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ORIGINAL PAPER

Changes in the content of free phenolic acids and antioxidative capacity of wholemeal bread in relation to cereal species and fermentation type

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Abstract
Only low molecular, simple phenolic acids and their dimers can be easily absorbed by intestinal cells. In this study, the changes in free (unbound) phenolic acids and antioxidative capacity were tracked from flour, through bread to final in vitro bread hydrolysate. The initial material of the study included wholemeal flours made of common wheat, spelt wheat and rye, fermented with baker's yeast *Saccharomyces cerevisiae* or by the use of baking starter made of lactic acid bacteria *Lactobacillus casei* and *L. brevis* cultures with *S. chevalieri* yeast. A significant overall increase in free phenolic acids in breads and their hydrolysates was found, with the highest increase found for rye samples. The impact of the fermentation type was not consistent, showing additional crucial factors of used flours, which can affect final results. The free phenolic acid content in all the samples was correlated with the antioxidant capacity.

Keywords Dough fermentation · Bread · Free phenolic compounds · Ferulic acid · Antioxidative capacity · In vitro digestion

Introduction

Bread is a staple food in western countries despite the fact that over the past few decades its consumption has fallen significantly. Current consumption ranges from ca. 100 g/day (ca. 2–3 slices) in the UK, Italy, Denmark and Germany [1–3] to ca. 150 g a day in Poland [4]. Despite a relatively small share in the diet, it still contributes more than 10% of adults' daily intake of protein, thiamine, niacin, folate, iron, zinc, copper, and magnesium; one-fifth of fibre and calcium intakes; and more than one-quarter of manganese intake [3]. In addition to this, bread, especially made of wholemeal flour, is a source of low molecular health-promoting compounds such as sterols, tocols, alkylresorcinols, and phenolic compounds. Among them, phenolic acids are the most abundant, but their overall content is highly variable according to grain species (common wheat, spelt wheat, rye, etc.), grain cultivar, condition of cultivation as well as the type of used flour (wholemeal vs. white flour) [5–7]. As a result, white breads contain only ca. 14 µg/g, while wholemeal breads contain up to 1400 µg/g of phenolic acids in dry matter [8]. Considering that the daily intake of phenolic compounds assayed, for example, in a typical American diet is close to 1000 mg [9], wholemeal bread significantly contributes to this intake.

The health benefits of phenolic acids are primarily associated with their antioxidant properties, but these acids also show anti-ulcer, anti-diabetic, anti-cancer, anti-inflammatory, anti-aging, anti-microbial, cardioprotective, hepatoprotective and neuroprotective activities [10, 11]. The majority of these activities have phenolic compounds which reach systemic circulation [12]. Hydroxycinnamic acids (e.g., ferulic) are especially quickly absorbed from gastrointestinal tract (from stomach, jejunum, ileum and colon) and are detectable, for example, within 10 min in human blood plasma after oral administration [13]. However, only a small amount of cereal phenolic acids are easily accessible from the digestive tract since the majority (up to 99%) exist as esters, ethers and amides with other grain components (especially with arabinoxylans) and they create a lignocellulose matrix resistant to digestion [13, 14]. Only relatively low

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molecular, simple phenolic acids and their derivatives (like dimers of ferulic acid) can be absorbed by the intestinal cells, which exhibit intracellular phenolic acid esterase activity and, which increases the amount of phenolics accessible in the upper gastrointestinal tract [15, 16].

The options of increasing the content of easily accessible phenolic acids in cereal products have been investigated by many researchers. In general, reduction of the size of bran particles or microfluidization promotes the liberation of free phenolic acids from cereal grain products during processing and digestion [17, 18]. A significant increase in their content was also observed when grain was germinated [19]. The use of arabinoxylan-degrading enzymes, fermentation processes, or both, also produce enhanced amounts of free phenolic acids. For example, wheat bran fermentation with yeast combined with enzyme treatment (cell wall degrading enzymes: mainly xylanase, cellulase, β-glucanase and feruloyl esterase) for 20 h at 20 °C led to a ca. fourfold increase in free ferulic acid level [20]. Similarly, Turner et al. [21] revealed that the use of the commercial Ultraflo L preparation containing feruloyl esterase (from *Humicola insolens*) increased the free ferulic acid content of wheat grain samples five–sixfold. Konopka et al. [5] observed that the use of commercial baking starter LV1 SAF Levain for the preparation of wheat and rye sourdough bread increased the content of free ferulic acid up to 13.5% of total bound ferulic acid. In the cited study, the highest increase was noted in bread made from wholemeal rye flour. Bacteria strains of this baking starter (*L. casei* and *L. brevis*) have a lower ability to decompose free phenolic acids. In contrast, a significantly reduced content of free phenolic acids was determined in breads produced using sourdough composed of a mixture of *L. plantarum* E-78076 + *L. brevis* E-95612 + *Candida milleri* C-96250 [22] or of *L. plantarum* TMW 1460 and *L. hammesii* DSM 16381 [23]. In these sourdoughs (both rye and wheat), monomeric phenolic acids were converted to various vinyl and ethyl-derivatives.

As previously mentioned, the health-promoting functionality of phenolic acids results from their antioxidant properties. These properties are usually determined in extracts made using aqueous organic solvents (like alcohol–water mixtures). However, Delgado-Andrade et al. [24] stated that the use of the in vitro digestion of bread is a critical stage that allows the release of a large amount of compounds with antioxidant properties. The cited authors found the antioxidant capacity of these extracts from oral and gastric digestion stages might be more useful for nutritional purposes than the values determined in aqueous organic extracts. However, to date, the results of the antioxidant capacity of various breads are still scarce and generally not consistent.

The aim of the present study was to compare the impact of selected types of wholemeal breads (made of common wheat, spelt wheat, and rye flours), produced under the impact of two methods of dough fermentation (using baker's yeast or sourdough followed by baker's yeast) and simulated bread digestion (in vitro assay) on the changes of free phenolic acid contents and antioxidative capacity using ORAC and DPPH tests, respectively. These changes were tracked from flour, through bread-to-bread digestion product.

Materials and methods

Materials

Three commercial wholemeal flours [common wheat (CWF), spelt (CSF) and rye (CRF)] and laboratory-prepared wholemeal flour (LWF) of Opatka cv. (common wheat) were used in this study. Grain of Opatka cv. was ground into flour in a Falling Number 3100 mill. The basic quality characteristics of the used flours are presented in Table 1. The moisture content was determined based on PN-EN ISO 712:2012 [25] method and ash content according to ICC method 104/1, ICC 1990 [26]. The falling number was determined in a Falling Number Apparatus 1600 (Petren, Sweden) according to Standard PN-EN ISO 3093:2010 [27]. An amylograph test was conducted in a Brabender amylograph (type 800145) according to Standard PN-EN ISO 7973:2016-01 [28]. The content of wet gluten was determined according to PN-EN ISO 21415-2:2015 [29] on the gluten testing line type SZ-1 (ZBPP Sp. z o.o., Bydgoszcz, Poland). The pH was determined on-line by a HI 9125 pH-meter, equipped with an HI 1083B electrode (Hanna Instruments, Cluji-Napoca, Romania). Titratable acidity (TTA) was determined according to Koistinen et al. [22]. The baker's yeast *S. cerevisiae*, the baking starter LV1 SAF Levain containing a mixture of *L. casei and L. brevis* cultures (2% in total) with *S. chevalieri* yeast (98%) and commercial flours were purchased from a local market in Olsztyn.

Wheat bread making process

Preparation involved dough preparation and baking a total of four variants of bread. Their formulations are shown in Table 2. Briefly, to obtain sourdough, a 1% concentration of baking starter relative to the total flour weight was used. Sourdough was prepared with a yield of 200% (175 g of flour and 175 g of water). These ingredients were carefully mixed, then allowed to ferment for 24 h at 30 °C with continuous mixing using a laboratory water bath shaker (357 type; Elpan, Lubawa, Poland). Bread dough was prepared by adding the rest of the flour and water (according to dough sample variants), salt and yeast. Dough was kneaded using a laboratory dough kneading machine (GM-2; Bydgoszcz, Poland) for 5 min. The fermentation process was performed using a proofing chamber (PL 10 type; Warmia, Grudziądz,

Table 1 Main characteristics of used cereal flours and prepared sourdoughs

	Wholemeal flour			
	LWF	CWF	CSF	CRF
Moisture content (%)	12.37b	12.52a	11.74c	12.52a
Ash content (%)	1.91d	1.97a	1.94bc	1.92c
Falling number value (s)	415a	259c	346b	207d
Wet gluten content (%)	8.87c	24.48b	29.21a	n.a.
Amylograph viscosity (BU)	930a	181d	658b	395c
Initial temperature of gelation (°C)	60c	63bc	64.5a	54.5d
Final temperature of gelation (°C)	87a	78c	83.3bc	65d
Titratable acidity (mL of 0.1 M NaOH/10 g of flour)	6.75c	3.25d	7.95a	7.55b
Sourdough pH	4.57b	4.44c	4.60b	5.21a
Sourdough titratable acidity (mL of 0.1 M NaOH/10 g of sourdough)	21.76b	17.07c	22.40a	15.05d

n.a. not analysed; different letters in the same line indicate statistically significant differences ($P \leq 0.05$), obtained by the ANOVA with Duncan test ($n = 3$)

Table 2 Formulations of prepared breads

Variant	Type of flour	Flour (g)	Yeast (g)	Sordough (g)	Salt (g)	Water (g)	Dough yield (%)
1	LWF, CWF, CSF	350	10.5	–	3.5	192.5	155
2	LWF, CWF, CSF	175	3.5	175		17.5	
3	CRF	350	10.5	–		202.5	165
4	CRF	175	3.5	175		27.5	

Poland). Breads from wheat flour (wheat 1, wheat 2, spelt) were fermented for a total of 110 min in a 85 ± 2% relative humidity and 31 °C. After 80 min, dough was mixed for 1 min and fermented for another 30 min. In turn, rye breads were fermented for 60 min at a 85 ± 2% relative humidity at 35 °C. Finally, 250 g samples of wheat dough and 350 g samples of rye dough were manually sheeted and rolled, proofed up to an optimum volume increase, then baked (230 °C, 30 min) in an electric oven (PL 10 type; Warmia).

Cooled bread was manually divided into crust and crumb. Separated crumb was subsequently lyophilized (Hetosicc freeze dryer Cd 13-2-type; Heto, Birkerod, Denmark). All lyophilisates were ground (laboratory mill type A 10; IKA Labortechnik, Staufen, Germany) and stored at − 20 ± 2 °C until further use (not longer than 2 months).

In vitro digestion

Reagents

Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to Minekus et al. [30] (Table 3). To adjust pH to needed values, HCl (6 mol/L) and NaOH (1 mol/L) were used. α-Amylase (type IX-A, Sigma-Aldrich) was mixed with SSF to achieve 150 U/mL. Pepsin solution (porcine gastric mucosa, 3200–4500 U/mg protein, Sigma-Aldrich) made up with SGF to achieve a 25,000 U/mL solution. Pancreatin (from porcine pancreas, 4×USP, Sigma-Aldrich) was mixed with SIF to achieve an 800 U/mL solution. Bile salts (porcine, Sigma-Aldrich) made up with SIF, 60 mg/mL.

Table 3 Preparation of digestion fluids stock solutions (made up to the volume 200 mL)

Constituent	Stock concentration (mol/L)	SSF pH 7	SGF pH 3	SIF pH 7
		Volume of stock (mL)	Volume of stock (mL)	Volume of stock (mL)
KCL	0.5	7.55	3.45	3.4
KH_2PO_4	0.5	1.85	0.45	0.4
$NAHCO_3$	1	3.4	6.25	21.25
NaCl	2	–	5.9	4.8
$MgCl_2(H_2O)_6$	0.15	0.25	0.2	0.55
$(NH_4)_2CO_3$	0.5	0.03	0.25	–

Simulated in vitro digestion method

Lyophilized breads were subjected to digestion according to the method described by Minekus et al. [30]. In the oral phase, 5 g of grounded sample was mixed with 3.5 mL simulated salivary fluid, 0.5 mL α-amylase solution (type IX-A, Sigma-Aldrich), 25 µL 0.3 M $CaCl_2$ and 975 µL of water. After 2 min, the following were added to the bolus: 7.5 mL of simulated gastric fluid, 1.6 mL of pepsin solution (Sigma-Aldrich), 5 µL of 0.3 M $CaCl_2$, 1 M HCl (to achieve pH 3.0) and water to obtain 10 mL of total added fluids at this stage. Digestion occurred in a shaking incubator (Incu-Shaker Mini, Benchmark, Sayreville, NJ, USA) for 2 h at 37 °C. After gastric phase reagents were added to start the intestinal phase, the following were added to the chyme: 11 mL of simulated intestinal fluid, 5 mL of pancreatin solution, 2.5 mL of bile salt solution, 0.15 mL of 0.3 M $CaCl_2$, 1 M NaOH (to achieve pH 7.0) and water to obtain 20 mL of total added fluids at this stage. The intestinal digestion was conducted in the shaking incubator for 2 h in 37 °C. Immediately after digestion, the samples were snap-frozen using liquid nitrogen and then lyophilized. After lyophilisation, the samples were grounded and used for further analysis.

Determination of phenolic acid content

The phenolic acids were determined by the RP-HPLC technique according to the method described by Skrajda-Brdak et al. [31]. The extraction of free phenolic acids was performed in triple, by adding to 1 g of finely milled grain 10 mL of 80% methanol and sonication for 15 min using an ultrasonic bath (InterSonic, Olsztyn, Poland). After extraction samples were centrifuged in Eppendorf centrifuge, type 5810R (Eppendorf, Hamburg, Germany). Supernatants were collected and evaporated to dryness at temperatures below 50 °C in a vacuum evaporator (Büchi, type R-210; Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved in 30 mL of deionized water acidified to pH 2. Phenolic acids were extracted by fourfold extraction with 20 mL of ethyl acetate. Total phenolic acids were extracted after 4 h alkaline hydrolysis, which was performed by adding to 0.5 g of finely milled grain 30 mL of 2 mol NaOH and mixing. After that time samples were acidified to pH 2, with 6 mol HCl. Samples were centrifuged and supernatants were subjected to extraction of total phenolic acids fourfold with 30 mL of ethyl acetate. Collected ethyl acetate extracts were evaporated in a vacuum evaporator, re-dissolved in 1 mL of methanol and subjected to chromatographic separation. Chromatographic separation was performed on an Agilent Technologies (Santa Clara, USA) 1200 series system fitted out with a photodiode detector with a Waters XBridge C18 column (Milford, MA, USA) (150 mm × 2.1 mm, 3.5 µm) at 30 °C. A gradient elution program was employed, using water/formic acid (99.85/0.15, v/v) (solvent A) and acetonitrile/formic acid (99.85/0.15, v/v) (solvent B) as elution solvents. The flow rate was 0.5 mL/min with a 40 min gradient elution program as follows: 0–3 min 1% B; 3–15 min, 1–10% B; 15–25 min, 10–60% B; 25–27 min, 60–80% B; 27–30 min, 80% B; 30–33 min, 80–1% B and was stable until 40 min. The detection was performed at the wavelength of 260 (*p*-OH-benzoic, vanillic, syringic acid) and 320 nm (*p*-coumaric, ferulic, sinapic acid). Phenolic acids were identified by comparison with absorption spectra of the reference phenolic acids (Sigma-Aldrich, Saint Louis, USA). The content of phenolic acids was determined from calibration curves of reference standards and expressed as µg per 1 g of a sample dry mass (DM). The R^2 value for all identified phenolic acids was ≥ 0.9988. Phenolic acid recovery was in range of 91–99%. The LOD for *p*-OH-benzoic, vanillic, syringic acid was 0.05 µg/mL, while for *p*-coumaric, ferulic and sinapic acid was 0.025 µg/mL. The LOQ for *p*-OH-benzoic, vanillic, syringic acid was 0.17 µg/mL, while for *p*-coumaric, ferulic and sinapic acid was 0.08 µg/mL. Calculated accuracy was as follows: *p*-OH-benzoic acid—102.33%, vanillic acid—102.44%, syringic acid 101.60%, *p*-coumaric acid—104.59%, ferulic acid—102.72% and sinapic acid 107.99%.

Preparation of extracts from flour, bread and in vitro bread hydrolysate

Finely milled samples (0.2 g) were triple-extracted with 80% methanol (1.5 mL) and centrifuged. Supernatants were collected, then evaporated to dryness at temperatures below 50 °C in a vacuum evaporator. The residue was dissolved in 1 mL of methanol and used to determine the antioxidant potential.

DPPH

The antioxidant capacity of samples was determined by the DPPH Radical Scavenging Assay, according to Konopka et al. [32] with some modifications. The extracts were added to a DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.2 mmol/L in methanol) and the mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm against methanol using a FLUOstar Omega multi-mode microplate reader (BMG LABTECH, Offenburg, Germany). The antioxidant capacity was determined based on a curve of % DPPH radical scavenging activity of different Trolox concentrations in methanol and expressed as µM TE (Trolox Equivalent) per 1 g of sample DM.

ORAC

The ORAC test was carried out according to the method described by Huang et al. [33] with some modifications. The assay was performed using the FLUOstar Omega multimode microplate reader. 25 µL of extracts and pure solvent (blank sample) were placed in wells of a black 96-well plate with 150 µL of 10 nM fluorescein (in 75 mM phosphate buffer, pH 7.4). The plate was pre-incubated at 37 °C for 15 min, and 25 µL of 153 mM AAPH (2,2′-azobis-(2-methylpropionamidine) dihydrochloride, in 75 mM phosphate buffer, pH 7.4) was added to each well. The fluorescence intensity was measured automatically by the reader (excitation at 485 nm, emission at 540 nm) every 1 min for 6 h. The ORAC values were calculated using the differences of areas under the curves of fluorescence decay between the blank and sample (net area under the curve). Trolox was used as a standard. The results are expressed as µM TE per 1 g of sample DM.

Statistical analysis

All chemical analyses were done in triplicate and analysed using Statistica 13.0 PL software (StatSoft, Kraków, Poland) at a significance level of $P \leq 0.05$. The differences in sample phenolic acid compositions and antioxidant potential were determined using ANOVA with a Duncan test. Additionally, the Pearson's correlation coefficient was measured to determine the relationship between antioxidant potential and phenolic acids.

Results and discussion

Total and free phenolic acids in used flours

The total phenolic acid content varied among tested wholemeal wheat flours and reached values from 268 µg/g DM in flour of CSF to 430 µg/g DM in flour of LWF (Table 4). These contents were rather low, since the HEALTHGRAIN project value determined for wheat grain ranges from 326–1171 µg/g DM [34]. Similarly, wholemeal rye flour contained 422 µg/g DM of phenolic acids, and this value was below the range of 491–1082 µg/g DM determined for rye grain [35]. However, it is worth knowing that the minimal concentration of phenolic acids determined in other studies is close to 200 µg/g DM [31, 36], while the highest concentration is close to 3200 µg/g DM in wheat grain [37] and to 2400 µg/g DM in rye grain [38]. The highest values were found in grain from biotic stresses during plant cultivation [7].

The phenolic acid fraction was composed of six phenolic acids (Table 4). Among them ferulic acid prevailed, reaching a share from 79% (rye) to 83% (spelt). This acid was followed by ca. 3–22% and 5–12% shares of *p*-coumaric and sinapic acids, respectively, accompanied by a ca. 5–7% share of sum of benzoic acids (*p*-OH benzoic, vanillic and syringic) in wheat flours and a ca. 2% share of *p*-OH benzoic and vanillic acids in rye flour. A similar composition of phenolic acids in wheat and rye grain was previously found by other studies [31, 38, 39].

Table 4 Total and free phenolic acids content in flours used in the study (µg/g of dry matter)

	Flour			
	LWF	CWF	CSF	CRF
Free phenolic acids				
p-OH benzoic	0.30b	0.30b	0.26c	0.32a
Vanilic	0.71c	1.25a	1.17ab	1.14b
Syringic	n.d.	1.30a	0.78b	n.d.
p-Coumaric	0.95a	0.36b	0.19c	0.91a
Ferulic	0.55d	1.38c	1.98b	3.10a
Sinapic	0.12d	0.60b	0.36c	1.73a
Total phenolic acids				
p-OH benzoic	10.57a	2.73c	3.24b	2.79c
Vanilic	19.82a	6.20b	6.31b	6.89b
Syringic	n.d.	6.45a	4.99b	n.d.
p-Coumaric	95.19a	8.32c	8.84c	30.26b
Ferulic	281.75b	222.1c	221.77c	332.26a
Sinapic	22.93c	27.49b	22.54c	49.36a
Sum of total phenolic acids	430.26a	273.29b	267.7b	421.56a

Different letters in the same line indicate statistically significant differences ($P \leq 0.05$), obtained by the ANOVA with Duncan test ($n = 3$); *n.d.* not detected

Only a small part of these compounds existed in an unbound (free) state. The content of free phenolic acids varied from 2.63 µg/g DM (LWF) to 7.24 µg/g DM (CRF) (Fig. 1), representing from 0.5 to 1.9% of total phenolic acids, respectively. In comparison, the free phenolic acid fraction in bread wheat grain can range from 3.1 to 6.7 µg/g, with an average value of 4.2 µg/g [40], while in spelt grain this value can be close to 21 µg/g [31]. In rye flour, ferulic and sinapic acids had the highest share among free phenolic acids. For wheat flours, the shares of individual phenolic acids were highly variable. Free syringic acid was absent in LWF and rye flours.

Free phenolic acids in breads and their in vitro hydrolysates

The free phenolic acid content in breads varied from 9.21 µg/g DM in sourdough fermented bread of LWF to 50.09 µg/g DM in sourdough fermented rye bread (Fig. 1). The increase of free phenolic acids in relation to initial

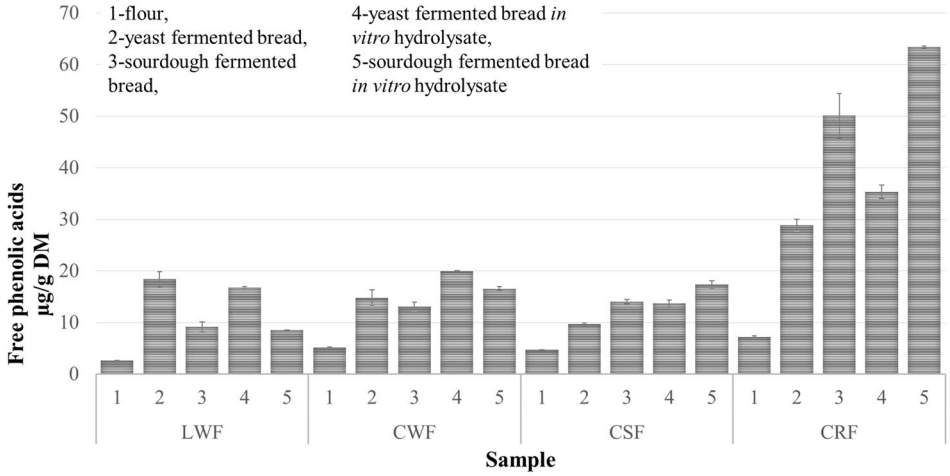

Fig. 1 Content of free phenolic acids in flours, breads, and breads hydrolysates

Table 5 Free phenolic acids content in breads (µg/g of dry matter)

	Bread			
	LWF	CWF	CSF	CRF
Yeast fermented bread				
p-OH benzoic	1.66a	0.73c	0.57d	0.93b
Vanilic	4.37a	2.26c	2.23bc	2.74b
Syringic	n.d.	1.33a	0.76b	n.d.
p-Coumaric	1.54b	0.73c	0.47d	3.45a
Ferulic	7.94b	7.74b	4.68c	15.76a
Sinapic	2.93b	2.00bc	0.96c	6.01a
Sourdough fermented bread				
p-OH benzoic	3.24a	1.59c	1.40c	2.51b
Vanilic	4.13a	2.19b	2.49b	3.89a
Syringic	n.d.	1.43a	1.12a	n.d.
p-Coumaric	n.d.	0.31b	0.23b	1.28a
Ferulic	0.98c	4.67b	6.49b	26.59a
Sinapic	0.87b	2.93b	2.31b	15.81a

Different letters in the same line indicate statistically significant differences ($P \leq 0.05$), obtained by the ANOVA with Duncan test ($n = 3$); *n.d.* not detected

Table 6 Free phenolic acids content in bread in vitro hydrolysates (µg/g of dry matter)

	Bread hydrolysate			
	LWF	CWF	CSF	CRF
Yeast fermented bread				
p-OH benzoic	1.49a	1.69a	1.67a	1.68a
Vanilic	4.38a	2.97b	3.06b	3.17b
Syringic	n.d.	2.03a	1.21b	n.d.
p-Coumaric	1.39b	0.86c	0.49d	4.38a
Ferulic	6.94c	9.77b	5.76d	19.01a
Sinapic	2.63b	2.63b	1.55c	7.12a
Sourdough fermented bread				
p-OH benzoic	2.39b	2.47b	2.16b	3.52a
Vanilic	4.64b	2.72c	2.94c	8.33a
Syringic	n.d.	2.04a	1.67a	n.d.
p-Coumaric	n.d.	0.42b	0.42b	1.9a
Ferulic	0.88d	5.53c	7.24b	32.58a
Sinapic	0.64d	3.43b	2.97c	17.08a

Different letters in the same line indicate statistically significant differences ($P \leq 0.05$), obtained by the ANOVA with Duncan test ($n = 3$); *n.d.* not detected

content in flour was two–sevenfold. In general, the highest increase was found in breads produced from flours, characterised by the highest initial content of total phenolic acids. The type of fermentation had an opposite effect on spelt and rye breads and on both common wheat breads. For spelt and rye breads, sourdough fermentation favoured the accumulation of free phenolic acids contents, while this type of fermentation diminished the accumulation of free phenolic acids in breads made of common wheat. Regardless of the fermentation type, free benzoic acids were preferentially accumulated in breads, especially *p*-OH benzoic and vanillic acids (Table 5).

After in vitro digestion of prepared breads, the determined content of free phenolic acids varied from 8.55 µg/g DM in the sourdough fermented LWF bread sample to 63.4 µg/g DM in the sourdough fermented rye bread sample (Fig. 1). These values were from three to ninefold higher than in initial flours and simultaneously in three (from four) hydrolysates of CWF, spelt and rye breads ca. 30% higher than in corresponding breads. Only hydrolysates of both LWF breads showed a similar content of free phenolic acids as in corresponding breads. Taking into account the composition of used flours, the highest overall

increase was observed in the content of *p*-OH benzoic and vanillic acids (Table 6). This was especially visible in rye bread hydrolysates, in which the final concentration of these acids exceeded the total initial value in flour.

The results of this study showed that breads and their hydrolysates are more abundant in the content of free phenolic acids, then respective flours (Fig. 1). However, the final content of these compounds in bread is the resultant of simultaneous processes of their release from cell walls, decomposition by native flour and microflora enzymes, thermal decomposition during baking and repeated binding by surrounding carbohydrates and proteins.

The main cause of the increase in the content of free phenolic acids is the activity of phenolic acid esterases during the fermentation stage. This activity was confirmed in selected lactic acid bacteria (LAB) and yeast strains. For example, Hole et al. [41] demonstrated that *L. acidophilus* LA-5, *L. johnsonii* LA1 and *L. reuteri* SD2112 strains can increase the free phenolic acid contents in oat (from 4.13 to 109.42 µg/g DM) and barley grain (from 2.55 to 69.91 µg/g DM) samples. Similarly, Ripari et al. [23] demonstrated that selected combinations of *L. hammesii* DSM 16381 with two strains of the *L. plantarum* (LM01 and PM4) effectively reduced the content of bound ferulic acid in wheat sourdough (in some samples, bound ferulic acid was almost completely liberated into free and conjugated forms). Native cereal grain also exhibits feruloyl esterase activity [42].

In turn, the decrease in concentration of free phenolic acids proceeds through their enzymatic and thermal degradation and by physical and chemical bonding. Liberated ferulic, *p*-coumaric and caffeic acids are toxic for Gram-positive bacteria under acidic conditions [13, 43, 44]. However, some LABs, particularly *L. plantarum* and *L. brevis* [45, 46], have a high tolerance to phenolic acids. This resistance is explained by the ability of LABs to convert phenolic acids to metabolites of reduced activity [23]. For example, ferulic acid is reduced to dihydroferulic acid and then decarboxylated to 4-vinyl-quaiacol, or decarboxylated and reduced to 4-ethyl-quaiacol [47]. In these transformations, a crucial role is the ability of a microbial strain to produce phenolic acid decarboxylase. Such activity was found in diverse fungi, yeast and bacteria (more details in Bhuiya et al. [48]). According to Ripari et al. [23], the use in wheat sourdough preparation of combinations of *L. hammesii* DSM 16381 with selected strains of *L. plantarum* results in efficient conversion of ferulic acid into vinyl- and ethyl-derivatives.

Another possible reason for the loss of liberated phenolic acids is their heat-liability. Ferulic acid, like other cinnamic acids, under heat treatment decarboxylates to produce ring-substituted styrenes/vinylbenzenes [49]. Complete decomposition of ferulic and sinapic acids occurs at approx. 175 °C [50, 51]. Liazid et al. [51] determined, that the greater the number of hydroxylic-type substituents, and the smaller the number of metoxylic-type substituents, the easier it is for degradation of phenolic acid to take place. Since bread crumb can reach maximally 100 °C during baking, phenolic acids can be degraded only in crust. However, other authors found that decomposition of phenolic acids can occur at lower temperatures. Tanchev et al. [52] recorded thermal degradation of gallic and protocatechuic acids at temperatures of 101–121 °C, at a solution pH in the range of 3–5. This suggests the possibility of higher thermal phenolic acid degradation in sourdough breads.

Apart from enzymatic and thermal degradation, free phenolic acids may also be redistributed and repeatedly bound through covalent or non-covalent bonds by components of dough and bread [53–57]. Phenolic acids can be covalently bound with proteins [57], non-covalently entrapped in the hydrophobic core of polysaccharides (mainly amylose) helices [58, 59] or covalently bound with starch molecules through the esterification of three hydroxyl groups from a glucose unit [60]. Bound phenolic compounds are more resistant to extraction. According to Fares et al. [55] and Menga et al. [61] free *p*-OH benzoic acid is primarily susceptible to binding during cereal grain processing.

Changes of antioxidative capacity from flour, through bread to bread hydrolysate

The results of antioxidative capacity are presented in Fig. 2. In general, the values of the DPPH assays were lower than ORAC assays and varied from 1.02 to 14.44 µM TE/g DM in the case of DPPH assay and from 6.11 to 34.77 µM TE/g DM for the ORAC assay. Both assays showed that rye flour, rye breads and rye digests had the highest antioxidative capacity, reaching the values of 7.92, 11.37–14.44, and 10.72–6.25 µM TE/g DM in DPPH assay, and of 11.72, 23.10–30.40, and 34.77–29.65 µM TE/g DM in the ORAC assay, respectively. The impact of the type of fermentation was relatively little visible among breads analysed using the DPPH assay, while in the ORAC assay, all breads prepared with the use of sourdoughs had a higher antioxidative capacity than variants using yeast only. Comparing breads and their digests, an opposite effect was in both assays. In the DPPH assay, all bread hydrolysates showed diminished antioxidative capacity, while in the ORAC assay almost all bread hydrolysates showed higher antioxidative capacity. The correlation coefficient between ORAC values and free phenolic acids the coefficients varied from $r = 0.73$ ($P \leq 0.05$) to $r = 0.96$ ($P \leq 0.05$).

The levels of antioxidative capacity values in ORAC and DPPH assays found in current study are close to those previously mentioned by Michalska et al. [62], Yu et al. [63] and Yu and Beta [64]. In all studies, the results of the ORAC assay are clearly higher than for the DPPH assay. For example, white wheat bread made with yeast has an

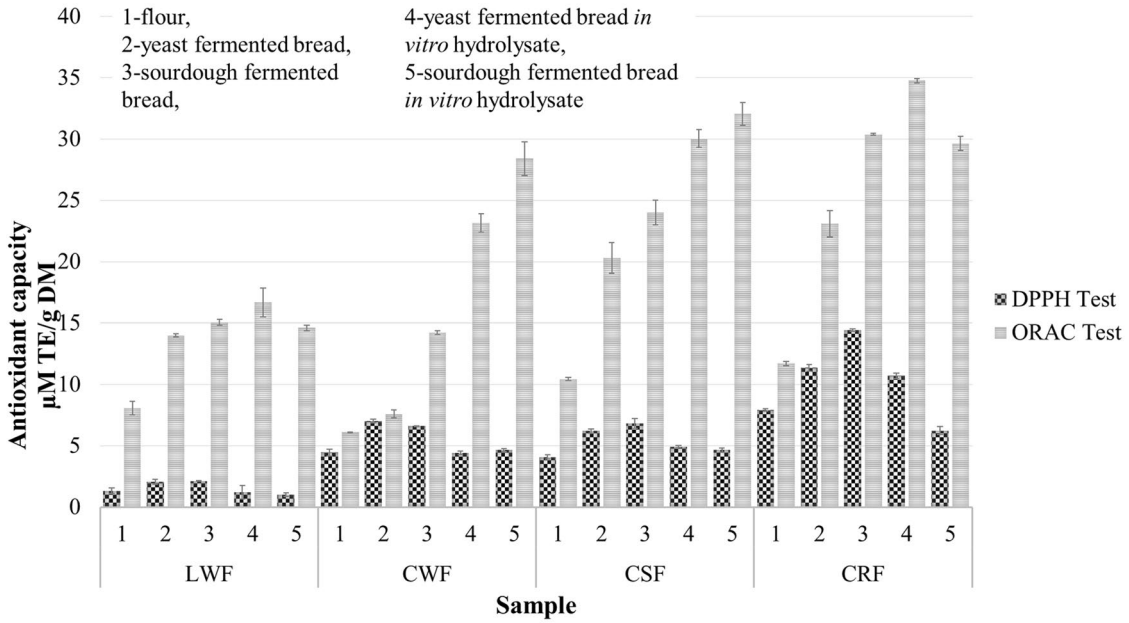

Fig. 2 Antioxidant capacity of flours, breads, and bread hydrolysates

antioxidant capacity from ca. 2 µM TE/g in DPPH assay [65] to ca. 50 µM TE/g in ORAC assay [66]. Similarly, Yu et al. [63] determined approx. 10–20-fold higher values of antioxidant capacity of wheat bread extract in the ORAC assay than in the DPPH assay. The determined differences may be a result of the distinct mechanisms of these assays. For ORAC, hydrogen atom transfer (HAT) mechanism occurs, while DPPH proceeds by single electron transfer (SET) [67]. It seems that the HAT mechanism predominates for flour, bread and bread digestion.

Conclusions

The wholemeal flours used in the current study exhibited a relatively low level of total and free phenolic acids. In all cases, breads were more abundant in free phenolic acids than in corresponding flour. However, the final content of these compounds is the result of simultaneous processes of their release and decomposition by native flour and/or microflora esterases and decarboxylases, thermal decomposition and repeated binding by surrounding chemicals. The overall increase of free phenolic acids determined in all samples indicated the prevalence of cell wall degradation processes. In general, free benzoic acids were preferentially accumulated in all breads. The additional increase of free phenolic acid contents in bread hydrolysates showed that digestion with α-amylase, pepsin and pancreatin enzymes favours the solubilisation of phenolic acids bound by proteins, polysaccharides or other bread compounds. The content of free phenolic acids positively correlated ($r > 0.73$) with the antioxidative capacity of extracts measured in the ORAC assay. In contrast, the results of the DPPH assay were not related to the final concentration of phenolic acids.

It should be noted that the impact of the fermentation type on the content of free phenolic acids was not consistent. This indicates that there are additional crucial factors determining the final results, such as flour quality indices, initial total content of phenolic acids, activity of native enzymes, etc.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with animal or human subjects.

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