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EFFECT OF NATURAL ANTIOXIDANT MIXTURES ON MARGARINE STABILITY

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In spite of their efficiency, the use of synthetic antioxidants such as tert-butylhydroquinone (TBHQ) has been questioned because of their possible carcinogenic effects. The purpose of this study was to establish a mixture of natural antioxidants that provides the optimum oxidative stability for margarine. Antioxidant treatments included 10 various mixtures (F_1- F_{10}) containing 100-500 ppm tocopherol mixture (Toc), 100-200 ppm ascorbyl palmitate (AP), 100-200 ppm rosemary extract (Ros) and 1000 ppm lecithin(Lec) along with a control or F_0 (with no antioxidant) and F_{11} containing 120 ppm TBHQ. The effect of antioxidant mixtures on the stability of margarine samples during an oven test (60±1°C), rancimat test at 110°C and storage at 4°C was evaluated. The final ranking of the natural antioxidant mixtures was as follows: F_2, F10>F_5, F9>F_8>F_1, F3, F4>F_6, F_7.Considering the results of this research and ranking criteria, F_2 (200 ppmAp + 200 ppmRos) and F_{10} (200 ppmRos + 200 ppm Toc +1000 ppm Lec) were recommended as substitutes for TBHQ to maintain the quality and increase the shelf-life of margarine.
Keywords: margarine, oxidative stability, shelf-life, antioxidant, auisidine

INTRODUCTION

Margarine is a water-in-oil emulsion containing a minimum of 80% fat and a maximum of 16% water and about 4% additives. During storage of oils, fats and foods containing fat, lipid oxidation is one of the principle causes of food quality deterioration. Margarine is no exception to this rule since it has a high fat content. In order to retard or prevent the oxidative deterioration and extend the shelf-life of many food products, addition of antioxidants is necessary (Abdalla and Roozen, 1999; Iranian Standard, 1999; Zandi & Gordon,1999; Codex Alimentarius Commission, stan 32,2001; O'Brien, 2004; Robbins and Sewalt, 2005). Synthetic antioxidants such as tert-butyl-hydroquinone (TBHQ), are being used regularly because they are highly efficient in preventing the oxidation of oils and fats. Despite their effectiveness, the use of synthetic antioxidants is restricted in several countries, because of their possible toxicity, metabolism, absorption and accumulation in the body and also their carcinogenic effects (Eskin and Robinson, 2001; Hras *et al.*, 2000). Many studies have evaluated the effects of several natural antioxidants on the oxidative stability of food systems.

α, γ and δ-tocopherols (Toc) are the most abundant natural antioxidants among the plant lipids (Hamilton *et al.*, 1998; Chu and Hsu,1999; Hras *et al.*, 2000). Amongst the herbs, rosemary (Ros) from the Labiatae family has attracted the greatest attention (Frankel *et al.*, 1994; Zandi and Ahmadi, 2000; Naciye *et al.*, 2008). Since rosemary extract is lipophilic, it is easily incorporated into oils and fats to prevent oxidation (Chu and Hsu,1999; Hras *et al.*, 2000). Lecithin (Lec) which is generally used as an emulsifier in food processing, can improve the dispersion of active antioxidants in emulsion systems and inhibits the propagation step of free radicals in the autoxidation mechanism by reacting with various free radicals. Lecithin has been widely used as synergist in combination with phenolic antioxidants (Koga and Terao,1995; Saito and Ishihara, 1997; Bandarra *et al.*, 1999). Ascorbyl palmitate is usually used in food products to prevent or retard the oxidation of oils and fats and it is preferred to ascorbic acid because of its greater solubility in oil (Hamilton *et al.*, 1998).

The most important commercial natural antioxidants currently being used are: tocopherols, ascorbyl palmitate, rosemary extract and lecithins. As lipid oxidation occurs via multiple mechanisms and no single antioxidant can prevent all the steps in the oxidation process, a mixture of antioxidants must be

used to create a synergistic effect (Koga andTerao,1995; Bandarra *et al.*, 1999; Hras *et al.*, 2000).

With regard to the role of edible oils in human health, the vulnerability of oils and fats to oxidative deterioration, the possible undesirable effect of synthetic antioxidants on consumers' health, and awareness of harmful effects of synthetic compounds must all be taken into account during the production of margarine. As margarine is a relatively new product in Iran, the potential difficulties mentioned above must be overcome during its production so that it can be introduced to the market as a safe substitute for butter. The purpose of this work was to test mixtures of natural antioxidants in margarine and select the combination that is most efficient in preserving its quality and which can be used as a substitute for TBHQ, which is currently being used as a synthetic antioxidant.

MATERIALS AND METHODS

Materials

Refined, bleached and deodorized sunflower oil with no added antioxidant was purchased from Kesht-o-Sana'at-e-Shomal, Iran. Refined, bleached, deodorized and antioxidant-free palm stearine was donated by PORIM (Palm Oil Research Institute of Malaysia).
Tocopherol mixture (α, γ and δ), rosemary extract, TBHQ and emulsifier (mono and di-glycerides) were purchased from Danisco, soy lecithin was obtained from ADM (Archer Daniels Midland), citric acid and potassium sorbate and other analytical grade chemicals and solvents were purchased from Merck Co. β-Caroten (dissolved in edible oils) was obtained from Roche (Switzerland), vitamin A and D_3 were purchased from BASF (Germany), sodium casienate, low fat milk powder and diacetyl were obtained from Iran Caseinate, Moghan Co. (Iran) and Roberte (Iran).

Sample Preparations

Lipid Phase Preparation
The lipid phase was prepared by heating the margarine base (20% palm stearin and 80% sunflower oil) to a temperature of at least 5.6°C above the melting point (melting point of palm stearin: 45°C) before adding the oil soluble ingredients. These ingredients included the emulsifier (0.5%), vitamins A and D_3 (0.01%), β-caroten (0.003%), flavor (0.02%) and antioxidants. The antioxidant mixtures were added to the oil phase as follows: F_0: control (with no added antioxidant); F_1: 500 ppm Toc + 100 ppm Ap; F_2: 200 ppm Ros + 200 ppm Ap; F_3: 200 ppm Toc + 200 ppm Ros; F_4: 200 ppm Ap + 200 ppm Toc + 100 ppm Ros; F_5: 100 ppm Toc + 1000 ppm Lec; F_6: 250 ppm Toc + 1000 ppm Lec; F_7: 500 ppm Toc + 1000 ppm Lec; F_8: 100 ppm Ap + 500 ppm Toc + 1000 ppm Lec; F_9: 200 ppm Ros + 100 ppm Ap + 1000 ppm Lec; F_{10}: 200 ppm Ros + 200 ppm Toc + 1000 ppm Lec; F_{11}: 120 ppm TBHQ.

Aqueous Phase Preparation
The aqueous phase was prepared separately. The skim milk powder (1%) was reconstituted, pasteurized and cooled to 45°C. The water soluble ingredients were added which included salt (0.5%), potassium sorbate as preservative (0.02%), sodium caseinate (1%) and citric acid (0.06%).

Emulsion Preparation
The oil and aqueous phases were blended together with high-shear agitation at 45°C to form a water-in-oil emulsion. The water phase was added to the oil phase in order to promote the formation of a water-in-oil emulsion. After preparation, the emulsion was transferred to an agitated holding tank that supplied the chilling units in which the emulsion was agitated 7 minutes.

Chilling and Crystallization
After preparation of emulsion, in order to achieve desirable consistency, it was transferred into a laboratory scale worker unit. The shaft in the worker unit revolves at about 35rpm with a residence time of approximately 3 minutes, it continued until large crystals disappeared. The temperature of margarine adjusted at 9-11°C in the worker unit.

Filling and Final Crystallization
The crystallized margarine emulsion was filled in 250 g poly-ethylene cups and stored in freezer (-18°C) for 48 hours to allow complete crystallization.

Chemical Analysis
Acid value, iodine value and melting point of oils and margarine samples were determined as described previously (AOCS Methods,1997).

Preparation of methyl esters and GC analysis of samples In order to prepare fatty acids methyl esters, the oil phase of samples was separated from aqueous phase and heated to above its melting point. 1 g of the oil phase was added to 10 ml heptane in a test tube, and then the mixture was converted to fatty acid methyl esters by treatment with 0.5 ml KOH in methanol. It was shaked for 30 seconds, the upper layer which

contained methyl esters was separated and analyzed using GC system.

ISO methods(ISO Methods, 2002 a,b) of analysis were used in these determinations. The GC system (Varian 3800/Autosampler) was equipped with a FID detector and capillary column (Cp splitter-88 Fame, 100 m 25 mm 25 m) and was operated under the following conditions: FID: 270°C, oven: constant temperature at 175°C, injector : 250°C and constant flow rate of carrier gas (N_2): 0.6 ml/min.

Oven Test

The samples were transferred in beakers to an oven maintained at 60±1°C. Oxidative stability was determined by measuring peroxide and anisidine values every 5 days over a 25-day period. Primary oxidation products were determined by peroxide measurements (AOCS,1997). Formation of secondary oxidation products was measured using the p-anisidine value (AOCS,1997). The induction period was considered to be the number of days required for the peroxide value of the samples to reach 20 meqO_2/kg of fat (Economou *et al.*, 1991). This is in agreement with the general acceptance that oils become rancid at peroxide values greater than 20 (Economou *et al.*, 1991; Hras *et al.*, 2000; Pokorny *et al.*, 2001).

Rancimat Test

The induction period of the oil phase of the samples was determined by Rancimat (110±1°C). The effectiveness of the antioxidants mixtures was expressed as the stabilization factor:

$$SF=IP_{inh}/IP_0$$

where IP_{inh} is the induction period in the presence of an inhibitor and IP_0 is the induction period of the non-inhibited system (Hras *et al.*, 2000; Yanishlieva and Marinova, 1996).

Shelf-life evaluation

The shelf-life of samples stored in the refrigerator (4±1°C) was measured by determination of peroxide values in the fresh sample and in samples stored for 2, 4, 8, 12, and 14 weeks after production. The induction period in the refrigerator was considered to be the number of days required for the peroxide value of the samples to reach 5meq/kg of fat (Economou *et al.*, 1991; ISIRI,1999).

Ranking of antioxidant mixtures

The antioxidant mixtures were ranked on the basis of several factors including the number of the days needed for the peroxide levels in the samples to reach 20 at 60°C (Economou *et al.*, 1991; Hras *et al.*, 2000; Pokorny *et al.*, 2001) and to reach a PV of 5 at 4°C (ISIRI,1999), the stabilization factor (at 110°C) and also the economic aspects of using natural antioxidants.

Statistical analysis

All determinations were carried out in triplicate. The results obtained for peroxide and anisidine values were subjected to analysis of student's t-test with a significance level of $p<0.05$, The results of the induction period data, stabilization factor and ranking points were analyzed using the Mann Whitney test. *P* value less than 0.05 were considered statistically significant.

RESULTS

The peroxide, anisidine and iodine values together with the melting point of the oils and also the data for the control sample (F_0) are presented in Table 1. The fatty acid composition of the oil samples was within the normal range i.e.: 16:0 (8.46%), 18:0 (3.68%), 18:1 (22.16%), 18:2c (63.10%), 18:2t (1.38%), 18:3 (0.2%), 20:0 (0.11%), 20:1 (0.35%) and 0.56% of other fatty acids for sunflower oil; the fatty acid composition of palm stearine was as follows: 16:0 (53.36%), 18:0 (5.40%); 18:1 (33.70%), 18:2 (6.24%), 18:3 (0.43%) and 0.87% of other fatty acids (Fig.1). In the case of margarine, the corresponding values were as follows: 16:0 (171.15%), 18:0 (4.34%), 18:1 (24.98%); 18:2c (51.26%), 18:2t (0.89%), 18:3 (0.26%), 20:0 (0.09%), 20:1 (0.23%) and 0.80% of other fatty acids.

Table 1. Physical and chemical characteristics of the samples of oils and margarine

Samples	Peroxide value (meq/kg)	Anisidine value	Iodine value(g/100g)	Acid value (mg/g)	Melting point (°C)
Sunflower Oil	0.2±0.035	1.24±0.002	131.87±0.78	0.04±0.0030	-
Palm Stearine	0.75±0.054	2.04±0.006	38.65±0.19	0.01±0.0004	49.0±0.22
Margarine	0.4°±0.040	1.50±0.003	111.5±0.45	0.03±0.0025	36.5±0.16

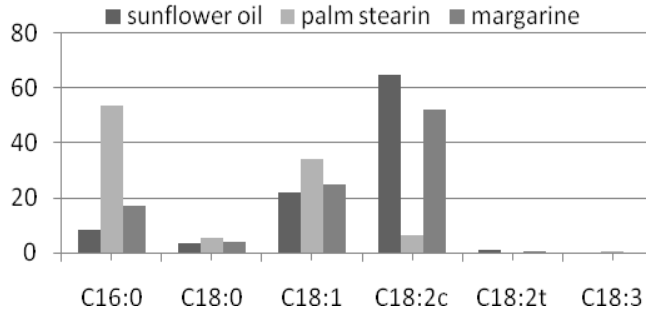

Fig.1. Fatty acids profile of starting oils and margarine(%)

The peroxide and anisidine values of the samples treated at 60°C showed that F_2, F_5,F_9, F_{10} and F_{11} had the greatest antioxidant activity ($p>0.05$). On the fifth day of the oven test (Fig. 2), the peroxide value of the oil phase of F_2 (8.19meq/kg) was the lowest among all the samples ($p<0.05$). F_8 and F_{11} (12.1 and 12.4meq/kg) showed the lowest peroxide value ($p<0.05$). There was no statistically significant difference between the antioxidant activity of F_8 and F_{11} on the fifth day ($p>0.05$).

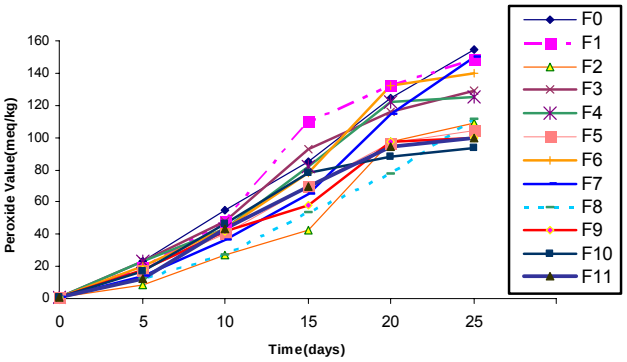

Fig.2. Peroxide value of margarine samples during storage at 60°C

By days 10 and 15, F_2, F_8 and F_9 contained a significantly lower level of primary oxidation products compared to the control sample and also the other samples ($p<0.05$). By days 20 and 25, the antioxidant mixtures in F_2, F_5, F_8,F_9, F_{10} and F_{11} showed the greatest antioxidant activity compared to the other antioxidant mixtures ($p<0.05$).

Anisidine value of control (F_0) and F_1 by day 25 was 25.75 and 19.28 meq/kg (Fig.3) which had statistically significant difference with F_{11} (TBHQ) ($p<0.05$). By day 25,anisidine value of F_2 (9.82), F_5 (10.30) and F_9 (10.35) had no statistically significant difference with F_{11} (9.77)($p>0.05$). This is in agreement with findings of Hras *et al.* (2000) and Chu and Hsu (1999). On the same day anisidine value of F_3, F_4 and F_8 reached to 14.20, 12.17 and 11.82 meq/kg, respectively. The results of statistical analysis showed that anisidine value of margarine samples containing these antioxidant mixtures had statistically significant difference with F_{11} ($p<0.05$). By increasing the concentration of Toc, but not Lec, in F_5 (100 ppm Toc+ 1000 ppm Lec), F_6 (250 ppm Toc+ 1000 ppm Lec) and F_7 (500 ppm Toc+ 1000 ppm Lec) at the end of the incubation period at 60 °C (day 25), the anisidine value reached to 10.30, 16.32 and 25.09 meq/kg, respectively, F_6 and F_7 had statistically significant difference with F_{11} ($p<0.05$). It seems that by increasing the concentration of Toc from 100 ppm to 500 ppm, Toc shows prooxidative activity. The anisidine value of F_{10}(Ros+Toc+Lec) by day 25 reached to 8.12 and had statistically higher antioxidant activity compared to F_{11} ($p<0.05$). Totally,Changes in anisidine value in the oven test (Fig. 3) Showed that F_9 (Ros+Ap+Lec), F_{10} (Ros+Toc+Lec) and F_{11} (TBHQ) had the highest antioxidant activity compared to the other treatments ($p<0.05$).

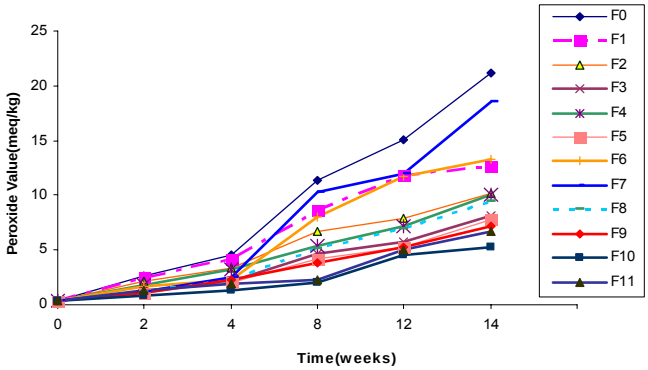

Fig.3. Anisidine value of margarine samples during storage at 60°C

The induction period of the samples (Table 2) was used as an appropriate criterion for evaluating antioxidant activity (Economou *et al.*, 1991; Hras *et al.*, 2000; Pokorny *et al.*, 2001). In this regard, the highest antioxidant activity in the oven test was associated with F_2 (Ros+Ap) and F_8 (Toc+Ap+Lec) in which the peroxide value reached 20 meq/kg after 8 days and was statistically different from that achieved with F_{11} (TBHQ). Other antioxidant mixtures (F_1, F_3, F_4, F_6 and F_9) demonstrated lower activity ($p<0.05$) and the activity of F_7 and F_{10} was not significantly different from that of F_{11} ($p>0.05$).

Table 2. Criterion used in ranking antioxidant mixtures

Samples	Time (days) to reach PV=20 at 60°C	SF at 110°C	Time (days) to reach PV=5 at 4°C	Cost of antioxidant mixtures ($)
F_0 (control)	4.00[a*]	1.00[a]	29[a]	0.00
F_1	4.98[a]	1.41[a]	34[a]	0.07
F_2	8.00[b]	2.74[b]	71[b]	0.05
F_3	4.30[a]	1.69[a]	45[a]	0.05
F_4	4.10[a]	1.70[a]	50[a]	0.06
F_5	5.00[a]	2.77[b]	84[c]	0.07
F_6	4.95[a]	1.48[a]	44[a]	0.09
F_7	6.10[c]	1.35[a]	38[a]	0.12
F_8	8.00[b]	2.28[b]	56[b]	0.13
F_9	5.10[a]	2.96[c]	84[c]	0.10
F_{10}	6.00[c]	2.63[c]	99[c]	0.11
F_{11}	6.20[c]	2.23[c]	90[c]	0.05

[a-d] Values that are significantly different in each column according to the student t-test and Mann-Whitney test (p<0.05).

The results of the Rancimat test (110°C) were expressed as the stabilization factor (SF) in Table 2. The SF of samples F_9 (Ros+Ap+Lec), F_{10} (Ros+ Toc+Lec) and F_{11} were not statistically significantly different from one another (p>0.05). Samples F_2 (Ros+Ap), F_5 (100 ppm Toc+Lec) and F_8 (Ap+Toc+Lec) had similar SFs and ranked as the second most effective group. The other samples including F_1, F_3, F_4, F_6 and F_7, did not show substantial antioxidant activity in this test (rancimat test).

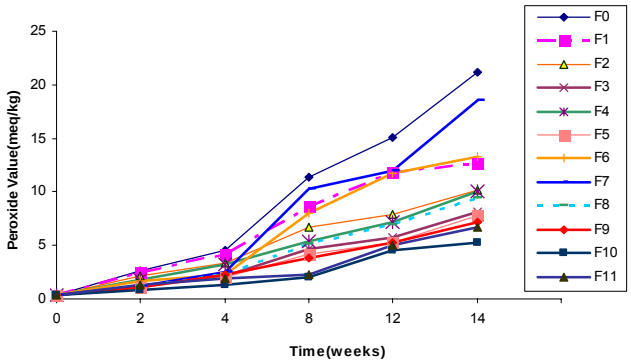

Fig.4. Peroxide value of margarine samples during storage at 4°C

At 4°C, the peroxide value of the control and samples F_1, F_2,F_3, F_4, F_6 and F_7 reached the point of rancidity (5meq/kg) after 8 weeks while samples F_5, F_9, F_{10} and F_{11} did not become rancid after this period (Fig. 4). The shelf-life of samples F_5, F_9, F_{10} and F_{11} was not statistically significantly different (p>0.05) but their shelf-life was longer than that of the other samples (p<0.05) (Table2). The shelf-life of margarine samples stored at 4°C was variable and ranged between 1 month (control, F_1 and F_7) and 3 months (F_{10} and F_{11}).

DISCUSSION

Margarine samples contained less than 1% trans fatty acids (0.89%) and were of high nutritional value (PUFA/SFA+TFA=2.34). According to the changes in peroxide values in the samples over the 25 days of the oven test, it appeared that F_2 (Ap+Ros), F5 (100 ppm Toc+1000 ppm Lec), F_8 (Toc+Ap+Lec) and F_{10} (Ros+Toc+Lec) had the highest antioxidant activity (p<0.05) and comparing F_2 and F_8, the effect of F_2 was significantly (p<0.05) higher compared to F_8. This is in agreement with the findings of Hras *et al.* (2000), Chu *et al.* (1999) and Haak *et al.* (2008) who reported that Ap+Ros, Toc+Ap+Lec and Ros+Toc+Lec had higher antioxidant activity than the other natural antioxidant mixtures tested in their studies. Also, the results of another study (Djenene *et al.*, 2002) demonstrated that combination of ascorbic acid with rosemary and the combination of tocopherol and ascorbic acid extended the shelflife of the product. In anisidine test, F_{10} showed statistically significant lower level of secondary oxidation products compared to the control and other samples. This result is in agreement with the results reported by Chu *et al.* (1999), Hras *et al.* (2000) and also with the work of Beddows *et al.*(2002) who found that the optimum effect against racidity in sunflower oil was with a mixture of ascorbyl palmitate and rosemary. Also, the results of another study (Fang and Wada, 1993) demonstrated that a mixture of tocopherol and rosemary extract had a significantly stronger antioxidant effect rather than tocopherol alone and rosemary alone. According to the findings of Hras *et al.* (2000) the mixture of rosemary extract and ascorbyl

palmitate extends the induction period required to reach a peroxide value of 20meq/kg in sunflower oil under test conditions, which indicates a strong synergism between these two natural antioxidants (Hras *et al.*, 2000). On the other hand, lecithin had a synergistic effect with tocopherol (Chu and Hsu,1999). The rancimat test indicated that the antioxidant combination of tocopherol and rosemary decreased lipid oxidation (Haak *et al.*, 2008; Govaris *et al.*, 2008) and also in agreement with the findings of Chu *et al.* (1999), Erkan *et al.* (2008), Frutos and Hernandez-Herrero (2005), Hras *et al.* (2000) and Judde *et al.* (2003) in which rosemary extract was found to have a higher phenolic content than other natural antioxidants.

The results of this research has shown that certain natural antioxidant mixtures can be used as substitutes for TBHQ to improve the shelf-life of margarine thus eliminating the need to use synthetic antioxidants as preservatives in food products which can accumulate in body organs and cause cancers and tumors. The results of the tests on margarine samples containing added natural antioxidants were in accordance with one another and indicated that F_1, F_3, F_4, F_6 and F_7 had lower antioxidant activity compared to the other samples. The shelf-life of these samples was less than 2 months which caused them to become rancid more rapidly than the other samples, they also showed the lowest SFs in the Rancimat test. F_5 extended the shelf-life of the samples to 12 months and showed high antioxidant activity in all other tests. The final ranking of the natural antioxidant mixtures was as follows: F_2> F_{10}> F_5, F_9 > F_8> F_1, F_3, F_4> F_6 and F_7 (Table 3).

With regard to the adverse effects of synthetic antioxidants on consumers' health and the economic aspects of the results of this research, F_2 (200 ppm Ros+200 ppm Ap) and F_{10} (200 ppm Ros+ 200 ppm Toc+ 1000 ppm Lec) are recommended as substitutes for TBHQ to maintain the quality and increase the shelf-life of the margarine. It is also recommended that further research on a larger scale should be carried out on this topic.

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Table3. Ranking[1] of antioxidant treatments

Samples	Ranking in oven test (60°C)	Ranking in SF(110°C)	Ranking in Shelf-life (4°C)	Economical value	Final Ranking
F_0(control)	3	3	3	-	9
F_1	3	3	3	1	10
F_2	1	2	2	1	6
F_3	3	3	3	1	10
F_4	3	3	3	1	10
F_5	3	2	1	1	7
F_6	3	3	3	2	11
F_7	2	3	3	3	11
F_8	1	2	2	3	8
F_9	3	1	1	2	7
F_{10}	2	1	1	2	6
F_{11}	2	1	1	1	5

1."1" is considered as the best in each column.

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