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Microspheres of chitosan for controlled delivery of brassinosteroids with biological activity as agrochemicals

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ABSTRACT

Chitosan microspheres (CS) were prepared by simple coacervation–cross-linking in the presence of sodium tripolyphosphate. Two synthetic analogues of brassinosteroids (BA) and diosgenin derivatives were dissolved in ethanol or methanol and encapsulated into CS microspheres. The extent of encapsulation was dependent on both the nature of the steroid and the solvent. For BA and derivatives encapsulation varied from 10% to 50%. Microspheres were characterized by FTIR spectroscopy. Particle sizes ranged from 790 to 1470 μm. Differential scanning calorimetry revealed the effect of the BA encapsulated on the thermal behaviour of the CS. In vitro release studies performed in ethanol and water at pH 7 indicated drug release dependence on the extent of encapsulation and the nature of the BA. Almost constant release rate was observed in all cases during the first 10 h. Release was always greater in ethanol than in water.
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1. Introduction

Brassinosteroids (BS) are steroid plant hormones with important regulatory functions and impact on, among other physiological processes, growth, xylem differentiation and disease resistance (Altmann, Coll-Garcia, Lisso, & Müssig, 2006). These plant hormones have potential uses as agrochemicals promoting the vegetable growth i.e. the productivity of the crops, due to their specific ability to stimulate cell enlargement and division, accretion of biomass in plants, yield and quality of seeds and modulation of plant stress responses (Alonso, Cabrera, Coll, Jomarrón, & Robaina, 1995; Asami et al., 2009). Several brassinosteroids have been employed for controlling insects and plagues affecting the plants due to their antiecdysteroid activity (Bhardwaj, Khurma, Ohri, & Sohal, 2007; Dinan, Voigt, & Whiting, 2001). However these potential benefits are not completely expressed in plants because the brassinosteroids are quickly metabolised and periodical applications are often needed increasing the economical cost of their employment. It is envisaged that if the duration of their action may be prolonged their use as agrochemicals, specifically to achieve the described beneficial effects, would be made more feasible.

Chitosan (CS) is a cationic linear polysaccharide composed essentially of β(1 → 4) linked glucosamine units together with some proportion of *N*-acetylglucosamine units. CS occurs rarely in nature. It is generally obtained by extensive deacetylation of chitin, a homopolymer of β(1 → 4) linked *N*-acetyl-D-glucosamine, present in the shells of crustaceans, molluscs, the cell walls of fungi, and the cuticle of insects (Muzzarelli, 1997). CS is a biocompatible, biodegradable, nontoxic, and mucoadhesive polymer, which makes it attractive for applications in medicine and pharmacy (Chandy & Sharma, 1990; Minami & Shigemasa, 1995; Muzzarelli, 1985; Rinaudo, 2006). For instance, there are numerous scientific reports and patents on the preparation of CS microspheres and microcapsules for the development of controlled release of therapeutic drugs (Acosta, Argüelles-Monal, Peniche, & Peniche, 2003; Aminabhavi, Patil, Rokhade, & Shelke, 2007; D'ath et al., 2006; Keshavayya, Kulkarni, & Kulkarni, 2007). CS is degraded by lysozyme and chitosanase (Matsuhashi et al., 1997). The former occurs in mammals, and the latter is found in plants and insects (Jenieux, 1997).

The antifungal activity of CS (Hirano & Nagao, 1989) and its ability to promote metabolic changes in plants allows it to influence favorably on the development of crops, inducing increased germination and greater yields (Fristensky, Hadwiger, & Riggleman, 1984).

In recent years CS has been employed as a delivery matrix for the release of several non-steroidal agrochemicals in agriculture (Dunn et al., 1990; Shtilman & Tsatsakis, 1993; Shtilman et al., 2006). In this context, use of CS for encapsulating BS will make possible to bring about the reported positive qualities of the polysaccharide as germicide, fungicide and stimulator of seeds germination, root development and disease resistance (Bumgardner et al., 2007;

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Hewajulige, Sivakumar, Sultanbawa, Wijesundera, & Wilson-Wijeratnam, 2007) together with the above mentioned effects of BS on plants.

The present article reports on the encapsulation of two novel synthetic (BA) brassinosteroids analogues (Agüero, Alonso, Bernardo, Coll, & Pérez, 2006; Alonso et al., 1995) with reported biological activity in chitosan (CS) microspheres to achieve their controlled delivery. The encapsulation of some derivatives of dicarboxylic steroidal acids in CS microspheres is also studied for comparison.

2. Experimental

2.1. Materials

Chitosan (deacetylation degree, D.D = 85.2% determined by ^{1}H-NMR, $M_v = 2.55 \times 10^5$) was obtained by extensive deacetylation of chitin isolated from shells of common lobster (*Panulirus argus*) at the Center of Biomaterials of the University of Havana, Cuba.

The analogues of brassinosteroids (DI-31, S-7) and diosgenin employed to obtain the steroidal monoesters of dicarboxylic acids were supplied by the Center of Natural Products at University of Havana.

The solvents and reagents employed were purchased from Sigma–Aldrich and used without further purification.

The structures of diosgenin, monosuccinate of diosgenin (MSD), monoitaconate of diosgenin (MID), monomaleate of diosgenin (MMD) and BA (DI-31 and S-7) are shown in Fig. 1.

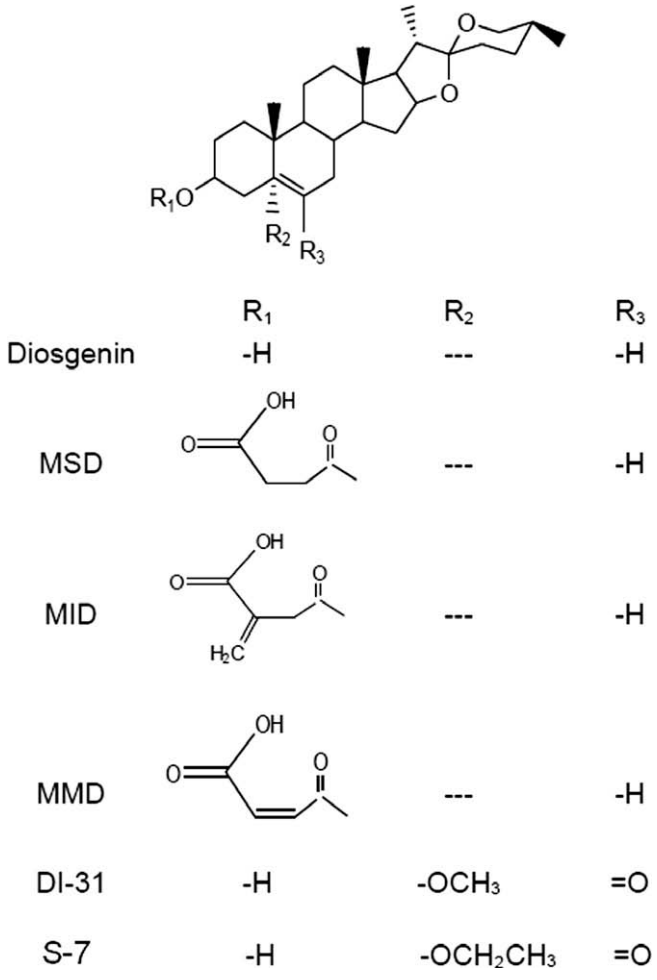

Fig. 1. Structure of diosgenin, diosgenin monoesters (MSD, MID, MMD) and brassinosteroids analogues DI-31 and S-7.

2.2. Microsphere preparation

Chitosan microspheres were obtained by a simple coacervation procedure. To this end a 2% (w/w) CS solution in 2% (v/v) aqueous acetic acid was added dropwise to a 10% (w/v) NaOH in methanol. Dropping was performed using an automatic encapsulator provided with a push–pull syringe pump and a syringe with a 22-gauge needle. The microspheres were rinsed with deionised water to pH < 8 and then cross-linked in 1% (w/v) sodium tripolyphosphate aqueous solution at room temperature for 1 h. After cross-linking, microspheres were rinsed with deionised water and transferred into ethanol or methanol, as required.

The microspheres were added into ethanolic or methanolic solutions of the studied brassinosteroids analogues, DI-31, S-7 and monosuccinate of diosgenin (MSD), monoitaconate of diosgenin (MID), and monomaleate of diosgenin (MMD). They were kept at 35 °C with magnetic stirring for 4 h and left to stand for 12–16 h. Afterwards the microspheres were filtered and washed several times with chloroform, ethyl acetate, and methanol and dried at atmospheric pressure for 24 h.

When using ethanol as solvent the steroid concentration was 6 mg mL^{-1}, and the experiment is designated as F1. The steroids concentration in the methanolic solutions of the loading experiments was 1 mg mL^{-1} (experiments F2).

2.3. Characterization

The CS microspheres loaded with the studied steroids were characterized by FTIR spectroscopy using a Perkin–Elmer FTIR spectrophotometer with 32 scans and 4 cm^{-1} resolution. Samples were prepared by KBr pellet method.

The microspheres size and size distribution were determined with an optical microscope Nikon Eclipse E-400. The morphology of microspheres was studied by scanning electron microscopy (SEM) with a TESCAN 5130 SB microscope. Samples were coated with Au-Pd using a POLARON SC 7620.

The DSC studies were performed with a Perkin–Elmer Differential Scanning Calorimeter Pyris 1 and analyzed with the Pyris 1 software (version 6.0.0.033).

The DSC studies were conducted with sample weights of approximately 8 mg, under a nitrogen dynamic flow of 20.0 ml min^{-1} and a heating–cooling rate of 10 °C min^{-1} (Dockal, Santos, & Soares, 2003). The samples were deposited in aluminum capsules and hermetically sealed. Indium was used to calibrate the instrument. Enthalpy (ΔH in J/g dry weigth) and peak temperature were computed automatically. The samples were heated and cooled from −30 to 300 °C.

2.4. Estimation of loading capacity and in vitro drug release of microspheres

25 mg of CS microspheres, which were either empty (placebo) or loaded with the steroids investigated, were stirred at 40 °C in 0.1 N acetic acid for 24 h. The resulting solutions were filtrated and the amount of encapsulated steroids was estimated by the recorded UV absorbance of aliquots at 245 nm (DI-31), 250 nm (S-7, MMD), 280 nm (MSD), and 284 nm (MID) using an Ultrospec 2100 Pro UV spectrophotometer. The concentration of the solution was determined from a previously obtained calibration curve. Blank CS microspheres were used as placebo. The amount of encapsulated compound is expressed as percent loading (g of brassinosteroids or steroidal monoesters in 100 g of microspheres). The reported loading percentage is expressed as the mean ± standard deviation of three experiments.

The in vitro release of the BA and diosgenin derivatives from the CS microspheres was studied by measuring the corresponding re-

lease profiles using UV detection of the delivered steroid. 25 mg of steroid-loaded CS microspheres were placed in a volumetric flask containing PBS (pH 7.0) in water or ethanol at total volume of 25 mL and incubated at 30 °C with constant agitation at 100 rpm. 1 mL of solution was periodically taken from the flask and replaced with fresh solution to maintain a constant volume. The UV absorbance of aliquots at the corresponding wavelength was determined and the concentration of the solution was determined as above. These studies were conducted in triplicate for each sample.

3. Results and discussion

3.1. Preparation of the placebo and steroid-loaded CS microspheres by simple coacervation/cross-linking with sodium tripolyphosphate (TPP)

The use of the automatic encapsulator for preparing CS microspheres by a simple coacervation procedure allowed to obtain spheres with quite regular diameters of approximately 1000 μm. They were cross-linked by the formation of a polyelectrolyte complex with TPP in order to increase their strength and stability.

The method employed for loading the steroids into the CS microspheres involved mild and non-destructive conditions, which are preferred when the compounds to encapsulate are temperature sensitive. The studied steroids are moderately polar and only slightly water soluble, but readily dissolved in alcohol. Therefore, they were dissolved in ethanol or methanol, as indicated, for their loading into CS particles. The loading percentages are reported in Table 1. Best results are generally obtained with the steroids dissolved in methanol, even though the steroid concentrations used were six times smaller. In all cases the loading determined never exceeded 55%, which may be attributed in part to dissolution of the steroids during washings. It is worth noting, however, the remarkable increase in loading attained when diosgenin was functionalized with dicarboxylic acids.

3.2. Optical microscopy

The CS and steroid-loaded CS particles obtained by the coacervation procedure had spherical shape. After drying in air their size was significantly reduced and some of them showed a disk like shape when inspected with the optical microscope. The size distribution of the microspheres was evaluated after measuring the diameter of 85–90 particles. The mean values varied from 790 to 1470 μm. The results are reported in Table 2. It can be observed that CS microspheres containing S-7, MSD, and MID presented bimodal size distributions. The particle sizes obtained are considerably bigger than those reported in other studies (Acosta et al., 2003; Aminabhavi et al., 2007; D'ath et al., 2006) but are not unusual for microspheres produced by the coacervation procedure employed in the present study. The existence of bimodal distributions in three of the systems prepared is probably due to a variation of the experimental parameters during the microspheres preparation.

3.3. Scanning electron microscopy

Fig. 2 and 3 show the SEM images of CS microspheres loaded with brassinosteroid analogues and diosgenin derivatives. The SEM of DI-31 and S-7 loaded CS microspheres presented different morphology when compared to the diosgenin monoesters loaded microspheres. The BA loaded microspheres presented wrinkles on the surface showing a very porous surface at higher magnifications. The derivatives loaded microspheres (Fig. 3) had smooth surface morphologies exhibiting a more compact surface at higher magnifications. These differences in surface morphology may be attributed at the uncontrolled drying conditions and the effect of the specific loaded substance.

Table 1
Steroids loading (in weight-%) of the studied BA and monoesters in CS microspheres.

Conditions	Diosgenin	MSD	MID	MMD	DI-31	S-7
F1	<5	9	34	45	33	12
F2	28	40	44	53	27	34

Table 2
Size distribution ± standard deviation (μm) and frequency (%) of the BA and monoesters loaded CS microspheres.

Sample	Diameter (μm)	Frequency (%)	Diameter (μm)	Frequency (%)
CS-DI-31	1300 ± 100	100	–	–
CS-S-7	870 ± 70	29	1 200 ± 100	71
CS-MSD	860 ± 70	37	1 240 ± 60	63
CS-MID	1000 ± 100	39	1 400 ± 70	61
CS-MMD	1360 ± 90	100	–	–

3.4. FTIR spectroscopy

The FTIR spectra of brassinosteroids analogues DI-31, S-7 and the monosuccinate, monoitaconate, and monomaleate of diosgenin loaded CS microspheres are shown in Figs. 4 and 5. Also included is the spectrum of placebo CS microspheres for comparison.

The IR spectrum of CS microspheres displayed distinctive absorption bands at 2942–2784 cm^{-1} (aliphatic C–H stretching band), 1657 cm^{-1} (Amide I), and 1597 cm^{-1} (–NH_2) bending and 1321 cm^{-1} (Amide III). The absorption bands at 1154 cm^{-1} (antisymmetric stretching of the C–O–C bridge), 1082 and 1032 cm^{-1} (skeletal vibrations involving the C–O stretching) are characteristic of its saccharide structure (Argüelles et al., 1999).

The spectra of brassinosteroids-CS microspheres are dominated by the CS peaks due to excess of CS over the BA in the loaded microspheres (see Table 1). The spectra of DI-31 and S-7 loaded CS microspheres show a rather intense and narrow band at 1700 cm^{-1}, which is absent in the spectrum of the empty CS microspheres. These bands are overlapping the Amide I and the –NH_2 bands at 1650 and 1597 cm^{-1}, respectively, producing a broad band ranging from 1700 to 1500 cm^{-1}. The observed hypsochromic shift of these peaks is probably the result of an interaction of the hydroxyl and/or carbonyl groups of the BA with the amine group of CS molecules. Similar observations were reported by D'ath et al., 2006 for the complex formation between polyphenolic compounds in olive leaf extract and CS.

These observations also apply to the spectra of the three steroidal monoesters MSD, MID and MMD loaded CS microspheres as shown in Fig. 5. However, the peak shift of the amino bands is less pronounced in the spectra of the monoesters indicating a probably less important interaction between the carboxyl/carbonyl groups of the monoesters with CS, possibly due to intermolecular association between monoester units.

3.5. Differential scanning calorimetry

The thermal behaviour of CS samples has been shown to be strongly dependent on the natural source (Dockal et al., 2003), the preparation conditions and the purity of samples (Mau, Yang, & Yen, 2009). Nevertheless, the main thermal effects presented by a CS from a particular source can be interpreted on the basis of the behaviour of chitosans from other sources.

For instance, the endothermic peak temperature and ΔH of fungal chitosan are 143.6–149 °C and 68.2–79.9 J/g, respectively (Mau & Yen, 2007), whereas shrimp chitosan presents endothermic peak temperature and ΔH of 143.8–148.5 °C and 183.5–216.1 J/g,

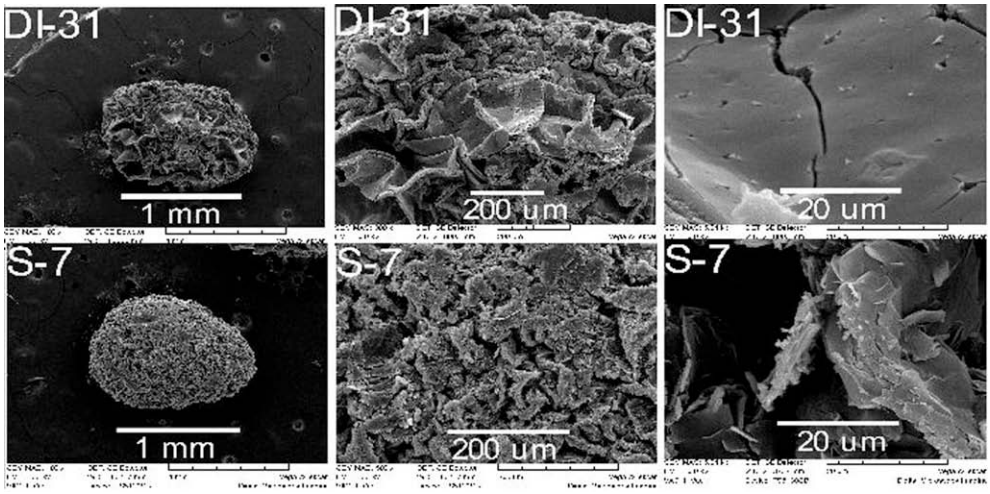

Fig. 2. Scanning electron micrographs of brassinosteroids analogues DI-31 and S-7 loaded chitosan microspheres at 100×, 500×, and 5000× magnifications.

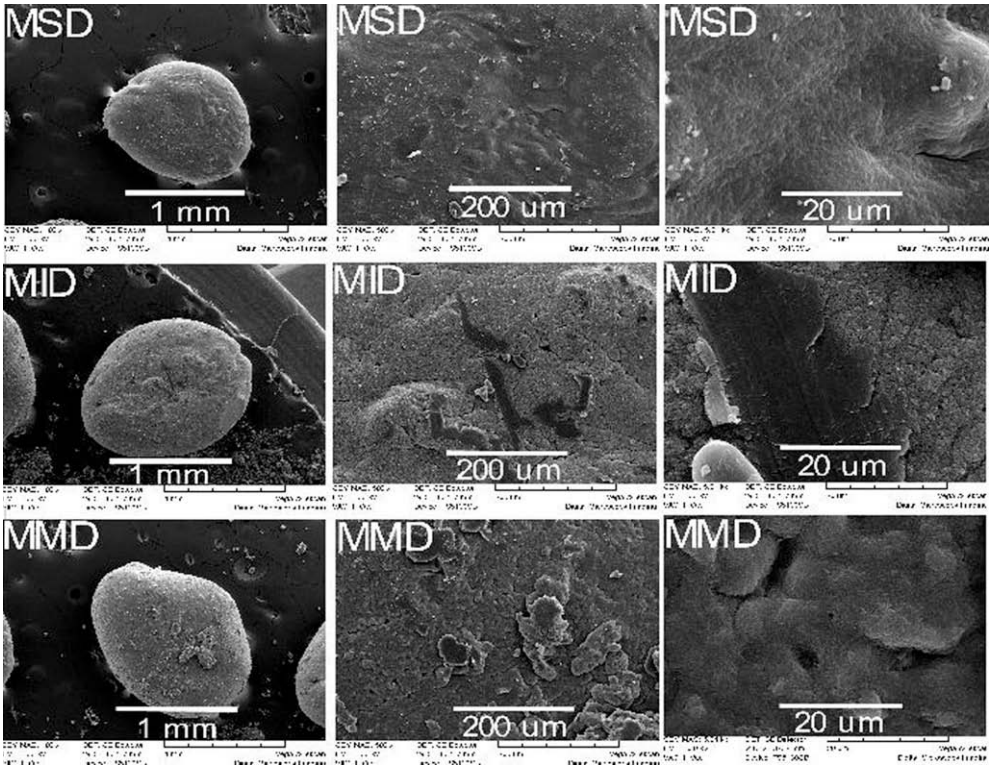

Fig. 3. Scanning electron micrographs of steroidal monoesters MSD, MID and MMD loaded chitosan microspheres at 100×, 500×, and 5000× magnifications.

respectively (Kittur, Prashanth, & Tharanathan, 2002). Melting endothermic peaks were reported at 152.3–159.2 °C with onset and completion temperatures of 124.7–126.4 and 220.5–226.2 °C, respectively for crab chitosans. The value of the associated ΔH (111.0–125.2 J/g) was found dependent on the reaction conditions (Mau et al., 2009).

The DSC curves of CS microspheres and the steroid-loaded CS microspheres obtained under nitrogen in the temperature range from −30 to 300 °C are shown in Fig. 6. The DSC of TPP cross-linked chitosan microspheres (Fig. 6Ia and IIa) show three endothermic peaks at 105.5, 167.4, and 181.1 °C, respectively. Their onset and completion temperatures are listed in Table 3, together with their associated peak enthalpy (ΔH). The total ΔH of the three effects is 115.1 J/g. These endothermic effects must result mainly from the melting and dissociation of chitosan crystals, by comparison with the aforementioned reports for crab chitosans (Mau et al., 2009).

The DSC of the steroid-loaded CS microspheres (Fig. 6Ib, c and IIb–d) show an intense endothermic peak between 187–213 °C (see Table 3) with associated ΔH of 61–180 J/g. These peaks can be result from the melting of chitosan with the encapsulated steroid and dissociation of CS chains.

3.6. Release profiles of the steroids-loaded CS microspheres

The drug release behaviour of the studied steroidal compounds from the CS microspheres was evaluated by performing in vitro release experiments at pH 7 (PBS) in water and ethanol solutions to simulate the conditions of the agrochemical formulations and veg-

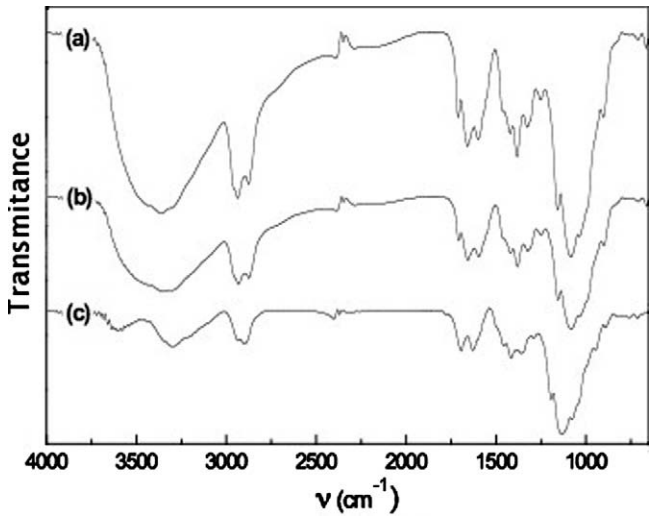

Fig. 4. Infrared spectra of (a) CS-S-7, (b) CS-DI-31, and (c) CS-TPP.

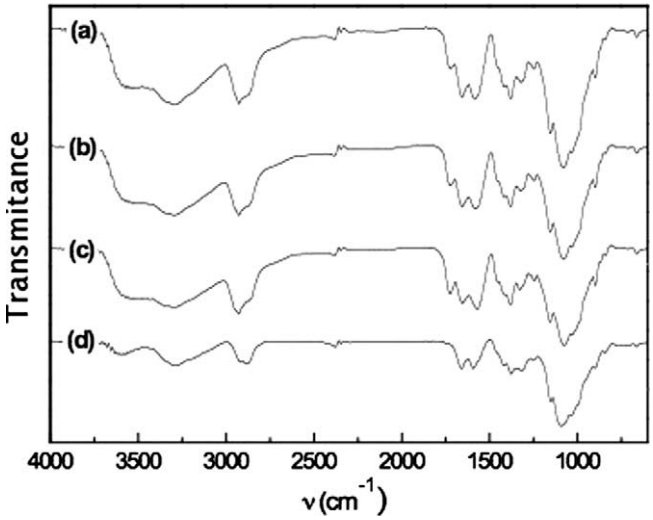

Fig. 5. Infrared spectra of (a) CS-MMD, (b) CS-MID, (c) CS-MSD, and (d) CS-TPP.

etable absorption. The release profiles of steroids, expressed as % cumulative release against time for steroid-loaded CS microspheres of formulations F1 (Table 2) are shown in Figs. 7 and 8.

Table 3
Thermal properties of TPP cross-linked CS microspheres and main endothermal effects steroids-loaded CS-TPP microspheres.

Microspheres	Endotherm (°C)			
	Onset	Peak	Completion	ΔH (J/g)
CS-TPP	127.9	150.5	155.7	10.2
	155.7	167.4	177.2	26.2
	177.2	181.1	195.3	78.9
CS-S-7	181.1	187.8	196.1	107.6
CS-DI-31	208.2	213.1	221.3	60.6
CS-MSD	186.8	194.7	203.4	91.7
CS-MID	197.9	205.5	217.1	179.6
CS-MMD	183.4	197.2	201.4	99.9

In all cases a sustained release of the steroids is obtained, which is characterized by an almost constant release rate (zero order kinetics) during the first 10 h. As expected, release was always higher in ethanol than in water, due to the greater solubility of the steroids in alcohol.

It is interesting to note that quite different release patterns were obtained for the encapsulated steroids, in spite of their similar chemical structure. This might be the result of different interactions of the steroids with the chitosan matrix. In the case of the diosgenin monoesters, an interaction of their carboxylic groups (Fig. 1) can be expected with the amino groups of chitosan. However, the general trend was that the substances which were less efficiently encapsulated were released faster and almost to completion. This is the case for S-7 as compared with DI-31 and for CS-MSD in comparison with CS-MMD and CS-MID.

4. Conclusions

The synthetic brassinosteroid analogues DI-31 and S-7 and diosgenin derivatives (monosuccinate, monoitaconate, and monomaleate of diosgenin) were loaded into TPP cross-linked chitosan microspheres. Higher loading percentages were obtained using ethanolic solutions of the steroids. Although, in all cases the loading never exceeded 55%, a remarkable increase in loading was attained when diosgenin was functionalized with dicarboxylic acids. Sustained release profiles were obtained for all the studied encapsulated steroids. The steroids which were less efficiently encapsulated were released faster and almost to completion. These results indicate that by introducing the appropriate changes in the brassinosteroids structure it would be possible to design an efficient chitosan based delivery system for the sustained release of these compounds for their application as agrochemicals.

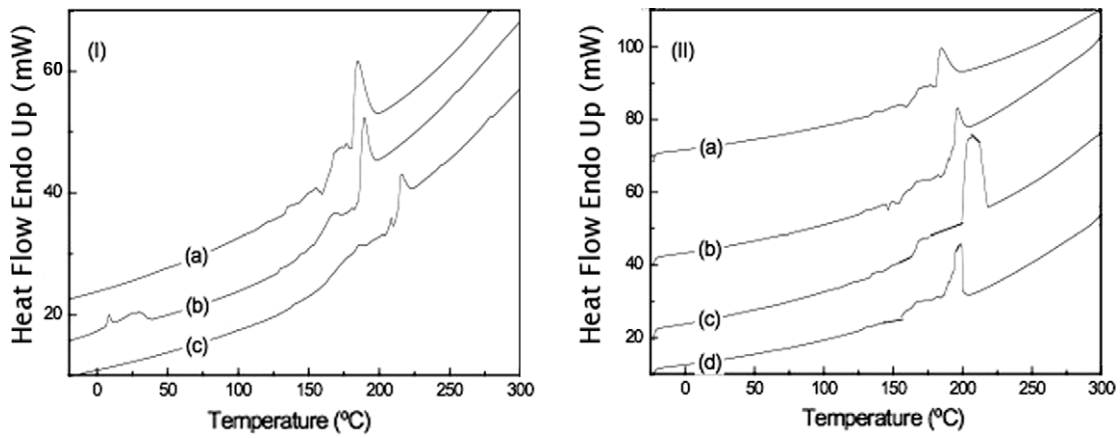

Fig. 6. DSC Curves of chitosan and steroids-loaded chitosan microspheres. I: (a) CS-TPP, (b) CS-S-7, (c) CSI-DI-31; II: (a) CS-TPP, (b) CS-MSD, (c) CS-MID, (d) CS-MMD.

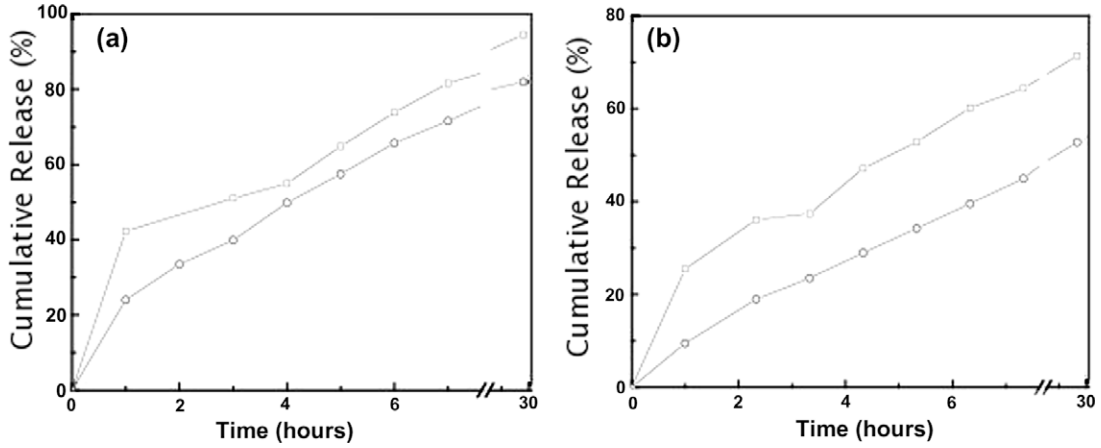

Fig. 7. In vitro release profiles at 30 °C of (□) S-7 and (○) D-31 loaded chitosan microspheres: (a) in ethanol and (b) in PBS solution (pH = 7.0).

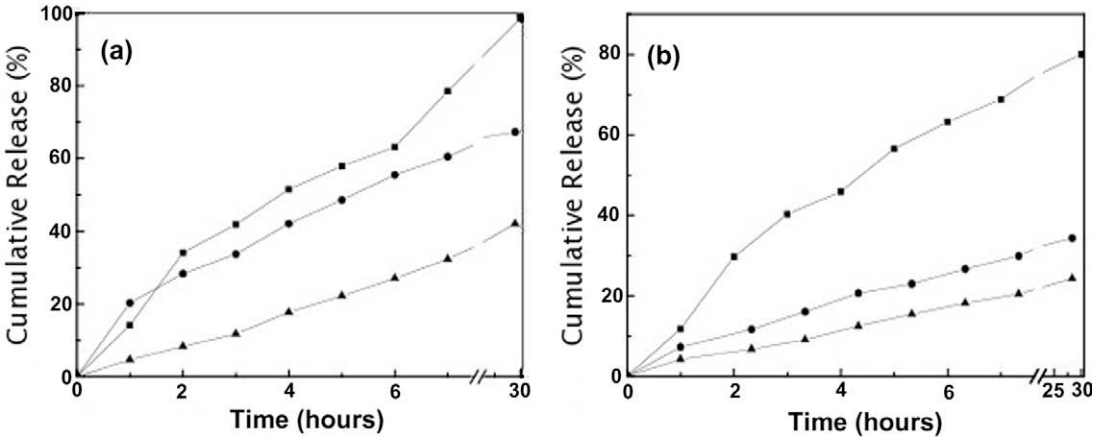

Fig. 8. In vitro release profiles at 30 °C of (■) CS-MSD, (●) CS-MMD and (▲) CS-MID, loaded chitosan microspheres: (a) in ethanol and (b) in PBS solution (pH = 7.0).

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