Preparation and characterization of polymeric nanoparticles loaded with the flavonoid luteolin, by using factorial design

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Abstract

Nanoparticles containing luteolin flavonoid were prepared by using interfacial polymer deposition following solvent displacement. The formulation was optimized using factorial design. The parameters studied were the type of polymer [poly(-caprolactone) and poly(lactic-co-glycolic acid)], nature oil (isodecyl oleate and oleic acid) and the quantity of luteolin. Nanocapsules and nanospheres were also prepared and evaluated. Colloidal suspensions were characterized by evaluating pH, the particle size, the zeta potential, the morphological aspect and the kinetic release. A new High Performance Liquid Chromatography method was developed and validated in order to quantify luteolin in colloidal suspension allowing the analyses of the absolute recovery, entrapment efficiency and the kinetic release. The luteolin-absolute recovery ranged from 61.6% to 95%; entrapment efficiency was nearly 100% in all formulations and the particle sizes were smaller than 185.5 nm. The nanoparticles prepared with isodecyl oleate show a negative zeta potential. On the other hand, when oleic acid was utilized, the zeta potential was positive. The nanoparticles prepared by using isodecyl oleate have a more perfect spherical shape with a regular surface and form, homogeneity, and lower size dispersion. Nanocapsules and nanospheres have a similar release mechanism of pure diffusion according with Korsmeyer-Peppas's model.

Keywords: Luteolin, polymeric nanoparticles, characterization, factorial design, release kinetics.

Introduction

Since the beginning of human civilization, herbs have been an integral part of a human diet and medicine, valued for their culinary and medicinal properties. However, with the development of patent pharmaceuticals in the first part of the twentieth century, herbal medicine lost ground to new synthetic products touted by scientists and physicians to be more effective and reliable [1]. Nowadays, a new and renovated interest toward herbal medicines is observed. Some of the reasons which justify this fact are: (i) the sale of herbal medicines and their secure use have considerably increased over the last 10 years; (ii) herbal medicines are used to treat a wide range of problems; (iii) the development of new diseases, with severe complications, for which there is still no appropriate treatment; (iv) the belief that herbal remedies are innocuous, in contrast to conventional drugs; (v) the idea that what is natural can only be good, *etc.*[2-3]. Among herbal medicines, extracts rich in flavonoids have elicited particular interest. Flavonoids are naturally-occurring plant polyphenols characterized by a diphenylpropane structure (C6-

C3-C6) found in abundance in vegetables, fruits, nuts, seeds, herbs, spices, flowers, red wine and tea. They possess a wide range of biological activities and could be used to prevent diseases. Research and application of flavonoids in functional foods and in nutraceutical and pharmaceutical industries have been areas of great interest especially because they demonstrate a remarkable array of biochemical and pharmacological actions including anti-inflamatory, anti-oxidant, cytostatic, apoptotic and estrogenic activities [4-5].

Among the flavonoids, luteolin (3′,4′,5,7-tetrahydroxyflavone) (Figure 1), found in a wide range of plants such as in celery, green pepper, perilla leaf, chamomile tea, broccoli, and carrots, exhibits several biochemical activities and capable of enhancing insulin sensitivity [6-8]. Furthermore, luteolin is permeable to the brain-blood barrier, making it applicable to the therapy of central nerve system diseases [9].

The effectiveness of nutraceutical products in preventing diseases depends on preserving the bioavailability of the active ingredients.

Unfortunately, the concentrations of polyphenols that appear to be effective *in vitro* are often of an order of magnitude higher than the levels measured *in vivo* [10]. The activity and potential health benefits of the nutraceuticals, including polyphenols might be limited due to insufficient gastric residence time, low solubility and/or permeability within the gut, instability under conditions encountered in the gastrointestinal tract or processing and storage that result in small amount of the molecules available following the oral administration [10]. The delivery of these compounds therefore requires product formulators to maintain the active molecular form up to the time of consumption and preserve the stability, bioactivity and bioavailability. These features are the central goal of nanoparticle systems [11-12].

In this context, the present study aimed to encapsulate luteolin in polymeric nanoparticles to overcome the disadvantages of its instability, protect the compound and increase its dispersal in aqueous medium and thereby improve the bioavailability. The nanoparticles which contain luteolin were prepared by interfacial polymer deposition following solvent displacement, which is known as the nanoprecipitation method proposed by Fessi et al.[11]. This method involves the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium and allows the preparation of nanocapsules (NC) when a small volume of nontoxic oil is incorporated into the organic phase or nanospheres (NS) when the oil is omitted [11-12].

Biodegradable nanoparticles are frequently used to improve the therapeutic value of various water-soluble/insoluble medicinal drugs and bioactive molecules by improving bioavailability, solubility and retention time. These nanoparticle–drug formulations increase drug efficacy, specificity, tolerability and therapeutic index of corresponding drugs. At the same time they reduce the patient's expenses, risks of toxicity and have many advantages such as the protection of premature degradation and interaction with the biological environment, and enhancement of intracellular penetration [13].

Novel formulations using the nanoencapsulation method have been successful when applied to plant active compounds and extracts enhancing stability, sustained delivery and pharmacological activity [14]. For example, the nanoprecipitation technique using Eudragit®E and polyvinyl alcohol as carriers was applied to nanoencapsulate the flavonoid naringenin, reported to induce cytotoxicity and apoptosis in various human cancer cell lines [15]. Naringenin application in cancer is limited due to low aqueous solubility and instability that results in suboptimal pharmacokinetics and poor bioavailability at the tumor sites [16]. The obtained narigenin nanoparticles showed obvious advantages over free naringenin in HeLa cells such as dose-dependent cytotoxicity, alterations in mitochondrial membrane potential, apopptotic morphological changes, increased intracellular ROS and lipid peroxidation [17]. Quercetin nanoparticles were developed for controlling ischemia-induced cerebral oxidative damage using PLA as polymer and phosphatidyl ethanolamine. An increased neuroprotective effect was demonstrated, such as the protection of endogenous antioxidant enzymes against ischemia reperfusion induced oxidative damage, when nanoencapsulated quercetin was used in comparison with free quercetin or empty nanocapsules [18].

In addition, injectable daidzein-loaded solid lipid nanoparticles (SLNs) with PEGylated phospholipid as stabilizer were prepared by the homogenization method to overcome problems related to its poor oral absorption and bioavailability in order to improve its pharmacological activity on cardio-cerebrovascular diseases. The daidzein encapsulated in SLNs could significantly increase circulation time compared with orally administrated daidzein or intravenoulsy delivered daidzein in solution in Sprague–Dawley rats. Furthermore, the daizein-loaded nanoparticles showed a better effect on cardiovascular and cerebrovascular systems of the anesthetic dogs and a protective effect on rats with ischemia-reperfusion injury model [19].

Another successful example is the nanoencapsulation of the green tea catechin (-)-epigallocatechin gallate (EGCG), a flavonoid that possesses anti-oxidant, anti-inflammatory, cardioprotective, neuro-protective and anti-cancer properties, but with the therapeutic potential limited by its poor systemic absorption following oral consumption due to its degradation in gastrointestinal tract. EGCG was encapsulated in chitosan-tripolyphosphate nanoparticles. It was demonstrated in Swiss Outbread mices that the administration of these nanoparticles enhanced the plasma exposure of total EGCG by a factor 1.5 in comparison with EGCG in solution, and a 2.3 fold increase in the apparent exposure of EGCG in jejunum [20].

Combining both the polymer-based nanoencapsulation technique and phythochemicals, including flavonoids may be a useful approach for enhancing delivery, stability and bioavailability and improving the therapeutic application of these compounds in a number of diseases.

The optimized formulation was identified by using factorial design. A 2^3 full factorial design was carried out. Factorial designs are commonly adopted in pharmaceutical research which is concerned with the effects of formulation variables and their interactions on response variables, yielding the most information from small number of experiments.

Material and Methods

Luteolin was obtained from Caymann Chemicals (Steinheim, Germany). The polymers poly(-caprolactone) average M_w 65.000 and poly(lactic-co-glycolic acid) (65:35) average M_w 40,000-75,000, oleic acid and the sorbitan monostearate (Span®60) were bought from Sigma Aldrich (Steinheim, Germany). Polysorbate 80 (Tween®80) and isodecyl oleate were obtained from chemistry importer Delaware (Porto Alegre, Brazil). The used solvents acetonitrile and methanol were HPLC grade purchased from J.T. Baker (Phillipsburg, USA). The fosforic acid, sodium chloride, potassium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained from J.T. Baker as well. Water was produced in house by Milli-Q System (18M<OMEGA>) (Millipore Corporation, Watford, UK).

Preparation of nanoparticles

The nanoparticles were prepared using solvent displacement method, which involves the precipitation of a preformed polymer (nanoprecipitation) as proposed by Fessi et al. [11]. Briefly, exact quantities of polymer, luteolin, span®60 and oil (only in nanocapsules) were accurately weighed and dissolved in acetone at 45℃. Then, the organic phase was poured into Tween®80 aqueous solution, which was under moderate magnetic stirring by using a peristaltic pump at 10% (PumpPro TPM 600 55RPM, Waton-Marlow, Wilmington, UK) at ambient temperature. In due course, the colloidal suspension was maintained for 10 minutes under moderate magnetic stirring. Subsequently, the solvent and the excess of water were removed by using a rotary evaporator (R-21, Büchi, Switzerland). The formulations are described in Table 1. The final volume of each colloidal dispersion was fixed in 25mL.

Experimental design

The experiments were performed by the nanoprecipitation method using a 2^3 full factorial design. In this experiment, three independent variables were studied in two levels: low and high, which were represented by the transformed values of –1 and +1, respectively. The independent variables were: the kind of both polymers and oils and the quantity of luteolin used. The quantity of span®60 and tween®80; the volume of the solvent and water, and the velocity of both the magnetic stirring and the transfer of the organic phase into the aqueous solution were kept constant to minimize fluctuations. Values of these selected variables are shown in Table 1. The repeatability in the nanoparticle preparation was evaluated through Relative-Standard Deviation (RSD%) calculations for 3 random formulations prepared in duplicate. The dependent variables of factorial design were: pH, particle size (PS), zeta potential (ZP), the luteolin absolute recovery (Rec%) and its entrapment efficiency (EE%). The best selected formulation found was specifically adjusted according to the presence (200.0mg, 0.8 % m/v) or not of oil (0.0 mg). The quantity of the organic solvent, tensoactives and water were the same as described in Table 1, and the amount of Luteolin was 1.25mg.

Quantification of luteolin content in polymeric nanoparticles

Luteolin was analyzed by High-Performance Liquid Chromatography (HPLC). HPLC analysis was performed by using an Agilent Technologies Liquid Chromatography 1200 Series configured with a degasser G1322A, quaternary pump G1311A, autosampler G1329A, column oven G1316A and UV detector G1314B. The reversed-phase procedure made use of a stainless steel Phenomenex® Gemini C18 column (150x4, 6mm i.d., 5μm particle size, s/no 292494-8, Torrance, CA, USA). The control of the HPLC system, the acquired and processed data collection was performed by Agilent Technologies EZCrom SI software (G6702AA, s.n.08021502300). Chromatographic analysis was performed in isocratic mode. The mobile phase consisted of acetonitrile and formic acid 0.05% (35:65; v/v). The flow rate was 1.2 mL min^{-1} for 6 min with an injection volume of 20µl. All experiments were performed at 220 nm and the column temperature was maintained at 35℃.

Preparation of standard and sample solutions for method validation

Primary stock standard solution of luteolin (1.00µg mL^{-1}) was prepared by accurately dissolving 10.0mg of luteolin into 10.0mL of methanol in a volumetric flask (Pirex®). The working solution (100µg mL^{-1}) was obtained by diluting 10 times the standard solution into methanol. The sample curve solutions were prepared by diluting the working solution to 5.00, 10.0, 20.0, 40.0, 60.0, 80.0 and 100µg mL^{-1}. They were obtained by mixing a sufficient quantity of the working solution into methanol towards a final volume of 1.00mL. All solutions and dilutions were prepared in triplicate. These solutions were used to perform the calibration curve, linearity, range, limit of detection (LOD) and limit of quantification (LOQ) of the method. Three other solutions (6.00, 50.0 and 90.0µg mL^{-1}) were prepared in quintuplicate in the same way in order to investigate the accuracy and precision of the method. The linearity was determined through the calculation of the linear regression from the peak area *vs.* the concentration plot of the seven standard solutions using the linear least squares methodology, and through the analysis of respective response factors (i.e. the peak area divided by the concentration of each standard sample). The limits of detection (LOD) and quantification (LOQ) were mathematically calculated through the relation between the standard deviation (*sd*) of the calibration curve and its slope (*S*), by using the multiplier suggested in the ICH standard [21]. The LOD and LOQ were calculated from the following equations: $\text{LOD} = [\,3.3 \times sd/S\,]$ and $\text{LOD} = [\,10 \times sd/S\,]$.

Pretreatment of luteolin samples for HPLC analyses

In order to obtain real quantitative values of luteolin in colloidal suspension, it was necessary to develop two different pretreatment methods; a specific one to assay the total content of luteolin in colloidal suspension (Rec%), and another one to quantify the luteolin associated with the nanoparticles (EE%). The total content of luteolin was determined by the opening of nanoparticles dispersed in a colloidal suspension, following the procedure described below: 0.9mL of acetone was added to 0.1mL of colloidal suspension and left to rest for 2 hours. After the resting period, the solution was subjected to centrifugation at 14,000rpm for 30min at 30℃ (Centrifuge 5810 R, Eppendorf®, Hamburg, Germany). Finally, 0.5mL of solution was dried in Speedvac (Savant Speedvac Plus SC 10 A, Farmingdale, USA) and the luteolin was resuspended in 0.2mL of methanol being analyzed by HPLC.

The entrapment efficiency of luteolin nanoparticles was determined by analyzing the concentration of the free unloaded compound in the aqueous phase of the colloidal suspension. Centrifugation was carried out using the cellulose acetate tube filter of the 0.22μm pore membrane (Costar®Spin-X®, Corning

Inc.). Approximately 0.5mL of nanoparticle dispersion was placed into the outer chamber of the filter assembly. The assembly was then centrifuged at 5,000rpm for 15min at 15°C. The nanoparticles along with the encapsulated compounds remained in the outer chamber whereas the aqueous dispersion medium containing the free unloaded compounds were moved to the sample recovery chamber through the filter membrane. After separation, 0.3mL of aqueous dispersion medium was dried. Finally, the product was resuspended in 0.2mL of methanol and the amount of the free luteolin in the dispersion medium was estimated by HPLC. The entrapment efficiency was subsequently calculated as the equation follows: EE% = [(total quantity of luteolin – quantity of free luteolin in the aqueous medium) / total quantity of luteolin] x 100.

Characterization of suspensions

The physicochemical analysis of nanoparticles was carried out immediately after their preparation. After preparing all the colloidal suspensions, the pH values were determined by using a potentiometer (B474 Micronal, São Paulo, BR). The analyses of the particle size (PS) of the nanoparticles were performed by photon correlation spectroscopy (PCS). The PCS measurements in nanometers were carried out at room temperature at a fixed angle of 90°. This technique yields the mean particle hydrodynamic diameter (PS) and the polydispersity index (PI), which are a dimensionless measure of the broadness of the PS distribution. The values of both the PS and the ZP were measured by using Zetatrac (Microtrac Inc., USA), which was controlled by Microtrac Flex V.10.5.0 software (Microtrac Inc., USA). For PCS and zeta potential (ζ, milivoltz) measurements, 0.1mL of each colloidal suspension was diluted into 10.0mL of ultrapure water and 10mM NaCl, respectively. The values reported are the mean values for each nanoparticle formulation. These measures were carried out at 25°C.

The nanoparticles were dried on a polished metal support and then gold-sputtered; afterwards, they were examined by the Scanning electron microscopy (SEM) (Philips XL 30 FEG) at 10 kV, using different magnifications until 10,000 times.

In vitro release studies of luteolin from nanocapsules

In vitro release studies of luteolin from nanocapsules in colloidal suspension were carried out after some changes in the dialysis bag diffusion technique, previously described by Levy and Benita (1990) [22]. Dialysis bags (Dialysis tubing cellulose membrane, 0.4 in, Sigma) with 1.0mL of the luteolin colloidal suspension were sealed and dropped into 1L of phosphate buffered saline (PBS) at pH = 7.4. This solution was kept under continuous magnetic stirring. The PBS was prepared by dissolving the following salts: 8.00g NaCl; 0.20g KCl; 1.44g $Na_2HPO_4.2H_2O$ and 0.24 g of KH_2PO_4 for each liter of distilled water. The whole system was kept at 35°C and on continuous stream at 3.0mL min^{-1}. The stream was controlled by a peristaltic pump (PumpPro TPM 600 55RPM, Waton-Marlow, Wilmington, UK). At given time intervals, a dialysis bag was withdrawn from the stirred releasing medium

and the luteolin content was directly assayed by the previously validated HPLC method. The values reported were the mean values corresponding to three different batches.

Results and Discussion

Characterization of polymeric nanoparticles loaded with luteolin

The nanoprecipitation method was conducted through the precipitation of a preformed polymer from an organic solution which was previously solubilized, with the diffusion of this organic solvent into the aqueous medium. In the aqueous phase, the polymers get insoluble, precipitating immediately leading to the formation of nanoparticles [11]. All formulations presented a macroscopic homogeneous aspect of a bluish white milky opalescent fluid (Tyndall effect) as a result of the nanoparticle formation as soon as the acetone was diffused into the aqueous phase, in agreement with the results previously reported in literature for other nanoparticle systems [11,23]. This observation confirms the formation of nanoparticles. This technique proved to be simple as well as easily reproducible.

An important result obtained in this work was the increase luteolin dispersion in water. The low solubility of luteolin may result in its poor permeation across the intestinal epithelial cells and gastrointestinal tract, which may compromise its clinical efficiency. In the formulations proposed for this study, it was possible to prepare colloidal suspension containing up to 100µg mL^{-1} (349.4µM) of the luteolin. This result is 75% higher than the water dispersion of luteolin obtained by Kim et al. (2008) through cyclodextrin complexation [24].

Analytical validation HPLC method

Reliable analytical data are a prerequisite for the correct interpretation of nanoparticle characteristics when under evaluation. The investigation upon of Rec%, EE% and kinetic release was just possible by using the HPLC method. The applicability of the method was checked by analytical validation according to the International Conference on Harmonization [21]. The specificity of the method was evaluated by comparing the chromatogram analyses of prepared nanoparticle samples containing and not containing the luteolin (Figure 2). No interfering peak was observed in 3.87min in the blank chromatogram (retention time of luteolin) in a work wavelength of 220 nm. In this sense, the method may be considered to be specific for the desired analysis since no interference peaks were observed.

The analytical calibration curves ($n = 3$) were linear over the concentration range from 5.00 to 100µg mL^{-1} (Figure 3). The linearity was assessed through calculating the regression equation ($y = a.x \pm b$) and the correlation coefficient (r^2) by the least squares method, where: $y = 89.19(\pm0.07).x - 67.21(\pm26.03)$ and $r^2 = 0.9999$. The y is the area of chromatographic peak, and x the concentration of standard solution in µg mL^{-1}. The standard

deviations of the values of *a* and *b* are indicated in parentheses. The r^2 value which is greater than 0.999 indicates a good linearity for the range of the proposed work. The quality of linearity was also evaluated through the analysis of the response factor (Figure 3). The response factors revealed a 5.50% RSD among all levels of concentration patterns. Therefore, samples may be adequately analyzed within the concentration range of the proposed method. Accuracy was analyzed by calculating the average percentage recoveries for the Luteolin at three different concentrations (6.00, 50.0 and 95.0µg mL^{-1}). These solutions were used to calculate both precision and accuracy. The precision was represented by Relative Standard Deviation (RSD). RSD for repeatability at each concentration level of standard solutions intra-day (*n = 15*) and inter-day (*n = 3*) were lower than 3.68% and 2.79%, respectively. These results indicate good precision of the analytical method. The total Rec% (accuracy) and its RSD found were 97.7±2.15%, showing strong agreement between the experimental and theoretical values. Detailed results for the three concentration levels which were tested are shown in Table 2.

The LOD and LOQ were calculated in accordance with the intercept standard deviation (*sd* = 21.91) and the slope of the calibration curve (*S* = 89.19). They were found to be 0.81 and 2.45µg mL^{-1}, respectively. The validated method was successfully applied in the evaluation of both the Rec% and the EE% of luteolin in polymeric nanoparticles. The pretreatments showed to be effective for the desirable analyses. It was possible to obtain recoveries up to 100% with regard to the linear calibration curve. These results are fundamental to analyze whether the target analyte was efficiently nanoencapsulated, its solubility and stability increased, as well as the kinetic release and the tested formulation efficacy. The quantitative results are described in Table 3.

Factorial design evaluation for Luteolin formulations

Biological action of several herbal medicines can be modified in agreement with their formulations. Their solubility, gastrointestinal stability and release profile can be improved by controlling the type of formulation employed. In this case, studies to prepare efficient formulations are necessary whenever the best features of nanoparticles are desired. Thus, the aim of this study was also to develop and evaluate processes and parameters to prepare nanoparticles loaded with luteolin by using factorial design.

The 2^3 factorial design had three independent variables which resulted in eight experiments. Furthermore, other three random experiments (02, 04 and 07) were carried out in order to evaluate the method capacity of reproducing the results. Thus, the total number of experiments was eleven. The analysis of the experimental scattering revealed a good reproducibility. Data dispersion for all dependent variables was smaller than 4.4% showing satisfactory repeatability of the nanoparticle preparation process. In this way, the experimental variations among the formulations were considered to be existent just when the calculation effects were higher than 4.4%.

In these experiments, the ideal type of polymer and oil, the quantity of luteolin to obtain the best colloidal dispersion and

luteolin stability were evaluated. The fixed variables which are described in Table 1 were kept constant. Their quantitative values were previously defined prior to the literature review [23], another group work [25] and experimental compatibility evaluation for luteolin.

The polymers evaluated, PCL and PLGA, are often employed as support to deliver drugs, either as NS (monolithic devices) or NC (reservoir devices). These polyester polymers have widely been used by the pharmaceutical industry because of their biocompatibility, dissolution capacity into organisms and mechanical properties which are suitable for such applications, and their biodegradability by micro and macroorganisms [26,27]. The spherical shape of nanoparticles was confirmed by using the scanning electron microscope (SEM). In order to obtain good resolution through SEM, it was necessary to remove all the water from the colloidal suspensions after they were added to the polished metal support. This process caused the gathering of nanoparticles and a polymeric pellicle was formed as a result. However, it was possible to observe remaining nanoparticles on the polymeric pellicle (Figure 4). The morphological analysis through SEM revealed that nanoparticles prepared by using isodecyl oleate had a better formation of spherical particles which shown regular surface and form, homogeneity, and lower dispersion in their sizes. On the other hand, nanoparticles prepared by using oleic acid shown poor formation of particles with higher dispersion in their sizes. It is likely that nanoparticles prepared by using oleic acid were less stable in both processes: the nanoparticle formation and the drying onto polished metal support. The formed negative charge in the edges of produced micelles when oleic acid is applied can justify the loss in colloidal dispersion stability.

The photomicrographs obtained did not show formation of crystals. Crystals may grow when the compound to be nanoencapsulated exceeds its solubility level or due to a great difference between the particle sizes. The absence of the crystals was confirmed by SEM, Rec% and EE% of the method.

The dependent variables utilized to investigate the colloidal dispersion were pH, PS, PI and ZP. On the other hand, the dependent variables, Rec% and EE%, were applied in the stability and kinetic release studies. The results of dependent variables are illustrated in Table 3.

The effect of each variable and their interactions were calculated according to the following equation: Effect of variable = $_+$ − $_-$, where $_+$ and $_-$ are the average of pH, PS, ZP, Rec% and EE% at the higher (coded as +1) and lower (coded as –1) levels, respectively. Figure 5 shows the calculated effects for the standard data for the performed 2^3 factorial design. On normalization the greatest values were taken as 100 and the rest were scaled proportionately.

The oils utilized are two potent chemical permeations which are widely used in commercial formulations. Isodecyl oleate ($C_{28}H_{54}O_2$) is neutral-water insoluble ester (no-ionic) which is employed as emulsifier and it can favor the substance inclusion as well as improving the active compound stability. In addition, it is a well-known emollient showing fluidity and causing no irritation

when widely used in skin formulations [28]. On the other hand, the oleic acid ($C_{18}H_{34}O_2$) is a monounsaturated fatty acid anionic which is used as a tensoactive forming micelles in aqueous phase. Two specific quantities of luteolin were investigated in order to evaluate its stability through Rec%, and the EE% during the preparation of nanoformulations.

The pH value is directly related to formulation compounds. The type of polymer and oil could cause effects in pH values. The pH values ranged from 5.3 to 5.7. However, regardless the prepared formulation or evaluated interaction effects, no significant effect was observed. This can be indicated by the small slope of the arrows in Figure 5A.

The PS can have important consequences in pharmaceutical applications for nanoparticles. Initially, all formulations were prepared by using oil which led to the preparation of only nanocapsules which showed average particle sizes of 130.6 nm. The range in PS values was from 72.4 to 185.5 nm. Although the types of the oils utilized in this work were different from the ones normally applied in literature (caprylic/capric triglyceride, mineral oil, *etc.*), the PS results are in agreement with previous studies described in literature which applied the nanoprecipitation technique, i.e., nanocapsules with PS smaller than 300 nm [23,29]. As so far isodecyl oleate and oleic acid have not been reported to prepare polymeric nanoparticles loaded with flavonoids. The PS in NC is limited by the size of the drops which are immediately created (oil core) when the organic phase is transferred into the aqueous phase. The oil core is controlled by the flow through which the organic phase is poured into aqueous solution, as well as the magnetic stirring and viscosity of the aqueous phase [29]. These factors have not undergone variations. In this case, the main particle size variations only depended on the type of polymers (PCL and PLGA) and oils (isodecyl oleate and oleic acid) used. In due course, through the calculation of the effects, it was possible to observe that PLGA and oleic acid decreased 16.9% and 33.6% the PS value, respectively (Figure 5C). Oleic acid is a *cis*-monoinsatured fatty acid which reduces the diameter of micelles. PLGA may form mainly dipole-dipole interactions with PLGA or with other groups, and PCL may make Van der Waals interactions as a rule. These different intermolecular interactions may lead to a singular way of how the polymers precipitate into the aqueous phase. However, by analyzing the calculation of the effects for the interactions of 2nd and 3rd-order ($x_1.x_2$; $x_1.x_3$; $x_2.x_3$ and $x_1.x_2.x_3$), it could be observed that only the interaction between the higher luteolin quantity and PLGA showed a slight increase (13.1%) in PS value. This effect is observed when the quantity of luteolin and the type of oil are at the same level (1.25mg luteolin-isodecyl oleate or 2.50mg luteolin-oleic acid). Thus, both types of oils and polymers could be used to prepare nanocapsules smaller than 250nm loaded with luteolin.

Regarding the size of nanoparticles, not only is their average value (nm) important, but also their size variability. A metric for size variability is the PI, a unitless quantity derived from the cumulate analysis and equivalent to the relative variance of the distribution [30]. In other words, the PI represents the homogeneity of nanoparticle size distribution. All formulations obtained a PI lower than 0.200. The PI average was 0.110, and the highest was 0.144. Values of polydispersity index lower than 0.200 describe narrow-average distribution and are considered to be a homogenic suspension. They are desired to keep the stability of colloidal dispersion without the formation of microparticles or precipitates [31]. The colloidal suspensions prepared using PCL, PLGA didn't precipitate even after five months. This narrow particle size distribution circumvents creation of luteolin concentration gradient between small and large nanoparticles which could lead to luteolin crystallization onto large particles, i.e., crystal growth known as Ostwald ripening [32].

Zeta Potential, likewise PS and PI, is a very important parameter in order to evaluate the colloidal suspension. When nanoparticles are prepared, the formation of an electrical double layer occurs surrounding the nanoparticles when in solution. The electrostatic potential at this "slipping plane" boundary is called the zeta potential and is related to the surface charge of the nanoparticle [33]. Accordingly, the zeta potential indicates the degree of repulsion between similarly charged particles in dispersion and it is a parameter widely used to predict colloidal suspension stability. Nanoparticles with a zeta potential between +10 and –10 mV are considered approximately neutral. On the other hand, nanoparticles with ZP higher than +30 mV or lesser than –30mV are considered to be very stable in the dispersion medium [32-33]. The formulations showed zeta potential ranging from 12.7 to 32.6 mV (using values in module). Two main effects were observed: all formulations prepared by using oleic acid have a positive ZP value, and the PLGA decreased the ZP in 42.3%, as it could be seen through the calculation of the effect between PCL and PLGA interaction. For this reason, PCL was considered to be better than PLGA at preparing stable colloidal suspension. The use of oleic acid caused a slight increase in ZP (8.6%). No other calculation of the effect showed significant effect over ZP.

For pharmaceutical use, a colloidal suspension must have a minimal tendency to agglomerate, which could create a hard cake. For this reason, tween®80 was used as steric stabilizer [32]. The absolute average recovery was 80.9%, and the lower and the higher values were 61.6 and 94.3%, respectively. The main effect was observed for the independent variable "quantity of luteolin" which showed a decrease of 12.4% when the amount of luteolin increased from 1.25 to 2.50mg. The 2nd order ($x_1.x_2$) interaction also depicted a reduction of 11.0% when the independent variables x_1 and x_2 were at the same level, i.e., PLC/1.25mg of luteolin or PLGA/2.50mg of luteolin. However, the 3rd order ($x_1.x_2.x_3$) interaction did not show significant variations.

Regardless the Rec% observed, there was no difference in EE%. All formulations showed an entrapment efficiency greater than 97%. The concentration of luteolin did not influence this variable. This result is a consequence of the low solubility of luteolin, which prefers both the oily core and polymeric interaction instead of the aqueous medium. As a consequence, the formation of colloidal dispersion increases the dispersion of luteolin in the aqueous medium.

In view of such results and the calculated cost, the best formulation was defined by using PCL and isodecyl oleate. Afterwards, the features of nanoparticles in the presence (NC) or not (NS) of isodecyl oleate, formulations 09 and 10, respectively, were investigated. Both experiments showed Rec% and EE% higher than 89.1% (Table 4). The main difference was the particle size between nanocapsules and nanospheres. The nanospheres were nearly 50% smaller. A slight improvement could also be observed in nanocapsules regarding the ZP, Rec% and EE%. Thus, the best formulation may be selected evaluating PS and by the physiological barriers which the nanoparticles need to transpose.

Release kinetics

The release assays of the formulations 09 and 10 were carried out in PBS medium at 35℃, which was kept under magnetic stirring and continuous stream to avoid saturation of the system. In this assay, the luteolin passes through the pores of the membrane while the nanoparticles do not, hence allowing the observation of the luteolin diffusion. The PBS butter and controlled temperature were selected in order to imitate the human biologic system. During the assay, aliquots were collected at pre-established times and the luteolin was quantified by HPLC. The results were expressed in terms of the percentage release (Figure 6).

The release profiles of NC and NS were similar and did not show statistic difference. The percentage of luteolin, which was released at the first sampling time of 0.5 hour were 34.5 and 43.1% for NS and NS, respectively. These two formulations revealed a fast release kinetic during the first two hours, which was approximately 63%. Such a fact probably occurs through the fast migration of luteolin adsorbed on the polymeric nanoparticle surface or positioned in the superficial polymeric layer [34]. After four hours, the formulations showed constant and slow release rate, probably because of the existence of internal interactions between luteolin and the polymer of the nanoparticles [34]. As a result, there may be a reduction on the release rate. Naturally, this behavior took place in the continuous stream at 3.0mL min^{-1}. The total percentage of release after fifteen hours for both formulations was 87%.

Mathematical modeling (zero order, first order, biexponential) was applied to obtain information about the kinetics of luteolin dissolution and release behavior. After adjusting these models, the best profile was selected based on the highest correlation coefficient (r^2). According to these criteria, the zero order was the best model to describe the dissolution and release profiles for nanoencapsulated luteolin, showing two specific tendencies: a fast one between 0 and 2 hours, and another slow one between 2 and 15 hours. In face of this result, the release behavior from nanocapsules of PCLwas determined through the theoretical model of Korsmeyer-Peppas for drugs release in polymeric systems [35]. This model is applied for anomalous behaviors of release, which was described by $M_t/M_\infty = K.t^n$ where M_t/M_∞ is a fraction of release in a given time t, n and K are the exponent of liberation and release factor, respectively [35]. Korsmeyer-

Peppas's model is usually used to analyze the release of a drug of a polymeric matrix when the release behavior is not well known or when one or more types of phenomena are involved.

The construction of the graph through $ln(M_t/M_\infty)$ in function of $ln(t)$ provides the exponent (n) and the release constant (K, time^{-n}). The empiric values of n, which were determined for luteolin in NS and NC, were 0.387 and 0.267, respectively, were obtained from the initial portion of the curve (%released < 80%). The release constants were 0.963 and 0.994, respectively. When the n values are smaller than 0.45 ($n \le 0.45$), the mechanism of release refers to a process of pure diffusion (Fickian diffusion) [35]. Nanoparticles loaded with luteolin depicted $n \le 0.45$ in accordance with the zero order mechanism, which was previously observed.

Conclusions

The results from our study show that homogeneous nanoparticle suspensions loaded with luteolin can be prepared through preformed polymers by using interfacial polymer deposition following solvent displacement. The prepared formulations and their physico-chemical characteristics were evaluated through factorial design. The polymers PCL and PLGA, oils isodecyl oleate and oleic acid, as well as the charge of luteolin were investigated. This strategy allowed us to prepare stable colloidal suspensions. The nanoparticles were obtained mainly by use of PCL and isodecyl oleate. Small, spherical and submicron sized nanoparticles were obtained. The range in PS values for NC was from 72.4 to 185.5 nm. NS showed an average particle size of 77.3 nm.

In order to characterize the Rec% and EE% in colloidal suspension, a HPLC method was developed and validated. This method allowed the assessing of the occurrence of loss and degradation processes of luteolin, EE% and the kinetic release. The method showed a limit of quantification of 2.45µg mL^{-1}. It was also possible to prepare colloidal suspensions containing up to 100µg mL^{-1} (349.4µM) of the luteolin. The Rec% (luteolin content) and EE% were higher than 90 and 98%, respectively, in the best formulations.

The suggested method of dialysis for the analysis of kinetic release of luteolin was efficient and showed reproductive results *in vitro*. It could be used in routine experiments. Luteolin release from nanoparticles appeared to have two components: an initial rapid release, due to surface-associated luteolin, followed by a slower exponential release of the luteolin dissolved in the core. NC and NS of PCL depicted the same release profile. These nanoparticles are quite stable in aqueous dispersion and can be further optimized to be used as an efficient luteolin delivery agent.

Acknowledgments

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Table 1. Investigated variables in polymeric nanoparticles formulations loaded with luteolin evaluated in 2^3 full factorial design.

Variables	Low level (− 1)	High level (+ 1)
x_1, Polymer 75.0 mg (0.3 % m/v)	PCL	PLGA
x_2, Luteolin	1.25 mg (0.005 %, m/v)	2.50 mg (0.010 %, m/v)
x_3,Oil 300.0 mg (1.2 % m/v)	Isodecyl oleate	Oleic acid
Fixed variables		
Acetone volume	14,0 mL	
Span®60	40.0 mg (0.16 %, m/v)	
Aqueous phase volume	27,0 mL	
Tween®80	40.0 mg (0.16 %, m/v)	

PCL: poly(-caprolactone) PLGA: poly(lactic-co-glycolic acid). (%, m/v): after the organic solvent was removed and the final volume water fixed in 25mL.

Table 2. Results of precision and accuracy intra and inter days, for three different concentrations of luteolin.

Nominal Concentration (µg mL^{-1})	Precision[a]			Accuracy
	Day 1 (n = 5)	Day 2 (n = 5)	Day 3 (n = 5)	Inter – Day (n = 15)
	Conc. µg mL^{-1}/ RSD (%)			(%)
6.00	5.87/1.27	5.65/3.41	5.99/3.68	97.3
50.0	48.09/0.30	48.38/1.20	50.41/2.47	97.9
95.0	92.27/0.47	92.31/0.39	94.85/0.31	98.0

[a] Mean concentration found (µg mL^{-1})/RSD.

Table 3. Characterization of luteolin loaded PCL and PLGA nanocapsules prepared through 2^3 full factorial design.

Exp	Independent variables			Dependent variables[a]				
	x_1	x_2	x_3	pH	PS (nm)	ZP (mV)	(Rec%)	(EE%)
01	−1	−1	−1	5.57±0.09	185.5±6.38	-28.4±0.7	82.2±3.0	97.8±1.0
02	+1	−1	−1	5.73±0.11	165.9±4.01	-16.0±0.5	94.3±1.7	98.0±1.2
03	−1	+1	−1	5.69±0.09	157.2±5.55	-29.4±0.9	75.3±2.2	98.8±1.8
04	+1	+1	−1	5.53±0.17	138.1±5.03	-12.7±0.8	61.6±1.8	98.5±1.6
05	−1	−1	+1	5.48±0.04	106.0±5.09	32.6±1.1	85.0±2.9	97.6±1.6
06	+1	−1	+1	5.38±0.07	72.4±6.37	18.8±0.5	85.6±0.7	98.0±2.2
07	−1	+1	+1	5.30±0.10	136.3±8.68	29.6±0.6	89.2±1.5	98.8±0.8
08	+1	+1	+1	5.45±0.08	83.0±7.09	16.7±1.1	74.1±1.4	98.7±0.3

[a]*Triplicate mean values of analyses and their standard deviations (mean±SD). PS, particle size; ZP, zeta potential; REC%, absolute recovery and; EE%, entrapment efficiency.*

Table 4. Characterization of nanocapsules and nanospheres of PCL polymer loaded with luteolin.

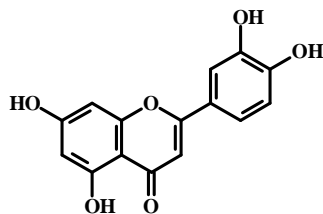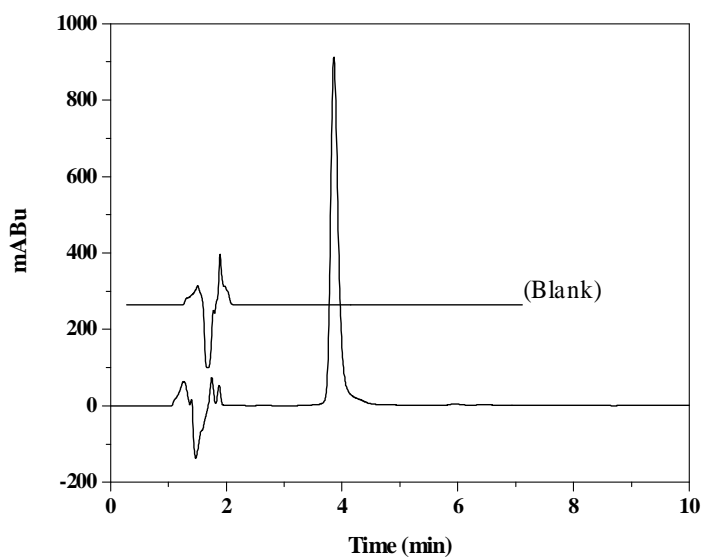| Experiment	pH	PS (nm)	ZP (mV)	(Rec%)	(EE%)
With oil[a]	5.77±0.13	161.8±6.61	-31.2±0.7	89.1±1.4	90.2±7.5
Without oil	5.89±0.15	77.3±4.01	-26.5±0.5	94.7±5.8	95.0±0.1
[a]*Oil: isodecyl oleate. Triplicate mean values of analysis and their standard deviations (mean±SD)*					

Figure 1. Luteolin structure.

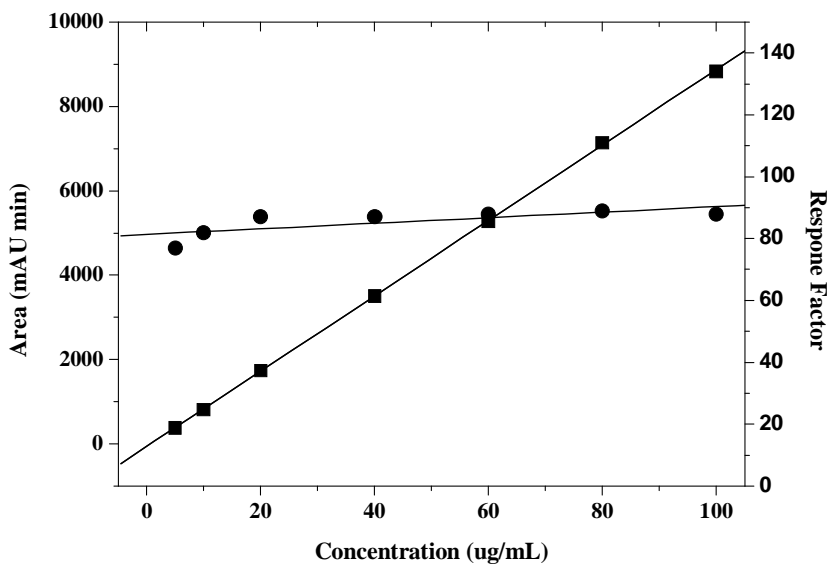Figure 2. Typical chromatogram of a colloidal dispersion of polymeric nanoparticles with and without (blank) luteolin.

Figure 3. Linearity for the quantitative method to Luteolin analysis by HPLC. ( ) Calibration curve obtained with luteolin standard solutions ($n = 21$); ( ) Luteolin response factor *vs.* concentration of luteolin standard solution ($n = 21$).
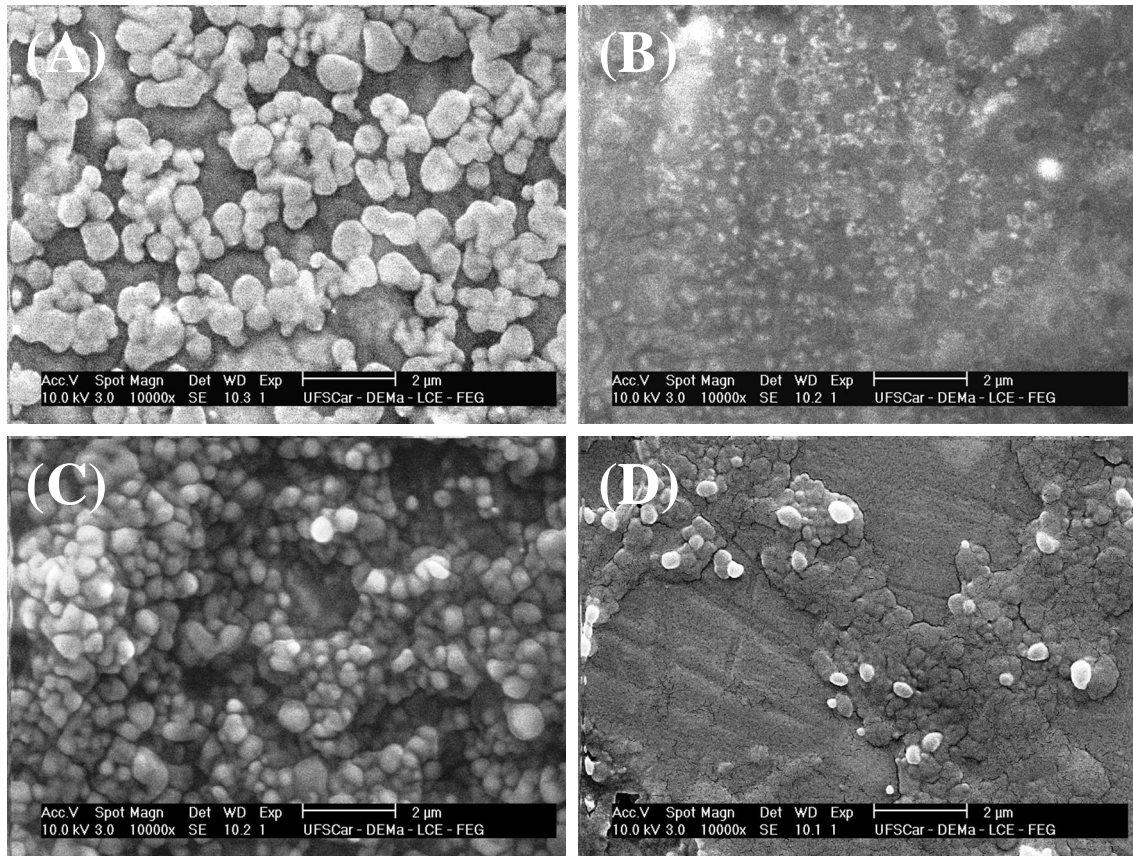
Figure 4. Scanning electron microscope images of nanocapsules containing (A) PLC/isodecyl oleate; (B) PCL/oleic acid; (C) PLGA/isodecyl oleate and (D) PLGA/oleic acid. The photomicrographs were taken with 10,000 x magnification.
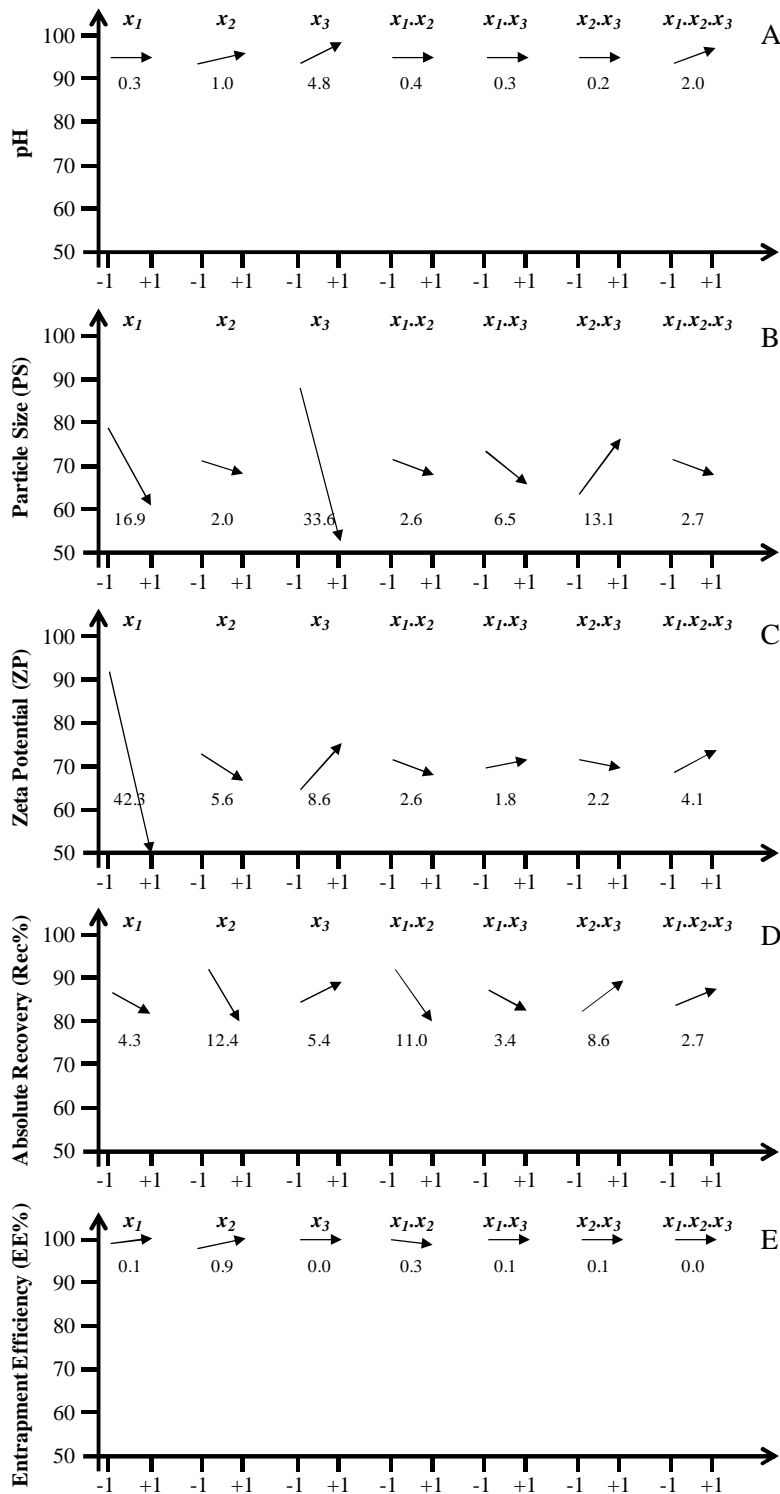
Figure 5. Calculated effects of the experiments performed by 2^3 factorial designs. Arrows inside the figure represent the calculated effects in percentage after normalization of the data. It is important to observe that the inclination of the arrows indicates the amplitude of the effects.
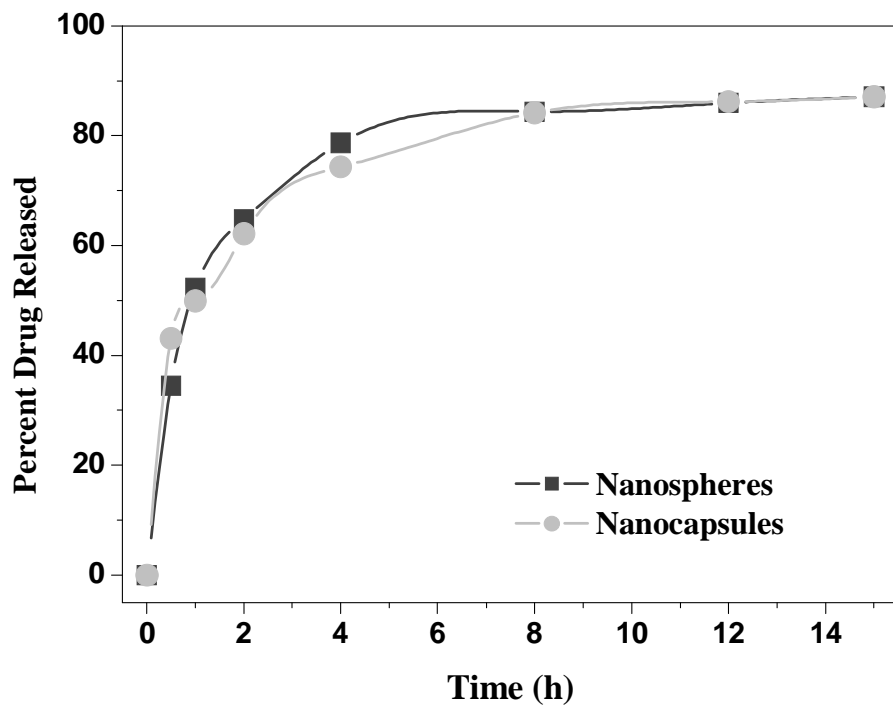
Figure 6. In vitro release profile of luteolin from ( ) nanospheres and ( ) nanocapsules.