Postharvest applications of clove essential oils on dry seeds stored under simulated warehouse conditions

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ABSTRACT

Clove essential oil was applied in postharvest trials on peanuts, beans, apricot kernels, and lentils stored in jute bags, and on wheat, maize, rice, and rape kept in silos, at ambient temperature (20°C). Eugenol, accounting for 85.6% of clove essential oil, has been used to assess clove essential oil residues after treatments in dry seeds. Two trials at different concentration and storage time were carried out. Immediately after treatment eugenol residues were under the instrumental limit of detection (LOD, 0.5 µg/Kg), except for wheat, peanuts, and rice (0.03, 0.10, 0.24, 0.23 mg/kg, respectively), and increased till a maximum after 2 weeks, except peanuts and apricot kernel which peaked after 30 days (12.69, and 0.47 mg/Kg, respectively). Sensory analysis showed that ventilation of the seeds allowed to decrease eugenol residue values under the flavor perception capacity of the in house panel (0.10 mg/kg), thus not affecting the organoleptic characteristics of the seeds.

Introduction

Plant extracts are used for a wide variety of purpose all over the word. Among the different uses their antimicrobial activity has led to pharmaceutical, natural therapies and food preservation products, including new and safer pesticide formulations to control postharvest diseases of horticultural crops (1–5). Essential oils are plant extracts constituted by volatile compounds, which have recently attracted a great deal of attention for their antimicrobial activity against a broad range of bacteria and fungi (6–11), and have been utilized in the food industry (12, 13). The application of essential oils and their constituents to extend storage and shelf life of foods, by limiting growth or survival of microorganisms, is an interesting challenge to decrease the use of synthetic pesticides. However, the lack of knowledge about the fate and behavior of the individual essential oils and their constituents represent a clear obstacle to their employment on a commercial scale. Moreover, the intense aroma of essential oils, even at low concentrations, can cause negative organoleptic effects exceeding the threshold acceptance of consumers (14).

Clove (*Eugenia caryophyllata*) which is represented by the immature un-opened flower buds of the clove tree, has been widely used as a spice, and in popular medicine for various therapeutic applications (15), as remedy for indigestion, atherosclerosis (16), diarrhea (17), ringworm (18), athlete's foot (19) and other fungal infections, especially in Asian countries. Its main constituent is represented by eugenol (4-Allyl-2-methoxyphenol) accounting from 60% to 90% (20, 21).

In recent years clove essential oil has been included in Annex I Directive 2009/82/EC (22), and has been registered in EU for postharvest indoor applications on apples, pears and peaches by drenching with clove oil solution at 450 g/hL (European Food Safety Authority 2012) (23), Various studies have documented the potential of clove oil as a natural antimicrobial agent for postharvest management to control decay and prolong storage and shelf life of horticultural crops (24–29).

Dry seeds do not require special storage conditions and treatments like other horticultural crops, but some parameters, as storage temperature, relative humidity, and duration, seed moisture content, and fungal contamination levels, should be monitored (30).

Cereal panicles (the grain bearing heads) are infected and damaged by fild fungal pathogens during crop growth and maturation, and harvested grain is constantly at risk of infection from post-harvest pathogens during the entire grain processing and storage period. During storage the moisture inside the grain kernels, establishes equilibrium with the air outside to generate a relative humidity level which may promote the growth and metabolic activity of deteriorating organisms, including fungi molds (30).

In this study, we developed and validate a novel liq- uid/solid extraction and gas-chromatography ion trap mass-spectrometry (GC/ITMS) method for the determina- tion of clove essential oil residues on dry seeds. The residue levels and decline curves of eugenol were investigated on peanuts, beans, apricot kernels, and lentils stored in jute bags, and on wheat, maize, rice, and rape kept in silos, at ambient temperature (20°C). Moreover sensory analysis carried out by an in-house panel, evaluated the organoleptic acceptance of treated samples and eugenol threshold limit.

Experimental

Plant material and storage conditions

Clove essential oil was obtained by steam distillation of clove dried fl wer buds, for 1 hour using a semi-industrial stainless steel apparatus (SISS) of 80 liter of capacity. The essential oils were recovered directly using a micropipette from above the distillate without adding any solvent. The essential oils were stored with anhydrous sodium sulfate in dark vials at 4°C. Solutions of 1% (v/v) oil were prepared in n-hexane before GC\MS analysis.

Trials were carried out on eight dry seed species: maize (*Zea Mais*), wheat (*Triticum durum*), lentils (*Lens culi- naris*), peanuts (*Arachis hypogaea*), beans (*Phaseolus vul- garis*), apricot kernels (*Prunus dulcis*), rice (*Oryza sativa*) and rape (*Brassica napus*). Seeds of dry peanuts, beans, apricot kernels, and lentils were stored in a temperature monitored area, in 10 Kg jute bags, whereas the seeds of dry wheat, maize, rice and rape were kept in a 4.68 m^3 silos. Seeds were stored at ambient temperature (20°C) to simulate commercial storage conditions.

Evaluation of the decrease of clove essential oil concentration after applications

Pieces of bibulous paper (5.0 × 5.0 cm × 3 mm) were soaked with aliquots of pure clove essential oil, to reach a concen- tration of 10 μg/cm^2, and 5 μg/cm^2 and placed in different part of empty silos. Ten paper samples were collected after 24 hours of conditioning, and every day for 2 weeks to deter- mine the decline curve. After sampling the singles bibulous papers were placed in 10 mL headspace vials, which were immediately clamped and stored at 5°C before analysis.

Eugenol residues and persistence on stored dry seeds

Trial I. Jute bags and silos surface were sprayed with aliquots of clove essential oil to reach concentrations of 30 μg/cm^2, than filled with the dry seeds, and stored for 30 days. Jute bags were filled with beans, lentils, peanuts, and apricot kernels, whereas silos were filled with maize, rice, wheat, and rape. Eugenol residue analyses were performed 3 hours after spray (day 0), and after selected periods dur- ing storage (Tables 3 and 4). Last samples collected were used for residue and sensory analysis. Sensory analysis was performed immediately after collection and after 1 hour of ventilation away from direct sunlight exposure. The in-house sensory panel consists of five members, and the rating scale included a 4-point linear scale, ranging 0 to 3 (0 absent, 1 light, 2 medium, 3 high).

Trial II

Based on the results of Trial I, the seeds showing the higher residues after the 30 days of storage, were subjected to a second experiment with concentration of clove essential oil reduced by a half (15 μg/cm^2) and storage period extended to 60 days. Eugenol analyses were performed 3 hours after spray (day 0), and after selected periods during storage (Table 5). All trials were performed in triplicate, and samplings were carried out according to the EC 152/2009 (31). After collection, samples were immediately placed in sealed
bags and stored at 5°C before analysis.

Chemicals

Eugenol, and methyl eugenol (internal standard, i.s.) were analytical standards (Fluka, Milan, Italy) at 95% certified purity. Hexane of residue analysis grade, and anhydrous Na_2SO_4 were purchased from Carlo Erba (Milan, Italy). Stock standard solutions of eugenol, and methyl eugenol were prepared dissolving 10 mg of the analytical standard in 10 mL of hexane and thereafter stored in a glass screw capped flask at –20°C. Working standard solutions of eugenol, and methyl eugenol were obtained by appropriate dilutions of the stock solutions with hexane and were used as spiking solutions as well. Matrix matched standards were prepared at the same concentrations as that of the calibration solutions by adding the appropriate amounts of standards to control matrix extracts.

Sample extraction

About 10 g of homogenized seed samples were weighed in a 40 mL screw capped tube with 10 mL of hexane and 5 g of anhydrous Na_2SO_4. The tube was agitated for 15 minutes in a rotary shaker, and the organic phase was transferred in a vial and analyzed in GC-ITMS without any cleanup step. In the case of muddy mixture the vials were subjected to centrifugation at 13,000 RPM for 5 min- utes before injection.

Bibulous paper samples were extracted with 10 mL of hexane directly added in the headspace vial with a syringe, introducing the syringe needle through the cap septa, without opening the vial. The vial was agitated for 5 minutes in vortex, and the solution was immediately injected into the GC-ITMS for the analysis.

Recovery assay

Untreated samples of maize, rice, wheat, lentils, pea- nuts, beans, apricot kernel, and rape, were spiked prior to extraction by adding an appropriate volume of stock standard solution of eugenol to reach concentrations of 0.012 mg/Kg and 20.00 mg/Kg of eugenol, and processed according to the above described procedure. The recovery assays were replicated four times.

GC/ITMS analysis

A Varian CP 3800 (Varian, Inc., Palo Alto, CA, USA) Gas-chromatograph, couplet with a Varian CP 7800 autosampler, and a 2000 ITMS mass spectrometer, was used. The capillary column was a DB-5MS (5% phen- ylmethylpolysyloxane, 30 m × 0.25 mm; film thickness 0.25 μm) (J&W Scientifi Fisons, Folsom, CA). The injector and the MS interface were set at 100°C and 180°C, respectively. The oven was programmed as follows: 60°C (1 min- ute) till 150°C (3°C/min). Helium was the carrier gas at 1 mL/min; the samples (1 μL) were injected in split mode (1:20). For clove essential oil analysis MS conditions were as follows: ionization mode EI from 50–450 amu. The oil components were identified by comparison of their relative retention times with those of standard references, and by computer matching against commercial library (32, 33) and homemade library mass spectra made up from pure substances and component of known oils. Quantitative analysis of each oils component (expressed as percentages) was carried out by peak area normalization measurement. Quantitation of eugenol was obtained with the internal standard method (methyl eugenol). MS conditions were: electron ionization mode, in selected ion storing (EI – SIS), m/z 164 a, and 178 a for eugenol, and methyleugenol, respectively (Figure 2).

Method validation

The experimental method was validated calculating the relative standard deviation (RSD%) of repeatability and intermediate precision, recovery, and linearity. Repeata- bility (r) involved the repeated analysis of six samples each day, while intermediate precision (IP) was calculated by the analysis of six samples/day in six different days.

Each sample belongs from an independent experiment. The matrix effect was assessed by comparing the analytical responses of eugenol standards dissolved in hexane with those prepared with control matrix extracts. The method limits of quantitation (LOQ) and determination (LOD) were calculated as ten-times, and three-times the signal- to-noise ratio.

Resultsanddiscussion

Clove essential oil composition

The GC_MS analysis of clove essential oil has allowed the identification of twelve compounds of which six accounted for 99.8%, namely: 2-heptanone, eugenol, β-caryophyllene, α-humulene, eugenyl acetate, and caryophyllene-oxide. The concentration of eugenol in our samples was of 85.6%, followed by β-caryophyllene (6,57%), eugenyl acetate (4.69%), α-humulene (1.88%), caryophyllene-oxide (0.94%), and 2-heptanone (0.13%), while only traces of α-pinene, 1–8 cineolo, β-ocimene, linalool, methyl eugenol, and α-copaene (< 0.02%) could be detected (Figure 1).

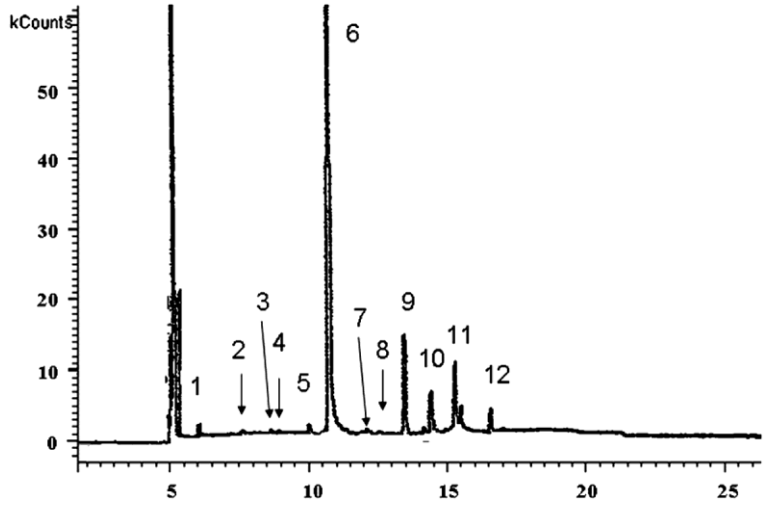

Figure 1. total ion current Gc-itMs chromatogram of clove essential oil. notes: (1) 2-hetanone, (2) α-pinene, (3) 1-8 cineolo, (4) β-ocimene, (5) linalolo, (6) Eugenol, (7) methyl-eugenol, (8) α-copaene (9) β-caryophyllene, (10) α-humulene, (11) eugenol acetate, (12) caryophillene-oxide.

Table 1. Eugenol recovery from the selected dry seed samples after application of clove oil at 0.01 or 20.00 mg/Kg active ingre- dient (a.i.), instrumental limits of quantification (loQ), and deter- mination (loD), and correlation coefficient (r^2 ± rsD%).

	Eugenol concentration (mg Kg^{-1})	
	0.01	20.00
Seed samples	Recovery (%)[a]	
Peanut	91.9 ± 6.2	92.5 ± 3.9
Bean	81.1 ± 5.5	82.5 ± 7.2
lentil	76.8 ± 5.2	83.5 ± 6.1
Maize	90.6 ± 5.4	85.7 ± 4.8
apricot kernel	84.7 ± 2.8	92.3 ± 6.6
Wheat	79.3 ± 3.9	81.8 ± 6.5
rice	81.7 ± 5.6	84.4 ± 4.5
rape	78.2 ± 3.4	82.7 ± 7.1
loD (mg/Kg)	0.0005	
loQ (mg/Kg)	0.012	
r^2	minimum 0.9937	max 1.000
rsD%	24	

Table 2. repeatability (rsD%) and intermediate precision (rsD%) of eugenol instrumental response at two different spiking levels of control seed samples.

	Clove oil concentration (mg/Kg a.i.)			
	0.01	20.00	0.01	20.00
	Repeatability[a]		Intermediate precision[b]	
Seed samples	RSD%		RSD%	
Peanut	7.2	9.9	7.5	11.8
Bean	6.7	5.6	8.1	5.0
lentil	5.9	7.4	9.2	8.1
Maize	8.3	6.3	11.1	9.3
apricot kernel	5.7	9.1	7.2	7.1
Wheat	6.4	7.6	9.3	8.2
rice	7.6	5.7	10.5	9.9
rape	8.7	6.6	7.4	10.8

notes: [a]n = 6; [b]n = 36.

notes: [a]Mean values ± standard deviation (n = 4).

Analytical method

The method developed in this study allowed good recov- eries ranging between 76.8 (lentil at 0.01 mg/Kg) and 92.5% (peanut at 20.00 mg/Kg), with RSD_{max} of 7.2% (bean), demonstrating good accuracy, and according to EC SANCO/10684/2009 guidelines values (Table 1) (34). Eight-point standard calibration curves ranging between 0.01 mg/L and 36.50 mg/L showed a good coefficient of determination (r^2) ranging between 0.9937 and 1.000, with RSD% max of 24% (Table 1). No interfering peaks were observedinthechromatographicrangeofinterest(Figure2), and no cleanup step was necessary. The LOD and LOQ were adequate for our GC-ITMS analysis (Table 1).

Instrumental repeatability and intermediate pre- cision, calculated as reported above, showed good results for all tests with RSD% ≤ 11.8 according to EC SANCO/10684/2009 (Table 2) (34).

The quantitative analysis was carried out in EI-SIS, allowing the exclusion of the possible matrix effect on the detection and quantification of the active ingredient.

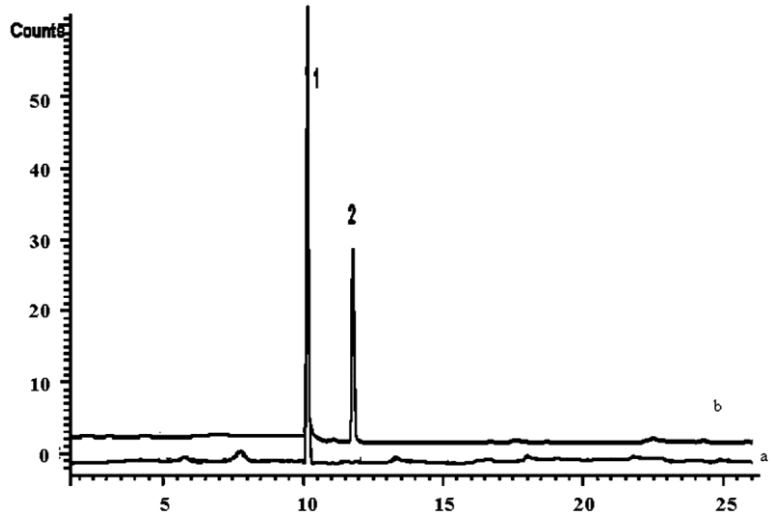

Figure 2. Gc-itMs chromatogram of rape extract for the determination of clove essential oil in selected ion storing mode (sis). Eugenol (m/z 164) and methyl-eugenol (m/z 178). notes: (1) methyl eugenol (s.i.); (2) eugenol. (a) rape extract chromatogram after 30 days + 1 hour aeration, and (b) rape extract chromatogram after 30 days, after clove oil spray treatment at 30 μg/cm^2.

Decrease of clove essential oil in bibulous paper after application in the Silos

Eugenol residues in the bibulous paper averaged 2.93 ± 0.16, and 5.30 ± 0.12 μg cm^2 after 24 hours of clove oil treatment at 5 μg/cm^2 or 10 μg/cm^2, respectively. Residues decreased in both cases, following a first-order kinetic (r^2 about 0.940) in the first week with half life time (t ½) of about 10 days, and remaining almost stable during the sec- ond week, with overlapping values in the two experiments (Figure 3).

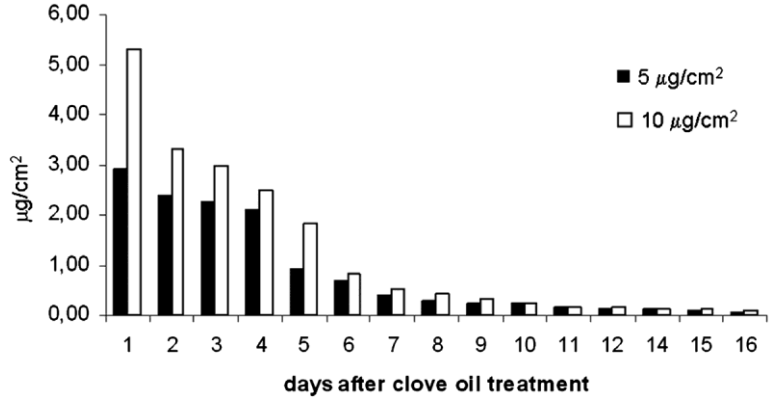

Figure 3. Decrease of eugenol residues (μg/cm^2) in bibolous paper layers treated with clove essential oil at 5 μg/cm^2 or 10 μg/cm^2 and placed on the silos internal surface.

Eugenol residues and persistence on stored dry seeds

Trial I. At time 0 (T = 0), 3 hours after spraying the stor- age devices, eugenol residues were under the instrumental LOQ for beans, lentils, maize, and apricot kernels. Wheat, and peanuts showed a residue of 0.03, and 0.10 mg/Kg, respectively, while rice and rapes showed simi- lar values around 0.23 mg/Kg (Tables 3 and 4). Eugenol residues increased in all commodities in the first 2 weeks of storage, with the highest values detected in rice (17.05 ± 2.10 mg/Kg), and wheat samples (14.49 ± 1.56 mg/Kg) (Table 4). Peanuts and apricot kernel residues peaked after 30 days with values of 12.69 ± 3.79 and 0.47 ± 0.16 mg/Kg (Table 3), respectively. Beans samples showed the lowest values during storage, with eugenol residues never exceed- ing 0.09 mg/Kg, while lentil showed an increase during the first 15 days till 0.59 ± 0.08 mg/Kg, and decreasing very slow after 30 days to 0.30 ± 0.02 mg/Kg (Table 3). Peanuts, wheat, and rice showed the higher residues after the 30-day experiment.

Trial II

In order to better evaluate eugenol behavior in peanuts, wheat, and rice seeds, a second experiment was carried out using half concentration of clove essential oil solu- tion (15 μg/cm^2) for a longer period of storage (60 days). The distribution behavior of eugenol residues followed a similar trend to Trial I. At T = 0 the residue levels were near the LOQ for all species, than increased in the first 15 days but with values lower than in the Trial I. Peanuts and wheat peaked after 30 days (0.26 ± 0.03, and 0.43 ± 0.06 mg/Kg, respectively), while rice reached the highest concentration of eugenol after 15 days (10.10 ± 0.15 mg/ Kg). In the second month of storage rice showed stable levels of about 6.5 mg/Kg, while wheat decreased slowly till 0.20 ± 0.02 mg/Kg, and peanut showed a drop of the values till 0.08 ± 0.02 mg/Kg (Table 5) response to clove essential oil treatments (25, 27, 29). The reported method allowed reliable analysis of eugenol residues in dry seed storage. The analysis of the bibulous papers showed, after 24 hours of conditioning in the silos, Sensory analysis was carried out at the end of the Trial I, 30 days after treatment. Seeds were analyzed by the in-house panel two times, immediately after collecting from the storage devices, and after one hour of ventilation in open air at ambient temperature (25°C).

Table 3. Eugenol residues (mg/Kg) after postharvest treatment (30 μg/cm^2 clove essential oil) on dry seeds of peanuts, beans, apricot kernels, and lentils stored in jute bags for 30 days at 20°c. analyses were performed 3 hours after spray (day 0), after select- ed periods during storage and subsequent seed ventilation for 1 hour in open air (oa).

Storage	Eugenol residues (mg/Kg ± RSD%)			
(days)	Peanut	Bean	Apricot kernel	Lentil
0[a]	0.10 ± 0.01[b]	< loQ	< loQ	< loQ
3	0.14 ± 0.03	< loQ	0.08 ± 0.01	0.15 ± 0.02
7	1.65 ± 0.17	0.06 ± 0.01	0.22 ± 0.06	0.49 ± 0.12
15	10.75 ± 3.33	0.09 ± 0.01	0.40 ± 0.14	0.59 ± 0.08
30	12.69 ± 3.79	0.06 ± 0.01	0.47 ± 0.16	0.30 ± 0.02
30+1 h oa	0.05 ± 0.00	< loD	< loD	< loD
	sensory evaluation			
30	3	0	1	1
30+1 h oa	0	0	0	0

notes: [a]3 hours after treatment; [b]Mean values ± standard deviation (n = 3). loQ = instrumental limit of quantification.

Table 4. infl e of clove oil spray treatment at 30 μg/cm^2 on eugenol residues (mg/Kg) in dry seeds of wheat, maize, rice, and rape kept in silos for 30 days at 20°c. analyses were performed 3 hours after spray (day 0), after selected periods during storage and subsequent seed samples removal for 1 hour in open air (oa).

(days)	Wheat	Maize	Rice	Rape
0[a]	0.03 ± 0.01[b]	< loQ	0.23 ± 0.08	0.24 ± 0.04
3	0.63 ± 0.07	0.51 ± 0.13	0.60 ± 0.03	0.53 ± 0.11
7	5.61 ± 0.07	2.08 ± 0.62	9.73 ± 0.38	1.82 ± 0.38
15	14.49 ± 1.56	2.63 ± 0.24	17.05 ± 2.10	3.48 ± 0.83
30	0.70 ± 0.08	0.24 ± 0.10	13.92 ± 1.08	2.30 ± 0.37
30+1 h oa	< loD	< loD	0.07 ± 0.01	< loD
	sensory analysis			
30	1	1	3	2
30+1 h oa	0	0	0	0

notes: [a]3 hours after treatment; [b]Mean values ± standard deviation (n=3). loD = instrumental limit of determination.

Table 5. infl e of clove oil spray treatment 15 μg/cm^2 on euge- nol residues (mg/Kg) in wheat, and rice kept in silos, and peanut kept in jute bags for 60 days. analyses were performed 3 hours after treatment (day 0) and after selected periods during storage.

Storage	Eugenol residues (mg/Kg ± RSD%)		
days	Peanut	Wheat	Rice
0[a]	0.01 ± 0.01[b]	0.02 ± 0.01	0.28 ± 0.02
3	0.08 ± 0.01	0.37 ± 0.07	0.48 ± 0.10
7	0.18 ± 0.02	0.23 ± 0.07	7.47 ± 0.05
15	0.15 ± 0.02	0.32 ± 0.05	10.10 ± 0.15
30	0.26 ± 0.03	0.43 ± 0.06	6.51 ± 0.04
45	0.07 ± 0.01	0.31 ± 0.01	6.10 ± 0.02
60	0.08 ± 0.02	0.20 ± 0.02	6.65 ± 0.02

notes: [a]3 hours after treatment; [b]Mean values ± standard deviation (n = 3).

All samples, except beans, revealed the presence of eugenol with different threshold scale from 1 (light) to 3 (high), while after 1 hour of air ventilation, they were all classified with 0 (absence) (Tables 3) and 4. Comparing residue analysis data and sensory evaluation it was evinced that rice and peanut had the higher sensory values (3) and the higher residues, rape was classified 2 with a residue of 2.30 mg/ Kg, while wheat, maize, apricot kernel, and lentil were classified 1 with eugenol residues values ranging from
1.30 mg/Kg to 0.70 mg/Kg. The only sample which received the values 0 after the first panel was represented by the beans which showed a residue of 0.06 mg/Kg. After ventilation all samples received the value 0 being all residues below the LOD, with the exception of peanuts (0.05 mg/Kg) and rice (0.07 mg/Kg) (Tables 3 and 4).

Postharvest treatments have the main issue to keep high the quality of stored horticultural crops. The concept of quality does not include only the nutraceutical, organo- leptic and visual characteristics of the crop but also its toxicological characteristics, including pesticide residue levels. For this reason punctual and appropriate analysis should be established to evaluate the residues of the pes- ticides both synthetic and botanical, used to lessen pest contamination of crops during postharvest. While many studies have been performed to determine the residue levels of synthetic pesticides used in pre-or postharvest treatments of fruits and vegetables, much less has been done on dry seeds.

Regarding clove oil, any data on the residue level could be found in literature, while several studies were found dealing with the efficacy and postharvest a decrease of almost 50% of the residues, and thereafter a lower rate of decrease till constant values in the second week.

This behavior is probably due to the volatility of eugenol, until the system did not come into equilibrium. Eugenol residues in our samples vary among the dif- ferent seeds, but this behavior was not related to the wax content of the seeds nor to the type of container used to store the different crops. The seeds were not treated directly with the clove essential oil, therefore the growth in concentration was related to the diffusion of eugenol in the seeds stored inside the silos and the jute bags. Sensory analysis showed that in order to detect eugenol in the seeds its amount should be higher than 0.10 mg/Kg, After storage the seed ventilated had eugenol residues below the limit of flavor perception of the in-house panel, and therefore did not adversely affect the organoleptic characteristics of the seeds. For this reason ventilation before commercial packing, could be the right option to eliminate eugenol flavor. The results of this study showed that clove oil may have significant commercial application for postharvest pest management of dry seeds, without impairing their organoleptic characteristics.

Disclosure statement

No potential conflict of interest was reported by the authors.

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