https://dx.doi.org/10.21577/0103-5053.20210126

J. Braz. Chem. Soc., Vol. 33, No. 1, 85-95, 2022

Article

Quality Control for Lignin and Gelatin Microcapsules Loaded with Orange Essential Oil

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Sustainable natural product-based microstructured systems and biopolymers are strong candidates for use in crop protection. Lignin and gelatin microcapsules loaded with orange essential oil were developed with spray-drying in order to enhance its potential. We evaluated the microparticle controlled release mechanisms, biological effects, structural and thermal properties. A quantitative method using headspace-gas chromatography was developed and evaluated for the controlled release of the essential oils. Controlled release studies showed a linear relationship between the biopolymer and essential oil concentrations when retaining volatile compounds. Thermal analyses demonstrated increases in essential oil stability when microencapsulated, especially for lignin as a biopolymer. These results showed that biopolymer type was the main factor influencing quality analysis parameters. Finally, microcapsules loaded with orange essential oil were applied in a bioassay, and showed gains in toxicity against *Spodoptera frugiperda* compared to non-encapsulated oil.

Keywords: orange essential oil, microencapsulation, spray-drying, HS-GC-FID analyses, *Spodoptera frugiperda* control

Introduction

Driven by the growing demand to develop robust products with low environmental impact for crop protection, many studies[1,2] have sought the application potential of essential oils as formulation components in the development of eco-friendly pesticides. Essential oils have been evaluated for their repellent and insecticide action and low toxicity against non-target organisms.[1,3] One essential oil which has drawn attention because of its major compound's biological effects (terpene D-limonene) is orange essential oil (OEO). This oil can be found in large quantities at a low cost.[4]

Despite its potential use in pest control, the number of commercial formulations based on essential oils is still low due to volatility, low aqueous solubility, and structural instability, which results in low persistence in nature and reduced action against target pests.[1-3] Microencapsulation is a technique that may enable the commercial use of essential oils by promoting a more significant residual effect on controlled release systems, as well as preventing their degradation and volatilization.[1]

Among the encapsulation methods of essential oils, spray-drying stands out for its compatibility with labile materials and low operational cost.[5] These characteristics, associated with the use of biopolymers, favor efficient formulations against pest insects with low toxicity to the environment.[1] However, some biocompatible industrial products such as gelatin and lignin,[6,7] have very diverse chemical characteristics which can generate different controlled release profiles. So far, these have only been modestly considered for essential oils encapsulation aiming for applications in pest control.

Costa *et al.*[8] evaluated the influence of rice starch porous spheres, inulin, and gelatin/sucrose biopolymers on the encapsulation of oregano essential oil by spray-drying. They demonstrated that different release behaviors could be obtained when using different biopolymers. In this study,

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Editors handled this article: Teodoro S. Kaufman and Brenno A. D. Neto (Associate)

gelatin/sucrose microparticles showed a faster essential oil release with higher antioxidant and antimicrobial activity. In contrast, the microcapsules containing rice starch and inulin ensured increased stability. Thus, we note that the use of microencapsulated of essential oils is directly influenced by their release in the environment, the adequate encapsulation method, the biopolymers used, and the application of control steps that allow evaluation of the interaction between these factors. Menossi *et al.*[1] and Pavela and Benelli[2] indicate that the choice of appropriate quality control parameters on the encapsulation process, as well as the use of analytical methods to ensure the quality of formulations are important factors for scalability and application in the field. Thus, stability and controlled release analyses have been carried out in parallel with biological assays to evaluate the quality of formulations containing essential oils.[9-11]

Based on this, the specific goals of this work are to develop gelatin and lignin microcapsules loaded with orange essential oil and characterize the microparticles as a function of controlled release, and evaluate the effect of biopolymer type on morphology, particle size distribution, and thermal properties. A specific analytical method by static headspace-gas chromatography was developed for quantitative analyses, providing information such as recovery, encapsulation, and controlled release. Finally, we evaluate the microparticle biological activities and the relationship between the dose to the biological assays against *Spodoptera frugiperda* caterpillars. This pest insect was selected for this study since it causes a considerable loss in Brazilian yields.[12,13]

Experimental

Chemicals

The D-limonene 99% (m m^{-1}) (Sigma-Aldrich, St. Louis, United States of America) and menthol (Arora Chemicals, São Marcos, Brazil) were used as analytical standard and internal standard (IS), respectively. Acetone (Panreac, Barcelona, Spain), *p*-cymene 99% (m m^{-1}) (Sigma-Aldrich, St. Louis, United States of America), mineral oil (EMD Chemicals Inc., Gibbstown, United States of America), and ultrapure water were used as solvents. Gelatin type B (Synth, Diadema, Brazil) and lignin (Suzano, São Paulo, Brazil) were used as biopolymers. The orange essential oil was obtained by cold pressing orange peels obtained from the region of Santa Cruz do Rio Pardo-SP (Agroterenas S/A Citrus, Santa Cruz do Rio Pardo, Brazil) and was used as a volatile core material. The specifications of the OEO were as follows: density at 25 °C: 0.842 g mL^{-1}, the refractive index at 20 °C: 1.473, aldehydes: 1.4%, and chemical profile composed mostly of D-limonene (77.5%), β-myrcene (11.1%), and α-pinene (3.99%).[14]

Microparticles preparation

Emulsions were prepared by adding a fixed mass of biopolymer (1,500 mg) into 30 mL of ultrapure water in Erlenmeyer (125 mL) under magnetic stirring (20,000 rpm, 60 s) using a disperser Ultra-Turrax (IKA T10basic, Wilmington, United States of America) according to Aguiar *et al.*[14] The emulsions were evaluated in different OEO concentrations in relation to biopolymer mass (28 and 56% m m^{-1}). The system was kept under agitation at 20,000 rpm for 60 s at a temperature of 20 ± 1 ºC and immediately transferred to the spray-dryer. Gelatin and lignin emulsions without the addition of OEO (controls) were also prepared.

The emulsions were transferred into a spray-dryer (mini Spray-Dryer BÜCHI, B 290, Flawil, Switzerland) equipped with a drying chamber of 500 mm × 200 mm and an atomizer with a 0.7 mm nozzle. The best operational spray-dryer parameters were obtained using the following conditions: inlet air temperature of 150 ºC, outlet air temperature of 63 ± 2 ºC, feed flow rate of 0.15 L h^{-1}, drying airflow of 536 L h^{-1}, and air injection flow of 8 m^3 h^{-1}. The spray-drying parameters were previously obtained, maximizing the OEO encapsulation efficiency and yields by fractioned factorial design.[14] The resulting dried microparticles were collected and stored at 8 °C, and the yield encapsulation (%, m m^{-1}) was calculated according to the equation S1 in the Supplementary Information (SI) section.

Headspace gas chromatography analyses (HS-GC-FID)

All samples were first submitted to extraction by static headspace (HS) using a PALSyr HS 2.5 mL for combi-PAL AOC-5000. The injection sample volume was 1,000 µL. Gas chromatography (GC) analyses were carried out with a Shimadzu GC-2010 Plus apparatus coupled with a flame ionization detector (HS-GC-FID) (Shimadzu Corporation, Kyoto, Japan). The analyses were carried out using a ZB-Wax capillary column (30 m × 0.25 mm inner diameter (i.d.)) coated with polyethylene glycol (0.25 µm phase thickness) (Phenomenex, Torrance, United States of America). The oven temperature gradient was programmed at 40 °C for 1 min, rising at 5 °C min^{-1} to 170 °C (1 min). Injector temperature was 170 °C in a split injection mode (15:1). The carrier gas was helium (1 mL min^{-1}). We used synthetic air (300 mL min^{-1}), hydrogen (40 mL min^{-1}), and nitrogen (30 mL min^{-1}) for FID.

Quantification of microencapsulated orange essential oil by HS-GC-FID

We analyzed encapsulation efficiency (%, m m^{-1}) using the HS-GC-FID and evaluated it according to the quantity of D-limonene, the major component of OEO. Samples were prepared after dissolving 25.0 mg of powder microparticles in 1.00 mL of acetone for 30 min. For each 800 µL of dispersion, we added 100 µL of menthol solution (10.0 mg mL^{-1}). A total of 10 µL from the resulting mixtures were transferred to a headspace vial (10 mL), homogenized by 15 min at 75 °C and 500 rpm in a dry-block oven, and analyzed by HS-GC-FID.[14]

We prepared two calibration curves for OEO quantification using extracts from gelatin and lignin as matrices. This protocol was used as matrix-matched calibration curves. Thus, we prepared standard stock solutions of D-limonene and menthol in acetone at 5.00 and 8.00 mg mL^{-1}, respectively. Afterward, these solutions were diluted in the aqueous extracts of the matrices obtaining the analytical curves at the concentration of 2.50×10^{-2}, 7.50×10^{-2}, 0.250, 0.750, 2.00, and 3.00 µg mL^{-1} of D-limonene, and 1.00 µg mL^{-1} of IS. The extracts of matrices were prepared by the dispersion of 0.0250% (m v^{-1}) of the powder gelatin or lignin control in water (see "Microparticles preparation" sub-section).

After HS-GC-FID analyses of these solutions, the calibration curves were plotted according to instrumental response, the ratio between the areas of the different analyte concentrations and IS area. All calibration curves were prepared in triplicates. The determined calibration curves for the gelatin and lignin matrices are presented in equations 1 and 2, respectively:

$$y = 1.972x - 0.086 \quad (1)$$

$$y = 1.212x - 0.0285 \quad (2)$$

The curves were linear in the selected concentrations range with coefficients of determination (r^2) greater than 0.99. The encapsulation efficiency (%, m m^{-1}) was calculated using the equation S2 in the SI section.

In vitro release analysis of microparticles

For the analysis of the controlled release of the encapsulated OEO in the products obtained by spray-drying, a headspace vial (10 mL) containing 25 mg of the dry product and 10 µL of the IS (menthol 1.00 mg mL^{-1}) was sealed with an aluminum cap and septa of PTFE/SIL and submitted to headspace extraction (40 ºC and 500 rpm) and quantification by GC-FID. We evaluated the release of the encapsulated OEO during 0, 15, 30, 45, 60, and 90 min.

Aiming for the potential application in the control of *S. frugiperda* caterpillars, we also performed a controlled release study for the encapsulated product when dispersed in an aqueous medium. Therefore, 25 mg of the recovered spray-dried product was transferred into an Eppendorf tube (1,500 µL) and dispersed using 1,000 µL of ultrapure water and 100 µL of the IS (menthol 11.0 mg mL^{-1}). Each Eppendorf tube was sealed and submitted to vortex homogenization until the complete dispersion of material (90 s). Subsequently, 10 µL were transferred to a headspace vial (10 mL), sealed, and submitted to headspace extraction at 40 ºC, 500 rpm, and analyzed by GC-FID. We evaluated the release of the encapsulated OEO during 0, 15, 30, 45, and 60 min.

Quantification of products by controlled release

For OEO quantification in the controlled release studies, we prepared four calibration curves for each biopolymer, evaluating the solvent effect in the headspace extraction. We used acetone, mineral oil, *p*-cymene, and ultrapure water as solvents. Therefore, standard stock solutions of D-limonene (5.00 mg mL^{-1}) and menthol (8.00 mg mL^{-1}) prepared and diluted with acetone, mineral oil, *p*-cymene, and ultrapure water. The dilutions were performed to obtain mixed solutions and analytical curves in concentrations of 2.50×10^{-2}, 7.50×10^{-2}, 0.250, 0.750, 2.00, and 3.00 µg mL^{-1} of D-limonene, and 1.00 µg mL^{-1} of IS. All curves were prepared in triplicates.

For the HS-GC-FID analysis, we also investigated the matrix biopolymeric effect on OEO quantification. In these analyses, the calibration curves were prepared in acetone, mineral oil, and *p*-cymene, adding 10 µL of the mixed solution of D-limonene and menthol into a headspace vial (10 mL) and 25 mg of the powder gelatin or lignin control (see "Microparticles preparation" sub-section). The sample was then homogenized for 30 min, at 40 ºC and 500 rpm. Subsequently, 1,000 µL of the vapor phase was injected into GC-FID. The prepared calibration curves in water were similarly built, except that 25 mg of the powder gelatin or lignin control were previously mixed at 1,000 µL of water, and only 10 µL of the resulting suspension was transferred into the headspace vial.

The calibration curves were plotted, and through least-square regression, we calculated the linear regression equation, the limit of detection, and quantification (equations S3 and S4, SI section). The OEO release (%, m m^{-1}) and partition coefficients were also calculated (equations S5 and S6, SI section). When evaluating the *in vitro* release mechanism of microencapsulated OEO, we

determined the coefficients concerning the following kinetic models: zero and first order, Higuchi, and Korsmeyer-Peppas (equations S7, S8, S9, and S10, SI section).[15]

Morphological evaluation of microparticles

The surface morphology of the microparticles was investigated by scanning electronic microscopy (SEM). Powder microcapsules were added on a double-sided adhesive carbon tape, submitted to metallization with gold/palladium, and analyzed by FEI Inspect S50 microscope (Torrance, United States of America) operating at 25 kV and magnifications between 200 and 50,000×. The particle size distribution was measured by the analysis of the diameter of approximately 300 particles using the ImageJ software.[16]

Powder microparticles thermogravimetric analysis

Thermogravimetric (TG) and derivative thermogravimetric (DTG) curves were obtained for powder microcapsules using a simultaneous thermal analyzer PerkinElmer (STA 6000, Waltham, USA). It was used 10 mg of the sample, and an alumina crucible at a heating rate of 10 °C min^{-1} ranging from 40 to 800 °C under an inert atmosphere, using N_2 (5.0) in a flow rate of 50 mL min^{-1}. The TG data and its first derivative were processed by software Origin 9.0.[17]

Biological assay

Bioassays were carried out against *S. frugiperda* caterpillars at the second instar, which were obtained from rearing at our bioassay laboratory. Caterpillars collected in the field were also added at every three generations to preserve the genetic variability of the population. In this bioassay, we prepared artificial diets using non-encapsulated OEO at 10 mg g^{-1} to the diet and microencapsulated OEO at 0.10, 1.00, and 10 mg g^{-1}.[18] The incorporation of non-encapsulated or microencapsulated OEO into the diet occurred at 40 ºC. Gelatin and lignin controls and water were incorporated into the diet as negatives controls. Neem (*Azadirachta indica*) extracts were used in the bioassays as a positive control. All bioassays were kept under a temperature of 26 ± 2 ºC and a photoperiod of 14 h of light and 10 h of darkness. Each treatment was performed using five applications with each repetition consisting of four caterpillars (n = 20). The evaluations were carried out every two days until the development to adult form. The recorded biological parameters were: pupal mass, mortality, and eggs produced. The insect was considered dead when it did not respond to the touch of a brush.

Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons of means by Tukey's test (5% probability) was performed using the SPSS software.[19]

Results and Discussion

Insecticide formulations containing essential oils need reduced volatilization, increased stability, and persistence of biological effects after crop application.[2] Hence, we proposed to obtain microcapsules loaded with OEO using spray-drying. Table 1 shows the best formulation compositions for orange essential oils, their encapsulation efficiencies, and yields.

We observed that the reduction in OEO concentration in the emulsion increased retention after the drying process, with encapsulation efficiencies higher than 85% for both biopolymers (formulations 2 and 4, Table 1). Carmona *et al.*[20] also observed a similar effect for the encapsulation of orange essential oil using whey protein and maltodextrin as biopolymers. We found that an increase in the essential oil load resulted in a reduction of encapsulation efficiency due to an insufficient amount of biopolymer to form a robust wall around the OEO inside the aqueous dispersion containing the produced powder. Additionally, this increase in essential oil load also favored its loss by evaporation, with a reduction in the encapsulation yield (Table 1).[20,21] Moreover, we observed encapsulation efficiency improvements in formulations containing gelatin

Table 1. Evaluated formulations for microencapsulated orange essential oil, containing encapsulation efficiencies and yields

Formulation	Biopolymer / mg		Essential oil[a] / (%, m m^{-1})	EE / (%, m m^{-1})	YE / (%, m m^{-1})
	Gelatin	Lignin			
1	1500	–	56	48.2	34.9
2	1500	–	28	95.6	51.8
3	–	1500	56	37.5	49.3
4	–	1500	28	86.9	73.6

[a]The concentration of the essential oil was calculated based on the mass of the biopolymers. EE: encapsulation efficiency; YE: yield efficiency.

rather than lignin, regardless of the OEO concentrations under the same conditions for spray-drying (Table 1). This result can be justified by the better emulsifying capacity of gelatin, which favors the formation of walls around the OEO in an aqueous medium before the drying step, increasing retention.[22]

Once we obtained the formulations by spray-drying described in Table 1, we proceed with quality control steps. We evaluated the effects of biopolymer type and the OEO load on the controlled release, stability, and maintenance of the biological activities against *S. frugiperda* caterpillars. We developed a method of direct analysis using headspace extraction and gas chromatography for controlled release, as we will describe below.

Calibration curves for controlled release studies using headspace-gas chromatography

A headspace-gas chromatography is a well-accepted method for the analysis of volatile compounds in complex matrices.[23] However, despite the advantages offered by the method for studying controlled essential oil release, such as automation and extraction without a pretreatment step, the sample matrix may have a significant effect on the analytical signal and instrumental sensitivity.[24,25] Regardless of the biopolymeric matrix used (e.g., the non-volatile biopolymers used for OEO encapsulation) and solvents used to prepare calibration curves, these matrix components, or their combination, may interfere directly in the diffusion, volatility, and adsorption of the analyzed OEO.[24,26]

In this case, to get the optimal analytical conditions for the quantification of OEO during the *in vitro* controlled release studies in powder microcapsules, we performed the calibration curves using matrix combinations to minimize possible failures in the quantitative analysis protocols by using headspace-gas chromatography. Therefore, the analytical calibration curves were constructed using both a solvent and a matrix of gelatin or lignin. As solvents, we selected acetone, mineral oil, and *p*-cymene to evaluate their influences during the headspace extraction. We choose acetone since it is a volatile and a commonly used solvent in preparing essential oils samples and mineral oil due to its low density and volatility.[27] In contrast, we selected *p*-cymene because it is not as volatile as acetone and can be used as a green solvent.[28] Therefore, we obtained individual calibration curves for D-limonene and the IS for each solvent, analyzing their linear equations (Table 2). The variance analysis and residual plots are illustrated in Table S1 and Figure S1, respectively, in the SI section.

In these analyses, we found that the variance of each curve to the regression equations was significant and that there was no lack of fit in the linear models. As such, all equations were linear with $r^2 > 0.99$ to both evaluated biopolymers (Table 2).

These results indicate a linear D-limonene release regardless of the solvent type; however, a better release rate to the headspace phase was obtained when using mineral oil as a solvent for both biopolymers. Liu and Wene[29] reached similar outcomes in preparing calibration solutions in mineral oil, reducing the partitioning in perfume samples. The different analytical sensibility we observed was due to the higher volatility of acetone and *p*-cymene compared to mineral oil under heating. Vapor pressures from acetone and *p*-cymene in the gas (headspace) phase increased with temperature, dominating the headspace volume in the vial and decreasing the D-limonene concentration when reaching equilibrium, therefore reducing the analytical sensitivity to the quantification by GC-FID.[26]

The choice of mineral oil instead of acetone or *p*-cymene as solvent was also supported by the lowest quantification obtained and limits of detection in the analytical method (Table 2). Therefore, in this study, mineral oil is the most appropriate solvent for the preparation of calibration curves for the quantification of OEO in powder microcapsules for controlled release studies. We also observed that in mineral oil, the calibration curve prepared in lignin presented

Table 2. Calculated analytical parameters for the GC-FID methods concerning the prepared calibration curves in acetone, mineral oil, *p*-cymene, and water

Biopolymer	Solvent	Linear regression	r^2	LOD / (μg g^{-1})	LOQ / (μg g^{-1})
Gelatin	acetone	$y = 4.83x + 0.42$	0.998	9.84	29.8
	mineral oil	$y = 7.39x + 7.60$	0.994	6.43	19.5
	p-cymene	$y = 3.86x + 1.03$	0.997	12.3	37.3
	water	$y = 80.6x + 34.3$	0.993	0.590	1.79
Lignin	acetone	$y = 6.10x + 1.34$	0.997	7.79	23.6
	mineral oil	$y = 12.3x + 1.34$	0.995	3.86	11.7
	p-cymene	$y = 2.43x + 0.69$	0.995	19.6	59.3
	water	$y = 75.7x + 25.3$	0.992	0.629	1.91

r^2: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification.

around 60% better analytical and calibration sensitivities than gelatin (Table 2). This result suggests a better affinity between gelatin and OEO than lignin.

We prepared calibration curves only in water when analyzing the controlled release of gelatin and lignin microcapsule samples, as dispersion in water for foliar application are standard procedures in the field. The obtained linear regression (Table 2) presented good fits between the variation of GC analytical signals and the nominal concentrations with $r^2 > 0.99$ and no lack-of-fit for the models (Table S1, SI section). Similarly, what we observed for mineral oil, the lower water volatility associated with low solubility of OEO components in an aqueous medium allowed an increase in the analytical responses of D-limonene. This fact could be verified when we compared the linear regression values and the limits of quantification and detection (Table 2).

Effect of biopolymers on *in vitro* controlled release

Powder microcapsules

After establishing the best protocol for quantification, we used the calibration curves in mineral oil to evaluate the controlled release of OEO from gelatin and lignin powder microcapsules. We conducted the headspace extraction using 40 ºC and 500 rpm to simulate Brazilian environmental field conditions.[13]

As illustrated in Figure 1a, the release curve from non-encapsulated OEO reaches a gas phase equilibrium before 15 min. The stabilization of the formulated product vapor pressure in the headspace extraction occurred after 60 min, with 16, 4.6, 0.66, and 0.40% of OEO released regarding formulations 1, 2, 3, and 4 (Table 1), respectively. In the same headspace exposition time, the non-encapsulated OEO had already decreased 71% of its initial concentration. Comparing the quantitative profiles of OEO in the headspace phase from microencapsulated and non-encapsulated samples suggests that the formulated products acted as controlled release systems.

The release profiles from powder formulations illustrated in Figure 1a also demonstrated the difference between gelatin and lignin. Among gelatin microcapsules, formulation 1 showed the highest microencapsulated OEO release rate of after 90 min, releasing more than 52 mg g^{-1} of their content. Meanwhile, formulation 2 showed an OEO release of 19 mg g^{-1} under the same time/temperature effect. The analysis of partition coefficients also confirmed these release profiles (Table 3). A lower partition coefficient is related to a higher concentration of OEO in the gas phase (headspace); therefore, a higher release of microencapsulated material.

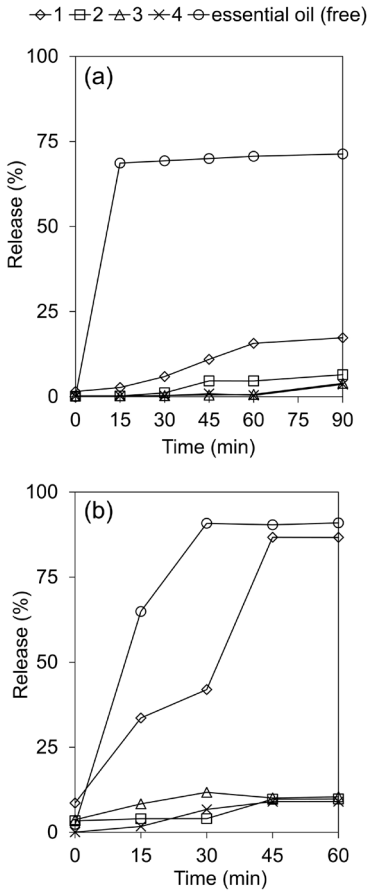

Figure 1. The release profile of microencapsulated orange essential oil as a function of different matrices and time ranging from (a) 0 to 90 min in powder and (b) 0 to 60 min in aqueous medium. In the figures, the formulations prepared using gelatin are described as 1 (56% essential oil) and 2 (28% essential oil). The formulations prepared in lignin are described as 3 (56% essential oil) and 4 (28% essential oil). Formulations described as gelatin and lignin are essential oil-free (controls).

Table 3. Partition coefficients (K) for headspace extraction of orange essential oil in gelatin and lignin microparticles in powder and dispersed in the aqueous medium

Biopolymer	Formulation (Table 1)	Partition coefficient (K)	
		Powder	Aqueous medium
Gelatin	1	5.77	1.15
	2	15.5	10.2
Lignin	3	25.5	7.26
	4	27.5	11.0
Orange essential oil (free)		1.40	1.10

Formulation 1 was obtained using a higher concentration of OEO (56% m m^{-1}, Table 1) in relation to the mass of the biopolymer. In this case, the lower proportion of biopolymer promotes the formation of thin wall membranes decreasing the protection of the volatile matrix. The smaller biopolymer proportion may also favor cluster (Figure 2a) and release of OEO under the effect temperature/time,

according to Hsieh *et al*.[30] and Carmona *et al*.[20] In addition to that, the non-encapsulated OEO excess may be adsorbed onto microcapsule surfaces, favoring the volatilization and release of the OEO non-encapsulated.[20]

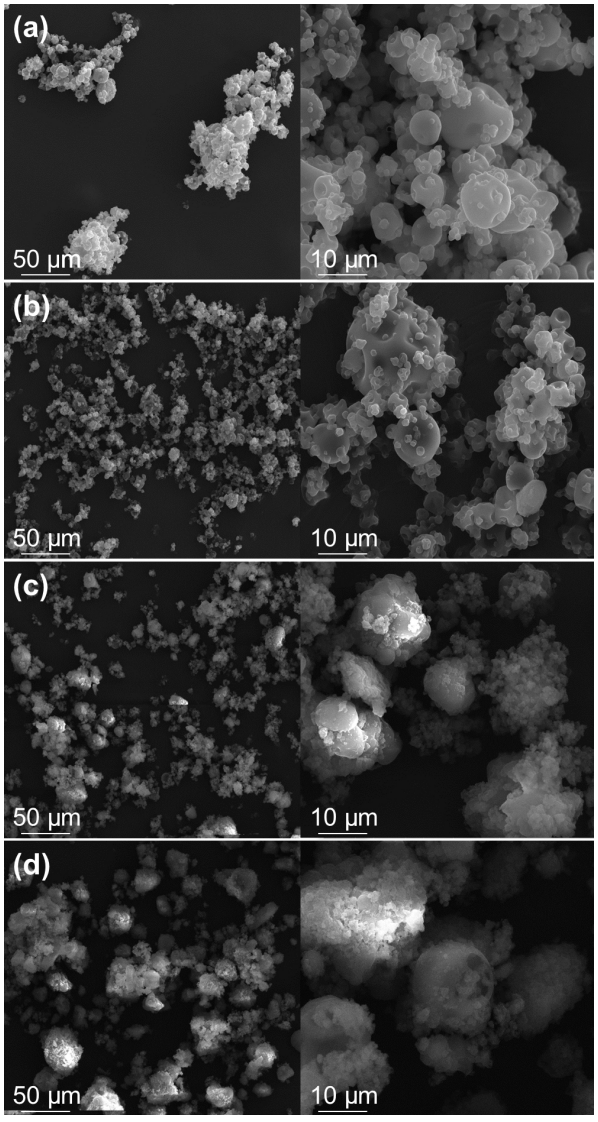

Figure 2. Microphotograph of microparticles loaded with orange essential oil. Gelatin microparticles prepared with 56% (a) or 28% (b) of the essential oil (m m^{-1}). Lignin microparticles were prepared with 56% (c) or 28% (d) of the essential oil (m m^{-1}). Magnification ranging between 1,000× and 5,000×.

In regard to gelatin formulations, lignin 3 and 4 showed a lower microencapsulated OEO release rate (1 and 2), releasing 12 and 11 mg g^{-1} of OEO after 90 min, respectively (Figure 1a). The SEM photograph analyses of the OEO microparticle (Figure 2) explain these observed differences. We found that gelatin microcapsules had a spherical tendency with a continuous and smooth surface (Figures 2a and 2b). In contrast, lignin microcapsules showed a rough structure (Figures 2c and 2d). Despite the spherical imperfections, lignin is a highly resistant aromatic biopolymer due to intermolecular forces that render matrix walls more compact which promote better resistance and protection against OEO volatilization.[31]

For the evaluation of powder microparticles OEO release mechanisms, we analyzed the determination coefficients for different kinetic models (Table 4). We found that determination coefficient values showed a mathematical model of zero and first order as the best fits between the kinetic release of encapsulated OEO in gelatin and lignin powder microparticles, respectively. These models presented determination coefficient values more similar to 1 (Table 4). As demonstrated by Li *et al*.[32] evaluating D-limonene nanoemulsions, the zero-order kinetics is compatible with OEO release with a constant rate, independent of its concentration.[15] On the other hand, we obtained first-order kinetic for lignin powder microcapsules, which indicates a release mechanism dependent on the amount of OEO inside microcapsules. In this case, the release amount of orange essential oil decreases over time.[15]

Gelatin and lignin microcapsule dispersion in the aqueous medium

Monitoring the controlled release in the aqueous medium, we observed that 91% of the emulsified and non-encapsulated OEO sample was in equilibrium in the headspace phase before 30 min at 40 ºC (Figure 1b).

Table 4. The release kinetic parameters of microencapsulated orange essential oil

Biopolymer	Formulation (Table 1)	Coefficient of determination (r^2)									
		Powder					Aqueous medium				
		Zero order	First order	Higuchi	Korsmeyer-Peppas	n	Zero order	First order	Higuchi	Korsmeyer-Peppas	n
Gelatin	1	0.94	0.88	0.84	0.85	0.56	0.93	0.87	0.86	0.96	0.56
	2	0.90	0.83	0.78	0.81	0.99	0.80	0.84	0.63	0.53	0.23
Lignin	3	0.67	0.88	0.42	0.42	0.41	0.82	0.75	0.91	0.96	0.30
	4	0.66	0.79	0.43	0.43	0.43	0.91	0.76	0.87	0.99	1.19

n: release exponent, indicative of the mechanism of microencapsulated bioactive compound release.[33]

Meanwhile, for the microencapsulated OEO, the headspace gas equilibrium occurred between 30 and 45 min for formulations 2, 3, and 4, with 9.7, 10, and 9.0%, respectively, for the evaluated OEO content (Figure 1b). These results show gains in stability for these formulations which decrease OEO volatilization. Formulation 1 presented stability gains, reducing OEO volatilization in powder form (Figure 1a); however, this gain was not observed in aqueous medium (Figure 1b), where it released 87% OEO content into the headspace phase under 45 min.

We calculated the values of the partition coefficients through the quantification of the released OEO into the gas phase from aqueous samples (Table 3). Similar to what is verified for powder microparticles, prepared formulations with the highest OEO content (formulation 1 and 3 of gelatin and lignin, respectively) also presented a higher transfer rate to the headspace phase when compared to formulations 2 and 4 during the aqueous medium analyses.

As we already verified for powder formulations, the OEO microparticles prepared with 56% (m m^{-1}) resulted in a thinner biopolymeric wall and smaller essential oil/biopolymer interactions than materials obtained with 28% (m m^{-1}). Moreover, higher OEO concentration may favor the essential oil coalescence, promoting heterogeneous particles with more size distribution (Figures S2a, S2b, S2c, and S2d, SI section). Formulations 1 and 3 showed more than 75% of the microparticle sizes ranging from 2.5 to 6.6 μm and 2.5 to 4.6 μm, respectively. When dispersed in the aqueous medium, larger particles are also more unstable, releasing essential oil early into the headspace phase.[34] This effect was particularly pronounced for the gelatin formulations with lignin (Table 3). These results highlight the importance of biopolymer type and quantity protecting essential oil and defining the controlled release mechanism.

The difference in the release profiles for gelatin and lignin microparticles formulated using OEO 56% (m m^{-1}) was associated with the larger hydrophilicity of gelatin, which in contact with water promotes polymeric chain swelling and the encapsulated OEO release.[35] Compared to gelatin, lignin is less soluble in water, with better OEO retention.[36] Note that compounds with high partition coefficient values favor the liquid or solid phase. These results are also supported by mathematical models (Table 4).

The Korsmeyer-Peppas release kinetics best described the relationship between the encapsulated OEO release in formulations 1, 3, and 4, with determination coefficients (r^2) closer to 1. In these cases, the diffusional exponent (n) values were used to describe the release mechanism (Table 4). For example, the n = 0.56 indicates that the release rate of OEO in formulation 1 depends simultaneously on the swelling of the gelatin in contact with water and non-Fickian transport diffusion processes.[33,35] However, with a decrease in the OEO concentration in gelatin microparticles (formulation 2), the release mechanism is then governed by a diffusion process, with a slower release rate (Figure 1b).[33,35] In lignin formulations, the decrease in OEO concentration remodeled the release mechanism from a system controlled by lignin erosion to being mainly controlled by a diffusion process (formulation 3, n = 0.30) (formulation 4, n = 1.19). Therefore, a high proportion of lignin hinders the OEO diffusion mechanism.[33,35]

Effect of the biopolymers on the thermal stability from microencapsulated OEO

The thermogravimetric analysis (TGA) allowed us to evaluate the thermal stability of microparticles loaded with OEO. In Figure 3a, we observed non-isothermal decomposition curves with mass loss in two stages. This thermal profile was observed for all samples.

The first stage occurred in the interval between 59-125 °C (gelatin) and 48-100 °C (lignin) for all sample controls and microparticles loaded with OEO. In this first stage, there was a mass loss of 11 and 3% for gelatin and lignin formulations, respectively, which was related to moisture vaporization and loss of volatile compounds from microcapsule surfaces with adsorbed OEO.[37]

A second stage was observed starting at 200 °C, as highlighted in the first derivative of the TGA curve (Figure 3b). The maximum degradation temperatures of the controls and microparticles loaded with OEO occurred at 331 (gelatin) and 363 °C (lignin), showing a total mass loss equivalent to 42 and 31% (m m^{-1}), respectively. This second mass loss was related to the thermal decomposition of biopolymer molecules and encapsulated compounds.[37] The similarity in the thermogravimetric events among controls and microparticles loaded with OEO showed that the final formulation was consistent with the initial material preparation, indicating that formulation by spray-drying did not affect the thermal properties of both biopolymers, even after changing OEO content (Figure 3b).

We also observed thermal stability gains on the OEO in gelatin and lignin microcapsules through thermogravimetric analysis, since the biopolymers start to decompose at temperatures higher than pure OEO, showing a maximum degradation temperature of 127 ºC according to Kringel *et al.*[38] Moreover, by the difference in maximum degradation temperature, we observed that lignin presents better thermal stability than gelatin, which may be associated with the higher compaction capacity of the lignin biopolymer (Figures 2c and 2d). Such factors can decrease permeability to heat and moisture in microcapsules, increasing their resistance.

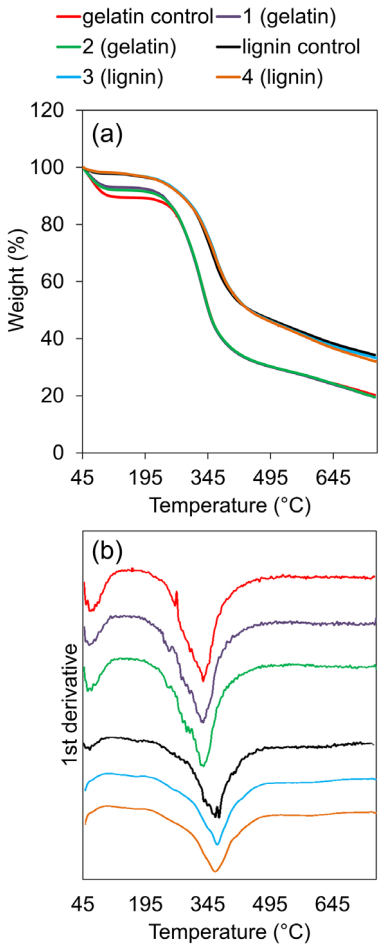

Figure 3. Thermograms TGA (a) and the first derivative of the curve of TGA, DTG (b). In the figures, the formulations prepared using gelatin are described as 1 (56% essential oil) and 2 (28% essential oil). The formulations prepared in lignin are described as 3 (56% essential oil) and 4 (28% essential oil). Formulations described as gelatin and lignin control are essential oil-free.

Biological assays of microencapsulated OEO against *Spodoptera frugiperda*

Based on controlled release profiles and thermal stability information, we selected the gelatin and lignin formulations that were prepared using the lowest OEO concentration (formulations 2 and 4, Table 1) for biological analyses. These two formulations presented the slowest controlled release profile, prolonged retention, and residual effects from OEO. The experimental data are described in Table 5.

We could verify that feeding caterpillars with an artificial diet containing non-encapsulated or microencapsulated OEO did not significantly differ in pupal weight compared to control groups ($p > 0.05$). The pupal weight and egg numbers were not presented for treatment using neem extract, once the caterpillars did not reach advanced development stages, showing 100% mortality in the larval phase (Table 5).

In treatments using gelatin microparticles, we verified a reduction in the average oviposition from females fed with diets containing formulations at 1.0 and 10 mg g^{-1} of OEO ($p < 0.05$) compared to a gelatin control. When we used lignin microparticles, a reduction in oviposition occurred for all treatments (Table 5). The decrease in egg numbers contributes to the insect pest's population reduction. We also observed that by adding gelatin microparticles into the diet, the probability of survival of the treated caterpillars gradually decreases as the concentration of OEO increased up to 1.0 mg g^{-1} (Table 5).

The addition of gelatin and lignin microparticles loaded with OEO initially increased the dietary ingestion in the first 48 h. After 96 h in contact with the artificial diet containing

Table 5. Biological assay for *Spodoptera frugiperda* evaluating gelatin and lignin microparticles loaded with orange essential oil. Pupal mass, number of eggs, and mortality to *Spodoptera frugiperda* insects fed with artificial diet

Treatment	Active compound concentration[a] / (mg g^{-1})	Mass of pupae / mg	Number of eggs	Mortality / %		
				48 h	96 h	196 h
Water	–	253 ± 20.7^{A}	1,360^{C}	0	5	0
Gelatin	–	235 ± 25.9^{A}	2,280^{H}	0	10	22
	0.10	223 ± 16.3^{A}	2,240^{GH}	0	0	35
	1.0	206 ± 15.6^{A}	1,450^{D}	0	5	42
	10	229 ± 16.4^{A}	1,730^{E}	0	0	25
Lignin	–	230 ± 15.5^{A}	730^{A}	0	15	29
	0.10	250 ± 14.8^{A}	890^{B}	0	10	22
	1.0	235 ± 19.0^{A}	860^{B}	0	5	16
	10	245 ± 13.4^{A}	1,990^{F}	0	0	25
Orange essential oil (free)[b]	10	231 ± 17.2^{A}	2,210^{G}	0	5	0
Neem extract[c]	0.30	–	–	0	45	100

[a]D-Limonene; [b]non-encapsulated orange essential oil; [c]neem extract used as positive control. Values followed by equal letters do not differ by Tukey's test at 5% probability.

OEO microcapsules, we observed a feeding deterrent effect on *S. frugiperda* caterpillars, diminishing diet consumption.

The associated toxicity with OEO microencapsulation also changed *S. frugiperda* behaviors attempting to escape from their containers with the artificial diet. This behavior was not observed for the controls. Furthermore, after 144 h of *S. frugiperda* exposition to the diet containing encapsulated OEO, they became immobile until pupal stage. This behavior has already been related to a neurotoxic knock-down effect from neural functions blockage, such as neurotransmitters.[39] We also observed a poor pupal and adult moth formation, as well as changes in their integument (see Figure S3, SI section). Cruz *et al*.[40] demonstrated that D-limonene, mainly in the association with other volatile organic compounds, prompted nutritional disorders which triggered indirect negative effects in *S. frugiperda* reproduction.

Conclusions

Results presented in this study demonstrate that the controlled release of microcapsules loaded with orange essential oil powder and dispersed in an aqueous medium can be successfully evaluated using headspace-gas chromatography. The quantitative analytical method provided a better understood of biological, morphological, and physical-chemistry phenomena associated with microparticles loaded with essential oils.

The analytical method by headspace-gas chromatography we developed, and the matrix effect in the release and stability studies, showed that orange essential oil microencapsulation resulted in higher thermal stability and retention of volatile organic compounds. Gelatin and lignin showed different affinity to orange essential oil. Due to that, their microparticles presented different release mechanisms in powder and aqueous medium. The stability and controlled release mechanism in powder lignin microparticles were similar, regardless of orange essential oil quantity. On the other hand, gelatin microparticles presented different release mechanisms according to their used orange essential oil content. The release mechanisms showed that the orange essential oil release depends mainly on diffusional transport and relaxational properties of the biopolymer chain. Biological assays demonstrated toxicity gains for orange essential oil after its microencapsulation, with mortality of *S. frugiperda* caterpillars from low to moderate and addition to phagodeterrent and repellent effects. Thus, these data include the first step in developing controlled delivery systems for the slow release of products containing orange essential oil for insect pest control as *S. frugiperda*.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This work was supported by the National Council for Scientific and Technological Development-CNPq (grant numbers 465357/2014-8, 403302/2013-7, 306109/2018-2, 429404/2018-2); the São Paulo Research Foundation-FAPESP (grant numbers 2012/25266-9, 2018/21201-8, 2014/50918-7); and Coordination for the Improvement of Higher-Level Personnel (CAPES) (Finance Code 001). This research is grateful to Suzano S/A for the lignin used in this work.

Author Contributions

Maria Clara S. Aguiar was responsible for the methodology, investigation, formal analysis, writing original draft, review and editing; Bárbara E. Denadae for the investigation; Paulo R. S. Bittencourt and Fernando R. Scremin were responsible for the investigation, and writing review and editing; Maria Fátima G. F. Silva and João B. Fernandes for the resources, and funding acquisition; Moacir R. Forim was responsible for the conceptualization, methodology, writing review and editing, resources, and funding acquisition.

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Submitted: July 15, 2021
Published online: September 9, 2021