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Optimization of conditions for milk-clotting enzymes extraction from *Solanum aethiopicum* seeds through response surface methodology

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ABSTRACT

S. aethiopicum is a cultivated plant, known over the West and Central Africa. Its seeds have proteinases which possesses activity (MCA). In this work, response surface methodology was used to optimize the extraction of milk-clotting enzyme (MCE) according to its adequacy to be employed as substitute of rennin. Box-Behnken design was applied to evaluate the effects of three independent variables, namely NaCl concentration, extraction duration and pH on extraction efficiency (EE) of MCE. Results indicated that the actual data were satisfactorily fitted into second-order polynomial model. The independent variables, the linearity and quadratics of NaCl concentration, extraction duration and pH as well as interactions between NaCl concentration and extraction duration had a significant effect on EE of MCE. The optimal conditions to obtain the highest extraction efficiency of MCE from S. aethiopicum seeds were 5.5 % (w/w) of NaCl concentration, 27 h of extraction time and pH 5.3. Under these conditions, the experimental values agreed with the predicted ones. The results in this work were helpful for preparation of crude enzymes from S. aethiopicum plant in view to use it in cheese technology.

Key Words: extraction efficiency, milk-clotting enzyme, optimization, response surface methodology, *S. aethiopicum*

INTRODUCTION

Milk-clotting enzyme is the first active agent in cheese-making. Rennin is the most widely used in the world. The worldwide increase of cheese production, the incidence of bovine spongiform encephalopathy, and the prohibition of recombinant calf rennet by many countries, have reduced both supply and demand of calf rennet [1]. Moreover, in developing countries such as Cameroon, slaughtering of young ruminants is forbidden. Thus, calf rennet becomes shortage and costly for local farmers. That has led to an increase in the demand for alternative sources of coagulant [2]. Results on new proteinases from plant for milk-clotting revealed its growing interest for the dairy technology [1]. The plant coagulants are used at artisanal scale and farm-house; they are easily

used in cheese-making. It is an interesting mean for dairy farmers in developing countries. The use of plant proteinases as milk coagulants is very fascinating, because they are natural enzymes and can be used to produce cheese for lacto-vegetarian consumers and ecological markets [3]. In Iberian Peninsula, the plant coagulants from *cynara cardunculus*, *Cynara humilis* and *cynara scolymus* have been used for centuries in cheese-making [4]. Recently, in Africa, enzymes from *Solanum dobium* berries were used to make the soft white cheese [5-6-7]. Berries of similar species, *S. aethiopicum* possessed also proteinases with milk-clotting activity [8].

S. aethiopicum (fig.1.) is taxonomically belonging to the *Solanaceae* family, and *Solanum* genus. It is an annual plant that grows in vast areas of Cameroon. It is a woody herb with a solid erect stem, black in color and about 150 to 180 cm high. The leaves are alternate, long petiole, simple, ovate, acuminate at the apex and pale green in color, while the rootlets are brown; the roots are about 10 mm thick and 25 cm long. The white flowers are hermaphrodite. The berries are grouped in clusters to one side of the stem or to the branch. Its fruits are round in shape, being 1.5 cm in diameter. Unripe fruits are green, while the mature fruits are red. The seeds are flat at the edges circular at the sides, clear in color and about 3 mm in diameter [9-10]. The unripe and ripe fruits are eaten, cooked or uncooked as tomato; while leaves are consumed like black nightshade [9-11].

It was reported that ripe fruits of *S. aethiopicum* could be a source of milk-clotting enzymes used as an alternative to rennin [8]. Therefore, taking *S. aethiopicum* fruits to prepare coagulant for cheese-making is an interesting attempt for local dairy farmers: this coagulant is natural, easily produced, available and cheap. Enzyme preparation from *S. aethiopicum* berries can be used as rennin substitute, only if the preparation releases proteinases which possess high milk-clotting activity (MCA) and a low general proteolytic activity (PA). The ratio of MCA to PA is used as a suitability index for an enzyme in cheese-making [12]. The measure of this index indicates the optimum extraction conditions: it is the extraction efficiency (EE) of milk-clotting enzymes (MCE).

Nomenclature

ANOVA	Analysis of variance	R^2	The coefficient of determination
BBD	Box-Behnken Design	RSM	Response Surface Methodology
CV	Coefficient of variation	*S.*	*Solanum*
EE	Extraction efficiency	X_1	Sodium chloride concentration (w/v)
MCA	Milk-Clotting Activity (U/mL)	X_2	Extraction duration (h)
MCE	Milk-clotting enzymes	X_3	pH
PA	Proteolytic Activity (U/mL)	Y	Extraction efficiency of MCE

The extraction of milk-clotting enzymes from *S. aethiopicum* berries can be affected by many factors, such as part of berries, extraction solutions, time of extraction, and pH among others. The effects of these parameters were emphasized [8]; however, the information on interactions among them was insufficient.

Such interactions, if significant, will be used to optimize the variables; in order to identify optimal and operational conditions for a suitable coagulant from *S. aethiopicum* berries. Response surface methodology (RSM) is a statistical method that uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equations [13]. This experimental methodology can generate a mathematical model and optimize the process levels [14]. So far, available publications on extraction of MCE with RSM are very limited.

The objective of this work was to investigate the effects of sodium chloride, duration and pH on the EE of MCE, and to optimize these parameters with consideration to the response by RSM.

Fig.1. Berries of *S. aethiopicum*

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant material

The ripe fruits of *S. aethiopicum* (fig.1.) were purchased locally. The berries were sorted, dried, cleaned, divided into berry flesh-coats and berry seeds, disinfected with sodium hypochloride (10% v/v) [15]. Berry seeds were utilized as raw material for extraction of crude milk-clotting enzymes.

2.1.2. Coagulation substrate

Raw skimmed milk of zebu (*Bos indicus*) was used as coagulation substrate. Milk was purchased from the Ngaoundere Station of Cattle Farming and Milk Production. It was skimmed by centrifugation at 3,000 g and 30°C for 30 min. This skimmed milk was stored at 4°C with sodium azide (0.2 g/L) added as preservative.

2.1.3. Zebu whole casein

The skimmed milk was precipitated at pH 4.6 with 1M HCl. The precipitate was washed three times with distilled water. It was solubilized at pH 7.0 with 1M NaOH and dialyzed against distilled water at 4°C for 24 hours [16]. This casein was used as substrate to determine the proteolytic activity.

2.1.4. Chemicals

Sodium chloride, acetic acid, sodium hydroxide, sodium acetate, trichloro-acetic acid, sodium phosphate and potassium phosphate were from Sigma Chemical Co (St. Louis, Mo, USA). The rest of chemicals were of analytical grade.

2.2. Methods

2.2.1. Extraction of milk-clotting enzymes

10 g of *S. aethiopicum* seeds was immersed into 100 mL of the extraction solution (using different concentrations of NaCl in acetate buffers). NaCl concentration (3 to 7 % w/v), extraction duration (h) and pH (0.1 M of acetate buffers pH 5.0 to 6.0) were designed according

to the preliminary results. The extraction processes were performed at 4°C in the refrigerator for 12 to 36 h. Every extract was filtrated using whatman No. 1 filter paper and maintained at 4°C, then used for determination of MCA and PA.

2.2.2. Determinations

2.2.2.1. Milk-clotting activity

The milk-clotting activity of each enzyme preparation from *S. aethiopicum* was determined following the procedure described by International Dairy Federation [17]. Extracts were added at a proportion of 1 mL per 10 mL of the skimmed milk. The clotting point was determined by periodic manual rotating of the test tube, at very short time intervals. The clotting time was recorded when discrete particles were discernable. One milk-clotting unit (U) was defined as the amount of protein that coagulates 10 mL of skimmed milk at 30° C in 100 s [18]. The milk-clotting activity of each extract was measured, assuming that all the soluble proteins from the extract were enzymes which clot milk at 30° C.

MCA (U/mL) = (100/CT) x S/E

CT: the clotting time (s); S: the zebu raw skim milk (mL); E: the enzyme volume (mL).

2.2.2.2. Proteolytic activity

Proteolytic activity was determined according to Silva and Malcata procedure [18], with some modifications. Casein 1% (w/v) was subjected to hydrolysis at 30°C in 100 mM phosphate buffer (pH 6.7). The hydrolysis was initiated by the addition of 1mL of each extract to 10 mL of casein solution. The reaction was stopped after 1 h by heating at 100°C for 5 min. The proteolytic activity was quantified by evaluating the soluble peptides in 6% (w/v) trichloroacetic acid (TCA). 1mL of each sample was treated with 5% (w/v) TCA at a volumetric ratio of 1:2; the mixture was allowed to settle for 10 min, and then centrifuged at 7,500g for 30 min. The absorbance of supernatant was measured at 280 nm. An appropriate control was prepared, in which the TCA was added before the extract. One unit of proteolytic activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 280 nm, under the assay conditions. Proteolytic activity was as follows:

P.A. (U/mL) = (ΔAbs_{280nm} x 10 x dilution factor)/ (E x t).

*Where $\boldsymbol{\Delta Abs_{280nm}}$ is the variation of absorbance between assay and control, **E** is the volume of crude enzyme solution; **t** is the time of reaction.*

2.2.2.3. Extraction efficiency of MCE

The EE of MCE is a suitability index of an enzyme preparation to be used as alternative of rennin [12]. It is calculated by the ratio of milk-clotting activity (MCA) to proteolytic activity (PA).

2.2.3. Experimental design

The experiments were designed according to the Box-Behnken Design (BBD) [19] with five replicates at the central point, to determine the response pattern and then to establish a model [20].Three independent variables used in this study were (X_1) sodium chloride concentration (% w/v), (X_2) extraction duration (h) and (X_3) pH, with three levels for each of them. The selection of ranges within which each factor varies, was based on preliminary experimental

results and literature data [21]. The independent variables, symbols, levels and real values of independent variables are shown in table 1. Response (EE of MCE) at each design point was recorded in table 2. The experiments were randomized to minimize the effects of unexplained variability in the observed responses due to extraneous factors.

Table 1- Independent variables and their levels employed in central composite design

Independent variable	Symbol	Level	Real value
NaCl concentration (%)	X_1	-1 0 1	3 5 7
Extraction duration (h)	X_2	-1 0 1	12 24 36
pH	X_3	-1 0 1	5.0 5.5 6.0

Conversion from coded variable to dimensionless variable is given by the following equation:

$X_i = \Delta X_i.x_i + X_0, \qquad x_i = 1, 2, 3$ (1).

Where $\mathbf{x_i}$ and $\mathbf{X_i}$ are the coded and real value of the independent variable **i**, $\mathbf{X_0}$ the real value of the independent variable **i** at the central point and $\mathbf{\Delta X_i}$ the step change of $\mathbf{X_i}$ corresponding to a unit variation of the coded value.

Table 2 – Box-Behnken Design arrangement and response

	Variable levels[a]			Responses[b]
Run	X_1	X_2	X_3	Y
1	-1	1	0	8.68
2	1	-1	0	7.75
3	0	0	0	11.4
4	0	0	0	11.79
5	1	1	0	11.65
6	-1	0	-1	7.88
7	0	-1	1	8.62
8	0	0	0	11.32
9	1	0	-1	9.07
10	0	0	0	11.62
11	-1	0	1	9.91
12	1	0	1	11.58
13	0	1	-1	9.07
14	0	-1	-1	6.58
15	-1	-1	0	6.15
16	0	0	0	11.4
17	0	1	1	10.25

a: coded variables.
b: Y = EE of MCE

A second-order polynomial equation used to express as a function of the independent variables is given below:

$$Y = b_0 + \Sigma\, b_i X_i + \Sigma\, b_{ii} X_i^2 + \Sigma\, b_{ij} X_i X_j \qquad X_i = 1, 2, 3 \qquad i \neq j \qquad (2)$$

Where **Y** represents the measured response; variables, $\mathbf{b_0}$, $\mathbf{b_i}$, $\mathbf{b_{ii}}$ and $\mathbf{b_{ij}}$ are the regression coefficients of variables for the intercept, linear, quadratic and interaction terms, respectively.

2.2.4. Statistical analysis

All the data were analyzed using Statgraphics (version 7.1 1994) and Statistica SDATA Directory (version 6.0) software program. The test of statistical significance was based on the total error criteria. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model.

RESULTS AND DISCUSSION

3.1. Effects of independent variables on response

The effects of independents variables: $\mathbf{X_1}$ (NaCl concentration), $\mathbf{X_2}$ (extraction duration) and $\mathbf{X_3}$ (pH) on EE of MCE were represented in table 3. The results of this table showed that linear ($\mathbf{X_1}$, $\mathbf{X_2}$ and $\mathbf{X_3}$), quadratic ($\mathbf{X_1^2}$, $\mathbf{X_2^2}$ and $\mathbf{X_3^2}$) and interaction ($\mathbf{X_1X_2}$) terms of model had a significant effect on Y (EE of MCE) at the 5% ($\mathbf{X_1X_2}$), 1% ($\mathbf{X_1}$, $\mathbf{X_1^2}$ and $\mathbf{X_3^2}$) and 1‰ ($\mathbf{X_2}$, $\mathbf{X_3}$ and $\mathbf{X_2^2}$) level. Positive coefficients for a linear ($\mathbf{X_1}$, $\mathbf{X_2}$ and $\mathbf{X_3}$) and interaction ($\mathbf{X_1X_2}$ and $\mathbf{X_1X_3}$) revealed an effect to increase **Y**, while negative coefficients for quadratic ($\mathbf{X_1^2}$, $\mathbf{X_2^2}$ and $\mathbf{X_3^2}$) as well as interaction ($\mathbf{X_2X_3}$) showed an effect to decrease **Y**.

Response surface analysis of the data in table 2 shows that the relationship between the EE of MCE and extraction parameters was quadratic with high regression coefficient, $R^2 = 0.97$ (table 3). Response surface plot and their corresponding contour plot for the extraction efficiency of MCE were given in figs.2-4a&b.

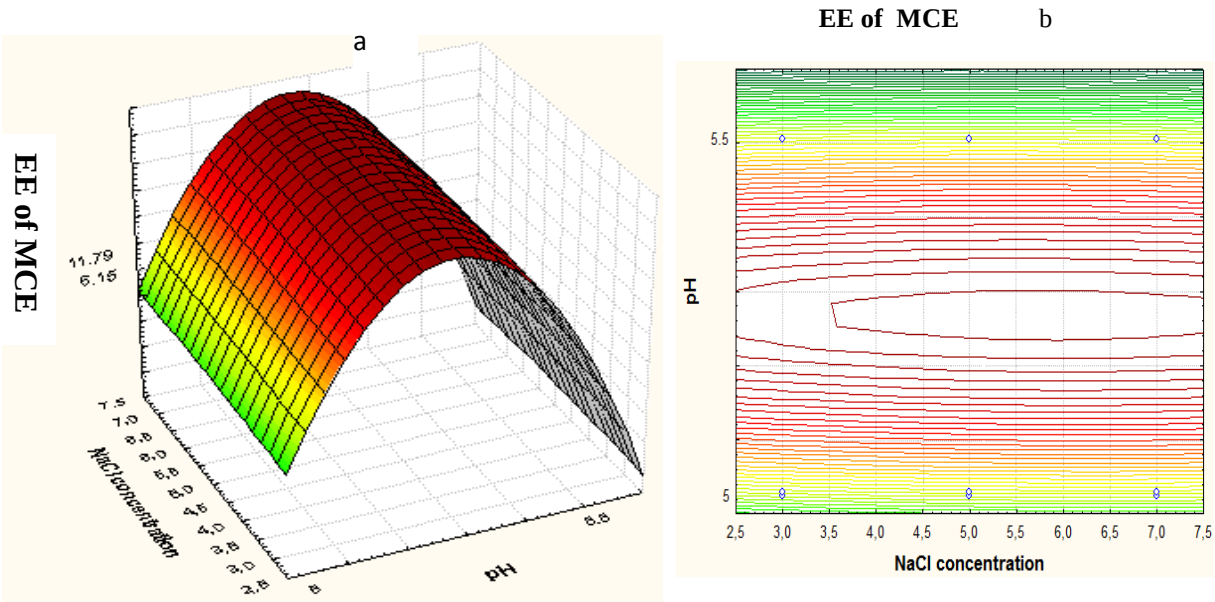

Fig.2. Response surface (a) and contour (b) plots showing the effect sodium chloride (NaCl) concentration (w/v) and pH on EE of MCE. Extraction time was constant at 24.0 h

The interaction between pH (5.5) and NaCl concentration (5%) influenced positively the EE of MCE from *S. aethiopicum* seeds (fig.2a&b). Further augmentation of pH led to the decrease of the EE of MCE. The results in fig. 3a&b showed that the interaction between extraction time and NaCl concentration influenced positively the EE of MCE, when the pH was constant at 5.5. The EE first increased with augmentation of both NaCl concentration and extraction time approximately 6% and 28 h respectively and thereafter decreased. Concerning the fig.4a&b, there was a negative relationship between EE and interaction duration-pH, when they were increased simultaneously and NaCl concentration maintained at 5% (w/v).

Different extractant solutions such as sodium chloride, potassium chloride, calcium chloride and others are often used to extract MCE directly from vegetable tissues. However, sodium chloride is the most popular extractant used for enzymatic preparation due to high extractability, and employed as ingredient in cheese-making [4-5-6-7-8-15-16-18].

The results indicate that the EE of MCE from *S. aethiopicum* seeds increased with the increasing of NaCl concentration from 3 to 6% (w/v). With further increase of NaCl concentration, the EE did not increase. The extraction of MCE could be attributed to the fact that, NaCl increases the ionic strength of extractant solution which facilitated the release of proteinases from *S. aethiopicum* seeds. However, at higher ionic strength, release of active MCE decreased so that for this study, the NaCl concentration of 5% (w/v) was chosen as center point in RSM.

Extraction duration had an important impact on the EE of MCE from *S. aethiopicum* seeds. As we know, mass transfer rate of analyte from matrix plays a key role in the efficiency of extraction [22]. In classical extraction, the mass transfer rate is controlled by the diffusion process which is time-dependent. In the diffusion controlled process, the recovery of analyte keeps increasing along with the extension of time [23]. It explains the positive influence of duration on EE of MCE in this work. However, higher time could lead to the release of active MCE as well as their inhibitors which unfortunately decreased the extraction efficiency. Thus, the extraction duration for this study was 24 h at center point in RSM.

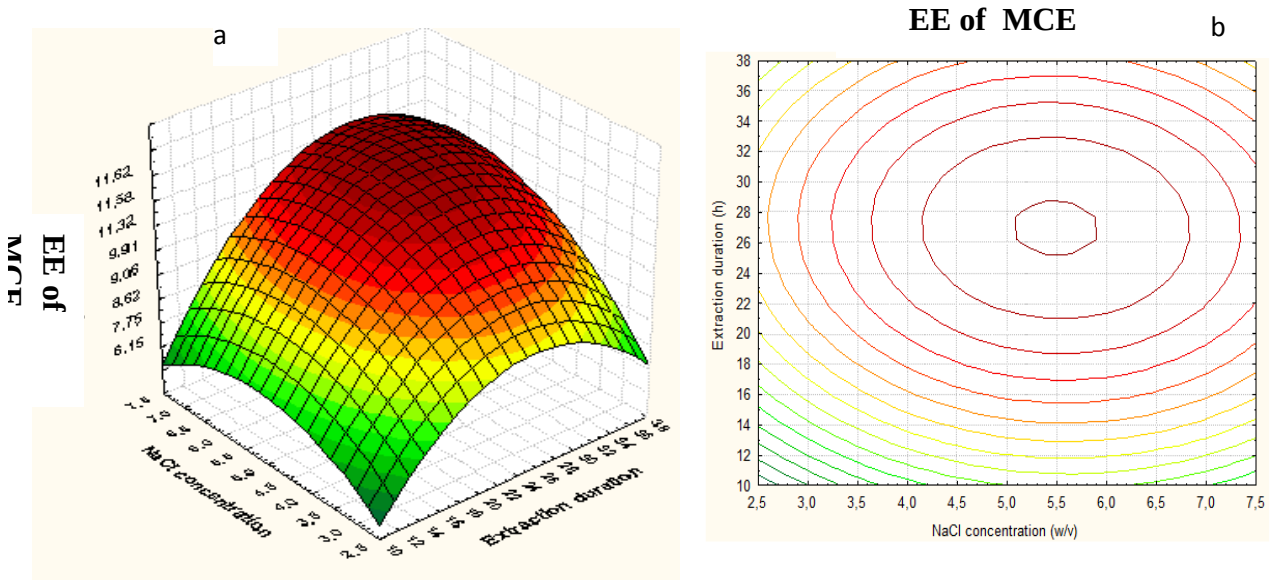

Fig.3. Response surface (a) and contour (b) plots showing the effect sodium chloride (NaCl) concentration (w/v) and extraction duration (h) on extraction EE of MCE. PH was constant at 5.5

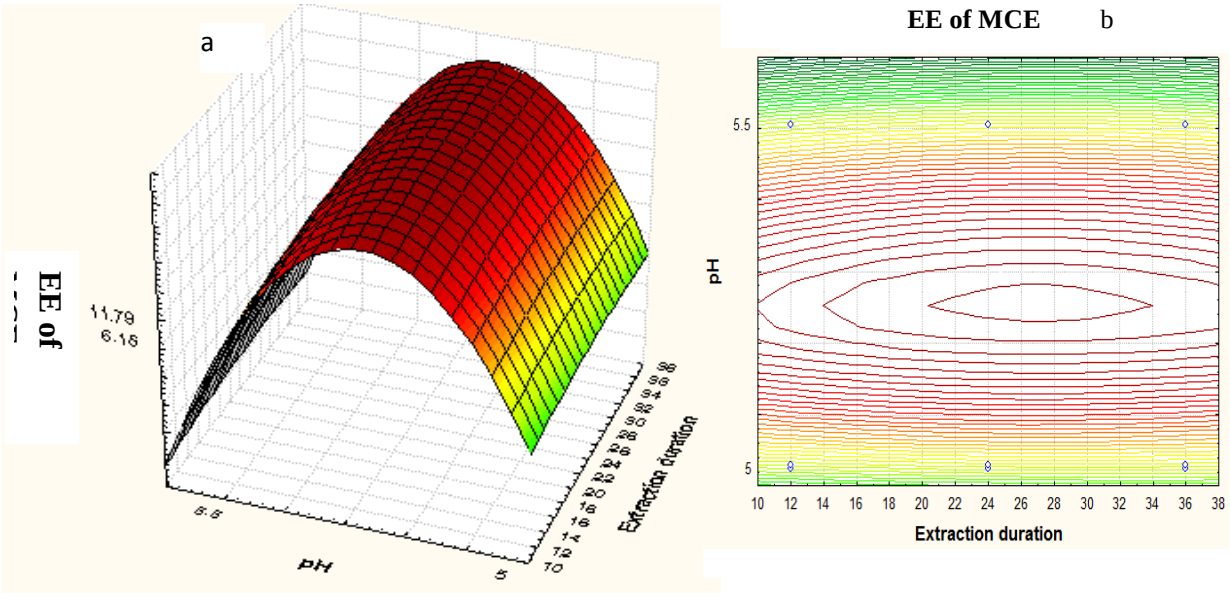

Fig.4. Response surface (a) and contour (b) plots showing the pH effect and extraction duration (h) on EE of MCE. Sodium chloride (NaCl) concentration was constant at 5% (w/v)

Crude proteinases from *S. aethiopicum* seeds as other enzymes are pH-instable protein whose activity can be limited at certain values of pH. There was a relationship between the pH and the EE of MCE. MCA and PA were pH-dependent. When the pH varied from 5.1 to 5.5, the EE of MCE increased, while it decreased remarkably with a further augmentation in pH (fig. 4). The cause was that, there was a close link between the EE of MCE and both MCA- PA. The preliminary experimental results in this study indicated that the highest EE of MCE from *S. aethiopicum* was obtained at the pH 5.5. These results are not in accord with the observations made with MCE from similar plant species (*S. dobium*) [6]. These authors indicated that the optimal pH was 5.0. However, the EE of MCE did not depend only on the pH-variation, but the interactions between pH and the other independent variables (NaCl and duration). RSM identified the statistical significance of these interactions. Therefore, the pH for this work was 5.5 at the center point in RSM to model the extraction process.

3.2. Fitting the model

The experimental conditions and corresponding response (EE of MCE) values were used to calculate the coefficients of the second-order polynomial equation (table 3). The mathematical model representing the EE of MCE as a function of independent variables was expressed by the following equation under the experimental conditions:

$$Y = 11.508 + 0.93\ X_1 + 1.3175\ X_2 + 0.9725\ X_3 - 0.98525\ X_1^2 - 1.96525\ X_2^2 - 0.91525\ X_3^2 + 0.3425\ X_1X_2 + 0.1175\ X_1X_3 - 0.2125\ X_2X_3 \quad (3)$$

Y is the EE of MCE; whereas $\mathbf{X_1}$, $\mathbf{X_2}$ and $\mathbf{X_3}$ are respectively NaCl, duration and pH.

The significance of the regression coefficients and results of ANOVA were presented in table 3. According to the F-test, **Y** was significant at 1‰ confidence level. The CV (%) is defined as the ratio of the standard error of the estimate to the mean value response. It is a measure of the reproducibility of the model. Model **Y** is rationally reproducible because their CV is lower than 10% and determined to be 4.6%. This CV indicated higher reliability of the experiments performed [24].

Table 3. Regression coefficients of the predicted second-order polynomial model for the EE of MCE

EE of MCE			
Terms	Regression coefficients	F-value	Standard error
b_0	11.5073		0.087
Linear			
b_1	0.929678	180.98**	0.138
b_2	1.31914	364.36***	0.138
b_3	0.97048	197.21***	0.138
Quadratic			
b_{11}	-0.984698	106.86**	0.191
b_{22}	-1.96495	425.50***	0.191
b_{33}	-0.914635	92.19**	0.191
Interaction			
b_{12}	0.343401	12.35*	0.195
b_{13}	0.118973	1.48	0.195
b_{23}	-0.213276	4.76	0.195
R^2	0.97		
CV (%)	4.6		

** Significant at the 5% level; ** Significant at the 1% level; *** Significant at the 1‰ level*

The coefficient of determination, R^2 is defined to be of the explained variation to the total variation, and it is a measurement of the degree of fitness [25]. Model can fit well with actual data when R^2 is near to the unit. ANOVA showed that R^2 value of the model was determined to be 0.97. This implied that the sample variation of 97% for the EE of MCE was attributed to the independent variables; and only 3% of the total variation could not be explained by the model. These above results and the fact that p-value of the model was less than 0.0001 showed that the resultant second-order polynomial model adequately represented the experimental data.

Table 4. Optimum conditions of extraction, predicted and experimental EE of MCE

	Optimum conditions		Extraction	Efficiency of MCE
NaCl concentration (%)	Extraction duration (h)	pH	Predicted value	Experimental value[a]
5.5	27	5.3	11.68	11.3 ± 0.5

[a] experiment was repeated three times

3.3. Optimization of extraction parameters

In general, optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, which makes the test of the model adequacy essential [26]. The extraction parameters would have been optimum if the MCA had the highest possible value for the lowest value of PA, this corresponds to the maximum EE of MCE.

The optimum condition of MCE extraction from *S. aethiopicum* seeds depended on NaCl (% w/v), duration (h) and pH, were obtained by RSM. According to the surface and contour plots of optimizing response (EE of MCE) as a function of above parameters, optimum zone was generated. It is the area in which every point would represent the combination of extraction parameters that would give the optimum EE of MCE. Thus, the point giving maximum EE of MCE was obtained at 5.5% (w/v) NaCl, 27 h and pH 5.3 (table 4).

Under these optimal conditions, the EE of MCE was 11.3 ± 0.5, while it was predicted by RSM models to be 11.68. These values were not significantly different at the 5% level. These results indicated that the actual value was found to be in conformity with the predicted one.

CONCLUSION

The most important findings of this work can be resumed as follows:
- Experimental results of the three variables (NaCl concentration, extraction duration and pH) affected significantly the EE of MCE.
- The mathematical model gave a high regression coefficient (R^2 = 0.97) and p-value less than 0.0001, which implied a good agreement between predicted and actual values of MCE EE from *S. aethiopicum* seeds. It confirmed a good generalization of the second-order polynomial model.
- Under optimal conditions (5.5% (w/v) NaCl, 27 h and pH 5.3) the EE of MCE from *S. aethiopicum* seeds was 11.3± 0.5, which was consistent with the predicted value at 95% confidence level.
- These results were helpful for the extraction of MCE from *S. aethiopicum* seeds. However, further study on the purification and characterisation of these MCE is necessary to explore the best conditions to use it in dairy technology.

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