OPTIMISING A PROCESSING PROTOCOL FOR A SAFE AND VITAMIN C RICH CONCOCTION USED BY UGANDANS TO ALLEVIATE SYMPTOMS OF COVID-19 .

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Abstract

Background:

When the COVID-19 outbreak was declared a pandemic on 12 March 2020, desperate Ugandans who could not afford exorbitant fees for treatment of COVID-19 resorted to using cannabis and other local herbs to treat the deadly disease. One of the popular concoctions used was a mixture of garlic, lemons, ginger, onion, and red pepper. The main objective was to optimize the processing protocol for a safe and vitamin C-rich concoction.

Methodology:

A centralized statistical screening design was applied to optimize the processing conditions. The processing conditions, including temperature and time, were optimized to maximize vitamin C retention while ensuring microbial safety.

Results:

The concoction that was prepared at the optimized conditions, 83 °C for 5 minutes, had a vitamin C retention of 69.51% and a microbial load of 0 CFU/ml. Confirmation runs were performed at 83 °C for 5 minutes and the observed responses coincided well with the predicted values given by the optimization technique.

Conclusion:

To best preserve the concoction's vitamin C content and, at the same time ensure a safe microbial load, it should be prepared at 83 °C for 5 minutes.

Recommendation:

Further analysis to be conducted on the optimization protocol for toxicity and effectiveness of the concoctions.

Keywords: Optimization, Temperature, Vitamin C, microbial load, concoction, time, COVID-19,
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1. Background of the study

The Director-General of the World Health Organization (WHO) declared the COVID-19 outbreak a pandemic on 12 March 2020. The burden of the COVID-19 pandemic in Uganda led to people seeking alternative medicine as preventive and treatment options. On 21 June 2021, the National Drug Authority informed the public that COVIDEX had been notified to be sold in licensed drug outlets for supportive treatment in managing viral infections but not as a cure for COVID-19. Currently, there is no specific drug for

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the treatment of COVID-19, and the main treatment methods are supportive and symptomatic treatment (Chen et al., 2020).

Apart from COVIDEX, several other natural remedies were used by Ugandans to prevent COVID-19 or alleviate symptoms of the disease. Among these remedies was a popular concoction that was prepared as a mixture of garlic, ginger, lemon, onion, and hot pepper. These fruits and vegetables are good sources of vitamin C in their raw form. However, heating these fruits and vegetables reduces their content of vitamin C and other nutrients, such as antioxidants. For example, onion, which has been used in traditional medicine for a long time to treat various conditions and infections and was obtained to destroy the avian influenza virus (H9N2,) loses its effectiveness when prepared in certain ways. The preparation method is crucial, as boiled or fried onions are relatively ineffective (Demeke et al., 2021).

No specific clinical data is available to confirm the use of vitamin C during the pandemic. However, recent research, including a review article on the protective role of vitamin C in the management of COVID-19, concludes that vitamin C possesses positive impacts on curing infections and may play a protective role in the current COVID-19 pandemic by boosting the immune system. Considering the role of vitamin C, it would be imperative to administer vitamin C to manage severe COVID-19 (Uddin et al., 2021).

Unlike many species, such as cats and dogs, which can biosynthesize their vitamin C supply, humans cannot do so because human cells cannot perform the crucial last step of vitamin C biosynthesis, the conversion of l-gulono-g-lactone into ascorbic acid, which is catalyzed by the enzyme gluconolactone oxidase (Bender, 2020). Humans, therefore, require an exogenous source of vitamin C (Hirschmann & Raugi, 1999). Many fruits and vegetables contain vitamin C, but an excess amount of heat can destroy the vitamin completely. Vitamin C begins to denature at temperatures as low as 30 °C (Popova, 2019). The deficiency of vitamin C in humans causes a major disease called scurvy. Scurvy is characterized by impaired wound healing; oedema; hemorrhage in the skin, mucous membranes, internal organs, and muscles; and weakening collagenous structures in bone, cartilage, teeth, and connective tissues (Devaki et al., 2017). The hemorrhage is due to the deficient formation of intercellular substances.

Concoctions or fresh juices made from fruits may be contaminated with microbes from raw materials, juice machines, handlers, and unhygienic conditions. Fruit juices have a pH in the acidic range (<4.5) serving as an important barrier to microbial growth. However, food-borne pathogens such as *E. coli* and Salmonella survive in the acidic environment of fruit juices due to acid stress response (Aneja et al., 2014). The main microbial contaminants in fruit juices are *Staphylococcus aureus*, *E. coli*, Kebsiella, *Vibrio cholera*, Streptococcal spp., and *Candida albicans* (Iqbal et al., 2015).

This study aims to optimize a processing protocol that preserves the concoction's vitamin C content while also ensuring its microbial safety.

2. Methodology

2.1. Generation of a design model using Design-Expert

A Central Composite statistical screening design (Parajo et al., 1992) was applied to optimize the processing conditions. The processing conditions, including temperature and time, were optimized to maximize vitamin C retention while ensuring microbial safety. As shown in Table 6, a randomized quadratic design model of twenty experiments with four centre points was performed by selecting two factors, cooking temperature (A) and time (B). The responses were microbial load measured in CFU/ml and vitamin C content measured in mg/ml. The statistics generated were examined via Design-Expert (Version 13, Stat-Ease Inc., Minneapolis, MN, USA) statistical software. Every experiment was carried out in randomized order to reduce the bias effects of uncontrolled factors.

2.2. The precision capability of the design model

The precision capability of a design model was performed through a visual check of the Fraction

Table 1: Shows the model of different processing conditions that were used for process optimization

		Factor 1	Factor 2
Std	**Run**	**A: temperature (°C)**	**B: time (min)**
18	1	80	10
3	2	90	5
2	3	70	5
5	4	70	15
12	5	90	10
19	6	80	10
11	7	90	10
16	8	80	15
15	9	80	15
6	10	70	15
10	11	70	10
8	12	90	15
14	13	80	5
13	14	80	5
20	15	80	10
7	16	90	15
9	17	70	10
1	18	70	5
4	19	90	5
17	20	80	10

of Design Space (FDS) graph. As shown in Figure 1, the model's fraction of design space score was 0.82. Stat-Ease, Inc. recommends an FDS score of at least 0.8 or 80% for exploration and optimization. Analysis of variance (ANOVA) and response surface analysis was used to determine the statistical significance of the model. The adequacy of the model was predicted through the ANOVA ($P < 0.05$) and regression analysis (R2). The response surface plot demonstrated the relationship between the responses and independent variables.

2.3. Raw material collection

Samples of ginger (Zingiber officinale), garlic (Allium sativum), lemon (Citrus limon), onions (Allium cepa), and hot pepper (Capsicum annuum) were purchased from Wandegeya food market. Well-grown green vegetables or fruits with no injuries were selected during collection.

2.4. Sample preparation

The fruits were gently rubbed while held under plain running water. A clean vegetable brush was used to scrub the fruits. The fruits were dried with a paper towel. Ten pieces (30.4 g) of garlic, two lemons, four thumb-size pieces (120 g) of ginger, one onion (60 g), and five red peppers (1.4 g) were weighed and blended with 1 liter of water. Samples were made by measuring 10 ml of this mixture. Each sample was labeled according to the related cooking duration and temperature.

2.5. Thermal treatment

Samples were heated at different water bath temperatures (70 °C, 80 °C, and 90 °C) for different times (5 minutes, 10 minutes, and 15 minutes). A water bath was preset to the required temperature and often checked with a thermometer to ensure that it matched the desired test temperature. Twenty runs of thermal treatment were performed. Four additional thermal treatment

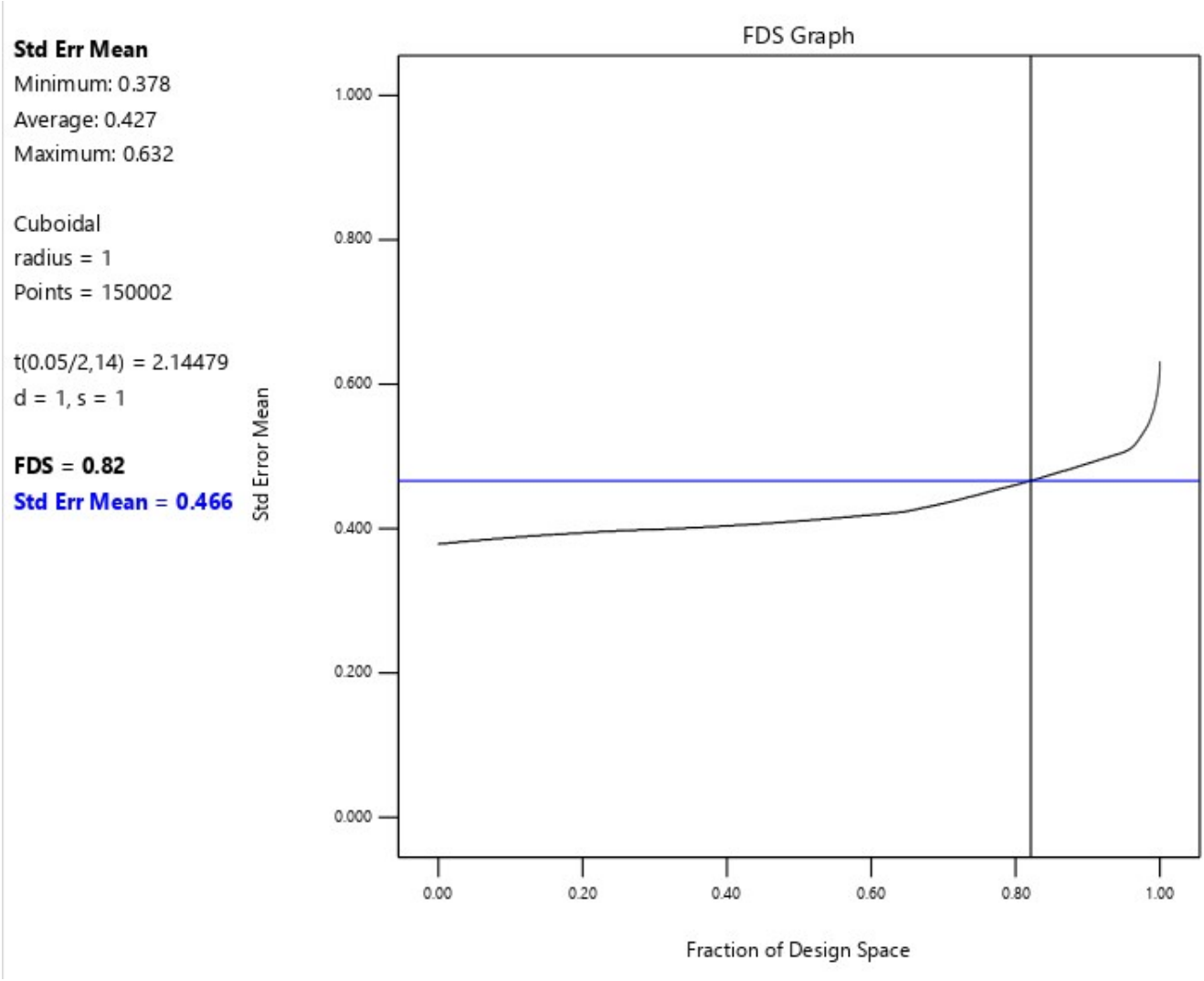

Figure 1: Fractionof Design Space (FDS) graph

runs were performed at 83 °C for 5 minutes for the confirmation step.

2.6. Determination of vitamin C content in the samples

The principle underlying the method for determining vitamin C is based on the vitamin's oxidizing and strong reducing powers (Pearson, 2021).

These properties of vitamin C are related to the enediol group in the ascorbic acid molecule. Vitamin C can be oxidized by oxidizing agents such as iodine, methylene blue, and dichloro-indophenol solution, and any of these reactions may be a means of its determination.

The 2, 6-dichlorophenol-indophenol (DCPIP) solution is vital as it can be explicitly made for determining ascorbic acid. It is a red dye in an acid solution and blue in a neutral or alkaline solution. It is entirely colorless in its reduced form.

Ascorbic acid content was determined using the 2, 6- dichlorophenol-indophenol titration method described in the Association of Office Analytical Chemists (1996), in which L-ascorbic acid was used to prepare a standard solution (1 mg/mL). The ascorbic acid concentration was calculated by comparison with the standard and expressed as mg/ml. 2,6-Dichloroindophenol Titrimetric Method (AOAC Method 967.21, 45.1.14).

2.7. Standardizing DCPIP

To standardize DCPIP, 0.0509 g of ascorbic acid was weighed into a 100 ml volumetric flask

and diluted to the mark with extracting solvent. 10 ml of the solution was pipetted into a 100 ml volumetric flask and diluted to the mark using the extracting solvent. 2 ml of the new formulation was pipetted into each of the four conical flasks. 5 ml of extracting solvent was added to each conical flask and titrated against DCPIP until a rose pink persisted for one minute. Titer values were recorded and used to calculate the DCPIP equivalents.

2.8. Extraction process

The extraction solvent was prepared as follows;

- Of phosphoric acid was measured into a bottle using a measuring cylinder
- 400 ml of acetic acid was measured using a measuring cylinder and added to the bottle with 90 ml of phosphoric acid to obtain the extraction solvent.
- Standardized DCPIP was put in a burette which had been fixed on a burette clamp stand. The initial reading on the burette was recorded.
- 5 ml of the formulation sample was pipetted and put into a 50 ml volumetric flask. Then the extraction solvent was added up to the mark. The contents of the 50 ml volumetric flask were shaken vigorously for 5 minutes, which aids in the optimization of the extraction of vitamin C from other juice components.
- Steps 2 and 3 were repeated for all the sample formulations in duplicates.

2.9. Analysis

Pipette 5 ml from the volumetric flask into a clean, dry conical flask and titrate it with the standardized DCPIP from the burette while swirling until you obtain a pale pink color that is stable on swirling for a minute or 60 seconds.

Read off and record the final burette reading.

Repeat the analysis steps in duplicates for all the formulation samples.

NOTE

Ensure that the glassware is clean and dry throughout to prevent water from interfering with the analysis because vitamin C is soluble in water.

Calculate the % Vitamin C in mg/100 ml of juice= (modal net titre*equivalent weight of DCPIP*total volume*100)/ (volume of sample pipetted*volume of sample titrated)

2.10. Microbial Analysis

The total colony counts were determined on plate count agar (PCA) by the pour plate technique. An inoculum (one milliliter) of a known dilution was pipetted into each of the 5 sterile Petri dishes. About 15 to 20 milliliters of PCA were poured over the inoculum and mixed slowly by moving the Petri dishes in clockwise, anticlockwise, and sideways directions. The agar was left to set. The Petri dishes were inverted and incubated at 37 °C for 24 hours.

2.11. Preparation of diluent

The diluent was prepared by dissolving one strength Ringer's tablet in 500 milliliters of distilled water in a conical flask. 9 ml of diluent were transferred into diluent bottles using a pipette and then autoclaved at 121 °C for 15 minutes at 15 psi.

2.12. Preparation of PCA

23.5 g of PCA medium powder was weighed, dissolved in one liter of distilled water, and autoclaved at 121 °C for 15 minutes. It was then cooled to 45 °C in a water bath.

2.13. Total colony counts

Colonies were counted after 24 hours of incubation. The plates with colony-forming units ranging from 30 to 300 were considered for counting as the colonies less than 30 would have run into more significant statistical inaccuracy, and the colonies greater than 300 would have been tedious to count.

Calculation of the microbial counts

Microbial counts were calculated using the formula below (Maturin & Peeler, 2001).

C=$\frac{\sum x}{V[n1+n2(0.1)]} \times \frac{1}{d}$Was the number of microbial units

$\sum$ was the sum of all counted colonies.

n_1 was the number of Petri dishes at the first count.

n_2 was the number of Petri dishes at the second count Was the dilution factor at the first count

3. Results and Discussion

3.1. Optimization of the Processing conditions

Response values obtained from the twenty runs were tabulated, as shown in table 2 below, and fed into the Design-Expert software for optimization. The optimization module searched for a combination of factor levels that simultaneously satisfied the criteria placed on each of the responses and factors. The factors were cooking temperature and time, while the responses were vitamin C content and microbial load. The criteria were to minimize the microbial load while maximizing the vitamin C content. Also, the microbial range was set between 0 and 25 CFU/ml.

Response surface methodology was used to optimize processing conditions, and figures 3 and 4 show the response surface plots. Figure 5 shows how vitamin C content varies in the acceptable response outcomes. Figure 6 shows how the microbial load varies in the acceptable response outcomes.

3.2. The suggested optimum cooking temperatures and times.

Design-expert suggested nine optimal processing conditions. Table 3 shows nine combinations of cooking temperature and time that simultaneously satisfy the criteria placed on each of the responses and factors. The best optimal combination predicted was 83 °C temperature and 5 minutes, which would retain 1.782 mg of vitamin C per milliliter with a microbial load of 0 CFU/ml.

3.3. The selected optimum cooking temperature and time

The best optimal combination predicted was a temperature of 83 °C and a cooking time of 5 minutes which would retain 1.782 mg of vitamin C per milliliter of the concoction and ensure a microbial load of 0 CFU/ml. The findings of this optimization process are similar to previous investigations by Igwemmar et al., who recommended that the vegetables and fruits be cooked for short periods (five minutes) to minimize the loss of vitamin C.

The ramps shown in figure 5 are a graphical view of each optimal solution. Desirability is an objective function that ranges from zero outside the limits to one at the goal. The numerical optimization finds a point that maximizes the desirability function. For several responses and factors, all goals get combined into one desirability function (Stat-Ease, 2022). A desirability of 0.989 shows that the lower and upper limits are closely set relative to the actual optimum.

3.4. Confirmation of the optimal outcome

Confirmation is intended to be used to confirm that the model can predict actual outcomes at the optimal settings determined from the analysis. Montgomery (2017) recommends that a confirmation experiment be performed to verify the good performance of the optimal operating conditions. Confirmation compares the prediction interval of the model to a follow-up sample's average. The model has confirmed if the sample's average is inside the prediction interval. Confirmation is usually done at or near factor settings recommended by numerical optimization.

Additional Four runs (Table 4) were conducted at the selected optimal processing conditions of 83°C, 5 minutes. The response means of those four runs were compared to the prediction interval. The data means for the microbial load (0), and vitamin C (1.768) were within the 95% prediction interval of 1.731 to 1.832 and -19 to 19 respectively, at α of 0.05.

4. C onclusion:

The optimization results revealed that preparing the concoction at 83 °C for five minutes retains the highest amount of vitamin C at a microbial load safe for human consumption. To best preserve the concoction's vitamin C content and, at the same time ensure a safe microbial load, it should be prepared at 83 °C for 5 minutes.

Table 2: The response values, i.e., vitamin C content and microbial load from runs conducted at randomized processing conditions of the design model

Std	**Run**	**Factor 1 A: temperature (°C)**	**Factor 2 B: time (min)**	**Response 1 vitamin c (mg/ml)**	**Response 2 microbial load (CFU/ml)**
18	1	80	10	1.488	2
3	2	90	5	1.736	0
2	3	70	5	1.798	102
5	4	70	15	1.705	57
12	5	90	10	1.302	2
19	6	80	10	1.488	1
11	7	90	10	1.333	0
16	8	80	15	1.457	0
15	9	80	15	1.426	0
6	10	70	15	1.674	57
10	11	70	10	1.643	97
8	12	90	15	0.620	0
14	13	80	5	1.767	3
13	14	80	5	1.798	3
20	15	80	10	1.488	2
7	16	90	15	0.620	0
9	17	70	10	1.674	67
1	18	70	5	1.798	150
4	19	90	5	1.736	3
17	20	80	10	1.488	1

Table 3: The suggested optimal processing conditions

Number	temperature	time	vitamin c	microbial load	Desirability	
1	**83**	**5**	**1.782**	**0**	**0.989**	**Selected**
2	83	5	1.781	-1	0.989	
3	84	5	1.781	-1	0.988	
4	84	5	1.78	-2	0.988	
5	84	5	1.778	-3	0.986	
6	83	5	1.783	1	0.971	
7	83	5	1.785	2	0.938	
8	82	5	1.785	3	0.92	
9	77	15	1.474	0	0.775	

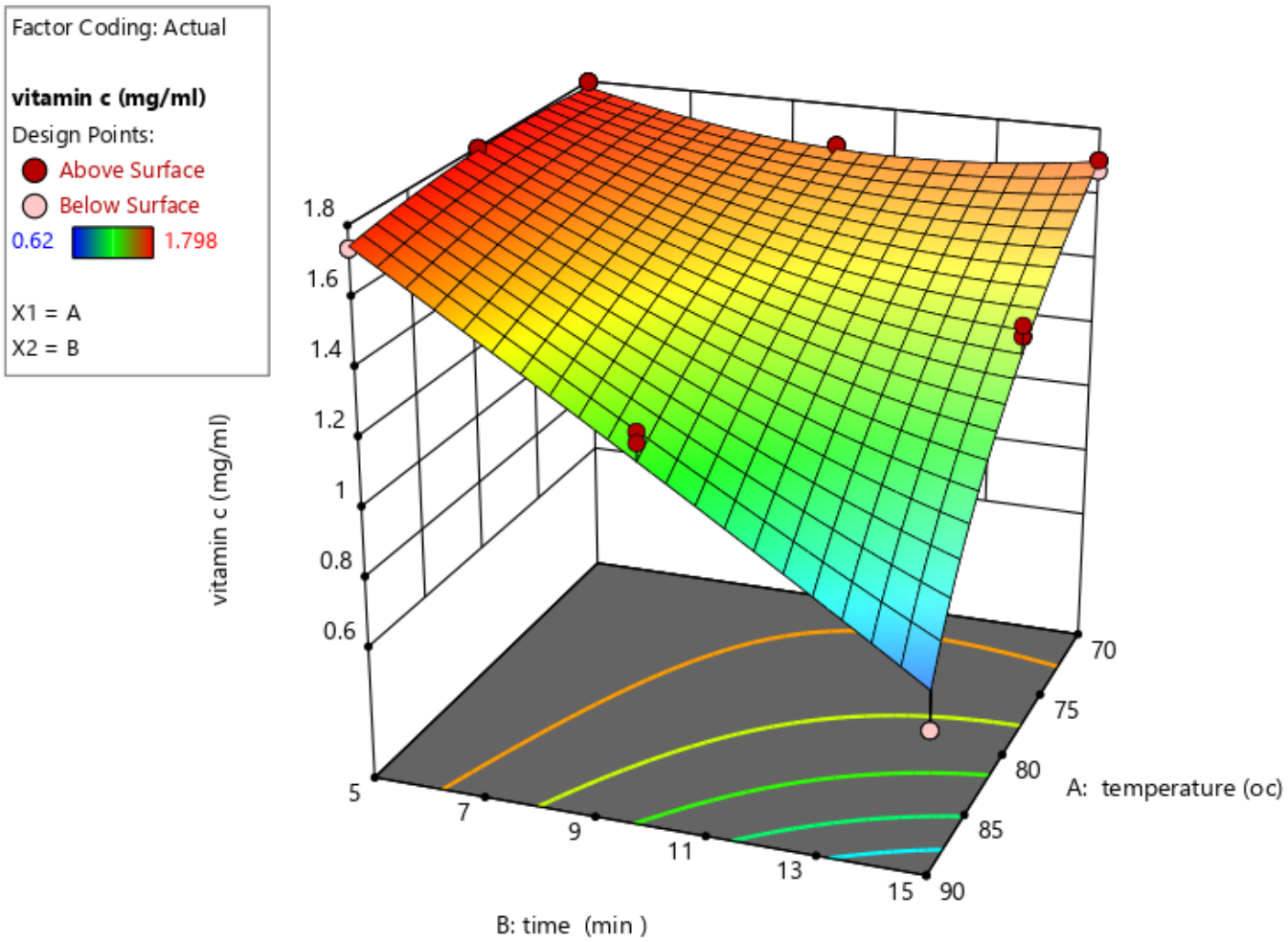

Figure 2: Response surface plot showing the effect of temperature and time on the amount of the vitamin C retained

Table 4: The results from validation runs conducted at 83 °C for 5 minutes

runs	vitamin c	microbial load
1	1.789	0
2	1.767	1
3	1.789	0
4	1.767	0
mean	1.768	0

5. Recommendation:

Further analysis to be conducted on the optimization protocol for toxicity and effectiveness of the concoctions.

6. Acknowledgement:

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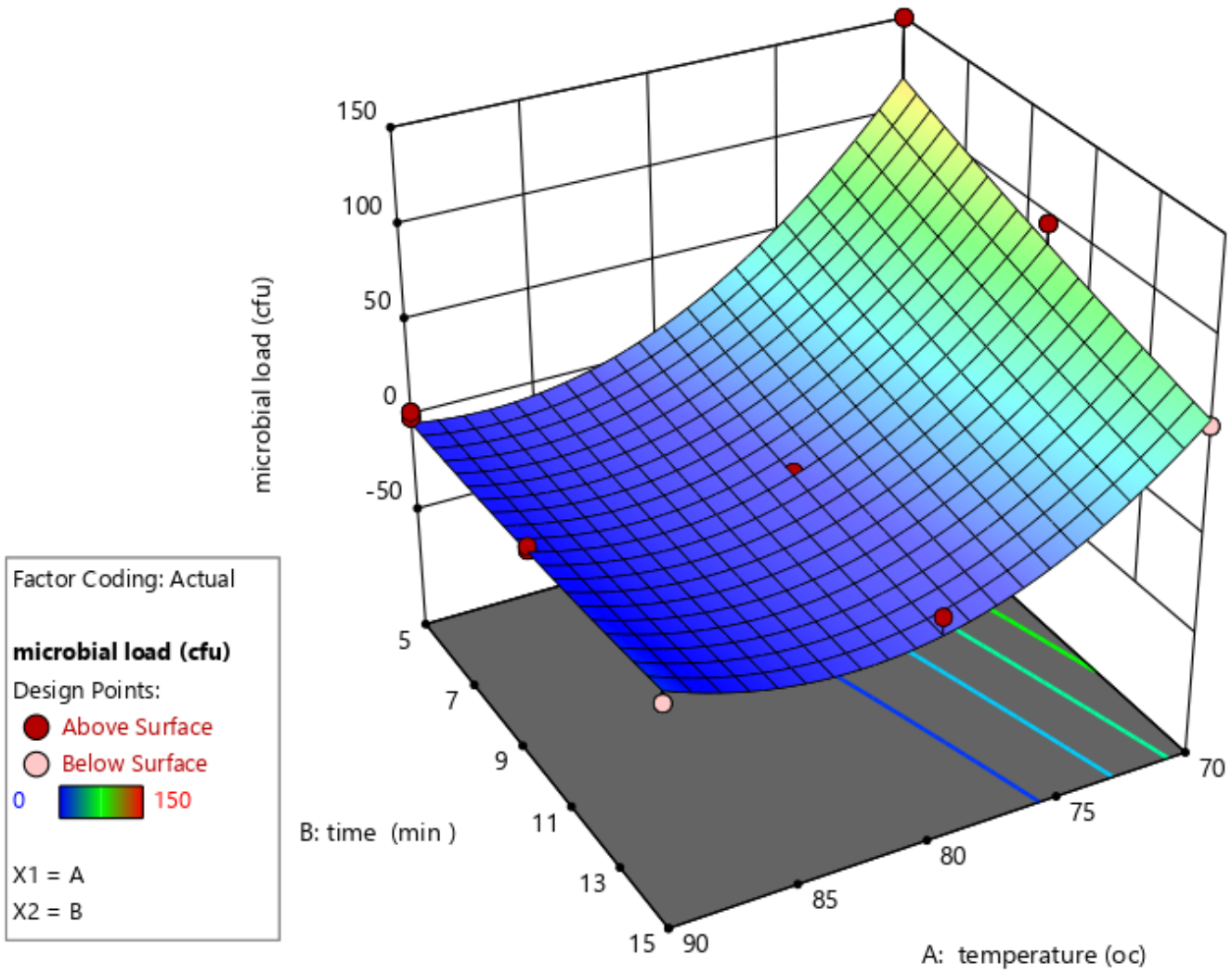

Figure 3: Response surface plot showing the effect of temperature and time on the microbial load of the concoction

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7. List of Abbreviations:

PCA- Plate count agar
CFU- Colony forming unit
DCPIP- Dichlorophenol-indophenol
COVID 19-Coronavirus disease of 2019

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This work received no financial support.

9. Conflict of interest:

All authors declare no conflict of interest.

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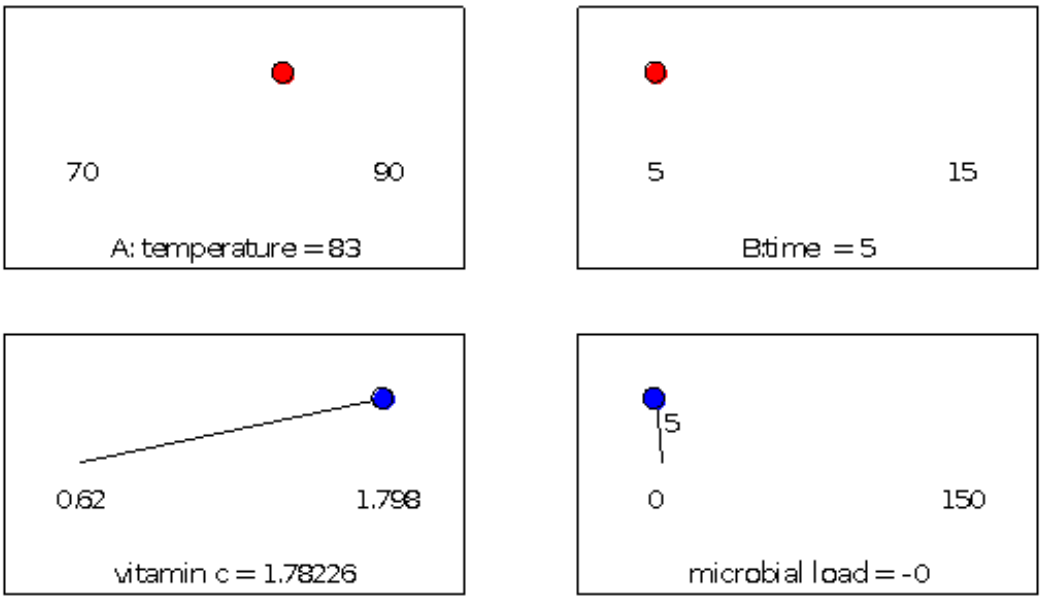

Figure 4: Selected optimal processing conditions and the predicted response values

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