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Functional Breads Enriched With Amaranth Flour and Grape Pomace Peels: Protein Fraction Bioaccessibility and Antioxidant Properties

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ABSTRACT

The overall objective of this work was to evaluate the effect of the addition of amaranth flour (***AF***) and/or red grape pomace peels (***GP***) on the protein fraction bioaccessibility and antioxidant activity of phosphate buffer soluble fractions. Breads containing 20% w/w ***AF***, 5% w/w ***GP***, or a mixture of both, which had shown acceptable sensory characteristics in previous studies, were subjected to simulated gastrointestinal digestion (SGID) using the INFOGEST consensus protocol. The original and digested products were characterized, focusing on the peptide/polypeptide fractions and their in vitro antioxidant properties. ***AF***-containing bread showed the presence of high molecular weight compounds in the SDS-PAGE profiles, probably due to albumin/gluten protein interaction, and small increases in phosphate buffer (35 mmol. L^{-1}, pH 7.8) solubility with a decrease in molecules > 10 kDa. While the presence of GP also modified the electrophoretic profile, appearing some new bands (< 45 and < 20 kDa) and also caused a diminution of < 10 kDa PBS-soluble molecules. After SGID, the proteolysis degree (TNBS method) was lower for ***GP***-containing breads (17%–18% vs. 21%–25%). Digests from breads added with ***AF*** and/or ***GP*** also presented high molecular compounds. The presence of ***AF*** and/or ***GP*** improved the antioxidant potency (IC_{50} values for ORAC and ABTS scavenging decreased by half or even more) for breads before SGID. After SGID, the antioxidant potencies of the buffer soluble fraction increased much more without effect due to the addition of ***AF*** or ***GP***. Amaranth protein appears to play a protective role over polyphenols during gastrointestinal digestion.

1 | Introduction

Wheat bread is a staple food, and refined wheat flour (***WF***) is the most commonly used basic ingredient for this product. However, from a nutritional perspective, this flour has poor protein quality due to its low levels of essential amino acids such as lysine and threonine, and a low content of fiber and other bioactive components. Based on this, many studies attempt to improve the nutritional characteristics of wheat flour by incorporating new functional ingredients to develop healthier and more nutritious breads. Nutritionally improved bread should have a lower glycemic index than regular white bread, be a source of good-quality protein, and contain dietary fiber, micronutrients, and antioxidants. In addition to this, the challenge is to achieve adequate technological quality and sensory properties for high consumer acceptance of these products (Miranda-Ramos et al. 2019; Hoehnel et al. 2020). Among the used alternatives, we can mention the partial replacement of wheat flour with legume flour (Gallo et al. 2021) or pseudo-cereals (Miranda-Ramos et al. 2019; Sanz-Penella

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et al. 2013) that can provide proteins that complete the nutritional profile of wheat, increasing their nutritional quality, as well as dietary fiber and other bioactive components, including antioxidants.

Grape pomace is the main solid organic by-product of the wine industry, and its main components are seeds, skin, stems, and pulp residues. Many phenolic compounds have been identified in grape pomace, the most abundant of which are anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols, flavonols, and stilbenes. The antioxidant properties of these components have been extensively studied (Beres et al. 2017). Previous work has postulated that white wheat bread could be a suitable matrix for the addition of grape pomace, providing fiber and antioxidant compounds. Although it has been postulated that the inclusion of a high-fiber ingredient could strengthen the structure of bread dough, reducing extensibility and affecting bread volume and texture, previous work has shown that the inclusion of 5% pomace (red grape, after fermentation) significantly influenced aroma, flavor, and appearance, but no significant impact was observed on the overall acceptability of the products (Tolve et al. 2021).

In the first part of the present study, breads fortified with 20% w/w amaranth flour (***AF***) and/or 5% w/w non-fermented red grape pomace skin (***GP***) were prepared. They presented good physicochemical properties and improved nutritional quality (Rodríguez et al. 2024). Thus, in an 80/20 ***WF/AF*** mixture, the protein digestibility-corrected amino acid score of lysine would be increased by about 12% compared to ***WF***. Also, the total amount of dietary fiber changed due to the addition of ***AF*** and/or ***GP***, with an increment of the insoluble fiber proportion, which can have healthy effects. In addition, ***AF*** introduced various phenolic compounds, such as (−)-epigallocatechin, flavanones, phloridzin, and stilbenes, while the addition of ***GP*** provided anthocyanins, caftaric acid, and a higher concentration of (−)-epigallocatechin, increasing antioxidant properties. An important proportion of these phenolic compounds in the breads would remain or even increase their concentration by release after gastrointestinal digestion, being bioaccessible to be further absorbed or transformed by the microbiota in the colon (Rodríguez et al. 2024). In addition to the aforementioned improvements, another aspect to be studied in these breads is the contribution of water-soluble antioxidant compounds, primarily peptides. This kind of property has been previously demonstrated in amaranth protein isolate and flour (Rodríguez et al. 2020). The objective of this study was to molecularly characterize the polypeptide/peptide fraction of the original products and their gastrointestinal digests, and to study their antioxidant properties, evaluating the effect of the addition of an American protein source, such as amaranth and a by-product of the wine industry.

2 | Experimental Procedures

2.1 | Chemicals

Alfa-amylase from Bacillus subtilis (10,070, 57.4 U/mg), aprotinin, blue dextran, bovine bile salts B3883, hippuric acid, porcine gastric mucosa pepsin P6887 (3200–4500 U/mg), porcine pancreatin 4XUSP P1750, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and 2,4,6-trinitrobencenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other reagents were of analytical grade.

2.2 | Preparation of Breads

The study used *Amaranthus mantegazzianus* seeds from Río Cuarto National University (Córdoba, Argentina). ***AF*** was obtained by grinding unpeeled seeds in a Udy cyclone mill (1 mm mesh). Unfermented Cabernet Sauvignon grape pomace from a sparkling wine production (Finca Lignac, San Rafael, Mendoza, Argentina) was dried in an oven at 40°C; the seeds were manually separated, and the remainder was ground and sieved to obtain a grape peel powder ***GP*** (particle size < 0.177 mm). Salt, sucrose, and dry brewer's yeast were obtained from a local market.

The bread formulation (320 g of composite flours, 210 mL of tap water, 3 g of salt, 15 g of sucrose, and 3.5 g of dry brewer's yeast) was according to Rodríguez et al. (2024). The following breads were studied: Control bread (***C***): 100% ***WF***; bread based on a mixture of ***WF/AF*** (80/20 w/w) (***A20***); bread based on a mixture of ***WF/GP*** (95/5 w/w) (***GP5***); bread based on a mixture of ***WF/AF/GP*** (75/20/5 w/w) (***A20GP5***). Proximal composition, physicochemical evaluation, and sensory properties of breads were evaluated previously and are available at Rodríguez et al. (2024).

2.3 | Simulation of Gastrointestinal Digestion (SGID)

The COST-INFOGEST static SGID protocol (Brodkorb et al. 2019) was used to digest ***C***, **A20, GP5**, and ***A20GP5*** (50 g) according to Rodríguez et al. (2024). The pH of the gastric and intestinal phases was monitored. The gastric phase was stopped by neutralizing the pH. In the case of complete digestion, samples that had undergone gastric and then intestinal digestion were then heated at 80°C for 10 min to inactivate the enzymes and stop hydrolysis. Control non-hydrolyzed samples (***Cc***, ***A20c***, ***GP5c***, and A20GP5c) were obtained using oral, gastric, and intestinal fluids without enzymes. A reagent blank (b_R, no sample) was also prepared. The obtained gastrointestinal digestion products (***Cd***, ***A20d***, ***GP5d***, and ***A20GP5d***) were frozen or freeze-dried. The total protein content in the digests was determined by the micro-Kjeldahl method followed by a colorimetric determination (Nkonge and Balance 1982).

2.4 | Protein Content and Solubility

Suspensions (20–125 mg mL^{-1}) of freeze-dried samples in 35 mmol L^{-1} phosphate buffer (pH: 7.8) were prepared (agitation at 500 rpm, 37°C, 1 h, Eppendorf Thermomixer) and then centrifuged (10,000 × g, 10 min, room temperature, Spectrafuge 24D, Lab Net International), preserving the supernatant. Soluble-protein concentration was determined by the Lowry method (Lowry et al. 1951). Due to interference from high phenolic

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content in the Lowry assay, soluble protein in GP was quantified using the Kjeldahl method (AOAC 2000). Official Method 955.04), using a nitrogen-to-protein conversion factor of 6.25 (Cebrián et al. 2024).

2.5 | Protein Hydrolysis Degree

The protein hydrolysis degree (HD) was measured in the digests using the TNBS method (Adler-Nissen 1979) according to (Rodríguez et al. 2020). HD was calculated as follows: $HD\% = 100 \times ([-NH_2]_h\text{-}[-NH_2]_0)/([-NH_2]_\infty\text{-}[-NH_2]_0)$; where $[-NH_2]$ indicates the concentration of free amino groups in the non-hydrolyzed (0) or the hydrolyzed samples (h). $[-NH_2]_\infty$ was estimated as $1/M_{aa} \times (1 + f_{Lys}) \times C_{prot}$, where M_{aa} is the average molecular weight of amino acids in proteins, f_{Lys} is the proportion of lysine in these proteins, and C_{prot} is the protein concentration; the lysine fractions were obtained from amino acid composition data for wheat and amaranth proteins (Bressani 1994; Kasarda et al. 1978; Bressani et al. 1993) and considering their proportion. ***GP*** was not considered in this calculation, given its low proportion in the formulations and the lack of amino acid composition data.

2.6 | Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoretic profiles of flours, freeze-dried breads, and their partial (gastric phase) and final (intestinal phase) digests were analyzed in tricine and glycine-SDS PAGE gels.

2.6.1 | Glycine-SDS-PAGE

(Laemmli 1970). Freeze-dried samples were dissolved in sample buffer (0.0625 mol. L^{-1} Tris, 2% SDS, 10% v/v glycerol) and centrifuged. Runs were performed in a Mini-Protean II (BIO-RAD) equipment using 120 and 40 g L^{-1} acrylamide for separating and stacking gels, respectively. Electrophoresis was carried out at constant current (30 mA per gel) at room temperature. Gels were stained with Coomassie Brilliant Blue R-250 (1 g L^{-1}). Silver staining was performed to increase analytical sensitivity (Blum et al. 1987). The Gel Analyzer 2010a program (http://www.gelanalyzer.com) was utilized to determine molecular weights (MW).

2.6.2 | Tricine-SDS-PAGE

(Schägger 2006). Freeze-dried samples were dispersed in sample buffer (0.375 mol L^{-1} Tris–HCl, pH 7, 75 g L^{-1} glycerol, 30 g L^{-1} SDS, 0.125 g L^{-1} Coomassie blue G 250) and centrifuged before loading. For separating, spacing, and stacking gels, runs were performed using 160 (6 mol L^{-1} urea), 100, and 40 g L^{-1} acrylamide, respectively. The buffer solutions listed below were employed: 0.1 mol. L^{-1} Tris–HCl, pH = 8.9 (anode buffer); 0.1 mol. L^{-1} Tris, 0.1 mol. L^{-1} tricine, 1 g. L^{-1} SDS, pH = 8.25 (cathode buffer); and 1 mol. L^{-1} Tris–HCl, 1 g. L^{-1} SDS, pH: 8.45 (gel buffer). A Mini Protean II Dual Slab Cell (BIO-RAD) was used for the runs. Silver and Coomassie Brilliant Blue R-250 (1 g. L^{-1}) were used to stain the gels.

2.7 | Gel Filtration Chromatography

A Superdex Peptide 10/300 (GE Healthcare, exclusion limit: 10 kDa, optimal separation range 0.1–7 kDa) column and an AKTA Purifier (GE Healthcare Bioscience AB) were used to analyze and separate the phosphate buffer-soluble fractions using gel filtration FPLC. The chromatographic conditions were as follows: room temperature; injection volume: 200 μL; column total volume (V_t: 24 mL); mobile phase: 35 mmol. L^{-1} phosphate buffer, pH: 7.8; flow: 0.5 mL.min^{-1}. The following expression for the molecular mass (MM) determination was obtained by calibrating with Blue Dextran for exclusion volume (V_0: 7.6 mL), aprotinin (6.5 kDa), vitamin B12 (1.355 kDa), and hippuric acid (0.179 kDa): $\log MM = 4.80 - 3.33 \times K_{av}$ (r^2: 0.9781), where K_{av} is $(V_e\text{-}V_0)/(V_t\text{-}V_0)$, Ve is the elution volume. Detection was carried out at 210 nm.

2.8 | Antioxidant Activity

Antioxidant activity of the buffer-soluble fractions of flours, breads, and their gastrointestinal digests was evaluated by two in vitro assays:

2.8.1 | Oxygen-Radical-Absorbance Capacity (ORAC)

(Orsini Delgado et al. 2015). Fluorescein solution (53.3×10^{-6} mmol. L^{-1} in 35 mmol. L^{-1} phosphate buffer, pH 7.8, 150 μL) was mixed with 25 μL of sample, phosphate buffer (negative control NC), or Trolox (positive control), and pre-incubated (37°C, 10 min). AAPH (160 mmol. L^{-1} in phosphate buffer, 25 μL) was added, and the reaction mixture was incubated (37°C, 45 min). Fluorescence intensity was measured (λ_{exc}: 485 nm, λem: 535 nm, Synergy HT–SIAFRT multidetection microplate reader, Biotek Instruments, USA) every 1 min to obtain the fluorescein decay curve and the area under the curve (AUC). A blank without AAPH was performed, and scavenging % was calculated as: ROO· scavenging $\% = [(AUC_S - AUC_{NC})/(AUC_B\text{-}AUC_{NC})] \times 100$; where S: sample, B: blank, NC: negative control. Trolox (6.25–75 μmol. L^{-1}) was used as a reference compound.

2.8.2 | ABTS Radical Cation ($ABTS^{+\cdot}$) Discoloration Assay

(Tironi and Añón 2010). $ABTS^{+\cdot}$ was produced by the reaction of 7 mmol. L^{-1} ABTS solution with 2.45 mmol. L^{-1} potassium persulfate (final concentration) in the absence of light, at room temperature, for at least 16 h before use. The solution was diluted in water for an absorbance of 0.70 ± 0.02 at 734 nm. 100 μL were mixed with 1 mL of ABTS+ solution in a 48-well plate, and the absorbance at 734 nm was measured in a Synergy HT-SIAFRT multi-detection microplate reader (Biotek Instruments, USA). Scavenging % was calculated as: $[(A_{NC0} - A_{St}) - (A_{NC0} - A_{CNt})/A_{NC0}] \times 100$; where: A_{NCt} and

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A_{NC0} are the absorbances of NC at $t = 15$ min and $t = 0$, respectively; and A_{St}: absorbance in the presence of the sample at $t = 5$ min. Trolox (0.05–0.20 mmol. L^{-1}) was used as a reference compound.

2.9 | Statistical Analysis

Measurements were performed at least in triplicate. Data were analyzed by ANOVA (General Linear Model Procedure). When significant differences were detected, mean values were evaluated using Tukey's test, or the t-test was used for pairwise comparisons of means (95% confidence). Statgraphics Centurion XVI software was used.

3 | Results & Discussion

According to earlier findings (Rodríguez et al. 2024), breads with acceptable technological qualities may be made by substituting 20% of ***AF***, 5% of ***GP***, or a mixture of these two for ***WF***. The study found that adding ***AF*** and ***GP*** to wheat bread formulations improved its texture, color, and volume without compromising its quality. The additions increased firmness, chewiness, and gumminess, but did not significantly affect volume or elasticity. ***GP*** reduced crumb brightness and increased redness, while ***AF*** had a milder impact. These breads also have improved nutritional qualities; for example, they contain more fiber and protein. Furthermore, ***AF*** and ***GP*** may offer antioxidant properties resulting from bioactive substances, such as phenolic compounds, as previously studied (Rodríguez et al. 2024), and/or peptides. The protein bioaccessibility and the antioxidant properties associated with peptide fractions are covered in the section that follows.

3.1 | Protein Characterization of Flours and Breads

Polypeptide/peptide profiles of flours and breads were analyzed by glycine and tricine-SDS-PAGE. The amaranth protein profile in ***AF*** (13.6% ± 0.4% w/w proteins) presented typical bands of > 66 kDa, 53, and 57 kDa (globulins and/or globulin-P); 45, 38, 32, and ≤ 20 kDa (globulin and albumin fractions) (Figure 1A.I,B.I, *lane 2*). ***WF*** (11.7% ± 0.5% w/w proteins) presented a lower solubilization of proteins in the electrophoresis sample buffer, showing bands of MW ≤ 30 kDa, probably corresponding to albumins and globulins, and some faint bands between 30 and 45 kDa, probably associated with gliadins (Siddiqi et al. 2020) (Figure 1A.I,B.I, *lane 1*). In the case of ***GP***, it was not possible to detect bands, both in reducing and non-reducing conditions, even with silver staining in glycine gels (Figure 1A.I, *lane 3*), and only a faint band around 20 kDa in tricine gels (Figure 1B.I, *lane 3*). Since ***GP*** presented a considerable total protein content (6.1 ± 0.9% w/w) but almost no polypeptides could be detected in the electrophoreses, it is likely that the protein was interacting with polyphenols and other fiber components, forming large aggregates that fail to solubilize even in the denaturing and reducing conditions of the sample electrophoresis buffer. The protein-polyphenol interaction can occur through non-covalent bonds, which generally result in reversible interactions, or covalent ones generating irreversible interactions, affecting the secondary, tertiary, crystal structures, and microstructure of native proteins. High phenolic concentration causes saturation of the protein binding sites, which adversely affects protein functionality such as solubility (Sun et al. 2022).

The total protein content in the four types of breads was similar, around 8% w/w (Table 2). The amount of protein loaded per lane was optimized until distinct bands were detected. For flour and bread samples (with and without 2-ME), 40 μg of total protein was sufficient. However, in the case of breads, it was necessary to use silver staining to increase sensitivity, indicating a lower solubilization of proteins in the sample buffer than in the case of ***WF***, as expected after the formation of the dough. These findings were consistent with the reduced protein solubility in breads, as will be described later. ***C*** exhibited the characteristic ***WF*** bands, both in the presence and absence of 2-ME (Figure 1A.I,A.II, *lane 4*). ***A20*** (Figure 1A.I, *lane 5*) also showed many of the characteristic ***AF*** bands, especially those with MW 38 kDa, some more lightly between 45 and 66 kDa, and those lower than 14 kDa. The molecules that did not enter the gel showed greater intensity, and polypeptides between 20 and 14 kDa presented lower intensity with respect to ***C***. In addition, the ***A20*** profile in the presence of 2-ME (Figure 1A.II, *lane 5*) showed a higher presence of MW < 30 kDa polypeptides. These facts agree with the interaction of amaranth albumins with wheat glutenin through disulfide bonds postulated by Miranda-Ramos et al. (2019). ***GP***-containing breads presented increased bands in the range of > 40 and < 66 kDa and < 20 kDa, which diminished in the presence of 2-ME (Figure 1A, *lanes 6* and *7*). These polypeptides could correspond to molecules derived from ***WF***, presumably gliadins and albumins (Siddiqi et al. 2020), that, in the presence of ***GP***, can be better solubilized than in the case of ***C***. The presence of ***GP*** produced a difference in the pH values of the breads, which were 5.7 for ***C*** and ***A20*** versus 4.3 and 4.6 for ***GP5*** and ***A20GP5***, respectively. It has been reported that a decrease in the pH of breads and the consequent increase in the positive charges could lead to the unfolding of gluten proteins and promote their solubilization, and to an increased activity of endogenous proteases (Tolve et al. 2021). Further, the formation of soluble protein-polyphenols complexes, which could be disaggregated with 2-ME, cannot be ruled out. Several works reported the interaction between wheat proteins, mainly gluten proteins (gliadins and glutenins), and phenolic compounds (Girard and Awika 2020). In addition, the presence of ***AF*** produced a higher intensity in bands that do not enter the gel and a diminution of the < 20 kDa band (in the presence of 2-ME) in ***A20GP5*** (Figure 1A.II, *lane 7*) with respect to ***GP5*** (Figure 1A.II, *lane 6*). Previously, we have described that the presence of ***AF*** induced a diminution of extracted anthocyanin compounds in the ***A20GP5*** bread, suggesting interactions with other compounds, probably proteins from ***AF***, since an opposite effect was observed in ***GP5***. The insolubilization of tannin–protein complexes is greatest near the isoelectric point of the protein; so, it could be favored in ***A20GP5*** since its pH (4.6) was quite similar to the isoelectric point of the amaranth proteins (Rodríguez et al. 2024).

The solubility of the protein components in 35 mmol. L^{-1} phosphate buffer (pH 7.8) was, as expected, very low in the case of ***WF*** and higher in the case of ***AF*** (Table 1) with values like those reported

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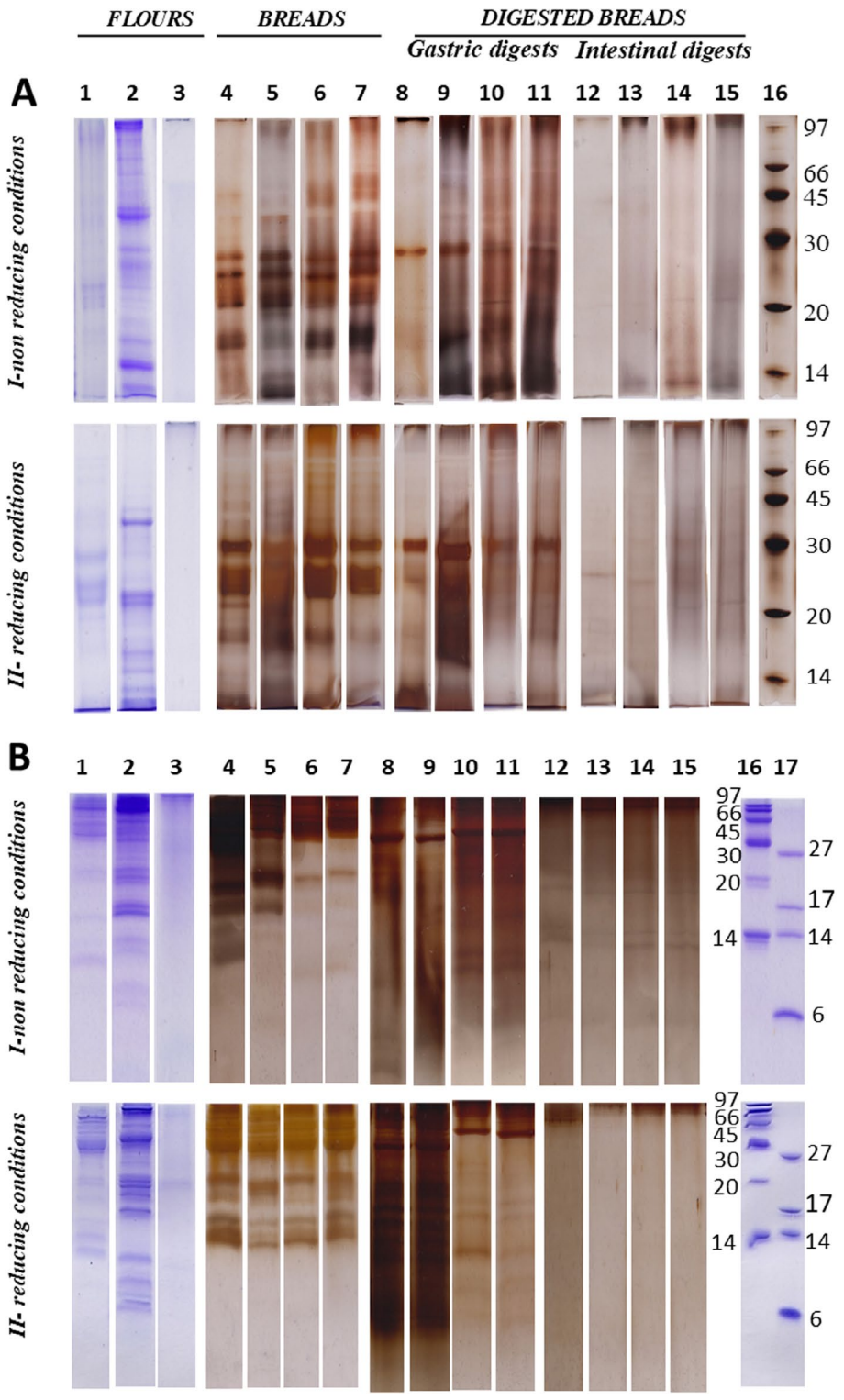


FIGURE 1 | Glycine-SDS-PAGE (A) and tricine-SDS-PAGE (B) in the absence (I) or presence (II) of reducing conditions. Flours: Lane (1) wheat flour (***WF***), lane (2) amaranth flour (***AF***), lane (3) grape peels flour (***GP***); Breads: Lane (4) ***C***: Control bread, lane (5) ***A20***: Mixture ***WF/AF*** (80/20 w/w), lane 6) ***GP5***: Mixture ***WF/GP*** (95/5 w/w), lane (7) ***A20GP5***: Mixture ***WF/AF/GP*** (75/20/5 w/w). Gastric digests: Lane (8) ***Cg***, lane (9) ***A20g***, lane (10) ***GP5g***, lane (11) ***A20GP5g***; Intestinal digests: Lane (12) ***Cd***, lane (13) ***A20d***, lane 14) ***GP5d***, lane (15) ***A20GP5d***; lane (16) low molecular weight standards LMW; lane (17) very low molecular weight standards VLMW. Lanes 1 to 3 were stained with Coomassie Brilliant Blue R-250, lanes 4 to 15 were stained with silver.

in previous studies (Rodríguez and Tironi 2023). The soluble protein concentration of ***GP*** could not be determined by the Lowry reaction as in the other cases because of the high concentration of phenolic compounds interfering, generating abnormally high values. Therefore, in this case, it was determined by the Kjeldhal method, obtaining a value of about 30% (Table 1). This low value

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TABLE 1 | Flours, breads and digested breads total and soluble protein content in 35 mmol. L^{-1} phosphate buffer.

Samples	Total protein (g/100 g sample)	Soluble protein (mg/mL)[1]	Protein solubility (g/100 g total protein)
Flours			
WF	11.7 ± 0.5	1.4 ± 0.1 (90 mg/mL)	12.8 ± 0.4
AF	13.6 ± 0.4	1.4 ± 0.1 (20 mg/mL)	58 ± 1
GP	6.1 ± 0.9	1.1 ± 0.1 (60 mg/mL)	30 ± 4
Breads			
C	7.9 ± 0.7	0.5 ± 0.1 (125 mg/mL)	5 ± 1[a]
A20	8 ± 0.3	0.9 ± 0.1 (125 mg/mL)	8.7 ± 0.6[b]
GP5	7.6 ± 0.4	0.8 ± 0.1 (125 mg/mL)	7 ± 1[a,b]
A20GP5	8 ± 0.2	0.9 ± 0.1 (125 mg/mL)	9.0 ± 0.6[b]
Digested Breads			
Cd	13.1 ± 0.5	3.1 ± 0.7 (40 mg/mL)	66 ± 7[A]*
A20d	13.2 ± 0.5	3.4 ± 0.9 (40 mg/mL)	62 ± 6[A]*
GP5d	13.5 ± 0.2	3.1 ± 0.6 (40 mg/mL)	56 ± 6[A]*
A20GP5d	13.4 ± 0.6	2.7 ± 0.3 (40 mg/mL)	51 ± 4[A]*

Note: Results are expressed as the mean value ± SD. Different letters (lowercase for breads and capitals for digested breads) indicate significant differences ($p < 0.05$). * Indicates differences in protein solubility between each bread and its corresponding digest.
[1]Protein concentration (Lowry method) in phosphate buffer–soluble fractions prepared from dispersion with different sample concentrations (which are indicated in parentheses). Soluble ***GP*** protein was determined by the Kjeldhal method due to phenolic interference in the Lowry method.

is correlated with an insolubilisation by protein-polyphenol interaction as postulated above. In the breads, the solubility of the components in phosphate buffer was drastically reduced with respect to the starting flours (Table 1) due to the formation of the gluten network and the heat treatment. ***C*** had the lowest solubility ($p < 0.05$), with no significant differences among the other three breads (although a tendency to increase could be observed in the presence of ***AF***). A possible explanation for these results is that the addition of ***GP*** and even more of ***AF*** decreased interactions among gluten-forming proteins, which remained in a soluble form, which agrees with the electrophoretic observations.

The molecular exclusion chromatographic profiles of the phosphate buffer-soluble fractions were analyzed using a Superdex Peptide 10/300 column, which allows the resolution of components < 10 kDa. Figure 2 (top panel) shows typical profiles for ***WF*** and ***AF***. When the chromatograms were normalized by soluble protein concentration (Figure 2, *column* ***A***), ***WF*** showed a majority proportion of components larger than 6.5 kDa, as well as components with MW around 1.36 kDa. It should be noted that to obtain the chromatograms, it was necessary to start from different concentrations of the flours in the dispersions, much higher for **WF**, given its lower protein solubility. When the chromatograms were normalized for the same flour concentration in the starting dispersions (Figure 2, *column* ***B***), it was again found that much more ***AF*** components were solubilized compared to ***WF***, with MW throughout the range, although with a higher proportion of polypeptides over 6.5 kDa. On the other hand, it was also observed that only components smaller than 1.36 kDa were detectably solubilized in the case of ***GP***, which could correspond to different types of components with absorption at 210 nm, including polyphenols.

The soluble fractions obtained from the breads (Figure 2) showed a marked decrease in the components with MW > 6.5 kDa compared to the flours, probably related to the decrease in solubility detected in these products due to the formation of the gluten network. Differences in the proportion of some peaks were observed among the different breads. **C** and ***GP5*** (to a lesser extent) presented a higher proportion of MW > 10 kDa and of the peak around 1.36 kDa in correlation with what was observed in ***WF***. Therefore, the presence of ***AF*** in the breads led to a lower amount of phosphate buffer-soluble molecules between 6.5 and 10 kDa, and some differences also in the proportions of molecules with MW < 1.36 kDa. For example, the shoulder near the 1.36 kDa peak—approximately at 0.6 kDa—shows a marked intensification in the ***A20*** bread compared to the control (***C***), and a more moderate increase in ***A20GP5*** relative to **GP5**, which indicates that the presence of ***GP*** limits the solubility of this protein fraction. This observation further supports the hypothesis of potential interactions between amaranth and pomace proteins. The presence of ***GP*** also contributed to a decrease in soluble molecules < 10 kDa. Raczkowska and Serek (2024) review highlights that fruit pomace, rich in polyphenols and dietary fiber, can influence the structural and functional properties of bread, further supporting the likelihood of protein–polyphenol interactions in such composite formulations.

3.2 | Effect of the SGID Process and Characterization of the Digests

The SGID of the four types of bread was carried out using the COST-INFOGEST consensus method (Brodkorb et al. 2019) in

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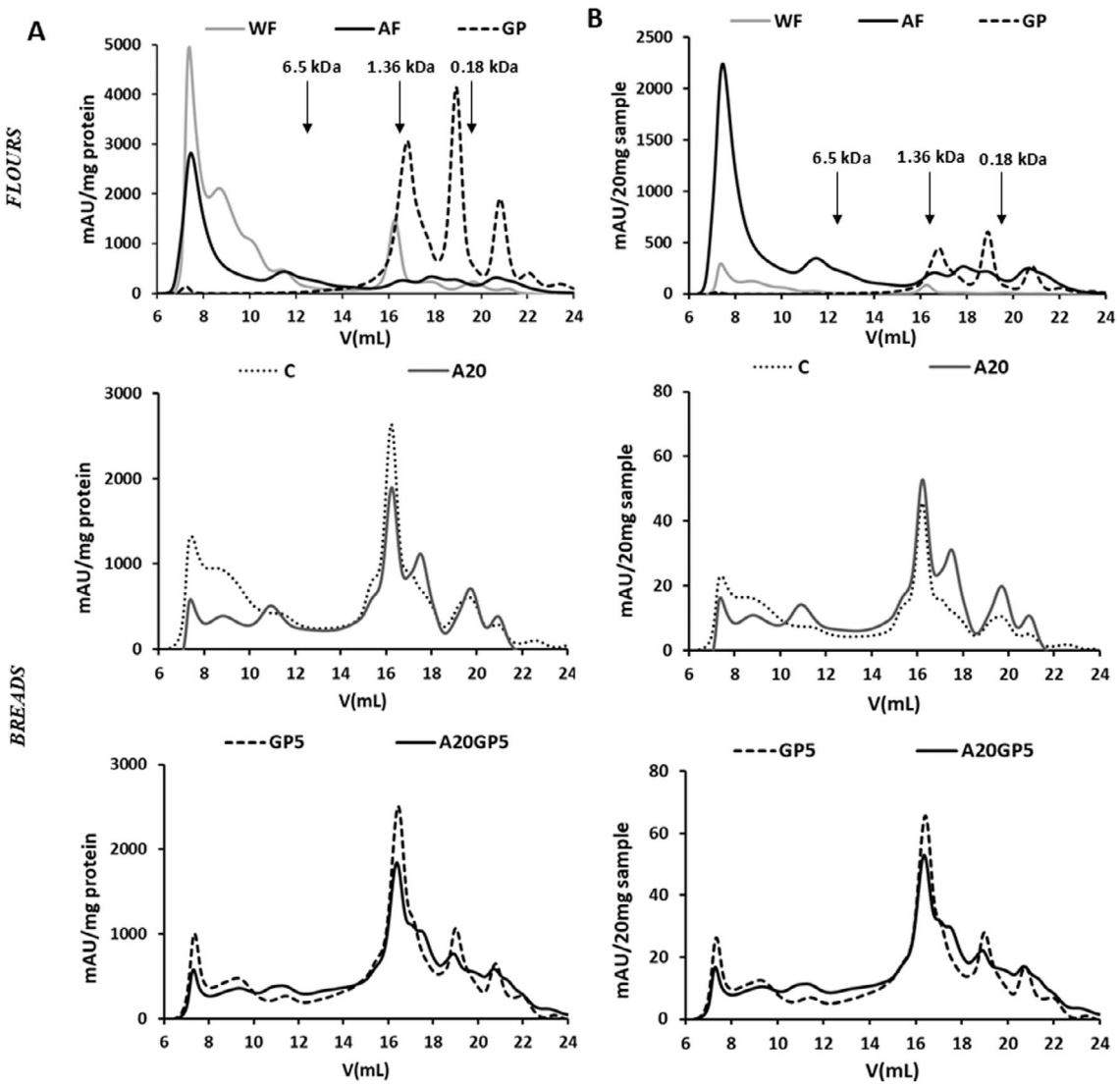


FIGURE 2 | Flours and breads FPLC gel filtration chromatograms (Superdex Peptide 10/300 GL) of phosphate buffer-soluble fractions. A: Signal (mAU) normalized according to the soluble protein content. B: Signal (mAU) normalized to a mass of 20 mg sample in the initial dispersion. ***WF***: Wheat flour, ***AF***: Amaranth flour, ***GP***: Grape peels flour. Breads: ***C***: Control, ***A20***: Mixture of ***WF/AF*** (80/20 w/w), ***GP5***: Mixture ***WF/GP*** (95/5 w/w), ***A20GP5***: Mixture ***WF/AF/GP*** (75/20/5 w/w).

TABLE 2 | Proteolysis degree (HD) in simulated gastric and intestinal digestion.

Sample	Gastric phase	Intestinal phase
Cd	3.7 ± 0.7^{b}	$25 \pm 1^{C+}$
A20d	2.5 ± 0.7^{ab}	$21 \pm 1^{B+}$
GP5d	1.5 ± 0.3^{a}	$18 \pm 1^{A+}$
A20GP5d	2.9 ± 0.1^{b}	$17 \pm 1^{A+}$

Note: Results are expressed as the mean value ± SD. Different letters in the same column indicate significant differences ($p < 0.05$) among samples. + Indicates significant difference in HD % in the gastric phase and after complete gastrointestinal digestion.

a bioreactor. Figure 4 shows the evolution of pH throughout the gastric and intestinal phases, presenting differences between the different breads. Throughout the gastric phase, the pH increased, probably due to the free amino groups released from the pepsin-digested protein (Gallo et al. 2021). The presence of ***AF*** and, to a greater extent, ***GP*** in the breads produced a smaller and slower increment of the pH value when compared to ***C***; this effect could be associated with a lower pH in ***GP***-containing breads as well as a lower liberation of free amino groups. In fact, the degree of proteolysis (HD, TNBS method) in the gastric phase presented low values, between 1.5% and 3.7% for all breads, but with ***GP5*** having the lowest value ($p < 0.05$) (Table 2). During the intestinal phase, the two breads containing ***AF*** (***A20*** and ***A20GP5***) showed lower pH increase, which could be related to a greater buffering capacity for ***AF***-containing products. After intestinal digestion, as expected, a significant increase ($p < 0.05$) of HD was observed for all breads. However, breads containing ***GP*** showed significantly ($p < 0.05$) lower values (Table 2). HD values were comparable to those obtained by Hoehnel et al. (2020), where a control wheat bread and a high-protein bread formulation (wheat flour was partially replaced by high-protein ingredients from faba bean, carob, and gluten) were analyzed using a different SGID protocol than the one used in the present work.

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They subjected bread samples to pepsin digestion (enzyme/substrate ratio: 1:50 w/w) at 37°C and pH 1–2 for 1 h. This was followed by pancreatin digestion (enzyme/substrate ratio: 1:10 w/w) at 37°C and pH 7–8 for 1 h, 3 h, or 24 h, to achieve short-, medium-, and long-term digestion, respectively. After the different intestinal phase times, the degree of proteolysis determined by the TNBS assay showed values of 14%, 18%, and 25% for the control bread and 17%, 23%, and 31% for the high-protein bread, respectively. Furthermore, the HD values obtained for all breads are significantly lower than those obtained for ***AF*** submitted to the same protocol (Rodríguez and Tironi 2023), which is due to the presence of different proteins (lower digestibility of wheat proteins), processing effects, gluten network formation, and the presence of other components. It is widely known that polyphenols can bind to proteins, including digestive proteases such as pepsin and trypsin. Binding of polyphenols to proteases can have several effects: inhibition, as demonstrated in most studies, activation, or no effect. Pepsin activity has been shown to be inhibited by several flavonoids, such as epigallocatechin gallate (EGCG), catechin, and baicalein. Trypsin has been shown to be inhibited by condensed tannins, with a clear relationship between the degree of polymerization of procyanidins and the enzyme inhibition observed. Trypsin has also been inhibited by different phenolic acids and by EGCG. In fact, EGCG also inhibited pancreatic lipase and phospholipase A2 (Pérez-Gregorio et al. 2020). Therefore, partial inhibition of digestive enzymes could be a possible explanation for the lower degrees of proteolysis recorded in ***GP***-added breads. This effect could also be related to the protein-polyphenol interaction in the bread matrix. Such interactions have been reported with various proteins of plant origin, including amaranth and wheat gluten proteins. Świeca et al. (2013) showed that the addition of wheat bread with onion skin polyphenols (containing mainly quercetin) significantly reduced protein digestibility, and insoluble complexes were observed.

When SDS-PAGE of samples from the gastric and intestinal phases of SGID (with and without 2-ME) was performed, a greater total protein (60 μg) amount than in the case of flours and breads was observed, and silver staining was required to detect the bands. The electrophoretic profiles of the gastric digests showed the disappearance or decrease of most of the bands in all the breads, remaining one band around 30 kDa with higher intensity (in all cases) and some bands below 20 kDa (except for ***Cd***) (Figure 1A.I, *lanes 8*, *9*, *10*, and *11*). After the intestinal phase, the electrophoretic profiles showed an almost complete disappearance of the polypeptides, leaving only high MW aggregates in the upper part of the gel, which presented much higher intensity in ***A20d***, ***GP5d***, and ***A20GP5d*** and some very faint bands with MW < 25 kDa and also < 14 kDa (Figure 1A.I, *lanes 12*, *13*, *14*, and *15*) that disappeared under reducing conditions (Figure 1A.II, *lanes 12*, *13*, *14*, and *15*). These profiles suggested that high molecular mass complexes formed in breads in the presence of ***AF*** and, to a greater extent, ***GP*** would not be attacked during SGID, and/or that other complexes could be formed under gastrointestinal conditions.

After SGID, phosphate buffer-soluble proteins increased significantly ($p < 0.05$) with respect to the breads, reaching protein solubility values of about 60% (Table 1). It should be noted that soluble polypeptides/peptides concentrations were determined using the Lowry method, which measures peptides of 3 or more amino acids, so dipeptides and free amino acids that could appear after SGID are not considered in this measure. When performing the Tukey test for comparison of means, the solubility of the four digests did not show significant differences ($p > 0.05$) (Table 1), but when a less demanding statistic such as LSD was applied, the protein solubility of ***A20GP5d*** showed a significant decrease ($p < 0.05$) with respect to ***Cd***. Gel filtration chromatograms of the these soluble fractions from gastrointestinal digests are shown in Figure 3C. Comparing with the corresponding gel filtration profiles of the breads (Figure 2), SGID produced the disappearance of molecules > 6.5 kDa, with mainly the increase of molecules with MW between 6.5 and 1.36 kDa (Ve about 12 and 16 mL), and some changes in the area corresponding to compounds with MW < 0.4 kDa (Figure 3). These findings showed a significant appearance of peptides with MW < 6.5 kDa, especially in the range of 4–0.5 kDa (corresponding to length chains in the range of 36–4 amino acids) in correlation with previously reported degrees of proteolysis and increases in protein solubility in gastrointestinal digests. Only small differences in the intensities of some peaks were observed between the digests of the four breads, such as a higher proportion of molecules > 10 kDa and a "shoulder" in the majority peak corresponding to molecules with MW close to 1.4 kDa in the ***GP*** breads.

3.3 | In Vitro Antioxidant Activity

The phosphate buffer-soluble fraction from ***AF*** showed higher antioxidant power (lower IC_{50} value expressed as soluble protein concentration) by the ORAC method, but lower (higher IC_{50} value) for $ABTS^{+\cdot}$ neutralization than ***WF*** (Figure 5A). The IC_{50} value was even lower for ***GP*** in both assays, indicating higher antioxidant potency. However, as previously mentioned, the presence of phenolic compounds that have been solubilized in buffer, together with the proteins, cannot be ruled out as exerting part of the antioxidant activity. In this sense, the IC_{50} value expressed as protein concentration would not be representative of the real situation. Based on this and the different samples' compositions, it was decided to also express the antioxidant potency as a function of the dry mass of the sample.mL^{-1} (starting point for the preparation of the soluble fractions). When the antioxidant power was expressed with respect to the dry mass of the flours, it became evident that for both methodologies, significantly lower ($p < 0.05$) mass of the starting sample of ***GP*** with respect to ***AF*** and of ***AF*** with respect to ***WF*** were required (Figure 5B), newly indicating that ***GP*** contained soluble components with higher antioxidant power than ***AF***, and the latter contained soluble compounds with greater antioxidant power than ***WF***.

C and ***A20*** breads showed similar potency for both methods when the IC_{50} was expressed as a function of soluble protein concentration, while the breads added with ***GP*** showed a significantly ($p < 0.05$) higher antioxidant potency (Figure 5A). In agreement with what was observed for the flours, when expressed with respect to dry mass, the substitution of 20% ***WF*** by ***AF*** produced a significant increase ($p < 0.05$) in the ORAC and $ABTS^{+\cdot}$ potencies of the breads (***A20*** versus ***C***), while the breads with ***GP*** presented an additional increase in the $ABTS^{+\cdot}$ scavenging activity (Figure 5B).

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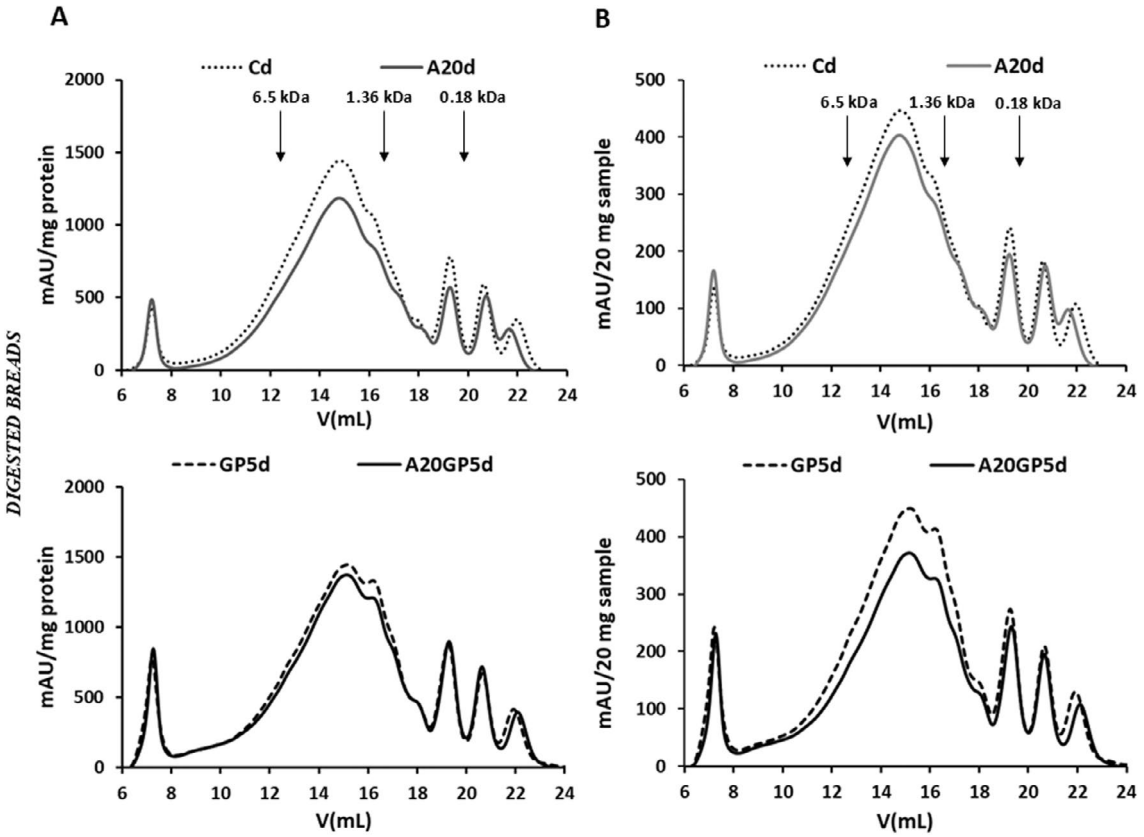


FIGURE 3 | Intestinal digests FPLC gel filtration chromatograms (Superdex Peptide 10/300 GL) of phosphate buffer-soluble fractions. A: Signal (mAU) normalized according to the soluble protein content. B: Signal (mAU) normalized to a mass of 20 mg sample in the initial dispersion. Breads intestinal digests: ***Cd***, ***A20d***, ***GP5d***, ***A20GP5d***.

When IC_{50} values were expressed as protein concentration (*mg protein.mL*$^{-1}$) of the soluble fraction, a significant increase ($p < 0.05$) in the ORAC potency was registered for ***Cd*** with respect to ***C***; ***Cd*** reached an IC_{50} value similar ($p > 0.05$) to that of ***A20d***. ***A20GP5d*** also increased the ORAC potency with respect to the corresponding undigested breads. In the case of $ABTS^{+\cdot}$ scavenging, ***Cd*** and ***A20*** did not present significant differences after SGID. On the other hand, ***GP5d*** significantly decreased ($p < 0.05$) the $ABTS^{+\cdot}$ scavenging potency with respect to **GP5**, while ***A20GP5d*** significantly increased this value with respect to ***A20GP5***. In general terms, we can say that the increases in antioxidant power (except in the case of ***GP5d***) would be mainly due to the appearance of molecules < 6.5 kDa described above, mainly peptides. It is remarkable that ***A20GP5d*** exhibited a potency expressed as soluble peptide/polypeptide concentration significantly higher ($p < 0.05$) than the rest of the breads by both antioxidant methods (Figure 5A). As previously described, chromatographic evaluation of these soluble fractions showed some minor differences among them (Figure 3). However, this does not mean that the molecular composition of all digests is the same, and future identification of the peptides and other components, such as polyphenols, present in each case will be necessary.

When IC_{50} values were expressed as the *dry mass of digest*×*mL*$^{-1}$ (Figure 5B), these values were in all cases (both antioxidant assays, all bread digests) significantly ($p < 0.05$) lower than the corresponding undigested breads, indicating an increase in potency. It should be noted that, in the starting mass of these soluble fractions, the digestion reagents contribute approximately 15% of the weight in solids, while the remaining 85% comes from the digested bread matrix, so that if these values were expressed as *dry mass of digested bread*×*mL*$^{-1}$ they would be even lower. Furthermore, the ORAC and $ABTS^{+\cdot}$ power of all digests did not present significant differences when IC_{50} values were expressed in terms of dry mass of digest ($p > 0.05$) (Figure 5B). These results show that this form of expression of IC_{50} values magnified the differences due to the SGID process but does not allow demonstrating the small differences between the digests of the different breads recorded when IC_{50} was expressed as a function of the concentration of soluble peptides/polypeptides.

Additionally, results showed that, although the antioxidant capacity (ORAC and $ABTS^{+\cdot}$ neutralization) of the 35 mM phosphate buffer (pH = 7.8) soluble components from ***AF*** was higher than that of ***WF***, this is not reflected in the breads and their gastrointestinal digests, probably due to the low proportion of ***AF*** in the formulations and to changes that the ***AF*** protein components may undergo in the bread matrix. We are not aware of any other work to date on the antioxidant activity of the soluble aqueous fractions from gastrointestinal digests of amaranth and/or grape peels fortified breads. In our previous study (Rodríguez et al. 2024), we evaluated the antioxidant activity of various organic extracts (methanol/water 70/30, methanol/HCl 97/3, and methanol/acetone/water 7/7/6) from the same fortified breads after SGID to analyze bioaccessibility and antioxidant properties of phenolic compounds.

A similar situation was recorded with the addition of ***GP***, an ingredient with high antioxidant capacity, which, at the

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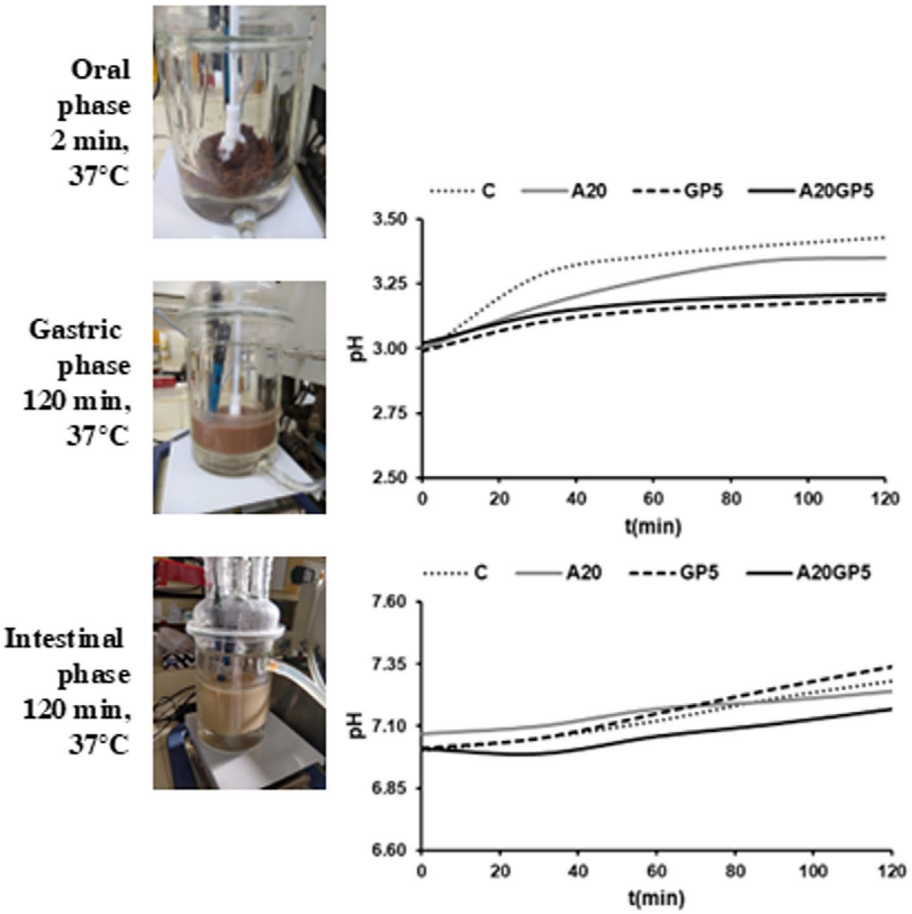


FIGURE 4 | pH changes throughout the simulated gastrointestinal digestion (SGID) stages in various bread formulations. ***C***: Control, ***A20***: Mixture of ***WF/AF*** (80/20 w/w), ***GP5***: Mixture ***WF/GP*** (95/5 w/w), ***A20GP5***: Mixture ***WF/AF/GP*** (75/20/5 w/w).

concentration used in these breads, did not generate significant changes in the antioxidant activity of the buffer-soluble fractions of gastrointestinal digests. Rocchetti et al. (2021) measured the antioxidant capacity of soluble samples of breads added with grape pomace before and after gastric and intestinal digestion using the FRAP and ABTS assays. The inclusion of pomace (5% and 10%) in the bread determined an increase in FRAP and ABTS values (expressed as μM TE/100 g dry basis) that was mainly attributed to the anthocyanins present in the pomace. After the gastric and intestinal phases of SGID, these authors reported a decrease in ABTS and FRAP values, which was explained by complex interactions occurring between phenolic compounds and other components of the food matrix, their chemical characteristics (i.e., degree of glycosylation), and different stability during simulated gastrointestinal digestion conditions. On the other hand, these authors did not consider the possible contribution of other substances, such as peptides, to the antioxidant activity in these fractions.

In other ways, in our previous work (Rodríguez et al. 2024), we demonstrated that, after SGID, the addition of ***AF*** in breads increased hydroxycinnamic acids, flavonols, and antioxidant activity of ethanol extracts; the addition of ***GP*** enriched hydroxycinnamic acids, anthocyanins, and hydroxybenzoic acids, maintaining the antioxidant potential, while the combined addition of ***AF*** and ***GP*** exposed mainly anthocyanins and flavanols and enhanced antioxidant activity. An important proportion of phenolic compounds in breads would remain or even increase their concentration by release after SGID. The joint presence of ***AF*** and ***GP*** in the breads changes the phenolic composition and antioxidant activity after SGID, as well as the existence of interactions between the polyphenolic components and other ***AF*** components (possibly proteins) was observed. In light of these results and integrating both studies, we can conclude that the joint addition of ***AF*** and ***GP*** to breads produced an increase in the antioxidant activity of bioaccessible compounds soluble in phosphate buffer (mainly peptides, although the presence of phenolic compounds cannot be ruled out), and also to an increase in the antioxidant potency of compounds that can be solubilized in methanolic solution (mainly phenolic compounds, although the presence of peptides, for example, cannot be ruled out). These effects could be related to the interactions established between amaranth polypeptides/peptides and phenolic compounds that could protect the latter from gastrointestinal conditions but also to form aqueous soluble complexes with improved antioxidant activity. Differently, wheat proteins did not show producing a similar effect.

4 | Conclusions

This study investigated the impact of fortifying bread with amaranth flour and red grape pomace peels on the antioxidant activity and bioaccessibility of the aqueous protein fraction.

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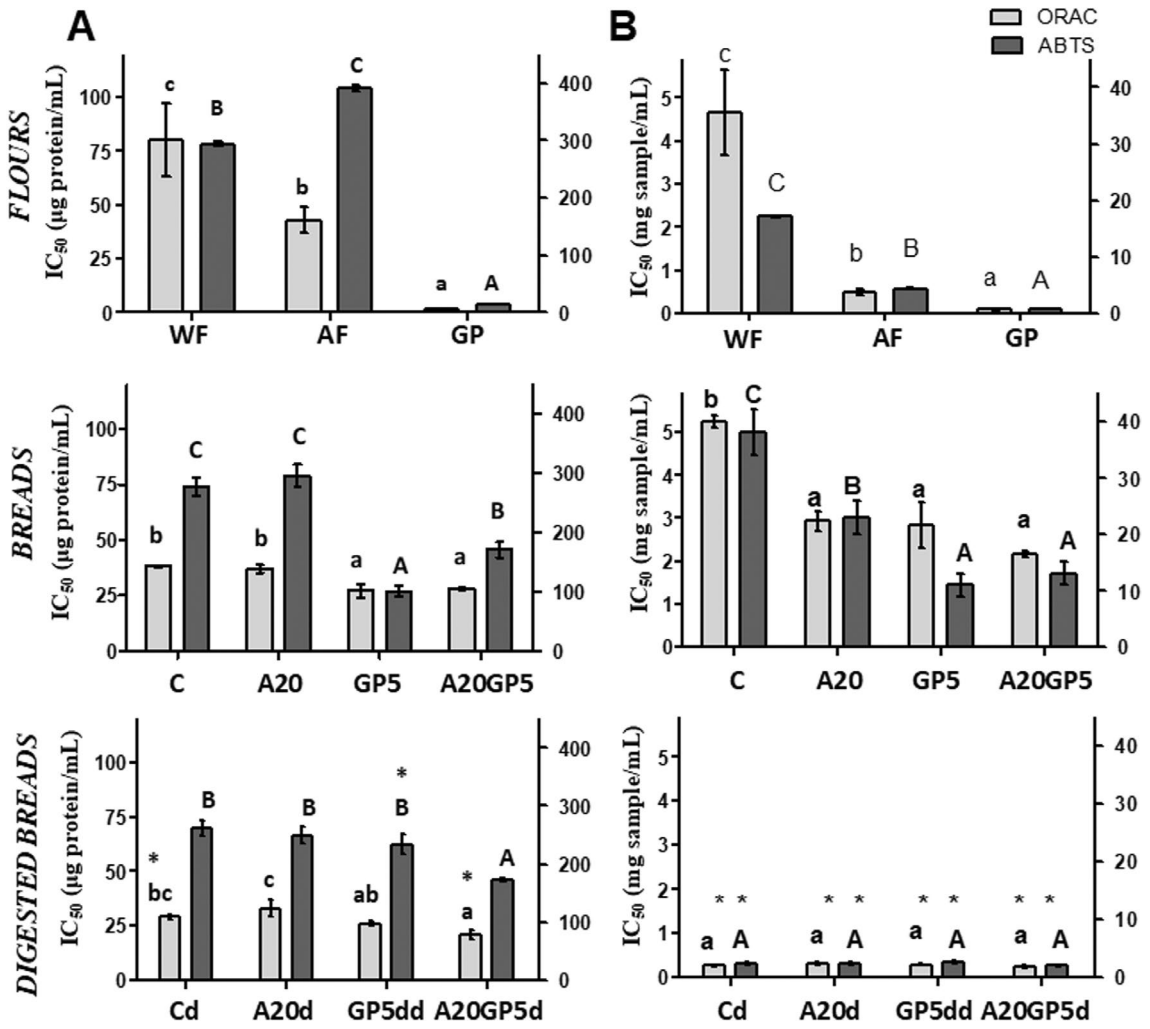


FIGURE 5 | Antioxidant activity of phosphate buffer soluble fractions: ORAC (left axis, light gray bars), $ABTS^{+\cdot}$ scavenging (right axis, dark gray bars). Results are expressed as: A: IC_{50} (µg protein.mL^{-1}); B: IC_{50} (mg sample (dry basis).mL^{-1}). (1) Flours: ***WF***: Wheat flour, ***AF***: Amaranth flour, ***GP***: Grape peels flour. (2) Breads: ***C***: Control, ***A20***: Mixture of ***WF/AF*** (80/20 w/w), ***GP5***: Mixture ***WF/GP*** (95/5 w/w), ***A20GP5***: Mixture ***WF/AF/GP*** (75/20/5 w/w). (3) Intestinal digests of breads ***Cd***, ***A20d***, ***GP5d***, ***A20GP5d***. Results are expressed as mean value ± SD. Different letters indicate significant differences ($p < 0.05$) among flours (1), breads (2) or digested breads (3): Lower case for ORAC, upper case for $ABTS^{+}$; * indicates a significant difference between a bread and its corresponding digest ($p < 0.05$).

In breads, amaranth proteins would modify some characteristics at the molecular level due to the interaction of albumins with gluten-forming wheat proteins, the formation of high-molecular-mass complexes, and small increases in solubility with a decrease in molecules > 10 kDa in the phosphate-buffered fraction. When ***GP*** is also present, amaranth proteins would interact with phenolic compounds, primarily anthocyanins. These molecular changes in the bread matrix would also produce some small modifications in the digests obtained by SGID. This study also compared the in vitro antioxidant activity of flours, breads, and their gastrointestinal digests using two methodologies. The presence of ***AF*** and/or ***GP*** induced an increase in the antioxidant potency of the buffer-soluble fractions of the breads. After SGID, these ingredients added separately did not cause increases in antioxidant activity of phosphate-buffered bioaccessible compounds, but the presence of both together produced improvements in the antioxidant activity of the soluble aqueous fraction and, according to previous studies, also in the soluble methanol fraction, suggesting beneficial interactions between amaranth polypeptides/peptides and phenolic compounds. In conclusion, bread added with 20% amaranth flour and 5% grape skins with improved nutritional quality and good physicochemical and textural properties does not present important modifications in protein bioaccessibility but a potential improvement in the antioxidant activity of bioaccessible compounds. Amaranth proteins and their gastrointestinal digestion products would play an important role in that due to their own activity and/or their interaction with phenolic compounds. These breads are presented as potential functional foods whose bioactivity should be further studied in the future.

Author Contributions

Mariela Rodríguez: investigation; Methodology; Formal analysis and preparation of figures and tables; Writing – original draft; Writing – review and editing. **Federico Bianchi:** formal analysis; Writing – review. **Barbara Simonato and Corrado Rizzi:** conceptualization; Resources; Funding acquisition; Project administration; Writing – review. **Valeria A. Tironi:** conceptualization; Methodology; Supervision; Writing – original draft; Writing – review and editing.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

References

Adler-Nissen, J. 1979. "Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid." *Journal of Agricultural and Food Chemistry* 27, no. 6: 1256–1262. https://doi.org/10.1021/jf60226a042.

AOAC. 2000. *Official Methods of Analysis*. 17th ed. Association of Official Analytical Chemists.

Beres, C., G. Costa, I. Cabezudo, et al. 2017. "Towards Integral Utilization of Grape Pomace From Winemaking Process: A Review." *Waste Management* 68: 581–594. https://doi.org/10.1016/j.wasman.2017.07.017.

Blum, H., H. Beier, and H. Gross. 1987. "Improved Silver Staining of Plant Proteins." *Electrophoresis* 8: 93–99.

Bressani, R. 1994. "El Amaranto y su Potencial en la Industria Alimentaria." *Alimentos Hoy* 7, no. 7: 15–19.

Bressani, R., E. De Martell, and C. De Godinez. 1993. "Protein Quality Evaluation of Amaranth in Adult Humans." *Plant Foods for Human Nutrition* 43: 123–143. https://doi.org/10.1007/BF01087917.

Brodkorb, A., L. Egger, M. Alminger, et al. 2019. "INFOGEST Static in Vitro Simulation of Gastrointestinal Food Digestion." *Nature Protocols* 14, no. 4: 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Cebrián, M., A. García-Roldán, J. Ibarruri, J. Zufía, and P. Jauregi. 2024. "Biorefinery Approach for an Integrated Valorisation of Grape Pomace in Distilleries." *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-024-06213-w.

Gallo, V., A. Romano, B. Miralles, et al. 2021. "Physicochemical Properties, Structure and Digestibility in Simulated Gastrointestinal Environment of Bread Added With Green Lentil Flour'." *LWT- Food Science and Technology* 154: 112713. https://doi.org/10.1016/j.lwt.2021.112713.

Girard, A. L., and J. M. Awika. 2020. "Effects of Edible Plant Polyphenols on Gluten Protein Functionality and Potential Applications of Polyphenol–Gluten Interactions." *Comprehensive Reviews in Food Science and Food Safety* 19, no. 4: 2164–2199. Portico. https://doi.org/10.1111/1541-4337.12572.

Hoehnel, A., J. Bez, I. Petersen, et al. 2020. "Enhancing the Nutritional Profile of Regular Wheat Bread While Maintaining Technological Quality and Adequate Sensory Attributes." *Food & Function* 11, no. 5: 4732–4751. https://doi.org/10.1039/D0FO00671H.

Kasarda, D., C. Nimmo, and G. Kohler. 1978. "Proteins and the Amino Acid Composition of Wheat Fractions." In *Wheat Chemistry and Technology*, edited by Y. Pomeranz, 227–299. American Association of Cereal Chemists.

Laemmli, U. 1970. "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4." *Nature* 227, no. 5259: 680–685. https://doi.org/10.1038/227680a0.

Lowry, O., N. Rosebrough, A. Farr, and R. Randall. 1951. "Protein Measurement With the Folin Phenol Reagent." *Journal of Biological Chemistry* 193: 265–275.

Miranda-Ramos, K., N. Sanz-Ponce, and C. Haros. 2019. "Evaluation of Technological and Nutritional Quality of Bread Enriched With Amaranth Flour." *LWT - Food Science and Technology* 114: 108418. https://doi.org/10.1016/j.lwt.2019.108418.

Nkonge, C., and G. M. Balance. 1982. "A Sensitive Colorimetric Procedure for Nitrogen Determination in Micro-Kjeldahl Digests." *Journal of Agricultural and Food Chemistry* 30, no. 3: 416–420. https://doi.org/10.1021/jf00111a002.

Orsini Delgado, M. C., M. Galleano, M. C. Añón, and V. Tironi. 2015. "Amaranth Peptides From Simulated Gastrointestinal Digestion: Antioxidant Activity Against Reactive Species." *Plant Foods for Human Nutrition* 70, no. 1: 27–34. https://doi.org/10.1007/s11130-014-0457-2.

Pérez-Gregorio, R., S. Soares, N. Mateus, and V. de Freitas. 2020. "Bioactive Peptides and Dietary Polyphenols: Two Sides of the Same Coin." *Molecules* 25, no. 15: 3443. https://doi.org/10.3390/molecules25153443.

Raczkowska, E., and P. Serek. 2024. "Health-Promoting Properties and the Use of Fruit Pomace in the Food Industry—A Review." *Nutrients* 16: 2757. https://doi.org/10.3390/nu16162757.

Rocchetti, G., C. Rizzi, M. Cervini, et al. 2021. "Impact of Grape Pomace Powder on the Phenolic Bioaccessibility and on *in Vitro* Starch Digestibility of Wheat Based Bread." *Food* 10, no. 3: 507.

Rodríguez, M., F. Bianchi, B. Simonato, C. Rizzi, A. Fontana, and V. Tironi. 2024. "Exploration of Grape Pomace Peels and Amaranth Flours as Functional Ingredients in the Elaboration of Breads: Phenolic Composition, Bioaccessibility, and Antioxidant Activity." *Food & Function* 15, no. 2: 608–624. https://doi.org/10.1039/D3FO04494G.

Rodríguez, M., S. García Fillería, and V. Tironi. 2020. "Simulated Gastrointestinal Digestion of Amaranth Flour and Protein Isolate: Comparison of Methodologies and Release of Antioxidant Peptides." *Food Research International* 138: 109735. https://doi.org/10.1016/j.foodres.2020.109735.

Rodríguez, M., and V. Tironi. 2023. "Chemical and Cell Antioxidant Activity of Amaranth Flour and Beverage After Simulated Gastrointestinal Digestion. Role of Peptides'." *Food Research International* 173: 113410. https://doi.org/10.1016/j.foodres.2023.113410.

Sanz-Penella, J., M. Wronkowska, M. Soral-Smietana, and M. Haros. 2013. "Effect of Whole Amaranth Flour on Bread Properties and Nutritive Value." *LWT- Food Science and Technology* 50, no. 2: 679–685. https://doi.org/10.1016/j.lwt.2012.07.031.

Schägger, H. 2006. "Tricine-SDS-PAGE." *Nature Protocols* 1, no. 1: 16–22. https://doi.org/10.1038/nprot.2006.4.

Siddiqi, R., T. Singh, M. Rani, D. Sogi, and M. Bhat. 2020. "Diversity in Grain, Flour, Amino Acid Composition, Protein Profiling, and Proportion of Total Flour Proteins of Different Wheat Cultivars of North India'." *Frontiers in Nutrition* 7: 141. https://doi.org/10.3389/fnut.2020.00141.

Sun, X., R. Sarteshnizi, and C. Udenigwe. 2022. "Recent Advances in Protein–Polyphenol Interactions Focusing on Structural Properties Related to Antioxidant Activities." *Current Opinion in Food Science* 45: 100840. https://doi.org/10.1016/j.cofs.2022.100840.

Świeca, M., U. Gawlik-Dziki, D. Dziki, B. Baraniak, and J. Czyż. 2013. "The Influence of Protein–Flavonoid Interactions on Protein Digestibility *in Vitro* and the Antioxidant Quality of Breads Enriched With Onion Skin." *Food Chemistry* 141, no. 1: 451–458. https://doi.org/10.1016/j.foodchem.2013.03.048.

Tironi, V., and M. C. Añón. 2010. "Amaranth Proteins as a Source of Antioxidant Peptides: Effect of Proteolysis." *Food Research International* 43, no. 1: 315–322. https://doi.org/10.1016/j.foodres.2009.10.001.

27719693, 2025, 4, Downloaded from https://aocs.onlinelibrary.wiley.com/doi/10.1002/sfp2.70035 by University Degli Studi Di Verona, Wiley Online Library on [04/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Tolve, R., B. Simonato, G. Rainero, et al. 2021. "Wheat Bread Fortification by Grape Pomace Powder: Nutritional, Technological, Antioxidant, and Sensory Properties." *Food* 10, no. 1: 75. https://doi.org/10.3390/foods10010075.

27719693, 2025, 4, Downloaded from https://aocs.onlinelibrary.wiley.com/doi/10.1002/sfp2.70035 by Universita Degli Studi Di Verona, Wiley Online Library on [04/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License