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Simultaneous Mitigation of Acrylamide, 5-HMF, and Browning Formation by Combining Three Additives in Commercial Crackers

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Abstract Bakery foods are the main sources of dietary exposure of acrylamide and 5-hydroxymethylfurfural (5-HMF). Applicable and convenient strategies for mitigation the formation of these neo-contaminants are in great demand. To better understand the potential of combined mitigating strategies on acrylamide, 5-HMF, and browning formation, three additives (sodium bicarbonate [SBC], sodium metabisulfite [SMBS], and citric acid [CA]) were optimized using a Box-Behnken design, and their effects as well as the resulting sensory properties were evaluated. SBC significantly mitigated acrylamide and 5-HMF generation ($P < 0.05$), and also increased total color changes (ΔE) ($P < 0.05$), while CA exerted an opposite effect as it promoted the formation of acrylamide and 5-HMF but decreased ΔE ($P < 0.05$). SMBS limited the formation of acrylamide and ΔE ($P < 0.05$). In addition, SBC and SMBS reduced acrylamide formation synergistically, and SMBS was the primary factor in decreased browning intensity. Interestingly, SBC inhibited 5-HMF formation via an improved pH value, whereas its inhibition on acrylamide formation were not obstructed by its effect of raising pH. With optimized levels of SBC (1.64%), SMBS (0.11%) and CA (0.50%), the concentrations of acrylamide and 5-HMF in cracker samples were 91.27 ± 11.26 μg/kg and 15.58 ± 0.35 mg/kg respectively, demonstrating 97.60% and 62.72% inhibition rates. Cracker samples with ΔE (4.41 ± 0.11) showed the desirable bright yellow color. This study presents a concrete example of how the control and optimization of selected operative parameters may mitigate multiple specific natural/process contaminants in final food products, while preserving a satisfactory sensorial range.

Keywords: *acrylamide, 5-hydroxymethylfurfural, total color changes, sodium bicarbonate, sodium metabisulfite, citric acid*

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1. Introduction

Potato (*Solanum tuberosu*m L.) is the world's most important non-grain food crop and is central to global food security. Increasing global potato consumption drives a demand for processed potato-based products. Among them, potato-based snacks (e.g., chips, crackers, French fries) comprise the largest snack food sector in all markets [1], due to their desirable taste, texture, convenience, and long shelf life. However, they are also blamed for their contribution to the dietary intake of acrylamide and 5-hydroxymethylfurfural (5-HMF), which pose potential health risks.

Acrylamide and 5-HMF can be generated from a sequence of thermal-triggered chemical reactions during snack food manufacturing. Acrylamide (C_3H_5ON) can be formed as a result of the Maillard reaction between asparagine and carbonyl compounds (reducing sugars), especially in asparagine- and carbohydrate-rich foods [2,3]. *In vivo* and *in vitro* studies of acrylamide toxicity confirm that it is mutagenic, carcinogenic, and neurotoxic [4,5]; and can disrupt skeletal development [6]. Moreover, dietary exposure of acrylamide has been identified as a public health concern due to its association with multiple cancers [7,8,9]. 5-hydroxymethylfurfural (5-HMF, $C_6H_6O_3$) is another notable compound formed by Amadori products degradation during the Maillard reaction, or by the acid-catalyzed thermal dehydration of fructose and sucrose [3].

From a toxicological point of view, 5-HMF exhibits potential genotoxic and mutagenic activities following metabolic activation to 5-sulphoxymethylfurfural [10,11]. Although the toxicity of 5-HMF to humans is still questionable, 5-HMF reduction practices are gaining importance.

Several methods have been explored by either decreasing chemical precursors or by impeding Maillard reaction pathways. Processing strategies play a critical role, as acrylamide formation occurs during heat treatment. Reducing thermal intensity during processing is an expedient approach for the mitigation of acrylamide and 5-HMF formation, but may decelerate the Maillard reaction rate and degrade the sensory properties of the product [12]. Alternatively, additive agents have been used to inhibit the chemical reactions that generate acrylamide and 5-HMF. Suman et al. reported a significant (50-70%) reduction in acrylamide content in wholegrain and cocoa biscuits by using sodium bicarbonate (SBC) leavening agent. This reduction was attributed primarily to the consequent pH variations [13]. Other pH-related acidulant such as lemon juice solution (540 ± 0.58 μg/kg) was more effective in reducing acrylamide content in fried potato chips than ginger garlic (720 ± 0.43 μg/kg) and mint leaf (770 ± 0.33 μg/kg) solutions [14]. Amino acids such as lysine and glycine have also been recommended for reducing acrylamide formation in potato-based snacks, as they either form adducts with acrylamide through the Michael addition reaction or compete with asparagine for carbonyls during the Maillard reaction. However, these studies focused primarily on improving inhibition efficiency. A limited number of reports have examined related harmful components (e.g., 5-HMF) in the reactions. On the other hand, compared to single mitigation strategies, joint efforts should be considered for sugar- and asparagine-enriched products such as potato and cereal, as the overall efficacy of combined mitigation strategies may exceed the sum of single measures. Nevertheless, the interactive effects among different mitigating measures on acrylamide and 5-HMF formation have been reported infrequently. Moreover, despite recent progress in elucidating methods to decrease acrylamide content, most studies have been conducted on potato model systems instead of real food products, thus raising questions regarding the practical applicability of their findings.

SBC, sodium metabisulfite (SMBS), and citric acid (CA) are the legal additives for bakery foods in China. SBC and CA are usually used as the leavening agents and acidity regulators in biscuits and bread, while SMBS is frequently used as the bleaching agent and antioxidant in biscuits and starch products. They are also effective inhibitors of Maillard reaction and the consequent formation of acrylamide, 5-HMF and browning. SBC and CA protonate the nucleophilic non-protonated amine ($–NH_2$) of asparagine to non-nucleophilic protonated amine ($–NH_3^+$) and block the nucleophilic addition of asparagine with electrophilic carbonyl carbon of reducing sugar that inhibits Maillard reaction and subsequently the acrylamide formation [15]. SMBC is frequently used as an inhibitor of browning in Maillard reaction because the neucleophilic sulfur atom binds to the carbonyl group of sugars before the amino groups [16].

The aim of our study was to examine the levels of reducing acrylamide and 5-HMF formation in commercial potato-based crackers, with a focus on the effects of combining additive treatments (SBC, SMBS, and CA). Response surface methodology was performed to evaluate additive parameters with an experimental Box–Behnken design, and the interaction effects among these three agents were evaluated. Meanwhile, total color changes (ΔE) and browning index (BI), based on the Commission Internationale de l'Elcairage (CIE) values (L^*, a^*, and b^*), were used to quantify the browning intensity resulting from Maillard reaction. The partial sensory properties of the crackers, including hardness and fracturability, were also characterized.

2. Materials and Methods

2.1. Materials and Reagents

Food-grade cracker materials of potato flakes, wheat flour, cassava starch, sucrose, salt, dried milk powder and shortening were kindly gifted by Zhejiang Little Prince Food Co., Ltd. Food-grade SBC, SMBS and CA were purchased from a food additive market in Hangzhou. Standard preparations (≥95 % by HPLC) of acrylamide, acrylamide-d3, and 5-HMF were purchased from Sigma-Aldrich (Merch, Germany). All other chemical reagents were of analytical grade and obtained from Aladdin Reagent Corporation (Shanghai, China).

2.2. Cracker Sample Preparation

Cracker samples were prepared at laboratory scale according to the following optimized recipe: 600.0 g potato flakes, 160.0 g wheat flour, 60.0 g cassava starch, 63.0 g sucrose, 7.0 g salt, 50.0 g milk powder, 60.0 g shortening (1000 g in total). It is the recipe of a cracker produced by Zhejiang Little Prince Food Co. Ltd., using 0.82% ammonium bicarbonate as the leavening agent. Ammonium bicarbonate has been shown to contribute, promote, and accelerate acrylamide formation in bakery products. Therefore, based on the results of our preliminary experiments, ammonium bicarbonate was replaced with SCB (0.82 -1.64%), SMBS (0.05 - 0.25%), and CA (0.1-0.5%) at levels determined according to a three-factor, three-level Box-Behnken Design (Table 1). Cracker samples produced from the optimized recipe with 0.82% ammonium bicarbonate were used as the control.

All ingredients in each recipe were mixed with 500 mL distilled water using an electronic mixer (HM-945, Donlim, China) for 5 min, followed by 5 min of hand kneading. Dough was then cut into round discs with a height of 2 mm and a diameter of 5 cm and baked at 200 °C for 5 min in an oven (T4-L326F, Media, China). Three consecutively replicated bakings were conducted to obtain cracker samples from each recipe. Baked cracker samples were collected for texture analysis, and then ground and passed through a 60 mesh sieve, sealed tightly, and stored at -18 °C.

Table 1. Levels of Sodium Bicarbonate (SBC), Sodium Metabisulfite (SMBS) and Citric Acid (CA), and Dough pH Values of Different Runs of the Three-Factor, Three-Level Box-Behnken Design.

Run	SBC/%	SMBS/%	CA/%	pH
1	0.82	0.05	0.3	7.39 ± 0.10
2	1.23	0.15	0.3	7.48 ± 0.08
3	1.64	0.05	0.3	8.05 ± 0.06
4	0.82	0.15	0.1	7.65 ± 0.09
5	1.23	0.15	0.3	7.51 ± 0.08
6	1.23	0.05	0.1	7.86 ± 0.09
7	0.82	0.25	0.3	7.19 ± 0.07
8	1.23	0.15	0.3	7.52 ± 0.08
9	1.23	0.15	0.3	7.51 ± 0.10
10	1.23	0.25	0.1	7.73 ± 0.08
11	1.23	0.25	0.5	7.29 ± 0.09
12	1.64	0.15	0.1	7.8 ± 0.06
13	1.23	0.15	0.3	7.5 ± 0.08
14	1.64	0.25	0.3	7.93 ± 0.06
15	0.82	0.15	0.5	6.96 ± 0.08
16	1.23	0.05	0.5	7.55 ± 0.06
17	1.64	0.15	0.5	7.69 ± 0.09
Control[a]	/	/	/	6.83 ± 0.06

[a]: 0.82% ammonium bicarbonate was used as the leavening agent.

2.3. pH Value of Dough

pH values of dough preparations before baking were measured according to the reported method [17]. Briefly, 5 g dough samples were soaked in 45 ml distilled water for 1 h. The suspensions were homogenized with a handled ultrasonicator (Ultrasonic processor FXID: 6m2003, Fangxu, China) for 5 min. The pH values of the suspensions were determined with a pH meter equipped with a glass electrode (FE28-Standard, Mettler, Switzerland).

2.4. Cracker Sample Characterization

2.4.1. Determination of Acrylamide

To extract acrylamide, 2 g ground cracker samples, 0.1 ml acrylamide-d3 aqueous solution (5 μg/ml) as internal standard, and 9 ml ultrapure water were homogenized in a 50 ml centrifuge tube. Then, 10 ml acetonitrile and 5 ml n-hexane were added to the homogenate, followed by vortex mixing for 1 min. After adding 1.5 g anhydrous sodium acetate and 6 g anhydrous magnesium sulfate to the centrifuge tube, extracting was conducted at room temperature for 1 h with shaking (60 rpm). The extract was centrifuged at 10000 rpm, 4 °C for 8 min to separate the acrylamide into the acetonitrile phase. An aliquot of 3 ml acetonitrile phase was cleaned with an MCX solid phase extraction cartridge. The filter was collected and dried under a nitrogen stream. The residue was re-dissolved in 0.3 ml ultrapure water and filtered through a 0.45 μm filter before the chromatograph analysis.

The chromatographic separation of acrylamide was performed by using the UPLC-ESI-MS system (Waters Acquity, USA). The UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) was maintained at 30°C. The injection volume for each sample was 10 μl. Using 0.1% formic acid in water (A) and methanol (B) as mobile phase, a gradient elution was performed with flow rate of 0.2 ml/min and gradual changing of the mobile phase as follows: 0 ~ 3.0 min, 0% B; 4.0 ~ 5.0 min, 95% B; 5.2 ~ 9.0 min, 0% B. Electrospray ionization mass spectrometric detection was conducted in positive ion mode. The capillary voltage was 3.0 kV and the cone voltage was 30 V. The source temperature was 150 °C. Nitrogen was used as the desolvation gas at 1000 L/h with the temperature of 500 °C. For the transitions, m/z 72 (acrylamide) and m/z 75 (acrylamide-d3), m/z 55 (acrylamide) and m/z 58 (acrylamide-d3) were used with collision energies all set to 15 V.

2.4.2. Determination of 5-HMF

5-HMF was extracted by suspending 1 g ground cracker samples in 10 ml ultrapure water and ultrasonic homogenizing for 30 min. The extracts of 5-HMF were clarified by adding 0.5 ml each of Carrez I and Carrez II solutions, standing by for 5 min, then centrifuging at 8000 g for 10 min. Supernatants were collected and filtered through 0.45 μm filters for chromatograph analysis.

A Shimadzu HPLC system (LC-2030C 3D Plus) was employed to quantitatively analyze 5-HMF concentrations in cracker sample extracts. The mixture of methanol: water (15% v/v) was used as the mobile phase, and the Nucleosil C18 column (250 × 4.6mm, 5 μm) was maintained at 25 °C. The injection volume for each sample was 10 μl. An isocratic elution program was performed with the flow rate of 1.0 ml/min. The Diode Array detector was set at 280 nm. Quantification was performed by an external standard calibration of 5-HMF (0 ~ 50 μg/ml).

2.4.3. Color Measurement

Color of the cracker samples (in powder) was measured with a tristimulus colorimeter (ColorFlex EZ, HunterLab, USA). Ground cracker samples were loaded into a quartz sample cup and then tested in duplicate. Three color parameters, L^*(lightness/darkness), a^*(redness/greenness), b^*(yellowness/blueness) were directly displayed in the color model. The ΔL (L^* - L^*_0), Δa (a^* - a^*_0) and Δb (b^* - b^*_0) were calculated to describe color changes in three color scales, where L^*, a^*, b^* were the color values from cracker samples with different recipes, and L^*_0, a^*_0, b^*_0 from the control sample of crackers. Total color changes (ΔE) were calculated as follows: $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{0.5}$. Browning index was also calculated based on the CIE values of L^*, a^*, b^*, for describing the browning intensity of cracker samples: Browning index = 100 × (k - 0.31)/0.17, where $k = (a^* + 1.75L^*)/(5.645L^* + a^* - 3.012b^*)$ [18].

2.5. Texture Analysis

Cracker samples with uniform size and shape were selected for texture analysis. Puncture tests were conducted using a texture analyzer TA-XT2 (Shanghai, China) equipped with a spherical probe (P/0.25S) and a 10 kg load cell. The parameters for analysis were pretest speed of 10 mm/s, test speed of 1 mm/s, post-test speed of 1 mm/s, compression distance of 10 mm, and threshold force of 5 g. The force exerted to break the cracker is defined as hardness (g), and the linear distance between the first and the last fracture registered is termed

fracturability (mm). The mean value was calculated from 6 independent assays of each sample.

2.6. Analysis of Sulfur Dioxide

Residual concentrations of sulfur dioxide in cracker samples were analyzed according to the method of Pharmacopoeia of the P. R. China 2020, using a distillation apparatus consisting of a flask with a liquid adding funnel and inert gas inlet, a back-condenser, and two successive distillate collecting tubes. 10 g ground cracker samples and 350 ml redistilled water were added to the flask. 50 ml of 3% hydrogen peroxide solution was used to collect the distillate (SO_2), by adding 3 drops 2.5% alcoholic methyl red solution as an indicator. The apparatus was conditioned by exposure to N_2 (0.2 L/min) for 10 min. After 10 ml HCl (6 M) was added, the contents of the flask were gently boiled and distilled under 0.2 L/min N_2 for 90 min. The distillate was titrated with 0.01 M NaOH solution. The residual concentrations in the cracker samples were then calculated based on the equivalent weight rule between reactants. A control experiment was performed to deduct the sulfur dioxide contents in background.

2.7. Box-Behnken Design

The Box-Behnken design is a suitable methodology for ascertaining the effects of formulation ingredients (factors) and their associated effect on responses (dependent variables) [19]. In the present study, the levels of SCB (X_1), SMBS (X_2), CA (X_3) in cracker recipes are the input factors, while the concentrations of acrylamide and 5-HMF, ΔE, hardness, and fracturability of cracker samples are reported as the response variables. The layout of the Box-Behnken design, composed of 17 trials including 5 central points was given by the Design Expert software program. The response variables were fit to the quadratic model, which was expressed with the following polynomial equation:

$$Y_Z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1 X_2 + \beta_5 X_1 X_3 + \beta_6 X_2 X_3 + \beta_7 X_1^2 + \beta_8 X_2^2 + \beta_9 X_3^2 \quad (1)$$

Where Y_Z are the response variables (Y_1, acrylamide; Y_2, 5-HMF; Y_3, ΔE; Y_4, hardness; Y_5, fracturability), while X_1, X_2, and X_3 are the input factors. β_0 is the intercept and $\beta_1 \sim \beta_9$ are the regression coefficients.

Analysis of variance (ANOVA) was done to evaluate the fitness of response variables, and to investigate and validate the factors which mitigate the formation of acrylamide and 5-HMF, and affect the organoleptic properties of the cracker samples. Mathematic equations were developed to predict the response variables. Then, optimization was performed by using multi-response desirability function.

2.8. Statistical Analysis

Response surface experimental design, data analysis, and generation of surface plots were performed by using Design Expert® 11 (Stat-Ease, Minneapolis, MN, USA). Pearson's two-tailed correlation coefficient analysis was also conducted to determine the correlation among variables, by using the software package IBM SPSS statistics 19.0 (IBM Corporation, Armonk, NY, USA). All statistical parameters were evaluated at $P < 0.05$.

3. Results

3.1. Description and Transformation of Response Variables

Remarkable changes in the concentrations of acrylamide (Y_1), 5-HMF (Y_2), and ΔE (Y_3) were observed among cracker samples with different levels of SBC, SMBS and CA. As shown in Table 2, Y_1, Y_2, and Y_3 ranged from 15.37 ± 1.79 ~ 3283.99 ± 265.30 μg/kg, 9.37 ± 0.63 ~ 35.79 ± 1.36 mg/kg and 2.39 ± 0.11 ~ 11.41 ± 0.15, respectively. The browning indices of the cracker samples lied in 50.67 ± 0.71 ~ 64.77 ± 0.86 (as shown in Table S1.). ΔE was in accordance with the browning index, because significant positive correlation was determined between these two variables (r = 0.904, P = 0.000). Herein, ΔE was used in this study to measure the browning development in the cracker samples.

Table 2. Acrylamide and 5-HMF Concentrations, Total color changes (ΔE) of the Cracker Samples

Run	Y_1[a]	Y_1'[b]	Y_2[c]	Y_3[d]
1	3283.99 ± 265.30	8.10 ± 0.08	35.79 ± 1.36	5.84 ± 0.16
2	105.94 ± 12.18	4.66 ± 0.11	23.73 ± 1.00	4.63 ± 0.15
3	400.75 ± 42.97	5.99 ± 0.11	11.74 ± 0.64	11.41 ± 0.15
4	177.70 ± 12.48	5.18 ± 0.07	31.79 ± 1.17	2.39 ± 0.11
5	102.55 ± 10.77	4.63 ± 0.11	22.87 ± 0.96	3.89 ± 0.16
6	634.98 ± 53.52	6.45 ± 0.09	19.72 ± 1.24	11.10 ± 0.16
7	441.52 ± 33.01	6.09 ± 0.08	33.92 ± 1.39	3.75 ± 0.17
8	114.76 ± 7.08	4.74 ± 0.06	21.03 ± 0.98	4.29 ± 0.11
9	114.97 ± 9.33	4.74 ± 0.08	23.19 ± 1.15	4.53 ± 0.16
10	22.50 ± 2.34	3.11 ± 0.11	23.62 ± 1.14	3.02 ± 0.07
11	119.34 ± 14.14	4.78 ± 0.12	19.37 ± 1.06	2.43 ± 0.14
12	42.55 ± 5.45	3.75 ± 0.13	9.37 ± 0.63	8.13 ± 0.14
13	141.80 ± 9.31	4.95 ± 0.07	22.66 ± 1.04	4.54 ± 0.13
14	15.37 ± 1.79	2.73 ± 0.12	15.49 ± 0.84	3.55 ± 0.15
15	2156.59 ± 173.36	7.68 ± 0.08	32.56 ± 1.40	3.58 ± 0.22
16	1384.71 ± 100.85	7.23 ± 0.07	27.61 ± 1.15	4.94 ± 0.14
17	37.19 ± 3.85	3.62 ± 0.11	14.68 ± 0.88	3.53 ± 0.16
Control	3491.65 ± 254.87	8.16 ± 0.07	40.35 ± 1.73	/

[a]: Y_1: Acrylamide concentration (μg/kg).
[b]:Y_1':Ln[Acrylamide] (μg/kg).
[c]: 5-HMF concentration (mg/kg).
[d]: total color changes, ΔE.

The ratios of maximum to minimum for Y_1, Y_2, and Y_3 response were 213.66, 3.82, and 4.78, respectively. A ratio < 10 is usually acceptable. The ratio of 213.66 suggests high heterogeneity and non-normal distribution of the acrylamide data, which was confirmed by the Kolmogorov-Smirnov test ($P < 0.05$). High variation of response variables may result in the poor performance and low quality of response surface methodology optimization [20]. Therefore, Y_1 was transformed into the natural log scale (signed as Y_1'), to stabilize variance and to achieve log-normal distribution. This can be written as follows: $Y_1' = \ln(Y_1 + a)$, where a is constant and taken to be zero.

Differences in hardness (Y_4) and fracturability (Y_5) of the cracker samples were much less than Y_1 ~ Y_3, which ranged from 419.78 ± 31.69 ~ 675.53 ± 107.86 g and 0.41 ± 0.07 ~ 1.10 ± 0.23 mm, respectively, as shown in Table S1. The ratios of maximum to minimum were 1.61 and 2.68, respectively.

3.2. ANOVA of Responses Variables

Table 3. ANOVA of Response Y_1', Y_2 and Y_3

Y_1': Ln[acrylamide]				
Source	SS[a]	F-value	*P*-value	VCR[b]
Model	37.87	297.30	< 0.0001	
X_1-SBC	14.99	1059.21	< 0.0001	39.48
X_2-SMBS	15.29	1080.19	< 0.0001	40.27
X_3-CA	2.89	204.32	< 0.0001	7.61
X_1X_2	0.3933	27.79	0.0012	1.04
X_1X_3	1.73	122.26	< 0.0001	4.56
X_2X_3	0.1975	13.95	0.0073	0.52
X_1^2	0.433	30.59	0.0009	1.14
X_2^2	1.84	129.79	< 0.0001	4.85
X_3^2	0.0006	0.0424	0.8427	0.00
Residual	0.0991			0.26
Lack of Fit	0.0353	0.7393	0.5814	
Pure Error	0.0637			
Cor Total	37.97			
R^2	0.9974			
Adjusted R^2	0.9940			
Y_2: 5-HMF				
Source	SS[a]	F-value	*P*-value	VCR[b]
Model	929.33	70.21	< 0.0001	
X_1-SBC	856.57	582.44	< 0.0001	91.16
X_2-SMBS	0.7565	0.5144	0.4965	0.08
X_3-CA	11.81	8.03	0.0253	1.26
X_1X_2	7.90	5.37	0.0536	0.84
X_1X_3	5.15	3.50	0.1034	0.55
X_2X_3	36.84	25.05	0.0016	3.92
X_1^2	1.18	0.8027	0.4000	0.13
X_2^2	4.29	2.92	0.1314	0.46
X_3^2	5.33	3.63	0.0986	0.57
Residual	10.29			1.10
Lack of Fit	6.17	2.00	0.2567	
Pure Error	4.12			
Cor Total	939.63			
R^2	0.9890			
Adjusted R^2	0.9750			
Y_3: ΔE				
Source	SS[a]	F-value	*P*-value	VCR[b]
Model		48.79	< 0.0001	
X_1-SBC	15.29	58.64	0.0001	13.15
X_2-SMBS	52.72	202.22	< 0.0001	45.33
X_3-CA	12.91	49.53	0.0002	11.10
X_1X_2	8.33	31.94	0.0008	7.16
X_1X_3	8.39	32.17	0.0008	7.21
X_2X_3	7.74	29.68	0.001	6.65
X_1^2	0.6789	2.60	0.1506	0.58
X_2^2	7.86	30.15	0.0009	6.76
X_3^2	0.5732	2.20	0.1817	0.49
Residual	1.82			1.56
Lack of Fit	1.47	5.46	0.0673	
Pure Error	0.3581			
Cor Total	116.31			
R^2	0.9943			
Adjusted R^2	0.9870			

[a] ss= Sum of Squares. [b] VCR= Variance contribution ratio (%).

To explain the relationship between input factors and response variables, data of Y_1', Y_2, Y_3, Y_4, and Y_5 were collected to fit the quadratic model. The statistical ANOVA results for the quadratic polynomial model are provided in Table 3. Herein, the higher F-value and lower *P*-value (< 0.05) of the model associated with the larger *P*-value of lack of fit (P > 0.05) and the multiple correlation coefficient (R^2) close to 1 indicate a well-fitting regression model [19].

As shown in Table 3, the *P*-value of models of these three response variables were < 0.0001, and the *P*-value of lack of fit test for Y_1', Y_2, and Y_3 were 0.5814, 0.2567 and 0.0673, respectively. The high R^2 values of 0.9974, 0.9781, 0.9843 indicated that the models failed to explain only 0.26%, 2.19%, and 1.57% of total variations of Y_1', Y_2, and Y_3, respectively. The values of adjusted R^2 (0.9940, 0.9750, and 0.9870) were closed to the values of R^2. All of these suggested that Y_1', Y_2, and Y_3 fit the quadratic model well.

However, the data of hardness (Y_4) and fracturability (Y_5) failed to fit the quadratic model. Neither fit the two-factor interaction or linear models, suggesting that the changes of texture data were not significantly related to the input factors within the experimental ranges.

3.2.1. Diagnostics of the Model Adequacy

The following equations (2) – (4) were developed to predict response Y_1', Y_2, and Y_3 in coded factors.

$$Y_1' = 4.75 - 1.37X_1 - 1.38X_2 + 0.60X_3 - 0.32X_1X_2 - 0.66X_1X_3 + 0.22X_2X_3 + 0.32X_1^2 + 0.66X_2^2 - 0.01X_3^2 \quad (2)$$

$$Y_2 = 22.70 - 10.35X_1 - 0.31X_2 + 1.22X_3 + 1.40X_1X_2 + 1.13X_1X_3 - 3.04X_2X_3 + 0.53X_1^2 + 1.01X_2^2 - 1.13X_3^2 \quad (3)$$

$$Y_3 = 4.37 + 1.38X_1 - 2.57X_2 - 1.27X_3 - 1.44X_1X_2 - 1.45X_1X_3 + 1.39X_2X_3 + 0.40X_1^2 + 1.37X_2^2 - 0.37X_3^2 \quad (4)$$

The linearity relationship between the actual and the predict data (Figure 1a - Figure 1c), associated with high values of signal to noise ratio (60.92, 28.91 and 22.60) demonstrated adequate precision of the models for predicting Y_1', Y_2, and Y_3 within the variable ranges chosen in this study.

3.2.2. Effects of the Input Factors on the Responses

The variance contribution rate was calculated to reflect the importance of input factors. The larger the calculated values, the larger its weight on the response. Additionally, a positive sign of coefficient signifies the collegial effect while a negative sign signifies the opposite effect of the factor on response variables.

As reported in Table 3, for Y_1' response, the variance contribution rates of the linear terms X_1 and X_2 were 39.62% and 40.33% respectively, much larger than that of X_3 (7.61%). Associated with the negative signs of the coefficients of X_1 and X_2, it suggested that SBC and SMBS were the main factors which mitigated acrylamide

formation in the cracker samples, while CA promoted the formation of acrylamide to some extent. SBC was the dominant factor that inhibited the formation of 5-HMF, with evidence that the linear term X_1 contributed 92.17% of total variations of Y_2. While adding CA favored 5-HMF generation in cracker samples. The effect of SMSB on 5-HMF formation was negligible, with the variance contribution rates of 0.08. Adding SMBS and CA both limited the development of browning in cracker samples, while adding SBC favored the browning development.

3.2.3. The Interaction among the Input Factors

Three-dimensional (3D) response surface plots were drawn to illustrate the interaction effects between the two factors when the third factor was kept at the center point, as shown in Figure 2.

Y_1' decreased with increasing SBC and SMBS levels in the experimental range, when CA was kept at 0.3% (Figure 2a). Adding CA attenuated the effects of SBC and SMBS on acrylamide migration, as lower Y_1' was observed when CA was at the lower level (Figure 2a and 2b). Additionally, in our preliminary experiments, acrylamide concentrations in cracker samples were respectively 2667.91 ± 188.30 μg/kg and 274.06 ± 23.75 μg/kg, when SBC was added alone into the optimized recipe at levels of 0.82% and 1.64%, respectively. The acrylamide concentration was reduced to 15.37 ± 1.79 μg/kg with the combined using of SBC and SMBS in the Run 14 of Box-Behnken design (Table 2), despite adding CA might promote acrylamide formation. These results suggested the synergy of SBC and SMBS on the mitigation of acrylamide formation.

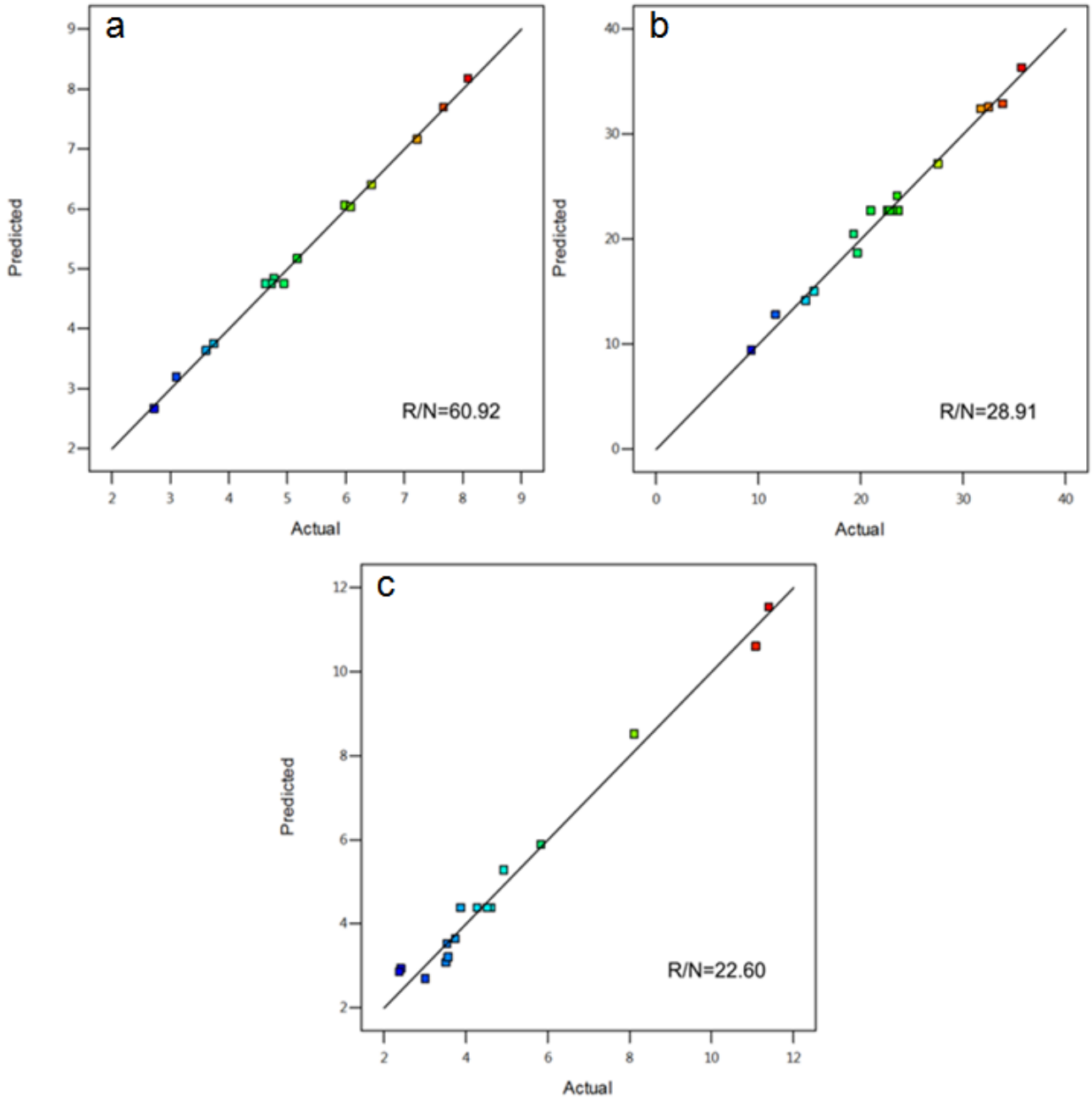

Figure 1. Predicted vs actual plots illustrating the goodness of fitting. (a): Ln[Acrylamide] (Y_1'); (b): 5-HMF (Y_2); (c): ΔE (Y_3). R/N: ratio of signal to noise

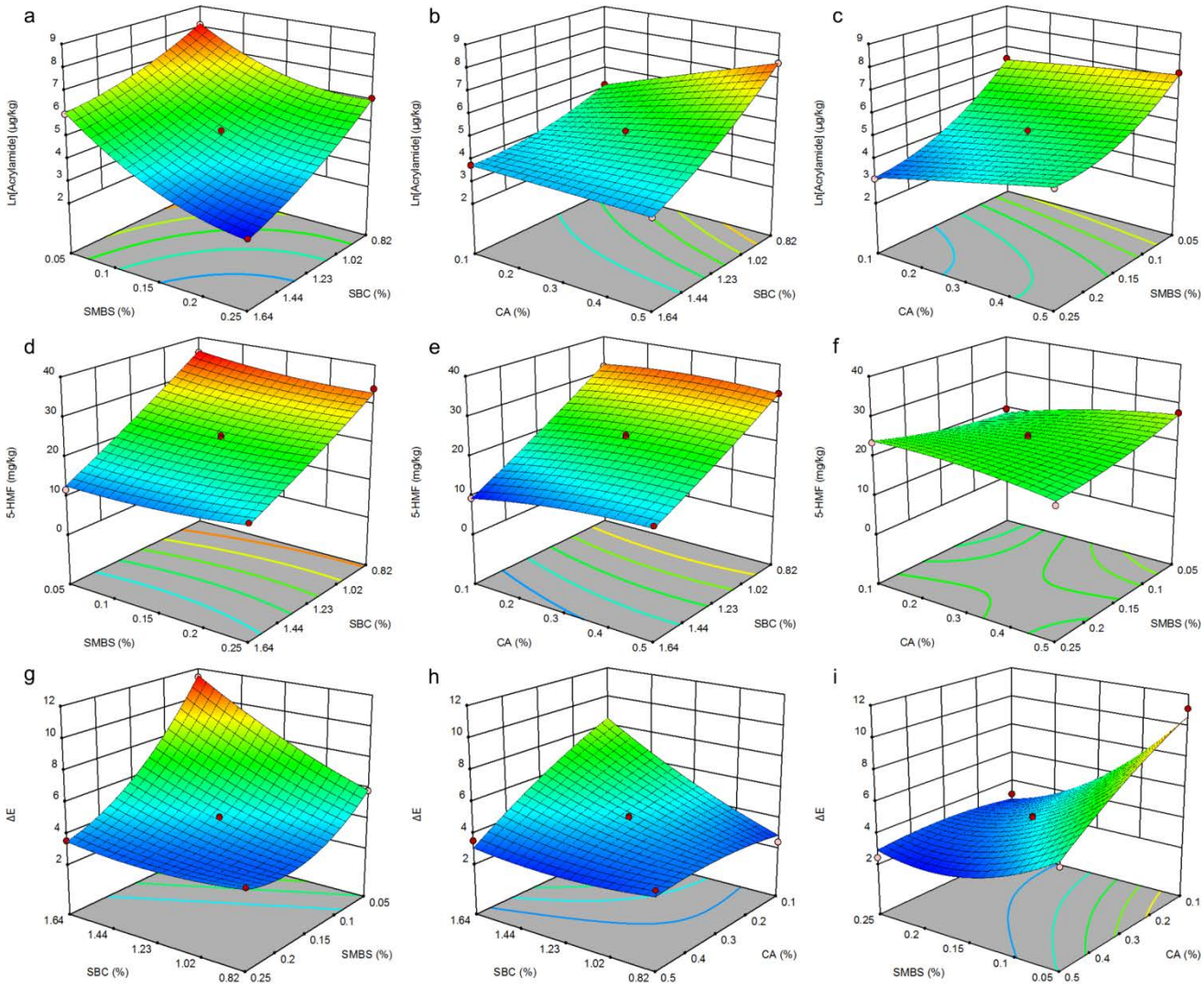

Figure 2. Response surface plots predicting with the quadratic model as the function of the response variables and described by the Equation (2)–(4). (a)–(c): 3D plots of Ln[Acrylamide] (Y_1'); (d)–(f): 3D plots of 5-HMF (Y_2); (g)–(i): ΔE (Y_3)

The 3D plots of Y_2 are shown in Figure 2d-Figure 2f. Significant interaction was only detected between X_2 and X_3 ($P < 0.05$). When SBC level was kept at the central point, the response Y_2 increased with the increasing levels of CA when SMBS was at the low level (0.05%), but decreased with increasing CA levels when SMBS was at the high level (0.25%) (Figure 2f).

The 3D plots of response Y_3 are shown in Figure 2g - 2i. Lower ΔE was identified when $X_1 < 1.23\%$ and $X_2 > 0.15\%$ (Figure 2g and 2h). The effect of CA on ΔE was more obvious when SBC was at high levels (1.64%) and SMBS was at the low level (0.05%) (Figure 2h and 2i).

3.3. Effects of Dough pH on the Response

In the present study, the pH values of dough samples were in the range of 6.96 ~ 8.05 with combined using SBC, CA and SMBS. Spearman's correlation analysis was conducted to determine the main factors related with dough pH, and the effects of dough pH on the response variables. The results showed that the SBC level was the main factor related with the dough pH (r = 738, $P = 0.001$), followed by the CA level (r = - 0.504, $P = 0.040$). The dough pH was negatively related with Y_1' (r = - 0.816, $P = 0.000$) when using the SMBS level as control variable, because the SMBS level was not significantly related with the dough pH (r = - 0.230, $P = 0.375$) but contributed the most to acrylamide reduction (Table 3). Dough pH was also negatively correlated with Y_2 (r = - 0.708, $P = 0.001$), but was positively related with Y_3(r = 0.548, $P = 0.023$). These results suggested that raising dough pH might reduce acrylamide and 5-HMF formation, but promote the browning development.

3.4. Multi-response Optimization and Verification

3.4.1. Optimization Using Desirability Function

Desirability function of Design Expert software was employed to determine the optimum levels of SBC, SMBS and CA for lowering acrylamide and 5-HMF concentrations and browning intensity. Constraints were given to the factors and the measured responses, as shown in Table 4. Responses Y_1', Y_2, and Y_3 were targeted for minimization. Because SMBS may cause sulfur dioxide residue and thereby increase the risk of asthma in sensitive individuals, the level of SMBS was set as low as possible to diminish the residual concentration of sulfur dioxide in cracker samples.

Table 4. Constraints for Optimum Levels of SBC, SMBS and CA

	Goal	Lower limit	Upper limit	Importance
SBC	In range	0.82	1.64	3
SMBS	Minimize	0.05	0.25	5
CA	In range	0.10	0.50	3
Ln[Acrylamide]	Minimize	2.73	8.1	5
5-HMF	Minimize	9.37	35.79	3
ΔE	Minimize	2.39	11.41	3

The optimum levels of SBC, SMBS and CA for minimum formation of Ln[Acrylamide], 5-HMF and ΔE were 1.64%, 0.11% and 0.50%, respectively. The desirability values of Y_1', Y_2, Y_3 and combination were 0.70, 0.79, 0.78 and 0.74, respectively. The desirability bar graph is reported in Figure 3.

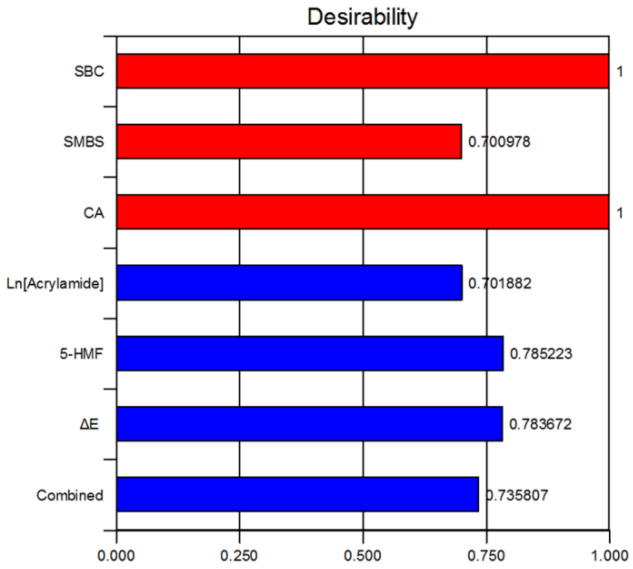

Figure 3. Desirability Bar Graph for Each Factor, Responses and Combination of All Responses

3.4.2. Verification Testing

To validate the optimization, cracker samples were made in triplicate with SBC, SMBS and CA at the optimized levels, as shown in Figure 4. Acrylamide and 5-HMF concentrations and ΔE were measured and compared with the predicted values, as shown in Table 5. The biased percentages were less than 5% for all response variables. While the bias was 12.95% on the base of actual acrylamide concentration.

The formation of acrylamide and 5-HMF in the verification test was inhibited by 97.27% and 62.72% respectively, compared with the concentrations of acrylamide and 5-HMF in the control cracker sample (Table 2). The cracker samples from the verification test were in bright yellow, which is a desirable color for bakery foods. The hardness and fracturability were 436.38 ± 37.82 g and 0.95 ± 0.10 mm respectively, which were within the experimental ranges. The residual content of sulfur dioxide was 67.37 ± 1.86 mg/kg. More effort is needed to diminish the level of residual sulfate, though the present amount was less than the benchmark level for biscuit products (0.1 g/kg) stipulated in the Chinese food criterion GB 2760.

Figure 4. Cracker samples from the triplicate verification test

Table 5. Verification Testing Data

	Predicted values	Actual values	Bias
Ln[Acrylamide]/μg/kg	4.33	4.51 ± 0.12	4.21%
Acrylamide/μg/kg	76.02	91.27 ± 11.26	12.95%
5-HMF/mg/kg	15.04	15.58 ± 0.35	3.59%
ΔE	4.34	4.41 ± 0.01	1.62%
Sulfur dioxide/mg/kg	/	67.37 ± 1.86	/
Hardness/g	/	436.38 ± 37.82	/
Fracturability/mm	/	0.95 ± 0.10	/

4. Discussion

pH value is the strong modulator for Mallard reactions and impacts the formation of acrylamide and 5-HMF. Acidity regulators, such as CA, acetic acid, and sodium acid pyrophosphate has been widely used to limit acrylamide levels in various foods [21]. Acidification might mitigate acrylamide formation by decreasing the generation of methylglyoxal and glyoxal, but promote 5-HMF formation by enhancing the generation of 3-dexoyglucosone [22], while alkalization favored the increasing of α-dicarbonyl compounds formation and the decreasing of 3-dexoyglucosone [23]. However, when using SBC as the leavening agent, over 50% acrylamide mitigation was achieved in various foods, though adding SCB increasing the pH values of the matrix before thermal-processing [13,22,24]. It seemed that pH values were not the determined factors for acrylamide formation when SBC adding into the formulation. Recently, Gülcan et al. reported that over 50% of acrylamide formation in bread was reduced by using CO_2 as the baking atmosphere, while the acrylamide formation was almost blocked with SO_2 as the baking atmosphere [25]. During baking, both SBC and SMBS decomposed and generated CO_2 and SO_2, respectively. The CO_2 filled in the baked foods, made the porous texture, and might have acted as an inert and inhibiting atmosphere that mitigated acrylamide formation in the cracker samples. The observed synergistic effect of SBC and SMBS on acrylamide mitigation in the present study might be due to the collegial effects of the generated CO_2 and SO_2.

Tas & Gökmen reported that alkalization favored the decreasing of 3-dexoyglucosone [23], the important intermediate related with 5-HMF formation [26,27]. Kavousi et al. also found that a higher amount of 5-HMF was formed at a lower pH at frying temperature in model system [28]. Sung & Chen reported that pH of cookies increased to 10.33 during 10 min-baking when 0.8% SBC was used as a leavening agent, due to SBC decomposing into sodium carbonate [29]. All of these results suggested that alkalinization caused by SBC drastically reduced 5-HMF formation.

During the thermal processing of foods, browning develops primarily as the result of Maillard reaction and caramelization [30]. Decreasing total color changes and increasing the brightness of carbohydrate-rich foods by adding SMSB or using a SO_2 baking atmosphere have been documented previously [28,31,32], because the nucleophilic property of sulfur atoms facilitated the preferential binding of SO_2 to the carbonyl groups of sugars rather than amino groups [25]. Previous studies have reported that a pH increase from 4.0 to 8.0 strongly enhanced the polymerization of carbonyl compounds generated during caramelization [33,34]. Therefore, the mitigation of browning in the cracker samples probably resulted from sulfur-mediated inhibition of the Maillard reaction and pH-mediated inhibition of caramelization.

5. Conclusions

By employing crackers as a model analyte, a strategy for mitigating the formation of acrylamide, 5-HMF and browning by combining three food additives was proposed by a Box-Behnken design and a desirability function approach. Optimal mitigation was obtained with SBC (1.64%), SMBS (0.11%), and CA (0.5%), without compromising the sensory quality of the product. The combined additives mitigated 5-HMF and browning formation by sulfur-mediated inhibition of the Maillard reaction and pH-mediated inhibition of caramelization. The mitigation of acrylamide formation resulted from the synergistic effects of SBC and SMBS. The inhibition of SBC on acrylamide formation was not obstructed by its effect of raising pH. More efforts are needed to understand the mechanism.

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Supplement

Table S1. Hardness (g), Fracturability (mm) and Browning Index (BI) of Cracker Samples from Different Runs.

Run	Hardness /g	Fracturability/mm	Browning index
1	503.13 ± 82.83	0.66 ± 0.06	60.36 ± 0.81
2	519.13 ± 78.15	0.56 ± 0.06	55.42 ± 0.80
3	457.38 ± 77.43	0.80 ± 0.12	64.10 ± 0.88
4	534.90 ± 46.31	0.63 ± 0.26	53.07 ± 0.76
5	531.65 ± 65.80	0.59 ± 0.08	54.04 ± 0.79
6	494.33 ± 38.59	0.82 ± 0.12	64.77 ± 0.86
7	499.33 ± 47.18	0.77 ± 0.09	49.26 ± 0.68
8	439.60 ± 51.62	0.75 ± 0.15	55.85 ± 0.80
9	608.17 ± 53.88	0.63 ± 0.05	55.75 ± 0.75
10	498.58 ± 71.30	0.73 ± 0.13	55.15 ± 0.79
11	556.28 ± 25.03	0.72 ± 0.10	51.69 ± 0.68
12	675.53 ± 107.86	1.10 ± 0.23	63.16 ± 0.83
13	635.28 ± 54.62	0.84 ± 0.19	57.10 ± 0.79
14	421.23 ± 85.98	0.44 ± 0.07	55.42 ± 0.75
15	542.33 ± 75.09	0.80 ± 0.04	52.72 ± 0.72
16	419.78 ± 31.69	0.53 ± 0.08	57.06 ± 0.78
17	468.50 ± 14.28	0.41 ± 0.07	54.71 ± 0.78
Control	529.36 ± 43.95	0.69 ± 0.07	50.67 ± 0.71