Agric Res J ***62*** *(3): 314-323, June 2025*
DOI: 10.5958/2395-146X.2025.00038.7

BEETROOT (*Beta vulgaris* L.) POWDER AS NATURAL COLORANT FOR VALUE ADDITION OF DOUGHNUTS

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ABSTRACT

Beetroot (*Beta vulgaris* L.) can be potentially used as a natural colorant as an alternative to the artificial colorants used in the food industry which will not only act as a natural colorant but will help in developing products which are nutritionally superior. In the present study, beetroot powder with highest peak in terms of wave number was used to develop doughnuts. Value addition of doughnuts with beetroot powder was highly acceptable at 10 percent supplementation. Crude protein, fibre and ash content of doughnuts significantly increased upon supplementation with beetroot powder. Total phenols, flavonoids, betalains and antioxidant activity of doughnuts significantly increased to 2-6 times when supplemented with beetroot powder. Betalains content of beetroot powder supplemented doughnuts was 287.36 mg/100g. Storage study showed that total phenols, flavonoids and antioxidant activity of products developed from stored beetroot powder significantly decreased but not as much deemed to be non-beneficial for consumption.

Keywords: Beetroot powder, Colorant, Doughnuts, Health, Steam blanching, Value addition

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Artificial colorants are extensively available in the market where red color is one of the most widely used. Many food industries, catering establishments, restaurants and bakery owners prefer artificial colorants for the food preparations like red-velvet cake, cookies, strawberry ice-cream and other desserts. These artificial colorants contain a number of chemicals and can produce oxidative stress within the body (Nayak and Mishra, 2020). Oxidative stress occurs as a result of the productions of free radicals which cannot be neutralized by the defense system of our body and have a vital role in the pathogenesis processes that can cause pulmonary injury, neuro-degeneration, several cancers and cardiovascular disorder (Chen, 2020). Liver carcinogenesis can be caused by food colours including tartrazine, erythrosine, and carmoisine. The two most prevalent food colours that are unlawfully added are triphenylmethanes and azo dyes. The metabolism of azo dyes, which are extensively employed in industry, results in their hazardous action. Active aromatic amines that target DNA are created as a result of enzyme-mediated azo reduction (Mishra, 2020). Having an alternative natural colorant which is safer and health friendly to use is a need of the hour. Fruits and vegetables are known to have abundance of phytonutrients which possess protection against oxidative stress, responsible for onset of various angiogenic diseases. Deep colored fruits and vegetables have been reported to be good sources of phenolics including flavonoids, anthocyanins and carotenoids and are recognized as healthy to human body. Several epidemiological studies have also been marked for the protective role of fruits and vegetables consumption against various chronic diseases (Oliviero and Fogliano, 2016). Beetroot is a red rooted vegetable belonging to the *Chenopodiaceae* family and recognized as health promoting food due to presence of essential components such as total polyphenols, flavonoids, ascorbic acid and betalains that promote health (Chhikara *et al*., 2019). Beetroot is one of the top ten vegetables with the highest antioxidant potential (El-Beltagi *et al*., 2018). Betalains are the main pigments found in beetroot that add to its antioxidant potential. They are the nitrogenous pigments which are categorized into two compounds namely betaxanthins (yellow pigment) and betacyanins (red pigment). Consumption of beetroot extract has been seen exhibiting increased

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Date of receipt: 28.11.2023, Date of acceptance: 24.05.2025

plasma nitrates thus helps decreasing blood pressure in older adults (Pinheiro *et al*., 2024). Red beetroot may have a significant future application in the food industry as a functional food ingredient with anti-inflammatory and antioxidant properties (Yi *et al*., 2017).

High moisture content of beetroot limits its shelf life as well as its ease of storage and transportation. Preservation methods like drying can be an effective measure for increasing the shelf life of beetroot and results in reduction of storage space required (Seremet *et al*., 2020). Drying is a widely used food preservation process where removal of water minimizes plenty of the moisture-driven deterioration reactions affecting the product quality. In the literature, a wide range of drying treatments have been applied to beetroot which includes freeze-drying (Kerr and Varner, 2019), microwave heat drying (Shokry, 2018), foam mat drying (Ng and Sulaiman, 2018) and spray drying (do Carmo *et al*., 2018). In this context, present work employed the use of beetroot powder developed by using a very economical steam method namely blanching with refrigeration as a pre-treatment prior to convective drying. This method is cost effective as it is an alternate to the above mentioned expensive techniques used for retention of the pigments.

Due to valuable nutritional and dietary properties of beetroot its demand and consumption has increased in recent years (Esatbeyoglu *et al*., 2015; Moding *et al*., 2018). Dehydrated beets (beet powder) as colorant were described under section 73.40 of title 21-Food and Drugs in Code of Federal Regulations (CFR) of United States Food and Drug Administration (FDA, 2009). Beetroot powder can be used as a promising vehicle for the nutritional improvement of bakery products and enhancement of their functional properties. Alshehry *et al*. (2021) developed beetroot powder cookies rich in minerals, antioxidants and pigments to supplement schoolchildren who may be anaemic. In addition to nutrients, the sensory quality is one of the leading selling points of food products. In this perspective, since the supplementation of products with non-wheat flour presumably contributes some alteration to the product quality, the present research aimed to evaluate the sensorial, nutritional and biochemical properties of doughnuts supplemented with beetroot powder at different levels. Effect of storage of beetroot powder on bioactive composition of doughnuts was also studied.

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MATERIALS AND METHODS

Preparation of beetroot powder

Beetroot was subjected to optimized method of blanching followed by peeling, slicing and drying as described by Kaur *et al*. (2021). The developed powder was further stored in HDPE bags at refrigeration temperature of 4°C.

Formulation and standardization

Functional doughnuts using standard recipes (Kochhar and Kaur, 2015) by supplementing varying levels of beetroot powder were prepared. For experimental samples of doughnuts, beetroot powder was incorporated at 2.5, 5, 7.5 and 10 percent along with the control products which were prepared with 100 percent of refined wheat flour. Doughnuts were prepared using standardized recipes in the bakery laboratory of department of Food and Nutrition, College of Community Science, Punjab Agricultural University, Ludhiana.

Sensory attributes

The sensory acceptance was assessed by using a 9-point hedonic scale (Wichchukit and O'Mahony, 2015) with scores ranging from 'extremely dislike-1' to 'extremely like-9' by a semi-trained panel of 10 judges from department of Food and Nutrition and Department of Food Science and Technology, Punjab Agricultural, Ludhiana. The product was evaluated based on its appearance, color, texture, flavor, taste and overall acceptability. The most acceptable beetroot powder supplemented doughnut formulation along with its control sample was analyzed for nutritional and biochemical parameters.

Nutritional and biochemical analysis

Proximate composition

The highly acceptable experimental doughnuts along with their control samples were dried in hot air oven and milled with a grinder (Philips Grinder HL 1631/00) until a homogenous fine powder was obtained and were analyzed for proximate composition (moisture, protein, fat, ash, carbohydrates and energy) using standard AOAC (2010) method.

Extraction of bioactive compounds

Phenols and flavonoids

Extraction of 1 g of sample was done with 20 ml of 80 % methanol by shaking for three hours in an orbital shaker. The extract was centrifuged at 10000 rpm for 5 minutes. The supernatant was filtered

through Whatman No. 1 filter paper. The volume was made to 50 ml with 80% methanol. The methanolic extract was stored at -20° C till analysis. The methanolic extract was analyzed for total phenols, flavonoids and antioxidant activity.

Total phenols

The total phenolic content in the samples was determined using colorimetric method described by Singleton *et al*. (1999).

Flavonoids

Flavonoid content in the samples was determined using the Aluminium chloride colorimetric method of Zhishen *et al*. (1999).

Antioxidant activity

Total antioxidant activity was determined using DPPH assay as described by Brand-William *et al*. (1995). For control, there was no sample extract added to the DPPH solution. Scavenging activity was calculated as follows:

DPPH radical−scavenging activity % =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A is the absorbance at 517 nm

Betalains

Betalains content was estimated by spectrophotometric method described by Bucur *et al*. (2016). Five grams of the sample was weighed in a beaker and stirred for 10 minutes in 15 ml of distilled water followed by filtration into a 50 ml volumetric flask. This extraction was repeated 3 times and stock solution was made upto 50 ml volume. Absorption was read at 25ºC ± 1 using solutions prepared from stock solution by dilution (1:2) with distilled water at 536 nm and 486 nm for betacyanins and betaxanthins, respectively. Betalains content was calculated using the following equation.

Betacyanins/ Betaxanthins (mg/g) =

$$\frac{A \times DF \times MW \times VD}{\varepsilon \times L \times W}$$

where, A= Absorption value

D= Dilution factor

V_d= Sample solution volume (ml)

L= Path length (1cm) of the cuvette

W= weight of the sample (g)

Betacyanins (MW = 550 g/mol, ε = 60000L/ (mol*cm)

Betaxanthins (MW=308/mol, ε = 48000L/ (mol*cm)

Color analysis

The color characteristics of highly acceptable experimental doughnuts were measured using Hunter lab colorimeter. Calibration of the instrument was done with standard black and white ceramic tile prior to taking the measurements. Values of L*, a* and b* were measured where L* indicates lightness read from 0 (opaque or black) to 100 (transparent or white), a positive a* denotes redness while a negative indicates greenness and a positive b* specifies yellowness whereas a negative b shows blueness of the sample. The data were evaluated based on L* a* b* values.

Storage analysis

The developed beetroot powder was stored at 25°C and relative humidity 50 % for a period of 90 days in high density polyethylene bags. Doughnuts were developed again using stored beetroot powder and were subjected to sensory evaluation as well as analysis for phenols, flavonoids and antioxidant activity.

Statistical analysis

All the trials were carried out in triplicates. The data collected from each sample was computed using SPSS (Statistical Package for Social Sciences, version 23.0) for employing mean values and standard error. The significant difference between sensory attributes was computed using Kruskal Wallis test whereas comparative analyses were carried out by post hoc Maan-Whitney U-test. The significant difference between control and experimental samples was compared using t-test.

RESULTS AND DISCUSSION

Beetroot powder can be used as a natural colorant with tremendous antioxidant properties and can be used as an ingredient to meet the requirement of healthier foods. Results of FTIR analysis in previous study (Kaur *et al*., 2021) showed that the peak ranging from 1627.6 to 1632.1 cm^{-1} which was credited to the amine group affirmed the presence of nitrogenous compounds name betalains in the beetroot powder. Keeping in view the results of FTIR analysis, wave numbers (Fig. 1) of the powder developed by steam blanching with refrigeration were

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highest and was chosen to be used in the present study to supplement refined wheat flour and its usage resulted in great improvement in sensory attributes as well as nutritional composition. Song *et al*. (2016) reported that beetroot powder can be considered a good material to improve the sensory properties as well as the quality characteristics of the sponge cake. The data pertaining to the sensory attributes of doughnuts have been summarized in Table 1 and Fig. 2. Four treatments namely E1, E2, E3, E4 along with control doughnuts were subjected to sensory analysis for appearance, color, texture, taste, flavor and overall acceptability parameters. The results for sensory evaluation of doughnuts showed that E4 had the highest overall acceptability. In a study conducted by Lim (2018), overall acceptability mean score of bread developed from beetroot juice and green tea was 7.5 on 9-point hedonic scale. Interestingly, doughnuts with maximum supplementation of beetroot powder were highly acceptable in terms of appearance, color, texture, flavor and taste. No undesirable effect on any of the sensory parameter was reported. Rather, beetroot powder gave a characteristic sweet flavor to the products which was again relished by all the panelists.

The proximate composition and energy values for control and highly acceptable experimental doughnuts are presented in Table 2. The moisture content of doughnuts decreased with the supplementation of beetroot powder. There was significant ($p<0.05$) difference in the moisture content of doughnuts. This indicates that products enriched with beetroot powder would have longer shelf life than control samples. In terms of protein percentage, the values of samples supplemented with beetroot powder significantly increased ($p<0.01$). Nagib and Zidan (2019) found that the cake supplemented with 15 percent beetroot powder contained significantly higher content of protein and fibre as compared to the control cake. Protein quality as well as concentration is one of the quality determinants in bakery products, as coagulation and strengthening of gluten structure occurs during baking. The protein

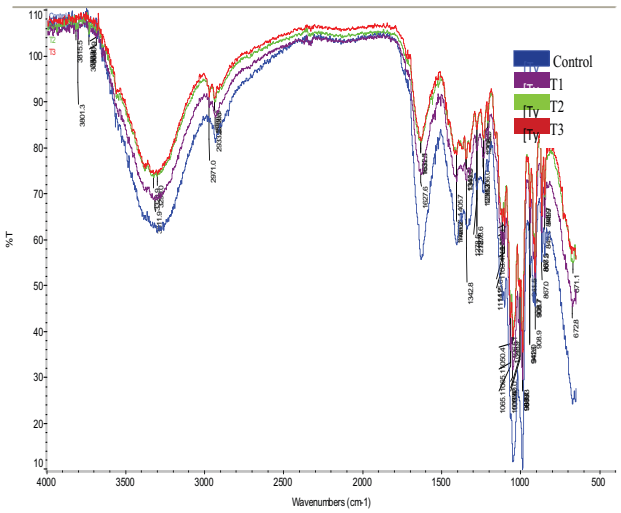


Fig. 1. FTIR Spectral cm^{-1} of beetroot powder with steam blanching treatments (Kaur *et al.*, 2021)
Control- No pre-treatment
T1- Hot water blanching with cold water immersion
T2- Steam blanching with cold water immersion
T3- Steam blanching with refrigeration

content of doughnuts increased by 2.88 percent, as compared to its respective control samples. This increase could be attributed to the replacement of protein rich beetroot powder with refined wheat flour. In addition, concentrating effect of frying can also be considered as process of dehydration (Bordin *et al*., 2013). It has also been reported that moisture content has a direct impact on the fat content when deep frying is involved. During deep frying, water content of crust will evaporate and move out of the food, enough water has to be migrated from the core of the food to the crust in order to continue the vapor flow thus leaving voids for the fat to enter. Thus, more the loss of moisture more is the fat uptake. It was found that fat content also increased with supplementation of beetroot powder but the increase in fat content was very less and was statistically non-significant for doughnuts. Minor fat increase of 0.43 percent was observed for doughnuts supplemented with beetroot powder due to increased fat content of beetroot powder as compared to the wheat

Table 1. Sensory evaluation of doughnuts prepared using beetroot powder

Product	Appearance	Color	Texture	Flavor	Taste	Overall acceptability
Control	8.30 ±0.26	8.40 ±0.22	8.60± 0.16	8.50 ±0.15	8.50 ±0.16	8.46 ±0.17
E1	8.10 ±0.23	8.00 ±0.14	8.40 ±0.16	8.30 ±0.14	8.30 ±0.15	8.22 ±0.14
E2	8.50 ±0.16	8.30 ±0.21	8.40 ±0.22	8.40 ±0.16	8.50 ±0.16	8.42 ±0.14
E3	8.60 ±0.16	8.50 ±0.16	8.60 ±0.16	8.60 ±0.16	8.60 ±0.16	8.58 ±0.12
E4	8.70 ±0.15	8.60 ±0.16	8.60 ±0.16	8.70 ±0.15	8.70 ±0,15	8.66 ±0.12

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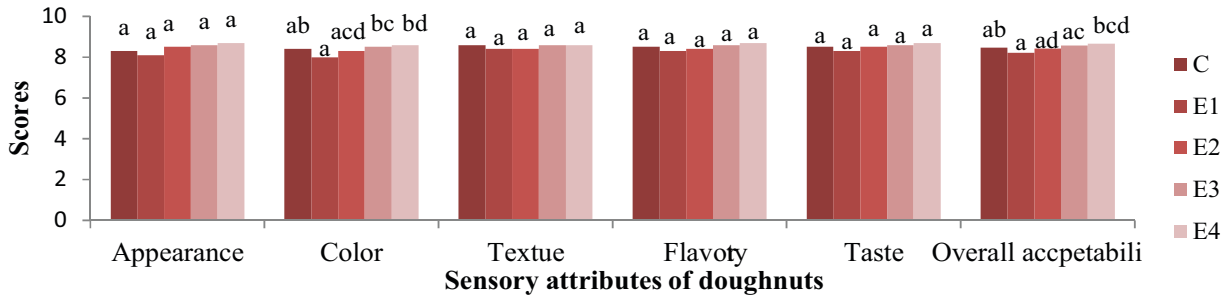


Fig. 2. Sensory attributes of functional doughnuts developed by supplementing beetroot powder

Different alphabets on bars indicate, for each product, significant difference among different supplementation levels following Maan-Whitney U-test *(p<0.05)*

Table 2. Proximate and biochemical composition of the developed products

Parameter	Control	Experimental	*t-value*
Moisture (%)	23.95±0.07	23.48±0.43	*12.90***
Crude protein (%)	12.12±0.08	12.47±0.15	*8.42***
Crude fat (%)	9.30±0.02	9.34±0.01	*1.12*^{NS}
Crude fibre (%)	0.36±0.01	0.75±0.06	*13.19***
Total ash (%)	1.72±0.01	2.46±0.02	*31.42***
Carbohydrate (g)	52.52±0.10	51.69±0.03	*11.91***
Energy (Kcal)	342.36±0.29	339.91±0.32	*5.57***
Total phenols (mg/100 g)	41.45±0.69	95.63±1.08	*42.04***
Flavonoids (mg/100 g)	7.43±0.14	31.08±0.89	*25.78***
Total Antioxidant Activity (%)	37.03±0.19	45.58±1.07	*7.82***
Betalains			
Betacyanins (mg/100g)	ND	182.79±0.62	*293.66***
Betaxanthins (mg/100g)	ND	104.56±0.66	*156.21***
Total Betalains (mg/100 g)	ND	287.36±1.29	*222.64***

**Significant at *p<0.01*
*Significant at *p<0.05*, NS: Non-significant
ND: Not detected
Experimental cake supplemented with 25 percent beetroot powder
Experimental cookies, bread and doughnuts supplemented with 10 percent beetroot powder

flour. Different types of fat can be added in the manufacturing of bakery products as they improve taste, appearance, air retention and dough plasticity (Mamat and Hill, 2014). The relative increase in fat content of beetroot powder supplemented products indicates that these would be more palatable as compared to control samples as fat improves palatability. On the other hand, supplementation of beetroot powder led to a significant increase in crude fibre where experimental doughnuts had 2-fold higher crude fibre in comparison to the control samples. Beetroot powder is a rich source of crude fibre and significantly (*p<0.01*) contributed to a steady increase with the supplementation. The ash content is an indication of minerals present in food substances. A significant increase 43.02 percent was observed in the ash percentage of the doughnuts where refined flour was replaced with beetroot powder. A possible reason for this trend is the higher content of minerals in the beetroot powder used to substitute refined wheat flour. This implies that beetroot powder supplementation could be rationally utilized as an edible ingredient where mineral and fibre content is of great importance. Similar findings were reported by Lucky *et al*. (2020), where protein, fat and total dietary fibre were found to be more in beetroot supplemented cake as compared to the plain cake when supplementation increased from 0 to 20 percent. However, such supplementation of beetroot powder in doughnuts also resulted in significantly (*p<0.01*) lower values of carbohydrates and energy.

Several studies have indicated supplementation of bakery products with different flours such as apple peel flour, grapes, and pomegranate (Kirbas

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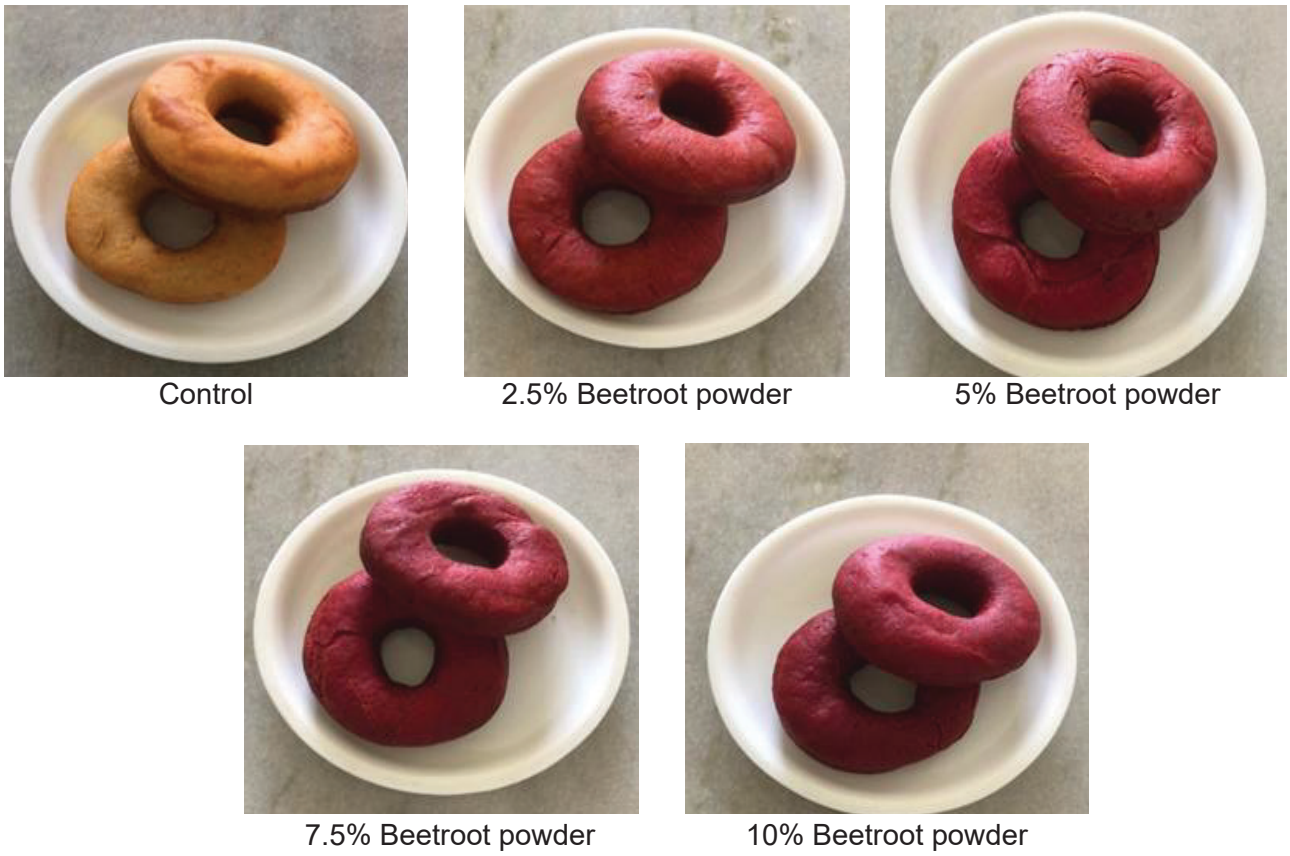


Fig. 3. Doughnuts developed by supplementing beetroot powder

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et al., 2019; Maner *et al*., 2017; Saeidi *et al*., 2018). In the present study, red beetroot powder developed by steam blanching with refrigeration prior to drying was chosen for the supplementation of refined wheat flour in doughnuts. Total phenols and flavonoids content of doughnuts was estimated and results are described as mg gallic acid and mg quercetin equivalent per 100 g fresh weight, respectively (Table 2). The phenolic content, flavonoids, antioxidant activity and betalains were positively co-related with the supplementation of beetroot powder. Our results showed that, experimental doughnuts containing beetroot powder showed significantly ($p<0.05$) higher total flavonoid, total phenolic content and antioxidant activity evaluated in comparison with the control doughnuts. In the present study, there was more than 2 times increase of total phenols in beetroot doughnuts as compared to the control sample due to high amount of phenolic compounds present in beetroot powder. Flavonoid content of doughnuts also increased by approximately 4 times as compared to the control samples (Table 2). Antioxidant activity of doughnuts was 23.08 percent higher in beetroot powder supplemented products as opposed to control products. Antioxidant activity of doughnuts could have been modified by active oxidative enzymes present in ingredients of compounds used in development of doughnuts or oxidized by ambient oxygen. In this case, enzymatic activities initiated with the addition of water, while a substantial incorporation of oxygen occurred during the initial dough mixing. It is known that antioxidant active compounds might be damaged or degraded as a consequence of the thermal process such as baking. Overall, thermal effect did not cause huge loss of antioxidant compounds. This can be explained by the fact that baking or frying results in the formation of products of maillard reaction which are known to have anti-oxidative properties and contributes to the antioxidant activity of the product (Shen *et al*., 2019). The increase in total antioxidant capacity of control products prepared from beetroot powder could also be partially explained by an increased release of bound phenolics upon baking. Wheat grain phenolic compounds are present in the bound form and exert lower antioxidant capacity in comparison with its hydrolyzed and isolated free forms and when substituted by beetroot powder resulted in higher antioxidant activity. Farhan *et al*. (2024) also stated that use of beetroot powder supplemented candies

which are rich in fibre and antioxidants are considered to be a viable in preventing the associated health challenges such as cancer and dysentery. According to recent reports in the literature, powdered functional ingredients contribute to the polyphenol content of biscuits (Shafi *et al*., 2022, Dundar, 2022, Mitrevski *et al*., 2023). Cookies made with 15% banana peel and bee pollen powder in place of wheat flour showed Total Phenolic Contents of 0.921 and 4.01 mg GAE/g, respectively. The results of our study cannot be directly related to the ones in literature because of the product difference. Nonetheless, it can be said that beetroot powder, when used as a functional ingredient in bakery items, has a much greater effect on the overall amount of polyphenols than the other functional ingredients discussed in the literature. Betalains are the main pigments responsible for the characteristic red to magenta hues seen in the doughnuts (Fig. 3). Betalains are yellow to red pigments abundantly found in beetroot having multiple benefits for human consumption, antioxidant potential being the foremost (Babarykin *et al*., 2019). They are highly sensitive, water-soluble, nitrogenous pigments which can be easily degraded by light, oxygen and high temperature. Various studies have demonstrated that mild thermal processing is beneficial for betalain preservation. This is attributed to betalain degradation by isomerisation, decarboxylation or cleavage during heat processing (Ravichandran *et al*., 2013; Raikos *et al*., 2016). Besides, mild heating conditions may also confer protection against betalain degradation by inactivating endogenous enzymes responsible for pigment degradation and color losses such as peroxidases which are abundant in beetroot. The beetroot powder used in the present study had been particularly optimized by steam blanching and drying which subsequently has resulted in the tremendous coloring properties. Betalains content in functional doughnuts (E4) was found to be 287.36 mg/100 g. Mitrevski et al. (2023) also studied that betalain, total phenols, flavoinoids and antioxidant activity increased in biscuits when spelt flour was replaced with beetroot powder. There are many different kinds of colorants in the market today that are used to color food products. However, as a result of growing consumer knowledge that synthetic coloring agents can have serious negative health effects, natural coloring is becoming more and more significant and popular. Hence, the worldwide demand for natural colorants is increasing due to increased awareness on their therapeutic properties, being also considered a matter of high relevance to the worldwide economy, society and public health (Vinha *et al*., 2018). Therefore, the consumers value naturally colored food as a boon for their health. Although, carotenoids have also been recognized for their antioxidant and coloring abilities but their use is limited due to their insolubility in water and high susceptibility to oxidation (Oliveira, 2019). In recent studies (Aykln-Dinçer *et al*., 2021; Costa *et al*., 2017, Mereddy *et al*., 2017), it has been seen that the use of beetroot powder and juices offers a great way to extend the shelf life of meat and meat products while reducing the need for (and possibly replacing) synthetic additives because of the high content of betalains (strong, deep purplish-red pigments with antioxidant activity), phenolic compounds (antioxidants), and inorganic nitrate.

Color values of functional bakery products

In bakery products, L* (luminosity) coordinate plays a significant role as it signifies the lightness of the product. The crumb of a product will be similar to the color of the ingredients used as it does not reach as high temperature as crust (Rodriguez- Sandoval *et al*., 2017). Higher the L* value, lesser is the darkness. L* coordinate of doughnuts was found to be 36.14 (36.10) whereas a* coordinate indicates redness of a product which was found to be 9.89 for doughnuts. The a* coordinate increased with the addition of beetroot powder, denoting a change from greenish to magenta hues. The addition of beetroot powder had a darkening effect on the doughnuts as they contain compounds darker than refined wheat flour. Negative b* coordinate corresponds to the blueness of the sample which was found to be -12.65 for doughnuts.

Effect of storage on bioactive compounds of functional doughnuts

Total phenols content of doughnuts decreased from 95.63 to 82.17 mg GAE/100 g. Antioxidant activity of doughnuts decreased from 45.58 to 40.36 percent. Phenol and antioxidant content of doughnuts supplemented with stored beetroot powder was observed to have decreased by 14.07 and 11.45 percent, respectively. Flavonoids content of doughnuts decreased from 31.08 to 24.24 mg QE/100 g. Flavonoid content of doughnuts was found to be decreased by 22 percent. It was also observed that storage resulted in loss of phenols, flavonoids and antioxidant activity. A decrease in these compounds during storage may result in loss of protective health benefits, making the product

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less effective for consumers. Degradation of these compounds also resulted in slight deterioration of the freshness and quality of the finished products. These results affirm that beetroot powder can be stored for a period of three months without much deterioration and degradation of its bioactive compounds and can still be good enough to be used in preparation of products.

CONCLUSION

The present study focused on the potential use of beetroot powder developed by using steam blanching with refrigeration as it contained maximum functional groups upon FTIR analysis. Formulation and sensory analysis of the functional doughnuts prepared by using beetroot powder revealed that supplementation even at the highest levels i.e. 10 percent was highly acceptable. The addition of beetroot powder in bakery products formulation seems to be well suited for coloring the products as well as for enhancing their biochemical and antioxidant potential. The doughnuts developed from the stored beetroot powder were still good source of bioactive compounds and had good antioxidant properties. Beetroot powder can be used to replace the carcinogenic synthetic colorants which are being used by the catering establishments, food-industries as well as bakeries. However, preparation and addition of beetroot powder may increase cost of production of the product which could limit its commercial viability, especially if the potential benefits are not demonstrated. Moreover, betalain stability is of great concern as it can only be used as a colorant in low-acid foods and can degrade very easily on high temperatures. In context of the present study, beetroot powder supplemented doughnuts were found to be highly acceptable and resulted in positive implications for both the formulation and consumer response with additional health benefit. A future study could assess how supplementation of beetroot powder based doughnuts can affect the overall nutrient content and the bioavailability of the nutrients in human health.

Authors' contribution

Conceptualization of research work and designing of experiments (NK, KG); Execution of experiments and data collection (SK, PA, NK); Data analysis and interpretation of results (ISG, SK, NK); Preparation of manuscript (SK, NK).

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