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Pilot scale assessment for seed protein enrichment of gluten-free breads at varying water content levels and after protein modification treatments

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Abstract

Three different seed protein concentrates were utilized in protein enrichment of gluten-free breads and their influence on texture, volume, color, and visual parameters were evaluated. First, protein concentrates (PC) were obtained from black cumin, grape seed, and pumpkin seed meals. For the original recipe, inclusion of proteins lowered loaf volumes, while volumes were improved when water content was increased (8% or 15%). Considering color, pumpkin seed protein concentrate (PSPC) samples demonstrated the highest level of similarity to controls, while black cumin protein concentrate samples were distinctly darker. Protein concentrates significantly increased firmness and decreased springiness of the crumb with the exception of PSPC samples, while further water inclusion enhanced the firmness attribute. When Maillard conjugation or TGase treatments were administered, firmness increased after both treatments, however, treated proteins enhanced cell distribution and homogeneity. The results showed current PCs can be utilized in enrichment of gluten-free breads.

Practical applications

Gluten-free bread formulations generally lack the texture and satiety of normal breads, especially due to the technical functionality of gluten proteins. Based on plant protein enrichment, a range of gluten-free breads were manufactured with increased protein content. The current findings on the textural attributes and water-holding capacities are applicable to various baked goods. Protein supplementation can also lower glycemic index in bread formulations.

1 | INTRODUCTION

Wheat is the grain that is most commonly used for bread production and bread making quality of wheat is related to the concentration and quality of gluten proteins. These proteins form a thin film layer during dough kneading, retain CO_2 produced by the yeast during the fermentation, and hence, raise the dough. While glutenins in wheat gluten are critical for the elasticity and mechanical strength of the dough (Xu, Bietz, & Carriere, 2007), gliadins are influential on the viscous characteristics and extensibility (Don, Lichtendonk, Pfijter, & Hamer, 2003a, 2003b).

Celiac disease is an autoimmune disorder and requires the elimination of gluten containing foods from the diet. In celiac patients, the regular consumption of gluten could lead to serious intestinal damage (Marco & Rosell, 2008a). Utilization of nongluten proteins in bread manufacture could enhance technical and sensory qualities, while preventing the celiac related damage and lowering glycemic load, which is relatively high in hydrocolloid based nongluten flour mixes (Sahagún, Benavent-Gil, Rosell, & Gómez, 2020). Consequently, a variety of studies have been conducted on the production of various gluten-free bread formulations (Föste et al., 2014; Rodriguez Furlan, Padilla, & Campderros, 2015; Vijaykrishnaraj, Roopa, & Prabhasankar, 2016).

Gluten-free breads have unique structural characteristics that lead to gritty mouth feel, due to a low hydration rate. Due to this observation, there have been major efforts to improve the texture of gluten-free formulations (Gallagher, Gormley, & Arendt, 2004). For example, Storck et al. (2013) studied protein-enriched gluten-free breads in the presence of transglutaminase (TGase) treatments and found that specific volume and crumb texture were enhanced when TGase treatment was combined with albumin or casein addition. Under appropriate conditions, foaming characteristics of proteins could be enhanced via Maillard based glycation (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001).

Black cumin (BC) (*Nigella sativa*) is a valuable and annually flowering medicinal plant from Ranunculaceae family (Baydar, 2009) which is native to the East Mediterranean countries, South Europe and Asia Minor (Baydar, 2009; Baytop, 1999). Currently, BC is also cultivated in the Middle East, North Africa, and Asia (Durani et al., 2007). According to Commodity Trade Statistics Database, the global consumption of BC was estimated to be 187,000 tonnes in 2011. Previously, defatted black cumin meal was used in the preparation of flat bread to deliver required adult daily intake of zinc, potassium, phosphorous, iron, and copper while the protein content of the flat bread was also enhanced. Defatted black cumin meal was reported to demonstrate twice the water-holding capacity of whole wheat flour, although its inclusion minimally affected final viscosity and water absorption by whole wheat flour (Osman et al., 2014). Along with milled sesame seeds and white rice samples, milled black cumin seeds were utilized in pan bread formulations (Al-Subhi, 2014). Addition of milled black cumin or sesame seeds enhanced the nutritional quality of the pan breads and also lead to acceptable quality products (Al-Subhi, 2014).

Pumpkin seed proteins (PSP) have a good potential of utilization in food formulations due to their health promoting effects such as anticarcinogenic activity, prevention of protein malnutrition, and inhibition of blood coagulation (Bucko et al., 2015). Furthermore, antidiabetic (Quanhong, Ze, & Tongyi, 2003), antifungal, antibacterial, anti-inflammatory (Caili, Huan, & Quanhong, 2006), and antioxidant activities (Nkosi, Opoku, & Terblanche, 2006) were demonstrated. Their utilization in bread formulations and nutritional supplements has been previously documented (El-Soukkary, 2001). Addition of pumpkin seed flour to gluten-free rice bread resulted in modified textural properties including decreased crumb springiness, cohesiveness, and resilience and increased crumb hardness and chewiness, while the influence on tested sensory attributes was slight (Dabash, Burešová, Tokár, Zacharová, & Gál, 2017).

Grape (*Vitis vinifera L.*) seeds (GS) constitute another abundant source for seed proteins, since grapes are among the most heavily cultivated fruits at 69 million tons annually (FAOSTAT, 2011). The seeds account for approximately 2%–3% of the total harvest and its protein content is approx. 10%–13% (Fantozzi, 1981). In winemaking, for example, roughly 13% of grape weight is converted to grape pomace, which is the major by-product and about 38%–52% of the pomace is derived from GS on a dry weight basis. After winemaking or cold press oil processing, GS by-products could be utilized as a source of plant proteins that demonstrate nutritional and technical value (Zhou, Zhang, Liu, & Zhao, 2011). In a study by Mironeasa, Iuga, Zaharia, Dabija, and Mironeasa (2017), GS flour brought to various sizes was combined with wheat flour in order to improve rheological properties of the dough. Since the rheological properties of the enriched dough was highly dependent on particle size, GS flour could be potentially utilized in a range of baked goods.

In this study, cold press deoiled meals of BC, PS, and GS were valorized in order to generate protein concentrates (PC), which in turn were used in protein enrichment of gluten-free bread products and the influence of protein enrichment on the textural properties was investigated. In the hopes to enhance the performance of seed proteins, Maillard conjugation, and TGase treatments on PCs were also studied. In any case, protein concentrate manufacture or modification treatments did not require the usage of any toxic chemicals or organic solvents. Consequently, current techniques are adaptable to industrial bread manufacture practices.

2 | MATERIALS AND METHODS

2.1 | Materials

Cold press deoiled meals of BC, PS, and GS meals were generously donated by Oneva (Neva Foods Ltd., İstanbul, Turkey), a local manufacturer of cold press oils. Commercial gluten-free flour mix containing corn starch, rice flour, sugar, leavening agents (sodium bicarbonate, sodium acid pyrophosphate), and thickeners (pectin, xanthan gum) was purchased from İHE, İstanbul, Turkey. Composition details were listed by the manufacturer at the following link: (http://www.ihe.istanbul/u/colyaklilar-icin-gnk-64). In addition, commercial salt, sunflower oil, and compressed yeast (Marmara Maya, Turkey) were also used in bread manufacture.

Transglutaminase was acquired from Tito (İstanbul, Turkey). All chemicals used (i.e., HCl, NaOH, NaCl, glucose, Tris, Z-Gln-Gly, $CaCl_2$, hydroxylamine, and $FeCl_3$) were of reagent grade and purchased from Sigma-Aldrich. The protein and moisture contents of black cumin protein concentrate (BCPC), pumpkin seed protein concentrate (PSPC), and grape seed protein concentrate (GSPC) were found to be approx. 54.7%, 83%, and 30.1%, and 0.8%, 5.7%, 7.6%, respectively. In addition, the oil and ash contents of the protein concentrates were found to be 22.3%, 4% and 5.4% and 1.3%, 1.7%, and 5.5%, respectively.

2.2 | Methods

2.2.1 | Preparation of seed protein concentrates

In all cases, further solvent based extraction was avoided in order to preserve the structural and functional characteristics of protein concentrates. Protein manufacture was based on the alkali extraction–isoelectric precipitation technique. This technique relies on the solubilization of protein molecules at basic pH, which is followed by the isoelectric precipitation at acidic pH values. Based on this approach, protein concentrates were produced using the method of Boye et al. (2010) with slight modifications (Coşkun, Çakır, Vahapoğlu, & Gülseren, 2019). Briefly, deoiled meal samples were dispersed in water (1:15, wt/vol) and the pH of the medium was brought to pH 9.5 using a NaOH solution (1 M). The dispersions were kept stirred (Isolab magnetic stirrer, Ref. No: 61302001 Germany) at 500 rpm for 1 hr at ambient temperature (22 ± 1°C). Immediately afterwards, the dispersions were centrifuged at a rate of 4,200× *g* (Mixtasel-BL centrifuge, Abrera, Barcelona, Spain) for 30 min. The supernatant containing the solubilized proteins was collected and the medium pH was brought to pH 4.5 using 1.0 N of HCl in order to induce isoelectric precipitation. To ensure the complete separation of precipitating proteins, the supernatant was once again centrifuged at 4,200× *g* for 30 min. The pellet was collected and immediately frozen at −20°C. Frozen samples were lyophilized using a Teknosem TRS 2/2V freeze drier (Teknosem Corp., İstanbul, Turkey) for approx. 24 hr (−58°C, approx. 10^{-3} mbar operation pressure). The moisture contents of BCPC, PSPC, and GSPC were found to be approx. 0.8, 4.6, and 4.4%, respectively. In the lyophilizates prepared from 10 g of meal sample for BC, PS, and GS, 3.38, 0.67, and 0.37 g of protein concentrates were obtained on a dry weight basis, respectively.

2.2.2 | Modification of protein functionality

Maillard conjugation and TGase treatments were performed in order to modify the functional properties of the protein concentrates. For modified protein enriched bread formulations, only a single water level was applied (i.e., 345 ml).

Modification of protein functionality by Maillard conjugation

Maillard conjugation was carried out under optimum wet processing conditions tested in the preliminary runs (1:2 protein: glucose ratio by weight, pH 7, 15 min 100°C) (data not shown) (Coşkun, Çağlar, Çakır, & Gülseren, 2019). First, lyophilized protein concentrate (1.5%) was kept stirred in pH 12 water to facilitate dissolution for 1 hr at ambient temperature pH was adjusted to pH 12 with 10 M of NaOH. Immediately afterwards, glucose was dissolved in the solution. Using a water-bath, the resulting mixture was then allowed to stand at 100°C for 15 min in order to induce Maillard reaction based conjugation. The mixture was removed from the water-bath; the container was rapidly cooled on ice to stop the reactions. Once ambient temperature was reached, the medium was adjusted to pH 7 and a final ionic strength of 100 mM using appropriate volumes of sodium phosphate buffer. The resulting solution was used in the manufacture of protein enriched gluten-free breads as described below.

Modification of protein functionality by TGase treatments

In the case of TGase treatments, protein concentrate dispersions were prepared (1.5%). Immediately afterwards, 50 mg of commercial TGase was used for each percentage of the protein samples and the solution was kept stirred at 37°C for 16 hr using a magnetic stirrer (Anuradha & Prakash, 2009). The resulting solution was then used in the manufacture of protein enriched gluten-free breads. In all cases concerning the use of modified proteins, the protein concentration was kept constant regardless of the extent of conjugation with glucose or the relative activity of the enzyme.

Commercial TGase activity was determined according to the method of Zeeb et al. (2013) with some modifications. Using Tris–buffer (200 mM, pH 6.0) as a solvent, the mixture of the substrate (Z-Gln-Gly, 12 mg•ml^{-1}), 100 mM of hydroxylamine, 10 mM of glutathione (reduced), and 5 mM of $CaCl_2$ was prepared. The mixture was incubated at 37°C for 5 min using a thermomixer (MIULAB Thermo Shaker Incubator, 1,000 rpm) to ensure thermal homogeneity. About 30 μl of enzyme solution (100 mg•ml^{-1}) was added to initiate the reaction. The reaction was rapidly stopped by TCA addition (12% (wt/vol), 500 μl) 10 min after the initiation step. Finally, a $FeCl_3$ solution (5% (wt/vol), 500 μl) prepared in HCl (100 mM) was added and the absorbance values were measured at 525 nm (Optima SB-3000 UV/VIS, spectrophotometer). TGase enzyme from Sigma (T5398) was used as a reference. TGase activity was defined based on the performance of the reference enzyme at 37°C and pH 7.0, where a transglutaminase activity of 0.1 unit•mg^{-1} was measured for the commercial enzyme utilized here.

2.2.3 | Protein content analysis

The protein content of the protein concentrates was determined according to AOAC Official Methods 920.87) (AOAC, 2003) (% N × 6.25). Based on the protein content data, necessary adjustments were made to ensure a constant level of protein inclusion in the bread formulations.

2.2.4 | Bread making

Bread making, color analysis, texture profile analysis (TPA), and loaf volume analysis were conducted at Polen Foods Ltd (İstanbul, Turkey). Preliminary farinograph analyses indicated that protein concentrates had strong influence on dough properties (data not shown). Consequently, the water content utilized in bread baking was applied at three different levels. First, 300 ml of water, 300 g of gluten-free flour, 3.6 g of salt, and 12 g of pressed yeast were used for the production of gluten-free bread. In the following set of experiments, water content was increased by 8% and 15% to 324 and 345 ml, respectively, while the other parameters were kept constant. The recipes for the bread formulations were summarized on Table 1. Protein enrichment was carried out with BCPC, GSPC, and PSPC

TABLE 1 Gluten-free bread formulations

Ingredient	Control	BC	GS	PS
Water (ml)	300	300	300	300
Water level increased by 8% (ml)	324	324	324	324
Water level increased by 15% (ml)	345	345	345	345
Gluten-free flour (g)	300	300	300	300
Salt (g)	3.6	3.6	3.6	3.6
Compressed yeast (g)	12	12	12	12
Sunflower seed oil (g)	18	18	18	18
BCPC (g)	–	8.22	–	–
GSPC (g)	–	–	15	–
PSPC (g)	–	–	––	5.42

Abbreviations: BC, black cumin; BCPC, black cumin protein concentrate; GS, grape seed; GSPC, grape seed protein concentrate; PS,pumpkin seed; PSPC, pumpkin seed protein concentrate).

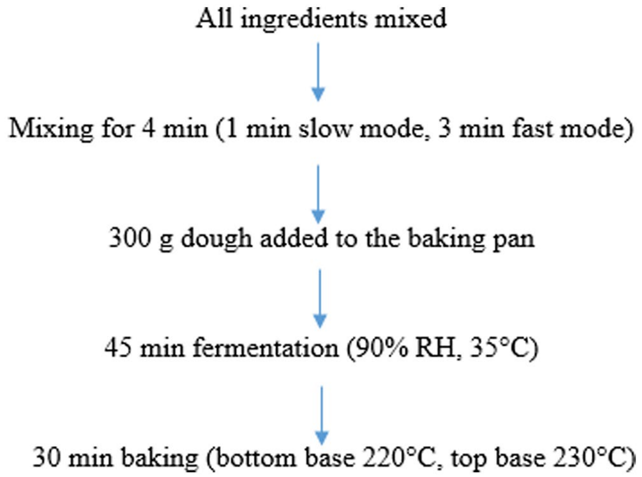

FIGURE 1 Bread making flowchart

samples. Including the control samples and three different protein concentrates, four sets of samples were prepared at three different water levels leading to the preparation of 12 different bread formulations. The level of protein enrichment was 1.5% in all cases.

For the production of gluten-free breads, all the ingredients were placed in the same set-top mixer (Kenwood set top mixer, major titanium, palette beater). After 3 min of fast and 1 min of slow mixing, a 300 g sample was withdrawn from the dough and placed in the cake molds used for gluten-free bread manufacture and kept in the fermenter for 45 min (Tecnomac, Juniorlev, Italy). The bottom and top were baked at 220 and 230°C, respectively, for 30 min using an industrial baking oven (Wiesheu Wolfen GmbH, Germany). After baking, the loaves were removed from the pans, and cooled at ambient temperature (21°C ± 1). Bread making procedures were summarized on Figure 1.

2.2.5 | Color analysis

The crumb color of gluten-free bread samples was determined using Hunter Lab ColorFlex EZ spectrophotometer (Hunter Associate Laboratory, Murnau, Germany). The instrumental calibration was carried out using a white standard calibration plate. The color attributes were expressed in CIE-Lab space as L^* (whiteness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness) (Jafari, Koocheki, & Milani, 2017).

2.2.6 | Loaf volume analysis

Loaf volumes of gluten-free breads were determined using a displacement technique. First, a certain amount of hemp seeds was placed into the container until overflow. Then, the hemp seeds were removed from the container, and the bread was placed in the container. Finally, the gaps were filled with hemp seeds to calculate the loaf volume based on the difference between the total volume and volume of remaining hemp seeds (ml) (Hamid & Luan, 2000).

2.2.7 | Texture profile analysis

TPA of gluten-free bread samples was performed using a texture analyzer (TA-XT2 plus, Stable Micro Systems, UK), according to the working standardized program of the supporting company, at a test speed rate of 1 mm/s. Bread crumb samples were taken from the center of the loaf at a height of 2 cm and was pressed to reach 25% of strain using a P/236 R probe (cylindrical probe with a radius of 36 mm) for 60 s. The calculations were performed by using the Texture Exponent software provided by the manufacturer. The analysis was performed 2 hr after baking when cooling of the crumb was complete (Ziobro, Witczak, Juszczak, & Korus, 2013).

2.2.8 | Statistical analysis

The data obtained were analyzed by using SPSS statistical software (version 25, SPSS Inc., Chicago, USA). One-way ANOVA with post hoc Tukey's tests were carried out to compare groups (i.e., differences in groups and between groups). Data were expressed as sample mean ± standard deviation. In all cases, at least three replicates for the samples were analyzed.

3 | RESULTS AND DISCUSSION

3.1 | Bread making and loaf volume analysis

Gluten-free breads produced with varying water levels (300, 324, or 345 ml) and enriched with BCPC, GSPC, or PSPC at a protein concentration level of 1.5% were shown in Figure 2. Visually, BCPC and

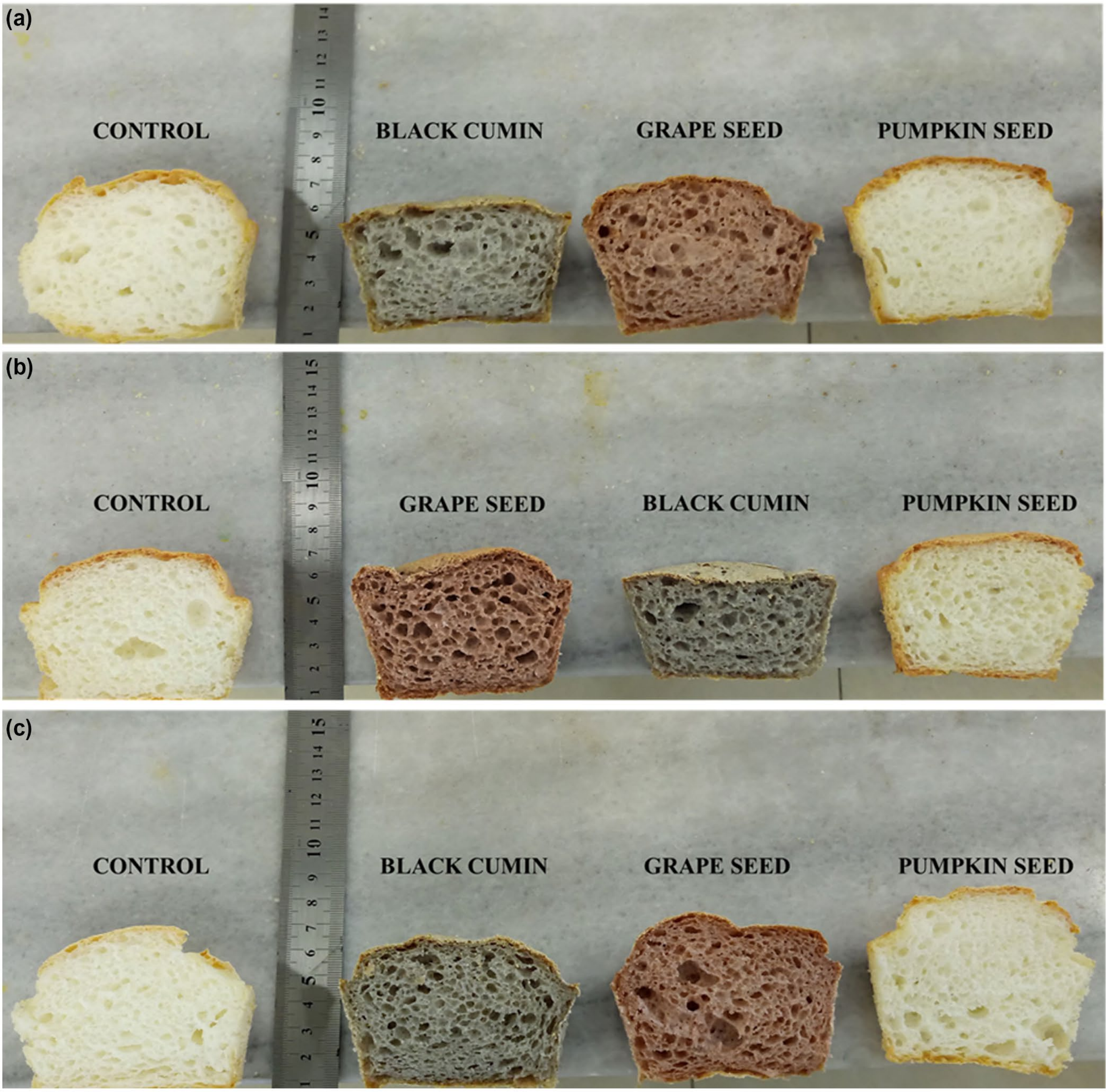

FIGURE 2 Baked gluten-free breads enriched with protein concentrates grape seed, black cumin, and pumpkin seed protein concentrates at varying water content levels. (a) Original water content (300 ml). (b) water content increased by 8% (324 ml). (c) water content increased by 15% (345 ml)

GSPC enriched samples had lower volumes compared to control and PSPC samples (Figure 2a). The differences in volume could possibly be attributed to the water-holding property of the proteins (Aprodu, Badiu, & Banu, 2016; Renzetti, Dal Bello, & Arendt, 2008). GSPC enriched gluten-free bread had higher volume than the BC counterpart but still had less volume than the control bread. The volume of PSPC enriched gluten-free bread only slightly decreased compared to the control, while this change was not statistically significant (Table 2). The differences between the concentrates might be attributed to the differences in protein characteristics as well as the impurities in each protein concentrate.

The composition of all three seeds studied here are characterized by considerable concentrations of protein and carbohydrates. For example, crude protein and crude carbohydrates account for approx. 20% and 37% in the black cumin seeds, respectively (Nergiz & Ötleş, 1993). Approximately, 39% of crude protein and 10% of carbohydrates were detected in the kernels of pumpkin, while these figures were not quite different than that of the whole seed (Alfawaz, 2004). Grape seed had a lower protein: carbohydrate ratio (8.2% to 37%, on dry weight basis) (Kamel, Dawson, & Kakuda, 1985) rendering protein extraction difficult and lowering its extraction efficiencies. In all current investigations, seed meals had significant contents of ash, fiber, and oil. Since the ash

TABLE 2 Loaf volume analysis results for gluten-free breads (ml)

Sample	Original water level	Water level increased by 8%	Water level increased by 15%	Maillard conjugation	TGase treatment
Control	920 ± 20.9^{a}	790 ± 28.5^{c}	715 ± 11.3^{e}	1,010 ± 22.5^{f}	940 ± 25.0^{i}
BC	380 ± 30.6^{b}	565 ± 25.1^{d}	800 ± 15.2^{e}	710 ± 33.8^{fh}	660 ± 31.2^{k}
GS	465 ± 16.3^{b}	850 ± 34.0^{c}	1,000 ± 20.0^{e}	880 ± 18.7^{g}	860 ± 20.6^{i}
PS	850 ± 20.0^{a}	860 ± 22.7^{c}	950 ± 15.3^{e}	905 ± 29.1^{fg}	905 ± 13.3^{i}

Note: The data represent the average of three independent experiments with ± standard deviation. One-way ANOVA post hoc Tukey's tests were used to verify significance ($p < .05$).

$^{a\text{-}i}$The significance level is $p < .05$ in all cases.

Abbreviations: BC, black cumin protein concentrate fortified gluten-free bread; GS, grape seed protein concentrate fortified gluten-free bread; PS, pumpkin seed protein concentrate fortified gluten-free bread.

and fiber components are mostly water soluble and/or dispersible, it is not possible to completely remove them from protein concentrates that were prepared through aqueous processing. As an example, the presence of fiber in bread mixes could be anticipated to limit the access of other molecules to water (Osman et al., 2014), which in turn could affect the structural attributes of the baked product negatively.

As summarized above, the loaf volume of gluten-free bread samples enriched with BCPC, GSPC were lower than control and PSPC sample at the original water level (i.e., 300 ml). As the water level increased, various changes in the volumes of the enriched gluten-free breads were observed (Table 2). For example, loaf volume of BCPC, GSPC, or PSPC enriched gluten-free breads were 380, 465, and 850 ml, respectively, whereas loaf volume of control sample was 920 ml for the original water level. However, loaf volumes of BCPC, GSPC, and PSPC enriched gluten-free breads were 800, 1,000, and 950 ml, respectively, when the water level was increased by 15%. When the water level was increased by 15% though, all breads had statistically similar loaf volumes ($p > .05$). The reduction in the volume of the control bread seen in Figure 2c could be attributed to the relative reduction in protein concentration.

In the previous literature, the specific volumes of gluten-free breads were shown to increase primarily due to the water retention capacity of proteins (Sanchez, Osella, & de la Torre, 2004). Although BC meal samples had high water-holding capacities (WHC), their influence on WHC of the final mixture in the manufacture of flat bread formulations was minimal. The authors attributed this finding to the low hydration rate of the BC meals (Osman et al., 2014). According to Ziobro, Witcak, Juszczak, and Korus (2013), the addition of pea protein had no significant influence on gluten-free bread volume, while soy protein decreased the volume and lupine protein increased it. The key factor seemed to be the composition and physicochemical characteristics of protein used for supplementation, which could swell and denature at high temperatures providing structural support to starch and hydrocolloids used in gluten-free bread manufacture.

Previously, Ribotta et al. (2004) reported that production of gluten-free bread formulation containing soybean flour resulted in a low bread volume when it was baked at 200°C. Alvarez-Jubete, Auty, Arendt, and Gallagher (2010) produced gluten-free bread with amaranth, quinoa, and buckwheat and reported that these samples had larger cell volumes than the control sample. Proteins tend to lower the number of missing cell walls created during processing (Scanlon & Zghal, 2001) and their inclusion may alter the textural attributes in the final product, including cell volume.

Gluten-free breads produced with modified protein concentrates were shown in Figure 3. In this particular case, protein dispersions were subjected to Maillard conjugation or TGase treatments in order to modify their functional characteristics. During the modification treatments, protein concentration in the samples was kept constant regardless of the extent of conjugation with glucose or the relative activity of the enzyme.

In the original set of samples including protein enriched samples or controls (Figure 2), the cells were large and not homogeneously distributed as qualitatively observed to the naked eye, whereas in the modified protein enriched samples (Figure 3), these properties were improved by Maillard conjugation or TGase treatments. This situation could be explained by the enhanced ability of modified proteins to hold CO_2 (Ziobro et al., 2013). First, as the leavening agent generates CO_2, it will diffuse throughout the liquid phase due to the gas concentration gradient. Consequently, the gas cell nuclei expand and overall density of the dough is reduced (Ribotta et al., 2004). In this stage, the foaming capacity of the dough is critical for the formation of the gas network (Moore, Heinbockel, Dockery, Ulmer, & Arendt, 2006). Protein addition has a potential to enhance foaming characteristics. Ziobro et al. (2013) reported that high specific volume of the albumin bearing bread could be attributed to the foaming capacity of protein molecules, which is obviously highly protein dependent. Volumes for modified protein bearing samples were also listed in Table 2. Gluten-free breads that were enriched with Maillard conjugates of BCPC had lower volume than the other samples but the pores were more homogeneous and smaller (Figure 3a, Table 2, $p < .05$). The other two enriched samples had statistically similar volume as the control sample. Loaf volumes of the nonmodified protein containing breads were higher at similar water addition level (i.e., 345 ml) (Table 2).

In the case of TGase treated and protein enriched gluten-free breads, volumes of the PSPC and GSPC enriched samples were comparable to the control sample, whereas BCPC enriched sample had lower volumes. However, the volume for TGase treated BC sample was statistically different from the control samples.

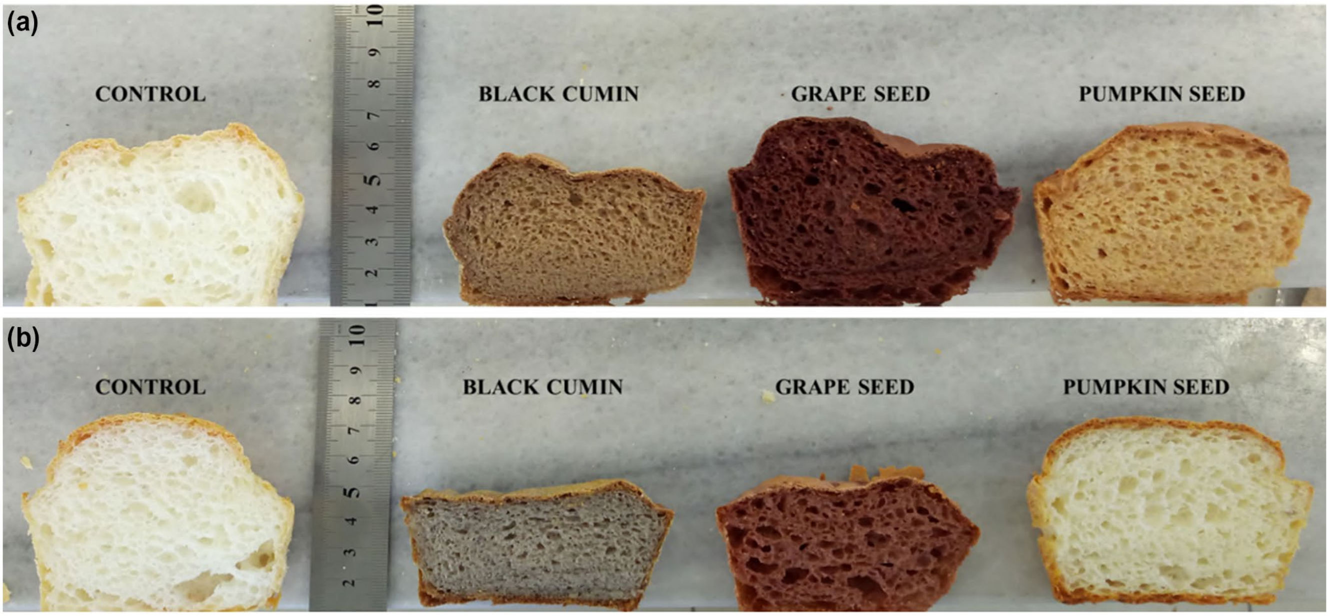

FIGURE 3 Baked gluten-free breads enriched with modified protein concentrates of black cumin, grape seed, and pumpkin seed. (a) Maillard conjugation. (b) TGase treatment

TGase treated BCPC, GSPC, and PSPC enriched gluten-free breads had 660, 860, and 905 ml of loaf volume, respectively. The decrease in the volume produced by TGase treated samples (i.e., in comparison to nonmodified protein bearing samples) might be due to the increase in the molecular weight and flexibility loss in protein molecules that occur due to enzymatic cross-linking (Marco & Rosell, 2008a, 2008b), since cross-links between glutamine and lysine residues inhibit the expansion of gas cells during the fermentation (Mohammadi, Azizi, Neyestani, Hosseini, & Mortazavian, 2015). However, BCPC samples had smaller pores and the pores were more homogeneously distributed compared the control sample (Figure 3b). These findings were consistent with the data of Bonet, Blaszczak, and Rosell (2006) obtained in wheat doughs containing TGase treated soy flour. Consequently, although the addition of TGase treated protein concentrates mostly decreased the bread volume, the distribution, and size of air cells in the bread samples were, however, improved by Maillard conjugation or TGase treatments.

3.2 | Textural parameters

Firmness and springiness are important sensory attributes of bread formulations, including gluten-free breads. Firmness of the products is primarily due to the cross-links between glutens and partly solubilized starch (He & Hoseney, 1990). In the absence of gluten, molecular interactions change, which in turn affects the firmness attribute. Springiness is the ability of the bread sample to go back to its original shape soon after being pressed down by the crumb and is generally associated with freshness and elasticity. Consequently, high quality breads will attain high springiness values (Matos & Rosell, 2012). Low springiness values are indicative of brittleness and the tendency of the bread to crumble upon slicing (McCarthy, Gallagher, Gormley, Schober, & Arendt, 2005). Since gluten-free breads are often characterized by a crumbly, brittle texture, increased springiness will be regarded as beneficial (Alvarez-Jubete et al., 2010).

Textural parameters such as firmness (g) and springiness (%) were determined for BCPC, GSPC, and PSPC enriched gluten-free bread samples (Figure 4). In this context, firmness and springiness values of control, BCPC, GSPC, and PSPC enriched gluten-free breads were 310.5, 2,455, 659.7, 168.08 g, and 65.5%, 49.9%, 55%, 70.8% for the original water level (i.e., 300 ml), respectively. Consequently, at the original water level protein enriched samples had a poor performance compared to the control bread excluding PSPC enriched gluten-free bread. However, as the water level increased in all cases firmness and springiness attributes improved (Figure 4, $p > .05$), which meant it was possible to define an optimum water level where the enriched samples performed favorably.

Previously, Phongthai, D'Amico, Schoenlechner, and Rawdkuen (2016) reported that addition of rice bran and egg albumin to the gluten-free bread formulations increased firmness values. Furthermore, the extent of increase in firmness was proportional to the amount of protein added (Kittisuban, Ritthiruangdej, & Suphantharika, 2014). As protein enrichment was practiced in gluten-free bread manufacture, the gas cell walls within the bread crumb were thickened leading possibly to increased firmness (Rodriguez Furlanet al., 2015). Phimolsiripol, Mukprasirt, and Schoenlechner (2012), Schoenlechner, Mandala, Kiskini, Kostaropoulos, and Berghofer (2010), Crockett, Ie, and Vodovotz (2011), and Sahagúnet al. (2020) also reported that addition of protein increased firmness values of gluten-free breads. Ziobro et al. (2013) analyzed soy enriched gluten-free bread

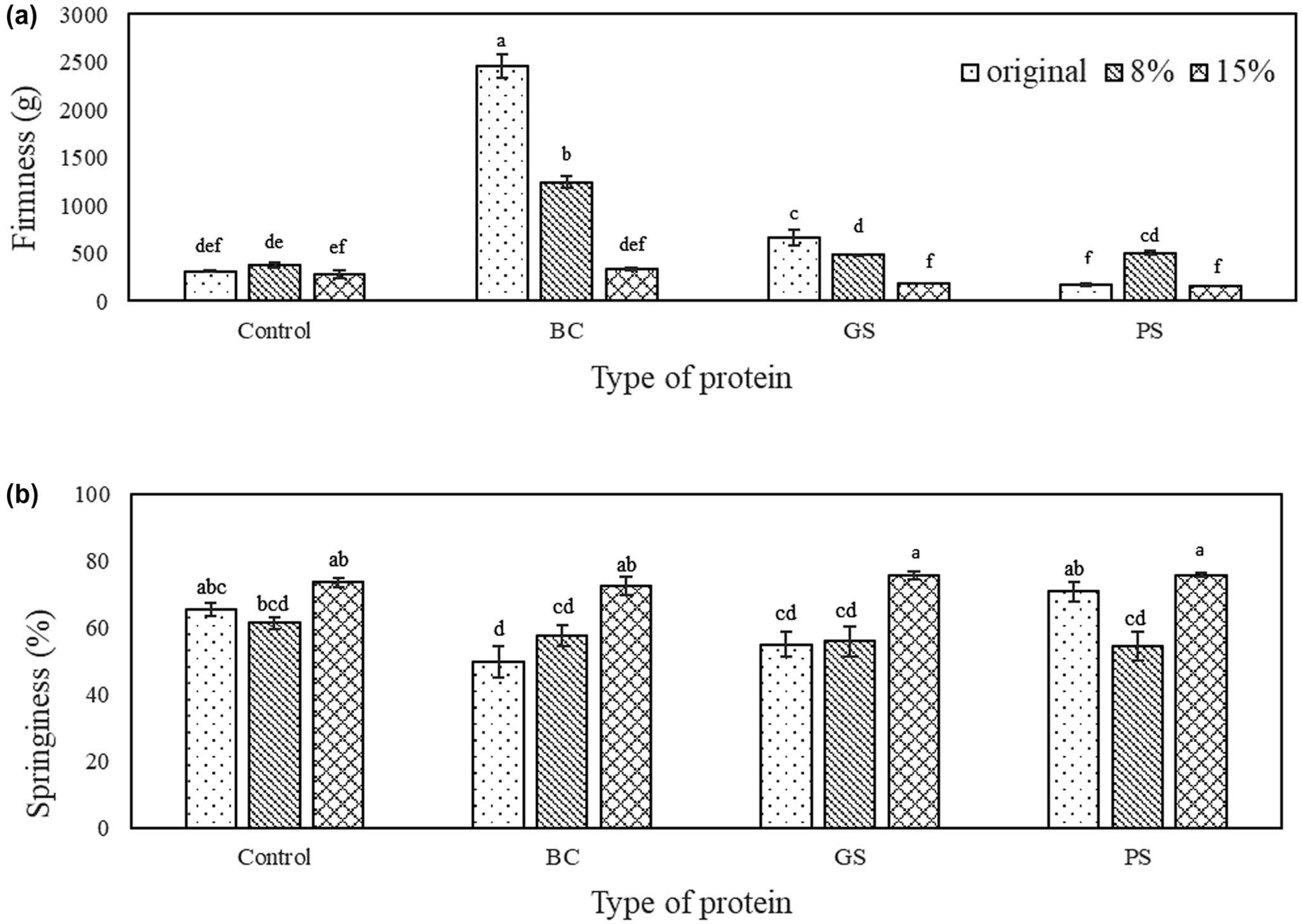

FIGURE 4 Textural parameters of gluten-free control bread and gluten-free breads enriched with protein concentrates. BC, black cumin; GS, grape seed; PS, pumpkin seed protein concentrates. (a, b) represent firmness (g) and springiness (%), respectively. Presented data and error bars represent the sample mean of three replicated experiments and standard deviations, respectively

and they reported springiness of the soy protein enriched gluten-free bread was smaller than the control sample. According to our results, in all cases, the springiness values were lower than the control except for the gluten-free bread enriched with PSPC. However, when the water level increased by 15%, the springiness values were found to be statistically similar to the control sample (Figure 4b, $p > .05$). Consequently, we report that addition of extra water has a potential in determining the firmness and springiness attributes of protein enriched gluten-free breads. Most of the differences in springiness values were not statistically significant.

Firmness (g) and springiness (%) values of modified protein enriched breads were also tested for samples including Maillard conjugates of BCPC, GSPC, and PSPC and their TGase treated counterparts (Figure 5). In this context, firmness value of Maillard conjugate control and enriched BCPC, GSPC, and PSPC conjugate samples were 152.6, 302.1, 345.9, and 282.8 g, respectively, indicating a clear rise in firmness attribute due to Maillard conjugation (Figure 5a). This process renders protein surface more hydrophilic, which possibly enhances their ability to bind increasing amounts of water, thereby leading to enhanced firmness. TGase treatment further increased the firmness values of enriched samples with the exception of PSPC sample (Figure 5a). The changes in springiness values were though limited regardless of the type of protein utilized or the modification treatments applied (Maillard conjugation or TGase treatment) (Figure 5b).

In previous studies, Basman, Köksel, and Ng (2002) reported that TGase addition increased firmness values possibly due to the formation of an over strong dough after excessive cross-linking. Similarly, Marco and Rosell (2008a) found that enriched gluten-free bread containing soybean protein concentrate and treated with TGase demonstrated higher firmness values than the control sample. According to our results, firmness values increased especially in the case of BCPC and GSPC enriched gluten-free breads due to the modification treatments. However, modified protein inclusion minimally affected springiness.

3.3 | Color parameters

First of all, in this study, no further efforts were made to remove the natural coloring agents from the protein concentrates. Color

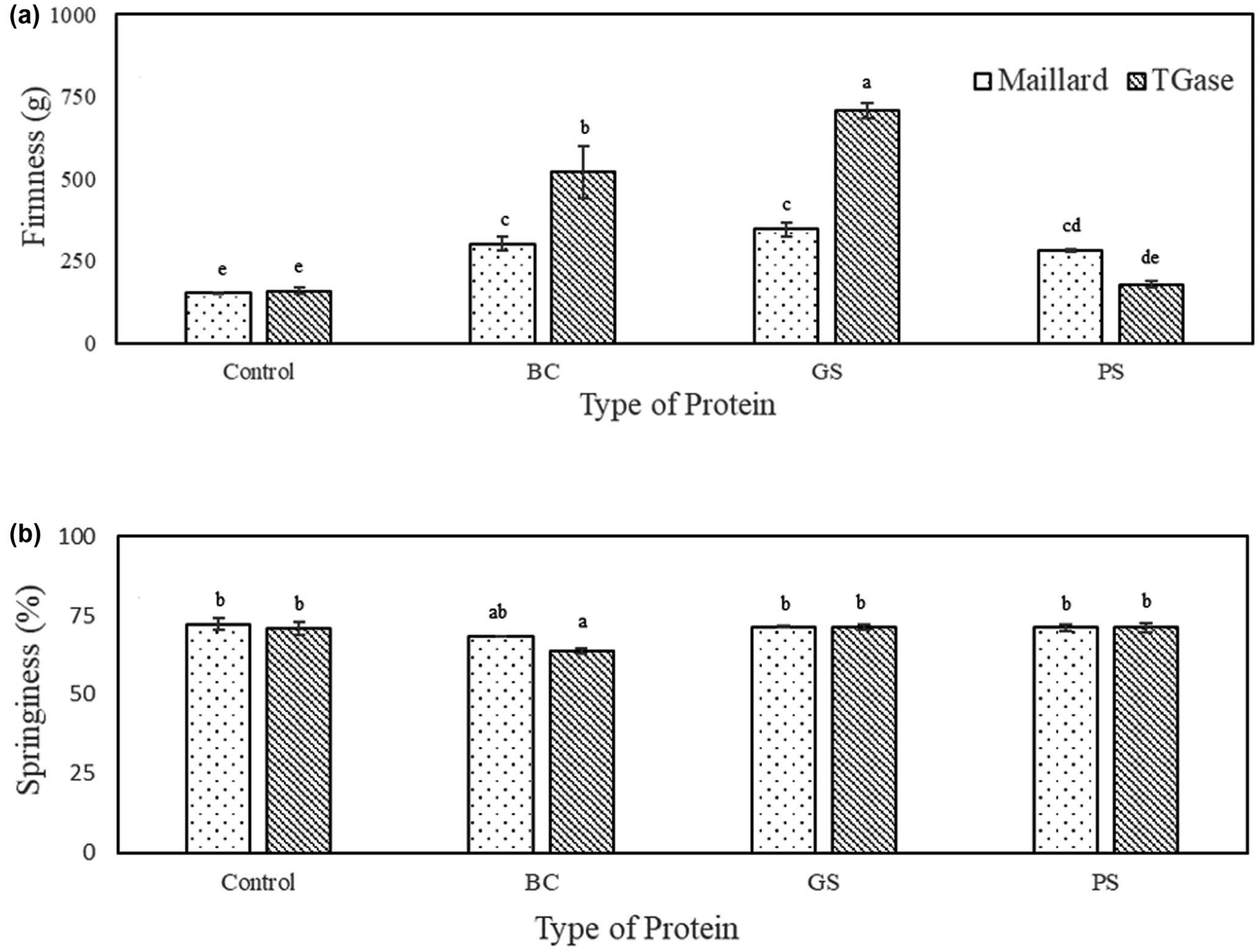

FIGURE 5 Textural parameters of gluten-free control bread and gluten-free breads enriched with modified protein concentrates. BC, black cumin; GS, grape seed; PS, pumpkin seed protein concentrates. (a, b) represent firmness (g) and springiness (%), respectively. Presented data and error bars represent the sample mean of three replicated experiments and standard deviations, respectively

parameters for protein enriched gluten-free breads were shown in Table 3. Based on the L^* values, BCPC enriched gluten-free bread was clearly the darkest sample followed by GSPC sample and the control sample. L^* value for the PSPC sample was comparable to the control sample (i.e., statistically similar L^* value). Similar trends were also observed for the b^* values. Since a^* values indicated redness, GSPC sample was considerably more red than the other samples and the differences in a^* values were statistically significant for GSPC in comparison to all other samples. Gluten-free bread samples enriched in Maillard conjugates of all three protein concentrates were darker than the control sample. After TGase treatments, BCPC and GSPC enriched gluten-free breads were darker than control bread, whereas PSPC enriched gluten-free bread was similar to the control bread ($p > .05$). As expected, Maillard conjugation lead to the formation of darker colors in the products.

Ziobro et al. (2013) stated that enrichment by pea protein, collagen, and lupine protein was characterized by significantly darker bread color. However, addition of soy protein did not significantly affect this value. Aguilar, Albanel, Minarro, and Capellas (2015) reported that chickpea and tiger nut enriched gluten-free flour resulted in darker crust, possibly due to the Maillard reactions in situ. Phongthai et al. (2016) reported that addition of rice bran protein concentrate to the gluten-free bread decreased the lightness value.

In summary, supplementation of gluten-free bread with BCPC, GSPC, and PSPC resulted in lower loaf volumes. However, as the water level increased, it was possible to attain higher volumes. The results of firmness and springiness tests showed that enriched breads generally demonstrated higher firmness and lower springiness values. However, these values could also be corrected by water addition. Cells distribution and homogeneity increased when BCPC, GSPC, and PSPC were modified via Maillard reaction or TGase treatments. Enrichment also affected color attributes. However, since the generated colors are representative of the natural color of the seeds that were utilized, consumer appeal might still remain strong in such products. Protein supplemented gluten-free breads had darker color with the exception of PSPC enriched samples.

TABLE 3 Color parameters of gluten-free bread crumb

Sample	Nonmodified protein concentrates			Maillard conjugation treated protein concentrates			TGase treated protein concentrates		
	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
Control	54.59 ± 0.8^{f}	2.43 ± 0.1^{k1}	23.34 ± 0.1^{r}	76.84 ± 0.4^{g}	−1.47 ± 0.0^{m}	10 ± 0.0$^{\text{oɳ}}$	78.59 ± 0.6^{g}	−1.43 ± 0.1^{m}	9.89 ± 0.1$^{\text{oɳ}}$
BC	23.68 ± 0.9^{a}	2.05 ± 0.0^{k1}	8.73 ± 0.0$^{\text{ɳ}}$	39.89 ± 0.2^{d}	7.68 ± 0.6^{j}	22.55 ± 0.7^{r}	46.9 ± 0.1^{e}	3.56 ± 0.6^{k}	11.69 ± 0.4$^{\text{oɳ}}$
GS	30.60 ± 1^{b}	7.68 ± 0.3^{j}	12.03 ± 0.4^{o}	24.90 ± 0.1^{a}	18.85 ± 0.2^{h}	17.26 ± 0.0^{p}	35.5 ± 0.7^{c}	18.77 ± 0.7^{h}	16.89 ± 0.9^{p}
PS	53.93 ± 0.5^{f}	1.71 ± 0.2^{1}	22.62 ± 1.9^{r}	53.57 ± 0.0^{f}	11.08 ± 0.7^{i}	32.1 ± 0.7^{s}	76.6 ± 0.5^{g}	−1.10 ± 0.1^{m}	15.65 ± 0.9^{p}

Note: The data represent the average of three independent experiments with ± standard deviation. One-way ANOVA post hoc Tukey's tests were used to verify significance ($p < .05$).

$^{a\text{-}ı}$The significance level is $p < .05$ in all cases.

Abbreviations: BC, black cumin protein concentrate fortified gluten-free bread; GS, grape seed protein concentrate fortified gluten-free bread; PS, pumpkin seed protein concentrate fortified gluten-free bread.

4 | CONCLUSION

In this study, BCPC, GSPC, and PSPC manufactured from cold press meals were used in gluten-free bread applications. Usage of plant proteins can safely enhance the protein content of such products along with potential enhancements in technical properties of the gluten-free breads. In the hopes to enhance technical and sensory characteristics, the influence of Maillard conjugation and TGase treatments on protein concentrates were also investigated.

It is highly desirable to enrich wheat flour or bread mixes with other nongluten, high protein concentrates to produce an alternative products satisfying consumer demand. In general, protein supplementation requires individual optimization for each and every other concentrate, because of the differences in various characteristics such as water binding capacities. As the water level increased, it also induced a rise in loaf volume and enhanced the texture of gluten-free breads as characterized by the firmness and springiness attributes.

While firmness increased after Maillard conjugation or TGase treatments, springiness was much less affected by these treatments. However, it was possible to enhance the distribution and homogeneity of the cells in the loaves by the utilization of modified protein concentrates.

Although the sensory attributes of the bread samples were not investigated in detail, brief analysis by our group indicated that there were no significant differences in taste when compared with the control sample (data not shown). Further sensory investigations employing a trained panel need to be carried out. Recently, Ziobro, Juszczak, Witczak, and Korus (2016) enriched the gluten-free breads with pea and lupin proteins and reported improved sensory parameters providing more acceptable color and smell. Similarly, Witczak, Juszczak, Ziobro, and Korus (2016) enriched gluten-free breads with potato protein and observed improvements in the sensory attributes.

The results of this study showed that BCPC, GSPC, and PSPC are valuable resources that can be utilized in protein fortification of gluten-free breads. Further investigations on the utilization of these concentrates in the normal (i.e., gluten bearing) bread formulations are currently underway in our labs.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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