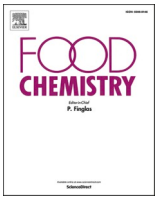
Food Chemistry 462 (2025) 140925

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Grape pomace and pecan shell fortified bread: The effect of dietary fiber-phenolic compounds interaction on the *in vitro* accessibility of phenolic compounds and *in vitro* glycemic index

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ARTICLE INFO

Keywords:
Agro-industrial byproducts
Functional bread
Bioactive compounds
Scanning Electron microscopy (SEM)
Bioaccessibility
Fourier transform infrared spectroscopy (FTIR)

ABSTRACT

Grape pomace (GP) and pecan shell (PS) are two by-products rich in phenolic compounds (PC), and dietary fiber (DF) that may be considered for the development of functional baked foods. In this study, four formulations with different GP:PS ratios (F1(8%:5%), F2(5%:5%), F3(5%:2%), F4(0%:5%), and control bread (CB)) were elaborated and characterized (physiochemical and phytochemical content). Also, their inner structure (SEM), changes in their FTIR functional group's vibrations, and the bioaccessibility of PC and sugars, including an *in vitro* glycemic index, were analyzed. Results showed that all GP:PS formulations had higher mineral, protein, DF (total, soluble, and insoluble), and PC content than CB. Additionally, PC and non-starch polysaccharides affected gluten and starch absorbance and pores distribution. *In vitro* digestion model showed a reduction in the glycemic index for all formulations, compared to CB. These findings highlight the possible health benefits of by-products and their interactions in baked goods.

1. Introduction

Interest in the consumption of bioactive compounds (BC), such as PC and DF, has increased in recent years. These compounds occur widely in the plant kingdom and many beneficial health effects, such as anti-cancer, antioxidant, cardioprotective, and blood glucose regulator, have been attributed to them (Kris-Etherton et al., 2002; Subiria-Cueto et al., 2021). Nowadays, there has been a tendency to acquire these compounds through alternative or unconventional sources. Agro-industrial by-products, which are considered industrial waste, have been reported as important sources of PC and DF (O'Shea et al., 2012). A practical way to exploit the properties of by-products has been by incorporating them into food products to increase their nutritional value and health benefits (Subiria-Cueto et al., 2021). In this sense, GP is the most abundant by-product generated during winemaking, and its composition contains a large amount of low molecular weight PC with antioxidant and anti-inflammatory properties, as well as soluble and insoluble DF (Carmona-Jiménez et al., 2018). Similarly, PS, the by-product of the pecan nut industry, is an important source of insoluble fiber and highly polymerized PC such as proanthocyanidins, which also present antioxidant and anticarcinogenic activity (Do Prado et al., 2009). One strategy that has been proposed to integrate both by-products into people's diets is to use bakery products as a vehicle for these bioactive compounds. Considering that bread is widely consumed in the world, and that some of its ingredients (refined wheat flour and sugar) can be related to the development of health problems such as

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https://doi.org/10.1016/j.foodchem.2024.140925
Received 28 April 2024; Received in revised form 17 August 2024; Accepted 18 August 2024
Available online 22 August 2024

obesity and diabetes (Bautista-Castaño & Serra-Majem, 2012), several studies have proposed the development of bakery products with beneficial properties for the consumer's health using non-conventional flours (Czajkowska-González et al., 2021). However, some challenges that must be considered are the physical and chemical impacts on baking products generated by PC, DF, and their interactions (Cauvain, 2012; Edwards, 2007). Despite the evidence that these BCs increase antioxidant capacity or improve sensory properties in baked goods, unexpected alterations may occur in other characteristics. Some studies have reported that there is a direct connection between the bioaccessibility of bioactive compounds with the presence of DF and PC (Czajkowska-González et al., 2021; González Aguilar et al., 2017; Jakobek & Matić, 2019). Depending on the compounds involved, there will be different physicochemical interactions that could modify multiple health benefits (Subiria-Cueto et al., 2021). In this study, it has been hypothesized that GP and PS increase the content of BC in bread formulations and modulate the bioaccessibility of PC and sugars during *in vitro* digestion. In this way, the effect of the combination of both PS and GP flours was tested to identify physicochemical, phytochemical and functional changes in different bread formulations. In the same way, the release of biological relevant compounds in a digestive *in vitro* system was evaluated to understand the possible effect of PC and DF interactions in the bread matrix.

2. Materials and methods

2.1. Materials

Cabernet Sauvignon GP samples were donated by Grupo Alximia, S. A. de C.V. in Valle de Guadalupe, Baja California, México. PS (*carya illinoensis*) was facilitated by Procesadora La Nogalera, S.A. de C.V. in Ciudad Juárez, Chihuahua, México. Both by-products were oven-dried (Isotemp oven, Fisher Scientific®, Waltham, MA, USA) (55 °C), grounded (Jiawanshun®, hc-1000, China), sieved (420 μm) and stored in vacuum bags until use, as reported by Subiría-Cueto et al. (2022). The wheat flour (WF), baking yeast, salt, sugar and vegetable oil were purchased in the local markets at Ciudad Juárez, Chihuahua, México.

2.2. Bread formulations with GP and PC

The substitution percentages of WF by PS and GP flours were established according previously published studies (PS: 5%, GP: 5–8%) (Aardema et al., 2016; Coria-Oliveros, 2020; Rocchetti et al., 2021). Control bread (CB) was prepared using an automatic bread machine (Hamilton Beach©, mod. 29,881, VA, USA). Ingredients used were: 394.7 g of wheat flour (WF), 6.6 g of salt, 12.1 g of sugar, 4.2 g of dry bakery yeast, 26.4 g of vegetable oil and 250.0 g of distilled water. All ingredients were mixed, fermented, and baked, according to the manufacturer's instructions. Four fortified breads were prepared, in which WF (394.7 g) was partially substituted by different amounts of GP and PS (GP%:PS%): F1 (8%:5% (31.6:19.7 g)), F2 (5%:5% (19.7:19.7 g)), F3 (5%:2% (19.7:7.8 g)), F4 (0%:5% (0:19.7 g)) and control bread (CB) (0%:0%). All by-products flour were added after the first lauded. After baking, bread products were cooled down, freeze-dried, grounded, and stored at −20.0 °C in vacuum bags until use. Preliminary sensory analyses were carried out with 10 non-trained participants from the Chemical Biological Science Department. Six different formulations were tested, and only those formulations that showed neutral or higher evaluations (higher than 5 on a 9-point scale score) were selected.

2.3. Physicochemical properties and phenolic content

Physicochemical analysis of bread samples was determined according to the AOAC methodologies for moisture (925.10 AOAC, 2000), protein (920.87 AOAC, 2000), fat (935.38 AOAC, 2000), ashes (923.03 AOAC, 2000), total carbohydrates (calculated by difference), total, soluble (SDF) and insoluble (IDF) dietary fiber (985.29 and 991.42 AOAC, 2000). pH was determined by mixing 5 g of each sample with 50 mL of distilled water and using a potentiometry (Fisherband™ accumet™, AB15 plus, Westford, MA, USA). The extraction of free PC was carried out by mixing 2 g of bread sample with 20 mL methanol: HCl (97:3, *v/v*), sonicated (30 min), centrifugated (3500 rpm for 15 min), recovered the supernatant and stored at −4 °C. The extraction was carried out two times and both supernatants were combined. The conjugated PC were extracted from the residue of the free PC. This residue was mixed with NaOH (3 M, 1:10, *w/v*), incubated in a water bath (80 °C for 4 h), pH was adjusted to 2.0 with HCl (37%), and centrifugated (2465G for 15 min). Supernatants were recuperated, and volume taken to 100 mL with water and stored at −4 °C. Both extracts (free and conjugated) were analyzed by Folin-Ciocalteu method, and total phenolic compounds (TPC) were determined as the summatory of free and conjugated PC (de La Rosa et al., 2011b; Muñoz-Bernal et al., 2021; Tolve et al., 2021). Flavonoids and condensed tannins were determined by the aluminum chloride method (de La Rosa et al., 2011a) and DMAC (Dimethylacetamide) method (Muñoz-Bernal et al., 2021). Results are reported as gallic acid equivalents (GAE) per gram of sample in dry weight (DW) for total, free, and conjugated PC, and catechin equivalents (CE) per gram of sample in DW for flavonoids and condensed tannins.

2.4. FTIR spectroscopy

FTIR spectroscopy was used to evaluate the vibrational bands of molecular bonds and identify the functional groups present in each bread sample using an ATR-FTIR spectrophotometer (Thermo Scientific® Nicolet mod. 6700, Walthman, MA, USA), with a resolution of 4 cm^{-1} in a spectral range of 4000 to 400 cm^{-1}. 10 mg of lyophilized and pulverized samples were deposited in a germanium STR crystal and analyzed, collecting 60 scans. A background spectrum was obtained by collecting 60 scans of the STR crystal (cleaned with isopropanol). The obtained spectra were processed for noise reduction, baseline correction, and normalization using the OPUS software (Escobar-Puentes et al., 2022; Rojas-Yañez et al., 2022).

2.5. Morphological studies and pore sizes by scanning electron microscopy (SEM)

Slices of bread samples were prepared for surface studies using SEM. Each bread slice formulation was carefully lyophilized to prevent any type of fracture. Cubes of 5X5X5 cm were cut from each bread and placed on the base of a JEOL JSM-7000F field emission SEM (Tokyo, Japan) using double-sided carbon tape. Measurements were collected at x20, x100, x500, and x1000 magnifications with a beam acceleration voltage of 15 kV. ImageJ© (National Institute of Health Image, MD, USA) analysis software was used to measure the size, frequency, and distance between pores (Ahlborn et al., 2005; Rojas-Yañez et al., 2022).

2.6. Color analysis

The color of the bread samples was measured with a reflectance colorimeter (Konica Minolta® Chroma Meter, mod. CR-400, Tokyo, Japan) using the CIEL* a* b* color system. Twenty measurements in different parts of the crust and crumb were collected, and the total color difference (ΔE) was calculated using Eq. 1 (Tolve et al., 2021).

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (1)$$

where L_0, a_0, and b_0 are the values of the fortified bread formulations; and L, a and b are the value of CB.

2.7. In vitro digestion system and phenolic bioaccessibility for bread samples

The PC and sugars released after the *in vitro* digestion model was carried out following the methodologies of Muñoz-Bernal et al. (2023) and Kopf-Bolanz et al. (2012) with slight modifications. The test consisted of simulating the oral, gastric, and intestinal stages of the human tract. For the oral stage, 300 mg of grounded lyophilized bread formulation samples were mixed with 3.0 mL of salivary solution (KCl 0.93 mg/mL, KSCN 0.08 mg/mL, KH_2PO_4 2.72 mg/mL, $NaHCO_3$ 0.67 mg/mL, NaCl 0.24 mg/mL, $MgCl_2(H_2O)_6$ 0.06 mg/mL, $CO(NH_2)_2$ 0.04 mg/mL, $CaCl_2(H_2O)_2$ 0.29 mg/mL, mucin II 1.0 mg/mL, human salivary α-amylase 0.004 mg/mL, and lysozyme 0.02 mg/mL) adjusted at pH 6.8 and 2.25 mL of distilled water. The samples were incubated at 37 °C with constant shaking at 70 rpm, and after 5 min, a volume of 500 μL was taken and stored at −20 °C. For the gastric stage, six mL of gastric solution (KCl 2.62 mg/mL, NaCl 2.4 mg/mL, KH_2PO_4 0.12 mg/mL, $CO(NH_2)_2$ 0.01 mg/mL, $NaHCO_3$ 2.18 mg/mL, $MgCl_2(H_2O)_6$ 0.12 mg/mL, NH_4Cl 0.05 mg/mL, glucuronic acid 0.2 mg/mL, glucosamine 0.33 mg/mL, galactose 0.32 mg/mL, $CaCl(H_2O)_2$ 0.09 mg/mL, mucin II 1.4 mg/mL, bovine serum albumin (BSA) 1 mg/mL and pepsin 2.5 mg/mL) was added to the previous solution (oral stage) and pH adjusted to 1.3, and incubated during 120 min at the same temperature and rpm. At the end of the gastric stage, 500 μL of each sample were taken and stored at −20 °C. Finally, for the intestinal stage, 3.0 mL of bile juice ($CaCl_2(H_2O)$ 0.54 mg/mL, BSA 1.8 mg/mL, KCl 0.5 mg/mL, KH_2PO_4 2.42 mg/mL, $NaHCO_3$ 1.6 mg/mL, NaCl 1.92 mg/mL, $CO(NH_2)_2$ 0.23 mg/mL, $MgCl_2(H_2O)_6$ 0.07 mg/mL, NaH_2PO_4 3.75 mg/mL, and bile 60 mg/mL) and 6.0 mL of pancreatic solution ($CaCl_2(H_2O)_2$ 0.09 mg/mL, mucin III 1.4 mg/mL, BSA 1 mg/mL, pancreatin 18 mg/mL, KCl 0.5 mg/mL, KH_2PO_4 0.11 mg/mL, $NaHCO_3$ 3.61 mg/mL, NaCl 1.92 mg/mL, $CO(NH_2)_2$ 0.11 mg/mL and $MgCl_2(H2O)_6$ 0.07 mg/mL) were added to the previous solution (gastric stage). The samples were incubated during 120 min at 37 °C and shaking at 70 rpm. At the end of the incubation, 500 μL of each sample was taken and stored at −20 °C.

For the determination of PC released in the oral and gastric stages, stored samples (500 μL) were passed through a SPE (C-18), washed with 5.5 mL of distilled water to remove reducing sugars, and PC recovered with 2 mL of methanol. Afterwards, the recovered PC were analyzed with the Folin-Ciocalteu method, and results reported as mg of GAE per mg of bread (mg GAE/g bread DW). To determine the PC released in the intestinal stage, a reverse dialysis process was carried out. Inside a 50 mL plastic tube containing 20 mL of the solution resulting from the intestinal stage, a dialysis bag containing bile juice (0.166 mL), salivary (0.166 mL), gastric (0.333 mL), and pancreatic solution (0.333 mL) was inserted. The tube was closed properly and incubated at 37 °C with shaking at 70 rpm for 2 h. Afterwards, dialysis bag was recovered, inner solution went through a SPE (C-18) in the same conditions as oral and gastric stages, and PC recovered were quantified with Folin-Ciocalteu method, and results were expressed as mg GAE/g bread.

The PC bioaccessibility percentage was determined by using the following eq. 2:

$$\%Bioaccessibility = (RPC/TPC) \times 100 \quad (2)$$

where, RPC were the total amount of released phenolic compounds after the *in vitro* digestive system and TPC were the total phenolic compounds (free and conjugated) in the bread (Rocchetti et al., 2021).

The amount of released reducing sugars (RRS) were quantified by the DNS (dinitrosalicylic acid) method (Saratale et al., 2020). 150 μL of each stored sample (oral, gastric, and intestinal stage) was mixed with 300 μL of DNS reagent, incubated at 100 °C for 10 min, and absorbance determined at 540 nm in a microplate reader (Bio Rad®, mod. xMark, CA, USA). Results were expressed in milligrams of glucose per gram of lyophilized bread sample.

2.8. Kinetics of released sugars and in vitro glycemic index

During the intestinal stage of the *in vitro* digestion process, a series of aliquots were taken at different times, to generate a sugar release curve of each bread formulation (Ferrer-Mairal et al., 2012). Samples were taken every 5 min during the first 30 min of the intestinal digestion stage, and then every 15 min for 180 min. Subsequently, the reducing sugars present in these samples were analyzed utilizing the DNS method (Saratale et al., 2020). The sugar content at different times were used to determine the kinetic behavior and calculate the area under the curve (AUC) with the support of Graphpad Prism 6 software. The AUC of CB was considered as a glucose reference against the rest formulations in order to calculate the predictive glycemic index (Eq. 3) (Goñi et al., 1997).

$$In\ vitro\ glycemic\ index = \frac{AUC_{Bread\ formulation}}{AUC_{Control\ bread}} \quad (3)$$

2.9. Statistical analysis

Physicochemical properties, phytochemical content, color analysis, porosity studies, and the release of phenols and sugars in the *in vitro* digestion model were determined in three breads and each bread was analyzed by triplicate. In the case of FTIR and SEM analyses, one bread was prepared, and three different slices were evaluated. One-way analysis of variance (ANOVA) and Fisher LSD was performed for the comparison of mean values. To evaluate correlations between variables, a Person's correlation coefficient was carried out. All tests were performed with significance at $p < 0.05$ using the statistical software Graphpad Prism 6® (MA, USA). Results were expressed as mean values ± standard deviation (SD).

3. Results and discussion

To evaluate the effect of fortifying a wheat bread with low and high molecular weight PC, and soluble and insoluble dietary fiber, on its physicochemical and structural properties, as well as in its *in vitro* glycemic index, in the present study wheat flour was partially replaced with different amounts of GP (low molecular weight PC and soluble fiber) and PS (high molecular weight PC and insoluble fiber) flours. Initially, six GP-PS partially substituted formulations were prepared, but after the sensory analysis, only four, that scored higher than the neutral-score were selected for the rest of the study (Fig. S1).

3.1. Physicochemical properties and PC of bread formulations

The physicochemical properties and PC content of the fortified bread formulations are listed in Table 1. F1 was statistically higher in moisture and ashes, while F2 and F3 were slightly higher in protein, but not statistically significant. DF content increased with higher contributions of GP and PS. This trend was observed across all formulations, with F1 containing the highest amount of TDF, IDF, and SDF. Interestingly, F4, which only contains PS flour, showed the highest IDF/SDF ratio, which agrees with the higher IDF content in PS. Considering that total moisture in the bread samples were not statistically different, until lyophilization, the difference in moisture content could be attributed to the capacity of TDF (IDF & SDF) to bind water molecules. This characteristic has been reported in other bakery products, attributing to SDF and cellulose the ability to increase hydration (Alongi et al., 2019; Pomeranz et al., 1977). For the ash content, it has been reported that both by-products contain Ca, Cu, Mg, Na, K, Fe, Mn, P, S and Zn. In this way, these minerals could be present in the bread samples (Flores-Córdova & Sánchez-Chávez, 2016; Lachman et al., 2013). The pH value for all GP containing formulation was lower than control, due to the presence of phenolic acids imparted by the GP (Pérez-Ramírez et al., 2018; Subiría-Cueto et al., 2022).

Table 1
Physicochemical properties and PCs of bread samples.

	CB	F1	F2	F3	F4
Physicochemical properties					
Total moisture (g/100 g)	31.9 ± 2.0^{a}	34.2 ± 3.5^{a}	30.5 ± 2.8^{a}	31.9 ±5.1^{a}	29.5 ± 7.2^{a}
Freeze-dried Moisture (g/100 g)	2.2 ± 0.0^{c}	2.9 ± 0.1^{a}	2.0 ± 0.0^{c}	2.7 ± 0.3^{b}	1.7 ± 0.6^{d}
Ashes (g/100 g)	2.1 ± 0.0^{c}	2.9 ± 0.0^{a}	2.7 ± 0.0^{b}	2.7 ± 0.1^{b}	2.9 ± 0.1^{a}
Fat (g/100 g)	1.8 ± 0.1^{a}	1.9 ± 0.2^{a}	1.9 ± 0.1^{a}	1.8 ± 0.2^{a}	1.7 ± 0.1^{a}
Protein (g/100 g)	10.1 ± 0.1^{c}	10.6 ± 0.2^{c}	11.8 ± 0.2^{a}	10.9 ± 0.5^{bc}	11.0 ± 0.3^{ab}
Total carbohydrates (g/100 g)	83.8 ± 0.3^{a}	81.7 ± 0.3^{cd}	81.6 ± 0.1^{d}	82.0 ± 0.8^{bc}	82.7 ± 1.1^{b}
Total dietary fiber (g/100 g)	4.7 ± 0.1^{e}	14.4 ± 0.4^{a}	12.6 ± 1.3^{b}	9.6 ± 0.5^{c}	7.4 ± 0.7^{d}
Insoluble fiber (g/100 g)	3.3 ± 0.3^{c}	10.1 ± 1.4^{a}	8.8 ± 2.4^{ab}	7.3 ± 0.7^{b}	5.8 ± 1.0^{b}
Soluble fiber (g/100 g)	1.4 ± 0.2^{c}	4.3 ± 0.9^{a}	3.8 ± 1.5^{ab}	2.3 ± 0.2^{ab}	1.6 ± 0.9^{bc}
IDF/SDF ratio	2.4	2.3	2.3	3.2	3.6
pH	5.5 ± 0.0^{b}	4.7 ± 0.0^{d}	5.1 ± 0.0^{c}	5.0 ± 0.01^{c}	5.6 ± 0.0^{a}
Phenolic compounds content					
FPC (mg GAE/g)	0.3 ± 0.1^{e}	4.1 ± 0.5^{a}	1.2 ± 0.0^{b}	0.8 ± 0.07^{c}	0.6 ± 0.0^{d}
CPC (mg GAE/g)	1.7 ± 0.7^{e}	27.1 ± 2.4^{a}	13.7 ±5.4^{b}	4.5 ± 0.7^{d}	7.2 ± 2.7^{c}
TPC (mg GAE/g)	2.1 ± 0.8^{e}	31.2 ± 2.9^{a}	15.0 ±5.4^{b}	5.3 ± 0.8^{c}	7.8 ± 2.7^{d}
Flavonoids (mg CE/g)	3.9 ± 0.5^{b}	3.8 ± 0.7^{b}	5.8 ± 0.4^{a}	6.2 ± 0.5^{a}	5.9 ± 0.6^{a}
Condensed tannins (mg CE/g)	n.d.	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.1^{b}

Values from physiochemical properties and phenolic compounds content are expressed as mean ± standard deviation in dry weight (DW). Freeze-dried moisture represents the remanent humidity of bread after samples were lyophilized; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), Free Phenolic Compounds (FPC), Conjugated Phenolic Compounds (CPC), Total Phenolic Compounds (TPC), Gallic Acid Equivalents (GAE), Catechin Equivalents (CE). Different superscript per line indicates statistical differences ($p < 0.05$).

The fortification of bread with GP and PS largely increased the total phenolic compounds content (TPC), F1 was statistically higher than the rest of the formulations, while CB presented the lowest content (Table 1). Interesting, F1, which had the highest GP and PS substitution presented higher FPC compared with previously published studies GP, while the rest of the formulations were within the range (1.3–2.07 mg GAE/g), and PS (4.0 mg GAE/g) (Rocchetti et al., 2021; Tolve et al., 2021; Villasante et al., 2022). However, it is important to consider that formulations F1, F2 and F3 contain both by-products. As expected, conjugated PC were higher than free PC in all formulations, showing the relevance of quantifying both free and conjugated PC (Shahidi & Ambigaipalan, 2015). This may indicate a molecular interaction between PC with other complex compounds of the food matrix, such as lipids, proteins, or carbohydrates is undertaken (Jakobek, 2015). Some studies have reported that PC can bind to the polar part of fatty acids, to proteins by non-covalent bonds, and to carbohydrates by hydrogen bonds (Angelino et al., 2017; Jakobek, 2015; Lachman et al., 2013). Equally, it has been reported that PC can covalently bind to vegetable cell wall derivatives, including pectin and cellulose in DF (Shahidi & Yeo, 2016). In relation to flavonoids, it was observed that their content was affected by the presence of GP and PS. It has been hypothesized that other compounds in the food matrix could be interfering. Various studies indicate that gluten and starch can bind covalently and non-covalently to catechins and tannins (Xu et al., 2019). For the condensed tannins, they barely were quantified in bread formulations, while CB were not detected. This same characteristic was reported in other foods fortified with GP and PS (Girard et al., 2016; Rocchetti et al., 2021; Villasante et al., 2022). The relevance of the PC content lies in their capacity to increase beneficial effects in bake products and how they could interact with other molecules from food matrix. In this way, it is possible to anticipate chemical or physical changes, including interferences in the release of bioactive compounds during human digestion.

3.2. Physical properties of bread samples

The partial substitution of WF by GP and PS flours modified the color, height, and crumb structure of the bread samples (Table 2). The height of the samples at fresh weight (FW) decreased as the amount of by-product flour was added to the sample from 14.05 cm for CB to 9.2 cm for F1. This reduction in the bread's height can be attributed to the reduction of gluten content due to the partial substitution of WF by GP and PS. In this way, the capacity of the dough to retain CO_2 is reduced and affects the inner porous structure (Edwards, 2007). The reduction of breads height can also be explain in terms of the amount of TDF (mainly IDF) and PC, considering that the presence of TDF affects starch hydration, increase the weight of the drought, and PC could inhibit fermentative enzymes (He et al., 2020; Tolve et al., 2021).

Scanning electron microscopy (SEM) was used to analyze the morphological and structural pore changes of the crumb at x20, x100 and x1000 magnifications in bread samples in DW (Fig. 1 and Table 2).

Table 2
Physical characteristics and pores distribution in bread samples.

	CB	F1	F2	F3	F4
Height (cm)	14.6 ± 0.8^{a}	9.5 ± 0.4^{d}	10.6 ± 1.1^{cd}	11 ± 0.6^{c}	12.5 ± 0.3^{b}
Pore size at x20 (mm)	0.74 ± 0.6^{a}	0.54 ± 0.3^{a}	0.61 ± 0.4^{a}	0.54 ± 0.3^{a}	0.59 ± 0.4^{a}
Pore distance at x20 (mm)	0.85 ± 0.6^{a}	0.77 ± 0.7^{a}	0.83 ± 0.7^{a}	0.40 ± 0.2^{a}	0.30 ± 0.1^{a}
Reported pores at x20					
<0.1 mm^2	60	1230	267	100	191
0.1–0.5 mm^2	3	5	1	5	9
0.5–1.0 mm^2	0	0	4	3	2
Reported pores at x100					
<0.1 mm^2	0	2048	0	53	390
0.1–0.5 mm^2	11	84	88	16	86
0.5–1.0 mm^2	5	6	13	4	13
>1.0 mm^2	2	4	12	4	7
L*					
Crust	67.4 ± 3.4^{a}	36.9 ± 2.5^{d}	38.6 ± 3.0^{cd}	42.2 ± 4.5^{c}	50.7 ± 1.7^{b}
Crumb	64.8 ± 2.1^{a}	34.8 ± 1.6^{d}	37.8 ± 0.5^{c}	40.0 ± 1.0^{b}	43.7 ± 4.4^{b}
a*					
Crust	−0.2 ± 1.9^{d}	7.8 ± 1.3^{a}	7.0 ± 0.4^{b}	5.6 ± 0.5^{c}	5.7 ± 0.4^{c}
Crumb	−2.7 ± 2.0^{d}	5.8 ± 0.4^{a}	5.5 ± 0.1^{b}	4.3 ± 0.4^{c}	4.3 ± 0.3^{c}
b*					
Crust	30.5 ± 3.4^{a}	14.2 ± 1.8^{d}	15.0 ± 1.1^{cd}	17.1 ± 2.2^{bc}	20.5 ± 1.2^{b}
Crumb	17.9 ± 1.1^{a}	12.3 ± 0.5^{c}	12.9 ± 0.1^{c}	12.9 ± 0.5^{bc}	14.6 ± 1.0^{b}
ΔE*					
Crust	nd	35.5 ± 6.1^{a}	33.5 ±5.0^{a}	29.1 ± 2.4^{ab}	20.3 ±5.0^{b}
Crumb	nd	31.6 ± 3.8^{b}	40.3 ± 0.4^{a}	26.2 ± 3.4^{c}	22.5 ±5.0^{c}

Values are expressed as mean ± standard deviation; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), not determined (nd). Height and color parameters were analyzed in fresh samples, while pores data were obtained in lyophilized samples. Different superscript per line indicates statistical differences ($p < 0.05$).

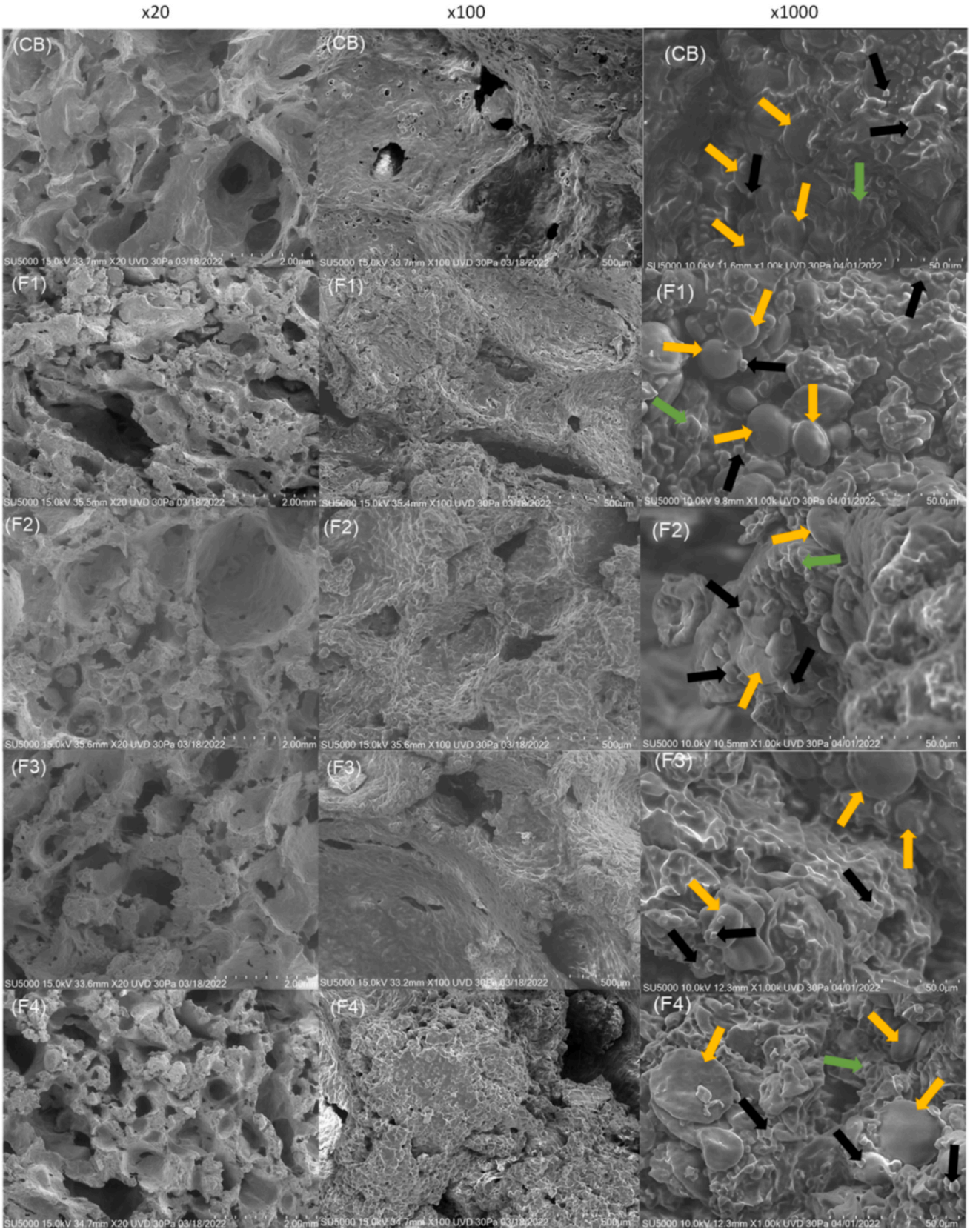

Fig. 1. Scanning electronic microscopy images of bread formulations (DW). Figures at Left panel: x20 SEM images. Center panel: x100 SEM images. Right panel: x1000 SEM images. Yellow arrows indicate starch granules; Black arrows indicate soluble proteins; Green arrows indicate fiber residues; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), Dry Weight (DW). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As observed in Fig. 1, CB exhibited a higher number of bigger pores presenting a notably rough appearance, while fortified breads presented a higher number of small pores. Upon magnification to x100, finer details emerged, unveiling smaller micropores and smoother surfaces. In contrast, F1 and F2 showcased large, irregular, and compact pores at x20, which transformed into smaller, deeper pores at x100. While F1 and F3 boasted similarly sized pores, F2 exhibited slightly larger and more widely spaced pores. F4 displayed slightly larger, circular pores, with a less smooth surface than other formulations. The x1000 magnifications were performed on pulverized bread samples. In all formulations it was possible to identify starch granulates (yellow arrows), soluble protein (black arrows), and fiber residues (green arrows). Both type A (16.1 to 19.0 μm) and type B (8.4 to 11.8 μm) starch granules were observed. With the support of ImageJ© software (National Institute of Health Image, MD, USA) the size and pore distribution were measured, finding that CB had the largest pores (0.74 mm) against fortified formulations (0.54 to 0.61 mm) (Table 2). However, no statistical difference in the distance between pores among bread samples was observed. Through a surface analysis at x20 and x100 magnifications, it was possible to classify pores by their surface area. For all breads, the most abundant pores were those with the smallest surface area (smaller than 0.1 mm^2). Once again, it was confirmed that the pore size is directly affected by the presence of GP and PS. According to Angelino et al. (2017), PC could interrupt the disulfide bonds of gliadin and glutenin proteins in the gluten network. These modifications weaken the network's ability to retain CO_2 in the fermentation, and therefore the pore size is compromised (Biduski et al., 2021). Also, there is a possibility that gluten was not hydrated enough, which it is another reason to present a low stability in the bread structure (Xu et al., 2019). Similarly, the presence of DF also affects the size of the bread. It has been reported that DF interacts with water decreasing the weight and volume of the bread (Kurek & Wyrwisz, 2015).

When the color of the fortified bread (FW) was analyzed (Table 2), it was found that CB had the highest L* values in crust and crumb, indicating higher luminosity. L* value decreased for all fortified formulations, been this reduction larger for samples containing GP. Both a* and b* increased in the fortified formulations. Bread samples with GP (F1, F2 & F3) were more brownish with higher a* values but lower b* values compared to a sample containing only PS (F4), which presented higher L* and b* and lower a* values. These physical properties evidence the impacts of each by-product, specifically GP. These results can be explained in terms of the by-product flour, considering that GP presented a brownish pigmentation, that has been attributed to the anthocyanins and tannins present in this by-product (Tolve et al., 2021; Yu & Smith, 2015). These differences in L*, a*, and b* can be better observed by determining the color differences (ΔE). In both crust and crumb, it was observed a smaller ΔE, indicating that PS produced a lesser color change. However, all formulations presented values of >3.0 in crust and crumb, indicating that the color difference compared to control is perceptible to the human eye (Sharma et al., 2005).

3.3. FTIR spectroscopy

The FTIR spectra of WF, both by-products' flours and ethanolic extracts (GP and PS), and all the bread formulations (control and F1-F4 in DW) are shown in Fig. 2. The main signals observed for WF were at 1640 cm^{-1} (C═O bonds), 1510 cm^{-1} (C—N bonds), and 3260 cm^{-1} (N—H bonds stretching) (Fig. 2A). These signals correspond to amide groups I (1653 cm^{-1}), II (1500 cm^{-1}), A (3300 cm^{-1}), and B (3100 cm^{-1}), which are associated with gluten and glutamine proteins in wheat (Cortez, 2020; Sivam et al., 2012). The signal at 1020 cm^{-1} corresponds to the C—O and C—C bonds stretching present in the glucose chains of starch. On the other hand, the spectrum at 2880 cm^{-1} corresponds to asymmetric stretching vibrations of C—H bonds of hydrocarbon chains of lipids or carbohydrates (Cortez, 2020; Sivam et al., 2013). PS presented a signal at 1020 cm^{-1} corresponding to β-glycosidic bonds of cellulose or

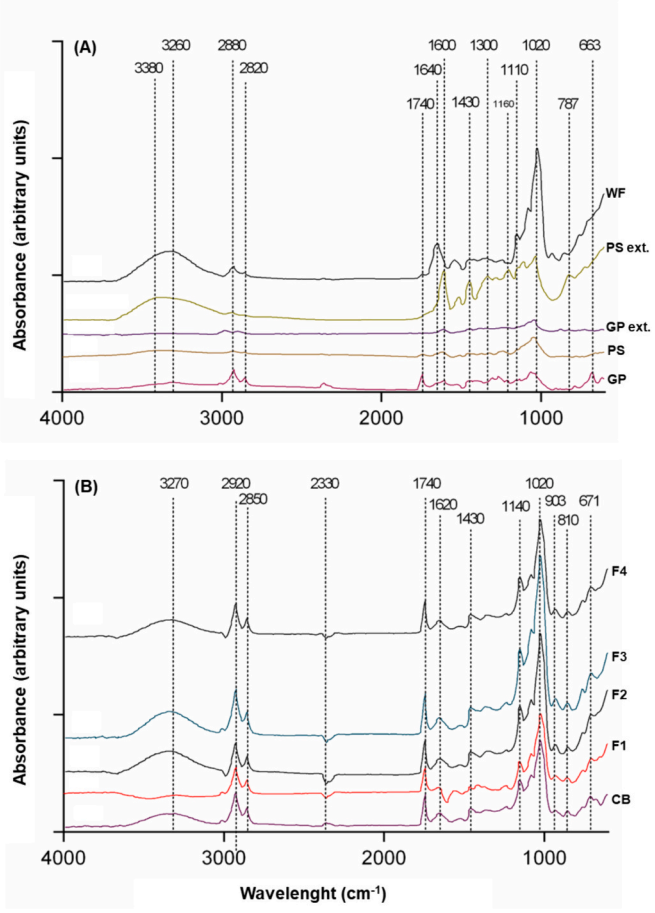

Fig. 2. FTIR spectra of food ingredients (A: WF, GP, and PS flour and their ethanolic extracts) and bread samples at dry weight (B: F1, F2, F3, F4 and CB). Wheat Flour (WF), Pecan Shell extract (PS ext.), Grape Pomace extract (GP ext.), Pecan Shell (PS), Grape Pomace (PC), Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%).

hemicellulose and at 1600 cm^{-1} associated with stretching of COO- groups and aromatic rings. In the same way, the spectra in the range of 1470 cm^{-1} and 1430 cm^{-1} are assigned to glucose C—H bonds or vibrations of phenolic rings (Konsolakis et al., 2015). On the other hand, in GP were detected different vibrational stretches for C—H bonds (2880 and 2820 cm^{-1}), C═O for tartaric acid (1740 cm^{-1}), and C—C for phenolic acids (1400 and 1600 cm^{-1}) (de Oliveira et al., 2018; Lucarini et al., 2020; Sivam et al., 2013). In the case of the extracts of the by-products, peaks close to 1000 cm^{-1} were identified for CH groups of aromatic compounds (Ping et al., 2012; Sivam et al., 2013). These results may indicate that both methanolic extracts present different compositions, in agreement with a previously reported study (Subiría-Cueto et al., 2022).

In Fig. 2B, the main spectral bands of the different bread formulations are shown, in which no important differences between samples were observed. The reported peaks correspond to C—C and C—O bonds for starch (1020 $cm^{-1)}$, and ester and C═O stretching bonds for lipids or lignin (1740 cm^{-1}) (Cueto et al., 2018; Punnadiyil et al., 2016). Signals at 2920 and 2850 cm^{-1} corresponding to C—H bonds stretching from lipids or other macromolecules. The signal at 3270 cm^{-1} indicates the presence of amide A and B of gluten proteins (Sivam et al., 2013; Weibiao & Hui, 2014). Signals in the range of 1200 to 800 cm^{-1} correspond to C-O-H bending and C-O-C stretching of glycosidic and β-1,4 bonds. Likewise, on that wavelength, it has been detected aromatic rings of phenolic acids (Sivam et al., 2013). In this way, it is feasible that interactions between PC and TDF derivatives were taking place in bread

formulations.

3.4. Release of PC and sugars after in vitro gastro-intestinal digestion

After the consumption of the bakery products, a large quantity of monomeric and oligomeric carbohydrates (reducing sugars) and PC are released and absorbed increasing glycemic response and making PC bioavailable (Coe et al., 2013). The release of PC and reducing sugars during an *in vitro* digestive model are shown in Fig. 3. In Fig. 3A, it is possible to observe that no significant amount of PC were released in the oral stage, except for F1 and F2 formulations, which presented the highest phenolic content (Table 1). In the gastric stage, there was a partial release of PC, mainly in F1 and F2 formulations. The largest amount of PC released from bread samples after *in vitro* digestion occurred in the intestinal stage. F1 (1.78 ± 0.3 mg GAE/g bread DW) and F2 (2.04 ± 0.08 mg GAE/g bread DW) showed the largest release of PC, followed by F3. F4, which was only fortified with PS, showed a similar PC release to CB. This could be because PS presented a higher amount of proanthocyanidins and IDF than GP, and consequently lesser PC bioaccessibility. The low release of PC in the oral stage may be the result of the short incubation time, causing a low salivary amylase activity (Velderrain-Rodríguez et al., 2014). On the other hand, in gastric stage there was a partial degradation of food matrix by the stomach acidity conditions and pepsin. However, the low content of released PC indicate that may be bound to resistant digestion components (Quirós-Sauceda et al., 2014). It has been considered that the difference in phenols released in the intestinal stage is due to the presence of pancreatin and the longer incubation period. Pancreatin contains different enzymes, such as of pancreatic α-amylase, trypsin, elastase, and lipases, which promote the degradation of complex molecules (carbohydrates, lipids, and proteins). In this way, PC were able to be released from bounded PC-macromolecules complexes (Jakobek, 2015). The gastrointestinal environment plays a fundamental role to hydrolyze organic molecules, however TDF can resist this degradation conditions. It has been observed that TDF (soluble or insoluble) can attach several molecules, such as PC, and modify their bioaccessibility, including PC (Saura-Calixto, 2011). In this way, the slight differences in PC bioaccessibility of the fortified breads could be relate them to their TDF contents (Table 1).

Table 3 shows the PC bioaccessibility percentages for all formulations. CB showed the highest values, followed by F3, F2, F4 and finally F1. It is important to consider not only the bioaccessibility percentage, but also the PC of each formulation (Table 1). In this way, all formulations except F4 showed higher amount of released PC, compared to CB. Each formulation presented different bioaccessibility percentages, which can be linked to the interactions between PC and TDF. The lower PC released observed in F4 could be explain considering that this sample showed the highest IDF/SDF ratio. These results are in agreement with several studies that evaluated the effect of the interactions of PC with cellulose, lignin hemicellulose (Agama-Acevedo et al., 2016; Ciolacu et al., 2012; Phan et al., 2015). Understanding molecular interactions and their relationship to bioaccessibility is crucial. This allows us to evaluate the bioavailability of compounds and anticipate their possible health effects.

Table 3
In vitro glycemic index of fortified breads.

Formulation	Bioaccessibility of phenols (%)	AUC	*In vitro* Glycemic index	Apparent glycemic index*
CB	43.8 ± 1.8%^{a}	75,377 ± 445.4^{a}	1.00^{a}	1.00
F1	5.7 ± 2.1%^{d}	61,047.5 ± 1014.6^{b}	0.81 ± 0.008^{b}	0.74
F2	13.6 ± 2.2%^{c}	58,691.5 ± 3728.5^{b}	0.78 ± 0.050^{b}	0.80
F3	25.7 ± 0.1%^{b}	57,871 ±50.9^{b}	0.77 ± 0.003^{b}	0.79
F4	11.4 ± 1.7%^{c}	59,180.5 ± 1331.4^{b}	0.79 ± 0.013^{b}	0.77

* Data calculated from released sugars in the intestinal stage of *in vitro* digestive system. Values are expressed as mean ± standard deviation. Bread samples were in DW; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), Area Under the Curve (AUC), Dry Weight (DW). Different superscript per column indicates statistical differences ($p < 0.05$).

Fig. 3B shows the RRS at the three stages for all bread samples reported as mg of glucose/g bread. According to the figure, only a small quantity of glucose was released at the oral and gastric stages, and no significant differences were observed between CB and fortified breads. This trend shares similarity to the PC released, indicating that during the oral and gastric stages, no significant release of these compounds is observed. The highest glucose released occurred in the intestinal stage, being CB the sample with the highest content (223.53 ± 11.4 mg of glucose/g bread DW). F2, F3, and F4 showed similar glucose content, statistically lower than CB, while F1 presented the lowest glucose levels with 157.94 ± 9.3 mg glucose/g bread DW. Similar results were reported for GP bread (Coria-Oliveros, 2020). Interestingly, a slight (non-significant, $r = -0.433$, $P = 0.107$) inverse correlation between released PC and RRS was observed, which may indicate that at higher phenolic content, lower glucose is released. This phenomenon could be related to the ability of some PC to inhibit digestive enzymes. It has been reported

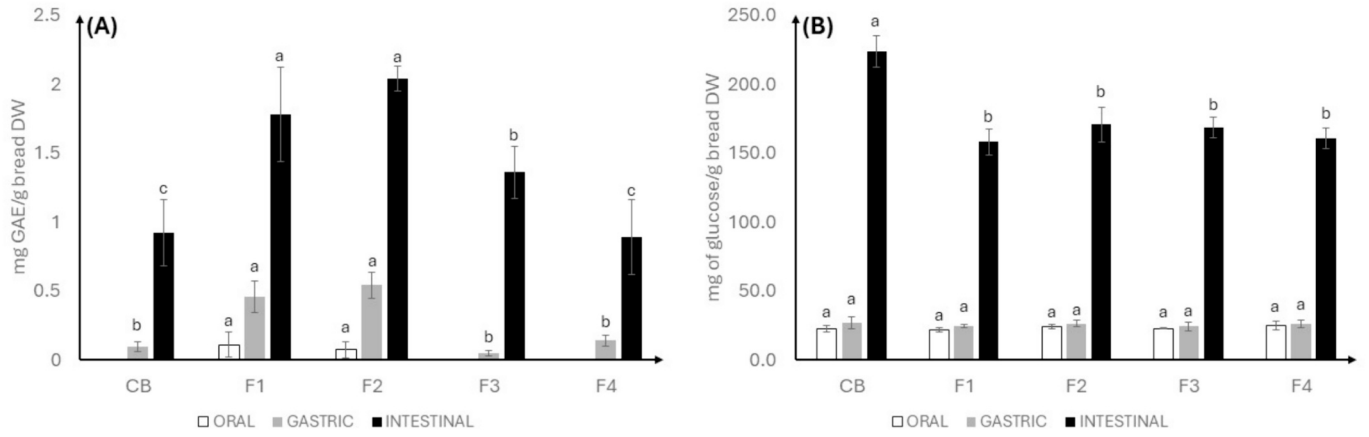

Fig. 3. PC (A) and sugars (B) released during the *in vitro* digestion stages. Values are expressed as mean ± standard deviation; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), Gallic acid equivalent (GAE), Dry Weight (DW). Different letters indicate statistical differences ($p < 0.05$) between the same *in vitro* digestive stages.

that phenolic acids (gallic, ellagic, tannic acids) and flavonoids (quercetin, luteolin, and condensed tannins) can inactivate α-amylase and α-glucosidase (Martinez-Gonzalez et al., 2017). Considering the high amount of PC reported in GP and PS, and fortified bread samples, this could explain the differences in RRS observed in the different formulations. Similarly, it has been hypothesized that the inclusion of TDF in bread could also influence glucose release. In this sense, it has been reported that cellulose, hemicellulose, lignin and pectin reduce the glycemic response (Mudgil & Barak, 2013).

3.5. Glucose release kinetics and in vitro glycemic index

In order to determine the glucose-release kinetic, the incubation period in the intestinal *in vitro* digestion experiments were extended for up to 180 min and aliquots were taken at different times, and glucose content determined by the DNS assay. Fig. 4 shows the glucose content during the 180 min period. Interesting, CB showed higher initial glucose levels, in agreement with Fig. 3B, where CB showed higher oral and gastric glucose content. This higher glucose content was maintained during the 180 min. Interestingly, no differences in glucose content was observed after 45 min, indicating that the glucose release kinetic ended. All fortified breads showed lower initial glucose content, and release during all the experiment, and no statistical differences were observed among samples. Fortified formulations showed a lower glucose release kinetic reaching a plateau at 120 min, indicating that the glucose releases ended at this time. These results reveal that the formulations added with by-products have the capacity to modulate the release of sugars in the digestive tract. This effect has been attributed to TDF because it can bind to carbohydrate monomers (glucose), or by the action of PC to inactivate polysaccharide hydrolytic enzymes (Martinez-Gonzalez et al., 2017; Palafox-Carlos et al., 2011).

To determine the *in vitro* glycemic index (GI), the area under the curve (AUC) of each kinetic was calculated and CB was considered the standard reference, and results are reported in Table 3. Interestingly, the AUC and glycemic index of the formulations did not differ significantly from each other, but they did differ from CB. This indicates that there was a 19 to 23% reduction in the glucose released during the intestinal stage. Even though this *in vitro* GI do not represent the *in vivo* process, it can be used as an approximation to calculate GI of new foods (Ferrer-Mairal et al., 2012; Li et al., 2021). These results were similar to those reported by Rocchetti et al. (2021) for GP fortified bread. To calculate a faster *in vitro* GI, we calculated an apparent GI as the ratio of the total glucose released during all the *in vitro* digestion for all fortified samples against CB (Fig. 3B), and results are depicted in Table 3, Interesting, both *in vitro* GI an apparent GI showed similar results, indicating that this apparent GI can be used as an approximation to the *in-vivo* studies. Some studies have determined the *in vivo* glycemic response of fortified breads with other by-products (milling by-products and pomegranate peel), observing a reduction on the glycemic response (36.9 and 28.0% GI reduction) (García et al., 2023; Pontonio et al., 2017). These findings support the information obtained in the *in vitro* digestion experiments, corroborating the relevance of GP and PS in the development of healthy food products.

4. Conclusions

The inclusion of GP and PS in the formulations resulted in an increase in proteins, ashes, PC, TDF in fortified breads, without modifying their sensory acceptability. In nutritional terms, F1 showed the highest values in most of the physicochemical properties, including TPC. IDF/SDF ratio was only increased in F3 and F4 formulations. The use of GP and PS decreased the height of all fortified breads between 24 and 35% compared to CB, while their color presented brownish tones and reddish colors with low luminosity. In the same sense, the size of the pores decreased between 17 and 27%. Through FTIR analysis, changes in the absorbances of functional groups belonging to gluten and starch were identified, evidencing molecular interactions between PC and TDF. The *in vitro* digestion showed that the addition of PS and GP by-products increased the PC release for all fortified samples. Also, there was an observable change in the release of sugars, which was attributed to the interactions of TDF with gluten and PC with digestive enzymes. Finally, the glucose release kinetics in the intestinal stage corroborated a 19–23% reduction of glucose release in all formulations, using *in vitro* glycemic index. This information supports the potential use of GP and PS by-products to develop food products with functional and technological characteristics and health effects.

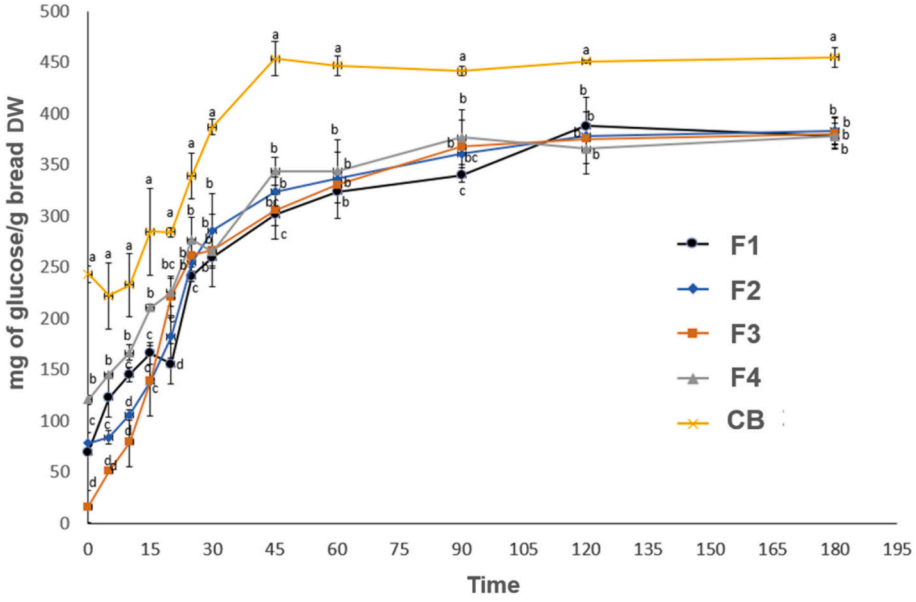

Fig. 4. Glucose release kinetics in the intestinal stage for *in vitro* glycemic index determination. Values are expressed as mean ± standard deviation; Control Bread (CB), F1 (GP:8%, PS 5%), F2 (GP: 5%, PS: 5%), F3 (GP: 5%, PS: 2%), F4 (GP: 0%, PS: 5%), Dry Weight (DW). Different superscript per column indicates statistical differences ($p < 0.05$).

CRediT authorship contribution statement

Rodrigo Subiria-Cueto: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Hortensia Reyes-Blas:** Methodology, Data curation. **Imelda Olivas-Armendáriz:** Methodology, Data curation. **Abraham Wall-Medrano:** Visualization, Methodology, Data curation. **Gustavo Adolfo González-Aguilar:** Writing – review & editing, Methodology, Investigation. **Laura A. de la Rosa:** Writing – review & editing, Project administration, Funding acquisition. **Nina del Rocío Martínez-Ruiz:** Writing – original draft, Investigation, Conceptualization. **Emilio Alvarez-Parrilla:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank CONAHCYT (CB-2016-286449) for financial support. Carlos Rodrigo Subiría-Cueto thank CONAHCYT for their postgraduate scholarships.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140925.

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