PRODUCTION AND CHARACTERISATION OF BIODEGRADABLE PLA NANOPARTICLES LOADED WITH THYMOL TO IMPROVE ITS ANTIMICROBIAL EFFECT

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Highlights

- PLA nanoparticles loaded with Thymol were prepared for the first time.
- The PLA was found to be the key variable in optimizing the nanoparticle preparation.
- The nanoparticles exhibited a high storage stability over a wide range of pHs.
- The antimicrobial activity of the nanoparticles was tested on apple pieces.

Abstract

Thymol is widely recognised as an antibacterial compound. However, its use in food technology is hindered by its high volatility, its light sensitivity and its low solubility in water. To overcome these drawbacks, the preparation of nanoparticles using polylactic acid (PLA) is proposed in this study.

The average size of the nanoparticles and thymol encapsulation efficiency parameters were studied using different PLA and thymol concentrations. Furthermore, the morphology, the storage stability of the nanoparticles at several pHs and the *in vitro* thymol release profile were also studied, as well as their thermal degradation profile. Finally, their antimicrobial activity on a real food model was measured, using for this purpose apple pieces previously inoculated with *E. coli*.

The PLA was found to be the key variable in optimizing the nanoparticle preparation, producing spherical nanoparticles with a thymol encapsulation efficiency of 60.3 ± 8%. These nanoparticles showed a high storage stability at several pHs and improved antimicrobial properties in comparison with the non-encapsulated thymol.

Keywords

PLA; thymol; encapsulation; nanoparticles; antimicrobial properties; storage stability; thermal degradation; release profile.

1. Introduction

The aromatic and medicinal properties of the plants of the genus *Thymus* have been widely recognized. These plants are usually used to make tea, as a flavouring agent and for medicinal purposes (Stahl-Biskup and Sáez, 2003).The results reported reveal that the main constituent obtained from the aerial parts of the plant are geraniol, linalool, gamma-terpineol, carvacrol and thymol (Piccaglia et al., 1993). Thymol (2-isopropyl-5-methylphenol) is a volatile compound which is a phenolic derivate of the terpene 3-hidroxy-p-cymene and its production by the plant is associated with part of its defensive strategy against phytopathogenic microorganisms. Numerous studies have reported the antibacterial and fungicidal properties of thymol (Andrade-Ochoa et al., 2015; Džamić et al., 2015; Hernández-Hernández et al., 2014). For this reason, this compound has received the attention of the food industry, thymol having been used as an antimicrobial agent and authorized by the Food and Drugs Administration (FDA) of the USA as generally recognised as safe (GRAS). However, there are technical limitations which hinder its use, such as the high volatility of thymol at room temperature, its high light sensibility and its low solubility in water (Mastelić et al., 2004).

One way to overcome these problems is the preparation of nanoparticles which contain thymol. In the form of nanoparticles, the amount of thymol introduced into the food matrix could be increased above its solubility limit, since the Brownian movement of the nanoparticles prevents their sedimentation in the lower layers of the food (Huang, 2012). Furthermore, the high surface-area ratio of nanoparticles enhances their contact with the microorganisms, and the protective environment provided could limit the volatility of the thymol and its light-induced degradation. For this reason, several authors have prepared protein-based nanoparticles which incorporate thymol. To prepare these nanoparticles, the main protein used was zein, since both zein and thymol are soluble in ethanol and the particles can be prepared by antisolvent precipitation (Rosa et al. (2015), Li et al. (2012), Zhang et al. (2014) and Chen et al. (2015)). However, the use of biodegradable synthetic polymers instead of proteins to encapsulate thymol remains unstudied, although their use to prepare nanoparticles is a topic that has received a lot of attention from the scientific community. These synthetic polymers have some advantages with respect to proteins: they are produced with a high level of purity, the experiments investigating their use have excellent reproducibility and they do not produce any antigenic effect (Nair and Laurencin, 2005). In particular, polylactic acid (PLA) has been widely used in biomedicine and it is considered a good material to prepare micro and nanoparticles, since the particle size and shape can be controlled so as to satisfy the preparation requirements. Furthermore, in this type of nanoparticle, the active agent is usually homogeneously dispersed within the PLA matrix, which is positively related to the appropriate liberation of the active agent (Lee et al., 2016).

To the best of our knowledge, this study documents the first preparation of PLA nanoparticles containing thymol. The production of these nanoparticles was evaluated using different PLA and thymol concentrations, and then they were characterised by evaluating their morphology, encapsulation efficiency, stability at several pHs and thermal degradation profile. The antimicrobial properties of the nanoparticles obtained were tested on apple pieces previously inoculated with *E. coli*.

2. Materials and methods

2.1. Materials

The PLA (180 kDa) was produced by NatureWorks (4032D). The following reagents were acquired in Sigma-Aldrich (St Louis, USA): thymol (ref. T0501), dichloromethane (DCM, ref 270997), polyvinyl alcohol (PVA, ref P8136), buffer Trizma® pH 7.0 (ref. T1819), 2,2-diphenyl-1-picrylhydrazyl (DPPH, ref. D9132) and Nutrient Broth (NB, ref. 70149NB). The agar was acquired in VWR (Pensilvania, USA).

2.2. Preparation of PLA nanoparticles

Nanoparticles were prepared following the single emulsion preparation technique (Lee et al., 2016) with slight modifications: a 1% PVA solution was prepared and saturated with thymol. In order to prepare this solution, the PVA at the required concentration was heated in a water bath at 90 °C until it was completely dissolved. This solution was cooled to room temperature, 2 mg/mL of thymol was added and the mixture was stirred overnight. Then, the solution was filtered to remove the non-solubilised thymol using a vacuum pump and Nº1 Whatman paper. The filtration process was repeated twice.

Three different amounts of PLA were dissolved in 7.5 mL of DCM, and for each of these PLA concentrations, three different amounts of thymol were dissolved in the same DCM volume. These percentages of thymol with respect to the weight of PLA, as well as the precise amounts of thymol and PLA tested in each case are shown in Table 1.

Table 1. Amounts of PLA and thymol dissolved in the organic phase and tested to optimize the nanoparticle preparation.

	Thymol		
PLA	**66%**	**100%**	**133%**
100 mg	66 mg	100 mg	133 mg
150 mg	100 mg	150 mg	200 mg
200 mg	133 mg	200 mg	266 mg

This DCM solution was carefully poured into 30 mL of the previously prepared PVA solution. The mixture was ultrasonicated for 2.5 minutes using the Sonopuls HD 2070 system (Bandelin, Germany) and the MS 73 probe, at a frequency of 20 kHz and applying a sonication amplitude of 90% (100% corresponds to 212 µm). This sonication amplitude corresponds to an ultrasonic intensity of 80 W/cm^2. During the sonication process, the sample was kept in ice to avoid temperature increase. To remove the DCM contained in the preparation, the emulsified solution was kept in a low-pressure medium at 40 °C for 40 minutes using a rotavapor (Büchi R-205, Büchi Labortechnik, Essen, Alemania). During the DCM evaporation, part of the thymol was able to pass from the nanoparticles to the PVA medium. It was precisely to avert this problem that the PVA solution was saturated with thymol, as was described previously.

After the DCM evaporation, the sample was centrifuged at 13000 rpm for 20 minutes to precipitate the nanoparticles. The supernatant, which contains the PVA and is saturated with thymol, was removed by decantation and it could be reused to prepare new nanoparticles. The same volume of distilled water was added to the sediment, and the nanoparticles were resuspended and centrifuged again at 4000 rpm for 5 minutes. In this case, the sediment was discarded to remove PLA aggregates, the nanoparticles remaining in suspension.

2.3. Nanoparticle size measurement and thymol content

The average size of the nanoparticles and their polydispersion index (PDI) were measured using dynamic light scattering (DLS, Nanosizer ZS, Malvern, UK).

The amount of thymol encapsulated was measured as follows: the nanoparticle suspension was centrifuged at 13000 rpm for 20 minutes and the supernatant was removed and replaced with the same volume of ethanol (96°). The sediment, which contains the nanoparticles, was dispersed in the ethanol with the aid of the sonicator system. Then, the sample was centrifuged again at 13000 rpm for 20 minutes. During this process and due to the high solubility of thymol in ethanol, the active agent was extracted from the nanoparticles. Finally, 0.1 mL of this ethanolic solution was diluted with 9.9 mL of fresh ethanol. The absorbance of the resulting solution was measured at 275 nm using a spectrophotometer (Spekol 1500, Analytik Jena AG, Jena, Alemania). Previously, known concentrations of thymol in ethanol were measured at this wavelength to calculate a calibration curve. The encapsulation efficiency (EE%) was calculated according to the following equation.

$$\mathrm{EE\%} = \frac{\text{thymol encapsulated}}{\text{thymol added in the DCM solution}} \times 100 \qquad (1)$$

2.4. Nanoparticle morphology

A drop of the nanoparticle solution was placed on a copper grid and negatively stained with a drop of phosphotungstic acid solution 2% (w/v). The micrographs were obtained using a transmission electron microscope (TEM, JEOL-2000 EX-II, Tokyo, Japan) operated at 200 kV. Furthermore, micrographs obtained using a scanning electron microscopy (SEM, JSM-6610LV, JEOL, USA) were also performed. In this case, a drop of the sample was dried on a glass microscope slide and coated with gold. The surface morphology of the nanoparticles was observed at 20 kV.

2.5. Nanoparticle storage stability at various pHs

A freshly prepared solution of nanoparticles was centrifuged at 13000 rpm for 20 minutes and the supernatant was discarded. The sediment, which contains the nanoparticles, was resuspended in a buffer solution at pH 4.0 (phosphate-citrate), or at pH 7.0 (Trizma®), or at pH 9.0 (sodium carbonate-bicarbonate) and stored at 5 °C. The buffer concentration was 0.01 M in all cases. Periodically, the average size of the nanoparticles and the thymol content were measured.

2.6. Thermogravimetric analysis (TGA)

The samples tested were lyophilized for 24 hours whilst applying a pressure of 0.33 mbar. The TGA curves were carried out in an SDTA851e TGA analyser (Mettler-Toledo, Switzerland) from 30 °C to 700 °C, under a nitrogen atmosphere. The heating rate was 10 °C/min. In this case, four samples were tested: the nanoparticles loaded with thymol; the nanoparticles prepared as described in section 3.2, but without the addition of thymol (unloaded nanoparticles); the thymol and the PLA.

2.7. *In vitro* thymol release profile

To evaluate the thymol release profile, 10 mL of a freshly prepared solution of nanoparticles was placed in a dialysis tube of 10 kDa MWCO, which was placed in a thermostatically controlled, 15 L capacity water bath (ref. 3001373, J. P. Selecta, Barcelona, Spain). The water in the bath was under agitation due to a water stream provided by a pump. Several temperatures were tested, and periodically one aliquot of 0.5 mL was taken from the dialysis tube and the thymol content in the nanoparticles measured. To characterise the thymol release profile, the data obtained were modelled using a first order equation (eq. 2) and the Korsmeyer-Peppas equation (eq. 3) (Siepmann and Peppas, 2001).

$$\frac{\mathrm{Mt}}{\mathrm{M}\infty} = 1 - \mathrm{e}^{-\mathrm{kt}} \qquad (2)$$

$$\frac{\mathrm{Mt}}{\mathrm{M}\infty} = \mathrm{k} \times \mathrm{t}^{\mathrm{n}} \qquad (3)$$

Where $\frac{Mt}{M\infty}$ is the accumulated percentage of thymol released at each time (t), *k* is the release constant of thymol and *n* is the diffusion exponent that characterises the release mechanism of the thymol.

2.8. Nanoparticle antimicrobial properties

To compare the antimicrobial properties of thymol and the nanoparticles loaded with thymol, apple pieces weighing 1 g were inoculated with the non-pathogenic *E. coli* CECT 101 strain. This strain was incubated at 30 °C for 48 hours using NB medium supplemented with 2% agar. To prepare the liquid inoculum, *E. coli* was incubated in NB liquid medium for 10 hours, at 30 °C with orbital stirring (250 rpm).

For all these treatments, each piece of apple was inoculated with 100 µL of *E. coli* and left for 2 minutes. The *E. coli* concentration used for this experiment was 10^5 UFC/mL, which might be considered high, but the strong antimicrobial properties of thymol made it necessary so as to be able to detect variation in the ability to decrease *E. coli* growth on the apple pieces between different preparations. After the inoculation, the apple pieces were submerged for 1.5 minutes in 5 mL of an aqueous solution that contained thymol or nanoparticles loaded with thymol. To solubilise the thymol, crystals of this active agent were added to distilled water at a concentration of 2 mg/mL, stirred overnight and filtered using nº1 Whatman paper. The concentration of dissolved thymol was determined by mixing 0.1 mL of this filtered solution with 9.9 mL of ethanol and measuring the absorbance at 275 nm, in accordance with the methodology described in section 2.3. To investigate the antimicrobial effect of this solution, three thymol concentrations were tested: 0.94 mg/mL, which corresponds to the maximum concentration of thymol dissolved in water, 0.5 mg/mL and 0.1 mg/mL. The same concentrations of thymol were used for the nanoparticle samples. After the treatment, the apple pieces were hermetically closed in polyethylene boxes and stored at 5 °C until their analysis. To follow the growth of the *E. coli* on the apple surface, samples were taken at different times and mixed with 9 mL of NaCl 0.7%. Then, the samples were homogenised using a stomacher and cultured in NB-agar medium. After 24 hours of incubation at 30 °C the *E. coli* colonies were counted and the results obtained expressed as $\log_{10}$ UFC/g.

2.9. Statistical analysis

Experiments were performed in triplicate and are shown as the mean value ± standard deviation of three independent experiments (n = 3). Least significant differences (LSD) were calculated by

Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

3. Results

3.1. Size, polydispersity index, thymol recovered and encapsulation efficacy of the nanoparticles prepared

There are several parameters that could have a significant effect on the preparation of these nanoparticles and have the capacity to modify their average size and the efficiency with which they encapsulate thymol. In this sense, the variables which should be considered would include the amount of DCM and water, the time and type of stirring used to obtain the emulsion (ultrasounds or mechanical stirring), the amount of energy applied during the process, the type and concentration of surfactant used to stabilise the emulsion, and the amounts of PLA and of thymol. In our previous tests it was observed that, among all these parameters, variations in the PLA and thymol concentrations produced changes in the properties of the nanoparticles in a meaningful and straightforward way, and for this reason they were considered to be key variables for this process.

Figure 1A indicates the average size of the prepared nanoparticles and their PDI, whilst in Figure 1B the amount of thymol encapsulated and the encapsulation efficiency are shown. According to Figure 1A, these values suggest that, in broad terms, the higher the concentration of PLA in the organic phase, the greater is the average size of the nanoparticles obtained. A similar trend was observed when the amount of PLA was kept constant and the concentration of thymol was increased. The latter effect was particularly pronounced in the 200 mg PLA samples. Furthermore, in all cases the PDI values obtained were low enough to be considered satisfactory, being even lower than those obtained by other authors in the preparation of similar PLA nanoparticles (Roussaki et al., 2014; Wrona et al., 2017). As regards Figure 1B, the thymol recovered from the nanoparticles followed a similar trend to the average size, in that increasing the concentration of PLA and thymol led to an increase in the amount of thymol recovered. However, the encapsulation efficiency showed a different trend to the recovered thymol parameter, and the highest values obtained were from the 150 mg PLA preparations. In this case, the 100 mg PLA preparations showed the lowest encapsulation efficiency values, probably because the amount of PLA was low enough to produce nanoparticles with low internal density and high porosity, which were incapable of retaining the thymol. In the case of the 200 mg PLA nanoparticles, the density effect may have been reversed, and so, due to their high density, most of the nanoparticles could have precipitated out by the end of the last centrifugation (5000 rpm, 4 min.), which was carried out in accordance with the preparation methodology described in the materials and method section.

Taking into account all these results, and although the concentration of thymol and PLA produced variations in the size of the nanoparticles, in all the tested preparations this parameter was maintained within the 220 - 260 nm range. Since this could be considered a narrow range, it might be more effective to optimize the encapsulation efficiency in order to prevent loss of reagent and to increase the amount of thymol recovered. From the results shown in Figure 1B, the best preparation was seen to be that obtained using PLA and thymol concentrations of 150 mg. Furthermore, the PLA concentration resulted more relevant than the thymol concentration to maximize the efficacy of encapsulation parameter.

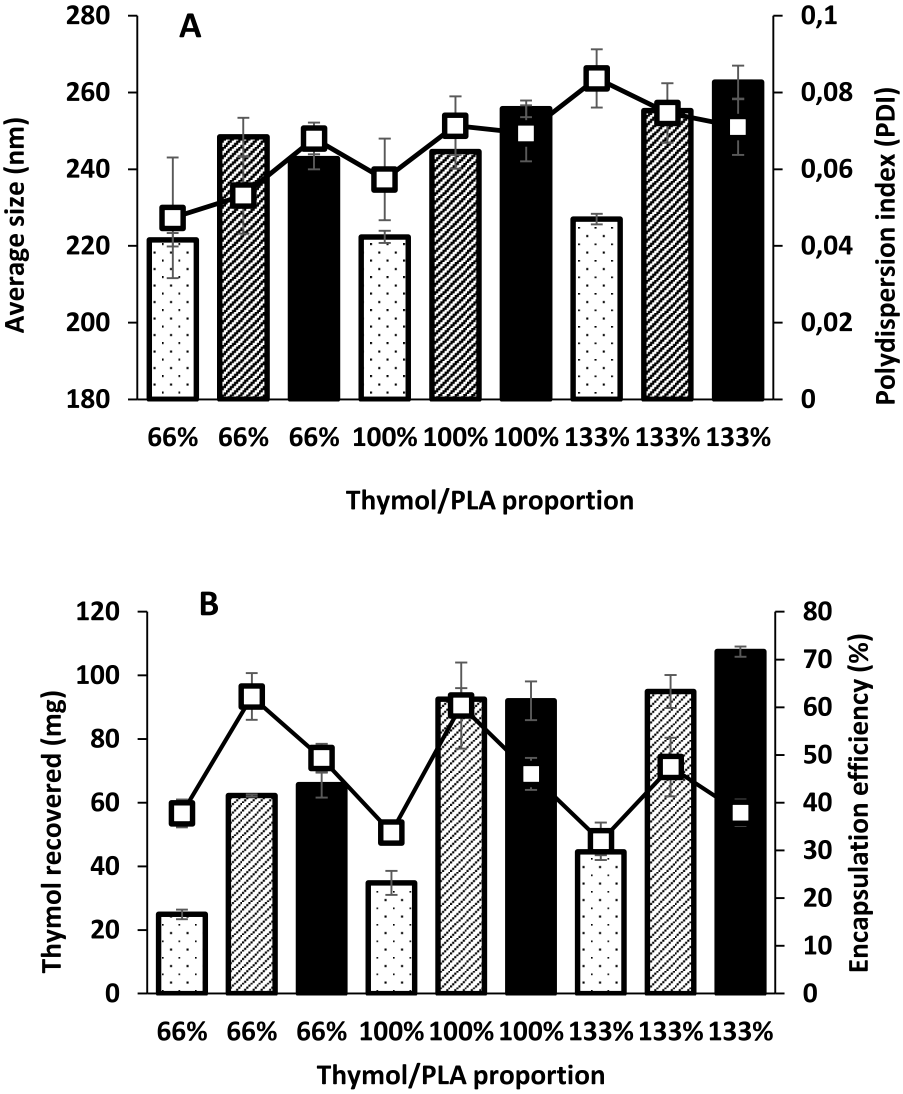

Figure 1. Parameters of interest for the characterisation of the nanoparticle preparations with respect to the PLA and thymol concentrations. Dotted bars correspond to the nanoparticles prepared using 100 mg de PLA, striped bars 150 mg of PLA and solid bars 200 mg of PLA. A: Average size (bars) and polydispersity index (line) of the nanoparticles prepared. B: Thymol recovered (bars) and encapsulation efficiency (line) of the nanoparticles prepared.

3.2. Nanoparticle morphology

The TEM and SEM micrographs of the nanoparticles prepared with 150 mg of PLA and several concentrations of thymol are shown in Figure 2. In all the cases, spheres with a smooth surface were observed. The size of the nanoparticles varied between 240 and 260 nm, corroborating the values obtained by DLS provided in section 3.1. The morphology of the nanoparticles prepared using proteins or polysaccharides is not usually so regular, and may have a rougher appearance, which could have some influence on the release profile of the active agent. Wattanasatcha et al. (2012) produced nanoparticles of ethylcellulose/methylcellulose loaded with thymol and with an almost spherical morphology. Similar results were obtained by Xue et al. (2014) preparing nanodispersions of lecithin, gelatin and thymol.

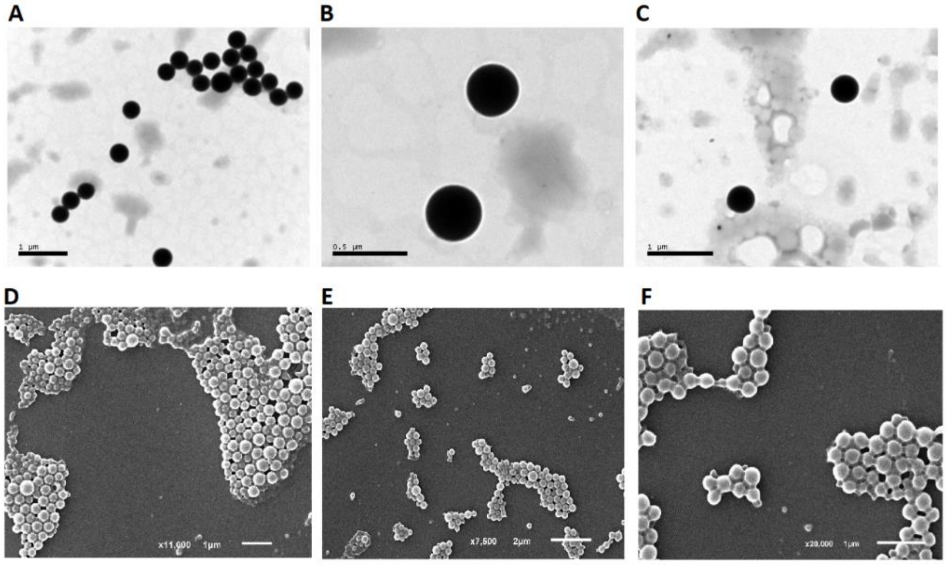

Figure 2. TEM (A, B, C) and SEM (C, D, E) micrographs of the nanoparticles prepared using 150 mg of PLA and 100 mg (A, D), 150 mg (B, E) and 200 mg (C, F) of thymol.

3.3. Nanoparticle storage stability at several pHs

The storage stability of the nanoparticles was tested at several pHs. For this purpose, the average size and the thymol encapsulated were evaluated every 10 days for 40 days. The results obtained are shown in Table 2. In all the tested samples, during a period of 40 days no changes were detected in the size of the nanoparticles at any pH. As regards the thymol encapsulated, at basic pH a slight decrease in this parameter was detected. In the case of the nanoparticles stored at pH 7.0 and 4.0, this decrease was even less pronounced. This could be due to the deprotonation of the phenolic hydroxyl group of the thymol at basic pH (Wu et al., 2012), which would increase its solubility in water, therefore enhancing the movement of the active agent from the nanoparticle to the aqueous medium. In any case, the decrease detected in the thymol loaded within the nanoparticles in 40 days can be considered low at all the pHs tested. Finally, it should be remembered that the encapsulation of thymol in protein-based nanoparticles is usually pH-dependent, since the proteins tend to aggregate or solubilize at a specific range of pH. In this case, the PLA nanoparticles do not exhibit this inconvenience and can be used over a wide range of pHs.

Table 2. Storage stability of the nanoparticles loaded with thymol at several pHs for 40 days.

	Average size (nm)					Thymol encapsulated (%)				
	Day 1	**Day 10**	**Day 20**	**Day 30**	**Day 40**	**Day 1**	**Day 10**	**Day 20**	**Day 30**	**Day 40**
pH 4.0	241.3 ± 1.3[a]	242.7 ± 2.7[a]	240.15 ± 0.2[a]	239.1 ± 2.2[a]	238.0 ± 2.5[a]	100[a]	95.8 ± 0.6[b]	96.4 ± 0.6[b]	96.0 ± 1.0[b]	96.5 ± 1.0[b]
pH 7.0	238.7 ± 1.3[a]	241.0 ± 0.5[a]	238.9 ± 1.8[a]	238.1 ± 1.5[a]	239.5 ± 1.2[a]	100[a]	98.2 ± 0.7[b]	98.9 ± 0.8[b]	98.0 ± 1.2[b]	97.3 ± 0.6[b]
pH 9.0	238.6 ± 0.1[a]	240.8 ± 1.5[a]	238.8 ± 1.8[a]	240.4 ± 2.0[a]	239.0 ± 1.0[a]	100[a]	96.7 ± 0.8[b]	95.5 ± 1.3[b]	96.2 ± 0.9[b]	92.2 ± 1.4[c]

Different letters in the same row indicate significant differences ($P<0.05$).

3.4. TGA analysis

The TGA analysis of the lyophilized nanoparticles prepared using 150 mg of PLA and the same amount of thymol, the nanoparticles control without thymol, the reactive thymol and the PLA are shown in Figure 3A. The derivative of the TGA curves (DTG) obtained are shown in Figure 3B. In these Figures it can be observed that the pure thymol is degraded completely in one single stage and over the temperature range 65-190 °C, showing a maximum rate of degradation at 158 °C. The PLA was degraded in a similar way as the thymol but at temperatures between 280 and 370 °C. The nanoparticles control, without thymol, showed a degradation curve similar to that found for the pure PLA, but divided into two stages. The first of them corresponds to PLA degradation, from 280 °C to 370 °C and the second one, from 390 °C to 470 °C, corresponds to PVA degradation (Yu et al., 2003). The degradation of the nanoparticles loaded with thymol was produced in three stages (Figure 3B). While the second and third stages are similar to those found for the control nanoparticles, the first stage could be attributed to the degradation of thymol, but it is offset to the right in comparison with that found for the pure thymol. In fact, this first degradation stage takes place from 120 °C to 250 °C, with a maximum degradation rate at 182 °C. This increase in the temperature of degradation of the encapsulated thymol could occur due to its inclusion in the nanoparticles, which provide a protective environment. Furthermore, it is significant that the amount of thymol in these nanoparticles is only around 10% of the weight of the lyophilized nanoparticles. This decrease in the thymol content could be due to the evaporation of thymol during the lyophilization process, since this active agent is a highly volatile essential oil and the drying process involves the application of low pressures for a relatively long time.

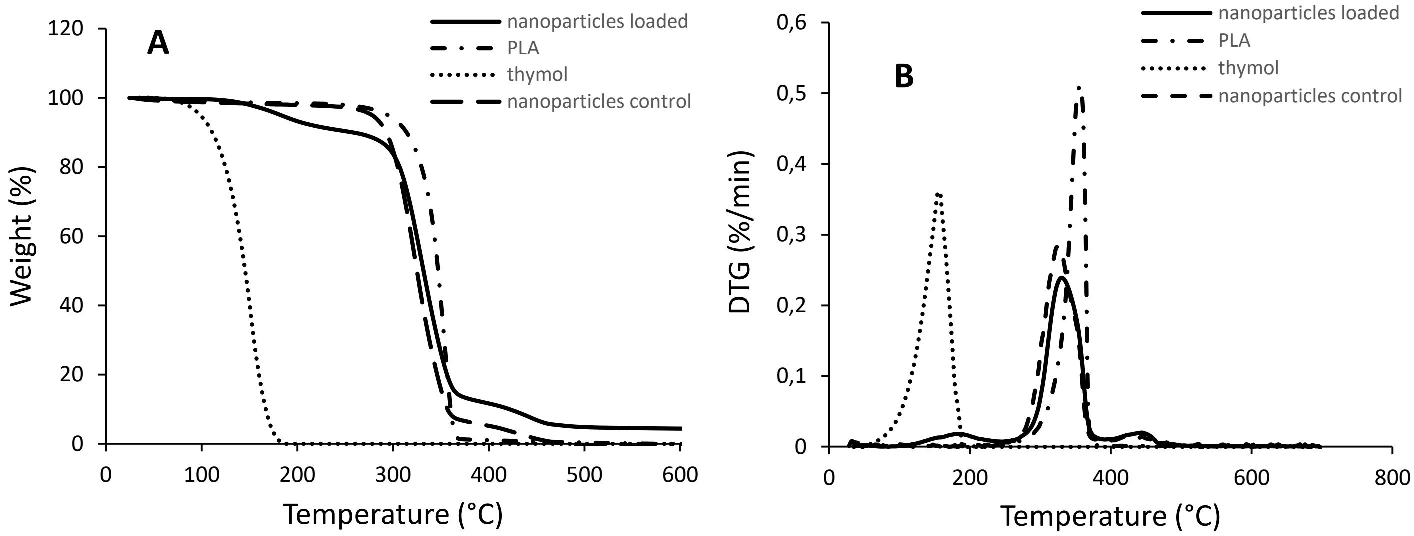

Figure 3. TGA (A) and DTG (B) curves of the nanoparticles loaded with thymol, the nanoparticles control without thymol, the reactive thymol and the PLA.

3.5. *In vitro* thymol release profile

The thymol release profiles of the loaded (150 mg of PLA and of thymol) nanoparticles at different temperatures were determined and are shown in Figure 4. Although this type of experiment has been designed principally to investigate the behaviour of injectable encapsulated drugs in the bloodstream, it can also provide valuable information about the state of the active agent inside the nanoparticle matrix and could provide evidence about possible interactions between the nanoparticle polymers and the active agent. According to this Figure, the thymol diffuses rapidly from the nanoparticles into the aqueous medium at 35 °C, releasing 93% of the active agent in 6 h. In the same time, if the temperature is lowered to 22 °C, 80% of the thymol is released. However, at 15 and 5 °C, the release of thymol over 6 hours decreases to 60%. As was to be expected, the rate of thymol release is temperature-dependent. Both at 35 and 22 °C the release of thymol for the first 2 hours is fast, and then a slight decrease in this release rate was detected. This first burst effect in 2 hours could be due to the release of the thymol adhered to the surface of the nanoparticles and to the thymol that is within the nanoparticles, but in the more external layers (Yadav and Sawant, 2010). The geometry of the nanoparticles and their high surface/volume ratio could be the reason for this first fast release. From this point, the thymol released came from the inner layers of the nanoparticles.

Furthermore, during the first hour at 15 and 5 °C there is a slight delay in the thymol release. This slow release could be due to the presence of free thymol remaining in the solution containing the nanoparticles. At the beginning of the test this thymol would be in the process of diffusing out of the dialysis tube, whereas at the highest temperatures tested, the thymol cleared quickly from the dialysis tube. Once the thymol excess disappears from the dialysis tube, the active agent is released quickly between the first and the fourth hour, in the case of the 15 °C experiment, and between the first and third hour when the temperature is lowered to 5 °C. Then, the thymol in the inner layers of the nanoparticles is also released, but at a lower speed. In any case, and bearing in mind that the antimicrobial and antioxidant properties of the thymol are concentration-dependent, this initial burst effect is desirable, since it allows an initial increase in the amount of free thymol in the medium.

There are several empirical models that can easily be applied to release profiles similar to those shown in Figure 4 and that have been widely reported in bibliography. In this case, the Korsmeyer-Peppas model was used, and the results obtained are shown in Table 3. To apply this model, only the data from the release curve up to 60% thymol release were considered (Siepmann and Peppas, 2012). Furthermore, neither the first hour in the experiments at 35 and 22 °C, nor the first two hours in those at 15 and 5 °C were considered. This omission was due to the distortion produced in the model by the burst effect at the two highest temperatures tested, and the delay detected in the thymol release at the two lowest temperatures. In this model, the value of the exponent "n" could be used to interpret, to some extent, the process of the mobilization of the thymol from the interior of the nanoparticle to the aqueous medium. Thus, if "n" is equal to 0.43, the diffusion of the active agent follows Fick's law. On the other hand, if the "n" value varies between 0.43 and 0.85, then the transport mechanism is catalogued as anomalous, being a mixture of Fickian diffusion and the movement of thymol produced by swelling of the nanoparticle. If alternatively, the value of "n" is equal to 0.85, then the transport is caused mainly by the nanoparticle swelling (Ritger and Peppas, 1987). Bearing this in mind and referring to Table 3, in all the cases in this experiment the mechanism of transport is anomalous, except at 5 °C, where due to the decrease in the temperature, the diffusion mechanism was hindered and the transport produced by the nanoparticle swelling becoming more important.

In Table 3 are also shown the first order kinetic equation parameters obtained from the experimental data. These results showed that in all the cases the thymol release can be adjusted to this type of kinetic with a high coefficient of determination (R^2), which suggests that this release is dependent on the thymol concentration. Furthermore, and as was expected, the "k" value decreased when the temperature also decreased, showing that the release of this active agent at low temperatures is slower in comparison with the same release at high temperatures.

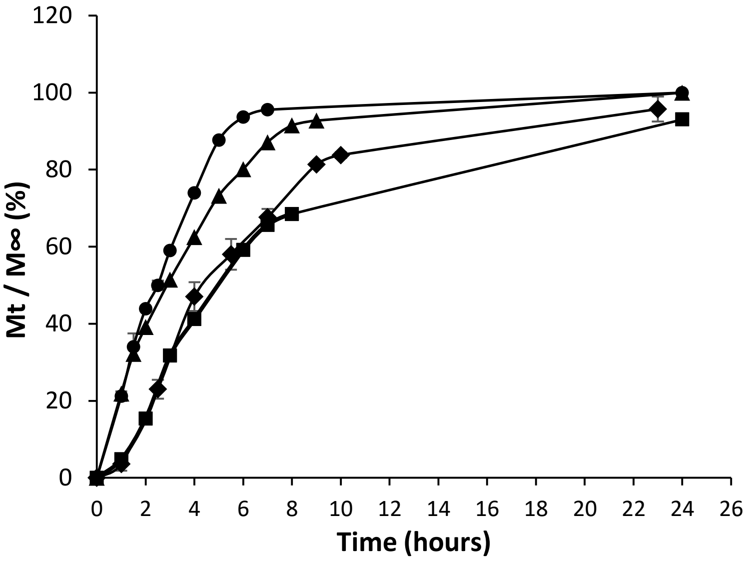

Figure 4. Thymol release from the nanoparticles prepared using 150 mg of PLA and 150 mg of thymol at 35 °C (circles), 22 °C (triangles), 15 °C (rhombi), and at 5 °C (squares).

Table 3. Parameters of interest obtained from Figure 4.

	First order		**Korsmeyer-Peppas**	
	R^2	**k**	**R^2**	**n**
35 °C	0.94	0.40	0.99	0.77
22 °C	0.98	0.28	0.99	0.67
15 °C	0.97	0.17	0.99	0.60
5 °C	0.97	0.14	0.98	0.85

3.6. antimicrobial properties of the nanoparticles

In Figure 5 is shown the effect of the aqueous solution with free thymol and the thymol encapsulated in nanoparticles on *E. coli* growth. In the samples treated with free and encapsulated thymol at 0.94 mg/mL, it was not to possible detect *E. coli* growth during the 14 days of treatment, so the thymol at this concentration was able to kill all the bacterial population inoculated. On the other hand, the solutions with a thymol concentration of 0.1 mg/mL showed a behaviour similar to that found in the control sample, it being impossible to detect differences between the encapsulated and non-encapsulated thymol. Therefore, the lowest thymol concentration tested did not have any effect on the growth of *E. coli*. However, in the samples treated with 0.5 mg/mL of thymol it was possible to detect a difference between the nanoparticles and the free thymol. While the free thymol was seen to be more effective than the nanoparticles in the initial days of the experiment, the encapsulated thymol produced a continuous decrease in *E. coli* growth during the 14 days of the experiment, proving to be more effective than the free thymol in the long-term. This could be due to the nanoparticles providing an environment that protects the thymol from evaporation, maintaining its effect for longer.

The superficial application of the nanoparticles loaded with thymol on the surface of a solid food could be a more realistic use for them than the typical applications found in the bibliography, which are mainly focused on the antibacterial properties of nanoparticles in liquid foods (Chen et al., 2014; Chen et al., 2015; Pan et al., 2014; Shah et al., 2012). Although from a scientific point of view, the use of liquid food to study the antibacterial properties of thymol-loaded nanoparticles is very interesting, in a practical way it requires a homogeneous dispersion of the thymol throughout the volume of the liquid, and in this case the flavour of the thymol could affect the organoleptic properties of the product, even though this active agent is encapsulated. However, the use of these nanoparticles to protect the surface of solid food requires only its immersion in a nanoparticle suspension, which limits the contact of the product with the active agent while the nanoparticles maintain their capacity to protect the thymol and release it over time.

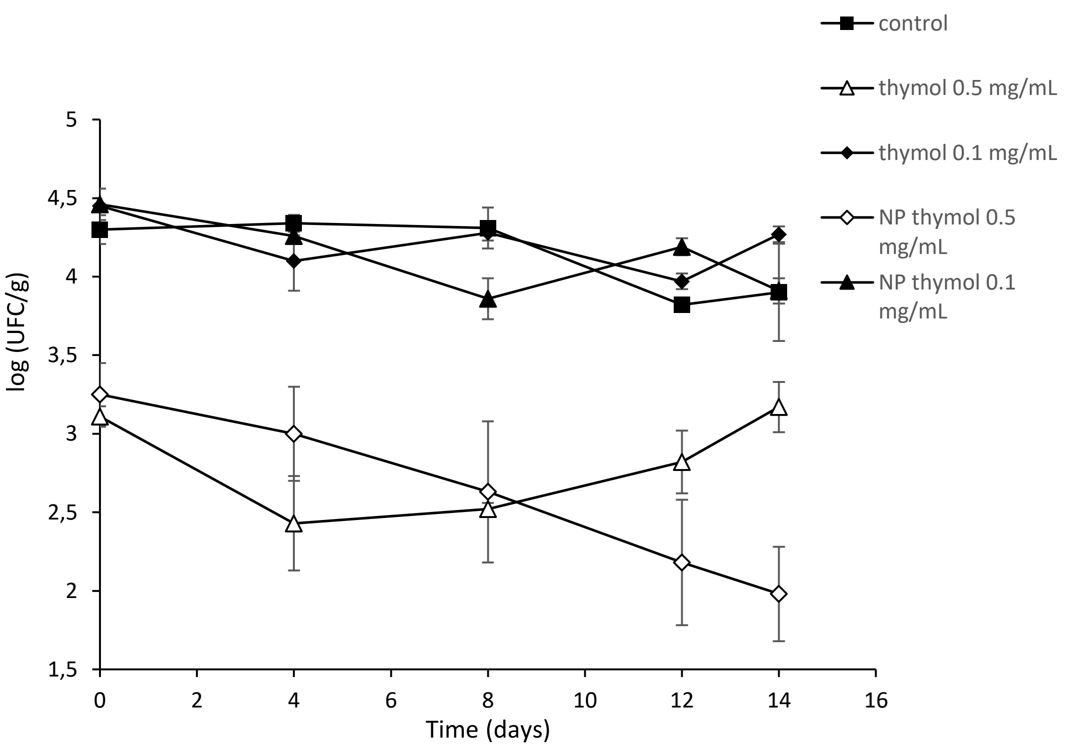

Figure 5. Growth of *E. coli* CECT 101 on apples treated with solutions containing free thymol and nanoparticles loaded with thymol.

4. Conclusions

In the tests performed using different PLA and thymol concentrations, the amount of PLA was found to be a key parameter to optimize the preparation of the thymol-loaded nanoparticles. These nanoparticles exhibited a spherical morphology, with a smooth surface and high storage stability over a wide range of pHs. Precisely, this stability at acidic and basic pHs could be considered an advantage in favour of these PLA nanoparticles when compared to those prepared using proteins and/or carbohydrates. Furthermore, their regular morphology and the homogenous arrangement of the active agent in the core are other relevant features that other biopolymer-based nanoparticles lack, and that have an important effect on the way the nanoparticles release the active agent. However, as can be seen from the TGA curves of the lyophilized PLA nanoparticles shown previously, a large proportion of the thymol evaporated during the lyophilization process, although this inconvenience could be compensated by the great stability of the nanoparticles in an aqueous suspension. Finally, using apple pieces inoculated with *E. coli* as a real food model, the PLA nanoparticles showed the capacity to maintain the thymol activity for 14 days, probably limiting its evaporation from the wet surface of the apple and favouring its release over time. The use of thymol-loaded nanoparticles to protect the surface of solid foods could be a more realistic use for them than their incorporation in liquid foods, since the first use limits the amount of thymol necessary to produce its antibacterial effect, minimising the strong flavour of the thymol.

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