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# CURCUMIN-LOADED POLYSACCHARIDES-BASED COMPLEX PARTICLES OBTAINED BY POLYELECTROLYTE COMPLEXATION AND IONIC GELATION. I-PARTICLES OBTAINING AND CHARACTERIZATION

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**KEYWORDS** : Polysaccharides microparticles, Polyelectrolyte complexation, Ionic cross-linking, Curcumin immobilization

**ABSTRACT**

Curcumin has essential therapeutic benefits, but it is insoluble in water and thus has low bioavailability. This study aimed to immobilize curcumin into new polysaccharide-based microparticles (gellan, i-carrageenan, and chitosan) to increase its stability and bioavailability. Curcumin-loaded complex microparticles were obtained from three polysaccharides, of different ionic character, by ionic cross-linking and polyelectrolyte complexation. The immobilization efficiency was between 85.75% and 97.25%. The microparticles were characterized morphologically by SEM, and it was observed that the microparticles containing the i-carrageenan had a more pronounced porosity of the matrix. The swelling degree values at pH = 7.4 were superior to those obtained at pH = 6.8 or pH = 2 and depend on both the cross-linking degree and particles morphology. The polysaccharides microparticles, curcumin, and constituent polysaccharides were characterized by FT-IR spectroscopy. The curcumin release kinetics was studied in three different pH media, and the release efficiency ranged between 65.1% and 97.9% at pH = 7.4, between 60.2% and 82.2% at pH = 6.8 and between 56.1% and 64.0% at pH = 2. These microparticles can be intended for oral administration, having as therapeutic target the colon, for the controlled release of curcumin, since they can overcome the gastric barrier without the degradation of the active principle, which is protected by the polymer matrix.

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# 1. Introduction

The pharmacological effects of curcumin were highlighted by numerous studies concerning its actions in the prevention and treatment of chronic diseases such as arthritis, cancer, depression, and neuro-vegetative diseases [1–9]. More than 100 therapeutic targets have been reported, and the number and diversity of the curcumin's biological benefits are numerous, ranging from anti-inflammatory, antioxi-dant, antiviral to antitumor effects [10].

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are characterized by chronic inflammation of the gastrointestinal tract. The highest prevalence of disease reported in 2017 in Europe was in Norway (505 cases of ulcerative colitis per 100,000 people), in Germany (322 cases of Crohn's disease per 100,000 people) and in North America in the USA (286 cases of ulcerative colitis per 100,000 persons) as well as in Canada (319 cases of Crohn's disease per 100,000 persons) [11]. The purpose of treating chronic IBD is to reduce inflammation that triggers the signs and symptoms. In the best cases, this can lead not only to the improvement of the symptoms but also to the induction and maintenance of long-term remission, which leads to reduced risks of complications. The treatment for IBD usually involves either drug therapy or surgery. Aminosalicylates, corticosteroids (are ineffective in maintaining remission), immunomodulators (generally not recommended due to cessation of action and toxicity) are used as medication. In some cases of perianal Crohn's disease, antibiotics such as ciprofloxacin and metronidazole can be used in combination with other medicines. Currently, there are no therapeutic strategies capable of significantly changing the history of IBD.

Nutrition therapy has interesting possibilities for treatment, and curcumin has gained interest due to its pharmacological action and its properties. The primary mechanism by which curcumin mediates these effects is related to the nuclear factor Kappa-light-chain-enhancer (NF-KB) suppression. Also, curcumin activity includes suppression of interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α), two essential cytokines in regulating inflammatory responses. For these important activities, curcumin was considered a potential treatment in the inflammatory bowel disease [12,13]. Curcumin is primarily metabolized by reduction (in the intestine under the action of CurA reductase) and conjugation after oral administration. The curcumin, and its reduced metabolites, are almost exclusively conjugated in plasma to glucuronic acid and sulfate [14–16].

Curcumin, (1E, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, is a compound extracted from *Curcumalonga* (Turmeric) with significant benefits for the human body. Depending on its origin and soil conditions, turmeric contains between 2 and 9% curcuminoids - a group of compounds including curcumin, as the major component, demethoxycurcumin, bisdemethoxycurcumin and cyclic curcumin (the minor component) [17,18]. Curcuminoids exist in two tautomeric forms, of which the bis-ketone is predominant in the acidic or neutral medium as well as in the solid-state, while the enolic form is predominant in alkaline solution. Figure S1 (Supplementary material) presents the curcuminoids structures.

The major drawback of curcumin is its water insolubility and, thus, low bioavailability in cells [19]. Clinical studies have shown that the human organism well tolerates curcumin in doses up to 8

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g/day. Increasing the dose up to 12 g/day resulted in adverse effects in 30% of patients. The dose-dependent adverse reactions reported were: (a) gastrointestinal disorders (b) infertility (at men) (c) inhibition of Hepcidin synthesis (d) iron chelator - causes a decrease in iron level in the body, may lead to anemia, (e) transient growth of liver enzymes; (f) suppression of platelet aggregation; (g) contact dermatitis and hives when administered in topical formulations. The best patient compliance was achieved when curcumin was administered in doses of 2–4 g/day because a large number of capsules or the increase in the size of the pills becomes clinically impractical, especially in the elderly population. Epidemiological data suggest a low incidence of gastric cancer in India is due to the high consumption of curcumin. The estimated curcumin dose in the diet of those who consume large amounts of turmeric daily is 0.15 g/day. In the absence of long-term clinical trials, this dose may be considered appropriate when curcumin is used for long periods. This dose of curcumin is similar to that recommended by the World Health Organization but ten times lower than that generally recommended in dietary supplements [20,21]. Adverse effects may also be due to the association of standard curcumin with piperine (a black pepper extract). Previous research studies on the side effects of piperine have shown that it can, lead to gastric disorders, to adverse effects on fertility, and can inhibit the therapeutic effect of some drugs if administered simultaneously [20,22,23]. The attenuation or elimination of these disadvantages has been attempted by preparing formulations based on micelles, liposomes, polymeric nanoparticles, complexes, emulsions [24,25]. Polymer matrices can protect curcumin from adverse environmental conditions; improve the half-life of the bioactive compound, thus increasing its bioavailability both *in vitro* and *in vivo* [26].

An increased number of studies report the encapsulation/incorporation of curcumin into nanoparticles based on natural polymers, such as chitosan, ghatti gum or synthetic polymers: poly(ethylene glycol), poly (lactic acid), poly(*N*-vinyl pyrrolidone), poly(lactic-co-acid glycolic acid) in order to obtain various pharmaceutical formulations [27–31].

Additional types of formulations involve the formation of inclusion complexes with cyclodextrins and phosphatidyl-choline as well as the encapsulation in lipid nanoparticles or liposome and micro/nano-emulsions [32].

Chitosan, gellan, and i-carrageenan are among the polysaccharides having a protective role as well as a controlled release behavior of the active principles and can be used to obtain curcumin-loaded particles.

Chitosan is a semi-crystalline linear natural cationic polymer obtained by the alkaline deacetylation process of chitin and is composed of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan (*N*-acetyl-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-D-glucan (D-glucosamine) [33,34]. Previous studies have shown that through curcumin immobilization in chitosan microparticles, cross-linked with sodium tripolyphosphate (TPP), the stability, plasma concentration, and bioavailability of curcumin have increased [35–37]. Another study investigates the curcumin encapsulation in chitosan nanoparticles that have been complexed by adding them to a matrix composed of a mixture of alginate and k-carrageenan, leading thus to an increased bioavailability of the bioactive compound [38]. The nanoparticles released 95% of the curcumin in 7 h, and the efficiency of the process depends on the amount of carrageenan present in the particles. Other curcumin-loaded

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chitosan-based nanoparticles were prepared, and the target site of these systems, with mucoadhesive properties, was the colon. In alkaline pH, the amino groups of the chitosan are deprotonated and therefore are unlikely to exert their mucoadhesive functionality entirely based on electrostatic interactions. However, these curcumin-loaded nanoparticles have demonstrated significant mucoadhesiveness, indicating that curcumin probably contributes to this effect [39].

Gellan is a linear anionic biopolymer with repeating tetrasaccharide sequences consisting of two β-D-glucose residues, a β-D-glucuronic acid residue, and α-L-rhamnose in a 2:1:1 ratio [40]. Even if gellan is resistant to enzymes such as pectinase, amylase, cellulase, papain, and lipase [41], significant degradation occurs in the presence of galactomannans, which facilitates the release of bioactive components from the polymeric system into the colon fluids [42,43]. Yang et al. [44] prepared and evaluated spherical particles obtained from chitosan and gellan, by ionotropic gelation and polyelectrolyte complexation, using calcium chloride as a cross-linker, for the encapsulation and controlled release of proteins. Higher gellan concentrations combined with vacuum drying of the microparticles slow down the rapid release of the protein at pH = 1.2. However, a sustained release was observed at pH = 6.8, and an efficient release has been found at pH = 7 [44]. In another study, the gellan and pectin particles, cross-linked with $AlCl_3$, were prepared for the resveratrol immobilization and release in the colon. The immobilization efficiency was 76%, and the release kinetics showed that the most significant amount of bioactive components was released at pH = 6.8 [45].

I-carrageenan belongs to the family of high molecular weight sulfated linear hydrophilic galactans consisting of alternating units of D-galactopyranose and 3,6-anhydro-galactose (3,6-AG) linked by alternating α-1,3 and β-1,4-glycosidic bonds [46]. The properties of carrageenans are mainly influenced by the number and position of the sulfate ester groups but also by the 3, 6-anhydro-galactose content [46–48]. The pH-responsive particles prepared by polyelectrolyte complexation with i-carrageenan and chitosan were obtained for albumin delivery and oral administration. Increased efficacy in release occurred at pH = 7.5 [49].

This study aimed to prepare new formulations by immobilizing curcumin into polysaccharide particles in order to increase its stability and bioavailability.

These polysaccharides were ionically cross-linked with magnesium acetate (in the case of gellan and i-carrageenan) and sodium sulfate (in the case of chitosan). The originality of our study is provided by the nature of the cross-linking agents, for the achievement of new complex hydrogel microparticles, by the use of gellan mixed with i-carrageenan to form the polyelectrolyte complex with chitosan, and also by the immobilization of curcumin in the obtained microparticles. The particles obtained were morphologically characterized by scanning electron microscopy, and the swelling behavior evaluation was performed in three aqueous mediums of different pH. Curcumin release occurs in the stomach and intestine but degrades significantly in the presence of an enzyme, galactomannans, which is present in the colonic fluids. I-carrageenan was used because on each structural unit, it contains two sulfate ester groups, and in mix-ture with the gellan, it leads to stable structures with adequate porosity. The porosity of the polymer matrix can be controlled by varying the concentration of the cross-linking agent. By immobilization of curcumin in chitosan

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micro/nanoparticles (ChPs), its bioavailability increases, and chitosan can form polyelectrolyte complexes with the mixture of gellan and i-carrageenan, thus leading to stable, gastro-resistant particles. On the other hand, the gellan/carrageenan matrix, in which the curcumin-loaded ChPs were included, constitutes an additional barrier to the release of curcumin, leading thus to a more sustained/controlled release.

# 2. Materials and methods

## 2.1. MATERIALS

I-carrageenan (M = 541,100 Da) with two $-\mathrm{HSO}_3^-/mol$ (464 Da) groups; chitosan having a degree of deacetylation of 75% (M = 190–310 kDa and a viscosity of 200–800 cPs) were purchased from Sigma Aldrich. Gellan ($\mathrm{M_w} = 2 - 3 \times 10^5$ Da) was purchased from Kelkogel. Curcumin powder (extracted from *Curcumalonga*), sodium sulfate, magnesium acetate tetrahydrate, Tween 20, and hydrochloric acid (37%) were purchased from Sigma Aldrich. Chemical Company (Romania) provides ethanol 98%, monobasic sodium phosphate, dibasic sodium phosphate.

## 2.2. PREPARATION OF THE COMPLEX PARTICLES

The preparation method of the curcumin-loaded ComPs, schematically illustrated in Figure 1, based on the formation of polyelectrolyte complexes between polysaccharides, involves two steps.

In the first step, curcumin-loaded ChPs were prepared as follows: 40 ml of 0.05% chitosan solution in 0.06 M acetic acid and 16 ml of 0.1% $Na_2SO_4$ solution were prepared. The ionic cross-linker solution ($Na_2SO_4$ 0.1%) was added over the chitosan solution. Then, 200 mg of curcumin was dissolved in 20 ml of absolute ethanol under stirring, and the solution was added dropwise to the previously obtained chitosan solution during ultrasonication (Sonics and Materials sonicator, Vibra Cell). Different times for ultrasonication were used: 4 min, 8 min, and 12 min to obtain different sizes of the ChPs.

The suspension of ChPs of different sizes containing curcumin was maintained at 40 °C in an ultrasonic bath for 1 h in order to evaporate the alcohol and to disperse the formed micro/nanoparticles and then maintained at the same temperature on a magnetic stirring plate (250 rpm) until complete evaporation of the alcohol. Finally, the ChPs were separated by centrifugation, washed three times with bi-distilled water, and re-suspended in 100 ml bi-distilled water. If not used immediately, the suspension was kept at 4 °C, in the dark, in closed containers.

In the second step, gellan or gellan mixed with 10%, 20%, or 30% i-carrageenan (w/w) was dissolved in 20 ml bi-distilled water at 80 °C to obtain a 2% (w/v) solution. The solution was cooled at 40 °C, and then 20 ml of the suspension of curcumin-loaded ChPs (prepared in the previous step) was added dropwise under vigorous stirring (6000 rpm) using an Ultraturrax homogenizer. This suspension was then extruded through a needle (23 Gauge) into 100 ml of different concentrations

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of magnesium acetate solution. In these conditions, both the instantaneous formation of the polyelectrolyte complex at the surface of the ChPs and the ionic cross-linking of the gellan (or of the gellan/i-carrageenan mixture) occurs. The ComPs were maintained for 3 h in the cross-linking solution for stabilization and then were separated by filtration, dried at 25 °C on filter paper in Petri dishes, and stored in sealed containers at 4 °C in the dark until further characterizations were performed. The coding of the samples, the experimental program used, and the curcumin encapsulation efficiency obtained are shown in Table 1.

***Figure 1.*** *Schematic representation of the preparation process of obtaining the polysaccharides ComPs with immobilized curcumin.*

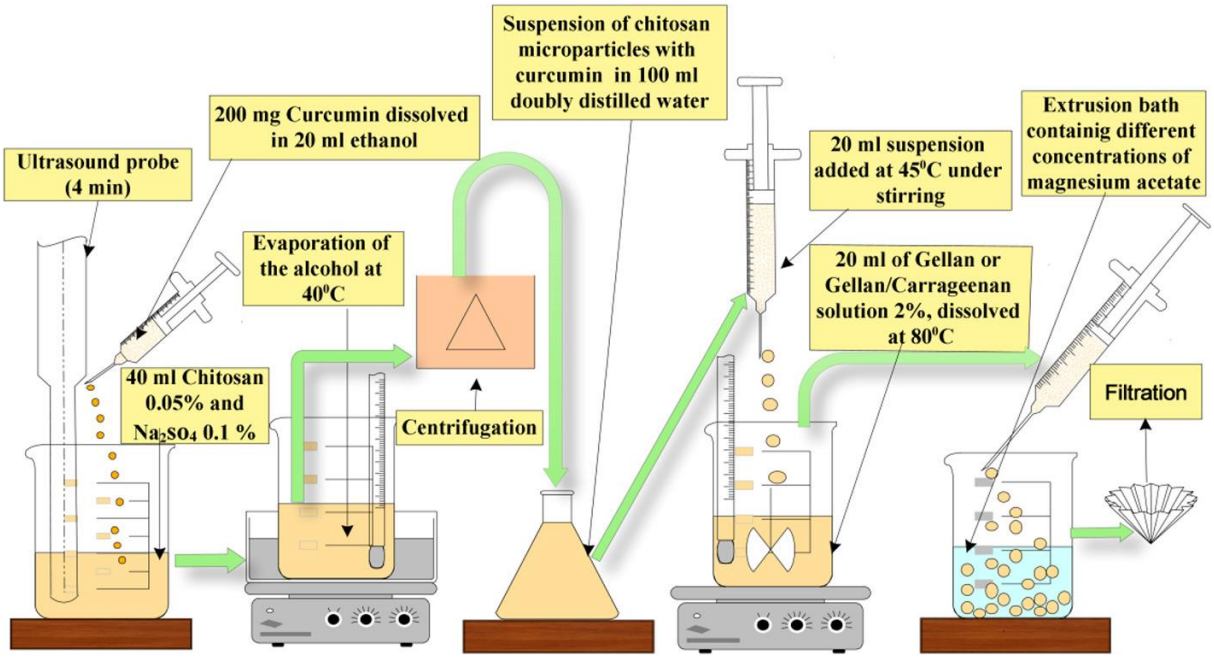

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***Table 1**. Experimental program.*

| Sample | Gellan (%) | I-Carrageenan (%) | Magnesium acetate solutions concentrations (%) | Encapsulation efficiency (%) |
|---|---|---|---|---|
| P1[a] | 100 | 0 | 1 | 97.25 |
| P2[a] | | | 2 | 91.5 |
| P3[a] | | | 3 | 87.23 |
| P4[b] | 70 | 30 | 2 | 85.71 |
| P5[c] | 80 | 20 | 2 | 90.4 |
| P6[d] | 90 | 10 | 2 | 94.2 |

[a] The composition of the polysaccharide particles was: 89% gellan and 11% chitosan; the volume of magnesium acetate solution was 100 ml.

[b] The composition of the polysaccharide particles was: 26.7% i-carrageenan, 62.3% gellan, and 11% chitosan; the volume of magnesium acetate solution was 100 ml.

[c] The composition of the polysaccharide particles was: 17.8% i-carrageenan, 71.2% gellan, and 11% chitosan; the volume of magnesium acetate solution was 100 ml.

[d] The composition of the polysaccharide particles was: 8.9% i-carrageenan, 80.1% gellan, and 11% chitosan; the volume of magnesium acetate solution was 100 ml.

## 2.3. CHARACTERIZATION OF THE PARTICLES

### 2.3.1. FTIR SPECTROSCOPY FOR THE OBTAINED PARTICLES

FT-IR spectra were obtained for free curcumin, chitosan, gellan, ChPs, and as well as for the ComPs P5C. The spectra were recorded on a Bruker Vertex FT-IR spectrophotometer over a frequency range of 4.000–400 $cm^{-1}$ at a resolution of 2 $cm^{-1}$.

### 2.3.2. PARTICLE SIZE ANALYSIS BY LASER DIFFRACTOMETRY

The mean diameter of the ChPs with curcumin immobilized and their dimensional distribution was analyzed by the laser beam diffractometry using the SHIMADZU-SALD 7001 diffractometer. Measurements were performed on micro/nanoparticle suspensions in bidistilled water at 24 h after preparation. The suspensions were soni-cated before measurements on a Bandeli Sonorex sonication bath for 10 min at room temperature. The accuracy of the results was also ensured through its agitation system in the measuring cell of the device, which holds the particles in suspension as well as performing several consecutive determinations. Three determinations were performed for each sample.

### 2.3.3. SCANNING ELECTRON MICROSCOPY (SEM)

Curcumin-loaded ComPs were characterized by scanning electron microscopy (SEM) to determine their morphology and to prove that curcumin-loaded ChPs were immobilized in the gellan or gellan/i-carrageenan matrix. The ComPs were dried, cut in cross-section, metalized with gold using a sputter deposition device, and analyzed using a Vega Tescan instrument.

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### 2.3.4. SWELLING DEGREE

The obtained ComPs have a hydrogel character, so it was considered useful to determine their capacity to retain water - usually quantified by the swelling degree (Q, %). This feature is essential because water determines the network meshes size increase formed on one side by ionotropic gelation of the chitosan and the other side of the gellan or gellan/i-carrageenan matrix, facilitating more or less the diffusion of curcumin from the hydrogel matrix. For ComPs obtained, the Q (%) values were determined gravimetrically. Three solutions, simulating physiological fluids, were used: PBS at pH = 6.8 (simulating the intestinal fluid) and at pH = 7.4 (simulating the pH of the blood or the pH of the colon fluid), and a solution simulating the gastric fluid at pH = 2, which was obtained from 0.1 N hydrochloric acid, 10 mM NaCl and bidistilled water.

A precisely weighed quantity (0.05 g) of dry ComPs ($M_{dry}$) was immersed in 5 ml of a swelling agent, at 37 °C. Periodically, the swelling agent was removed by filtration, and the particle surface was buffered with filter paper to remove the excess of liquid. The weight of the swollen ComPs ($M_{swollen}$) was determined. The amount of swelling agent retained by the ComPs ($M_{swelling\ agent}$) was the difference between the weight of swollen ComPs ($M_{swollen}$) and that of dry ComPs ($M_{dry}$). After weighing, the samples were reintroduced into the swelling agent (always 5 ml), and the operation was repeated until equilibrium was reached. The swelling degree was expressed as the ratio between the amount of swelling agent present in the ComPs at each time interval and the amount of entirely dried ComPs (Eq. (1)).

$$Q(\%) = \frac{M_{sweeling\ agent}}{M_{dry}} \times 100 \quad (1)$$

The determination of the swelling degree was done in triplicate. Data are given as mean value ± confidence interval at 95%.

### 2.3.5. ENCAPSULATION EFFICIENCY

The curcumin calibration curve in ethanol was drawn using solutions with concentrations ranging between 1 and 5 µg/ml. The absorbance of these solutions was recorded with a BOECO-S22, UV–Vis spectrophotometer at the wavelength of 425 nm.

The calibration curve equation was $y = 0.1951 \times (R^2 = 0.9996)$.

Curcumin was extracted in ethanol (V = 10 ml) from a known amount of curcumin-loaded dry ComPs. Based on the calibration curve, the curcumin quantity within the ComPs was determined. In this case, the efficiency of encapsulation was:

$$Ei\ \% = m_{cf}/m_i \times 100 \quad (2)$$

where $m_{cf}$ is the amount of curcumin in the ComPs determined spectro-photometrically, and $m_i$ is the initial weight of the curcumin. Three determinations were performed for each sample, and the errors were within ±3%.

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#### 2.3.6. RELEASE KINETICS OF CURCUMIN FROM THE COMPLEX PARTICLES

It was studied in three different pH media, such as PBS at pH = 7.4 (specific to blood and colon fluids), pH = 6.8 (specific for the intestinal fluid) and pH = 2 (solution prepared from NaCl 10 mM and 0.1 N HCl, simulating the pH in the gastric environment). In this study, a weighed quantity of dry ComPs was immersed in 20 ml of solution at pH = 7.4, pH = 6.8 or pH = 2 under slight stirring (150 rot/min) at 37 °C, in the dark, in closed containers, and the amount of curcumin released was determined at a wavelength of 425 nm (BOECO-S22, UV–Vis spectrophotometer).

Since the curcumin is a hydrophobic substance and is very poorly soluble in aqueous media, 1% (w/w) of Tween 20 was added to the solution in which the release kinetics is carried out [45]. Based on the calibration curves for free curcumin solutions at pH = 7.4, pH = 6.8 and pH = 2 with 1% Tween 20, the quantity of curcumin from the release medium was determined periodically.

The equations of these curves were the following:

- for the calibration curve of curcumin at pH = 7.4: $y = 0.1201x$ ($R^2 = 0.9987$)
- for the calibration curve of curcumin at pH = 2: $y = 0.0856x$ ($R^2 = 0.999$)
- for the calibration curve of curcumin at pH = 6.8: $y = 0.1089x$ ($R^2 = 0.9977$)

To determine if the highest quantity of curcumin from ComPs could be released in the colon medium at pH = 7.4, it was also study the release kinetics with the same samples in different pH mediums, consecutively modified: for 2 h at pH = 2, then for 3 h at pH = 6.8 and after for 4 h at pH = 7.4. For this assay, a certain quantity of dry ComPs was weighed and then immersed in the release medium at pH = 2 for 2 h. After 2 h, the samples were filtered, washed three times with bidistilled water, and then immersed in the release medium at pH = 6.8 for3 h and finally atpH = 7.4 for4 h. The samples from the release medium were taken periodically to determine the quantity of released curcumin. The determination of the release efficiency was done in triplicate. Data are given as mean value ± confidence interval at 95%.

# 3. Results and discussions

## 3.1. PARTICLES PREPARATION

Two polyanions (gellan, i-carrageenan) and a polycation (chitosan) were used to obtain the polymer matrix for the encapsulation of curcumin, and their selection was determined by the fact that they are natural, non-toxic polymers capable of forming polyelectrolyte complexes. Also, they can gently form gels by ionic cross-linking in the presence of polyvalent ions (cations or anions), thus avoiding the use of covalent cross-linkers, most often toxic.

The particles obtained by ionic gelation with Na2SO4 are composed of chitosan, in which curcumin was encapsulated by co-precipitation. It was already demonstrated that the encapsulation of curcumin in ChPs is beneficial and results in improved stability and bioavailability when administered orally [34,50]. The dark yellow color of CPs is a visual confirmation of the active

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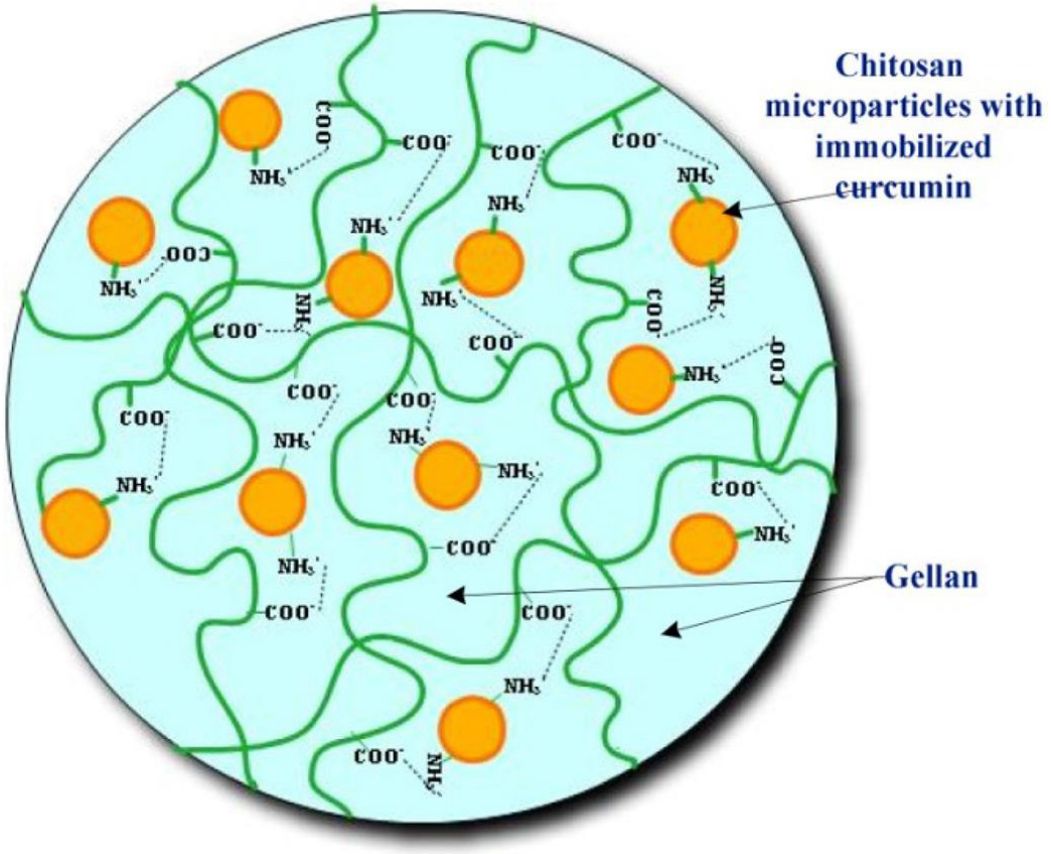
principle encapsulation. The obtained ChPs were incorporated then in the gellan or gellan/i-carrageenan matrix. A viscous solution of the two polysaccharides mixture was obtained, and it was extruded in a bath that contains magnesium acetate solution of different concentrations. The polyelectrolyte complex was formed by electrostatic interactions between the chitosan free amine groups from the surface of microparticles and the carboxylic or sulfate groups of the two anionic polysaccharides. Magnesium acetate participates in the ionic cross-linking of the carboxylate groups from gellan and sulfate groups from carrageenan, respectively. These groups are not involved in the chitosan complexation but contribute to the increased mechanical stability of the obtained ComPs. It has to be mentioned that the attempt to work with gellan/i-carrageenan mixtures in which the percentage of i-carrageenan exceeds 30% (w/w) led to the formation of unstable particles. Previous research reported in the literature has demonstrated that the potassium ions are efficient for i-carrageenan cross-linking. However, high potassium levels are not desirable in medical applications because they can cause severe arrhyth-mias and muscle weakness due to hyperkalemia [51].

The formed ComPs were spherical, stable, and had an average diameter of 3 mm in swollen state and 0.5–1.0 mm when dried. The schematic representation of curcumin-loaded ChPs in a gellan matrix, for example, is illustrated in Figure 2.

***Figure 2**. Schematic representation of the polyelectrolyte complex formed by interactions between amine groups of the curcumin-loaded ChPs with the carboxylic groups from gellan.*



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## 3.2. FT-IR SPECTRA OF THE PARTICLES

Figure 3(a) shows the FT-IR spectra for chitosan, curcumin, and curcumin-loaded ChPs, and Figure 3(b) shows the FT-IR spectra for ComPs, gellan, and carrageenan.

Figure 3(a and b) indicates that in the FT-IR spectrum of the curcumin-loaded ChPs are found the characteristic absorption bands of the components. Thus, broadband from 3420 $cm^{-1}$ could be attributed to the absorption of the \\OH groups from chitosan (Figure 3a) to the phenol groups from curcumin as well as to the —$NH_2$ groups of chitosan. The absorption peaks corresponding to the functional groups of curcumin were observed at much lower intensities such as 1627 $cm^{-1}$ (aromatic moiety C=C stretching vibration), and 1513 $cm^{-1}$ (—C—O— and —C=C— vibration band) indicating that the main curcumin peaks were retained when it was incorporated in the ChPs, and these results suggested that it was incorporated into the ChPs. It was also observed that the peak at 1541 $cm^{-1}$ of the chitosan was shifted in the curcumin-loaded ChPs spectrum at 1587 $cm^{-1}$ and can be attributed to the intermolecular interactions that could occur between components. Other absorption peaks characteristic of curcumin are slightly shifted from characteristic values, suggesting the fact that some interactions, like hydrogen bonds, occur between the phenolic groups of curcumin and amino groups of chitosan and prove that curcumin was immobilized in the polymer matrix.

In the spectra presented in Figure 3b, including the P5C sample, it was found a broad absorption band, between 3500 $cm^{-1}$ and 3600 $cm^{-1}$, specific for the alcoholic-OH groups (from polysaccharide) and phenolic groups (from curcumin). The peak at 1436 $cm^{-1}$ in the P5C sample spectrum is slightly shifted compared with the curcumin-specific absorption band (1428 $cm^{-1}$) and proves that the polyphenol is present in the composition of the particles. The intense band at around 1024 $cm^{-1}$ in the gellan spectrum, was assigned to the C—O—C stretching vibrations (from the glycosidic cycle). It was found that this band is slightly shifted in the carrageenan spectrum (1069 $cm^{-1}$) and the P5C particles spectrum at 1042 $cm^{-1}$, proving the fact that the P5C sample composition contains all the polysaccharides used (this absorption band was not observed in the curcumin spectrum). Finally, in the P5C sample spectrum, a specific band of the sulfate group from the carrageenan is slightly shifted from 846.1 $cm^{-1}$ to 875 $cm^{-1}$.

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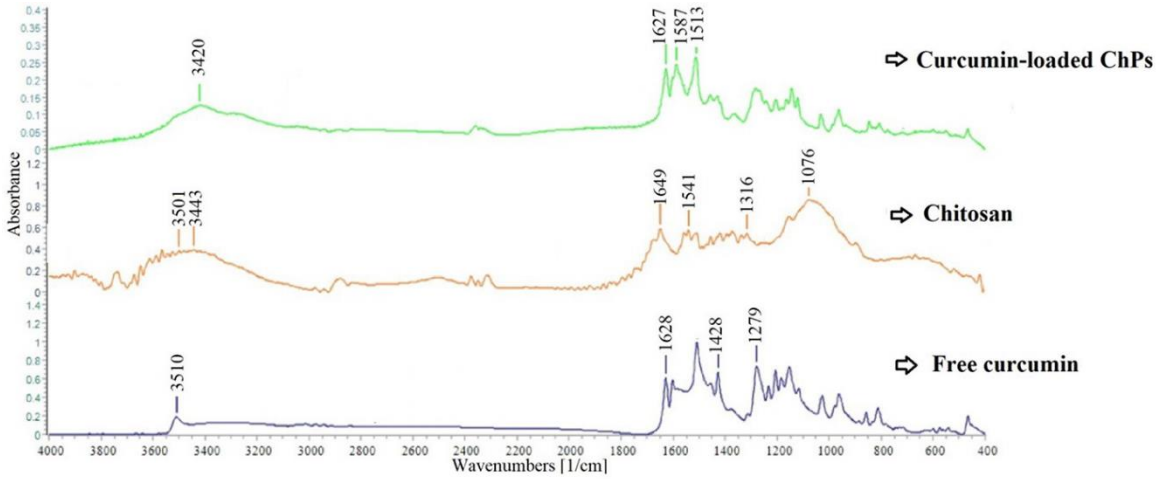
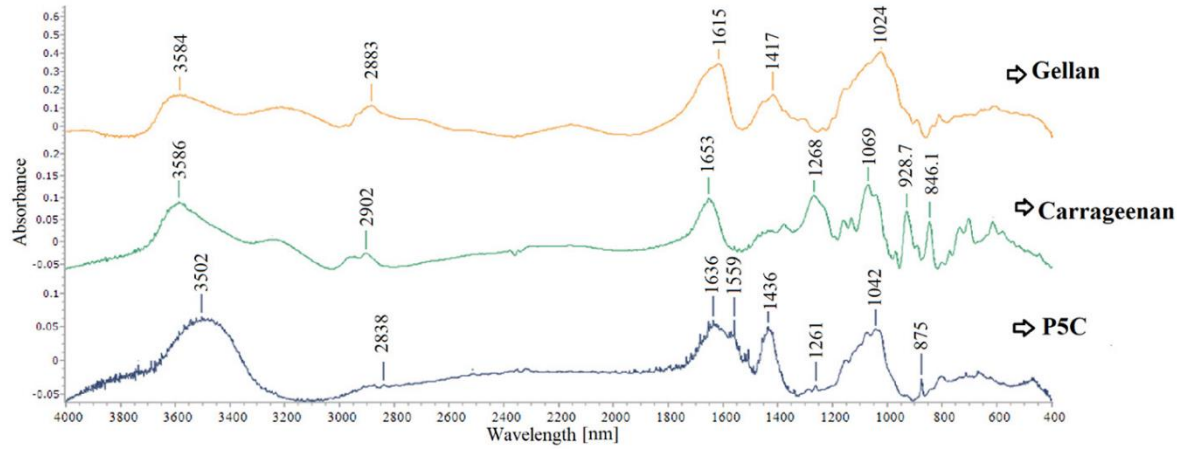
***Figure 3**. FTIR spectra for (a) ChPs containing curcumin, curcumin, chitosan, and (b) P5C ComPs, curcumin-loaded ChPs, carrageenan, and gellan.*




(a)




(b)

***Table 2.** The particle's mean diameter obtained after different durations of ultrasound treatment.*

| Nr. Crt. | Ultrasonication time (min) | Mean diameter (μm) | Standard deviation |
|---|---|---|---|
| 1 | 4 | 1.445 | 0.138 |
| 2 | 8 | 0.376 | 0.174 |
| 3 | 12 | 0.154 | 0.190 |

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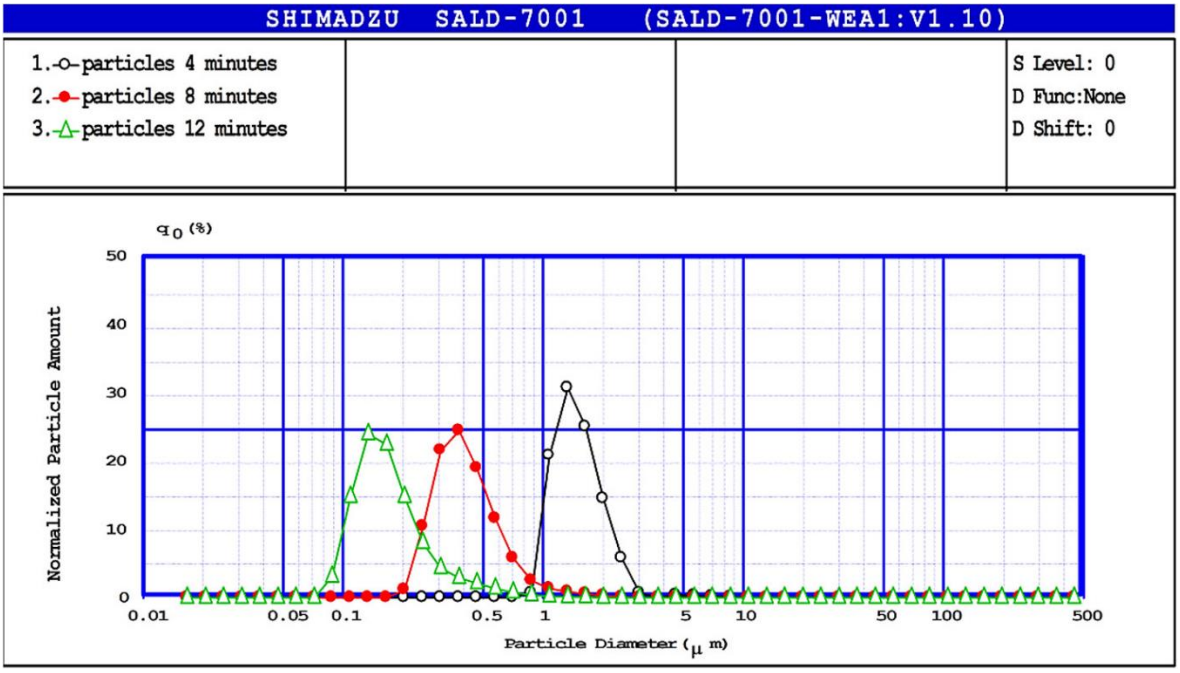
## 3.3. DETERMINATION OF THE PARTICLES MEAN DIAMETER BY LASER DIFFRACTOMETRY

The mean diameter and dimensional polydispersity for curcumin-loaded ChPs were determined by laser diffractometry. Three types of particles obtained under ultrasonication conditions at different durations were analyzed to evaluate the influence of the duration of the ultrasound treatment. An expected effect was observed, namely the decrease of the mean particle diameter with increasing the time of the ultrasound treatment, while the sample retains a quite polydisperse character. Therefore for the samples obtained after 4 min of ultrasonication treatment, the particle's mean diameter was around 1.45 µm; after 8 min of ultrasonication treatment, it was 0.38 µm, and after 12 min it was 0.15 µm. Table 2 presents the particle's mean diameter for three ultrasound treatment durations as well as the standard deviation (the analyze was performed in triplicate for all samples). In Figure 4, the dimensional polydispersity curves of the samples obtained after 4 min, 8 min, and 12 min of ultrasound treatment are illustrated. It was observed that as the time of the ultrasound treatment increases both the size of the nanoparticles and the dimensional polydispersity decreases, as expected.

***Figure 4**. Dimensional polydispersity curves of curcumin-loaded ChPs using different ultrasonication durations determined by laser diffractometry.*



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## 3.4. COMPLEX PARTICLES MORPHOLOGY

Figure 5 presents scanning electron microscopy photos performed for the P1C sample on the cross-section.

The morphology of the gellan or gellan/carrageenan ComPs containing curcumin-loaded ChPs was highlighted by scanning electron microscopy. The gellan matrix presents a fibrillar structure, specific to this polysaccharide. The curcumin-loaded ChPs included in the gellan matrix maintain their spherical shape, are dispersed in large number in the gellan matrix in which they were firmly anchored, and their size was of around 2–3 μm. The diameter was higher than the one of the same microparticles determined by laser diffractometry (1.445 μm). A possible explanation was that around them, an additional layer of gellan was deposited by polyelectrolytic complexation with the ChPs leading to this increase.

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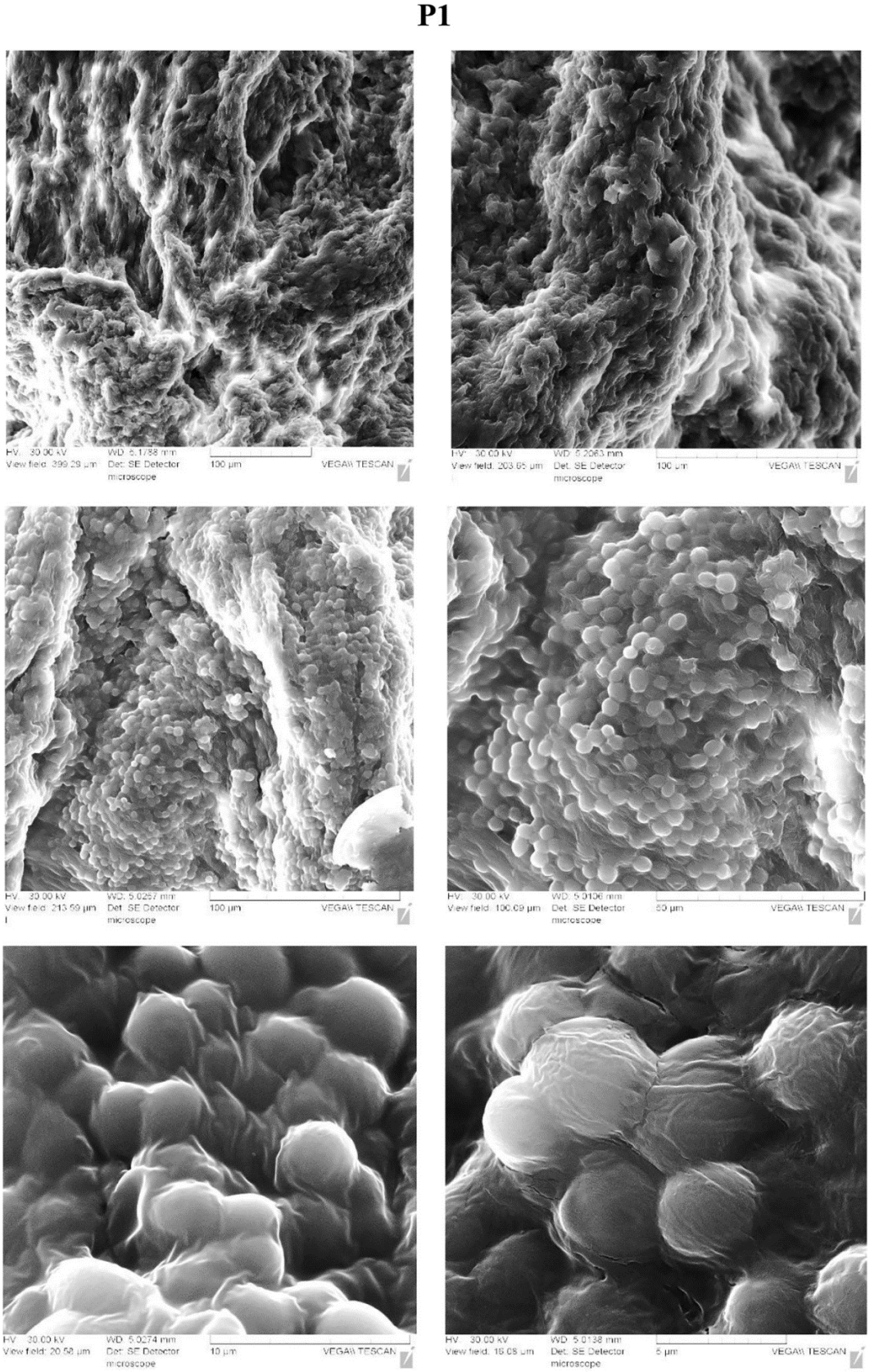
***Figure 5**. Scanning electron microscopy images in cross-section for the P1C sample (the ultrasound treatment for obtaining curcumin loaded ChPs was 4 min).*



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## 3.5. SWELLING BEHAVIOR IN AQUEOUS MEDIA

The swelling behavior of the ComPs in different aqueous media was evaluated by determining the evolution in time of the swelling degree (Q, %). The evaluation of this feature was necessary because the swelling degree determines the diffusion of the active principle from the particles and thus influences its release. A higher swelling degree value, induced by a lower crosslinking density and a higher porosity, will lead to a more pronounced diffusion of the active principle from the polymer matrix [52]. The swelling degree (Q), as a function of time, was determined gravimetrically for samples P1C, P2C, P3C, P4C, P5C, and P6C in PBS (pH = 7.4 and pH = 6.8) and in solution simulating intestinal gastric fluid (pH = 2). These results are illustrated in Figure 6.

As expected, the maximum swelling degree decreases in the order: P1C > P2C > P3C, an effect due to a higher cross-linking degree achieved by increasing the concentration of the magnesium acetate solution, therefore of the crosslinking ion. From Figure 6, it occurs that Q values were higher at pH = 7.4 than at pH = 6.8 or pH = 2 because the ComPs contain gellan in a predominant quantity (see Table 1). Therefore, the basic pH induces the formation of carboxylate anions from the acid groups that did not participate in the cross-linking with $Mg^{2+}$ ions, which leads to electrostatic repulsions between the polysaccharide chains and has an effect on the relaxation of the network facilitating the diffusion of higher amounts of water. El-Sherbiny and Smyth [53] reported similar conclusions for curcumin-loaded PLGA nanoparticles with chitosan-grafted-PEG or chitosan [53].

The pKa value of the carboxylic groups in gellan is about 3.5, whereas this value is close to 2.6 for the sulfate groups in carrageenan. At pH > pKa, the carboxylic groups are deprotonated, the hydrogen bonds are not formed, electrostatic repulsion occurs, and the hydrophilicity of the hydrogel increases, which leads to a higher swelling degree and an intensified release rate of the active principle [54]. The crosslinking degree for the P1C sample was lower than for P2C and P3C sam-ples, which means that many carboxylic groups in the gellan matrix were free. At pH = 2, the number of hydrogen bonds increased in the P1C sample, leading to lower adsorption of the swelling medium in the first 2–3 h. These intermolecular attraction forces predominantly have as consequence the polymer-polymer interactions and not medium-polymer interactions resulting in a smaller amount of medium absorbed by these hydrogel particles [55]. The temperature of37 °C can lead to improved absorption of the medium at pH = 2, and some of the hydrogen bonds formed may be cleaved as a result of molecular motions [56]. The protonation of the free amino groups from the surface of the chitosan particles leads to an increased swelling degree value after 2 h at pH = 2. Figure 6c shows that the swelling degree values for the P2C and P3C samples (with a higher cross-linking degree) were close but higher than the swelling degree values of the P1C sample in the first hours of the kinetic study, effect determined by the lower number of formed hydrogen bonds. The higher cross-linking degree and protonated carboxylic groups induce a slow diffusion of the solution at pH = 2 inside of the hydrogel particles in the case of P2C and P3C samples. Figure 6c shows that, at pH = 2, for the P3C sample, the swelling degree tends to reach the equilibrium after about 2 h, whereas this equilibrium is reached after about 5 h for the sample P3C. The literature indicates that the cleavage of hydrogen bonds takes place in time after a first-order kinetic, which could explain the non-specific

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kinetics of the swelling degree at pH = 2 for the P1C sample compared with P2C and P3C samples [56].

Curcumin incorporated in the ComPs is more soluble in alkaline solutions and could allow the adsorption ofa higher quantity of buffer solution at pH = 7.4. Even if at pH = 7.4 can uptake a considerable amount of solution, ComPs did not start to disintegrate, and their weight did not decrease probably because of the interactions between the two polymers (gellan or gellan mixed with i-carrageenan and chitosan) which were responsible for the formation of ComPs with sufficiently high mechanical resistance [57]. It can be observed that the values of Q% decrease at pH = 6.8 compared with the ones from pH = 7.4 because the electrostatic repulsions are lower but remain relatively high with values between 831.25% and 1646%.

At acidic pH, high levels of swelling could be expected due to the presence of chitosan, which will be protonated and will lead to electrostatic repulsions between the macromolecular chains, allowing thus the penetration of a higher amount of water. However, the ChPs having a small size and being strongly immobilized in the gellan or gellan/i-carrageenan matrix, respectively, cannot bring a high intake to the maximum swelling degree. Moreover, the lower swelling degree can be explained as a consequence of the hydrogen bonding between the carboxylic (or sulfate) groups and the —OH groups in the dominant polysaccharides [58]. The swelling degree value for sample P4C, P5C, P6C were higher than for sample P2C due to the presence of i-carrageenan in the polymer matrix, which gives it a higher porosity, and hence the diffusion of more significant amounts of water into the ComPs [53]. Even if the differences between the swelling degree values of the ComPs containing carrageenan are not substantial, there was a slight tendency of them to decrease with reducing the quantity of this polysaccharide in their composition (P6C < P5C < P4C), regardless of the pH value of the aqueous medium in which was determined.

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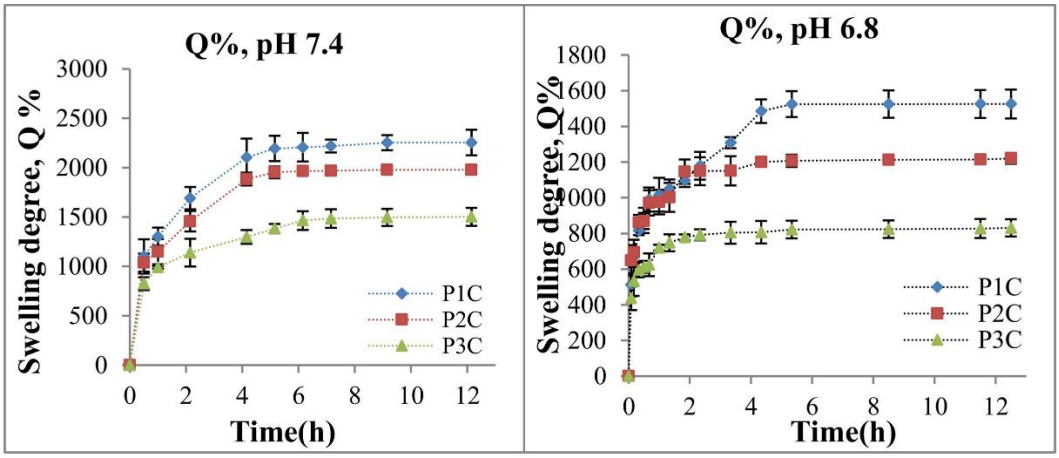
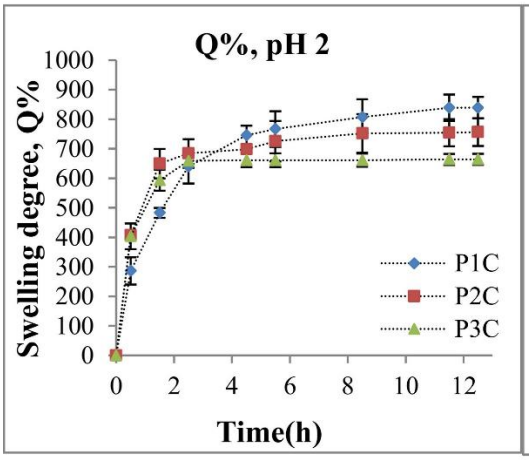
***Figure 6.*** *Time variation of the swelling degree for the P1C, P2C, P3C samples in (a) PBS atpH = 7.4, (b) PBS atpH = 6.8 (c) in solution which simulates the gastric fluid atpH 2 and for the P2C, P4C, P5C, P6C samples in (d) PBS at pH = 7.4, (e) PBS at pH = 6.8 (f) in solution which simulates the gastric fluid at pH = 2. Data are given as mean value ± confidence interval at 95%.*



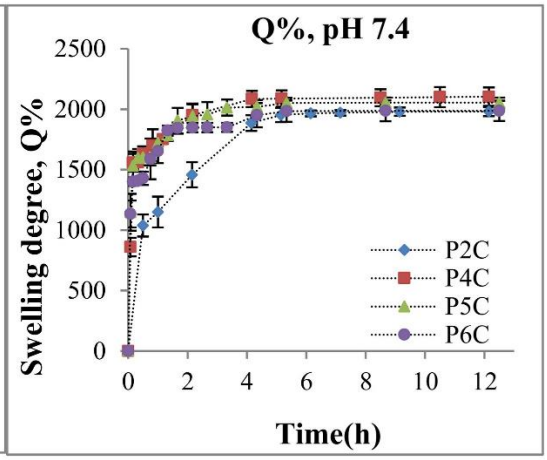

(a)



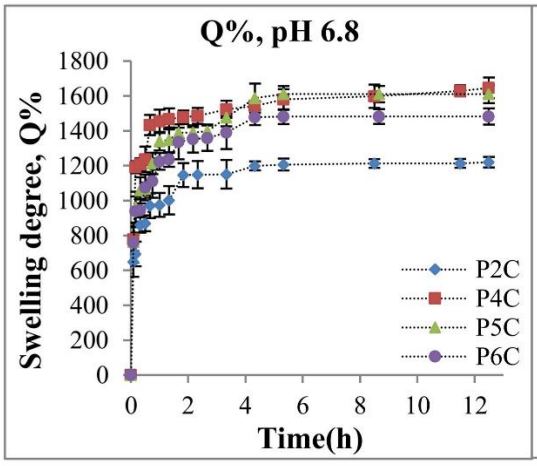

(b)



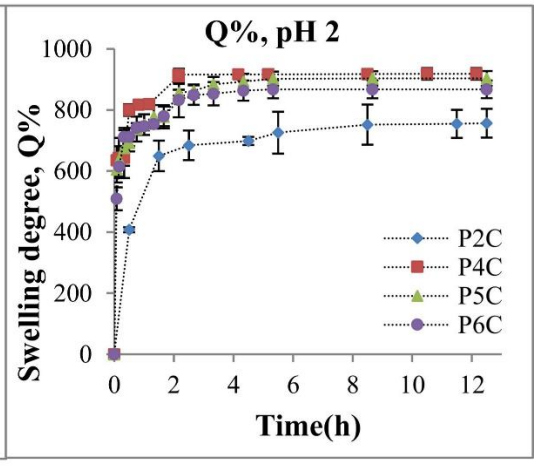

(c)




(d)




(e)




(f)

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## 3.6. IMMOBILIZATION EFFICIENCY

From Table 1, it appears that the curcumin encapsulation efficiency (EE %) was between 87.23% and 97.25%, for the ComPs with the polymeric matrix formed only of gellan. Moreover, it can be noted that EE % increases with decreasing the concentration of magnesium acetate, used as ionic cross-linker (P1C-P3C). Thus, it can assert that the immobilization efficiency of curcumin-loaded ChPs in the gellan matrix increases when the polymeric matrix has a lower cross-linking degree, and the network meshes are more flexible, larger, and a higher amount of curcumin-loaded ChPs can be included. With the increase of the cross-linking degree, the polymer network becomes denser, and the amount of curcumin-loaded ChPs incorporated in the polymer matrix was lower. For the P2C, P4C, P5C, and P6C samples with the same cross-linking degree but with different concentrations of i-carrageenan, it can be observed that the immobilization efficiency in almost all the cases decreases when the quantity of i-carrageenan increases.

Compared to gellan, carrageenan has two sulfate groups capable of ionic gelation, at each structural unit, and gellan only one. As a result, the network formed in the presence of carrageenan may be denser, especially as a result of reducing the amount of gellan. It is expected, therefore, that the encapsulation efficiency of curcumin (in the ChPs) will increase with decreasing the amount of carrageenan. The curcumin immobilization efficiency for the P4C, P5C samples with 30%, and 20% i-carrageenan in composition was lower. According to the ComPs preparation method, after ionotropic gelation of the gellan/carrageenan matrix with curcumin-loaded ChPs, they were stored for 3 h in the gelling medium (magnesium acetate solution) for maturation. During this period, some of the immobilized curcumin may diffuse into the aqueous magnesium acetate solution, and the diffusion was more intense as the porosity of the ComPs was higher; this morphological characteristic of the ComPs being correlated with the amount of carrageenan in their composition. The porosity increases with the amount of this polysaccharide in the matrix. Even if the gellan/carrageenan matrix was denser, the diffusion of curcumin was more intense due to the higher porosity. In our previous research studies, it was demonstrated that i-carrageenan induces to the polymeric matrix a higher porosity, and the diffusion of the bioactive compound immobilized depends on this [59].

## 3.7. CURCUMIN RELEASE KINETICS

Curcumin release kinetics were studied at physiological temperature (T = 37 °C), in three different pH media: PBS (pH = 7.4 and pH = 6.8) and in solution simulating the gastric fluid at pH = 2. The curcumin release curves from the analyzed samples and the influence of different factors on release efficiency are presented in Figures 7, 8, and 9.

Figure 7 shows the influence of pH and cross-linking degree on the curcumin release kinetics in the analyzed samples. Figure 8 shows the influence of pH and porosity on curcumin release kinetics from the analyzed samples. Figure 9 shows the influence of curcumin-loaded micro/ nanoparticle size and pH on curcumin released kinetics in the analyzed samples.

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From Figures 7, 8 and 9 it appears that the maximum amount of curcumin released, independent of the particles type (i.e., release efficiency as given in Table 3), was higher in the slightly alkaline medium (pH = 7.4) than at acidic pH (pH = 2) and the values of release efficiency at pH = 6.8 was between these two. This effect correlates very well with the swelling degree values of the analyzed ComPs, for mediums with different pH values. Obviously, at pH values in a slightly alkaline domain, where the swelling was maximal (effect explained above), the maximum amount of curcumin released was higher compared to the one released in more acidic mediums, facilitating the diffusion of curcumin from the ComPs.

In the case of P1C, P2C, P3C samples, presented in Figure 7, the curcumin release occurred at a higher rate up to 1440 min after which the released amount begins to drop to an equilibrium point at about 9360 min, remaining constant after that at pH = 7.4, pH = 6.8 and pH = 2. Moreover, for all pH values, the release efficiency for the P1C, P2C, and P3C samples, with gellan ComPs containing the curcumin-loaded ChPs, increases with decreasing the cross-linking degree. The same behavior is also observed in Figure 8 for P4C, P5C, and P6C samples at pH = 6.8 and pH = 7.4. The higher release efficiency in the alkaline medium was the consequence of a more intense swelling of the polymer matrix, which allows the network meshes to increase their size, and therefore the diffusion of the bioactive principle was intensified.

The curcumin diffusion from P2C, P4C, P5C, and P6C particles (Figure 8) depends on the i-carrageenan amount within the ComPs, therefore of the porosity of the ComPs. At pH = 2 after 5040 min, the curcumin release kinetic has a different behavior compared to the curcumin released kinetics at pH = 6.8 or pH = 7.4. At pH = 2, after 6420 min for all analyzed samples, curcumin release efficiency from ComPs was almost similar for all the samples. After 9360 min, it was observed that the curcumin release efficiency was maximum for P2C samples, and it decreased with increasing of the carrageenan concentration in the ComPs so that the lowest release efficacy value was observed for the P4C particles. Increasing the percent of i-carrageenan in the composi-tion of the ComPs has the effect of increasing the cross-linking degree (see previous explanation); therefore, the intensification of the curcumin release from the more cross-linked matrices should proceed slower. Carrageenan, however, induces a higher porosity in the polymeric matrix in which the ChPs containing curcumin were immobilized, which has the effect of intensifying the diffusion of the active principle through the pores. Between the two opposite effects induced by carrageenan, it turns out that the one due to the increase of the porosity was dominant, which explains the obtained results.

On the other hand, carrageenans are susceptible to depolymerization by acid hydrolysis. At high temperatures and low pH, this can quickly lead to the complete loss of functionality [57]. Previous carrageenan degradation studies have shown that only 0.1% of glycosidic linkages are damaged after kappa-carrageenan samples have been maintained for 3 h at pH = 1.2 and 37 °C [46,60].

From Figure 9, it was observed that the curcumin release efficiency depends on the size of the ChPs containing curcumin incorporated in the gellan or gellan/carrageenan matrix; their size decreases with increasing the ultrasonication duration. For this analysis, the P4C sample was used in which the ChPs obtained after different ultrasonication times of 4 min, 8 min, and 12 min were incorporated.

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The curcumin release efficiency increases with pH increasing and with a decreasing of the particle size. Thus, for P4C ComPs containing ChPs with immobilized curcumin with a size of approximately 150 nm (ultrasonicated for 12 min), a maximum curcumin release efficiency was obtained independent of the pH of the medium used. The effect was due to the growth of the specific surface of the ChPs containing curcumin, with the increase of the ultrasound treatment duration. Their contact surface with the release medium increases with decreasing the diameter, facilitating the release of an increasing quantity of active principle. More details concerning the curcumin transport and release mechanism in the polymer matrix can be obtained by using the Ritger-Peppas kinetic model [61]:

$$\frac{M_t}{M_\infty} = k1 \times t^n \quad (3)$$

The values of the exponential factor n are listed in Table 3.

From Table 3, it appears that for almost all ComPs samples, from which the curcumin has been released, the exponential diffusion factor n exhibit values equal or very close to 0.5, indicating a Fickian-type diffusion. Furthermore, the amount of active ingredient released is directly proportional to the time until a release efficiency of around 60%, of the total released drug, was reached, percent found ideal in the mathematical models used to describe the controlled release drug delivery systems [62,63].

The success ofa colon-specific drug delivery system depends on the physicochemical properties of the drug, the type of administration system, all other factors that may influence the gastrointestinal transit time, and the degree of interaction between the drug and the digestive tract [64,65]. Also, the oral delivery system should protect the drug against release into the stomach and the small intestine [64]. The colon can also be a potential target for the systemic absorption of several medications used for the treatment of non-colon disorders [64].

Preclinical and clinical data on oral administration of curcumin revealed its low systemic bioavailability and increased susceptibility to metabolic activity, and previous research has shown that only 2.30 ± 0.26 μg/ml curcumin was found in plasma after oral administration of 10 g of curcumin. Curcumin undergoes extensive metabolic changes in the intestine and liver, which prevent systemic use of curcumin to treat various diseases. A variety of materials such as natural or synthetic polymers and lipids have been used to prepare delivery systems that effectively cross the gastrointestinal epithelium, and thus curcumin can reach the colon and systemic circulation with the recommended therapeutic doses [66].

The purpose of curcumin encapsulation in polysaccharides-based particles was, therefore, to facilitate the oral administration of curcumin, ensuring that the stomach barrier is overcome and, consequently, the presence of curcumin to a large extent in the colon. Gellan was chosen as the predominantly quantitative polymer for the preparation of these ComPs due to its stability in the strongly acidic environment of the stomach and its resistance to enzymes in the gastrointestinal tract. Due to the generally short time of food storage in the stomach (emptying the stomach takes 2–6 h) and in the small intestine (3–5 h), it is ensured that these curcumin-loaded ComPs will pass into the colon.

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Upon their passage through the stomach and small intestine, the release of a relatively low quantity of curcumin is possible, as demonstrated by the above described kinetic release studies. It was interesting to study the release of curcumin from three types of ComPs by sequentially placing them first in a solution at pH = 2 that simulates the stomach medium, then in PBS solution at pH = 6.8 that simulates the intestinal fluid and then in PBS at pH = 7.4 that simulates the colon environment. For release in an acidic environment, the duration was set at 2 h (comparable to that in the stomach). Then, the ComPs were placed immediately in a release medium with pH = 6.8 for 3 h because this is the time in which 50% of all food passes through the small intestine and then the ComPs were placed in a solution at pH = 7.4 for 4 h (residence time in the colon ranges from 4 to 72 h). The kinetic curves obtained are shown in Figure 10, which illustrates a different behavior for the P2C, P3C, and P4C particles. In fact, after removing the ComPs from the acidic and intestinal environment, and placing them in a slightly alkaline medium, the curcumin release was practically intensified.

The total amount of curcumin released after around 10 h, in the two successive media, exceeds the maximum amount of curcumin released from the same three particle types, also after 10 h, either in acidic medium, intestinal medium or slightly alkaline medium only (see Figures 7, 8, 9).

It should also be noted that after approximately 10 h during which the ComPs remain in the digestive tract, the release of curcumin from the P4C ComPs was practically complete (over 60% of curcumin could be released into the colon). This result confirms our starting hypothesis and demonstrates the protective role of the polymeric matrix on curcumin in overcoming the stomach barrier; therefore, these curcumin-loaded formulations can be suitable for the delivery of this active principle.

Other results regarding the protective role of the polysaccharides matrix nature on the stability of curcumin in the action of degrading factors (light, pH, complexation, etc.) will be reported in a subsequent paper.

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***Figure 7.*** *Curcumin release kinetics (in terms of efficiency) from ComPs based on gellan as a function of time for P1C, P2C, P3C samples: in PBS atpH = 7.4 (a) in PBS atpH 6.8 (b) and at pH = 2 (c).*

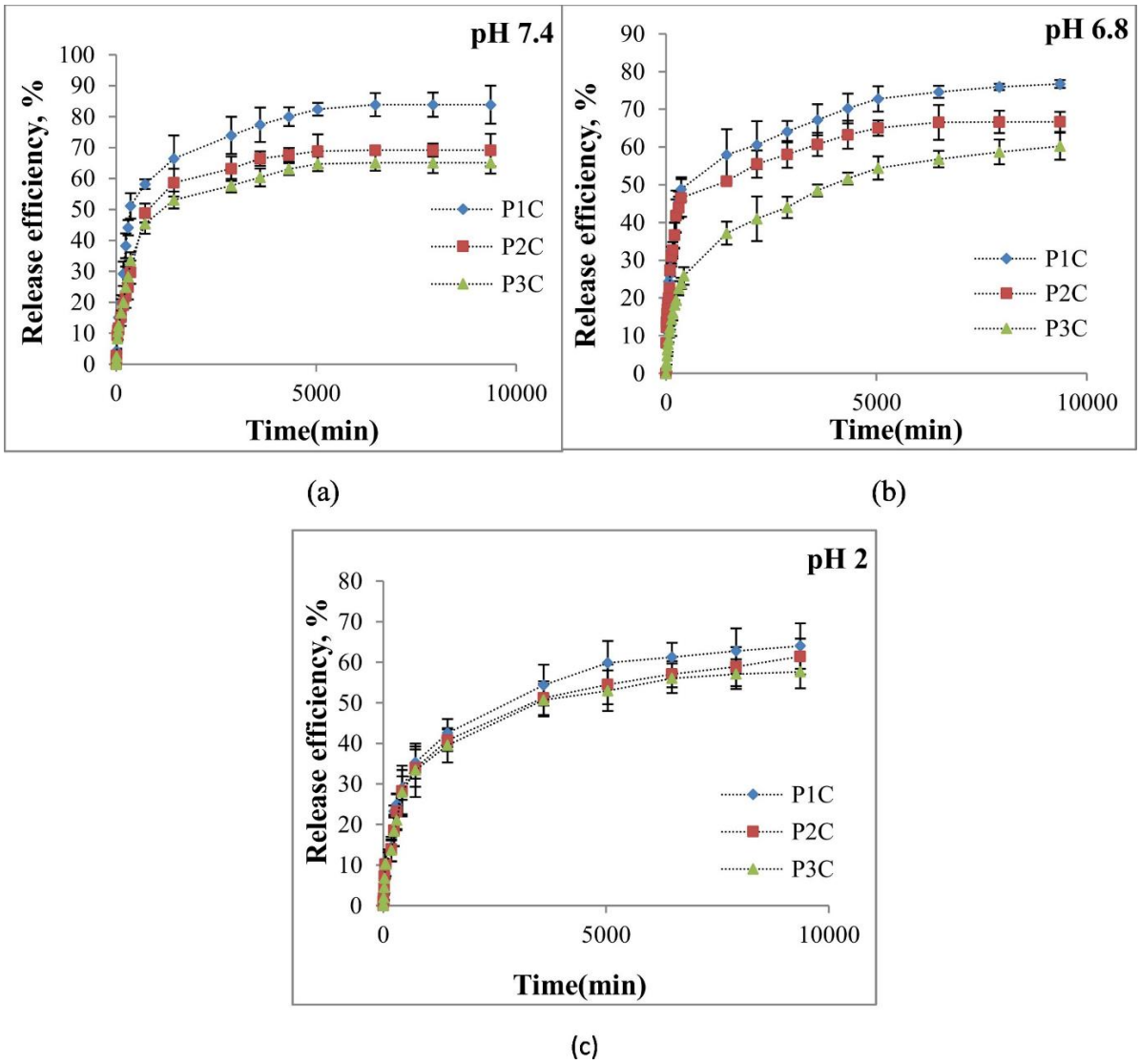

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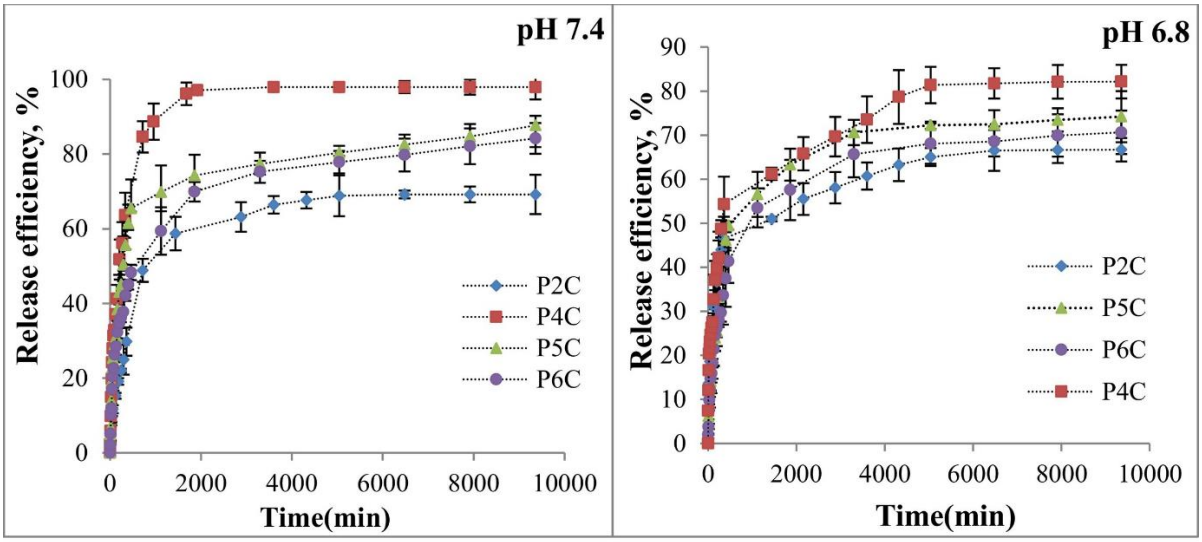
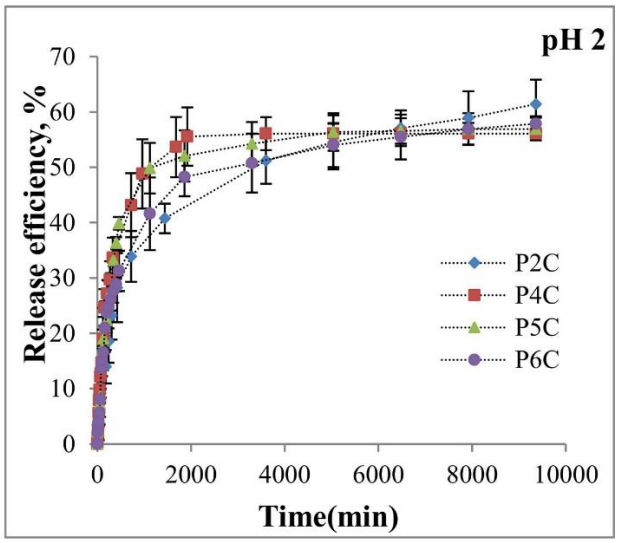
***Figure 8**. Curcumin release kinetics (in terms of efficiency) from polysaccharides ComPs as a function of time for P2C, P4C, P5C and P6C samples: in PBS atpH = 7.4 (a) in PBS atpH 6.8 (b) and at pH = 2 (c). Data are given as mean value ± confidence interval at 95%.*




(a) (b)




(c)

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***Figure 9**. Curcumin release kinetics (in terms of efficiency) from P4C polysaccharides ComPs which contains curcumin-loaded ChPs obtained at different durations of ultrasonication as a function of time: in PBS at pH = 7.4 (a) in PBS at pH 6.8 (b) and pH = 2 (c). Data are given as mean value ± confidence interval at 95%.*

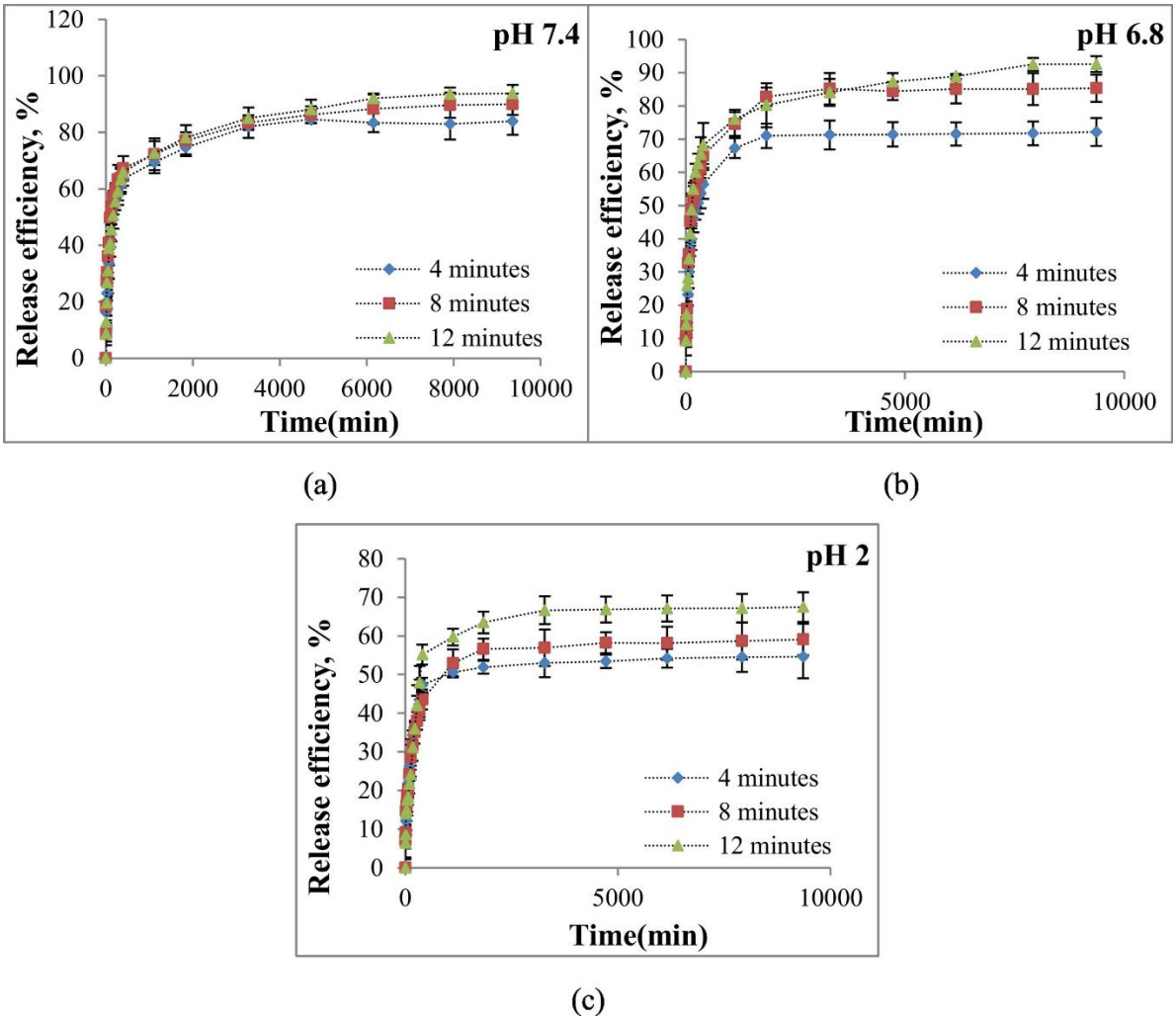


***Table 3.** The release efficiency and the values of the exponential factor n from the Ritger-Peppas kinetic model.*

| Sample | Release efficiency (%) | | | Release time (min) | Exponential factor, n | | | $R^2$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH = 7.4 | pH = 6.8 | pH = 2 | | pH = 7.4 | pH = 6.8 | pH = 2 | pH = 7.4 | pH = 6.8 | pH = 2 |
| P1 | 83.87 | 76.73 | 63.99 | 9360 | 0.49 | 0.43 | 0.52 | 0.93 | 0.99 | 0.96 |
| P2 | 69.18 | 66.69 | 61.41 | | 0.51 | 0.4 | 0.49 | 0.95 | 0.98 | 0.92 |
| P3 | 65.1 | 60.22 | 57.73 | | 0.54 | 0.53 | 0.52 | 0.89 | 0.94 | 0.94 |
| P4 | 97.94 | 82.15 | 56.08 | | 0.5 | 0.41 | 0.53 | 0.94 | 0.97 | 0.81 |
| P5 | 87.67 | 74.2 | 56.91 | | 0.51 | 0.51 | 0.5 | 0.96 | 0.98 | 0.85 |
| P6 | 84.19 | 70.64 | 57.87 | | 0.51 | 0.49 | 0.52 | 0.91 | 0.93 | 0.92 |

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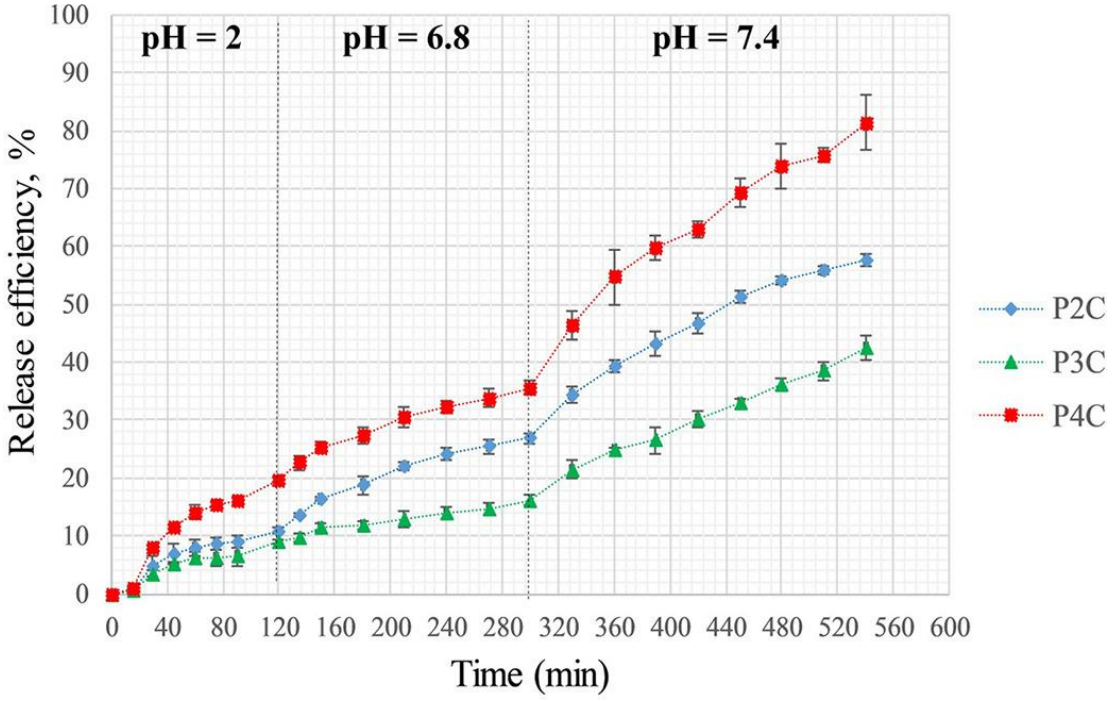
***Figure 10.*** *Kinetics curves for the curcumin release from P2C, P3C, and P4C particles respectively in the acidic medium at pH = 2 (2 h), in PBS at pH = 6.8 (3 h) and then in the slightly alkaline medium in PBS at pH = 7.4 (4 h). Data are given as mean value ± confidence interval at 95%.*




## 4. Conclusions

Curcumin-loaded spherical polysaccharide-based ComPs were obtained using a method based on the polyelectrolyte complexation of the constituent polysaccharides and ionotropic gelation in the presence of divalent metal ions.

Curcumin encapsulation efficiency was higher than 85.7% and increased when the cross-linking density or the amount of i-carrageenan decreases. Moreover, the swelling degree and the amount of curcumin released from the ComPs increase when the cross-linking degree decreases or the concentration of i-carrageenan from the ComPs increases. The presence of i-carrageenan in the composition of the polymer matrix results in a different morphology, characterized by increased porosity, which leads to an intensification of the diffusion process. Independent of the ComPs type, curcumin was released according to specific kinetic profiles, in pH = 2, pH = 6.8 and pH = 7.4 mediums, with higher efficiency in the latter.

Finally, it can be admitted that these polysaccharides, used to obtain these carrier particles, have a protective role on curcumin, allowing it to overcome the gastric barrier and to be absorbed more efficiently into the colon.

Supplementary data to this article can be found online at
https://doi. org/10.1016/j.ijbiomac.2019.12.247

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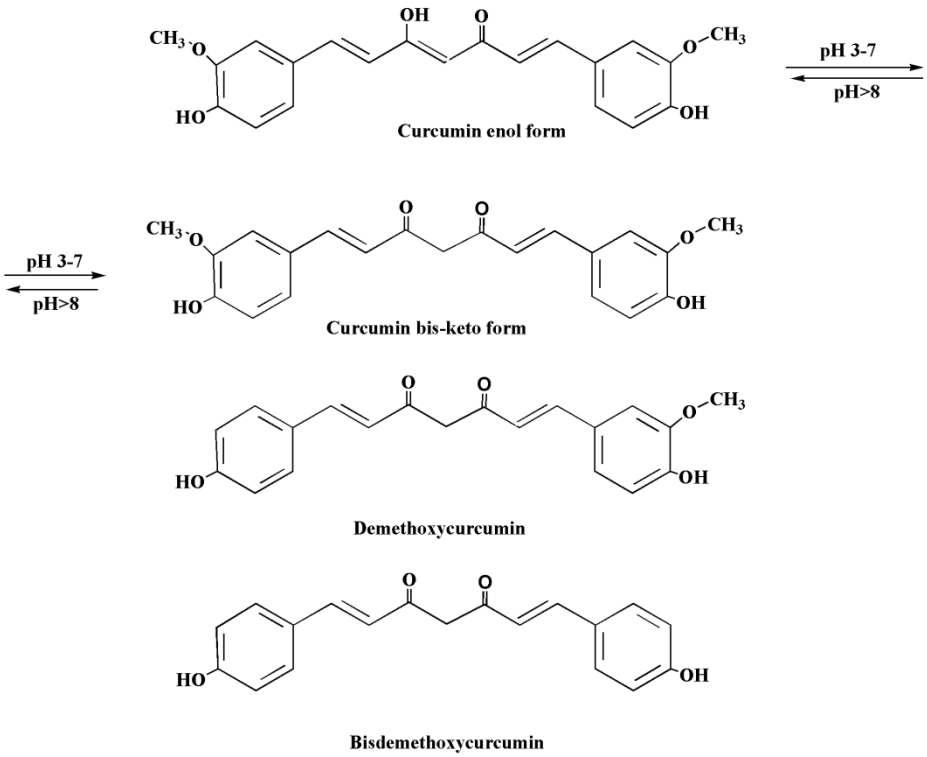
Figure S1: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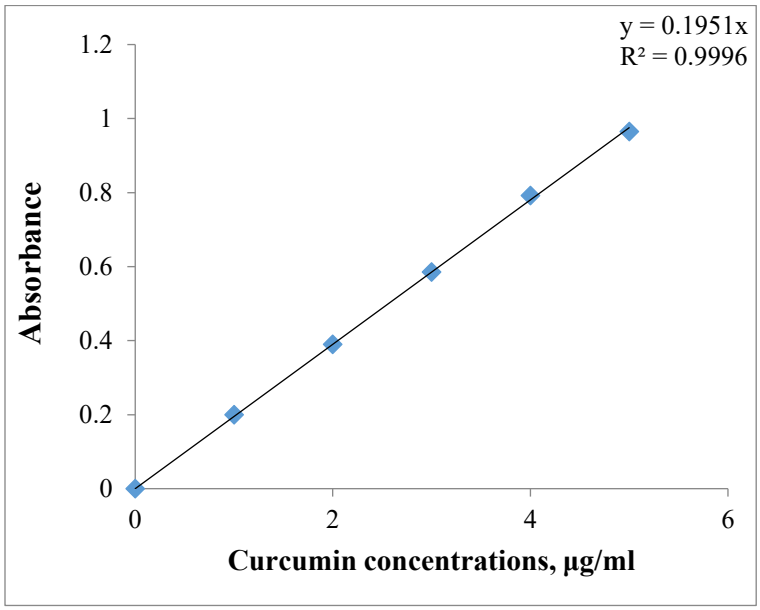
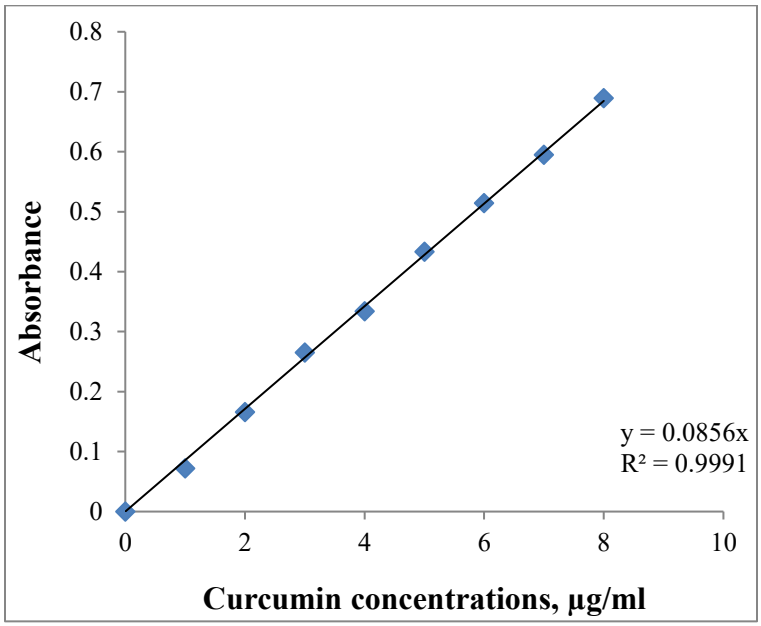
Curcumin enol form
pH 3-7
pH>8
Curcumin bis-keto form
Demethoxycurcumin
Bisdemethoxycurcumin

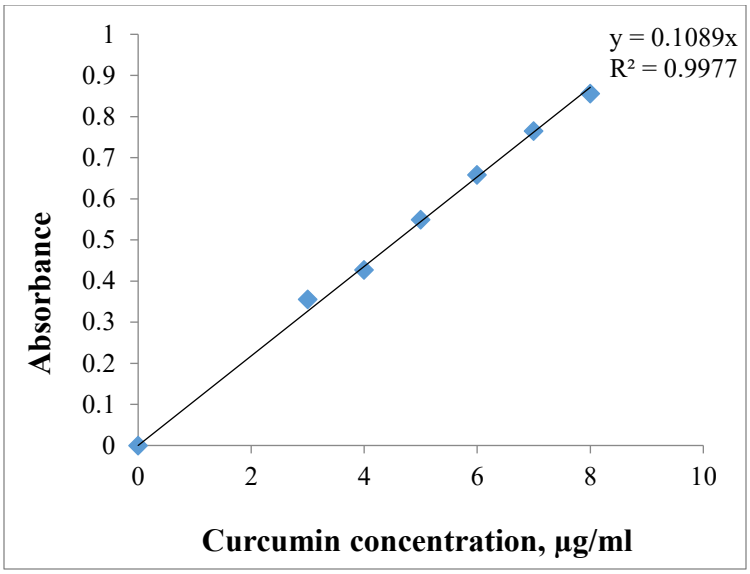
Figure S 2:


y = 0.1951x
R² = 0.9996
Absorbance
Curcumin concentrations, µg/ml
0
0.2
0.4
0.6
0.8
1
1.2
0
2
4
6

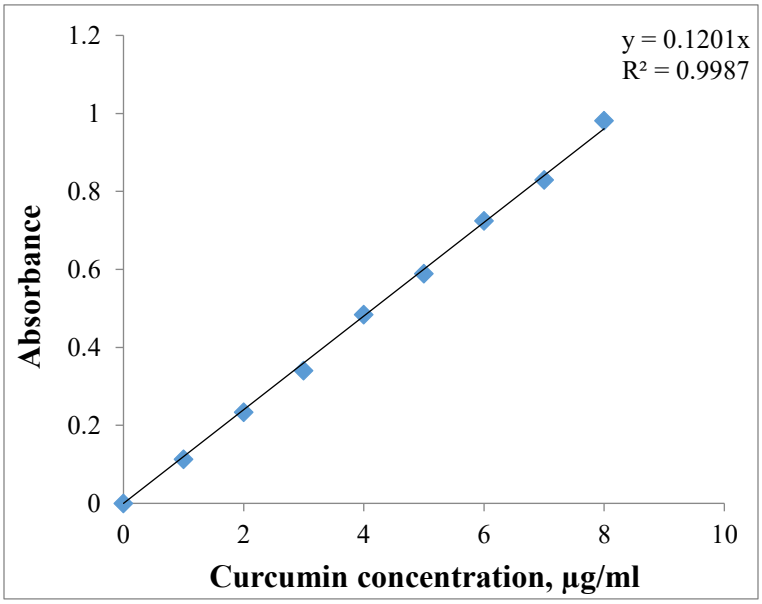
(a)


y = 0.0856x
R² = 0.9991
Absorbance
Curcumin concentrations, µg/ml
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10

(b)


y = 0.1089x
R² = 0.9977
Absorbance
Curcumin concentration, µg/ml
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
2
4
6
8
10

(c)


y = 0.1201x
R² = 0.9987
Absorbance
Curcumin concentration, µg/ml
0
0.2
0.4
0.6
0.8
1
1.2
0
2
4
6
8
10

(d)