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Effects of liposomal natural and synthetic antioxidants on oxidative stability of soybean oil

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ABSTRACT

Given the importance and role of edible fats in human health and their sensitivity against oxidative degradation the one hand, and the adverse effect of synthetic antioxidants on consumers health while increasing their awareness on the other hand, this study aimed to improve the effectiveness of the natural antioxidant ascorbyl palmitate, its stability and control release by nanoencapsulation of ascorbyl palmitate using \nanolipososmes, and further its comparison with tert-butylhydroquinone and butylated hydroxy toluene as synthetic antioxidants in soybean oil. Results of this study showed that utilization of 200 ppm tert-butylhydroquinone, 600 ppm ascorbyl palmitate and 250 ppm butylated hydroxy toluene in nanoliposome form had the greatest oxidative stability in soybean oil respectively. These results indicated that the use of nanoliposomes encapsulated antioxidants improve the antioxidant efficacy and that ascorbyl palmitate perform as efficiently as butylated hydroxy toluene, making it suitable to be used as a natural antioxidant. The antioxidant encapsulation in nanoliposomes is a practical approach for expedient protection of these compounds in food systems, while at the same time increasing their performance and stability in food applications.

Keywords: *ascorbyl palmitate, oxidation stability, soybean oil, nanoliposome, tert-butylhydroquinone.*

1. INTRODUCTION

Lipid oxidation leads to formation of unpleasant rancid flavors and color change as well as reduction of nutritional value of fat and oil [1]. Also, oxidation of biological molecules has been reported to be involved in some diseases, such as cancer, cardiovascular disease, cataracts, diabetes, joint rheumatoid arthritis and mutation [2, 3]. Different methods are used to prevent oxidation, including the use of antioxidants, oxygen removal by vacuum or use of inert gas as well as using low temperatures and dark storage [4]. Among these strategies, using antioxidants is perhaps the most frequently used method. Although these substances are naturally associated as antioxidants with some oils (such as tocopherols), today, to prevent oxidation of oils, synthetic antioxidants with phenolic compounds are often used due to their strong antioxidant properties [5]. Despite the fact that synthetic antioxidants work efficiently during thermal processes and storage conditions, their use is being controversial due to their toxicity and food safety issues [6-8]. The most common synthetic antioxidants used in the food industry are butylated hydroxy toluene (BHT) butylated hydroxyanisole (BHA) and butylated hydroxy toluene (TBHQ) [9].

Due to the adverse effects of synthetic antioxidants, (e.g. mutagenic effects, toxicity and carcinogenicity), use of natural antioxidants is suggested such as polyphenols, vitamin antioxidants including ascorbic acid and tocopherol, vitamin A and beta-carotene with protective effects against chronic diseases], cancer [11)], diabetes, cardiovascular disease [12], Alzheimer's, cataracts, and mutagenicity [13]. Ascorbic acid is considered as an excellent antioxidant [14], since it keeps electrons away from the enzymes responsible for oxidation and other oxidizing agents [15]. Ascorbyl palmitate (AP) is a naturally occurring antioxidant that is comparable to TBHQ in terms of antioxidant strength and is obtained from the combination of ascorbic acid and palmitic acid. Ascorbic acid is not fat soluble, but ascorbic palmitate has the ability to dissolve in fat. Thus, their combination is a fat soluble antioxidant [16].

AP was recognized as a safe substance in 1982 for protection against chemical degradation without any specific restrictions [17]. It has been reported that the use of AP reduces undesirable color, peroxide value and crosslinking. This compound is fat soluble and is known to be more effective than BHA or BHT in the prevention of oxidative reactions of oils [18]. AP acts as a synergist with tocopherols during the storage of oils [17]. Effectiveness of these valuable ingredients in food can be enhanced by micro-encapsulation of antioxidants. Many studies have conducted that for encapsulation of antioxidants and other active ingredients in lipid-based carrier systems to increase their therapeutic potential due to facilitated intracellular delivery and prolonged retention time inside the cell [8]. The controlled release of active agents and an increase in solubility of these compounds are some of the important advantages of their use. For this aim, fats should be melted, then the fat soluble component should be dissolved in it, and thereafter, a high-pressure homogenization is applied to the fused fat phase in the presence of surface active agents at 5 to 10° C above the fat's melting point [8]

The antioxidant encapsulation in nanoliposomes is not only a valuable solution for protecting these compounds in food systems,

but also increase their performance and stability in food applications. The other points of the liposomal carrier systems are the ability to control and release of the components [19]. Encapsulation of bioactive compounds, such as food antioxidants, provides their optimal protection against environmental and chemical changes such as enzymatic activity, pH and temperature fluctuations [8, 20].

In order to increase the potential effectiveness of natural antioxidants of AP, its stability and control release during the storage time, the present study aimed to investigate the effect of nano-encapsulated AP antioxidant and control its release by using nano-liposomes compared to synthetic antioxidants TBHQ and BHT in soybean oil.

2. MATERIALS AND METHODS

Soybean oil without antioxidant (neutralized, bleached, deodorized) with certain components and control sample were prepared. Fatty acids i.e. Eicosapentaenoic acid (EPA) and Docosahexaenoic hexanoic acid (DHA) from Novelty Edge Co. (Puchong, Malaysia), Soybean phospholipids from IMCOPA (*Santo André*, Brazil), 1,1,3,3-Tetraethoxypropane and thiobarbituric acid from Sigma-Aldrich (Spruce St. St. Louis, MO) were purchased. Chemicals including methanol, chloroform, n-hexane, ethanol, Iron (III) chloride, BHT and glycine were provided from Merck Co. (Darmstadt, Germany).

Liposome Preparation by Mozafari Method.

Empty and encapsulated liposomes were prepared according to the method used by Colas et al. (2007). The mixture of liposomal ingredients was prepared including the preheated (up to 30 °C) soybean polar lipids (PLs), PUFAs (a 2:3 Ratio of DHA: EPA) with 2: 0.4 mass ratio was prepared by adding distilled water and glycerol (final concentration of 2% v/v) (preheated up to 30 °C) and heated at 30 ° C on a hotplate for 60 minutes at 1000 rpm. The preparation process was carried out in a six-baffled glass vessel. Compared to the conventional method, liposomes were prepared by direct hydration and without solving PL and FAs in organic solvents. Liposomal samples were kept under nitrogen for one hour at 25 °C for better annealing and stability [8].

Evaluation of oil stability.

The amount of free fatty acids was measured by the titration method reported in the American Association of Oil Chemists, AOCS (Ca5a-40) [21] Peroxide number was measured by spectrophotometry (ferric thiocyanate method) [22]. Iodine number was estimated by Hanus method (AOAC 920/158) [23].To determine the oxidation stability index, 3 g of oil samples were tested at 120 °C by using 743 Rancimat with airflow rate being 15 L/h [24]. Spectrophotometric (UV-Vis spectrophotometer, England) method was used according to AOCS standard at 530 nm, in order to determine the Anisidine value (21)

Statistical Analysis.

Results were analyzed in a completely randomized design. The effect of antioxidants alone and with nanoliposome on the oxidative stability of soybean oil was analyzed. The treatments included the control samples, and antioxidants i.e. AP (600 ppm), BHT (250 ppm), and TBHQ (200 ppm). The tests were performed in 3 replications. Data were analyzed by SPSS software version 19 and Duncan's multiple range test performed at 95% confidence level.

3. RESULTS

The purpose of this study was to investigate the effect of AP as a natural antioxidant in the form of nanoliposomes, which is used as a carrier of functional foods. It is also compared to synthetic antioxidants BHT and TBHQ. For this purpose, 200 ppm of TBHQ alone and another 200 ppm TBHQ with 600 ppm nanoliposome, 250 ppm BHT alone and another 250 ppm with 600 ppm nanoliposome, and finally 600 ppm AP alone and another 600 ppm AP with 600 ppm nanoliposome were incorporated into soybean oil and the oxidative evaluation factors were investigated. Soybean oil is liquid in a relatively wide range of temperature with a high unsaturated fatty acid content. The presence of a relatively high amount of up to 8.7% of the linolenic acid [18: 3], lead to its susceptibility to oxidation [24]**.**

Iodine value.

The iodine value is an indicator of the number of unsaturated bonds in the oil. Also, oil spoilage due to oxidation is directly related to iodine number. Under the same conditions, oils with double or multiple bonds are more susceptible to oxidative degradation [25]. As shown in Fig. 1a, the iodine value of all oil samples were about 120 to 130 on day zero, however, during a storage period of 45 days at 25 °C, the iodine number of all oil samples reduced. The highest amount of iodine reduction was observed in the oil sample without antioxidants. Among the antioxidants used, TBHQ had the greatest effect on the iodine value, while, there was no any significant difference ($\alpha \leq 0.05$) between BHT and AP. Comparison of the use of antioxidant alone and its use in nanoliposome form, showed that the addition of antioxidant in nanoliposome form increased the antioxidant effect; in all the added nanolipososme antioxidants the iodine value had a lower reduction and remained relatively high. Among all soybean oil treatments, the sample containing 200 ppm TBHQ in nanoliposome form had the highest iodine value, followed by nanoliposomal BHT and AP, respectively. The control sample had the lowest levels of iodine value during the storage time. It should be noted that unlike TBHQ, AP is not composed of pure antioxidant compounds. However, natural antioxidant contents of AP are similar to weaker synthetic antioxidants, e.g. BHT, in terms of the presence of phenolic compounds. Fig. 1b shows the effect of antioxidants on the iodine value at 180 °C. As shown, a temperature of 180 °C of temperature reduced the iodine value in soybean oil, and during the 45-day maintenance at 25 °C, the iodine value of all oil samples were reduced. The highest amount of iodine value reduction was observed in the oil sample without antioxidants. The effect of antioxidants on preventing the

reduction of iodine value had a similar trend with those results at 25 °C. Among the used antioxidants, nanoliposomal TBHQ had the greatest effect on reducing the iodine value, followed by nanoliposomal BHT and AP.

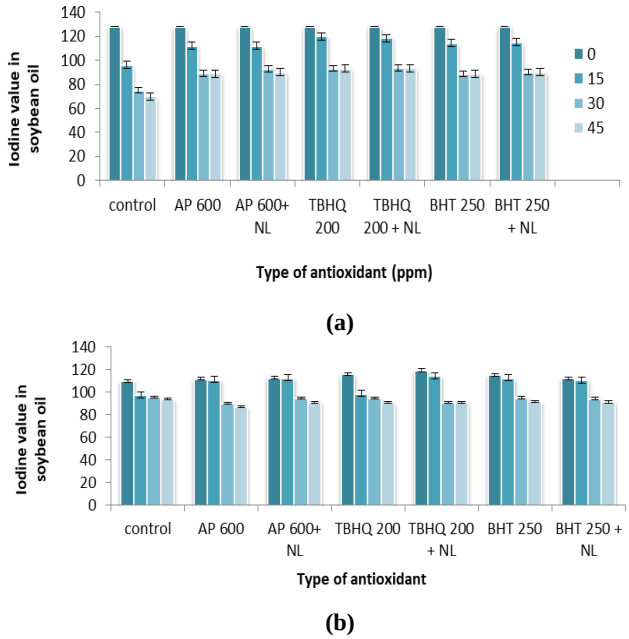

Figure 1. The effect of antioxidants on the iodine value of soybean oil at 25 °C (figure a) and 120 ºC (figure b) for 45 days.

Peroxide value.

As a qualitative indicator of oils, peroxide value is usually associated with chemical spoilage and its evaluation should be carried out at the beginning of the oxidation. This value varies depending on the type of the oil, its degree of non-saturation, the time of storage and the packaging quality in different oils [26]. For high quality fats and oils, the peroxide value is less than 5, usually 0 to 3. Its increase to a level higher than 10 indicates the oxidative degradation of oils [27]. Fig. 2a and 2b show the results of the average peroxide values of soybean oil samples in the presence of antioxidants at two different temperatures. The peroxide value on the first day was zero. As shown, the peroxide value increased until 15th day and then it decreased. On the contrary, on the 15th day, all treatments showed less peroxide values compared to those of control samples. However, sample containing 200 ppm of nanoliposomal TBHQ showed the lowest peroxide value followed by the sample with 250 ppm nanoliposomal BHT, and 600 ppm nanoliposomal AP. These results indicated that nanoliposomal TBHQ has the highest antioxidant effect, followed by nanolipossomal BHT and AP. The effect of adding antioxidants on peroxide value of soybean oil at 180 °C indicated that by increasing the temperature, the peroxide value of soybean oil increases. In addition, the use of nanoliposomal antioxidants had a greater effect on reducing the peroxide value of soybean oil compared to that of antioxidant alone. Hydroperoxides are products of the reaction between oxygen and unsaturated fatty acids, generated during the initial phase of oxidation. As a result by passing the time, the level of these compounds increased to a certain extent, and the secondary phase of oxidation begins where these compounds decomposed quickly to volatile aldehydes and ketones and consequently peroxide value decreases. Thus, by increasing storage time, the peroxide value decreased. Similarly, in some studies, peroxide value reduction through storage time was attributed to the products formed by the decomposition of some hydroperoxides [28]. On the 15th day all treatments showed lower peroxide values compared to those of control, however, the sample containing 200 ppm of nanoliposomal TBHQ, showed least peroxide values followed by sample containing 250 ppm BHT and 600 ppm nanoliposomal AP respectively. These results indicated that the nanoliposomal TBHQ has the highest antioxidant activity, and nanoliposomal BHT and AP equally have the highest effect without significant difference (α>0.05). The results of adding the antioxidants on the sunflower oil at 180 °C indicated that with increasing temperature, the peroxide value increased, which is consistent with the findings of [29] As the temperature increases, the rate of oil oxidation increases and the duration of the induction phase decreases. In general, every 10 °C increase in temperature leads to a decrease in the induction phase by a certain coefficient, called standard coefficient. The oxidation process, in which the triglycerides are oxidized and peroxides are formed, is one of the most important reactions that occur during the heating of the oils. The results of this study show that the peroxide formation exceeded the standard limit in soybean oil at 180 °C. This oil contains a high percentage of unsaturated fatty acids compared to other oils and as a consequence, the rate of free radicals formation is faster. Thus, at 180 °C the peroxide concentration exceeds the limit and acceleratedly continues in a chain reaction. However, the use of antioxidants can attenuate this trend. In addition, as indicated in this study, the use of antioxidants in nanoliposome form was more efficient in reducing the peroxide value. In a study by Liolios et al. (2009), nanoliposomal compounds (carvacrol, thymol), proved being more active than the pure compounds, and their antimicrobial activities were significantly increased after their encapsulation in nanoliposomes [30]. In another study, Satyanarayana et al. (2000) indicated that AP had a greater effect on preventing the hydroperoxide production in peanut oil used as deep fat frying oil compared to BHA, BHT and PG as synthetic antioxidants. In addition to better oxidation prevention of ascobyl palmitate, the peroxide value reduced during the storage time [31].

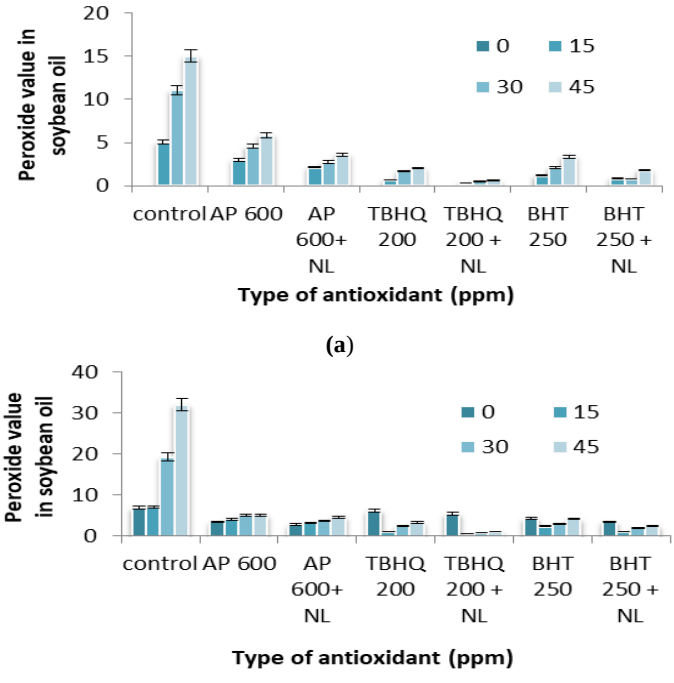

Figure 2. The effect of antioxidants on the number of soybean oil peroxide at 25 ° C (figure a) and 120 ºC (figure b) for 45 days.

Acid value.

This value is used to quantify the acidity of a substance and is an indicator of the oil quality [30]. It actually shows the level of released fatty acid during the hydrolysis and is a function of oil freshness, the degree of hydrolysis and oxidation (Fig. 3a and 3b). As shown in both Figures, the acid value increased in all samples during the 45-day storage time. Nevertheless, the use of antioxidants had a significant effect on the reduction of the acidification rate of soybean oil. The use of antioxidants in the form of nanoliposomes intensified the antioxidant effects of AP, BHT, and TBHQ, and thus, in their presence the acid number decreased. The control sample had the highest acid number and the sample with the lowest acid number was contained 200 ppm of nanoliposomal TBHQ followed by samples with 250 ppm nanoliposomal BHT along with 600 ppm nanoliposomal AP without any significant difference ($\alpha>0.05$). Acid value of soybean oil at 180 ° C and 45 days of storage indicated that the acidity of soybean oil on the first day was about 0.6 mg/g potassium hydroxide in soybean oil, nevertheless, there is an increasing trend in acid number of the samples by higher temperature and more storage time. This increase is much more significant in the control samples compared to oil samples containing antioxidants. As a result of hydrolytic degradation and oil oxidation, the triacylglycerol molecule decomposes into glycerol and free fatty acids, resulting in an increase in the acid number of the oil. It is noteworthy that in soybean oil, even after 8 hours of heating at 180 °C and 45 days of storage, the acid number will not go higher than the limit (maximum 2). The use of antioxidants as nanoliposomes had a higher effect on reducing the acid value of soybean oil. Comparison of antioxidants indicated the nanoliposomal TBHQ as the most efficient followed by BHT and AP, respectively.

Anisidine value.

Anisidine value is an indicator of secondary oxidation products, especially aldehydes [32]. Our results showed that the use of antioxidants reduced the anisidine value of soybean oil. Nanoliposomal TBHQ had the highest effect ($\alpha\leq0.05$) among antioxidants followed by nanoliposomal BHT and AP ($\alpha>0.05$), respectively. It should be noted that all the results of the Rancimat test confirmed the results of the peroxide test. Therefore, the comparison of the antioxidant properties of AP in oil with synthetic antioxidant TBHQ and BHT indicated that the TBHQ perform better than BHT and the natural antioxidant, AP, with no significant difference ($\alpha>0.05$) between BHT and AP (Fig. 4a). Addition of antioxidants in nanoliposome form significantly increased the antioxidant activities in both natural and synthetic antioxidants. Evaluation of anisidine index at 180 °C showed that increase in temperature accelerates this index and increase in storage time caused higher anisidine number which is due to the development of the process of thermo-oxidation at this temperature. The lowest number of anisidine at this temperature was observed in the sample with nanoiposomal TBHQ followed by AP and BHT ($\alpha>0.05$), and the highest value was observed in control sample after 45 days of storage (Fig. 4b).

Oxidative stability.

As shown in Fig. 5a and 5b, the highest oxidation stability in soybean oil samples is the one with 200 ppm nanoliposomal TBHQ followed by samples containing 250 ppm nanoliposomal BHT and 600 ppm nanoliposomal AP without significant difference ($\alpha\geq0.05$). In addition, the highest stability was observed in this sample against oxidation compared to the control during the 45 days storage time. The control sample of soybean oil showed the lowest oxidation stability by passing time. Soybean oil samples containing the antioxidants TBHQ, BHT and AP had the highest oxidation stability time.

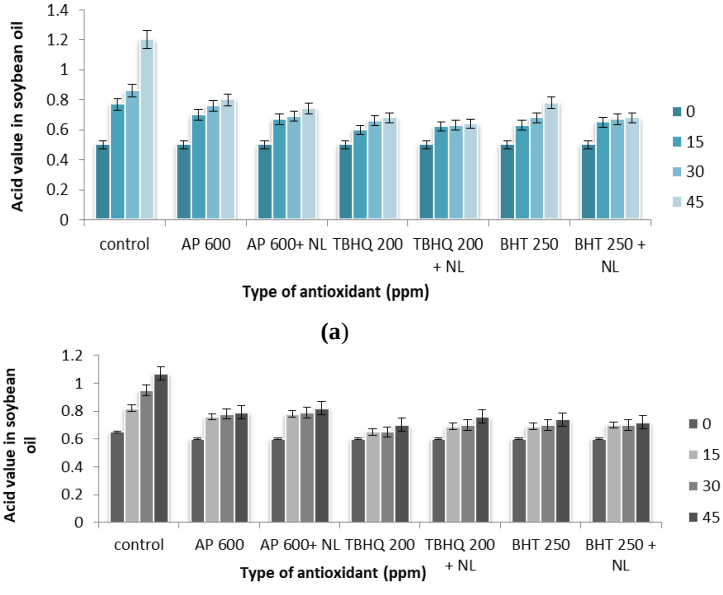

Figure 3. The effect of antioxidants on the acid number of soybean oil at 25 °C (figure a) and 180 ˚C (figure b) in 45 days.

An increase in temperature of up to 180 ˚C significantly reduced the oxidative stability of soybean oil, however, the use of antioxidants alone or in nanoliposome form, increased the oxidative stability of the oil samples, while those with nanoliposome had more significant effects. Nanoliposomes containing antioxidants are more stable than free antioxidants, due to the lipophilic characteristics of antioxidant and phospholipid in nanoliposome that create strong bonds between them and reduce the antioxidant permeability through the membrane wall [18, 33].

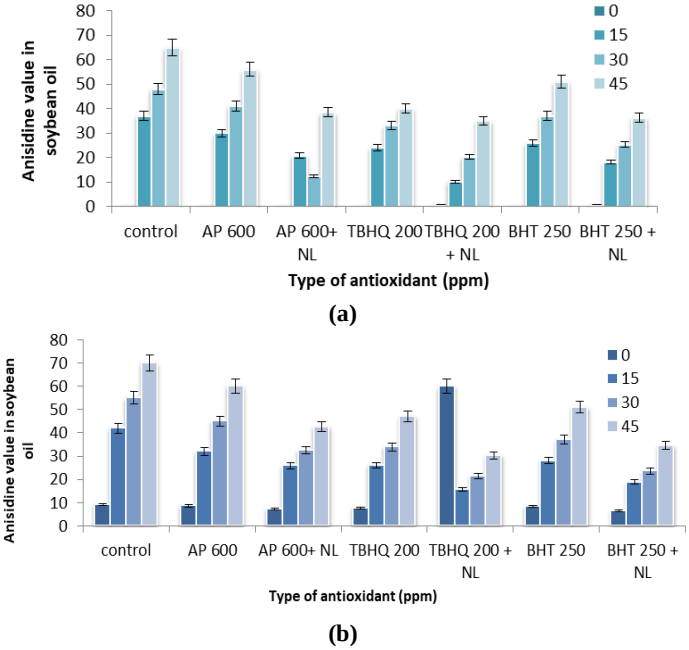

Figure 4b The effect of antioxidants on the anisidine value in soybean oil at 25° C (figure a) and 120 ºC (figure b) for 45 days.

Holser (2012) in his study showed that among docosahexaenoic acid (DHA) and α-linolenic acid (ALA) only the one containing DHA nano-liposomes had the minimum oxidative degradation and its products, after 20 minutes of heating at 120 ° C [34]. The use of this technology in food and food products is expected to help improve the shelf life of the food additives and their developments. Although bioanalysis of fats and oils are well known, concerns still exist about the presence of the nanoparticles in food and feed formulations. The results of a study by Liolios et al. (2009) showed that the use of carvacol and thymol nanoliposomes increases the oxidative stability of peanut oil, possibly due to the encapsulation of the compounds responsible for the linkage between lipophilic parts in the two layers of lipids and thus liposome fixation [30].

According to the studies, the most important natural antioxidants which are technically utilized today in the oil industry are as follow: tocopherols, AP, rosemary extract and lecithin. Different antioxidants follow different mechanisms or pathways under various stress conditions. These include preventive oxidants, chain breaking antioxidants; inhibiting the formation of free lipid radicals; quenching single oxygen species; reducing hydro peroxides and converting them into stable compounds; inhibiting prooxidative enzymes; chelating metals and converting metal pro-oxidants into stable products and through synergism with other antioxidants [35]. Since lipid oxidation occurs by a complex set of mechanisms, and there is no a single antioxidant that can prevent all oxidation steps and keep the sample oxygen free, a combination of antioxidants can be utilized for better synergistic impact [29, 37].

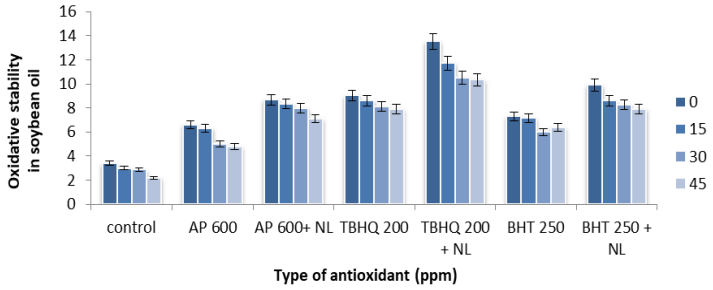

Figure 5a. The effect of antioxidants on the oxidation stability of soybean oil at 25 °C for 45 days.

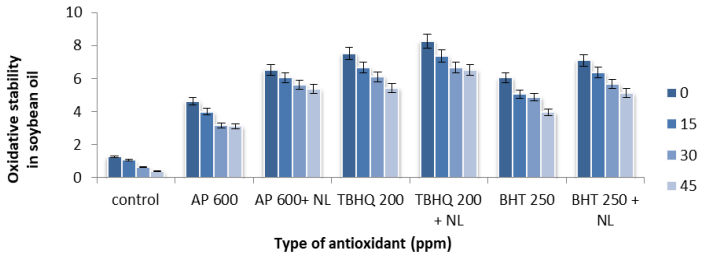

Figure 5b. The effect of antioxidants on the oxidation stability of soybean oil at 120 ºC for 45 days.

4. CONCLUSIONS

Study of the effects of using antioxidants alone and in nanoliposome form, showed that in soybean oil, the use of 200 ppm nanoliposomal TBHQ has the greatest effect on oxidative stability of the sample, followed by 250 ppm BHT and 600 ppm AP. The results confirmed that the use of antioxidants encapsulated in nanoliposomes can increase the antioxidant performance. The highest antioxidant activity was observed following usage of TBHQ as the strongest synthetic antioxidant. Antioxidant properties of BHT and AP showed no significant difference (α>0.05), suggesting AP as a natural antioxidant being a suitable substitute for synthetic antioxidants. Observation of this study is expected to assist in improving the shelf life of oil.

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