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Eugenol diffusion coefficient and its potential to control *Sitophilus zeamais* in rice

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Given the insecticidal potential of eugenol as a fumigant, this work aimed to determine the diffusion coefficient of eugenol emanating from a pure standard solution (99%), as well as from clove essential oil (*Eugenia caryophillata* Thunb. (Myrtaceae)) through rice grain; to chemically analyse the volatile composition of commercially available eugenol and clove essential oil; and to evaluate the mortality of *Sitophilus zeamais* Motschulsky (Coleoptera: curculionidae) after exposure to eugenol inside a test chamber filled with rice. The solid phase microextraction method of extracting and quantifying eugenol by gas chromatography presented a good analytical response for the quantification of the analyte. There was no significant difference between the diffusion coefficient of eugenol diffusing from pure eugenol or from clove essential oil. The diffusion coefficient of eugenol through rice with the conditions herein adopted is $1.09 \times 10^{-3}\,cm^2\,s^{-1}$. The characterization of clove essential oil confirmed the presence of eugenol as its major component (74.25%). A difference was observed in the composition of the distinct phases evaluated. The exposure of adult *S. zeamais* to diffused eugenol from pure eugenol over seven days resulted in significantly higher mortality rates (~37%) than eugenol diffused from clove essential oil (~11%). No differences in mortality rates were observed in individuals placed at different positions inside the test chamber during eugenol fumigation.

Plant essential oils' extraction, composition, and bioactivity have been widely studied over the last years. The composition of the essential oils extracted from a wide variety of plants have been catalogued[1,2], and the behavioural and physiological bioactivities of these botanicals against insect pests have been evaluated in stored products[2,3], and it has been found that the extraction mode can directly interfere with essential oil yield and composition[4,5]. Thus, essential oils and essential oil-based pesticides could be used for food protection in alternation or combination with other pesticides, reducing the overall amount of applied pesticides and avoiding or reducing the possibility of developing resistance in pest populations[6]. Concerning the application of essential oils in crop protection and food safety, clove essential oil (CEO) (*Eugenia caryophyllata* Thunb. (Myrtaceae)), specifically its major component eugenol (Fig. 1), has attracted attention from researchers due to its potential as an insecticide[7–10] and fungicide[11–14] and its antimicrobial[15–18] and antioxidant[19,20] properties. Moreover, clove bud, leaf, and stem oil and eugenol have been generally recognized as safe (GRAS) as a direct human food ingredient[21]. Similarly, eugenol, as an active substance with no maximum residue level (MRL) required for several food applications, has been approved by the European Commission[22].
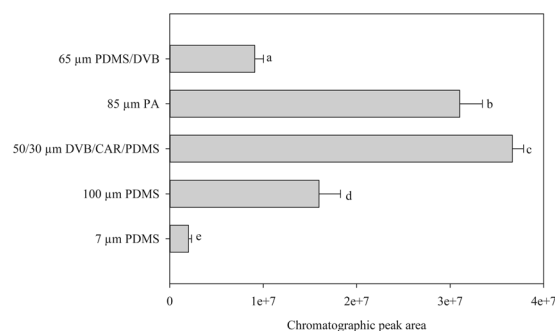
Using plant essential oils for crop protection and food safety could bring valuable advantages, since many of the oils tested on insects to date seem to present multiple modes-of-action and sites-of-action, which accounts for the multiple ranges of pesticidal actions and sublethal effects[23–25]. Generally, essential oils and their major constituents are relatively nontoxic to mammals and are environmentally nonpersistent. While there is a well-established worldwide production and trade chain for perfume and flavouring industries that could extend essential oils' applications[6], little work has been done on the application of essential oils for both crop protection and food safety.

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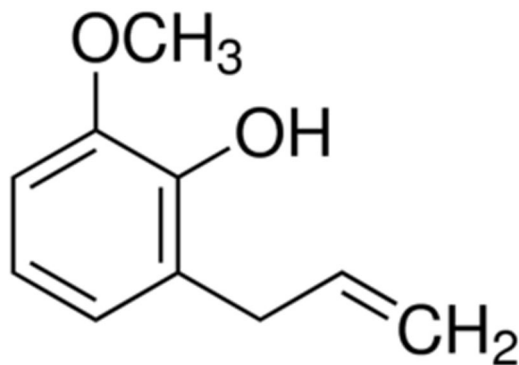
Figure 1. Eugenol (4-allyl-2-methoxyphenl) CAS N° 97-53-0.

Figure 2. Effect of fibre coating on extraction efficiency of eugenol (230 µL L^{-1}). Error bars indicate the standard deviation.

One of the steps after identifying potential plant essential oils with the required bioactivity for crop protection and food safety is to evaluate whether these substances could be realistically applied to crop protection and food safety[2,26]. Most of the previous papers that have investigated the potential of eugenol or clove essential oil as a biopesticide used only a small amount of grain or used bioassays with only insects[7,27,28], undervaluing the efficiency of the biopesticide on more grain. In using plant essential oils as a fumigant for the protection of stored product commodities, it is important to determine the parameters that would allow the application to be scaled up. Isa and colleagues[29] used available parameters, mathematical modelling and numerical simulation to study phosphine distribution in a cylindrical silo containing grain. Among the required parameters is the diffusion coefficient, which reflects the rate a substance will move through the medium and away from its release point, thus making it available to kill pests along the way[30].

Considering the insecticidal potential of clove essential oil and its major component eugenol as a fumigant in the control of insect pests in stored grain, the present work had the following aims: to evaluate the diffusion coefficient of eugenol from both a high-purity product and from CEO through rice grain; to characterize the different phases of the fumigation process; and to evaluate the mortality of *Sitophilus zeamais* Motschulsky (Coleoptera: curculionidae) after fumigation with a high-purity eugenol product and with CEO in a prototype of approximately 5.5 kg of rice.

Results

Eugenol sampling from the air.
Based on a higher chromatographic peak area, the fibre coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) was more efficient in terms of its chromatographic responses than the other fibres (Fig. 2). In addition, the chromatographic responses of the analyte extracted in triplicate with the DVB/CAR/PDMS fibre showed good repeatability with a low coefficient of variation (CV = 3.27%). The comparison between the chromatographic peak area of the five tested fibres showed a statistically significant difference between the five fibres' response ($\alpha = 0.05$).

The extraction time of eugenol by the fibre coated with DVB/CAR/PDMS was determined based on the response profile of the chromatographic peak area over time. There was no statistically significant difference ($\alpha = 0.05$) between fibre's responses after 120, 240, 300 or 360 seconds of exposure to the same concentrations of eugenol (Fig. 3). The peak area of the chromatographic response after 120 seconds of exposure was approximately 97% of the maximum peak area reached. Thus, the exposure time of the SPME fibre was chosen based on the chromatographic responses and to guarantee a short interval time between the measures in the diffusion experiment.
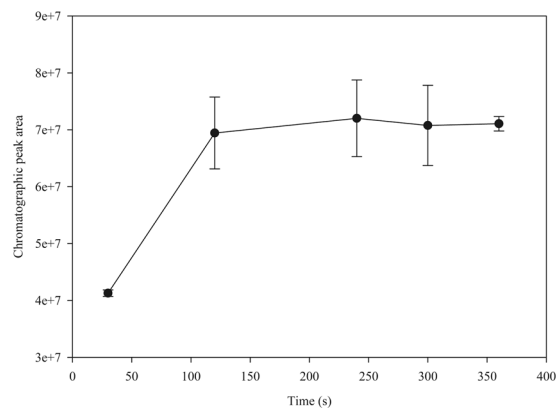
Figure 3. Effect of exposure time on the chromatographic peak areas of eugenol ($230\,\mu L\,L^{-1}$) extracted by $50/30\,\mu m$ *Stableflex* fibre coated with DVB/CAR/PDMS. Error bars indicate the standard deviation.
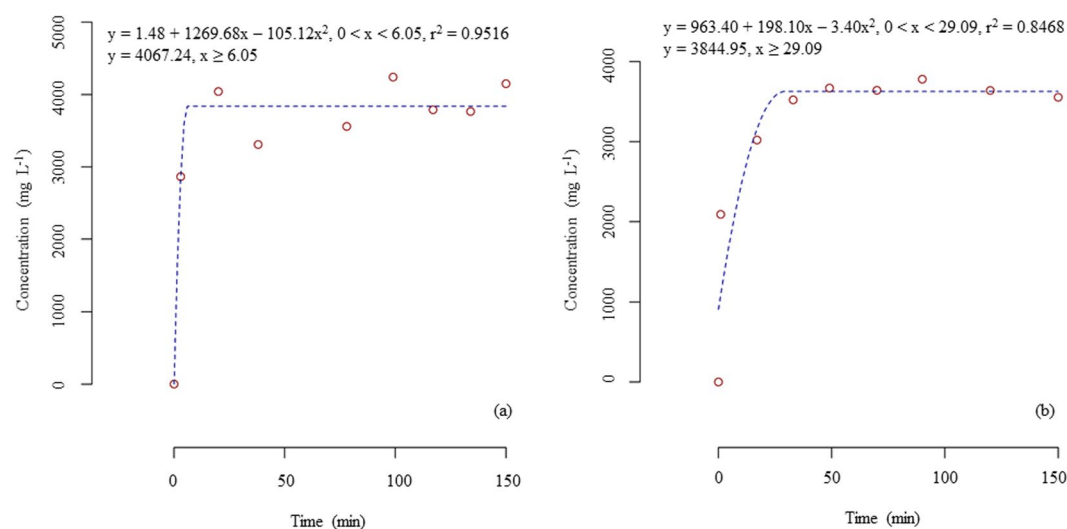

Figure 4. Saturation profile in the Büchner flask with volatilized eugenol from (**a**) eugenol 99% and (**b**) clove essential oil.

Method validation. The calibration curve of the SPME-GC/FID method was based on the chromatographic response of a *Stableflex* fibre of $50/30\,\mu m$ coated with DVB/CAR/PDMS and exposed to nine different concentrations of eugenol (0.41, 1.23, 4.08, 8.15, 16.29, 148.05, 287.28, 1160.30, and $4391.13\,mg\,L^{-1}$) for 120 seconds in duplicate. The calibration curve of eugenol ($y = 994.64 \times -1557$) showed good linearity and strong correlation between the concentrations and peak area within the range of 2.62 to $4391.13\,mg\,L^{-1}$ ($r^2 = 0.9988$). The LOD and LOQ values, calculated according to Eqs 1 and 2 [31,32], were 0.87 and $2.62\,mg\,L^{-1}$, respectively. The precision and accuracy of the method were evaluated at the concentrations of 148.05, 287.28 and $1160.30\,mg\,L^{-1}$. The relative precision was evaluated by recovery assays that varied from 97.96 to 101.88%. The accuracy of the method was attested by a coefficient of variation (CV) lower than 8.30%.

Saturation in the Büchner flask. Prior to determining the diffusion coefficient, it was necessary to determine the saturation in the Büchner flask (internal volume: 283 mL) containing volatilized eugenol from its liquid phases (15 mL) (EUG and CEO). The saturation time was 6.05 minutes, and the concentration of the volatilized eugenol was $4067.24\,mg\,L^{-1}$ when using EUG as the liquid phase (Fig. 4a). The saturation time was 29.09 minutes, and the concentration of the volatilized eugenol was $3844.95\,mg\,L^{-1}$ for the CEO as the liquid phase (Fig. 4b). Thus, prior to opening the connecting valves for the diffusion experiments, the liquid phases were kept in agitation in the Büchner flask with the connecting valve closed for 10 and 30 minutes for the EUG and CEO, respectively.

Diffusion of eugenol. The prototype geometric parameter, β, was $3.66 \times 10^{-3}\,cm^{-2}$. The slope of the adjusted diffusion equation (Eq. 3) for the eugenol from EUG was 2.40×10^{-4} (Fig. 5a), while that from CEO was 2.2×10^{-4} (Fig. 5b). However, there was no statistically significant difference between the slopes for eugenol from
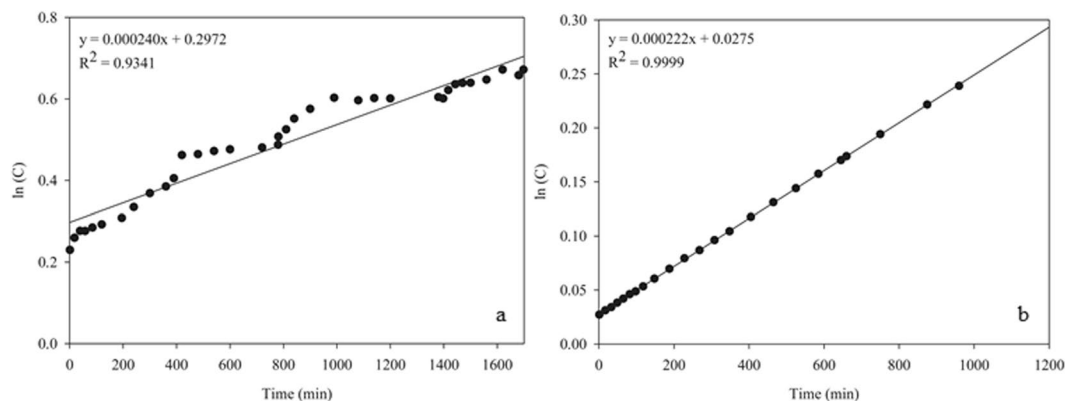
Figure 5. Adjusted diffusion equation for volatilized eugenol from (**a**) eugenol 99% and (**b**) clove essential oil through rice grain.

Compound	Retention time (min)	KI measured	KI reference[60]	ΔKI (%)	Eugenol 99% (EUG)			Clove essential oil (CEO)		
					Liquid phase (%)	Volatile phase (%)	After diffusion (%)	Liquid phase (%)	Volatile phase (%)	After diffusion (%)
Eugenol	30.55	1371	1356	1.11	100.00	100.00	100.00	74.25	57.78	100.00
β-Caryophyllene	33.24	1415	1418	0.21	—	—	—	13.55	37.08	—
α-Caryophyllene	35.01	1445	1452	0.49	—	—	—	2.50	4.11	—
Epizonarene*	39.06	1513	1501	0.80	—	—	—	0.47	1.03	—
Caryophyllene oxide*	42.35	1570	1582	0.76	—	—	—	0.60	—	—
Hexadecanol*	58.24	1869	1880	0.59	—	—	—	8.62	—	—

Table 1. Relative percentage composition of the clove essential oil and eugenol 99% in different phases. KI: Kovats index, ΔKI: percent difference of measured values of KI compared to reference values. *Tentatively identified.

the EUG and CEO diffusion equations ($\alpha = 0.05$). Hence, a common slope, 2.39×10^{-4}, was used to determine the diffusion coefficient of eugenol through rice as $D = 6.53 \times 10^{-2}\,cm^2\,min^{-1}$ ($1.09 \times 10^{-3}\,cm^2\,s^{-1}$).

Chemical composition of the liquid and volatilized phases.
The chemical characterization of the liquid phases by GC/MS identified six different constituents in the CEO with eugenol confirmed as its major constituent (74.25%) (see Supplementary Fig. S1). As expected, only eugenol was identified at the EUG liquid phase. The proportion of the constituents of the CEO volatile phase was slightly different from that identified at the CEO liquid phase (Table 1).

Bioassay.
Mortality data that was corrected by Abbott's formula using insect mortality in the control condition showed that fumigation with EUG for 7 days reached 36.49% *S. zeamais* mortality, whilst fumigation with CEO for the same time period reached 11.12% mortality. Both EUG and CEO fumigation for 4 days reached a maximum of 1.3% *S. zeamais* mortality. Although the 7-day treatment showed a significant difference between the use of EUG and CEO ($\alpha = 0.05$), no difference was observed in the 4-day treatment. Insects placed in distinct positions (top or bottom) of the prototype did not show any significant difference in mortality rates ($\alpha = 0.05$) in the same treatment duration and liquid phase (Fig. 6).

Discussion
The extraction efficiency of SPME fibres depends on the polarity of the analyte to be determined and its affinity towards the distinct adsorbent materials coating the fibres. For eugenol, the SPME fibre coated with three materials (DVB/CAR/PDMS) was the most efficient to extract the analyte. Fibres coated with DVB/CAR/PDMS have also been used to extract the volatile compounds in lavender[33] to analyse *Melaleuca alternifolia* essential oil[34] and *Angelica dahurica* essential oil[35]. On the other hand, fibres coated with PDMS/DVB have been used to analyse the essential oil from *Tanacetum vulgare* L.[36], and fibres coated with PDMS have been used to analyse *Eucalyptus cinereal* essential oil[37]. These results reinforce the importance of choosing the appropriate SPME fibre, since the material coating the fibre is a primary issue when selecting a fibre. Similarly, the exposure time of the fibre to the analyte should be carefully determined. The optimized exposure time to analyse the compounds in lavender and *Angelica dahurica* essential oil were 15 and 10 minutes, respectively[33,35]. The shorter exposure time observed for eugenol can be related to the simplicity of the matrix and the ability of the DVB/CAR/PDMS fibre to retain eugenol.

The validation of the analytical method aimed to evaluate its performance in quantifying eugenol. Given the chromatographic peak areas, calibration curves were constructed linking the areas of the analyte with its
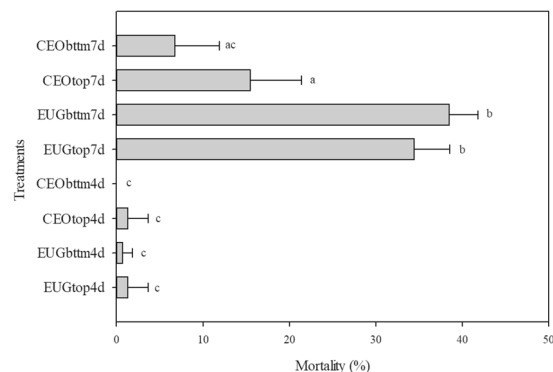
Figure 6. Mortality of *S. zeamais* treated with eugenol 99% (EUG) or clove essential oil (CEO), located at the top (top) or bottom (bttm) of the prototype for 7 or 4 days (7 d or 4 d). Error bars indicate the standard deviation. Data points followed by the same letter are not significantly different according to post hoc Tukey's test at p < 0.001.

concentrations (0.41–4391.13 mg L^{-1}). The linear regression of the calibration curve to analyse eugenol by SPME-GC/FID showed a strong linear correlation between the concentrations and peak areas within the studied range. Whilst the limit of detection is the lowest detectable concentration of an analyte, the limit of quantification is the lowest concentration that can be determined within a given level of uncertainty[31,38]. In general, the aim of method validation for pesticide residues in food is to reach at least the maximum residue level (MRL) of the pesticide in the matrix. However, as eugenol is generally recognized as safe (GRAS)[21] and no MRL is applied[22], we looked for LOQ and LOD capable of satisfactorily quantifying the eugenol in the diffusion experiment; an LOQ and LOD of 0.87 and 2.62 mg L^{-1}, respectively, were enough to successfully do so. In chromatographic methods, a good analytical method should reach a precision from 70 to 120% and accuracy is expected to be lower than 20%[31,39]. Thus, the SPME-GC/FID quantifying eugenol with a precision between 97.96–101.88% and accuracy lower than 8.30% fits the requirements of a robust analytical method. Therefore, the validation results demonstrate the reliable performance of the method.

Although we used two liquid phases as sources for volatile eugenol, there was no significant difference between the diffusion coefficients of eugenol from either of these sources. The real factor related to the diffusion of eugenol was the volatilized eugenol itself, regardless of its source. This result could be explained by the eugenol saturation in the Büchner flask prior to opening the valve and proceeding with the diffusion experiment. There are differences in the volatilization of eugenol from either CEO or EUG, as noted by the varying saturation times of eugenol in the Büchner flask with from either source. However, the saturation prior to the diffusion experiment eliminated the dependence on the kinetics of the volatilization of eugenol from the liquid phases. Thus, the diffusion coefficient was determined based solely on the eugenol diffusion through rice grain regardless of the volatilized eugenol's source.

The diffusion coefficient of eugenol through rice grain is almost seven times lower than the diffusion coefficient of the major constituent of mustard oil, allyl isothiocyanate (AITC), through maize[40]. Compared to phosphine, the diffusion coefficient of eugenol is approximately 145 times lower than the former[29,41]. The observed differences between the coefficients of diffusion may be related to the vapor pressure of each compound. Eugenol has lower vapor pressure (0.022 mmHg at 25 °C) and a lower diffusion coefficient (1.09 × 10^{-3} cm^2 s^{-1}), sub-cooled phosphine shows higher vapor pressure (655.55 mmHg at −90 °C) and a higher diffusion coefficient (1.59 × 10^{-1})[41], while the AITC shows an intermediate vapor pressure (5.43 mmHg at 25 °C) and intermediate diffusion coefficient (7.20 × 10^{-3})[40] closer to eugenol.

Identifying the constituents of the essential oils is important to provide information that could support the findings regarding the effects of biopesticides' use. For fumigation, it is important to characterize the volatilized phase induced by the essential oil because it is this phase that will be in direct contact with the grain (and pests) rather than the liquid phase of the essential oil[8,11,42]. As expected, the characterization of both liquid and volatile phases of the EUG solely identified eugenol and confirmed it as the major constituent of the liquid phase of CEO. In addition to eugenol, five other compounds were either identified or tentatively identified at the liquid phase of the CEO (see Supplementary Figs S2 to S7), but only three remained present at its volatile phase. Only eugenol was identified on the top of the column of grain when EUG or CEO were used to fumigate (Table 1). The authors suppose that either some compounds might have been adsorbed to the column of rice grain or were present in such a low concentration after diffusion that the analytical method could not properly identify the compounds at the top of the column of grain.

The characterization of the liquid phase of the CEO agrees with observations in previous work[4,19,43]. Eugenol is well-known as the major constituent of CEO, along with other compounds commonly identified in the composition of this essential oil[4,19,44–46]. The second most abundant constituent of the CEO was β-caryophyllene, also known as caryophyllene. Similar to eugenol, caryophyllene is permitted to be directly added to food for human consumption[47]. Moreover, several studies have shown caryophyllene to have anti-diabetic, anticarcinogenic, antioxidant, and antimicrobial properties[48–50].

The volatile phase promoted by the CEO presented constituents in different proportions than those observed for the liquid phase. Although eugenol remained the major constituent at the CEO volatile phase, its presence

dropped from 74.25% at the liquid phase to 57.78%, while the presence of β-caryophyllene increased from 13.55 to 37.08%. This behaviour may be explained by the difference in the vapor pressure of eugenol and β-caryophyllene. While the eugenol vapor pressure is 0.022 mmHg (25 °C), that of β-caryophyllene is 0.031 mmHg (25 °C); the higher vapor pressure of a substance is, the more volatile it is expected to be. These differences can influence the bioactivity of CEO and whether it is used as fumigant or used in direct contact with the pests. Eugenol, β-caryophyllene, and α-caryophyllene have already been identified as the three constituents of CEO with the highest occurrence at the volatile phase[11]. Eugenol has also been identified as the major constituent of the volatile phase promoted by the essential oil of some species of cinnamon[51].

Although four days of fumigation with CEO or EUG were not enough to kill more than 1.3% of *S. zeamais*, the mortality of the insects reached almost 40% after seven days. The comparison between the treatments with EUG and CEO for seven days showed that the former resulted in higher mortality. Prior works have already attributed higher bioactivity to EUG than CEO. Lee *et al.*[7] concluded that a lethal dose to 50% (LD_{50}) of *Sitophilus oryzae* treated with eugenol was 50.7 μL L^{-1}, while $LD_{50} > 150$ μL L^{-1} when using the clove essential oil.

Other previous work have evaluated the acute toxicity of the eugenol to pests of stored grains[7,27,28]. However, these works have been conducted with experimental designs that neglected the effect of the diffusion of the essential oil, or its major constituent, through grain. We hypothesize that the diffusion of eugenol through grain may impact the acute toxicity of eugenol. High mortality of *S. oryzae. Tribolium castaneum, Oryzaephilus surinamensis, Rhyzopertha dominica*, and *Callosobruchus chinensis* was achieved by fumigation with eugenol, however, the fumigation experiment was conducted solely with a small amount of stored food[28]. Similarly, Lee *et al.*[7] evaluated the mortality of *S. oryzae* subjected to treatment with clove essential oil and eugenol, but solely with a small, unspecified amount of rice in the cage. Comparing the results of previous work with the present results reinforce the importance of the fumigant's diffusion to evaluate whether a new product could realistically be used to protect stored food. As suggested by Silva and colleagues[52] for the application of ozone gas (O_3) in high volumes of rice grain, the use of external forces to induce the flow of gas through the column of grain could overcome the issue raised by the low diffusion coefficient of eugenol, improving its distribution and increasing its protective effect on grain.

Notwithstanding this, clove essential oil and its constituents have shown important behavioural and physiological effects on pests of stored grains. Food consumption by *S. zeamais* adults significantly dropped when the insects were exposed to sublethal doses of eugenol. Moreover, the growth rate, food consumption, and food utilization of *T. castaneum* larvae and adults were also reduced[53]. Additionally, the physiological toxicity of eugenol may have inhibited feeding in *S. oryzae, T. castaneum, and R. dominica* adults[54]. In addition, the total number of *S. zeamais* that emerged from the parental generation exposed to sublethal concentrations of clove essential oil decreased as the essential oil concentration increased[55]. Indeed, the octopaminergic system seems to mediate the insecticidal activity of eugenol[56].

Our study determined the eugenol diffusion coefficient through rice using a validated chromatographic method to quantify eugenol. Moreover, the chemical composition of the distinct phases observed during the fumigation process was characterized. A bioassay was conducted to verify the influence of eugenol diffusion on the mortality of a major pest of stored grains. Compared to similar studies carried out previously, this bioassay was conducted in conditions that were closer to a realistic application of essential oil as a fumigant to protect stored commodities. The results of this work are a key step towards bringing the basic science of biopesticides to real applications at the farm or industry level to guarantee food quality and safety.

Methods

Chemicals. Eugenol ReagentPlus® 99% and acetonitrile Chromasolv® ≥ 99.9% were purchased from Sigma-Aldrich (St. Louis, MO, USA). The clove essential oil was purchased from Mundo dos Óleos (Brasília, DF, Brazil). According to the manufacturer, the clove essential oil was obtained by steam distillation of *Eugenia caryophyllata* leaves.

Eugenol sampling from air. The solid phase microextraction (SPME) technique was used to sample the volatilized eugenol and to quantify it in a gas chromatograph equipped with a flame ionization detector (GC/FID) (GC2014, Shimadzu, Japan). The SPME fibres were handled with a manual sampler purchased from Sigma-Aldrich (St. Louis, MO, USA). The affinity of five different fibres coated with the following materials was evaluated: divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm *Stableflex*, PDMS/DVB 65 μm fused silica, PDMS 7 μm fused silica, polyacrylate (PA) 85 μm fused silica, and PDMS 100 μm fused silica. All SPME fibres were purchased from Sigma-Aldrich (St. Louis, MO, USA). To evaluate the extraction capacity, twenty-two millilitres of eugenol solution in acetonitrile (230 μL L^{-1}) were added to a headspace vial along with a stir bar and immediately sealed. With the solution continuously agitated by the stir bar, the needle of the SPME was inserted in the headspace vial and the fibres were exposed for two minutes to the headspace with eugenol at the same conditions. This procedure was done to guarantee the feasibility of SPME for quantitative analysis in nonequilibrium situations[57]. After adsorption, the SPME fibre was retracted, the manual holder transferred to the chromatograph and the fibres immediately inserted into the injector port of the chromatograph where they remained exposed until the end of the run to avoid any carryover effects. Blank checks were run periodically to ensure the absence of contaminants or residuals in the fibre.

The sampling time for the extraction of eugenol was determined based on the profile of the chromatographic responses to the fibre over time, using the fibre with the higher capacity to extract the analyte. The fibre was exposed to 230 μL L^{-1} of eugenol in acetonitrile for 30, 120, 240, 300, and 360 seconds.

Method validation. The SPME method was validated based on the following parameters of merit: linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. The calibration curve of the

method was constructed based on the chromatographic responses of nine different concentrations of eugenol (0.41, 1.23, 4.08, 8.15, 16.29, 148.05, 287.28, 1160.30, and 4391.13 mg L^{-1}). To achieve different concentrations, the eugenol was diluted in acetonitrile in 20 mL vials, mixed in a vortex for 1 minute, and then the fibre was immediately exposed to the eugenol. The chromatographic injections, performed twice, were made with the SPME manual holder, inserting the needle of the SPME fibre directly in the injector port of the chromatograph and exposing the SPME fibre to the carrier gas (Nitrogen at 2.03 mL min^{-1}) where it remained exposed until the end of the run to avoid any carryover effects. Blank checks were run periodically to ensure the absence of contaminants or residuals in the fibre. The linearity was then evaluated by the correlation coefficient obtained from the linear regression of the calibration curve. In brief, the limit of detection refers to the lowest amount of analyte that can be detected but may not be possible to exactly quantify within good statistical parameters. On the other hand, the limit of quantification refers to the amount of the analyte that can be quantified within suitable precision and accuracy. The LOD and LOQ were calculated according to the technical requirements for registration of pharmaceuticals for human use[32] by the International Conference of Harmonisation (ICH) which is widely applied for method validation of analytical methods[31,58] (Eqs 1 and 2, respectively).

$$\text{LOD} = 3.3 \ \sigma/s \tag{1}$$

$$\text{LOQ} = 10 \ \sigma/s \tag{2}$$

In Eqs 1 and 2, σ is the standard deviation of response and s is the slope of calibration curve.

The precision was assessed by the coefficient of variation (CV) and repeatability. Accuracy was assessed from six-repetition recovery assays at three different concentration levels[31].

GC/FID analysis. The chromatographic conditions for quantification of eugenol by GC/FID were as follows: the injector temperature was fixed at 220 °C, the detector was operated at 270 °C, the initial column temperature was 40 °C with a heating rate of 30 °C min^{-1} up to 240 °C, and this temperature was fixed for 1 minute; the total running time was 8 minutes. The separations were performed on a 30-metre long DB-5 capillary column (Agilent Technologies, Palo Alto, CA, USA) having an inner diameter of 0.25 mm and film thickness of 0.10 μm. Nitrogen was used as carrier gas at 2.03 mL min^{-1}. After each assay, the SPME fibres were continuously subjected to thermal desorption at the injector port until there was no adsorbed analyte that could interfere with the subsequent assays.

Experimental apparatus for diffusion experiment. Diffusion of eugenol was evaluated through 5.5 kg of rice (O*ryza sativa* L.) (BRS MG Rubelita variety) purchased from EPAMIG (Belo Horizonte, MG, Brazil) and having a moisture content of 11.0% wet basis (w.b.). Both clove essential oil (CEO) and eugenol 99% (EUG) were used to generate the volatilized eugenol that diffused through the rice. An adaptation of the prototype of the Stokes diaphragm cell[59] was used to determine the diffusion coefficient of eugenol through rice (Fig. 7). A Büchner flask (internal volume of 283 mL) was fitted at the base of the diffusion column (height × diameter of the column of rice: 50 × 15 cm) with a rubber septum at the side opening and connected to the diffusion column through a ball valve. An amount of 15 mL of EUG or CEO and a magnetic stir bar were added to the Büchner flask, according to each experiment (see Supplementary Fig. S8). For both liquid phases, the magnetic bar was stirred slow enough to keep the liquid phase homogeneous and in constant agitation.

Saturation in a büchner flask. The eugenol saturation in the Büchner flask was evaluated through regular measures of the eugenol concentration at the lateral opening of the Büchner flask whilst the ball valve was closed, and the stir bar agitated the solution. This procedure was performed to ensure an atmosphere with constant concentration of eugenol and that the volatilization of eugenol was not kinetically limiting its diffusion through the column of grain.

Diffusion of eugenol. After the Büchner flask was saturated with eugenol, the valve connecting it with the diffusion chamber was opened to allow the diffusion of eugenol through the column of rice. From that moment, the eugenol concentration was measured at the top of the grain column using the previously optimized and validated SPME method. All the experiments were repeated three times and performed in a climatized room with temperature at 21 ± 2 °C. The mathematical solution to determine the diffusion coefficient using an adaptation of Stoke's diaphragm cell (Eqs 3 and 4) is based on the concentration gradient and spatial parameters of the prototype[59]. The proposed solution posits that the concentration of the diffusing substance remains constant at the bottom chamber (below the connecting valve between the Büchner flask and the column of grain). To ensure this condition, we evaluated the time taken for the eugenol to saturate in the Büchner flask and monitored its concentration during the diffusion experiment.

$$D = \frac{1}{\beta t} \ln\left(\frac{C^0_{eug,bttm} - C^0_{eug,top}}{C_{eug,bttm} - C_{eug,top}} \right) \tag{3}$$

$$\beta = \frac{A}{h}\left(\frac{1}{V_{base}} + \frac{1}{V_{topo}} \right) \tag{4}$$

In Eqs 3 and 4, D is the diffusion coefficient (cm^2 min^{-1}), $C^0_{eug,bttm}$ is the initial eugenol concentration at the bottom chamber of the prototype (mg L^{-1}), $C^0_{eug,top}$ is the initial eugenol concentration at the top of the column of grain (mg L^{-1}), $C_{eug,bttm}$ is the eugenol concentration at time t at the bottom chamber of the prototype (mg L^{-1}),
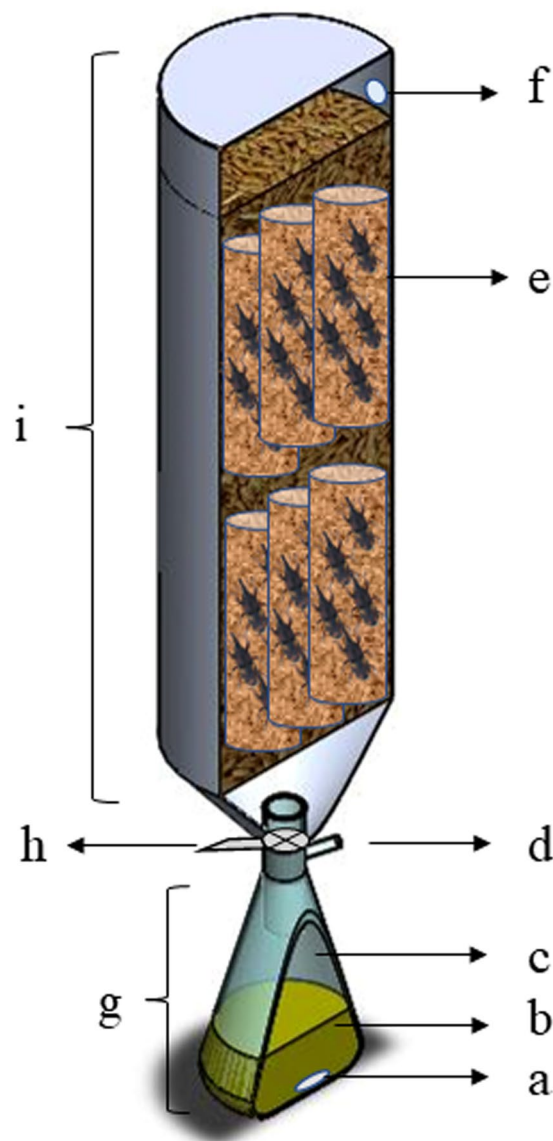
Figure 7. Scheme of the experimental apparatus of the diaphragm cell: (**a**) magnetic stirring bar, (**b**) liquid phase (eugenol or clove essential oil), (**c**) headspace of the Büchner flask with the volatile phase, (**d**) lateral opening of the Büchner flask sealed with a rubber septum for sampling of the volatile phase, (**e**) cages with grain and insects used for the bioassays, (**f**) rubber septum for sampling of the volatile phase after passing through the column of grain, (**g**) the bottom chamber of the prototype, (**h**) ball valve connecting the parts of the system, and (**i**) upper chamber of the prototype.

$C_{eug,top}$ is the eugenol concentration at time t at the top of the column of grain (mg L^{-1}), β is the prototype geometric parameter (cm^{-2}), A is the cross-sectional area to diffusion (176.71 cm^2), h is the height of the column of grain (50 cm), V_{bttm} is the volume of the base of the prototype (below the connecting valve) (1068.52 cm^3), and V_{top} is the volume of the top portion of the prototype (above the connecting valve) (9984.36 cm^3). The initial eugenol concentration at the bottom chamber of the prototype ($C^0_{eug,bttm}$) was considered as the saturation concentration of the Büchner flask. During the diffusion experiment, the eugenol concentration in the Büchner flask was monitored to yield $C_{eug,bttm}$. As expected, the initial concentration of eugenol at the top of the column of grain ($C^0_{eug,top}$) was confirmed to be zero since the valve was closed and eugenol was neither supposed to diffuse through the closed valve nor emanate from non-treated grain. $C_{eug,top}$ was determined by gas chromatography over time.

Chemical composition of the liquid and volatilized phases. The three distinct phases were characterized by: the liquid phase (clove essential oil or eugenol), the volatile phase promoted by the liquid phase at the Büchner flask, and the volatile phase at the top of the column of grain. The analyses were conducted in a gas chromatograph coupled to a mass spectrometer (GC/MS) (GC7820A-5977B, Agilent, United States) to identify the constituents of each phase. Additionally, an alkane standard solution C7–C30 at 1000 μg mL^{-1} in hexane (Sigma

Aldrich, St. Louis, MO, USA) was injected for retention index calculation and confirmation of the compounds identified[60].

GC/MS analysis.   The GC/MS was operated in full scan mode (mass acquisition range m/z 50–450) using ionization energy of 70 eV. The gas chromatograph was operated in a split-ratio of 20:1 with an injector temperature of 220 °C. The initial column oven temperature was 60 °C with a heating rate of 2 °C min^{-1} up to 200 °C, followed by an increase of the heating rate for 5 °C min^{-1} up to 250 °C. Helium was used as the carrier gas with a column flow of 1.2 mL min^{-1}. The total data acquisition time was 80 minutes. The separations were performed on an HP-5 ms capillary column (Agilent Technologies, Palo Alto, CA, USA) 30 m × 0.25 mm of inner diameter × 0.25 μm film thickness with stationary phase 5% diphenyl/95% dimethyl polysiloxane. CEO and EUG were diluted in acetonitrile to 50 μL L^{-1} and 1 μL of each liquid phase was injected by the auto injector AOC-20i (Agilent, United States) to the chromatograph. The SPME method was used to extract the volatile phases and the fibre was kept at the injector port of the chromatograph during the entire run. The solvent cut time was set to 4 minutes. The constituents of each phase were identified by mass spectrum comparison with the NIST mass spectra database (version 14.0) and confirmed by Kovats Index (KI) calculation and comparison with the literature[60]. The KI of each constituent was calculated based on the retention times of the constituent and the alkanes of the standard solution.

Bioassay.   The effect of the diffusion during the volatile phase was evaluated based on the mortality of *S. zeamais* in rice fumigated with CEO or EUG. Rice infested with 50 non-sexed *S. zeamais* adults aged up to two weeks were put in cylindrical cages. The top and bottom of the cages were made of a perforated metal plate to allow the diffusion of eugenol through the cages. The cages were 20 cm long and were arranged vertically in the prototype (Fig. 7). Thus, the cages were placed in two positions at the prototype: bottom or top. A thin layer (approximately 1 cm) of rice grain was placed between the upper and the lower cages. At each position, three cages were put side by side and each cage was considered as an experimental unit. The empty space of the prototype was filled with rice free of insects. Mortality was evaluated after 4 and 7 days from the beginning of the fumigation. Insects were considered dead when no normal reaction was expressed after being touched with a brush. The control treatment was performed with the same procedure, except that the grain was not treated with any insecticide product, only atmospheric air. The connecting valve of the prototype (Fig. 7), open to free atmospheric air, was used for control. According to Abbott's formula[61], the mortality of insects subjected to the atmospheric air was used for mortality correction of the insects submitted to eugenol or clove essential oil treatment.

Statistical analysis.   The chromatographic responses of each tested SPME fibre was compared using analysis of variance (ANOVA) followed by Tukey test when significant differences were observed at p < 0.001. To evaluate the extraction time profile of the SPME fibre, the chromatographic response at each time was compared using the ANOVA followed by Tukey test when significant differences were observed at p < 0.001. Sigma Plot (Systat Software Inc., San Jose, CA, USA) was used to conduct these analyses.

Data obtained from the eugenol saturation in the Büchner flask was assessed by the quadratic plateau function performed using the package easynls for R software (R Core Team, Vienna, Austria).

The comparison between the diffusion coefficients obtained for eugenol from the liquid phases (EUG or CEO) was performed by analysing the significant difference between the regression equations obtained by the diffusion[62] of each liquid phase. The significant difference between the regression equation (Eq. 3) coefficients was also briefly tested, and a common estimator was found when the coefficients were not significantly different.

The mortality of *S. zeamais* after 4 and 7 days after fumigation with CEO and EUG was corrected using Abbott's formula[61]. The mortality of the insect resulting from the fumigation with CEO and EUG after exposure to each period was compared using the ANOVA followed by Tukey test when significant differences were observed at p < 0.001.

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Author Contributions

L.R.A.F., F.F.H. and M.E.L.R.Q. advised and outlined the experimental design. L.R.A.F. provided the reagents and insects used in the study. L.H.F.P. and F.F.H. conducted the experiments, performed statistical analysis, and drafted the first version of the text. M.E.L.R.Q. assisted with chromatographic analysis. M.V.d.A.S. assisted with the experimental apparatus and prepared the figures. L.R.A.F., A.H.S. and F.F.H. reviewed and edited the manuscript. All authors approved the manuscript.

Additional Information

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