Slow Release of Pheromones to the Atmosphere from Gelatin−Alginate Beads

IDO YOSHA,[†] ARNON SHANI,[†] AND SHLOMO MAGDASSI*,[§]

Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel, and Casali Institute of Applied Chemistry, Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Alginate−gelatin beads with dispersed droplets of a model pheromone, dodecyl acetate, were prepared as a vehicle for slow release of pheromones into the atmosphere over a prolonged period of time. The beads are prepared in two steps, the first being preparation of an oil-in-water emulsion composed of dodecyl acetate as the oil phase and gelatin as the emulsifier, which provides steric stabilization. After mixing with alginate solution, this emulsion is embedded within beads by simple electrostatic cross-linking. It was found that bead porosity at micrometric and nanometric scales plays an important role in controlling the release of dodecyl acetate.

KEYWORDS: Pheromones; slow release; calcium alginate; beads; emulsions; gelatin

INTRODUCTION

Pest management is an integral part of modern agriculture and its efficacy can, in many cases, make the difference between success and failure of the grower. The damage caused by pests is estimated to be between 35 and 40% of all crops grown (*1*).

Integrated pest management (IPM) (*2*) and integrated crop management (ICM) (*3*) are the principal concepts in pest management around the world, which is moving toward more environmentally friendly agriculture.

Pheromones (*4*) are used in ICM for monitoring pest populations (mostly insects), mass trapping, and mating disruption (*5−7*). The pheromones, in all methods of application, are released into the atmosphere from devices that are spread in the field. Among the devices developed for the slow release of pheromones are rubber septa, dispensers, impregnated cardboard, hollow fibers, and polyethylene tubes (*8*); all are applied by hand. The release of pheromone is usually required over a period of several weeks, up to about 3 months.

The possible formation of a sprayable slow-release formulation for the sex pheromone of the Mediterranean corn borer was recently reported by Vlieger (*9*). It was found that by using a biodegradable polymeric resin as microsphere reservoirs, the concentration of the pheromone was sufficiently high for >30 days. Atterholt et al. (*10*) reported on the use of paraffin wax and biopolymers such as starch and soy protein isolate. For practical applications the pheromone delivery system should meet various requirements, such as adhesion to leaves or fruits, high water fastness, stability in the application tank, and, above all, compliance with all regulatory issues. The latter requirement may be achieved, for example, by using edible biopolymers.

Alginate is an edible, linear polysaccharide extracted from brown seaweed and is widely used as a rheology control agent in industry. The polymer consists of 1,4-linked β-D-mannuronate (M) and α-L-guluronate (G) residues arranged in a nonregular blockwise pattern, consisting of three types of blocks: MM, GG, and MG. The structure of alginate, its chemical and physical properties (*11*), and its ability to form hydrogels in the presence of multivalent cations (*12−14*) (mostly calcium) have been extensively studied. The cations serve as cross-linkers between the functional groups of the polymer to form a mechanically and thermally stable gel (*15*).

Gelatin (*16*) is obtained by partial hydrolysis of collagen, the main protein component in skins, bones, hides, and other animal body tissues. The type of hydrolysis by which gelatin is produced determines its isoelectric point. Gelatin type B is produced by an alkaline process and has an isoelectric point between pH 4.6 and 5.2. Gelatin is not a single chemical substance; the main constituents of gelatin are large and complex polypeptide molecules of the same amino acid composition as the parent collagen, covering a wide range of molecular weights.

Dodecyl acetate was used as a model molecule for a water insoluble, volatile pheromone. We used dodecyl acetate as a pheromone component as well as a model compound for many other pheromone components, for both its similar polarity and its volatility. This chemical is a component of insect sex pheromones, in some cases the major active component and in others a minor one, although biologically active (*17*). The availability and low cost of the chemical enabled us to use it in all of our studies and draw conclusions based on the various and vast range of results.

In the present study we report on a new system for the slow release of pheromones into the atmosphere, which is based on alginate−gelatin beads containing embedded microscopic droplets of pheromone.

* Author to whom correspondence should be addressed (telephone + 972-2-658-4967; e-mail magdassi@cc.huji.ac.il).
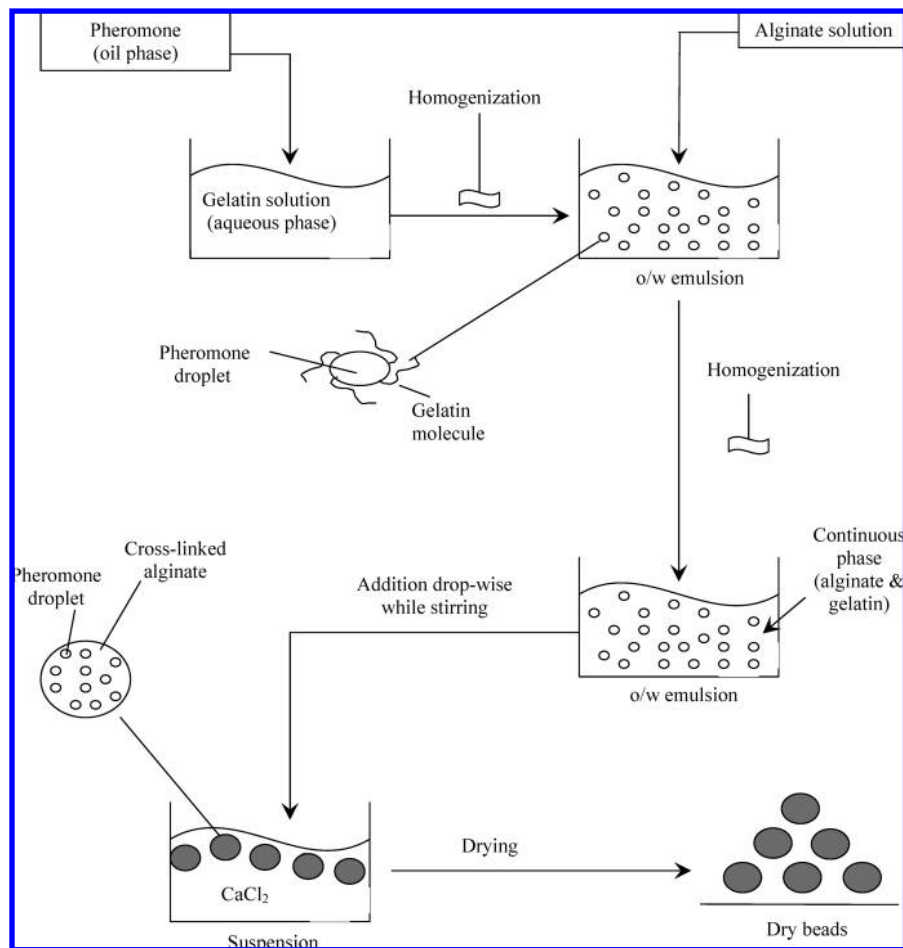† Ben-Gurion University of the Negev.
§ The Hebrew University of Jerusalem.

Figure 1. Schematic presentation of preparation of the beads.

MATERIALS AND METHODS

Materials. High-viscosity alginate (*Macrocystis pyrifera*), Rhodamine B isothiocyanate (RITC) (Sigma, St. Louis, MO), dodecyl acetate (97% purity $C_{12}OAc$), 1-dodecanol (98% purity), fluoresceine isothiocyanate (FITC) (90% purity) (Aldrich, Milwaukee, WI), gelatin type B 75 Bloom (Sigma), $CaCl_2$ dihydrate (99% purity) (Sigma), NaN_3 (99% purity; Riedel-deHaën, Seelze, Germany), ethanol abs. (Frutarom, Haifa, Israel), and tetradecanol (97% purity; Aldrich) were used without any further purification.

Methods. *Preparation of the Beads.* Beads were prepared (**Figure 1**) through the following steps (18): (a) Dodecyl acetate ($C_{12}OAc$) was emulsified with demineralized water containing 0.13% w/w NaN_3 as a preservative for 5 min at 10200 rpm (Diax 900, Heidolph, Schwabach, Germany), using gelatin type B 75 Bloom as the surface-active agent. It should be noted that by this method, 100% of the pheromone should be present within the droplets. After an emulsion was formed, it was mixed (10200 rpm, 3 min) with a solution of sodium alginate at various concentrations. (b) The mixture was added dropwise at a rate of 10 mL/min with a syringe (self-made pneumatic syringe equipped with 11 needles, 21G × 1.5 in.) to a 0.25% w/w solution of $CaCl_2$, which served as the cross-linking agent. The beads were left in the solution for a known period of time and then filtered and washed once with demineralized water. Beads of 1–1.5 mm in diameter were obtained after drying in a climate chamber (CEO 910W-4, Lunaire, Williamsport, PA), under a controlled regimen of air speed, temperature, and humidity.

Scanning Electron Microscopy. The morphology of the dry beads was evaluated using scanning electron microscopy (JEOL, JSM 35 CF, Japan).

Mercury Porosimetry. The porosity of the beads was measured by a mercury porosimeter (Micromeritics, Poresizer 9320, Norcross, GA).

Confocal Microscopy. Fluorescence imaging of the emulsions and the beads was performed using a confocal microscope (Zeiss, LSM 510, Thornwood, NY). FITC fluorescence labeling of gelatin was

performed according to the procedure described by Vinetsky and Magdassi (*19*), and dodecyl acetate droplets were colored by dissolving Nile Red (10 μM) in the liquid prior to emulsification.

Evaluation of Dodecyl Acetate Release from the Beads. The beads (1.5–2 mm in diameter) were left to dry at room temperature overnight and then carefully weighed in equal portions and placed in a climate chamber (CEO-910W-4 with revolving trays, Lunaire) under a regimen of 0.5 m/s air flow speed, 35 °C temperature, and 3–5% humidity.

Two samples of each formulation were taken from the chamber every few days. The beads were placed in a vial, and 2 mL of ethanol was added to each vial. The beads were manually crushed and left overnight on a test tube roller to allow complete extraction of the dodecyl acetate by the ethanol. Aliquots were filtered using a 0.45 μm nylon filter (Tracer, Barcelona, Spain) and injected into a gas chromatograph at the following conditions: detector (FID) temperature, 280 °C; carrier gas (N_2) flow, 1.6 mL/min; injector temperature, 280 °C; column head pressure, 10 psi; chromatographic method, 100 °C/1 min, 30 °C/min, 250 °C/2 min., 30 °C/min 280 °C/1 min; internal standard, tetradecanol.

RESULTS AND DISCUSSION

The first stage of preparation of the slow-release formulation was emulsification of the dodecyl acetate using gelatin. Gelatin was selected because it is a surface-active biodegradable polymer, which may also function as a component of the polymeric bead matrix. We used gelatin type B to prevent precipitation with the alginate, which is added at a later stage.

To use the gelatin, we first evaluated its ability to form and stabilize emulsions in which the dispersed phase is dodecyl acetate. Preliminary experiments indeed showed that oil/water (O/W) emulsions can be obtained by using only gelatin as the emulsifier.
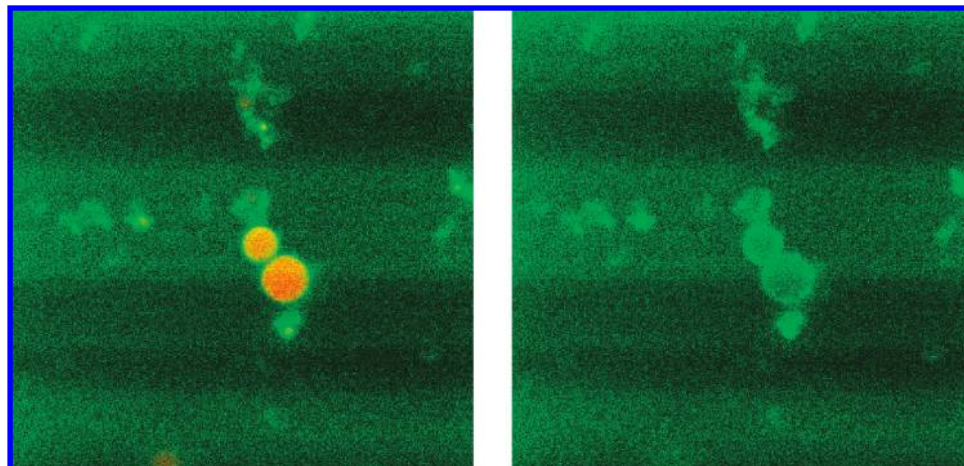
Figure 2. Laser confocal microscope image of the emulsion. Gelatin (green) can be seen adsorbed on the hydrophobic pheromone droplet (red core).
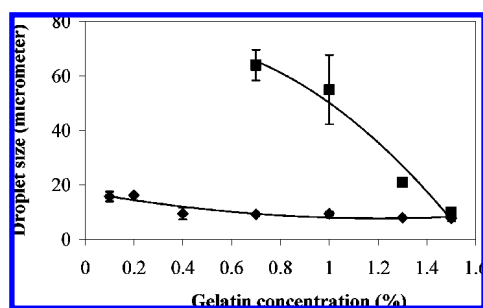

Figure 3. Stability of the emulsion as represented by the average size (μm) of emulsion droplets versus gelatin concentration: (◆) droplets after preparation of the emulsion; (■) droplets after 2 weeks.

To confirm that gelatin stabilizes the emulsion through adsorption, and not through gelling of the continuous aqueous phase, laser confocal imaging of an emulsion was performed while using a fluorescent hydrophobic dye, Nile Red, dissolved within the droplets. FITC-labeled gelatin was dissolved in the aqueous phase. As shown in **Figure 2**, the red spots, which are the emulsion droplets, are surrounded by a green halo of the gelatin. This indicates that the gelatin is surrounding the dodecyl acetate droplets and at the same time is also present at the continuous phase of the emulsion, in agreement with a previous study (*19*).

The role of gelatin in stabilizing the emulsion was investigated by measurement of the emulsion's average droplet size as a function of gelatin concentration immediately and 2 weeks after preparation of the emulsion. As shown in **Figure 3**, the average droplet size decreases with the increase in gelatin concentration, and by comparison of the droplet size after storage, it appears that the emulsion becomes stable at gelatin concentrations >1.5 wt %.

To determine the mechanism by which the gelatin stabilizes the emulsion, ζ potential was measured. The ζ potential of emulsion with gelatin concentration of 0.5−2.5 wt % was in the range from −6 to −4 mv, at pH 5.6. The small negative ζ potential of the emulsion is due to the adsorbed gelatin (gelatin type B, which has an isoelectric point slightly below the pH in which the emulsion is prepared). A typical potential of an electrostatically stabilized emulsion ranges around 30−40 mV (positive or negative). The low values that were measured suggest that the steric stabilization mechanism governs the emulsion stability.

Once it was found that the required gelatin concentration is 1.5 wt %, beads were formed by mixing the emulsion with alginate solution, followed by dropping it as discrete droplets into a $CaCl_2$ cross-linking solution, as described under Materials and Methods. The SEM of a typical bead with a diameter of 1−1.5 mm is shown in **Figure 4**, whereas the cross-section photo of the bead indicates that it is composed of micrometer-sized pores divided by a three-dimensional network of polymeric walls. The observed pores are probably formed due to evaporation of the dodecyl acetate droplets during drying of the bead prior to evaluation by the SEM.

Release of the Volatile Component. Preliminary studies indicated that the dodecyl acetate is released from the bead over a period of several weeks. For a potential use in agriculture, the release of pheromones should continue for about 2−3 months, and therefore further systematic evaluation of the factors that govern the release rate was performed.

The effect of alginate concentration in the beads on the release rate of dodecyl acetate is shown in **Figure 5**. Five bead formulations having various concentrations of alginate were prepared, and the residual content of dodecyl acetate in the beads was evaluated over time. The following preparation parameters were kept constant: dodecyl acetate concentration was 10% w/w of the emulsion, gelatin concentration was 1%, $CaCl_2$ concentration of the cross-linking solution was 0.25% w/w, and the cross-linking duration was 30 min. In general, it was found that the release of dodecyl acetate was dependent on alginate concentration: as alginate concentration increased, the residual dodecyl acetate in the beads at a given time increased (the release rate decreased).

It was found after 30 days (**Figure 6**) that almost all of the dodecyl acetate was released at alginate concentrations below 0.5%. Above 0.7% alginate the beads retained about 55−70% of the dodecyl acetate. Analysis of the data did not yield a unified release order for all of the beads, indicating that the release mechanism may vary at different alginate concentrations.

The effect of gelatin concentration on the release rate was studied for beads prepared with various gelatin concentrations and, for comparison, with one emulsion that was prepared without gelatin (using a synthetic emulsifier, Tween 80 ethoxylated sorbitan mono oleate). The following preparation parameters were kept constant: alginate concentration, 1%; dodecyl acetate, 10% w/w of the emulsion; $CaCl_2$ concentration, 0.25% w/w; cross-linking duration, 30 min. As shown in **Figure 6**, it was found that up to a gelatin concentration of 1%, the release of dodecyl acetate decreased with the increase of gelatin concentration. Above 1% gelatin the release did not change
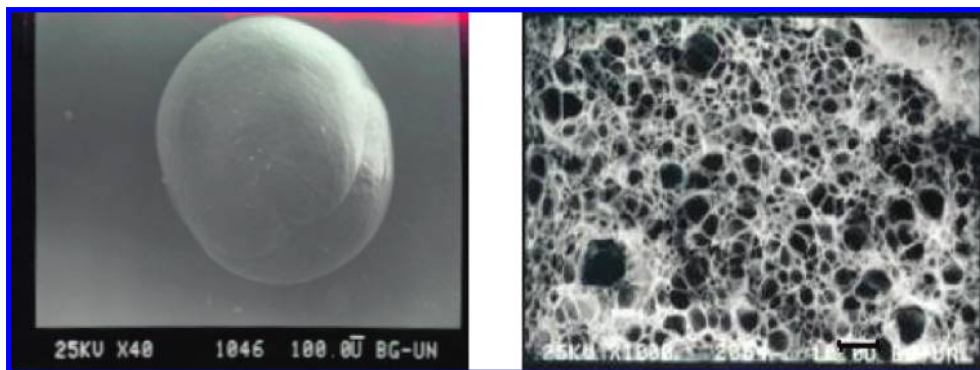
Figure 4. Electron micrograph of a bead (left) and of a cross section of a bead (right, scale bar = 10 μm).
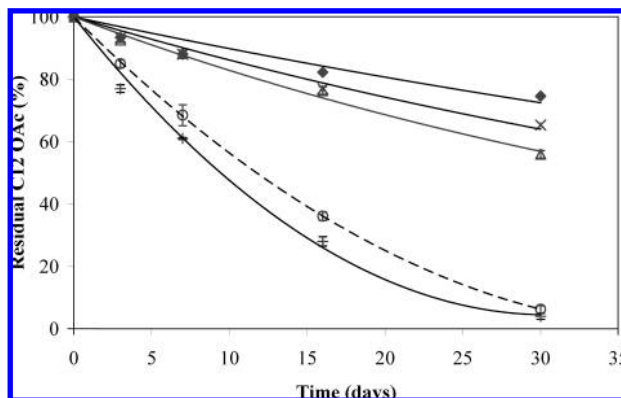

Figure 5. Residual dodecyl acetate, as a function of time, for beads with various alginate concentrations: (◆) 1%; (×) 0.8%; (△) 0.7%; (○) 0.5%; (+) 0.3%.
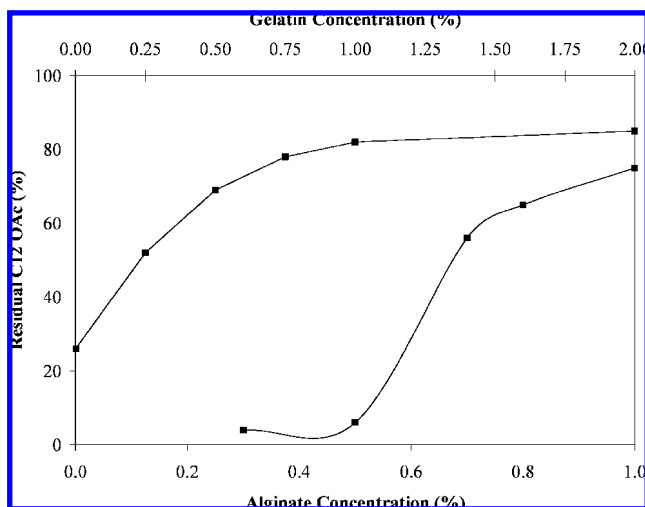

Figure 6. Residual dodecyl acetate as a function of gelatin concentration on day 30 (upper *X*-axis) and as a function of alginate concentration on day 30 (lower *X*-axis).

Figure 7. (Top) Effect of alginate concentration on the total porosity of the beads and on the average pore size: (slashed bars) 0.5 and (dotted bars) 1%. (Bottom) Effect of gelatin concentration on the total porosity of the beads and on the average pore size: (dotted bars) 0.25, (checkerboard bars) 0.5, and (striped bars) 1%.

significantly. Therefore, it appears that the overall effect of increasing the biopolymer concentration is to prolong the release of the dodecyl acetate.

Physical Properties of the Beads and Their Effect on the Release Rate. If the release mechanism is based on diffusion of the volatile component out of the beads through the walls of the pores, it is expected that the release will decrease with the increase in alginate and gelatin concentration, and indeed this is what was found, as described above.

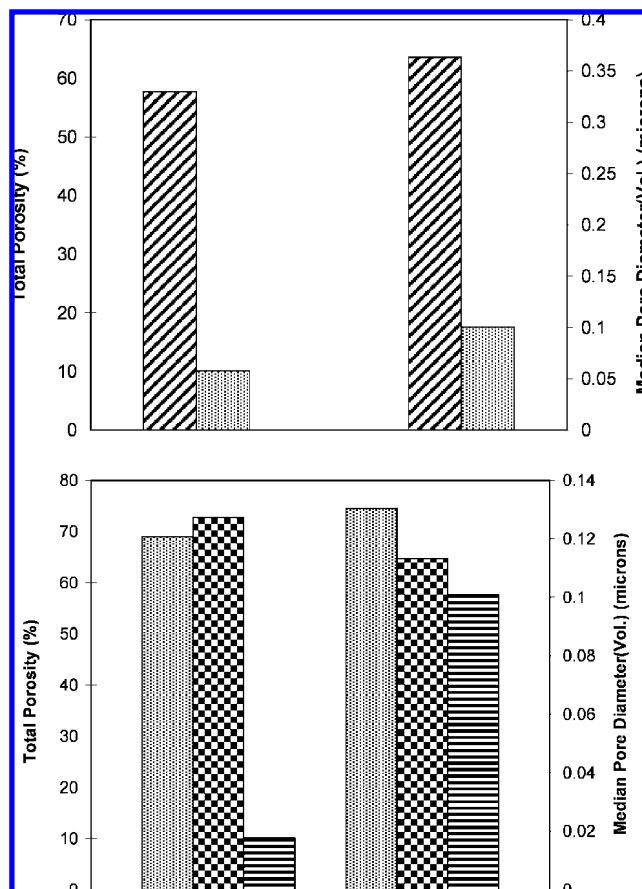To quantitatively evaluate the effect of bead porosity on the release rate of dodecyl acetate, we measured the porosity of beads prepared with various alginate concentrations, using a mercury porosimeter. The instrument compresses mercury into the matrix pores under pressure and measures the average pore size in the matrix, as well as its total porosity.

As shown in **Figure 7**, it appears that, in general, the total porosity is about 60−70% at alginate or gelatin concentration up to 0.5% and decreases to about 10% at 1% alginate or gelatin concentration. In addition, the average pore sizes are about 350 and 100 nm at 0.5 and 1% alginate, respectively. These porosity results may explain the differences in release of dodecyl acetate in samples prepared at various gelatin and alginate concentrations. It should be noted that the pores observed by SEM (**Figure 4**) are in the range of 1−10 μm, which are actually the voids occupied by the pheromone droplets. The average pore size is

Pheromone Release to the Atmosphere

J. Agric. Food Chem., Vol. 56, No. 17, 2008 **8049**

less dependent on gelatin concentration, and it is about 100−130 nm for the three concentrations studied.

Therefore, it can be concluded that the beads contain pores of two sizes: (a) micrometer-sized pores (observed by SEM), which are determined by the size of the embedded dodecyl acetate droplets, and (b) nanometer-sized pores that exist within the walls of the large pores. It is expected that the size of the pores within the walls is determined by the cross-linking process of the alginate and by the composition of the matrix.

In conclusion, a new system for slow release of pheromones into the atmosphere has been developed, containing beads composed of edible polymers. The preparation of the beads is very simple and reproducible, and upscaling was already performed in our laboratories in kilogram quantities.

Confocal microscopy with fluorescent markers shows that the gelatin is present both at the O/W interface and dissolved within the aqueous phase of the emulsion. Thus, the beads are composed of dodecyl acetate droplets dispersed within a polymeric matrix composed of both gelatin and cross-linked alginate. These beads release the dodecyl acetate into the atmosphere through the polymeric walls from which the beads are composed.

The release of dodecyl acetate from the beads may be controlled by changes in bead porosity by varying the alginate and gelatin concentrations. These beads, due to their large size at present, still require further study toward obtaining an effective delivery system for pheromones in agriculture, by both size reduction and adjustment of spraying instruments. We expect that this type of delivery system will be suitable for protection of apples, pears, grapes, and other fruits and vegetables, such as tomatos and potatoes, and even for forest pest control.

LITERATURE CITED

(1) Pimentel, D. Pesticides and world food supply. *Chem. Br.* **1991**, 646–648 (July).

(2) Kogan, M. Integrated pest management: historical perspectives and contemporary developments. *Annu. Rev. Entomol.* **1998**, *43*, 243–270.

(3) Copping, L. The crop-protection industry. *Chem. Ind. (London, U.K.)* **2001**, *20*, 500–501.

(4) Nordlund, D. A. Semiochemicals: a review of terminology. In *Semiochemicals, Their Role in Pest Control*; Nordlund, D.A., Jones, R. L., Lewis, W. J., Eds.; Wiley: New York, 1981; pp 13−28.

(5) Bakke, A.; Lie, R. Mass trapping. In *Insect Pheromones in Plant Protection*; Jutsum, A. R., Gordon, R. F. S., Eds.; Wiley: New York, 1985; pp 67−87.

(6) Campion, D. G.; Critchley, B. R.; McVeigh, L. J. Mating disruption. In *Insect Pheromones in Plant Protection*; Jutsum, A. R., Gordon, R. F. S., Eds.; Wiley: New York, 1985; pp 89−119.

(7) Wall, C. Monitoring and spray timing. In *Insect Pheromones in Plant Protection*; Jutsum, A. R., Gordon, R. F. S., Eds.; Wiley: New York, 1985; pp 39−66.

(8) Weatherstone, I. Principles of design of controlled-release formulations. In *Behavior Modifying Chemicals for Insect Management*; Ridgway, R. L., Silverstein, R. M., Inscoe, M. N., Eds.; Dekker: New York, 1990; pp 93−112.

(9) de Vlieger, J. J. Development of a sprayable slow release formulationfor the sex pheromone of the Mediterranean corn borer *Sesamia nonagroides*. *Environmentalist* **2008**, *28*, 45–48.

(10) Atterholt, C. A.; Delwiche, M. J.; Rice, R. E.; Krochta, J. M. Study of biopolymers and paraffin as potential controlled release carriers for insect pheromones. *J. Agric. Food Chem.* **1998**, *46*, 4429–4434.

(11) Moe, S. T.; Draget, K. I.; Skjåk-Bræk, G.; Smidsrød, O. Alginates. In *Food Polysaccharides and Their Applications*; Stephen, A. M., Ed.; Dekker: New York, 1995; pp 245−285.

(12) Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D. Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Lett.* **1973**, *32*, 195–198.

(13) Smidsrød, O. Molecular basis for some physical properties of alginate in gel state. *Faraday Discuss. Chem. Soc.* **1974**, *54*, 263–274.

(14) Skjåk-Bræk, G.; Grasdalen, H.; Smidsrød, O. Inhomogeneous polysaccharide ionic gels. *Carbohydr. Res.* **1989**, *10*, 31–54.

(15) Smidsrød, O.; Haug, A.; Lian, B. Properties of poly(1,4-hexuronates) in gel state. *Acta Chem. Scand.* **1972**, *26*, 71–78.

(16) *Kirk-Othmer Encyclopedia of Chemical Technology, III, Vol. 18*; Wiley: New York, 1980; pp 711−719.

(17) Arn, H.; Toth, M.; Priesner, E. *List of Sex Pheromones of Lepidoptera and Related Attractants*, 2nd ed.; International Organization for Biological Control: Montfavet, France, 1992.

(18) Shani, A.; Magdassi, S.; Yosha, I. Polymer-based water-insoluble beads for sustained release of insect pheromones. PCT Int. Appl. WO0032043, June 8, 2000.

(19) Vinetsky, Y.; Magdassi, S. Formation and surface active properties of microcapsules based on gelatin−sodium dodecyl sulfate interactions. *Colloids Surf. A* **1997**, *122*, 227–235.