ISSN: 2347-467X, Vol. 11, No. (3) 2023, Pg. 1166-1176

Current Research in Nutrition and Food Science

www.foodandnutritionjournal.org

Designing of A Synergistic Mixture of Natural Antioxidants Through Statistical Approaches for Enhancing the Oxidative Stability of Sardine Oil

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Abstract

Given the diverse routes of oxidation and a variety of oxidation products, the right combination of antioxidants is expected to exhibit synergistic effects in retarding refined sardine oil oxidation. In this study, a full factorial design (2^4) was utilized to choose a combination of natural antioxidants which exhibit interactive effect and response surface modelling (RSM) was used to identify the optimal concentration of the selected antioxidant mixture which exhibit synergistic effect. Catechin and resveratrol showed a strong interactive effect among the four natural antioxidants (sinapic acid, vanillic acid, catechin, and resveratrol) studied in sardine oil stored for 50 days at 25ºC under darkness. Two optimal concentrations of interactive antioxidants were found through RSM. Catechin and resveratrol at 0.5 mM and 0.625 mM respectively, exhibited a strong synergistic effect whereas, at 0.5 mM and 3.7 mM respectively, showed prooxidant effect. This is the first of its kind report on the formulation of a synergistic antioxidant mixture for retarding oxidation using statistical approaches.

Article History
Received: 30 June 2023
Accepted: 16 October 2023

Keywords
Antioxidants;
Optimization;
Prooxidants;
Sardine Oil.

Introduction

Long-chain n-3 and n-6 polyunsaturated fatty acids (PUFA) play a significant role in the food and pharmaceutical industries owing to their ability to impart numerous benefits to human health. For instance, n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to prevent blood clots, alleviate depression and reduce the risk of heart disease.[1] n-3 PUFAs are particularly susceptible to hydrogen abstraction due to lower bond-dissociation energy (80 kcal/mol), resulting in oxidation processes.[2] In addition, fish oil oxidation occurs by various factors (light, metal ion, heat) that produce a variety of primary

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Doi: https://dx.doi.org/10.12944/CRNFSJ.11.3.21

and secondary oxidation products that continue to participate in oxidation reactions. Light causes the acceleration of oxidation by singlet oxygen quenching, a non-radical electrophilic that reacts with double bonds of unsaturated fats and oils.[3] Similarly, metal ions reduce the activation energy of oxidation by hydrogen abstraction. Different oxidation mechanisms produce a variety of low molecular weight volatiles, ultimately demeaning their nutritional value.[4,5] Some pathways produce undesirable low molecular weight volatiles that have human sensory threshold values as low as 0.01ppb[4] thereby contributing to oil rancidity. While the benefits and importance of n-3 PUFA-rich oil as prophylactic components are well addressed, their translation to clinical applications is not sufficiently explored perhaps due to the poor oxidative stability of these compounds.

Generally, the application of synthetic antioxidants have been carried out as a pre-requisite step in developing PUFA-based formulations.[6] Despite their ability to achieve the intended application, synthetic antioxidants remain a debatable area owing to their role as carcinogens.[7] Hence, natural antioxidants are constantly being explored as an alternative to synthetic antioxidants.[8] Antioxidants can scavenge free radicals and reactive oxygen species, chelated metal ions, quench secondary oxidation products etc. with varied rates.[2] Every natural antioxidant prevents oil oxidation through divergent mechanisms. For instance, antioxidants have a lower reduction potential to donate hydrogen to free radicals.[9] Similarly, bond-dissociation energies for O-H of phenolic antioxidants are low (70-80 kcal/mol), making them thermodynamically favorable to scavenge free radicals.[10] Considering the fact that the oil undergoes multiple simultaneous diverse mechanisms of oxidations, combining two or more antioxidants of different natures is expected to be a logical and feasible solution. However, combining two or more antioxidants not necessarily offer higher oxidative stability, instead they may only exhibit additive action. In some cases they may exhibit prooxidant effect as well. Whereas, if the right antioxidants are chosen at right concentrations, their cooperative action displays a stabilizing effect more significant than the sum of individual antioxidant effects. Such mixture is called as synergistic mixture and it is seen often with herbal extracts rich in diverse groups of antioxidants. Several reports of Rosemary extract and other natural extracts containing mixture of antioxidants imparting higher oxidative stability to the edible oil are reported.[11] However, studies on the formulation of such mixtures using pure antioxidants to produce synergistic antioxidant mixture is rather scarce.

Full factorial design (FFD) is a robust statistical technique used for screening variables for their main and interactive effects. Once the variables are selected based on their main and interactive effects, response surface methodology (RSM) can be adapted to determine the optimal concentration of the variables which gives the highest desired response. RSM has been predominantly used in optimizing the extraction of natural antioxidants from their sources. However, its applications in exploring the interactive effect of different natural antioxidants at different concentrations is a relatively understudied process. Only recently, the importance of these statistical approaches in understanding the combined effect of antioxidants has been gaining attention.[6] The current study aims to employ statistical methods to identify the best combination of antioxidants to have an interactive effect on sardine oil. We aim to achieve this by initially choosing four natural antioxidants based on our previous studies8. We further apply these statistical tools to identify the appropriate concentration of some carefully chosen natural compounds to have dominant antioxidant effect rather than prooxidant effects.

Materials and Methods

Materials

Crude sardine oil without antioxidants was obtained from Raj Fishmeal and oil company, Malpe, Karnataka, India. Refined sardine oil was prepared by a method developed in our lab earlier and stored at -20ºC.[12] Catechin hydrate (≥98%), Resveratrol (≥ 99%), Sinapic acid (≥98%), Vanillic acid (≥97%), and p-Anisidine (≥99%) were purchased from Sigma-Aldrich, India. Potassium iodide and Starch were purchased from Merck, India. 2,2,4-Trimethylpentane (99%), Acetic acid glacial (99.5%), and Sodium thiosulphate anhydrous (97%) were purchased from Loba Chemicals, India

Oxidative Stability Experiments

A calculated amount of different antioxidants were dissolved in ethanol and were added to 15 ml amber glass vials. Then the solvent was driven off by

nitrogen purging. Required amount of refined fish oil was added to each vial to achieve the desired final concentration of antioxidants and homogenized for 15 min. The concentration of antioxidants were decided based on the previous reports[13,14,15] to avoid any possible prooxidant effect. All the samples were stored in the incubator at 25ºC in contact with atmospheric air under darkness. The samples were withdrawn on five day intervals until the 50th day and the peroxide and p-anisidine values were determined. All the tests were performed in triplicates and the mean values were reported.

Characterization of the Oil

The physico-chemical properties of the crude and refined fish oil were determined before the beginning of oxidative stability experiments. The total concentration of n-3 PUFA (EPA + DHA) in the refined oil was 3.73 g/kg, before the commencement of oxidative stability studies. The crude sardine oil was refined and stored in a freezer at -20°C.[8] Peroxide value (AOCS Cd 8b-90) and p-Anisidine (AOCS Cd 18-90) value was determine.[15] The total oxidation (TOTOX) values were calculated based on the peroxide and p-anisidine values.[16]

Table 1: Levels of different natural antioxidants used in a full factorial design

Independent Variables	Coded Symbol	Variable Levels		
		Low (-1)	Middle (0)	High (+1)
Catechin (mM)	X_1	0.2	0.35	0.5
Resveratrol (mM)	X_2	0.2	0.35	0.5
Sinapic acid (mM)	X_3	0.2	0.35	0.5
Vanillic acid (mM)	X_4	0.2	0.35	0.5

Full Factorial Design (FFD) Experiments

A (2^4) full factorial design taking four variables (antioxidants) at two levels, augmented with three center point experiments was implemented in 19 experimental runs. Based on our preliminary screening studies comprising ten natural antioxidants,[8] four (catechin, resveratrol, sinapic acid, and vanillic acid) were chosen for this study. The factor settings (low and high) were established based on previous studies and they were set far enough from each other (0.2 mM & 0.5 mM) to identify the effect of individual antioxidants. The factor settings of the four chosen antioxidants are given in Table 1. TOTOX values were taken as response for analyzing the performance of these antioxidants in imparting oxidative stability to the n-3 PUFA containing refined sardine oil. The experimental design matrix was generated using Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA) software (Supplementary Table 1). Furthermore, the results of these experimental runs were analyzed using the same software.

Response Surface Methodology (RSM)

Based on the results of FFD experiments two factors were selected (catechin and resveratrol) for further optimization experiments. Central composite design (CCD) was applied to model the efficacy of antioxidants chosen. TOTOX value measured during the storage period was taken as the response. All the antioxidants were tested at five levels, through 13 experiments comprising four factorial points, two axial points (α = 1.414) and seven replicates at the center point. In the first set of experiments, catechin at 0.25 to 0.75mM and resveratrol at 3 to 4mM concentration range was taken (Table 2). In the second set of experiments, catechin at 0.25 to 0.75mM and resveratrol at 0.25 to 1mM concentration range was taken (Table 2). The results obtained from the design were fitted in a second-order polynomial equation,

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ j>1}}^{n=1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \varepsilon$$

Where Y is the responses, X_i and X_j are the independent variables, β_0, β_i, β_{ii}, and β_{ij} are the intercept terms, coefficients, quadratic coefficients, and coefficients of interaction effects, respectively. ε is a random error. For the regression analysis of the experimental data, Design Expert 11.1.2

(Stat-Ease, Minneapolis, MN, USA) was used. The models developed from the RSM experiment were analyzed using ANOVA, and the model variables were considered significant at $p < 0.05$. The polynomial equation developed above was used to build surface plots. Furthermore, by using the optimizer tool, optimal values were predicted using Design expert software.

Table 2: Levels of natural antioxidants used in central composite design in model 1 & model 2

Model 1						
Independent Variables	**Coded Symbol**	**Variable Levels**				
		Low (-1)	**Middle (0)**	**High (+1)**	**-α**	**+α**
Catechin (mM)	X_1	0.25	0.5	0.75	0.146	0.853
Resveratrol (mM)	X_2	3	3.5	4	2.792	4.207
Model 2						
Independent	**Coded Symbol**	**Variable LevelsVariables**				
		Low (-1)	**Middle (0)**	**High (+1)**	**-α**	**+α**
Catechin (mM)	X_1	0.25	0.5	0.75	0.146	0.853
Resveratrol (mM)	X_2	0.25	0.625	1	0.094	1.155

Results and Discussions

Oxidation of n-3 PUFA rich oil follows a chain of reactions resulting in primary and secondary oxidation products. Primary oxidation products are generally characterized by peroxide estimation, and the extent of secondary oxidation is identified by measuring the p-anisidine value. To facilitate a holistic indication of oxidation, peroxide and p-anisidine values were presented as total oxidation (TOTOX) values, as mentioned in the previous section.

A single antioxidant is generally known to prevent oxidation caused by a specific mechanism in oil. Accordingly, the single antioxidant tend to retard a specific mechanism or mechanisms of oxidation based on its physicochemical properties.[17,18] However, n-3 PUFA containing oil undergoes oxidation via diverse mechanisms initiated by several components and proceeds simultaneously at different rates. Hence, combining different antioxidants can complement each other and improve the overall performance of the antioxidants in retarding oxidation.[19,20] Given the complexity of preventing oxidation in n-3 PUFA oil and improving the antioxidant effectiveness, there has been a shift towards adding similar and independent antioxidants that result in a common overall effect on improving the stability by distinct pathways. In bulk oil systems, the understanding and the precise knowledge of interaction of added antioxidants and oil components in the presence of environmental factors (light, oxygen, moisture etc.) are limited. Under these circumstances, statistical tools such as FFD and RSM comes handy. These statistical techniques integrates the statistical designs and regression modeling techniques, and provide optimization methods which can be used to obtain mathematical model equations. These model equations correlates each independent variable with the response function. Such an empirical model equation can be used to identify the most significant variables or identify the concentration of those significant variables to get the best possible response.

Full Factorial Design (FFD) Analysis

Refined fish oil added with calculated quantity of antioxidants based on the experimental design was analyzed for its oxidative stability. It is well-established fact that antioxidants exhibit

antioxidant and prooxidant properties when used in concentrations beyond a certain limit.[21] At appropriate concentrations, antioxidant combinations can be expected to exhibit a synergistic effect. The experimental design comprising four natural antioxidants, each at two levels of concentration along with the mean values of the responses in three replicate (± standard deviation) is presented in Supplementary Table 1. On the basis of the response values, regression equation in terms of coded values was developed as follows,

Table 3. Analysis of variance (ANOVA) for the full factorial model

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	6979.00	10	697.90	4.21	0.0346	significant
A-Catechin	1668.74	1	1668.74	10.06	0.0157	significant
B-Resveratrol	1922.84	1	1922.84	11.59	0.0114	significant
C-Sinapic Acid	35.41	1	35.41	0.2133	0.6582	
D-Vanillic Acid	170.31	1	170.31	1.03	0.3448	
AB	1338.37	1	1338.37	8.06	0.0251	significant
AC	4.20	1	4.20	0.0253	0.8781	
AD	303.33	1	303.33	1.83	0.2185	
BC	847.81	1	847.81	5.11	0.0583	
BD	370.55	1	370.55	2.23	0.1788	
CD	317.44	1	317.44	1.91	0.2092	
Curvature	1632.55	1	1632.55	9.84	0.0165	
Residual	1161.73	7	165.96			
Lack of Fit	1161.73	5	232.35			
Pure Error	0.0000	2	0.0000			
Cor Total	9773.28	18				

$$Y_{TOTOX}=186.72-10.21X_1-10.96X_2-1.49X_3+3.26X_4-9.15X_1X_2-0.5124X_1X_3-4.35X_1X_4+7.28X_2X_3+4.81X_2X_4+4.45X_3X_4$$

Where, X_1 is catechin, X_2 is resveratrol, X_3 is sinapic acid and X_4 is vanillic acid. ANOVA of the model developed by regression for the FFD experiments shows that the model was significant ($p<0.05$). The Regressions coefficients (R^2) of 0.85 with the F and P value of the model 4.21 and 0.0346 respectively, indicating that the developed model is fitting well with the experimental data. In addition, 9.84 was the "curvature F value" implying the presence of significant curvature in the design space. From the ANOVA (Table 3), it can be inferred that both catechin and resveratrol showed main effect (p value of 0.0157 and 0.0114, respectively), whereas, the sinapic acid and vanillic acid fail to show the main effect ($p>0.05$). Catechin which showed a significant main effect, fail to show any interactive effect with sinapic acid and vanillic acid. Similarly, resveratrol also did not show an interactive effect with sinapic acid and vanillic acid. However, catechin and resveratrol showed a significant interactive effect as well (p-value of 0.0251). Catechin, a condensed tannin is known to suppress oil oxidation through metal chelation and free-radical scavenging activity,[12] whereas the resveratrol is believed to possess free-radical scavenging activity.[22] Based on the different antioxidation mechanisms offered by the catechin and resveratrol and the main and interactive effects of these antioxidants, it was hypothesized that the synergistic effect prevails for the prevention of oxidation of sardine oil.

Response Surface Methodology (RSM)

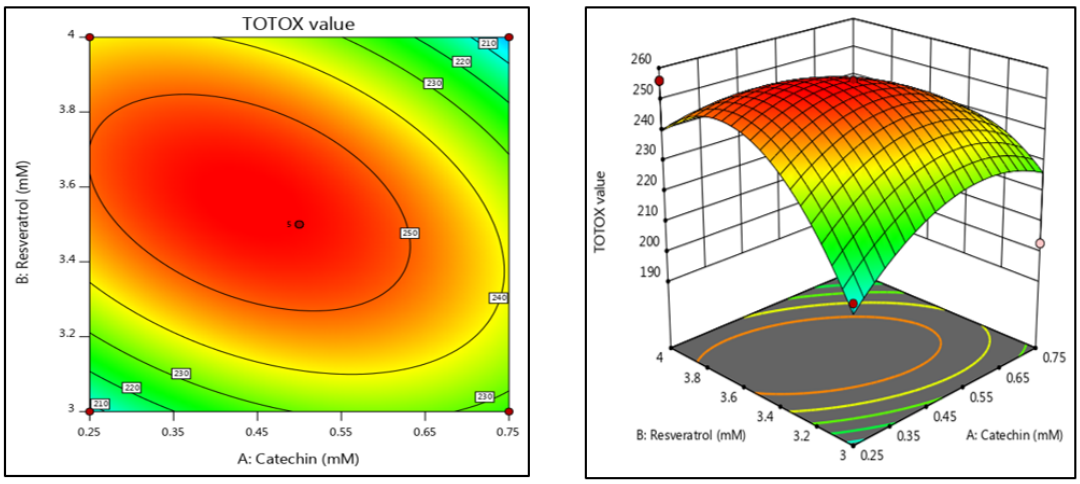
The catechin and resveratrol were identified as antioxidants which provides the synergistic effect based on the FFD and further experiments were designed to obtain the optimum concentration of the antioxidants to provide the lowest TOTOX value using RSM. The concentration range of catechin

was chosen in the range of 0.146 to 0.853mM and resveratrol in the range of 2.792 to 4.207mM were considered as independent variables (Table 2) and the TOTOX value was the response of the oxidation process (RSM-Model 1). A total of 13 experimental runs were conducted and the response obtained were tabulated in Supplementary Table 2, along with the residuals. The quadratic equation is generated in coded units for the response (TOTOX value) and is given as follows,

$$Y_{TOTOX} = 255.93 - 4.74X_1 + 2.08X_2 - 14.22X_1X_2 - 13.37X_1^2 - 23.55X_2^2$$

Table 4: The ANOVA of natural antioxidants for the response surface model 1

Source	Sum of Squares	df	Mean	F-value	p-value	Remarks
Model	5630.97	5	1126.19	4.23	0.0431	significant
A-Catechin	179.75	1	179.75	0.6756	0.4382	
B-Resveratrol	34.61	1	34.61	0.1301	0.7290	
AB	808.45	1	808.45	3.04	0.1248	
A^2	1242.91	1	1242.91	4.67	0.0675	
B^2	3858.10	1	3858.10	14.50	0.0066	
Residual	1862.44	7	266.06			
Lack of Fit	1862.44	3	620.81			
Pure Error	0.0000	4	0.0000			
Cor Total	7493.41	12				



(a)

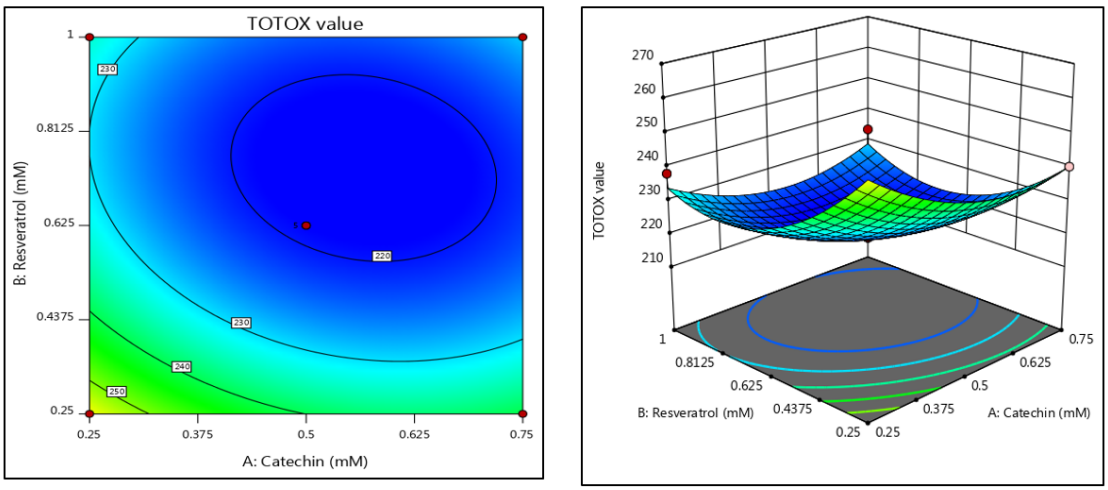
Fig.1: Contour and surface plots of the increase in the TOTOX values in response to the variation in catechin and resveratrol concentration, from the model equation 1

Where X_1 represents catechin and X_2 represents resveratrol. ANOVA indicates that the quadratic model developed is significant ($p<0.05$). The model presented an F-value of 4.23, and determination coefficient (R^2) of 0.7515 implying the model is significant (Table 4). The contour plots show that the design space is adequate and the global optima lies in the center of the design space. The convex surface plots show that the response (TOTOX) was being maximized instead of getting reduced with increasing concentration of the antioxidants (Figure 1). Thus surface plots imply a prooxidant effect of these two natural antioxidants at the tested concentrations. However, the degree of increasing of

TOTOX value is highly influenced by the increasing concentration of resveratrol and almost independent of the increasing concentration of catechin, which was observed from the contour plot (Figure 1). The optimizer tool available in Design Expert software predicted a maximum TOTOX value of 256 at the catechin and resveratrol concentrations of 0.25 and 4.0mM, respectively. The validation experiments of the oxidative stability conducted for 50 days as explained earlier in triplicate at the predicted antioxidants concentrations and found that the experimental result is within 5% variation of predicted TOTOX value. The contribution of resveratrol on the prooxidant effect was confirmed by analyzing the TOTOX value of the oil with constant catechin concentration of 0.25mM and increasing the resveratrol concentration in the range of 0.25 to 4mM (Figure 1). Hence, it is hypothesized that the synergistic effect of oxidative prevention may be possible by decreasing the concentration of catechin and resveratrol.

Table 5: The ANOVA of natural antioxidants for the response surface model 2

Source	Sum of Squares	df	MeanSquare	F-value	p-value	Remarks
Model	2223.43	5	444.69	6.17	0.0167	significant
A-Catechin	216.52	1	216.52	3.01	0.1266	
B-Resveratrol	565.30	1	565.30	7.85	0.0265	
AB	28.80	1	28.80	0.3998	0.5473	
A^2	472.04	1	472.04	6.55	0.0376	
B^2	1105.15	1	1105.15	15.34	0.0058	
Residual	504.26	7	72.04			
Lack of Fit	504.26	3	168.09			
Pure Error	0.0000	4	0.0000			
Cor Total	2727.68	12				



(b)

Fig.2: Contour and surface plots of the decrease in the TOTOX values in response to the variation in catechin and resveratrol concentration, from the model equation 2

As the aim of this entire exercise was to find a synergistic mixture of natural antioxidants, which can enhance the oxidative stability of the sardine oil significantly, one more RSM study was conducted by designing the experiments (RSM-Model 2) with a lower concentration range for

the resveratrol and the similar concentration of catechin (Table 2). Accordingly, experiments were conducted at the unaltered catechin concentrations (0.146 to 0.853 mM) with the changed resveratrol concentration range from 0.094 to 1.155 mM. Total of 13 experimental runs were conducted and the response obtained were tabulated in Supplementary Table 3. The quadratic equation is generated in coded units for the response (TOTOX value) and is given as follows,

$$Y_{TOTOX} = 218.93 - 5.20X_1 - 8.41X_2 + 2.68X_1X_2 + 8.24X_1^2 + 12.60X_2^2$$

The negative coefficient of the linear terms and the positive second-order coefficients indicating the combination of antioxidants at the appropriate concentration can reduce the response (TOTOX) of the system. ANOVA indicates that the quadratic model developed is significant ($p<0.05$). The model presented an F-value of 6.17, and determination coefficient (R^2) of 0.8151 implying the model is significant (Table 5). The contour plots show that the design space is adequate and the global optima lies in the center of the design space. The concave surface plots show that the response was getting reduced as the variables varied with each other (Figure 2). Thus, surface plots show a synergistic response of these two natural antioxidants at the tested concentrations. Using the optimizer tool available in Design Expert software was exploited with the goal of minimizing the response (TOTOX) by varying the concentration of both the antioxidants. A TOTOX value of 218 was predicted at catechin and resveratrol concentrations of 0.5 and 0.625mM concentration, respectively. Further, oxidative stability studies conducted for 50 days as explained earlier in triplicate, validated the predicted values with less than 5% variation.

Thus, two natural antioxidants having the ability to interact each other while retarding oxidation by FFD and later, RSM was implemented to study the collective behavior of selected antioxidants at different concentrations in a bulk oil system. By conducting RSM at two different sets of concentrations of selected antioxidants (catechin and resveratrol), it was proved that the same set of antioxidants can act as prooxidants at higher concentrations whereas they exhibit synergistic activity at optimal concentrations. The combination showed prooxidant effect in the first study (RSM model 1) where resveratrol was taken at higher level (Figure 1), whereas it showed synergistic effect while it was taken at the lower level (RSM Model 2) (Figure 2). Even though both the antioxidants synergistically contribute to the prooxidant effect at their higher concentrations, the prooxidant effect was mainly contributed by the increasing resveratrol concentration (Figure 1). Hence the catechin concentration was taken in the range of 0.146 to 0.853mM in both the RSM studies (Model 1 and 2). Melo *et al*.[23] had made similar observation while reporting their study on the antioxidant effect of grape rachis extract - α-tocopherol combination in soybean bulk oil system. They had reported a synergistic interaction between grape rachis extract (1% w/w) and a lower concentration of α-tocopherol (0.25% w/w) and prooxidant effect at lower concentration of rachis extract (0.25%w/w) and higher concentration of α-tocopherol (1.0% w/w). Incidentally the grape rachis extract also contains significant amount of resveratrol. Unfortunately, studies on the effect of resveratrol alone on oxidative stability of bulk oil system is scarce. Resveratrol is a stilbene natural phenolic compound which exhibit free-radical scavenging mechanism, whereas catechin exhibit both free-radical and metal-chelating mechanism. Under optimal concentrations, this mixture exhibits synergism to inhibit the oxidation of sardine oil.

Considering the fact that the oil undergoes multiple simultaneous diverse mechanisms, combining two or more antioxidants of different natures is expected to be a logical and feasible solution. The phenomenon of sacrificial oxidation of an antioxidant and the concept of synergism, where the cooperative action displays a stabilizing effect more significant than the sum of individual antioxidant effects often seen in herbal extracts rich in diverse groups of antioxidants strengthen this logic.[24] Two mechanisms can achieve synergism; Homosynergism involves two or more antioxidants with the same mechanism of action. One antioxidant acts as a primary antioxidant, and the other antioxidant acts as a synergist.[25] Heterosynergism has two or more antioxidants with different mechanisms. Metal chelators with free radical scavengers often show synergism. Phospholipids which are metal chelators, inhibit metal-catalyzed oxidation, thereby produce lower levels of radicals to be reduced by free radical scavengers.[26] Such antioxidant combinations utilize different or the same mechanism to suppress

oxidation for a long duration.[27] Though several literatures have explored the application of natural antioxidant extracts like rosemary extract,[28] the contribution of individual components of these extracts to the synergistic effect was never explored in detail. While it is well established that antioxidants work in synergy to either exhibit a prooxidant effect or an antioxidant effect, the critical concentration at which this synergistic antioxidant becomes prooxidant has not been studied. It is crucial to understand the prooxidant mechanisms and their causes to efficiently design appropriate concentrations of antioxidants to control bulk oil oxidation. For instance, tea polyphenols were identified to exhibit antioxidant activities at 0.01% and prooxidant effect at 0.04% concentrations respectively.[29] Thus, in terms of concentration levels, antioxidant properties are more dominant at lower levels and prooxidant effects may dominate at higher levels. Another possible cause for prooxidant mechanism can be attributed to pH, location and metal-catalyzed phenolic oxidation in case of highly heterogenic systems like emulsions.[30] To the best of our knowledge, this is the first report to develop a synergistic formulation of antioxidants using statistical approaches.

There is an escalating appeal for n-3 PUFA based products in the food and nutraceutical industry that is preceded by the need to address these products' oxidative stability.[6] While standard antioxidant strategies involving synthetic antioxidants, natural antioxidants, and antioxidants from novel sources continue to be the focal point of several researchers, there is a perspective shift toward utilizing the existing antioxidants by the best feasible strategies for maximum protection against oxidation. Using existing antioxidants at appropriate concentrations in combinations is expected to provide synergistic/ interactive effects on improving the oxidative stability of n-3 PUFA rich oils.[19] However, there exists a myriad of effective antioxidant choices and possible concentrations for the effective interactive antioxidant effect to retard oxidation. While antioxidants are expected to have a syngergistic antioxidant effect, many cases have reported the prooxidant activity as well.[3] The possibility of a molecule to exhibit antioxidant or prooxidant properties when used in combinations is determined by several factors with concentration of the compounds as a primary influencer.[3,19] As the existing studies are limited to exploring the interactive effect from natural extracts and their combinations, there is a sine qua non to initiate formulation of appropriate antioxidant combinations to progress in this field further. To determine the superlative antioxidants and their finest concentrations, applying statistical tools can be considered an effective choice to limit the number of experimental trials and identify the best combinational effects. The current article represents such a statistical approach for this application. Only recently have the importance of these statistical approaches in understanding the combined effect of antioxidants have been gaining attention.[6] The current study has demonstrated the application of statistical tools to formulate desired combination of antioxidants to exhibit synergistic effects. The results of this study further emphasize on the importance of critical antioxidant concentrations to achieve the desired synergy in the sardine oil system. While these studies effectively determine the best antioxidant combinations and their concentrations, they do not necessarily provide the mechanism behind such interactive effects. That can be addressed in the future by conducting structure-activity relationship studies, the physical location of antioxidants in the oxidation system and their lipophilicity.[19]

Conclusions

Antioxidant combinations are considered more effective than their individual counterparts on account of their ability to exhibit different mechanisms and synergistic effects. In this study, phenolic antioxidants were combined to analyze their interactive effect. Catechin and resveratrol showed a significant effect ($p<0.05$) in FFD experiments in the 50 days of storage at 25ºC. RSM was employed to determine the optimal concentrations of the mixture through two sets of studies. Catechin (0.5mM) and resveratrol (0.625mM) expressed a significant synergistic effect ($p<0.05$) in enhancing the oxidative stability of the sardine oil. We further established that the same combination of antioxidants can exhibit both prooxidant effect and synergistic antioxidant effect, concentrations being the most important parameter. For instance, compared with the control, a combination of catechin (0.5mM) and resveratrol (0.625mM) reduced the 41.65% TOTOX value in the 50 days' study. Whereas, higher concentration of the same mixture exhibited a prooxidant effect in the bulk oil system. To the best of our knowledge, the current study is one of the preliminary research

works to demonstrate the application of statistical tools to develop antioxidant formulation in sardine oil.

Acknowledgement
Sumit Mishra is supported by scholarship for doctoral course by the National Institute of Technology Karnataka (N.I.T.K.), Surathkal, India.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflicts of Interest
Authors declare no conflict of interest

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