http://dx.doi.org/10.5935/0103-5053.20160155

J. Braz. Chem. Soc., Vol. 28, No. 1, 126-135, 2017.
Printed in Brazil - ©2017 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Use of Lignins from Sugarcane Bagasse for Assembling Microparticles Loaded with *Azadirachta indica* Extracts for Use as Neem-Based Organic Insecticides

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Microcapsules of sugarcane bagasse lignin loaded with organic extracts of neem (*Azadirachta indica*) were prepared and evaluated as potential bioinsecticides. Lignins were extracted and modified by oxidation and acetylation reactions providing different biopolymers. Afterwards, they were characterized through several analytical techniques. The formulations were initially prepared as colloidal suspension of lignin nanoparticles, which were then spray-dried. The products were submitted to quality control protocols using high performance liquid chromatography (HPLC), thermal and photochemical degradation studies, followed by biological assays against *Spodoptera frugiperda* and *Diatraea saccharalis*. The formulations showed increases in both thermal and photo stability of approximately 40% compared with control samples. All the formulated microparticles were efficient against the insects evaluated and required a shorter time to achieve 100% mortality than the controls. The use of sugarcane bagasse lignins to prepare microparticles improved the resistance of neem extracts against abiotic factors, retaining the biological activity of this biopesticide.

Keywords: lignin, microparticle, biopesticide, biorational control, stability

Introduction

Insect pests have an important effect on the efficient production of food and animal feeds worldwide, demanding robust control strategies that depend on synthetic agrochemicals.[1] However, although their application has been effective in controlling of some pest species, their extensive and sometimes indiscriminate use has resulted in many social and environmental detrimental impacts.[2] An alternative model is the use of naturally occurring insecticides (biopesticides) to either replace or complement the use of synthetic compounds through the development of new formulations for the control of insect pests. These biopesticides include wyerone acid (*Vicia faba*), azadirachtin (*Azadirachta indica* L. Juss), nicotine (*Nicotiana tabacum*), pyrethrins (*Tanacetum cinerariaefolium*), tetrahydroberberine (bulbous *Corydalis* sp.), etc.[3]

Significant production, regulation, and enforcement problems must be overcome, however, to obtain commercially viable biopesticides. In addition, their stability during storage and the reproducibility of their effectiveness must be evaluated before they can be reliably marketed. One of the main limitations in the use of natural products for crop protection is their short residual period, where several extrinsic factors may affect the stability of the product or the active natural compound. For instance, results from field experiments have shown that crude *A. indica* extracts applied to crops only remain active for 3 days.[4] Environmental

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instability of molecules such as azadirachtin results in a lack of reproducibility of the biological activity, which directly translates into a lack of confidence in these natural products by farmers. Thus, together with the identification of active molecules such as natural insecticides, there is a need to develop formulations that allow these products to be used in a convenient and effective manner.

A more sophisticated approach to the formulation of agrochemicals involves nano- and micro-encapsulation. The active compounds are enclosed within a polymeric structure that improves physicochemical parameters, such as resistance to extrinsic factors, increased water solubility for organic compounds, and controlled release, while maintaining the activity of the product.[5] However, so far, most of the polymers used for the production of micrometric particles are synthetic and/or non-biodegradable. For formulations in which natural insecticides are used, natural polymers should be used to encapsulate the insecticide so as to prepare completely natural biopesticides and to maintain their environmentally friendly characteristics.[6]

Within this context, lignins are inexpensive biopolymers that can be used to develop new biopesticides. Lignins are complex branched phenolic heteropolymers resulted from the oxidative polymerization of three main types of monolignol alcohols such as *p*-coumaryl, coniferyl, and sinapyl alcohol.[7] Apart from their interesting chemistry and natural origin, lignin-derived products can indirectly affect positively the environment even after field application, as their degradation contributes to the formation of soil humic substances, black carbon and other aromatic substances.[8]

Biomass from higher plants is the largest available renewable source for the production of bioproducts. In Brazil, approximately 425 million tons of sugarcane bagasse are produced *per* year; this is usually burned for the co-generation of heat and electricity, but could be used in different products.[9] This burning could be considered to be a sub-optimal application of this substrate because a valuable set of possible molecules and products can be derived from the lignocellulosic material.[10] Besides this apparent availability, so far only a handful of attempts of using this inexpensive material as polymeric substrate for micro and nanoencapsulation.[11-13]

This study aimed to develop a new environmentally friendly biopesticide containing azadirachtin-rich neem extracts and formulations encapsulated in nano- and microparticles prepared using lignins from sugarcane bagasse. The final products were assessed to determine any gains in stability and its efficacy in the biological control of the insect pests such as *Spodoptera frugiperda* and *Diatraea saccharalis* when compared with conventional preparations.

Experimental

Reagents and solvents

Neem seed kernels (*Azadirachta indica*), neem oil, and sugarcane bagasse (*Saccharum officinarum*) were provided by Baraúna Ind. Co. Ltd. (Catanduva, SP, Brazil). Ethanol, *n*-hexane, methanol, dichloromethane and ethyl acetate of analytical-reagent grade were obtained from Panreac (Barcelona, Spain). Acetonitrile (HPLC-grade) was purchased from J. T. Baker (Ecatepec, Mexico). Ultrapure water was produced in our laboratory using a Milli-Q System (< 18 MW cm^{-1}) (Millipore, Bedford, MA, USA). Sodium hydroxide, anhydrous pyridine and acetic anhydride (analytical-reagent grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO-d_6) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

Neem extracts

Neem extracts were obtained using a modified version of the method described by Da Costa *et al.*[14] in which air-dried neem seed kernels were first extracted three times with *n*-hexane (1:25 m/v, 60 min) to remove any impurities; after the last filtration the solid material was extracted five times with ethanol (1:25 m/v, 720 min). The alcoholic extracts were combined and the solvent was removed under reduced pressure. The enriched azadirachtin fraction was obtained from the alcoholic extract using liquid-liquid extraction. The alcoholic extract (20.0 g) was solubilized in 400 mL H_2O:MeOH (3:1 v/v), which was then fractionated with *n*-hexane (6 × 300 mL) and ethyl acetate (6 × 300 mL); the latter extract was used in this work (F-EtOAc). The azadirachtin contents in the final ethyl acetate fraction and in neem oil were determined by reversed-phase high performance liquid chromatography (HPLC) as described by Forim *et al.*[15] An Agilent 1200 Series system was used in which 20 µL volumes were injected into a C_{18} column (Zorbax Eclipse XDB-C_{18}; 150 × 4.6 mm internal diameter, 5 µm) with an isocratic solvent system containing ACN:H_2O (35:65) at a flow rate of 1.0 mL min^{-1}, followed by ultraviolet (UV) detection at 217 nm.

Extraction and modification of lignins from sugarcane bagasse

Sugarcane bagasse was initially washed with hot water (80 °C), dried in a circulating air oven for 4 days at 40 °C until 10% (m/m) moisture content, and then milled.

Sugarcane bagasse was delignified via a soda-pulping process to separate the lignin present in biomass, following the method of Ibrahim *et al.*[16] and Rocha *et al.*[17] with some modifications. Briefly, 20.0 g of bagasse were immersed in 200 mL of aqueous 0.5 mol L^{-1} NaOH (1:10 m/v) and heated in a stainless-steel autoclave for 1 h at 120 °C. The delignified material was filtered at the end of the reaction to obtain a black liquor and the pH was adjusted to 5.5 with 1.0 mol L^{-1} HCl. The solvents were reduced to a small volume and then hemicellulose and silicon compounds were precipitated by adding three volumes of 95% ethanol (v/v). The ethanol present in the resulting liquor was removed and the pH was lowered to 1.5, forcing the precipitation of lignin. Finally, the lignin (referred to as Lig-Alk) was filtered, washed, and dried.

The black liquor (200 mL) obtained after the delignification step was used directly in the oxidation process adding to reflux 14% H_2O_2 (m/v) at pH 13 for 2 h at 98 °C.[18] The oxidized lignin (Lig-Ox) was then purified and precipitated as described previously. A third kind of lignin (Lig-Ac) was obtained through the acetylation of Lig-Alk. This reaction was carried out by dissolving 1.0 g of lignin in 10.0 mL of anhydrous pyridine and 20.0 mL of anhydrous acetic anhydride, keeping the reaction under an inert atmosphere and agitating for 48 h at 28 °C. The reaction system was then transferred to a water-bath and 20.0 mL of dichloromethane/MeOH solution (1:1 v/v) was added.[19] The resulting solution was fractionated in a separating funnel with 2.0 mol L^{-1} HCl until pyridine had been completely removed. The organic solution was then washed with 10.0 mL of water and dried.

Structural characterization of lignin

The obtained lignins were characterized by Fourier transform infrared spectroscopy (FTIR; Bomem MB-102; ABB, Zurich, Switzerland), thermogravimetric analysis (TGA; TGA-Q50; TA Instruments, New Castle, DE, USA), and matrix-assisted laser ionization-time of flight mass spectrometry (MALDI-TOF MS; Bruker Autoflex Speed, Bruker Daltonics, Germany) and the morphology was evaluated by scanning electron microscopy (SEM; Philips XL-30 FEG; Philips, Amsterdam, the Netherlands).

Preparation of lignin/neem colloidal nanoparticles and silica-based biopesticide microparticles

The nanoparticles were prepared as a colloidal dispersion in an aqueous medium containing biopolymer nanoparticles. Good results were obtained when the nanoparticles were formulated with neem oil (3.5 g), the F-EtOAc fraction of the neem extract (0.3 g), lignin (1.5 g) and a surfactant (Tween®80, 0.67 g), using the nanoemulsion/solvent displacement method and the interfacial deposition nanoencapsulation process.[20] Colloidal silicon dioxide (Aerosil®200, 1.5 g) was then added to the colloidal suspension and homogenized with magnetic stirring. This mixture was fed into a spray-dryer (Büchi B-290, Büchi Labortechnik, Switzerland) operated using a 0.7 mm atomizer nozzle, with air as the atomizing gas and a sample feed rate of 0.75 mL min^{-1}, an atomizing air flow of 530 L h^{-1} at a 90% capacity aspirator (38 m^3 h^{-1}), and with inlet and outlet temperatures of 120 ± 4 and 60 ± 4 °C, respectively.

Characterization of microparticle spray-dried powder

The yields were calculated by summing the weights of all the components in the colloidal suspensions, omitting the water content. The total amount of marker azadirachtin in the microparticles was determined by HPLC.[20] Samples (25.0 mg) were dispersed in 5.0 mL of acetone for 60 min at room temperature with constant stirring. After dissolution of the polymer, 1.0 mL of this solution was centrifuged at 18,500 × g for 30 min at 25 °C; 0.5 mL of the supernatant was then dried under vacuum, solubilized in 0.5 mL of methanol, and determined by HPLC. Chromatographic analyses were carried out using an Agilent 1200 Series system with a Zorbax Eclipse XDB C_{18} column (150 × 4.6 mm internal diameter, 5 µm particle size), using H_2O:ACN (65:35, v/v) as the mobile phase, an oven temperature of 30 °C and a flow-rate of 1.0 mL min^{-1}, followed by UV detection at 217 nm.

The surface morphology of the microparticles in the powder was determined by SEM (Philips XL 30 FEG). The samples were sprinkled onto SEM stubs covered with double-sided carbon tape and then sputter-coated with gold. The photomicrographs were obtained at magnifications ranging from 1000 to 50,000 times.

Photo and thermal stability assay of lignin microparticles

The microparticles in the powder were stored for 120 h inside a controlled environment chamber. Two samples of non-encapsulated neem extracts were used as controls. The first control was exposed to the environment as microparticle samples; the other control was kept inside the chamber and protected from UV radiation. Other neem extracts were formulated, together with additives for photochemical protection, such as Tinogard TL (BASF,

Mississauga, Canada) (0.1% m/m) and Mirustyle X-HP (Croda, Yorkshire, UK) (2%, m/m).

The samples were incubated in a chamber irradiated with four UV lamps (Philips 15 W actinic lamps; l = 45.0 cm; w = 2.6 cm; Philips) under constant irradiation (6153 mW m^{-2}) at an energy of 22.15 kJ m^{-2} h^{-1} at 30 ± 2 °C. The samples were spotted 25 cm away from the UV lamps and stirred every day to maintain a uniform exposure to both humidity and UV radiation. The chamber was totally covered by mirrors (l = 60.0, h = 40.0, and w = 60.0 cm). Samples were frequently withdrawn at specific time intervals for stability analysis. Thermal analyses were carried out in an oven at 60 °C. We used a non-encapsulated neem fraction at the same oven temperature and at room temperature as controls. For both assays, the samples were removed successively at specific times and analyzed by HPLC.[20]

Efficacy studies using biological assays

The biological efficacy of the formulated products was evaluated against *Spodoptera frugiperda* and *Diatraea saccharalis*. Both assays were carried out at the Bioassay Laboratory, Department of Chemistry, UFSCar, at 25 ± 1 °C, a relative humidity of 70 ± 5%, and with a photo period of 12 h.

The *S. frugiperda* diet was prepared as described by Kasten *et al.*[21] with the studied products added at the end at a temperature of about 50 °C. Each treatment consisted of a mass of formulated product corresponding to a known quantity of azadirachtin (the active compound). Controls were prepared using empty microparticles containing only the neem F-EtOAc fraction or water. The experiment was performed according to Matos *et al.*[22] The duration of the larval and pupal phases, pupal mass and mortality were evaluated. The data were evaluated using an analysis of variance technique (ANOVA). Comparison between the mean values of each treatment (n = 15) was carried out using the Tukey test at a 95% confidence interval. All assays were evaluated daily.

D. saccharalis caterpillars were collected in corn and sugarcane crops and were kept in glass tubes (2.5 × 8.5 cm) on a specific diet.[23] The insects were submitted to two different biological assays, supplied either in an artificial diet or in rhizomes of sugarcane. The assay using an artificial diet described by Hensley and Hammond,[24] followed the same methodology as for *S. frugiperda*. The second assay was performed using larvae (2nd instar) reared in plastic flasks (200 mL) and fed with rhizomes of sugarcane (10 × 3.0 cm) previously sprayed with the formulated products or controls. Each treatment was carried out using 15 insects (n = 15).

Results and Discussion

Lignin extraction

Micro- and nano-structured lignin particles are an interesting class of biopolymer, not only from an environmental point of view, but also as a result of their aryl characteristic composition. The properties of biopolymers obtained from lignins depend on the heterogeneous monomeric composition of the initial plant matrix and on the extraction method. The variable phenylpropanoidic structure and size are responsible for a general hydrophobic behavior, which could result in better efficiency for the encapsulation of nonpolar compounds. However, it is also possible to chemically modify the polar hydroxyl groups to tune the hydrophobicity, depending on the application.[25,26] The phenolic acid moieties present in lignin structures are known for their antioxidant properties and may also provide photo and thermal stability, properties that are of great interest regarding to prolonged and controlled release.[27,28]

Using the alkaline method described, the total yield of precipitated lignin (Lig-Alk) was 7.2% (m/m) according to sugarcane bagasse weight. The process also produced a final hazard-free liquid residue consisting only of saline water. The lignin obtained was a brown powder, which was used for further chemical modifications. Acetylation on lignin (Lig-Ac) was performed with overall yield of 86.1%, corresponding to 6.2% (m/m) of original biomass. Oxidized lignin (Lig-Ox) was obtained in total yield of 5.5% (m/m) after precipitation (overall yield of 76.4%), less than Lig-Ac, which might be related to partial hydrolysis of some acid-labile acetyl groups.

Lignin characterization

All samples were characterized to evaluate the different properties arising from each treatment. Lignins with functional groups might have different interaction mechanisms with the active compounds to be encapsulated, resulting in different liberation kinetics and biological activity.

As a starting point, we evaluated the feasibility of MALDI-TOF MS for the determination of the size of Lig-Alk (Figure 1); this revealed an oligomeric molecular pattern. No distinguishable signal was observed below *m/z* 1000; the most intense peak was at *m/z* 1054.7351 indicating a pentamer. Lower intensity peaks within the cluster also indicated minor amounts of other monomers and substitutions, highlighting the complexity of the composition of the matrix. Higher order lignin oligomers were also observed in lower intensities, with major

increments between the most intense peaks of each cluster found 210 Da apart, indicating a guaiacyl-syringyl lignin composition with mono- and dimethoxylated monomers and coniferyl and sinapyl alcohols, in agreement with the composition of non-woody angiosperm lignin and corroborating the analysis of sugarcane using other methods.[29]

FTIR analyses are useful in confirming the success of acetylation and oxidation reactions in promoting structural modifications.[26,30] The FTIR spectra (Figure 2) showed the different profiles among alkaline, acetylated and oxidized lignin samples. As expected, the intensity of the O–H stretching around 3400 cm^{-1} was increased in oxidized lignin as a result of the formation of new oxygenated functional groups; it was lowered in the acetylated samples as a result of esterification. Although there was a sharp band in Lig-Alk in the 2800-3600 cm^{-1} region related only to the hydroxyl group, both other lignins showed a very broad signal, characteristic of substances bearing hydroxyl and/or carboxyl functional groups, especially in Lig-Ox, thus confirming the changes. An increase in the intensity of bands around 1700 cm^{-1} related to C=O stretching in both Lig-Ox and Lig-Ac, with the latter showing a small red shift compared with the former, indicates that the bond vibration corresponds to different classes of compounds: esters and carboxylic acids, respectively. A stronger band at 1230 cm^{-1} attributed to C–O stretching confirms the increase in carbonyl groups and new C–O bonds in both the oxidized and acetylated samples, particularly the latter.

TGA analysis revealed similar profiles for all samples and showed two major thermal events. The first occurred at temperatures ranging between 150 and 250 °C and is related to an increase in the oxygen content and a decrease in the hydrogen content, suggesting the oxidation of the aliphatic portions of the molecule. The second event occurred at temperatures of 300-480 °C and showed an increase in carbon content, with the increased liberation of volatiles such as H_2O, CO, CO_2, CH_3OH and CH_4 (Figure 3).[31]

Similar thermal behavior was observed in all samples indicating analogous structural features. It is evident from differential thermogravimetric (DTG) analysis that Lig-Ac has its maximum weight loss in slightly lower temperatures when compared to both Lig-Alk and Lig-Ox. This probably occurs due to the increased volatility of acetylated molecules compared to free hydroxyls. In contrast, the oxidation process enhances the thermal stability of Lig-Ox, as can be inferred from the increase in the temperature of both thermal events in these samples. Nevertheless, highest mass loss is observed for Lig-Ox after the first event. To the best of our understanding, this observation is due to the

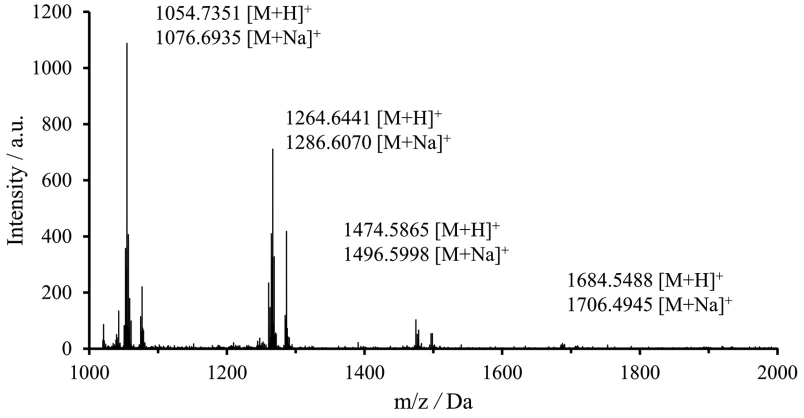

Figure 1. MALDI-TOF mass spectrometry profile of sugarcane lignin (Lig-Alk) sample extracted through a soda-pulping process.

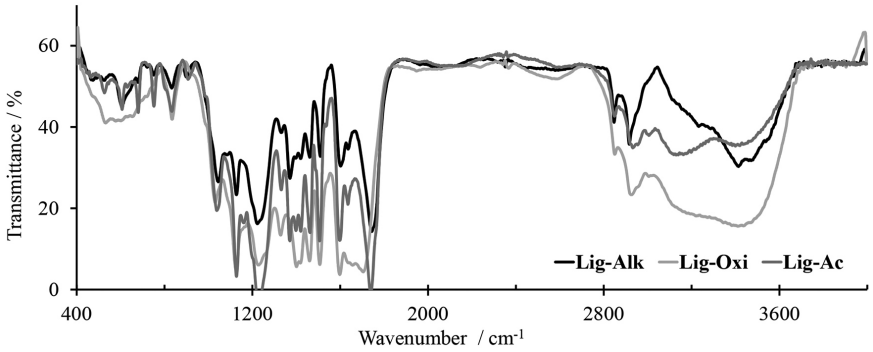

Figure 2. FTIR spectra of extracted lignin from sugarcane (Lig-Alk) and chemically modified lignins (Lig-Ox and Lig-Ac).

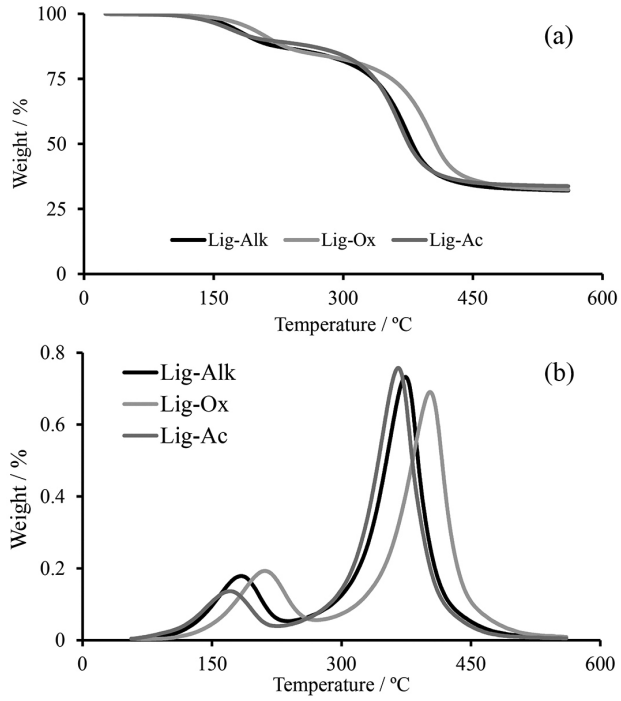

Figure 3. (a) Thermogravimetric analysis (TGA); (b) differential thermogravimetric (DTG) of obtained lignins.

fact that the maximum percentage is calculated according to the initial mass of the sample. Given the fact that the Lig-Ox have already been oxidized, additional oxygen atoms promotes slightly increase in its mass, therefore, increasing the mass of the remaining product.

The largest discrepancy among the samples was observed at the onset temperature of the second event, where Lig-Ox showed a 20-30 °C increase compared with the other samples, again suggesting an enhancement in the thermal stability. However, their T_{max} values are close enough to indicate that, after the thermal reactions began, similar reaction behavior occurred in all the samples. It is known that the thermal stability of lignins arises mainly from hydrogen bonding.[32] The lignins in this study showed low thermal stability compared with other studies, with values similar to other materials rich in guaiacyl-syringyl moieties,[33] further confirming the characteristics of the material. Oxidized lignins have shown slightly higher values for both the onset and maximum temperature of the two thermal events, which may be related to the increased thermal stability of the lignin as a result of new classes of compounds that can form hydrogen bonds. These results are in agreement with other experiments showing an increase in thermal events in so-called thermostabilized lignins that had undergone thermal oxidation.[31]

The morphology of the lignins was analyzed by SEM (Figures 4A1-C1). The photomicrographs show rough surfaces for Lig-Alk and Lig-Ox (Figures 4A1 and 4B1, respectively), indicating that the oxidation process did not result in strong morphological modification. However, a clear morphological change was seen in the acetylated sample (Figure 4C1), which showed a smoother surface than the other two lignins. This may be the result of a larger number of van der Waals interactions for polymers with complex nonpolar characteristics.

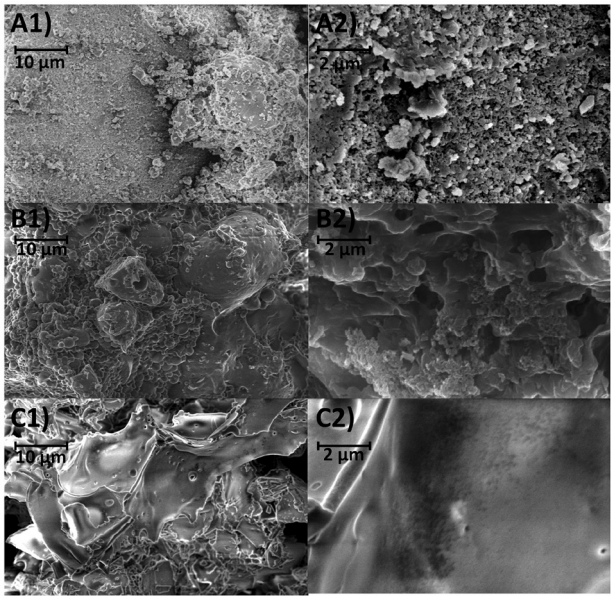

Figure 4. Scanning electron micrographs of lignins (A1) Lig-Alk; (B1) Lig-Ox; and (C1) Lig-Ac; and lignin microparticles loaded with neem extracts formulated with (A2) Lig-Alk; (B2) Lig-Ox; and (C2) Lig-Ac. 1, 2,000×; and 2, 10,000×.

Collectively, these results demonstrate the plethora of options which can be used to confirm the chemical modification of the sugarcane lignin structure, allowing the tuning of several desirable parameters, such as hydrophobicity and a microporous structure. This material was then used to produce nano- and microparticles loaded with neem extracts. The various characteristics allowed us to correlate several structural and chemical characteristics with the performance of the final encapsulated bioproducts.

Preparation and characterization of silica/lignin/neem microparticles EtOAc

The F-EtOAc fraction of neem was used to prepare the microparticles, so as to enable control of the azadirachtin content in the formulated products. This allowed us to carry out the biological assays with the same dose of active compound, so that a direct result was obtained with respect to the activity and content of the active compound (azadirachtin), irrespective of whether it was encapsulated. The F-EtOAc neem fraction and oil neem contained 556.4 and 195.0 mg kg^{-1} azadirachtin, respectively, and the

dose of products applied in the biological assays ranged from 2.0 to 5.0 μg azadirachtin. All the microparticles prepared in powder form showed an absolute recovery for azadirachtin of > 96% as well as high encapsulation efficiency, indicating almost no loss of the active compound during the micro-encapsulation process, regardless of the matrix used. The quantity of azadirachtin in the final microparticles powder were 56.3, 62.0, and 65.6 mg kg^{-1} to formulations using Lig-Alk, Lig-Ox, and Lig-Ac, respectively. Quantitative analyses were performed by fitting the value of area produced by the analyte azadirachtin (retention time: 10.28 minutes) in each chromatogram in a calibration curve constructed by using linear regression of a set of solutions of azadirachtin standard in six different concentrations, following previously described methodology.[15,20] Chromatograms used in quantification are displayed in Figure 5.

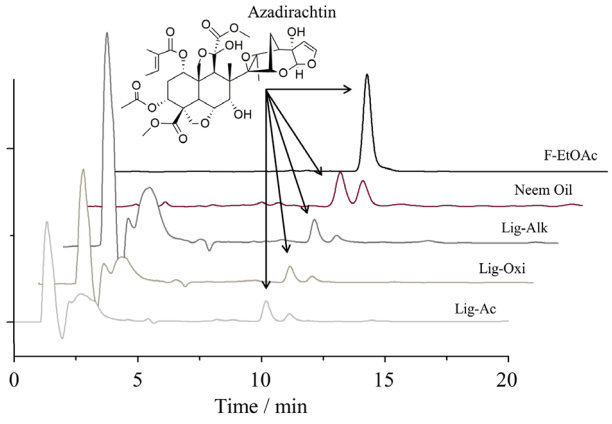

Figure 5. HPLC analyses of azadirachtin in samples of F-EtOAc, neem oil and formulations with Lig-Alk, Lig-Oxi and Lig-Ac.

All the powder materials contained clusters of spherical microparticles with particle sizes ranging between 1 and 2 μm. The microparticles of Lig-Alk (Figure 4; A2) were more homogeneous than those of Lig-Ox (Figure 4; B2) and Lig-Ac (Figure 4; C2); the latter presented as layers rather than spheres.

Photo and thermal stability gain assays

The loaded microparticles were submitted to photo and thermal stability tests to determine their efficiency in protecting the active principle from environmental stress (Figure 6). The neem F-EtOAc fraction was extremely sensitive to both thermal and photo degradation, with around 30 and 50% of the active compound consumed within 120 h, respectively. Commercial additives were not effective in promoting azadirachtin resistance. However, when azadirachtin was encapsulated using lignins, excellent results were observed and the formulations were able to sustain photodegradation with almost no loss in active content (Figure 6a). The formulations without additives showed better protection than those with commercial protectants, indicating that the microparticle itself behaves as an excellent photo protectant. With respect to the thermal stability, there was a loss of only 10-15% in the azadirachtin content of the microparticles, similar to the control group at room temperature, whereas the non-formulated neem fraction had a thermal degradation nearer to 50% (Figure 6b). These results show that the production of lignin microparticles enhanced the photo and thermal stability of azadirachtin formulations.

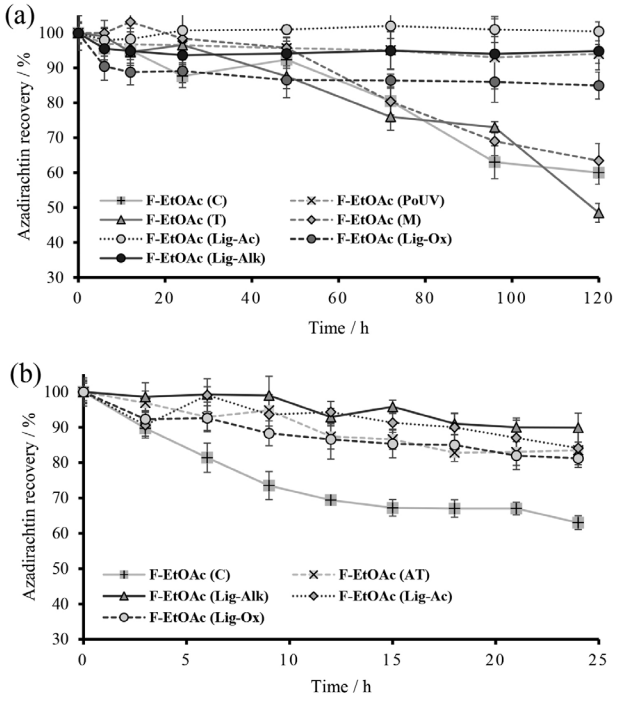

Figure 6. (a) Photo stability and (b) thermal stability of microencapsulated azadirachtin and control sample. F-EtOAc, ethyl acetate neem fraction; C, control; PoUV, protected from UV radiation; T, Tinogard; M, Mirustyle; AT, ambient temperature.

Biological assays

All formulations were assayed against *S. frugiperda* and *D. saccharalis*, highly polyphagous pest insects known to devastate entire plantations, causing severe damage in economically important crops such as corn, rice, sorghum and sugarcane. Each diet was assembled to have final azadirachtin concentrations of 2.0 or 5.0 μg kg^{-1}. Table 1 gives the results for the *S. frugiperda* assay, with mean values for the larvae, pupal phase and pupal mass of *S. frugiperda* fed with an artificial diet and treated with lignin formulations.

The pure lignins used for encapsulation did not show any appreciable activity. The insects fed on a diet containing

Table 1. Mean duration of larval and pupal phase and pupal mass of *S. frugiperda*

Treatment (μg of azadirachtin)	Mean ± SE duration of larval phase / days	Mean ± SE duration of pupal phase / days	Mean ± SE pupal mass / mg
Lig-Alk (5 μg kg^{-1})	26.9 ± 9.75^{cd}	0	0
Lig-Alk (2 μg kg^{-1})	31.1 ± 5.81^{c}	0	0
Control Lig-Alk	13.2 ± 2.86^{d}	9.65 ± 1.38^{a}	92.6 ± 54.1^{a}
Lig-Ac (5 μg kg^{-1})	26.5 ± 11.4^{c}	0	0
Lig-Ac (2 μg kg^{-1})	29.3 ± 8.69^{c}	0	0
Control Lig-Ac	34.1 ± 8.77^{bc}	9.00 ± 2.09^{a}	67.3 ± 19.8^{b}
Lig-Ox (5 μg kg^{-1})	28.9 ± 8.24^{cd}	0	0
Lig-Ox (2 μg kg^{-1})	33.4 ± 10.0^{c}	0	0
Control Lig-Ox	16.1 ± 5.33^{d}	8.95 ± 1.56^{a}	128 ± 31.4^{a}
F-EtOAc (5 μg kg^{-1})	33.6 ± 8.56^{c}	0	0
F-EtOAc (2 μg kg^{-1})	39.4 ± 12.6^{bc}	0	0
Control F-EtOAc	12.6 ± 3.67^{d}	9.80 ± 1.2^{a}	139 ± 40.2^{a}

Temperature: 25 ± 10 °C, relative humidity: 70 ± 5%, photophase: 12 h; SE: standard error; the letters relate to the Tukey's test at 5% probability.

pure lignins reached the adult phase with similar values of pupal days and mass, except for Lig-Ac, which, although it did not kill the larvae, hindered their growth and lowered their final mass, confirming that the polymer did not show any insecticidal activity on *S. frugiperda*. An artificial diet containing both non-encapsulated fraction (neem F-EtOAc) and microencapsulated formulations displayed similar activities, resulting in a prolonged larval period and 100% mortality. This demonstrates that the microcapsules are able to retain the insecticide in sufficient quantities to promote the desired activity, while retaining their enhanced photo and thermal stabilities.

For *D. saccharalis*, two different assays were performed to simulate diverse field conditions. The first assay was carried out using an artificial diet at nominal azadirachtin contents of 2 and 5 μg; in the second experiment, formulations with 5 μg of azadirachtin were sprayed into sugarcane pieces before exposing the plant to the insect (Figure 7).

In the first experiment, complete mortality of insects fed with Lig-Ac formulations was observed within 8 days at both concentrations and with 12 days for the Lig-Alk and Lig-Ox formulations. The enriched fractions were as effective as the lignin formulations in achieving complete mortality, but only after 16 days; insects from the control experiments reached the adult phase. In the second experiment, in which insects were fed with sugarcane chunks doped with the formulations, a prolonged life cycle was seen for *D. saccharalis*. Although the control group was not able to complete its life cycle, even after the completion of the experiment (60 days), there was no mortality among the insects of this group. All the other assays achieved 100% mortality. The activity of the

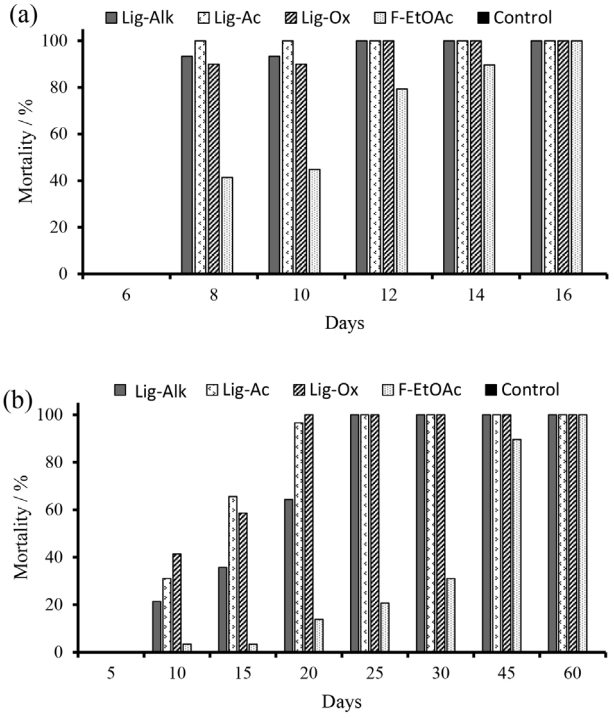

Figure 7. Assay for *D. saccharalis* using (a) artificial diet; (b) sugarcane rhizomes as the food source.

formulations was superior to that of the positive controls (neem fraction not encapsulated), achieving complete mortality in a maximum of 23 days. In this assay, both Lig-Ox and Lig-Ac formulations showed similar efficacy, reaching complete action in 20 days, whereas the Lig-Alk formulations took 25 days. For comparison, at the same time, treatment with enriched fractions showed only 20% mortality, reinforcing the efficiency of the biologically based microparticle formulations of azadirachtin.

Conclusions

In summary, sugarcane bagasse lignin was extracted using a rapid and residue-free process, with an acceptable overall yield. Chemical modifications in the structure showed the feasibility of tuning the physical and chemical properties. Analytical characterization using several different techniques proved to be efficient in elucidating the physicochemical structure of the polymers and in improving our understanding of the relation between chemical modifications and microparticle behavior. High encapsulation efficiency was obtained for azadirachtin regardless of the lignin used and the method of preparing the formulations was successful, generating microparticles that greatly improved the thermal and photo stability of the active component. Assays conducted in two important crop pests proved the enhancement in the efficacy of the insecticidal activity of azadirachtin with respect to controlled release and the avoidance of degradation. This technique opens up a new path for the development of completely green pesticides made from biodegradable polymers and biopesticides for the safe and efficient control of pest insects in agriculture.

Acknowledgments

The authors would like to thank the CNPq (National Council of Technological and Scientific Development, 477051/2012-0; 301427/2012-7, 563063/2010-6), FAPESP (São Paulo Research Foundation 2011/11860-5; 2014/50827-1; 2011/00970-4; 2010/52312-8), INCT-Biorational Control of Plague Insects, CAPES (Coordination for the Improvement of Higher Level of Education Personnel), and Prof Edson Rodrigues Filho (LABioMMi) for support during several different projects.

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Submitted: January 31, 2016
Published online: May 19, 2016

FAPESP has sponsored the publication of this article.