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Effect of micro-encapsulated antioxidant formulations on mycobiota, residual levels, sensory analyses and insect pest attack in stored peanuts

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ABSTRACT

The *in situ* effect of microencapsulated 2(3)-tert-butyl-4 hydroxyanisole (BHA) on stored peanuts (*Arachis hipogaea*) intended for human consumption was evaluated. Peanut were stored unshelled in flexible containers called "big bags" that were made of polypropylene raffia. 100 kg of peanuts were used in each big bag and stored in refrigerated cells (< 18 °C) for about 5 months in two different peanut processing companies during 2015/2016 period. Fungal populations, aflatoxin accumulation, BHA residues, acidity and fatty acid profile, sensory analyses, insect damage and environmental factors variation, were evaluated. At the end of the storage period, significant ($p < 0.05$) fungitoxic effects of the BHA formulation were observed in the order of 30 and 15% for the first and second company, respectively. *Cladosporium,* yeasts*, Penicillium, Fusarium, Alternaria* and *Aspergillus* were the main fungal isolates. No aflatoxins were found for both companies and years evaluated. In addition, taste of the peanuts was not significantly affected ($p < 0.05$) by formulation used and insect damage was always lower than 3%. However, different levels of BHA were detected throughout the experiment in the two companies, with final levels of 2.5 for the C1 and 275 ng BHA/g peanuts in C2. Formulation did not affect acidity and organoleptic properties of peanuts. These results show that BHA formulation could be used as part of alternative strategy for control of fungal contamination storage period.

1. Introduction

Peanut (*Arachis hypogaea* L.) cultivated in Argentina is known worldwide for its quality. In this country around 80% of peanut production is exported, accounting for 25% of annual global of these oil seed exports (USDA, 2015). Peanuts comprise 6 different products, with blanched ones being the most important for export (49%) followed by shelled peanuts (38%) and then peanut oil (10%) with Argentina being the main exporter of peanut confectionery (Blengino, 2015). Quality of peanuts and by-products, from the field to the table, should ensure fresh and long-lasting flavor, ideal texture, and outstanding nutrition without any risk for health. However, peanut storage in Argentina extends from 3 to 6 months, period during which its quality is susceptible to be decreased by chemical, physical and biological factors (Passone et al., 2014). One important chemical risk of this food is the presence of aflatoxins (AFs) due to contamination with toxigenic fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus*. These are frequently isolated form peanuts during storage (Bhattacharya and Raha, 2002; Gonçalez et al., 2008; Nakai et al., 2008). Mycotoxins are associated the presence of fungal inoculum on susceptible substrates. Usually, they can be produced in ripening peanuts in soil, especially under drought stress in the field and also during transport and storage where conditions are suitable for their production. Despite the absence of direct correlation between the extent of mould growth and mycotoxin production, prevention of fungal growth effectively minimizes the risk of mycotoxin accumulation (Garcia et al., 2009).

On the other hand, insect contamination in food commodities is an important quality control problem of concern for food industries (Nesci et al., 2011). Stored products of agricultural origin are attacked by > 600 species of beetles, 70 species of moths, and about 355 species of mites causing quantitative and qualitative losses (Rajendran, 2002). Major insect pests of stored peanuts include the groundnut bruchid *Caryedon serratus* (Olivier), *Oryzaephilus surinamensis* (L.), *Plodia interpunctella* (Hübner), and *Tribolium castaneum* (Herbst) (Rajendran and

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Sriranjini, 2008). In addition, interactions between biotic and abiotic factors may promote the formation of a moldy substrate and toxin accumulation in stored peanuts.

To prevent mycotoxin contamination, control of growth of mycotoxigenic moulds and pests is necessary. Both biological contaminant factors are commonly controlled using synthetic fungicides and insecticides (López et al., 2004; López-Malo et al., 2000). However, continuous and indiscriminate use of chemical preservatives in foods and feeds, can lead to toxic effects for consumers and to the development of resistances in microorganisms (López et al., 2004). On the other hand, butylated hydroxyanisole (BHA) is a food grade antioxidant that has been used extensively for many years as antioxidants to preserve and maintain freshness, nutritive value, flavor and color of food and animal feed products (JECFA, 1996). Besides, this antioxidant showed antifungal and insecticidal effects on stored peanuts (Nesci et al., 2011, Passone et al., 2007; Passone et al., 2008a, 2008b; Passone et al., 2009). However, analysis of antioxidant residual levels in stored peanuts shows a rapid reduction, probably due to the environmental and biological factor interactions (Passone et al., 2008c). Girardi et al. (2015) applied BHA microencapsulation technology to protect food grade antioxidants from the action of different environmental factors such as temperature and water activity (a_w). Besides, Garcia et al. (2016) showed that 20 mM of this BHA formulation completely inhibited *Aspergillus* section *Flavi* development, and therefore, AFs accumulation, with 95% mortality of the studied pest (*Oryzaephilus surinamensis*). While a lower dose (10 mM) decreased growth of both organisms and toxin levels with 50% mortality for the insect. However, results of both studies were obtained at a laboratory scale where environmental factors are more controlled. The aim of this work was evaluate the *in situ* effect of microencapsulated BHA on stored peanuts intended for direct consumption, stored in big bags for five months, assessing: a) total fungal population; b) aflatoxin B_1 accumulation; c) antioxidant residue levels; d) acidity and fatty acid profile; e) sensory analysis; f) insect damage; g) environmental variations, in two different peanut processing companies of south of Córdoba, Argentina, during 2015/2016.

2. Materials and methods

2.1. Estimation of mould populations in peanut samples

Subsamples of 10 g of each sample were finely milled and diluted with 90 mL peptone-water (0.1%) and shaken during 20 min at room temperature (25 ± 2 °C). After that, serial decimal dilutions until 10^{-3} were made. An aliquot of 0.1 mL of each dilution per sample was spread in duplicate on the surface of two solid media: dichloran rose Bengal (DRBC) and dichloran glycerol 18% (DG18) (Pitt and Hocking, 1997; Samson et al., 2010). Plates were incubated in darkness for 5–7 days at 25 ± 2 °C. The colonies were counted and populations expressed as colony-forming units per gram (CFU/g) of peanut. Macro and microscopic identification of fungal genera were made according to (Samson and Frisvad, 2004; Samson et al., 2010). Samples were analyzed in triplicates.

2.2. Aflatoxin B_1 analyses in peanut samples

2.2.1. Aflatoxin B_1 extraction

1 kg of each sample was mix in a blender with 2 L of water during 3 min. 75 g of the mixture was homogenized also in blender with 75 mL of methanol (99.8%) for 2 min. They were filtered twice with filter paper (Whatman N° 1) and transferred to 2 mL vials. Aflatoxin concentration of the samples was determined by high performance liquid chromatography (HPLC).

2.2.2. Aflatoxin B_1 detection and quantification

Aflatoxin B_1 was detected and quantified by using a HPLC system (Agilent, Hewlett Packard, series 1100, separation module) and a C_{18} column (5 µm LiChrospher 100 RP-18 Merck 125 mm length and 4 mm diameter Darmstadt, Germany) and a pre-column (5 µm Hypersil ODS Agilent, length and 4 mm diameter). Water: methanol: acetonitrile (66.6: 16.7: 16.7) mixture was used as the mobile phase at a flow rate of 1 mL/min and an injection volume of 30 µL. Fluorescence Detector module (G1321A, series N° E40505866) was used for fluorescence detection (λexc 365 nm; λem 435 nm). A post column photochemical derivatization system (Kobra Cell, corriente 100 mA.) was used. Mycotoxin was quantified on the basis of the HPLC fluorimetric response compared with that of a range of mycotoxin standards. Detection (LOD) and quantification (LOQ) limits of the analytical method were 1.5 ng/g and 4.5 ng/g, respectively.

2.3. Extraction and quantification of BHA residues in peanut samples

Extraction, detection and quantification of BHA in peanut samples were made according to Passone et al. (2008c): subsamples of 5 g of peanut kernel were shaken with 10 mL of acetonitrile on an orbital shaker for 10 min. After that, the grains were separated from the extract by filtration by repeating this procedure three times. At the last extraction, samples were sonicated (Elma D-78224 SINGEN) during 15 min. An aliquot of 1 mL was taken from each extraction and combined to obtain a final volume of 3 mL. Each sample was analyzed in triplicate.

Extracts were analyzed using HPLC system (Waters 2696 separations module, Waters, Milford, USA). Chromatographic separations were performed on a C18 reverse phase stainless steel column (5 µm LiChrospher 100 RP-18 Merck125 mm length and 4 mm diameter, Darmstadt, Germany), connected to a pre- column (20 × 4.6 mm id, 5 µm particle size, Phenomenex). Mobile phase used was methanol:acetonitrile:water (35:35:30), at a flow rate of 1.5 mL/min. BHA was detected by UV (Waters 2998) at 280 nm and quantified using a software integrator (Empower, Milford, MA, USA). Antioxidant levels were calculated by comparing the area of the chromatographic peak of samples with those of standard calibration curve which were 0.1, 1, 10, 100, 250, 500, 750 and 1000 ng/mL. Limits of detection and quantification were 0.4 ng/g and 1.2 ng/g.

2.4. Determination of acidity and fatty acid profile in peanut samples

Methodology based on AOAC 940 and ISO 660 for determination of free fatty acid content was used. For this purpose, extraction was carried out by pressing the oil, using a hydraulic press. Between 4 and 20 g of oil were placed in an Erlenmeyer, to which were added 30 mL of 99.5% ethyl alcohol, 30 mL of sulfuric ether and 4 drops of phenohphthalein (1%). After that mix was stirred and titrated with a solution of 0.1 N of sodium hydroxide (NaOH), until the solution color changed to wards pink. Percentage of acidity was calculated as:

$$Acidity\ (\%) = \frac{\text{Volume of NaOH consumed} \times \text{Normality of NaOH} \times 28.24}{\text{Oil weight (g)}} \quad (1)$$

Fatty acid profile was determined by gas chromatography. For this, 10–12 drops of the oil previously obtained by pressing were placed in a test tube and 2 mL of a 1 M KOH-methanol solution was added. The mixture was shaken in a vortex for 30 s and placed in a thermostatic bath until solution became clear (about 5 min at 70 °C). Subsequently, 6 mL of n-hexane was added, shaken manually for 20–30 s and centrifuged during 5 min at1800 rpm. Supernatant was recovered and fatty acids quantified by gas chromatography. Results were expressed as percentage of oleic, linoleic acid, and ratio of Oleic acid/Linoleic acid (O/L).

2.5. Sensory analysis in peanut samples

A peanut subsample of 500 g was roasted at 100 °C for 1 h and