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EFFECT OF SUBSTITUTION OF WHEAT FLOUR WITH SPINACH POWDER ON PHYSICO-CHEMICAL, NUTRITIONAL AND SENSORY QUALITIES OF COOKIES

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ABSTRACT

Wheat flour cookies are commonly made products identified for their high carbohydrate content; however, they contain a few functional ingredients and provide limited health benefits. In this study, spinach leaves (*Spinacia oleracea* L.) were utilized as a functional ingredient in cookie formulation due to their high macro and micronutrients. The spinach leaves were blanched, dried in a tray dryer at 65°C for 5 hours, and then ground to obtain spinach powder (SP). The effect of replacement of wheat flour with 0% (control), 5%, 10%, 15%, and 20% of SP on physicochemical, nutritional, and sensory qualities of cookies was subsequently investigated. The result revealed that the SP consisted of 32.13% protein, 27.84% carbohydrate, 17.60% ash, 6.92% fat, 9.54% fiber, and 5.97% moisture. The replacement of wheat flour with increased level of SP in cookies resulted in a decrease of spread ratio of cookies. On the other hand, the hardness of cookies was increased with 43.27 N of the maximum hardness of cookies containing 20% SP. In addition, the substitution of wheat flour with SP at levels ranging from 5% to 20% has demonstrated a significant improvement in the proximate composition of cookies, including protein, fat, ash and fiber in comparison to the control ($p \leq 0.05$). The incorporation of SP at various levels in the cookies led to a significant improvement ($p \leq 0.05$) in total phenolic, acrylamide, melanoidins, and antioxidant activity. The sensory evaluations indicated that cookies containing 15% SP were highly accepted; however, it is a crucial to consider the limitations associated with acrylamide content (carcinogenic compound). The cookie formulation containing 10% SP has been identified as optimal, as it results in acrylamide levels below the limits set by Commission Regulation (EU) 2017/2158. The cookies substituted with 10% SP exhibited 2.72 mg GAE/g of total phenolic content, 5.32 mg/mL of IC₅₀ DPPH radical scavenging activity, 3.58 g/100 g of melanoidins, and 328.12 µg/kg of acrylamide. Furthermore, the partial replacement of wheat flour with 10% SP in the cookies, when stored for at least 2 months, ensures that the levels of microorganisms are still within the standards of Thai Community Product for cookies. This study emphasizes the potential of utilizing SP to enhance the nutritional value of the cookies.

Key words: Acrylamide, Antioxidant, Cookie, DPPH, Melanoidins, Nutrient, Proximate, Phenolic, Spinach

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INTRODUCTION

Spinach (*Spinacia oleracea*) is a leafy green vegetable which belongs to the Amaranthaceous family [1]. It is rich in macronutrients and micronutrients, such as protein, carbohydrate, fiber, vitamins, chlorophylls, carotenoids, fatty acids, and minerals (especially, calcium and iron) [2-6]. In addition, the consumption of spinach reduce the risk of the oxidative stress produced by aging, cardiovascular disease, inflammatory effect, and cancer due to the containing antioxidant content [6]. Wheat flour cookie is a widely baked product characterized by high carbohydrate, although it has low functional ingredients and offers limited benefits for health. Acrylamide is a toxic and carcinogenic compound formed through the Maillard reaction during high-temperature baking and other food processing methods conducted above 120°C. The bakery products (such as cookies, breads and cakes) contain the major source of acrylamide [7]. The bakery industry has experienced an increase in demand for baked goods that are nutritionally fortified and have sensory attributes with health benefits [1]. However, the levels of acrylamide in bakery and the risk that resulting intake of acrylamide have on human health. Consequently, there is an increasing demand of enriching cookies with nutritious ingredients to improve their physical and nutritional characteristics. Research indicates that inclusion of plant materials, such as green tea [8], jasmine rice grass [9], and holy basil leaves [10], enhance the functional qualities of cookies. Thus, enhancing the functional and sensory attributes of baked goods presents an attractive approach to address the current demands of consumers. Moreover, the high yield and cost-effective nature of spinach is consistently overlooked, and incorporation of spinach into flour for cookie production represents a significant initiative. In addition to enhancing the cookie type, it offers a unique flavor, color and increases nutritional value of the cookies. Even though scientists share an in-depth knowledge of the nutritional properties of fresh spinach, there is a lack of processed foods utilizing spinach powder in the market. This study investigated the impact of incorporating spinach powder at various concentrations into wheat flour to enhance the production, utilization, and development of spinach-enriched products with outstanding nutritional value and improved flavor, by analyzing the physicochemical properties and sensory attributes of cookies. The findings could lead to the commercial production of spinach and its integration into the development of healthy food products for consumers.

MATERIALS AND METHODS

Materials

Spinach leaves were purchased from local supermarket in Chiangmai, Thailand. The ingredients of cookies consisted of wheat flour (United Flour Mill Public, Thailand), salt (Thai refined salt, Thailand), unsalted butter (Orchid, Thailand), sugar (Mitr phol



sugar, Thailand), baking powder (Imperial, Thailand), milk powder (Dairy Rich, New Zealand) vanilla flavour (Winner, Thailand), and eggs (Charoen Pokphand Group, Thailand). These ingredients were bought from local market in Bangkok, Thailand.

Spinach Powder (SP) Preparation

Spinach leaves were thoroughly washed with tap water and the excess water was removed using salad spinner. The washed leaves were blanched in hot water at 100°C for 2 minutes and then immediately immersed in an ice-water bath. The excess water was removed and the blanched leaves were dried using a Tray dryer (KluayNamThai, Thailand) at 65°C for 5 hours. The dried leaves were then blended using a blender (Philips HR2118/02, Indonesia), finely ground and sieved to obtain particles with 177 µm. The spinach powder (SP) was stored in a vacuum-sealed foil pouch at 4°C for further analysis.

Cookie Preparation

The cookies preparation was carried out according to the methods of Wattanakul *et al.* [10] with modifications. The cookie ingredients consisted of wheat flour (400 g), salt butter (250 g), sugar (4 g), egg (55 g), baking powder (15 g), milk powder (25 g) and vanilla flavor (20 g). The SP was substituted at 0%, 5%, 10%, 15%, and 20% levels of wheat flour. The ingredients were mixed to obtain a uniform dough using a stand mixer (5KPM5EER KitchenAid, USA) for 13 minutes with the medium speed. The dough was subsequently rolled out and cut into circular shapes with a diameter of 36 mm and a thickness of 8 mm. The cookies were then baked in an oven (Kitchenmall, Thailand) at 180°C for 15 minutes.

Water Activity and Color Measurement

The water activity of the powder was measured at 25°C using a water activity meter (LabMaster-AW, Novasina, Switzerland) and the impact of presence and absence of SP on cookie color was performed using the L*, a*, b* system, where L* refers to the lightness component, a* (intensity of red (+) and green (-)) and b* (intensity of yellow (+) and blue (-)). Cookies were analyzed in terms of the referred parameters using an UltraScan Vis spectrophotometer (UltraScan Vis, Hunterlab, USA).

Spread Ratio

The cookies were determined for diameter (cm) and thickness (cm) using a Vernier caliper and the spread ratio of cookies was calculated following the equation 1.

$$\text{Spread ratio} = \frac{\text{width (cm)}}{\text{thickness (cm)}} \quad (1)$$

Texture Analysis

The hardness of cookies was evaluated with the method of Wattanakul *et al.* [10] using a TA. XT Plus texture analyzer (Stable Micro Systems, UK) equipped with



Exponent software version 6.1.21.0. The test setting included a probe HDP/3PB, load cell of 5 kg, pre-test speed of 1.0 mm/s, test speed of 3.0 mm/s, post-test velocity of 10.0 mm/s, and an initial probe distance of 25 mm. The maximum force (N) required to break the cookie.

Proximate Analysis

Proximate analysis of SP and cookies was carried out in accordance with the method established by the Association of Official Analytical Chemists [11]. The moisture content was evaluated using hot air oven drying method (AOAC 925.10). Crude fat was determined using Soxhlet extraction (AOAC 922.06). The determination of protein was assessed using Kjeldal method (AOAC 981.10). Ash content was determined using muffle furnace at 550°C until the ash was obtained (AOAC 945.38). The fiber content was obtained using method of Sarawong *et al.* [9]. Carbohydrate was calculated using the difference as described in equation 2.

$$\text{Carbohydrate g/100g} = 100 - [\text{moisture} + \text{ash} + \text{protein} + \text{fat} + \text{fiber}] \quad (2)$$

Cookie Extraction for Analyses of Total Phenolic Content and Antioxidant Activity

The cookie extraction was performed using a slightly modified method of Kraboun *et al.* [12]. The cookie sample (100 g) was extracted with 200 mL of methanol. The mixture was then shaken on a shaking incubator (NB-205VL, N-BIOTEK, Korea) at 170 rpm for 24 hours and the supernatant was filtrated through a filter paper. The extraction was repeated twice using the method described above. The solvent was evaporated using an evaporator (R-205, Büchi, Switzerland). The cookie extract was kept in an amber glass bottle at -20°C for further analyses.

Determination of Total Phenolic Content

The total phenolic compounds were measured using the methods described by Mildenerger [13] with modifications. The cookie extracts were prepared with their initial concentration of 25 mg/mL and then diluted to 12.5 mg/mL. In a microplate, the following was mixed per well: 15 µL of sample, 170 µL of water, 12 µL of Folin-Ciocalteu reagent (2 N), and 30 µL of sodium carbonate (200 g/L). The microplate was incubated in the dark for 1 hour at room temperature. After that, 73 µL of water was added in the microplate. The absorbance was measured at 765 nm using a microplate reader (Thermo Fisher Scientific, USA). The total phenolic content (TPC) was calculated based on a linear standard curve of gallic acid. The result was presented as gallic acid equivalents (GAE).



DPPH Radical Scavenging Assay

The DPPH radical scavenging ability was determined according to Kraboun *et al.* [14] with some modifications. Each 2-12 mg/mL of cookie extracts (40 μ L) was mixed with 200 μ L of 0.02 mM DPPH solution and 4 mL of methanol, respectively. The mixtures were incubated at ambient temperature for 15 minutes and the absorbance was measured at 517 nm using UV-Vis Spectrophotometer (GENESYS 20, Thermo Fisher Scientific, USA). The ability of DPPH radical scavenging was expressed in mg of equivalent Trolox per milliliter of sample (mg Trolox/mL).

Acrylamide Assay

Acrylamide was quantified with a modified method based on Rufián-Henares *et al.* [15]. The cookie sample (450 mg) was dissolved in 5 mL of water. Subsequently, 100 μ L of [$^{13}\text{C}_3$]-labelled acrylamide methanol solution (10 mg/L) was added into the sample suspension. The mixture was then vortexed for 1 minute. Consequently, 750 μ L of each Carrez I and Carrez II were added into the mixture and vortexed for 10 seconds. The mixture was left at 35°C for 10 minutes. It was subsequently centrifuged at 2,400 *g* for 15 minutes at 4°C. The supernatants were separately aliquoted into the microtubes and then frozen for further analysis. Prior to acrylamide analysis, the supernatant was thawed and centrifuged at 10,000 *g* for 10 minutes at room temperature. The supernatant (1 mL) was then filtered using an Oasis HLB cartridge (Waters, Milford, USA). The initial seven droplets were eliminated and the remaining was transferred into the vials. LC-MS was carried out using an Agilent 1100 liquid chromatography coupled with an Agilent Quadrupole MS detector (Agilent Technologies, USA). The separation was performed using an Inertsil ODS-3V analytical column (5 μ m, 4.6x250 mm) (GL Sciences, Japan) at 32°C with isocratic elution. The injection volume was 60 μ L and the mobile phase consisted of a water to formic acid ratio of 99.8 to 0.2, with a flow rate of 0.6 mL/min. The results were acquired using a selected ion monitoring mode. Acrylamide and $^{13}\text{C}_3$ -acrylamide were detected at *m/z* 72.1 and 75.1, respectively. The calibration curve of acrylamide was obtained using external standard solutions varying from 1 μ g/L to 100 μ g/L. The result was presented as μ g/kg of sample.

Melanoidins Assay

Melanoidins were determined using a slightly modified method of Kraboun *et al.* [12]. The molecular structure of melanoidins has never been identified yet and the melanoidin standard is currently unavailable. Consequently, the standard calibration curve was generated using the cookies extract as a source of melanoidins. The standard curve of melanoidins was measured at the wavelength of 420 nm. A stock solution was prepared using a 2:1 ratio of cookie extracts to distilled water (2:1). The stock solution was sequentially diluted 5 times. The melanoidin content in each



dilution was determined using the modified Lambert-Beer equation, as shown in equation 3

$$C = \frac{A}{ab} \quad (3)$$

Where

C = Melanoidins

A = Absorbance of extract solution

b = Path length in the spectrophotometer (cm)

a = Specific extraction coefficient (L.g⁻¹cm⁻¹)

The value of “a” was 1.1289 L. g⁻¹cm⁻¹. The standard calibration curve was generated by plotting absorbance values against melanoidin content. For each sample, A 1:9 dilution was assessed for each sample. Melanoidins were spectrophotometrically measured at 420 nm. The melanoidins content was quantified as mg/kg of sample.

Sensory Evaluation

Fifty untrained panelists assessed the sensory attributes of cookies with a 9-point hedonic scale. All samples were assigned three-digit random codes and randomly served to the assessors. The assessors scored the samples on a scale from 1 to 9 (1= dislike extremely, 9 = like extremely). Ethical approval for the participation of human participants in this study was obtained by the Naresuan University-Network of Research Ethics Committee (COA No. 0042/2023 and NREC No. 0044/2023 on December 26th, 2023).

Microbial Evaluation for Storage Stability of SP Cookie

The most preferred cookies with the presence of SP were selected based on their physicochemical properties and sensory assessment for the storage study. The cookies were stored in polyethylene pouches at room temperature (25±2°C). The cookie storage was analyzed over a period of 0, 7, 14, 30, 45, and 60 days. The microbiological analyses, including total plate count, total yeast and mold count, were conducted in accordance with the standard methods described in the Food and Drug Administration-Bacteriological Analytical Manual (FDA-BAM) [16]. The results were quantified and expressed in colony-forming units (CFU)/g.

Statistical Analysis

The experimental results were presented as mean ± standard deviation of three replications. A completely randomized design was employed to analyze the physicochemical data; a randomized complete block design (RCBD) was applied for the sensory evaluation data. An analysis of variance was performed using IBM SPSS Statistics 27.0 (IBM Corp., Armonk, NY, USA). Whenever a significant F value ($p \leq 0.05$) was found, the Duncan’s multiple range test was used to identify significant differences among means.



RESULTS AND DISCUSSION

Physical properties of cookies with a substitution of SP

The partial substitution of wheat flour with various amounts of SP in the cookies impacted the physical properties (Figure 1 and Table 1). The water activity of cookies is a crucial for predicting their stability and safety in regard to microbial growth and lipid oxidation rates [17]. The International Commission on Microbiological Specification for Food states that the minimum water activity in the most foods is 0.85 [18]. This result found that the water activities of all measured cookies were in the range of 0.42 to 0.45 (Table 1). Hence, the cookies could be assumed to be microbiologically stable, because their water activity values were all less than the minimum threshold value. Similarly, Liu *et al.*[17] found low the water activity (0.38-0.42) of cookies with the addition of amaranth and navy bean flour, while Sirisha *et al.*[19] observed a low water activity (0.34-0.36) in spinach incorporated foxtail millet biscuit. The influence of SP substitution in cookies on color properties are shown in Figure 1 and Table 1. The L*, a*, and b* values of cookies were associated with the quantity of SP in the cookie ingredients. The color characteristics of cookies indicated that greater amounts of SP in the cookies led to a notable decrease in L* (lightness), a* (redness) and b* (yellowness) (Table 1). These findings align with those of literature reviews [3,8,9,10,20,21]. The L*, a* and b* values of cookies incorporating green tea powder [20], jasmine rice grass [9], spinach [3,19] and Holy Basil leaves [9] exhibited a decrease compared to the cookies containing only wheat flour. The replacement of wheat flour with increased quantities of SP in cookies resulted in a decrease of spread ratio. Conversely, they enhanced the hardness of cookies (Table 1). The maximum of hardness of the cookies containing 20% SP was measured at 43.27 N. Earlier studies have also revealed that with the addition of fiber dilute the gluten which results in the development of hard dough [3]. Similarly, Khan *et al.* [21] and Aljobair [22] demonstrated that the incorporation of spinach powder in chapati premixes and clove powder in cookies increased the hardness. Thus, the spread ratio and hardness of cookies are influenced due to fiber content. This observation was consistent with the findings of Sarawong *et al.* [9] and Wattanakul *et al.* [10], who indicated that the fiber content in jasmine rice grass and holy basil leaves contributed to the spread ratio and softness of cookie. Similarly, Galla *et al.* [3] revealed that a higher quantity of spinach, which is rich in fiber, led to a reduced spread ratio, but increasing the hardness of cookies.

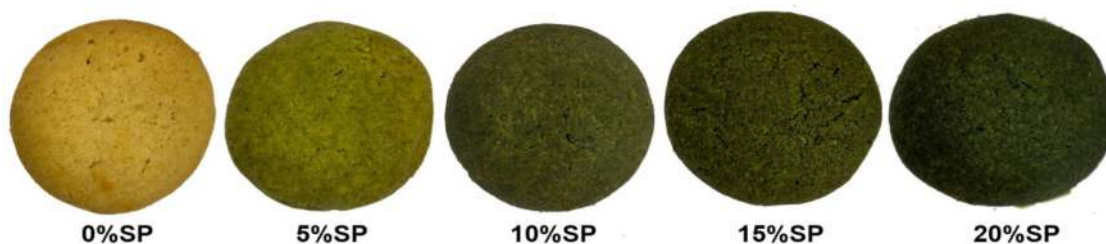


Figure 1: Characteristics of cookies with different levels of spinach powder (SP)

Proximate composition of cookies with a substitution of SP

The chemical composition of spinach can vary based on several factors, such as seasoning of harvesting and the area of growing. The nutrient composition of SP and cookies containing 0-20% of SP are showed in Table 2 and Table 3, respectively. Spinach powder possessed good quantities of protein (32.13 g/100 g, DW), carbohydrate (27.84 g/ 100g, DW), ash (17.60 g/100 g, DW), fiber (9.54 g/100 g, DW), fat (6.92 g/100 g, DW), and moisture (5.94 g/100 g, DW). Furthermore, the spinach serves as a rich source of galactolipid containing omega-3 and mega-6 fatty acids [4,5,23,24]. The proximate composition of SP exhibited several variations when comparing the research findings to the existing literature. Galla *et al.*[3] found differences in proximate composition in dried SP at 55°C for a duration of 8 hours resulting in the following percentages: 8.60% moisture, 18.81% ash, 5.69% fat, 27.81% protein, 8.82% fiber, and 30.28% carbohydrate. In addition, the moisture content of cookies varied between 4.02% and 4.18%. The inclusion of SP in the cookie formulation led to a reduction in moisture content. However, there were no significant difference in moisture content when compared to control ($p>0.05$). The replacement of wheat flour with SP at levels ranging from 5% to 20% has demonstrated a significant improvement in the nutritional composition of cookies, including protein, fat, ash and fiber in comparison to the control cookie. The enhancement of the proximate composition of cookies after the incorporation of SP could be attributed to the abundance of these substances in spinach. Similarly, previous reports indicated that substituting wheat flour with Thai Jasmine rice grass [9] and holy basil leaves [10] in biscuits resulted in an increase in protein, lipid, fiber, and ash content, as well as with the addition of red and green spinach in bake product [25].

Total phenolic content and DPPH radical scavenging (Ic_{50}) of cookies with a substitution of SP

The TPC for both the control and the inclusion of SP in cookies is presented as Table 4. The incorporation of SP at various levels in the cookies led to a significant improvement ($p\leq 0.05$) in the TPC. The control cookie demonstrated the lowest TPC

at 0.23 mg GAE.g⁻¹ DW, whereas the cookies with SP substitution exhibited higher TPC values between 1.65 and 2.92 mg GAE.g⁻¹, DW. The spinach powder demonstrated an excellent TPC of 11.63 mg/g, DW [26]. The replacement of 5%, 10%, 15%, and 20% with SP resulted in an increased in TPC by approximately 7.2, 11.8, 12.1, and 12.7 times, respectively, compared to the control. This finding aligned with the studies of Salehi [27], Sowmya *et al.* [28], Wattanakul *et al.* [10] and Sarawong *et al.* [9]. They showed that the incorporation of fruit and vegetable powders, including mango peel, mango kernel, blueberry, grape seed, basil leaves and jasmine rice grass, into the cookie formula resulted in an increase of TPC in cookies. Similar to the TPC results, there was a significant difference ($p \leq 0.05$) in the DPPH radical scavenging activity between the control cookie and the inclusion of SP of cookies. The IC₅₀ values of DPPH radical scavenging activity were observed to range from 4.98 to 12.12 mg/mL, as shown in Table 4. These results indicated that the control exhibited the highest IC₅₀ value of DPPH free radical scavenging activity, whereas the 20% SP demonstrated the lowest IC₅₀ value. In comparison to the control, the substitution of 5%, 10%, 15%, and 20% SP resulted in a decrease in the IC₅₀ of DPPH radical scavenging activity by approximately 1.1, 2.3, 2.3, and 2.4 times, respectively. In addition, Tiveron *et al.* [29] indicated that the lowest extract concentration to reduce the initial amount of DPPH radical by 50% of spinach was 22.87 mg/mL. Similarly, Mišan *et al.* [30] illustrated that the obtained IC₅₀ value depended on the level of vitalplant (*F. Cortex*, *Menthae piperitate folium*, *C. fructus*, and *P. fructus*) incorporated into the cookie formulation. The cookies enriched with a higher level of vitalplant demonstrated a reduction in the IC₅₀ value of DPPH free radical, compared to the control. Thus, the results indicated that substitution of wheat flour with SP could have a positive effect on health benefits, especially antioxidant activity.

Acrylamide and melanoidins of cookies with a substitution of SP

The acrylamide content for cookies is presented as Table 4. The Maillard reaction during high-temperature baking involves the interaction of the amino acid group of free asparagine with the carbonyl groups of reducing sugars, resulting in the formation of a Schiff-base. This process leads to the formation of 3-aminopropionamide through decarboxylation, which is subsequently degraded to form acrylamide as a carcinogen [7]. The results indicated that acrylamide content in cookies varied between 265.11 and 698.33 µg/kg. The increased amount of SP resulted in an increase in acrylamide content. Similar results were observed in bakery products that replaced wheat flour with lentil flour [32]. They revealed that the higher concentration of lentil flour led to an increase of acrylamide content in breads and cookies. The commission Regulation (EU) 2017/2158 stated that the acrylamide levels in biscuits and wafer must not exceed 350 µg/kg [7]. Hence, only cookies incorporated 5-10% SP meet with the standards set by the commission



Regulation (EU) 2017/2158. In addition, López-Ruiz *et al.* [33] demonstrated that the incorporating dietary fiber, especially pectin, was suggested to mitigate acrylamide in cookies and reduce the bioaccessibility of acrylamide in gastrointestinal tract. The reduction of acrylamide in cookies could be due to the fact that negative fiber (i.e. pectin and k-carrageenan) traps the asparagine in an egg-box structure and avoids its reaction with reducing sugars to produce acrylamide [33]. Melanoidins are produced in the final stage of the Maillard reaction, occurring during the cooking and thermal processing of food. They are associated with several health benefits, such as antioxidant, antimutagenic, antimicrobial, and prebiotic properties [34,35]. The results demonstrated that the melanoidins content in cookies ranged from 1.46 to 7.14 g/100 g. Obviously, the formation of acrylamide content in SP cookies was related to the melanoidins content (Table 4). These findings were in line with Kraboun *et al.* [12], who reported that an increase of acrylamide content correlated with a higher level of melanoidins in roasted broken rice. Furthermore, the average daily intake of melanoidins in the diet is approximately 10 g, indicating their potential contribution to the daily dietary antioxidant requirement [35].

Sensory evaluation of cookies with a substitution of SP

The sensory evaluation of cookies is presented in Table 5. These findings suggested that the cookies with up to 5% SP incorporation did not show a significant difference from the control in terms of appearance, color, odor, hardness, taste, and overall acceptance. The level of above 5% SP cookies resulted in noticeably darker appearance (Figure 1) and less preferred by panellists (Table 5). The inclusion of SP (10-20%) led to a reduced texture score compared to the control. The analysis of texture attribute suggested that the incorporation of SP at levels up to 5% in the formulation did not impact the sensory score. In the case of biscuits and chapati premixes, it was observed that adding more than 5% SP led to a decrease in the texture sensory score [3,20]. On the other hand, Waseem *et al.* [36] revealed that the presence of SP at a concentration of 2.5% in Chapattis significantly affected the hardness score in comparison to the control. These results indicated that the substitution of wheat flour with up to 15% of SP did not impact the odor and overall acceptability among panelists, with the exception of cookies containing 20% SP. Even though cookies with 15% SP exhibited nutrient composition and overall acceptability, it is a crucial to address the limitations related to acrylamide content, which is classified as carcinogen. Therefore, the formulation with 10% SP in cookies was identified as the optimal level according to sensory evaluation and physicochemical properties in this study.

Effect of storage time on microorganism of cookies

The cookie formulation with 10% SP was identified as the optimal level based on its physicochemical property and sensory attribute. The shelf-life of cookies substituted



wheat flour with 10% SP was evaluated over a storage period of 60 days at room temperature, with the findings presented in Table 6. Thai Community Product Standards (TCPS 118/2555) state that the microbiological quantities in cookies must be less than 1×10^4 CFU/g in total plate count and less than 100 CFU/g for total yeast and mold [37]. Over the storage period, the bacterial counts reached a maximum of 2.73×10^3 CFU/g, while the yeast and mold counts increased to 0.92×10^2 CFU/g by the end of storage duration. However, all microorganisms did not exceed Thai Community Product Standards (TCPS 118/2555). Therefore, the cookie with the presence of 10% SP in PE bags can be maintained at 25°C for at least 2 months, during which the levels of microorganisms remain within the standards established by of Thai Community Product for cookies.

CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

This study pointed out the potential of SP as a significant ingredient in the development of nutritionally improved and sensory-acceptable cookies. The addition of SP in cookies enhanced protein, fiber, galactolipid, ash, TPC, melanoidins, and antioxidant activity. The SP incorporation in cookies resulted in a significant decrease in color parameters, spread ratio, while hardness of cookies increased in comparison to the control. Although the sensory evaluations indicated that cookies containing 15% SP were highly favored, it is an essential to consider the limitations associated with acrylamide content (carcinogenic compound). The formulation containing 10% SP in cookies has been selected as the optimal cookie formulation. It can be stored at room temperature for a minimum of 2 months when packaged in polyethylene bag, while the total plate count and total yeast and mold count still remained below the recommended thresholds of Thai Community Product for cookies. However, further study is an essential to fully understanding the *in vitro* and *in vivo* digestion of cookies for better comprehension and utilization in functional food formulations of cookies.

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Conflict of interest statement

The authors do not have any conflict of interest.

Authors' contributions

Wipavan Julaya: Conceptualization, Methodology, Formal analysis, Data curation, Writing (original draft). **Narissara Uthai:** Conceptualization, Methodology, Formal analysis, Data curation, Writing (original draft). **Racharat Yampuang:** Methodology, Formal analysis. **Kitisart Kraboun:** Methodology, Formal analysis. **Noppamas**



Poolcharoensil: Methodology, Formal analysis. **Doungtip Kaikaew:** Methodology, Formal analysis. **Chonthira Sarawong:** Methodology, Formal analysis. **Jutarat Wattanakul:** Conceptualization, Methodology, Formal analysis, Data curation, Validation, Supervision, Writing (original draft), Writing (review and editing).



Table 1: Physical properties of cookies made from wheat flour substituted with spinach powder (SP)

Level of SP (%)	Aw ^{ns}	L*	a*	b*	Spread ratio	Hardness (N)
0	0.43 ± 0.003	70.70 ± 1.26 ^a	3.33 ± 0.39 ^a	27.52 ± 0.80 ^a	4.22 ± 0.08 ^a	29.72 ± 3.18 ^c
5	0.42 ± 0.003	53.82 ± 1.62 ^b	-1.23 ± 0.53 ^b	23.22 ± 0.78 ^b	3.99 ± 0.13 ^b	34.47 ± 4.56 ^b
10	0.44 ± 0.003	45.26 ± 1.38 ^c	-1.23 ± 0.48 ^b	16.61 ± 1.16 ^c	3.92 ± 0.03 ^b	37.21 ± 2.68 ^b
15	0.42 ± 0.001	42.54 ± 1.54 ^d	-3.13 ± 0.26 ^c	15.25 ± 0.53 ^d	3.86 ± 0.01 ^b	38.28 ± 2.03 ^b
20	0.45 ± 0.001	38.08 ± 1.49 ^e	-4.02 ± 0.14 ^d	11.09 ± 0.58 ^e	3.66 ± 0.10 ^c	43.27 ± 3.45 ^a

Data were expressed as a mean ± SD and analyzed using ANOVA followed by a Duncan test with a significant level at $p \leq 0.05$, $a > b$, ns: no significant difference

Table 2: Proximate composition of spinach powder (SP)

Composition	Content (g/100 g, DW)
Moisture	5.97 ± 0.09
Carbohydrate	27.84 ± 2.09
Protein	32.13 ± 1.28
Fat	6.92 ± 0.50
Ash	17.60 ± 1.03
Fiber	9.54 ± 0.79

Data were expressed as a mean ± SD; DW means dry weight

Table 3: Proximate compositions of cookies made from wheat flour substituted with 0%, 5%, 10%, 15% and 20% of spinach powder (SP)

Level of SP (%)	Moisture* ^{ns} (g/100g)	Carbohydrate (g/100g)	Protein (g/100g)	Fat (g/100g)	Ash (g/100g)	Fiber (g/100g)
0	4.10 ± 0.07	71.95 ± 0.18 ^e	5.49 ± 0.10 ^e	16.91 ± 0.09 ^e	0.92 ± 0.03 ^e	0.63 ± 0.04 ^e
5	4.04 ± 0.07	66.13 ± 0.10 ^d	7.04 ± 0.07 ^d	18.02 ± 0.04 ^d	1.74 ± 0.05 ^d	3.03 ± 0.03 ^d
10	4.18 ± 0.16	59.85 ± 0.56 ^c	8.44 ± 0.14 ^c	19.21 ± 0.20 ^c	2.64 ± 0.04 ^c	5.67 ± 0.10 ^c
15	4.02 ± 0.04	54.09 ± 0.31 ^b	9.84 ± 0.13 ^b	20.43 ± 0.07 ^b	3.49 ± 0.02 ^b	8.14 ± 0.11 ^b
20	4.03 ± 0.10	47.91 ± 0.30 ^a	11.42 ± 0.07 ^a	21.47 ± 0.19 ^a	4.41 ± 0.11 ^a	10.76 ± 0.12 ^a

Data were expressed as a mean ± SD and analyzed using ANOVA followed by a Duncan test with a significant level at $p \leq 0.05$, $a > b$, ns: no significant difference



Table 4: Total phenolic content, DPPH radical scavenging activity, melanoidins and acrylamide of cookies made from wheat flour substituted with 0%, 5%, 10%, 15% and 20% spinach powder (SP)

Level of SP (%)	TPC (mg GAE/g)	IC ₅₀ of DPPH (mg/mL)	Melanoidins (g/100g)	Acrylamide (µg/kg)
0	0.23 ± 0.01 ^a	12.12 ± 0.22 ^c	1.46±0.02 ^d	265.11±2.42 ^d
5	1.65 ± 0.01 ^b	10.61 ± 0.35 ^b	1.52±0.02 ^d	276.19±3.19 ^d
10	2.72 ± 0.04 ^c	5.32 ± 0.22 ^a	3.58±0.01 ^c	328.12±3.17 ^c
15	2.78 ± 0.03 ^c	5.17 ± 0.21 ^a	6.12±0.02 ^b	512.23±5.19 ^b
20	2.92 ± 0.01 ^d	4.98 ± 0.08 ^a	7.14±0.02 ^a	698.33±7.82 ^a

Data were expressed as a mean ± SD and analyzed using ANOVA followed by a Duncan test with a significant level at $p \leq 0.05$, a>b; GAE: gallic acid equivalent

Table 5: Sensory acceptability of cookies made from wheat flour substituted with 0%, 5%, 10%, 15% and 20% of spinach powder (SP)

Level of SP (%)	Attributes					
	Appearance ^{*ns}	Color	Odor	Hardness	Taste	Overall acceptability
0	8.10±0.76	7.43±0.50 ^c	7.27±0.91 ^a	8.00±0.59 ^a	8.20±0.85 ^a	8.40±0.62 ^a
5	8.00±0.59	7.53±0.57 ^c	7.37±0.76 ^a	7.93±0.58 ^a	8.07±0.69 ^a	8.23±0.43 ^a
10	7.93±0.45	7.70±0.53 ^{bc}	7.10±0.76 ^a	7.33±0.55 ^b	7.37±0.49 ^b	8.17±0.70 ^a
15	7.97±0.56	7.97±0.72 ^{ab}	7.03±0.67 ^a	7.23±0.63 ^b	7.27±0.64 ^b	8.20±0.48 ^a
20	8.27±0.64	8.23±0.73 ^a	6.57±0.90 ^b	5.73±0.83 ^c	6.37±0.96 ^c	7.27±0.87 ^b

Data were expressed as a mean ± SD and analyzed using ANOVA followed by a Duncan test with a significant level at $p \leq 0.05$, a>b; ns: no significant difference

Table 6: Microbial count of cookies made from wheat flour substituted with 10% spinach powder (SP) during storage at 25 ± 2°C for 0, 7, 14, 30, 45 and 60 days

Storage time (days)	Total plate count (CFU/g)	Yeast and mold (CFU/g)
0	2.02 × 10 ³	0.51 × 10 ²
7	2.13 × 10 ³	0.56 × 10 ²
14	2.21 × 10 ³	0.68 × 10 ²
30	2.36 × 10 ³	0.78 × 10 ²
45	2.55 × 10 ³	0.86 × 10 ²
60	2.73 × 10 ³	0.92 × 10 ²

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