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Optimisation of the marination process for frozen-thawed squid rings using response surface methodology

Optimierung des Marinierprozesses für gefrorene-aufgetaute Tintenfischringe unter Verwendung der Response-Surface-Methodik

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Summary

The frozen-thawed squid rings were marinated by using different formulations of the mineral water and lemon juice at different times at 4°C. Analyses of microbe counts (total mesophilic bacteria (TMB), total psychrotrophic bacteria, lactic acid bacteria, yeast-mould, Enterobactericeae, coliform, fecal coliform, *E.coli, S.aureus, Vibrio* spp.), pH and sensory factors were conducted during the marination process. Increasing the fraction of lemon juice in the marinade solution resulted in a decrease in the number of TMB and a lower squid ring pH. Increasing the fraction of lemon juice in the marinade solution increased the growth of lactic acid bacteria. Faecal coliform, *E.coli, S.aureus* and *Vibrio* spp. were not observed in any of the marinated or non-marinated samples. Response Surface Methodology (RSM) was used to optimise the marination conditions, including the content of the marination liquid (lemon juice+mineral water), the marination time (1,3,6,12,24,48,72 h) and the pH. The recommended optimal conditions for marinating frozen-thawed squid rings at 4°C are as follows: 41.2 ml lemon juice+61.7 ml mineral water/100 g squid rings, pH 4.4, 1 h marination time. Additionally, Good agreement was found between the experimental values and those estimated from the RSM model for the pH (r^2=0.89), total mesophilic bacteria count (R^2=0.93) and lactic acid bacteria count (r^2=0.84). Given the desirable sensory qualities of the resulting squid rings, these optimal marination conditions can be evaluated in the seafood-processing industry.

Keywords: Optimisation, marination, squid, response surface methodology

Introduction

Seafood is highly perishable because it supports the growth of specific spoilage microorganisms. The application of processing technologies can extend the shelf-life of seafood products (Boziaris et al., 2013). One such method is marination in a solution that typically contains salt, spices, sugar, plant extracts, oil and acids such as fruit juice, vinegar, or wine (Behera et al., 2020). The marinated products can be packaged in sauces, marinade solution or vegetable oil to maintain the nutritional quality of the seafood and to extend the shelf-life. Marination can also improve the taste and textural characteristics of the products (Cherifi and Sadok, 2016). The salt and acid content of the marinade solution, method of preparing the raw materials and the storage conditions of the marinated product are essential to the quality, sensory characteristics and shelf-life of the products (Simat et al., 2011). Marinated products are enjoyed by many people, and their consumption is increasing (Björkroth, 2005).

Cephalopods, specifically Coleoidea (squid, cuttlefish and octopus), have been consumed by humans worldwide and across different food cultures for millennia. These organisms are generally good nutritional sources of proteins, omega-3 fatty acids, minerals and micronutrients, and their fat content is low (Mouritsen and Styrbaek, 2018). Squid products have become more and more popular with consumers in recent years due to their high nutritional values and desirable sensory properties (Cui et al., 2020). They are common in the Aegean, Marmara and Mediterranean coasts of Turkey. However, squid meat is very tough and hard to splinter due to the high content of insoluble myostromin (11.0%), which also negatively influences the acceptance of squid by consumers (Xiao et al., 2021). Therefore, marination of squid before consumption is highly desirable.

Marinated squid are used in highly-valued, ready-to-eat products with high nutritive value. Consumption of marinated sea products such as squid has increased considerably in the last decade in many European countries (Guldas and Hecer, 2012). Squid products are sold as fresh, frozen or in salads that are marinated with other sea products in most of the countries. Mostly, squid rings are preferred as fried by consumers. After purchasing from fish markets, many of frozen squid products are marinated at home by consumers in a solution of sugar, carbonate, mineral water and lemon juice. After marinating, the prefererred cooking method for squid rings is frying. But, this marination process takes the times of consumers before the frying process. So, it is essential that marination process for squid rings should be optimized and this type of products should be taken its place in markets.

Although many studies have investigated the marination of fish (Kilinc and Cakli, 2004a; Kilinc and Cakli, 2005a; Kilinc and Cakli 2005b; Baygar, et al., 2010; Caglak and Karslı, 2015; Ucak and Gokoglu, 2016; Kaya and Basturk, 2018), few have focused on marinated seafood products (Cakli et al., 2005; Kilinc et al., 2008; Ozogul et al., 2008). However, no studies have reported standardised marination methods for frozen-thawed squid rings. Therefore, the optimisation and standardisation of squid marination would be of great value not only to consumers, but also to the seafood processing industry for increasing the consumption of squid products. Accordingly, this study aims to determine the optimal conditions for the marination of frozen-thawed squid rings using response surface methodology (RSM).

Material and methods

Material and the preparation of marinade formulations

Frozen squid rings *(Loligo vulgaris)* were acquired from a fish processing factory in the Izmir province of Turkey. They were transported to the laboratory at the Fish Processing Technology of Ege University Fisheries Faculty within 20 minutes using a cold chain, where they were immediately divided into groups and marinated under varied conditions. The marinade solutions varied in lemon juice and mineral water content but had the same concentrations of sugar and carbonate. The ratio of marinade solution to squid rings was 1:1 for samples G9 to G29 and 2:1 for samples G30 to G36. The solution contents for marinated and non-marinated squid rings are shown in Table 1. All marinated and non-marinated squid rings were stored at 4°C.

Analysis methods

Microbiological analysis

Marinated and non-marinated samples (10 g) were placed in 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany). Samples diluted 1:10 and homogenised for 1 minute using a stomacher (IUL, Barcelona, Spain). A 1-mL aliquot of inoculum was placed into the 9 mL of peptone water (total dilution, 1:100). Further dilutions were then

TABLE 1: *The formulations of the groups of marinated and non-marinated squid rings.*

Frozen squid rings		1 hour	3 hour	6 hour	12 hour	24 hour	48 hour	72 hour
Non-marinated		Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Marinated	100 g squid ring +10 ml lemon juice + 90 ml mineral water+0.5 g sugar+0.5 g carbonate	Group 9	Group 10	Group 11	Group 12	Group 13	Group 14	Group 15
	100 g squid ring +90 ml lemon juice + 10 ml mineral water+0.5 g sugar+0.5 g carbonate	Group 16	Group 17	Group 18	Group 19	Group 20	Group 21	Group 22
	100 g squid ring+50 ml lemon juice + 50 ml mineral water+0.5 g sugar+0.5 g carbonate	Group 23	Group 24	Group 25	Group 26	Group 27	Group 28	Group 29
	100 g squid ring+100 ml lemon juice + 100 ml mineral water+0.5 g sugar+0.5 g carbonate	Group 30	Group 31	Group 32	Group 33	Group 34	Group 35	Group 36

Group 1: Frozen squid rings.

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prepared from the stock 1:100 solution and added to sterile Petri dishes. Microorganisms were inoculated into each Petri dish, and the appropriate medium for each species of microorganism was added. The cultures were incubated at the appropriate temperature for each species of microorganism. At the end of the incubation, colonies on petri dishes ranging from 30 to 300 were counted according to the method of Harrigan and McCance (1976).

Mesophilic and psychrotrophic bacteria counts. Bacteria were counted using the pour plate method using plate count agar (Merck) as the growth medium. Dishes were incubated at 30°C for 24–48 h and 7°C for 10 days, respectively (Harrigan and McCance, 1976).

Enterobacteriaceae counts. Bacteria were counted using the double agar layer method. Violet red bile dextrose agar (Merck) was used as the medium, and the dishes were incubated at 30°C for 25 h. After incubation, the dark red colonies were counted as Enterobacteriaceae (Harrigan and McCance, 1976).

Coliform bacteria counts. Violet red bile agar (Merck) was used as the medium using the double agar layer method, and the dishes were incubated at 30°C for 24 h. For faecal coliform counts, bacteria were inoculated into brilliant green lactose broth and assayed for gas production in the tubes at 44–45°C after 48 h (ICMSF, 1986).

E. coli counts. Using the spread plate method, plates with eosin methylene blue lactose sucrose agar (Merck) were inoculated with 0.1 mL of inoculum and incubated at 37°C for 24 hours. Bright green colonies were considered *E. coli.* according to the method of Mossel and Moreno (1985).

Staphylococcus aureus counts. From each dilution of inoculum, 0.1 mL was inoculated onto Baird Parker agar (Merck) supplemented with egg yolk tellurite emulsion. The inoculum was spread onto the BPA using the spread plate method. The dishes were then incubated at 37°C for 30 h (Mossel and Moreno, 1985).

Yeast and mould counts. Using the pour plate method with yeast extract glucose chloramphenicol agar (Merck), the inoculated dishes were incubated at 25°C for 3–5 days (Anonymous, 2000).

Lactic acid bacteria counts. Using the double agar layer method with De Man, Rogosa Sharpe agar (Merck), the inoculated petri dishes were incubated at 30°C for 2–3 days (Baumgart et al., 1986).

Vibrio bacteria counts. Using the spread plate method with triosulfate citrate bile sucrose agar, 0.1 mL of inoculum taken from each dilution was inoculated onto the agar. The inoculum was spread onto the agar, and the dishes were incubated at 20°C for 3–5 days (Serratore et al., 1999).

All microbiological analyses were performed in triplicate from each marinated and non-marinated sample.

pH analysis

Samples (10 g) from each marination process and non-marinated controls were dissolved in 10 mL of distilled water. The pH of each sample was determined using a Hanna pH metre (HANNA-Hl 2211, Leighton Buzzard, England) in triplicate according to the method of Bongiorno et al., (2018).

Sensory analysis

Sensory analysis of marinated squid rings was carried out using a 9-point hedonic scale that assesses general acceptability, colour, odour and texture (Tomac et al., 2017). Ten panellists aged 25–45 years from the Fish Processing Technology of Fisheries Faculty of Ege University participated in the analysis. All marinated squid samples (≈10 g) were served in random order to each panellist. The panellists were asked to give each sample a numerical score of 1 to 9, according to the following criteria: 9, excellent; 8, very good; 7, good; 6, between good and middle; 5, middle; 4, threshold score; <4, unacceptable, in which the scores ranged from disliked to extremely disliked as 3, 2 and 1, respectively.

Statistical analysis and mathematical modelling of RSM

Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) Version 25.0 software (IBM SPSS Statistics 25). Results were compared with respect to the squid ring marination time and marinade formulations. One-way analysis of variance (ANOVA) (Montgomery and Runger, 2003) was used to determine statistically significant differences ($p < 0.05$) in results according to marination time. For ANOVA, the homogeneity assumption was checked using the Levene test and, the normality assumption was checked using the Kolmogorov–Smirnov test. In cases where these assumptions were not met, the non-parametric Kruskal-Wallis test was used. To identify differences in sensory analysis results between groups, the Friedman S-test and two dependent sample sign tests were performed as non-parametric tests (Gamgam and Altunkaynak, 2017).

The RSM is a collection of mathematical and statistical techniques that is useful for the modelling and analysis of problems in which the response of interest is influenced by several variables and the objective is to optimise this response (Montgomery and Runger, 2003). The first step in RSM was to find a suitable approximation for the true functional relationship between the response (Y is the response; pH, total mesophilic (TMC), lactic acid bacteria count (LBC) and the set of independent variables (lemon juice, mineral water and marination time). Lemon juice and mineral water were tested at 4 levels (10, 90, 50, 100 mL) and the marination time at 7 levels (1, 3, 6, 12, 24, 48, 72 h).

Results and discussion

The shelf-life, as measured by sensory attributes, is considered to be 10 days for cuttlefish in ice and 9 days for squid in ice (Vaz-Pires and Seixas, 2006). Vaz-Pires et al. (2008) advised that the consumer should not consume any fresh or chilled cephalopods after 10 days of storage. On the other hand, Paarup et al. (2002) reported that the cooked mantles of whole and gutted squid were rejected after 10 and 12 days of storage, respectively, due to ammoniacal off-odours. Tomac et al. (2017) reported that squid rings stored at 4°C spoiled after 3 days according to sensory acceptability. Docillo and Racuyal (2017) stated that marinating squid *(Sepioteuthis lessoniana)* with Sprite soda before the smoking process enhanced its flavour, resulting in the highest level of acceptability as compared to squid treated with beer or vinegar. Compared to fresh/chilled/cooked cephalopods, marinated products not only have a longer shelf-life but also have good sensorial characteristics. For this reason, in the study squid rings were marinated using the different marination formulations to determine the most suitable formulation for the marinated squid rings. The groups of the marinated and non-marinated squid rings are given in Table 1.

In this present study, frozen-thawed squid rings were marinated in sugar and carbonate with different formulations of lemon juice and mineral water. Marinated and non-marinated samples were stored at 4°C for different times (0, 1, 3, 6, 12, 24, 48 and 72 h) (Table 1). The microbiological and pH changes after thawing of marinated and non-marinated squid rings are shown in Table 2. The total mesophilic bacteria count of non-marinated squid rings reached 8.65 log cfu/g after thawing at 4°C for 72 hours (G8), and the total psychrotrophic bacteria count of frozen-thawed non-marinated squid ring reached 1.63 log cfu/g after thawing at 4°C for 72 hours (G8).

The total mesophilic bacteria count of the non-marinated squid rings was 2.65, 3.03, 3.25, 5.24, 6.91, 8.65 log cfu/g for the groups G3, G4, G5, G6, G7, G8 respectively. A significant difference ($p < 0.05$) was observed between the groups of non-marinated squid rings stored at 4°C. Thawed squid rings exceeded the microbiological limit according to the ICMSF (1986) for consumption after 72 h at 4°C (limit, 7.0 log cfu/g for fresh and frozen fishery products). Kilinc et al. (2021) reported that the aerobic plate count of frozen-thawed squid rings was reached 3.27 log cfu/g after thawing at 4°C for 8 h. The results of our study are consistent with those of Kilinc et al. (2021) regarding the increasing total mesophilic bacteria count of squid rings during thawing. For processed fishery products, the consumption microbiological limit was 6.0 log cfu/g according to the ICMSF (1986). The total mesophilic bacteria count of G14 (6.04 log cfu/g) exceeded the microbiological consumption limit (6.0 log cfu/g) after 48 h of marination, whereas other groups (G21, G28 and G35) were not exceed this limit at this time. Additionally, the total mesophilic bacteria count of G36 was still determined as 4.62 log cfu/g after 72 h of marination. Statistically significant differences ($p < 0.05$) were observed between time points in the marination process. In all groups, the total psychrotrophic bacteria count increased during marination, reaching 1.92, 1.95, 1.83 and 1.74 log cfu/g at the end of the marination process for G15, G22, G29 and G36, respectively. Our results are in close accordance with those of Lapa-Guimaraes et al. (2002), who reported that the total psychrotrophic bacteria count of squid *(Loligo plei)* was still below the 6.0 log cfu/g limit after 16 days of storage in ice.

We observed that marination of frozen-thawed squid rings inhibited the growth of total mesophilic bacteria count. The total mesophilic bacteria count of groups (G9, G10, G16, G17, G23, G24, G25, G30, G31, G32, G33) were <1 log cfu/g. However, not all types of microorganisms were inhibited by marination; acid-tolerant bacteria survived and grew during the marination process. Therefore, the total mesophilic bacteria count of marinated squid rings reached 7.45, 6.43, 6.68 and 4.62 log cfu/g for the groups G15, G22, G29 and G36, respectively, after 72 h of marination at 4°C. After 48 and 72 hours of marination, the LBC of marinated squid rings reached 1.50

TABLE 2: *The microbiological and pH changes of squid rings during marination process.*

G	TPBC (log cfu/g)	TMBC (log cfu/g)	LBC (logcfu/g)	CBC (log cfu/g)	EBC(logcfu/g)	YMC (logcfu/g)	pH
1	<1 ^{A}	<1 ^{A}	<1 ^{A}	<1 ^{A}	<1 ^{A}	<1 ^{A}	7.06±0.02 ^{A}
2	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	7.10±0.01 ^{B1}
3	<1 ^{A1}	2.65±0.05 ^{B1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	7.10±0.01 ^{B1}
4	<1 ^{A1}	3.03±0.21 ^{C1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	7.12±0.03 ^{B1}
5	<1 ^{A1}	3.25±0.57 ^{D1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	7.16±0.01 ^{C1}
6	1.54±0.00 ^{B1}	5.24±0.60 ^{E1}	<1 ^{A1}	<1 ^{A1}	1.54±0.00 ^{B1}	<1 ^{A1}	7.22±0.07 ^{D1}
7	1.61±0.17 ^{C1}	6.91±0.06 ^{F1}	1.56±0.04 ^{B1}	<1 ^{A1}	1.61±0.17 ^{C1}	<1 ^{A1}	7.21±0.02 ^{D1}
8	1.63±0.11 ^{C1}	8.65±0.12 ^{G1}	1.68±0.01 ^{C1}	1.02±0.00 ^{1}	1.96±0.54 ^{D1}	<1 ^{A1}	7.26±0.01 ^{E1}
9	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	6.70±0.13 ^{A2}
10	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	6.64±0.02 ^{B2}
11	<1 ^{A1}	2.55±0.05 ^{B2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	6.68±0.05 ^{B2}
12	<1 ^{A1}	2.98±0.09 ^{C2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	6.55±0.07 ^{C2}
13	1.55±0.04 ^{B1}	4.51±0.03 ^{D2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A2}	1.58±0.06 ^{B2}	6.54±0.01 ^{C2}
14	1.74±0.12 ^{C2}	6.04±0.46 ^{E2}	1.50±0.02 ^{B2}	<1 ^{A1}	<1 ^{A2}	1.67±0.05 ^{C2}	6.69±0.03 ^{D2}
15	1.92±0.03 ^{D2}	7.45±0.42 ^{F2}	1.65±0.04 ^{C2}	<1 ^{A2}	<1 ^{A2}	1.92±0.03 ^{D2}	6.67±0.03 ^{B2}
16	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.83±0.06 ^{A3}
17	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.60±0.02 ^{B3}
18	<1 ^{A1}	3.22±0.62 ^{B3}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	1.51±0.04 ^{B2}	3.86±0.02 ^{A3}
19	1.51±0.03 ^{B2}	3.58±0.04 ^{B3}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	1.72±0.04 ^{C2}	3.61±0.03 ^{B3}
20	1.75±0.08 ^{C2}	4.30±0.00 ^{C3}	<1 ^{A1}	<1 ^{A1}	<1 ^{A2}	2.00±0.30 ^{D3}	3.85±0.37 ^{A3}
21	1.77±0.06 ^{C2}	5.22±0.25 ^{D3}	1.54±0.04 ^{B1}	<1 ^{A1}	<1 ^{A2}	2.13±0.62 ^{E3}	3.87±0.37 ^{A3}
22	1.95±0.03 ^{D2}	6.43±0.56 ^{F3}	1.77±0.02 ^{C3}	<1 ^{A2}	<1 ^{A2}	2.26±0.65 ^{F3}	3.82±0.02 ^{A3}
23	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	4.28±0.03 ^{A4}
24	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.89±0.02 ^{B4}
25	<1 ^{A1}	<1 ^{A4}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.83±0.12 ^{C4}
26	<1 ^{A1}	2.77±0.02 ^{B4}	1.51±0.02 ^{B2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.68±0.04 ^{D4}
27	1.51±0.05 ^{B3}	4.66±0.31 ^{C4}	1.80±0.07 ^{C2}	<1 ^{A1}	<1 ^{A2}	1.54±0.03 ^{B4}	4.11±0.04 ^{E4}
28	1.70±0.04 ^{C3}	5.49±0.59 ^{D4}	1.86±0.08 ^{C3}	<1 ^{A1}	<1 ^{A2}	1.74±0.08 ^{C4}	3.83±0.06 ^{C4}
29	1.83±0.05 ^{D3}	6.68±0.09 ^{E4}	2.10±0.16 ^{D4}	<1 ^{A2}	<1 ^{A2}	2.04±0.21 ^{D4}	4.13±0.02 ^{E4}
30	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.74±0.09 ^{A5}
31	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.95±0.04 ^{B5}
32	<1 ^{A1}	<1 ^{A4}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.40±0.04 ^{C5}
33	<1 ^{A1}	<1 ^{A5}	<1 ^{A}	<1 ^{A1}	<1 ^{A1}	<1 ^{A1}	3.48±0.02 ^{D5}
34	<1 ^{A4}	3.12±0.25 ^{B5}	1.51±0.02 ^{B2}	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	3.38±0.02 ^{E5}
35	1.66±0.11 ^{C4}	3.58±0,13 ^{C5}	1.61±0.03 ^{C4}	<1 ^{A1}	<1 ^{A2}	<1 ^{A1}	3.52±0.03 ^{F5}
36	1.74±0.08 ^{C4}	4.62±0.04 ^{D5}	1.93±0.45 ^{A5}	<1 ^{A2}	<1 ^{A2}	<1 ^{A1}	3.38±0.02 ^{E5}

n=3; The results were given as the mean value of three experiments. The results are shown as X ± SD. G; Groups, TPBC;Total psychrotrophic bacteria count, TMBC;Total mesophilic bacteria count, SAC; S. aureus bacteria count, CBC; Coliform bacteria count, EBC; Enterobactericeae count, FCBC; Fecal coliform bacteria count, YMC; Yeast and mould count. The different capital letters A–G in the same column show the difference ($p<0.05$) in the same groups according to the marination time. The different number 1–5 in the same column show the difference ($p<0.05$) between the groups in the same marination time.

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and 1.65 log cfu/g for G14 and G15 and 1.54 and 1.77 log cfu/g for G21 and G22, respectively. In contrast, the LBC of G29 and G36 increased to 2.10 and 1.93 log cfu/g, respectively, after 72 hours of marination. Lactic acid bacteria is reported to grow and survive in acidic conditions (Kindossi et al., 2015). Bunruk et al. (2013) studied the microbiological, physical, chemical and sensory qualities of oyster meat treated with garlic juice (0.2 mL) and then marinated with Sa-tay condiment (8%, wt/wt) and stored at 4°C±2°C. The authors reported that the mesophilic and lactic acid bacteria of the juice-treated oyster increased with increasing storage time. Similarly, the lactic acid bacteria count of marinated squid rings increased during marination in our study.

The quality and safety parameters of mixed marinated seafood salad containing European squid *(Loligo vulgaris),* sea snail *(Rapana thomasiana),* common cuttlefish *(Sepia officinalis),* common octopus *(Octopus vulgaris)* and shrimp *(Parapenaeus longirostris)* were reported as still acceptable after storage at 4°C for months (Ozogul et al., 2008); furthermore, no pathogenic bacteria (*Staphylococcus aureus, Escherichia coli* and *Salmonella*) were detected in the seafood salad. Bunruk et al. (2013) reported that the juice-treated oysters had low counts of psychrophilic bacteria, coliforms, faecal coliforms and other pathogenic bacteria (*Vibrio* spp., *Escherichia coli, Salmonella* spp. and *Staphylococcus aureus*) throughout the storage period. Sensory evaluation and microbiological analysis of 17 processed seafood products stored at 4°C (Boziaris et al., 2013) showed that predominant spoilage microorganisms were yeasts and lactic acid bacteria, followed by *Staphylococcus* spp, Enterobacteriaceae and total coliform, all of which were below the detection limit in all of the seafood products tested. In accordance with these studies, we detected no *S. aureus,* Enterobacteriaceae, faecal coliforms, *E. coli,* or *Vibrio* spp. in any of the marinated squid samples.

The pH of the raw squid meat was 6.5 (Geng et al., 2019), whereas the pH of squid samples was over 7.0 (Grygier et al., 2020). The pH of squid was reported to increase significantly with increased tumbling time (Gokoglu et al., 2017), with the highest pH of 7.5 observed after 6 h of tumbling. The addition of salt to the medium is reported to increase the pH of squid (Gokoglu et al., 2017). Another study reports that the squid pH increased with storage time, from 7.23 to 7.55 and 7.81 after 4, 8 and 12 days of refrigerated storage (Gou et al., 2010). Similar to the reports of Gou et al. (2010) and Grygier et al. (2020), we observed that the pH of squid rings increased from 7.06 ± 0.02 at baseline to 7.26 ± 0.01 after 72 h of storage at 4°C (Table 2). The pH of marinated products should be maintained between 4.1 and 4.5 (Çetinkaya, 1997) to prevent the growth of pathogenic and spoilage bacteria, which occurs above pH 4.5. Below the pH value of 4.5, pathogenic microorganisms can not possible to be developed in marinateted products (Kilinc and Cakli, 2004b). The pH of marinated products is decreased by the inclusion of acidic solutions and longer marination times.

The pH of marinated products is decreased by the inclusion of acidic solutions and longer marination times. In the pH values of G30 to G36 changed from 3.74 ± 0.09 to 3.38 ± 0.02. Increasing the ratio of lemon juice gave rise to a decrease in the pH during marination as 6.70, 3.83, 4.28, and 3.74, for the groups (G9, G16, G23, and G30) after 1 hour of marination. These results are consistent with those reported above (Kilinc and Cakli, 2004a; Kilinc and Cakli, 2005a; Cakli et al., 2005; Kilinc et al., 2008).

Our sensory analysis results show that the non-marinated squid rings exhibited a decrease in sensory quality scores by the end of storage at 4°C (Table 3).

The sensory quality scores of marinated squid rings also changed with increasing marination time. The sensory quality of group G36 but not G15 and G29 was found to be acceptable after 3 days of marination. The general accepta-

TABLE 3: *The sensory changes of squid rings during marination process.*

G	Colour	Odour	Texture	General Acceptability
1	8.5±1.08^{A}	8.3±0.95^{A}	7.0±0.67^{A}	8.1±0.74^{A}
2	7.4±0.70^{B1}	7.6±0.70^{B1}	6.8±0.42^{B1}	8.1±0.74^{A1}
3	6.5±0.53^{C1}	7.2±0.92^{C1}	6.7±0.48^{BC1}	7.3±0.82^{B1}
4	6.4±0.52^{C1}	6.5±0.71^{D1}	5.7±0.48^{BC1}	6.8±0.63^{C1}
5	6.0±0.67^{D1}	6.2±0.42^{E1}	6.6±0.52^{C1}	6.5±0.53^{C1}
6	5.3±0.67^{E1}	5.8±0.63^{F1}	5.3±0.67^{CD1}	5.1±0.74^{D1}
7	4.4±0.52^{F1}	4.3±0.48^{G1}	4.0±0.82^{D1}	4.7±0.67^{E1}
8	3.0±0.82^{G1}	3.0±0.67^{H1}	3.1±0.88^{D1}	3.0±0.67^{F1}
9	7.6±0.52^{A1}	7.6±0.53^{A1}	7.8±0.79^{A2}	7.5±0.53^{A2}
10	7.4±0.52^{A2}	7.1±0.74^{B1}	7.1±0.88^{B2}	7.3±0.48^{A1}
11	6.7±0.48^{B1}	6.3±0.67^{C1}	6.4±0.70^{C2}	6.4±0.70^{B2}
12	5.7±0.48^{C2}	5.5±0.53^{D2}	5.5±0.53^{D2}	5.5±0.53^{C2}
13	5.0±0.67^{D1}	5.0±0.82^{E2}	5.1±0.57^{E2}	4.5±0.85^{D2}
14	4.5±0.74^{E1}	4.4±0.52^{F2}	4.5±0.53^{F2}	3.6±0.52^{E2}
15	3.6±0.74^{F1}	3.6±0.52^{G2}	3.8±0.63^{G2}	3.4±0.70^{E2}
16	7.5±0.53^{A1}	7.5±0.53^{A1}	7.8±0.79^{A2}	8.2±0.63^{A1}
17	7.1±0.53^{B2}	7.0±0.63^{B2}	7.1±0.74^{B2}	7.5±0.53^{B1}
18	6.2±0.63^{C3}	6.9±0.57^{C2}	6.6±0.57^{C3}	6.9±0.63^{C3}
19	5.7±0.32^{D3}	5.8±0.47^{D3}	5.7±0.67^{D3}	6.0±0.47^{D3}
20	5.0±0.57^{E2}	5.0±0.63^{E4}	5.1±0.48^{E3}	5.2±0.63^{E3}
21	4.5±0.41^{F2}	4.6±0.48^{F3}	4.7±0.42^{F3}	4.6±0.67^{F3}
22	4.0±0.52^{G2}	4.1±0.67^{G3}	4.3±0.48^{G3}	4.1±0.42^{G3}
23	8.5±0.53^{A2}	7.9±0.74^{A2}	7.5±0.74^{A3}	7.9±0.74^{A3}
24	8.0±0.82^{B3}	7.6±0.52^{A3}	7.1±0.74^{A2}	7.5±0.48^{B2}
25	7.5±0.53^{C4}	6.6±0.25^{B1}	6.8±0.74^{B3}	6.9±0.67^{C34}
26	6.8±0.63^{D4}	5.9±0.48^{C2}	5.9±0.74^{C3}	5.8±0.48^{D4}
27	6.0±0.48^{E3}	5.2±0.00^{D4}	5.3±0.57^{D3}	5.1±0.57^{E3}
28	4.2±0.42^{F1}	4.8±0.52^{E3}	4.8±0.79^{E3}	4.4±0.52^{F3}
29	3.7±0.48^{G1}	3.9±0.88^{F4}	3.9±0.82^{E4}	3.9±0.74^{G3}
30	8.5±0.85^{A2}	7.9±0.74^{A2}	7.8±0.63^{A3}	8.8±0.63^{A3}
31	8.3±0.67^{B3}	7.9±0.74^{A4}	7.5±0.97^{B3}	7.9±0.63^{B2}
32	7.7±0.67^{C4}	7.3±0.52^{B1}	7.0±0.67^{C3}	7.5±0.85^{C4}
33	7.0±0.71^{D5}	6.9±0.79^{C2}	6.6±0.57^{D3}	7.0±0.63^{D4}
34	6.5±0.71^{E4}	6.1±0.79^{D4}	6.2±0.48^{E4}	6.8±0.57^{E3}
35	5.5±0.53^{F2}	5.3±0.67^{E3}	5.9±0.57^{F4}	5.9±0.71^{F3}
36	5.0±0.53^{G3}	5.1±0.74^{F5}	5.3±0.48^{G5}	5.2±0.88^{G3}

n=3; The results were given as the mean value of three experiments. The results are shown as X ± SD. G; Groups. The different capital letters. A–H in the same column show the difference ($p<0.05$) in the same the groups according to the marination time. The different number 1–5 in the same column show the difference ($p<0.05$) between the groups in the same marination time.

bility scores of the marinated squid rings were 3.4, 4.1, 3.9 and 5.2 for groups G15, G22, G29 and G36, respectively, after 72 h of marination. As a score below 4 was considered unacceptable, G22 and G36 were found to be consumable after 72 h of marination. The mean sensory scores of G36, in terms of colour, odour, texture and general acceptability were significantly higher than the other samples ($p < 0.05$) and thus was rated the most preferred by the panellists. The effects of the marinade content and marination time on sample sensory quality were consistent with those of previous studies (Melendo et al., 1997; Kilinc and Cakli, 2004a; Kalahrodi et al., 2021).

RSM has been used successfully to determine optimal conditions and procedures in food processing (Can and Ersan, 2013). Cankiriligıl et al. (2020) optimised the hydrolyzation procedure for amino acid analysis of fish meat using RSM. In another study, the process of beef tenderisation using bromelain was optimised using RSM (Zainal et al., 2013). The optimal conditions for the hydrolyzation of turkey meat were determined by RSM to be pH 5.42 and 50.09°C for 1.08 hours (Wang and Shahidi, 2018). RSM was also used to optimise the content of chicken marinade using a Box-Behnken centre design (Wei et al., 2017). The optimal marination conditions for cassava fish (*Pseudotolithus* sp.) fillets were shown by RSM to be salt ratio of 10g /100 g, citric acid concentration 2.5 g/100 g and a marination time of 6 h (Kindossi et al., 2015). Hu et al. (2014) investigated the tenderisation of jumbo squid *(Dosidicus gigas)* meat using ultrasound. In the study, RSM was used to predict the tenderisation effect of various heat treatments. Sivaraman et al. (2016) used RSM to determine the antioxidant activity of squid protein hydrolysates prepared with papain. Other authors have used RSM to optimise hydrolysis conditions such as hydrolysis time, enzyme to substrate ratio and reaction temperature (Fang et al., 2012). However, no study has investigated the optimal marinade solutions and marinade times for treating squid rings before frying.

In this study, the first step in applying RSM was to find a suitable approximation for the true functional relationship between the response (Y) and the set of independent variables. Three independent variables (lemon juice, mineral water and time) were considered. The lemon juice and mineral water variables were tested at 4 levels (10, 90, 50 and 100 mL), and the time variable was tested at 7 levels (1, 3, 6, 12, 24, 48 and 72 hours). The levels of the independent variables in the model were suitable for determining the presence of curvature in the model. Therefore, the use of a second-order model given equation (1) was appropriate.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

In this equation, Y is the response (pH, TMC, LBC) and X_i is the independent variable (lemon juice, mineral water and marination time), $ß_c$ is the constant term, $ß_i$ is the linear effect of the independent variable, $ß_{ii}$ is the quadratic effect of the independent variable and $ß_{ij}$ is the interaction effect of the independent variable on the dependent variable. ϵ is the error term. The estimation values of the coefficients in the models created for each response are shown in Table 4.

TABLE 4: *The estimation values of the coefficients in the models created for each response.*

Coefficient	Ph	TMC	LAB
$\widehat{\beta_0}$	7.21319**	0.995982 *	-0.289259
Linear			
$\widehat{\beta_1}$	-0.09041**	-0.020190	0.023237**
$\widehat{\beta_2}$	0.00011	-0.004287	-0.003548
$\widehat{\beta_3}$	-0.00592	0.211610**	0.042734**
Quadratic			
$\widehat{\beta_{11}}$	0.00058**	0.000207	-0.000270**
$\widehat{\beta_{22}}$			
$\widehat{\beta_{33}}$	0.00008	-0.001499**	-0.000222
Interaction			
$\widehat{\beta_{12}}$	-0.00003	-0.000154	0.000091*
$\widehat{\beta_{13}}$	0.00045**	-0.000242	0.000026
$\widehat{\beta_{23}}$	-0.00052**	-0.000057	0.000006
r^2	0.89	0.93	0.84

Indices 1,2, and 3 refer to the variables of lemon juice, mineral water and marination time, respectively. $Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j + \varepsilon$. In here, Y is response (pH, TMC, LAB), X_i's are independent variables (lemon juice (birimi), mineral water (birimi) and marination time (h)), β_0 is the constant term. *coefficients significant at $p - value < 0.05$, **coefficients significant at $p - value < 0.01$.

Regression analysis and model optimisation were conducted using the Minitab programme. According to the data in Table 4, we concluded that the pH was significantly affected ($p < 0.01$) by both the linear (negatively) and quadratic (positively) effects of the lemon juice (X_1 and X_{11}). The interaction of the lemon juice and the marination time (X_{13}) significantly and positively affected the pH ($p < 0.01$), while the interaction of the mineral water and the marination time (X_{23}) significantly and negatively affected the pH ($p < 0.01$) (Table 4). The estimation model accounts for 89% (r^2=0.89) of the variability in the pH. In other words, the quadratic effect of the marination time and the marinade formulation had a significant influence on the pH of the marinated squid samples. The model was determined as adequate and explains 89% of the variations in the pH. The TMC was significantly ($p < 0.01$) affected by both the linear (positively) and quadratic (negatively) effects of marination time X_3 and X_{33}) (Table 4). The estimation model explains 93% (r^2 =0.93) of the variability in TMC.

Figure 1 shows the effect of variables considered in this study on the pH of marinated squid rings. According to the coefficients given in Table 4, the lemon juice content of the marinade solution and the pH of the squid rings were inversely related (Figure 1(A)). The combined effect of the increasing lemon juice and the marination time significantly affected the pH values. These relationships are clearly seen in Figure 1(B). The negative effect of the mi-

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FIGURE 1: *pH values of marinated frozen-thawed squid rings affected by the independent variables. (A) Lemon juice and mineral water (marination time= 36.5). (B) Lemon juice and marination time (mineral water=50). (C) Mineral water and marination time (lemon juice=50). Linear, quadratic and interaction effects of independent variables (lemon juice, mineral water, marination time) on pH values of squid rings are seen. Lemon juice content of the marinade solution and the pH values of squid rings were inversely related. The interaction effect of lemon juice and the marination time was significant (p<0.01). Mineral water and marination time have negative effect on pH values.*

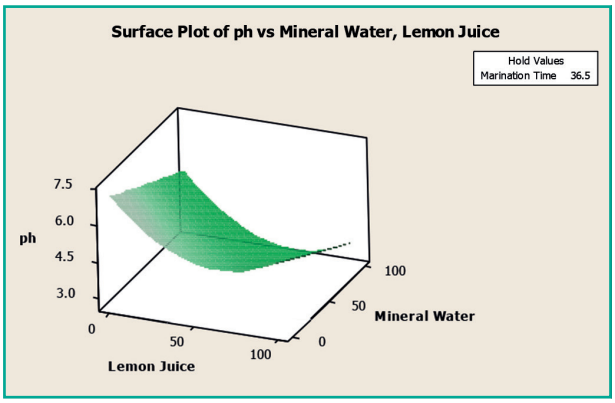

FIGURE 1A: *The effect of lemon juice and mineral water on pH values of squid rings.*

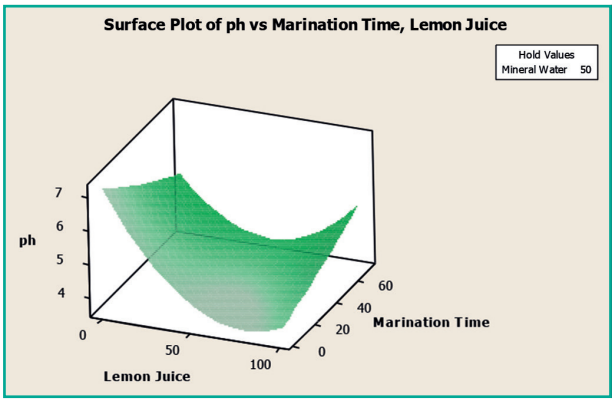

FIGURE 1B: *The effect of lemon juice and marination time on pH values of squid rings.*

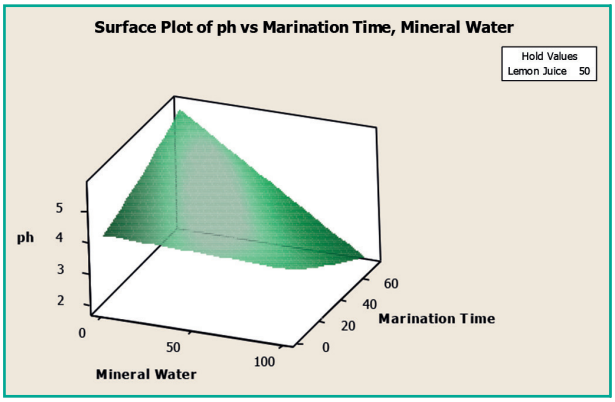

FIGURE 1C: *The effect of mineral water and marination time on pH values of squid rings.*

neral water and the marination time on the pH are seen in Figure 1(C). Considering all of these effects, optimisation study results are as follows. For a target pH of 4.4 (range, 4.1–4.5), the global solution was X_1=41.2, X_2=61.7, X_3=1. Thus, the marination time with lemon juice was the variable most affected by the target value. The optimal marination conditions required to obtain a pH of 4.4 (within the acceptable limits) was determined as a marination time of 1 h, marinade solution of 41.2 mL lemon juice in 61.7 mL mineral water/100 g squid rings.

The TMC was significantly ($p < 0.01$) affected by both the linear (positively) and quadratic (negatively) effects of marination time (X_3 and X_{33}) (Table 4). The estimation model explains 93% (r^2=0.93) of the variability in total mesophilic bacteria count. Figure 2 shows the evolution of total mesophilic bacteria count in marinated squid rings, depending on the variables considered in this study. The regression coefficients showed that the marination time had significant linear and quadratic effects ($p \leq 0.01$) on the total mesophilic bacteria count of the squid rings. The model was determined the highest adequate and also could be explained about 93% of the variations in the total mesophilic bacteria count of samples.

The TMC was significantly affected by the marination time (Table 4). Therefore, Figure 2(B) and Figure 2(C) show similar structures. From these figures, it can be seen that low total mesophilic bacteria counts can only be obtained with short marination times. The lactic acid bacteria count was significantly affected ($p < 0.01$) by both linear (positively) and quadratic (negatively) effects of lemon juice (X_1 and X_{11}) (Table 4).

The marination time also significantly affected ($p < 0.01$) the lactic acid bacteria count in a linearly positive manner. The interaction of the lemon juice and the mineral water (X_{12}) significantly ($p < 0.05$) and positively affected the lactic acid bacteria count. The estimation model explains 84% of the variability in lactic acid bacteria counts. In other words, there was a strong and significant influence of the linear effect of marination time and the marinade solution ratio on the lactic acid bacteria count of the marinated squid samples. The model was determined to be adequate and could explained 84% of the variations in lactic acid bacteria counts.

Comparison of lactic acid bacteria counts in marinated squid rings according to the variables considered in this study is shown in Figure 3. Because of the positive linear and negative quadratic effect of lemon juice, an increasing and then decreasing structure is seen in Figure 3(A). At a mineral water content of 50 mL a positive effect of both lemon juice and marination time on lactic acid bacteria growth was clearly seen (Figure 3B). Figure 3(A) and Figure 3(B) show the quadratic effect of lemon juice, while Figure 3(C) shows the linear effect of marination time. Low lactic acid bacteria counts were only possible with low lemon juice content and short marination times.

Regression models were used in this study to estimate the effects of the marination parameters on the quality of marinated squid rings. The fit of the models is expressed by the coefficient of regression (r^2), which was determined to be 0.89, 0.93 and 0.84 for the pH, total mesophilic bacteria count and lactic acid bacteria count, respectively. Thus, the models explained 89%, 93% and 84% of the variability in the responses.

FIGURE 2: *TMC values of marinated frozen-thawed squid rings affected by the independent variables. (A) Lemon juice and mineral water (marination time= 36.5). (B) Lemon juice and marination time (mineral water=50). (C) Mineral water and marination time (lemon juice=50). Linear, quadratic and interaction effects of independent variables (lemon juice, mineral water, marination time) on TMC of squid rings are seen. Marination time had significantly ($p<0.01$) linear and quadratic effect on TMC of squid rings.*

FIGURE 3: *Lactic Acid values of marinated frozen-thawed squid rings affected by the independent variables. (A) Lemon juice and mineral water (marination time= 36.5). (B) Lemon juice and marination time (mineral water=50). (C) Mineral water and marination time (lemon juice=50). Linear, quadratic and interaction effects of independent variables (Lemon juice, mineral water, marination time) on LAB of squid rings are seen. The lemon juice has both linear and quadratic effect on LAB of squid rings. The interaction effect of lemon juice and marination was signicant ($p<0.05$). Marination time was significant ($p<0.01$) linear effect on LAB of squid rings.*

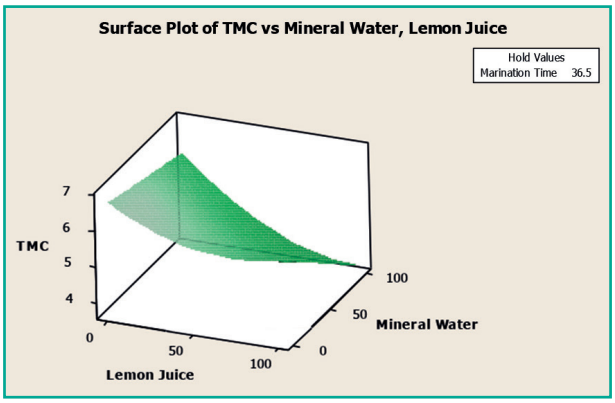

FIGURE 2A: *The effect of lemon juice and mineral water on TMC of squid rings.*

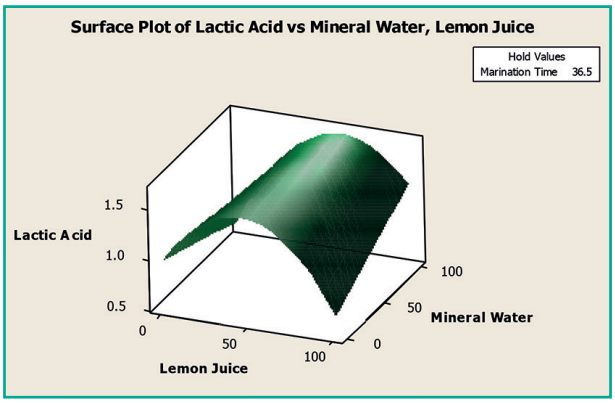

FIGURE 3A: *The effect of lemon juice and mineral water on LAB of squid rings.*

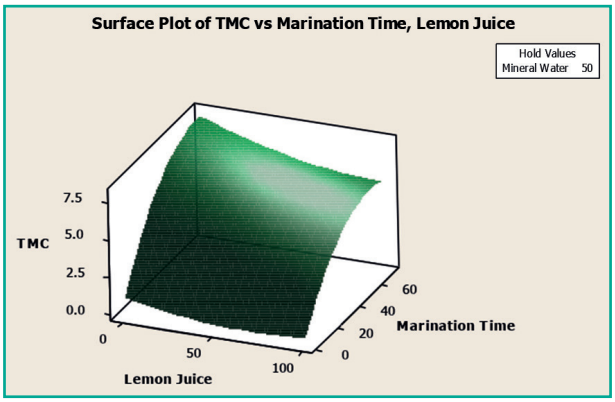

FIGURE 2B: *The effect of lemon juice and marination time on TMC of squid rings.*

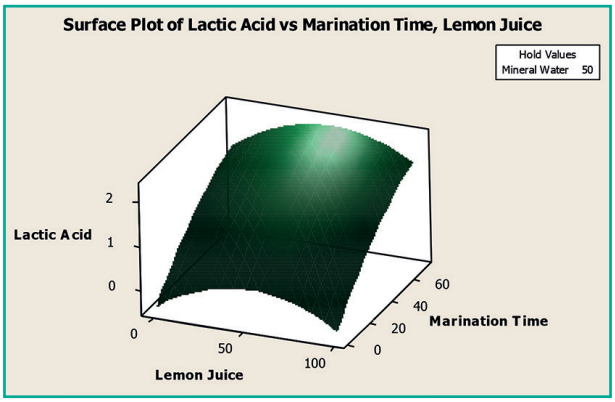

FIGURE 3B: *The effect of lemon juice and marination time on LAB of squid rings.*

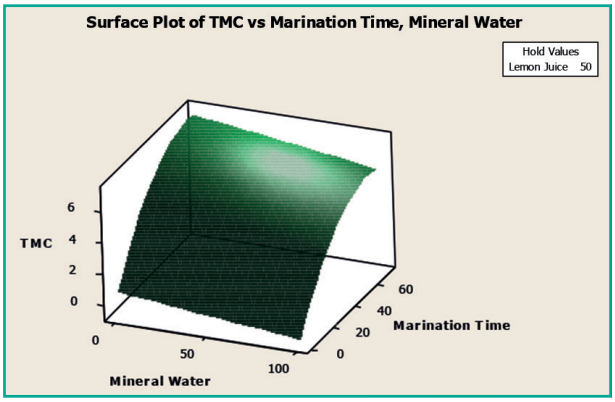

FIGURE 2C: *The effect of mineral water and marination time on TMC of squid rings.*

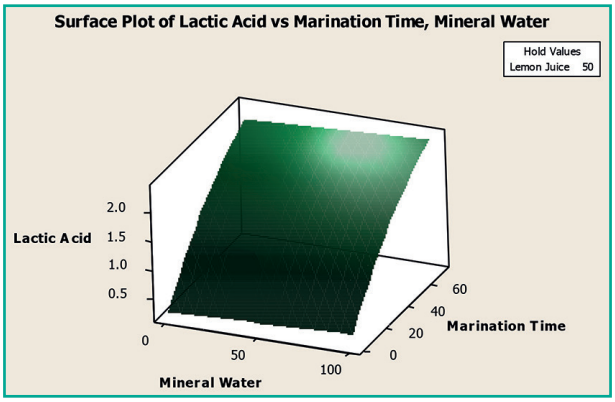

FIGURE 3C: *The effect of mineral water and marination time on LAB of squid rings.*

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Conclusion

RSM was used to predict the optimum processing conditions (marination time, marinade solution content and pH) and their interactions to optimise the marination process for squid rings. The recommended optimal conditions for marinating frozen-thawed squid rings at 4°C are as follows: marinade solution containing 41.2 mL lemon juice+61.7 mL mineral water/100 g squid rings, pH 4.4 and a marination time of 1 h. The results showed that these three variables significantly influenced the quality of the marinated squid rings. The effects of the marination process variables (time of marination, marinade solution content and pH) on the frozen-thawed squid rings were investigated using an RSM model, revealing the following r^2 values: pH, 0.89; total mesophilic bacteria count, 0.93; and lactic acid bacteria count, 0.84. Thus, good agreement was found between the experimental values and those estimated using the RSM model. These optimal marination conditions for frozen-thawed squid rings can be evaluated for the production of industrial marinated squid products. This type of industrial seafood products would be very practical and preferable by consumers because of eliminating the marination process of squid rings at home before frying process of squid rings.

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Author statement

Berna Kılınç: formal analysis, writing the original draft, content and design of the microbiology analysis. Fevziye Nihan Bulat: formal analysis, writing the original draft, content and design of the microbiology analysis. Sevcan Demir Atalay: formal analysis, statistical analysis of the data.

Conflict of interest

The authors have no conflict of interests to declare.

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