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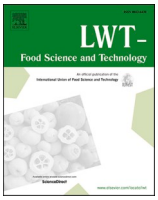

Release and possible mechanism of extractable and non-extractable polyphenols from highland barley bran using chemical and physiological methods

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ABSTRACT

Highland barley bran (HBB) is rich in extractable polyphenols (EP) and non-extractable polyphenols (NEP). Previous studies have generally used chemical solvents to extract EP and NEP and assess their functional properties. However, EP and NEP obtained using chemical method do not indicate phenolic compounds profiles released in the body. This study used chemical (acetone and acid hydrolysis) and physiological methods (*in vitro* gastrointestinal digestion and colonic fermentation) to treat HBB and investigated the differences between the two methods in releasing EP and NEP. Results showed that chemical method yielded a higher total phenolic content (EP + NEP = 718.25 mg GAE/100 g) than physiological method (634.62 mg GAE/100 g). More phenolic compounds, particularly ferulic acid oligomers, were released by chemical method and exhibited stronger total antioxidant capacities than those released by physiological method. Moreover, structural analysis indicated two methods used different mechanisms to release EP and NEP, with chemical method releasing polyphenols by drastically disrupting the cellular structure of the aleurone layer and physiological method releasing polyphenols by degrading cell walls. Therefore, a possible release mechanism was proposed. These findings highlight the importance of physiological method in accurately reflecting polyphenol bioaccessibility, which is critical for evaluating their potential health benefits.

1. Introduction

Polyphenols are secondary metabolites in plants that have important functional properties, such as antioxidant, antiaging, and antidiabetes effects (Aravind, Wichienchot, Tsao, Ramakrishnan, & Chakkaravarthi, 2021). Polyphenols are generally divided into extractable polyphenols (EP) and non-extractable polyphenols (NEP). EP can be extracted using aqueous–organic solvents, while NEP require hydrolysis methods (e.g., acid and alkaline hydrolysis) for their extraction (Martins, Rodrigues, Mercali, & Rodrigues, 2022; Saura-Calixto, 2012). Previous studies have mostly used chemical solvents to obtain EP and NEP and assess their functional properties (Huang et al., 2023; Thondre, Ryan, & Henry, 2011). However, the health benefits of EP and NEP are associated with their release in biological fluids, and EP and NEP obtained using chemical solvents do not accurately represent the profile of phenolic compounds released under physiological conditions. Moreover, Saura-Calixto, Serrano, and Goñi (2007) found that using the chemical method to indicate the amounts of EP and NEP released in the human intestine overestimated their actual values because a portion of polyphenols was not accessible during digestion. To better understand the

Abbreviations: EP, extractable polyphenols; NEP, non-extractable polyphenols; HBB, highland barley bran; A-EP, extractable polyphenols extracted with acetone; A-NEP, non-extractable polyphenols extracted with acid hydrolysis; G-EP, extractable polyphenols released by *in vitro* gastrointestinal digestion; C-NEP, non-extractable polyphenols released by *in vitro* colonic fermentation; LOD, limit of detection; LOQ, limit of quantification; DFA, dehydrodiferulic acids; TriFA, dehydrotriferulic acids; TetraFA, dehydrotetraferulic acid..

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bioaccessibility of polyphenols, Saura-Calixto (2012) proposed to study EP and NEP via a physiological method. EP are polyphenols that are released in the small intestine under physiological conditions, while NEP are polyphenols that are barely released in the small intestine, reach the colon with dietary fibers, and are released by colon microbiota. Previous studies have compared the amounts of EP and NEP released by the two methods (Pérez-Jiménez & Saura-Calixto, 2005; Saura-Calixto et al., 2007). However, to the best of our knowledge, information regarding the differences in phenolic compositions and antioxidant capacity of EP and NEP obtained by the two methods is currently unavailable.

Highland barley, a variety of barley, contains high amounts of β-glucan, γ-aminobutyric acid, polyphenols, phytosterols, and other bioactive substances and has received a lot of attention (Li, Du, Tu, Zhang, & Wang, 2022). These bioactive substances are not evenly distributed in the grain and are mainly concentrated in the bran (Xi et al., 2023). Highland barley bran (HBB) is a by-product of processing and is usually discarded directly or as low-value animal feed (Xi et al., 2023). Recovering bioactive substances from HBB achieves its high-value utilization, contributes to the circular economy, and reduces waste and the burden on the environment (Feng et al., 2024). Extensive studies have proved that HBB is rich in EP and NEP (He et al., 2023; Li et al., 2023). However, these studies have mainly explored the release of EP and NEP using chemical method, lacking comparison with those released using the physiological method. Furthermore, previous studies have shown that different methods released EP and NEP by loosening or destroying the food matrix structure (Wang, Nie, et al., 2022; Xu et al., 2020). However, the effects of chemical and physiological methods on HBB structure remain unclear. Therefore, this study aims to (1) release EP and NEP from HBB using chemical and physiological methods, (2) compare the differences in phenolic profiles and antioxidant capacity, and (3) analyze the structural changes of HBB and explore the associated release mechanism.

2. Materials and methods

2.1. Chemicals

Simulated salivary, gastric, and intestinal fluids were purchased from Coolaber (Beijing, China). α-Amylase (10 080, 0.83 mkat/g), pepsin (P7000, 4.18 mkat/g), pancreatin (P1750, 1.67 mkat/g), and LC–MS grade methanol and acetonitrile were purchased from Merck (Shanghai, China). LC grade methanol and calcofluor white (fluorescent brightener 28) were purchased from Macklin (Shanghai, China). Folin–Ciocalteu reagent (1 mol/L), porcine bile salts, 1.1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ), Dulbecco's phosphate buffered saline, and standards (16 phenolic compounds) were purchased from Solarbio (Beijing, China). All standards were dissolved in LC–MS grade methanol to prepare 1 mg/mL stock solutions for quantitative analysis. Other chemicals were of analytical grade.

2.2. Samples preparation

Highland barley kernels (No. Kunlun 14, Xining, China) were removed from the pericarp, intermediate layer, and one or three layers of aleurone using a pearling machine (Satake, Tokyo, Japan) to obtain HBB. The HBB yield was 21.28 g/100 g. HBB was defatted with hexane (w:v, 1:5) at ambient temperature (~23 °C) for 12 h, ground, passed through a 60-mesh sieve, and finally stored at −20 °C.

2.3. Chemical extraction of EP and NEP

2.3.1. EP: supernatant after extraction with different aqueous–organic solvents

Three solvents [methanol−aqueous solution (7:3, v:v), ethanol−aqueous solution (7:3, v:v), and acetone−aqueous solution (7:3, v:v)] were used to extract EP to select the suitable solvent for extraction. HBB (2.00 g) was mixed with 30 mL of each solvent. The mixtures were extracted through sonication at 300 W for 30 min and then centrifuged (4002×*g*, 15 min) (Arigò, Česla, Šilarová, Calabrò, & Česlová, 2018). The supernatant was removed, and the residue was re-extracted twice using the above solvents. The obtained supernatant was combined and evaporated (35 °C) to remove the organic solvent. The remaining aqueous phases after evaporation, namely, M-EP, *E*-EP, and A-EP, were lyophilized and redissolved in 10 mL of methanol−aqueous solution (7:3, v:v).

2.3.2. NEP: supernatant after acid or alkaline hydrolysis

The acetone−aqueous solution was a more effective solvent for EP extraction. The remaining HBB after acetone extraction was used for further NEP extraction. The HBB was hydrolyzed by adding HCl (2 mol/L, 30 mL) at 90 °C for 2 h or by adding NaOH (2 mol/L, 30 mL) for 2 h at ambient temperature (Wang, He, et al., 2022). Subsequently, the mixtures were adjusted to pH 7.00 ± 0.20 and centrifuged (4002×*g*, 15 min). The collected supernatants, namely, A-NEP (HCl extract) and B-NEP (NaOH extract), were lyophilized and redissolved in 10 mL of methanol−aqueous solution (7:3, v:v).

The EP and NEP extracts were filtered using 0.22 μm nylon membrane filters (Jinteng, Tianjin, China).

2.4. Physiological method for the release of EP and NEP

2.4.1. EP: supernatant after in vitro gastrointestinal digestion (G-EP)

The assay was modified according to the method of Minekus et al. (2014) and Pérez-Jiménez and Saura-Calixto (2005). The simulated salivary fluid was warmed to 37 °C before use, and α-amylase was dissolved in simulated salivary fluid to prepare a solution (12.50 μkat/mL). HBB (2.00 g), α-amylase solution (1.0 mL), and simulated salivary fluid (9.0 mL) were respectively added to a 50 mL tube, and the tube was then incubated in a bath incubator (MiuLab, Hangzhou, China) at 37 °C for 5 min to simulate oral digestion. Subsequently, simulated gastric fluid (8.2 mL), pepsin in simulated gastric fluid (0.42 mkat/mL, 1.6 mL), and HCl (1 mol/L, 0.2 mL) were added to the tube and incubated for 2 h to simulate gastric digestion. The small intestine digestion was achieved by adding simulated intestinal fluid (12.5 mL), pancreatin in simulated intestinal fluid (13.34 μkat/mL, 5 mL), and porcine bile salts (0.04 mg/mL, 2.5 mL) to the tube and then incubated for 6 h. The mixture was centrifuged (3247×*g*, 20 min), and the digestible supernatant (G-EP) was lyophilized and redissolved in 10 mL of deionized water.

2.4.2. NEP: supernatant after in vitro colonic fermentation (C-NEP)

The experiment was conducted according to a previously described method (Pérez-Burillo et al., 2019). The medium was prepared as follows: 15 g peptone was dissolved in nearly 1 L of water, the pH was adjusted to 7 with NaOH (1 mol/L), and the volume was adjusted to 1 L using water. L-cysteine (312 mg) and sodium sulfide (312 mg) were dissolved in 2 mL NaOH (1 mol/L) and the volume was adjusted to 50 mL using water. Subsequently, 1 mg/mL resazurin sodium salt was prepared. They were mixed in the ratio of 1 L, 50 mL, and 1.25 mL to obtain the medium. The fecal samples were donated by three volunteers (20–30 years old) with no history of intestinal diseases or antibiotic administration for at least 3 months. These samples were mixed in a ratio of 1:1:1 to prepare 32 g/100 mL fecal slurry using Dulbecco's phosphate buffered saline. The indigestible residue after *in vitro* gastrointestinal digestion (0.50 g), supernatant after small intestine digestion (325 μL), medium (7.5 mL), and fecal slurry (2 mL) were added to a 50 mL tube at 37 °C for 48 h for anaerobic fermentation. The experiment was conducted in the anaerobic chamber (Xingda Co., Ltd, Henan, China) filled with N_2. The assay contained two control groups: the mixture without fecal slurry (control 1) and the mixture without indigestible residue (control 2). The fermentation broth (C-NEP) was centrifuged, lyophilized, and redissolved in 10 mL of deionized water.

The G-EP and C-NEP extracts were filtered through 0.22 μm

Table 1
Phenolic contents and antioxidant capacities of EP and NEP obtained by chemical and physiological methods in HBB.

	chemical method		physiological method	
	A-EP	A-NEP	G-EP	C-NEP
phenolic contents				
phenolic content (mg GAE/100 g)	266.77 ± 10.63^{c}	451.48 ± 42.56^{a}	319.59 ± 8.09^{b}	315.03 ± 28.15^{bc}
total phenolic content (mg GAE/100	718.25 ± 33.59		634.62 ± 28.15	
antioxidant capacities				
DPPH antioxidant capacity (mg Vc/100 g)	15.13 ± 0.95^{d}	183.13 ± 14.19^{a}	46.33 ± 2.06^{c}	112.99 ± 13.78^{b}
total DPPH antioxidant capacity (mg Vc/100 g)	198.25 ± 13.44		159.32 ± 8.35	
FRAP antioxidant capacity (μmol FE/ 100 g)	604.85 ± 26.44^{d}	3005.68 ± 315.40^{a}	1162.58 ± 37.99^{c}	1632.01 ± 118.75^{b}
total FARP antioxidant capacity (μmol FE/ 100 g)	3610.52 ± 290.86		2794.58 ± 80.83	

Note: EP, extractable polyphenols; NEP, non-extractable polyphenols; HBB, highland barley bran; A-EP, extractable polyphenols extracted with acetone; A-NEP, non-extractable polyphenols extracted with acid hydrolysis; G-EP, extractable polyphenols released by *in vitro* gastrointestinal digestion; C-NEP, non-extractable polyphenols released by *in vitro* colonic fermentation. Values with different superscript letters in the same row are significantly different from each other ($p < 0.05$).

polyethersulfone membrane filters before the next analysis. HBB treated with chemical and physiological methods was freeze-dried at 1 Pa and −69 °C (Haier, Qingdao, China) for 2 d to be used for structural analysis.

2.5. Total phenolic content

The total phenolic content was determined based on the method of Wang, He, et al. (2022). Phenolic extracts (1.0 mL) were mixed with 0.5 mL Folin–Ciocalteu reagent and 3.0 mL of Na_2CO_3 solution (20 g/100 mL). After 15 min, 10.0 mL of deionized water was added to the mixture, and the absorbance at 760 nm was measured using a UV–visible spectrophotometer (Metash, Shanghai, China) after incubating in darkness for 1 h. The total phenolic content was calculated based on the gallic acid standard curve.

2.6. Qualitative analysis of phenolic compounds

Qualitative analysis of the phenolic compounds was conducted using Waters ACQUITY ultra-high-performance liquid chromatography (UPLC) equipped with Xevo G2 quadrupole time-of-flight (Q-TOF) spectrometer. This experiment was performed following the method of Xia et al. (2022) with slight modifications. Phenolic extract (5 μL) was injected into the UPLC system with formic acid in water (0.5 mL/100 mL, A) and acetonitrile (B) as mobile phases. The flow rate was set as 0.3 mL/min, and the elution gradient was as follows: 0–2 min, 5–10 mL/100 mL B; 2–10 min, 10–30 mL/100 mL B; 10–17 min, 30–40 mL/100 mL B; 17–19 min, 40–70 mL/100 mL B; 19–20 min, 70–100 mL/100 mL B; 20–26 min, B; 26–26.1 min, 100–5 mL/100 mL B; 26.1–30 min, 5 mL/100 mL B. The sample was separated in a Waters ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm), and the column temperature was 40 °C. The conditions of the mass spectrometer were as follows: electrospray ionization source, negative ion mode, capillary of 2.5 kV, source temperature of 120 °C, desolvation temperature of 450 °C, cone gas flow of 50 L/h, desolvation gas flow of 800 L/h, collision energy of 10–30 V, and mass range of 50–1200 *m/z*.

The data acquired by UPLC-Q-TOF were imported into the Waters UNIFI system to process. For identified phenolic compounds, the error between the calculated and observed *m/z* of identified compounds was set to ±5 ppm. The final identification of phenolic compounds was based on UNIFI results, PubChem database, and previous studies.

2.7. Quantitative analysis of phenolic compounds

HPLC equipped with a 1260 infinity II variable wavelength detector (Agilent Technologies, Beijing, China) was used for the quantitative analysis of individual phenolic compounds. Phenolic extract (10 μL) was injected into the HPLC system. Formic acid in water (0.1 mL/100 mL, A) and methanol (B) were used as mobile phases with a flow rate of 1.0 mL/min. The elution gradient was as follows: 0–8 min, 12–30 mL/100 mL B; 8–28 min, 30–60 mL/100 mL B; 28–29 min, 60–12 mL/100 mL B; 29–30 min, 12 mL/100 mL B. In addition, an Agilent ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm) was used to separate the phenolic compounds, and the column temperature was set at 30 °C. Absorbances were measured at 280 and 330 nm. The retention time of each phenolic compound was determined by injecting the corresponding standard into the HPLC system. The limit of detection (LOD) and limit of quantification (LOQ) of compounds were determined by 3 and 10-times signal-to-noise ratios, respectively.

2.8. Determination of antioxidant capacity

2.8.1. DPPH assay

The DPPH assay was performed using the method of Wang, He, et al. (2022). Before the experiment, 60 μmol/L DPPH solution was prepared using ethanol. Phenolic extracts (0.2 mL) were mixed with 4.8 mL DPPH solution, and the changes in absorbance of the mixture were recorded at 515 nm. Ascorbic acid (Vc) was used to plot a standard curve for calculating DPPH antioxidant capacity.

2.8.2. Ferric reducing antioxidant power (FRAP) assay

This assay was performed as per a previously described method (Wang, He, et al., 2022). Acetate buffer (300 mmol/L, pH 3.6), $FeCl_3 \bullet 6H_2O$ (20 mmol/L), and TPTZ solutions (10 mmol/L) in HCl (40 mmol/L) were prepared. They were mixed in a ratio of 10:1:1 to obtain the ferric–TPTZ solution, which was preheated to 37 °C before use. Phenolic extracts (0.2 mL) were mixed with 4.8 mL ferric–TPTZ solution, and the changes in the absorbance of the mixture were recorded at 595 nm. Ferrous sulphate ($FeSO_4$) was used to plot a standard curve for calculating FRAP antioxidant capacity.

2.9. Structural characterization

2.9.1. Fourier transform infrared (FTIR) spectrometry

HBB (1 mg) and KBr (100 mg) were mixed, ground, and then pressed into a 1–mm pellet. The transmittance of the sample was scanned via FTIR spectrometry (Bruker, Germany) in the range from 4000 cm^{-1}–400 cm^{-1}.

2.9.2. X-ray diffraction (XRD) analysis

The HBB crystalline structure was determined using an XRD instrument (Bruker, Germany). The voltage was set at 40 kV, and the diffraction angle (2θ) was scanned from 5 to 40° (Xi et al., 2023).

2.9.3. Scanning electron microscopy (SEM)

The HBB was fixed on the stage using a double-sided tape and sprayed with gold (MC1000, Hitachi, Japan). The morphological

Table 2
Phenolic compositions of EP and NEP obtained by chemical and physiological methods in HBB.

				chemical method		physiological method	
identification	RT (min)	[M-H]$^-$ (m/z)	Main MS/MS fragmentation	A-EP	A-NEP	G-EP	C-NEP
(−)-epigallocatechin	0.74	305.0679	109.0392 304.0549	-	-	-	+
rosmarinic acid	0.94	359.0759	135.0540	-	+	-	+
myricetin	1.31	317.0307	178.9980	-	-	-	+
gallic acid	1.32	169.0134	125.0214	-	+	-	+
syringic acid	1.69	197.0451	138.0307 153.0547	+	-	-	-
vanillic acid	1.85	167.0349	123.0445	-	+	-	-
dihydroquercetin	1.98	303.0507	125.0242 285.0482	+	-	-	-
epicatechin gallate	1.98	441.0815	125.0242 271.0602	+	-	-	+
protocatechuic acid	2.11	153.019	109.0273	+	+	-	+
chlorogenic acid	2.32	353.0894	191.0556	+	-	-	-
vanillin	2.4	151.0395	136.0161	-	+	-	-
4-hydroxybenzoic acid	2.93	137.024	94.0364	+	+	+	-
L-epicatechin	3.09	289.0722	109.0283 123.0447 125.0239 137.0238 179.0325 245.0818	+	-	+	+
caffeic acid	3.66	179.0344	135.0445	+	+	-	-
rutin	4.04	609.147	447.0917	+	+	+	-
kaempferol-3-rutinoside	4.82	593.1496	207.0659	+	-	+	-
p-coumaric acid	4.96	163.04	119.0496	-	+	+	-
luteolin 7-*O*-glucoside	4.97	447.093	285.0389	+	-	-	-
isoorientin	4.98	447.0937	327.0509 357.0612	-	-	+	-
orientin	4.98	447.0936	327.0508	-	-	+	-
trans-cinnamic acid	5.54	147.045	103.0548	-	-	-	+
ferulic acid	5.74	193.0507	134.0368 149.0600 178.0267	-	+	+	+
cosmosiin	5.76	431.0982	269.0431	+	-	+	-
vitexin	5.9	431.0979	121.0285 283.0598 311.0598	+	+	+	+
8-5noncyclic DFA	5.97	385.0927	159.0443 282.0895	-	+	-	-
8-8noncyclic DFA	6.46	385.0931	341.1034	-	-	+	+
8-8tetrahydrofuran DFA	6.77	403.1051	136.0159 148.0526 207.0660 233.0453	+	+	-	-
naringin	6.91	579.1703	271.0612	+	-	-	-
neohesperidin	7.62	609.1824	301.0711	+	-	-	+
8-8cyclic DFA	8.32	385.0928	282.0893 326.0784	+	-	+	+
4-*O*-5 DFA	8.42	385.0928	193.0505 282.0896 326.0774 341.1062	+	+	-	+
8-8tetrahydrofuran/5-5 TriFA	8.59	595.149	551.1541	-	+	-	-
8-5noncyclic/5-5 TriFA	8.93	577.1366	489.1551 533.1451	-	+	-	-
quercetin	9.36	301.0361	151.0031	-	-	-	+
8-5cyclic DFA	9.54	385.092	267.0660 282.0890 297.1126 326.0787 341.1034	+	+	+	+
8-5decarboxylated DFA	9.78	341.103	239.0718 267.0660 282.0890 326.0787	+	+	-	+
5-5/8-8cyclic TriFA	10.04	577.1367	489.1549 533.1457	-	+	-	+
5-5/8-8noncyclic TriFA	10.5	577.1359	489.1535 533.1452	-	+	-	-
8-8cyclic/8-*O*-4 TriFA	11.2	577.1337	314.1165	-	-	-	+
kaempferide	11.27	299.0565	284.0318	+	-	+	-
4-*O*-8/5-5/8-5noncyclic TetraFA	11.65	769.1788	725.1891	-	+	-	-

Note: EP, extractable polyphenols; NEP, non-extractable polyphenols; HBB, highland barley bran; RT, retention time; A-EP, extractable polyphenols extracted with acetone; A-NEP, non-extractable polyphenols extracted with acid hydrolysis; G-EP, extractable polyphenols released by *in vitro* gastrointestinal digestion; C-NEP, non-extractable polyphenols released by *in vitro* colonic fermentation; -, not detected; DFA, dehydrodiferulic acids; TriFA, dehydrotriferulic acids; TetraFA, dehydrotetraferulic acid.

structure was observed using SEM (SU8010, Hitachi, Japan) at 10 kV and 1000× magnification.

2.9.4. Confocal laser scanning microscopy (CLSM)

HBB was fixed with glutaraldehyde solution (3.5 mL/100 mL, dissolved in phosphate buffered saline with a pH of 7.2) for 12 h and osmic acid solution (1 g/100 mL, dissolved in water) for 2 h. The sample was dehydrated with different ethanol gradients and embedded in resin. The polymerized sample was cut into 1−μm−thick sections using a microtome (Cnoptec, Chongqing, Beijing). Polyphenols possess autofluorescence properties (Zhang et al., 2019), and the polyphenol distribution in HBB was observed via Carl Zeiss 880 fluorescent microscopy (Oberkochen, Germany) at 405 nm. The sections were stained with 10 mg/100 mL Calcofluor white aqueous solution for 10 min to observe the cell wall structure at 405 nm and 40× magnification (Tagliasco et al., 2022).

2.10. Statistical analysis

Each experiment was performed three times independently, and the results are expressed as mean ± standard deviation. Microsoft Excel 365 was used for data analysis, SPSS 26 was used for analysis of variance with Duncan's test, and GraphPad Prism was used for graphing.

3. Results and discussion

3.1. EP and NEP contents obtained by chemical and physiological methods

To establish an effective chemical method for EP and NEP extraction, different organic solvents and hydrolysis methods were used in the study. Results indicated that EP contents in methanol−aqueous, ethanol−aqueous, and acetone−aqueous solutions were 203.85, 180.16, and 266.77 mg gallic acid equivalents (GAE)/100 g, respectively. This indicated that acetone was efficient in extracting EP, consistent with the result reported by Thondre et al. (2011). Acid hydrolysis yielded a significantly ($p < 0.05$) higher NEP content (451.48 mg GAE/100 g) than alkaline hydrolysis (227.81 mg GAE/100 g). In previous studies, acid hydrolysis was applied to release NEP from dietary fiber. This hydrolysis disrupted the glycosidic bonds between NEP and polysaccharides and cleaved a certain amount of ester and ether bonds when subjected to high-temperature treatment (Shi et al., 2020; Wang, Yang,

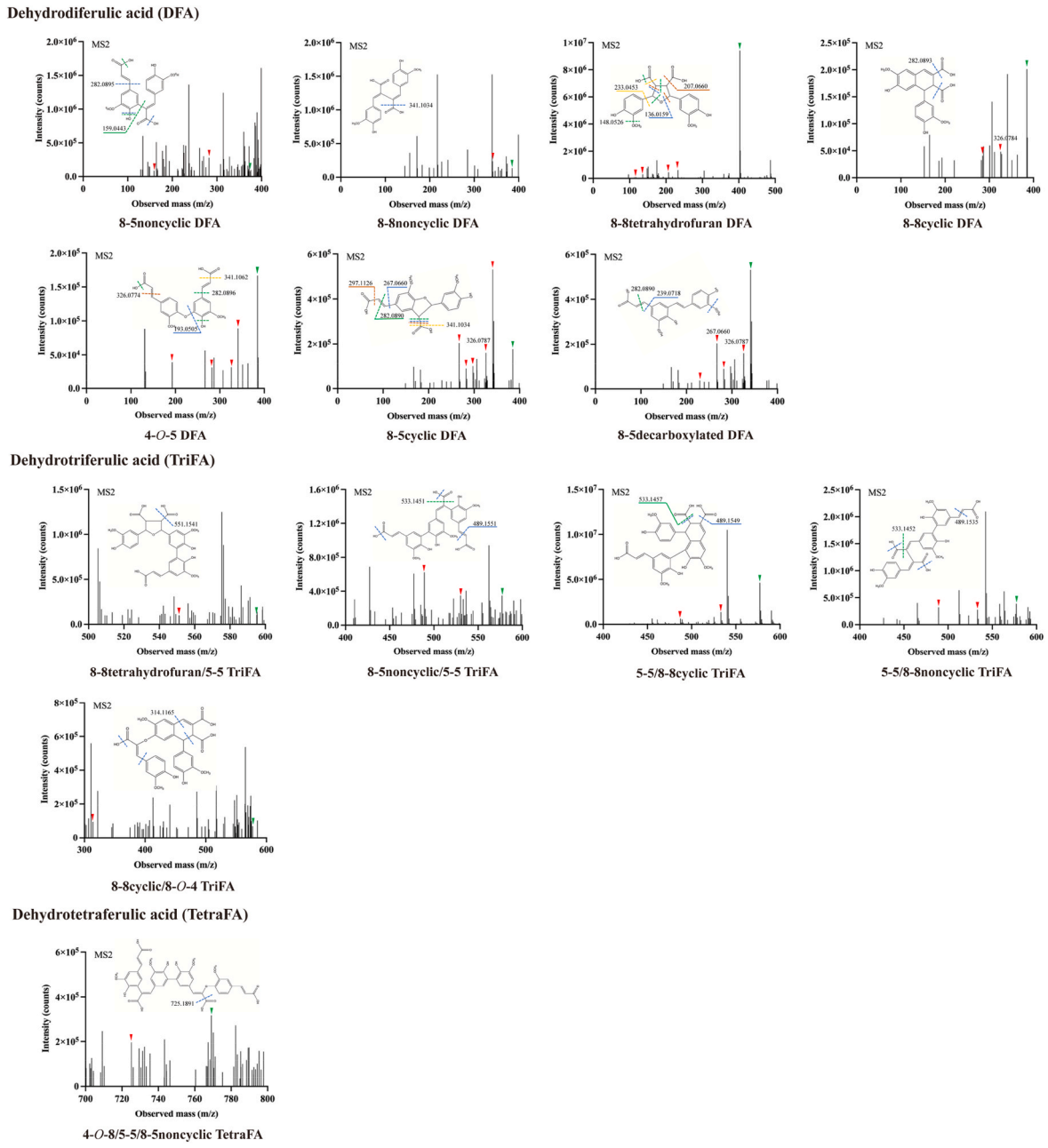

Fig. 1. MS/MS spectra and fragmentation pathway of ferulic acid oligomers in negative ion mode.

Yao, Xu, & Sun, 2012). Moreover, Arranz and Calixto (2010) found that acid hydrolysis was more effective than alkaline hydrolysis in releasing NEP from wheat bran. Therefore, acetone combined with acid hydrolysis was employed to extract EP and NEP (Table 1).

As shown in Table 1, the content of G-EP released during *in vitro* gastrointestinal digestion was 319.59 mg GAE/100 g, which was significantly ($p < 0.05$) higher than A-EP content. Pérez-Jiménez and Saura-Calixto (2005) found similar results in oat bran. This was probably because digestive enzymes hydrolyzed the starch and proteins, loosening the HBB structure and contributing to the partial release of polyphenols that acetone could not extract. HBB enters into the colon after small intestine digestion, and NEP are released under the action of colon microbiota and enzymes secreted by them (Zhang et al., 2023). NEP content in fermentation broth was lower than that in A-NEP. The total phenolic content in HBB is the sum of EP and NEP (Martins et al., 2022). The total phenolic contents obtained using chemical and physiological methods were 718.25 and 634.62 mg GAE/100 g, respectively. This indicated that a portion of polyphenols in HBB was inaccessible, and if total phenolic content obtained through the chemical method is used to reflect its bioaccessibility in humans, the actual value is overestimated.

3.2. Identification of phenolic compounds using UPLC-Q-TOF

Table 2 presents the phenolic compounds identified using UPLC-Q-TOF in HBB. The phenolic compositions of EP in acetone differed from those in digestive fluid, and there were differences in NEP compositions obtained via acid hydrolysis and colon fermentation. A total of 33 and 28 phenolic compounds were identified in phenolic extracts obtained by chemical and physiological methods, respectively.

A total of 21 phenolic compounds were detected in A-EP: 5 phenolic acids, 5 dehydrodiferulic acids (DFA), and 11 flavonoids. For NEP extracted by acid hydrolysis (A-NEP), more phenolic compounds were released, especially for *p*-coumaric acid, ferulic acid, 8-5noncyclic DFA,

Table 3
Contents (μg/g) of EP and NEP obtained by chemical and physiological methods in HBB.

phenolic compounds	calibration curve	R^2	LOD (μg/mL)	LOQ (μg/mL)	chemical method		physiological method	
					A-EP	A-NEP	G-EP	C-NEP
gallic acid	y = 23.751x+1.4075	0.998	0.02	0.07	<LOD	1.39 ± 0.10^{b}	<LOD	13.31 ± 1.53^{a}
protocatechuic acid	y = 13.906x+3.1764	0.997	0.05	0.18	29.29 ± 0.91^{a}	3.97 ± 0.50^{c}	<LOD	11.42 ± 1.70^{b}
(−)-epigallocatechin	y = 0.9958x+4.5543	0.9966	0.30	1.06	<LOD	<LOD	<LOD	259.71 ± 38.28
4-hydroxybenzoic acid	y = 13.696x+2.5346	0.9972	0.02	0.08	3.01 ± 0.10^{b}	66.09 ± 9.91^{a}	4.48 ± 0.43^{b}	<LOD
L-epicatechin	y = 5.1485x+1.0118	0.9999	0.07	0.24	36.61 ± 1.37^{b}	<LOD	29.86 ± 1.39^{c}	69.41 ± 6.71^{a}
vanillin	y = 42.972x+5.18	0.9979	0.04	0.15	<LOD	20.15 ± 2.60	<LOD	<LOD
epicatechin gallate	y = 12.283x+1.065	0.999	0.03	0.14	4.03 ± 0.63^{b}	<LOD	<LOD	38.15 ± 3.65^{a}
p-coumaric acid	y = 42.143x+6.7656	0.9981	0.01	0.02	<LOD	8.74 ± 0.70^{a}	8.93 ± 0.28^{a}	<LOD
ferulic acid	y = 27.589x+20.31	0.9979	0.01	0.04	<LOD	318.73 ± 4.78^{a}	45.00 ± 0.61^{b}	40.22 ± 0.33^{c}
vitexin	y = 11.982x+2.8209	0.9993	0.05	0.16	1.90 ± 0.51^{d}	45.81 ± 2.18^{a}	8.88 ± 0.79^{c}	36.58 ± 0.02^{b}
rutin	y = 5.1594x+0.048	0.9996	0.08	0.25	5.27 ± 0.62^{c}	9.57 ± 0.79^{a}	7.48 ± 0.95^{b}	<LOD
neohesperidin	y = 16.286x+1.4938	0.998	0.03	0.10	0.60 ± 0.01	<LOD	<LOD	<LOQ
rosmarinic acid	y = 14.471x-13.399	0.9985	0.06	0.21	<LOD	13.76 ± 0.72^{b}	<LOD	33.71 ± 0.03^{a}
cosmosiin	y = 15x+1.4217	0.9986	0.04	0.12	1.72 ± 0.25^{a}	<LOD	1.35 ± 0.39^{a}	<LOD
myricetin	y = 10.738x-0.1116	0.9991	0.08	0.28	<LOD	<LOD	<LOD	<LOQ
kaempferol-3-rutinoside	y = 8.4158x+0.2728	0.9999	0.09	0.31	2.56 ± 0.48^{b}	<LOD	10.59 ± 1.56^{a}	<LOD
total					85.00 ± 0.40^{c}	488.22 ± 9.84^{a}	116.56 ± 3.32^{b}	502.51 ± 22.42^{a}

Note: EP, extractable polyphenols; NEP, non-extractable polyphenols; HBB, highland barley bran; LOD, limit of detection; LOQ, limit of quantification; A-EP, extractable polyphenols extracted with acetone; A-NEP, non-extractable polyphenols extracted with acid hydrolysis; G-EP, extractable polyphenols released by *in vitro* gastrointestinal digestion; C-NEP, non-extractable polyphenols released by *in vitro* colonic fermentation. Values with different superscript letters in the same row are significantly different from each other ($p < 0.05$).

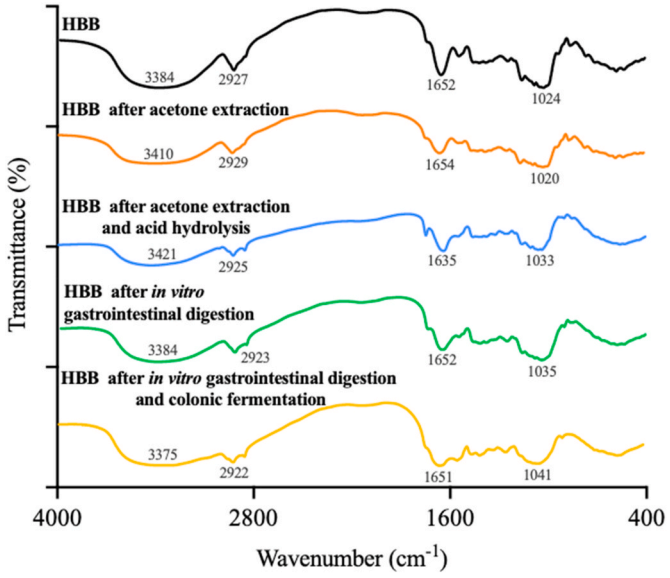

Fig. 2. FTIR spectra of HBB after treatment with chemical method (acetone extraction and acid hydrolysis) and physiological method (*in vitro* gastrointestinal digestion and colonic fermentation). HBB, highland barley bran.

dehydrotriferulic acids (TriFA, 8-8tetrahydrofuran/5-5 TriFA, 8-5noncyclic/5-5 TriFA, 5-5/8-8cyclic TriFA and 5-5/8-8noncyclic TriFA), and 4-*O*-8/5-5/8-5noncyclic dehydrotetraferulic acid (TetraFA). Fig. 1 presents the identification of ferulic acid oligomers using UNIFI software. Studies have shown that hydroxycinnamic acids, particularly ferulic acid, can form DFA, TriFA, and TetraFA in the presence of peroxidase or laccase. These ferulic acid oligomers mediate covalent cross-linking between cell wall polymers, especially polysaccharides and polysaccharides or polysaccharides and lignin, forming ester, ether, and glycosidic bonds (Bento-Silva, Patto, & Bronze, 2018). Their mediated cross-linking is important for cell wall structure. The presence of ferulic acid and its oligomers in extracts suggested that the HBB structure may be disrupted.

A total of 14 phenolic compounds were detected in G-EP obtained via *in vitro* gastrointestinal digestion. Compared with A-EP, *p*-coumaric and ferulic acids were found in G-EP. However, phenolic acids (syringic, protocatechuic, chlorogenic, and caffeic acids) and flavan-3-ols (epicatechin gallate) were not detected. This may be because these phenolic compounds are sensitive to pH conditions and are transformed or degraded during exposure to the gastrointestinal environment (Velderrain-Rodríguez et al., 2014). Tagliazucchi, Verzelloni, Bertolini, and Conte (2010) found that caffeic acid levels decreased by 24.9% after gastric and small intestine digestion. The released EP were absorbed in the small intestine; however, EP compositions affected interactions between EP and digestive enzymes and between EP and cell membranes, which further influenced their bioaccessibility (Rocchetti et al., 2022). During colonic fermentation, a total of 19 phenolic compounds were released, ferulic acid oligomers such as 8-8noncyclic DFA, 8-8cyclic DFA, 4-*O*-5 DFA, 8-5cyclic DFA, 8-5decarboxylated DFA, 5-5/8-8cyclic TriFA and 8-8cyclic/8-*O*-4 TriFA were detected. Studies have shown that some microbiota in the colon secrete esterases that release ferulic acid and its oligomers from polysaccharides (Pereira et al., 2021; Zhang, Zhang, Li, Deng, & Tsao, 2020). Moreover, compared with those in A-NEP, trans-cinnamic acid, neohesperidin, myricetin, quercetin, and flavan-3-ols ((−)-epigallocatechin, epicatechin gallate, and L-epicatechin) were identified in C-NEP. Li et al. (2024) found that myricetin and quercetin were released from highland barley during colonic fermentation. The released NEP were further transformed by microbiota such as *Bacteroides*, *Eubacterium*, and *Enterococcus*, thus mediating the growth of microbiota that can utilize these compounds (Zhang et al., 2020). Additionally, NEP with different structures produced different metabolites. For instance, the main metabolites of ferulic and gallic acids were hydroxyhippuric acid derivatives and pyrogallol, respectively, and they may have different bioaccessibility and bioavailability in the body (Bresciani et al., 2016; Rocchetti et al., 2022). In summary, NEP compositions affected the growth of microbiota and the type of metabolites.

3.3. Quantification of phenolic compounds using HPLC

Table 3 presents the contents of EP and NEP obtained by chemical and physiological methods in HBB. In A-EP, L-epicatechin and protocatechuic acid were the main phenolic compounds, whereas ferulic acid exhibited the highest content (318.73 μg/g) in A-NEP. He et al. (2023) found that ferulic acid was the most abundant phenolic compound in NEP of HBB. In addition, ferulic acid was the main phenolic compound released during *in vitro* gastrointestinal digestion, with a content of

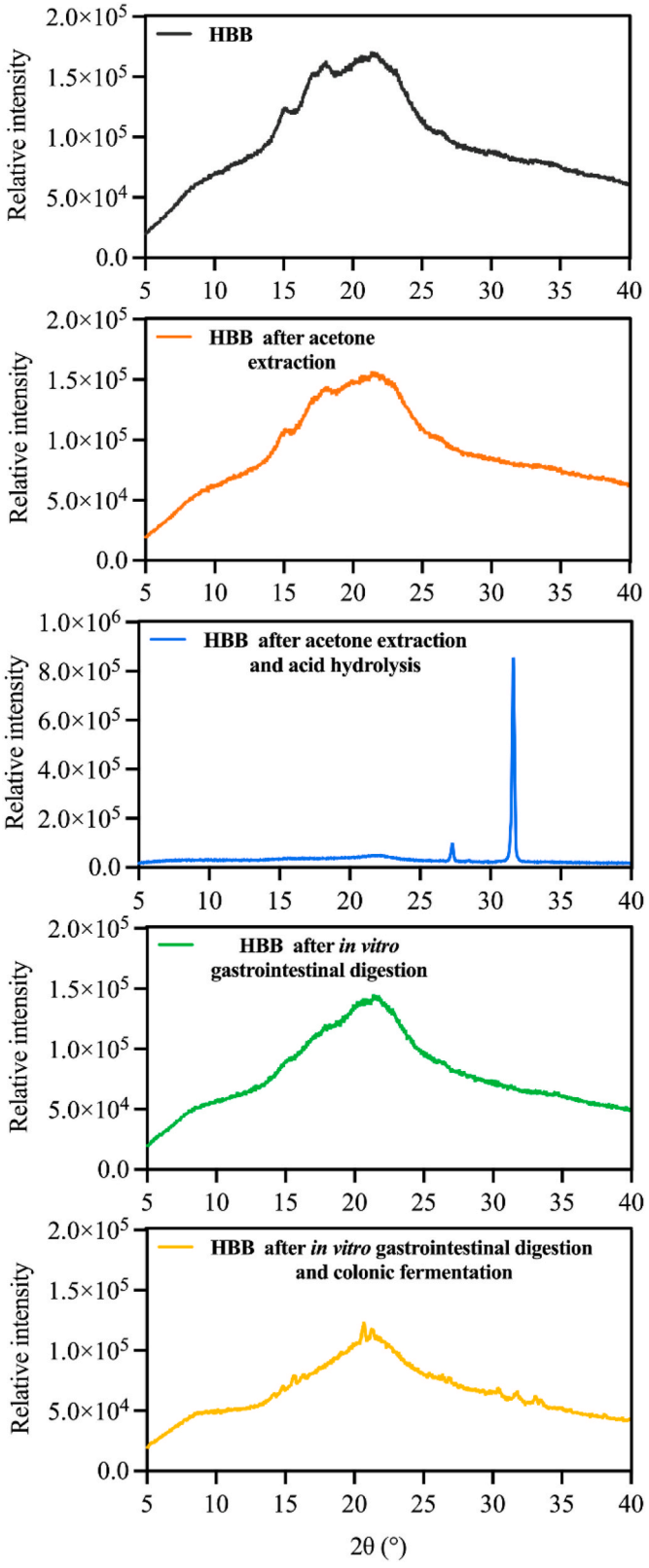

Fig. 3. XRD spectra of HBB after treatment with chemical method (acetone extraction and acid hydrolysis) and physiological method (*in vitro* gastrointestinal digestion and colonic fermentation). HBB, highland barley bran.

45.00 μg/g. However, the amount of ferulic acid was 40.22 μg/g in C-NEP, significantly ($p < 0.05$) lower than that in A-NEP, possibly due to its transformation in the colon. Studies have shown that the phenolic compounds released in the colon undergo a series of transformations to form small molecules of phenolic and nonphenolic compounds by colon microbiota (Rocchetti et al., 2022; Zhang et al., 2020). Zhang et al. (2023) proposed the possible catabolic pathways for the transformation of ferulic acid into gallic acid via dehydrogenation, dihydroxylation, and demethylation, and gallic acid undergoes further dehydroxylation to form protocatechuic acid. Moreover, myricetin and quercetin can undergo the transformations of ring cleavage and α-oxidation to form protocatechuic acid (Li et al., 2024). As shown in Table 2, the contents of gallic and protocatechuic acids in C-NEP were significantly ($p < 0.05$) higher than those in A-NEP, indicating phenolic compounds may have been transformed. The amount of NEP released may affect its metabolite concentration, further affecting its biological activity (Saura-Calixto, 2012). In summary, the contents of EP and NEP released in the body differed from those extracted using chemical solvents.

3.4. Antioxidant capacities of EP and NEP obtained by different methods

As shown in Table 1, the DPPH and FRAP values of EP released by *in vitro* gastrointestinal digestion were higher than EP extracted with acetone−aqueous solution. Pérez-Jiménez and Saura-Calixto (2005) found similar results in oat bran. This may be because the G-EP has a higher phenolic content than A-EP. The total antioxidant capacities of EP and NEP obtained by chemical methods were higher than those obtained via physiological method, with DPPH and FRAP values of 198.25 mg Vc/100 g and 3610.52 μmol FE/100 g, respectively. This indicated that the total antioxidant capacities of EP and NEP in chemical solvents did not accurately reflect the antioxidant effects they exert *in vivo*. The released EP and NEP during digestion exhibited antioxidant activities to maintain the health of the gastrointestinal tract (Zhang et al., 2019). The absorbed polyphenols and their metabolites entered the blood circulation and further improved the antioxidant capacity of the blood plasma, preventing diseases caused by oxidative stress (Zhang et al., 2020).

3.5. FTIR and XRD analyses

FTIR spectra of the samples are shown in Fig. 2. In untreated HBB, the peak at 3384 cm^{-1} was related to the –OH and –CO stretching vibration of phenolic compounds, cellulose, or hemicellulose (Tian et al., 2024). The peak at 2927 cm^{-1} corresponded to the C–H stretching vibration of cellulose (Xiong et al., 2021). The peak at 1652 cm^{-1} was caused by the presence of C=O in aromatic compounds (Xi et al., 2023). The peak at 1024 cm^{-1} was attributed to the C–O stretching in oligosaccharides (Xiong et al., 2021). The spectral profiles of treated HBB were similar to those of the corresponding untreated HBB. However, the intensities and positions of the main characteristic peaks differed. After acetone extraction, no significant change in the positions of the main characteristic peaks of HBB was observed. However, peaks at 3384 and 1024 cm^{-1} were blue-shifted after acid hydrolysis, suggesting that acid hydrolysis significantly affected the functional groups of HBB. The intensity of HBB peaks significantly decreased after *in vitro* gastrointestinal digestion and colonic fermentation. These results indicated that the chemical and physiological methods affected the HBB structure.

As shown in Fig. 3, untreated HBB exhibited diffraction peaks at 15.0°, 17.2°, 21.7°, and 33.4° (2θ), which corresponded to the pattern of cellulose type I (Murata et al., 2015). No significant changes in the shape of the diffraction peaks in HBB were observed after acetone extraction. However, further acid hydrolysis led to significant decreases in the signal intensities of these peaks in HBB, and new sharp diffraction peaks appeared at 27.3 and 31.6° (2θ), indicating that acid hydrolysis disrupted the HBB crystalline structure. Huang et al. (2023) obtained similar results in wheat bran. The intensities of characteristic diffraction peaks decreased after *in vitro* gastrointestinal digestion. Several small

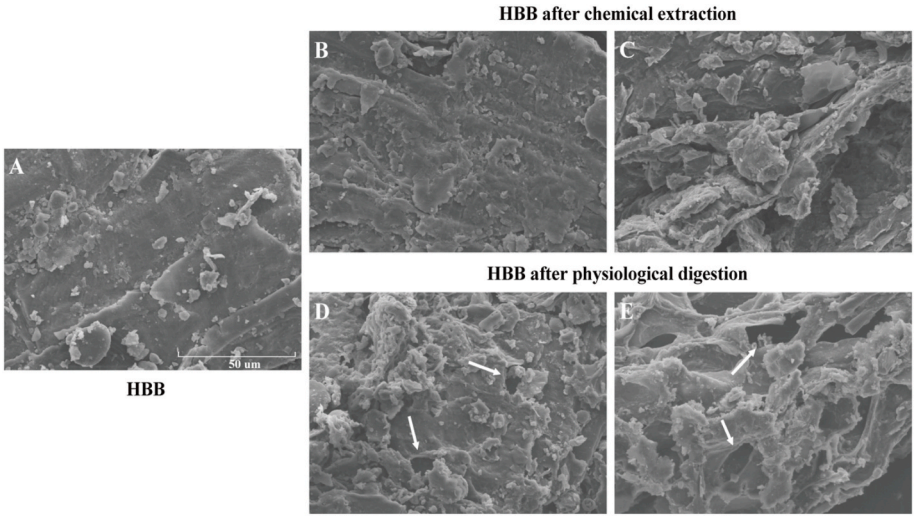

Fig. 4. SEM images of HBB after treatment with chemical and physiological methods (1000 ×). A, HBB; B, HBB after acetone extraction; C, HBB after acetone extraction and acid hydrolysis; D, HBB after *in vitro* gastrointestinal digestion; E, HBB after *in vitro* gastrointestinal digestion and colonic fermentation. HBB, highland barley bran.

diffraction peaks appeared in HBB after colonic fermentation, indicating that the physiological method altered the HBB crystalline structure to a certain extent.

3.6. HBB morphological structure

To investigate the release mechanism of EP and NEP, the morphological structure of HBB before and after treatment by the two methods were observed. As shown in Fig. 4A, untreated HBB had a complete and smooth structure, with some blocky and starch particles attached to the surface. The HBB surface after acetone extraction became rough, and exhibited a loose and fragmented structure after acid hydrolysis (Fig. 4C). A similar phenomenon was observed in wheat bran treated with acid hydrolysis (Huang et al., 2023). The HBB structure after *in vitro* gastrointestinal digestion exhibited cavities (white arrows), and some cracks on the surface were observed; the structure then formed a honeycomb structure after colonic fermentation (Fig. 4E). The observations were consistent with the results of Arenas and Trinidad (2017), which was related to the degradation of dietary fiber. In gastrointestinal digestion, pH is the main cause of dietary fiber degradation, and digestive enzymes also contribute to the decomposition of dietary fiber structure (Yuan et al., 2019). In the colon, microbiota-producing glycoside hydrolases degrade dietary fiber, leading to the disintegration of the overall structure of HBB (White, Lamed, Bayer, & Flint, 2014; Zhang et al., 2019). These results indicated that the two methods released EP and NEP by damaging and degrading the HBB structure, respectively.

3.7. HBB microstructure and polyphenol distribution

HBB has three fractions: pericarp, intermediate layer, and aleurone layer. As shown in Fig. 5A0, the aleurone layer structure, which is a lattice-like cell wall, was clearly observed compared with the other fractions. Therefore, changes in the aleurone layer structure after treatment with the two methods were investigated. The aleurone cell walls without treatment were intact, and broken cell walls were observed (white arrow), which were caused by pearling and grinding during sample preparation. After acetone extraction, no significant change was observed in the aleurone cell walls. The cellular structure of the aleurone layer was completely disrupted after acid hydrolysis, thus releasing more NEP. Faint fluorescence was observed, as shown in Fig. 5C2. These results indicated that the chemical method completely released EP and NEP from HBB.

After *in vitro* gastrointestinal digestion, the aleurone cell walls became diffuse, and this phenomenon was more pronounced after colonic fermentation, indicating cell wall degradation. In addition, Tydeman et al. (2010) found that carrot cell walls were gradually solubilized after gastrointestinal digestion and colonic fermentation. Moreover, the autofluorescence of the corresponding polyphenols decreased. As shown in Fig. 5E, HBB treated with *in vitro* digestion and colonic fermentation retained its cellular structure, indicating that the physiological method was milder than the chemical method. In conclusion, chemical and physiological methods had different degrees of destructive effects on the cellular structure of the aleurone layer during the extraction of EP and NEP.

3.8. Possible release mechanisms of polyphenols

Based on the results of the structural analysis, the chemical and physiological methods used different mechanisms to release EP and NEP. Based on the studies of Gökmen, Serpen, and Fogliano (2009) and Wang, Li, Ge, and Lin (2020), Fig. 6 presents a schematic of the polyphenol distribution in the aleurone layer, including free polyphenols (A), polyphenols trapped in dietary fiber (B), polyphenols that physically (C) and chemically (D) interact with dietary fiber, and polyphenols trapped in intact cells (E). Hence, a possible release mechanism for polyphenols was proposed. During sample preparation, highland barley kernels were pearled to obtain bran, and the aleurone cells were partially destroyed during pearling. The free polyphenols (A fraction) in the broken cells were extracted using acetone. Acid hydrolysis destroyed the cellular structure of the aleurone layer and released the remaining polyphenols. In this process, glycoside bonds between dietary fiber and NEP were attacked by protons and eventually broke (Shi et al., 2020). Moreover, the ester bonds were broken, thus, ferulic acid and its oligomers were released. Based on the CLSM images, the ether bonds were disrupted, so the polyphenols in the HBB were completely released. For the physiological method, cell walls were degraded under the action of pH (2–7) and digestive enzymes, resulting in the release of A and B fractions, and even C and D fractions were partially released. Therefore, the polyphenols content released from the small intestine digestion (G-EP) was higher than that of acetone (A-EP). Furthermore, EP may include not only free polyphenols but also polyphenols that interact with dietary fiber, and Martins et al. (2022) proposed a similar conclusion. In colonic fermentation, polyphenols interacted with cell wall polymers (C

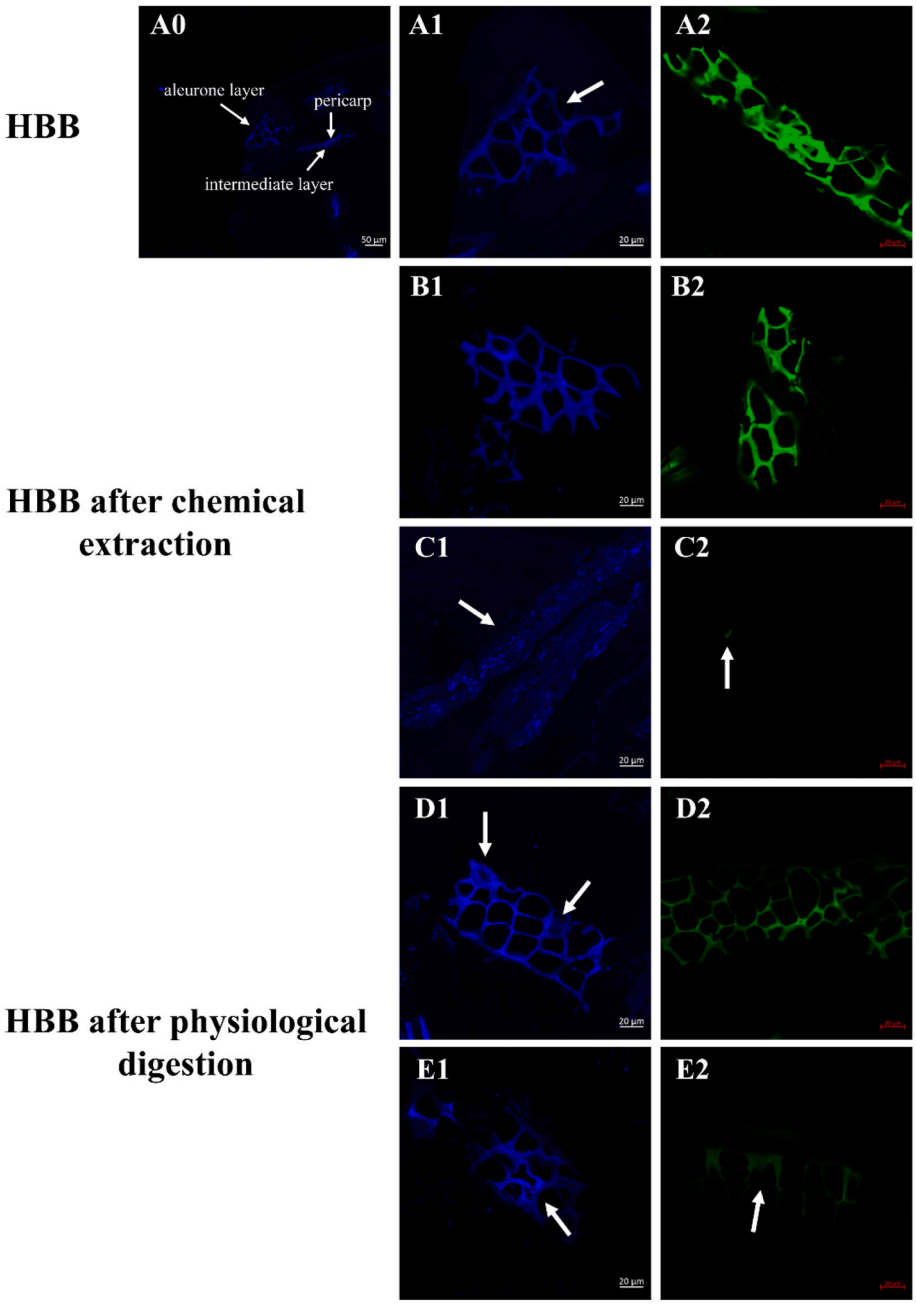

Fig. 5. CLSM images of HBB after treatment with chemical and physiological methods. A0, HBB; A1-A2, the aleurone layer; B1-B2, the aleurone layer after acetone extraction; C1-C2, the aleurone layer after acetone extraction and acid hydrolysis; D1-D2, the aleurone layer after *in vitro* gastrointestinal digestion; E1-E2, the aleurone layer after *in vitro* gastrointestinal digestion and colonic fermentation; HBB, highland barley bran. A1-E1, cell walls were stained in blue color; A2-E2, polyphenols were stained in green color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and D fractions), and polyphenols trapped in the intact cell (E fraction) were released during this process. The presence of carbohydrate hydrolases and esterases in the colon cleaved the glycosidic and ester bonds, respectively. Because HBB is a multicomponent system, excluding other components to prove the release mechanism of polyphenols requires further exploration.

4. Conclusions

This study compared the differences between chemical and physiological methods in releasing EP and NEP. Results indicated that the total EP and NEP contents released by the chemical method were higher than those released by physiological method. Observation via microscopy revealed that the two methods exhibited different degrees of destructive effects on the cellular structure of the aleurone layer. Therefore, a possible release mechanism was proposed that helps to understand the types of polyphenols that would be released from HBB. These findings demonstrate that EP and NEP extracted through chemical method do not reflect their release *in vivo*. Moreover, the study substantiates that HBB is rich in polyphenols and has potential health benefits, which can promote the development of whole grain or bran-rich products.

CRediT authorship contribution statement

Xueqing Wang: Writing – original draft, Methodology, Conceptualization. **Giorgia Purcaro:** Writing – review & editing, Supervision. **Bei**

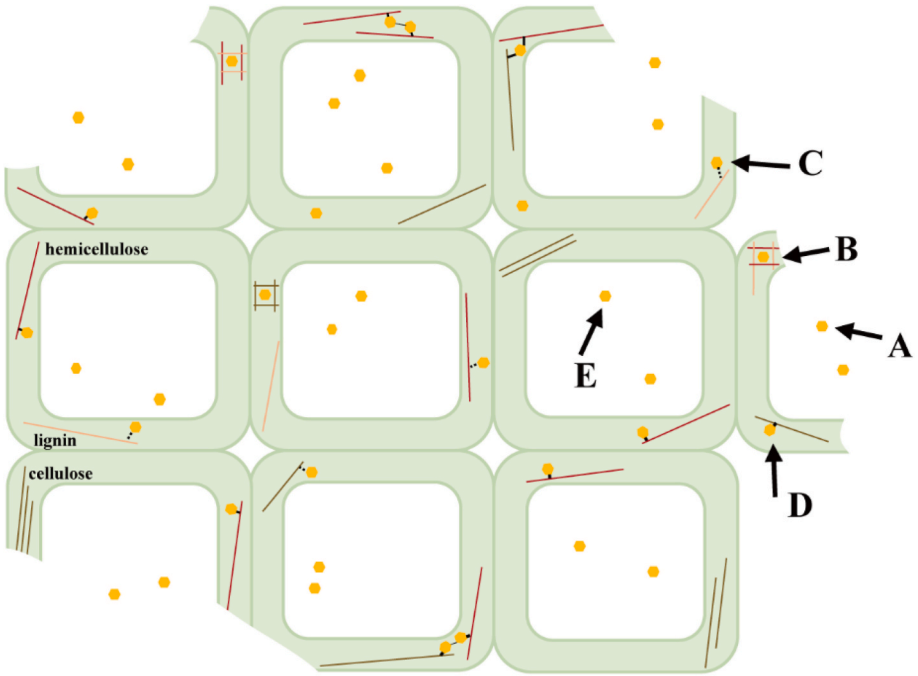

Fig. 6. Schematic diagram of the distribution of polyphenols in the aleurone layer. A, free polyphenols; B, polyphenols trapped in dietary fiber; C, polyphenols that physically interact with dietary fiber; D, polyphenols that chemically interact with dietary fiber; E, polyphenols trapped in intact cells.

Fan: Resources, Formal analysis. **Li-Tao Tong:** Validation, Resources. **Liya Liu:** Validation, Resources. **Jing Sun:** Validation, Resources, Methodology. **Yonghu Zhang:** Resources, Funding acquisition. **Fengzhong Wang:** Supervision, Project administration, Funding acquisition. **Lili Wang:** Writing – review & editing, Supervision, Conceptualization.

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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.

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