· radical scavenging assays to determine the main antioxidant compounds isolated from sorghum were developed. These results will further help develop sorghum varieties containing polyphenols relevant to the potential prevention of human diseases.

KEYWORDS: *antioxidants, HPLC-DPPH, polyphenols, sorghum, UHPLC-PDA−MS/MS*

1. INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most-produced cereal in the world, with Africa being the major producing region (>40% of world production).[1] Sorghum is well-suited for cultivation in the semiarid and subtropical regions of Africa as it is one of the most drought-tolerant cereal crops.[2−10]

People with celiac disease suffer from symptoms associated with an immune reaction to gluten/gliadin proteins found in all *Triticum* species and closely related cereals such as wheat barley and rye.[11] Sorghum does not contain toxic gliadin-like peptides, making sorghum a safe food for people who suffer from celiac disease.[12,13]

Over the past decade, the potential role of sorghum in human health and disease prevention has gained increased research attention due to its polyphenol content.[2,3,6,9,14−17] Polyphenols are common to most plants and have been widely investigated for their health benefits related to their antioxidant capacity,[18−20] that is their ability to scavenge free radicals and modulate various signaling pathways relevant to disease prevention.[21]

Phenolic compounds can be divided into free, esterified, and insoluble-bound forms, depending on whether they occur in the free form or are covalently bound to other molecules such as fatty acids, pectin, cellulose, arabinoxylan, and structural proteins.[22] On the basis of their chemical structure, polyphenols can be divided into different classes, the most common of which are (I) flavonoids/isoflavonoids, (II) phenolic acids, (III) lignans, and (IV) stilbenes.[23] Furthermore, multiple polyphenols can be combined to form larger polymers of polyphenols. A good example in sorghum is flavan-3-ols combining to make condensed tannins, also known as proanthocyanidins.[24]

Depending on the variety of sorghum, the grains can contain high levels of various phenolic compounds such as phenolic acids, flavonoids, and condensed tannins.[21,25] Polyphenols serve an important role in natural plant defense against pathogens and pests; for example, the presence of condensed tannins in sorghum increases tolerance to bird predation.[26] Among cereal grains, sorghum is the most diverse in terms of the types and amounts of the major polyphenols,[26] thus providing the intriguing opportunity to expand the role of cereal-derived polyphenols in human health. The phenolic compounds found in sorghum grain are more abundant and diverse compared to other main cereals such as wheat, barley, maize, and rice.[27] Pericarp color in sorghum can be affected by polyphenol composition, genetic variation, and environmental factors.[28] Tissue culture studies indicate that some compounds in different classes of polyphenols can potentially prevent different pathologies that affect humans such as cancer, aging,

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Table 1. List of Sorghum Varieties

variety color	variety name	source	supplied by	specific character
white sorghum	DSM 3-410	Richardson Seeds Ltd.	M. Malin	food-grade
red sorghum	DSM IF-41912	Richardson Seeds Ltd.	M. Malin	food-grade
black sorghum	DSM 212-2311	Richardson Seeds Ltd.	M. Malin	food-grade

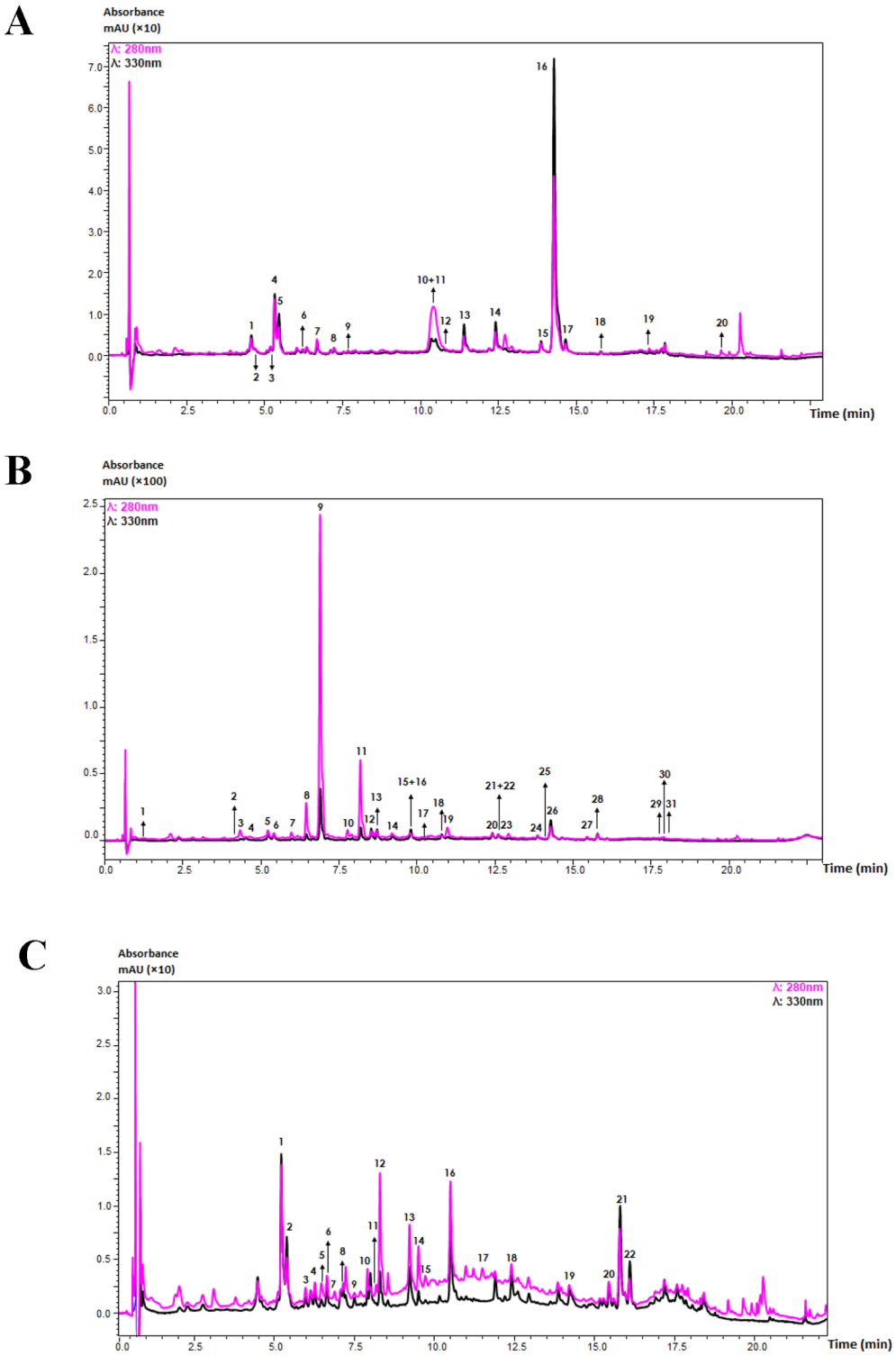

Figure 1. RP-UHPLC-PDA chromatograms (purple line, λ: 280 nm; black line, λ: 330 nm) of hydroalcoholic extracts of (A) white, (B) red, and (C) black sorghum.

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Table 2. UHPLC-PDA-IT-TOF Characterization of the Hydroalcoholic Extract of White Sorghum

peak	Rt	compound	$[M-H]^-$	[MS/MS]	error (ppm)	molecular formula
1	4.60	N^1−N^4-dicaffeoyl-spermidine I	468.2142	332.1663	−1.92	$C_{25}H_{31}N_3O_6$
2	4.72	1-*O*-caffeoyl-2-*O*-glucosylglycerol I	415.1296	253.0715	−0.48	$C_{18}H_{24}O_{11}$
3	5.21	1-*O*-caffeoyl-2-*O*-glucosylglycerol II	415.1273	253.0697	+6.26	$C_{18}H_{24}O_{11}$
4	5.36	N^1−N^4-dicaffeoyl-spermidine II	468.2159	332.1663	+1.5	$C_{25}H_{31}N_3O_6$
5	5.50	2-*O*-caffeoylglycerol	253.0719	161.0329	+0.4	$C_{12}H_{14}O_6$
6	6.27	N^1−N^8-caffeoyl-feruloyl-spermidine	482.2298	306.1857	+0.2	$C_{26}H_{33}N_3O_6$
7	6.76	1-*O*-coumaroylglycerol	237.0772	163.0380	+1.69	$C_{12}H_{14}O_5$
8	7.34	saccharide	371.0992	249.0667	+2.16	$C_{16}H_{20}O_{10}$
9	7.70	apigenin-7-*O*- hexoside I	431.1003	269.0461	+1.39	$C_{21}H_{20}O_{10}$
10	10.40	apigenin-7-*O*- hexoside II	431.0994	269.0462	+2.6	$C_{21}H_{20}O_{10}$
11	10.51	7,3′,4′,*S*′-tetrahydroxy flavanone	287.0568	151.0022	+2.44	$C_{15}H_{12}O_6$
12	10.79	chrysoeriol hexoside	461.1099	299.0055	+2.17	$C_{22}H_{22}O_{11}$
13	11.44	genkwanin	283.0622	268.0411	+3.53	$C_{16}H_{12}O_5$
14	12.49	1,3-*O*-dicaffeoylglycerol	415.1051	253.0697	+2.89	$C_{21}H_{20}O_9$
15	13.97	1,3-*O*-coumaroyl-caffeoyl-glycerol	399.1094	253.0743	+2.26	$C_{21}H_{20}O_8$
16	14.32	7,3′,4′- trihydroxyflavone	269.0430	225.0598; 197.0626	+6.69	$C_{15}H_{10}O_5$
17	14.72	chrysoeriol	299.0561	284.0344	0.00	$C_{16}H_{12}O_6$
18	15.47	1,3-*O*-dicoumaroylglycerol	383.1140	163.0429	+1.04	$C_{21}H_{20}O_7$
19	17.43	trihydroxy-octadecenoic acid	329.2329	311.1646	−1.21	$C_{18}H_{34}O_5$
20	19.25	dihydroxy-octadecenoic acid	313.2383	277.1823	−0.32	$C_{18}H_{34}O_4$

inflammation, obesity, and other chronic diseases.[16,23,29−32] Selecting varieties of sorghum with high concentrations of those unusually high polyphenol compounds could be an effective strategy to confer benefits to human health.

The aim of the present study is to compare the free phenolic profile and antioxidant activity in three different varieties of sorghum (white, red, and black), which were grown in the Mediterranean environment. This data can provide important information to farmers when it comes to selecting a sorghum variety with increased nutritional and health promoting properties.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Ultrapure water (H_2O) was obtained by a Milli-Q Direct 8 system (Millipore, Milan, Italy). Unless otherwise specified, all reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy).

2.2. Plant Cultivars. The sorghum cultivars and seed sources employed in this study are listed in Table 1. In 2018, full-field cultivation was carried out in San Bartolomeo in Galdo (BN) in the Fortore area located in the Campania Region, southern Italy (41°25′ N, 15°01′ E, and 597 m a.s.l.). Soil found in this region is predominantly clay loam, deep, and with a good water holding capacity.

2.2.1. Flour Sample Preparation. Sorghum samples were milled into flour using a two-roll mill (Chopin Moulin CD1) (Chopin S.A., Villeneuve la Garenne, France). After milling, the samples were sieved with a planetary sieve (Buhler AG, Uzwil, Switzerland) with a screen size of 120 μm^2.

2.2.2. Extraction of Free Polyphenols. Polyphenols were extracted from sorghum samples following the Kang et al. protocol,[33] with slight modifications. In brief, 2.5 g of each sorghum variety was extracted overnight at room temperature by continuous and gentle shaking, with 15 mL of aqueous 80% v/v methanol and 0.1% v/v hydrochloric acid solution being added. The obtained solution was centrifuged at 6000 rpm at 4 °C for 15 min. Then, the supernatant was collected and filtered through 0.45 μm nylon membrane filters and injected for reverse-phase (RP)-ultrahigh performance liquid chromatography (UHPLC) coupled to diode array detection (DAD) and mass/mass spectrometry (MS/MS) analysis.

2.3. LCMS-IT-TOF Parameters. UHPLC-ESI-IT-TOF analyses were performed on a Shimadzu Nexera UHPLC system coupled online to an ESI-IT-TOF mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC−MS data elaboration was performed by the LCMSsolution software (Version 3.50.346, Shimadzu).

The LC−MS analysis of sorghum extract was carried out on a Kinetex C18 150 mm × 2.1 mm × 2.6 μm (100 Å) column thermostated at 45 °C (Phenomenex, Bologna, Italy), monitoring the chromatograms at 280 and 330 nm. H_2O (A) and ACN (B), both acidified by acetic acid 0.1% v/v, were used as mobile phases, with the flow rate set at 0.5 mL min^{-1}. The analysis was performed in gradient elution as follows: 0.01−15.00 min, 2−30% B; 15.01−20.00 min, 30−70% B; 20.01−21.00 min, 70−98% B; 21.01−22.50 min, isocratic to 98% B; then 4 min for column equilibration.

MS detection was operated in negative ionization mode with the following parameters: detector voltage, 1.65 kV; curved desolvation line (CDL) temperature, 250 °C; block heater temperature, 250 °C; nebulizing gas flow (N_2), 1.5 L/min; drying gas pressure, 95 kPa. Full scan MS data were acquired in the range 150−1500 m/z, MS/MS experiments were conducted in the data-dependent acquisition, and fragments ions were acquired in the range 150−1000 m/z with collision-induced dissociation (CID) energy set at 50%.

The identification of investigated analytes was carried out by comparing their retention times and MS/MS data with those present in the literature. The molecular formulas of identified compounds were calculated by the Formula Predictor software (Version 1.12, Shimadzu). The following online databases were also consulted: ChemSpider (http://www.chemspider.com), SciFinder Scholar (https://scifinder.cas.org), and Phenol-Explorer (www.phenol-explorer.eu).

2.4. Quantitative Analysis. Instrumental calibration for the quantitative analysis was carried out using the external standard method. Kaempferol 3-*O*-glucoside, naringenin 7-*O*-glucoside, apigenin, catechin, and caffeic acid were selected as external standards.

Stock solutions were prepared in CH_3OH, and the calibration curves were obtained in concentration ranges of 10−250 $\mu g\ mL^{-1}$ (kaempferol 3-*O*-glucoside), 2.5−50 $\mu g\ mL^{-1}$ (naringenin 7-*O*-glucoside), 5−100 $\mu g\ mL^{-1}$ (apigenin), 5−200 $\mu g\ mL^{-1}$ (catechin), 0.5−10 $\mu g\ mL^{-1}$(caffeic acid), with the five concentration levels run in triplicate. Linear regression was used to generate a calibration curve plotting the peak areas against analyte concentrations. The quantification of compounds was expressed as milligrams per 100 g of dried sorghum.

2.5. Method Validation. The quantification method was validated in terms of linearity, precision, limit of detection (LOD), and limit of quantification (LOQ). Linear regression equation and coefficient correlation (R^2) were used to evaluate the linearity of the results. LODs and LOQs were calculated as follows:

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Table 3. List of Identified Compounds in the Red Sorghum Extract

peak	Rt	compound	$[M-H]^-$	[MS/MS]	error (ppm)	molecular formula
1	0.80	caffeic acid hexoside	341.1101	179.0572; 258.0729	−1.20	$C_{15}H_{18}O_9$
2	4.25	catechin	289.0724	205.0555	+0.70	$C_{15}H_{14}O_6$
3	4.30	epicatechin dimer	577.1384	407.0786; 289.0727; 425.0921	+0.53	$C_{30}H_{26}O_{12}$
4	4.63	epicatechin trimer	865.2024	577.1440; 287.0568	−0.67	$C_{45}H_{38}O_{18}$
5	5.20	N^1−N^4-dicaffeoyl-spermidine	468.2124	289.1522; 332.1636; 306.1817	+3.40	$C_{25}H_{31}N_3O_6$
6	5.43	1-*O*-caffeoylglycerol	253.0719	161.0275	−0.01	$C_{12}H_{14}O_6$
7	6.00	eriodictyol-di-*O*-hexoside	611.1625	287.0563; 449.1119	+0.33	$C_{27}H_{32}O_{16}$
8	6.49	eriodictyol-*O*-hexoside I	449.1092	287.0582; 151.0067; 269.0482	+0.67	$C_{21}H_{22}O_{11}$
9	6.95	eriodictyol-*O*-hexoside II	449.1086	287.0560; 151.0067; 269.0408	−0.67	$C_{21}H_{22}O_{11}$
10	7.81	naringenin hexoside I	433.1153	271.0630; 151.0069	+1.30	$C_{21}H_{22}O_{10}$
11	8.20	naringenin hexoside II	433.1148	271.0628; 151.0061	+1.85	$C_{21}H_{22}O_{10}$
12	8.55	luteolin hexoside I	447.0927	285.0408	−0.60	$C_{21}H_{20}O_{11}$
13	8.75	dihydroluteolin hexoside	445.0780	285.0409; 447.0918	+0.40	$C_{21}H_{18}O_{11}$
14	9.20	luteolin hexoside II	447.0936	285.0408	+0.67	$C_{21}H_{20}O_{11}$
15	9.79	apigenin hexoside	431.1003	269.0461	+1.80	$C_{21}H_{20}O_{10}$
16	9.80	eriodictyol-*O*-hexoside III	449.1091	287.1049; 151.0068	+0.45	$C_{21}H_{22}O_{11}$
17	10.22	chrysoeriol hexoside	461.1100	284.0322; 299.0544	+1.70	$C_{22}H_{22}O_{11}$
18	10.75	naringenin hexoside III	433.1154	271.0663; 151.0069,	+1.70	$C_{21}H_{22}O_{10}$
19	11.00	eriodictyol	287.0573	151.0070	+3.83	$C_{15}H_{12}O_6$
20	12.25	luteolin hexoside III	447.0927	285.0409	−0.60	$C_{21}H_{20}O_{11}$
21	12.42	1,3-*O*-dicaffeoylglycerol	415.1042	253.0721; 235.0544	+2.89	$C_{21}H_{20}O_9$
22	12.62	luteolin	285.0408	175.0399; 199.0393; 243.0264, 257.0442; 285.0408	+2.46	$C_{15}H_{10}O_6$
23	12.95	naringenin	271.0591	151.0023	+8.12	$C_{15}H_{12}O_5$
24	13.85	1,3-*O*-coumaroyl-caffeoyl-glycerol	399.1080	253.0732; 235.0616	+5.76	$C_{21}H_{20}O_8$
25	14.20	1,3-*O*-feruloyl-caffeoyl-glycerol	429.1084	253.0697; 193.0515	+6.00	$C_{22}H_{22}O_9$
26	14.30	7,3′,4′- trihydroxyflavone	269.0466	225.0571; 151.0074; 197.0637	+4.09	$C_{15}H_{10}O_5$
27	15.50	1,3-*O*-dicoumaroyl-glycerol	383.1133	163.0460; 237.0748	+7.31	$C_{21}H_{20}O_7$
28	15.80	1,3-*O*- coumaroyl-feruloyl-glycerol	413.1273	177.0585; 235,0622; 193.0500	+2.66	$C_{22}H_{22}O_8$
29	17.78	dihydroxy-octadecadienoic acid	311.2224	293.2118	−1.29	$C_{18}H_{32}O_4$
30	17.85	dihydroxy-octadecenoic acid I	313.2404	183.1395; 195.1323; 295.2276	+2.23	$C_{18}H_{34}O_4$
31	17.93	dihydroxy-octadecenoic acid II	313.2404	183.1395; 195.1323; 295.2276	+2.23	$C_{18}H_{34}O_4$

$$\mathrm{LOD} = 3.3 \times \frac{\sigma}{S} \quad (1)$$

$$\mathrm{LOQ} = 10 \times \frac{\sigma}{S} \quad (2)$$

where σ is the standard deviation of the response and S is the slope coefficient of the calibration curves. The precision of the method was evaluated by assessing the relative standard deviation (RSD) intraday and interday at three different concentrations, in triplicate.

2.6. Off-Line HPLC-DPPH Conditions. For the determination of the total antioxidant capacity of polyphenolic compounds isolated from sorghum samples, the acidified aqueous methanol extracts were diluted in the appropriate ratio (white and black sorghum, 0.05−1.5 mg mL^{-1}; red sorghum, 0.05−0.5 mg mL^{-1}), and the solutions thus obtained were added to DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.75 mM) in a 1:1 (v/v) ratio.[34] The mixture was briefly sonicated and then left to react for 30 min in the dark at room temperature. The sample was filtered and injected in the LC system with the same chromatographic conditions reported above, except running in isocratic mode with mobile phases (B/A) 60:40. In these conditions, all compounds eluted at the column dead time in a single chromatographic peak, and the DPPH peak area, monitored at 517 nm, was taken into account for the calculation. The blank control was prepared by diluting the DPPH solution with CH_3OH in a 1:1 ratio. IC_{50} values were calculated through linear regression analysis by interpolation using the Microsoft Excel (2016) software program.

The total DPPH radical scavenging activity of each sorghum extract was expressed as the trolox equivalent antioxidant capacity (TEAC).[35] The TEAC was calculated as follows:

$$\mathrm{TEAC} = \mathrm{IC}_{50}\ \text{of trolox}\ (\mu g\ mL^{-1})/\mathrm{IC}_{50}\ \text{of sample}\ (\mu g\ mL^{-1}) \quad (3)$$

However, for the determination of single compounds contribution to the antioxidant activity, the chromatographic separation of the reaction mixture was conducted in gradient mode as reported earlier, by incubating the sample with radical solution. The radical scavenging contribution of single compounds isolated from sorghum samples was calculated with the following equation:

$$\text{radical scavenging (RS\%)} = \{(\mathrm{PA}_{\mathrm{control}} - \mathrm{PA}_{\mathrm{spiked}})/\mathrm{PA}_{\mathrm{control}}\} \times 100\% \quad (4)$$

where $PA_{control}$ is referring to the sample peak area solution diluted with methanol, whereas PA_{spiked} is referring to the sample peak area resulting from mixing DPPH with the different extracts.

2.7. Online HPLC-DPPH Conditions. After chromatographic separation, the effluent of the UHPLC column was mixed online by a stainless steel tee union with the DPPH· solution and allowed into a coil reactor (standard stainless steel tubing 1.6 mm × 0.3 mm × 6.0 m, O.D. × I.D. × L). The column oven and reactor coil temperatures were both set to 45 °C. The radical solution was eluted by a LC-20 AT pump (Shimadzu, Milan, Italy) using as mobile phase 0.1 mM DPPH· solution and setting the flow rate to 0.5 mL min^{-1}.

In order to optimize the online postcolumn derivatization HPLC-DPPH assay, a standard mixture of polyphenolic compounds (catechin, chlorogenic acid, caffeic acid, epicatechin, and ferulic acid) and negative (phloridzin) and positive (trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) controls was employed (Figure S2).

The chromatograms of all sorghum varieties were acquired by setting the same instrumental parameters above-described and were monitored at 280 and 517 nm. The compounds that were able to quench the radical caused the signal to deviate from the baseline and were observed

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Table 4. RP-UHPLC-PDA-ESI-IT-TOF Identification of Polyphenols Isolated from Black Sorghum

peak	Rt	compound	$[M-H]^-$	[MS/MS]	error (ppm)	molecular formula
1	5.33	N^1−N^4-dicaffeoyl-spermidine	468.2167	306.1812; 332.1614	+5.77	$C_{25}H_{31}N_3O_6$
2	5.47	2-*O*-caffeoylglycerol	253.0733	161.0329	+5.03	$C_{12}H_{14}O_6$
3	6.14	quercetin-3,4′-*O*-di-ß-glucopyranoside	625.1757	301.0704	−1.7	$C_{27}H_{30}O_{17}$
4	6.27	N^1−N^8-caffeoyl-feruloyl-spermidine	482.2313	306.1795; 332.1600	−0.67	$C_{26}H_{33}N_3O_6$
5	6.48	7,3′,4′- trihydroxyflavone	269.0452	225.0597	−1.12	$C_{15}H_{10}O_5$
6	6.68	1-*O*-coumaroylglycerol	237.0768	163.0378	+0.01	$C_{12}H_{14}O_5$
7	6.88	luteolin hexoside	447.0926	285.0399	+2.1	$C_{21}H_{20}O_{11}$
8	6.91	eriodictyol-*O*-hexoside	449.1113	287.0578; 151.0062	+5.34	$C_{21}H_{22}O_{11}$
9	7.39	saccharide	371.1049	231.0609; 249.0667	+0.27	$C_{16}H_{20}O_{10}$
10	7.91	pyrano-3′,4′,5′,5,7-pentahydroxyflavone-(3 → 4)-catechin-7-*O*-glucoside	881.2201	432.0884; 594.1516; 746.1696	+2.5	$C_{45}H_{38}O_{19}$
11	8.17	naringenin hexoside	433.1164	271.0612	+2.31	$C_{21}H_{22}O_{10}$
12	8.34	pyrano-3′,4′,5′,5,7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside	883.2328	287.0648; 297.0637; 405.0628, 433.0970; 541.1080; 595.1401; 721.1801	+0.45	$C_{45}H_{40}O_{19}$
13	9.25	pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside I	867.2330	271.0671; 389.0776; 417.0997; 431.0624; 579.1625; 705.1798	+1.39	$C_{45}H_{40}O_{18}$
14	9.53	pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside II	867.2370	417.0974; 431.0624; 525.1335, 551.1191; 579.1501; 705.1826	+4.04	$C_{45}H_{40}O_{18}$
15	9.75	7-*O*-methyl catechin	303.0863	193.0603	+3.63	$C_{16}H_{26}O_6$
16	10.53	pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside	851.2400	389.0685; 401.1034; 551.1230; 563.1576; 689.1249	+1.53	$C_{45}H_{40}O_{17}$
17	11.58	7,3′,4′,5′-tetrahydroxy flavanone	287.0952	151.0486	+2.21	$C_{15}H_{12}O_6$
18	12.49	1,3-dicaffeoylglycerol	415.1051	161.0278; 253.0697	+5.06	$C_{21}H_{20}O_9$
19	14.24	1,3-*O*-feruloyl-caffeoyl-glicerol	429.1248	193.0603; 253.0697	+1.13	$C_{22}H_{22}O_9$
20	15.49	1,3-dicoumaroylglicerol	383.1133	163.0369; 219.0664	+4.96	$C_{21}H_{20}O_7$
21	15.82	1,3-coumaroyl-feruloyl-glicerol	413.1273	161.0329; 177.0599; 235.0616	+0.48	$C_{22}H_{22}O_8$
22	16.12	1,3-*O*-diferuloylglicerol	443.1412	193.0472; 207.0718; 235.0616; 249.0667	+1.65	$C_{23}H_{24}O_9$

as negative peaks. The change in peak area was proportional to the antioxidant activity of the analyzed compounds.

3. RESULTS

3.1. Qualitative Profiles of Sorghum Extracts. Figure 1 shows the chromatographic profiles of polyphenolic compounds isolated from white, red, and black sorghum varieties as indicated in Table 1. All tentatively identified compounds are listed in Tables 2−4.

The LC−MS/MS analyses highlighted that phenolamides and some classes of free polyphenols, such as phenolic acids, flavones, and flavanones, were present in the three investigated sorghum varieties. Proanthocyanidins have only been detected in red sorghum, while pyrano-flavanone-flavanol dimers have only been detected in black sorghum (Table 5).

In the white sorghum extract (Figure 1A and Table 2), the chromatographic peak **4** at m/z 468 showed a fragment ion at m/z 332 $[M-H-136]^-$, which suggests the presence of a caffeoyl group in the structure; therefore, it was tentatively proposed as N^1−N^4-dicaffeoyl-spermidine.[36,37]

Compound **5** showed at m/z 253 $[M-H]^-$ a fragmentation ion at m/z 161 $[M-H-92]^-$ produced by a loss of glycerol residue and was identified as 2-*O*-caffeoylglycerol.[38]

The peaks **9** and **10** were observed at different retention times in the chromatogram but all gave ions at m/z 431 $[M-H]^-$ with a MS/MS fragmentation pattern characterized by a base peak at m/z 269, corresponding to the loss of moiety of *O*-linked hexoside; thus, this compound was tentatively identified as isomers of apigenin hexoside.

Compound **14** showed a precursor ion at m/z 415 $[M-H]^-$ and provided fragment ions at m/z 253 $[M-H-162]^-$, deriving from the loss of a caffeoyl moiety. Chromatographic peak **14** was identified as 1,3-*O*-dicaffeoylglycerol.[39]

Compound **16** identified in the white sorghum hydromethanolic extract produced a precursor ion at m/z 269, fragments at m/z 225 $[M-H-CO_2]^-$, and a secondary fragment at m/z 197 $[M-H-CO_2-CO]^-$. This compound could be attributed to 7,3′,4′-trihydroxyflavone.[33]

Moreover, in the red sorghum extract (Figure 1B and Table 3), chromatographic peak **9** exhibited the precursor ion at m/z 449, but the loss of hexose moiety $[M-H-162]^-$ and the relative loss of water $[M-H-162-18]^-$ produced fragments at m/z 287 and 269, leading to its tentative identification as eriodictyol-*O*-hexoside.[40]

Finally, in the black sorghum extract (Figure 1C and Table 4), the compounds **10, 12, 13**, and **14** presented similar MS/MS fragmentation patterns, characterized by the loss of sugar moiety $[M-H-162]^-$ and catechin glucoside unit $[M-H-162-288]^-$, leading to their tentative identification as pyrano-flavonoid derivatives.[41] Moreover, we found that, in the black sorghum profile, 3-deoxyanthocyanidins derivates were also present, as they have a typical maximum absorbance of 486 nm (Section 1, Figure S1).[42,43]

3.2. Method Validation. The linear regression equations, R^2, % RSD, LODs, and LOQs values are shown in Table S2. For each standard, a linear relationship was obtained over the tested concentrations and the peak areas. In all cases, the correlation coefficients were equal or greater than 0.9994. The % RSD values ranged from 0.04 to 1.96, which indicate good method precision intraday and interday. Furthermore, good values of LOD and LOQ were observed.

3.3. Quantitative Profile of Polyphenolic Extracts of Sorghum Varieties. The quantitative profile of polyphenolic compounds isolated from the three investigated varieties of sorghum is shown in Table 6.

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Table 5. Chemical Classification of Biomolecules Identified in Hydroalcoholic Sorghum Extracts

			sorghum varieties		
class	sublclass	compounds	red	white	black
phenolic acid	hydroxycinnamic acids	caffeic acid hexoside	+		
		1-*O*-caffeoylglycerol	+		
		1,3-*O*-dicaffeoylglycerol	+	+	+
		1,3-*O*-coumaroyl-caffeoyl-glycerol	+	+	
		1,3-*O*-feruloyl-caffeoyl-glycerol	+		+
		1,3-*O*-dicoumaroyl-glycerol	+	+	+
		1,3-*O*- coumaroyl-feruloyl-glycerol	+		
		1-*O*-caffeoyl-2-*O*-glucosylglycerol		+	
		2-*O*-caffeoylglycerol		+	+
		1-*O*-coumaroylglycerol		+	+
		1,3-*O*-feruloylglicerol			+
		1,3-coumaroyl-feruloyl-glicerol			+
flavonoids	flavanols	catechin	+		
		7-*O*-methyl catechin			+
		pyrano-3′,4′,5′,5,7-pentahydroxyflavone-(3 → 4)-catechin-7-*O*-glucoside			+
		pyrano-3′,4′,5′,5,7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside			+
		pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside			+
		pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside			+
	flavonols	quercetin-3,4′-*O*-dibeta-glucopyranoside			+
	flavanones	naringenin hexoside	+		+
		eriodictyol	+		
		eriodictyol hexoside	+		+
		eriodictyol-di-*O*-hexoside	+		
		naringenin	+		
		7,3′,4′,5′-tetrahydroxy flavanone		+	+
	flavones	luteolin hexoside	+		+
		dihydroluteolin hexoside	+		
		apigenin glucoside	+		
		chrysoeriol hexoside	+	+	
		luteolin	+		
		7,3′,4′-trihydroxyflavone	+	+	+
		apigenin 7-*O*-hexoside		+	
		genkwanin		+	
		chrysoeriol		+	
	proanthocyanidin	epicatechin dimer	+		
		epicatechin trimer	+		
polyamines	phenolamides	N^1−N^4-dicaffeoyl-spermidine	+	+	+
		N^1-N^8-caffeoyl-feruloyl-spermidine		+	+
octadecanoids	other octadecanoids	dihydroxy-octadecadienoic acid	+		
		dihydroxy-octadecenoic acid	+	+	
		trihydroxy-octadecenoic acid		+	
carbohydrate		saccharide		+	+

The quantitative analysis highlighted that the white sorghum sample was rich in phenolamides (N^1−N^4-dicaffeoyl-spermidine, 7.86 ± 0.02 mg per 100 g of dried sorghum) and flavones compounds (apigenin-7-*O*-hexoside, 20.36 ± 0.15 mg/100 g; 7,3′,4′,-trihydroxyflavone, 25.47 ± 0.13 mg/100 g), while in the black sorghum extract, three abundant pyrano-flavonoid derivatives were quantified (pyrano-3′,4′,5′,5,7-pentahydroxy-flavanone-(3 → 4)-catechin-7-*O*-glucoside, 23.04 ± 1.25 mg/100 g; pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside, 13.67 ± 1.17 mg/100 g; pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside, 20.75 ± 0.09 mg/100 g). Among the 3-deoxyanthocyanidins, luteolinidin was the most abundant compound (Table S1).

Finally, the main compound identified in the red sorghum hydroalcoholic extract was eriodictyol-*O*-hexoside (260.05 ± 5.59 mg/100 g).

3.4. Antioxidant Activity Screening of Sorghum Grains by Off-Line and Online HPLC-DPPH Assays. In this work, to determine the total antioxidant activity of polyphenolic compounds isolated from sorghum samples, we carried out a DPPH free radical assay.

In detail, different amounts of acidified aqueous methanol were incubated with a 0.75 mM DPPH solution. The DPPH radical scavenging activity of each investigated sample was expressed as the Trolox equivalent antioxidant activity (TEAC), as reported in Table 7. Our results showed a greater antioxidant activity of red sorghum (TEAC value: 0.19) than the white (TEAC value: 0.07) and black sorghum (TEAC value: 0.06) samples.

In order to identify compounds responsible of antioxidant activity observed for the total extracts, we coupled the DPPH assay with a separation technique, such as liquid chromatog-

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Table 6. Quantitative Analysis of the Hydroalcoholic Extracts of the Three Sorghum Varieties

sorghum variety	peak	compound	quantity (mg per 100 g of dried sorghum)
white	1	N^1−N^4-dicaffeoyl-spermidine[a]	7.86 ± 0.02
	2	1-*O*-caffeoyl-2-*O*-glucosylglycerol[a]	4.12 ± 0.06
	10	apigenin-7-*O*- hexoside II[b]	20.36 ± 0.15
	14	1,3-*O*-dicaffeoylglycerol[a]	3.09 ± 0.01
	15	1,3-*O*-coumaroyl-caffeoyl-glycerol[a]	1.62 ± 0.04
	16	7,3′,4′- trihydroxyflavone[b]	25.47 ± 0.13
red	8	eriodictyol hexoside[c]	32.03 ± 2.71
	9	eriodictyol hexoside II[c]	260.05 ± 5.59
	11	naringenin hexoside[d]	32.14 ± 2.32
black	1	N^1−N^4-dycaffeoyl-spermidine[a]	7.42 ± 0.10
	2	2-*O*-caffeoylglycerol[a]	3.67 ± 0.39
	12	pyrano-3′,4′,5′,5,7-pentahydroxyflavanone-(3 → 4)-catechin-7-*O*-glucoside[e]	23.04 ± 1.25
	13	pyrano-eriodictyol-(3 → 4)-catechin-7-*O*-glucoside[e]	13.67 ± 1.17
	16	pyrano-naringenin-(3 → 4)-catechin-7-*O*-glucoside[e]	20.75 ± 0.09

[a]Caffeic acid. [b]Apigenin. [c]Kaempferol 3-*o*-glucoside. [d]Naringenin 7-*o*-glucoside and. [e]Catechin was used an as external standard.

Table 7. Trolox Equivalent Antioxidant Capacity (TEAC) of Sorghum Extracts

sorghum varieties	TEAC (IC_{50} of Trolox $\mu g\ mL^{-1}$ /IC_{50} of sample $\mu g\ mL^{-1}$)
red	0.19
white	0.07
black	0.06

raphy.[44] In this case, after reaction with the radical solution for 30 min at dark, the sample was analyzed by RP-UHPLC-PDA. The presence of potential antioxidants was indicated by a decrease in peak areas (UV/vis) compared to untreated sample.

In this contest, the crucial aspect is the ratio between the concentration of DPPH and the investigated extract. If an excess of DPPH is employed, every peak is reduced below detection limits; on the contrary, with an inadequate concentration of DPPH, no significant differences can be observed between treated and untreated samples. After several tests, we found that the best final conditions were obtained with 0.75 mM DPPH and (0.5 mg mL^{-1}) 1 mg mL^{-1} sample.

As can be observed in the Figure 2, our data showed that, in the red sorghum, the chromatographic peak area of eriodictyol-*O*-hexoside was significantly reduced by DPPH radical solution (RS value: 58.5%) as well as peak areas of pyrano-flavanone-flavanol dimers derivates and 7,3′,4′- trihydroxyflavone isolated from black (RS: 77.5% and 70.5%) and white sorghum (RS: 18.2%), respectively.

With the aim to rapidly assess the antioxidant potential of these polyphenolic compounds, we carried out a fast screening approach based on the coupling of chromatographic separation with postcolumn DPPH radical reaction, in which eluting compounds react with the radical solution in a reaction coil. Thus, antioxidants were detected as negative peaks by monitoring the decrease in the absorbance of the DPPH· trace at 517 nm, as it was reduced to the corresponding pale-yellow hydrazine derivative.

The reaction coil length, temperature, reaction time, and DPPH radical concentration were optimized to study the formation of negative peaks (data not shown). In brief, the analytical platform was optimized by employing a mixture of standard compounds and using trolox and phloridizin as positive and negative controls, respectively. Negative peaks were observed for all compounds except phloridzin. Phloridzin was the only compound in the mixture that did not possess any radical scavenging ability in agreement with data in the literature (Figure S2). As shown in Figure 3, our results highlighted that eriodictyol-*O*-hexoside isolated from the red sorghum sample is able to significantly quench the DPPH radical, generating a negative peak with an abundant area directly proportional to its antioxidant activity.

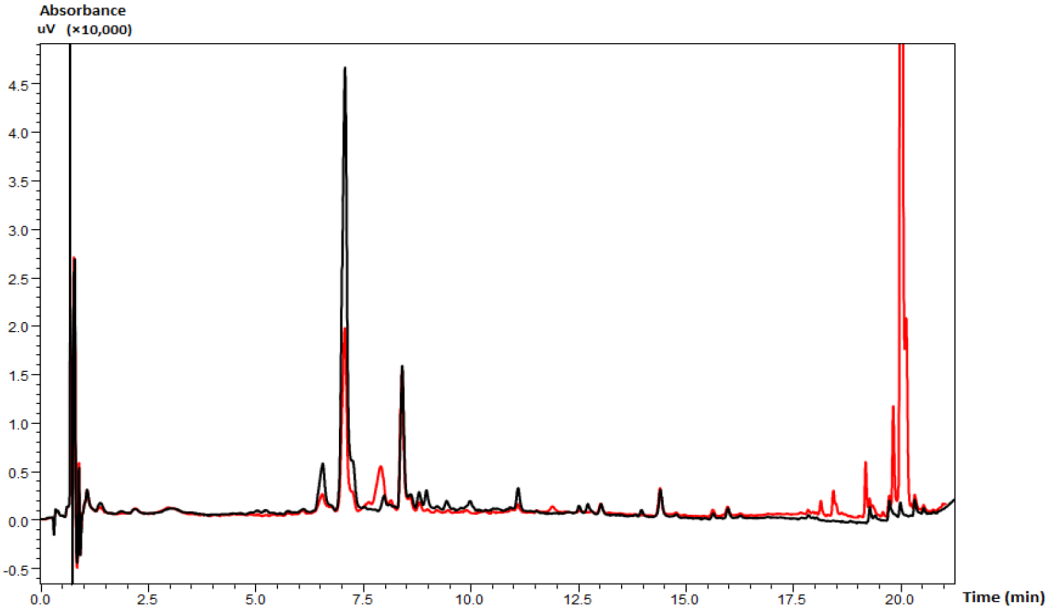

Figure 2. PDA chromatogram relative to the polyphenols isolated from red sorghum extract untreated (black line) and spiked with DPPH radical solution (red line).

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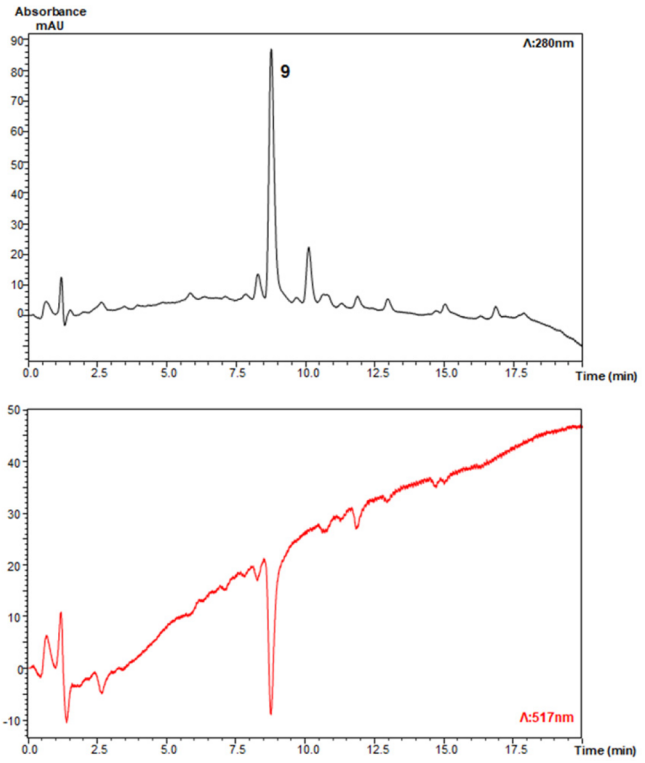

Figure 3. UV (black line, λ: 280 nm) and DPPH radical quenching (red line, λ: 517 nm) chromatogram of red sorghum extract.

4. DISCUSSION

The increase of sorghum consumption as a gluten-free food has potential health benefits in both the US[17] and the Mediterranean region,[9,45−47] suggesting that sorghum is a viable high nutrition crop that could be used as an alternative to gluten containing grains by a greater population.[26] Sorghum is unique among major cereal grains in that it has high levels of a diverse array of bioactive components not common to other cereals, with potential benefit to human health.[30,48,49] The phenolic compounds are the main bioactive compounds of sorghum and are present in all varieties of this cereal.[24] The most prominent group of beneficial sorghum bioactive components are the polyphenols, especially the flavonoids.[26]

In this work, we analyzed the content of free polyphenolic compounds and antioxidant activity in three distinct varieties of sorghum: a white-colored variety, a red-colored variety, and a black-colored variety grown in the Mediterranean environment.

As shown in Table 5, hydroxycinnamic acids and polyamines are present in all the varieties of analyzed sorghum. Major differences were found in the distribution of flavonoids. In fact, while flavones and flavanones are present in all three varieties of sorghum, proanthocyanidins, such as epicatechin dimer and epicatechin trimer, have been identified only in red sorghum and glycosides of pyrano-flavanone-flavanol dimers have been detected only in black sorghum.

After the analytical characterization of polyphenols isolated from three sorghum varieties, in the next step of this study, we evaluated their antioxidant activity.

Numerous spectrophotometric assays have been developed for the determination of the antioxidant properties of polyphenolic compounds isolated from sorghum such as radical scavenging effects on nitric oxide (NO), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, the oxygen radical absorbance capacity (ORAC), and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

In detail, Hong et al.[50] compared the phenolic composition and the antioxidant activity of novel sorghum black-seeded germplasms by different spectrophotometric cell-free assays and suggested that sorghum extracts are better antioxidants against less stable radicals, such as DPPH, then to more stable radicals like NO.

Awika et al.[51] used three different screening methods to measure the antioxidant activity of sorghum (*Sorghum bicolor*), highlighting that the ABTS and DPPH assays have a similar predictive power as ORAC on sorghum antioxidant activity but they are more cost-effective and simpler than ORAC assay.

Furthermore, the DPPH method, in addition to being faster, is characterized by less steps and reagents to prepare compared to the ABTS assay.

For these reasons, the DPPH radical scavenging assay was utilized to evaluate the antioxidant properties of the investigated vegetable samples. Our results showed that the acidified aqueous methanol extract of red sorghum presents a greater antioxidant activity in comparison to both white sorghum and black sorghum (Table 7), highlighting how eriodictyol-*O*-hexoside possesses considerable radical scavenging activity (>50%).

Regarding complex multianalyte samples, one of the DPPH assay limitations is represented from its inability to provide information regarding the individual antioxidant potential of different analytes. Contrariwise, if the assay is coupled with a separation technique, such as UHPLC, the method can be useful for the evaluation of individual contributions to the antioxidant activity of natural extracts. For this purpose, in this study, we have developed a precolumn DPPH assay for the identification of antioxidant compounds in three aforementioned sorghum varieties. In this analytical platform, after the reaction with the radical, the sample is injected, and if the compound possesses antioxidant activity, its UV/vis peak area would decrease.

The HPLC-DPPH assay showed that the compounds with greater radical scavenging activity were eriodictyol-*O*-hexoside (Figure 2), pyrano-flavanone-flavanol dimers derivates, and 7,3′,4′- trihydroxyflavone. These compounds are characterized by a catechol (3′,4′-dihydroxy) structure in the B-ring, a crucial element responsible for their high antioxidant activity.[52]

The most abundant polyphenolic compounds in sorghum belong to the class of flavonoids that can give the white, red, and black colors to the different varieties of sorghum depending on the genotype of the variety and the environment in which they are growing.[41] It has been shown that flavonoids, polyphenolic compounds present in all plants, are able to eliminate free radicals relevant to human disease.[21,48] Thus, it is appropriate to study the polyphenol content in cultivated sorghum varieties in the Mediterranean environment.

The starting point for establishing the health benefits of a food is the food's nutritional and chemical properties. In the case of sorghum, as with plant foods generally, the phytochemical component is of interest and reflects recent developments in the nutritional sciences.[21,48] As previously mentioned, the type of phytochemicals in some sorghum varieties has the capacity to neutralize free radicals. This antioxidant activity has been correlated to potential health beneficial properties such as antimicrobial,[53] reduced oxidative stress,[54] anti-inflammatory activity,[55] protection against cardiovascular disease, obesity, and diabetes,[15] and anticancer activity.[21,25,26,56]

The identification of new polyphenolic compounds in high quantities in sorghum varieties grown in the Mediterranean

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environment will help to determine the benefits of sorghum grains and bioactive phenolic compounds relevant to human health. Several studies have shown the health benefits of phenolic compounds contained in the sorghum grain such as anti-inflammatory activity and anticancer properties.[57−62]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.1c00115.

Discussion of identification of anthocyanins in the black sorghum extract, figures of chromatographic profile and UV and DPPH radical quenching chromatograms, and tables of RP-UHPLC-UV-ESI$^+$ identification and method validation parameters (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Paola Pontieri − *Istituto di Bioscienze e BioRisorse-UOS Napoli-CNR c/o Dipartimento di Biologia, Sezione di Igiene, Napoli 80134, Italy;* orcid.org/0000-0003-3468-0748; Email: paola.pontieri@ibbr.cnr.it

Authors

Giacomo Pepe − *Department of Pharmacy, University of Salerno, 84084 Fisciano, Italy;* orcid.org/0000-0002-7561-2023

Pietro Campiglia − *Department of Pharmacy, University of Salerno, 84084 Fisciano, Italy; European Biomedical Research Institute of Salerno, 84125 Salerno, Italy;* orcid.org/0000-0002-1069-2181

Fabrizio Merciai − *Department of Pharmacy and PhD Program in Drug Discovery and Development, University of Salerno, 84084 Fisciano, Italy*

Manuela Giovanna Basilicata − *Department of Pharmacy, University of Salerno, 84084 Fisciano, Italy*

Dmitriy Smolensky − *USDA-ARS, CGAHR, Manhattan, Kansas 66502, United States*

Matteo Calcagnile − *Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università del Salento, Lecce 73100, Italy;* orcid.org/0000-0001-9745-4583

Jacopo Troisi − *Theoreosrl - Spin off of the University of Salerno, Pugliano, SA 84090, Italy;* orcid.org/0000-0003-2962-7379

Roberta Romano − *Dipartimento di Ingegneria Civile, Chimica, Ambientale e dei Materiali (DICAM), Università di Bologna, Bologna 40131, Italy*

Fabio Del Giudice − *Bioteam Laboratory, Napoli 80129, Italy*

Mariarosaria Aletta − *DCSRSI SPR BIBLIOTECA, Napoli 80131, Italy*

Marco Guida − *Department of Biology, University of Naples Federico II, Naples 80126, Italy*

Pietro Alifano − *Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università del Salento, Lecce 73100, Italy;* orcid.org/0000-0003-3768-7275

Luigi Del Giudice − *Istituto di Bioscienze e BioRisorse-UOS Napoli-CNR c/o Dipartimento di Biologia, Sezione di Igiene, Napoli 80134, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsfoodscitech.1c00115

Author Contributions

□P.P. and G.P. contributed equally to this work.

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Notes

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