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Optimization of α-tocopherol and ascorbyl palmitate addition for the stabilization of sardine oil

R. Morales-Medina, P.J. García-Moreno✉, M.M. Muñío, A. Guadix and E.M. Guadix

Department of Chemical Engineering, University of Granada, 18071 Granada, Spain

✉Corresponding author: pjgarcia@ugr.es

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SUMMARY: The purpose of the present work was to optimize the addition of natural antioxidants (α-tocopherol and ascorbyl palmitate) for the stabilization of sardine oil rich in omega-3 PUFA. The optimal values for peroxide value (PV), which minimizes primary oxidation products, were obtained at low concentrations of α-tocopherol (50–207 ppm), high content of ascorbyl palmitate (450 ppm) and 50 ppm citric acid. On the other hand, optimal values for *p*-anisidine value (AV), which minimizes secondary oxidation products, were found at medium concentrations of α-tocopherol (478–493 ppm), high contents of ascorbyl palmitate (390–450 ppm) and 50 ppm citric acid. The conflicting effect of α-tocopherol on the individual optimization of PV and AV motivated the generation of a Pareto front (set of non inferior solutions) employing the weighted-sum multi-objective optimization technique.

KEYWORDS: *Antioxidants; Fish oil; Lipid oxidation; Optimization; Stabilization*

RESUMEN: ***Optimización de la adición de α-tocoferol y palmitato de ascorbilo para la estabilización de aceite de sardina***. El objetivo de este trabajo fue optimizar la adición de antioxidantes naturales (α-tocoferol y palmitato de ascorbilo) para la estabilización de aceite de sardina rico en omega-3 PUFA. Bajas concentraciones de α-tocoferol (50–207 ppm) combinadas con la adicción de antioxidantes secundarios como palmitato de ascorbilo (450 ppm) y ácido cítrico (50 ppm), minimizaron la formación de hidroperóxidos en el aceite de sardina estudiado. Sin embargo, los productos secundarios de oxidación se redujeron para concentraciones medias de α-tocoferol (478–493 ppm), altas de palmitato de ascorbilo (390–450 ppm) y 50 ppm de ácido cítrico. El efecto contradictorio de la concentración de α-tocoferol en la optimización individual del índice de peróxidos e índice de *p*-anisidina motivó la realización de una optimización simultánea que permite satisfacer la optimización de cada una de las variables individuales en el grado deseado.

PALABRAS-CLAVE: *Aceite de pescado; Antioxidantes; Estabilización; Optimización; Oxidación lipídica*

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1. INTRODUCTION

Fish oils are widely known because of the beneficial role they play in human health and nutrition. These properties are attributed to the high content of polyunsaturated fatty acids (PUFA), especially of the omega-3 PUFA family and more in particular to eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids (Fedačko *et al*., 2007). During the last decades, the anti-thrombotic,

anti-arrhythmic and anti-inflammatory effects of these fatty acids have been reported (Ruxton, 2011). Furthermore, omega-3 PUFA have been described to prevent diabetes, allergy and some types of cancer such as stomach, pancreatic, breast, prostate and colon cancers (Sidhu, 2003).

However, due to their high level of unsaturation these omega-3 PUFA are extremely susceptible to oxidative spoilage. In addition, the steps required for the extraction and processing of fish oils promote hydrolytic and oxidative reactions, which cause the production of free fatty acids, hydroperoxides and rancidity (Donnelly and Robinson, 1995). The mechanism of lipid oxidation consists of three main steps: initiation, propagation and termination reactions. In short, the first step takes place when an unsaturated lipid loses a hydrogen atom and produces free radicals due to the presence of initiators such as heat, light or ionizing radiation or metal ions. Then, free lipidic radicals react with oxygen so as to produce peroxyl radicals and lately hydroperoxides, which are known as primary oxidation compounds. Hydroperoxides are decomposed by the action of heat and/or metal ions yielding secondary oxidation compounds such as non-volatile and volatile aldehydes, alcohols, and tertiary oxidation products. These secondary oxidation products are responsible for undesirable odors and off-flavors (Shahidi and Zhong, 2010).

To prevent the oxidation of omega-3 PUFA, the stabilization of fish oils with natural antioxidants is one of the most common strategies followed. In the same manner that oxidation can be driven by several mechanisms, antioxidants can act by different pathways and are classified into two main groups: primary or chain-breaking and secondary or preventive antioxidants. The mechanism of chain-breaking antioxidants consists of donating hydrogen atoms to lipid radicals so as to convert radicals into stable products. Otherwise, secondary antioxidants act by a set of mechanisms such as scavenging oxygen, regenerating primary antioxidants, binding metal ions, absorbing ultraviolet radiation or deactivating singlet oxygen (Frankel, 2005). The most typical primary antioxidant is α-tocopherol, while the most common natural secondary (preventive) antioxidants are citric acid, which acts as a metal chelator, and ascorbyl palmitate, which can scavenge oxygen, among others (Frankel, 2009). The combinations of these three natural antioxidants has shown high efficiency for the stabilization of fish oils since synergic effects among them were reported (Olsen *et al.*, 2005, Aubourg *et al.*, 2004, Frankel, 2005). In view of the above, the study of the stabilization process is a crucial issue to be considered for the production of food-grade fish oil.

The aim of this work was to optimize the stabilization process for sardine oil under ambient conditions. Such a systematic study is not available in the literature. The influence of α-tocopherol and ascorbyl palmitate concentrations on free fatty acid contents and on oxidation properties (peroxide, p-anisidine and total oxidation values and induction period) was evaluated. Finally, a bi-objective optimization was performed because the optimum values of peroxide and p-anisidine were simultaneously pursued.

2. MATERIALS AND METHODS

2.1. Materials

Crude sardine oil was kindly donated by AFAMSA S.A. (Vigo, Spain), and had a composition of 19.7% (w/w) in EPA and 9.4% (w/w) in DHA. Citric acid, (±)-α-tocopherol and 6-O-Palmitoyl-L-ascorbic acid were acquired from Sigma-Aldrich Quimica SA (Madrid, Spain). In order to enhance the solubility in oil, citric acid granules were milled until achieving a size smaller than 200 μm. Hexane (95%), ethyl ether, ethanol, acetic acid (99.9%), isooctane, potassium hydroxide ethanolic solution (0.1 N) and sodium thiosulfate solution (0.0.1 N) were purchased from Panreac Química S.L.U. (Barcelona, Spain).

2.2. Preparation of the fish oil matrix

The fish oil matrix was obtained from crude sardine oil by chromatographic purification on an alumina-silica column according to the method described by Lin and Hwang (2002) with some modifications. A glass chromatographic column (5×50 cm) was packed with 100 g of silica gel (70–230 mesh, Merck) and 100 g of basic alumina (70–230 mesh, Merck) suspended in hexane (400 mL). The oil (200 g) was dissolved in an equal mass of hexane and passed through the column. The chromatographic column was wrapped with aluminum foil to prevent light-induced oxidation during the purification process, and the purified oil dissolved in hexane was collected in an aluminum foil-wrapped flask. The hexane was evaporated in a rotary evaporator at 40 °C under vacuum, and the oily residue was bubbled with a nitrogen stream. This process was carried out seven times. The oil from all the batches was homogenously mixed and stored at −20 °C until use.

2.3. Stabilization procedure

The desired amounts of antioxidants were incorporated into the fish oil matrix prepared previously by heating up to 95 °C and agitating vigorously for 1.5 h under high vacuum (15 mbar). A factorial experimental design comprising 18 runs was executed, in which the concentrations of α-tocopherol and ascorbyl palmitate were set at three levels and the storage times at two levels. In the case of α-tocopherol, the concentrations tested were 50, 550

and 1050 ppm, which are in the range of the concentrations described in previous studies: 50–1000 ppm (Drusch *et al*., 2008), 50–500 ppm (Zuta *et al*., 2007), 800 ppm (Olsen *et al*., 2005) and 50–500 ppm (Kulås and Ackman, 2001a). For ascorbyl palmitate the concentrations evaluated (50, 250 and 450 ppm) were also in the line of the concentrations studied in previously published works: 50–500 ppm (Drusch *et al*., 2008), 200 ppm (Olsen *et al*., 2005), 250 ppm (Kulås and Ackman, 2001a) and 100 ppm (Hamilton *et al*., 1998). In this study, a low concentration of α-tocopherol indicated values close to 50 ppm, a medium concentration indicated values close to 450 ppm and a high concentration values close to 1050 ppm. In the case of ascorbyl palmitate, a low concentration denoted values close to 50 ppm, medium concentration values were close to 250 ppm and high concentration values were close to 450 ppm. Finally, a fixed concentration of citric acid (50 ppm) was employed for each experiment according to Kulås and Ackman (2001a).

Two samples were prepared for each combination of antioxidants and they were stored in 100 mL amber bottles at 25 °C. Each bottle contained approximately 60 mL and no nitrogen was added to the samples. Samples were taken at 10 and 30 days for analysis. In addition, two samples of purified oil were subjected to the same protocol in an attempt to study the behavior of the oil without any antioxidant.

2.4. Determination of hydrolysis and oxidation properties

2.4.1. Free fatty acids

The free fatty acid (FFA) content of the oil samples was determined according to the standard ISO 660:2009 (ISO, 2009). This method is based on the titration of the oil, suitably diluted with an ethanol-ethyl ether mixture, with a potassium hydroxide solution employing phenolphthalein as the indicator. Analyses were carried out in triplicate and the results were expressed as percentage of oleic acid.

2.4.2. Peroxide, p-anisidine and total oxidation (Totox) values

The peroxide value (PV) of the oil samples was determined according to the standard ISO 3960:2007 (ISO, 2007). The PV method is based on the titration with a sodium thiosulfate solution of the oil diluted with an acetic acid-isooctane mixture and then treated with potassium iodide. Measurements were carried out in triplicate and the results were expressed as mili-equivalents per kg of oil.

The anisidine value (AV) of the oil samples was determined according to the standard ISO 6885:2006 (ISO, 2006a). The AV method is based on the reaction of *p*-anisidine diluted in acetic acid with the α– and β– unsaturated aldehydes (primary 2-alkenals) present in the oil. Analyses were carried out in triplicate and the results were expressed as 100 times the increment of absorbance produced by this reaction, measured at 350 nm.

Totox is a comprehensive oxidation index calculated from a weighted sum of peroxide value (PV) and *p*-anisidine value (AV) by applying Eq. 1:

$$Totox = 2 \cdot PV + AV \tag{1}$$

2.4.3. Rancimat induction period

The Rancimat induction period of the samples was determined according to the standard ISO 6886:2006 (ISO, 2006b). A Metrohm Rancimat model 743 (Methrom Instruments, Herisau, Switzerland) was used. A stream of filtered, clean, dry air at a rate of 20 $L \cdot h^{-1}$ was bubbled into 3 g of oil samples contained in reaction vessels. These vessels were placed in an electric heating block set at 90 °C. Effluent air containing volatile organic acids from the oil samples was collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water was continuously recorded and the induction period (IP) was automatically determined by the apparatus. Measurements were carried out in triplicate and the Rancimat induction period was expressed as resistance time (in hours) of the oil to oxidation.

2.5. Statistical analysis

The statistical analysis and the regression models were generated using the Statgraphics software (version 5.1). First, the output variables (Y: FFA, PV, AV, Totox and IP) were related to the input variables (X: concentration of α-tocopherol, concentration of ascorbyl palmitate and time of storage) by second degree polynomials as follows, Eq. 2:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{2} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \tag{2}$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables. It should be noted that no quadratic coefficient was employed for the time of storage since it was assayed at only two levels.

Next, the analysis of variance (ANOVA) was carried out. The significance of all the terms in the models was judged statistically by computing the *p*-value at a confidence level of $1-\alpha=95\%$. The regression coefficients were then used to generate contour maps and to find the optimal stabilization

conditions which maximize the quality of the oil in terms of oxidation parameters at 10 and 30 days of storage.

2.6. Bi-objective optimization

A problem of bi-objective optimization arises when two objectives must be fulfilled. In our case, it is desired to obtain minimum values for PV and AV in order to obtain the highest quality of the stabilized oil.

The concept of Pareto Front, related to the identification of an adequate solution, consists of a set of non inferior solutions, which are defined as those in which an improvement in one objective requires a degradation of another (Halsall-Whitney and Thibault, 2006). In this work, the Pareto Front was generated by employing the weighted-sum method (Kim, 2004). This method consists of expressing a comprehensive objective function (OBJ) as a linear combination of the individual objectives (PV and AV), by means of a weight factor (w), which quantifies the relative importance given to the accomplishment of each individual objective, Eq. 3:

$$OBJ=w\cdot(PV)+(1-w)\cdot(AV) \quad (3)$$

where $0\leq w\leq 1$

The Solver Tool, included in the MS Excel software, was chosen to carry out all the calculations required for the bi-objective optimization.

3. RESULTS AND DISCUSSION

3.1. Quality properties of fish oils

Table 1 presents the experimental values of free fatty acid content (FFA), peroxide (PV), *p*-anisidine (AV), Totox and induction period (IP) of the crude, purified, and control and stabilized oils produced.

It was observed that the purification process of the oil produced a significant reduction in the FFA content, which varied from 6.26 to 0.14%, and of the AV which was reduced from 17.18 to 2.17. However, the peroxide value was maintained practically constant at around 2.7 meq $O_2\cdot Kg^{-1}$ oil. Although it was reported that adsorption on silica-alumina reduces hydroperoxides in the oil, this high-purity oil also showed rapid oxidation (Lin and Hwang, 2002). Thus, it is suggested that a reduction in PV could have happened but the oil matrix may have suffered oxidation after the purification process. Regarding the fatty acid profile, no significant differences were observed between crude and purified oil (data not shown).

During the storage period, the FFA content of the stabilized oils did not increase but even suffered a slight decrease, Table 1. This indicates that hydrolysis of the stabilized oils did not occur due to their low moisture content and that FFA were possibly oxidized since they are more susceptible to oxidation than esterified fatty acids (Aidos *et al*., 2001).

Regarding oxidation products, PV was employed to determine the content of hydroperoxides for the stabilized oils. In Table 1, it is shown that PV increased from 2.79 meq $O_2\cdot Kg^{-1}$ oil for the purified oil to 30.49 and to 53.78 meq $O_2\cdot Kg^{-1}$ oil for the control samples after 10 and 30 days of storage, respectively. It was revealed that the addition of antioxidants led, in most cases, to stabilized oils with lower PV than the control samples. The PV levels of the stabilized oil samples ranged between 18.20 and 41.81 meq $O_2\cdot Kg^{-1}$ oil after 10 days of storage at 25 °C, while PV levels up to 69.84 meq $O_2\cdot Kg^{-1}$ oil were obtained after 30 days of storage. For 10 days of storage, low-medium concentrations of α-tocopherol combined with the maximum addition of ascorbyl palmitate (450 ppm) resulted in the lowest PV, 20.83 and 18.20 meq $O_2\cdot Kg^{-1}$ oil, respectively. In the case of 30 days of storage, the highest addition of ascorbyl palmitate assayed (450 ppm) combined with a low content of α-tocopherol (50 ppm) resulted in the maximum prevention of hydroperoxide formation, a PV of 31.55 meq $O_2\cdot Kg^{-1}$ oil. Table 1 also shows that at low and medium α-tocopherol concentrations (50 and 550 ppm), the PV decreased when the ascorbyl palmitate concentration increased. This fact can be explained by the synergic effect between ascorbyl palmitate and α-tocopherol. Several works reported that ascorbyl palmitate can regenerate α-tocopherol from its radical derivatives enhancing its activity and thus reducing the amount of α-tocopherol radicals available for participation in side reactions and further oxidation (Olsen *et al*., 2005, Frankel *et al*., 1994). On the contrary, at high concentration of α-tocopherol (1050 ppm) a detrimental effect in the PV was observed when increasing the concentration of ascorbyl palmitate. In previous studies (Zuta *et al*., 2007, Kulås and Ackman, 2001b), it was revealed that α-tocopherol can become prooxidant when used at a high concentration by regenerating peroxyl radicals, Eq. 4, and by participating in the chain transfer reaction, Eq. 5.

$$LOOH+A\cdot\rightarrow LOO\cdot+AH \quad (4)$$

$$A\cdot+LH\rightarrow AH+L\cdot \quad (5)$$

Where LOO· refers to the hydroperoxyde radical, AH is the α-tocopherol molecule, A· is the α-tocopherol free radical and L· lipid free radicals.

Moreover, citric acid (50 ppm) was employed as a metal chelator in order to reduce the metal-catalyzed decomposition of preformed hydroperoxides. In addition, it has a synergic effect with α-tocopherol, which protects this chain-breaking antioxidant against the metal-catalyzed initiation

Table 1. Experimental design and measured values for the response variables

Exp.	Citric acid (ppm)	α-tocopherol (ppm)	Ascorbyl palmitate (ppm)	Time at 25 °C (days)	FFA (% oleic)	PV (meq $O_2 \cdot Kg^{-1}$)	AV	TOTOX	IP (h)
Crude oil	–	–	–	–	6.26±0.02	2.67±0.20	17.18±0.56	22.52	–
Purified oil	–	–	–	–	0.14±0.01	2.79±0.30	2.17±0.74	7.75	0.31±0.05
Control 10	0	0	0	10	0.11±0.01	30.49±0.24	19.74±1.38	80.72	0.12±0.03
1	50	50	50	10	0.08±0.02	41.81±0.27	18.33±0.76	101.94	0.15±0.13
2	50	50	250	10	0.06±0.00	30.00±0.46	14.71±0.71	74.71	0.30±0.09
3	50	50	450	10	0.06±0.01	20.83±0.20	10.59±1.21	52.25	0.47±0.12
4	50	550	50	10	0.03±0.02	28.2±0.19	10.48±0.75	66.88	1.59±0.01
5	50	550	250	10	0.06±0.02	21.50±0.24	6.27±0.54	49.27	1.80±0.05
6	50	550	450	10	0.08±0.00	18.20±0.19	4.94±0.94	41.34	2.26±0.06
7	50	1050	50	10	0.11±0.01	27.82±0.34	10.5±1.66	66.14	2.06±0.04
8	50	1050	250	10	0.10±0.01	25.66±0.25	8.82±1.35	60.13	1.95±0.04
9	50	1050	450	10	0.00±0.00	35.00±0.10	12.99±0.32	82.99	0.68±0.02
Control 30	0	0	0	30	0.11±0.02	53.78±0.78	49.38±4.60	156.94	0.02±0.00
10	50	50	50	30	0.14±0.02	52.83±0.24	47.85±2.19	153.51	0.02±0.00
11	50	50	250	30	0.11±0.01	43.28±0.24	33.96±3.44	120.51	0.02±0.01
12	50	50	450	30	0.08±0.00	31.55±0.24	18.97±1.35	82.07	0.03±0.01
13	50	550	50	30	0.06±0.01	55.09±0.41	17.94±0.17	128.12	0.85±0.03
14	50	550	250	30	0.08±0.02	53.6±0.37	15.24±0.15	122.47	0.90±0.04
15	50	550	450	30	0.08±0.01	50.73±0.51	12.87±0.13	114.33	1.06±0.06
16	50	1050	50	30	0.11±0.01	62.45±0.64	20.37±1.68	145.27	1.33±0.02
17	50	1050	250	30	0.08±0.00	69.68±0.46	19.3±0.15	158.66	1.18±0.08
18	50	1050	450	30	0.09±0.01	69.84±0.30	34.35±1.48	174.03	0.05±0.01

FFA: free fatty acid content, PV: peroxide value, AV: *p*-anisidine value, Totox: total oxidation value, IP: induction period.

of oxidation. Nevertheless, when employed at low concentrations (i.e. 50 ppm), citric acid did not alter the concentration at which α-tocopherol became prooxidant (Kulås and Ackman, 2001b). The content of nonvolatile secondary oxidation products produced by the decomposition of hydroperoxides was monitored according to the *p*-anisidine value (AV). It was observed that an increase was produced in the AV from 2.17 for the purified oil to 19.74 and to 49.38 for the control samples after 10 and 30 days of storage, respectively. The stabilized oils presented lower AV than the control samples at the same time of storage. In this case, for both 10 and 30 days of storage, the medium concentration of α-tocopherol combined with a high addition of ascorbyl palmitate (450 ppm) allowed for the maximum reduction in the decomposition of hydroperoxides, with AV of 4.94 and 12.87, respectively. Although previous studies reported that ascorbyl palmitate may increase hydroperoxide decomposition by reducing trace metal ions present in the systems (Hamilton *et al.*, 1998, Olsen *et al.*, 2005), the opposite effect was found in this work. It might be explained by the fact that high concentrations of ascorbyl palmitate avoid prooxidant effects and that a better regeneration of the α-tocopherol radical prevented the decomposition of lipid hydroperoxides by Eq. 4 (Frankel, 2005).

With regard to the Totox index of the stabilized oils, it followed the same trend as PV at 10 and 30 days of storage, (Table 1). This was due to the higher weight given to PV compared with the weight of AV in its calculation, Eq. 1.

On the other hand, the Rancimat induction periods did not have a direct correspondence with PV and AV, apart from the stabilized oil obtained in experiment 6 which presented the minimum PV and AV and also had the maximum induction period after 10 days. This fact is attributed to the high temperature (90 °C) employed in the Rancimat test which did not allow for a comparison of the data obtained from this accelerated oxidation method and experiments at room temperature (Drusch *et al.*, 2008).

Regarding the fatty acid profile of the oils, no significant changes were observed after 10 or 30 days of storage (data not shown). Similar results were obtained by Aryee *et al*. (2012) for salmon oil after 45 days of storage at 25 °C in the dark.

3.2. Statistical modeling

The experimental data obtained for each measured variable were fitted to a quadratic model. Table 2 shows the polynomial coefficients for each surface response model and the associated *p*-value. Similar statistical analyses have already been used to optimize different stages in the processing of fish oil such as the extraction of oil from herring by-products (Aidos *et al*., 2003), and the bleaching of sardine oil (García-Moreno *et al*., 2013).

Table 2 reveals that PV, Totox and IP are highly dependent on the linear effect of the α-tocopherol concentration, with an associated probability $p<0.001$. Regarding ascorbyl palmitate concentration, its linear effect was only statistically significant for PV and Totox. On the other hand, the time of storage was the input variable with the highest influence on the measured variables, with its linear effect being statistically significant in the cases of PV, AV, Totox and IP. Quadratic effects were found to be significant only for the α-tocopherol concentration in the cases of PV, AV, Totox and IP. The interaction between α-tocopherol and acorbyl palmitate concentration resulted statistically significant for most of the variables (PV, AV, Totox and IP), whereas the interaction between α-tocopherol concentration and time of storage was found to be significant only in the cases of PV and Totox. The interaction between ascorbyl palmitate concentration and the time of storage was not significant for any of the output variables. It should also be noted that none of the experimental factors assayed was statistically significant for FFA.

In Table 2, it is also observed that the proposed quadratic models explain the variability of the data to a large extent, with coefficients of determination, R^2, being around 0.90 or higher for most of the output variables except for FFA.

In order to optimize the stabilization process, the measured variables PV and AV were chosen as appropriate objective functions to indicate the oxidative deterioration of the oil samples. Firstly, the goodness of the fit for these variables was proven by plotting the measured values against the predicted ones for PV (Figure 1a) and AV (Figure 1b). The data were correlated by means of a regression line whose equation is inserted into each figure. Also, the dotted lines representing a deviation of ±10% between experimental and predicted values are shown.

Secondly, contour plots for PV and AV at 10 and 30 days of storage were generated by means of the second-order models obtained by employing response surface methodology, as shown in Figure 2. These contour plots made it possible to optimize the quality of the stabilized oils in terms of oxidation products, obtaining minimum values for PV and AV. These optimum values were marked as circles in Figure 2. In Figure 2a1, the optimal value for PV after 10 days of storage, 18.56, was found at the α-tocopherol concentration of 207 ppm and at the highest ascorbyl palmitate concentration assayed (450 ppm). For the AV at 10 days of storage (Figure 2b1), the minimum value of 2.64 was obtained at a medium concentration of α-tocopherol (478 ppm) and at a high concentration of ascorbyl palmitate (390 ppm). In the case of 30 days of storage, Figure 2a2 shows the optimal value of PV, 33.56, which was obtained for the lowest concentration of α-tocopherol assayed (50 ppm) and the highest concentration of ascorbyl palmitate (450 ppm). Figure 2b2 depicts the minimum value of AV after 30 days of storage, 15.34, which was found at a

Table 2. Polynomial coefficients and *p*-values for the response variables

	FFA (% oleic)		PV (meq O_2·Kg^{-1})		AV		Totox		IP (h)	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	5.95E−02	–	4.06E+01	–	2.21E+01	–	1.03E+02	–	−2.58E−01	–
A: α-tocopherol, ppm	−5.58E−05	0.7356	−5.28E−02	0.0001	−6.01E−02	0.0634	−1.66E−01	0.0008	5.57E−03	0.0009
B: ascorbyl palmitate, ppm	−2.12E−06	0.2547	−6.84E−02	0.0020	−6.62E−02	0.1216	−2.03E−01	0.0004	3.84E−03	0.2948
C: Time, day	1.80E−03	0.0977	5.57E−01	<0.0001	9.21E−01	0.0003	2.03E+00	<0.0001	−1.37E−02	0.0051
AA	8.15E−08	0.2577	1.87E−05	0.0091	3.84E−05	0.0049	7.58E−05	0.0001	−2.89E−06	0.0038
AB	−6.51E−08	0.5987	7.10E−05	0.0001	6.64E−05	0.0056	2.08E−04	<0.0001	−3.73E−06	0.0201
AC	−1.22E−06	0.5459	1.31E−03	<0.0001	−2.57E−04	0.4137	2.36E−03	0.0001	−2.13E−05	0.3486
BB	−9.69E−08	0.8234	1.44E−05	0.6934	4.91E−05	0.4688	7.79E−05	0.3265	−3.67E−06	0.4527
BC	1.36E−06	0.7869	2.31E−04	0.5850	−3.83E−04	0.6221	7.95E−05	0.9290	−2.79E−05	0.6173
R^2	0.4478		0.9851		0.8815		0.9889		0.8747	

FFA: free fatty acid content, PV: peroxide value, AV: *p*-anisidine value, Totox: total oxidation value, IP: induction period.

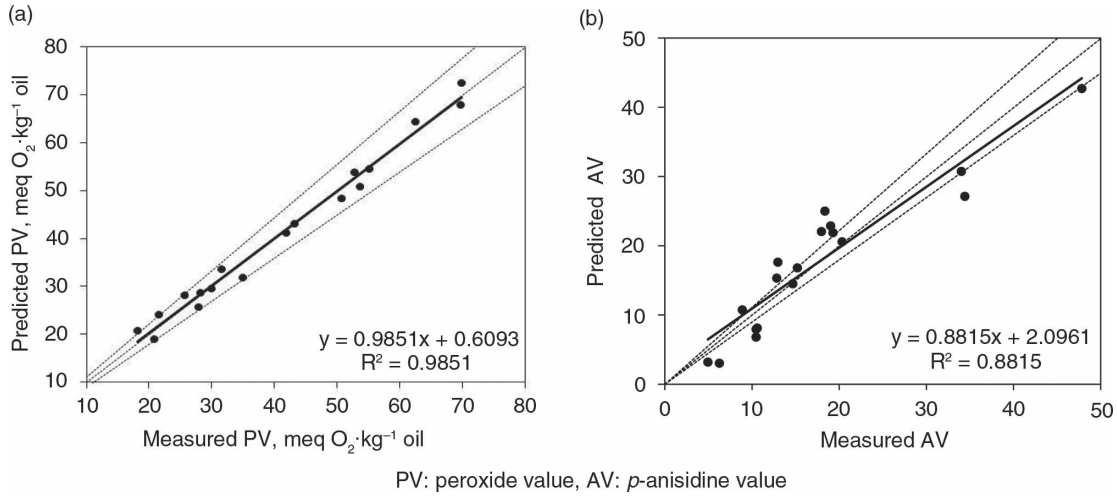

FIGURE 1. Correlation between predicted and measured values of PV (a) and AV (b).

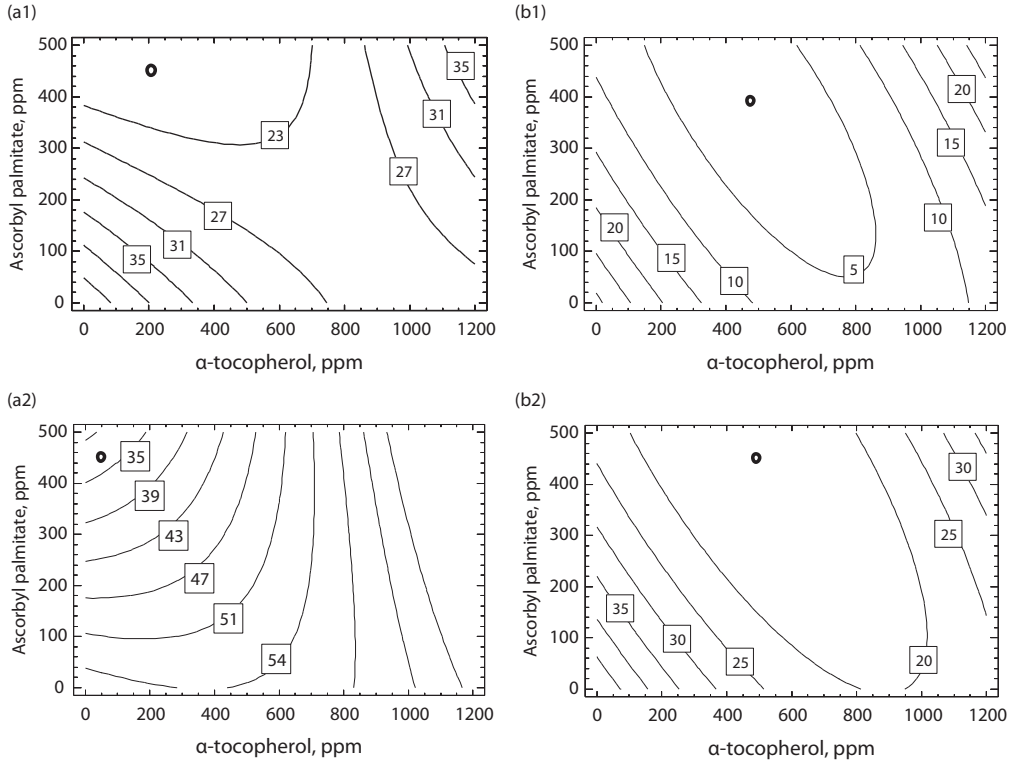

FIGURE 2. Contours plot for PV (a) and AV (b) at 10 days (1) and 30 days (2) of storage.

medium concentration of α-tocopherol (493 ppm) and the highest concentration of ascorbyl (450 ppm).

The results stated above indicate that in order to minimize the formation of hydroperoxides, low concentrations of α-tocopherol (50 and 207 ppm) combined with a high addition of ascorbyl palmitate (450 ppm) were required when evaluating the stabilized oil samples at 10 and 30 days of storage. Higher concentrations of α-tocopherol resulted in a prooxidative effect. Similar results were found by (Kulås and Ackman, 2001b) who reported an optimum α-tocopherol content of 100 ppm for the stabilization of bulk fish oils.

On the other hand, medium α-tocopherol concentrations (478 and 493 ppm), also combined with 450 ppm of ascorbyl palmitate, more effectively inhibited the formation of secondary oxidation products. This is attributed to the ability of

α-tocopherol to destroy hydroperoxides by forming either stable alcohols or inactive products by non-radical processes, such as a reduction or hydrogen donation (Kulås and Ackman, 2001a, Drusch *et al*., 2008, Frankel, 2005).

3.3. Bi-objective optimization

The conflict in the optimization of the two response variables selected (PV and AV) suggested the employment of a multi-objective optimization technique. Thus, a bi-objective optimization was carried out considering the data obtained for 30 days of storage, which better indicates the shelf life of the stabilized oils.

A Pareto Front, (Table 3), was generated using the weighted-sum method in order to find a set of non inferior solutions which satisfied both objectives to an adequate degree. The optimization of the objective function for each bound of the weight interval corresponds to the optimization of a single objective. It means that the minimization of PV for w=1 (row 1) and the minimization of AV for w=0 (row 12). Between these two values, each point of the non inferior solutions corresponds to a particular value of w, which varied at intervals of 0.1 (Table 3). Additionally, in row 5, a new solution was obtained, in which the weight of PV was twice the weight of AV, denoting the optimization of Totox. Each solution (PV, AV) is determined by a combination of factors inside the ranges of the independent variables (α-tocopherol and ascorbyl palmitate concentrations). It was observed that a decrease in AV (desired) implies an increase in PV (undesired). This trend is more pronounced at values of AV lower than 16.45.

TABLE 3. Set of optimal solutions (Pareto Front) and decision space for the bi-objective optimization problem

Row	w	PV (meq O_2·Kg^{-1})	AV	α-tocopherol (ppm)	Ascorbyl palmitate (ppm)
1	1.00	33.56	22.89	50	450
2	0.90	33.56	22.89	50	450
3	0.80	33.56	22.89	50	450
4	0.70	33.56	22.89	50	450
5	0.67	33.56	22.89	50	450
6	0.60	34.13	21.98	77	450
7	0.50	36.28	19.34	171	450
8	0.40	38.42	17.58	252	450
9	0.30	40.50	16.45	323	450
10	0.20	42.50	15.78	386	450
11	0.10	44.41	15.44	443	450
12	0.00	46.22	15.34	493	450

PV: peroxide value, AV: *p*-anisidine value.

Furthermore, the decision space, also shown in Table 3, was obtained from the Pareto Front by determining the optimal combination of experimental factors (α-tocopherol and ascorbyl palmitate concentration) for each weight factor w employed. As the weight factor w decreases from w=1 to 0, moving from row 1 to 12, the objective of minimum AV is favored over that of minimum PV. It is important to note that varying the weight factor from 1 to 0.67, did not produce any change in the optimal concentrations of α-tocopherol and ascorbyl palmitate, which were maintained at the same values as those obtained for the individual optimum of PV, 50 and 450 ppm respectively. Nevertheless, employing a weight factor (w) lower than 0.67 resulted in a higher optimal concentration of α-tocopherol (Table 3).

Therefore, selecting a single optimal solution from the Pareto Front (optimal concentration of α-tocopherol combined with 450 ppm of ascorbyl palmitate) will depend on the required characteristics of the stabilized oil. In this sense, assuming that AV is more closely related to flavor and odor deterioration than PV, medium α-tocopherol concentrations that combined with ascorbyl palmitate (450 ppm) and citric acid (50 ppm) which allow the minimization of AV should be preferred.

4. CONCLUSIONS

This study revealed that low concentrations of α-tocopherol (50–207 ppm) optimized the reduction of hydroperoxide formation in sardine oil when combined with sparing synergists like ascorbyl palmitate (450 ppm) and metal chelators such as citric acid (50 ppm). A higher α-tocopherol content resulted in a prooxidative effect. In terms of secondary oxidation products, medium α-tocopherol concentrations (450–493 ppm), which improved the destruction of hydroperoxides to stable products, were required to minimize AV.

As a consequence of the conflicting behavior of α-tocopherol concentration towards the accomplishment of the optimization objectives (minimization of PV and AV), a set of non inferior solutions (Pareto Front) which satisfied both goals was obtained by the weighted-sum method. Therefore, the final characteristics for the stabilized oil (optimal PV and/or AV) will determine the selection of a single solution inside the Pareto Front.

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