

Optimization of the Process Variables for Minimizing of the Aflatoxin M1 Content in Iranian White Brine Cheese

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ABSTRACT

A model capable of predicting the minimum content of Aflatoxin M₁ (AFM₁) in Iranian white brine cheese has been developed using a chemometric approach to determine the optimum processing conditions. Renneting temperature, cut size, stirring time, press time, curd size and saturated brine pH were all regarded as process variables. Three-dimensional response surface and contour plots were drawn. The minimum content of AFM₁ (116.9 ng kg⁻¹ dry matter) was predicted when the processing variables were: Renneting temperature= 40°C, Cut size= 0.5 cm, Stirring time= 10 minutes, Press time= 20 minutes, Curd size= 64 cm³, Saturated brine pH= 4.6. These values predicted for optimum process conditions were in good agreement with experimental data.

Keywords: Aflatoxin M₁, Chemometrics, Minimization, White brine cheese.

INTRODUCTION

Aflatoxins are a group of structurally related toxic compounds that belong to the group of mycotoxins. Aflatoxins are mainly produced by three species of mould, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* that may grow on a number of crops. Aflatoxin M₁ (AFM₁) and Aflatoxin M₂ are oxidative metabolic products of aflatoxin B₁ and B₂ produced by animals and are usually excreted in the milk of dairy cattle that have consumed aflatoxin contaminated feed. Aflatoxins have toxic and carcinogenic effects on humans and animals (FAO/WHO, 1998; Rothschild 1992).

A large number of studies have been carried out on the occurrence of AFM₁ in different types of cheeses. However, contrasting data have been reported on the influence of processing variables on AFM₁ re-

covery and its distribution between cheese and whey. Some authors observed that half or more of the AFM₁ was in the whey- 50%, 50%, 61%, 66%, and 100%, according to Grant and Carlson (1971), Stubblefield and Shannon (1974), Wiseman and Marth (1983a), Blanco *et al.* (1988), and Purchase *et al.* (1972), respectively. In contrast, others reported that most of the AFM₁ was with the curd ranging from 66% to 72%, from 73% to 77%, 80%, and 100% according to Marshaly *et al.* (1986), El Deeb *et al.* (1992), McKinney *et al.* (1973), and Allcroft and Carnaghan (1963), respectively.

According to Blanco *et al.* (1988) these contrasting results can be ascribed to different factors such as extraction technique, methodology, the type and degree of milk contamination, differences in milk quality, expression of the results, and the presence of a small portion of curd in whey, all of

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which could influence AFM1 concentration and the cheese manufacturing process.

However, this discrepancy in the results may be partially ascribed to the traditional approach to experimentation. The traditional approach is to change only one process factor at a time (OFAT), or one component in a formulation, and does not provide data on the interactions of factors (or components), a likely occurrence with food formulations and processes. Statistically based design of experiments (DOE) is a standard chemometric tool that provides validated models, including any significant interactions, for confident prediction of response as a function of the process factors. Using this tool, the simultaneous evaluation of processing factors can be achieved more efficiently than with the traditional one-factor-at-a-time (OFAT) approach.

In Iran, white brine cheese (Feta type) is a major item in the diet and, unfortunately, recent studies have revealed high occurrence of AFM1 in Iranian Feta cheese. Kamkar (2006) found that almost 82.5% of the cheese samples were contaminated and 60.6% of contaminated samples exceed the maximum tolerance limit ($0.25 \mu\text{g kg}^{-1}$) accepted by European Union. Obviously, this reflects high levels of contamination in raw milk and, due to the high heat stability of AFM1, the cheese making process must be modified and attention directed to the conditions that stimulate AFM1 transfer from curd to whey.

It is evident from the literature that no work has been reported so far for optimization of the process variables for white brine cheese. The present investigation was, therefore, undertaken to optimize the process variables, viz. renneting temperature, cutting size, stirring time, press time, curd size and saturated brine pH. This was aimed towards reduction of AFM1 in Iranian white brine cheese using a chemometric approach. A second-order model was used to generate contour plots for the extent of AFM1 in cheese curds.

MATERIALS AND METHODS

Cheese making

The brine cheese was manufactured for this study according to the method used in Iranian cheese making plants. White brined cheese was prepared from cow's milk. The milk was pasteurized at 72°C for 15 seconds and cooled to $32\text{--}35^\circ\text{C}$. CaCl_2 was added at a level of 150 mg kg^{-1} of milk followed by the addition of 1% starter culture (R-704, Chr, Hansen's Dairy Cultures, Denmark) 30 minutes before renneting. Cultures of *Streptococcus salivarius subsp. Thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were used as a starter. Commercial powdered microbial rennet (ChyMax, Chr, Hansen's Inc., Denmark: 183 International Milk Clotting Units (IMCU) ml^{-1}) was added to coagulate the milk samples. Coagulated milks were cut at two sizes (0.5 and 1 cm) and then stirred two times (at 10 and 20 minutes). The curds were pressed by using weights two times (at 1 and 2 hours) and were then cut into rectangular shapes of two sizes (64 and 256 cm^3) and soaked in sterile brine (22%, w/v) with two adjusted pH (4.6 and 6) for 16 hours. The curd pieces were then used for aflatoxin M1 analysis.

Experimental Design

The study is based on the hypothesis that the extent of AFM1 is functionally related to process variables, and attempts to fit a multiple regression equation describing the response, i.e. AFM1 content. Table 1 lists variables in a descending order of assumed importance as process variables.

A 2-level D-optimal fractional factorial design with resolution V was used. Resolution V designs will allow for accurate estimation of the main effects and two-factor interactions. The six factors (processing variables), levels and experimental design are given in Table 2.

Table 1. Variables and their levels for experimental design.

Independent Variables	Symbols		Levels	
	Coded	Actual	-1	+1
Renneting temperature, °C	^a <i>a a</i>	x_1	30	40
Cut size, cm	^b <i>b b</i>	x_2	0.5	1
Stirring time, min	^c <i>C c</i>	x_3	10	20
Press time, h	^d <i>d d</i>	x_4	1	2
Curd size, cm ³	^e <i>e e</i>	x_5	64	256
Saturated brine pH	^f <i>f f</i>	x_6	4.6	6

^a $(x_1 - 35) / (5)$; ^b $(x_2 - 0.75) / (0.25)$; ^c $(x_3 - 15) / (5)$; ^d $(x_4 - 15) / (5)$; ^e $(x_5 - 160) / (96)$; ^f $(x_6 - 5.3) / (0.7)$.

The aflatoxin contents of the raw milk samples used for cheese making are regarded as covariate and ranged from 43 to 59 ng L⁻¹.

Evaluation of AFM1

Determination of AFM1 was based on an enzyme-linked immunoassay using the Iscreen test kit (Tecna, Italy). This method is quick, reliable and cost effective for the estimation of AFM1.

Most of the reagents used were contained in the Iscreen test kit. AFM1 standard solutions used for the construction of the calibration curve were at levels of 0, 5, 10, 25, 50, 100 and 250 ng L⁻¹ and all included in the ELISA test kit.

Preparation of the samples was conducted according to the instructions for the Iscreen kit. Briefly, milk samples were chilled and then centrifuged for 10 minutes at 3,500 rpm (Heraeus Megafuge 1.0). One hundred µL of the skimmed milk was diluted with 400 µL of the sample diluent. An aliquot (100 µl per well) of this solution was used directly in the test. Curd and cheese samples (2 g each) were homogenized (Ultraturrax, IKA-Werke) and extracted with 15 mL dichloromethane. The suspension was then filtered and 3.75 ml of the extract was evaporated under a mild stream of nitrogen. The oily residue was redissolved in 750 µL extraction

buffer and was mixed thoroughly for one minute. After centrifugation for 15 minutes at 2,700 rpm, 50 µL of the methanolic phase was diluted with 200µl of dilution buffer and mixed gently. An aliquot of this solution was used in the test (100 µL well⁻¹).

Aflatoxin M1 standards or the prepared sample solutions were added to microtiter wells in duplicate. During incubation for 45 minutes at room temperature in the dark, the antibody binding sites were occupied proportionally to the aflatoxin M1 concentration. The liquid was then removed completely from the wells, which were washed four times with washing buffer. In the next step, any remaining free binding sites were occupied by the enzyme conjugate, which was added (100 µL) and incubated for 15 minutes at room temperature in the dark. Any unbound enzyme conjugate was then removed in a washing step. Developing solution (100 µL) was added to each well and incubated for 15 minutes at room temperature in the dark. Bound enzyme conjugate converts the colorless chromogen into a blue product. Then the addition of the stop reagent (500 µL per well) led to a color change from blue to yellow. The measurement was made photometrically at 450 nm.

In order to obtain AFM1 actual sample concentration in ng L⁻¹ for milk and ng kg⁻¹ for cheese, the concentration read from the calibration curve was further multiplied by a dilution factor 5 for milk and a dilution fac-

**Table 2.** Two-level fractional factorial design used to evaluate the effects of process variables on the aflatoxin M1 content of Iranian white brine cheese.

Run	Variable level						Response AFM1 (ng kg ⁻¹)
	A	B	C	D	E	F	
1	-1	-1	-1	-1	-1	1	212.011
2	-1	-1	-1	-1	1	-1	213.6846
3	-1	-1	-1	1	-1	-1	157.1293
4	-1	-1	1	-1	1	1	205.6766
5	-1	-1	1	-1	-1	1	137.2198
6	-1	-1	1	1	1	-1	147.2181
7	-1	1	-1	-1	1	1	208.4942
8	-1	1	-1	-1	-1	1	166.4252
9	-1	1	-1	1	1	-1	154.6239
10	-1	1	1	-1	-1	-1	160.7627
11	-1	1	1	-1	1	1	167.9522
12	-1	1	1	1	1	1	168.8892
13	1	-1	-1	-1	1	1	136.9447
14	1	-1	-1	-1	1	1	144.3451
15	1	-1	-1	1	-1	1	122.2758
16	1	-1	-1	1	1	-1	124.4093
17	1	-1	1	-1	-1	-1	133.5399
18	1	-1	1	-1	1	1	144.4958
19	1	1	-1	-1	-1	-1	148.3947
20	1	1	-1	-1	1	1	163.2476
21	1	1	1	-1	-1	-1	132.919
22	1	1	1	-1	-1	1	161.3869
23	1	1	1	-1	1	-1	143.4111
24	1	1	1	1	-1	-1	136.2858

tor 7.5 for cheese. Finally, to obtain AFM1 concentration in cheese samples on dry basis (ng kg⁻¹ of dry matter), the dry matter percentage of the cheese samples was multiplied by the concentration of AFM1. Dry basis AFM1 concentrations were used for statistical analysis.

Determination of Cheese Dry Matter

Dry matter of cheese samples were obtained by Iran's standard 1735 (ISIRI, 2003) for cheese and processed cheese.

Data Analysis

Multiple regression analysis was conducted to fit the model represented by the equation to the experimental data. Maximization or minimization of the polynomial thus fitted was then performed using nu-

merical methods.

Statistical analysis of the data was performed using MINITAB Statistical Software, Release 13.1. A Half-Normal plot and other plots were created using Statistica version 6.0 (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Diagnostic Checking of the Fitted Model

Linear and interaction effects can be represented graphically on a Half-Normal probability plot (Figure 1). This plot can be used to choose significant effects. A plot of the ordered values of a sample versus the expected ordered values from the true population will approximate to a straight line. Thus, if the effects represent a sample from a normal population, we would expect to see them form an approximate straight line on a

normal probability plot of the effects. Usually, only a few effects turn out to be significant. They show up as outliers on the normal probability plot.

As shown in Figure 1, renneting temperature (A), press time (D) and saturated brine pH (F) had the most significant main effects on the aflatoxin M1 contents of cheese. Among the interactive effects, the interaction of renneting temperature with press time (AD), interaction of renneting temperature with cut size (AB) and interaction of renneting temperature with stirring time (AC) are the most significant. The aflatoxin M1 content of raw milk samples (43 to 59 ng L⁻¹) used for cheese making had not significant effect on the aflatoxin M1 content of cheese samples ($P < 0.05$).

A full factorial polynomial model was fitted to the aflatoxin M1 content of the cheese curds. Where possible, stepwise deletion of terms was applied to remove the statistically non-significant terms, hence simplifying the model. However, when the exclusion of such terms from the model decreases R^2 (ad-

justed) and increases the estimator of the variance S , the term was included in the model. The estimated regression coefficients of the polynomial model were as follows:

$$y = 158.85 - 16.26 \times A + 3.21 \times B - 2.93 \times C - 11.69 \times D + 4.68 \times E + 7.18 \times F + 6.92 \times A \times B + 4.66 \times A \times C + 8.86 \times A \times D + 3.60 \times B \times D + 4.47 \times B \times F$$

where y is aflatoxin M1 content of the cheese curds (ng kg⁻¹ of dry matter); A, B, C, D, E and F are processing variables at their coded levels. Regression analyses of the fitted model indicated that this model accounted for more than 93.19% of the variations in the experimental data, which were found to be highly significant.

Analysis of a Variance

When a model has been selected, an analysis of variance is calculated to assess how well the model represents the data. An analysis of variance for the response is presented in Table 3. To evaluate the goodness

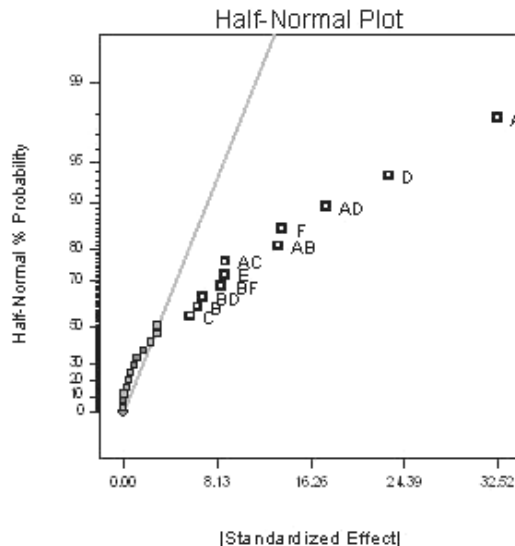


Figure 1. Half-Normal probability plot of effects.

Table 3. Analysis of variance for the proposed model.

Response	Source of variation	df	Sum of Squares	Mean Squares	F-value
Aflatoxin M1 content	Regression	11	16354.39	1486.76	29.62**
	Residual	12	602.41	50.20	
	Total	23	16956.80		



of fit of the model, an F -value test was conducted. The F -value for AFM_1 was 29.62. On this basis, it can be concluded that the selected model adequately represents the data for AFM_1 content in the cheese curds.

From analysis of the residuals, it is possible to conclude that they were randomly distributed around zero, and there was no evidence of outliers.

Conditions for Optimum Response

The model was useful in indicating the direction in which to change variables in order to minimize the extent of AFM_1 . The optimum conditions for yielding a minimum content of AFM_1 are presented in Table 4.

The minimum value of AFM_1 was found to be 116.9 ($ng\ kg^{-1}$ dry matter). The plots shown in Figures 2-5 are based on the afore-said model for AFM_1 (Equation 1) with four variables kept constant at the optimum level, and varying the remaining two within the experimental range.

As shown in Figure 2, aflatoxin M1 was decreased by increasing renneting temperature (A) from 30 to 40°C. By decreasing cutting size (B), the aflatoxin M1 quantity in cheese samples decreased and this reduction was more significant at elevated renneting temperatures. At a low cutting size (0.5 cm), increasing the renneting temperature from 30 to 40°C resulted in a 24.52% reduction in AFM_1 content while, at high cutting size (1 cm), the value of reduction was about

Table 4. Optimum conditions for minimum extent of AFM_1 .

Process variables	Coded values	Uncoded values
Renneting temperature, °C	+1	40
Cut size, cm	-1	0.5
Stirring time, min	-1	10
Press time, min	+1	20
Curd size, cm^3	-1	64
Saturated brine pH	-1	4.6

Minimum value of $AFM_1 = 116.9\ ng\ kg^{-1}$ of dry matter

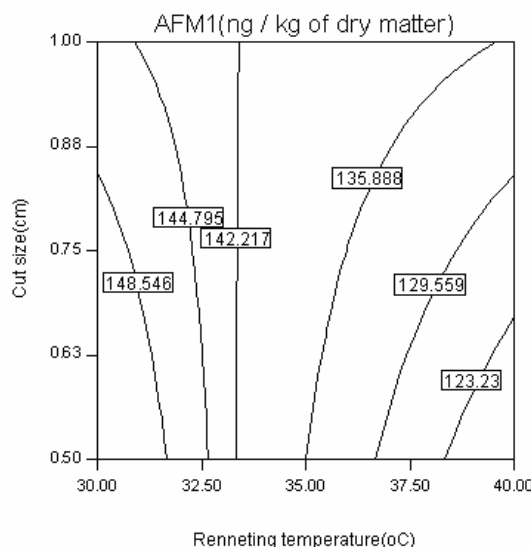


Figure 2. Contour plot showing the effect of renneting temperature (A) and cut size (B) on the aflatoxin M1 content of Iranian white brine cheese. The numbers inside the contours represent aflatoxin M1 content ($ng\ kg^{-1}$ of dry matter) of cheese samples.

7.05%. AFM₁ is a water-soluble compound (Van Egmond and Paulsch, 1986) and increasing of renneting temperature may increase its solubility in whey partition which, in turn, can reduce the AFM₁ content of the curd. Additionally surface area: volume ratio increases with reduction of cutting size, which, in turn, increases the flux of aflatoxin from curd to whey.

Figure 3 shows that aflatoxin M₁ decreases by increasing press time (D) and those cheese curds with high renneting temperatures pressed for a long time had significantly lower aflatoxin M₁ content. This effect can be attributed to the high levels of syneresis in curds that coagulated at high temperatures (Walstra *et al.*, 1999).

As shown in Figure 4, the cheese samples with low saturated brine pH (F) and a small cut size (B) had significantly lower aflatoxin M₁ and increasing the cut size at high saturated brine pH leads to increased aflatoxin M₁ content. However, at a low level of saturated brine pH, increasing the cut size had no effects on aflatoxin M₁ content. Aflatoxin M₁ is stable in an acidic environment (Wiseman and Marth, 1983b), so its reduc-

tion by lowering pH can not be attributed to the degradative effect pH. Aflatoxin M₁ can be associated with the casein molecule by means of hydrophobic bonds (Dosako *et al.*, 1980) and lowering of saturated brine pH may enhance the release of aflatoxin M₁ from the casein molecule. Furthermore, it is known that syneresis increases with a lowering of pH (Sundaram Gunasekaran and Ak, 2003) and high levels of whey as well as aflatoxin M₁ removed from curds.

As shown in Figure 5, aflatoxin M₁ content in the cheese samples with a high renneting temperature was not influenced by stirring time (C) but in the cheese samples with a low renneting temperature, aflatoxin M₁ content decreased with increasing the stirring time. It is known that cheese curds made at low temperatures show low syneresis and have a soft texture. Therefore, extending stirring time provides for a high release of whey as well as AFM₁ from the curds. However, curds formed at high renneting temperatures are firm due to the high level of initial syneresis and, therefore, extending stirring time in these curds has no significant effect on AFM₁.

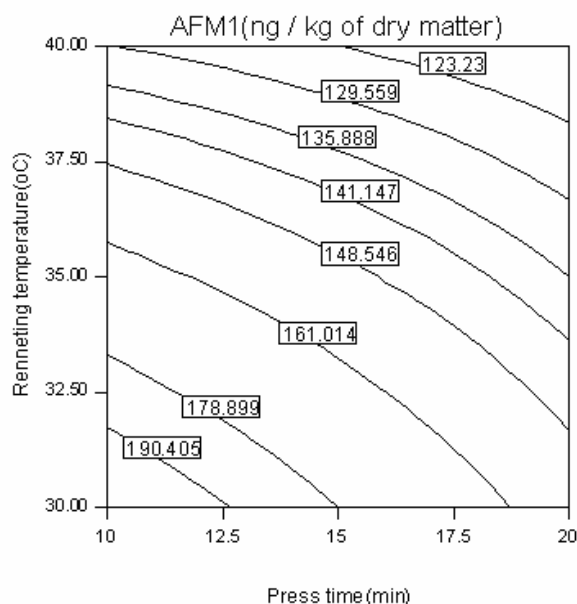


Figure 3. Contour plot showing the effect of renneting temperature (A) and press time (D) on the aflatoxin M₁ content of Iranian white brine cheese. The numbers inside the contours represent aflatoxin M₁ content (ng kg⁻¹ of dry matter) of cheese samples.

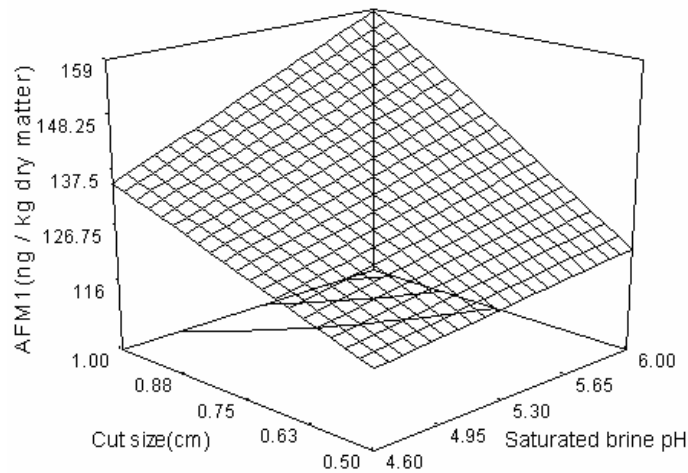


Figure 4. Surface plot showing the effect of saturated brine pH (F) and cut size (B) on the aflatoxin M₁ content (ng kg⁻¹ of dry matter) of Iranian white brine cheese.

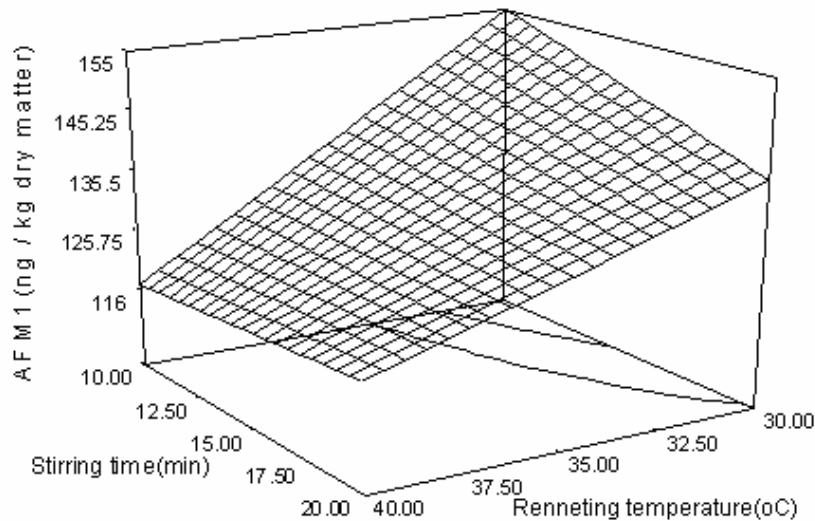


Figure 5. Surface plot showing the effect of stirring time (C) and renneting temperature (A) on the aflatoxin M₁ content (ng kg⁻¹ of dry matter) of Iranian white brine cheese.

CONCLUSION

The results showed that among the six studied processing variables, three variables (renneting temperature, press time and saturated brine pH) were the most significant factors affecting the aflatoxin M₁ content of Iranian white brine cheese. Aflatoxin M₁ content decreased with increasing the renneting temperature and press time. Lowering

the saturated brine pH also reduced the aflatoxin M₁ content of the cheese samples.

Taking account of all of the factors studied and based on numerical analysis of the statistical model, the optimum processing conditions for minimization of Aflatoxin M₁ in cheese curds were: Renneting temperature= 40°C, Cut size= 0.5 cm, Stirring time= 10 minutes, Press time= 20 minutes, Curd size= 64 cm³ and Saturated brine pH= 4.6.

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بهینه‌سازی متغیرهای فراوری به منظور کمینه کردن آفلاتوکسین M_1 در پنیر سفید ایرانی

ح. محمدی، م. علیزاده، م. ر. باری، ا. خسروشاهی و ح. تاجیک

چکیده

با استفاده از روشهای کمومتری مدلی برای پیش‌بینی حداقل مقدار آفلاتوکسین M_1 در پنیر سفید ایرانی ساخته شد و بر مبنای آن شرایط بهینه فراوری تعیین گردید. دمای رنت زنی، اندازه برش، زمان بهم زدن، زمان پرس، اندازه لخته و pH آب نمک اشباع به عنوان متغیرهای فراوری در نظر گرفته شدند. نمودارهای سه بعدی سطح پاسخ و کانتورپلاتها رسم گردیدند. حداقل مقدار آفلاتوکسین M_1 (۱۱۶/۹) نانوگرم در کیلوگرم ماده خشک (زمانی حاصل شد که متغیرهای فراوری در مقادیر زیر تنظیم گردیدند: دمای رنت زنی = ۴۰ درجه سلسیوس، اندازه برش = ۰/۵ سانتیمتر، زمان بهم زدن = ۱۰ دقیقه، زمان پرس = ۲۰ دقیقه، اندازه لخته = ۶۴ سانتیمتر مکعب و pH آب نمک اشباع = ۴/۶).