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Biostimulants for Sustainable Food Production: Effects of Wood Distillate to Fortify Chickpea Flour for Development of Functional Bakery Products

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ABSTRACT: In recent years, chickpea flour has been incorporated into various food products for improving their protein content and nutritional values. Based on the 'Farm to Fork' strategy, the need for the reduction of chemical pesticides is pushing the use of biobased products for cultivation. Wood distillate (WD), a byproduct of woody biomass pyrolysis, has emerged as a biostimulant with positive effects on crop yield and quality. To achieve functional bakery products with improved antioxidant capacity enriched with proteins, we use flour from WD-treated chickpeas. WD treatment significantly increases the content of polyphenols and proteins, such as vicilin- and legumin-like proteins. After a simulated gastrointestinal digestion of the biscuits, the released peptides are analyzed. An improved number of peptides (460) released by WD-treated chickpea flour cookies has been detected compared with the control (286). Chickpea flour from a biostimulated plant offers a new perspective for the production of nutritionally enriched bakery products.

KEYWORDS: *biostimulants, antioxidant properties, bakery products, pyroligneous acid, wood vinegar*

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INTRODUCTION

There is a strong body of science underpinning the health benefits from foods. The need of foods for improving health and possibly reduce the risk of diseases, while enhancing the overall well-being of the world population, led to the concept of functional foods. In line with the European Green Deal priorities, the Farm to Fork (F2F) strategy supports the development of functional food products and organic production systems using biostimulants with low impact on environment/climate.[1]

Chickpea (*Cicer arietinum* L.) seeds are an important source of dietary fibers, proteins, polyphenols, vitamins (i.e., vitamin B6 and vitamin E), and minerals (including manganese, iron, and phosphorus)[2,3] with antioxidant and anti-inflammatory properties.[4,5] Proteins derived from chickpeas have favorable functional assets, such as solubility, water and oil absorption capacity, emulsifiability, foaminess, and gelling properties.[6] These characteristics are closely linked to the amino acid composition and in turn to protein structure.[7] Consequently, chickpeas could represent an interesting source of vegetable proteins that could be exploited for the development of protein-enriched food ingredients.[8]

Several processes (i.e., grinding, chopping, hulling, sieving, sprouting, boiling, or soaking) are commonly used to make raw seeds edible and palatable and to increase their nutritional value. Although chickpeas can be consumed in their whole form, they are often subjected to primary processes to obtain flour.[9] Chickpea flour is characterized by a composition of proteins (~20%), lipids (~6%), carbohydrates (~62%), ash (~3%), and water (~11%).[10] Furthermore, the chemical composition and morphological characteristics (i.e., size, hardness, and density) of chickpea seeds play key roles in the performance of the milling process. Therefore, these factors influence the physical properties of the resulting flour and its applicability in various food products.[11]

Nowadays, chickpea flour has been incorporated into a variety of food products, including bread, pasta, and cakes, often in combination with other cereal flours. The addition of chickpea flour often improves the quality of cereal-based products, particularly in terms of protein content, nutritional values, and sensory properties.[9]

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The quality of the flour also depends on how chickpeas are cultivated. The F2F strategy indicates, as a key point, that by 2030, the use of chemical pesticides should be reduced by 50% and the use of synthetic fertilizers by 20%.[1] In line with these indications, several biobased products have been developed that are able to improve the yield and nutritional quality of crops, devoid of environmental impact. Among all the currently marketed biobased products, one of the greatest interests is wood distillate (WD).

WD is a byproduct derived from the pyrolysis process of wood biomass.[12] WD is mainly used as a biostimulant,[13] and we recently proved its effects on tomatoes' quality and composition.[14,15]

We herein report an investigation of the antioxidant profile and protein enrichment of chickpea flour from plants cultivated under biostimulation with WD. The preparation of nutritionally enriched cookies and the investigation of the bioaccessible peptides are also here described.

MATERIALS AND METHODS

Materials. Chickpea seeds, obtained from a local farm ('small chickpea from Arezzo', Tuscany, Italy), were placed in 50 mL tubes and subjected to a 3-day cold stratification at 6 °C. Following cold treatment, the seeds were sterilized with 3% (v/v) sodium hypochlorite (NaClO) for 2 min, followed by thorough rinsing with deionized water. Subsequently, the seeds were allowed to germinate in Petri dishes in the dark at 15 °C for 1 week.

All materials involved in the germination process, including Petri dishes, pipet tips, deionized water, tweezers, and filter papers, underwent sterilization with ultraviolet (UV) lamps for 1 h to prevent the growth of phytopathogens. The resulting seedlings were then transplanted into plastic pots ($10 \times 10 \times 12$ cm^3) filled with soil. After 2 weeks, 20 plants were transferred to the Botanical Garden of the University of Siena (Italy). Among these, ten plants were randomly selected for weekly foliar applications of 0.25% (v/v) chestnut (*Castanea sativa* Mill.) wood distillate (BioDea WD, Arezzo, Italy), while the remaining 10 plants served as control and were treated only with water. The experiment lasted 4 months (from March to July 2021), concluding when >50% of the plants had dried out. Subsequently, the aboveground biomass of these plants was harvested and transported to the laboratory, where each seed of both the control and WD-treated plants was removed from its pod and ground with a stainless mill to obtain two different flours.

Chemicals. Gallic acid, (+)-hydrated catechin, Folin−Ciocalteu reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium molybdate ($Na_2MoO_4 \cdot 2\ H_2O$), hydrochloric acid (HCl), sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), and 2,20-azino-bis(3-ethylbenzothiazolin-6-sulfonic) radical (ABTS) were purchased from Merck (St. Louis, MO). Ethanol and purified water were purchased from VWR (Chromasolv, VWR International Srl, Milano, Italy). All additives and mobile phases were liquid chromatography−mass spectrometry (LC-MS) grade and purchased from Merck (Milan, Italy).

Cookies Preparation. Three different types of cookies (**C**) were prepared using three different types of chickpea flours: (i) chickpea flour from control plants (not cultivated with WD) (**BF**), (ii) chickpea flour from **WDF**-treated plants, and (iii) commercial chickpea flour (**CF**). The corresponding cookies were named **BC**, **WDC**, and **CC**, respectively. For the preparation of these cookies, a common recipe was used, substituting the regular flour with the aforementioned flour, which represented the only protein source. The recipe is as follows: 80.0 g of flour, 20.0 g of corn starch, 20.0 g of saccharose, 50.0 mL of water, 3.5 g of commercial baking powder (ingredients: raising agents, disodium diphosphate E450i, sodium bicarbonate E500ii, corn starch), and 20.0 mL of sunflower oil. All of the ingredients were purchased from a local shop, except for **WDF** and **CF**. From these quantities, approximately 8 cookies for each type of flour were prepared. To prepare the cookies, sugar, oil, flour, starch, and baking powder were mixed in a bowl. Finally, water was added to eliminate lumps and make the mixture more homogeneous. Subsequently, the dough was divided into eight cookies. The divided doughs, of equal measure, were placed on a baking tray previously lined with parchment paper and shaped to be baked in the oven. The cookies were baked (BOSCH HRA574BB0 oven) at a temperature of 200 °C for 8 min. After cooling to room temperature (24 °C), baked biscuits were packed in sealed polyethylene bags and stored in a desiccator until the analyses were performed the same day of the preparation and after 4 and 10 days.

Cookies Extraction. Antioxidant properties of cookie samples (**WDC**, **BC**, and **CC**) were evaluated as a function of time by performing an extraction method described in the literature with some modifications.[16] The extraction was performed by suspending 1 g of cookie in 7 mL of a hydroalcoholic solution (ethanol/purified water 50:50 v/v). Subsequently, the mixture was sonicated for 15 min at room temperature. Then, the solution was centrifuged for 10 min at 8000 rpm. Once centrifugation was completed, the supernatant was collected and set aside for the analyses. The extraction procedure was performed as a function of time (t = 0, 4, and 10 days).

Total Polyphenol Content. The total polyphenol content (**TPC**) of the extract of flour and cookie extracts was assessed following a literature method with some modifications.[17] In a volumetric flask, 6 mL of each solution, 2 mL of Na_2CO_3 solution (2% w/v), and 1 mL of Folin−Ciocalteu reagent were combined. After 2 h at room temperature, the absorbance was measured by using a Jasco V-530 UV/vis spectrometer (Jasco, Tokyo, Japan) at 720 nm versus a control. This procedure was carried out in triplicate, and the TPC value was determined using a standard curve obtained with gallic acid (**GA**) within the range of 8−40 μM (R^2 = 0.9988). The **TPC** for each extract was expressed as milligrams of **GA** equivalent per gram of cookie (mg of **GAE**/g). The experiments on the cookies samples were performed as a function of the time (t = 0, 4, and 10 days).

Phenolic Acid Content. The quantification of phenolic acid content (**PAC**) in the extract of flours and cookies was conducted using a modified Arnov test.[18] In more detail, in a 10 mL volumetric flask, 1 mL of the extract solution, 1 mL of 0.5 mol L^{-1} HCl, 1.0 mL of NaOH (4.0% w/v), 1.0 mL of Arnov's reagent (composed of 0.1 mg mL^{-1} $NaNO_2$, and 0.1 mg mL^{-1} $Na_2MoO_4 \cdot 2\ H_2O$), and purified water were mixed. The absorbance was measured spectrophotometrically at 490 nm (Jasco V-530 UV/vis spectrometer, Tokyo, Japan). The **PAC** value was expressed as milligrams of **GA** equivalent per gram of cookie (mg of **GAE**/g), after establishing the corresponding calibration line. The experiments on the cookies samples were performed as a function of time (t = 0, 4, and 10 days).

Flavonoid Content. The measurement of flavonoid content (**FC**) in the extracts of flours and cookies was carried out using a spectrophotometric method, with some modifications based on a previously published procedure.[19,20] In a 5.0 mL volumetric flask, 0.5 mL solution of each solution was combined with 0.15 mL of $NaNO_2$ aqueous solution (15% w/v) and 2.0 mL of purified water. After 6 min, 0.15 mL of an $AlCl_3$ solution (10% w/v) was added. Subsequently (after an additional 6 min), 3 mL of NaOH (4% w/v) and purified water to reach a total volume of 5.0 mL were added. After 15 min at room temperature and in the dark, the absorbance of the solutions was measured using a spectrophotometer at 510 nm (Jasco V-530 UV/vis spectrometer, Tokyo, Japan). The recorded results were expressed in milligrams of catechin (**CT**) equivalent per gram of cookie (mg **CTE**/g), after establishing the corresponding calibration line (10.0−100.0 μM, R^2 = 0.9975). The experiments on the cookies samples were performed as a function of time (t = 0, 4, and 10 days).

Antioxidant Performances. To assess the scavenging potential of the flours and prepared cookies, different volumes of sample solution were combined with a solution containing ABTS radicals (2 mL). This mixture was then incubated for 5 min at 37 °C. Subsequently, the absorbance was measured using a spectrophotometer at 734 nm (Jasco V-530 UV/vis spectrometer, Tokyo, Japan).[21] The inhibition against radical specie was estimated according to eq 1

$$\text{inhibition}\ (\%) = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

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Table 1. Content of Total Polyphenols (TPC), Phenolic Acid (PAC), Flavonoid (FC), and Scavenger Activity (ABTS IC_{50}) of the Analyzed Flours[a]

Code	TPC (GAE mg g^{-1})	PAC (GAE mg g^{-1})	FC (CTE mg g^{-1})	ABTS IC_{50} (mg mL^{-1})
WDF	30.22 ± 1.35[a]	5.90 ± 0.26[a]	27.82 ± 1.11[a]	0.0031 ± 0.0001[a]
BF	8.38 ± 0.34[b]	2.68 ± 0.12[b]	13.50 ± 0.54[b]	0.0094 ± 0.0002[b]
CF	8.30 ± 0.33[b]	2.23 ± 0.10[c]	11.23 ± 0.31[c]	0.0114 ± 0.0003[c]

[a]Data represent mean ± SD (n = 3), with different letters in the same columns indicating statistically significant differences ($p < 0.05$).

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The scavenging activity of the sample was expressed in terms of IC_{50}. Ascorbic acid was used as a positive control. The experiments on the cookies samples were performed as a function of time (t = 0, 4, and 10 days).

LC-MS/MS Identification of Chickpea Biscuit Polyphenols. Extraction of polyphenolic compounds from cookie samples was performed according to the protocol previously described.[22] Briefly, samples of chickpea biscuits were ground into a fine powder, and 100 mg were extracted with 1 mL of a methanol/water/acetic acid solution (65:29:6, v/v/v). The mixture was shaken at 300 rpm at room temperature for 3 h using a Thermomixer comfort apparatus (Eppendorf, Hamburg, Germany). The extracts were then centrifuged at 14,000 rpm at 25 °C for 20 min (Eppendorf microcentrifuge 5424, Hamburg, Germany). The supernatants were collected and injected in LC-MS. Ultra-high performance liquid chromatography-high-resolution mass spectrometry/MS (UHPLC-HRMS/MS) analysis was performed on a Thermo Scientific Vanquish UHPLC coupled online to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization probe (HESI II) operating in negative and positive modes. The chromatographic separation was performed on a Luna Omega Polar C18 analytical column (100 × 2.1 × 1.6 μm) (Phenomenex, Bologna, Italy). The column temperature and flow rate of mobile phases were set at 40 °C and 0.3 mL/min, respectively. The mobile phases were H_2O (A) and ACN (B) both acidified with 0.1% HCOOH (v/v) with the following gradient: 0.01−10.00 min, 5−95% B; 10.01−12.00 min, isocratic to 95% B; 12.01−13.00 min, 5% B; then 5 min for column re-equilibration. MS was calibrated by Thermo Calmix Pierce calibration solutions in both polarities. Full MS (150−1500 m/z) and data-dependent MS/MS were performed at a resolution of 60,000 and 15,000 full width at half-maximum (fwhm) respectively; normalized collision energy (NCE) values of 15, 20, and 25 were used. Source parameters: Sheath gas pressure, 50 arbitrary units; auxiliary gas flow, 13 arbitrary units; spray voltage, +3.5 kV, −2.8 kV; capillary temperature, 320 °C; auxiliary gas heater temperature, 300 °C. The identification of investigated analytes was carried out by comparing their retention times and MS/MS data with those present in the literature. Data analysis and processing were performed using FreeStyle 1.8 SP2 and the commercial software Compound Discoverer v. 3.3.1.111 SP1 (Thermo Fisher Scientific, Bremen, Germany).

Identification of Bioaccessible Peptides after Simulated Gastrointestinal Digestion (GID) of Cookie Samples. *In Vitro GID.* In vitro digestion of the cookie samples (**WDC**, **BC**, and **CC**) was performed using the recently updated harmonized INFOGEST method.[23] Briefly, samples were exposed to simulated oral, gastric, and intestinal phases. Sample (1 g), simulated salivary fluid (SSF, 1 mL, KCl: 15.1 mM; KH_2PO_4: 3.7 mM; $NaHCO_3$: 13.6 mM; $MgCl_2(H_2O)_6$: 0.15 mM; $(NH_4)_2CO_3$: 0.06 mM; HCl: 1.1 mM; $CaCl_2(H_2O)_2$: 1.5 mM), and salivary amylase (75 U/mL) were added to a centrifuge tube and stirred for 2 min at pH = 7 and 37 °C (oral digestion). Next, simulated gastric fluid (SGF, 20 mL, KCl: 6.9 mM; KH_2PO_4: 0.9 mM; $NaHCO_3$: 25 mM; NaCl: 47.2 mM; $MgCl_2(H_2O)_6$: 0.12 mM; $(NH_4)_2CO_3$: 0.5 mM; HCl: 15.6 mM; $CaCl_2(H_2O)_2$: 0.15 mM) containing pepsin (2000 U/mL) was added to the previous mixture and stirred at 350 rpm for 2 h at pH = 3 and 37 °C (gastric digestion). Finally, the simulated intestinal fluid (SIF, 20 mL, KCl: 6.8 mM; KH_2PO_4: 0.8 mM; $NaHCO_3$: 85 mM; NaCl: 38.4 mM; $MgCl_2(H_2O)_6$: 0.33 mM; HCl: 8.4 mM; $CaCl_2(H_2O)_2$: 0.6 mM) containing trypsin (100 U/mL), chymotrypsin (25 U/mL), and pancreatin was added to the previous mixture and stirred for 2 h at pH = 7 and 37 °C (intestinal digestion). The reaction was stopped bringing the solution to pH 2. The mixture was centrifuged at 4000g at 4 °C for 10 min (Mikro 220R centrifuge, Hettich, Germany), filtered on 0.45 μm filters (Phenex RC membrane, Phenomenex, Bologna, Italy), lyophilized, and stored at −80 °C. The samples were subjected to solid-phase (SPE) extraction to purify and concentrate the digests. The peptide fraction was solubilized in distilled water and loaded on a Strata-X 33 μm Polymeric Reversed Phase SPE cartridge (500 mg sorbent and 760−820 m^2/g surface area; Phenomenex), previously equilibrated in distilled water, then eluted with MeOH and 2% (v/v) formic acid, and finally relyophilized and stored at −20 °C. Lyophilized samples were solubilized in a mixture of H_2O/ACN, 65:35 (v/v) before LC-MS/MS analysis.

LC-MS/MS Experimental Conditions. UHPLC-HRMS/MS analysis was performed on a Thermo Vanquish coupled online to an Orbitrap Exploris 120 Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide separation was performed in reversed-phase mode, with a Kinetex 2.6 μm EVO C18 100 Å, LC Column 150 mm × 2.1 mm (Phenomenex, Bologna, Italy) with a guard cartridge system (SecurityGuard ULTRA cartridges for EVO-C18, sub-2 μm and core−shell columns with 2.1 mm internal diameters). The column temperature was set at 40 °C and the flow rate was 0.2 mL/min. The mobile phase was (A): H_2O with 0.1% HCOOH (v/v) and (B): ACN with 0.1% HCOOH (v/v). The following gradient was employed: 0.01 to 2.00 min, isocratic to 2% B; 2.01 to 30.00 min, 2−25% B; 30.01 to 33.00 min, 25−95% B; 33.01 to 35.00 min, 95−95% B; 35.01 to 37.00 min, 95−2% B; 37.01 to 42.00 min, isocratic to 2% B. Five microliters was injected. The ESI was operated in positive mode. Full MS (100−1500 m/z) and data-dependent MS/MS were performed at a resolution of 60,000 and 15,000 fwhm, respectively. HCD collision energy values of 15, 20, and 25 were used. Source parameters: RF lens, 70%; sheath gas pressure, 40 arbitrary units; auxiliary gas flow, 15 arbitrary units; spray voltage, +3.5, −2.5 kV; ion transfer tube temperature, 320 °C; vaporized temperature, 300 °C; and auxiliary gas heater temperature, 300 °C.

Peptide Sequence Identification. Raw MS/MS data files were converted to mzXML format, and a free trial of PEAKS 11 software (Bioinformatics Solutions Inc., Waterloo, Canada) was employed for peptide sequence determination. Search was performed using a database (DB) search tool by searching against Swiss-Prot/UniProt database taxonomy *C. arietinum* (Chickpea) (Garbanzo) (cv. CDC Frontier), with an improved algorithm that validates and assists the database search with de novo sequencing results with the following settings enzymes: pepsin, trypsin, chymotrypsin; peptide charges from +1 to +4, monoisotopic precursor mass; fragmentation mode, CID (y and b ions); precursor mass tolerance, 10 ppm; fragment mass tolerance, 0.5 Da; carbamidomethyl cysteine was set as a fixed modification oxidation (M), and phosphorylation (S, T, Y) was used as dynamic modifications.

Statistical Analysis. The inhibitory concentration IC_{50} was calculated by nonlinear regression with the use of Prism GraphPad Prism, version 4.0 for Windows (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by a multi-comparison Dunnett's test was applied ($p < 0.05$). To improve data interpretation, multivariate data analysis based on hierarchical clustering and principal component analysis (PCA) was carried out using the online tool MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Hierarchical clustering was applied using a complete linkage clustering method with a Pearson distance measurement. The multivariate data matrix was analyzed after data autoscaling (data

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Figure 1. Cookies were derived from different flours: chickpea control flour (**BC**), WD-treated chickpea flour (**WDC**), and commercial chickpea flour (**CC**).

Table 2. Content of Total Polyphenolic (TPC), Phenolic Acid (PAC), and Flavonoids (FC), and Scavenger Activity (ABTS IC_{50}) of the Prepared Cookies[a]

time (days)	code	TPC (GAE mg g^{-1})	PAC (GAE mg g^{-1})	FC (CTE mg g^{-1})	ABTS IC_{50} (mg mL^{-1})
0	**WDC**	5.83 ± 0.25^{a}	1.40 ± 0.06^{a}	7.97 ± 0.31^{a}	0.067 ± 0.003^{a}
	BC	4.16 ± 0.18^{c}	0.90 ± 0.04^{b,c}	3.20 ± 0.14^{d}	0.141 ± 0.006^{d}
	CC	3.50 ± 0.13^{d}	0.83 ± 0.03^{c,d}	2.93 ± 0.11^{e}	0.156 ± 0.007^{e}
4	**WDC**	4.61 ± 0.19^{b}	0.99 ± 0.04^{b}	5.91 ± 0.25^{b}	0.085 ± 0.003^{b}
	BC	2.95 ± 0.12^{e}	0.83 ± 0.03^{c,d}	2.90 ± 0.11^{e}	0.158 ± 0.006^{e}
	CC	1.73 ± 0.07^{g}	0.69 ± 0.02^{e}	1.77 ± 0.07^{g}	0.163 ± 0.006^{e}
10	**WDC**	3.48 ± 0.14^{d}	0.91 ± 0.03^{d}	5.60 ± 0.14^{c}	0.104 ± 0.004^{c}
	BC	2.19 ± 0.09^{f}	0.72 ± 0.03^{e}	2.14 ± 0.09^{f}	0.191 ± 0.008^{f}
	CC	1.42 ± 0.05^{h}	0.62 ± 0.02^{f}	1.42 ± 0.06^{h}	0.168 ± 0.007^{e}

[a]Data represent mean ± SD (n = 3), with different letters in the same columns indicating the degree of statistically significant differences (p < 0.05).

were mean-centered and divided by the standard deviation of each variable). Boxplots and p-values associated with pairwise class comparisons for each bioactive compound were obtained using an in-house RStudio script (version 2022.12.0 + 353).

RESULTS

Antioxidant Performances of Chickpea Flours. The antioxidant performances of control flour **BF**, WD-treated flour **WDF**, and commercial flour **CF** in terms of total phenolic compounds, as well as phenolic acids and flavonoids, are reported in Table 1. Scavenger features were evaluated by the ABTS assay.

WDF showed a TPC value (30.22 GAE mg g^{-1}) almost four times higher than those of **BF** and **CF** flours. Similarly, **WDF** displayed the highest PAC content (5.90 GAE mg g^{-1}), with an increment almost 2.6 times higher compared to **BF** and **CF**. As far as the flavonoid content, **WDF** (27.82 CTE mg g^{-1}) proved to possess the highest value, two times higher than those of untreated and commercial flours. Scavenger profiles, investigated against ABTS radical species, displayed a scavenger activity of **WDF** 3.6 times higher than those of the **CF** and **BF** samples.

Antioxidant Performances of Chickpea Flour-Based Cookies. Three kinds of cookies (**CC**, **WDC**, and **BC**) were prepared based on **CF**, **WDF**, and **BF** flours (Figure 1).

Antioxidant properties of the cookies were evaluated and monitored as a function of the time (t = 0, 4, and 10 days), and the results are presented in Table 2.

The recorded TPC after baking (t = 0 day) ranged from 5.83 GAE mg g^{-1} (**WDC**) to 3.50 GAE mg g^{-1} (**CC**), indicating a 40% decrease (3.3 times lower in the cookies) in TPC concerning the values of starting flours. Similarly, a detrimental effect of heat was recorded for the PAC (3.8 times lower), while a more evident effect was verified in the FC value (7.8 times lower), confirming the highest heat sensitivity of the flavonoid molecules. When analyzed 10 days after cooking (t = 10 days), the TPC showed a more significant reduction between 40% (**WDC**) and 59% (**CC**) with respect to those values recorded at t = 0. Similarly, PAC and FC values slightly decreased after 10 days, and this decrement was more significant for the cookies made with **BF** and **CF**. Scavenger properties of the cookies showed that the IC_{50} of **WDC** was almost 1 order of magnitude higher compared with the other two cookies. In general, ABTS values experimentally calculated at t = 0 and t = 10 days highlighted that **WDC** showed an IC_{50} value (0.104 mg mL^{-1}) lower than those of **BC** (+84%) and **CC** (+62%), indicating a better antioxidant profile.

Phytochemical Composition of Chickpea Flour-Based Cookies. Full-scan HRMS-MS/MS data obtained for hydro-alcoholic extracts derived from chickpea cookie samples (**WDC**, **BC**, and **CC**) revealed the presence of at least 137 compounds, primarily belonging to the class of flavonoids (Flav), phenolic acids (PhAc), dipeptides, saponins (Sap), fatty acids (FA), and lipids (Table S1). Figure 2 shows a representative TIC (total ion current) chromatogram of an alcoholic extract of chickpea cookies analyzed in both ionization modes. UHPLC-HRMS/MS in negative ionization mode allowed the detection of different flavonoids, mainly kaempferol aglycones and phenolic acids.

Peak **46** was tentatively identified as a kaempferol pentoside-hexoside-deoxyhexoside, with a molecular ion $[M - H]^-$ at m/z 725 corresponding to the molecular formula $C_{32}H_{38}O_{19}$. In MS2, this compound produced fragment ions at m/z 579 $[M - H - 146]^-$ corresponding to the loss of deoxyhexosyl moiety and the subsequent loss of 324 Da resulted in the deprotonated aglycone ion at m/z 284.[24] MS spectra of peaks **39** and **44** showed a deprotonated molecular ion at m/z 609 corresponding to the molecular formula $C_{27}H_{30}O_{16}$. However, the loss of a

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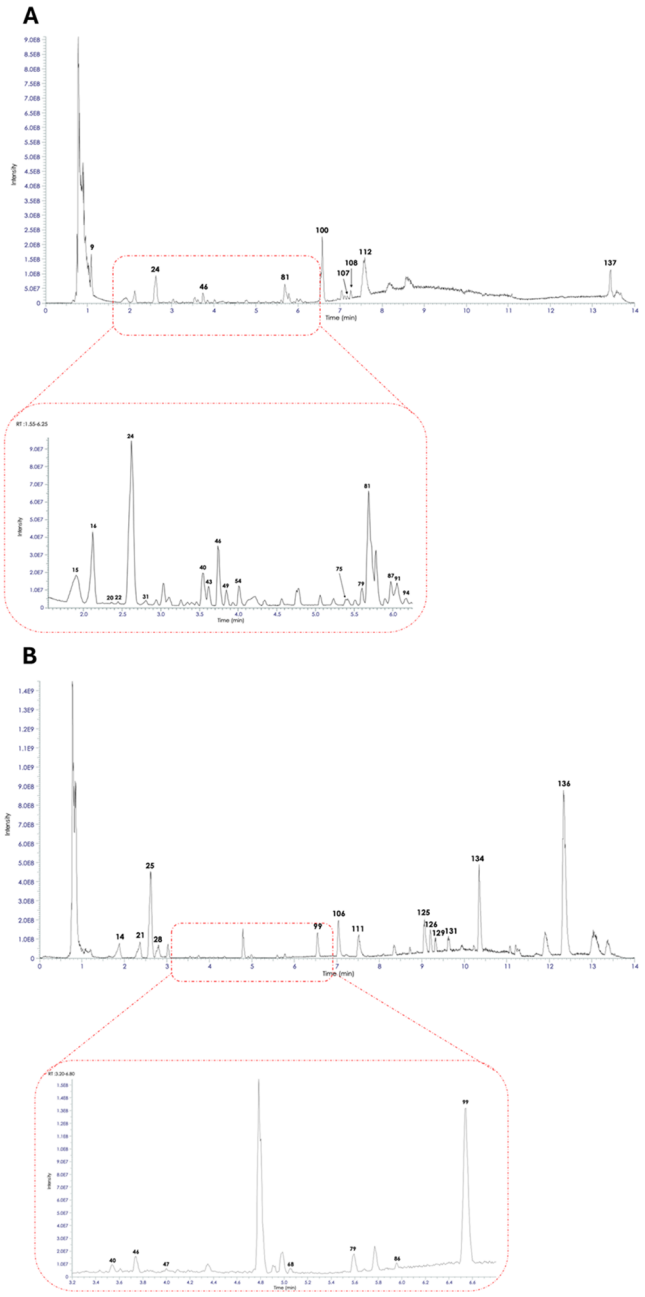

Figure 2. Representative TIC of chickpea cookie extracts in negative (A) and positive (B) ionization modes.

series of hexoside moieties (162 Da) gave fragments at m/z 447 [M − H − 162 − 162] and m/z 285 [M − H − 162 − 162 − 162], leading to their tentative identification as kaempferol dihexoside isomers.[24,25]

Primeveroside salicylic acid (peaks **16** and **23**) was the main phenolic acid identified in the hydroalcoholic extracts, with a molecular ion at m/z 431 corresponding to the molecular formula of $C_{18}H_{24}O_{12}$.[25] Different dipeptides were detected in negative ionization mode; peak **24** was tentatively identified as *N*-γ-Glutamylphenylalanine ($C_{14}H_{18}O_5N_2$), giving $[M - H]^-$ ions at m/z 293. MS/MS fragments at m/z 128 and m/z 275 were observed, corresponding to the loss of glutamine residue and water, respectively.[26] Among the 39 identified fatty acids, peaks **81**, **87**, **91**, and **94** were detected at different retention times in the chromatogram and exhibited the precursor ions at m/z 329 $[M - H]^-$. The MS/MS fragmentation pattern showed ions at m/z 311 $[M - H - H_2O]^-$, 293 $[M - H - 2H_2O]^-$, and 229 $[M - H - 100]^-$ corresponding to the loss of water and end-group HO−CH═CH$(CH_2)_3CH_3$, respectively.

These peaks were tentatively identified as trihydroxy-octadecenoic acid (TriHODE) isomers.[26] Moreover, peak **100** showed a fragmentation pattern at m/z 293 $[M - H]^-$ with ions at m/z 193 and m/z 99, likely resulting from cleavage of the C4−C5 bond and the loss of the hexanal end-group ($C_6H_{12}O$, 100 Da). This fragmentation pattern was tentatively attributed to (±)-gingerol ($C_{17}H_{26}O_4$).[27,28] TIC analyzed in positive ionization mode showed the presence of different saponins. $[M + H]^+$ ion at m/z 943 was tentatively identified as soyasaponin Bb (peaks **99**, $C_{48}H_{78}O_{18}$) with MS/MS fragments at m/z 797 and m/z 599 corresponding to the successive loss of deoxyhexosyl ($C_6H_{10}O_4$, 146 Da), hexosyl ($C_6H_{10}O_5$, 162 Da), and two water moieties (18 + 18 Da), respectively. Thus, following the loss of dHex + Hex + $2H_2O$, the unresolved portion was tentatively identified as aglycone with m/z 459.[29,30] LC-HRMS/MS analysis also indicated the presence of soyasaponin Bd (peak **79**, $C_{48}H_{76}O_{19}$, m/z 957), showing fragmented ions at m/z 811 $[C_{48}H_{76}O_{19}-dHex]^+$, m/z 649 $[C_{48}H_{76}O_{19}-dHex-Hex]^+$, and m/z 631 $[C_{48}H_{76}O_{19}-dHex-Hex-H_2O]^+$. A similar fragmentation pattern was observed for soyasaponin Be (peaks **92**, **97**, **105** $C_{48}H_{76}O_{18}$, m/z 941), which gave fragmented ions at m/z 795 $[C_{48}H_{76}O_{18}-dHex]^+$, m/z 633 $[C_{48}H_{76}O_{18}-dHex-Hex]^+$, and m/z 615 $[C_{48}H_{76}O_{18}-dHex-Hex-H_2O]^+$.[30,31] Maackiain (peaks **125** and **129**) showed a precursor ion at m/z 284 ($C_{16}H_{12}O_5$) and generated MS/MS base fragment ions at m/z 151 $[M + H - C_8H_7O_2]^+$, m/z 138 $[M + H - C_9H_7O_2]^+$, and m/z 109 $[M + H - C_{10}H_7O_3]^+$.[32] Two fatty acid amides were identified: oleamide (peak **134**), which showed a precursor ion at m/z 282 $[M + H]^+$ and the most common fragments associated were m/z 265 $[M + H - H_2O]^+$ and m/z 247 $[M + H - 2H_2O]^+$,[33] and erucamide (peak **136**) (m/z 338, $C_{22}H_{43}NO$), which exhibited a similar fragmentation pattern, wherein two consecutive losses of water molecules were observed, leading to the formation of two fragments at m/z 321 $[M + H - H_2O]^+$ and m/z 303 $[M + H - 2H_2O]^+$.[34]

Multivariate Analysis of Phytochemicals Identified in WDC, BC, and CC Cookies. A hierarchical cluster analysis (HCA) of the 137 compounds, detected by UHPLC-HRMS/MS analysis, shows a heat map of the distribution of major metabolites among **WDC**, **BC**, and **CC** samples. The color code, ranging from blue to red, represents the relatively lower or higher amount (by area) of a specific metabolite in a given sample (Figure 3).

The top 25 bioactive compounds were ranked based on results from ANOVA ($p < 0.05$) and used to create the heatmap. The list of 25 features includes Flavs, FAs, fatty acyls, Saps and PhAcs. The dendrogram at the top of the heatmap highlights the first level of separation between the **WDC** group and the remaining **CC** and **BC** groups, further divided in the second level of separation.

In the **WDC** sample, the species that contributed to the discrimination were FA_08, FA_10, FA_13, FA_16, FA_18, FA_19, FA_20, FA_34, FAcyls_02, Flav_05, Flav_08, PhAc_09, Flav_10, Flav_11, Flav_18, Flav_21, PhAc_09, and Sap_02 (see Table S1), which exhibited a higher normalized extracted ion chromatogram (XIC) area in this sample.

This pattern is in complete contrast to the **BC** sample, which instead upregulates FA_36, Flav_20, Flav_25, Flav_30, Flav_33, Flav_34, Flav_38, and Sap_01. The **CC** sample does

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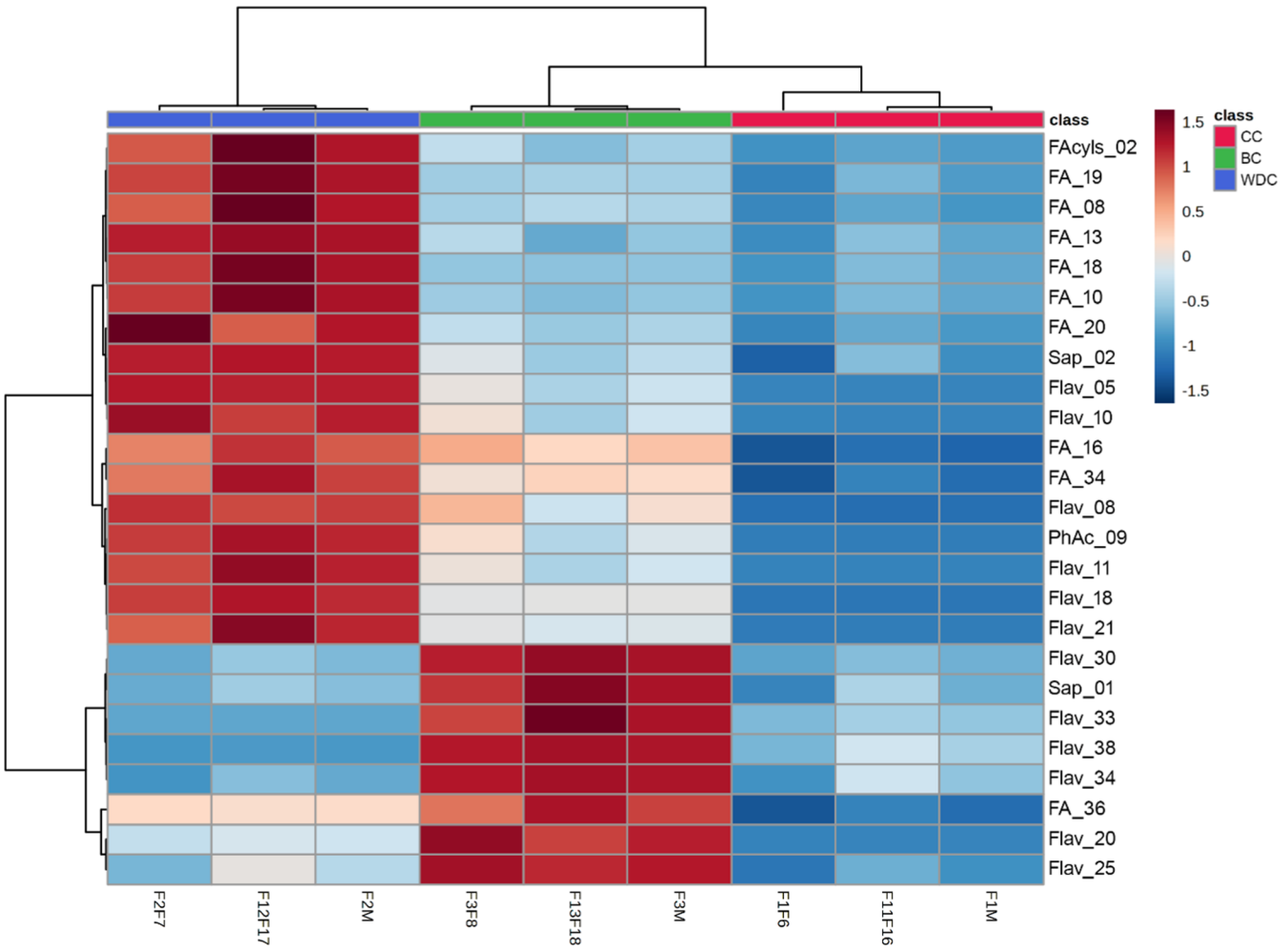

Figure 3. Hierarchical clustering heatmap of the top 25 up- and down-expressed bioactive compounds. In the heatmap, red and blue colors indicate higher and lower chemical abundance, respectively. Cookie samples (**WDC**, **BC**, and **CC**) are identified based on the color scale provided in the legend at the top right.

not show any significantly up-expressed compound compared to **BC** and **WDC** samples.

Top 25 bioactive compounds, highlighted in the heatmap, are further displayed using boxplots generated by an in-house RStudio script, as illustrated in Figure 4. Each individual plot displays, on the *y*-axis, the normalized XIC area ranges of the different molecules for each class. Additionally, the graphs show p-values obtained by comparing the area values of each class against every other class for each compound within the top 25.

Release of Peptides after Simulated Gastrointestinal Digestion. The simulated gastrointestinal digestion protocol allowed us to mimic the physiological and biochemical conditions and the sequence of events that occur during in vivo gastrointestinal digestion. The highly acidic environment in the stomach lumen determined the parent protein denaturation and consequently led to the exposure of the protein's peptide bonds. The consequent action of the gastroenzymes, such as pepsin, and the small intestine enzymes, such as trypsin, chymotrypsin, and pancreatin, allowed hydrolysis of proteins, generating several small peptides.

The peptide identification was carried out using UHPLC-Orbitrap-based tandem mass spectrometry (MS/MS). The base peak chromatograms relative to the gastrointestinal digestion of each chickpea cookie are reported in Figure S1.

MS/MS spectra were employed for sequence determination, and the complete list of peptides, including retention times, peptide sequences, precursor proteins, and masses, is reported in Tables S2−S4.

The identified peptides were 286 in **BC** samples, 460 in **WDC** samples, and 284 in **CC** samples, belonging to vicilin-, legumin- and provicilin-like proteins. Figure 5 shows the classes of peptides released after in vitro gastrointestinal digestion of chickpea cookies, clustered based on their molecular weight (Da). In particular, the peptides identified in the present study ranged from 6 to 18 amino acid residues, thus corresponding to molecular weights between 400 and 2000 Da.

DISCUSSION

It is widely recognized that WD can enhance the nutritional characteristics of various plant parts, including leaves, fruits, tubers, and seeds.[35−37]

The foliar application of 0.25% (v/v) WD to chickpea plants significantly boosted the protein composition and antioxidant profile of the flour obtained through seed milling.

Since WD is a complex product composed of >200 compounds, the specific mechanism by which it influences plant metabolism remains to be fully elucidated. In our recent research, we have suggested that WD may induce a particular

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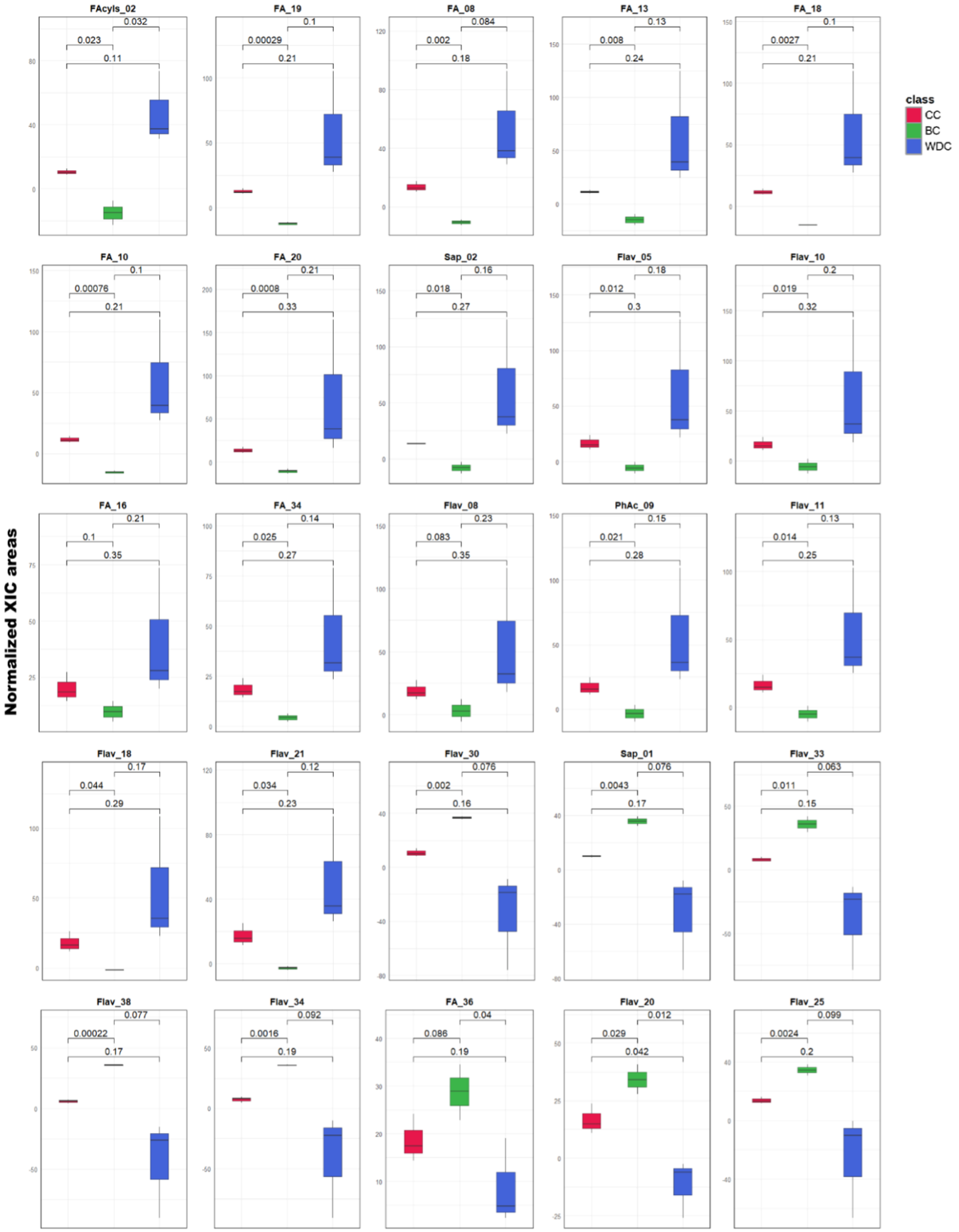

Figure 4. Boxplots of the top 25 bioactive compounds detected in the cookie samples (**WDC**, **BC**, and **CC**). Normalized areas were obtained from extracted-ion chromatograms (XIC). Boxplot uses boxes to show medians and interquartile ranges, with whiskers indicating the data range from minimum to maximum values.

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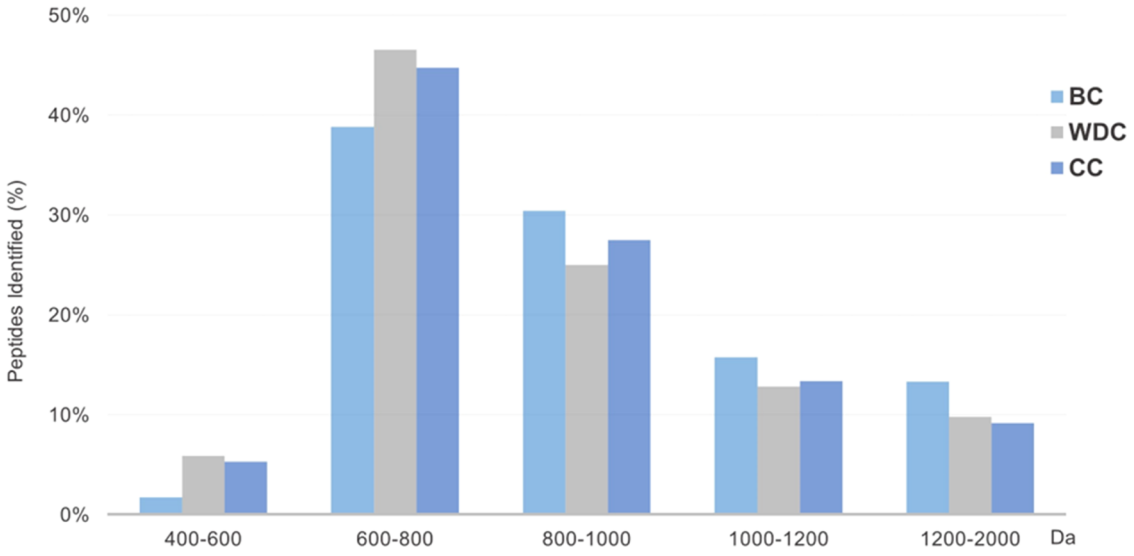

Figure 5. Distribution of relative molecular mass (400−2000 Da) for all peptides released after GID simulation of control flour (**BC**), WD-treated chickpea flour (**WDC**), and commercial chickpea flour (**CC**).

type of plant stress known as eustress, a mechanism leading to the activation of defense mechanisms and consequently leading to increased production of antioxidant compounds.[38]

In this study, seeds of chickpea plants grown with the foliar application of **WD** were successfully employed in the preparation of flour (**WDF**) with significant antioxidant properties.

Literature data displayed TPC values of untreated chickpea flours in the range 1.3−9.7 GAE mg g^{-1},[16] according to the recorded value for **BF** and **CF**. To increase the concentration of the phenolic compounds, different treatments were suggested, mainly involving fermentation processes.[39]

The antioxidant performance observed in this study showed a strong positive correlation between the TPC of the flours and the scavenger activities.[40] Interestingly, similar results were registered for **BF** and **CF**, a commercial/reference flour, while **WDF**, produced in the same conditions as those providing **BF**, showed a statistically significant increase in the TPC value.

The market of bakery products is constantly growing;[41] therefore, developing baked items with improved nutritional properties may appeal to customers, who are highly concerned about their food choices.

In the preparation of cookies, chickpea flour represented about 25% (w/w) of the dough, representing the only source of proteins, while the other ingredients did not contain compounds with a phenolic structure.

However, according to the literature data, the baking process has a negligible impact on the phenolic compounds, as well as phenolic acids and flavonoid molecules.[42]

A significant ($p < 0.05$) TPC reduction of the cookies has been mainly attributed to the Maillard reaction generated during baking with consequent loss of heat-sensitive molecules.[43]

For identification of accessible flavonoids, fatty acids, and saponines, UHPLC-HRMS/MS analysis was performed using various cookie samples, and 137 bioactive compounds were identified. The multivariate analysis confirmed a clear difference in the chemical composition among **WDC**, **BC**, and **CC** samples.

The presence of different classes of compounds in **WDC** (Figures 3 and 4), including the high amount of Flavs and FAs, can be attributed to the treatment with WD, responsible for the fortification of flour in terms of polyphenols and fatty acids.

In the field of functional foods, bioactive peptides are increasingly recognized as useful tools for improving body's health and preventing chronic diseases. Indeed, food proteins offer several health benefits through their interaction with specific biochemical pathways. Most of these activities are attributed to peptides encrypted in the parent protein sequences, which are released through digestion, absorbed intact by intestinal cells, and transported to their target tissues in sufficient quantities to exert nutritional benefits.[44]

Given that chickpeas are primarily consumed for their protein composition[45] and represent a potential source of functional peptides with medicinal properties, including antioxidant, antihyperlipidemic, and antiproliferative activities,[46] this study investigated the levels of the bioaccessible peptides from **WDF** cookies by simulating their gastrointestinal digestion.

The gastrointestinal digestion revealed that there were 284 and 286 peptides in **CC** and **BC** samples, respectively, while 460 peptides were identified in **WDC** samples. Proteins identified in **WDC** samples include vicilin-, legumin-, and provicilin-like molecules. The peptides identified ranged from 6 to 18 amino acid residues, thus between 400 and 2000 Da. It is very interesting to observe that **WDC**s contain a remarkable number of peptides ($n = 214$) with molecular weight distribution in the range 600−800 Da, being the bioaccessible ones.

Different approaches have been reported to enhance the quality of chickpea proteins, including germination dehulling, fermentation, hydrolysis, other chemical modifications, extrusion, and high hydrostatic pressure. These methods are independent and can be used to improve the nutritional qualities of the proteins.[9] To the best of our knowledge, our work investigates, for the first time, the effects of WD, used as a biostimulant for the production of chickpea flours, to produce bakery foods characterized by a higher amount of bioaccessible peptides. Referring to the antioxidant profile, **WDF**, compared to **BF** and **CF**, showed significant differences in terms of polyphenol molecules (Table 1 and Figure 3). WD treatment is responsible for these significant differences since chickpeas that generated **BF** and **WDF** were cultivated in the same climatic and edaphic conditions, using WD biostimulation. These data taken

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together confirm **WDF** as a key ingredient in producing functional bakery gluten-free products fortified with proteins that release bioaccessible peptides.

The development of new gluten-free products is growing; in addition to individuals who need to pursue a gluten-free diet, there has been a growing ask for gluten-free products from those aiming to pursue a healthy eating regimen.[47]

However, gluten-free products available on the market generally exhibit subpar cooking and sensory qualities. High-quality gluten-free products can be made using alternative ingredients but a correct balance of formulations and appropriate technological processes is crucial to address the changes in textural and sensorial properties that arise due to the absence of gluten.[48]

The development of baked products with chickpea flour as the main ingredient, replacing white wheat flour, is promising from nutritional and technological standpoints.[49] The use of chickpea flour may significantly affect the chemical composition, in terms of good quality proteins, and total antioxidant capacity value of the baked products.[41]

Noteworthy, chickpea pasta showed an important content of chickpea allergens and immunoglobulin IgE binding proteins, such as 7S globulin, 2S albumin, LTP, and PR-10, similar to hydrated chickpea seeds and cooked chickpeas. During boiling, more allergens from chickpea pasta were transferred to boiling water than chickpea seeds.[50] Furthermore, chickpea flour allowed the production of yeast-leavened bread with suitable functional and sensory properties and better protein quality when compared to the control.[51] Moreover, chickpea flour had a high water absorption index, low emulsion activity, pasting temperature, and degradation.[52] Thus, chickpea flour-based foods have been proposed to decrease the harmful complications of type-2 diabetes and other pathologies.[9,42]

Wood distillate is an environmentally safe biobased product stimulating plant growth and yield.[53,54] WD-treated chickpea plants displayed higher seed weight and diameter, and enhanced amount of starch, soluble proteins, polyphenols, and total antioxidant power, as well as many amino acids.[38] We demonstrated that this biostimulant, used for reducing chemical fertilizers, enhances chickpea plant growth and the overall nutritional properties of flour (**WDF**). **WDF**-based bakery products were developed, and they showed a much higher content of bioaccessible peptides. Thus, these biscuits could be used for treating different pathologies or in gluten-free diets. Furthermore, these bakery products can be consumed by vegans and vegetarians because they are enriched in proteins and antioxidants but produced without animal-derived ingredients.[55,56]

In conclusion, we demonstrated that chickpea flour derived from WD-treated plants has a higher content of polyphenols with respect to those of **CF** and **BF**. The obtained peptides maintained a similar trend with those of flour in terms of polyphenol content and antioxidant activity. The gastrointestinal digestion furnished the bioavailable peptides of vicilin- and legumin-like families. An improved number of peptides (460) released by WD-treated chickpea flour cookies has been detected, compared with the control (286). To the best of our knowledge, we demonstrated for the first time that WD-treated chickpea flour could be a valid solution to produce bakery products. This first research outlined new sustainable practices that can open new perspectives for the production of not only cookies but also other baked products in order to obtain new fortified foods with improved antioxidant properties and available peptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.4c00098.

Tentatively identified compounds from chickpea cookie (**WDC**, **BC**, and **CC**) extracts by LC-HRMS/MS analysis, including the retention time molecular formula, experimental molecular ions, mass error ($\Delta m/z$, ppm), and MS^2 product ions (Table S1); complete list of potential encrypted bioactive peptides identified in **BC** G.I. digest (Table S2); complete list of potential encrypted bioactive peptides identified in **WDC** G.I. digest (Table S3); complete list of potential encrypted bioactive peptides identified in **CC** G.I. digest (Table S4); base peak profiles acquired by LC-MS/MS of peptides released after simulated gastrointestinal digestion of chickpea cookies samples: (A) **BC**; (B) **WDC**; (C) **CC** (Figure S1) (PDF)

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¶R.F. and U.G.S. contributed equally to this work. G.C. and S.L. are colast authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BC:cookies from chickpea control flour; BF:chickpea flour from control plants; CC:cookies from commercial flour; CF:commercial chickpea flour; CT:catechin; F2F:Farm to Fork; FC:flavonoid content; GA:gallic acid; GAE:gallic acid equivalent; GID:gastrointestinal digestion; NCE:normalized collision energy; PAC:phenolic acid content; PCA:principal component analysis; TPC:total phenolic content; WD:wood distillate; WDC:cookies from WD-treated chickpea flour; WDF:chickpea flour from WDF-treated plants

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