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Antifungal impact of volatile fractions of *Peumus boldus* and *Lippia turbinata* on *Aspergillus* section *Flavi* and residual levels of these oils in irradiated peanut

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ABSTRACT

To investigate the antifungal properties of essential oil (EO) vapors from boldo and poleo on *Aspergillus* section *Flavi* and the residual levels of the oils in peanut, irradiated peanuts conditioned at three water activities (0.98, 0.95, 0.93) were treated with 2 and 3 μL/g of boldo and 3 and 5 μL/g of poleo. EO treatments produced the greatest impact on fungal growth parameters, followed by oil concentrations and a_W levels. The three main components in peanut exposed to oil vapors were piperitone oxide, α-terpinene and eucalyptol for boldo and β-caryophyllene epoxide, limonene and piperitenone for poleo. Residues of boldo and poleo EO were significantly decreased from 24.7 to 100% and from 26.6 to 99.7% at the end of the incubation period, respectively.
The application of nontoxic boldo oil as fumigant in the control of *Aspergillus* section *Flavi* may represent a potential alternative antifungal treatment, without significant residues after 35 days.

1. Introduction

Peanut is an economically important crop in Argentina, both domestically and internationally. Annual production in 2013 reached 0.9 million tons. Peanut exports have fluctuated between 0.44 and 0.68 million tons since 2011, ranking in first position since 2012 (SIIA, 2013). There are two reasons for the importance of Argentinean peanuts in the world market: the low internal consumption (270 g annual per capita) and quality that allow access to markets that are closed to other countries (Atayde et al., 2012; SIIA, 2013). Peanuts are rich in energy and contain health beneficial nutrients, minerals, antioxidants and vitamins, giving them an exceptional nutrient profile, that are essential for optimum health (Jubeen et al., 2012). Storage time and conditions prevailing during storage have a significant bearing on peanut quality which may be diminished by chemical, physical and biological factors.

The decrease in nutritional content of peanuts has also been related to the fungal growth during storage. *Aspergillus flavus* and *Aspergillus parasiticus* are among the major storage fungi found regularly in stored peanut (Atayde et al., 2012; Jubeen et al., 2012; Passone et al., 2010). Growth of these fungi is markedly affected by environmental factors, especially water activity (a_W) and temperature which are limiting factors in the functioning of ecosystems (Giorni et al., 2009). *A. flavus* and *A. parasiticus* cause food spoilage leading to production of toxic metabolites (aflatoxins) (Passone et al., 2010). In previous studies carried out on stored peanut in big bags and conditioned at four a_W levels, we reported, in five successive months of sampling, the prevalence of *Aspergillus* section *Flavi* aflatoxin producing strains (65.0 and 75.0%) (Passone et al., 2010). Despite the water stress conditions registered in the stockpiled warehouse throughout the storage period, peanut seed samples were contaminated with aflatoxins (AFs) ranging from 2.9 to 69.1 ng/g (Nesci et al., 2011). Aflatoxins in general and particularly aflatoxin B_1 (AFB_1) are genotoxic, immunotoxic and hepatocarcinogenic secondary metabolites (group 1) (IARC, 2002). Therefore, the final regulations proposed by the European Union for maximum levels of total AFs and AFB_1 in peanuts have been set at 4 and 2 ng/g, respectively (Commission Regulation, 2010).

Essential oils (EOs) are volatile oily liquids obtained from different plant parts and widely used as food flavors. In spite of having been long recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Erdoğan Orhan et al., 2012; Srivastava et al., 2011), the recent interest in alternative natural substances has led to a new scientific awareness of these extracts.

Peumus boldus Mol., commonly known as boldo, is an endemic plant from the central region of Chile which has been used in folk medicine due to various biological activities including anti-inflammatory, antipyretic,

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hepatoprotective, anti-carcinogenic and antioxidant (Backhouse et al., 1994; Gerhardt et al., 2009; Srivastava et al., 2011). Most of these biological effects can be attributed to its strong ability to scavenge free radicals (Srivastava et al., 2011). *Lippia turbinata* Griseb. (poleo), native of the west-central region of Argentina, is well known for its aromatic-medicinal properties being used as a digestive and antispasmodic to treat gastrointestinal disorders (Pascual et al., 2001).

Avila-Sosa et al. (2012) have demonstrated the potential of EOs added to edible films as antifungal agents by vapor contact and shown that antimicrobial activities of the different EO vapors could be achieved at lesser amounts than when applying the EOs in direct contact with a food surface. The authors suggest that smaller compounds such as monoterpenes present in *Lippia berlandieri*, *Cinnamomum verum* and *Cymbopogon citratus* EOs are most efficient when used as headspace volatiles. In recent studies, EOs were screened for antifungal effects by headspace volatile assays in peanut meal extract agar (Passone et al., 2013). Boldo EO (1.5 μL/mL) showed growth inhibition percentages in the order of 91.2%. However, a high dose of poleo EO (3.0 μL/mL) was necessary to achieve growth inhibition of aflatoxigenic strains in the order of 63.6%. *A. flavus* and *A. parasiticus* strains tended to show AFB_1 stimulation in the presence of sublethal doses (1–2 μL/mL).

Commission Regulation (EU) (2011) classified food additives into two categories; those whose conditions of use are strictly defined and those without any limitation particularly different plant species and their chemical compounds. As a result, there are now numerous lines of research aimed at the application of natural substances such as EOs in foods because they can be freely used as food additives. However, none of them has considered the EO residues on the food, which can directly affect the risk of losing antifungal activity during storage period due to the high volatility exhibited by these oils.

In view of this, the objectives of this study were: (a) to corroborate the effectiveness of boldo and poleo oils by vapor contact assay against *A. flavus* and *A. parasiticus* growth *in situ* on irradiated peanut under different environmental conditions (0.98, 0.95 and 0.93 a_W); (b) to determine the chemical profile of the EOs extracted from boldo and poleo spices; and (c) to quantify the levels of EO residues in peanut grains after two incubation periods.

2. Materials and methods

2.1. Fungal strains

A. flavus Link (RCP08108) and *A. parasiticus* Speare (RCP08299) were used in this study. These strains were originally isolated from stored peanut in Córdoba, Argentina, in August/December 2008 (Passone et al., 2010). These isolates were deposited in the *Aspergillus* section *Flavi* culture collection, Microbiology and Immunology Department of the National University of Río Cuarto. The fungi were maintained on slants of malt extract agar (MEA) at 4 °C and stored as spore suspensions in 1.5 g/L glycerol at −20 °C.

2.2. Essential oils

The plant species *P. boldus* Mol. (boldo) and *L. turbinata* (Griseb.) (poleo) used in this study were purchased in a dry form from a local market. The plant species were stored at −20 °C after harvest. The plant materials were obtained from dried leaves of *P. boldus* and dried leaves and stems of *L. turbinata* var. *integrifolia*. A portion (100 g) of material from each plant species was subjected to 3 h water-distillation, and the EOs were extracted by steam distillation on a laboratory scale (Figmay S.R.L.). The yield was 2.0 and 1.02 mL per 100 g of boldo and poleo, respectively. The EO was dried over anhydrous sodium sulfate and, after filtration, stored in sterilized amber vials at 4 °C until it was used (Passone et al., 2013).

2.3. Substrate

Irradiated peanut grains (7 kGy) with retained germinative capacity were used. The peanut grains were checked for sterility and absence of AFB_1. The sterile and AFB_1-free grains with an initial a_W of 0.483 were kept at 4 °C. The water activity of irradiated peanuts was adjusted by the aseptic addition of distilled water to kernels in sterile bottles. The bottles were held at 4 °C for 48 h with periodic hand-shaking during this time. The amount of water necessary to reach the different a_W levels (0.98, 0.95 and 0.93) was determined by the calibration curve generated. The water activity of the kernels was checked with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.).

2.4. Vapor contact assay

The effect of two EOs (boldo and poleo) on the growth of two aflatoxigenic isolates in irradiated peanut grains at three a_W levels and 25 °C was studied. Eighty grams of rehydrated, shelled and whole peanut grains were dispensed as a monolayer into sterile Petri dishes (14 cm diameter), which contained a glass ring (2 cm diameter) at the edge of the dish. The plates were inoculated centrally with a 5 mm diameter mycelial disk taken from the over-night culture of each isolate grown on MEA. Sterilized cotton was placed in the glass ring and each EO was added to the cotton, having no direct contact with the substrate (Bluma et al., 2009). Boldo oil doses were 2 and 3 μL per gram of peanut (μL/g), while poleo EO doses were 3 and 5 μL/g. The control plates (without essential oil) were inoculated following the same procedure. All Petri dishes were sealed with polyethylene film and incubated at a temperature of 25 °C until the colony reached the edge of the plate. Those treatments in which no fungal development was observed at 11 days were incubated for a maximum of 35 days. Tests were undertaken in quadruplicate and each colony was measured daily in two directions at right angles to each other to obtain the mean radii. The radii of the colonies were plotted against time, and a linear regression applied, in order to obtain the growth rate as the slope of the line. Lag phase for growth was calculated mathematically and defined as the time (days) in which each colony reached a diameter of 5 mm for each treatment, in relation to isolates, EOs and a_W (Bluma et al., 2009). In all cases, the experiments were carried out in at least four replicates per treatment. The growth of fungal cultures containing different concentrations of all EOs was compared with that of the control culture with no EOs. After growth was evaluated, all samples were frozen for later extraction and oil residue quantification.

2.5. Essential oil analyses

Gas chromatography/mass spectrometry (GC/MS) analyses of the main components of each EO were performed in a Perkin Elmer *Clarus 600* equipped with a 60 m × 0.25 mm ID (film thickness 0.25 μm) DB5 Perkin Elmer column. Turbo Mass software was used to control and acquire data from the GC–MS. Operating conditions were an initial oven temperature of 60 °C (held 5 min), 5 °C min^{-1} to 210 °C. Helium was used as carrier gas at 49.6 psi. Injector temperature and GC transfer line were maintained at 250 °C and 200 °C respectively. Ionization was carried out in the mass spectrometer under vacuum by electron impact with a −70 eV ionization energy. Chromatograms were acquired in "scan" mode scanning the quadrupole from m/z 50 to m/z 300 (scan time: 0.2 s, inter-scan time: 0.1 s).

The oil components were identified comparing the MS spectra with Libraries of NIST MS Search 2.0 program.

2.6. Extraction of residues of essential oils

After 11 and 35 days of incubation, boldo and poleo EO components from 144 peanut kernel samples conditioned at three a_W levels and inoculated with *A. flavus* (RCP08108) and *A. parasiticus* (RCP08299)

were determined in triplicate. According to literature data (Fonseca de Godoy et al., 2011), the best method for extraction of EOs from complex matrices such as peanut kernels, is the solid phase microextraction (SPME) using polydimethylsiloxane (PDMS) fiber cover of 100 μm (Supelco). To optimize this step, different conditions for the extraction process were studied by varying the exposure time (10, 20 and 30 min) and temperature (25, 40 and 50 °C) of PDMS fiber in the headspace of the sealed vials. For both oils, it was determined that the best experimental conditions for the extraction process were as follows: 3 g of peanuts were placed in a glass vial, which was then sealed and maintained at room temperature (20–25 °C) for 15 min and finally placed in a bath at 50 °C for 30 min exposed to the fiber.

2.7. Quantification of residues of essential oils

The extract was analyzed by GC/MS splitless mode by maintaining the fiber in the injection port for 10 min under the same conditions as those described in Section 2.6. For quantification of the residual oil, an external calibration curve was used. For the boldo oil treated samples, the compound selected was eucalyptol (MW: 154.25 g/mol; CAS: 470-82-6) and for the poleo oil treated samples the compound selected was limonene (MW: 136.23 g/mol; CAS: 5898-27-2) both quantified by GC/MS. The quantification curves in the range of sample concentrations were performed with these two compounds. Each concentration level of standard solution was analyzed in triplicate. Quantification was performed by reporting the measured integration areas in the calibration equation of the corresponding standards. The detection (LOD) and quantification (LOQ) limits of the analytical method for the two compounds were 0.05 and 0.30 ng/g for eucalyptol and 0.02 and 0.82 ng/g for limonene.

2.8. Data analysis

All data analyses were performed by ANOVA. *F*-value was found in ANOVA using SAS program 6.1 SAS Institute, Cary, NC (1998). A posteriori tests were performed using SigmaStat program Version 3.10. (Systat Software, Inc.). The significant differences for lag phase and growth rate were determined using Duncan's New Multiple Range Test at $p \leq 0.001$ level. Bonferroni t-test ($p \leq 0.001$) was employed to determine significant differences between treatments and control for oil residue levels.

3. Results

3.1. Antifungal activity of EO vapor phases

The effect of single factors as well as their two-, three- and four- way interactions on lag phase and growth rate was determined by ANOVA (Table 1). The major effect on lag phase was produced by EO treatments, followed by oil C and EO × C; while EO, a_W levels and EO × C were the factors that produced greatest impact on fungal growth rate.

Boldo EO was the most effective: at doses of 2 and 3 μL/g and at all a_W levels studied, it increased the lag phases from 2.8 days to more than 30 days, respectively, when it was compared with the untreated control (Table 2). The effects of poleo EO on this fungal parameter were highly dependent on medium a_W and oil concentrations. The lag phases of two fungal isolates were extended between 0.9 and 3.7 days by the dose of 5 μL/g of poleo oil, whereas it was observed that 3 μL/g of this EO produced either reductions or increases up to 2.3 days.

Saturated atmosphere of the volatile fraction of boldo at 3 μL/g caused complete inhibition of the growth of both fungal isolates at all a_W assayed and at 2 μL/g strongly reduced mycelial development (mean of 71.7%), except for *A. flavus* at 0.98 a_W (Fig. 1). Poleo EO showed low antifungal activity: at 5 μL/g it inhibited *A. flavus* RCP08108 growth rate by 44.9 and 46.8% at 0.98 and 0.95 a_W, respectively. Meanwhile, the low dose of poleo EO applied (3 μL/g) stimulated fungal growth, regardless of grain moisture conditions.

Table 1
ANOVA Test. Effects of essential oils (EOs) and their concentrations (C) on lag phase and growth rate of *Aspergillus* section *Flavi* isolates (I) grown on peanut grains conditioned at various a_W levels.

Source of variation	DF	Lag phase		Growth rate	
		MS	*F* value[a]	MS	*F* value
a_W	2	495414.15	22.41**	0.1511	106.05**
I	1	122603.27	5.55*	0.0037	2.64
EO	1	5790556.42	261.95**	0.2089	146.63**
C	2	2248718.05	101.73**	0.0639	44.84**
a_W × I	2	218344.82	9.88**	0.0321	22.52**
a_W × EO	2	26558.62	1.20	0.0156	10.94**
a_W × C	4	127151.19	5.75**	0.0223	15.64**
I × EO	1	82914.72	3.75	0.0028	1.97
I × C	2	59417.30	2.69	0.0093	6.49*
EO × C	2	1622604.04	73.40**	0.0524	36.74**
a_W × I × EO	2	52413.89	2.37	0.0061	4.31*
a_W × I × C	4	149901.11	6.78**	0.0113	7.95**
a_W × EO × C	4	113231.98	5.12**	0.0053	3.70*
I × EO × C	2	68602.38	3.10*	0.0011	0.80
a_W × I × EO × C	4	168628.21	7.63**	0.0056	3.91*
Error	108	22105.48		0.001	

DF: degrees of freedom; MS: mean of squares.
[a] Snedecor's *F* test.
* Significant at $p < 0.05$.
** Highly significant at $p < 0.001$.

3.2. Essential oil analyses

3.2.1. Chemical composition of the essential oils

The main components of boldo and poleo EOs were identified by GC–MS spectrometer analyses. Tables 3 and 4 show the composition percentages of the main components of each EO studied. α-Terpinene (30.61%) was the principal component of boldo, while σ-cymene and eucalyptol represented 28.49 and 12.85%, respectively in this oil. Limonene (48.83%), β-caryophyllene epoxide (18.06%) and piperitenone (7.67%) were the main components of poleo oil.

3.2.2. Results of the analytical method

The mean area percentages for the main compounds of boldo oil extracted at different temperatures and fiber exposition times from peanut matrix are shown in Table 5. These results showed that the best experimental conditions for the extraction process were at 50 °C with the fiber exposed for 30 min.

Piperitone oxide, α-terpinene and eucalyptol were present in 28.09, 18.84 and 12.34%, respectively in peanut exposed to vapors released by boldo oil (Table 3). These results showed that the major components in boldo are not the same as in peanut samples; however eucalyptol was found in the same proportions in both matrices (12.5%) and also it is not present in the peanut samples treated with poleo EO. Therefore,

Table 2
Effect of EO volatile fractions on the lag phase of *Aspergillus* section *Flavi* strains at different a_W.

Strains/doses (μL/g)	a_W	Lag phase (days) ± SD				
		C	Boldo		Poleo	
		0	2	3	3	5
RCP08108	0.98	..1.8 ± 0.4[a]	4.6 + 0.9[c]	–[d]	3.5 + 0.3[b]	4.8 + 0.5[c]
	0.95	..2.1 ± 0.8[a]	–[d]	–[d]	4.2 + 0.4[b]	5.8 + 0.7[c]
	0.93	..6.1 ± 0.3[a]	–[c]	–[c]	5.7 + 0.6[a]	7.0 + 0.8[b]
RCP08299	0.98	..2.5 ± 0.3[a]	>[b]	–[b]	3.8 + 0.8[a]	6.2 + 3.2[a]
	0.95	..2.7 ± 0.0[a]	19.6 + 17.7[b]	–[b]	3.4 + 0.2[a]	4.4 + 0.5[a]
	0.93	17.3 ± 14.4[a]	–[a]	–[a]	19.6 + 17.8[a]	21.0 + 16.2[a]

Key: ≥30 days. (–) Under these conditions the strains were not able to give visible mycelium. Mean values based on quadruplicated data ± standard deviation. *A. flavus* RCP08108, *A. parasiticus* RCP08299.
Data with the same letter for each a_W are not significantly different according to Duncan's New Multiple Range Test ($p < 0.05$).

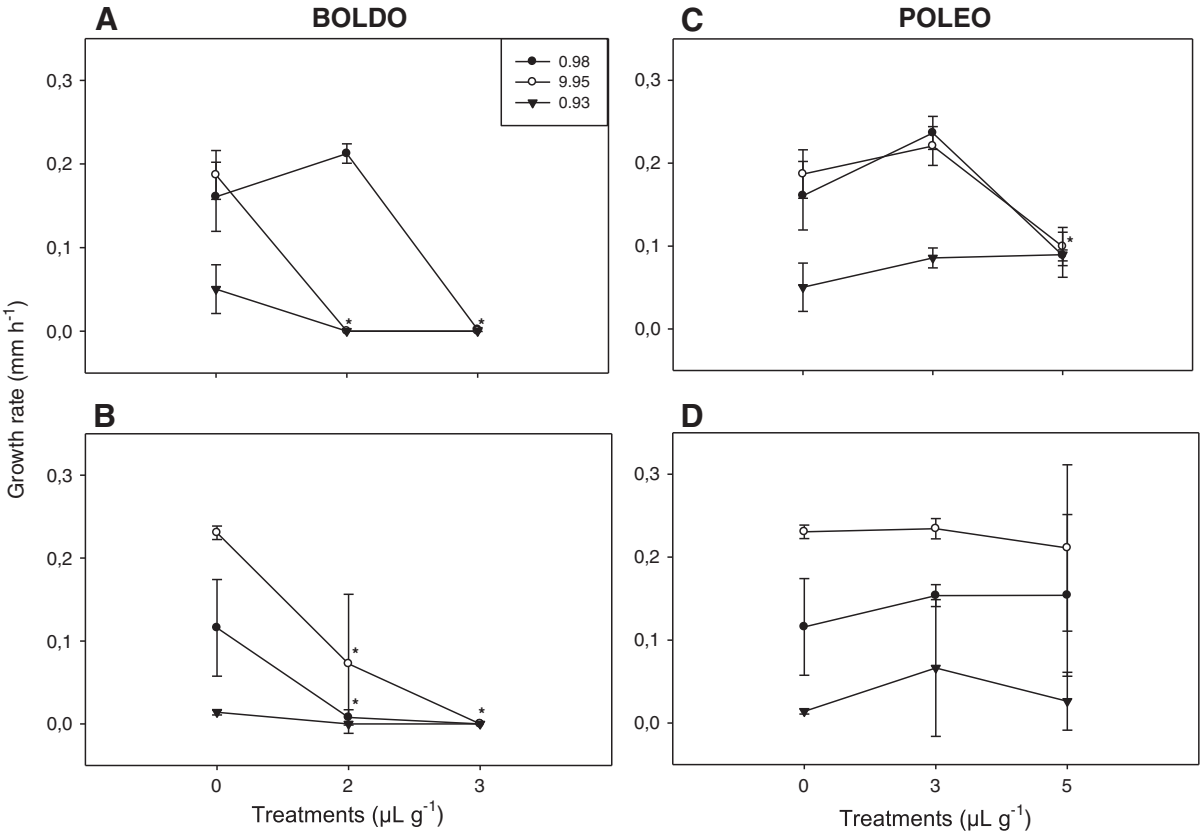

Fig. 1. Effect of EO volatile fractions on growth rate of (A, C) *A. flavus* (RCP08108) and (B, D) *A. parasiticus* (RCP08299) at different a_W levels on peanut grains. Mean values based on quadruplicate data with (*) for each a_W are significantly different to the control samples according to the Duncan New Multiple Range Test ($p \leq 0.001$).

this compound (eucalyptol) was used for the quantification of boldo oil in peanut samples.

Residue levels of peanut exposed to saturated atmosphere of volatile fraction of poleo oil mainly comprised β-caryophyllene epoxide (36.48%), limonene (21.83%) and piperitenone (16.79%) (Table 4). The main components in peanut samples treated with poleo oil were the same as in pure EO. Although the main compound was β-caryophyllene epoxide, limonene was used for the quantification of poleo EO as the first (β-caryophyllene epoxide) was found in peanut samples treated with boldo oil.

The parameters of the linear regression for eucalyptol and limonene over the whole concentration range for the EOs studied are shown in Table 6.

3.2.3. Residue levels of boldo and poleo oils in peanut samples

The effect of water activity (a_W), *Aspergillus* section *Flavi* isolates (I), incubation time (T), essential oil type (EO), oil concentrations (C) and their two-, three-, four- and five-way interactions on the residue levels of boldo and poleo oils was examined at a temperature of 25 °C. ANOVA results showed that oil concentrations in irradiated peanut grains were highly dependent on oil type followed by incubation time and oil doses applied (Table 7).

Comparing both EO analyzed, it is notable that boldo oil residues were about 7.4 and 5.5 times greater than poleo EO levels in peanut grains inoculated with *A. flavus* and *A. parasiticus*, respectively, regardless of a_W levels and incubation time (Fig. 2). These data also show that, with some exceptions (boldo 2 and 3 µL/g; 11 days; 0.93 a_W), the residues

Table 3
Relative percentage of main components of boldo oil and boldo treated peanut sample.

No.	RT[a]	Relative percentage (%)		Compounds
		Boldo oil	Peanut + boldo oil	
1	10.701	1.86	–	α-Pinene oxide
2	12.137	1.81	–	γ-Terpinene
3	12.352	0.52	–	β-Pinene
4	14.132	28.49	7.69	ρ-Cymene
5	14.262	3.62	–	(±)-Limonene
6	**14.418**	**12.85**	**12.34**	**Eucalyptol**
7	19.570	1.41	1.41	*trans*-Sabinene hydrate
8	21.685	30.61	18.84	α-Terpinene
9	23.646	10.21	28.09	Piperitone oxide
10	26.122	1.78	5.73	1,3,4-Eugenol methyl ether
11	31.419	2.64	8.57	(−)-β-Caryophyllene epoxide

[a] RT: Retention time.

Table 4
Relative percentage of main components of poleo oil and poleo treated peanut sample.

No.	RT[a]	Relative percentage (%)		Compounds
		Poleo oil	Peanut + poleo oil	
1	**14.392**	**48.83**	**21.83**	**(±)-Limonene**
2	20.735	2.83	–	Anisole, o-isopropenyl-
3	21.880	1.98	1.14	Piperitone oxide
4	22.906	7.31	–	Bornyl acetate
5	24.647	7.67	16.79	Piperitenone
6	25.197	1.05	–	Piperitenone oxide
7	26.142	1.79	–	1,3,4-Eugenol methyl ether
8	27.033	3.47	7.98	β-Caryophyllene
9	28.013	1.02	2.59	α-Caryophyllene
10	30.029	0.90	–	α-Bisabolene
11	31.489	18.06	36.48	(−)-β-Caryophyllene epoxide

[a] RT: Retention time.

Table 5
Area percentages of the main compounds from boldo oil treated peanut grains according to temperature variations and fiber exposition times.

Compounds	Retention time (min)	Relative area percentages (%) ($n = 3$)						
		Boldo oil	Peanut + boldo oil					
			30 min			50 °C		
			25 °C	40 °C	50 °C	10 min	20 min	30 min
ρ-Cymene	14.132	28.49	1.94	2.50	7.69	6.60	2.17	7.69
Eucalyptol	14.418	12.85	6.67	11.50	12.34	43.57	30.80	12.34
α-Terpinene	21.685	30.61	18.21	18.75	18.84	7.08	13.76	18.84
Piperitone oxide	23.646	10.21	26.80	27.09	28.09	0.74	11.99	28.09
(−)-β-Caryophyllene epoxide	31.419	2.64	12.04	n.d.	8.57	1.00	0.72	8.57

of boldo and poleo oils in peanut grains inoculated with *A. parasiticus* were on average 30.6 and 43.8% greater than those in samples inoculated with *A. flavus*.

When the effect of incubation time was analyzed, boldo oil residues in peanut inoculated with *A. flavus* and *A. parasiticus* were significantly decreased from 33.2 to 100% and from 24.7 to 62.1%, respectively at 35 days. Meanwhile poleo oil levels in the peanut matrix were reduced in the order of 26.6 to 99.7% at the end of the incubation period.

At 11 days of incubation both EO residues were higher (35.5%) in those peanut samples treated with the major oil dose, whereas at 35 days all oil concentrations were reduced to a similar level, regardless of substrate a_W conditions and fungal inoculum applied.

When the effects of peanut water availability on oil residues were analyzed, boldo and poleo oil concentrations were 38.4 and 33.3% higher at the lower a_W level tested (0.93 a_W) than at 0.98 a_W, regardless of incubation time and fungal specie.

4. Discussion

The results from our studies on *Aspergillus* section *Flavi* strains have shown that compounds released by boldo (2 and 3 μL/g) and poleo (5 μL/g) EOs were able to inhibit fungal growth on sterilized peanut grains, after adding them using the disk volatilization method and consequently generating a saturated atmosphere. Although, recently it has been found that a variety of EOs and plant extracts have the ability to inhibit fungal development of *Aspergillus* section *Flavi* (Bluma et al., 2009; dos Santos et al., 2012; Prakash et al., 2013; Tian et al., 2012; Vilela et al., 2009), very few authors have demonstrated their effectiveness *in situ*. Bluma et al. (2009) studied the antifungal activity of volatile fractions of boldo and poleo EOs in sterilized maize grains on *A. flavus* and *A. parasiticus* strains. Boldo oil (2.5 μL/g) showed the highest antifungal effect (38–54% of growth reduction/0.98 a_W) which was enhanced as substrate a_W decreased. The results of an *in situ* assay conducted by Prakash et al. (2013) on food system in storage containers for 12 months showed significant efficacy of the *Cinnamomum glaucescens* EO as disinfectant against *A. flavus* contamination, revealing 71.07% protection of chick pea seed samples fumigated with this oil.

In our study the main chemical components of EOs were α-terpinene and ρ-cymene for boldo and limonene and β-caryophyllene epoxide for poleo, most of the chemical components are terpenoids, including monoterpenes, sesquiterpenes, and their oxygenated derivatives. Terpenes are the active antimicrobial compounds of EOs. The marked action of oil components might be related to the low molecular weight and highly lipophilic nature, enabling easy passage through cell membranes to induce biological responses in *A. flavus* (Nogueira et al., 2010). Although the action mechanisms of this class of compounds are not fully understood, recently Tian et al. (2012) concluded that the plasma membrane and the organelles may be the important targets of the EO main components and that the main compounds of *Cinnamomum jensenianum* (eucalyptol and α-terpineol) may affect the above targets inducing considerable impairment of ergosterol biosynthesis by *A. flavus*, leading to an imbalance in the intracellular osmotic pressure, blockage of enzymatic reactions, leakage of cytoplasmic contents, and so on, eventually resulting in the death of the cell. Tyagi and Malik (2011) reported that higher percentage of monoterpene hydrocarbons (54.7%) present in the vapors than the oil (44.5%) could be responsible for the higher antifungal activity. However, this study showed that while α-terpinene and limonene (terpenes) were the main components of EOs, the antifungal effect could not be attributed only to the latter. It has been shown that a possible synergistic effect of minor components may represent an important part of the antimicrobial activity of EOs such

Table 6
Parameters of the linear regression.

Compounds	Parameters of the linear regression			Range of linear response (μg/g)
	Intercept (AU s)	Slope (AU s μg μL^{-1})	Correlation coefficient (R^2)	
Eucalyptol	1.00×10^6	5.00×10^7	0.984	3.03×10^{-3}–1.21
Limonene	5.06×10^5	3.00×10^7	0.9626	8.23×10^{-3}–1.09

Table 7
ANOVA Test. Significance of grain water condition (a_W), *Aspergillus* section *Flavi* isolates (I), incubation time (T), essential oil type (EO) and their concentrations (C) on peanut oil residua.

Source of variation	DF	MS	*F* value[a]
a_W	2	0.9679	17.45**
I	1	2.2540	40.65**
T	1	3.1036	55.97**
EO	1	10.7765	194.35**
C	1	2.2792	41.10**
$a_W \times I$	2	1.1280	20.34**
$a_W \times T$	2	0.0970	1.75
$a_W \times EO$	2	0.5336	9.62**
$a_W \times C$	2	0.1736	3.13*
$I \times T$	1	0.0158	0.29
$I \times EO$	1	0.8573	15.46**
$I \times C$	1	0.0065	0.12
$T \times EO$	1	0.9886	17.83**
$T \times C$	1	0.6394	11.53
$EO \times C$	1	1.5969	28.80**
$a_W \times I \times T$	2	0.3139	5.66*
$a_W \times I \times EO$	2	1.0042	18.11**
$a_W \times I \times C$	2	0.2316	4.18*
$a_W \times T \times EO$	2	0.0329	0.59
$a_W \times T \times C$	2	0.0564	1.02
$a_W \times EO \times C$	2	0.1891	3.41*
$I \times T \times EO$	1	0.0178	0.32
$I \times EO \times C$	1	0.0215	0.39
$T \times EO \times C$	1	0.1118	2.02
$a_W \times I \times T \times EO$	2	0.2807	5.06*
$a_W \times I \times T \times C$	3	0.1285	2.32
$a_W \times I \times EO \times C$	2	0.2667	4.81*
$I \times T \times EO \times C$	1	0.0277	0.50
$a_W \times I \times T \times EO \times C$	4	0.0946	1.71
Error	144	0.0554	

DF: degrees of freedom; MS: mean of squares.
[a] Snedecor's *F* test.
* Significant at $p < 0.05$.
** Highly significant at $p < 0.001$.

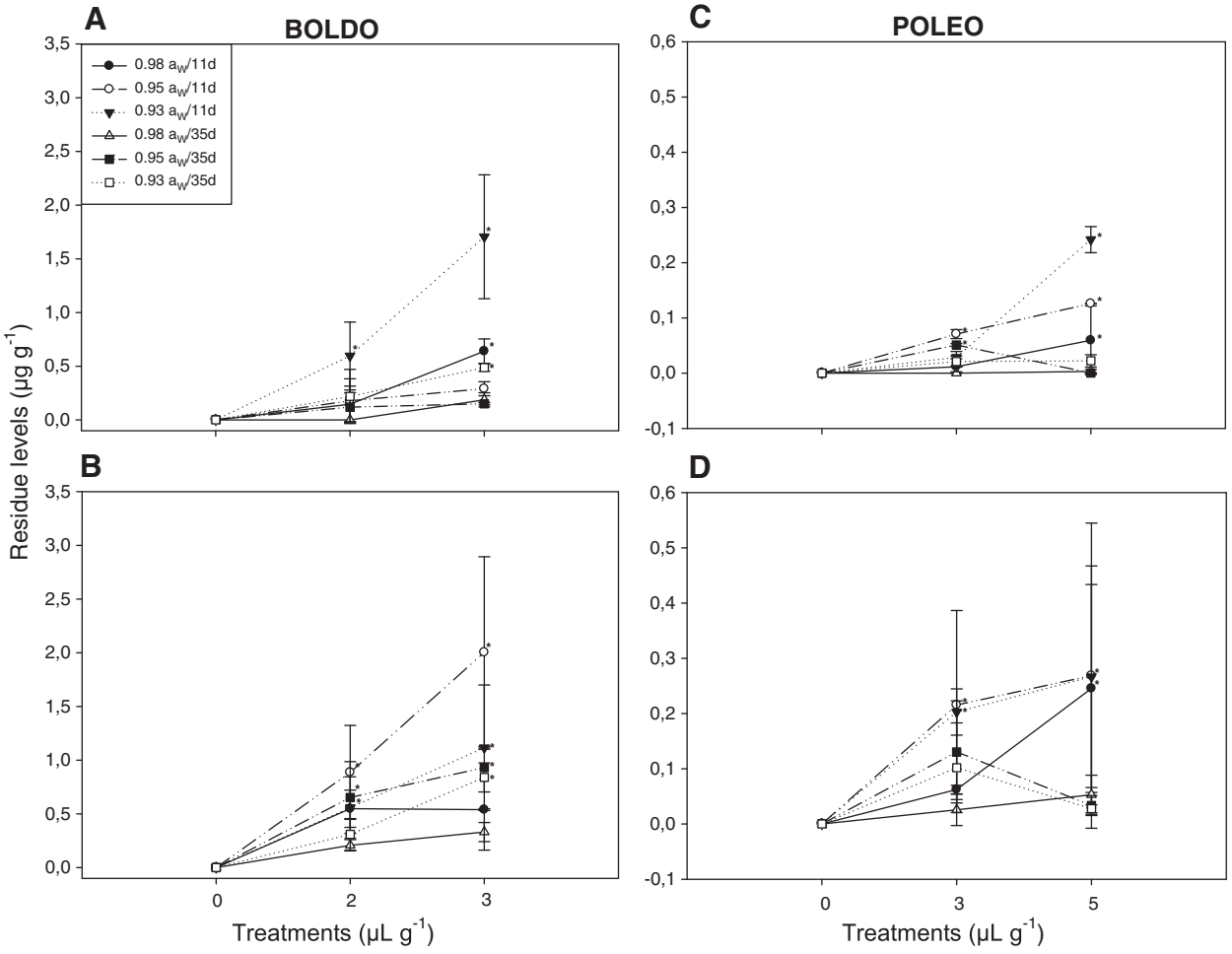

Fig. 2. Mean residue levels ± standard deviation of EOs in peanut grains conditioned at different a_W values, inoculated with (A, C) *A. flavus* (RCP08108) and (B, D) *A. parasiticus* (RCP08299) and incubated during 11 and 35 days. Data with (*) are significantly different from the values corresponding to the untreated peanut samples according to the Bonferroni Test ($p \leq 0.001$).

as piperitone oxide (ketone) and β-caryophyllene epoxide (ethers) that were the most abundant compounds in peanut treated with boldo and poleo, respectively. Eucalyptol was the major component (86.0%) in *Eucalyptus globulus* EO, but Vilela et al. (2009) reported that it demonstrated very poor fungicidal activity against *A. flavus* and *A. parasiticus* in both contact and headspace volatile exposure assays. In a recent study conducted in a real food system (table grapes), *Cinnamomum cassia* EO exhibited stronger antifungal activity than the main component (cinnamaldehyde) (Kocevski et al., 2013).

It is well known that EOs are able to quickly volatilize at ambient temperature. This characteristic gives EOs a great advantage from the point of view of application practicality and homogeneity, as it ensures that all food is protected from spoilage fungi with reduced or no organoleptic changes. Recently, dos Santos et al. (2012) reported that the application of a coating composed of chitosan and *Origanum vulgare* L. at sub-inhibitory concentrations preserved the quality of grapes as measured by their physical and physicochemical attributes. Therefore, as it is possible to apply boldo and poleo EOs as food flavorings without limitation (Commission Regulation (EU), 2011), it was considered important to determine the residues of these oils in peanut matrix. These important data allow estimation of the period of bioactivity of the oil, avoiding undesired effects, particularly accumulation of aflatoxins that may be caused by sublethal doses (Passone et al., 2013).

Consequently, this is the first study that evaluates the residue levels of the EO volatile fractions in peanut grains conditioned at different a_W, inoculated with strains of *Aspergillus* section *Flavi* and incubated for 11 and 35 days. Eucalyptol and limonene were the compounds used as markers to estimate the boldo and poleo oil concentrations in the samples. Although in our study boldo was applied at doses lower than those of poleo, the residues of the first oil were 83.6 and 90.4% higher at 11 and 35 days of incubation, respectively. On the one hand, this result could be due to the higher content of terpenoid compounds present in boldo oil (69.8 for boldo and 59.0% for poleo). On the other hand, considering that the antifungal effect of poleo EO was from zero to moderate when it was applied at the doses of 3 and 5 µL/g, respectively, it is probable that fungi were able to metabolize some of the volatile components of this oil; taking into account that the assay was performed under saturated atmosphere conditions and that the plates were sealed to avoid loss of volatile components. Similarly, Magan et al. (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. Moreover, considering the a_W effects, boldo and poleo oil residues were 35.8% higher at the lowest grain water content (0.93). This behavior could be also caused as a result of fungal development, since the highest growth rates for both *Aspergillus* strains were observed at 0.98 a_W. When the effect of the oil concentration was analyzed, the greater levels of boldo and poleo EO residues were obtained in the samples with the higher doses (3 and 5 µL/g, respectively) but only at 11 days of incubation. The other factor considered in this study was the influence of the time on oil residues. Considering the initial EO applications: 2 and 3 µL/g of boldo and 3 and 5 µL/g of poleo, 25.3 and 36.5% of boldo and 3.5 and 4.3% of poleo were recovered from peanut at 11 days of incubation, while at the second incubation period (35 days) the levels were reduced to a mean of 15.4 and 1.1%, respectively. In a previous study the residue levels of chemical antioxidants in peanut stored during 5 months were

analyzed periodically (Passone et al., 2008). Although 1802 μg/g of butylated hydroxyanisole and propyl paraben and 2204 μg/g of butylated hydroxytoluene were sprayed on in-pod peanuts, the residual levels of these chemicals in seeds were reduced from 5.84 to 0.32 μg/g at the end of the storage period.

In conclusion, this study demonstrated that the volatile components of boldo EO showed marked antifungal effect by completely inhibiting the growth of *A. flavus* and *A. parasiticus* strains in peanut grains and that these compounds were not significantly present in the substrate at 35 days. These data allow determination of the application intervals of boldo oil in the peanut storage system, considering: (i) the storage period of peanut; (ii) the grain a_W; (iii) the oil composition and (iv) the fungal growth rate. Therefore, the application of nontoxic boldo oil as fumigant in the control of *Aspergillus* section *Flavi* may be useful in reducing fungicide use. In addition, the antioxidant effect of polyphenols and terpenes present in this EO all help to conserve physical and chemical characteristics of stored grains.

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