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PREPARATION OF CHITOSAN – TRIPOLYPHOSPHATE NANOPARTICLES FOR THE ENCAPSULATION OF POLYPHENOLS EXTRACTED FROM ROSE HIPS

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The encapsulation of polyphenols in chitosan matrix can provide the stability of active components and their controlled release. Rose hips (*Rosa canina*) contain considerable amount of polyphenols related to strong antiradical activity. Natural extract of rose hips was encapsulated in chitosan-tripolyphosphate (CS-TPP) by ionotropic gelation method. The influence of chitosan-tripolyphosphate mass ratio on the physicochemical properties and entrapment efficiencies of nanoparticles was analysed in this work. It was found in this study that CS-TPP mass ratio affected zeta potential and mean size of polyphenols–chitosan nanoparticles. The encapsulation efficiency of rose hips extracts in CS-TPP nanoparticles was from 25.8 to 46.0 %. The release rate of polyphenols in vitro was investigated, too.

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1. Introduction

Natural products are important sources of biopolymer materials as: polysaccharides, polyphenols, polyesters, polyamides, and proteins, all of them playing an important role in biomedicine with applications in tissue engineering, regenerative medicine, drug-delivery systems and biosensors [1].

Chitosan is a polysaccharide derived from naturally occurring chitin. Its unique properties make it attractive for many industrial and biomedical applications (including controlled drug release, wound healing, nutrition supplements, water purification, removal of toxins, scaffolds for tissue engineering, and semipermeable membranes). Due to its pH dependent solubility, it forms stable films on various surfaces under neutral and basic pH conditions. Its amine groups serve for covalent attachment of biomolecules, and it can be co-deposited with other polymers or nanoparticles [2]. Composed of 2-amino-2-deoxy-β-D-glucan combined with glycosidic linkages, chitosan is considered as a perfect material for developing micro/nanoparticles [3]. Chitosan, is a more versatile form of this polysaccharide, which is the second most abundant natural polymer on earth after cellulose. An important application of chitosan is the development of drug delivery systems, with a regulated drug release rate and a reduced frequency of administration of the drug [4] due to its gel-forming ability in low pH range [5]. The hydrogen bonding and ionic interactions are responsible for the adhesive properties of chitosan and different substrates [6].

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The ionotropic gelation technique, as the most important technique for ionic cross-linking of chitosan with low molecular weight counterions, hydrophobic counterions and high molecular weight ions, involve sodium tripolyphosphate (TPP), too [7-10]. Chitosan microspheres as a potential carrier for drugs [11, 12], like anticancer agents and vaccines [13-16] are the most widely studied systems.

Polyphenols are valuable compounds possessing scavenging properties towards radical species and are involved in defence against ultraviolet radiation or aggression by pathogens [17] these abilities making polyphenols important for the treatment of various diseases like inflammation or cancer, but also for anti-ageing purposes in cosmetic formulations, or for nutraceutical applications. These properties are also responsible for a lack in long term stability, deficiency avoided by encapsulation methods described in the literature [18]. The reports on encapsulation of polyphenolic antioxidants by ionic gelation process are limited. Liang [19] prepared by this method chitosan nanoparticles loaded with tea polyphenol extract. The particles have been proved to be good nanosystems for slow release, the polyphenolic material being actively maintained. A comparative analysis about the encapsulation of yerba mate extract was done by Harris [20] who analysed chitosan tripolyphosphate nanoparticles (ionic gelation) and microspheres prepared by spray-drying.

This study investigated the preparation of CS-TPP nanoparticles loaded with polyphenolic rose hips extract by ionotropic gelation method. The effects of different preparation conditions on the particle size, zeta potential, encapsulation efficiency and release rate of polyphenols from CS-TPP nanoparticles were studied, too.

2. Methods

Materials. Rose hips were purchased from the local pharmacy and then pulverized and stored in a desiccator before extraction and analysis. Ethanol, Folin-Ciocalteau phenol reagent, DPPH (1,1-diphenyl-2-picryl-hydrazyl) were provided by Merck. Polyphenol standards (gallic acid, (+) catechin), CS of medium molecular weight, derived from crab shell and sodium triphosphate pentabasic (TPP) were purchased from Sigma-Aldrich. All the other reagents used in the experiments were of analytical grade.

Extraction of polyphenols from rose hips. Dried rose hips (10 g) were sonicated for 30 minute at room temperature in 100 mL of these solvents: absolute ethanol, water, EtOH: water (80:20; 50:50 v/v). Each extract was centrifuged at 4000 RPM for 20 min. The residue was re-extracted by repeating the procedure mentioned above. For spectrophotometric analysis the supernatants were combined and filtered, while for polyphenols encapsulation the 50 % aqueous ethanol combined supernatants where evaporated at 35 ^{0}C under vacuum, to remove solvents. The polyphenol extract was concentrated up to 20 % (w/v) of solid content.

Determination of total polyphenols. The concentration of total phenolics was measured by method described by [21] modified by [22]. One milliliter of sample or standard solutions of gallic acid was added to a 25 mL volumetric flask containing 9 mL of bidistilled water. A reagent blank using ddH_2O was prepared. One milliliter of Folin & Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added with vigorous mixture. The solution was immediately diluted to volume with ddH_2O and mixed thoroughly. After incubation for 90 min the absorbance versus prepared blank was read at 750 nm. Total phenolic contents of rose hips extracts were expressed as mg Gallic acid equivalents (GAE).

Determination of total flavonoids. Total flavonoids were measured by a colorimetric assay developed by [23]. One milliliter of sample or standard solutions of catechin was added to a 10 mL volumetric flask containing 4 mL ddH_2O. At zero time, 0.3 mL 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6 min, 2 mL 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 mL of ddH_2O and thoroughly mixed. Absorbance of the mixture was determined at 510 nm versus prepared water blank. Total flavonoids of rose hips extracts were expressed on a dry weight basis as mg/100 g catechin equivalents (CE).

Determination of antioxidant activity. DPPH scavenging activity was determined using a method of [24] modified by [25]. One hundred μM DPPH was dissolved in methanol. The rose hips extracts, 0.1 mL, were added to 2.9 mL of the methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm after 30 min. A control solution consisted of 0.1 mL of extraction solvent and 2.9 mL of DPPH solution was prepared. The radical stock solution was prepared fresh daily. The antioxidant activity percentage was calculated with the following equation:

$$\%, RSA = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \cdot 100 \quad (1)$$

where $A_{control}$ and A_{sample} are the absorbances of control and sample prepared as mentioned above.

Preparation of Nanoparticles. CS-TPP nanoparticles were prepared according to the procedure reporting previously [26]. CS, 1mg/mL was dissolved in 1% (w/v) acetic acid and sonicated before the solution became transparent. The addition of TPP solution, at a concentration of 1 mg/mL, to CS solution (pH=5), with stirring at room temperature, produced the formation of CS-TPP nanoparticles by ionic gelation mechanism. For the preparation of CS-TPP nanoparticles loaded with polyphenols, the extract of rose hips, 20 % (w/v) was added to chitosan solution before adding TPP solution.

Characterization of Nanoparticles. The measurements of particle size and zeta potential of nanoparticles were performed on a Zetasizer Nano-ZS on the basis of Dynamic light scattering (DLS) techniques.

Evaluation of Polyphenols Encapsulation. CS-TPP nanoparticles loaded with polyphenols were transferred into a VectaSpin Micro centrifuge filter. After centrifugation at 8000 RPM for 15 min, polyphenols penetrated through the membrane into centrifuge tube and the amount of polyphenols in ultrafiltrate was determined by Folin-Ciocalteau method. The encapsulation efficiency of polyphenols was calculated using formula no. (2).

$$EE,\% = \left(\frac{TPA_{Loading} - TPA_{Ultrafiltrate}}{TPA_{Loading}} \right) \cdot 100 \quad (2)$$

EE = encapsulation efficiency, %; $TPA_{Loading}$ = total amount of polyphenols loading; $TPA_{Ultrafiltrate}$ = amount of polyphenols in ultrafiltrate.

In Vitro Release of Polyphenols. The nanoparticles in the filter were diluted with buffer solution of pH=5.5 to 2 mL. The filter was incubated at 37 ^{0}C, at certain collection time the filters were centrifuged and the amount of polyphenols in each ultrafiltrate was determined as described above. The amount of extract released, determined as polyphenol content, and was estimated with the formula no. (3).

$$Release\ rate\ (\%) = \left(\frac{E}{E_0} \right) \cdot 100 \quad (3)$$

where E is the amount of polyphenols determined in the release medium and E_0 is the initial amount of polyphenols in the nanoparticles.

3. Results and discussion

Total phenolic content. In this study, the effect of ethanol concentration for the efficient extraction of antioxidative compounds from rose hips was investigated. The most efficient composition for polyphenols extraction was observed in 50% aqueous ethanol. The total phenolic

content varied from 185 to 4388 mg equivalent GAE/100 g of dry rose hips (Table 1), all these results being in good agreement with literature [27].

Total flavonoids content. Similarly, total flavonoids in rose hips extracts were determined from the regression equation of calibration curve and expressed as catechin equivalents mg CE/100 g dry rose hips. The 50% aqueous ethanol extract exhibited the highest flavonoids content of 2095 mg CE/100 g dry rose hips (Table1). Similar results were also reported by [28].

DPPH radical scavenging activity. The antioxidant activity of the rose hips extracts was determined using DPPH free radical scavenging assay. The RSA values of the extracts are presented in Table 1. The highest antioxidant activity (95.4 %) was observed in 80 % ethanol extract but also 50% ethanol extract presented a strong antioxidant activity. These results correspond with earlier reported by [29] who obtained for 8% rose hips aqueous infusions a RSA value of 67.2 %.

Table 1. The content of total polyphenols, flavonoids and antioxidant activity of rose hips extracts

Assay	Ethanol concentration, %			
	0	50	80	100
Total polyphenols (mg GAE/100 g)	3488.4	4387.6	1569.4	185.4
Total flavonoids (mg CE/100 g)	1652.7	2095.4	650.8	57.6
RSA (%)	78.6	86.4	95.4	57.0

The aqueous alcoholic mixture was employed in an attempt to extract as many compounds as possible. This is based on the ability of alcoholic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium to low-polarity constituents [30]. Since the polarity is increasing with ethanol concentration, the 50 % aqueous ethanol was the most adequate concentration for our experiments, in good agreement with literature [31].The binary solvent system demonstrated higher yield of phenolic compounds and flavonoids as compared to monosolvents, well recognized by the other reports [32].

Effect of CS-TTP Mass Ratio. CS-TPP mass ratio significantly influences the characteristics of CS-TPP nanoparticles. The particle size decreased with the increasing of CS-TPP mass ratio from 2:1 to 8:1, as shown in Figure 1. According to [33] it is known the ability of CS to quickly gel on contact with TPP relies on the formation of inter- and intramolecular crosslinking between the amino groups and the phosphate groups. This effect can be explained by the penetration of the drug molecule in chitosan network, activating hydroxyl sites and establishing physical–chemical electrostatic interactions and hydrogen bonds in the new system, in good agreement with literature data [34]. Besides all these, chitosan–TPP nanoparticles are mainly characterized by a positive zeta potential. Interaction is therefore strong towards any negative surface charge, or negatively charged oligoanions and polyanions. This type of interaction can be used to obtain strong adhesion and immediate immobilization on negatively charged surfaces [35]. On the other hand, interactions with anionic proteins and polymers may stimulate the aggregation or precipitation phenomena, limiting the applicability of some chitosan-TPP nanoparticle formulations. Recently presented studies outlined the stability of chitosan-TPP nanoparticles and particularly, the limitations of this particle stability using model body fluids [36]. When CS-TPP mass ratio was high (the available quantity of TPP was small), TPP might dominant inter- and intramolecular crosslink with CS to form small nanoparticles. As CS-TPP mass ratio declined the available quantity of TPP increased and the superfluous TPP would link the monoparticles to form larger nanoparticles.

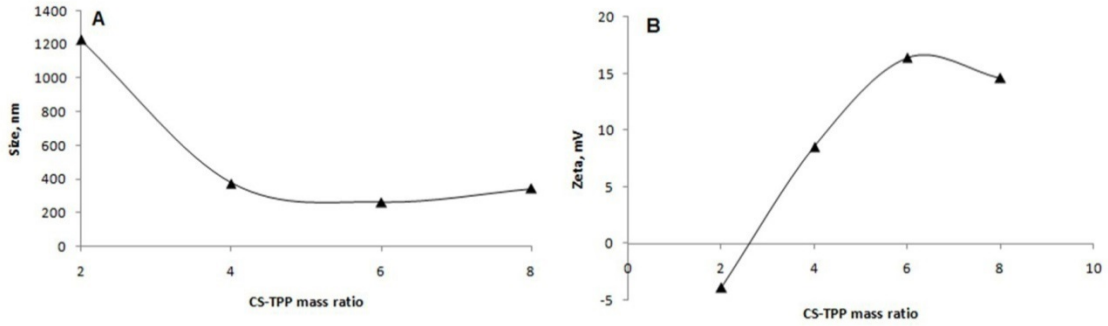

Fig. 1.Effect of CS-TPP mass ratio on particle size (A) and zeta (B)

The zeta potential decreased suddenly as the CS-TPP mass ratio decreased from 4:1 to 2:1, this decrease may be due to aggregation of the nanoparticles. A small increase of zeta potential values was obtained as the CS-TPP mass ratio increased from 4:1 to 8:1 which may be a consequence of less ionic cross-linking. The zeta potential of the CS-TPP nanoparticles obtained in the present study was about 15 mV.

Encapsulation efficiency and Release rate of rose hips polyphenols. In this study the effect of CS-TPP mass ratio, concentration of polyphenols on the encapsulation efficiency and in vitro release of polyphenols from CS-TPP nanoparticles loaded with rose hips extract, were investigated.

Table 2. The effect of CS-TPP mass ratio on the encapsulation efficiency

CS-TPP mass ratio	Encapsulation efficiency (%)
2:1	46.0
4:1	42.4
6:1	36.2
8:1	25.8

The effect of CS-TPP mass ratio on the encapsulation efficiency was investigated at the following mass ratios of 2:1, 4:1, 6:1 and 8:1. The encapsulation efficiency, as presented in Table 2, decreased from 46.0 % to 25.8 % as CS-TPP mass ratio increased. The obtained results are in concordance with previous studies where chitosan nanoparticles were used for nasal vaccine delivery [37] or for catechins delivery [33]. Thus, in last study mentioned the encapsulation efficiency of tea catechins decreased from 45.14 to 32.23 % as the CS-TPP mass ratio increased from 4:1 to 9:1.

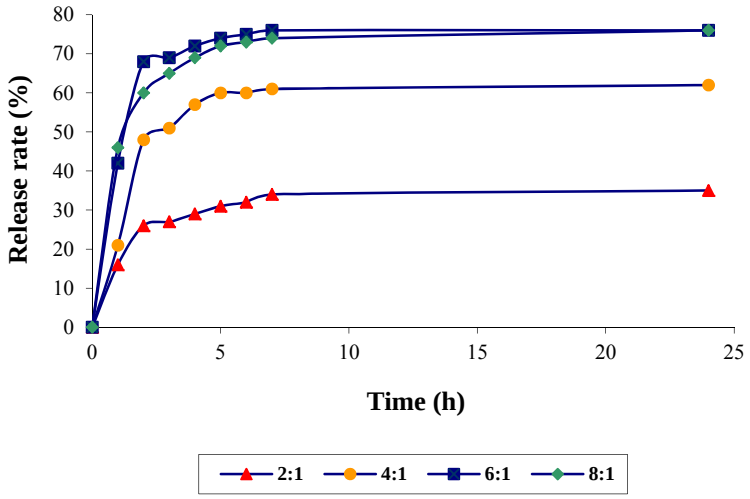

Fig. 2. Effect of CS-TPP mass ratio on the polyphenols release profile.

Polyphenols release rate increased with increase of CS-TPP mass ratio, thus the highest released rate was observed for CS-TPP mass ratio of 6:1. As the CS-TPP mass ratio decreased from 4:1 to 2:1, total release of polyphenols decreased from 62 to 35 %. The results obtained indicate that lower nanoparticles size can be related with good encapsulation efficiency and release rate.

Effect of polyphenols concentration. For the preparation of CS-TPP nanoparticles loaded with polyphenols the extract of rose hips (0.5-10 % v/v) was added to chitosan solution before the addition of TPP solution.

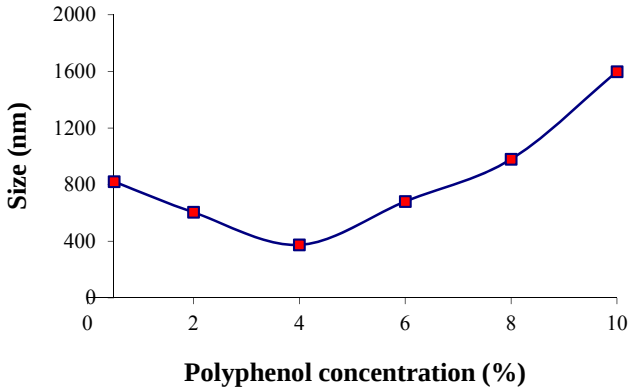

Fig. 3. Effect of polyphenols concentration on the nanoparticles size, CS: TPP mass ratio = 6:1

Nanoparticles size decreased when polyphenols concentration increased from 0.5 to 4%, after that the NPs size has increased with polyphenols concentration. For the CS-TPP nanoparticles loaded with 4% (v/v) polyphenols the mean particle size was smaller and may be attributed to a greater cross-linking density of the rose hips polyphenols induced by the interaction between CS matrix and rose hips polyphenols [38].

Type of interactions. The penetration of the drug molecule in chitosan network could occurs both by electrostatic interactions between $-NH_3^+$ groups and phosphate groups from sodium tripolyphosphate and hydrogen bonds between OH groups of polyphenols and OH groups of chitosan. A possible scheme of such system is represented in the Figure 4A.

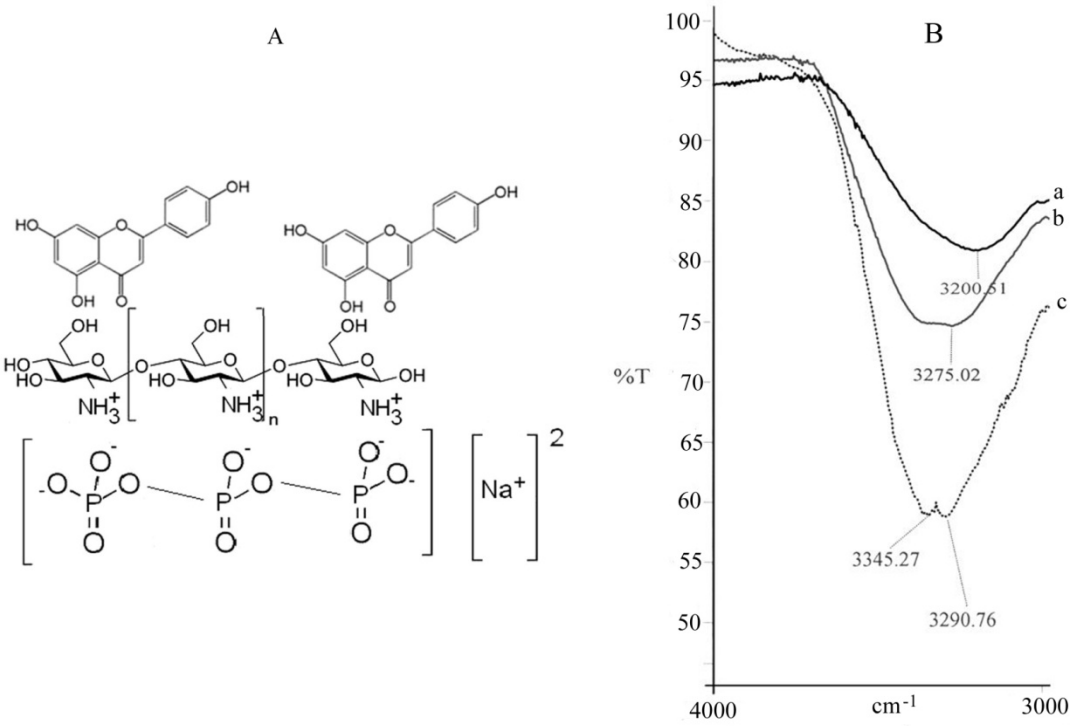

Fig. 4. Type of interaction (A) and FT-IR spectra (B) of CS (c), CS-TPP (b) and CS-TPP nanoparticles loaded with polyphenols (a)

FT-IR spectroscopy was used to investigate the interactions between chitosan-TPP nanoparticles and rose hips polyphenols by measuring the samples from 4000-650 cm^{-1} at a resolution of 1 cm^{-1}. In chitosan a splitted band (3345 cm^{-1} and 3290 cm^{-1}) could be observed, which is flattened by interaction with tripolyphosphate (3275 cm^{-1}) becoming weak and broad shoulder band at 3220 cm^{-1} after the interaction with polyphenols, as a proof for a hydrogen bond association process, Figure 4B. A large shift of 145 cm^{-1} in the O–H stretching to a lower frequency confirms that polyphenols are hydrogen bonded to chitosan and tripolyphosphate. In the neutral manifold, the typical strength of π-hydrogen bonds is considerably weaker (≤5 kcal/mol) than that of σ-hydrogen bonds (≈10 kcal/mol). By electronic spectrum a red shift of the O-H band is observed after the H bonding, with an energy of 3.71 kcal/mol, corresponding to a π-hydrogen bond [39].

4. Conclusions

The results of the present study confirmed that rose hips (*Rosa canina*) contained significant amounts of phenolic compounds. The total phenolic contents of extracts decreased from 50 % EtOH (4388 mg GAE/100 g dry fruits) to EtOH (185 mg GAE/100 g dry rose hips). Rose hips had strong antioxidative activity, tested by free radical scavenging activity, RSA was influenced by the extracting solvent in the following way from high to low: 80% EtOH > 50% EtOH: water > ethanol.

CS-TPP nanoparticles loaded with polyphenols were prepared by the ionic gelation method. The formation of appropriate CS-TPP nanoparticles loaded with polyphenols can be achieved by controlling the significant parameters including CS-TPP mass ratio, polyphenols concentration. The results obtained indicate that lower nanoparticles size can be related with good encapsulation efficiency and release rate. It was observed that the highest release rate was

achieved at a polyphenols concentration of 4% (v/v) and CS-TPP mass ratio of 6:1. Other parameters may also be optimized to achieve ideal controlled polyphenols delivery systems.

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References

[1] E.Khor, L. Lim, Biomaterials **24**, 2339 (2003).
[2] S.T. Koev, P.H. Dykstra, X. Luo, G.W.Rubloff, W.E. Bentley, G.F. Payne, R. Ghodssi, Lab on a Chip **10**, 3026–3042 (2010).
[3] T.M Aminabhavi, J. Control. Release **100**, 5 –28 (2004).
[4] J.Knapczk, L. Krowczynski, B. Pawlik, Z. Liber, Pharmaceutical dosage forms with chitosan, in G. Skjak-Braek, T. Anthonsen, P. Sandford (Eds.), Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications, Elsevier Applied Science, London, 665–669 (1984).
[5] J. Knapczyk, Int. J. Pharmaceut. **93**, 233 (1993).
[6] P. He, S.S. Davis, L. Illum, Int. J. Pharmaceut. **166**, 75-88 (1998).
[7] Y. Sawayanagi, N. Nambu, Chem. Pharm. Bull. **30**, 4464 (1982).
[8] M. George, T.E. Abraham, J. Control. Release **114**, 1-14 (2006).
[9] M.L.Lorenzo-Lamosa, C. Remuñán-López, J.L.Vila-Jato, M.J. Alonso, J. Control. Release **52**, 109 (1998).
[10] R. Bodmeier, K. Oh, Y. Pramar, Drug Dev. Ind. Pharm. **15**, 1475 (1989).
[11] L. Illum, Pharm. Res. **15**, 1326 (1998).
[12] V.R. Sinha, A.K. Singla, S.Wadhawan, R. Kaushik, R. Kumria, K. Bansal, S. Dhawan, Int. J. Pharmaceut. **274**, 1-33 (2004).
[13] M. Susan, I. Baldea, S. Senila, V. Macovei, S. Dreve, R.M. Ion, R. Cosgarea, Int. J. Dermatol. **50**, 280-286 (2011).
[14] S. Dreve, R. M. Ion, A. Muresan, R. Cosgarea, A. Filip, M. Perde, E.Indrea, D. Silipas, presented at the National Symposium "Days of the University of Medicine and Pharmacy Iuliu Hatieganu", 2006, Cluj-Napoca, Romania.
[15] S. Dreve, A. Muresan, A. Filip, D. Daicoviciu, S. Clichici, M. Perde-Schreppler, R.M. Ion, E. Indrea, T. D. Silipas, presented at the EuroNanoforum, 2007, Düsseldorf, Germania.
[16] R.M. Ion, S. Dreve, D.V. Brezoi, presented at the 14th International Conference "New technologies and products in machines manufacturing and technologies"- TEHNOMUS XIV, 4-5 May 2007, Suceava, Romania.
[17] S.M. Doncea, R. Stoica, R.M. Ion, I. Trandafir, V. Pavel, Bulletin of Petroleum-Gas University of Ploieşti **LXII 3A**, 143-148 (2010).
[18] A. Munin, F. Edwards-Levy, Pharmaceutics **3**, 793-829 (2011).
[19] J. Liang, F. Li, Y. Fang, W.Yang, X. An, L. Zhao, Z. Xin, L. Cao, Q. Hu, Colloids Surf. B Biointerfaces **82**, 297–301 (2011).
[20] R. Harris, E. Lecumberri, I. Mateos-Aparicio, M. Mengíbar, A. Heras, Carbohydr. Polym. **84**, 803–806 (2011).
[21] V.L. Singleton, J.J. Rossi, Am. J. Enol. Viticult. **16**, 144 (1965).
[22] D. Kim, S. Jeond, C.H. Lee, Food Chem. **81**, 321 (2003).
[23] J. Zhishen, T. Mengcheng, W. Jianming, Food Chem. **64**, 555–559 (1999).
[24] W. Brand-Williams, M.E. Cuvelier, C. Berset, Food Science and Technology **28**, 25-30 (1995).
[25] D.O. Kim, K.W. Lee, H.J. Lee, C.Y. Lee, J. Agr. Food Chem. **50**, 3713-3717 (2002).

[26] P. Calvo, C. Remuñán-López, J.L. Vila-Jato, M.J. Alonso, J. Appl. Polym. Sci. **63**, 125–132 (1998).
[27] E. Jabłońska-Ryś, M. Zalewska-Korona, J. Kalbarczyk, Journal of Fruit and Ornamental Plant Research **17**, 115-120 (2009).
[28] K.K. Chew, S.Y. Ng, Y.Y. Thoo, M.Z. Khoo, W.M. Wan Aida, C.W. Ho, International Food Research Journal **18**, 571-578 (2011).
[29] H. Kiliçgün, D. Altiner, Phcogmag. **6**, 238-241 (2010).
[30] V. Seidel, Initial and Bulk Extraction, in S. Sarker, Z. Latif, A. Gray (Eds.), Natural Products Isolation, Humana Press, New Jersey, 36-37 (2006).
[31] M. Savova, T. Kolusheva, A. Stourza, I. Seikova, Journal of the University of Chemical Technology and Metallurgy **42**, 295-300 (2007).
[32] R. Chirinos, H. Rogez, D. Campos, R. Pedreschi, Y. Larondelle, Sep. Purif. Technol. **55**, 217-225 (2007).
[33] B. Hu, C. Pan, Y. Sun, Z. Hou, H. Ye, X. Zeng, J. Agr. Food Chem. **56**, 7451-7458 (2008).
[34] J.L. Atwood, J.W. Steed (Eds.), Encyclopedia of Supramolecular Chemistry, Marcel Dekker, New York, Chapter 3, (2004).
[35] A.Vila, A. Sánchez, K. Janes, I. Behrens, T. Kissel, J.L. Vila Jato, M.J. Alonso, Eur. J. Pharm. Biopharm. **57**, 123–131 (2004).
[36] Q. Gan, T. Wang, C. Cochrane, P. McCarron, Colloids and Surfaces B: Biointerfaces **44**, 65-73 (2005).
[37] A.R. Mokarram, M.J. Alonso, Archives of Razi Institute, **61**, 13-25 (2006).
[38] L. Zhang, S.L Kosaraju, Eur. Polym. J. **43**, 2956–2966 (2007).
[39] N. Mikami, A. Okabe, I. Suzuki, J.Phys. Chem. **92**, 1858 (1988).