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International Journal of Pharmaceutics 264 (2003) 45–55
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization

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Received 10 October 2002; received in revised form 9 July 2003; accepted 9 July 2003

Abstract

Gelatin microparticles containing propolis extractive solution (PES) were prepared by spray-drying technique. The optimization of the spray-drying operating conditions and the proportions of gelatin and mannitol were investigated. Regular particle morphology was obtained when mannitol was used, whereas mannitol absence produced a substantial number of coalesced and agglomerated microparticles. Microparticles had a mean diameter of 2.70 μm without mannitol and 2.50 μm with mannitol. The entrapment efficiency for propolis of the microparticles was upto 41% without mannitol and 39% with mannitol. The microencapsulation by spray-drying technique maintained the activity of propolis against *Staphylococcus aureus*. These gelatin microparticles containing propolis would be useful for developing intermediary or eventual propolis dosage form without the PES' strong and unpleasant taste, aromatic odour, and presence of ethanol.

Keywords: Propolis; Gelatin; Spray-drying; Microparticle characterization; Optimization

1. Introduction

Research and development of various microparticulate systems as drug carriers have been done in the pharmaceutical field (Chen et al., 1987; Gupta and Hung, 1989) to make solid entities from oils, to control odour or taste, to protect drugs from moisture or oxidation, to alter solubility, to delay volatilization, and to prevent incompatibilities (Luzzi and Palmieri, 1985). Biodegradable and biocompatible materials such as gelatin (Esposito et al., 1996; Palmieri et al., 1996; Toledano and Magdassi, 1997; Vinetsky and Magdassi, 1997a,b; Franz et al., 1998; Morita et al., 2001), dextran (Lee et al., 1999; Stenekes et al., 1999), and collagen (Rossler et al., 1995) have been investigated on microencapsulation processes.

Gelatin is a natural material and its biocompatibility and the degradation to non-toxic and readily excreted products are the main attractive characteristics. But, being a soluble polymer, gelatin has to be modified to prepare drug delivery systems. Drying, cross-linking or co-lyophilization, for example, can be used to reach such modification (Morita et al., 2001; Vandelli et al., 2001). Gelatin is a water-soluble material having wall-forming ability when a mixture of ethanolic solution or volatile aroma, water, and wall-forming material is spray-dried (Lee et al., 1999).

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doi:10.1016/S0378-5173(03)00386-7

Spray-drying is extensively used in the pharmaceutical industry to produce raw drug or excipients or as microencapsulation process (Broadhead et al., 1992; Palmieri et al., 1994; Lee et al., 1999; Billon et al., 2000). This technique transforms liquid feed into dry powder in a one step, continuous particle processing operation and can be applied to a wide variety of materials (Broadhead et al., 1992). Spray-drying technique has inconveniences related to processing variables that must be well controlled to avoid difficulties such as low yields, sticking, or high moisture content. These are often encountered with laboratory scale spray-dryers (Broadhead et al., 1992; Billon et al., 2000). The optimization of spray-drying process involves the evaluation of parameters concerning both spray-dryer and feed formulation (Conte et al., 1994; Wendel and Celik, 1997; Lee et al., 1999; Billon et al., 2000).

Propolis (bee glue) is a strongly adhesive resinous bee hive product collected by honeybees that has been used by man since ancient times due to its pharmaceutical properties (Ghisalberti, 1979; Burdock, 1998). These include antimicrobial (Moreno et al., 1999; Koo et al., 2000; Sforcin et al., 2000; Marcucci et al., 2001; Santos et al., 2002), fungicidal (Kujumgiev et al., 1999; Murad et al., 2002), antiviral (Ghisalberti, 1979; Marcucci, 1995; Kujumgiev et al., 1999), antiulcer, immunostimulating, hypotensive (Marcucci, 1995; Burdock, 1998), inflammatory (Burdock, 1998; Song et al., 2002a,b), antioxidant (Moreno et al., 1999; Isla et al., 2001; Nagai et al., 2001), and cytostatic (Banskota et al., 2002) activities. Its chemical composition is complex and typically consists of waxes, resins, water, inorganics, phenolics, and essential oils, the exact composition depending upon the source plant(s) (Bankova et al., 1992; Marcucci, 1995; Markham et al., 1996; Burdock, 1998).

Alone or incorporated in another dosage form propolis ethanolic extractive solution is commonly utilized on therapeutics, namely wound healing, tissue regeneration, treatment of burns, neurodermatitis, leg ulcers, psoriasis, herpes simplex and genitalis, rheumatism and sprains, periodontal diseases, candidiasis, cheilitis, stomatitis, influenza, and cold. Some disadvantages are the strong and unpleasant taste, aromatic odour, and high ethanol concentration (Ghisalberti, 1979; Burdock, 1998). These disadvantages result in difficulties on packing, transport, and incorporation in another dosage form. The patient compliance to the therapeutics is committed too.

The objective of this study was to develop a spray-drying method to prepare propolis microparticles using gelatin as a wall-forming polymer and to characterize the obtained propolis microparticles.

2. Materials and methods

2.1. Materials

Propolis was collected at the experimental farm of Universidade Estadual de Maringá (UEM), Paraná State, Brazil. Type A gelatin, Royal (Brazil), was used without further purification. Methanol (Mallinckrodt; HPLC grade), water filtered through a Milli-Q apparatus (Millipore) and acetonitrile (Mallinckrodt; HPLC grade) were used for the High-Performance Liquid Chromatography (HPLC) mobile phase separation. Pharmaceutical grade standard, chrysin, was purchased from Sigma and it was used as external standard. Acetone (Merck; analytical grade), ethyl acetate (Merck; analytical grade), methanol (Merck; analytical grade), acetic acid (Merck; analytical grade), aluminium chloride (Merck; analytical grade), mannitol and ethyl alcohol (96°GL; pharmaceutical grade), Miller Hinton culture medium (Mikrobiologie), Vogel Johnson Agar culture medium (Difco) and telurite solution 1% (Difco) were also used.

2.2. Preparation and characterization of the propolis extractive solution (PES)

Propolis extractive solution was prepared with propolis/ethanol ratio of 30/70 (w/w) by turbo extraction (List and Schmidt, 1989), was filtered through filter paper and was made up to the initial weight with the ethanol. Exactly weighted 10 g of PES were concentrated on water bath (100 °C) with eventual shake. The concentrated material was dried on the Ohaus–MB 200 infrared analytical balance (110 °C). Three replicates were carried out to estimate the inherent variability of the determination.

The total flavonoids drift of PES was obtained using the technique according Franco et al. (2000). Three millilitres of distilled water, 3.0 ml of acetone, and 3.0 ml of PES were added in a separation funnel. This

mixture was three times extracted with 15 ml of ethyl acetate. Ethyl acetate was added to 50.0 ml S1. Exactly 1 ml of aluminium chloride ethanolic solution (2%, w/v) and methanolic solution of acetic acid (5%, v/v) was added into 10.0 ml of S1 to 25.0 ml MS. At the time, methanolic solution of acetic acid (5%, v/v) was added into 10.0 ml of S1 to 25.0 ml MS to be used as compensatory solution. After 30 min, MS was analysed by HITACHI U2000 spectrophotometer (λ = 425 nm). Six replicates were carried out to estimate the inherent variability of the determination and the total flavonoids drift of PES was calculated in grams of quercetin (specific absortivity = 500) obtained in 100 g of dried propolis.

2.3. Preparation of propolis microparticles

Propolis extractive solution was dispersed in a series of gelatin solutions, using dripping technique, at 25 °C and with magnetic agitation by 30 min (Bruschi and Gremião, 2001). The quantity of gelatin utilized was function of the PES DR% (Table 1). The final dispersions were spray-dried in a BÜCHI Mini Spray Dryer model B-191 (Büchi, Switzerland) through the nozzle using a peristaltic pump. The spray-drying conditions were dependent on the experiment (Table 1). The resultant dried products were collected and kept away from rehydration until further tests.

An experiment was developed using mannitol with the aim to reduce the microparticles aggregation. The utilized amount of mannitol was 20% (w/w) in relation to the total amount to be obtained.

2.4. Analysis of experiments

On the analysis of experiments two responses were considered: production yield and residual moisture content of spray-dried product. Residual moisture was measured with an Ohaus–MB 200 infrared analytical balance on a 3.0 g sample at 110 °C. Three replicates were carried out to estimate the inherent variability of the analysis.

2.5. Characterization of propolis microparticles

2.5.1. Scanning electron microscopic study

The spray-dried products were coated under argon atmosphere with gold/palladium and examined under a scanning electron microscope (JEOL JSM—T330A). The scanning electron photomicrographs (SEM) were evaluated.

2.5.2. Particle size analysis and distribution

The samples of propolis microparticles (PM) were subjected to particle analysis by a LEICA DMRXA optical microscopy with Leica *Qwin Image Analysis System*. Particles were placed on glass slide and the size measurements of microparticles were performed using Feret's diameter as parameter. Three thousand and five hundred microparticles were measured and the particle size distribution was estimated.

2.5.3. Assay for propolis trapping efficiency

The amount of propolis in the microparticles was determined by HPLC. A 15 mg sample of PM was added in acetonitrile to 25.0 ml and sonicated for 5 min. The dispersion was filtered through a membrane filter (pore size 0.20 μm, GTTP, Millipore). The propolis concentration in the clear solution was determined by HPLC quantifying the three major peaks (markers) of the obtained chromatogram. A reversed phase Chromsep RP C18 column (250 mm × 4.6 mm i.d., particle size 5 μm, Varian) was used, and the column temperature was maintained at 30 ± 0.1 °C. The mobile phase was methanol (A) and acetonitrile/water (2.5/97.5, v/v) (B), previously filtrated

Table 1
Spray-drying conditions and amounts gelatin tested

Experiment	Inlet temperature (°C)	Gelatin/PES DR% ratio (w/w)	Feed rate (%)	Aspiration (%)	Pressure (%)	Mannitol (%)
1	140	3/1	4	85	2	0
2	160	6/1	6	85	3	0
3	140	6/1	5	90	2	0
4	160	6/1	6	80	3	0
5	160	6/1	6	80	3	20

through a 0.45 μm PALL-Gelman membrane filter and degassed by ultrasound. After preliminary experiments, a gradient elution system was adopted: 0 min, 50% A and 50% B; 47 min, 80% A and 20% B; 50 min, 100% A and 0% B; 58 min, 100% A and 0% B; 62 min, 47% A and 53% B; 67 min, 47% A and 53% B. The flow-rate was 1.0 ml/min and the absorbance of the eluate at 310 nm was monitored. The HPLC system consisted of Prostar/Dynamax 2.4 two pumps, a Prostar automatic controller of flow, a Prostar 330 Ultraviolet-Visible Photo Diode Array spectrophotometric detector module, column oven, and a Star integrator system (Varian). Fixed loop injector (Rheodyne VS 7125, 100 μl) was utilized to carry the sample into the column. The entrapment efficiency for propolis of the microparticles was calculated using the three markers, comparing the results obtained from the PES with the PM.

2.5.4. Antibacterial activity evaluation

Staphylococcus aureus ATCC 25 923 was used to evaluate the antibacterial activity of PM. The strain was kept in laboratory. For experiment, *S. aureus* was inoculated in Miller Hinton medium culture (MH) at 37 °C/24 h and the microorganism standardization was carried out using spectrophotometer (Coleman) at 580 nm. The equipment was calibrated with distilled water and culture medium to zero of absorbance. The sample was adjusted to have 25% of transmittance.

About 150, 300, and 500 mg exactly weighted experiment 4 and experiment 5 PM samples were added in 2.0 ml of MH tube. Propolis extractive solution was sterilized by filtration using 0.22 μm membrane (Millipore) and was used as control. Exactly 2.0 ml of PES were added in 2.0 ml of MH tube. A tube without substance addition was used to negative control. 0.5 ml of standardized *S. aureus* solution were added in all tubes. After 37 °C/24 h, the samples were inoculated in with telurite solution Vogel Johnson Agar medium. The inoculated plates were incubated at 37 °C/24 h and the plates were visual examination of the bacterial growth.

3. Results and discussion

3.1. Characterisation of the PES

Propolis extractive solution dryness residue was 17.05 ± 0.18% with 1.08% of relative standard deviation (RSD). Total flavonoids drift was 1.98 ± 0.07% and its RSD was 3.41%.

3.2. Analysis of experiments

The influence of spray-dried conditions, gelatin amount, and mannitol presence was carried out. The PM formulations were covered in the collecting flask and the yields and moisture contents were measured. The yields varied from 28.46 to 51.48% for experiments 1–5, respectively (Tables 2 and 3). In parallel, moisture contents varied from 4.12 to 9.40% for experiments 1–5, respectively (Table 2).

The low gelatin concentration in the experiment 1 and the fixed operating conditions of the experiment 2 induced low yields by promoting large sticking in the drying chamber. The resulting products cannot be removed. For these products, high moisture content is

Table 2
Production yield and residual moisture content of spray-dried products

Preparation	Yield (%)	Moisture content (%)
Experiment 1	34.62	5.88
Experiment 2	43.32	9.40
Experiment 3	28.46	6.25
Experiment 4	44.80	6.54
Experiment 5	51.48	4.12

Table 3
Propolis trapping efficiency

Preparation	Spray-drying conditions	Drug trapping efficiency (%)		
		Peak 1	Peak 2	Peak 3
1	Experiment 4	42.24 ± 1.94	47.85 ± 0.87	41.28 ± 0.92
2	Experiment 5	50.96 ± 2.19	63.71 ± 5.69	39.61 ± 3.17

also recorded. On the other hand, the experiment 3 resulted good moisture content and very low yields. These results were probably promoted by small feed rate and spray pressure.

The experiment 4 resulted product with good yield and moisture contents. These results were probably influenced by combination of the liquid feed rate, the dryer inlet temperature, and spray pressure obtained. The experiment 5 yield obtained was better than experiment 4 yield (Fig. 2). The same spray-drying conditions were used, but mannitol was added in the formulation. Mannitol (20–90%, w/w) is utilized

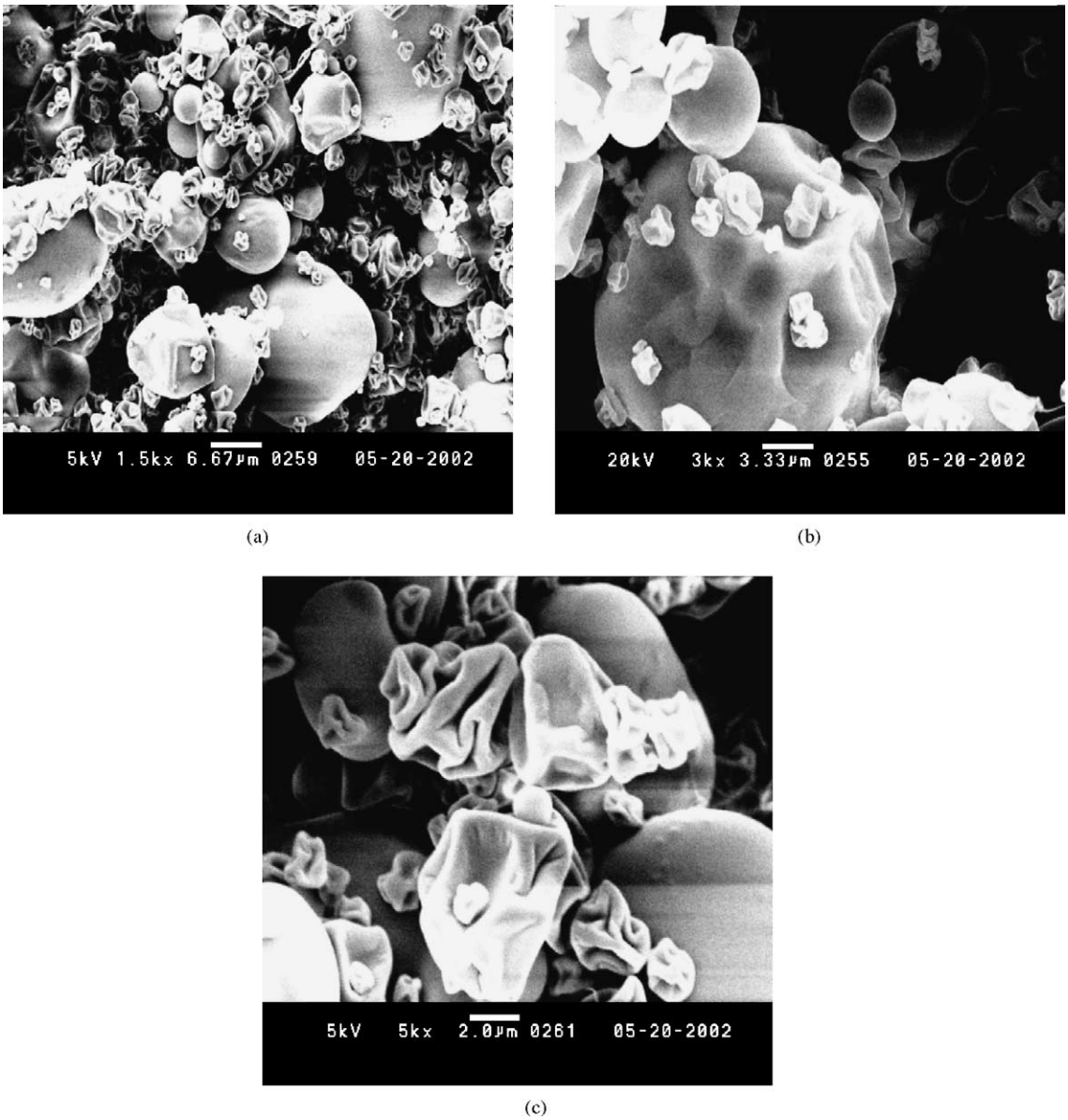

Fig. 1. SEM photomicrographs of spray-dried propolis microparticles showing the outer topology of experiment 4 conditions (without mannitol): (a) original magnification 1500×; (b) original magnification 3000×; and (c) original magnification 5000×.

in spray-dried formulations to improve spray-dried material characteristics and avoid microparticle sticking (Wade and Weller, 1994). Thus, the best responses were obtained with the experiments 4 and 5 conditions.

3.3. Scanning electron microscopy

The photomicrography of the PM without mannitol (experiment 4) is showed in Fig. 1. The most part of particles was small and had spherical shape,

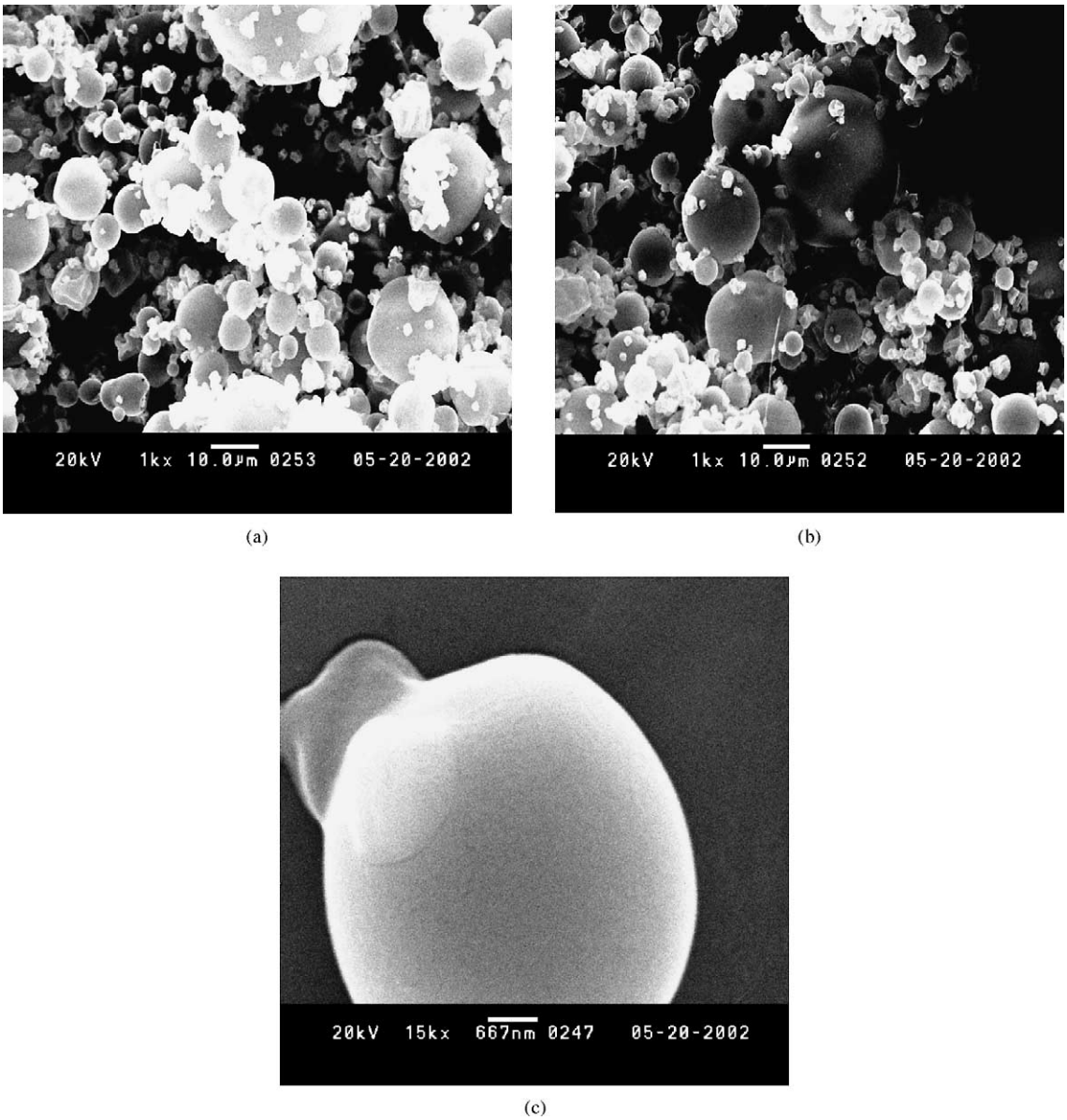

Fig. 2. SEM photomicrographs of spray-dried propolis microparticles showing the outer topology of experiment 5 conditions (with mannitol): (a) original magnification 1000×; (b) original magnification 1000× and; (c) original magnification 15,000×.

non-uniform surface, and were coalesced. These spray-dried products were more or less agglomerated. These results are in agreement with Palmieri et al. (1996) and Billon et al. (2000).

The SEM photomicrographs of spray-dried PM obtained with mannitol (experiment 5) showed a very smooth and uniform surface, and spherical shape (Fig. 2). A low number of coalesced microparticles and less agglomeration were observed. These results were probably obtained by mannitol added in the formulation, acting as a plasticizer and preserving the system hydration. For spray-drying microencapsulation, the presence of plasticizer is important since it promotes the formation of spherical and

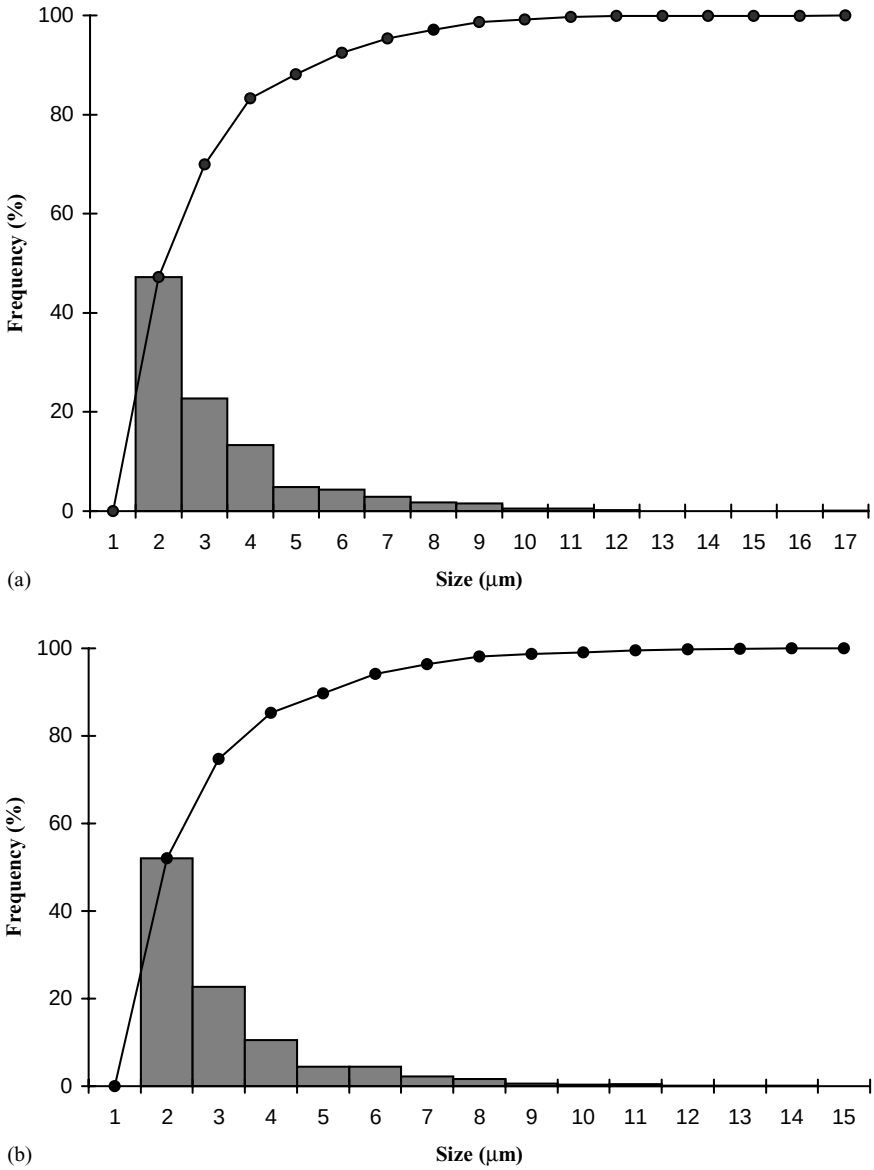

Fig. 3. Size distribution of propolis microparticles without mannitol (a) and with mannitol (b): size frequency distribution (bars) and size cumulative frequency distribution (line). The particle size class interval is 1.0 μm.

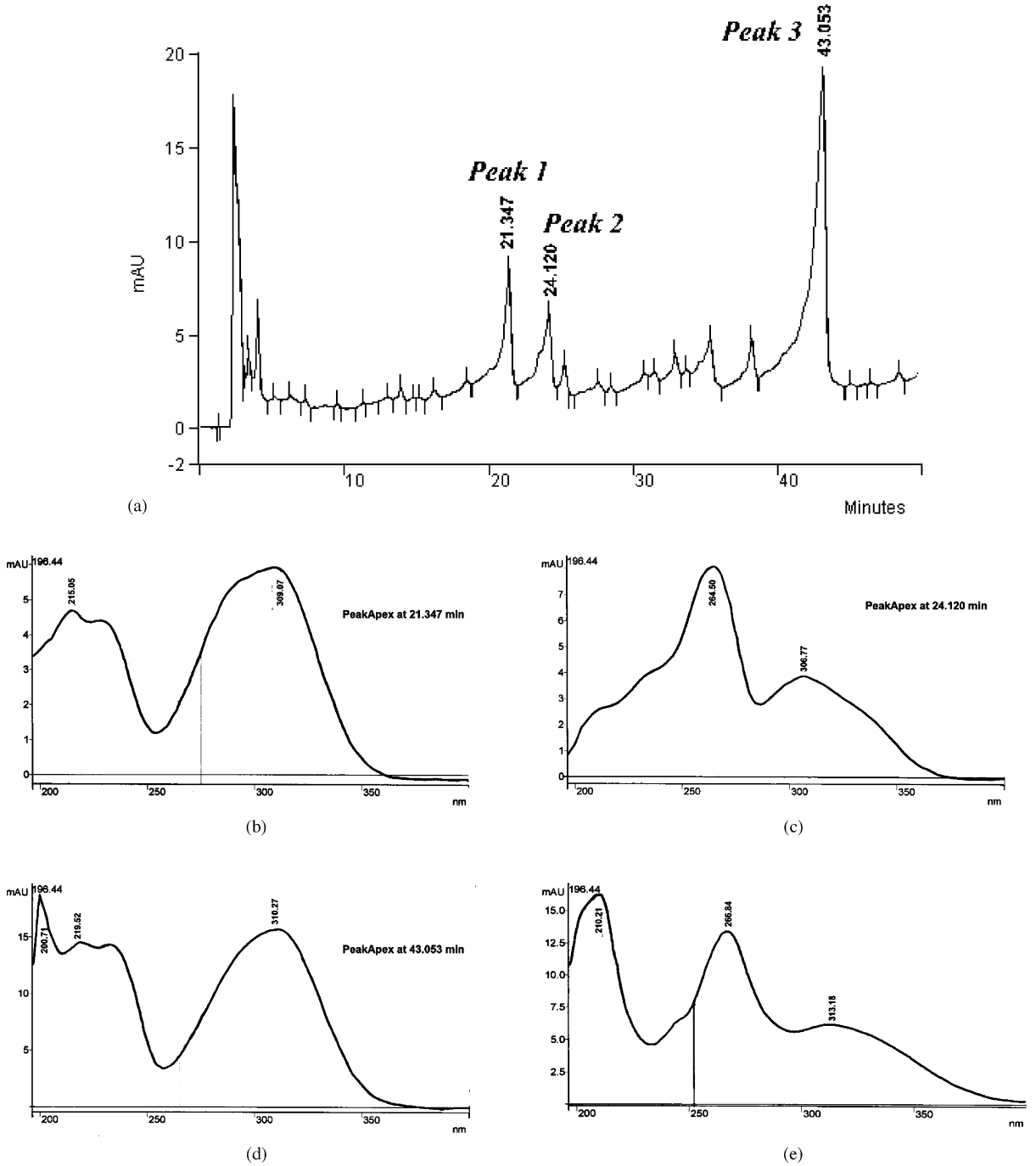

Fig. 4. HPLC chromatogram diode array spectrums 200–400 nm: (a) PES chromatogram; (b) diode array spectra of Peak 1 (21.347 min); (c) diode array spectra of Peak 2 (24.120 min); (d) diode array spectra of Peak 3 (43.053 min); and (e) diode array spectra of chrysin.

smooth-surfaced microparticles (Zhang et al., 2000). In this work, mannitol was added to reduce the microparticles agglomeration thus interacted with the gelatin, probably reducing the polymer chain contacts. This reduction can have decreased the rigidity of the three-dimensional film structure, resulting on a strong adhesion force between gelatin and propolis assuring an enough contact. Moreover, mannitol retains some water molecules linked to its own structure and could fill the intern empty space of the microparticles, preserving the hydration, avoiding depressions on the surface, and assuring a more uniform wall of the obtained microparticles.

3.4. Particle size analysis and distribution

Propolis microparticles were prepared by a spray-drying method using the conditions of experiments 4 and 5. The typical size distribution graphs are presented in Fig. 3. The microparticles have a mean diameter of 2.70 μm without mannitol (experiment 4) and 2.50 μm with mannitol (experiment 5). These results confirm that obtained structures are microparticles (Fig. 3).

3.5. Entrapment efficiency of propolis in microparticles

To calculate the entrapment efficiency for propolis of the microparticles, PES and PM were quantified by HPLC. Propolis extractive solution chromatogram showed three major peaks that were used as markers (Fig. 4). The UV spectra of the peaks with retention times of 21.347, 24.120, and 43.053 min indicated that these substances have similar behaviours to the chrysin and correspond, probably, to the flavonoids. The precise identification of these compounds is being investigated. Thus, the three major peaks were used as markers. For each peak, the propolis entrapment efficiency was calculated.

3.6. Antibacterial activity evaluation

The use of microparticle could enhance the therapeutic effect of biomedical materials or drugs. Baras et al. (2000) reported that microencapsulation preserved the crucial characteristics required to generate an effective humoral immune response of an antigen after a single-dose administration. Santoyo et al. (2002) showed that cidofovir-loaded microparticles could improve cidofovir topical therapy. Sforcin et al. (2000) evaluated the efficient antibacterial activity of PES on *S. aureus* strains. In this work, no bacterial growth was observed for PES and PM samples. Bacterial growth was only observed in the negative control. These results qualitatively demonstrate that the microencapsulation by spray-drying technique maintained the activity of the PM against *S. aureus*.

4. Conclusion

Gelatin microparticles containing propolis preparation by spray-drying method has been shown to be a feasible and inexpensive method. The characteristics and morphology of microparticles could be improved by a proper selection of spray-drying conditions, gelatin amount, and mannitol presence. Thus, these results were useful for developing of propolis dosage form without the PES' strong and unpleasant taste, aromatic odour, and high ethanol concentration.

Acknowledgements

The authors thank the financial support of CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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