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Residue levels of food-grade antioxidants in postharvest treated in-pod peanuts during five months of storage

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Abstract

In order to investigate residue levels of butylated hydroxyanisole (BHA), propyl paraben (PP) and butylated hydroxytoluene (BHT) during storage, eight-hundred kilograms of bulk peanuts were treated with the following antioxidant emulsions: BHA (1802 μg g^{-1}), BHA–PP (1802 μg g^{-1} + 1802 μg g^{-1}) M1 and BHA–PP–BHT mixtures (1802 μg g^{-1} + 901 μg g^{-1} + 2204 μg g^{-1}) M2 and (1802 μg g^{-1} + 1802 μg g^{-1} + 2204 μg g^{-1}) M3. Residues were determined in peanut pod and seed tissues at 1-month intervals during the storage. While the reduction levels of BHA and PP in pods at the end of the storage period ranged from 66% to 76%, BHT levels were decreased extensively (86%). Twenty-four hours after peanuts were treated, antioxidant emulsions effectively seeped into the seeds and low levels of these chemicals were detected during the assay. Residues of PP in seeds were lower (62%) than the other antioxidants. Although the doses used were higher than those approved for food-grade antioxidants in stored peanuts, the residue levels in seeds (32.8–0.02 μg g^{-1}) did not exceed the maximum residue limits during the storage period.

Keywords: Antioxidant; Butylated hydroxyanisole; Butylated hydroxytoluene; Propyl paraben; Peanut; Storage; HPLC

1. Introduction

Peanut (*Arachis hypogaea* L.) is an economically important crop in Argentina, its annual production reaching 350 million tons. Argentina ranks third in the world among peanut exporters. Most of the product is exported to the EU and the USA and the rest is consumed internally. Peanut seeds are grown and commercialized principally for human consumption and as raw materials for feedstuffs and for oil and flour production (SAGPyA, 2007). The peanut storage in our country extends from 3 to 6 months; during this period peanut quality is susceptible to diminish by chemicals, physical and biological factors.

The lipid material in nut kernel oils is highly unsaturated and susceptible to oxidation (Appelqvist, 1989). Significant changes occurred in the free amino acid fractions in storage periods up to 9 months in simulated commercial conditions (4 °C, 65% relative humidity) (Pattee, Young, & Giesbrecht, 1981a, 1981b). These free amino acids were probably subjected to oxygen-centered free radical attack and like the lipid oxidation processes in raw peanut; the amino acids combined with other molecules or underwent scission reactions to lower molecular weight species. The carbohydrate components, fructose, glucose, inositol, sucrose and stachyose were significantly affected by storage time while raffinose and ribose were not changed. Oxidative stability in raw peanuts decreased with storage time but not in a uniform manner across seed size (Pattee, Young, Pearson, Singleton, & Giesbrecht, 1982).

Food-grade antioxidants such as BHA, BHT, tertiary butylhydroquinone and PP are effective in preventing

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oxidation of nuts and nut products by delaying the development of oxidative rancidity because they are able to absorb the energy of the activated fat molecules (Lundberg, 1962; Sherwin, 1978) and thereby, increasing peanut shelf life. The rancidity process in peanuts stored at 63 °C was observed at the second day of storage. Nevertheless, in seeds treated with a BHA–BHT mixture, this process was delayed about of 18 days (Eastman Chemical Company, 2004).

The second aspect responsible for the reduction in stored peanut quality in Argentina is aflatoxin contamination. Toxicological studies on aflatoxins have shown that they are potent carcinogenic and teratogenic metabolites produced mainly by *Aspergillus flavus* and *A.parasiticus* and other species of *Aspergillus* section *Flavi* (IARC, 1993; Klich & Pitt, 1988; Kurtzman, Horn, & Hesseltine, 1987). Raw peanuts now entering the European Union's borders must have less than 4 ppb of total aflatoxin and not more than 2 ppb of B_1 (Commission of the European Communities, 2006). Previous studies carried out in Argentina demonstrated that peanut is a good substrate for the growth of aflatoxin-producers and therefore, it is prone to be contaminated with aflatoxins (Barros, Torres, & Chulze, 2003; Novas & Cabral, 2002; Vaamonde, Degrossi, Comerio, & Fernández Pinto, 1995).

Numerous researchers have focalized their studies in the role of phenolic antioxidants in preventing a great number of pathological disturbances associated to the generation of free radicals, such as atherosclerosis (Steinberg, 1992), brain dysfunction (Gordon, 1996) and cancer (Ames, 1983; Feskanich et al., 2000; Michels et al., 2000) and diverse effects on inflammatory diseases (Decharneux, Dubois, Beauloye, Warriaux-de Coninck, & Wattiaux, 1992). On the other hand, there have been studies showing that antioxidants could counteract many of the deleterious effects caused by mycotoxins ingestion in animals (Coulombe, Guarisco, Klein, & Hall, 2005; Klein, Van Vleet, Hall, & Coulambe, 2002) and more important it have been demonstrated the chemoprotective property of inhibit AFB_1 action (Garner, 1975; Kensler, Davis, & Bolton, 1994; Klein, Van Vleet, Hall, & Coulambe, 2003; Mgbodile, Holscher, & Neal, 1975; Talalay & Benson, 1982). Raw peanut seeds contain endogenous nonenzymatic antioxidants, such as α-tocopherol that is an excellent chain-breaking antioxidant, almost 100 times more effective against peroxyl radicals than BHT (Burton & Traber, 1990; Cobb & Johnson, 1973). Sanders et al. (1992) found that tocopherol content was consistently different in peanuts from various origins; these authors observed that peanuts produced in the USA had a consistently higher tocopherol content than peanuts produced in Argentina. Therefore, peanuts in Argentina not only must be in-pod stored for safety, but they also need to be supplemented with additional antioxidants. On the other hand, several antioxidants have been shown to be effective in controlling mycotoxigenic growth and mycotoxins accumulation (Farnochi, Torres, Magan, & Chulze, 2005; Joseph, Jayaprakasha, Selvi, Jena, & Sakariah, 2005; Selvi, Joseph, & Jayaprakasha, 2003). It was also demonstrated that different mixtures of BHA, PP and BHT were affective fungitoxicants by inhibiting aflatoxigenic fungi and their toxins accumulation in peanuts (Passone, Resnik, & Etcheverry, 2005).

Thus, the purpose of this work was to determine if food-grade antioxidant emulsions were able to penetrate peanut pods to reach the seeds and remain during a 5 month period under local storage conditions to prevent aflatoxigenic growth and aflatoxin accumulation and to evaluate if antioxidant residue values were within the Codex Alimentarius (2006) for BHT and BHA and for PP by Food and Drug Administration (FDA).

2. Materials and methods

2.1. Reagents and standards

All the antioxidant chemicals were obtained from Eastman Chemical Company (Kingsport, USA). These were benzoic acid, 2(3)-*tert*-butyl-4 hydroxyanisole (BHA); *n*-propyl *p*-hydroxybenzoate (PP) and 2,6-di (*tert*-butyl)-*p*-cresol (BHT). Stock solutions of BHA, PP and BHT (5 and 10 mmol L^{-1}) were prepared in ethyl alcohol-water (95: 5 v/v) and the appropriate concentration was sprayed to in-pod peanuts. Ethyl alcohol pro-analysis was obtained from Cicarelli (Santa Fe, Argentina). Acetonitrile (J.T. Baker® HPLC grade, Mexico City, Mexico) was used for extract antioxidant from the matrix (peanut pods and seeds) and the solvents that constituted the mobile phase were methanol (Tedia® HPLC grade, Fairfield, USA), acetonitrile, acetic acid (J.T. Baker® HPLC grade, Mexico City, Mexico) and water (Nanopure Diamond® HPLC grade, Barnstead International, USA) that was filtered through a cellulose nitrate filter (0.45 μm, μicroclar, Bs. As., Argentina).

2.2. Samples

Trials were conducted during 2004–2005, in Storage Company of south of Córdoba, Argentina. Eight-hundred kilograms of bulk peanut conventional cultivar (Runner variety) harvested in 2003–2004 for human consumption and checked for absence of aflatoxins was distributed in five containers (big hermetic bag called silos). While the silos were filled, all in-pod peanuts were treated with different antioxidant formulations using a dosage measure system. Silo 1 was treated with BHA (1802 μg g^{-1}), a mixture of BHA–PP (1802 μg g^{-1} + 1802 μg g^{-1}) M1 was applied on silo 2 and ternary mixtures with BHA–PP–BHT (1802 μg g^{-1} + 901 μg g^{-1} + 2204 μg g^{-1}) M2 and (1802 μg g^{-1} + 1802 μg g^{-1} + 2204 μg g^{-1}) M3 were applied on silos 3 and 4, respectively. The four experimental units were put in the stockpiled cell to maintain treated peanuts in the same environmental conditions than the peanut storage system.

2.3. Sampling procedures

Ten samples of 500 g from each silo were randomly selected of each experimental unit (200 Kg) using a compartmented sampling spear, which enabled samples to be taken from different depths. The first sampling was made at the start of the assay and then every 30 days during a 5 month period. The samples were collected in polyethylene bags, brought to the laboratory and analyzed immediately (within 24 h) for antioxidant residue levels. Peanut seeds and pods were separated, and were analyzed separately. Before analysis, the samples were dried during 24 h in a forced air system at 50 ± 2 °C.

2.4. Extraction of antioxidant residues

One gram of milled peanut pods was taken to evaluate the antioxidant content. The antioxidants were extracted with 15 ml acetonitrile by shaking the pods with the solvent for 10 min using a magnetic stirrer (Precytec A E 28, Bs. As., Argentina). The extraction procedure was repeated three times. In the last extraction, the samples were sonicated (Branson 8510, Danbury, USA) during 15 min and after that, pod samples were centrifuged (ANALEN 2036, Bs. As., Argentina) during 10 min at 3000 rpm. An aliquot (500 μL) of each extraction was taken and combined to obtain 1.5 mL final volume for each sample.

To carry out the antioxidant extraction from peanut seed tissue, 5 g of whole seeds were shaked with 10 mL acetonitrile in a magnetic stirrer during 10 min. The procedure was repeated three times. In the last extraction, the samples were sonicated during 15 min. An aliquot of each extraction (1 mL) was taken and combined to obtain 3 mL final volume for each sample.

The mean recoveries were calculated by spiking peanuts at different levels ranging from 240 to 1800 μg g^{-1}.

2.5. Quantification of antioxidant residues

Antioxidant content from peanut pod and seed tissues was determined by a HPLC system Agilent® (Santa Clara, USA) 1100 serie, included a degasser, an autosampler, a UV detector, a quaternary pump and a thermostatted column compartment connected to a HP ChemStation Plus Family Software®. Chromatographic separations were performed on a reversed phase Varian Microsorb (Palo Alto, USA) MV 100-5, C18 (250 mm × 4.6 mm ID, 5 μm) and on a guard column Thermo Electron Corporation® (Waltham, USA) BDS-Hypersil, C18 (10 mm × 4 mm ID, 5 μm). Solvents that constituted the mobile phase were A (methanol), B (acetonitrile) and C (acetic acid–water, 2: 98, v/v). The elution conditions applied were: 0–6 min, linear gradient from A: B: C (30: 0: 70, v/v/v) to A: B: C (65: 20: 15, v/v/v); 6–15 min, linear gradient from A: B: C (65: 20: 15, v/v/v) to 100% A; and finally, washing and reconditioning the column. The flow rate was 1.5 mL min^{-1} and the injection volume was 10 μL. The chromatographic separation was carried out at 25 ± 0.5 °C, maintaining the vials in the autosampler at 24 ± 0.5 °C. Antioxidants were monitored and quantified at 280 nm. Quantification was performed by reporting the measured integration areas in the calibration equation of the corresponding standards. The detection and quantification limits of the analytical method for the three antioxidants were 0.05 and 0.1 ng g^{-1} for PP, 0.76 and 1.52 ng g^{-1} for BHA and 0.88 and 1.76 ng g^{-1} for BHT.

2.6. Statistical analysis

Statistical analysis were made using SigmaStat program Version 3.10. Copyright© 2004 Systat Software, Inc. (San Jose, USA). Mean values of BHA, PP and BHT residues in peanut pod and seed tissues were determined by analyses of variance (ANOVA) ($P < 0.001$). Bonferroni test ($\alpha = 0.05$) was made to compare means of antioxidant residues to determine the significant differences between the first and subsequent sampling.

3. Results and discussion

3.1. Results of analytical method

The parameters of the linear regression over the whole concentration range for the antioxidants studied and the mean recovery percentages for antioxidants extracted from pods and seeds are shown in Table 1.

3.2. Residue levels of BHA, PP and BHT in peanut pods

The effect of storage (S) and antioxidant mixtures (T) on the residue levels of BHA, PP and BHT was examined at intervals of about 1 month during five months of storage at ambient temperature (16 ± 7 °C). ANOVA results showed that antioxidant concentrations in peanut pods were significantly affected by storage period and antioxidant mixtures (Table 2). Antioxidant residues in pods from silo 1 were decreased relatively faster during the first two months of storage, while the residues from silos 2, 3 and 4 were significantly reduced during the first month and thereafter at a slower rate until the end of storage period (Fig. 1). Prior to storage (time zero sample) the initial concentration of BHA on pods was 1181 μg g^{-1} when it was inoculated as single form, 2235 μg g^{-1} when this chemical was sprayed as binary mixture, while in pods treated with ternary mixtures initial levels were 1702 and 1575 μg g^{-1}. The original applied dose was at the rate of 1802 kg of BHA per ton of dry peanut pods. Therefore, a total of 93% of the applied dose of BHA was recovered at 24 h, while recovery levels of PP and BHT were 108% and 54%, respectively. Antioxidant mixtures significantly influenced ($P < 0.001$) BHA residues. It is notable that BHA levels in pods treated with ternary mixtures were higher (29%) than in silo 1. At the end of the storage period, the levels of BHA and PP in pods had decreased to 66–75% and 69–76% of the initial levels, respectively; and BHT to 86%.

Table 1
Parameters of the linear regression and recovery percentages for BHA, BHT and PP

Antioxidants	Parameter of the linear regression			Recovery percentages ($n = 30$)	
	Intercept (AU s)	Slope (AU s μg g^{-1})	Correlation coefficient (R^2)	Pods (%)	Seeds (%)
BHA	79	300	0.9998	92 ± 2	96 ± 7
BHT	26	186	0.99993	92 ± 4	71 ± 6
PP	133	429	0.9993	84 ± 2	82 ± 5

Table 2
Significance of storage time (S), treatments (T) and their interactions on pod antioxidant residues

Factor	DF[a]	MS[b]	F value
S	5	8410931	173**
T	3	5526301	114**
S × T	15	967251	20**
Error	432	48663	

[a] DF = degrees of freedom.
[b] MS = mean squares.
** Significant at $P < 0.001$.

It is known that BHA and BHT are able to volatilize at ambient temperature (Dziezak, 1986; Rajalakshmi & Narasimhan, 1995). Nevertheless, Cruz, Conde, Domínguez, and Parajó, 2007 demonstrated that the volatilization process of methanolic solutions of BHA and BHT was very slow at 100 °C. In addition, these authors observed lower specific antioxidant activities of non-volatilized fraction than the initial product. Hamama and Nawar (1991) detected 2,3′-di-*tert*-butyl, 2′-hydroxy, 4,5′-dimethoxydiphenyl ether, 2,2′-dihydroxy-5,5′-dimethoxy-3,3′-di-*tert*-butylbiphenyl and free radical species as the thermal decomposition products of BHA and BHT. On the other hand, Magan, Aldred, and Sanchis (2004) reported that *Aspergillus* and *Penicillium* spp. were able to metabolize a range of food-grade preservatives and that it occurred after 14 days of incubation. The addition of these factors could provide an explanation about the significant reduction of antioxidant residues observed in peanut pods.

3.3. Residue levels of BHA, PP and BHT in peanut seeds

Statistical analysis on antioxidant residues; storage period (S) and antioxidant mixtures (T) and two-way interac-

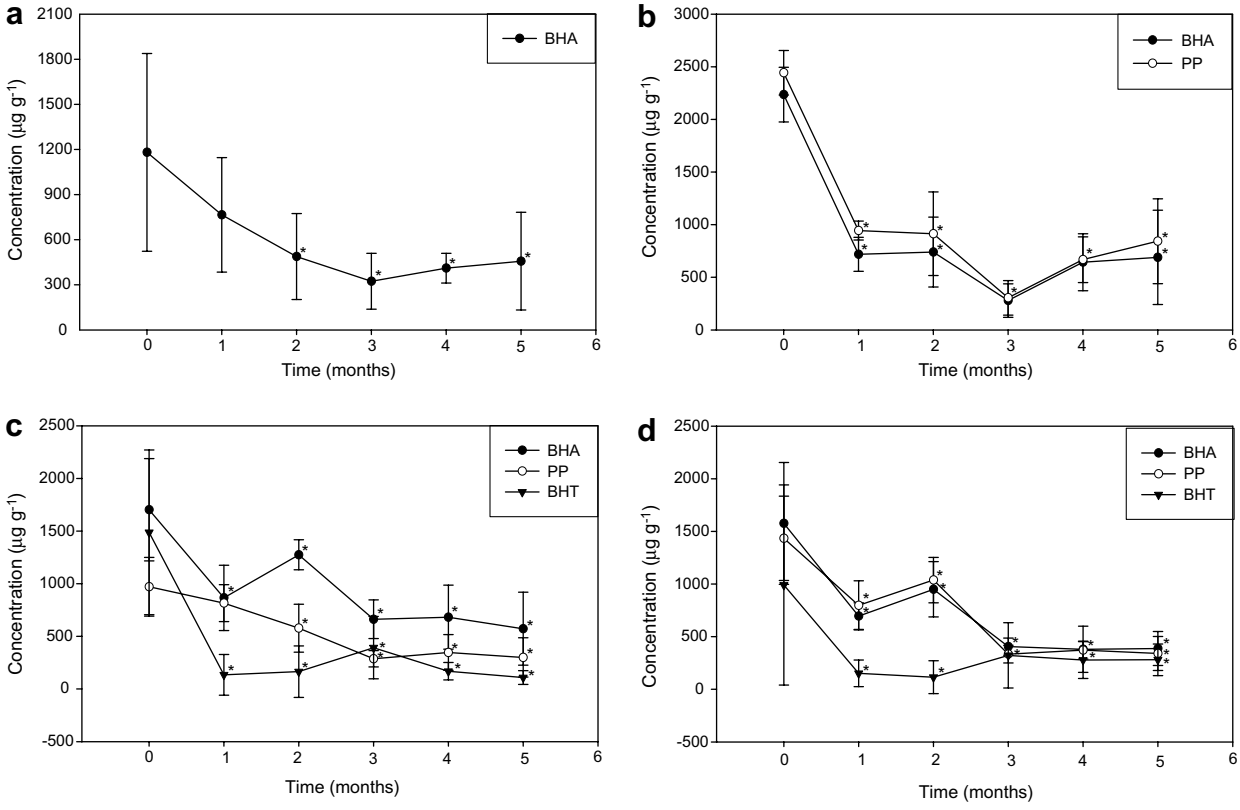

Fig. 1. Mean residue levels ± standard deviation of antioxidants in peanut pods at six sampling times: (a) silo 1, BHA (1802 μg g^{-1}); (b) silo 2, BHA–PP (1802 μg g^{-1} + 1802 μg g^{-1}); (c) silo 3, BHA–PP–BHT (1802 μg g^{-1} + 901 μg g^{-1} + 2204 μg g^{-1}); (d) silo 4, BHA–PP–BHT (1802 μg g^{-1} + 1802 μg g^{-1} + 2204 μg g^{-1}). Data with (*) are significantly different ($\alpha < 0.05$) from the values corresponding to the first sampling according to Bonferroni Test.

tions were statistically significant (Table 3). Considering the concentrations of BHA (1802 μg g^{-1}), PP (901 and 1802 μg g^{-1}) and BHT (2204 μg g^{-1}) applied on in-pod peanuts, only between 0.05 and 0.14%, 0.14 and 0.5% and 0.02 and 0.2%, respectively were initially (within 24 h) recovered from peanut seeds (Fig. 2). Although increases in BHA levels in seeds from silo 1 (15.7 μg g^{-1}/2° sampling), silo 2 (3.5 μg g^{-1}/5° sampling) and silo 4 (9.4 μg g^{-1}/5° sampling) were observed, in general, BHA concentrations were maintained relatively constant during the storage period in a mean level of 2.4 μg g^{-1}. Antioxidant peaks observed in samples from peanut pod and seed tissues, i.e.: levels of BHA when it was sprayed as binary mixture (2235 μg g^{-1}), could be attributed to an unsuitable homogenization of these preservatives when they were applied. Similarly, residues of BHT in seeds were stable with mean levels of 1.25 and 10.4 μg g^{-1} in silo 3 and 4, respectively. In seeds treated with ternary mixtures the mean level of PP was 1.3 μg g^{-1}, but the residue levels of this chemical were reduced slower (75%) until the end of storage period.

Table 3
Significance of storage time (S), treatments (T), and their interactions on seed antioxidant residues

Factor	DF[a]	MS[b]	*F* value
S	5	135	12**
T	3	483	43**
S × T	15	170	15**
Error	432	11	

[a] DF = degrees of freedom.
[b] MS = mean squares.
** Significant at $P < 0.001$.

While the residues of PP in pods were higher (35%) than BHA and BHT, the levels of this chemical (PP) in seeds were lower (62%) than the other antioxidants. This behavior could be attributed to the volatilization property of BHA and BHT at ambient temperature (Dziezak, 1986; Rajalakshmi & Narasimhan, 1995). Through volatilization, these chemicals could penetrate peanut pods during all storage period, providing protection to the seeds. On the contrary, PP levels observed in seeds could be attributed to the penetration occurred during 24 h post-treatment aided by the food-grade solvent (ethanol) used. In accordance with recently published data about antioxidant stability (Cruz et al., 2007) and the environmental conditions registered along the assay (16 ± 7 °C; water activity (a_W) 0.696 ± 0.013; pH 6.84 ± 0.16), it may be suggest that radical-scavenging capacity of BHA, PP and BHT was retained during the 5 months.

From a human health perspective, the Codex Alimentarius and the FDA allow the use of phenolic antioxidants in

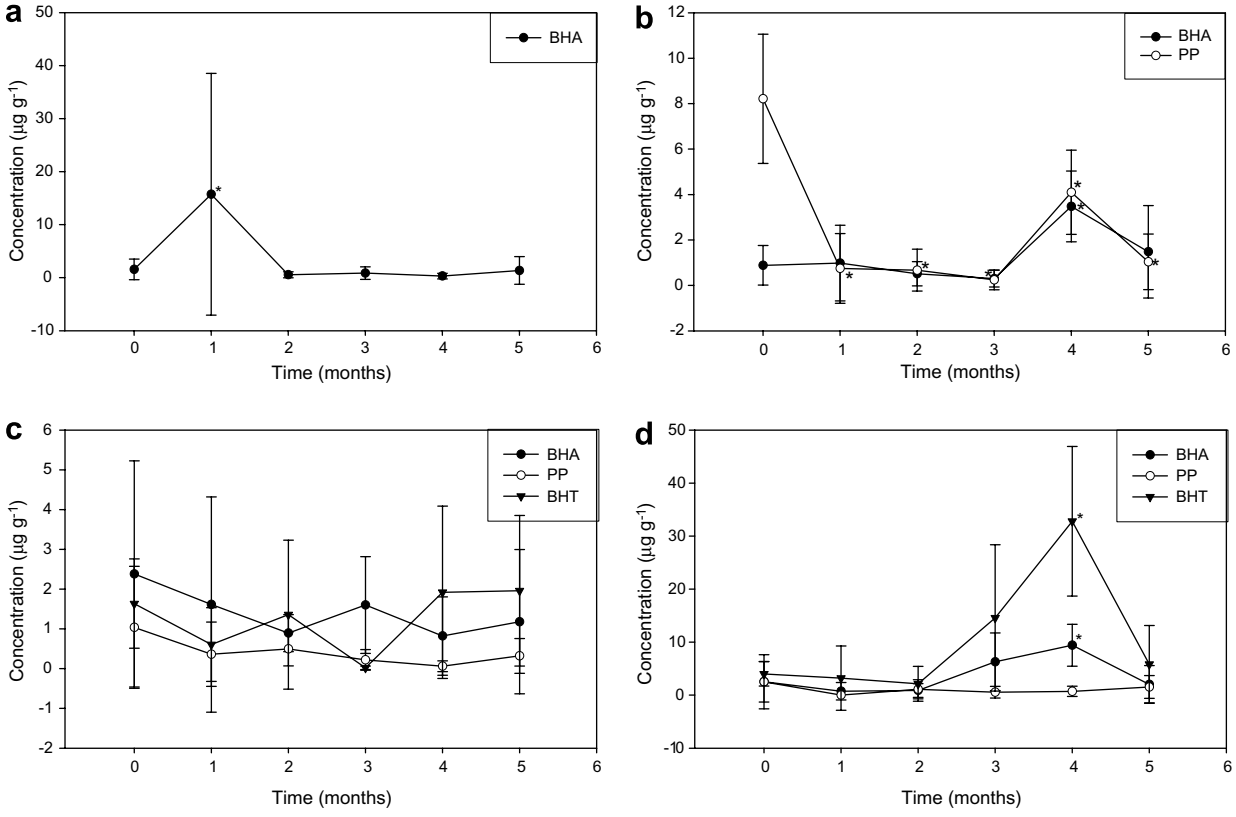

Fig. 2. Mean residue levels ± standard deviation of antioxidants in peanut seeds at six sampling times: (a) silo 1, BHA (1802 μg g^{-1}); (b) silo 2, BHA–PP (1802 μg g^{-1} + 1802 μg g^{-1}); (c) silo 3, BHA–PP–BHT (1802 μg g^{-1} + 901 μg g^{-1} + 2204 μg g^{-1}); (d) silo 4, BHA–PP–BHT (1802 μg g^{-1} + 1802 μg g^{-1} + 2204 μg g^{-1}). Data with (*) are significantly different ($\alpha < 0.05$) from the values corresponding to the first sampling according to Bonferroni Test.

foods and they are regarded as safe (GRAS) chemicals. The maximum usage level of single or multiple antioxidants approved by the more restricted legislation mentioned before, is 200 $\mu g\ g^{-1}$ based on the weight of the fat or oil (200 kg of antioxidant per ton of fat or oil). Considering that the oil content of peanut varies depending on the variety of this nut but ranges from approximately 40–50% (Appelqvist, 1989), therefore a dose of 100 $\mu g\ g^{-1}$ based on the total product weight could be applied. Although in the present study 1802 $\mu g\ g^{-1}$ of BHA and PP and 2204 $\mu g\ g^{-1}$ of BHT were sprayed on in-pod peanuts, the residual levels of these chemicals in seeds ranged from 5.84 to 0.32 $\mu g\ g^{-1}$ at the end of the storage period, which are within the levels allowed.

4. Conclusions

In the present work, BHA, PP and BHT residues extracted from stored peanut were significantly reduced ($P < 0.001$) during the storage period. Twenty-four hours after treating in-pod peanuts with BHA, with binary mixture of BHA–PP, or with ternary mixtures of BHA–PP–BHT, these antioxidant emulsions had effectively seeped into the seeds. Low levels of these chemicals were detected during the entire assay. Although doses of food-grade antioxidants higher than the allowed by Codex Alimentarius or FDA for oil nut content were added to the peanuts, the antioxidant residues detected in seeds were approximately 540 times lower than in pod, and did not exceed the maximum residue levels allowed. This study showed that food-grade antioxidants could be effectively applied on in-pod stored peanut to preserve the shelf life.

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