Pumpkin Seed-Fortified Functional Biscuits: Nutritional Enhancement, Antioxidant Capacity, and Consumer Acceptability

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Abstract
This has been driven by an increasing trend to promote food products that are health-based, thereby creating research interest from which widely consumed basketry foodstuffs can be fortified with plant-based products that are rich in nutrient content. The paper examined how functional biscuits made of pumpkin seed powder (PSP) of Cucurbita maxima can be produced and evaluated based on quality and content of the product and the substitution levels (0% T0, control, 10% T1, 20% T2, 30% T3, 40% T4) of wheat flour. All formulations were evaluated in proximate chemical composition, total phenolic content (TPC), antioxidant activity (DPPH and FRAP assays), physical parameters and sensory evaluation using a nine-point hedonic scale. PSP incorporation generated meaningful (p 0.05) dose circumstances of crude protein (7.21% to 14.87%), crude fat (8.43% to 17.62%), dietary fiber (1.98% to 4.76%), and ash (1.12% to 3.04%), whilst carbohydrate content dropped to 77.44% to 57.40%. Inhibition of DPPH increased to 61.8 and 74.3 mg GAE/100 g dry weight and TPC respectively. Progressive changes in lightness (L) and spread ratio decreasing as well as a textural hardness increasing with the increase in PSP were found through physical characterization. The sensory analysis revealed that the overall acceptability was at its highest level at T2 (20% PSP) with hedonic rating of 7.8 ± 0.4, but decreased as the substitution increased because of the negative perception of color, texture, and taste. The 20% PSP formulation was found to be optimal and it provided a 57% protein, 61% fat and 102% antioxidant activity increase compared to control with an even better consumer acceptance.

Keywords: pumpkin seed powder; functional biscuits; antioxidant activity; proximate composition; sensory evaluation; *Cucurbita maxima.*

ISSN 2072-3857

1. Introduction

The food industry has undergone a remarkable transformation in recent decades due to increased consumer awareness of the link between diet and the prevention of chronic diseases such as cardiovascular disease, type 2 diabetes, obesity, and some types of cancer. This has led to the emergence of the concept of functional foods, defined as:

"Traditional or processed foods that provide additional health benefits beyond their basic nutritional value due to the presence of bioactive compounds capable of promoting health or reducing the risk of disease" (5).

Sensory properties such as color, taste, aroma, and texture are crucial factors in consumer acceptance of bakery products. These properties are typically assessed using the nine-point Hedonic scale to determine the degree of consumer acceptance of a food product (22). Studies indicate that changes in product color or texture resulting from the addition of plant-based ingredients can directly influence consumer preferences for fortified biscuits (13).

Among food products suitable for fortification with functional ingredients, bakery products and biscuits are prime examples, given their widespread consumption, long shelf life, and the ease with which their composition can be modified to incorporate high-value ingredients without significantly altering their technological properties (13).

Pumpkin (Cucurbita spp.) is a globally important agricultural crop, with global production exceeding 25 million tons annually. Despite the widespread use of pumpkin fruit in the food industry, pumpkin seeds are often considered an underutilized byproduct, despite their high concentrations of protein, unsaturated fatty acids, minerals, and phenolic compounds with antioxidant activity (16).

Pumpkin seeds typically contain 25–37% protein and 40–50% fat, rich in unsaturated fatty acids such as linoleic and oleic acids. They are also a significant source of minerals like magnesium, zinc, and iron (20). Furthermore, they contain bioactive compounds such as tocopherols, phytosterols, phenols, and flavonoids, which contribute to enhanced antioxidant activity and reduced oxidative stress associated with many chronic diseases (11). Given these nutritional and functional properties, several studies have explored the use of pumpkin seed powder (PSP) in fortifying bakery products such as bread and biscuits to enhance their nutritional and functional value (2).

Studies indicate that replacing wheat flour with pumpkin seed powder at a rate of 10–25% can improve the nutritional value of the product while maintaining acceptable sensory characteristics for consumers (9). However, increasing the substitution levels may affect the dough properties and the biscuit's texture due to the loosening of the gluten network.

Therefore, this study aimed to develop functional biscuits fortified with pumpkin seed powder at different substitution levels for wheat flour (0, 10, 20, 30, and 40%) and evaluate the effect of this on the nutritional properties, antioxidant activity, and physical and sensory characteristics of the final product.

2. Materials and Methods

ISSN 2072-3857

2.1 Raw Material Procurement and Preparation of Pumpkin Seed Powder

Raw pumpkin seeds (Cucurbita maxima) were obtained as certified commercial agricultural supplier and were preprocessed using a strict and standardized pre-processing procedure before the process of producing the flour because compositional uniformity and microbiological safety of the product and the absence of extraneous contaminants that might affect the analytical validity. Receipt Seeds were reported by eye and mass sorted on a clean stainless-steel sorting table to eliminate shriveled or discoloured, or insect-damaged or otherwise defective seeds as well as any other foreign particulate matter such as scraps of pumpkin flesh, strands of fiber, or foreign debris. The sorted seeds were then washed two times with drinkable water at ambient temperature to remove the dust and soluble impurities on the seed followed by a single rinse with distilled water and then drained through a stainless-steel mesh colander. To eliminate surface moisture, the seeds were put on clean cotton cloth in a single layer and dried gently at the surface in 10-minutes time before being transferred to drying trays. The soaked seeds were placed in a force-conveyed oven (60 ± 2°C) in a single uniform layer over stainless steel mesh-bottomed drying trays and left to dry continuously over 24 hours which was found to provide enough time to completely dry the seeds to moisture levels less than 5% (w/w) - as determined by gravimetric assay - without causing thermal decomposition of heat-sensitive bioactive compounds such as tocopherols and phenolic acids. After drying, the seeds were then moved directly into the glass desiccators with anhydrous silica gel and cooled to ambient temperature, after which additional procedures were undertaken [17].

The dried seeds were treated to partial cold-pressing with a laboratory screw press to decrease the level of native oil in the seeds to that which was favorable to the production of stable flour. The nutritionally significant percentage of the desirable poly unsaturated fatty acid fraction and lipid soluble bioactive compounds such as tocopherols and carotenoids in the finished PSP were not completely deoiled. The partially deoiled seed cake expelled in the press underwent size reduction on a laboratory pin mill at 12,000 rpm and PSP with a fine particle size of 177 µm (80-mesh) stainless steel sieve was obtained after passing it through the sieve in sequence. The proximate composition of PSP was established in three replicates before the preparation of the biscuits to give the compositional background of interpreting the treatment effects showing the protein content of 32.4 ± 0.8%, crude fat 28.7 ± 1.1%, ash 4.8 ± 0.2%, dietary fiber 6.2 ± 0.4%, moisture content 4.6 ± 0.2%, and carbohydrate content 23.3 ± 0.9% on a dry weight basis. PSP was prepared freshly in polyethylene bags made of airtight laminated foils with nitrogen flushing to reduce lipid oxidation and kept as 4 ± 1^{o}C under light protection and consumed within fourteen days of preparation [8].

All other ingredients (refined all-purpose wheat flour, 72% extraction rate, 11.2 ±.3% DW protein content, unsalted butter), reconstituted whole egg powder, baking powder, pure vanilla extract, and food-grade sodium chloride were obtained in one reputable commercial food ingredient supplier to have as little inter-batch compositional variability as possible across the experimental production runs. The verification of all ingredients was done by certificate of analysis documents of the supplier that indicated adherence to relevant food safety and quality requirements. All dry ingredients were kept at 20 ± 2°C and relative humidity of 60 ± 5% under these conditions and butter was stored at 4°C, and all ingredients were utilized within described shelf life. The chemical composition of wheat flour was the proximate chemical composition of the PSP and finished biscuit samples was determined by the same AOAC methods and confirmed that the composition was similar to published values of 72% extraction wheat flour and offered the second

ISSN 2072-3857

compositional reference point to interpret the fortification effects [10].

2.2 Table 1. Nutritional composition of pumpkin seeds (per 100 g dry weight)

Component	Content
Protein	25–37 g
Fat	40–50 g
Carbohydrates	10–15 g
Dietary fiber	6–8 g
Magnesium	500–550 mg
Zinc	7–10 mg
Iron	8–9 mg
Phosphorus	1000–1200 mg
Potassium	800–900 mg

Sources.(20)

2.3 Biscuit Formulation Design and Production

The five biscuit formulations were prepared following a single-variable experimental design where the level of PSP substitution was the independent variable with all other formulation parameters being held constant across experimental treatments so that any difference in quality could be purely attributed to the effects of PSP incorporation. The substitution levels were: T0 (0% PSP, control with pure wheat flour), T1 (10% PSP), T2 (20% PSP), T3 (30% PSP) and T4 (40% PSP) to give a range of substitution levels that spans the substitution levels commonly studied in the published literature and extends out to higher levels where which a negative impact on quality is likely to be observed giving the entire response curve required to identify the optimal level. The standardized formulation basis per 100 g of total flour was: granulated white sugar 30g, unsalted butter 40g, reconstituted egg powder 10g, double-acting baking powder 1.5g, pure vanilla extract 0.5g, food-grade sodium chloride 0.5g, distilled water added as necessary to provide a consistent workable non-sticky dough of uniform consistency throughout all formulations with an incremental addition of water (generally 2-5 mL additional water was needed at higher P Table 1 gives the full formulation table.

Table 1. PSP formulations (per 100 g total flour basis) Ingredient composition of PSP fortified biscuit formulations.

Ingredient	T0 (0% PSP)	T1 (10% PSP)	T2 (20% PSP)	T3 (30% PSP)	T4 (40% PSP)
Wheat flour (g)	100.0	90.0	80.0	70.0	60.0
Pumpkin seed powder (g)	0.0	10.0	20.0	30.0	40.0
Granulated sugar (g)	30.0	30.0	30.0	30.0	30.0
Unsalted butter (g)	40.0	40.0	40.0	40.0	40.0
Reconstituted egg powder (g)	10.0	10.0	10.0	10.0	10.0
Baking powder (g)	1.5	1.5	1.5	1.5	1.5
Vanilla extract (g)	0.5	0.5	0.5	0.5	0.5
Sodium chloride (g)	0.5	0.5	0.5	0.5	0.5
Water (mL)	~15	~17	~19	~21	~23

The substitution levels (0, 10, 20, 30, 40%) were chosen based on the methodology of previous studies that used pumpkin seed flour in bakery products, which showed that the substitution range up to 40% allows for the study of the gradual effect of the functional component on the physical and sensory properties of the product (2).

PSP = Pumpkin Seed Powder; T0 = control (0% PSP); T1-T4 = successively increasing the PSP substitution rate.

The production of the biscuits was standardized by creaming according to protocols that were outlined in the literature concerning production of seed flour enriched biscuits and was applied uniformly to all five formulations and three separate production replicates. Every equipment was washed and dried using 70 % of ethanol solution and was airdried. Butter was allowed to condition at 18–20°C then creamed to permit the best fat crystal structure to incorporate air. The creaming was done in a laboratory stand mixer (5 L bowl capacity) at medium speed (setting 4 of 10) and timed at 5 minutes, yielding a pale and homogenous visibly aired mixture. The cream of butter-sugar mixture was reconstituted with egg powder solution and vanilla extract in a slow, steady stream with the mixer operating at low speed (setting 2), and the mixing process was to be continued until full emulsification was attained (2 minutes after all the eggs had been added). The flour content of each formulation (the right proportions of wheat flour, and PSP weighed to the nearest 0.1 g on an analytical scale) was mixed with the baking powder and sodium chloride and sifted 3 times through to a fine sieve to guarantee a uniform distribution of raising agent and removal of agglomerates. The dry mixture of ingredients filtered was added to the butter-egg mixture in two equal portions and mixed at low mixer speed (setting 2) with 30 seconds of mixing between additions, and the mixture then folded in by hand using a flexible silicone spatula to ensure that any unmixed material remaining at the bottom and sides of the bowl was incorporated. The finished dough was put into a disc, coated with a food-grade polyethylene film and left at ambient temperature (22 ± 2^{o}C) during 15 minutes to ensure that the gluten network relaxes and that the distribution of water is equalized throughout the dough matrix [3].

After the resting, the sheet of dough was sheeted between two parallel acrylic guide rails (5 mm height) to form a homogeneous sheet of 5 ± 0.2 mm thickness, confirmed by measuring 6 random points with a digital caliper after sheeting. The 50 mm diameter pieces of the biscuit were cut with a sharp edged stainless-steel cutter and placed on baking trays with silicone baking paper to allow the pieces not to stick to the surface and

ISSN 2072-3857

the heat to spread evenly over the biscuit base. The pieces of biscuits were placed at a distance of at least 20 mm to ensure that they would spread laterally freely in baking. The composition of all the formulations was baked using a preheated fan-assisted convection oven (Rational SCC61, Germany) at 175 ± 2°C during precise 15 minutes with an internal oven temperature checked using a calibrated platinum resistance thermometer probe. The baked cookies were taken out of the oven and allowed to cool on wire racks at room temperature to a specific period of exactly 30 minutes then later on to the sealed polyethylene bags to be analyzed and sensory assessed. All formulations were made on the three independent replicates on different days, and a new weighing of the ingredients was carried out to each replicate, and analytical measurements were carried out on samples taken separately on each replicate [2].

2.4Proximate Chemical Composition Analysis

The proximate chemical composition of PSP, wheat flour and all five final biscuit formulations was identified based on the standardized procedures of the Association of Official Analytical Chemists (AOAC, 2016) which were used throughout all samples and were done using an analytical triplicate with results given in a dry weight basis unless otherwise mentioned. Moisture was evaluated using the gravimetric oven-drying method (AOAC Method 925.10) in which 2.0 g samples were dried in a forced-air oven at 105 ± 2°C at the constant temperature of 105^{o}C and at a constant weight, which was considered to have reached constant weight after 4 hours. The amount of crude protein was identified with the use of the macro-Kjeldahl nitrogen digestion procedure (AOAC Method 960.52), in which 1.0 g of the sample was combined with 1.0 g of concentrated sulfuric acid in the presence of a mixture of copper sulfate/potassium sulfate to catalyze the digestion of protein, after which the ammonia was steam distilled into boric acid solution and back-titrated by using 0.1 M hydrochloric acid. The total nitrogen values were then transformed into protein content by the use of the species-specific conversion factor of 5.70 (wheat flour dominant samples [T0 and T1]) and 5.30 (PSP and PSP dominant samples), with a weighted average conversion factor being used in the calculation of intermediate formulations based on their proportions PSP content. The Soxhlet continuous extraction method (AOAC Method 920.39) was used to extract crude fat using petroleum ether (boiling point 40–60°C) as the extraction solvent and a reflux period of 6 hours on a 5.0 g sample dried by the moisture determination procedure previously. The fat percentage was determined by grammetrically by using the weight of lipid material left after the solvent has evaporated under low pressure [6].

The amount of ash was calculated as a result of dry combustion at 550 ± 10°C in a muffle furnace (AOAC Method 923.03) using 2.0 g samples in pre-weighed, pre-ignited porcelain crucibles subjected to 2 hours of heating at 550°C, followed by at least 5 hours of heating at 550°C at which time uniformly gray-white ash was produced free of black carbon particles. Endogen total dietary fiber was calculated using the enzymatic-gravimetric method (AOAC Method 985.29) with a commercial total dietary fiber assay kit (Megazyme International, Ireland) in which samples were subjected to the enzymatic-gravimetric method with successive treatment of heat-stable alpha-amylase, protease and amyloglucosidase to hydrolyze starch and protein fractions followed by the precipitation of the fiber with 78 % ethanol, filtration through pre-weighed sinter The total carbohydrate was determined arithmetically as the difference between the sum of all directly determined proximate fractions: carbohydrate (%) = 100- (moisture% + protein% + fat% + ash% + fiber%). The modified Atwater factors, 4 kcal/g protein, 9 kcal/g fat and 4 kcal/g carbohydrate were used to estimate the gross energy content [18].

ISSN 2072-3857

2.5 Total Phenolic Content and Antioxidant Activity Determination

The extraction of bioactive compounds in biscuits was done according to an optimised protocol that was based on the published procedures of phenolic extraction of baked cereal matrices. Biscuit samples were ground in a fine form in a laboratory micro-mill to pass a sieve. 250 µm sieve 1.0 g of ground material was added to the 20 mL 80% (v/v) aqueous methanol in 50 mL centrifuge tube. Extraction was carried out after ultrasonication (40 kHz, 200 W output) during 30 minutes at room temperature and then centrifugation at 5000 x g during 10 minutes at 4°C. The ensuing supernatant was quantitatively collected and the residue was re-extracted with an additional 10 mL of 80 percent methanol under identical conditions. To use in antioxidant assays, the two supernatants were combined and brought to a final volume of 30 mL by adding 80% methanol, and filtered using 0.45 -mu PTFE membrane syringe filters. All extracts were ready to be analyzed right after their preparation and kept on ice during the analysis to reduce the occurrence of phenolic oxidation and degradation [4].

Total phenolic content (TPC) was established by Folin-Ciocalteu colorimetric method whereby 0.5 mL of biscuit extract was put in a glass test tube and 2.5 mL of Folin-Ciocalteu solution was added, which is diluted 1:10 (v/v) using distilled water and 2.0 mL of 7.5% (w/v) sodium carbonate solution was added. The mix was vortex mixed and left to incubate at room temperature in complete darkness after 30 min, after which the absorbance was measured at 765 nm at reagent blank prepared with 80% methanol instead of the extract in a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). TPC was measured using a six-point gallic acid standard curve plotted in the 0-500 0g/mL range ($R^2 \geq 0.999$) and converted to milligrams of gallic acid equivalents per 100 grams of sample dry weight (mg GAE/100 g DW). A 0.5 mL biscuit extract was combined with 3.0 mL 0.1 mM DPPH freshly made in 80% methanol in an ambered glass tube, vortex-mixed, and incubated at room temperature in complete darkness (precisely 30 min) before the absorbance was measured at 517 nm, which was taken to measure DPPH radical scavenging activity. The percent inhibition percentage was determined as: % inhibition = [(A_control − A_sample) / A_control] × 100, where A control was A of DPPH solution with 80% methanol in place of extract. The FRAP assay was performed by making fresh FRAP working reagent with a 10:1:1 (v/v/v) mixture of 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) dissolved in 40 mM hydrochloric acid, and 20mL of ferric chloride hexahydrate solution. Biscuit extract 0.15 mL was mixed with 2.85 mL FRAP reagent and incubated at 37°C, for a period of 4 minutes, then the absorption at 593 nm was recorded. FRAP values were determined using a ferrous sulfate heptahydrate standard curve and given in µmol equivalents of Fe^{2+} per gram of dry weight [7].

2.6 Physical Quality Parameters

The physical characterization included characterization of spread ratio, instrumental color measurement, and textual hardness analysis of ten randomly selected intact biscuits of each production replicate of each formulation of the three production replicates, averaging the results of the ten individual measurements and the three production replicates. Spread ratio was checked by stacking: ten biscuits were stacked one on top of another and the total height of the stack measured with a digital vernier caliper with a precision of 0.01 mm and the stack was disassembled, the stack was then turned 90, and the stack was re-assembled to measure again, mean height per biscuit was the average of the two measurements and taken as the thickness, diameter was measured directly on the biscuit at two different orientations and averaged. Spread ratio was determined as the ratio of average diameter to average thickness whereby a high value meant more spreading in

ISSN 2072-3857

the baking process. The instrumentality of biscuit color was determined by a HunterLab ColorFlex EZ spectrophotometric colorimeter calibrated with standard white and black reference tiles prior to each measurement session with CIE L* (lightness, 0 = black to 100 = white), a* (−60 = green to +60 = red), and b* (−60 = blue to +60 = yellow) values determined on the surface of each sample of the biscuit at three random points. The colorimetric data were used to determine Browning Index BI = [100 × (x − 0.31)] / 0.17, where x = (a* + 1.75L*) / (5.645L* + a* − 3.012b*), to generate a single composite index of surface browning intensity whether each of the individual colorimetric coordinates is interpreted or not [1].

A TA-XT Plus Texture Analyzer (Stable Micro Systems, UK) with 35 mm diameter flat-ended aluminum probe (P/35) and a data acquisition rate of 500 pps was used to measure textural hardness with the following test parameters: pre-test speed 2.0 mm/s, test speed 1.0 mm/s, post-test speed 5.0 mm/s, trigger force 5 g, compression distance 5 mm and data acquisition rate 500 pps. The biscuits were put in a flat position on the base plate of the texture analyzer and squashed in the middle of the probe. Textural hardness was measured as the highest compressive force (Newtons, N) measured at the compression cycle and this was used to show the force needed to break the biscuit at the hardest point. The calculations of means were performed after ten measures of each formulation per replicate were performed and Grubbs test identified outliers at $p < 0.05$ [14].

2.7 Microbiological Analysis

The microbial quality of the manufactured biscuit samples was assessed to ensure product safety and storability. Microbiological tests were performed according to the standard methods outlined in the Association of Official Analytical Chemists (1) manual.

Total Plate Count

The total number of live bacteria was estimated using the standard plate count method. Ten grams of each biscuit sample were weighed, sterile ground, and mixed with 90 mL of sterile peptone water to obtain the first dilution (10^{-1}). Subsequently, a series of successive decimal dilutions were prepared, and 1 mL of each dilution was cultured on Plate Count Agar (PCA). The plates were incubated at 37°C for 24–48 hours. After incubation, the visible bacterial colonies were counted, and the results were expressed in colony-forming units per gram of sample (CFU/g). Yeast and Mold Count

Yeast and mold counts were estimated using Potato Dextrose Agar (PDA) acidified with tartaric acid to inhibit bacterial growth. One milliliter of the appropriate dilutions was spread on the surface of the medium, and the plates were incubated at 25°C for 3–5 days. After incubation, the developing colonies were counted, and the results were expressed in colony-forming units per gram (CFU/g).

Coliform Bacteria

Coliform bacteria were detected using Violet Red Bile Agar (VRBA). One milliliter of the appropriate dilutions was cultured in the plates and incubated at 37°C for 24 hours. After incubation, characteristic dark red colonies with a surrounding sedimentation zone were counted. These colonies were considered indicative of the presence of coliform bacteria, and the results were expressed in colony-forming units per gram of sample (CFU/g).

2.8 Sensory Evaluation

The sensory assessment of the products was carried out by a group of 25 semi-trained panelists selected among the academic staff and postgraduate studentsTheir ages ranged from 22 to 45 years. A 9-point Haddonian scale was used to assess color, taste, smell, texture, and overall acceptability. within the food science department and all reported to have prior experience in the consumption of biscuits as a normal dietary product, absence of any known food allergy or intolerance of any of the ingredients used in the manufacture of the biscuits and no known physiological or pharmacological condition that could impair

the ability to perform a sensory evaluation. The informed written consent to participate was given to all the panelists and they were told that the study was about the evaluation of biscuits of the different levels of pumpkin seed powder. An organized calibration procedure was carried out before formal testing during which panelists became acquainted with the nine-point hedonic scale by assessing reference samples and standard descriptors of each of the scale points, with special attention paid to the calibration of scale ends points (1 = Dislike Extremely; 9 = Like Extremely) and the middle (5 = Neither Like nor Dislike). The panelists received a briefing that they were expected to rate five sensory attributes, namely, color/appearance, aroma, taste/flavor, texture/mouthfeel, and overall acceptability on a scale, with no attribute being rated based on the score they provided on other attributes [16].

The preparation of the biscuit samples to be used in sensory evaluation was based on production replicate material that was freshly prepared in the morning of every session of evaluation and served within 4 hours upon packaging. The samples were two complete biscuits on a clean white ceramic plate and were marked with a randomly generated three-digit code. The randomization of sample presentation order among panelists was based on a Latin square design that ensured the first-order carry-over effects, whereas order was re-randomized each time the evaluation session occurred. Assessment in single sensory evaluation booths was done under standard conditions such as D65 daylight-balanced light at 500 lux intensity, ambient temperature 22 ± 1°C and individual ventilation to avoid cross-contamination of aroma stimuli amongst panelists at the adjoining positions. Between samples, palate-cleansing agents were administered (room temperature potable water and unsalted plain crackers) and a 2-minute gap between consecutive sample appraisals was imposed. The five formulations were assessed in one session each replicate, with the overall sensory assessment being done in three sessions on three different days with respect to the three production replicates and higher than 85% attendance of the panel in each session [18].

2.9 Statistical Analysis

The quantitative data are all given in terms of arithmetic mean (SD) of three independent production replicates, with each replicate giving one value of the mean calculated by three independent analytical determinations of chemical parameters or ten independent analytical determinations of physical parameters. IBM SPSS Statistics Version 26.0 (IBM Corp., Armonk, NY, USA) was used to conduct the statistical analysis. The Shapiro-wilk test was used to measure normality of all parameters measured and the use of the Levene test to measure homogeneity of variance, both assumptions were confirmed to be met ($p > 0.05$) before proceeding to an analysis of the parameters by parametric means. ANOVA was used to determine whether the overall effect of treatment depending on the level of PSP substitution on all the measured parameters was statistically significant using a significant level of 0.05. In the case of parameters with significant overall ANOVA, post-hoc pairwise mean variations were carried out with Duncan Multiple Range Test (DMRT) at the 0.05 significance level to obtain homogeneous subsets of treatment means whose results are represented by one superscript letter in the data tables where one homogenous subsets of means share no overlapping letter. The product-moment correlation coefficients of Pearson were determined to measure the intensity and direction of bivariate correlations between the substitution level of PSP and every quality parameter measured and between two quality parameters of specific nutritional or technological interest. The reported p-values are all two tailed [2].

3. Results and Discussion

3.1 Proximate Chemical Composition of Biscuits

As shown in Table 2, the overall chemical composition of each of the five formulations of biscuits analyzed (after baking and cooling) is given, and the data indicate that there is a general trend of statistically significant ($p \leq 0.05$), dose-dependent changes in compositions across the PSP substitution range that is entirely consistent with the compositional differences between PSP and wheat flour reported in Section 2.1. The moisture content dropped considerably and significantly between 3.82 ± 0.12% in T0 and 2.31 ± 0.09% in T4 with significant differences being observed between all the successive substitution levels. This loss of moisture indicates the diminution equilibrium moisture content of PSP in comparison with wheat flour under similar conditions of storage, which is explained by the lower starch content and the variation of hygroscopic behavior of constituent components of pumpkin seeds to those of the wheat flour polysaccharides. The theoretical usefulness of such moisture loss is spread to the shelf life of products as the reduced water activity linked to the lower moisture content in PSP-enriched biscuits is likely to delay microbial growth, slow down non-enzymatic browning in the course of storage, and preserve the textural crispness of endowments over longer times [10].

Table 2. Close chemical structure of PSP-enriched cookie in varied substitution percentage (% dry weight basis, mean ± SD, n = 3)

Parameter	T0 (0%)	T1 (10%)	T2 (20%)	T3 (30%)	T4 (40%)
Moisture (%)	3.82±0.12^{a}	3.41±0.10^{b}	3.05±0.08^{c}	2.67±0.11^{d}	2.31±0.09^{e}
Crude Protein (%)	7.21±0.18^{e}	9.46±0.22^{d}	11.34±0.31^{c}	13.12±0.28^{b}	14.87±0.35^{a}
Crude Fat (%)	8.43±0.24^{e}	11.27±0.33^{d}	13.58±0.41^{c}	15.74±0.38^{b}	17.62±0.44^{a}
Ash (%)	1.12±0.05^{e}	1.64±0.07^{d}	2.11±0.08^{c}	2.58±0.09^{b}	3.04±0.11^{a}
Crude Fiber (%)	1.98±0.09^{e}	2.74±0.12^{d}	3.41±0.15^{c}	4.12±0.14^{b}	4.76±0.17^{a}
Carbohydrates (%)	77.44±1.21^{a}	71.48±1.14^{b}	66.51±1.08^{c}	61.77±1.19^{d}	57.40±1.26^{e}
Energy (kcal/100g)	415.2±4.8^{d}	428.6±5.1^{c}	438.4±5.6^{bc}	447.1±5.9^{ab}	455.3±6.2^{a}

The difference between means in a row with varying superscript letters is considerably different at $p \leq 0.05$ (Duncan Multiple Range Test). Difference Calculated carbohydrates.

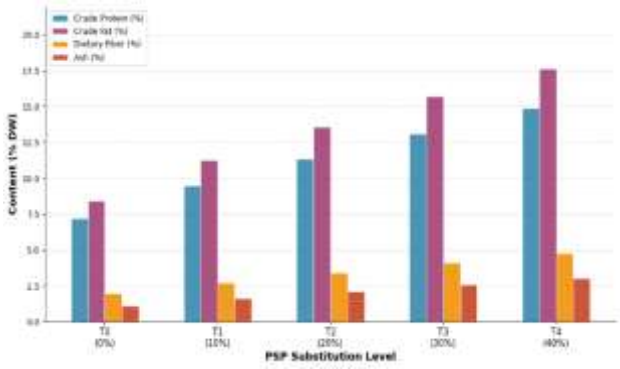

Figure 1. Impact of level of substitution of PSP proximate composition (protein, fat, fiber, and ash) of fortified biscuits.

The increase in the level of crude protein content was statistically significant ($p \leq 0.001$) and linear dose-dependent, between 7.21 ± 0.18% T0 to 14.87 ± 0.35 % in T4, which is equivalent to the increase in the protein content of 106 % in the entire substitution range. The mechanism behind the protein-enriching effect is relatively simple, as it involves the substitution of low-protein wheat flour (11.2% protein) with high-protein PSP

(32.4% protein) in the flour mix, the result of which is a biscuit with protein content comparable to that of nutritionally enriched categories of functional foods at T3 and T4 substitution levels. These results are largely consistent with the results of [1], which indicated protein enrichment up to about 15% in PSP-enriched biscuits within a 5-30% range of substitution, and those of [5]. which also indicated similar protein-enriching behavior in PSP-enriched cookies in wheat-pumpkin seed composite formulation. The potential nutritional importance of this protein enhancement is notably notable under the conditions of protein-energy malnutrition in developing countries and the increased requirement of people in developed markets to consume products with a higher amount of proteins as snacks, making PSP-enriched cookies a product that can be used within highly diverse settings of nutritional interventions [6].

The content of crude fat levels rose considerably ($p \leq 0.05$) when the amount in T0 was 8.43 ± 0.24% and in T4 was 17.62 ± 0.44%, which is 109% more than in T0. This qualitative nature of such fat increase has a nutritional advantage, since the pumpkin seed oil is characterized by a preponderance of polyunsaturated linoleic acid (omega-6) and monounsaturated oleic acid, producing a fatty acid profile with cardiovascular beneficial effects when taken in place of saturated fat-rich substitutes. But the quantitative increase in the proportion of fats, and especially the enrichment of polyunsaturated fatty acids which are naturally more vulnerable to oxidative rancidity than saturated fats, creates a consideration of storage stability which is not considered in the cross-sectional analytic design of the current study but has been specifically reported by [1]. as a limiting factor of the shelf life of high-PSP biscuits. The content of ash was found to rise between 1.12 ± 0.05% to 3.04 ± 0.11% over the range of substitution reflecting the increased mineral density of PSP and demonstrating that the dietary fiber increased significantly over the range of substitution, although dietary fiber under recommended amounts (25-38 g/day) is one of the most current nutritional deficiencies in the world. The proportions of carbohydrates in the diet dropped down to 57.40% as compared to 77.44% indicating that the fiber was enriched and that the fiber enrichment effect also contributed to the reduction of the proportions of carbohydrates in the diet as the dietary fiber is known to moderate the post-prandial rise of blood glucose by reducing hydrolysis of starch and absorption of glucose in the small intestine. Such trends are in line with the rates that were found by [8] in germinated pumpkin seed flour-enriched cookie and by [2]. in a wider profile of seed-enriched biscuits [13].

3.2 Discussion of Microbial Results

Microbiological tests showed a gradual decrease in the total bacterial count, as well as the counts of yeasts and molds, in the pumpkin seed powder-enriched biscuit samples compared to the control sample. This decrease can be explained by the high content of phenolic compounds and antioxidants in pumpkin seeds, which possess growth-inhibiting activity against many microorganisms. These compounds disrupt microbial cell walls and inhibit intracellular enzyme systems, thus reducing the ability of microorganisms to grow and reproduce.

Pumpkin seeds also contain a range of bioactive compounds, such as tocopherols, flavonoids, and phytosterols, which have been shown in numerous studies to inhibit bacterial and fungal growth, in addition to their role in reducing oxidation processes in food products. Therefore, the significant increase in antioxidant activity in the pumpkin seed powder-enriched samples can explain the relative decrease in microbial load in these samples. These results indicate that the use of pumpkin seed powder not only improves the nutritional value of biscuits, but may also contribute to enhancing microbial stability and

extending product shelf life, especially at moderate substitution levels that balance sensory quality and microbial safety.

3.3 Antioxidant Activity and Total Phenolic Content

Table 3 shows the antioxidant activity parameters and the total phenolic content of all formulations of the biscuits made based on methanolic extract prepared just before analysis. All three antioxidant indices including the TPC, DPPH inhibition and FRAP indices presented significant change ($p \leq 0.001$) and consistent dose-dependent change with increasing PSP substitution level, with significant difference found statistically between all adjacent treatment pairs in each assay system. The total phenolic content rose to about four times 18.6 ± 0.8 mg GAE/100 g DW in T0 to 74.3 ± 2.1 mg GAE/100 g DW in T4, DPPH inhibition increased from 23.4 ± 1.2% to 61.8 ± 2.4%, and FRAP values increased from 42.3 ± 1.8 to 118.7 ± 3.6 µmol Fe^{2+} eq/g DW across the same substitution range. The Pearson correlation analysis validated the exceptionally high positive linear relationships between TPC and DPPH inhibition ($r = 0.987$, $p = < 0.001$) and between TPC and FRAP values ($r = 0.993$, $p =< 0.001$), confirming that the phenolic compounds provided by PSP are the major drivers of the antioxidant action of enriched biscuits.

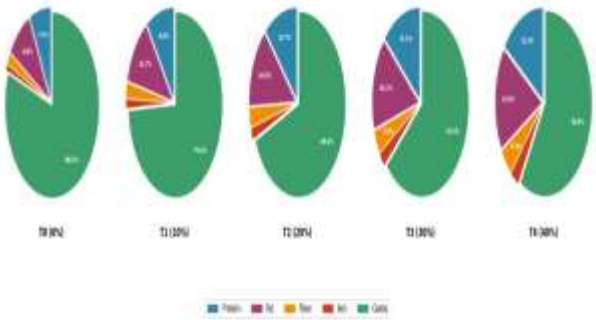

Figure 2. Proximate composition percentage (%) of biscuit formulations T0 to T4 obtained using pie chart analysis.

Table 3. Phenolic content and antioxidant activity of PSP-enriched biscuits (mean ±SD, n= 3)

Parameter	T0 (0%)	T1 (10%)	T2 (20%)	T3 (30%)	T4 (40%)
TPC (mg GAE/100g DW)	18.6±0.8^{e}	33.2±1.1^{d}	48.9±1.6^{c}	61.4±1.9^{b}	74.3±2.1^{a}
DPPH Inhibition (%)	23.4±1.2^{e}	36.7±1.8^{d}	47.3±1.9^{c}	54.9±2.1^{b}	61.8±2.4^{a}
FRAP (µmol Fe^{2+} eq/g DW)	42.3±1.8^{e}	64.1±2.3^{d}	83.6±2.8^{c}	101.2±3.2^{b}	118.7±3.6^{a}
β-Carotene (µg/100g DW)	3.2±0.4^{e}	12.7±0.8^{d}	24.3±1.2^{c}	36.8±1.6^{b}	49.4±1.9^{a}

TPC = Total Phenolic Content; GAE = Gallic Acid Equivalents; DW = Dry Weight. The means in a row having dissimilar superscript letters are highly varied ($p \leq 0.05$).

The extent of the DPPH inhibition effect observed within the PSP fortification range 23.4% in the control to 61.8% in 40% PSP, which is a 164% increase in the free radical scavenging capacity, is statistically strong and physiologically significant, and comparable to the reported trends in antioxidant fortification. The results were reported as high increases in

TPC and antioxidant activities in pumpkin seed flour-enriched wafers, which is also important to note that high levels of antioxidant activity were preserved after simulated in vitro gastrointestinal digestion, and bio accessible antioxidants fractions were still significantly high in fortified samples as compared to controls, thus supporting the in vivo relevance of antioxidant enrichment reported in the current study. Prior chromatographic studies have found the protocatechuic acid, caffeic acid, chlorogenic acid, ferulic acid, and several derivatives of quercetin and luteolin to be the primary phenolic compounds which have been linked to the antioxidant activity of pumpkin seed extracts and operate their antioxidant activity via both hydrogen atom transfer and single electron transfer mechanisms. The good relation between TPC and DPPH and FRAP values ($r > 0.98$) proves the fact that these phenolic compounds are the most important contributors to the total antioxidant capacity of PSP-enriched biscuits and these phenolics align with the well-established structure-activity scenarios of plant phenolics and radical-scavenging pathways [4].

The gradual rise in 3.2 μg/100 g DW in T0 to 49.4 μg/100 g DW in T4 is a nutritionally significant enrichment of this provitamin A carotenoid, a lipid-soluble antioxidant with singlet oxygen-quenching activity. Although these values are significantly lower than the levels of β-carotene in pumpkin flesh or high-carotenoid vegetables, they indicate significant additions to the carotenoid content of a product category that does not normally have significant levels of carotenoids, and they are present in the lipid matrix of the biscuit (provided by butter and pumpkin seed oil), and can be incorporated into micelles during intestinal digestion and be bioaccessible compared to carotenoids in aqueous or cell wall-bound plant matrices. This partial remaining of baking process of 175°C, which was not quantified in the current study when compared in relation to pre-baking dough content, is in line with the published findings of sensible thermal encapsulation or lipid-matrix-embedding stability of carotenoids during baking of biscuits under the same temperatures and time periods [17].

3.4 Physical Quality Parameters of PSP-Enriched Biscuits

Statistically significant ($p \leq 0.05$) and technologically explainable changes in all parameters of physical quality characterization of the PSP-enriched biscuits were observed that were consistent throughout the range of substitution, with the direction and magnitude of change being indicative of the compositional and structural implications of progressive wheat flour dilution in the biscuit matrix with PSP. The ratio of the spread varied greatly with 8.42 ± 0.21 in T0 to 5.87 ± 0.18 in T4 with the largest step wise decrease being between T0 and T1 (8.42 to 7.68), indicating that even the lowest level of PSP caused the dough to spread meaningfully. Reduction in spread ratio with increasing amount of incorporation of seeds is one of the most recurring physical changes reported in the biscuit fortification literature and is due mainly to diminution in wheat gluten dilution of the viscoelastic protein matrix that enables the flow and dispersion of dough during the initial stages of oven heating until the fixation of the biscuit structure by gelatinization of starch. [3]. also found similar changes in the ratio of pumpkin seed powder spread when using it to make biscuits, which was explained by both the dilution of gluten and the difference in rheology of the dough because of the redistribution of water by PSP fiber components, which is a mechanistic explanation of the rheological characteristics of PSP-enriched doughs in line with the rheological definition given by [9].

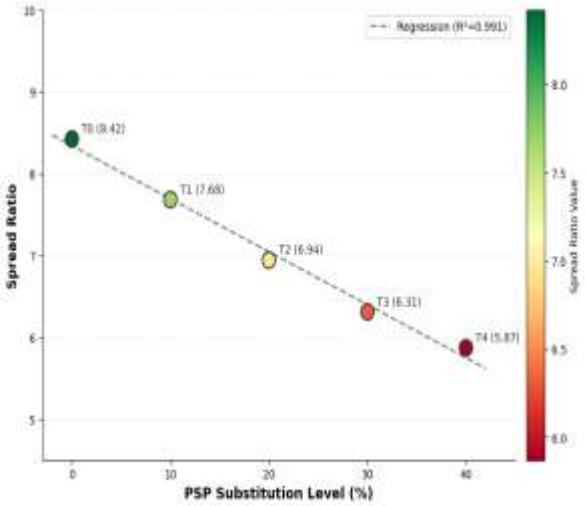

Figure 3. Correlation between the degree of substitution with PSP and the ratio of biscuit spreads with significant negative linear trend.

The analysis of the instrumental color indicated that there is a progressive statistically significant darkening of the color of the biscuits surface with the addition of PSP and it is expressed in terms of the shrinking of L in the range of 68.4 ± 1.2 (T0) to 51.3 ± 1.4 (T4), increasing a* values from 4.2 ± 0.3 to 8.7 ± 0.4, and increasing b* values from 22.8 ± 0.9 to 31.6 ± 1.1, and the corresponding changes The color darkening process of PSP-enriched biscuits is multifactorial: pigmentation of the dark greenish-brown hue of the pumpkin seed material inherently has a direct chromatic impact on the dough and biscuit color, whereas the high protein and reducing sugar content of PSP as compared to wheat flour encourages non-enzymatic pigmentation reactions during baking, producing further melanoidin chromophores which additional increase the surface darkening compared to pigment dilution process. In T1 and T2 substitution levels, this darkening also gave the attractive golden-amber color which was related to high scores of positive color acceptability in the sensory analysis (see Section 3.5). the darkening which was more extreme gave a dark brown look which panelist rated less well. The hardness of the texture also changed substantially, as in T0, the hardness was 18.4 ± 0.8 N, and in T4, it was 38.7 ± 1.4 N, and all of the neighboring substitution pairs were significant ($p \leq 0.05$). This hardening is progressive and is indicative of the shift towards a gluten-network-dominated texture in the control to a more fiber- and protein aggregate-dominated texture in the high-PSP level where the non-cohesive, particulate character of the PSP-enriched matrix gives way to a harder and more brittle fracture behaviour as compared to the softer and more cohesive control biscuit texture [1].

3.5 Sensory Evaluation

Although the highest antioxidant activity values were recorded at 40% PSP, this treatment did not achieve the highest sensory acceptability, as the evaluation scores decreased due to the darker color and firmer texture. In contrast, the 20% PSP treatment achieved a balance between nutritional value, antioxidant activity, and sensory acceptability, indicating that increasing functional value should be done without negatively impacting the product's sensory quality.

The provided data of sensory evaluation (Table 4) demonstrate a uniform and statistically highly-characterized trend of sensory reaction to PSP fortification which is of immediate practical importance to the correct choice of the level of substitution. The response pattern of all five senses attributes (color, aroma, taste, texture, and overall acceptability) was non-linear and inverted-U-

shaped throughout the range of PSP substitution, with scores ranging through T0 and T2 reaching a peak of T2 (20% PSP) and then decreasing dramatically at T3 and T4. This response pattern is the most informative answer that can occur in a product development context since it puts the clear statement of T2 as the peak of the sensory acceptability function and T3 and T4 as formulations in which the nutritional benefits of increasing the incorporation of higher PSP are overriding by the intolerable sensory effects. The total score of the hedonic scale (7.8 ± 0.4) at T2 was decidedly within the range of the hedonic scale (7.8 ± 0.4), indicating that the 20% PSP formulation would be well-received by the evaluation panel and would likely be anticipated to demonstrate meaningful consumer acceptance should it be introduced in the market [11].

Table 4. Sensory evaluation variables of PSP-enriched biscuits based on a nine-point hedonic scale (mean and SD, n = 25 panelists/session × 3 sessions).

Sensory Attribute	T0 (0%)	T1 (10%)	T2 (20%)	T3 (30%)	T4 (40%)
Color/Appearance	6.8±0.6^{c}	7.2±0.5^{b}	7.9±0.4^{a}	6.4±0.7^{cd}	5.1±0.8^{e}
Aroma	6.5±0.7^{c}	7.0±0.6^{b}	7.6±0.5^{a}	6.8±0.6^{b}	5.8±0.9^{d}
Taste/Flavor	6.9±0.5^{c}	7.3±0.4^{b}	7.8±0.3^{a}	6.6±0.6^{c}	5.2±0.8^{d}
Texture/Mouthfeel	7.1±0.5^{b}	7.4±0.4^{ab}	7.7±0.3^{a}	6.3±0.7^{c}	5.0±0.9^{d}
Overall Acceptability	6.8±0.6^{c}	7.2±0.5^{b}	7.8±0.4^{a}	6.5±0.7^{c}	5.3±0.8^{d}

Scale: 1 = Dislike Extremely; 5 = Neither Like nor Dislike; 9 Like Extremely. The difference between means in a row represented by distinct superscripts is very different ($p \leq 0.05$, DMRT).

The acceptability of color/appearance was in the most evident version of an Inverted-U form, with scores of 6.8 ± 0.6 in T0 to 7.9 ± 0.4 in T2, and thereafter, dropping significantly to 6.4 ± 0.7 at T3 and 5.1 ± 0.8 at T4. The increase of color acceptability at T1 and T2 compared to T0 is because of the appealing golden-amber Maillard browning that moderate levels of PSP formed that were likened by panelists to a more appealing, richer appearance of a biscuit than the slightly pale control. The steep drop of color scores in T3 and T4 is supported by the instrumental color values which indicate excessive darkening (L* = 55.8 and 51.3, respectively, with the browning index values of 68.4 and 79.6), indicating that the browning threshold where the consumer would regard the darkening in biscuits as an unwanted quality is between 20 and 30 % of PSP replacement under the production conditions in the current study. There was a similar pattern in texture acceptability, which peaked at T2 (7.7 ± 0.3), with values decreasing significantly to T3 (6.3 ± 0.7) and T4 (5.0 ± 0.9) as observed in the data of textural hardness where hardness increased gradually as a result of increasing the load gauge to 18.4 N (T0) to 38.7 N (T4). These results are very similar to those of Rrustemi et al. (2024), who also found similar sensory acceptability trends of pumpkin and flax by-product-enriched cookies, and of [11]. which found a peak overall acceptability of cookies with about 2025% incorporation of pumpkin flour as a wheat-composite product. The taste/flavor scores were highest at T2 (7.8 ± 0.3), where panelist verbal comments, as measured by open-ended post-reacting questionnaires, indicated that the mild nutty taste and slightly enriched sweetness of the 20% PSP biscuit were pleasant and typical whereas the higher level of seed taste and slight bitterness at T3 and T4 were rated as off flavor that did not match the expected taste profiles of a biscuit [16].

3.6 Identification of the Optimal Fortification Level

The combination of the data of the chemical composition, antioxidant activity, physical quality and sensory evaluation at all five levels of PSP substitution clearly indicates that T2 (20% PSP) was the best level of fortification

to produce nutritionally enhanced functional biscuits that at the same time meet consumer acceptability requirements. At this substitution level, biscuits had a protein value of 11.34 ± 0.31% (a 57.3% increase over the control), crude fat of 13.58 ± 0.41% (a 61.1% increase), dietary fiber of 3.41 ± 0.15% (a 72.2% increase), ash of 2.11 ± 0.08% (a 88.4% increase), DPPH antioxidant activity of 47.3 ± 1.9% inhibition (a 102.1% increase), TPC of 48.9 ± 1.6 mg GAE/100 g DW (a 162.9% increase), and FRAP of 83.6 ± 2.8 μmol Fe^{2+} eq/g DW (a 97.6% increase). All these coexisting measures of accomplishment, nutritional enrichment of more than 50% protein, over 70% fiber, and over 100% antioxidant activity, together with optimum sensory acceptability, constitute the operational definition of an optimal functional food formulation as perceived in the food science literature, and are consistent with the findings of [5].and [19], who both found substitution levels in the 15-25% range optimum in pumpkin seed-enriched biscuit and cookie formulations across different base formulations and production systems [18].

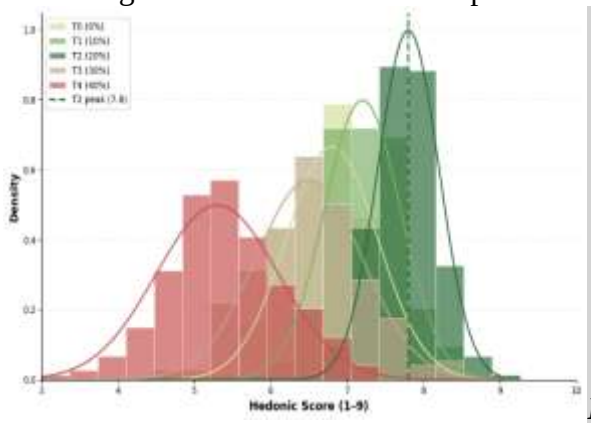

Figure 5. Mean scores of overall acceptability (PSP-fortified biscuit formulations T0 -T4).

The fact that the sensory scores drop at T3 and T4, even though all nutritional parameters have continued to improve, is an indication of the underlying conflict between nutritional fortification and sensory quality which is inherent in the incorporation of plant-based ingredients at high levels and is the primary technical limitation to the effectual enrichment of biscuits with plant-based ingredients. Even though certain consumer groups such as consumers with a more nutritionally aware profile, sports nutrition consumers, as well as health professionals might be willing to accept or even desire the richer sensory profile of higher-PSP formulations, in return receiving a better-quality nutritional profile, the mass-market consumer group to which commercial biscuit products are marketed depends on sensory acceptability and a formulation with a hedonic rating of below 6.0 score out of 9.0 points (Like Slightly), as seen in T4 in the present study is unlikely to embrace a more nutr The 20 % PSP substitution level that has been discovered in the current research is thus not only the scientifically calculated optimal point of the sensory acceptability curve but also the commercially viable upper limit of functional enrichment which can be obtained without repositioning the products to specialized nutrition market segments [15].

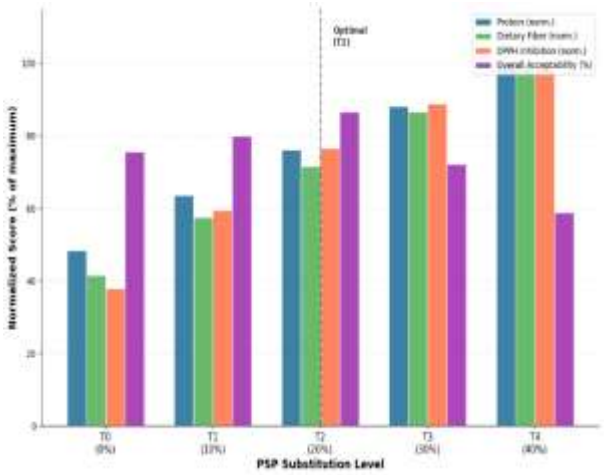

Figure 6. Normalized comparison of protein level, dietary fiber, DPPH level of inhibition, and general desirability at the various levels of PSP substitution that T2 is the best formulation.

4. Conclusions

This work has reported exclusive multi-parameter scientific findings that the powder of pumpkin seeds obtained through Cucurbita maxima can be effectively integrated into the formulations of biscuits at up to 40 percent substitution level to produce a continuum of products with sequentially better nutritional composition, antioxidant activity, and altered physical and sensory properties the 20 percent level of pumpkin seed powder substitution has unequivocally come out as the best formulation in the combined assessment criteria. At the 20 % substitution level, PSP-enriched biscuits provided nutritionally meaningful changes in protein content (+57.3%) and crude fat (+61.1%) and dietary fiber (+72.2%) and ash (+88.4%) in comparison with the control along with over 100 % improvement in the DPPH antioxidant activity (+102.1%) and almost threefold increase in total phenolic content (+162.9) with an overall score of highest hedonic rating of The physical characterization information proved that the spread ratios reduction and color darkening was moderate and acceptable at the 20 percent substitution level with the textual hardness falling within the range of consumer expectation of a nutritionally enhanced and slightly firmer product than the standard control product, the biscuit.

The practical upper limit of the nutritionally acceptable formulation space in which commercial functional development of biscuits should be focused is the identification of the clear peak of sensory acceptability at 20% PSP substitution, with statistically significant score fall at 30% and 40% substitution levels due to excessive color darkening, hardening of textures and intensity of flavours. Both better and more nutritionally sound, higher levels of substitution resulted in global scores of acceptability that were close to but not equal to the hedonic neutral midpoint (score 5.0 at 40% PSP) and could not be accommodated in the mainstream of a commercial environment. The results of the current research are highly supportive of the emerging literature on the subject of pumpkin seed flour enrichment of biscuits and similar items, which validates the significant nutritional enrichment potential of the method as well as the technical and sensorial limits that currently control its application. Future studies need to cover the stability of PSP-fortified biscuits during storage with particular reference to lipid oxidation kinetics at the 20 % substitution level, mineral bio accessibility of PSP-fortified biscuits in in vitro digestion models, the possibility of microencapsulation or antioxidant packaging of the product as an approach to extending the shelf life, and the research concerning consumer acceptance of the product in target market segment such as children, elderly consumers and health

ISSN 2072-3857

conscious adults based on increased consumer panel studies.

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