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PREPARATION AND CHARACTERIZATION OF LIGNIN MICROPARTICLES-IN-ALGINATE BEADS FOR ATRAZINE CONTROLLED RELEASE

--Manuscript Draft--

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Response to Reviewers:	Reviewer 1 The paper is highly recommended for publication in this journal but please consider the following comments and suggestions: 1. p.10 line 236 - "The dilution factor was taking..." - change taking into taken As the reviewer mentioned, the term “taking” was changed by “taken”. 2. p.11 line 244 - "Four atrazine standards....." - but you mentioned calibration (1, 5, 30, 60, 100 mg/L) in line 245 - kindly check and make necessary correction by mentioning "Five atrazine standards" instead of four.

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I N T E C

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Editor-in-Chief

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Thank you for your mail regarding our manuscript: "Preparation and characterization of lignin microparticles-in-alginate beads for atrazine controlled release", by C. A. Busatto, M. E. Taverna, M. R. Lescano, C. Zalazar and D. A. Estenoz, submitted for publication in Journal of Polymers and the Environment.

The manuscript now includes all of the last reviewer suggestions.

Hoping that you will now find this final version acceptable for publication, please receive my kind regards.

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PREPARATION AND CHARACTERIZATION OF LIGNIN MICROPARTICLES-IN-ALGINATE BEADS FOR ATRAZINE CONTROLLED RELEASE

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Abstract

The use of lignin as polymeric matrices for controlled release systems in agriculture is a promising alternative for its revalorization. In this work, different atrazine delivery systems were studied. Lignin derived from ionic isolation was used for the preparation of atrazine-loaded microparticles by the solvent extraction/evaporation and microfluidic techniques. Microparticles were also encapsulated in sodium alginate beads. Lignin microparticles prepared by microfluidics presented a larger particle size, higher encapsulation efficiency and a narrow size distribution. The *in vitro* release of atrazine was evaluated in water. Atrazine release from microparticles prepared by the solvent extraction/evaporation technique showed a significant burst release, and this effect was reduced by incorporating microparticles within alginate beads. In addition, the phytotoxicity of the systems was evaluated employing *Lactuca sativa* seeds. The phytotoxicity results showed that lignin-based formulations are safe according to the parameters evaluated, in contrast with commercial atrazine that resulted phytotoxic.

Keywords: lignin; microparticles; atrazine; controlled release

1. Introduction

Pesticides are widely used in agriculture to protect crops from potential yield losses. In commercial pesticide formulations, most of the active principle is lost to the environment and less than 1% remains on the target [1]. This fact has increased the interest in developing efficient and safe formulations to reduce the harmful effects of pesticides on the environment [1, 2]. In this direction, controlled release formulations present several advantages over traditional commercial formulations, such as reduction of pesticide use, protection of pesticides from environmental degradation, increased safety for users and the environment, and reduced leaching of pesticides in soil [3, 4]. The conventional encapsulation techniques for pesticides include ionotropic gelation [5], complex coacervation [6], *in situ* polymerization [7, 8], interfacial polymerization [9], nanoprecipitation [10], and solvent extraction/evaporation [11]. However, the size of particles is difficult to control and the size distribution can be broad via mechanical stirring, homogenization or ultrasonication [12]. Dispersion in size and morphology of the carriers may produce undesirable variation in the rate of particle degradation, drug stability, and the kinetics of drug release [13], decreasing the effectiveness of formulations. Fabrication of polymeric microparticles with controlled size and morphology can be achieved by microfluidics [14]. Using microfluidic devices, streams of immiscible fluids can be combined to generate highly monodisperse emulsion droplets, which allow a precise control over the size of final particles. Microfluidic is a promising technique for the development of pesticide delivery systems because it offers low-cost and easy-to-use platforms [13].

Atrazine is a triazine herbicide commonly used to prevent pre- and postemergence broadleaf weeds in crops [15]. However, several animal studies have related atrazine with a wide range of adverse health effects, including reproductive disruption and cancer [16, 17]. Based on the available evidence, the European Union banned the use of atrazine in 2004 although this herbicide is employed in many countries. Atrazine can be detected in soil, surface water, and groundwater at concentrations exceeding its maximum permissible limit [18]. Detection of atrazine in water is related to its intense usage, moderate persistence and mobility through the soil [19].

Several authors studied the herbicidal activity and phytotoxicity effects of atrazine delivery systems in plants and seeds compared to a commercial formulation. Grillo et al. [20] studied the incorporation of atrazine into poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) microspheres. The i*n vitro* experiments indicated that atrazine-loaded PHBV microspheres produce lower genotoxic effects than free atrazine in *Lactuca sativa* plants. Pereira et al. [11] prepared nanoparticles of poly(ε-caprolactone) (PLC) containing atrazine and evaluated their herbicidal activity and genotoxicity. The nanoparticle formulations were shown to be effective for the control of the target species and demonstrated that they were able to reduce the genotoxicity of the herbicide. Oliveira et al. [21] demonstrated that encapsulation of atrazine in PCL nanocapsules not only maintained the action mechanism of the herbicide, but also potentiated its herbicidal activity against mustard plants when compared with the effects of the commercial atrazine product. Thus, it was possible to reduce the atrazine dosage, without compromising the biological activity of the herbicide. De Oliveira et al. [22] prepared solid lipid nanoparticles containing atrazine and simazine. The results indicated that the formulations were more effective, compared to the commercial formulation, and caused no toxicity in non-target organisms (Z. *mays* plants and mouse

fibroblast cells). Chen and Wang [23] prepared atrazine-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) by the emulsion-solvent evaporation method. The results demonstrated that PLGA-NPs had a high encapsulation efficiency and slow release rate. Also, Sharma et al. [24] studied a nanosorbent for atrazine release based on hydroxyapatite, obtaining promising results related to the reduction of agricultural runoffs.

Andrade [25] investigated the effects of nanoencapsulated atrazine compared to free atrazine on biomarkers of the freshwater teleost *Prochilodus lineatus,* concluding that the exposure to free atrazine promoted changes in a greater number of biomarkers compared to encapsulated atrazine and thus indicating that the nanoencapsulation of the herbicide protected the animal from the effects of atrazine.

In recent years, natural polymers have gained considerable acceptance over synthetic polymers as matrices for controlled release formulations because of their eco-friendly nature, cost effectiveness, easy availability, and biodegradability [26]. The most commonly used are sodium alginate [27], cellulose [28], lignin [29, 30, 31], starch [32], and chitosan [33]. Among synthetic polymers, PCL [21], polylactic acid [12], poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate-valerate) (PHBV) [34] are the most frequently used matrices for pesticide encapsulation.

Lignin is the second most abundant polymer from biomass after cellulose and is the largest renewable source of aromatic groups in nature [35]. Nowadays, most commercial lignins are obtained as a by-product from lignocellulose treatments performed during pulp and paper processing [36]. Isolation of lignins using ionic liquids is a promising alternative because it is an ecofriendly method and they promote selective extraction of selected components. The physico-chemical characteristics of the different lignins can vary noticeably depending on the original source and extraction

method used [37]. The use of lignin for the synthesis of novel polymeric materials is the most promising alternative for its revalorization. In the last decades, several authors have reported the use of lignin as matrices for controlled release formulations of pesticides. In our previous work, the preparation of organosolv and ionic lignin microparticles for the controlled release of atrazine was studied [29]. About 98% and 95% of atrazine was released in 24 and 48 h approximately from organosolv and ionic lignin microparticles, respectively. In addition, atrazine mobility experiments in soil showed that atrazine-loaded microparticles could reduce leaching compared to a commercial formulation of free atrazine. In order to improve the efficiency of formulations by controlling the morphology of the particles and increasing the release time, different preparation techniques and matrices need to be evaluated. On the other hand, it is important to investigate the phytotoxicity of formulations to assess their safety and herbicidal activity.

In the present work, lignin derived from spruce and obtained from ionic isolation process was used for the preparation of atrazine-loaded microparticles. Lignin microparticles were prepared by the solvent extraction/evaporation and microfluidic techniques. Microparticles were also encapsulated in sodium alginate beads. The systems were characterized in terms of particle size and distribution, morphology, drug encapsulation efficiency and swelling behavior. The *in vitro* release of atrazine was evaluated in water. In addition, phytotoxicity of the systems was evaluated employing *Lactuca sativa* seeds in comparison with a commercial free atrazine formulation.

2. Experimental work

2.1 Materials

Ionic lignin from spruce was obtained through a patented isolation method that employs ionic imines. It was kindly provided to us by Dr. Stefan Saur and Prof. Dr. Willi Kantlehner from Hochschule Aalen (Aalen, Germany). Atrazine commercial formulation (≥ 90%, SYNGENTA) was used for control release and germination index experiments. Atrazine standard (98%, Chem Service Inc., USA) was used for High Pressure Liquid Chromatography (HPLC) calibration. The following chemical reagents were used as received: sodium alginate (NaAg, Todo droga), calcium chloride ($CaCl_2$, Sigma-Aldrich), polyvinyl alcohol (PVA; 205 kDa; 87.7% hydrolyzed; Sigma Aldrich), dichloromethane (DCM; Anedra), methanol (Cicarelli), tetrahydrofuran (THF, Cicarelli), acetonitrile (HPLC grade, Sintorgan), glacial acetic acid (analytical reagent, Anedra). Ultrapure water (0.055 μS cm^{-1}) was used to prepare all solutions. This water was obtained from an OSMOION purification equipment.

2.2 Characterization of lignin

The moisture, ash content, elemental composition and polymer molar mass distribution of ionic lignin were determined as detailed in our previous work [29].

The moisture and ash content were determined gravimetrically. Elemental composition including carbon (C), hydrogen (H), sulphur (S), and nitrogen (N) were performed by an elemental CHNSO analyzer (SerieII, Perkin Elmer). For polymer mass distribution, a Waters 1525 chromatograph fitted with an automatic injector (Waters 717plus) was used. The chromatograph was fitted with a set of Waters Styragel HR 4 E 7.6 × 300 mm columns and a differential refractometer detector (Waters 2414). The carrier solvent was THF at 1 mL min^{-1} and the system was operated at 25 ºC. Dry samples were dissolved

in 0.25 mL THF with a nominal concentration of 1 mg mL^{-1}. Injection volumes were 200 μL. Polyethylene glycol standards were used for the calibration.

2.3 Preparation of lignin microparticles by the solvent extraction/evaporation technique

Microparticles were prepared according to the solvent extraction/evaporation method described by Taverna et al. [29]. Briefly, ionic lignin (30 mg) was dissolved in 3 mL of DCM and the suspension was filtered through a 0.45 μm nylon microfiltration membrane (Microclar, Argentina) to remove the undissolved lignin. Atrazine standard was added to the solution at a concentration of 20% w/w with respect to the polymer, and the dispersion was sonicated for 1 min. Then, the solution was dropped onto a aqueous solution of PVA (1% w/v) and stirred at 500 rpm using a homogenizer (Kinematica Polytron PT 2500e, Switzerland) for 5 min. Afterwards, 70 mL of 0.3% w/v PVA solution was added and the emulsion was stirred for 30 min. The organic solvent was removed using a rotary evaporator (Büchi EL 130, Germany) for 2 h at room temperature. Solid microparticles were collected by centrifugation using a Hettich Universal 16 centrifuge (Germany) at 2000 rpm for 3 min, washed with water twice and lyophilized using a Telstar Cryodos 80 lyophilizer (Spain). Dried microparticles were stored in a desiccator at room temperature until further analysis.

2.4 Preparation of microparticles by microfluidics

Microparticles were prepared using a co-flow microfluidic device as described by Busatto et al. [38]. The system consisted of two concentric capillary tubes (tube 1 and tube 2) and a T-junction (Fig. 1). The fused silica tube 1 (inner diameter: 75 μm, outer diameter: 148 μm) associated with adjusted tubing sleeves (1/16'', inner diameter of 180 μm) was inserted into the T-junction (1/16'') along its main axis. This tube crosses the T-joint and ends in the center of the fused silica tube 2 (inner diameter: 250 μm, outer diameter: 356 μm). The T-junction allows the injection of the dispersed liquid

phase into the continuous phase. The continuous phase consisted of a PVA solution (1% w/v). For the preparation of the dispersed phase, ionic lignin (30 mg) was dissolved in 3 mL of DCM and the suspension was filtered through a 0.45 μm nylon microfiltration membrane (Microclar, Argentina) to remove the undissolved lignin. Atrazine standard was added to the solution at a concentration of 20% w/w with respect to the polymer, and the dispersion was sonicated for 1 min. The dispersed and continuous phases were injected using syringe pumps and the flow rates were 17 μL min^{-1} and 21 μL min^{-1}, respectively. The resulting emulsion was collected in a beaker containing 100 mL of ultrapure water and then placed in a rotary evaporator (Büchi EL 130, Germany) for 2 h at room temperature to evaporate the remaining solvent. The particles were then separated by centrifugation at 500 rpm for 2 min, and washed twice with ultrapure water. Finally, microparticles were lyophilized and stored for further analysis.

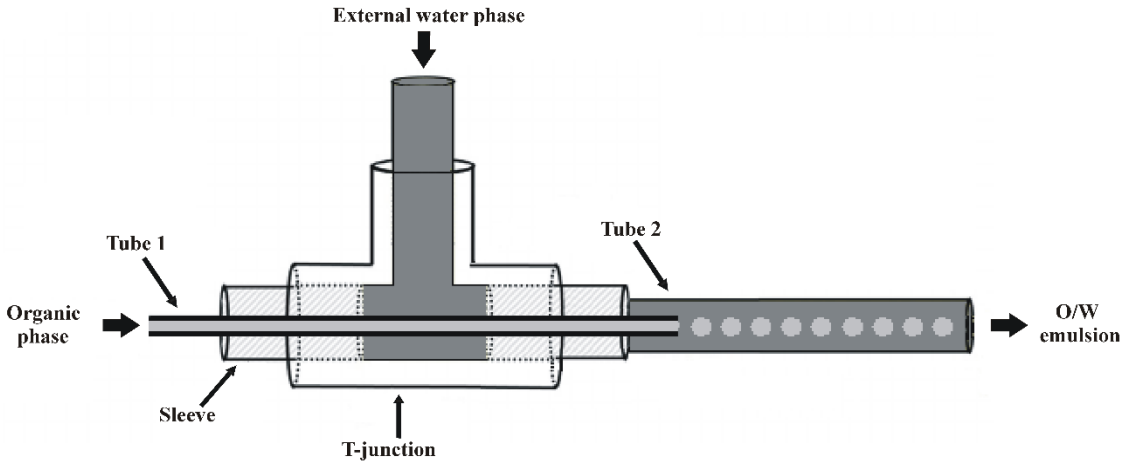

Fig. 1. Schematic representation of the microfluidic device used for lignin microparticles preparation.

2.5 Preparation of alginate beads containing lignin microparticles

About 4 mg of atrazine-loaded microparticles were dispersed in 0.5 mL of ultrapure water and vortexed during 1 min. Then, the dispersion was mixed with 1.5 mL of

sodium alginate solution in distilled water (2% w/v) and vortexed during 1 min. The resulting dispersion was added dropwise to a 30 mL gellant bath of 0.25 M $CaCl_2$ using a syringe pump. Alginate beads containing lignin microparticles were filtered, washed twice with ultrapure water and dried in an oven at 40 °C until constant weight.

2.6 Particle size determination

Microparticles were dispersed in water and observed in an optical microscope (DM 2500 M, Leica, Germany) coupled with a camera (LEICA DFC 290 HD). The mean particle diameter was determined using a free image processing program. Approximately 300 particles were measured for each formulation.

The average diameter of dry alginate beads was determined using a Leica S8 APO stereomicroscope (Leica AG, Wetzlar, Germany).

2.7 Particle morphology

The morphology of microparticles was studied by scanning electron microscopy (SEM). Samples were put over an aluminum stub and sputter coated with gold under argon atmosphere (SPI Supplies, 12157-AX) using soft conditions (two sputterings of 40 s each with an intensity of 15 mA). Microparticle morphology was examined using an acceleration voltage of 5 kV in a Phenom ProX microscope.

2.8 Swelling Kinetics

The water uptake was measured for alginate beads using a gravimetric procedure. A known mass of beads was placed in a Petri dish containing 10 mL of ultrapure water and incubated at 25 ºC. The swollen beads were removed at predetermined times and the excess water was blotted from the surface of the beads using filter paper. After that, they were weighted. Three replicates (5 beads in each replicate) were carried out. The swelling ratio (SR) was calculated by the following equation:

$$SR = \frac{W_s - W_d}{W_d} \cdot 100 \quad (1)$$

where W_s and W_d are the swollen and dry weight of hydrogel beads at time *t*, respectively.

2.9 Encapsulation Efficiency

Approximately 3 mg of microparticles were dispersed in 5 mL of methanol and stirred at 50 rpm during 24 h to extract the herbicide. Then, the samples were centrifuged at 2000 rpm during 3 min and atrazine concentration in the supernatant was measured by HPLC following the procedure detailed in the *In vitro atrazine release assays* Section. The assay was run in duplicate. Encapsulation efficiency (EE) was calculated as follow:

$$EE = \frac{A_e}{A_t} \cdot 100 \quad (2)$$

where A_e and A_t are the experimental and theoretical loads of atrazine in lignin microparticles, respectively.

2.10 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of atrazine and different lignins microparticles systems were acquired on a Shimadzu Model 8201 Fourier transform spectrophotometer in the frequency region of 4000-500 cm^{-1}. KBr pellets were prepared with 3 wt% of dry sample. Spectra were analyzed by Hyper IR software. Bands were assigned according to El Mansouri and Salvado [39] and Czaplicka et al. [40].

2.11 In vitro atrazine release assays

About 4 mg of atrazine-loaded microparticles, free or encapsulated in sodium alginate beads, were dispersed in 25 mL of ultrapure water and the vials were incubated at 25 °C. At predetermined times, 2 mL of samples were taken and replaced with an equal volume of water in order to maintain constant volume. The dilution factor was taken into account for calculations. Experiments were run in duplicate.

Atrazine quantification was performed by HPLC using an HPLC-UV/Visible Waters chromatograph equipped with an YMC-Triart C18 column (5 µm particle size, 4.6×250

mm; inner diameter × length) and a Waters 2489 UV–vis detector. Atrazine retention time was 5.32 min. The mobile phase consisted of an acetonitrile/water mixture (70:30 v/v) acidified with acetic acid at a flow rate of 1.0 mL min^{-1}. The column temperature and the detection wavelength were 25 °C and 221 nm, respectively. A calibration curve was performed in the 0–50 mg L^{-1} range. Five atrazine standards were used for calibration (1, 5, 30, 60, 100 mg L^{-1}). The limit of detection was 0.3 mg L^{-1} and the limit of quantification was 1 mg L^{-1}.

2.12 Phytotoxicity assays

Phytotoxicity assays were evaluated using an acute toxicity assay according to IRAM 29114 [41] and EPA 840.4200 [42] with slight modifications. Petri dishes with a diameter of 9.1 cm were used. A filter paper disc was placed at the bottom of each dish. 4 mL of beads dispersion or free atrazine solution in water were added and twenty seeds of the target species (*Lactuca sativa*) were sown in each Petri dish. In all experiments, a concentration of atrazine equivalent to 50 ppm was used in order to emulate an application rate of 2 kg ha^{-1}, which correspond to the recommended dose in field. In addition**,** distilled water was used as a control. The samples were run in duplicate. After a period of 3 days the root length of the germination seeds and the number of them were evaluated in terms of the Germination Index (GI) and the Elongation Root (RE). These parameters were analysed according to Ortega et al. [43], Zucconi et al. [44], Bagur-Gonzalez et al. [45] that proposed different toxicity tables for each of them.

GI and RE were calculated employing the following equations:

$$GI = \left(\frac{G}{G_c}\right) \cdot \left(\frac{RL}{RL_c}\right) \cdot 100 \quad (3)$$

$$RE = \frac{RL - RL_c}{RL_c} \quad (4)$$

where, G: number of germinated seeds in the sample; G_C: number of germinated seed in the control; RL: average root length in the sample; RL_C: average root length in the control.

Analysis of variance (one-way ANOVA) at 95% confidence level and subsequently Duncan multiple range test of average root length were carried out. Statistical analysis were performed using free software (R program version 2.3.3.3).

3. Results and discussion

3.1 Characterization of ionic lignin

According to our previous work [29], the ash and moisture content for ionic lignin were 0.6 and 23.34 wt%, respectively. Regarding elemental composition, C, N, S, H contents were 66.26, 0.26, 0.71 and 8.27%, respectively. The values obtained for $\overline{M}_w$ and $\overline{M}_n$ were 3718 and 768 g mol^{-1}, respectively. The values reported are in accordance with those reported in the literature [46].

3.2 Characterization of lignin microparticles and alginate beads

Lignin microparticles were prepared by the solvent extraction/evaporation (Exp. A) and microfluidic (Exp. B) techniques using similar experimental conditions. Table 1 shows the mean particle size and encapsulation efficiency of microparticles from Exps. A-E. The particle size was larger for microparticles prepared by microfluidics, and the coefficient of variation indicates a relative narrow size distribution compared to the conventional emulsion method. In addition, microparticles obtained by microfluidics presented higher encapsulation efficiency of atrazine probably due to differences in the diffusion rate of atrazine from the emulsion droplets to the aqueous phase during particle solidification. The emulsions prepared by the solvent extraction/evaporation method resulted in lower droplet size, decreasing the diffusion pathway of atrazine to

dissolve into the continuous phase, and thus resulting in lower entrapment efficiency. As it can be observed in Fig. 2, both microparticles exhibited a smooth surface and round shape. Microparticles prepared by microfluidics showed a better monodispersity in size.

Lignin microparticles were subsequently encapsulated into alginate beads in order to study the release behavior of these systems. Fig. 3 shows optical images of alginate beads (Exp. C) and alginate beads containing microparticles (Exp. D and E) prepared by both techniques. Alginate beads showed a smooth surface and a higher polydispersity than beads encapsulating microparticles. As it can be observed, microparticles were homogeneously distributed within alginate beads. Alginate beads containing microparticles presented a rough surface and greater sphericity probably due to the microparticles content. The particle size of alginates beads was not affected by the incorporation of microparticles.

Table 1. Characteristics of lignin microparticles and alginate beads

	Microparticles		**Alginate beads**	**Alginate beads containing microparticles**	
	Solvent evaporation	**Microfluidics**		**Solvent evaporation**	**Microfluidics**
Experiment	**A**	**B**	**C**	**D**	**E**
Particle size (μm)	25.3 ± 6.7	50.0 ± 3.0	999.3 ± 145.5	1061.5 ± 26.9	1025.1 ± 68.4
Coefficient of variation (%)	26.5	6.1	14.6	2.5	6.7
Encapsulation Efficiency (%)	47.4 ± 3.5	59.7 ± 8.2	-	-	-

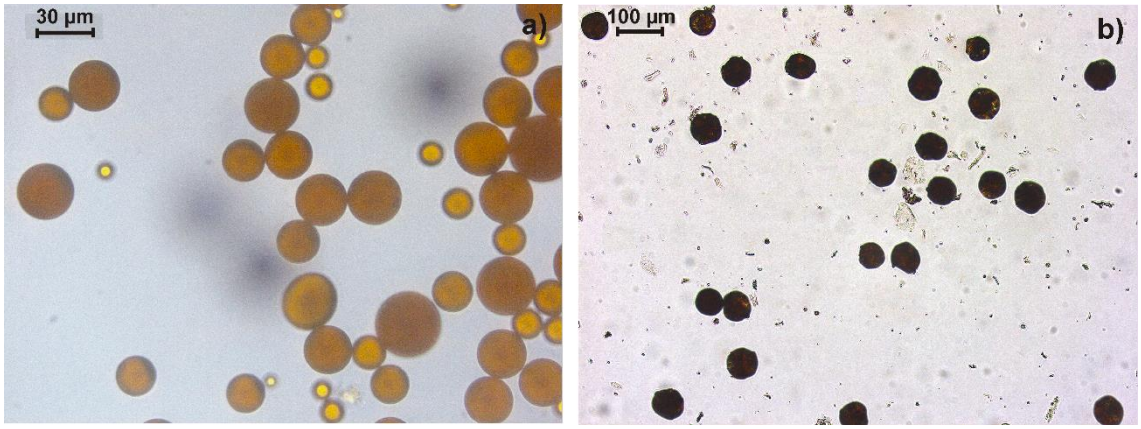

Fig. 2. Optical images of lignin microparticles prepared by the solvent extraction/evaporation method (a) and microfluidics (b).

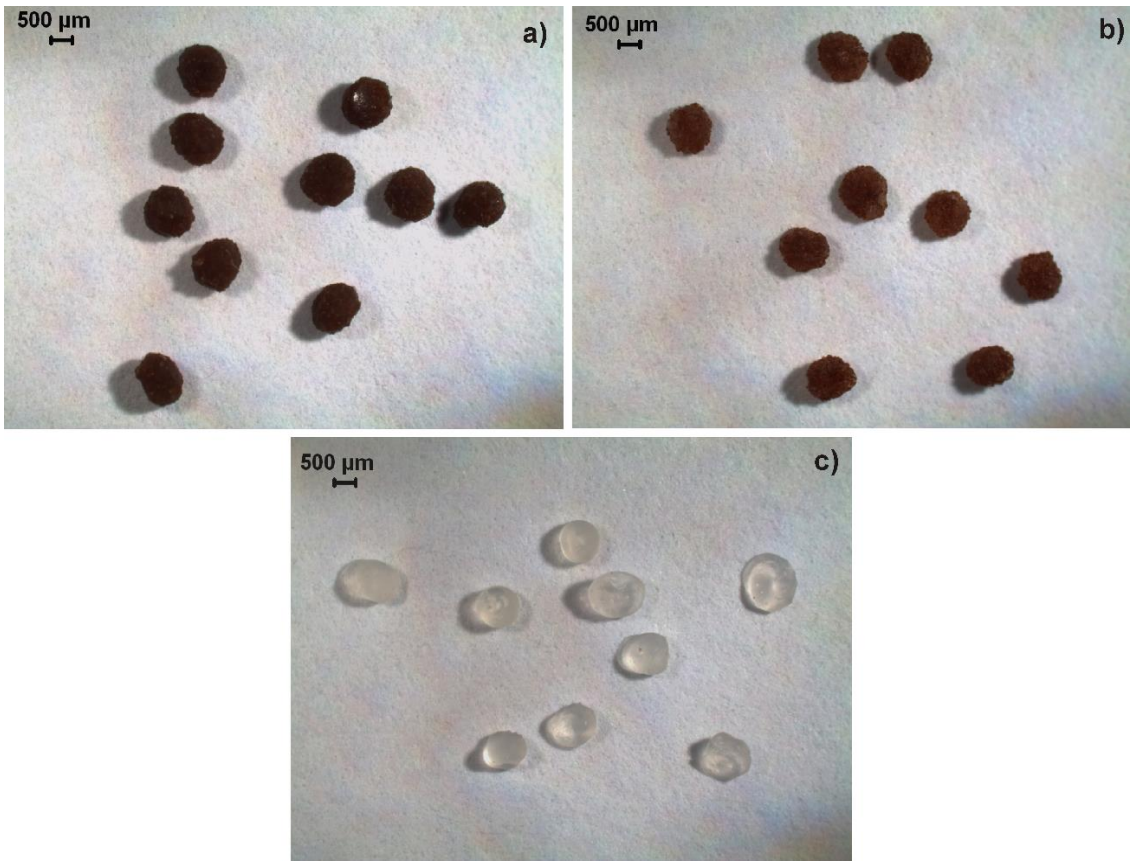

Fig. 3. Optical images of alginate beads: a) Alginate beads containing microparticles (Exp. D); b) Alginate beads containing microparticles (Exp. E); c) Alginate beads (Exp. C).

3.3 Morphology studies

The surface morphology of microparticles and alginate beads was studied by SEM and the micrographs are depicted in Figure 4. As previously observed in optical images, highly monodispersed microparticles were obtained by microfluidics. Alginate beads showed a smooth surface and some cracks and fissures appeared on the surface as a consequence of water removal during the analysis. As it can be seem in Fig. 4d-e, beads of Exp. D and E presented dispersed particles on their surface, indicating a homogeneous distribution of microparticles within the beads.

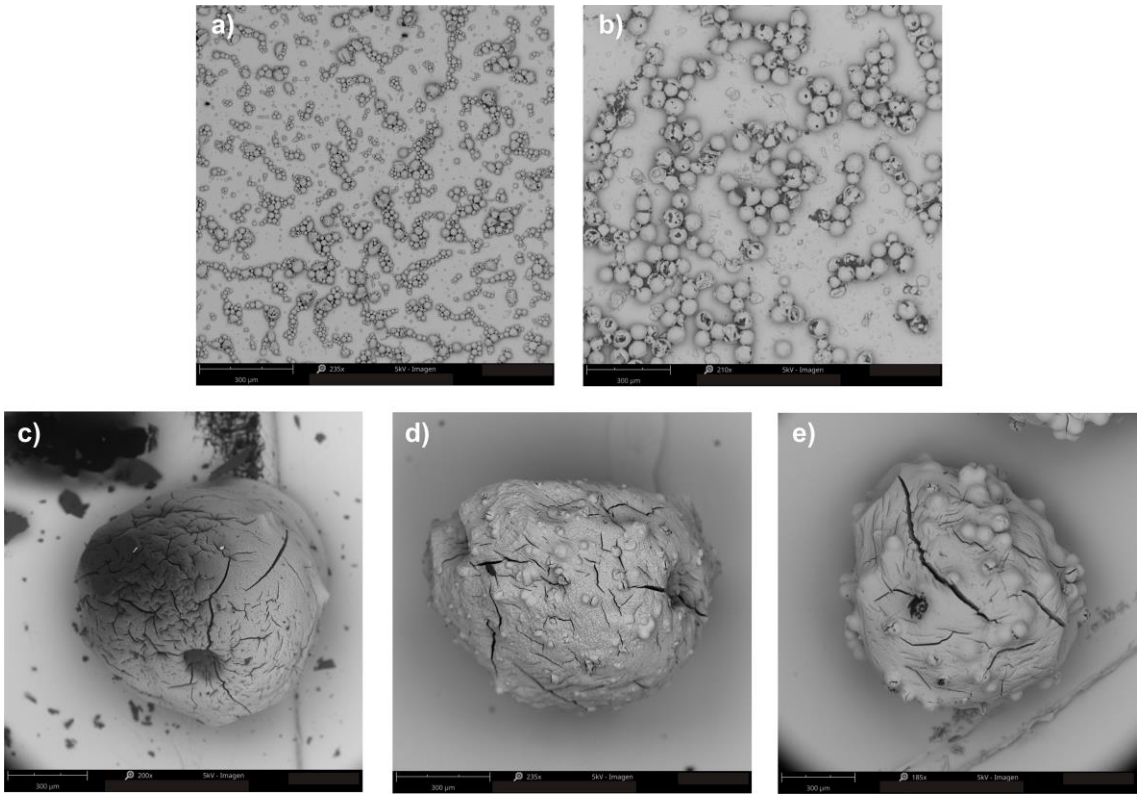

Fig. 4. SEM images of microparticles and microparticles-in-alginate beads: (a) Exp. A; b) Exp. B; c) Exp. C; d) Exp. D; e) Exp. E.

3.4 FTIR studies

Infrared spectra of atrazine, ionic lignin and atrazine-loaded microparticles (Exp. A and B) are presented in Fig. 5.

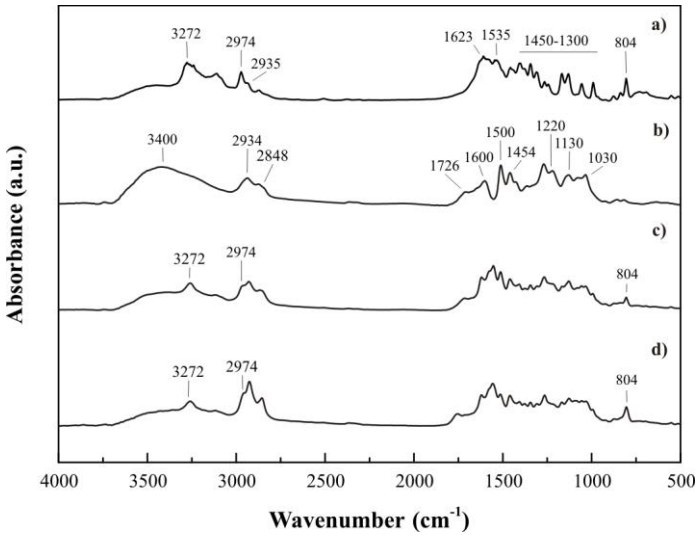

Fig. 5. FTIR analysis: a) atrazine; b) ionic lignin; c) atrazine-loaded microparticles (Exp. A); and d) atrazine-loaded microparticles (Exp. B).

The FTIR spectrum of atrazine exhibited a band at 3272 cm^{-1} which is attributed to the amine groups, while bands at 2974 and 2935 cm^{-1} were assigned to CH_3 and CH groups stretching vibrations [40]. The stretching vibrations of the 1,3,5-triazine ring appeared at 1623 and 1535 cm^{-1}. Several bands from 1450 to 1300 cm^{-1} were observed due to deformation vibrations of this ring. In addition, stretching vibrations of C–Cl groups were observed 804 cm^{-1}.

The ionic lignin spectrum showed bands at 3400 cm^{-1} related to OH groups, while bands at 2934 and 2848 cm^{-1} were attributed to C—H stretch in the methyl and methylene groups [39]. The carbonyl stretching vibrations appeared at 1726 cm^{-1}. Signals at 1500 and 1600 cm^{-1} correspond to the aromatic skeleton. Bands at 1454 cm^{-1}, and 1220 cm^{-1} were related to C—H deformation, and C—C, C—O, and C=O stretch, respectively.

The band at 1030 cm^{-1} was attributed to C-O stretching while the band at 1130 cm^{-1} was assigned to the C-O antisymmetric stretching.

The spectra of atrazine-loaded lignin microparticles exhibited characteristic bands of atrazine (3272, 2974 and 804 cm^{-1}) that confirm the successful encapsulation of the herbicide.

3.5 Water uptake

The water uptake curves of alginate beads are shown in Fig. 6. As it can be seen, they were characterized by a fast initial uptake of water, after which an apparent equilibrium or slow water uptake was observed. Alginate beads containing lignin microparticles (Exps. D and E) showed a similar water uptake behavior, with a higher water uptake compared to empty alginate beads (Exp. C). This fact can be explained by differences in the crosslinking degree of alginate beads caused by the incorporation of lignin microparticles. The presence of microparticles decreases the crosslinking points between calcium ions and carboxylic groups of alginate, increasing the distance between the polymeric chains and favoring the fluid absorption and the swelling of the systems [47].

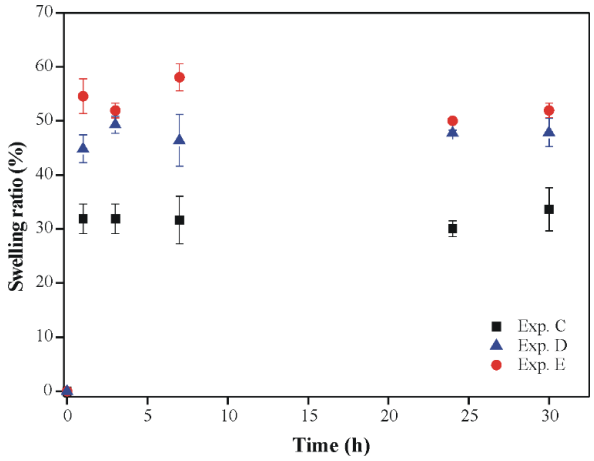

Fig. 6. Swelling ratio of alginate beads and alginate beads containing microparticles as a function of time.

3.6 Release assays

The controlled release of atrazine from the different formulations based on lignin microparticles was investigated. All systems released atrazine in a relatively slow rate and maintained its sustained release for longer periods. Fig. 7 illustrates the release profiles of atrazine from lignin microparticles prepared by the solvent extraction/evaporation and microfluidic techniques as a function of time (up to approximately 4 days). Analysis of the release kinetics curves indicated that a significant fraction of the encapsulated herbicide (about 40%) was release rapidly at the beginning of the experiment for microparticles obtained from the conventional emulsion method. After that, a sustained release of atrazine was observed, with nearly 100% of herbicide released after 2 days. In contrast, microparticles prepared by microfluidics presented a lower burst release (about 20% of the encapsulated herbicide) and a sustained release over 3 days approximately. This fact can be attributed to differences in particle size, since smaller particles exhibit a higher surface area and the initial pesticide release is related with pesticide located near the surface of microparticles. Moreover, microparticles prepared by microfluidics presented a relative narrow size distribution which can improve the control over drug release. Polydispersity in sizes is also one of the main causes of the initial pesticide release due to the presence of small microparticles that encapsulate a significant fraction of drug that is released more rapidly.

Lignin microparticles were also trapped within sodium alginate beads. A homogeneous distribution of microparticles was observed inside alginate beads. The release profiles of atrazine from microparticles contained in alginate beads are also shown in Fig. 7. As it can be noted, the initial burst release was significantly reduced for microparticles prepared by the conventional emulsion method. This behavior can be explained by the

longer diffusion pathways for atrazine generated by the alginate matrix. Alginate beads containing microparticles prepared by microfluidics showed a similar initial release rate of atrazine in comparison to free microparticles. However, after 24 h, the sustained release of atrazine was extended up to approximately 96 h. In the case of beads from Exp. D, atrazine was released more rapidly at longer times in comparison to beads from Exp. E. This fact could be related to microparticles diffusion from alginate beads to the surrounding medium due to the smaller mean particle size and higher polydispersity, accelerating atrazine release.

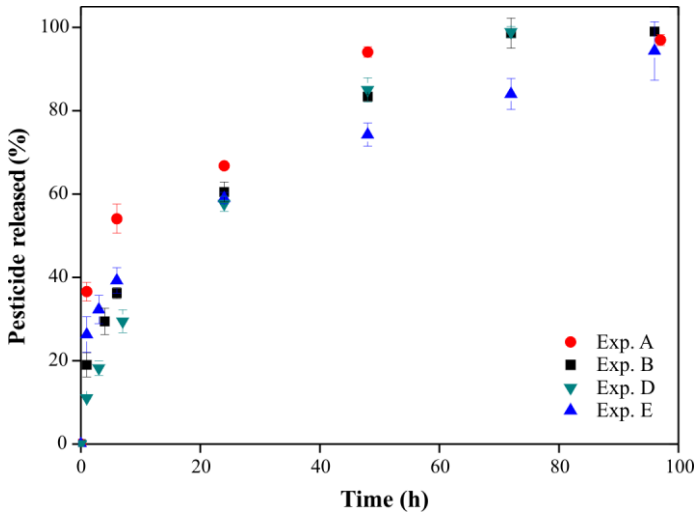

Fig. 7. Cumulative release of atrazine in water from lignin microparticles and alginate beads containing microparticles.

In order to investigate the mechanism of pesticide release from lignin microparticles, the release data were analysed with the following mathematical models: zero-order, first-order, Higuchi, and Korsmeyer-Peppas. The kinetic models for drug release were estimated using the following graphical plots: cumulative drug released vs. time (zero-order model), log drug remaining vs. time (first-order model), cumulative drug release

vs. square root of time (Higuchi model), and log cumulative drug release vs. log time (Korsmeyer model). The values of the regression coefficients (R^2) are presented in Table 2. It was found that the obtained data were fitted very well with the Higuchi model with R^2 values higher than 0.9672, indicating that Fickian diffusion is the main mechanism for atrazine release from lignin microparticles and alginate beads containing microparticles. To further characterize the release mechanism, the Korsmeyer exponent (*n*) was calculated. The value of *n* was between 0.1907 and 0.5241, indicating that the pesticide release mechanism from the different formulations was diffusion controlled [48].

Table 2. Kinetic models for atrazine release from lignin-based matrices

	Zero-order model	First-order model	Higuchi model	Korsmeyer model	
Exp.	R^2	R^2	R^2	R^2	n
A	0.6649	0.9131	0.9672	0.9905	0.1907
B	0.8663	0.9589	0.9993	0.9955	0.3667
D	0.9364	0.9368	0.9983	0.9978	0.5241
E	0.8457	0.9722	0.9981	0.9905	0.2728

3.7 Phytotoxicity assays

The root elongation bioassay is one of the most commonly used test method for environmental monitoring in terms of simplicity, rapidity and economy [49]. Plant and seed growth and development are largely influenced by environmental stimuli [50]. In particular, as germination is the first step of material exchange between the developing plant and the environment, both the number of germinated seeds and the root elongation are sensible parameters for phytotoxicity testing [49, 51]. Therefore, in order to evaluate

the reduction of the undesirable impacts of atrazine on the environment and the human health through the release technique presented in this work, a phytotoxicity test was performed employing *Lactuca sativa* seeds. The use of sustained release systems in agriculture offers several advantages over conventional techniques, including more prolonged action of the pesticide active principle. This may help to reduce the number of applications required and improve targeting, hence reducing the negative effect of high pesticide concentration in the environment. Fig. 8 shows photographs of the different assays performed after three days of incubation.

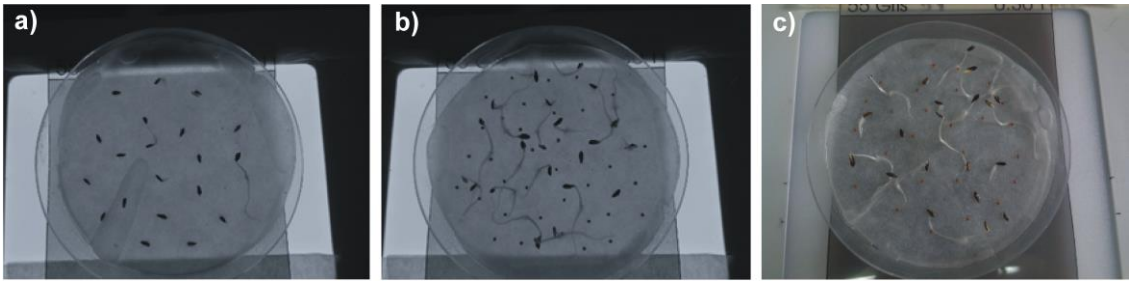

Fig. 8. Photographs of the phytotoxicity assays: a) Free commercial atrazine; b) Alginate beads containing microparticles (Exp. D); c) Alginate beads containing microparticles (Exp. E). All photographs were taken after three days of incubation.

The GI and RE were calculated after the assay for free commercial atrazine and alginate beads containing atrazine-loaded microparticles prepared by the solvent extraction/evaporation and microfluidic techniques. The results are shown in Table 3. These parameters are compared with the control (water) employing equations shown in Section 2.10. In addition, the average root length (RL) is shown for each system.

Table 3. Phytotoxicity assays parameters

Atrazine System	RL* (mm)	GI (%)	RE
Free commercial atrazine	6.32	28.4	-0.49
Exp. D: Alginate beads containing microparticles of solvent evaporation method	16.8	124.1	0.33
Exp. E: Alginate beads containing microparticles of microfluidics	12.7	70.7	0.01

*RL for control was 12.6 mm

Statistical significant differences between experiments were observed in terms of the average root length ($p>0.05$). The results of Duncan test showed that there are two homogeneous groups statistically different: *i)* free atrazine and *ii)* alginate beads of Exp. D, alginate beads of Exp. E and the control. According to this result, the average root

length for the experiment of free atrazine was lower than for encapsulated atrazine and the control, indicating no toxicity for formulations encapsulating atrazine. In addition, the average root length in experiments using microparticles and the control has no statistical differences, probably due to the N and S content of lignin that could increase the seed germination.

Comparing the values obtained based on the bibliography cited on Section 2.10, it can be demonstrated that free atrazine is phytotoxic (GI lower than 60%) [44]. According to Bagur-Gonzalez et al. [45], free atrazine toxicity is moderate (RE =-0.45) compared with alginate beads of Exp. D and Exp. E (RE = 0.33 and 0.01, respectively) that showed no toxicity. The phytoxicity assay reinforced the hypothesis that the release systems used in this work could help to reduce undesirable impacts on the environment with the plus benefit for the cultivation that the active principle release is prolonged with time.

4. Conclusions

Microparticles prepared by microfluidics presented a larger particle size, higher encapsulation efficiency and a narrow size distribution. The incorporation of microparticles within alginate beads allowed to reduce the burst release of free microparticles and to extent the release period. In addition, acute phytotoxicity effects of formulations were evaluated on *Lactuca sativa* seeds. The phytotoxicity results showed that lignin-based formulations are safe according to the parameters evaluated, in contrast with commercial atrazine that resulted phytotoxic. The studied lignin-based formulations could improve the effectiveness of pesticides and reduce its undesirable impacts on the environment.

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Figure 1

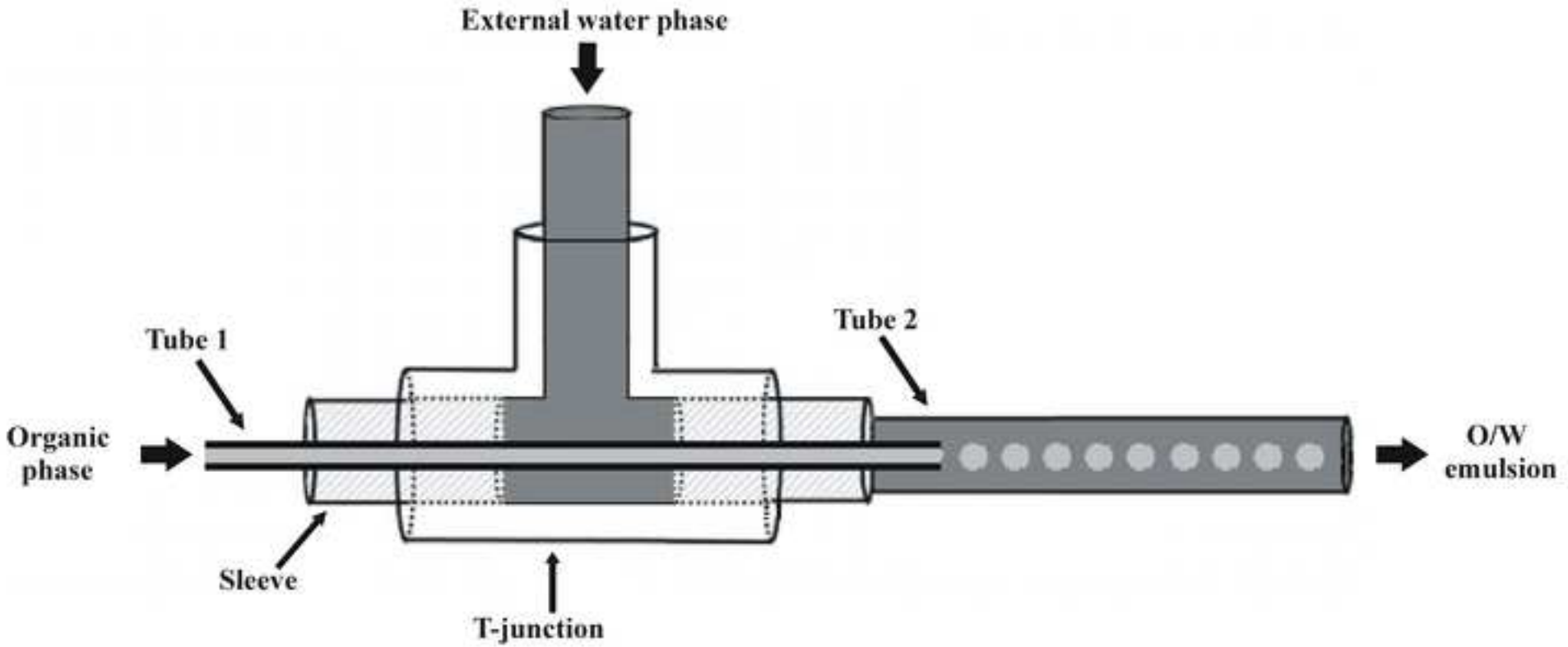

Figure 2

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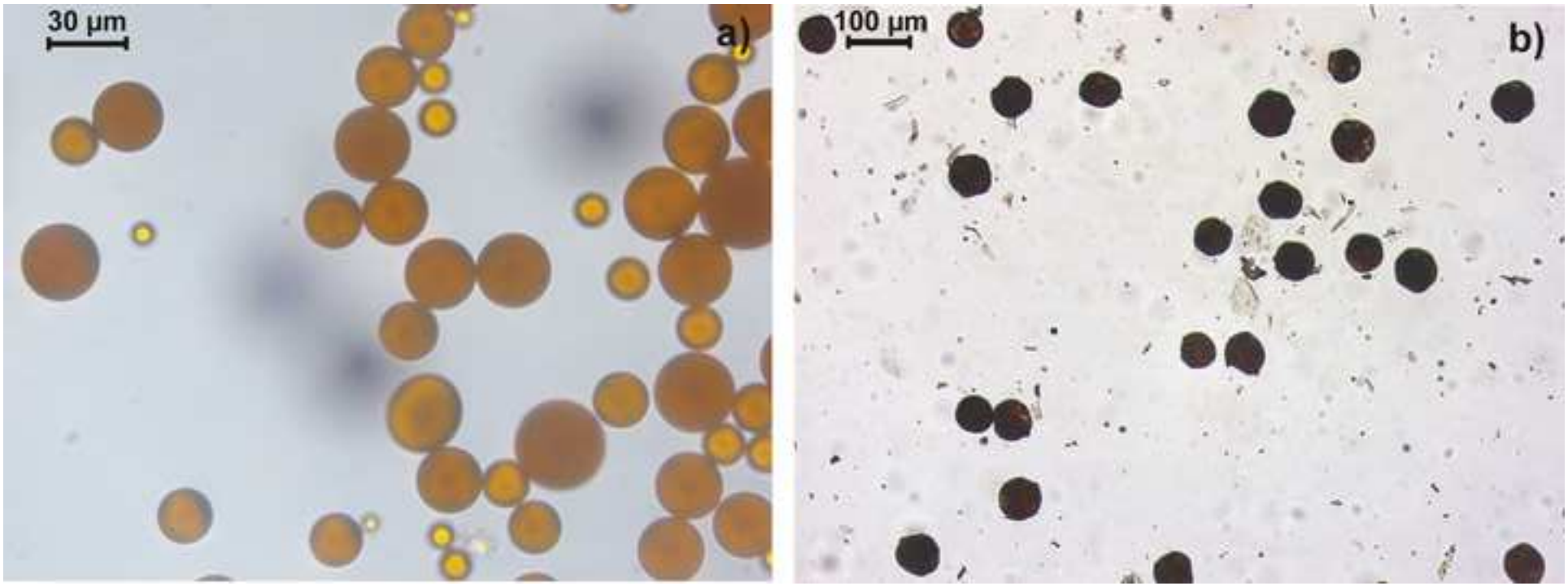

Figure 3

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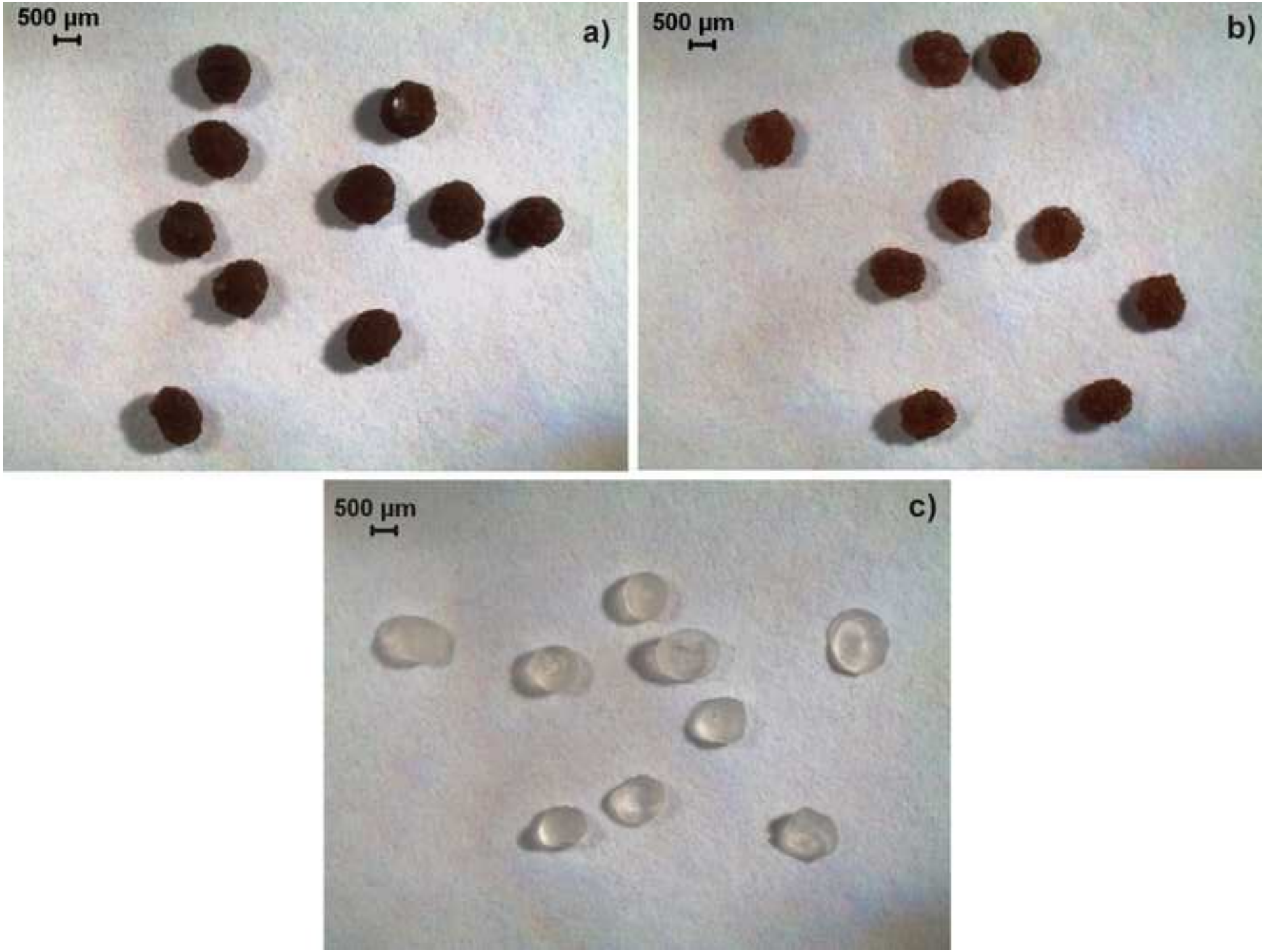

Figure 4

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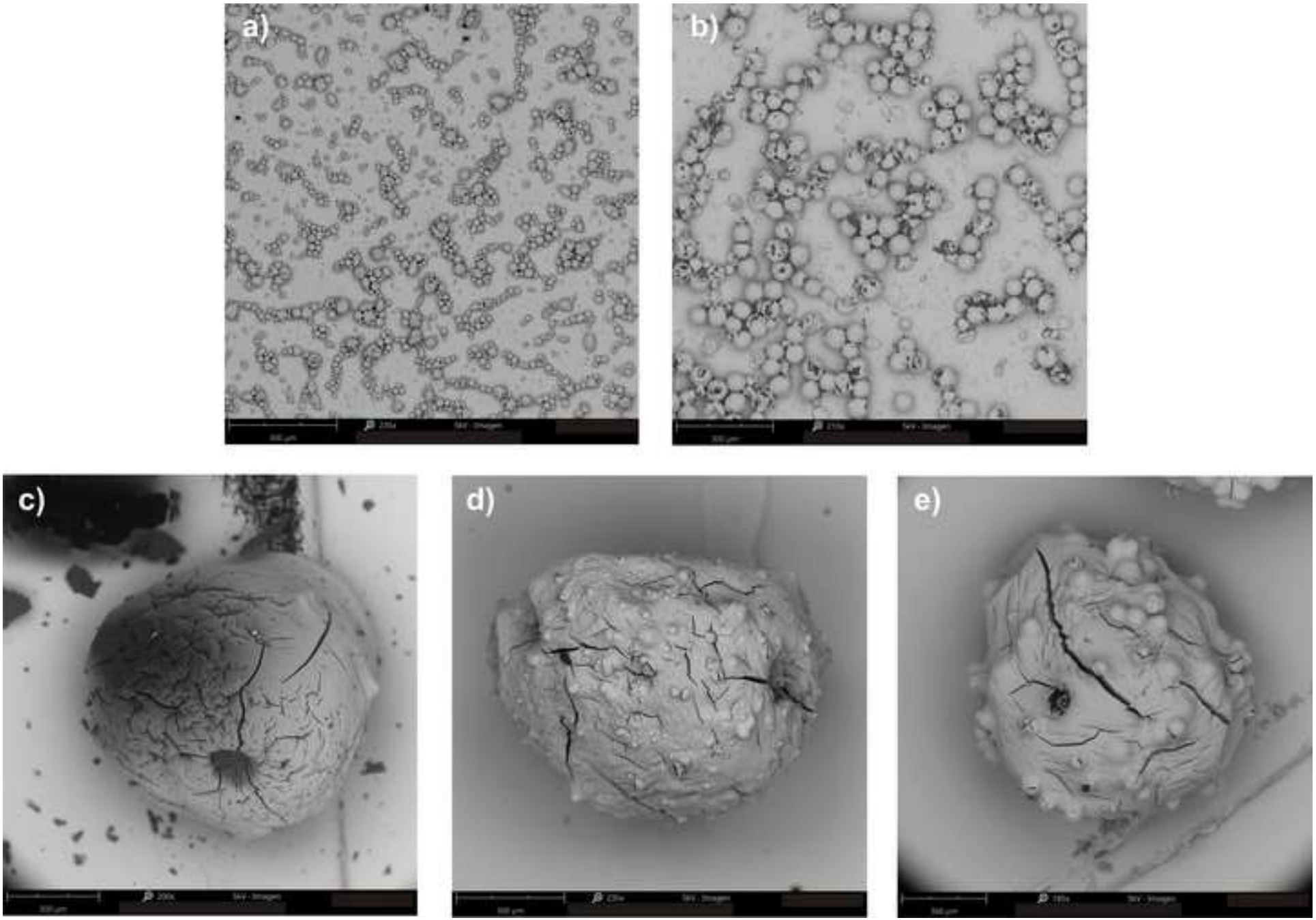

Figure 5

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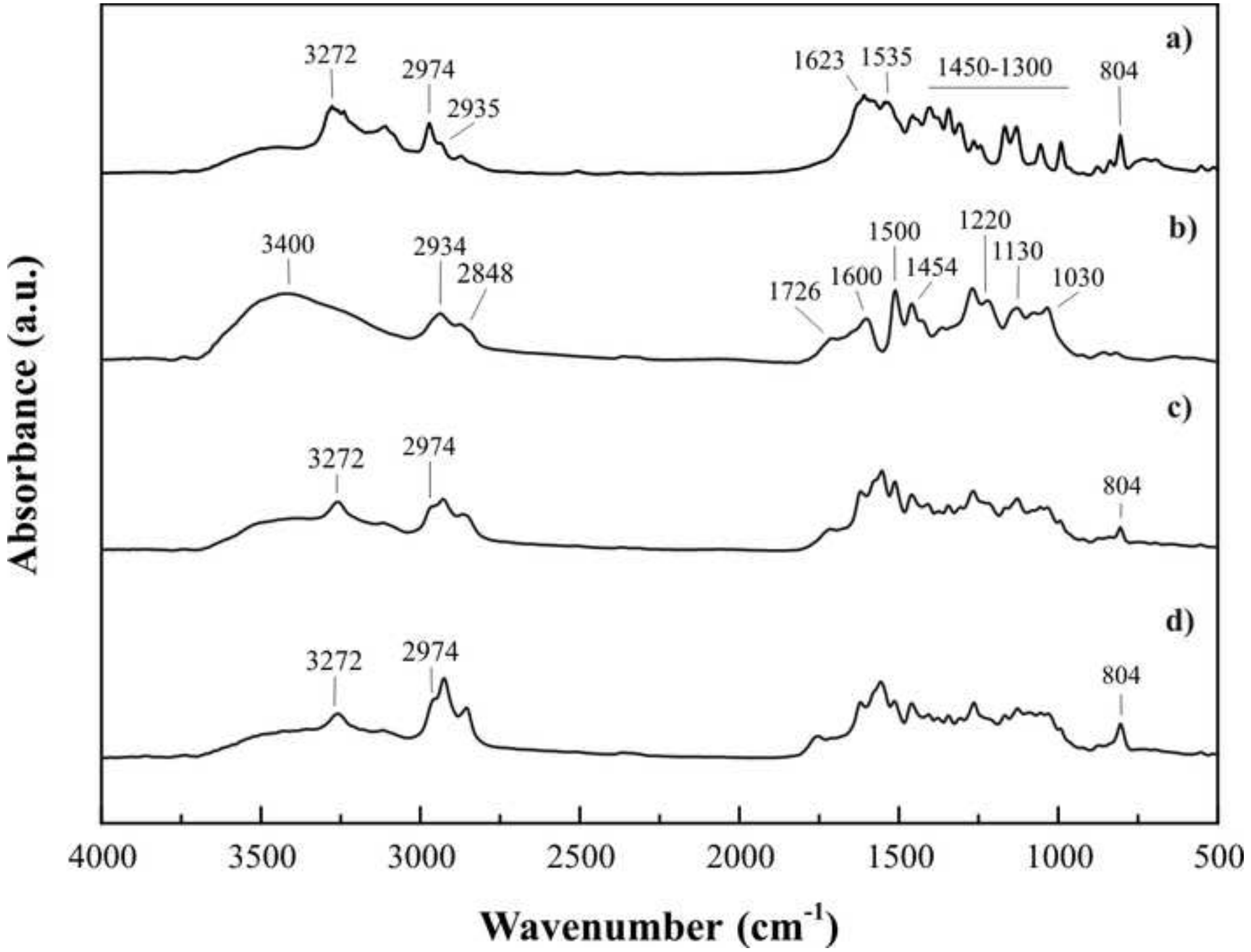

Figure 6

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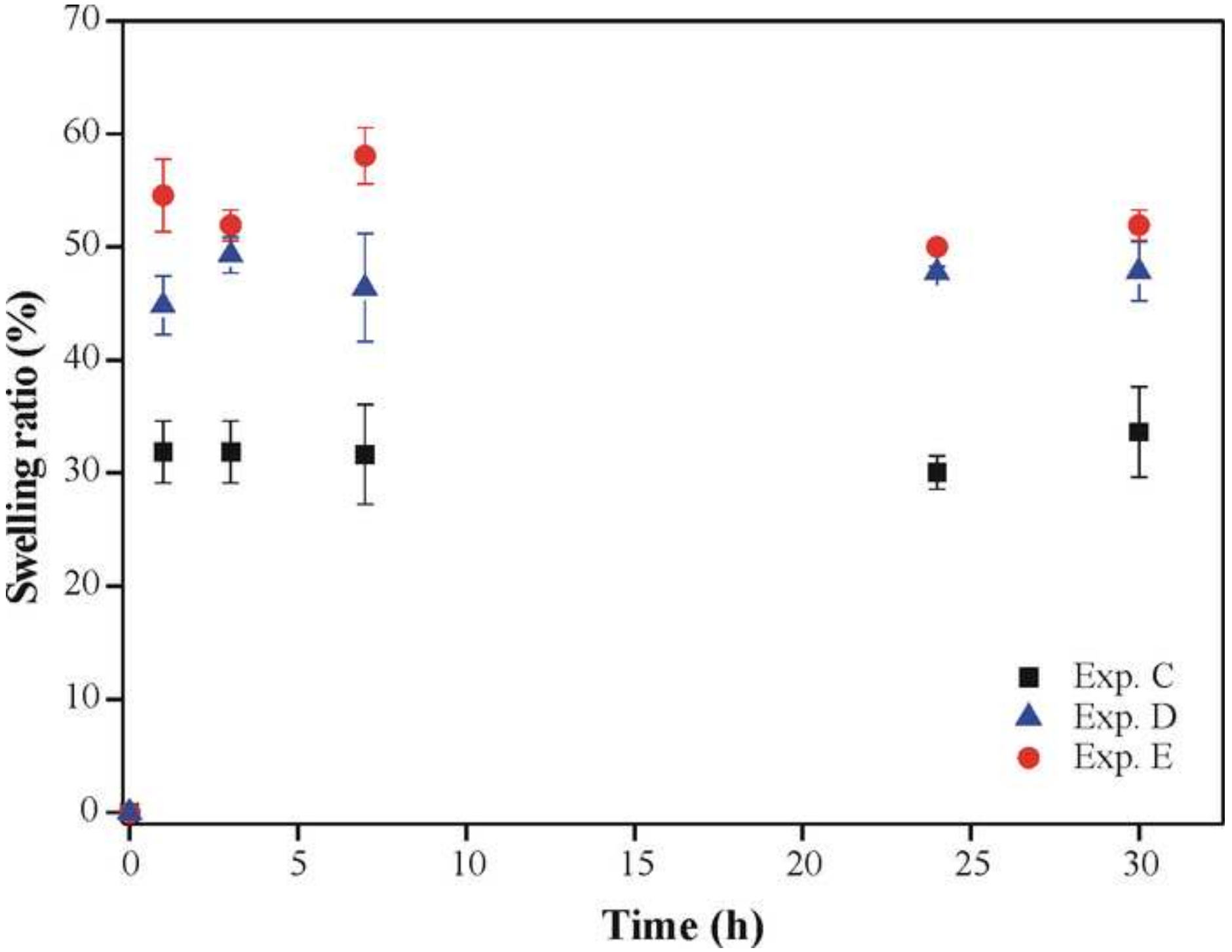

Figure 7

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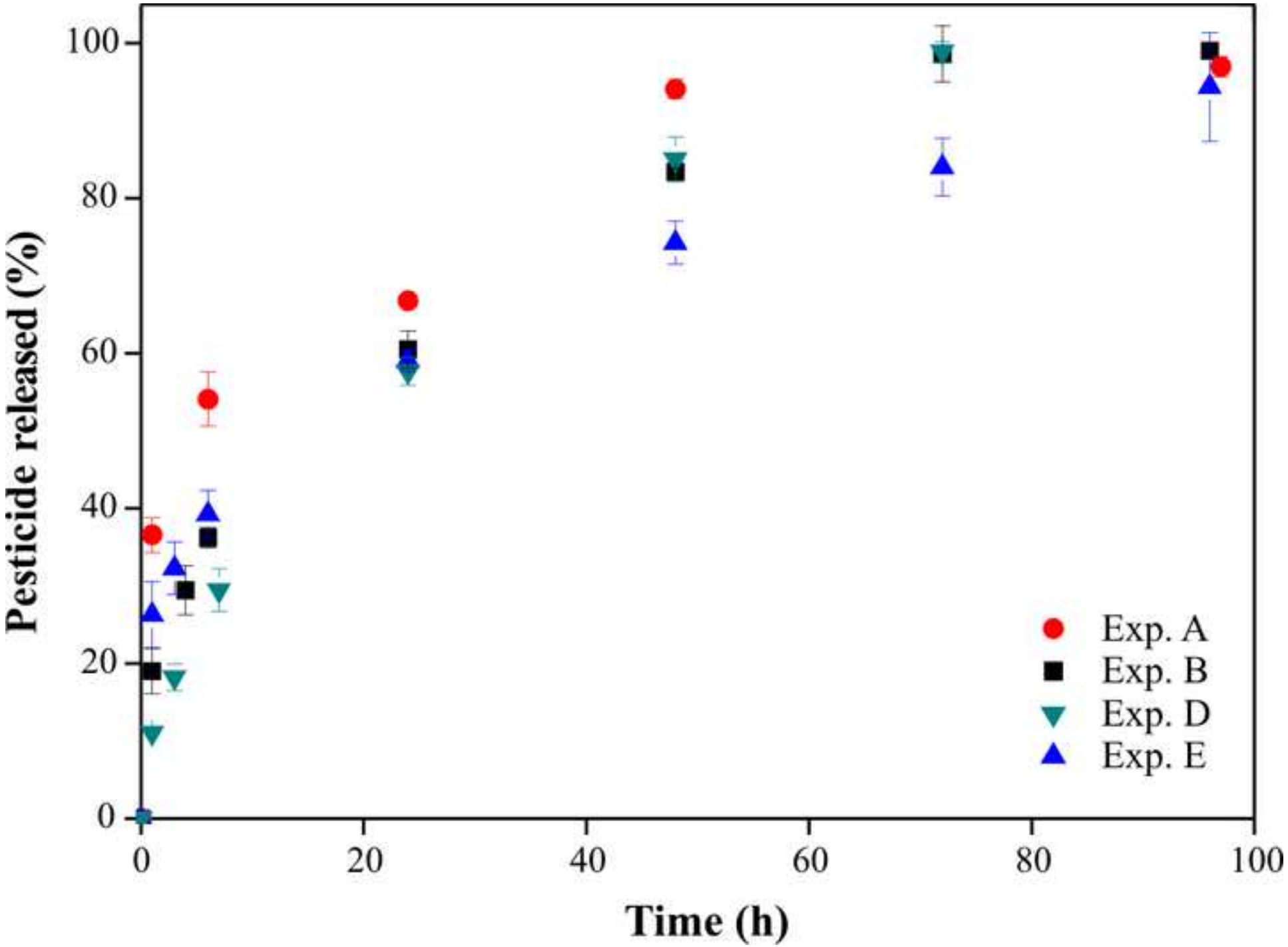

Figure 8

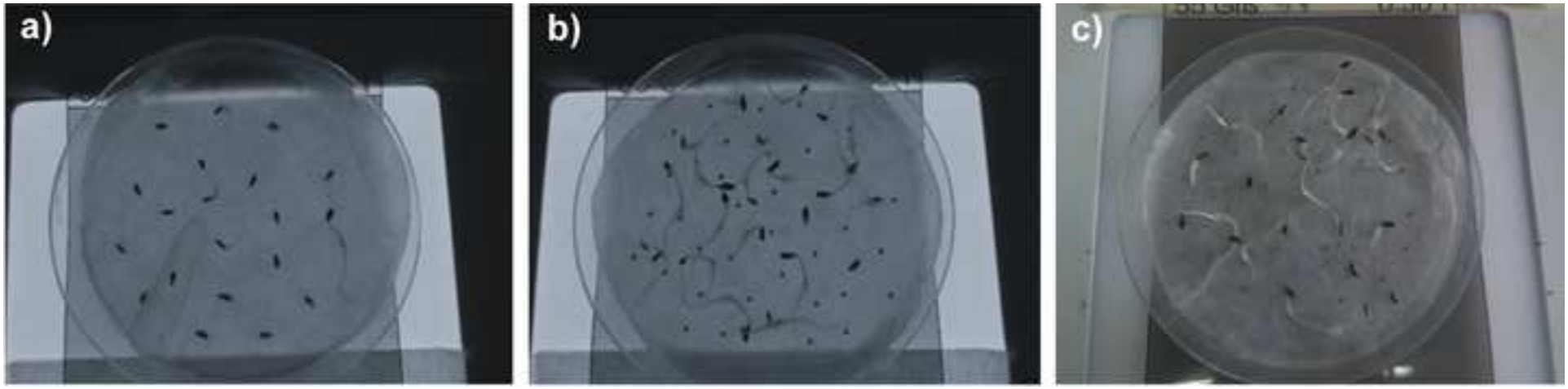

Table 1. Characteristics of lignin microparticles and alginate beads

	Microparticles		**Alginate beads**	**Alginate beads containing microparticles**	
	Solvent evaporation	**Microfluidics**		**Solvent evaporation**	**Microfluidics**
Experiment	**A**	**B**	**C**	**D**	**E**
Particle size (μm)	25.3 ± 6.7	50.0 ± 3.0	999.3 ± 145.5	1061.5 ± 26.9	1025.1 ± 68.4
Coefficient of variation (%)	26.5	6.1	14.6	2.5	6.7
Encapsulation Efficiency (%)	47.4 ± 3.5	59.7 ± 8.2	-	-	-

Table 2. Kinetic models for atrazine release from lignin-based matrices

	Zero-order model	First-order model	Higuchi model	Korsmeyer model	
Exp.	R^2	R^2	R^2	R^2	n
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*RL for control was 12.6 mm