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Impact of ancient cereals, pseudocereals and legumes on starch hydrolysis and antiradical activity of technologically viable blended breads

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ABSTRACT

Wheat flour replacement from 22.5% up to 45% by incorporation of ternary blends of teff (T), green pea (GP) and buckwheat (BW) flours provided technologically viable and acceptable sensory rated multigrain breads with superior nutritional value compared to the 100% wheat flour (WT) counterparts. Blended breads exhibited superior nutritional composition, larger amounts of bioaccessible polyphenols, higher anti-radical activity, and lower and slower starch digestibility. Simultaneous lower rapidly digestible starch (57.1%) and higher slowly digestible starch (12.9%) and resistant starch (2.8%) contents (g per 100 g fresh bread), considered suitable nutritional trends for dietary starch fractions, were met by the blend formulated 7.5% T, 15% GP, 15% BK. The associated mixture that replaced 37.5% WT, showed a rather lower extent and slower rate of starch hydrolysis with medium-low values for C_∞, and H_{90}, and lowest k, and intermediate expected Glycaemic Index (86). All multigrain breads can be labelled as source of dietary fibre (≥3 g dietary fibre/100 g bread).

1. Introduction

Grains are basic, ubiquitous and healthy raw materials, good source of carbohydrates – mainly starch and dietary fibre – providing excellent vectors for diversity and innovation. It raises a great deal of recent interest that ancient crops (Angioloni & Collar, 2011a), pseudocereals (Collar & Angioloni, 2014a) and legumes (Angioloni & Collar, 2012), besides wheat, constitute nutrient-dense and healthy grains with explicited breadmaking applications.

A slow release and absorption of glucose may be generated in a food matrix according to the processing conditions and surrounding ingredients (Lehmann & Robin, 2007), encompassing beneficial effects in the management of diabetes and hyperlipidemia (Jenkins, 2007). Native cereal starches are ideal sources of slowly digestible starch (SDS) (>50%), and the slow progressive digestion property is realized by a layer-by-layer inside–outside (radial) digestion process (Zhang, Ao, & Hamaker, 2006a). Mechanical and thermal treatments change the structure and digestibility of starch. Thermal treatments such as the cooking process completely destroys the semicrystalline structure of native starch granules and causes the loss of SDS and resistant starch (RS) and increases rapid digestible starch (RDS) (Zhang, Venkatachalam, & Hamaker, 2006b). In cereal products, the starch gelatinization extent, which is mainly controlled by the moisture level and the cooking time and temperature influences the formation of SDS (Englyst, Vinory, Englyst, & Lang, 2003). In bread dough, although formation of resistant starch (RS3) may occur in the high water-containing parts during cooling, a large portion of starch is gelatinized during cooking and induces a rapid digestibility of starch (Bravo, Englyst, & Hudson, 1998). In extruded cooked cereal products such as breakfast cereals, in addition to the thermal treatment, the high pressure and shear forces destroy the starch granular structure and increase its gelatinization extent, making it more available to amylolytic enzymes (Le François, 1989). On the contrary, in pasta, a dense protein network is formed, which limits the accessibility of α-amylase to the starch and restricts the diffusion of water molecules to the starch granules. As a consequence, a reduction of the extent of starch gelatinization takes place (Englyst, Kingman, & Cummings, 1992). In some biscuits with very low moisture levels during the treatment, the extent of gelatinization is reduced and partially intact granules and gelatinized starch co-exist, resulting in a higher content of SDS compared to breakfast cereals and baked products (Englyst et al.,

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2003). In many plant sourced foods, such as legumes and minimally processed cereal grains, starch granules are trapped within the plant cell walls (*e.g.* whole grains), which retard their degradation (Würsch, Del Vedovo, & Koellreutter, 1986). Disruption of the granule structure as by milling can increase the susceptibility to enzymatic degradation.

Legumes that are low glycaemic index foods, which generate slow and moderate postprandial glucose and insulin response, have been shown to decrease blood glucose responses compared to other cereal based foods (Tovar, Granfeldt, & Bjorck, 1992) such as whole bread. The digestibility of legume starch is much lower than that of cereal starch (Madhusudhan & Tharanathan, 1996). Cooked legumes are prone to retrograde more quickly, thereby lowering the process of digestion. The higher content of amylose in legumes, which probably may lead to a higher RS content, may possibly account for their lower digestibility. Also, legumes contain more of proteins than cereals, and protein–starch interaction in legumes may equally contribute to their decreased glycaemic responses (Geervani & Theophilus, 1981). Additionally, the presence of high amounts of dietary fibre and antinutritional factors such as phytates and amylase inhibitors may greatly influence the rate and extent of legume starch digestibility.

The current proposal is aimed at exploring the competences and exploiting the suitability in mixed wheat matrices of non-breadmaking whole grains with unique nutritional components (teff, green pea and buckwheat flours), to obtain novel and healthy fermented baked goods meeting the functional and sensory restrictions of viscoelastic breadmaking systems. Bread functional and nutritional profiles were assessed in quaternary wheat blended matrices, and compared with the wheat flour counterparts. Special emphasis will be placed on starch hydrolysis kinetics and relevant starch nutritional fractions in mixed grain matrices.

2. Materials and methods

2.1. Materials

Commercial flours from refined common wheat *Triticum aestivum* (WT), and whole teff *Eragrostis tef* (T), green pea *Pisum sativum* (GP), and buckwheat *Fagopyrum esculentum* (BW) were purchased from the Spanish market. Refined WT (70% extraction rate) of 356×10^{-4} J energy of deformation *W*, 0.64 curve configuration ratio P/L, 95% Gluten Index, 62% water absorption in Brabender Farinograph, was used. Ireks Vollsauer sour dough was from Ireks (Spain); Novamyl 10,000 a maltogenic thermostable α-amylase from Novozymes (Denmark); and calcium propionate, from Sigma–Aldrich (USA).

2.2. Methods

2.2.1. Bread making of wheat and wheat-based blended flours

Doughs and breads were prepared for WT as control, and wheat-based blended flours (T, GP, BW) by WT replacement from 22.5% up to 45%, and incorporation of ternary blends of T, GP and BW flours according to a Multilevel Factorial Design with the following attributes: three experimental factors (T, GP and BW flours) at 2 levels, coded 0 (7.5% wheat flour replacement) and 1 (15% wheat flour replacement), and 5 error degrees of freedom. The model resulted in 8 randomized runs in 1 block. A 3 digit bread sample code was set referring to low (0) and high (1) wheat flour replacement by T (1st digit), GP (2nd digit), and BW (3rd digit) flours in sample formulation, as it follows: 010, 001, 011, 000, 111, 101, 100, 110. Blended flours (100 g), water (62%, flour basis), commercial compressed yeast (4%, flour basis), salt (1.5%, flour basis), vegetable fat–margarine-(4%, flour basis), sugar (2%, flour basis), commercial sour dough (4%, flour basis), milk powder (5%, flour basis), Novamyl 10,000 (7.5 mg, flour basis), and calcium propionate (0.5%, flour basis) were mixed in a 10 kg mixer at 60 revolutions min-1 for 10 min up to optimum dough development. Fermented doughs were obtained after bulk fermentation (10 min at 28 °C), dividing (500 g), rounding, moulding, panning and proofing up to maximum volume increment (30 min at 28 °C), and were baked at 200 °C for 30 min to make control and blended breads.

2.2.2. Chemical and nutritional composition of flours and breads

Moisture, protein, ash and fat contents of commercial flours, control and blended breads were determined following the ICC methods (ICC, 1976–1996). Total, soluble and insoluble dietary fibre contents were determined according to the AOAC method 991.43 (AOAC, 1991). Three replicates were made for each analysis. Digestible carbohydrates were calculated by indirect determination as 100 − [Moisture + Protein + Fat + Ash + Dietary Fibre] (FAO, 2003). Resistant starch determination was performed according to AOAC Official Method 2002.02 (AOAC, 2000) by using Megazyme kit K-RSTAR 08/11. Bioaccessible phenol determination was carried out by conducting an "in vitro" digestive enzymatic mild extraction that mimics the conditions in the gastrointestinal tract according to the procedure of Glahn, Lee, Yeung, Goldman, and Miller (1998) and adapted by Angioloni and Collar (2011b) for breads.

The stable 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical was used to measure the radical scavenging capacity of flour and bread samples according to the $DPPH^{\bullet}$ method (Brand-Williams, Cuvelier, & Berset, 1995), modified by Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) and adapted. 2 g of flour, and 3 g of French bread (freeze-dried and milled <0.5 mm) were placed in a centrifuge tube (50 mL) and 20 mL of acidic methanol/water (50:50 v/v, pH 2) was added (10 mL for French bread). The tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500*g* for 10 min, and the supernatant was recovered. 20 mL of acetone/water (70:30, v/v) was added to the residue (10 mL for bread), and shaking and centrifugation were repeated. Both methanolic and acetonic extracts were combined and adjusted to 25 for bread or 50 mL with methanol. After gentle shaking, aliquots of 0.1 mL were taken, and 3.9 ml of a solution of DPPH 0.050 g/L (equivalent to 0.1268 μmol/mL) was added. Tubes were gently shaken, and 4 mL of each tube were added to a 4 mL cuvettes, and A515 nm was read at 1 min and every 5–10 min until the plateau was reached. A cuvette containing 4 mL of DPPH 0.494 μmol in methanol was read at the same periods. A blank of methanol was used. Lectures were taken in duplicated samples. Plots of μmol DPPH *vs* time (min) were drawn, and calculations were made to know the antiradical activity (AR). $AR = [([DPPH]_{INITIAL} - [DPPH]_{PLATEAU}) \times 100]/[DPPH]_{INITIAL}$.

2.2.3. Bread measurements

2.2.3.1. Enzymatic/biochemical determinations. In vitro starch hydrolysis kinetics and relevant starch fractions in blended breads was determined following the AACC (2005) method 32–40, adapted as previously described (Angioloni & Collar, 2011c). Rapidly digestible starch (RDS) and slowly digestible starch (SDS) were measured after incubation for 20 min and 120 min, respectively, as stated by Englyst et al. (2003). Total digestible starch (DS) was determined in the supernatant after 16 h of incubation while resistant starch (RS) was determined in the pellet as the starch remaining after 16 h incubation. The digestion kinetics and expected glycaemic index (eGI) of bread were calculated in accordance with the procedure followed by Chung, Liu, Pauls, Fan, and Yada (2008) based on the method established by Goñi, Garcia-Alonso, and Saura-Calixto (1997). A first order kinetic equation $[C = C_{\infty}(1 - e^{-kt})]$ was applied to describe the kinetics of starch hydrolysis, where *C*, C_{∞} and *k* were the hydrolysis degree at each time, the maximum hydrolysis extent and the kinetic

constant, respectively. The hydrolysis index (HI) was calculated as the relation between the area under the hydrolysis curve (0–16 h) of blended bread samples and the area of standard material from white bread (control) (Chung et al., 2008). The expected glycaemic index (eGI) was calculated using the equation proposed by Granfeldt, Björck, Drews, and Tovar (1992): eGI = 8.198 + 0.862HI.

2.2.3.2. Physico-chemical and sensory determinations. Colour determinations were carried out on bread crumb using a Photoshop system according to the method previously described by Angioloni and Collar (2009), and results were expressed in accordance with the Hunter Lab colour space. The Photoshop (Ps Adobe Photoshop CS5 extended) system (L, a, b colour coordinates) was previously calibrated using colour sheets from Pantone® Formula Guide (Pantone, Inc., USA). Pantone colour sheets (for calibration) and bread slices (for colour measurement) images were acquired at 300 pixel resolution with a ScanJet II cx flatbed scanner (Hewlett-Packard, USA). The scanner was held in a black box in order to exclude the surrounding light. All measurements (three slices per sample) were made in triplicate. Hunter Lab colour space parameters from Minolta colorimeter were calculated from the calibration linear equation Colorimeter *vs* Photoshop (Angioloni and Collar, 2009). Parameters determined were L (L = 0 [black] and L = 100 [white]), a ($-a$ = greenness and $+a$ = redness), b ($-b$ = blueness and $+b$ = yellowness), ΔE – total colour difference, and WI – whiteness index (Collar & Angioloni, 2014b).

Crumb grain characteristics were assessed in bread slices using a digital image analysis system. Images were previously acquired with a ScanJet II cx flatbed scanner (Hewlett-Packard, Palo Alto, CA, USA) supported by a Deskscan II software. The analysis was performed on 40 mm × 40 mm squares taken from the centre of the images. Data were processed using SigmaScan Pro 5 (Jandel Corporation, San Rafael, CA, USA). The crumb grain features evaluated were mean cell area, cells/cm^2, cell/total area ratio, wall/total area ratio and crumb area/total cell ratio (Collar, Bollaín, & Angioloni, 2005). In addition, area distribution and cell number distribution were counted, and percentage of cell were calculated according to pre-set cell size ranges: <0.4 mm^2, 0.4–1.0 mm^2, 1.0–10 mm^2, 10–20 mm^2.

Sensory analysis of fresh breads was performed with a panel of eight trained judges (four males and four females aged 24–55) using semi structured scales, scored 1–10 in which extremes (lowest: 1; highest: 10) were described for each sensory attribute according to Setser (1996). Evaluated attributes were grouped into visual, textural and organoleptic characteristics (Collar et al., 2005).

Bread primary and secondary mechanical characteristics (TPA in a double compression cycle) of fresh breads were recorded in a TA-XTplus texture analyser (Stable Micro Systems) using a 25 mm diameter probe, a 5 kg load cell, 50% penetration depth and a 30 s gap between compressions on slices of 25 mm width (Armero & Collar, 1998). For textural measurements, three slices of two freshly made breads were used for each sample.

2.2.4. Statistical analysis

Multivariate analysis of variance and non linear multiple regression analysis of data were performed by using Statgraphics V.7.1 program (Bitstream, Cambridge, MN). Multiple range test (Fisher's least significant differences, LSD) for analytical variables was applied to know the difference between each pair of means.

3. Results and discussion

Bread is a complex viscoelastic porous matrix, composed mainly of gluten/protein, starch, lipids and water, whose sensory, technological and nutritional final quality is multifactor dependent. The technological viability and sensory acceptability of blended bread

Table 1
Proximate chemical and nutritional composition[a] of flours (per 100 g flour, d.b.) and breads (per 100 g fresh blended bread).

Sample Code[b]	Protein[e] (g)	Total dietary fibre (g)	Insoluble dietary fibre (g)	Soluble dietary fibre (g)	Fat (g)	Ash (g)	DC[c] (g)	Energy (kcal)[d]	Moisture (g)
Flours									
Wheat	14.13 ± 0.05b	2.19 ± 0.12a	1.20 ± 0.09a	0.99 ± 0.25a	1.56 ± 0.09a	0.63 ± 0.09a	81.70	–	14.32 ± 0.10c
Green pea	25.12 ± 0.04d	14.56 ± 0.95d	8.50 ± 0.15d	6.05 ± 0.27c	1.27 ± 0.15b	2.58 ± 0.12c	56.63	–	8.17 ± 0.09a
Buckwheat	19.71 ± 0.06c	13.52 ± 0.38c	6.58 ± 0.25b	6.93 ± 0.36d	3.44 ± 0.18c	2.05 ± 0.19b	61.16	–	11.70 ± 0.18b
Teff	13.05 ± 0.02a	12.19 ± 0.49b	7.40 ± 0.36c	4.80 ± 0.30b	5.06 ± 0.09d	2.21 ± 0.09b	66.97	–	11.90 ± 0.09b
Breads									
010	11.9 ± 0.1b	3.3 ± 0.3b	1.9 ± 0.31b	1.42 ± 0.39b	3.5 ± 0.2a	–	47.8	277	33.4 ± 0.8c
001	11.7 ± 0.2b	3.3 ± 0.2b	1.85 ± 0.34b	1.49 ± 0.30b	3.6 ± 0.4a	–	48.5	280	32.9 ± 0.6bc
011	12.3 ± 0.1c	3.9 ± 0.3c	2.18 ± 0.39b	1.72 ± 0.28b	3.6 ± 0.2a	–	47.7	281	32.5 ± 0.9b
000	11.6 ± 0.3b	2.9 ± 0.3b	1.63 ± 0.45b	1.24 ± 0.25b	3.6 ± 0.1a	–	49.6	283	32.3 ± 0.4bc
111	12.2 ± 0.2c	4.3 ± 0.5c	2.42 ± 0.39b	1.88 ± 0.44b	3.8 ± 0.3a	–	47.8	283	31.9 ± 0.3b
101	11.7 ± 0.1b	3.8 ± 0.2c	2.13 ± 0.28b	1.67 ± 0.37b	3.8 ± 0.2a	–	51.4	295	29.3 ± 0.8a
100	11.7 ± 0.1b	3.3 ± 0.2b	1.9 ± 0.24b	1.41 ± 0.31b	3.7 ± 0.4a	–	50.8	290	30.5 ± 0.9a
110	12.2 ± 0.2c	3.8 ± 0.1c	2.21 ± 0.41b	1.63 ± 0.45b	3.7 ± 0.1a	–	48.1	282	32.2 ± 0.4bc
Control	11.1 ± 0.1a	1.4 ± 0.2a	0.83 ± 0.36a	0.59 ± 0.32a	3.4 ± 0.2a	–	51.2	283	32.9 ± 0.6bc

[a] Mean values ± standard deviation. Within columns, values (mean of three replicates) with the same following letter do not differ significantly from each other ($p > 0.05$).
[b] Three digit bread sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in sample formulation.
[c] DC: digestible carbohydrates calculated by indirect determination: DC = 100 − [Moisture + Protein + Fat + Ash + Dietary Fibre].
[d] Energy conversion: protein × 4 kcal/g; fat × 9 kcal/g; digestible carbohydrates × 4 kcal/g; dietary fibre × 2 kcal/g.
[e] Conversion factor from *N* to protein = 6.25.

Table 2
Physical characteristics of blended breads.

Characteristic	Blended bread samples[a,b]								
	010	001	011	000	111	101	100	110	Control
Volume and Textural									
Specific volume, mL/g	1.9 ± 0.2a	2.0 ± 0.1a	2.0 ± 0.1a	2.9 ± 0.2b	2.1 ± 0.2a	2.3 ± 0.3a	2.1 ± 0.1a	2.1 ± 0.1a	3.1 ± 0.2b
Hardness, *N*	22.8 ± 2.7d	20.6 ± 1.4d	25.0 ± 0.5e	15.8 ± 1.7íb	25.3 ± 0.1e	17.8 ± 2.1bc	18.7 ± 1.0 cd	18.1 ± 0.1c	8.7 ± 0.2a
Cohesiveness	0.599 ± 0.008 cd	0.630 ± 0.016e	0.521 ± 0.015a	0.609 ± 0.018de	0.499 ± 0.021a	0.592 ± 0.002c	0.635 ± 0.016e	0.541 ± 0.005b	0.695 ± 0.004f
Springiness	0.879 ± 0.002b	0.896 ± 0.001d	0.842 ± 0.001a	0.908 ± 0.026e	0.898 ± 0.084abcde	0.878 ± 0.008bc	0.885 ± 0.001c	0.858 ± 0.024a	0.955 ± 0.001f
Colour									
L	71.9a	70.6a	69.6a	72.2a	68.6a	69.1a	71.5a	70.5a	74.6b
a	2.9b	3.1b	3.9c	2.7b	4.1c	3.3b	3.1b	3.3b	1.1a
b	15.3b	14.9b	16.0b	15.0b	16.1b	15.3b	15.3b	15.7b	12.0a
Whiteness Index	67.9b	66.9b	65.4a	68.3b	64.4a	65.3a	67.5b	66.4b	71.9c
ΔE	4.7	5.3	7.0	4.2	8.0	6.8	5.0	6.0	–
Crumb grain									
Mean cell area, mm^2	0.17a	0.21a	0.24b	0.24b	0.25b	0.31c	0.20a	0.21a	0.27c
Max area, mm^2	9.4a	20.0d	13.7b	7.3a	11.8b	12.9b	12.0b	9.1a	16.4c
Cell area distribution, %									
<0.4 mm^2	34	25	20	22	20	14	27	24	22
0.4–1.0 mm^2	24	24	16	20	18	15	26	23	15
1.0–10 mm^2	42	44	59	59	60	64	44	54	50
10–20 mm^2	0	7	5	0	2	7	3	0	12
Cell number distribution, %									
<0.4 mm^2	90	87	88	86	87	84	87	87	88
0.4–1.0 mm^2	6	8	6	8	7	8	8	8	6
1.0–10 mm^2	4	4	6	7	6	8	4	5	5
Cell density, cells/cm^2	151d	131c	136	115b	123c	105a	126c	135c	102a
Cell to wall area ratio	25:75	28:72	33:67	28:72	31:69	33:67	25:75	29:71	27:73

[a] Mean values ± standard deviation. Within rows, values (mean of three replicates) with the same following letter do not differ significantly from each other ($p > 0.05$).
[b] Three digit bread sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in sample formulation.

Table 3
Starch hydrolysis kinetics, expected Glycaemic Index and relevant starch nutritional fractions of blended breads.

Characteristic	Blended bread samples[a,b]								
	010	001	011	000	111	101	100	110	Control
Starch hydrolysis kinetics									
C_∞	74.3 ± 1.3c	65.7 ± 0.9a	74.1 ± 0.9c	73.6 ± 1.3c	71.2 ± 1.2b	75.8 ± 0.8c	75.2 ± 0.7c	75.7 ± 0.8c	81.0 ± 0.9d
k	0.0686 ± 0.0032c	0.0825 ± 0.0091c	0.0477 ± 0.0013a	0.0797 ± 0.0062c	0.1106 ± 0.0085d	0.0599 ± 0.0031b	0.0593 ± 0.0029b	0.0821 ± 0.0079c	0.0720 ± 0.0081c
H_{90}, %	74.2 ± 1.1c	65.7 ± 1.2a	73.1 ± 0.8c	73.6 ± 0.8c	71.2 ± 0.6b	75.4 ± 0.9c	74.9 ± 0.9c	75.6 ± 0.6c	81.0 ± 1.1d
HI, %	90 ± 3b	81 ± 4a	92 ± 4b	91 ± 3b	88 ± 2b	94 ± 3b	93 ± 3b	93 ± 3b	100 ± 1c
eGI	86 ± 4b	78 ± 2a	86 ± b	87 ± 4b	84 ± 3b	89 ± 3b	88 ± 3b	89 ± 3b	94 ± 1c
Starch nutritional fractions (per 100 g bread, as is)									
RDS, g	57.8 ± 0.9b	58.4 ± 1.1b,c	56.4 ± 1.0b	56.2 ± 1.1a	60.0 ± 1.2c	59.6 ± 1.5b,c	62.5 ± 1.4c	54.3 ± 1.0a	68.5 ± 1.1d
SDS, g	8.4 ± 1.1c	5.4 ± 0.7b	17.5 ± e	5.7 ± 0.6b	2.3 ± 0.6a	11.8 ± d	12.6 ± d	4.5 ± 1.1b	7.5 ± 1.0c
DS, g	66.2	63.8	73.9	61.9	62.3	71.4	75.2	58.8	76.0
RS, g	2.4 ± 0.1b	2.7 ± 0.3b	2.9 ± 0.5b	2.8 ± 0.4b	2.5 ± 0.2b	2.3 ± 0.2b	2.5 ± 0.2b	2.2 ± 0.2a,b	1.8 ± 0.3a
TS, g	69	67	77	65	65	74	78	61	78

[a] Mean values ± standard deviation. Within rows, values (mean of three replicates) with the same following letter do not differ significantly from each other ($p > 0.05$).

[b] Three digit bread sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in sample formulation. RDS: rapidly digestible starch, SDS: slowly digestibly starch, eGI: expected glycaemic index, DS: digestible starch, RS: resistant starch, TS: total starch. C_∞: equilibrium concentration, k: kinetic constant, H_{90}: total starch hydrolysis at 90 min, *HI*: hydrolysis index. A first order kinetic equation [$C = C_\infty(1 - e^{-kt})$] was applied to describe the kinetics of starch hydrolysis C concentration at t time; C_∞ equilibrium concentration; k kinetic constant; t time. TS= DS + RS; DS= RDS + SDS.

matrices are explored first, prior to assess the "in vitro" starch hydrolysis kinetics, the relevant starch nutritional fractions and the anti-radical activity of blended breads *vs* wheat matrices.

3.1. Chemical and nutritional composition of single flours (WT, T, GP and BW) and quaternary blended breads

Single WT, T, GP and BW flours exhibited different chemical and nutritional profiles that resulted in quantitative different bread patterns regarding both chemical and nutritional composition (Table 1). Comparatively to wheat flour (T, GP and BW *vs* WT, per 100 g flour basis, d.b.), non-wheat flours, accounted for much higher protein with the exception of teff (13.05%, 25.12%, 19.71% *vs* 14.13%), similar or higher fat (5.06%, 1.27%, 3.44% BW, *vs* 1.56%), and ash (2.05–2.58% *vs* 0.63%) contents, and much higher total dietary fibre (12.19–14.56% *vs* 1.4%), and significantly lower digestive carbohydrates (57–67% *vs* 82%). Data are compatible with a superior nutritional profile for ancient cereals (Angioloni & Collar, 2011a), pseudocereals (Collar & Angioloni, 2014a) and legumes (Angioloni & Collar, 2012), as reported earlier.

Quaternary blended breads obtained by replacement of WT flour from 22.5% to 45% with mixed T, GP and BW flours, explicited (per 100 g fresh bread) compared to WT bread counterparts, similar protein (11.6–12.2% *vs* 11.1%) and fat (3.5–3.8% *vs* 3.4%) contents, but much higher total dietary fibre (2.9–4.3% *vs* 1.4%), insoluble (1.63–2.42% *vs* 0.83%) and soluble (1.2–1.9% *vs* 0.59%) dietary fibre sub-fractions, especially for bread samples with higher level of WT replacement (011, 111, and 110) by high-fibre non-wheat flours (Table 1). Blended bread samples contain about double to triple the fibre of the regular white bread, so that breads can be labelled as source of fibre (≥3 g DF/100 g food) according to Nutritional Claims for Dietary Fibre foods (Regulation (EC) No. 1924/2006).

3.2. Physical and sensory characteristics of blended breads vs wheat matrices

Bread crumb is a typical viscoelastic biopolymer foam system with cellular structure composed mainly of gluten/protein, starch, and water, and minor constituents such as lipids and non-starch polysaccharides in presence of other ingredients, additives and technological aids. Major breadmaking steps leading to bread from flour, significantly change dough viscoelasticity. In this research, 45% of WT replacement by combinations of non-gluten forming flours-T, GP, and BW was previously established as the maximum level of substitution that did not significantly hinder dough handling ability (data not shown) in terms of stickiness (<1 N), dough hardness (<85 N), cohesiveness (>0.3), adhesiveness (<160 N s) and springiness (>0.6). The simultaneous addition of T, GP and BW significantly decreased the bread volume (from 3.1 mL/g to 1.9–2.3 ml/g for most samples except for the bread with lowest level of WT replacement (000) that develops similar volume (2.9 mL/g) to control bread (Table 2). With respect to refined WT flour bread types, lower volume blended breads encompassed harder (15.8–25.3 N *vs* 8.7 N) and low cohesive crumbs (0.499–0.630 *vs* 0.695) with poorer springiness (0.858–0.908 *vs* 0.955) particularly for medium-high replaced blends (Table 2). Blended breads are all visibly different from control WT breads ($\Delta E \geq 3$) in crumb colour features, characterized by lower lightness L and Whiteness Index, more red (a positive) and yellow (b positive) colour tri-stimulus values, with no significant differences among mixed samples. Crumb pore uniformity and crumb grain structure were not significantly affected, though in the non-wheat flour supplemented breads the crumb quality slightly decreased *vs* control breads in terms of lower average cell size for most samples (0.17–0.25 mm^2 *vs* 0.27 mm^2) and higher cell density (up to 150 cells/cm^2 *vs* 100 cells/cm^2) with variable cell to wall area ratio

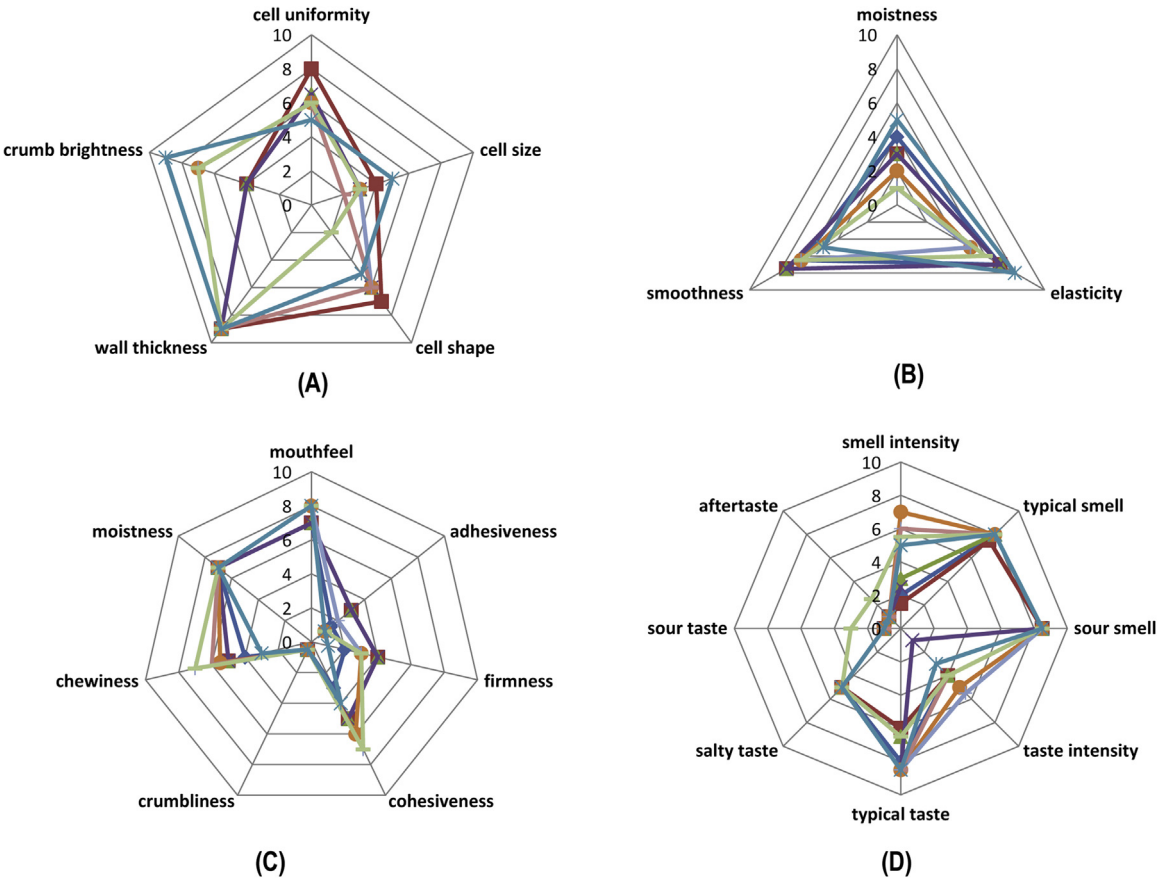

Fig. 1. Spider graphs of visual (A), tactil (B), biting (C), and flavour (D) sensory attributes of control and blended wheat-based breads. Three digit sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in samples.

(25:75–33:67 *vs* 27:73). Cell area and cell number distribution evidenced that 42–64% of total cell area is occupied by alveoli sized 1.0–10 mm^2, while at about 90% of cells sized ≤0.4 mm^2 (Table 2). Blended breads were scored significantly higher than refined WT control breads in both taste and smell intensity, tactil smoothness, visual cell uniformity and round shaped cells, and biting firmness, adhesiveness, cohesiveness, and chewiness (Fig. 1). In addition, blended breads deserved similar ratings as compared to WT control breads concerning visual wall thickness, biting mouth-feel, and typical smell.

3.3. *"In vitro" starch hydrolysis kinetics and anti-radical activity of blended breads vs wheat matrices*

Taking into account the nutritional added value derived from non-wheat flour incorporation into wheat bread formulation, especially dietary fibre (Table 1), and considering that blended matrices were technologically viable (Table 2) and sensorially scored higher than wheat breads in most attributes (Fig. 1), in vitro starch hydrolysis kinetics (Fig. 2) and relevant starch nutritional fractions (Tables 3–5), bioaccessible polyphenols, and anti-radical activity (Table 6) were determined.

3.3.1. *Starch hydrolysis kinetics*

The in vitro determination of carbohydrate digestibility to predict the glycaemic response of complex foods is of great interest since in vivo evaluations are invasive, labour-intensive and costly (Lehmann & Robin, 2007). In cereal products, the starch gelatinization extent, which is mainly controlled by the moisture level (Primo-Martin, Van Nicuwenhuijzen, Hamer, & Van Vliet, 2007)

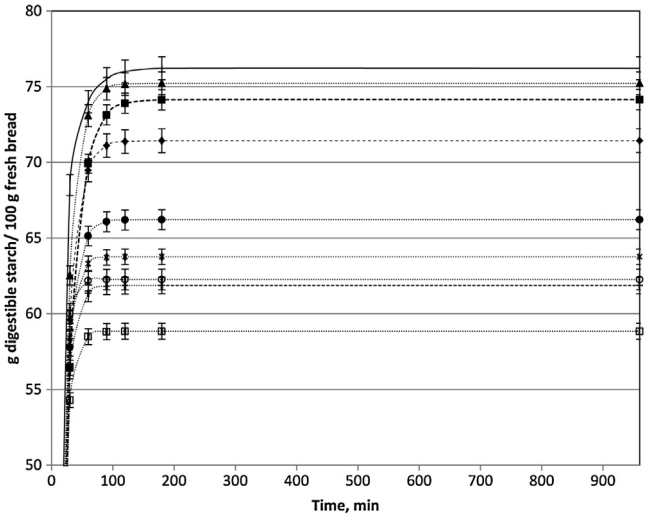

Fig. 2. Digestible starch hydrolysis kinetic curves (mean of three replicates) of control and blended wheat-based breads. A first order kinetic equation [$C = C_\infty(1 - e^{-kt})$] was applied to describe the kinetics of digestible starch hydrolysis. Three digit sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in sample formulation.

and the cooking time and temperature influences the formation of SDS (Englyst et al., 2003). In addition, amylose can complex with lipids hindering attack by hydrolytic enzymes more than is free carbohydrate (Nebesny, Rosicka, & Tkaczyk, 2004). Characteristics such as solubility and the presence of fibre, fat and protein all contribute to the rate of digestion (Dona, Pages, Gilbert, & Kuchel,

Table 4
Single significant effects ($p < 0.05$) of rate of wheat flour replacement by low (0) and high (1) dose of teff, green pea and buckwheat on starch hydrolysis kinetic parameters and relevant starch nutritional fractions of blended breads (per 100 g bread, as is).

Parameter	Level	Overall mean	Teff	$p < 0.05$	Green pea	$p < 0.05$	Buckwheat	$p < 0.05$
C_∞	0	73.2	71.9 ± 1.8	a	ns		74.7 ± 1.8	b
	1		74.5 ± 1.8	b			71.7 ± 1.8	a
k	0	0.0738	0.0696 ± 0.0073	a	0.0704 ± 0.0073	a	ns	
	1		0.0780 ± 0.0073	b	0.0773 ± 0.0073	b		
H_{90}	0	73.0	71.6 ± 1.7	a	ns		74.6 ± 1.7	b
	1		74.3 ± 1.7	b			71.4 ± 1.7	a
RDS (%)	0	58.2	57.2 ± 1.7	a	59.2 ± 1.7	b	ns	
	1		59.1 ± 1.7	b	57.1 ± 1.7	a		
SDS (%)	0	8.5	9.2 ± 1.5	b	ns		7.8 ± 1.5	a
	1		7.8 ± 1.5	a			9.2 ± 1.5	b
DS (%)	0	66.7	66.4 ± 0.2	a	68.0 ± 0.2	b	65.5 ± 0.2	a
	1		66.9 ± 0.2	b	65.3 ± 0.2	a	67.8 ± 0.2	b
RS (%)	0	2.6	2.7 ± 0.0	b	2.6 ± 0.0	b	2.5 ± 0.0	a
	1		2.4 ± 0.0	a	2.5 ± 0.0	a	2.6 ± 0.0	b

ns: non-significant, $p > 0.05$. For each parameter, within rows, values (mean of three replicates ± standard error) with the same following letter do not differ significantly from each other ($p > 0.05$).

2011). Through the process of retrogradation, gelatinized or solubilized starch can be transformed from an unstructured into a more ordered or crystalline state. This large physical change causes heat processed starchy foods to harden or become stale as they spontaneously approach a metastable state of lower free energy. This has been reported to decrease the GI value, due to an increased resistance to amylase (Chung, Lim, & Lim, 2006). Starch hydrolysis that follows first order kinetics (Frei, Siddhuraju, & Becker, 2003), proceeded at different rate and extent for blended samples compared to the WT flour counterparts (Table 3). The steady state kinetic constant (k) of amylolysis that has been proposed as a reliable index of the inherent susceptibility of flour starches to amylase hydrolysis (Frei et al., 2003) ranged from 0.0477 (011) to 0.1106 (111) in blended samples *vs* 0.0720 in control breads, evidencing from slower to faster hydrolysis kinetics, respectively, depending on bread formulation. C_∞ that corresponds to the equilibrium percentage of starch hydrolysed after 16 h, varied from 65.7 (001) to 74–76 for all the remaining blended breads except for the highly replaced sample 111 that showed intermediate values (71.2). Control breads underwent up to 81% of starch hydrolysis, so that all the non-wheat replaced samples showed a lower extent of starch hydrolysis despite at 90 min of reaction time, the equilibrium is

Table 5
Second order significant interactions ($p < 0.05$) of rate of wheat flour replacement by low (0) and high (1) dose of teff (T), green pea (GP) and buckwheat (BW) – design factors on starch hydrolysis kinetic parameters and relevant starch nutritional fractions of blended breads (per 100 g bread, as is).

Parameter	Level	Overall mean	T × GP	$p < 0.05$	T × BW	$p < 0.05$	GP × BW	$p < 0.05$
C_∞	00	73.2	69.7 ± 1.8	a	ns		ns	
	01		74.2 ± 1.8	bc				
	10		75.5 ± 1.8	c				
	11		73.4 ± 1.8	bc				
k	00	0.0738	0.0811 ± 0.073	bc	0.0742 ± 0.073	b	ns	
	01		0.0582 ± 0.073	a	0.0651 ± 0.073	a		
	10		0.0596 ± 0.073	a	0.0707 ± 0.073	ab		
	11		0.0964 ± 0.073	c	0.0853 ± 0.073	c		
H_{90}	00	73.0	69.6 ± 1.7	a	73.9 ± 1.7	b	ns	
	01		73.6 ± 1.7	bc	69.4 ± 1.7	a		
	10		75.2 ± 1.7	bc	75.3 ± 1.7	b		
	11		73.4 ± 1.7	bc	73.3 ± 1.7	b		
RDS (%)	00	58.2	57.3 ± 1.7	a	ns		59.4 ± 1.7	b
	01		57.11 ± 1.7	a			59.0 ± 1.7	b
	10		61.07 ± 1.7	b			56.0 ± 1.7	a
	11		57.15 ± 1.7	a			58.2 ± 1.7	b
SDS (%)	00	8.5	5.5 ± 1.5	a	7.0 ± 1.5	a	9.1 ± 1.5	b
	01		13.0 ± 1.5	b	11.4 ± 1.5	b	8.6 ± 1.5	ab
	10		12.2 ± 1.5	b	8.6 ± 1.5	a	6.5 ± 1.5	a
	11		3.4 ± 1.5	a	7.0 ± 1.5	a	9.9 ± 1.5	b
DS (%)	00	66.7	62.8 ± 0.2	b	64.0 ± 0.2	a	68.5 ± 0.2	d
	01		70.1 ± 0.2	c	68.8 ± 0.2	c	67.6 ± 0.2	b
	10		73.3 ± 0.2	d	67.0 ± 0.2	b	62.5 ± 0.2	a
	11		60.6 ± 0.2	a	66.8 ± 0.2	b	68.1 ± 0.2	c
RS (%)	00	2.6	2.8 ± 0.02	b	2.6 ± 0.0	c	2.6 ± 0.0	c
	01		2.7 ± 0.02	b	2.8 ± 0.0	d	2.5 ± 0.0	b
	10		2.4 ± 0.02	a	2.3 ± 0.0	a	2.3 ± 0.0	a
	11		2.4 ± 0.02	a	2.4 ± 0.0	b	2.7 ± 0.2	d

ns: non-significant, $p > 0.05$. For each parameter, within rows, values (mean of three replicates ± standard error) with the same following letter do not differ significantly from each other ($p > 0.05$). RDS: rapidly digestible starch, SDS: slowly digestibly starch, eGI: expected glycaemic index, DS: digestible starch, RS: resistant starch, C_∞: equilibrium concentration, k: kinetic constant, H_{90}: total starch hydrolysis at 90 min, HI: hydrolysis index. A first order kinetic equation [$C = C_\infty(1 - e^{-kt})$] was applied to describe the kinetics of starch hydrolysis.

Table 6
Bioaccessible polyphenols and anti-radical activity of blended breads.

Sample	Bioaccessible polyphenols[a]			Anti-radical activity[c]	
	mg gallic acid/100 g flour, as is	Δ with respect to flour content, %	Δ with respect to wheat bread content, %	Remaining μmol DPPH at steady state	%
Flours					
Wheat	380 ± 15c	–	–	0.1310 ± 0.010b	74.2a
Teff	303 ± 15a	–	–	0.1407 ± 0.009b	72.3a
Green pea	343 ± 17b	–	–	0.1451 ± 0.010b	71.4a
Buckwheat	365 ± 12b,c	–	–	0.0604 ± 0.008a	88.1b
Breads[b]					
010	442 ± 10b	20	7	0.3150 ± 0.016b	37.9
001	464 ± 11c	26	12	0.3009 ± 0.018b	40.7
011	482 ± 13c	31	16	0.2715 ± 0.017a	46.5
000	466 ± 15c	26	13	0.3102 ± 0.015b	38.8
111	464 ± 15c	28	12	0.2619 ± 0.019a	48.4
101	478 ± 18c	31	16	0.2655 ± 0.020a	47.7
100	443 ± 10b	30	7	0.2816 ± 0.016a	44.5
110	416 ± 12a	15	1	0.2876 ± 0.018a	43.3
Control	414 ± 15a	9	–	0.3427 ± 0.013c	32.4

[a] Mean values ± standard deviation. Within columns, values (mean of three replicates) with the same following letter do not differ significantly from each other ($p > 0.05$).
[b] Three digit bread sample code refers to low (0) and high (1) wheat flour replacement by teff: green pea: buckwheat flours in sample formulation.
[c] Corresponding to 12 mg flour or freeze-dried bread that consumed DPPH when 0.494 μmol of the free radical are initially available to react. The plateau was decided at 150 min of reaction.

already reached in almost all blended samples (Fig. 2) with similar values for C_∞ and H_{90} except for sample 011 (Table 3). Calculation of the samples hydrolysis indices (HI%), the proportion of flour starch that is theoretically digestible, by dividing the area under the hydrolysis curve of each blended sample by the corresponding area of the control sample (Table 3) pointed out the lowest value in sample 001 in good accordance with the lowest equilibrium percentage of starch hydrolyzed C_∞, and hence leading to the lowest eGI (78).

Multiple analysis of variance (MANOVA) provided information on the significant ($p < 0.05$) single and/or interactive effects of the rate (low and high) of wheat flour replacement by non-wheat flours T, GP and BW in blended breads on starch hydrolysis kinetics (Table 4). Increased doses of single T and BW led to opposite changes in starch hydrolysis kinetic parameters: single T encompassed higher C_∞ (71.9–74.5), k (0.0696–0.0780) and H_{90} (71.6–74.3), while BW lowered both C_∞ (74.7–71.7), and H_{90} (74.6–71.4) values, leading to faster and slower starch hydrolysis kinetics, respectively (Table 4). Simultaneous presence of both T and BW at lower (0) and higher (1) dose, respectively, slowed down hydrolysis kinetics giving the lowest k (0.0651) and H_{90} (69.4) values through a significant antagonistic effect (Table 5). The high protein content of BW flour *vs* T flour (Table 1) may obstruct enzyme attack by hindering enzyme accessibility due to protein–starch interactions in hydrated blended flours (Dona et al., 2010).

3.3.2. Relevant starch nutritional fractions

Categorized starch fractions based on the rate of glucose released and its absorption in the gastrointestinal tract include RDS, SDS and RS, defined here as the three consecutive nutritional fractions divided by reaction time when "in vitro" starch digestion takes place (Table 3, Fig. 2). RDS is the fraction of starch granules that cause a rapid increase in blood glucose concentration after ingestion of carbohydrates. The fraction of starch that is said to be RDS in vitro is defined as the amount of starch digested in the first 20 min of a standard digestion reaction mixture (Englyst et al., 1992). Although RDS is defined by experimental analysis of digestion in vitro, it has been reported that the rate of starch conversion to sugar follows similar kinetics in the human digestive system (Dona, Pages, Gilbert, & Kuchel, 2010). Values for RDS (g/100 g bread, as is) were all lower in blended breads (from 54.3% – 110 to 62.5% – 100) than in control WT (68.5%) breads (Table 3). In fact, increased T dose provided significantly ($p < 0.05$) higher RDS values (57.2% – 0 to 59.1% – 1), while GP increased dose led to lower RDS fraction (59.2% – 0 to 57.1% – 1) (Table 4). Simultaneous presence of both flours provided significant interactions in such a way that at higher levels of WT replacement by T (15%), higher amounts of GP (15%) are needed to keep RDS fraction at about 57%.

SDS is the fraction of starch that is digested slowly but completely in the human small intestine. From studies of in vitro digestion (Dona et al., 2010), it has been observed that there is a transition in the smoothness of the progress curves of reducing sugar production from RDS to SDS in good agreement with profiles in Fig. 2. SDS is defined as the starch that is digested after the RDS but in no longer than 120 min under standard conditions of substrate and enzyme concentration (Englyst et al., 1992). Blended breads explicited a wide range of SDS (g/100 g bread, as is) values ranging from 2.3% (111) to 17.5% (011), *vs* control breads that contained intermediate amounts (7.5%) (Table 3). Higher T presence decreased SDS formation (from 9.2% to 7.8%), while higher dose of BW favoured SDS accumulation (7.8–9.2%) (Table 4). Maximum SDS values 11.5–13% were achieved in breads when the pairs T/GP and/or T/BW do not exceed 22.5% of WT replacement in blended bread formulations (Table 5).

The fraction of starch that escapes digestion in the small intestine, and may be subject to bacterial fermentation in the large intestine, is termed RS. Blended breads contained similar amounts of RS (g/100 g bread, as is), regardless the formulation (from 2.2% to 2.9%), and in general higher than the content found in control breads (1.8%) (Table 3). Increased dose of either T or GP slightly decreased RS, while higher BW inclusion slightly increased RS (Table 4). The highest RS in blended breads was observed for the binary T/GP 00 and T/BW 01 including 7.5% T, 7.5% GP, and 15% BW (Table 5). Simultaneous lower rapidly digestible starch (57.1%) and higher slowly digestible starch (12.9%) and resistant starch (2.8%) contents (g per 100 g fresh bread), considered suitable nutritional trends for dietary starch fractions (Englyst et al., 2003), were met by the blend formulated 7.5% T, 15% GP, 15% BK (sample 011). The associated mixture that replaced 37.5% WT, showed a rather lower extent and slower rate of starch hydrolysis (Table 3, Fig. 2) with medium-low values for C_∞, and H_{90}, and lowest k, and intermediate expected Glycaemic Index (86). The incorporation of non-wheat flours into wheat bread formulation seems to reduce starch hydrolysis, probably because of their lower

starch and higher fibre and protein contents, especially for GP and BW flours (Table 1). The reduced rate and overall reduced starch digestibility of blended breads may be affected by the high content of viscous soluble dietary fibre components in legume matrices (Angioloni & Collar, 2012). In addition, high protein content particularly for GP flour (Table 1) can promote starch–protein interactions restricting enzyme attack as pointed out for lentils (Chung et al., 2008).

3.4. Bioaccessible polyphenols and anti-radical activity of blended breads vs wheat matrices

Bioaccessible polyphenol content (mg gallic acid/100 g flour, as is) of blended breads ranged from 416 mg to 482 mg, and were 1–16% larger ($p < 0.05$) than bioaccessible polyphenols determined in WT breads (414 mg), and higher with no exception than the content observed in flours (303–380 mg) (Table 6). Accumulation of bioaccessible polyphenols from flour to bread varied from 9% in control WT bread to 15–31% in blended breads (Table 6), in good accordance with previous results on multigrain blended breads (Angioloni & Collar, 2011b). Mechanical input during mixing and thermal treatment during baking may induce depolymerization of constituents, mainly fibre, and hence may favour bread accessibility to enzyme attack and the subsequent release of fibre-associated polyphenols. In addition, Maillard reactions that occur during bread baking can result in the synthesis of substances with antioxidant properties (Vogrincic, Timoracka, Melichacova, Vollmannova, & Kreft, 2010). Anti-radical activity was determined by the extent of the reduction of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical. Results expressed correspond to the remaining unreacted DPPH$^{\bullet}$ amount when 0.494 μmol of the free radical are initially available to react with methanol/acetone/water extracts from 12 mg flour or freeze-dried bread. The plateau (steady state) was decided at 150 min of reaction in all cases. Higher anti-radical activity for flours (71–88%) than for breads (32–48%) was observed (Table 6). It should be noticed the high anti-radical activity of BW flours (88%) as pointed out earlier (Angioloni & Collar, 2011b) that resulted in a concomitant higher anti-radical activity in blended breads with 15% of BW replacement (001, 011, 111, 101) (Table 6). The observation, can be ascribed to the changes occurring over breadmaking steps in terms of (a) the lipoxygenase catalyzed oxidation of polyunsaturated fatty acids that can lead to oxidation of phenolic compounds (particularly for the cinnamic acid derivatives) by coupled reaction due to substantial incorporation of oxygen in the dough during mixing (Eyoum et al., 2003), and (b) losses or degradation of phenolic compounds during baking (Angioloni & Collar, 2011b) as a result of the known susceptibility of phenolic acids and flavonoids to heat. In addition, it has been stated that dietary fibre and other compounds of proven resistance to the action of digestive enzymes, such as resistant starch, resistant protein, Maillard compounds and other associated compounds, may reduce the bread phenol bioaccessibility (Saura-Calixto, García-Alonso, Goñi, & Bravo, 2000). This is not the net result in this research, but analogous speculation can be applied to the loss of anti-radical activity from flours to breads, since non-wheat flours all have (Table 1) high dietary fibre content (>12%) and most of them, high protein content (>20%, GP and BW).

4. Conclusions

Wheat flour replacement from 22.5% up to 45% by incorporation of ternary blends of T, GP and BW flours provided technologically viable and acceptable sensory rated multigrain breads with superior nutritional value compared to the 100% wheat flour (WT) counterparts. Blended breads exhibited superior nutritional composition, larger amounts of bioaccessible polyphenols, higher anti-radical activity, and lower and slower starch digestibility, which extent was formulation dependent. Suitable nutritional trends for dietary starch fractions in terms of simultaneous low RDS (57.1%) and high SDS (12.9%) and RS (2.8%) contents (per 100 g fresh bread), were met by blends formulated 7.5% T, 15% GP, 15% BK, that replaced 37.5% WT. The associated breads showed a rather low extent and slower rate of starch hydrolysis with the medium-low values for C_{∞}, and H_{90}, lowest k, and intermediate eGI (86). Low and slow starch digestibility can be ascribed to the high protein and dietary fibre contents of non-wheat flours (especially GP and BW) that favour starch–protein interactions and constitute a physical interference in bread matrices, respectively, obstructing and delaying enzyme attack and subsequent starch digestion. All multigrain breads can be labelled as source of dietary fibre (≥3 g dietary fibre/100 g bread). The formulation based on WT:T:GP:BW flours, 62.5:7.5:15:15 fulfilled from 25% (men) to 40% (women) of dietary fibre, and from 54% (men) to 66% (women) of protein daily requirements (Otten, Hellwig, & Meyers, 2006), when a daily consumption of 250 g of bread is accomplished, following the WHO bread intake recommendation.

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