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Research article

Optimization of pasteurized milk with soymilk powder and mulberry leaf tea based on melatonin, bioactive compounds and antioxidant activity using response surface methodology

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ABSTRACT

Melatonin is a natural hormone which regulates human circadian rhythms and is presented in milk at low concentrations. To improve melatonin concentration and amounts of bioactive compounds in pasteurized milk (PM), soymilk powder (SMP) and mulberry leaf tea (MLT) were added using mixture design response surface methodology (RSM) and levels of SMP, MLT and raw milk (RM) were optimized. PM containing 4.50% SMP, 4.50% MLT and 88.80% RM gave the highest chemical compositions, bioactive compounds and antioxidant activity. Mathematical models of chemical compositions, bioactive compounds and antioxidant activity showed significant differences, whereas sensory attributes were not significantly different in all modeled parameters. Optimum levels were 3.90% SMP, 4.50% MLT and 89.40% RM. Verification of optimum proportions showed that experimental values of chemical compositions, bioactive compounds and antioxidant activity agreed with model predictions. Optimum PM contained melatonin (1.49 ng/mL), free tryptophan (0.26 μg/mL), and total phenolic content (0.72 mg GAE/mL) with high antioxidant activity when assayed by DPPH, ABTS and FRAP. Results suggested that mixture design RSM has the potential to optimize SMP, MLT and RM levels to obtain PM with increased amounts of bioactive compounds and high melatonin content.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone synthesized from the amino acid tryptophan via the 5-hydroxytryptophan and serotonin pathway found in the pineal gland of animals and plant tissues (Hattori et al., 1995; Lerner et al., 1958). Melatonin treats sleep disorders by alleviating insomnia for shift workers and ameliorating jet lag by controlling the human biological clock (circadian rhythms) (Arnao and Hernández-Ruiz, 2006; Howatson et al., 2012; Tan et al., 2002). Melatonin also displays antioxidant properties; it scavenges broad reactive oxygen species and it also increases the activity of antioxidative enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (Aguilera et al., 2015; Rodriguez et al., 2004).

High values of melatonin have been determined in several grains and plants (Hattori et al., 1995; Manchester et al., 2000). Soybeans are edible seeds and classified as legumes. They are processed into soymilk which is widely consumed in Asian countries. Soybeans have a unique smell, texture and taste, and also contain high protein, oil and bioactive compounds such as isoflavones, phenolics and various amino acids (including tryptophan, a precursor of melatonin synthesis) (Murch and Saxena, 2002; Tyug et al., 2010). Consumption of tryptophan-enriched cereal has been proven to improve sleep quality of the elderly while also increasing urinary antioxidant capacity (Bravo et al., 2013). Melatonin is present in plants, especially mulberry leaves (*Morus* spp.). Historically, these were used for animal feed but they are now utilized to brew mulberry leaf tea for human consumption. Mulberry leaves are a good source of bioactive compounds, with melatonin detected at concentrations ranging from 40.70 to 279.60 ng/g dw (Pothinuch and Tongchitpakdee, 2011).

Here, soymilk powder (SMP) and mulberry leaf tea (MLT) were prepared and added to raw milk (RM) to improve melatonin content, bioactive

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compounds and antioxidant activity in functional pasteurized milk (PM). Adding high levels of SMP and MLT improves bioactive compound levels and also impacts on physical and chemical properties of PM with high total solids and changes in color, appearance, texture and flavor which affect consumer acceptability. To achieve PM containing high melatonin and bioactive compounds with accepted quality, an experimental mixture design was used to model the response surface and optimize levels of SMP, MLT and RM. A mixture design is a type of response surface methodology (RSM) which is widely used in experiments where factors representing components of a mixture, and hence not independent of each other, are set to vary orthogonally, similarly to factorial designs. Response functions of the mixture as chemical compositions, bioactive compounds, antioxidant activity and sensory evaluation were estimated. Proportions were optimized based on response value criteria, and results were compared with predicted values via confirmatory trials.

2. Materials and methods

2.1. Chemicals and materials

Melatonin and tryptophan standard, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), and Folin-Ciocalteu phenol reagent (FCR) were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Methanol, at HPLC grade, was purchased from BDH (Poole, UK). Trolox standard (TE) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Fluka Chemicals (Buchs, Switzerland). Deionized water was obtained from Milli-Q Systems (Millipore Co., Bedford, MA, USA).

Good quality raw milk was collected from milk storage tanks of Kokko Milk Factory, Maha Sarakham Province, after raw milk delivery between 07.30 and 09.00 a.m. supplied by local dairy farms in the Maha Sarakham area. Homogenized raw milk in storage tanks was sampled to determine its characteristics including chemical composition and quality as contained fat (3.75%), protein (2.85%), solid not fat (SNF) (8.48%), total solids (TS) (12.23%) and standard plate count less than 1×10^5 cfu/mL. Soybeans (*Glycine max* (L.) Merrill) were purchased from a supermarket. Mulberry leaves (*Morus* spp.), Buriram 60 cultivar were collected from a local farm in Maha Sarakham Province, Thailand.

2.2. Material preparation

The collected raw milk was stored at 5 ± 2 °C before using to produce pasteurized milk following the formulas in Table 1. Mulberry leaves were prepared as mulberry leaf green tea before supplementing in milk. The leaves were cleaned, chopped (1.5×1.5 cm^2) and roasted at 50 ± 2 °C for 30 min. Rolled leaves were dried in the sun to final moisture content of approximately 10%. Soybeans were prepared for soymilk powder according to the procedure of Jiang et al. (2013) with slight modifications. The soybeans were soaked in water at a ratio of 1:3 w/v for 8 h at room temperature and then rinsed. Soaked soybeans were blended in water at 1:1 w/v for 1 min using a household blender (QH-900B1, Joyoung Co., Ltd., Hangzhou, China), and then filtered through two layers of cheesecloth. The residue was reblended at the same ratio. Maltodextrin (DE10) (1% w/v) was added to the soymilk and dried using a freeze dryer (Heto Power Dry PL3000, Czech Republic). Dried samples were stored at -20 °C prior to analysis.

2.3. PM formulation

To produce PM with high melatonin concentration and bioactive compounds, SMP and MLT were added to RM in different quantities. Our primary study determined that consumers accepted PM treatment when mixed at 4.00% SMP, 4.00% MLT and 89.80% RM. Here, response surface methodology was adopted utilizing user-defined mixture design. Optimization of the proportions was based on response values regarding chemical compositions, bioactive compounds, antioxidant activity and sensory evaluation. Quantities of the three components ranged as X_1 = soymilk powder (SMP) (3.50–4.50%), X_2 = mulberry leaf tea (MLT) (3.50–4.50%) and X_3 = raw milk (RM) (88.80–90.80%). Estimated components of $X_1 + X_2 + X_3 = 97.80\%$ with fixed basic formulations of sugar (2.00%) and food grade color (0.20%). PM formulation consisting of two center points is shown in Table 1.

Table 1. Treatments of the three components using mixture design.

Treatment	Formulation (%)			Sugar	Color
	SMP (X_1)	MLT (X_2)	RM (X_3)		
1	4.50	4.00	89.30	2.00	0.20
2	4.50	3.50	89.80	2.00	0.20
3	4.50	4.50	88.80	2.00	0.20
4	4.00	4.50	89.30	2.00	0.20
5	4.00	4.00	89.80	2.00	0.20
6	4.00	4.00	89.80	2.00	0.20
7	4.00	4.00	89.80	2.00	0.20
8	4.00	3.50	90.30	2.00	0.20
9	3.50	4.50	89.80	2.00	0.20
10	3.50	3.50	90.80	2.00	0.20
11	3.50	4.00	90.30	2.00	0.20

X_1 = SMP = soymilk powder, X_2 = MLT = mulberry leaf tea, X_3 = RM = raw milk.

An equation for the predictive model in linear, quadratic and cubic terms is presented as:

$$Y = \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3$$

where β_1, β_2, and β_3 represent constant coefficients of the linear model. Constant coefficients for the nonlinear model show as interaction of β_{12}, β_{13}, and β_{23} which represent the quadratic model, while β_{123} is a cubic model. Y is the predicted response function. X_1, X_2 and X_3 are the proportions of SMP, MLT and RM, respectively.

2.4. Preparation of PM mixed with SMP and MLT

MLT was brewed in one-quarter of the RM at 73 ± 2 °C for 30 min. The leaves were then removed by filtering through cheesecloth and the remaining three-quarters of RM, SMP and the other ingredients were added. The mixture was pasteurized at 65 ± 2 °C for 30 min. PM samples were quickly cooled, filled in sterilized bottles using the aseptic technique and stored at 5 ± 2 °C.

2.5. Chemical compositions and PM quality determination

Chemical compositions comprising protein, fat, solid not fat (SNF) and lactose were determined using an ultrasonic milk analyzer (Milkotronic, Stara Zagora, Bulgaria). Total solids (TS) content was calculated from fat and SNF. Color parameters L*, a* and b* were measured using a Minolta Chroma Meter CR-300 (Konica Minolta, Japan) and pH was determined using a pH meter (Mettler Toledo, USA).

2.6. Total plate count determination

Total microbial count was performed following the method of Saxena and Rai (2013). The microbial colony was counted and expressed in colony forming units per milliliter (cfu/mL).

2.7. Analysis of melatonin, free tryptophan, total phenolic content (TPC) and antioxidant activity

2.7.1. PM extraction

PM samples were extracted following the method of Alyaqoubi et al. (2014) with minor modifications. Extraction solvent was prepared as 1 N HCl in 95% methanol in the ratio 15:85 v/v. PM samples were extracted

with the solvent (1:4 v/v) and shaking incubated at 150 rpm, 15 °C for 4 h using a shaking incubator (Daihan Labtech, Model LSI-1005R, Korea). Supernatants were separated to analyze melatonin, free tryptophan, TPC and antioxidant activity.

2.7.2. Melatonin and free tryptophan content analyses

Extracted samples were purified to assess melatonin and free tryptophan contents using C18 solid phase extraction (SPE) cartridges (Waters, Milford, MA, USA) according to the method of Pothinuch and Tongchitpakdee (2011) and Cao, Murch, O'Brien, and Saxena (2006) with some modifications. Cartridges were activated by adding 10 mL of methanol and then 10 mL of deionized water, followed by the extracted sample. The cartridges were washed with 10 mL of 5% methanol, and 80% methanol was used to elute the retained compound in the cartridge. Extracted samples were filtrated through a nylon syringe filter (0.2 μm) (Whatman, USA) and then analyzed using a Shimadzu 20ADS liquid chromatograph; SIL-20AC HT autosampler; CTO-20AC column oven coupled with an LC-MS 8030 (Shimadzu, Japan). Chromatographic separation of each sample was performed using an InertSustain® C18 column (2.1 × 150 mm i.d., 3 μm) (GL Sciences Inc., Japan) with the mobile phase consisting of 0.45% formic acid (A) and acetonitrile (B). Elution gradient was controlled as mobile phase B (0.0 min, 20%) (5.00 min, 50%) (6.00 min, 100%) (9.00 min, 20%) and (10.00 min, 0%) with flow rate set at 0.25 mL/min, column temperature 30 °C and injected volume 2 μL. The MS/MS system consisted of a triple quadrupole mass spectrometer with an electrospray ionization (ESI) setting. Nitrogen (N) was used as the drying gas at a flow rate of 15 L/min and nebulizing gas at 3 L/min, interface temperature 350 °C, desolvation line (DL) temperature 250 °C and temperature of the heat block 400 °C. Melatonin and free tryptophan were identified using multiple reaction monitoring (MRM) with positive ion mode. Qualitative identification of precursor ions was achieved by scanning through argon gas, and product ions were created by collision-induced dissociation (CID). Transitions for melatonin were determined at m/z 233.2→174.2, Q1 pre-bias at -20.0, collision energy (CE) at -16.0 and Q3 pre-bias at -19.0. Free tryptophan was scanned at m/z 205.00→188.00, Q1 pre-bias at -30.0, CE at -12.0 and Q3 pre-bias at -22.0 with collision-induced dissociation gas at 230 kPa and interface voltage at 4.5 kV.

2.7.3. TPC analysis

TPC was determined following the method described by Singleton et al. (1999). The extracted (0.2 mL), 0.8 mL of Folin-Ciocalteu reagent in water ratio (1:10) and 1 mL of sodium carbonate (7.5% w/v) were added and mixed using a vortex mixer (Harmony, Model VTX-3000L, Japan), then left at room temperature for 2 h. The reaction was measured at 750 nm using a Libra S12 UV-vis spectrophotometer (Biochrom, Cambridge, UK) with results calculated as mg gallic acid (GAE)/mL.

2.7.4. Antioxidant activity

2.7.4.1. Free radical scavenging activity (DPPH). DPPH assay was determined following the method of Brand-Williams et al. (1995). The extracted (50 μL) and 1.95 mL of 1,1-diphenyl-2-picrylhydrazyl solution (6×10^{-5} mol/L) were mixed, left for 30 min and then measured at 517 nm. Results were expressed as mg Trolox (TE)/mL.

Table 2. Chemical compositions and quality of PM treatments.

Treatment	Formula (%)			Composition (%)					pH	Colony	Color values		
	SMP	MLT	RM	Fat	SNF	Lactose	Protein	TS		(cfu/mL)	L*	a*	b*
1	4.50	4.00	89.30	4.32 ± 0.04	10.40 ± 0.08	5.68 ± 0.08	4.88 ± 0.05	14.72 ± 0.06	6.65 ± 0.03	4.00 × 10	63.68 ± 0.16	-11.04 ± 0.08	20.66 ± 0.10
2	4.50	3.50	89.80	4.31 ± 0.02	10.39 ± 0.09	5.66 ± 0.05	4.90 ± 0.08	14.70 ± 0.10	6.67 ± 0.02	4.33 × 10	64.07 ± 0.37	-11.09 ± 0.24	20.85 ± 0.12
3	4.50	4.50	88.80	4.29 ± 0.07	10.42 ± 0.09	5.69 ± 0.02	4.91 ± 0.08	14.71 ± 0.12	6.50 ± 0.03	6.67 × 10	63.70 ± 0.21	-9.88 ± 0.26	20.12 ± 0.15
4	4.00	4.50	89.30	4.26 ± 0.06	10.26 ± 0.08	5.65 ± 0.04	4.80 ± 0.05	14.52 ± 0.14	6.65 ± 0.01	6.00 × 10	65.94 ± 0.68	-9.01 ± 0.30	18.41 ± 0.60
5	4.00	4.00	89.80	4.23 ± 0.06	10.25 ± 0.07	5.61 ± 0.11	4.78 ± 0.09	14.48 ± 0.11	6.51 ± 0.01	4.67 × 10	65.55 ± 0.35	-10.92 ± 0.17	19.81 ± 0.28
6	4.00	4.00	89.80	4.22 ± 0.02	10.23 ± 0.06	5.64 ± 0.04	4.77 ± 0.08	14.45 ± 0.07	6.54 ± 0.01	3.00 × 10	65.87 ± 0.19	-10.29 ± 0.08	19.77 ± 0.14
7	4.00	4.00	89.80	4.23 ± 0.08	10.22 ± 0.11	5.62 ± 0.05	4.75 ± 0.09	14.45 ± 0.11	6.57 ± 0.02	3.00 × 10	66.77 ± 0.42	-10.62 ± 0.10	19.18 ± 0.57
8	4.00	3.50	90.30	4.22 ± 0.08	10.11 ± 0.10	5.65 ± 0.08	4.74 ± 0.09	14.33 ± 0.03	6.53 ± 0.02	5.67 × 10	65.49 ± 0.12	-10.00 ± 0.07	18.99 ± 0.33
9	3.50	4.50	89.80	4.20 ± 0.05	10.12 ± 0.06	5.68 ± 0.06	4.67 ± 0.03	14.32 ± 0.09	6.57 ± 0.02	3.33 × 10	66.52 ± 0.05	-11.24 ± 0.11	20.40 ± 0.08
10	3.50	3.50	90.80	4.18 ± 0.03	10.07 ± 0.09	5.67 ± 0.07	4.65 ± 0.04	14.25 ± 0.12	6.64 ± 0.02	1.67 × 10	67.43 ± 0.03	-11.56 ± 0.60	20.00 ± 0.41
11	3.50	4.00	90.30	4.20 ± 0.05	10.10 ± 0.08	5.66 ± 0.06	4.67 ± 0.06	14.30 ± 0.12	6.67 ± 0.02	5.33 × 10	66.84 ± 0.69	-11.64 ± 0.34	19.03 ± 0.34

SMP = soymilk powder, MLT = mulberry leaf tea, RM = raw milk, SNF = solid not fat and TS = total solids. L* = light (+) and black (-), a* = red (+) and green (-), b* = yellow (+) and blue (-).

Table 3. Melatonin, free tryptophan, TPC and antioxidant activity of PM treatments.

Treatment	Formulation (%)			Melatonin	Free tryptophan	TPC	Antioxidant activity (mg TE/mL)		
	SMP	MLT	RM	ng/mL	μg/mL	mg GAE/mL	DPPH	ABTS	FRAP
1	4.50	4.00	89.30	1.61 ± 0.15	0.30 ± 0.02	0.68 ± 0.02	0.52 ± 0.01	0.80 ± 0.01	0.51 ± 0.02
2	4.50	3.50	89.80	1.70 ± 0.06	0.29 ± 0.02	0.68 ± 0.03	0.51 ± 0.01	0.72 ± 0.01	0.49 ± 0.02
3	4.50	4.50	88.80	1.93 ± 0.01	0.27 ± 0.01	0.73 ± 0.03	0.54 ± 0.01	0.82 ± 0.04	0.54 ± 0.03
4	4.00	4.50	89.30	1.61 ± 0.08	0.26 ± 0.01	0.72 ± 0.04	0.53 ± 0.01	0.78 ± 0.03	0.52 ± 0.02
5	4.00	4.00	89.80	1.32 ± 0.12	0.28 ± 0.01	0.71 ± 0.03	0.51 ± 0.04	0.72 ± 0.02	0.50 ± 0.01
6	4.00	4.00	89.80	1.38 ± 0.05	0.29 ± 0.01	0.71 ± 0.04	0.52 ± 0.01	0.73 ± 0.01	0.49 ± 0.01
7	4.00	4.00	89.80	1.37 ± 0.11	0.30 ± 0.01	0.70 ± 0.03	0.51 ± 0.01	0.74 ± 0.02	0.49 ± 0.02
8	4.00	3.50	90.30	1.16 ± 0.09	0.26 ± 0.01	0.68 ± 0.02	0.49 ± 0.01	0.67 ± 0.03	0.44 ± 0.01
9	3.50	4.50	89.80	1.18 ± 0.04	0.24 ± 0.01	0.72 ± 0.01	0.50 ± 0.01	0.76 ± 0.01	0.51 ± 0.01
10	3.50	3.50	90.80	1.03 ± 0.03	0.20 ± 0.01	0.67 ± 0.03	0.47 ± 0.01	0.63 ± 0.04	0.42 ± 0.02
11	3.50	4.00	90.30	1.20 ± 0.09	0.26 ± 0.01	0.70 ± 0.02	0.49 ± 0.02	0.69 ± 0.03	0.47 ± 0.02

SMP = soymilk powder, MLT = mulberry leaf tea and RM = raw milk.

2.7.4.2. Ferric reducing antioxidant power assay (FRAP). FRAP assay was evaluated following the method of Benzie and Strain (1996). FRAP reagent was prepared by mixing acetate buffer 0.3 M (pH 3.6) (A), 10 mM of tripyridyl-s-triazine (TPTZ) in 40 mM of HCl (B), and 20 mM of $FeCl_3.6H_2O$ (C) in the ratio A:B:C at 10:1:1 v/v/v and incubated at 37 °C for 30 min. The extracted (0.3 mL) and 1.7 mL of FRAP reagent were added, mixed and left for 60 min. The reaction was measured at 593 nm and results were reported as mg TE/mL.

2.7.4.3. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay (ABTS). ABTS assay followed the procedure described by Re et al. (1999). ABTS radical cation reagent was prepared by mixing 7.0 mM ABTS and 2.45 mM $K_2S_2O_8$ in the ratio 1:1 v/v and kept in the dark to avoid the light for 16 h. Then, ABTS reagent was diluted with methanol and measured at 734 nm (absorbance of 0.700 ± 0.005). The extracted (50 μL) and 1 mL of ABTS reagent were added, mixed and left for 6 min. The reaction was measured at 734 nm and results were calculated as mg TE/mL.

2.8. Sensory evaluation

The sensory study was approved by the Ethics Committee for Research Involving Human Subjects, Mahasarakham University (approval number 048/2016). Thirty untrained panelists comprising students of the Faculty of Technology, Mahasarakham University, Thailand agreed with the rules

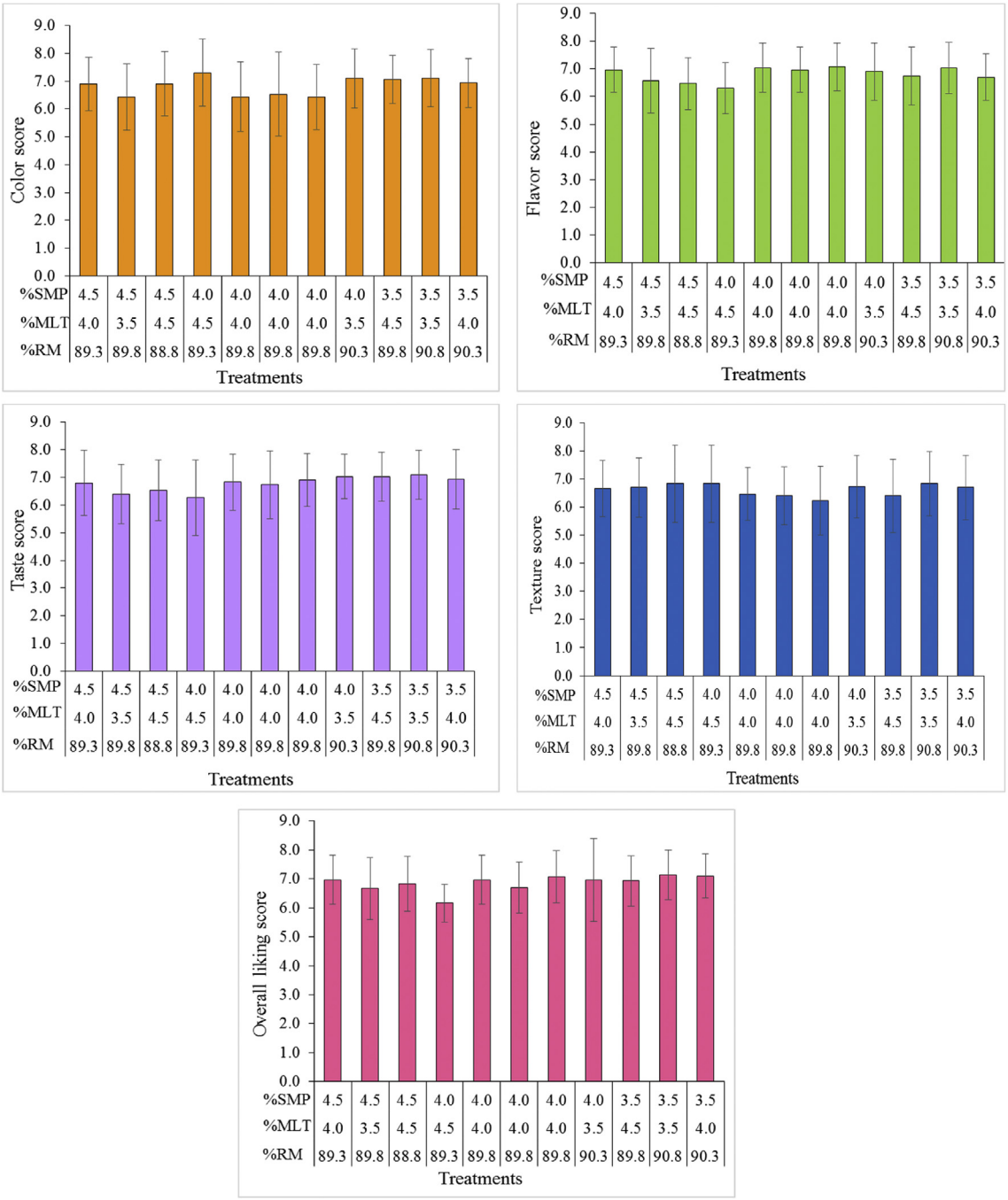

Figure 1. Sensory evaluation of PM treatments using mixture design. SMP refers to soymilk powder, MLT refers to mulberry leaf tea and RM refers to raw milk. Numbers 1 to 9 represent values as 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely.

of the study and participated in the experiment. All regularly consumed milk and had no allergies to PM products. Sensory attributes were evaluated as color, flavor, taste, texture and overall liking using a 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely) (Marsanasco et al., 2015).

2.9. Statistical analysis

The mathematical model was confirmed using analysis of variance (ANOVA) with statistical significance considered at $p < 0.05$. The regression coefficient (R^2) was accepted at higher than 0.80, and there was no significant lack-of-fit ($p > 0.05$). To confirm model adequacy, optimum proportions were verified for five replicates. Mixture design was analyzed using Design-Expert version 7.1.5 (trial program).

3. Results

3.1. Chemical compositions and quality of PM

Chemical compositions and quality of PM treatments are shown in Table 2. Highest fat, SNF, lactose, protein and TS were found in treatments consisting of highest SMP (4.50%), MLT (3.50–4.50%) and RM (88.80–89.80%). Low values were mainly found in PM containing low amounts of SMP (3.50%), MLT (3.50–4.50%) and RM (89.80–90.80%). Results indicated that increase in chemical compositions occurred by increasing the amount of SMP. This can be explained because soybeans are rich in protein, lipid and carbohydrate. After SMP was added, chemical concentrations of protein, fat and SNF distributed in milk increased. These results were supported by Kpodo et al. (2013) who reported that a combination of soy-peanut-cow milk increased protein and total solids content. The pH values of all PM treatments varied from 6.50 to 6.67, with total microbial count less than 1×10^2 cfu/mL. Color showed increasing lightness (L*) with least amount of SMP and MLT added. Greenness (a*) varied due to chlorophyll content released by MLT during the brewing process, whereas yellowness (b*) increased with increasing SMP. Saetan, Usawakesmanee, and Siripongvutikorn (2016) reported that *Cinnamomum porrectum* herbal tea infused in hot water influenced total chlorophyll and carotenoid content leached, while leaf extracts supplemented in yogurt decreased lightness but increased greenness (Shokery et al., 2017). Polyphenol added to milk increased greenness, while lightness decreased and yellowness remained constant; pH values of milk with and without polyphenol added were recorded at 6.56 and 6.73, respectively (Wegrzyn et al., 2008).

3.2. Melatonin, free tryptophan and TPC

Results for melatonin, free tryptophan and TPC of all PM treatments are shown in Table 3. Highest melatonin (1.93 ng/mL) and TPC (0.73 mg GAE/mL) were found in PM comprising highest amounts of SMP (4.50%) and MLT (4.50%), while lowest melatonin concentration (1.03 ng/mL) and TPC (0.67 mg GAE/mL) were found in the treatment consisting of 3.50% SMP and 3.50% MLT. Free tryptophan contents at 4.00–4.50% SMP (0.26–0.30 μg/mL) were higher than treatments consisting of 3.50% SMP (0.20–0.26 μg/mL). Our results showed that increasing amounts of mixture components, especially SMP and MLT, increased concentrations of these compounds whereas melatonin content in natural RM, in general, was only 14.45 pg/mL (Karunanithi et al., 2014). All components contained abundant bioactive compounds as melatonin, free tryptophan and phenolic content; therefore, high concentrations of these compounds were easily distributed and aggregated in both water and oil phases of milk. Our results concurred with Gad and El-Salam (2010) who reported that higher amounts of rosemary and green tea extracts in skim milk increased total phenolic content. Adding green tea or *Moringa oleifera* leaf extracts in yogurt increased total phenolic content compared with plain yogurt (Shokery et al., 2017).

3.3. Antioxidant activity

Antioxidant activity using DPPH, FRAP and ABTS assays are presented in Table 3 and showed a similar trend to bioactive compounds. PM at 4.50% SMP and MLT gave highest radical scavenging efficiency, whereas lowest radical scavenging was found in the treatment added with 3.50% of each component. Gad and El-Salam (2010) reported that antioxidant capacity of DPPH and FRAP increased with increasing levels of rosemary and green tea extracts in skim milk, with pasteurization at 65 °C for 15 min. Results trended similarly for phenol content. Adding

Table 4. Predicted models for composition and quality, bioactive components and antioxidant activity of PM treatments.

Response	Prediction equation	R^2	*p*-value	Lack-of-fit
Composition (%) and quality				
Protein	$0.27X_1+0.07X_2+0.04X_3$	0.9784	<0.0001	0.5886
Fat	$0.15X_1+0.05X_2+0.04X_3$	0.8848	0.0002	0.1136
Solid not fat	$0.40X_1+0.16X_2+0.09X_3$	0.9301	<0.0001	0.1200
Total solids	$0.55X_1+0.21X_2+0.13X_3$	0.9370	<0.0001	0.0840
Lactose	$13.92X_1+5.54X_2+0.10X_3-0.20X_1X_2-0.15X_1X_3$ $-0.06X_2X_3$	0.8485	0.0409	0.6696
pH	$-6.91X_1-23.76X_2 -0.03X_3+0.13X_1X_2+0.09X_1X_3+0.27X_2X_3$	0.8154	0.0644	0.1167
Total plate count	$14.77X_1+13.66X_2-0.78X_3$	0.2953	0.2466	0.3064
L*	$-2.30X_1+0.53X_2+0.81X_3$	0.8650	0.0003	0.7299
a*	$-398.95X_1+260.22X_2-0.27X_3+2.37X_1X_2+4.41X_1X_3$ $-2.93X_2X_3$	0.9116	0.0114	0.4823
b*	$+351.44X_1-61.48X_2+0.50X_3-4.24X_1X_2-3.85X_1X_3 +0.74X_2X_3$	0.6117	0.3151	0.2118
Bioactive component				
Melatonin (ng/mL)	$0.59X_1+0.26X_2-0.02X_3$	0.9046	<0.0001	0.0834
Free tryptophan (μg/mL)	$-4.87X_1-9.60X_2-0.03X_3+0.12X_1X_2+0.06X_1X_3+0.11X_2X_3$	0.9392	0.0046	0.7292
TPC (mg GAE/mL)	$8.64E\text{-}003X_1+0.05X_2+5.06E\text{-}003X_3$	0.8331	0.0008	0.2385
Antioxidant activity (mg TE/mL)				
DPPH	$0.04X_1+0.03X_2+2.44E\text{-}003X_3$	0.9255	<0.0001	0.5860
ABTS	$0.09X_1+0.11X_2-8.51E\text{-}004X_3$	0.9628	<0.0001	0.2099
FRAP	$0.05X_1+0.07X_2+1.89E\text{-}0.04X_3$	0.9423	<0.0001	0.3961

X_1 = SMP = soymilk powder, X_2 = MLT = mulberry leaf tea and X_3 = RM = raw milk.

higher amounts of SMP and MLT increased antioxidant activity because of the variety of bioactive compounds contained in these ingredients that can be distributed in milk. SMP showed high radical scavenging using Trolox equivalent antioxidant capacity (TEAC), beta-carotene bleaching (BCB) assay and ferric reducing antioxidant power (FRAP) (Tyug et al., 2010). Melatonin and its derivatives displayed potential effective antioxidant activity against radical mechanisms including transfer of both electron and hydrogen atoms (Rodriguez-Naranjo et al., 2012). Sawale, Singh, and Arora (2015) reported that antioxidant capacity increased in milk added with *Pueraria tuberosa* extract. Melatonin, phenolic compounds and free tryptophan present in milk also act as antioxidants. These compounds are considered to have amphiphilic properties as their structures dissolve easily in both nonpolar and polar phases. Therefore, these bioactive substances effectively inhibited lipid peroxidation and peroxyl/superoxide which cause cell damage and promote disease (Chen et al., 2003).

3.4. Sensory evaluation

Sensory evaluation of color, flavor, taste, texture and overall liking attributes was determined using a 9-point hedonic scale, with results presented in Figure 1. All attributes showed no significant differences for all PM treatments, and scores among each attribute were also not significantly different. Highest overall liking score was found in PM comprising 3.50% SMP, 3.50% MLT and 90.80% RM. Soybeans have a unique smell, flavor and color; they contain isoflavonoids which affect the appearance and flavor of PM. Therefore, adding high levels of SMP decreased sensory satisfaction of the panelists. Ismail, Ghoneem, EL-Boraey, Tabekha, and Elashrey (2017) reported that panelists recorded lowest scores of color, appearance, smell, taste, mouthfeel and overall acceptability for 100% soymilk yogurt because high levels of soy-yogurt gave a yellowish color and beany flavor compared with yogurt made from cow or buffalo milk mixed with 25% soymilk. Adding 0.2% of

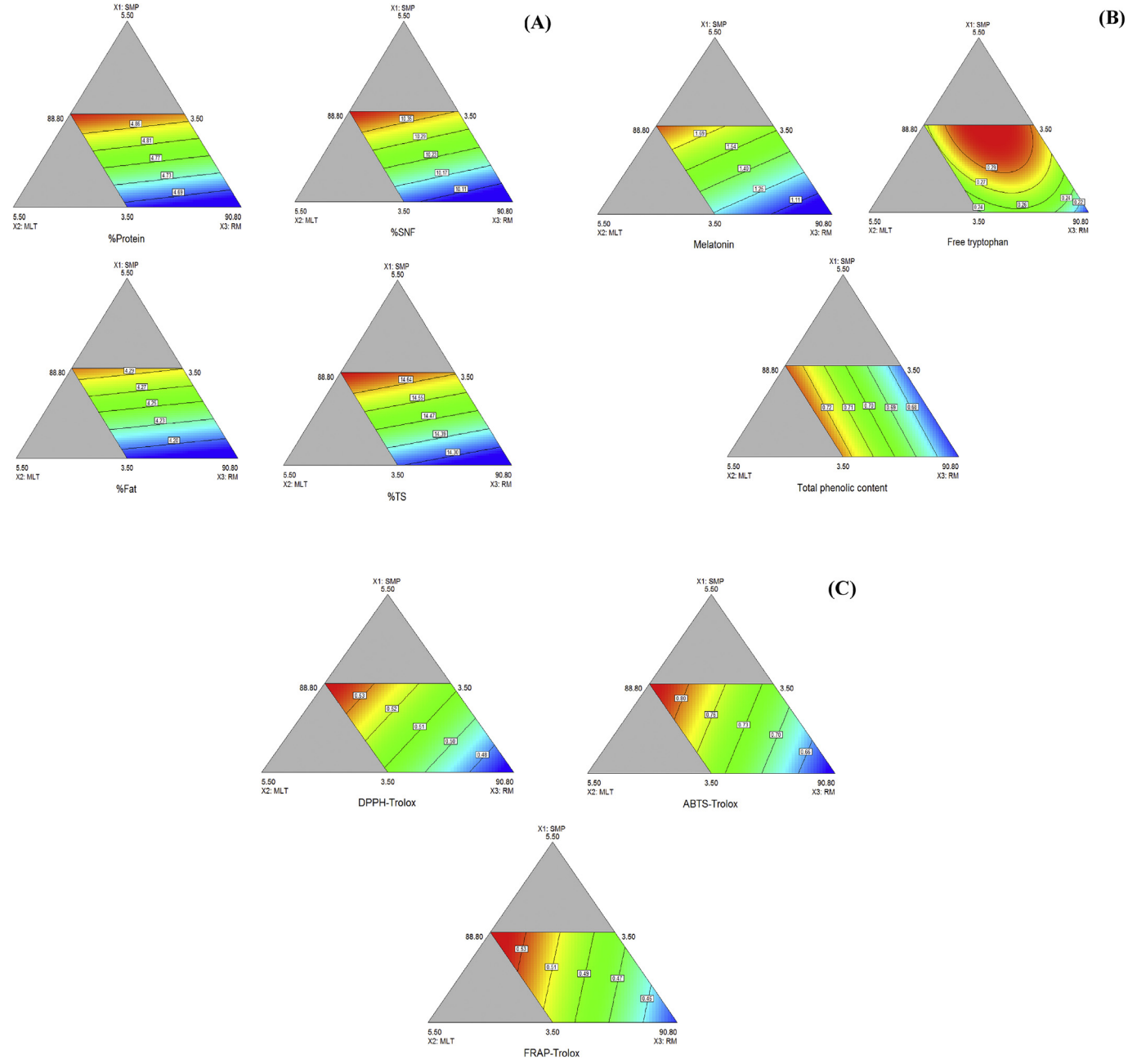

Figure 2. Contour plots for component composition interaction (A), melatonin, free tryptophan and TPC (B), and antioxidant activity using DPPH, ABTS and FRAP assays (C) of PM treatments.

Table 5. Predicted models for sensory assessment of PM treatments.

Response	Prediction equation	R^2	*p*-value	Lack of fit
Color	$13.23X_1+115.50X_2+0.39X_3-0.97X_1X_2-0.17X_1X_3-1.30X_2X_3$	0.5663	0.3884	0.0229
Flavor	$-14.27X_1-98.26X_2-0.08X_3+1.43X_1X_2+0.15X_1X_3+1.08X_2X_3$	0.6372	0.2757	0.0316
Taste	$-0.35X_1-0.14X_2+0.10X_3$	0.5088	0.0582	0.1113
Texture	$34.73X_1+70.49X_2+0.36X_3-0.65X_1X_2-0.41X_1X_3-0.81X_2X_3$	0.6171	0.3066	0.2490
Overall liking	$-355.89X_1-523.56X_2-1.50X_3+114.15X_1X_2+4.15X_1X_3+6.02X_2X_3-1.27X_1X_2X_3$	0.6797	0.3842	0.2957

X_1 = SMP = soymilk powder, X_2 = MLT = mulberry leaf tea and X_3 = RM = raw milk.

Pueraria tuberosa herb extract in milk slightly affected the astringent flavor (Sawale et al., 2015), and astringency was also impacted by isoflavonoids and polyphenols (Peleg et al., 1999).

3.5. Optimization of mixture proportion

Mathematical models and predicted equations of chemical composition and quality are shown in Table 4. Models of protein, fat, SNF and TS showed significant differences ($p < 0.05$) in linear terms, whereas lactose was presented as a quadratic model. Lack-of-fit showed no significant differences ($p > 0.05$) and regression coefficients (R^2) were higher than 0.8 for all of these response variables. The response variables pH, microbial count and color of yellowness (b*) could not be used for explanation/prediction because models associated with these had overall significance ($p > 0.05$); however, lightness (L*) and greenness (a*) values yielded significance for both linear and quadratic terms ($p < 0.05$). Highest positive coefficients for protein, fat, SNF, TS and lactose content were influenced by SMP followed by MLT and RM, respectively. Increasing SMP in PM increased protein, fat and TS while supplementing with MLT also influenced PM as it contains high phenolic contents, flavonoids and chlorophylls which affected L* and a* colors (Saetan et al., 2016; Wegrzyn et al., 2008). Response surfaces of component composition interaction are presented as contour plots in Figure 2A.

Analysis of model variance for melatonin, free tryptophan, TPC and antioxidant activity using DPPH, ABTS and FRAP assays presented significant differences ($p < 0.05$) in linear and quadratic terms, with lack-of-fit ($p > 0.05$) and $R^2 > 0.8$ for all response functions (Table 4). Increasing melatonin content in PM showed the largest contribution by SMP followed by MLT and RM, respectively. Free tryptophan increase was mostly enhanced by the combination of each component, especially SMP combined with MLT followed by MLT with RM. Regarding TPC and antioxidant activity, strong positive coefficients were realized by MLT followed by SMP and RM, respectively. Contour plots of the proportions are shown in Figure. 2B and 2C.

For sensory attributes, the model and R^2 gave no significant differences for all parameters of color, flavor, taste, texture and overall liking (Table 5). All attributes showed no significant differences at $p > 0.05$, $R^2 < 0.7$ and lack-of-fit of color and flavor showed at $p < 0.05$. These findings indicated that the models were not appropriate to predict and optimize all attributes. Combinations of SMP, MLT and RM had no effect on consumer satisfaction in terms of color, flavor, taste, texture and overall liking as the panelists were unable to distinguish any differences.

Using the models and response surfaces obtained, optimization was conducted under the criteria of achieving minimum values of fat, SNF and TS, combined with maximum values of protein, melatonin, free tryptophan, TPC and antioxidant activity. Optimum proportions were suggested as 3.90% SMP, 4.50% MLT and 89.40% RM, and these values were verified by five subsequent confirmatory trials, to confirm the adequacy of the model concerning the predicted responses. Predicted optimized values are compared with verified results in Table 6.

Table 6. Verification of optimum experimental proportions compared with predicted values.

Response	Experimental value	Predicted value	%CV
Composition (%)			
Protein	4.87 ± 0.07	4.77	1.47
Fat	4.31 ± 0.06	4.24	1.16
Solid not fat	10.32 ± 0.11	10.24	0.55
Total solids	14.63 ± 0.12	14.48	0.73
Bioactive component			
Melatonin (ng/mL)	1.46 ± 0.11	1.49	1.44
Free tryptophan (μg/mL)	0.23 ± 0.02	0.26	8.66
TPC (mg GAE/mL)	0.73 ± 0.04	0.72	0.98
Antioxidant activity (mg TE/mL)			
DPPH	0.50 ± 0.02	0.52	2.77
ABTS	0.80 ± 0.03	0.78	1.79
FRAP	0.53 ± 0.01	0.52	1.35

4. Conclusions

SMP and MLT both contain nutritive values and bioactive compounds including melatonin, free tryptophan and TPC which can be added to produce functional PM. Mixture design RSM was used to optimize levels of SMP, MLT and RM of PM to achieve high melatonin and bioactive compounds in PM. Supplementing SMP (4.50%) and MLT (4.50%) in RM (88.80%) gave highest increase in chemical compositions, bioactive components and antioxidant activity, whereas sensory evaluation of all attributes showed no significant effect on mixture proportions. Optimization suggested optimum proportions as 3.90% SMP, 4.50% MLT and 89.40% RM which provided maximum values of melatonin (1.49 ng/mL), free tryptophan (0.26 μg/mL), TPC (0.72 mg GAE/mL), antioxidant activity of DPPH (0.52 mg TE/mL), ABTS (0.78 mg TE/mL), and FRAP (0.52 mg TE/mL). Mixture design RSM determined the optimum mixture proportions to produce PM containing high melatonin and bioactive compounds. Regression equations of our model can be utilized to predict values of independent product variables to optimize process development.

Declarations

Author contribution statement

Jintana Sangsopha, Anuchita Moongngarm, Nutjaree Pratheepawanit Johns, Grigg Grigg: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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