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Effect of hydroxybenzoic acids antioxidants on the oxidative stability of sardine oil

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Abstract

The antioxidant capacities of three derivatives of hydroxybenzoic acids (Gentisic acid, protochatechuic acid and vanillic acid) in sardine oil were compared. Peroxide value, conjugated diene value, p-anisidine value and thiobarbituric acid reactive substances (TBARS) value were assessed to determine the oxidative stability provided by these substances to the sardine oil. Results showed that gentisic acid (2,5 dihydroxy benzoic acid) was the most effective of the chosen hydroxybenzoic acids in imparting oxidative stability to the sardine oil. Protochatechuic acid (3,4 dihydroxy benzoic acid) provided relatively less oxidative stability, while vanillic acid had no effect. Results from this work showed that the position of hydroxylation and methyl substitution influences the antioxidant capacity of the molecules in sardine oil. Furthermore, it was found that the extent of oxidative stability conferred by the antioxidants in lipid systems is influenced by several other physical and chemical factors as well.

Keywords: Antioxidants; Oxidative stability; Sardine oil; Hydroxybenzoic acids; Primary oxidation; Secondary oxidation

1. Introduction

Oils rich in n-3 polyunsaturated fatty acids have been recognised widely for their nutritional significance. However, the high susceptibility of these oils to oxidation, limits their utilisation as processed food and nutritional supplements [1]. The literature on the oxidative stability of n-3 PUFA rich oils are inconsistent, which was attributed to the variable fatty acid composition of oil [2]. Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are generally used to prevent oxidation in fish oil [3]. However, they are not considered safe due to their suspected role as carcinogenic promoters [4]. Hence, the use of natural antioxidants can be considered to improve the oxidative stability of n-3 PUFA rich oil. Studies on the extraction of many types antioxidants from natural sources are continuing to evolve along with their applications in many food products for diminishing free radicals [5]. After successful separation of antioxidants, researchers study the relationship between chemical structures and effectiveness of antioxidants from variable sources which may have anticarcinogenic activity along with antioxidant characters [6]. Though many antioxidants are available in nature, phenolics are found abundantly [5]. They not only provide good sensorial qualities and antioxidant activity, but also impart beneficial properties like antitumour, anti-mutagenic, antiviral, anti-inflammatory [7]. Thus, addition of phenolics to oil results in enhancement of nutritional properties, along with improvement of oxidative stability of oil.

Phenolic acids are one of the most persistent groups of antioxidants in plants and the structure of the antioxidant greatly influences the antioxidant power [6]. Hence, a correlation between their structure and activity in oil could be used to predict the effectiveness of an antioxidant. Further, the activity of antioxidants in lipid systems is influenced by external factors like hydrophilicity, interfacial structures [8]. Hence, it becomes critical to make a comparison of different structured phenolic acids to find the best possible compound. Of the wide variety of phenolic acids available in nature, minor attention has been directed to the antioxidant capacities of simple derivatives of benzoic acids [9]. Since, attempts are being for separating and purifying novel antioxidants from many sources [5], the current study on application of a particular class of antioxidants can help predict the effectiveness of an antioxidant in lipid system after their separation from natural source. This study attempts at

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understanding the effectiveness of the hydroxybenzoic acids in improving the oxidative stability of sardine oil. Because of the limited literature on the hydroxybenzoic acids and the abundance of these compounds in natural resources, current study provides additional choice of antioxidants for imparting stability in fish oil.

2. Materials and methods

Crude sardine oil was procured from a local seafood industry and was refined in our laboratory. The refined oil was stored at −20 °C without any added antioxidants prior to use. Gentisic acid, protochatechuic acid and vanillic acid and 1,1,3,3-tetramethoxypropane (malonaldehyde) were from Sigma (India). Potassium iodide, trichloroacetic acid, sodium thiosulphate and thiobarbituric acid were purchased in analytical grade from Loba Chemie. All solvents were of analytical grade and were purchased from Merck India.

2.1. Analytical methods

In order to perform the oxidation studies of sardine oil, 100 ppm of antioxidants dissolved in solvent were taken in glass vials. The solvent was evaporated by flushing with nitrogen and carefully calculated amount of oil was added. The samples were then homogenised for 15 minutes. The sample vials were stored at 37 °C and covered with aluminium foil and kept in contact with atmospheric air for 14 days. Analysis was performed for all samples periodically.

2.1.1. Peroxide value

Peroxide value measurement was done based on the standard AOCS method [10]. The peroxide value of oil is measured by Eq. (1) and expressed as milli equivalents of peroxide per 1000 g of oil.

$$Peroxide\ value = \frac{(S-B)*M*1000}{m} \quad (1)$$

S is the sample titre value, B is the blank titre value, M is the molarity of sodium thiosulfate used, m is the mass of test in g.

2.1.2. p-Anisidine value (pAV)

p-Anisidine value was determined according to AOCS [10]. The anisidine values were calculated as in Eq. (2)

$$pAV = 25*\frac{1.2As-Ab}{m} \quad (2)$$

As is the absorbance of oil after reaction with p-anisidine, Ab is the absorbance of oil in isoocatane, m is the weight of sardine oil used for analysis (g).

2.1.3. Conjugated diene (CD) value

Conjugated dienes are formed during oxidation of fats or oils and can be analysed by measurement of absorbance at 230–235 nm. The CD value in bulk sardine oil was measured based on the method described by Hopia et al. [11]. The absorbance of the lipid – isooctane solution was measured at 234 nm. Oxidation is directly proportional to increase in absorbance.

2.1.4. Thiobarbituric acid reactive substances (TBARS) value

Another significant assay for monitoring secondary oxidation products is the thiobarbituric acid reactive substances (TBARS) assay and it was performed as described by Buege and Aust [12] with minor modifications. The experiment is based on the reaction between malonaldehyde (MAD) which is a product of oxidation and thiobarbituric acid to give a red colour complex. Oil sample was mixed with TBARS reagent containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. A slight pink colour was developed on heating the mixture. The samples were then cooled under running water and centrifuged. The absorbance of the supernatant was read at 532 nm. A standard graph was constructed using 1,1,3,3-tetramethoxypropane (MAD).

2.1.5. Statistical analysis

The experimental data in triplicates were analysed by one way analysis of variance (ANOVA) using MiniTab17 software and samples with $p < 0.05$ were significant.

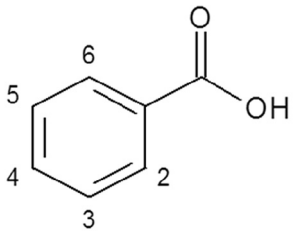
3. Results and discussion

Based on detailed literature study, the antioxidants were chosen from hydroxybenzoic acid group. The three chosen antioxidants from benzoic acid group showed variation in the structure based on the degree of hydroxylation and methylation and the position of functional group substitution (Fig. 1).

3.1. Peroxide value

The production of complex array of secondary products results from these colourless and odourless labile hydroperoxides [13]. Hence, determination of hydroperoxides was used to monitor primary oxidation of sardine oil in the presence of three derivatives of hydroxy benzoic acid (Fig. 1). All samples, including control showed a steady increase in the peroxide value during initial six days (Fig. 2). However, a faster increase was noted in later days, with control and vanillic acid sample showing maximum of 22.09 and 21.63 respectively.

Of all the benzoic acids tested, gentisic acid showed highest reduction in peroxide value of 25.13% (Table 1) which is consistent with the results obtained by Ashidate et al. [14] when



Gentisic	2 – OH, 5 – OH
Protochatechuic	3 –OH, 4 – OH
Vanillic	3 – OCH_3, 4 - OH

Fig. 1. Structure of hydroxybenzoic acids.

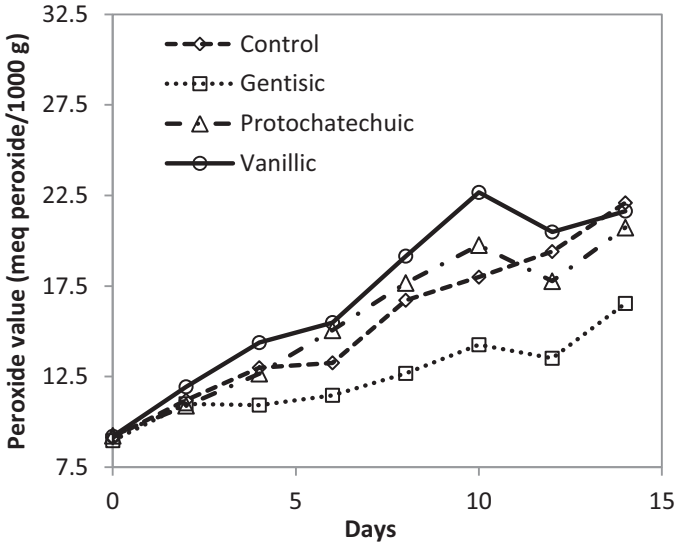


Fig. 2. Effect of 100 ppm of benzoic acid derivatives on the peroxide value of sardine oil stored for 14 days at 37 °C. Bars represent standard deviation (n = 3).

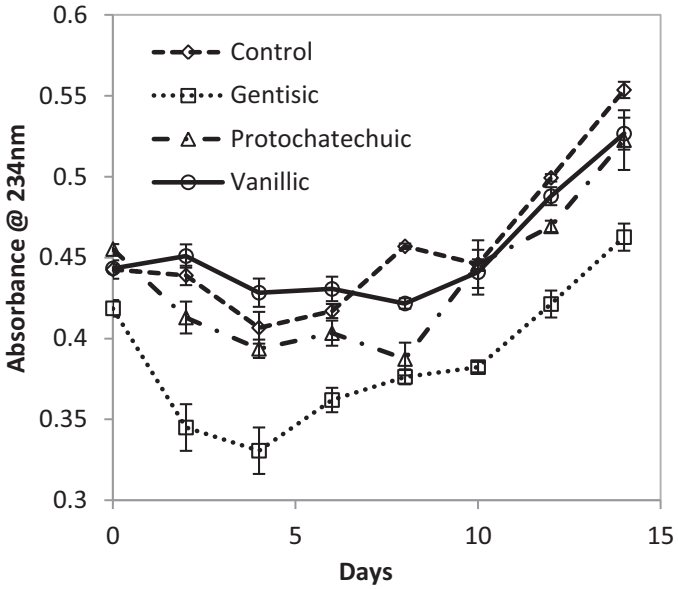


Fig. 3. Effect of 100 ppm of benzoic acid derivatives on the conjugated diene value of sardine oil stored for 14 days at 37 °C. Bars represent standard deviation (n = 3).

tested against human plasma, where cholesterol ester hydroperoxides were effectively scavenged. The reason for the high antioxidant activity of gentisic acid throughout the 14 day storage period (Fig. 2) was attributed to its high radical scavenging ability which in turn was because of its hydroxyl substitution at the 2 and 5 positions (Fig. 1) of the aromatic ring [6]. Protochatechuic acid showed a very moderate stability of 6.02% (Table 1) though it had the same number of hydroxyl substitution as gentisic acid (Fig. 1) but different position of substitution. Protochatechuic acid failed to prevent oxidation which could be related to the inefficient association of antioxidant molecules at the sight of oxidation [15] or due to the steric crowding of bulky peroxyl radicals around the substituent because of adjacent hydroxyl substitution [16]. Vanillic acid failed to show any significant reduction ($p > 0.05$) in sardine oil oxidation, but showed a slight pro-oxidant effect which was attributed to its low radical scavenging ability as a result of methoxy substitution [6]. Thus, methylation of 3-hydroxyl group has reduced the radical scavenging ability and increased the prooxidant effect [17].

Table 1
Effectiveness of each antioxidant on improving the oxidative stability of sardine oil at the end of 14 days storage period.

Antioxidants	% Decrease in the oxidation of sardine oil			
	Peroxide value	p-Anisidine value	CD value	TBARS value
Gentisic acid	25.13 ± 3.91[a]	11.48 ± 1.84[c]	16.43 ± 2.37[b]	25.70 ± 3.22[b]
Protochatechuic acid	6.19 ± 0.42[b]	Ns	5.5 ± 0.69[d]	Ns
Vanillic acid	2.05 ± 1.53[e]	Ns	4.8 ± 3.87[d]	Ns

Ns, not significant.
Percentage decrease was calculated by considering control to be 100% oxidised.
[a, b, c, d] Values with the same letter in each column were not significantly different ($p < 0.05$).

3.2. Conjugated dienes (CD) value

Lipid oxidation leads to the formation of isomeric derivatives of free radical with conjugated diene bonds resulting from the structural changes of pentadieonic double. These compounds show a maximum absorbance at 232 nm and hence can be used for the monitoring of lipid oxidation [13]. The antioxidant effect of gentisic acid was found to be high when compared to all other phenolic acids (Fig. 3), which is similar to the results obtained by Hradkova et al. [18]. This high antioxidant activity was related to the formation of hydrogen bond between carboxyl and hydroxyl groups [18]. Similar to peroxide value, the samples with vanillic acid showed negligible reduction in the conjugated dienes value (Table 1). Since the lipid peroxide molecules undergo rearrangement to form conjugated dienes, potent radical scavenger like gentisic acid showed the maximum activity. Thus, it can be concluded from Table 1 that the antioxidant that provides a good reduction in the primary oxidation of oil is gentisic acid which was the result of its good radical scavenging ability.

3.3. p-Anisidine value (pAV)

To have a better understanding of the progress of oxidation in sardine oil, measurement of secondary oxidation products along with primary oxidation products is necessary [13]. Calculating the p-anidine value (pAV) is one of the oldest methods for evaluating secondary oil oxidation. pAV is the measure of secondary oxidation products generated during decomposition of hydroperoxides. The formation of a Schiffs base, on reaction of aldehyde carboxy bond with the p-anisidine amine group, is used as the base of this analysis [19]. The results for the pAV of sardine oil added with

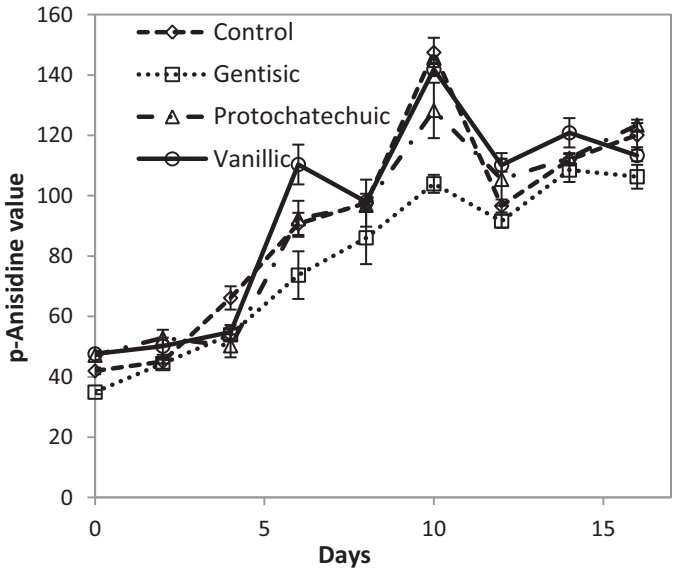


Fig. 4. p-Anisidine value of sardine oil stored at 37 °C for 14 days in the presence of 100 ppm of three different hydroxy benzoic acid derivatives.

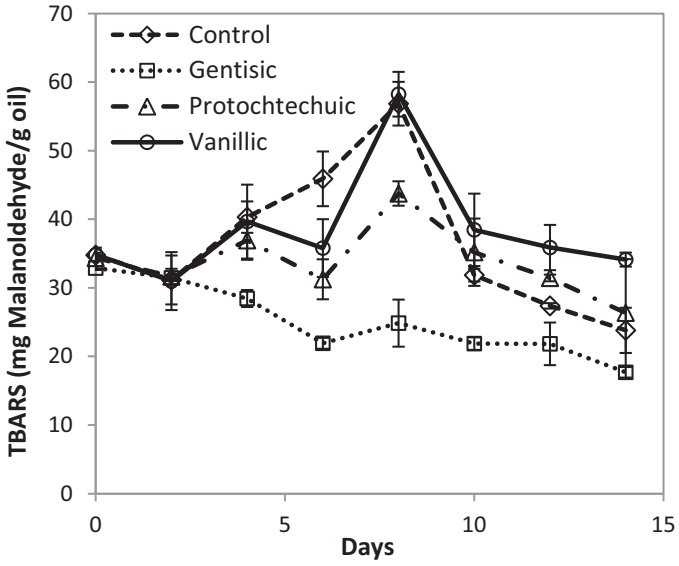


Fig. 5. TBARS value of sardine oil stored at 37 °C for 14 days in the presence of 100 ppm of three different hydroxy benzoic acid derivatives.

three different hydroxybenzoic acids are shown in Fig. 4. Only gentisic acid showed a significant effect ($p < 0.05$) of 11.48% (Table 1) on reducing the pAV when compared to the other hydroxybenzoic acids tested (Fig. 4) at the end of 14 days storage period. Nevertheless, protochatechuic acid and vanillic acid failed to show any significant reduction ($p > 0.05$) in pAV (Table 1). Thus, at the end of 14 days storage period, the descending order of sardine oil stability with added benzoic acid derivatives followed the order: gentisic acid > protochatechic acid > vanillic acid.

3.4. *Thiobarbituric acid–reactive substance (TBARS) value*

Malonaldehyde produced during the decomposition of the lipid hydroperoxides was measured to estimate the extent of secondary oxidation in sardine oil [6]. Along with the measurement of primary oxidation in oil, a better picture on sardine oil oxidation, with and without antioxidants, can be achieved using TBARS value. The effect of different benzoic acids in reducing TBARS value was calculated (Fig. 5). Of the three antioxidants chosen, gentisic acid showed a maximum of 26% reduction in TBARS value (Table 1). A clear antioxidant effect was also seen in protochatechuic acid until the 8th day of incubation after which the efficiency reduced. Among the three beozoic acids, gentisic acid had the highest antioxidant effect by reducing the TBARS value to 24.85, from that of a control value of 56.85 (Fig. 5). In case of samples with vanillic acid, no antioxidant effect was seen (Fig. 5), which is similar to the results obtained by Jung et al. [20]. Since the generation of secondary oxidation products occurs from lipid hydroperoxides, any potent radical scavenger can be expected to reduce the secondary oxidation as well. This explains the efficiency of gentisic acid and to some extent protochatechuic acid to reduce TBARS value. This could also indicate that gentisic acid is best suited for inhibiting primary oxidation of oil than secondary oxidation.

4. Conclusions

In conclusion, gentisic acid was found to be the most effective of the three structurally variant benzoic acids tested in improving the oxidative stability of sardine oil. The overall good performance of gentisic acid can be attributed to its radical scavenging ability. However, the position of hydroxyl group substitution can be considered a major reason for the same, indicated by the moderate effectiveness of protochatechuic acid. Due to the multiple mechanisms of oxidation in oil which in turn depends on the diversity in oil composition, it is crucial to evaluate individual antioxidant performance in oil in achieving oxidative stability during storage.

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