SHORT COMMUNICATION

OPTIMIZATION OF THE AMINOREDUCTONE FORMATION IN THE MAILLARD REACTION

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ABSTRACT

The optimization of process parameters for the production of aminoreductone (AR), a bioactive product formed in the initial stage of Maillard reaction was investigated using response surface methodology (RSM) and Box-Behnken design technique. The optimum process conditions were determined by analyzing the response surface of three-dimensional surface plot and solving the regression model equation with the Design Expert software. The optimum conditions include: heating time of 15 min, temperature of 112.85°C, pH of 8.33 and buffer concentration of 0.53 which were used to obtain the maximum AR yield (76.6 mM) in the model solution of lactose (0.3 M) and butylamine (0.3 M).

- Keywords: aminoreductone, Maillard Reaction, optimum condition, Box-Behnken design, response surfaces -

INTRODUCTION

Maillard reaction products are responsible for the change of color, taste, flavor and the nutritional value of food products (RAMONAITYTE *et al.*, 2009). Therefore, the Maillard reaction is the most important influence on food quality and acceptance (JAEGER *et al.*, 2010). However, the evaluation of the extent of the Maillard reaction is difficult with many parallel and consecutive reactions (MORALES and JIMENEZ-PEREZ, 1998). Several studies have reported detection methods for estimating the extent of Maillard reaction by the detection of an intermediate product as hydroxymethylfurfural (SHIMAMURA *et al.*, 2004) or the final polymerized products such as melanoidins (BOEKEL, 1998). In the early stage of the Maillard reaction, Aminoreductone (AR) is formed (BOEKEL, 1998). Therefore, the detection of AR would be more effective in the indication of the Maillard reaction and heat treatment of food than other methods (SHIMAMURA *et al.*, 2004). Thus, the role and characteristics of AR is, of great interest to food scientists.

A number of functionalities of AR such as an antioxidative activity, have been found (PISCHETSRIEDER *et al.*, 1998), a protective ability on photo-degradation of riboflavin in milk (TRANG *et al.*, 2008), and antimicrobial activities against pathogenic bacteria: *Helicobacter pylori* (TRANG *et al.*, 2009), *Pseudomonas aeruginosa* (PA), multi-drug resistant *Pseudomonas aeruginosa* (MDRP), *Escherichia coli* (EC), methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) (TRANG *et al.*, 2011, 2013). In the field of food technology, food scientists and producers always consider many factors that can contribute to a good and healthy product. As AR presents the potential properties in medical practices (TRANG et al., 2009, 2011) and contributes to food quality (KATSUNO et al., 2013), it can be used as a functional additive ingredient in food to improve the quality of food.

As a product of the Maillard reaction, the formation of AR depends on multiple parameters such as, the heating time, the heating temperature, the pH and the buffer concentration (BOEKEL, 1998). Conventionally, the formation of AR might be optimized using a single factor optimization to evaluate the optimum producing condition, which is relatively simple and does not require statistical analysis. However, the single variable optimization strategy is not only tedious, but can also lead to misinterpretation of results, especially since the interaction between different factors are overlooked (MANNAN *et al.*, 2007). The response surface methodology (RSM) as a combination of mathematical and statistical techniques was employed to overcome this major problem in the optimization study (LI *et al.*, 2008). In this method, statistically designed experiments used a small set of carefully planned experiments, to build models, evaluate the effects of factors and find the optimum conditions for desirable responses (LI *et al.*, 2002). It can simultaneously study several variables with a small number of observations, less time consumed and cost effects (DEEPAK *et al.*, 2008). For this reason, the purpose of this study is to optimize the technological conditions favoring the production of AR in a model system using a statistical approach: response surface methodology.

MATERIALS AND METHODS

Reagents

XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrezolium-5-carboxanilide) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Lactose monohydrate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). n-Butylamine was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest commercial grade available. Milli-Q water was used in all procedures.

Model solutions

The solutions containing lactose and butylamine were used as a model system of by AR production in the Maillard reaction (model solution). The solutions were prepared according to the previous report (SHIMAMURA *et al.*, 2004; TRANG *et al.*, 2011). Lactose monohydrate (0.3 M) and butylamine (0.3 M) were dissolved in phosphate buffer. One milliliter of the model so-

Table 1 - Factors in actual and coded levels for the Box-Behnken design.

No	Factors	Symbols	Coded and actual level		
			-1	0	+1
1	Heating temperature (°C)	A	90	110	130
2	Heating time (min)	B	5	15	25
3	pH	C	7	8	9
4	Buffer concentration (M)	D	0.3	0.5	0.7

lutions was heated under the indicated condition. Immediately after heating, the heated solutions were cooled in ice and used for the determination of AR formation.

Determination of aminoreductone formation

The formations of AR in the heated model solutions were determined using a XTT assay, performed in a 96-well microtiter plate according to the method described by Shimamura *et al.* (2011). Each well contained 60 μL of 0.5 mM XTT prepared with 0.2 M potassium phosphate buffer (pH 7.0) saturated with menadione. A sample (40 μL) was added to the well and after mixing in a microplate shaker at a speed of 500 rpm for 15 s, the difference in the absorbance between 492 nm and 600 nm was measured using a microplate reader (MPR A4i, Tosoh, Tokyo, Japan) as the absorbance at 0 min. After 20 min at room temperature, the difference in absorbance was again measured and the increase in the absorbance was recorded as the ability of a sample to reduce XTT (XTT reducibility). The concentration of AR was estimated by the following equation: $y = 0.606\ x + 0.046$, where x and y represent the concentration of AR (mM) and the reducibility of XTT, respectively (TRANG *et al.*, 2008).

Experiment design and procedure

Box-Behnken design with three levels (low, medium, and high, coded as -1, 0, and +1) is more efficient and easier to arrange and to interpret when compared with the others, such as the Plackett-Burman design, the central composite design and the Graeco-Latin square design (FRANCIS *et al.*, 2003). This statistical technique was therefore used in this study.

A total of 27 runs was used to optimize the producing parameters namely: pH, buffer concentration, heating temperature and heating time (BOEKEL, 1998). Factors in actual and coded levels considered in this study are listed in Table 1. The experiments were designed according to the Box-Behnken design using 24 axial points and three central points as shown in Table 2. Individual experiments were carried out in random order. The average of two replicated values of each run was taken as dependent variables or responses.

Design-Expert 7.1 (Stat-Easse, Inc., Minneapolis, MN, USA) was used for the experimental design, data analysis, quadratic model building, graph (three-dimensional response surface and contour) plotting and to optimize by desirability methodology.

Table 2 - Experimental design and results of the Box-Behnken design.

Run	Factor A (°C)	1 Factor 2 B (min)	Factor 3 C	Factor 4 D (M)	AR concentration (mM)
1	90	5	8	0.5	0.032
2	130	5	8	0.5	34.855
3	90	25	8	0.5	26.244
4	130	25	8	0.5	15.270
5	110	15	7	0.3	45.056
6	110	15	9	0.3	64.033
7	110	15	7	0.7	49.940
8	110	15	9	0.7	74.594
9	90	15	8	0.3	8.802
10	130	15	8	0.3	35.402
11	90	15	8	0.7	19.148
12	130	15	8	0.7	36.247
13	110	5	7	0.5	10.947
14	110	25	7	0.5	33.1
15	110	5	9	0.5	25.3
16	110	25	9	0.5	43.9
17	90	15	7	0.5	10.811
18	130	15	7	0.5	34.91
19	90	15	9	0.5	24.954
20	130	15	9	0.5	46.294
21	110	5	8	0.3	20.89
22	110	25	8	0.3	44.396
23	110	5	8	0.7	35.508
24	110	25	8	0.7	44.478
25[a]	110	15	8	0.5	76.657
26[a]	110	15	8	0.5	76.657
27[a]	110	15	8	0.5	69.672

[a] Center points

RESULTS AND DISCUSSIONS

Effects of individual factors on aminoreductone formation. Variables and factor levels of the experimental design

To optimize the variables of AR formation, the key factor affecting the AR formation as well as the range of experimental values must be determined. The mechanism of AR formation in the Maillard reaction in the model solution of lactose and butylamine has been investigated (TRANG *et al.*, 2011). Based on the results of those experiments, the solution of lactose and butylamine at 1:1 in concentration ratio (0.3:0.3 (M)) was the best model system for the formation of AR and was used in our previous studies (TRANG *et al.*, 2011). Thus, we used it in the production of AR in this study. The effects of individual factors in the formation of AR while keeping other variables constant were shown in Fig. 1. In the model solution consisting lactose and butylamine, the extent of the AR formation strongly relied on the heating temperature and heating time (Fig. 1a and 1b). This phenomenon was also similar to our previous studies (SHIMAMURA *et al.*, 2004; TRANG *et al.*, 2011). As soon as the maximum amount of AR was obtained, XTT value decreased. These might be referred to as the progress of the advanced reactions of AR that commonly takes place in the complicated sequences of the Maillard reaction during heating (TRANG *et al.*, 2011) or by the competition of isomerisation/degradation reaction to lactose at the high heating temperature (more than 100°C) leading to the reduction of lactose for the Maillard reaction (BOEKEL, 1998). The maximum value of AR obtained in Fig. 1a and 1b might also change depending on the change of heating time and heating temperature, respectively. Thus, the heating time (with a range of 5-25 min) and heating temperature (with a range of 90-130°C) were chosen as the main factors and their levels for the experimental design on the response surface methodology.

Boekel (1998) suggested that changes in pH can have an effect on reaction rates (BOEKEL, 1998). At the same, heating time and temperature, reactivity of sugar and amino group are also influenced by pH (MARTINS *et al.*, 2001). Similar results were also found in this study (Fig. 1c). In the Maillard reaction of lactose and butylamine

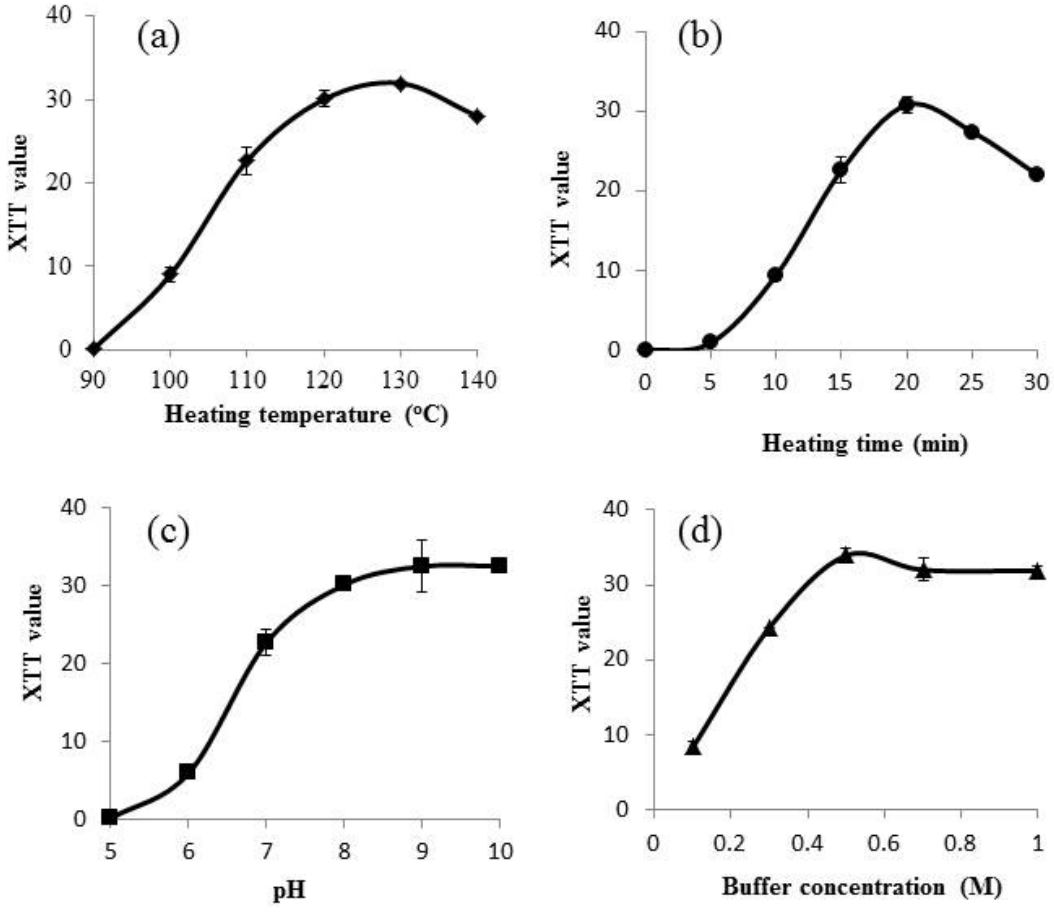

Fig. 1 - Effects of individual factors in the formation of aminoreductone.
The effect of heating temperature (Fig. 1a), heating time (Fig. 1b), pH (Fing. 1c), buffer concentration (Fig. 1d) on the AR formation while other factors were controlled (heating time of 15 min; heating temperature of 110°C; buffer concentration of 0.2 M and pH of 7).

Table 3 - Results of the regression analysis of the Box-Behnken design for Aminoreductone production.

Factor	Variable	Regression coefficient	F-value	P-value (Probability) > F
Model			**10.27**	**0.0001**
	b_0	+ 74.33		
	Linear			
A	b_1	+ 9.42	14.01	0.0028*
B	b_2	+ 6.65	7.00	0.0214*
C	b_3	+ 7.86	9.76	0.0088*
D	b_4	+ 3.44	1.87	0.1960
	Interaction			
AB	b_{12}	- 11.45	6.90	0.0221*
AC	b_{13}	- 0.69	0.025	0.8769
AD	b_{14}	- 2.38	0.30	0.5957
BC	b_{23}	- 0.89	0.042	0.8419
BD	b_{24}	- 3.63	0.70	0.4206
CD	b_{34}	+ 1.42	0.11	0.7503
	Quadric			
A2	b_{11}	- 33.26	77.65	< 0.0001*
B2	b_{22}	- 28.01	55.09	< 0.0001*
C2	b_{33}	- 11.90	9.94	0.0083*
D2	b_{44}	- 10.07	7.11	0.0205*

*, insignificant model terms.

at 110°C, 15 min of heating time, buffer concentration of 0.2M, the formation of AR rapidly reached maximum values from pH 5 to 8 and did not change much at higher pH values. The pH value that was higher, was used. The more complicated equipment design and handling for AR producing was required. Thus, the range of pH value from 7 to 9 for AR production was chosen for experimental design.

Limited data exist on the effects these buffers have on the Maillard reaction and the formation of AR. Besides, buffer agents were added in diverse foods to control the pH of the system (BELL, 1997). To find out the suitable conditions and establish the good model solution for the production of AR, the effect of phosphate buffer concentration in the formation of AR was also investigated. As shown in Fig. 1d, the rates of AR formation increased with the increasing phosphate buffer concentration from 0.1 to 0.5 and slightly decreased with higher concentrations. Similar observations of the increase in the Maillard reaction rate with the increasing buffer concentration was also presented in the model system of glycine and glucose (BELL, 1997). The results obtained in our study indicated that phosphate anion should be used as a catalytic compound for the production of AR. The range of buffer concentration from 0.3 to 0.7 which contains an optimized buffer concentration for AR production was chosen for experimental design.

The parameters of optimization for AR production were investigated using a Box-Behnken design under RSM. Parameters such as, temperature of 110°C, time of 15 min, pH of 8 and buffer concentration of 0.5 were chosen as center points from the above pre-screening on the effect of individual factors for the formation of AR.

Evaluation of aminoreductone formation

The design matrix of the factors is shown in Table 2, along with the experimental response values. Using the software Design Expert, the results of the experiment of AR formation were used to calculate the coefficients of the quadratic polynomial equations, which were used to predict the formation of AR.

The statistical model was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model was summarized (Table 3). As shown in Table 3, the Model's F-value of 10.27 and the p value of 0.0001 ($< \alpha = 0.05$) implied that the model was highly significant in which A, B, C, AB, A2, B2, C2, D2 ($p < 0.05$) are significant model terms (Table 3). Because D2 had a significant effect, the corresponding main effect of D is included in the regression model. After excluding these insignificant effects from the model and rerunning the software Design Expert, the model for AR production might be expressed by:

$Y = + 74.33 + 9.42*A + 6.65B + 7.86*C + 3.44*D - 11.45*A*B - 33.26*A^2 - 28.01*B^2 - 11.90*C^2 - 10.07*D^2$ where Y (yield) is the yield of AR (mM); A, B, C, D is the coded values of the heating temperature, the heating time, the pH and the buffer concentration, respectively; $R^2 = 0.9155$; adjusted-$R^2 = 0.8708$.

The goodness of the model can be checked by the determination coefficient R2 and the adjusted-R2. The determination coefficient R2 of

0.9155 indicated that the model could explain 91.55% of the variance (WANG and LU, 2005). The value of adjusted-R2 closed to R2 and 1, showed the good correlation between the experimental and predicted values. Thus, this model can be used to predict the formation of AR in the Maillard reaction.

Analysis of response surfaces

Three-dimensional response surfaces were plotted on the basis of the model equation by the Design Expert program to investigate the interaction among the variables and to determine the optimum condition of each factor for maximum AR

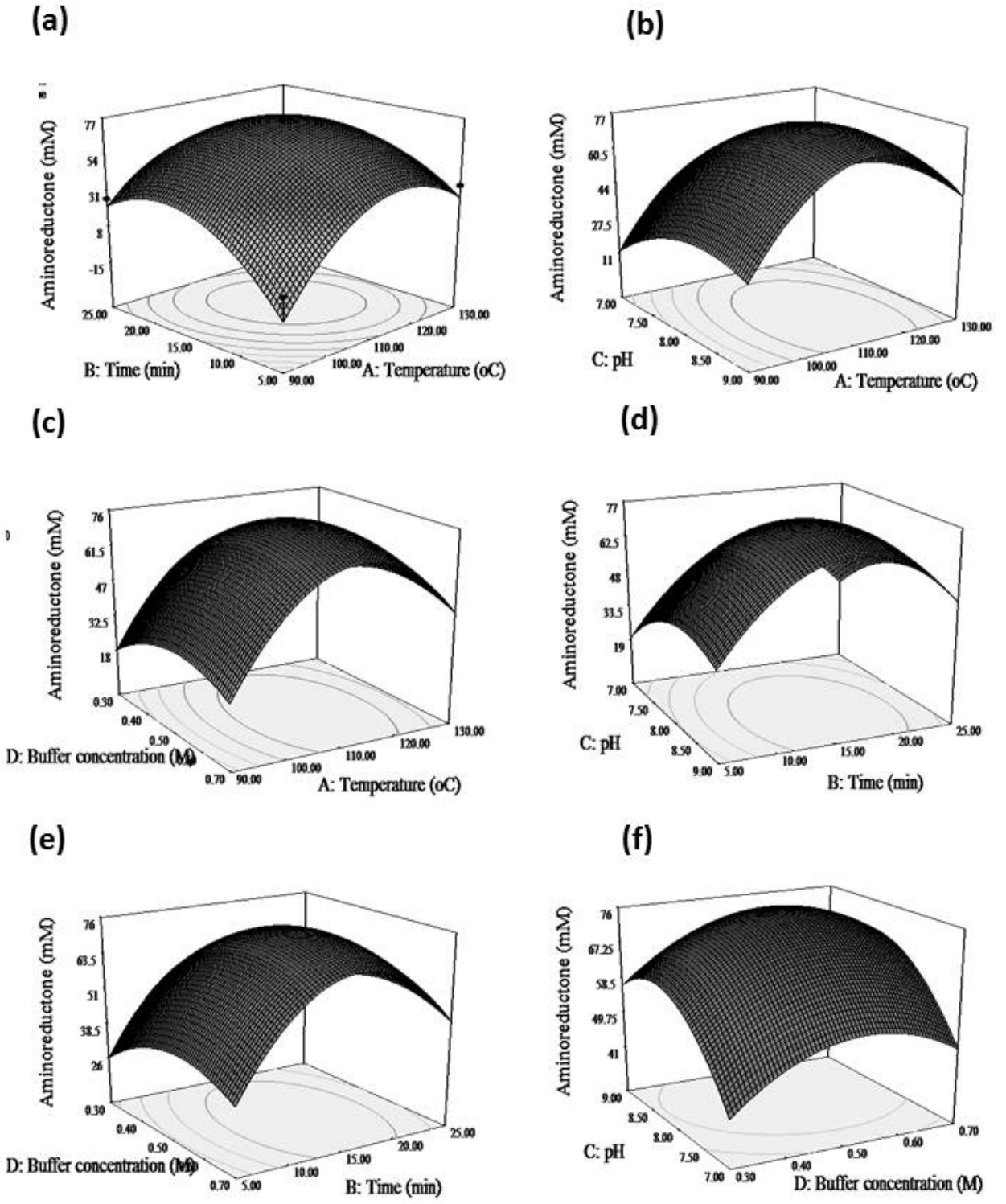

Fig. 2 -Response surface plot for Aminoreductone production.
The interaction between (a) heating time and heating temperature, (b) pH and heating temperature, (c) buffer concentration and heating temperature, (d) pH and heating time, (e) buffer concentration and heating time, (f) buffer concentration and pH.

production in the model solution of lactose and butylamine (Fig. 1). By keeping other variables at their center point values, three dimensional plots of two factors versus the AR formation were drawn (Fig. 2). Perturbation graph showed the effect of each independent factors on AR production while keeping other factors at their respective midpoint levels (Fig. 3). From the response surface (Fig. 2) and perturbation plot (Fig. 3), it is obvious that heating temperature and time had a significant effect on AR production compared with other variables. Although, pH was reported to influence the Maillard reaction (MARTINS *et al.*, 2001), the results of this study indicated an unimportant effect of pH on the formation of AR. The perturbation graph clearly showed that the two variables (buffer concentration and pH) did play any significant role in the AR production.

Optimization of conditions for aminoreductone formation

Based on the analysis of the response surface of the regression equation, the optimum process parameters were found to be 112.85°C for heating temperature 15 min for heating time with pH 8.33 and buffer concentration 0.53 M resulting in the predicted maximum AR formation which was 76.6 mM.

Validation of the models

The trail experiments were conducted under optimized process conditions with temperature of 112.8°C, heating time of 15 min, pH of 8.3 and buffer concentration of 0.5 M. The results of AR formation were founded to be 75.76 ± 0.02 mM, which was very close to the predicted AR formation obtained from the regression equation (76.6 mM). Thus, the model could be used to predict the AR content formed in the food during heating; and to find the suitable heating, condition (temperature and time) that favored the formation of aminoreductone in the specific food system which the pH and food components were clarified.

The chemistry underlying the Maillard reaction is very complex. It encompasses not only one reaction pathway, but a whole network of various reactions with thousand products (MARTINS *et al.*, 2001). By applying the RSM, the optimum process parameters for AR production were found. These were both helpful for studying further the application of AR in the health and medical fields, as well as providing provide useful information for identifying the technological conditions that favors the formation of AR as a functional ingredient in food.

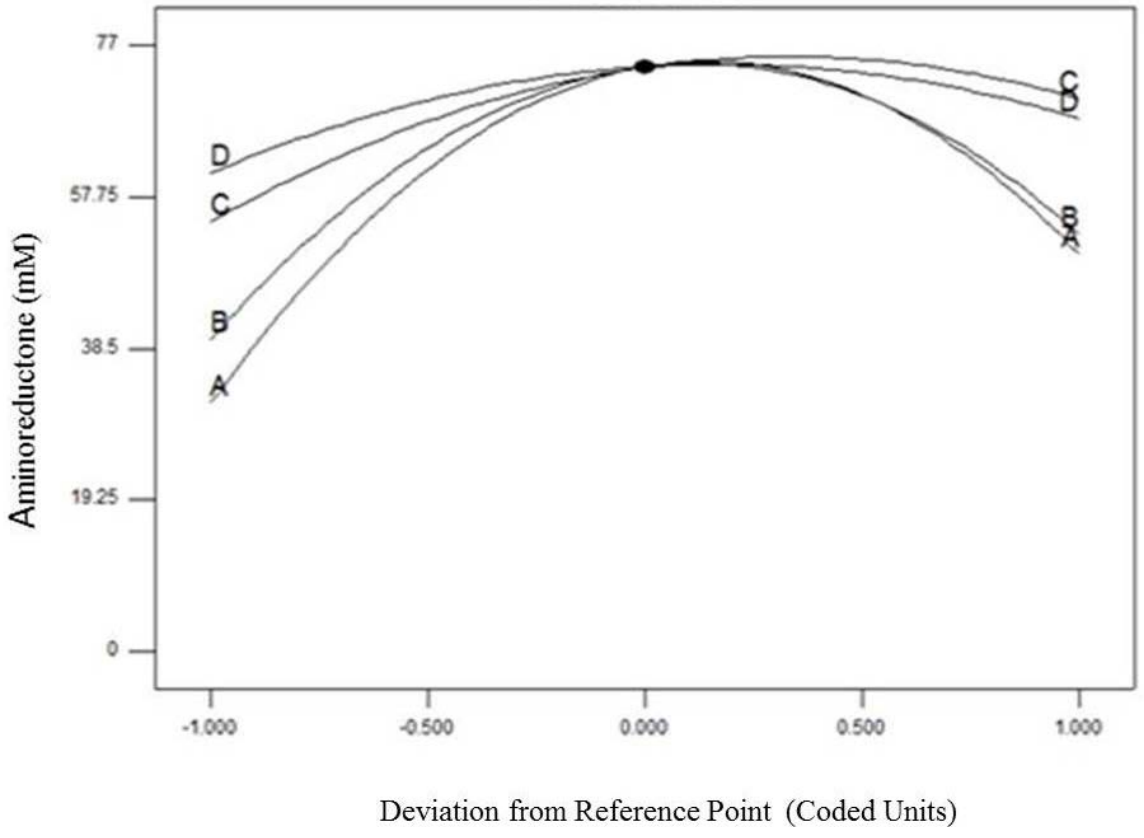

Fig. 3 - Perturbation graph showing the effect of each independent factors on Aminoreductone production while keeping other factors at their respective midpoint levels.
(A) heating temperature, (B) heating time, (C) pH, (D) buffer concentration.

CONCLUSIONS

In this study, RSM, Box-Behnken design was used to model and establish a regression equation between the response (AR formation) and four statistically significant factors: the heating time, the heating temperature, the pH, the buffer concentration. In four variables, the two factors of heating time and heating temperature showed the effect on the AR formation. Finally, the optimal solutions were sought on the basis of the influence of the four parameters in the formation of AR in the Maillard reaction. Predicted values obtained using the model equations were in agreement with the observed values. Heating time of 15 min, heating temperature of 112.85°C, pH of 8.33 and buffer concentration of 0.53 has been determined as optimum levels of the process parameters to achieve the maximum amount of AR formed in the Maillard reaction of lactose and butylamine. These optimum conditions were used to evaluate the trail experiment and the maximum yield of AR formation was recorded as 75.76 mM. The results indicated that optimization using RSM can be useful to control and predict the production of AR in the Maillard reaction. The results obtained from this study would be applied in AR production for therapeutic application as an efficient source of antimicrobial agents, an antioxidant compound in functional food.

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