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Loading natural emulsions with nutraceuticals using the pH-driven method: Formation & stability of curcumin-loaded soybean oils bodies

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Abstract

Previous studies have shown that the pH-driven method can be used to load curcumin into a variety of colloidal particles, including micelles, liposomes, lipid droplets, and oil bodies. This method is based on the increase in hydrophobicity and a corresponding decrease in water-solubility of curcumin when the pH changes from highly alkaline to acidic. In this study, we examined the physical and chemical stability of curcumin-loaded soybean oil bodies prepared using the pH-driven method. First, the impact of pH (from 6.5 to 8) on the stability of curcumin-loaded soymilk during storage was investigated at 4 ºC for 36 days. At this low storage temperature, more than 85% of the alkaline-sensitive curcumin was retained at all three pH values, without any evidence of color fading. The impact of holding temperature (4, 20, 37, and 55 ºC) on the physicochemical stability of the curcumin-loaded soymilks was then measured during storage at pH 7 for 14 days. At 4 and 20 ºC, the emulsions remained physically stable, most of the curcumin (> 90%) was retained, and there was no evidence of color fading. At the higher temperatures, however, the rate of curcumin degradation increased. For instance, around 30% and 70% of curcumin was lost when the soymilks were stored at 37 and 55 ºC, respectively. On the other hand, the soymilks remained physically stable throughout this period. This study provides valuable information about the loading of curcumin into pre-existing plant-based milks and creamers, which may be useful for developing a new category of functional foods and beverages.

Keywords: pH shift; soymilk; plant-based foods; curcumin; nutraceuticals

Introduction

Recently, there has been increasing interest in replacing animal-based and artificial ingredients in foods and beverages with plant-based alternatives [1]. This movement has largely been driven by the perceived benefits of plant-based foods on the health of both humans and the environment [2-4]. For this reason, the food industry is reformulating many of its existing products, as well as developing innovative new products, using plant-based components. One category of product that has been particularly successful is plant-based milks and creams, such as those based on soybean, cashew, coconut, almond, hemp, and oat [5]. These products are often designed to have a similar appearance, texture, and flavor as their dairy-based counterparts.

Many plant-based milks and creams are formed by breaking down plant structures to release oil storage bodies, such as soybeans, almonds or cashews [5]. These oil bodies are a natural form of colloidal particle that consists of a triglyceride core surrounded by a layer of phospholipids and proteins [6, 7]. These oil bodies contribute many of the desirable physicochemical attributes to plant-based milks, including their opacity, mouthfeel, and flavor profile. Like other types of colloidal particles, the aggregation stability of oil bodies is determined by the nature of the attractive and repulsive interactions acting between them, which depends on their surface chemistry and the prevailing environmental conditions, such as pH, ionic strength, and temperature [8, 9]. The formulation of stable plant-based milks and creams, therefore, depends on understanding the nature of the colloidal interactions acting between the oil bodies. In principle, the hydrophobic core of oil bodies can be used to solubilize and transport non-polar bioactive agents, such as oil-soluble vitamins and nutraceuticals, which could be used to fortify plant-based milks and creams with health promoting-components. The challenge, however, is to load the hydrophobic bioactives into pre-existing oil bodies.

Previous researchers have loaded curcumin into artificial seed oil bodies formed by homogenization of an oil and aqueous phase together in the presence of seed proteins and phospholipids [10]. In the present study, however, we focused on loading curcumin into the natural oil bodies present in soymilk. Our method, therefore, has the advantage that it leaves the oil bodies in natural plant-based sources intact. Curcumin is the main bioactive component found in turmeric, which is used as a coloring and flavoring agent in foods, as well as a nutraceutical [11-13]. Previous studies have shown that curcumin can be loaded into pre-existing colloidal particles, such as lipid droplets, using a variety of strategies. Crystalline curcumin in powdered form has been mixed with an oil-in-water emulsion and then the resulting mixture has been heated, which causes the curcumin to dissolve and move into the hydrophobic interior of the lipid droplets [14, 15]. However, holding the emulsions at elevated temperatures can promote droplet aggregation and curcumin degradation. Alternatively, curcumin can be dissolved within an organic solvent (such as ethanol) and then mixed with pre-existing colloidal particles, which should also cause the curcumin to move into the particles. But the utilization of organic solvents in the fabrication process is often undesirable because of the additional costs associated with removing them, as well as their potential to damage the environment [16, 17].

Recently, a simple, inexpensive, organic solvent-free method has been developed to load curcumin into various types of colloidal delivery system, including micelles, liposomes, nanoemulsions, and protein nanoparticles [18-22]. This pH-driven method is based on changes in the hydrophobicity, and therefore water-solubility, of curcumin when the pH is changed. At relatively low pH values (< pH 8), curcumin is a neutral non-polar molecule with a high oil-water partition coefficient and low water-solubility [23]. As the pH is raised above this value, a number of the hydroxyl groups on curcumin become progressively deprotonated, resulting in an

increase in negative charge, increase in hydrophilicity, decrease in oil-water partition coefficient, and rise in water-solubility. This phenomenon can be used to load pre-existing colloidal particles using a two-step process: (i) curcumin crystals are dissolved in a strongly alkaline solution; (ii) this solution is mixed with an acidified colloidal suspension. The final pH of the mixed system is around neutral or less, which causes the curcumin in the aqueous phase to become non-polar and move into the hydrophobic interior of the colloidal particles.

The objective of the current study was, therefore, to establish whether the pH-driven method could be used to successfully load curcumin into the soybean oil bodies within a commercial soymilk product. Moreover, we examined the impact of environmental stresses, such as storage pH and temperature, on the physical and chemical study of the curcumin-loaded soymilks. Furthermore, the gastrointestinal fate of the soymilk before and after storage was also investigated using an *in-vitro* digestion model. The results of this study should aid the design and formulation of more efficacious nutraceutical-enriched functional foods and beverages.

Materials & Methods

Materials

A commercial soy creamer, which contained 10% w/v fat, was purchased from a local supermarket (Silk, Whitewave Foods, Broomfield, Colorado). Curcumin powder (C2302, purity 95%) was produced by TCI Chemicals (Portland, OR). Sodium Hydroxide (SS266), hydrochloric acid (83.3 mM) and dimethyl sulfoxide (BP231) were obtained from Fisher Chemicals (Fair Lawn, NJ). Nile Red (N3013), fluorescein isothiocyanate isomer I (FITC, F1250), and sodium azide (S2002) were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO).

The *Nutrition Facts* label on the soymilk used in this study reported that the product had the following attributes: serving size (15 mL); calories per serving, 20 kCal; total fat, 1.5 g; total

carbohydrate, 2 g (1g sugar); and, protein, 0 g. The ingredients reported on the product label were: soymilk (filtered water and soybean), cane sugar, palm oil maltodextrin, contains 2% or less of: soy lecithin, natural flavor, tapioca starch, locust bean gum, and dipotassium phosphate. It should be noted that soybeans do contain some protein, which was presumably below the threshold required to appear on the nutrition facts label. This was important because it meant that their location could be determined using confocal fluorescence microscopy (see later).

Preparation protocol

Blank commercial soymilk

Initially, the effect of pH on the physiochemical properties of curcumin-free soymilks was examined by adjusting the pH from 2 to 10 using acid and base solutions. The particle characteristics of the soymilks were then measured using the methods described later.

Curcumin-loaded soymilk

The effect of particle characteristics on the properties of curcumin-loaded soymilks prepared using the pH-driven method was then examined. A stock curcumin solution (10 mg/g) was prepared by dissolving a weighed amount of curcumin powder into a sodium hydroxide solution (0.2 N NaOH). The alkaline curcumin stock solution was then added to a commercial soymilk (1:10 w/w) and the final system was rapidly adjusted to pH 6.5, 7.0 or 8.0 using a hydrochloric acid solution. The curcumin-loaded soymilks were then diluted with double distilled water to produce a final system containing 5 % oil, then stirred for 10 min at room temperature to ensure homogeneity. Finally, sodium azide (0.02%), which is a non-food grade preservative, was added to the system to inhibit any microbial growth. It should be noted that the pH-shift method would lead to the formation of some NaCl in the final samples due to the use of NaOH to create an alkaline solution and then addition of HCl to neutralize it. Based on the initial NaOH

concentration (0.2 M) and the dilution factor (1:10), we would expect the NaCl level in the final samples to be around 20 mM.

Storage study

The effect of pH on the chemical stability of curcumin within the soymilk was investigated when it was stored at 4 ºC for 36 days. Curcumin-loaded soymilks with three different pH values were prepared (pH 6.5, 7, and 8), poured into a series of glass test tubes (10 mL), and then sealed with a cap and parafilm to avoid evaporation and contamination. All samples were then incubated in the dark at 4 ºC to avoid photodegradation. Sealed samples were then taken and analyzed for each measurement throughout the storage period.

The impact of storage temperature (4, 20, 37 and 55 ºC) on the stability of the curcumin-loaded soymilk was then investigated at pH 7. A known volume of curcumin-loaded soymilk (10 mL) was placed within the glass test tubes and then stored in the dark. Again, samples were selected periodically for analysis to determine the impact of storage temperature on product stability.

Optical properties

The appearance and optical properties of the soymilks were determined using a digital camera and instrumental colorimeter (ColorFlex EZ 45/0-LAV, Hunter Associates Laboratory Inc., Virginia, USA), respectively. The colorimeter represented the optical properties of the samples using tristimulus color coordinates: L^*, a^* and b^* values. Here, L^* represents lightness (0) to darkness (100); a^* represents red (+) to green (-); and, b^* represents yellow (+) to blue (-). Samples (10 mL) were poured into a Petri dish and then illuminated using D65-artificial daylight using a 10^o standard angle and a black background.

Particle characterization

The particle size distribution (PSD) and mean particle diameter (D_{32}) of each sample was measured using a laser diffraction particle size analyzer (Mastersizer 2000; Malvern Instruments, Worcestershire, UK). The electrical characteristics (ζ-potential) of the particles in each sample were determined by measuring their electrophoretic mobility using a light scattering device (Nano-ZS, Malvern Instruments, Worcestershire, UK). Each sample was diluted with double distilled water with the same pH as their aqueous phase prior to measurement.

Microstructure analysis

A confocal scanning fluorescence microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) was used to investigate the microstructure of the samples. Each sample was stained with Nile red (1 mg/ mL ethanol) and FITC (1 mg/mL DMSO) to highlight the lipid (stained red) and protein (stained green) regions, respectively. All images were acquired using 200× magnification (20× objective lens and 10× eyepiece lens).

In-vitro study

A simulated gastrointestinal tract (GIT) was used to measure the bioaccessibility of the curcumin in the soymilks (pH 7) both before and after storage. This GIT model is based on one previously developed in our laboratory, which consists of simulated mouth, stomach and small intestinal conditions [24]. The curcumin-loaded soymilk was passed through the full GIT model and then the amount of curcumin solubilized within the mixed micelle phase was measured.

To simulate the mouth phase, a fixed volume of pre-warmed (37 ºC) curcumin-loaded soymilk (15 mL) was transferred into a pre-warmed (37 ºC) simulated artificial saliva fluid (15 mL), which was prepared by adding mucin (3 mg/mL) to artificial saliva solution. This mixture was then adjusted to pH 6.8 and placed within a temperature-controlled shaking incubator

operating at 100 rpm and 37 ºC for 30 seconds (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA). The resulting sample was then incubated under simulated gastric conditions. Pre-heated (37 ºC) simulated gastric fluid (30 mL), which contained 3.2 mg/ mL pepsin, was added to the same volume of the sample from the mouth phase (30 mL). The resulting mixture was then adjusted to pH 2.5 and incubated at 37 ºC for 2 hours with 100 rpm shaking. After that, the sample (60 mL) arising from the stomach phase was transferred into the small intestinal phase. Pre-heated simulated intestinal fluid (3 mL) and bile salt solution (7 mL) was added to the mixture and the pH was adjusted to neutral. Then, a freshly prepared pancreatic lipase solution (5 mL) was added to the mixture and the pH was adjusted back to neutral. The bile salt solution was prepared by dissolving porcine bile extract (53.57 mg/mL) into phosphate buffer (5 mM, pH 7.0), and the pancreatic lipase solution was prepared by adding pancreatic lipase (0.16 mg/mL) into phosphate buffer (5 mM, pH 7.0). Throughout the 2-hour small intestinal phase, an automatic titration unit (Metrohm, USA Inc.) was used to monitor and maintain the sample at pH 7.0.

Curcumin concertation determination

The concentration of the curcumin within the soymilk was determined using a UV/visible spectrophotometry method. First, the curcumin was extracted from the samples using acidified dimethyl sulfoxide (0.1% v/v 6N HCl) solution. Acidification was used to enhance the chemical stability of the curcumin. The absorbance of the curcumin solutions was measured using a UV-visible-spectrometer (Genesys 150, Thermos Fisher Scientific, Madison, WI) at a wavelength of

420 nm. The curcumin concentrations were then calculated from the measured absorbance values using a standard curve prepared using a series of samples of known curcumin level.

Encapsulation Efficiency

The encapsulation efficiency of the curcumin-loaded soymilks was calculated using the following equation:

$$\textit{Encapsulation Efficiency} = 100 \times C_{encapsulated} / C_{initial} \quad (1)$$

Here, $C_{initial}$ is the total concentration of curcumin initially added to the soymilk and $C_{encapsulated}$ is the concertation of curcumin present within the curcumin-loaded soymilk after preparation using the pH-driven method.

Stability and Bioaccessibility

After passing through the full GIT model, the resulting fluid was collected and divided into two fractions. One fraction, which was used without any further treatment, was taken to be the total digested phase. The other fraction, which was the supernatant collected after centrifugation, was taken to be the mixed micelle phase. The mixed micelle phase was prepared by centrifugation of the total digest at 18,000 rpm at 25 ºC for 50 min (Thermo Scientific, Waltham, MA) and then collecting the clear supernatant from the samples. The curcumin concentrations within the total digested phase (C_{digest}) and with the mixed micelle phase ($C_{micelle}$) were then determined using UV/visible spectrophotometry as described earlier.

The *bioaccessibility and stability* of the curcumin-loaded soymilk were then calculated using the $C_{micelle}$, C_{digest} and $C_{initial}$ values using the following expressions:

$$Bioaccessibility\ (\%) = 100\ \times \frac{C_{micelle}}{C_{digest}} \quad (2)$$

$$Stability(\%) = 100\ \times \frac{C_{digest}}{C_{initial}} \quad (3)$$

Here, the $C_{micelle}$ is the concentration of curcumin within the mixed micelle phase; the C_{digest} is the concentration of curcumin within the digest phase; the $C_{initial}$ is the concertation of curcumin within the soymilk system before digestion. The *Bioaccessibility* represents the fraction of curcumin solubilized in the mixed micelle phase, which is potentially available for absorption. The *Stability* represents the total amount of curcumin that survived after the soymilks were passed through the simulated GIT.

Kinetic study

The kinetics of curcumin degradation within the soymilk samples during storage was calculated using the Arrhenius equation:

$$k = A\, e^{-Ea/RT} \quad (4)$$

Here, k is the rate coefficient, A is the Arrhenius constant; E_a is the activation energy, R is the universal gas constant (8.314×10^{-3} kJ mol^{-1} K^{-1}); and T is the temperature in Kelvin.

Statistical analysis

All the data from this experimental study are displayed as the mean ± the standard deviation determined from measurements made on three freshly prepared samples. Statistical comparisons were carried out using dedicated mathematical software (SPSS version 6.0) and a significant difference was considered to occur when the *p*-value was lower than 0.05.

Results and discussions

Soymilk characteristics

The commercial soymilk used in this study consists of soybean oil bodies dispersed in an aqueous solution that contains various other ingredients, such as cane sugar, maltodextrin, soy lecithin, starch, locust bean gum, and dipotassium phosphate. Visually, the soymilk had a homogenous appearance with a bright milky color (Fig. 1a). Measurement of the pH of the

soymilk showed that it was slightly basic (pH 7.5). The mean particle diameter (D_{32}) of the soymilk determined by laser diffraction analysis was 367 ± 2 nm. Particle size distribution analysis showed that the majority of particles had diameters between about 0.1 and 4 μm, but that there was a small population of particles with larger dimensions (Fig 1b). The original soymilk had a relatively high negative surface potential (ζ = -39.8 ± 2.6 mV), which can be attributed to the presence of anionic phospholipids and proteins at the oil body surfaces [7]. The magnitude of the negative charge on the oil bodies should be high enough to generate a strong electrostatic repulsion that prevents them from aggregating with each other [25, 26].

Influence of pH on particle characteristics in soymilk

The impact of pH on the physicochemical properties and stability of the curcumin-free (blank) soymilk was characterized to establish whether it would remain stable during the pH-driven method and to determine the range of pH conditions where it would remain stable in commercial applications. The particle characteristics of these soymilks were measured after their aqueous phases had been adjusted to pH values ranging from 2 to 10 and then stored for several hours (Fig.1a). From pH 6 to 10, the soymilks remained visibly stable, with no evidence of phase separation. From pH 2 to 5, however, there was clear evidence of phase separation, with a curd-like whitish upper phase and a clear watery lower phase. These results show that the soymilks were highly unstable to aggregation and creaming under moderately acidic conditions.

The impact of pH on the mean particle diameter and ζ-potential of the particles in the soymilks were investigated using laser diffraction and electrophoresis analysis, respectively (Figs. 1c & 1d). From pH 10 to 7, the soybean oil bodies had a relatively high and constant negative surface potential (ζ ≈ -42 mV) and had relatively small and constant particle dimensions (D_{32} < 380 nm). When the pH was reduced to 6, the surface potential became slightly less

277 negative ($\zeta \approx -35$ mV) and the particle dimensions increased somewhat ($D_{32} \approx 578$ nm), which
278 suggested that a limited amount of oil body aggregation had occurred. When the pH was further
279 reduced into the range from 5 to 2, there was a pronounced increase in the mean particle
280 diameter ($D_{32} > 12$ μm), which indicated that extensive oil body aggregation occurred under
281 these conditions. The laser diffraction measurements were therefore consistent with the visual
282 observations of phase separation in the samples over this pH range (Fig. 1a). The origin of this
283 effect can be attributed to the decrease in the magnitude of the surface potential on the oil bodies
284 at lower pH values (Fig. 1c), which would have reduced the electrostatic repulsion between
285 them. Indeed, the ζ-potential went from negative at high pH values to positive at low pH values,
286 with a net charge of zero around pH 3.6. This effect can be attributed to progressive protonation
287 of the carboxyl groups ($-COO^- \rightarrow -COOH$) and amino groups ($-NH_2 \rightarrow -NH_3^+$) on the adsorbed
288 proteins and phospholipids as the pH was reduced. It should be noted, however, that the
289 commercial soymilk contained other components that could also have attributed to its electrical
290 characteristics, such as soy lecithin. Presumably, this ingredient was added to improve the
291 aggregation stability of the oil bodies, probably by adsorbing to any hydrophobic patches on
292 their surfaces.

293 **Influence of pH-driven method on soymilk properties**

294 The experiments described previously indicated that the soymilk remains physically stable at
295 relatively high pH values. In commercial products, the pH may vary somewhat depending on the
296 ingredients used to formulate them, as well as any chemical changes that occur during storage.
297 For this reason, we carried out the following experiments using soymilk samples with three
298 different final pH values: pH 6.5, 7.0 and 8.0. Soymilk samples above pH 8 were not evaluated
299 because food and beverage products rarely have such highly alkaline conditions.

Appearance

Digital photography images and instrumental colorimetry measurements were used to characterize the appearance of the curcumin-loaded soymilks at the three different pH values (Figs. 3a and 4). The photography images showed that the soymilk samples had a homogenous cloudy yellow appearance at pH 6.5 and 7, but were slightly more orange-colored at pH 8 (Fig 3a). The tristimulus coordinates of the soymilks also indicated that there were appreciable differences in their color depending on pH (Table 1). At pH 6.5 and 7, the soymilks had fairly similar color characteristics indicative of a creamy yellow appearance: L^* (≈ 82), a^* (≈ -8), and b^* (≈ 81). But, at pH 8, the soymilk had significantly lower L^* (≈ 77) and b^* (≈ 75) values and higher a^* (≈ 0.6) values (Table 1). This suggests that the soymilk at the highest pH was less light, less green, and less yellow than the ones at the lower pH values. This effect can be attributed to the pH-dependent color of the curcumin molecule. When the pH is increased to around the first pK_a value of curcumin (around pH 7.5 to 8.5), one of its hydroxyl groups becomes deprotonated, which leads to a change in color from yellow to orange [27-29]. This phenomenon may be important when formulating curcumin-loaded soymilk products with specific visual characteristics.

Curcumin concentration

The impact of the pH-driven method on the encapsulation efficiency of the curcumin within the soymilks was determined. A fixed amount of curcumin (250 μg/mL) was utilized to prepare all the nutraceutical-loaded soymilks using the pH-driven method. After loading, the encapsulation efficiencies of the curcumin in the soymilks were determined. At all three pH values, a relatively high and fairly similar encapsulation efficiency was obtained, *i.e.,* 91-94%

(Table 1). These results indicate that the pH-driven method was a successful approach for incorporating curcumin into pre-existing oil bodies.

Particle characteristics

The impact of using the pH-driven method for loading curcumin into the soybean oil bodies on the structural properties of the soymilk was also investigated. The loading of the curcumin into the oil bodies had no significant impact on their surface potential (Fig. 2a) or mean particle diameter (Fig. 2b) at any of the pH values used. There was little change in the surface potential and only a slight decrease in the mean particle diameter when the pH was increased from 6.5 to 8, which is consistent with the earlier experiments (Figs. 1a, c & d). Immediately after preparation, all the curcumin-loaded soymilks had a homogenous creamy yellow appearance (Fig. 3a), which suggests that they initially had good stability to aggregation and creaming. This was confirmed by measuring the microstructures of the soymilk using confocal microscopy (Fig. 3b), which showed that at all three pH values the soymilks contained relatively small oil bodies that were distributed evenly throughout the system. Fluorescent staining indicated that the oil bodies (stained red) were dispersed in an aqueous medium that contained proteins (stained green).

Effect of pH on storage stability of curcumin-loaded soymilk

For commercial applications, it is important that plant-based milks have a sufficiently long shelf life. For this reason, we measured the physiochemical stability of curcumin-loaded soymilks (pH 6.5, 7, and 8) during storage at 4 ºC in the dark. This temperature was selected because the milks would be expected to be stored in the refrigerator prior to utilization.

Appearance

After 36 days storage, all of the soymilks still had a creamy yellow/orange color but some phase separation was observed, with a watery serum layer being visible at the bottom of the test tubes (Fig 3a). This effect was attributed to the upward movement of the oil bodies due to gravity. The thickness of the serum layer was greatest for the soymilk at pH 6.5, which was probably because it contained the largest particles (Fig. 2b), presumably because some of the oil bodies had aggregated. Conversely, the soymilk at pH 8 was the most stable to gravitational separation, maintaining a uniform creamy yellow/orange appearance after storage. There did, however, appear to be a slight reduction in the intensity of the color in this sample after storage, as well as the formation of thin orange sediment at the bottom of the test tube (Fig. 3a). This orange sediment was assumed to be due to the formation and sedimentation of curcumin crystals within the soy milks. The decrease in the color-intensity of the soymilk at pH 8 may, therefore, have been because some of the curcumin had moved to the bottom of the samples and therefore did not contribute to their overall appearance. In addition, some of the curcumin may have chemically degraded during storage (see later).

The instrumental color coordinates of the curcumin-loaded soymilk were measured before and after storage (Fig. 5a, Table 1). The kinetics of color fading during storage is highlighted by plotting the normalized yellowness *versus* time because this was the dominant color coordinate: b^*/b_0^*, where b_0^* is the color coordinate at zero time. This value changed by less than 2% during storage, which suggests that there was little alteration in yellow color. There was also little change in the a^*-value of the soymilks (Table 1). Indeed, a^* increased from -8 to -6 for the soymilks at pH 6.5 and 7, which indicates a slight decrease in their greenish hue. Conversely,

the *a** of the soymilk at pH 8 decreased slightly from 0 to -1, which indicates that it became slightly more greenish.

Curcumin concentration

The concertation of curcumin remaining in the soymilk was also measured during storage to investigate the impact of pH on its chemical stability (Fig. 5b). After 36 days storage at 4 ^{o}C, the majority of curcumin was still present, with the precise amount depending on solution conditions (Table 1): pH 6.5 (91%), pH 7 (90%), and pH 8 (87%). Overall, these results suggest that curcumin was relatively stable to degradation under these storage conditions, but that the rate of degradation increased as the pH was raised, which is consistent with previous results [30].

Particle characteristics

Changes in the characteristics of the particles in the curcumin-loaded soymilks stored at different pH values were also determined (Fig. 4). The mean particle diameter of all the soymilks remained relatively constant during storage (Fig. 4a), suggesting that the oil bodies were stable to aggregation. The ζ-potentials of the particles in all the soymilks became slightly less negative (from around -40 to -35 mV) during storage (Fig. 4b), which suggests that there were some changes in the interfacial composition of the oil bodies during storage. Even so, the negative surface potential should still have been large enough to generate a strong electrostatic repulsion that inhibited the aggregation of the oil bodies. As mentioned earlier, we did see some phase separation (creaming) of the oil bodies after 36 days storage, particularly at the lower pH values. This result suggests that there may have been some weak flocs formed in the soymilk samples that promoted creaming due to the increase in particle size, but which were disrupted when the samples were diluted for the particle size measurements.

Effect temperature on the soymilk containing curcumin

Many commercial food and beverage products experience changes in their temperature during their manufacture, storage, and utilization. Consequently, it is important to establish the impact of temperature on the physicochemical properties and stability of the curcumin-loaded soymilk samples. For this reason, changes in the properties of the soymilk were measured when they were stored at 4, 20, 37, and 55 ºC for 14 days. The soymilk at pH 7 was selected for these studies because it was more stable to creaming than the pH 6.5 sample and more stable to curcumin crystallization than the pH 8 sample.

Appearance

The storage temperature had a pronounced impact on the overall appearance of the curcumin-loaded soymilks (Fig. 6a). The extent of color fading increased as the storage temperature increased. At 4 and 20 ºC, the curcumin was relatively stable to fading, with the soymilks maintaining a strong yellowish color, but at 37 and 55 ^{o}C there was clear evidence of color fading. In addition, the soymilks exhibited a greater degree of gravitational separation as the storage temperature was raised. The samples stored at 4 and 20 ºC were relatively stable to creaming during storage, but those stored at 37 and 55 ^{o}C had a thick cream layer at their surfaces after storage, which was attributed to the upward movement of the oil bodies due to the gravitational forces acting upon them.

Instrumental colorimetry analysis of the curcumin-loaded soymilk during storage also showed that the rate of color fading increased with storage temperature. The color stability of the soymilk samples was represented by plotting the relative change in their yellowness (b^*/b_0^*) over time (Fig. 7a). The L^*, a^* and b^* values of the soymilks before and after storage is shown in Table 2. Overall, color fading increased in the following order: 4 ºC ≈ 20 ºC < 37 ºC < 55 ºC

(Fig. 7a). The lightness (L^*) of all the soymilks remained greater than 90 % even after 15-days storage. The greenness (a^*) of the soymilks decreased by about 20% at 4 and 20 ºC, 30% at 37 ºC, and 50% at 55 ºC after 15-days storage. The yellowness (b^*) of the soymilks did not change appreciably (< 2%) at 4 and 20 ºC, but decreased by about 10% at 37 ºC, and 30% at 55 ºC. These results suggest that the curcumin should be stored at a relatively low temperature in order to minimize color fading.

Curcumin concentration

The fading of the curcumin-loaded soymilks was most likely due to the chemical instability of the curcumin molecule at elevated temperatures. We therefore measured the impact of temperature on the change in curcumin concentration during storage (Fig. 7b). The rate of curcumin degradation clearly increased with increasing temperature. After 15 days storage, less than 10% of the curcumin degraded at 4 and 20 ºC, around 35% at 37 ºC, and around 75 % at 55 ºC. The chemical transformation of the curcumin during storage would therefore account for the faster color fading observed at the higher temperatures.

A more detailed analysis of the reaction kinetics of curcumin degradation in the soymilks was obtained by applying the Arrhenius equation (Equation 4). This equation can be rearranged to give:

$$\ln(k) = \ln(A) - \frac{E_A}{RT} \quad (5)$$

Thus, the activation energy can be determined by plotting the logarithm of the reaction rate (k) versus the reciprocal of the absolute temperature ($1/T$) (Fig. 8). The reaction rate was determined at each temperature from plots of curcumin concentration *versus* time, assuming a first order reaction, *i.e.,* $C/C_0 = A \exp(-kt)$. This analysis indicated that the activation energy for curcumin degradation was around 48.4 kJ/mol.

Particle characteristics

The size and electrical characteristics of the particles in the soymilk samples were also measured during storage at the four different temperatures (Figs. 6b & 6c). Overall, the mean particle diameter remained relatively small (< 400 nm) and the ζ-potential remained highly negative (≈ –40 mV) throughout storage. These results suggest that the soymilk samples were relatively stable to coalescence or strong flocculation at all temperatures. Conversely, the fact that we did observe appreciable creaming at the higher storage temperatures suggests that some weak flocculation of the oil bodies may have occurred. However, the confocal microscopy images of the samples did not indicate that extensive oil body aggregation had occurred (Fig. 6d). An alternative explanation is that more rapid oil body creaming occurred at the higher storage temperatures because of the reduction in aqueous phase viscosity. The dynamic shear viscosity of water has been reported to be 1.6, 1.0, 0.69, and 0.50 mPa s at 4, 20, 37, and 55 ^{o}C, respectively (www.vaxasoftware.com). Thus, there is more than a 3-fold decrease in the aqueous phase viscosity from the lowest to highest storage temperatures used. According to Stoke's law, the creaming velocity of a spherical particle due to gravity is inversely related to the viscosity of the surrounding fluid, so a decrease in aqueous phase viscosity should promote faster creaming [31]. Overall, these results suggest that the physical stability of the soymilks is also improved by storing them at relatively low temperatures.

Bioaccessibility and stability

Finally, we examined the impact of encapsulation of curcumin within the soymilks on two of the main factors affecting nutraceutical bioavailability after ingestion: bioaccessibility and stability in the gastrointestinal tract (GIT) [32, 33]. The curcumin-loaded soymilk stored at pH 7 and 4 ºC was used for these experiments because it had the best physical and chemical stability. The

potential bioavailability of the curcumin was determined using a simulated GIT before and after storage of the soymilks (Table 3). As expected, the total fraction of curcumin within both the total digest and the mixed micelle phases were slightly (but significantly) higher before storage than after storage, which can be attributed to some curcumin degradation. The gastrointestinal stability and bioaccessibility of the curcumin were fairly similar before and after storage. About 82-85% of the curcumin survived passage through the simulated mouth, stomach, and small intestine phases, but only about 55-59% of the curcumin was solubilized within the mixed micelle phase and therefore available for absorption. It is possible that the remainder of the curcumin either formed crystals that precipitated or bound to insoluble protein complexes and was therefore not present in the mixed micelle phase. Nevertheless, these measurements show that a substantial fraction of the ingested curcumin should still be in a from that would be bioavailable. Having said this, it is important to note that the human gut contains many types of metabolic enzymes that can transform curcumin into different forms [34, 35], which was not considered in the current study. In future studies, it would therefore be useful to test the curcumin-loaded soymilks using animal or human feeding trials.

Conclusions

In summary, this study has shown that the pH-driven method can be successfully used to load curcumin into commercial soymilks and that the storage stability of the resulting systems depends on pH and temperature. The soymilks had good chemical stability when stored at refrigerator temperatures at pH 6.5, 7 and 8 for 36 days, but there was some change in the color of the curcumin-loaded soymilks at the highest pH. The soymilks were susceptible to phase separation due to creaming when stored at pH 6.5, which was attributed to oil body aggregation. They also showed evidence of phase separation due to the formation and sedimentation of

curcumin crystals when stored at pH 8. The physical and chemical stability of the curcumin-loaded soymilks (pH 7) was relatively high at 4 and 20 ºC, but decreased at higher temperatures of 37 and 55 ºC. Overall, our results suggest that curcumin-loaded soymilks prepared using the pH driven method should be stored at neutral pH at relatively low temperatures. We also showed that these samples had relatively good bioavailability in a simulated GIT model. Nevertheless, further studies are needed to test their bioavailability using *in vivo* animal or human feed studies, as well as to test their stability under the conditions found in real food products.

Funding

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Table 1. Influence of pH on curcumin-loaded soymilk: initial curcumin concentration, loading capacity, tristimulus color value (*L**, *a**, and *b**), mean particle diameter (D_{32}) and electrical characteristics (ζ- potential). Different letters represent significant differences (Duncan, $p< 0.05$).

	pH 6.5	**pH 7**	**pH 8**
Initial curcumin concentration (μg/mL)	231.6 ± 8.8 [a]	228.3 ± 7.4 [a]	235.4 ± 3.7 [a]
Loading capacity (%)	92.6 ± 3.2 [a]	91.3 ± 2.7 [a]	94.2 ± 1.4 [c]
L*	82.2 ± 0.2 [b]	82.4± 0.1 [b]	77.3 ± 0.5 [a]
a*	-8.44 ± 0.15 [a]	-8.01 ± 0.09 [a]	0.64 ± 0.68 [b]
b*	82.2 ± 0.5 [c]	80.3 ± 0.4 [b]	75.2 ± 0.7 [a]
D_{32} (μm)	0.47 ± 0.02 [b]	0.39 ± 0.01 [a]	0.38 ± 0.01 [a]
ζ- potential (mV)	-40.8 ± 1.3 [b]	-42.1 ± 2.0 [a]	-42.8 ± 0.9 [a]

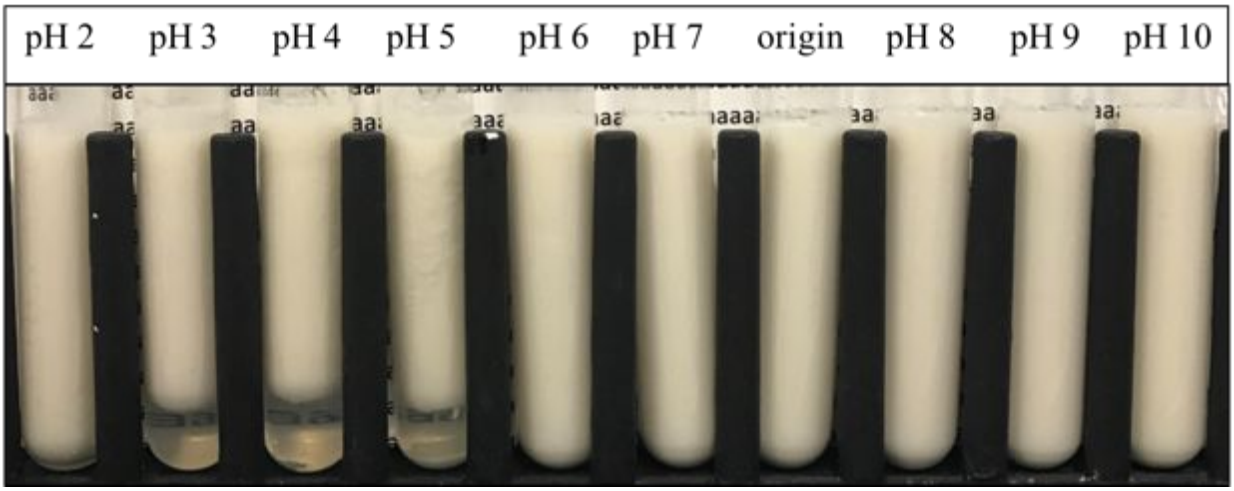
Table 2. Influence of temperature on the tristimulus color coordinates (L^*, a^*, and b^*) of samples before and after 15-day incubation. Different letters represent significant differences (Duncan, $p < 0.05$).

		4 °C	20 °C	37 °C	55 °C
Before Storage	L^*	82.41± 0.1 [a]	82.41± 0.1 [a]	82.41± 0.1 [a]	82.41± 0.1[a]
	a^*	-8.01 ± 0.09 [a]	-8.01 ± 0.09 [a]	-8.01 ± 0.09 [a]	-8.01 ± 0.09 [a]
	b^*	80.32 ± 0.40 [a]	80.32 ± 0.40 [a]	80.32 ± 0.40 [a]	80.32 ± 0.40 [a]
After Storage	L^*	80.86 ± 0.08 [c]	80.82 ± 0.64 [c]	78.70 ± 0.12	76.30 ± 1.00
	a^*	-6.58 ± 0.03 [c]	-6.53 ± 0.04 [c]	-5.40 ± 0.16 [b]	-3.74 ± 0.30 [a]
	b^*	80.10 ± 0.27 [d]	78.94 ± 0.53 [c]	72.02 ± 0.26 [b]	51.66 ± 1.86 [a]

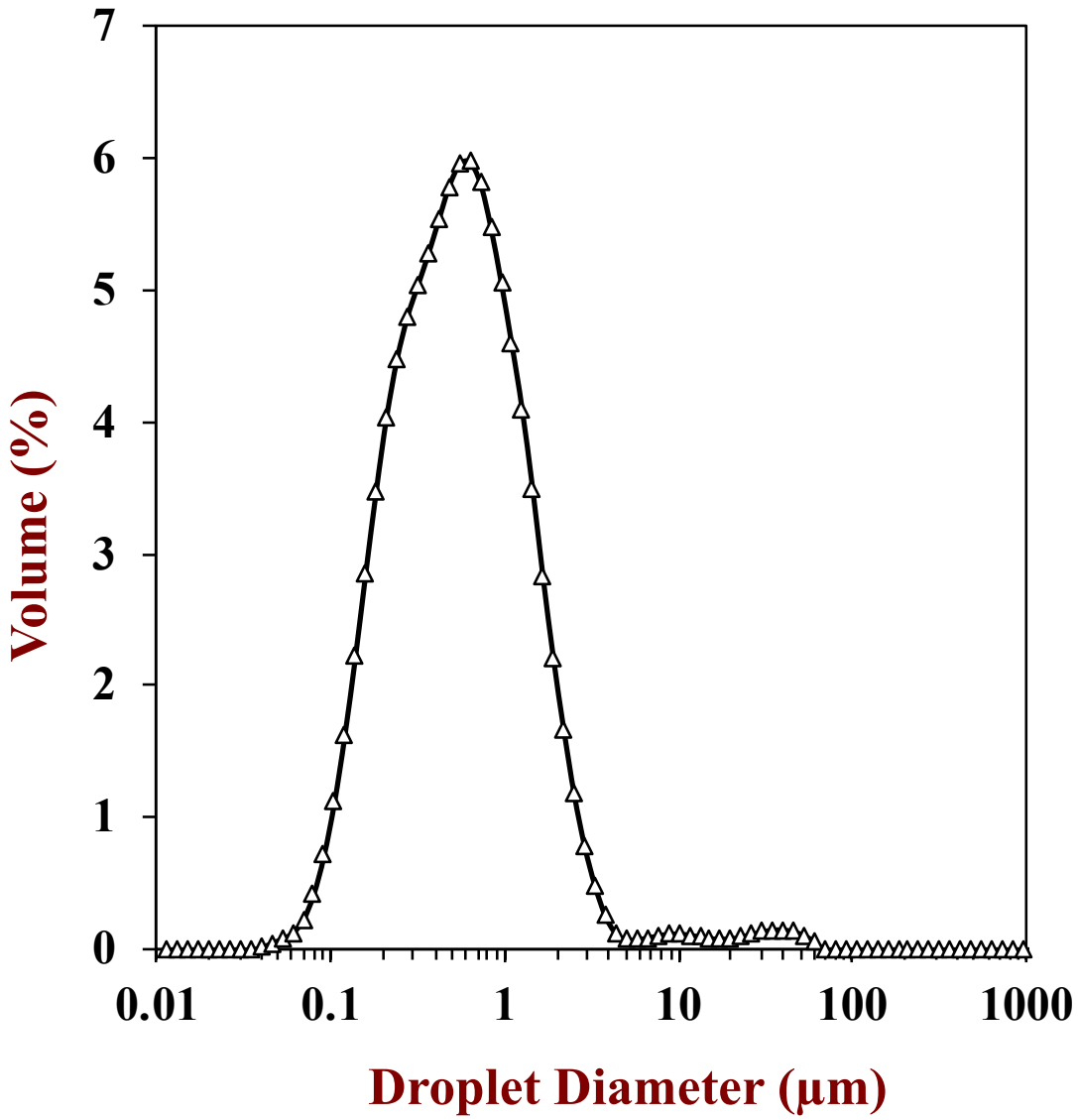
Table 3. Impact of storage at pH 7 on the gastrointestinal stability and bioaccessibility of curcumin in soymilks determined using a simulated gastrointestinal tract

	C_{Digest} (ug/mL)	$C_{Micelle}$ (ug/mL)	Stability (%)	Bioaccessibility (%)
Before Storage	186.80 ± 2.56	109.63 ± 18.13	81.87± 1.82	58.60 ± 9.08
After Storage	177.67 ± 1.43	97.23 ± 10.06	85.39 ± 3.14	54.76 ± 6.04

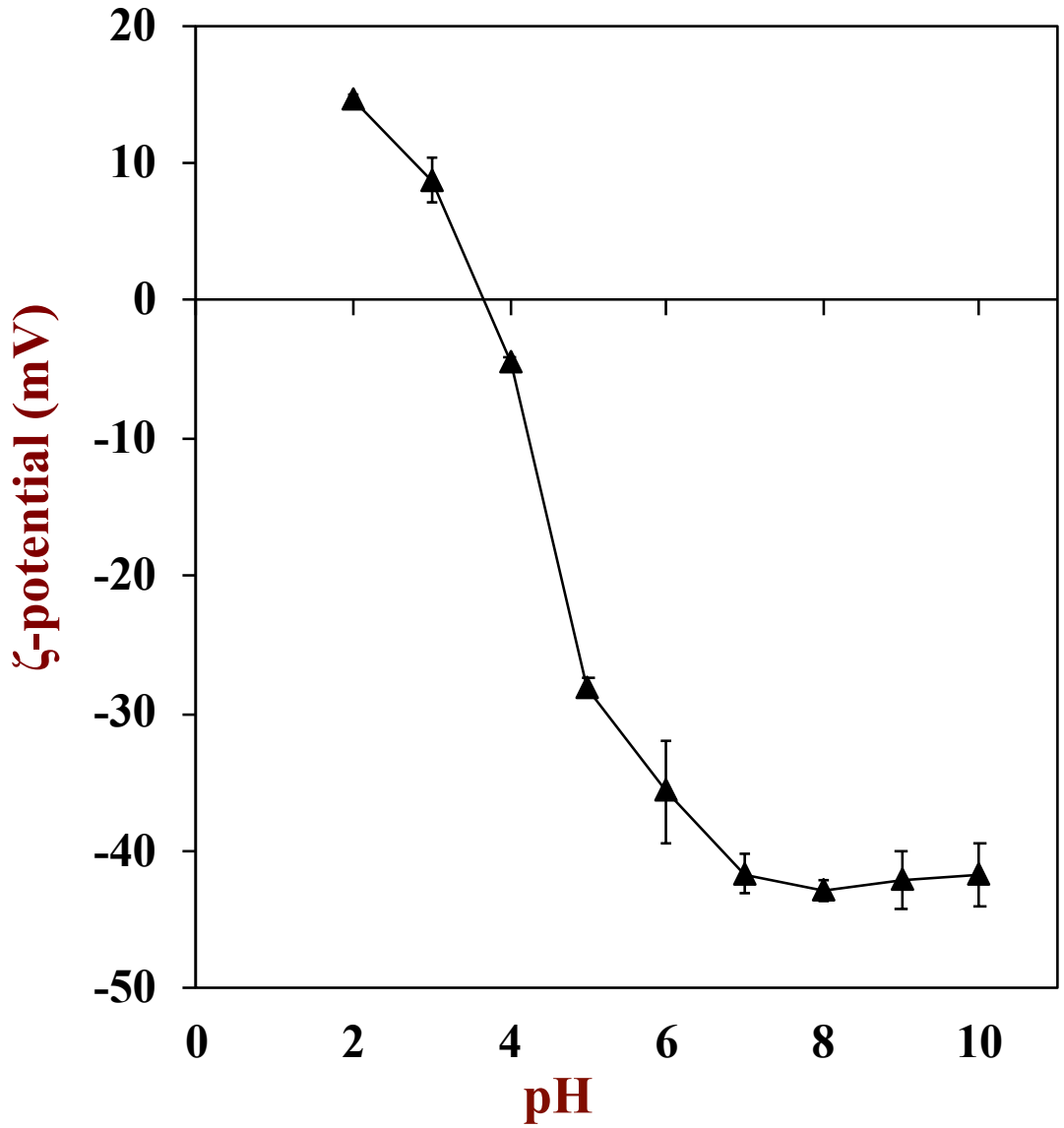
Fig. 1a



1b



1c



1d

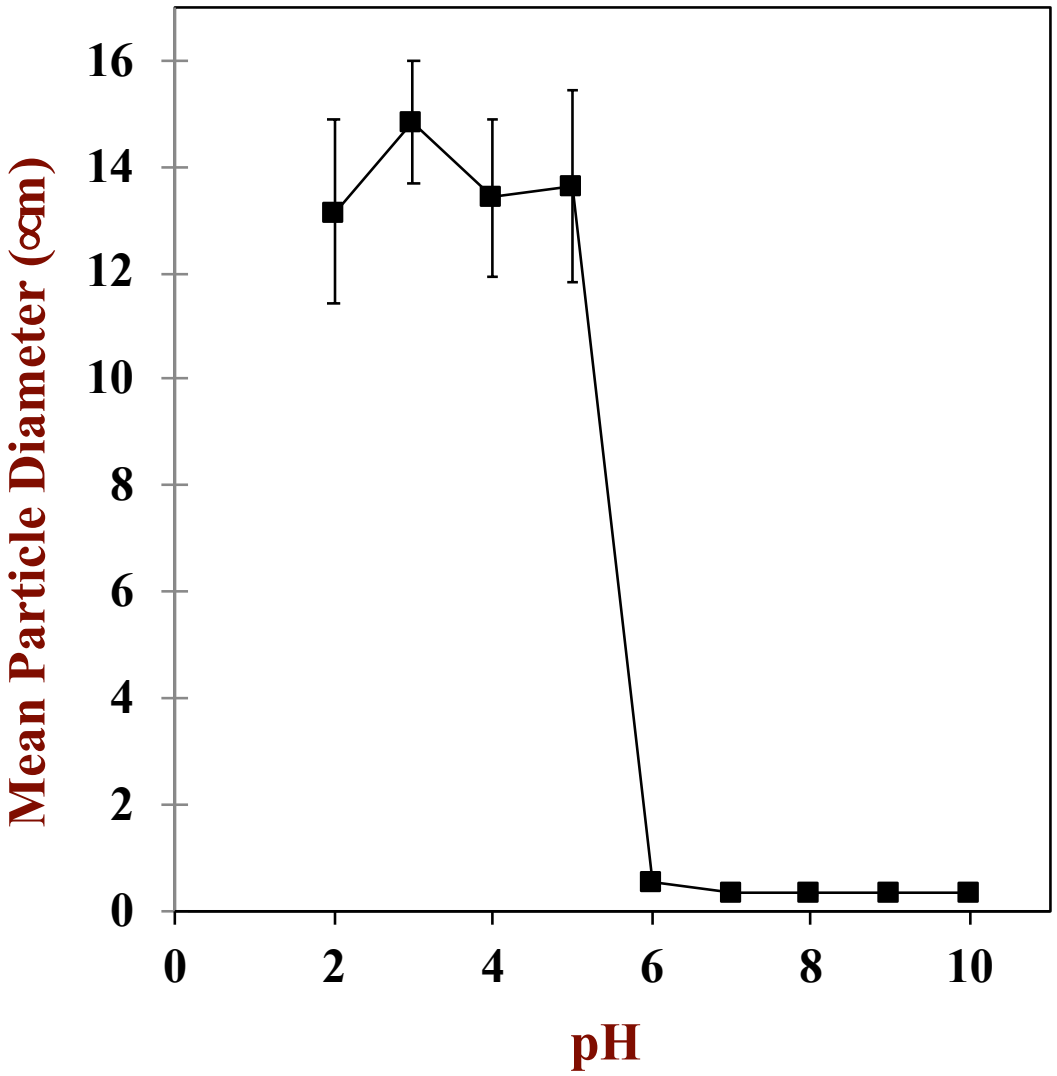


Fig. 1. a) Impact of pH on appearance of blank soymilk; b) particle size distribution of blank soymilk (pH 7.5); c) Impact of pH on electrical characteristics (ζ-potential) of blank soymilk; and d) Impact of pH on mean particle diameter (D_{32}) of blank soymilk.

Fig. 2 a

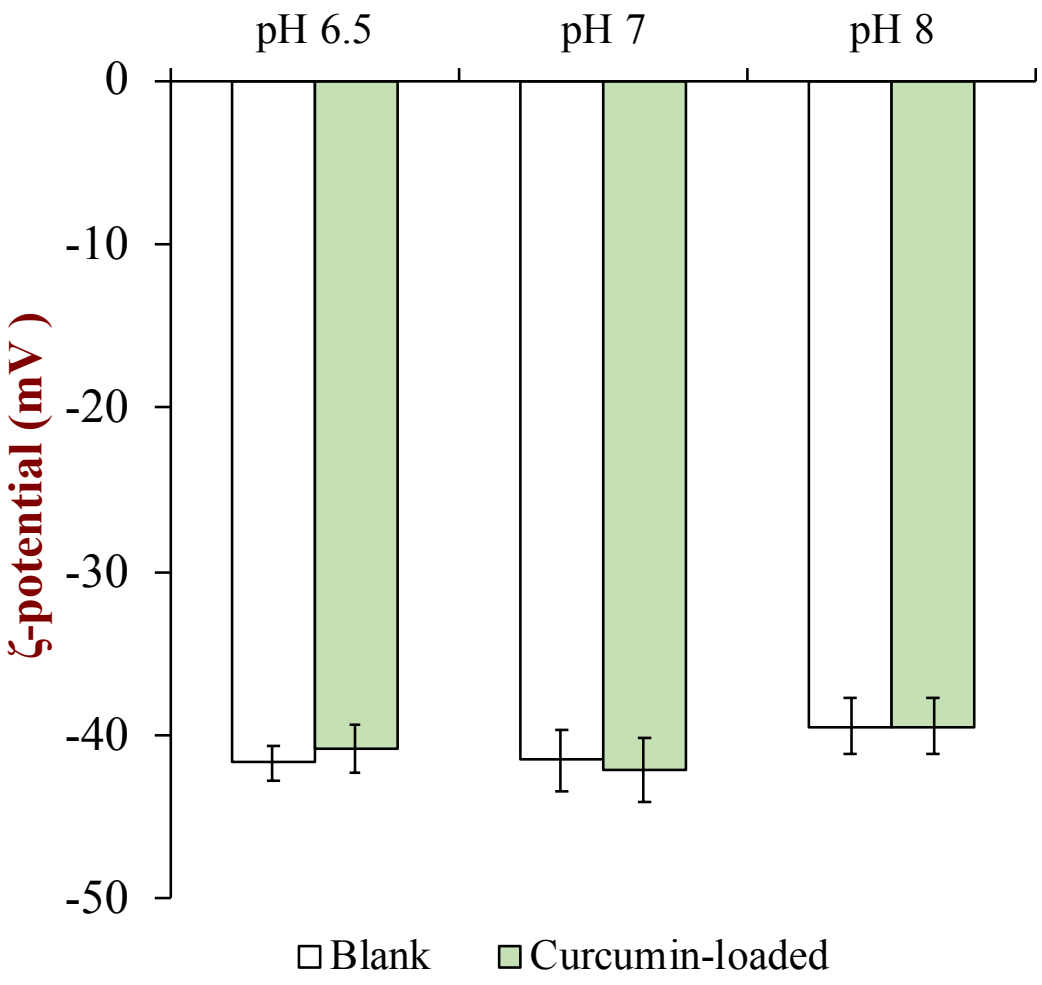

pH 6.5
pH 7
pH 8
0
-10
-20
-30
-40
-50
ζ-potential (mV)
Blank
Curcumin-loaded

Fig. 2b

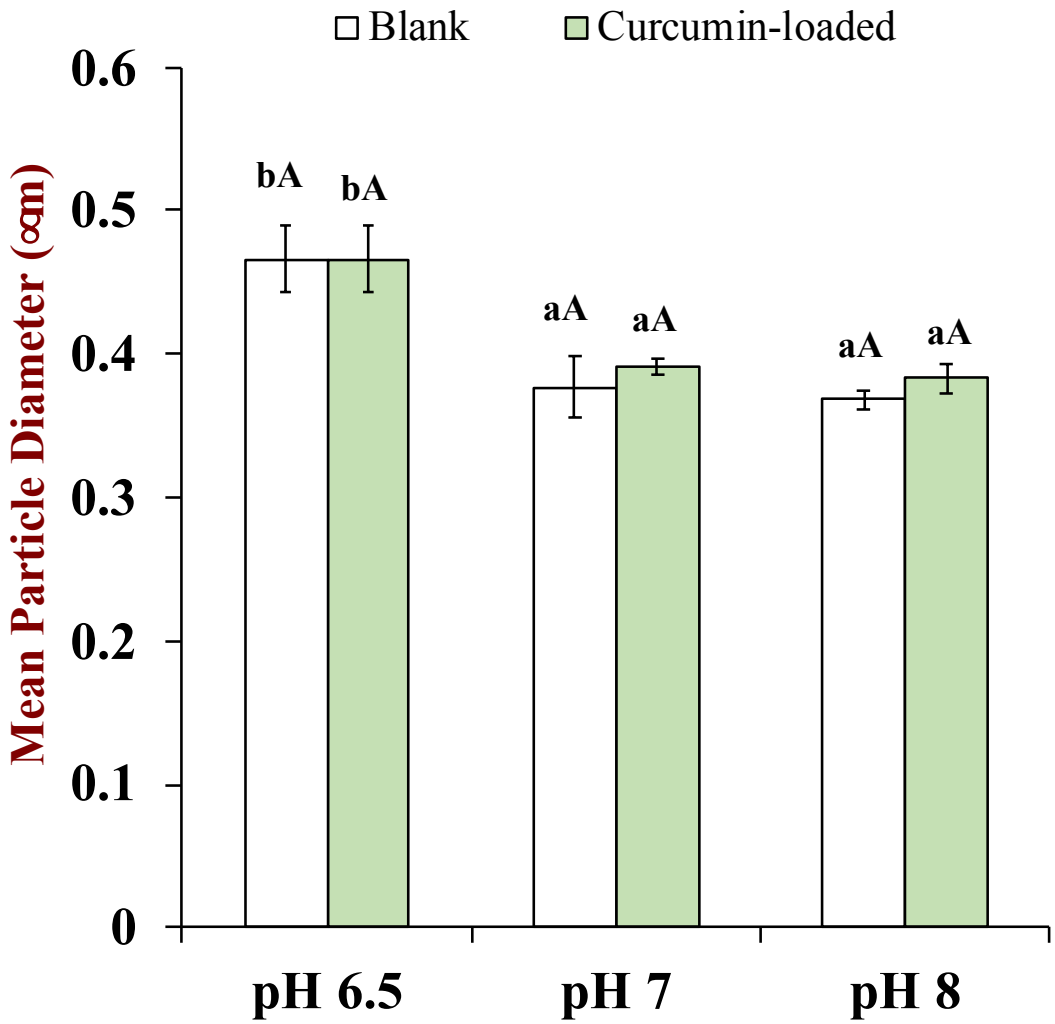


Fig. 2. The a) electrical characteristics (ζ-potential) and b) mean particle diameter (D_{32}) of the blank and curcumin-loaded soymilks at various pH values. Different lowercase letters indicate significant differences (Duncan, $p < 0.05$) within the same type of soymilk; different capital letters indicate significant differences between two types of sample (Duncan, $p < 0.05$).

Fig. 3 a

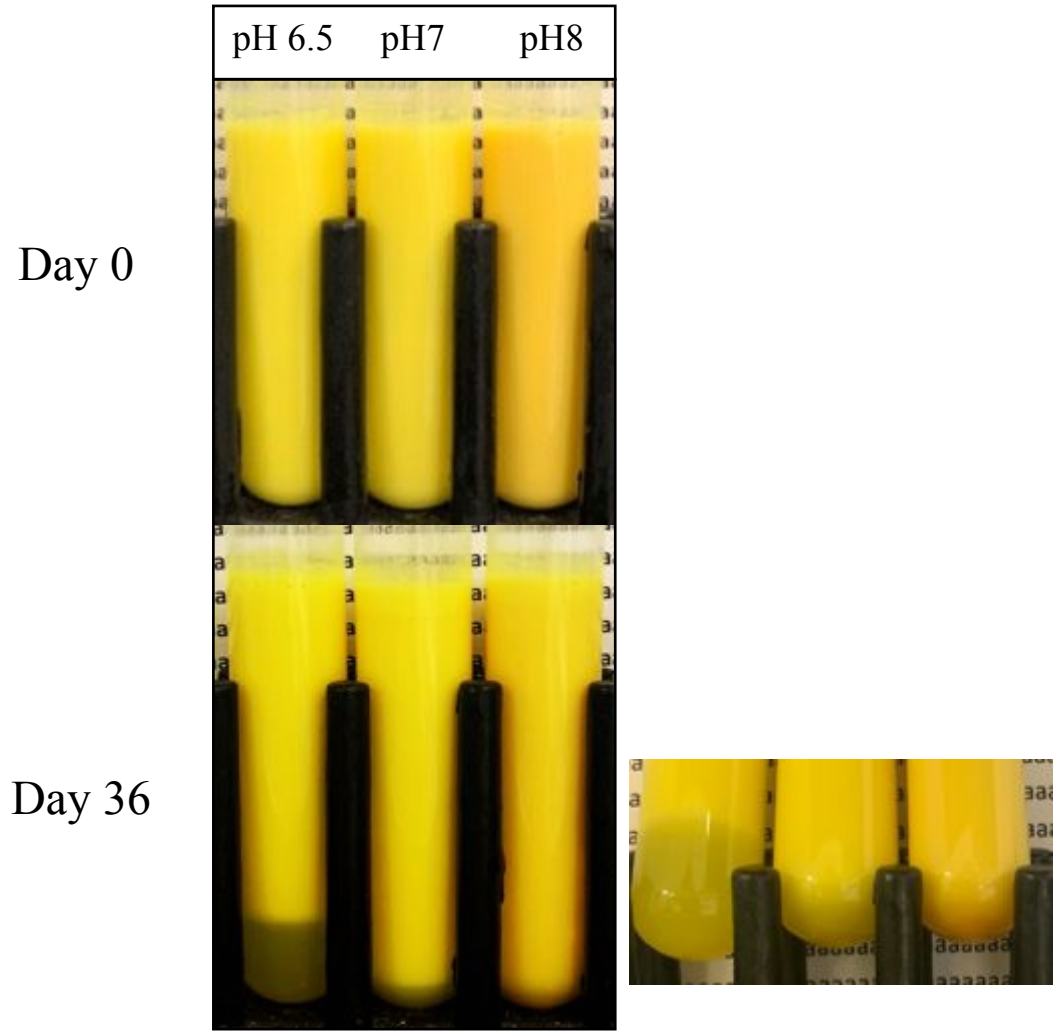


Fig. 3 b

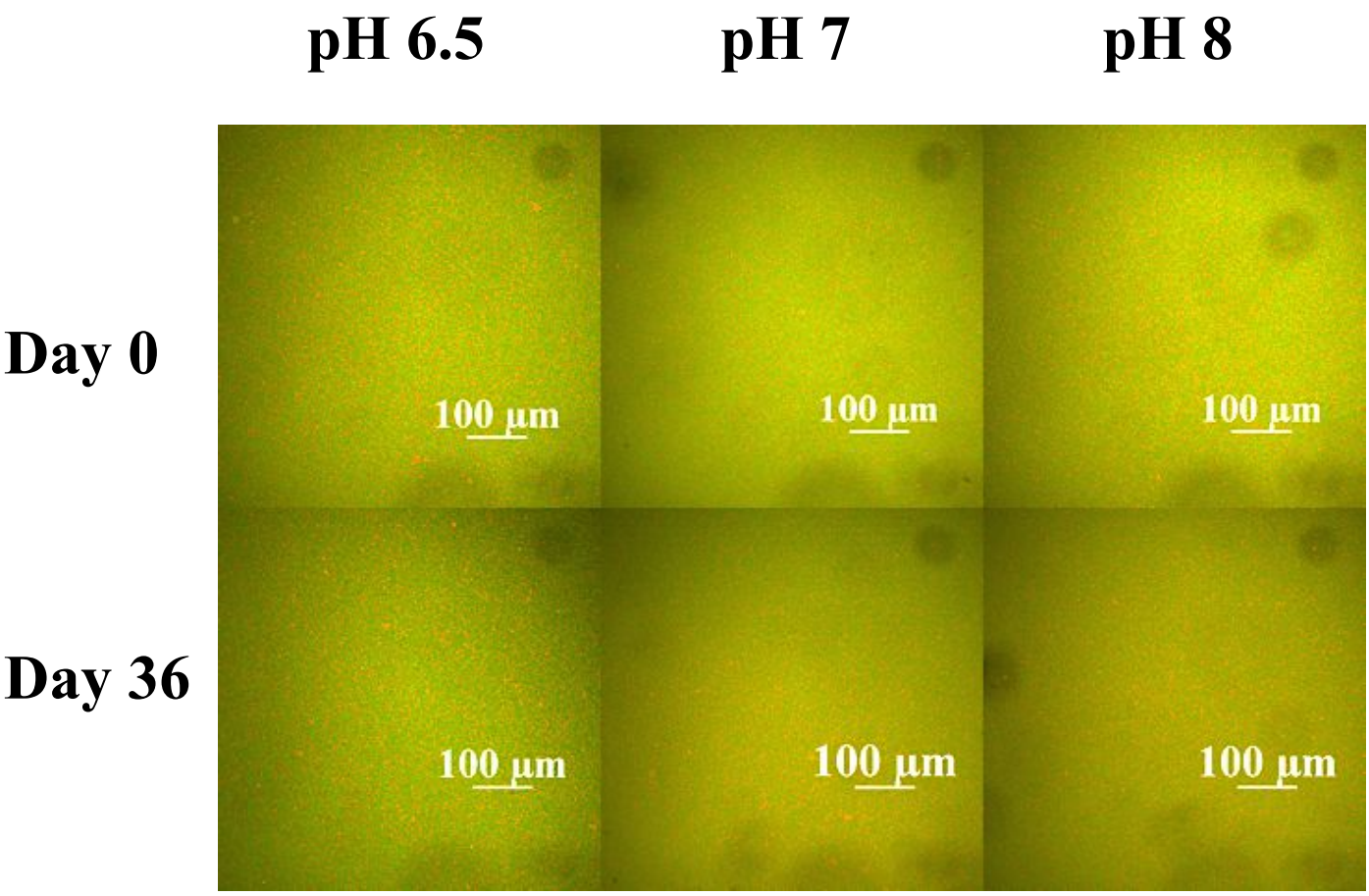
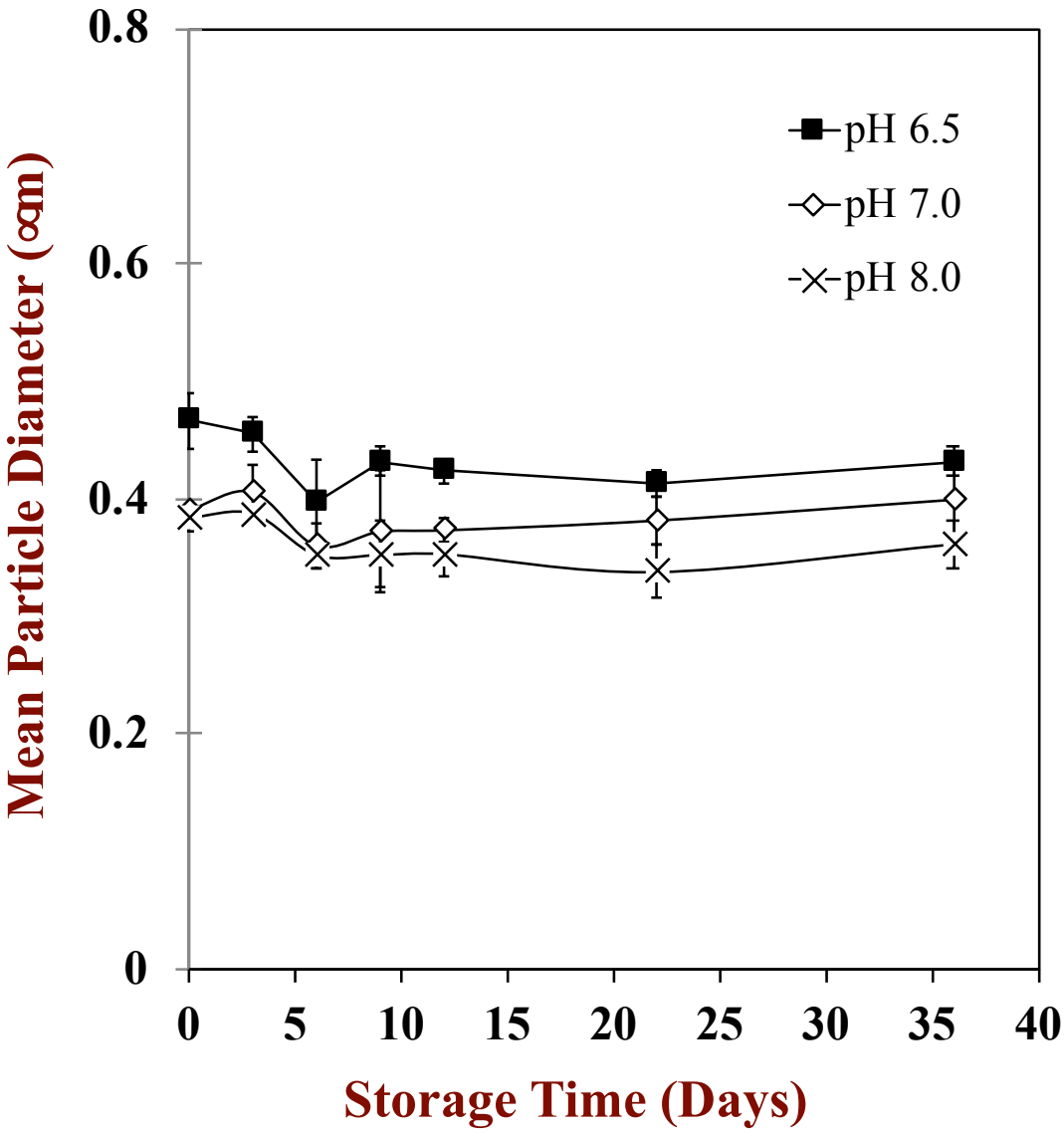


Fig 3. a) The appearances and b) microstructures of curcumin-loaded soymilks before and after storage at various pH values at 4 ºC for 36 days. The microstructure images were obtained using confocal fluorescence microscopy and the scale bars are 100 μm.

Fig. 4

4a


0.8
0.6
0.4
0.2
0
Mean Particle Diameter (œm)
pH 6.5
pH 7.0
pH 8.0
0
5
10
15
20
25
30
35
40
Storage Time (Days)

4b

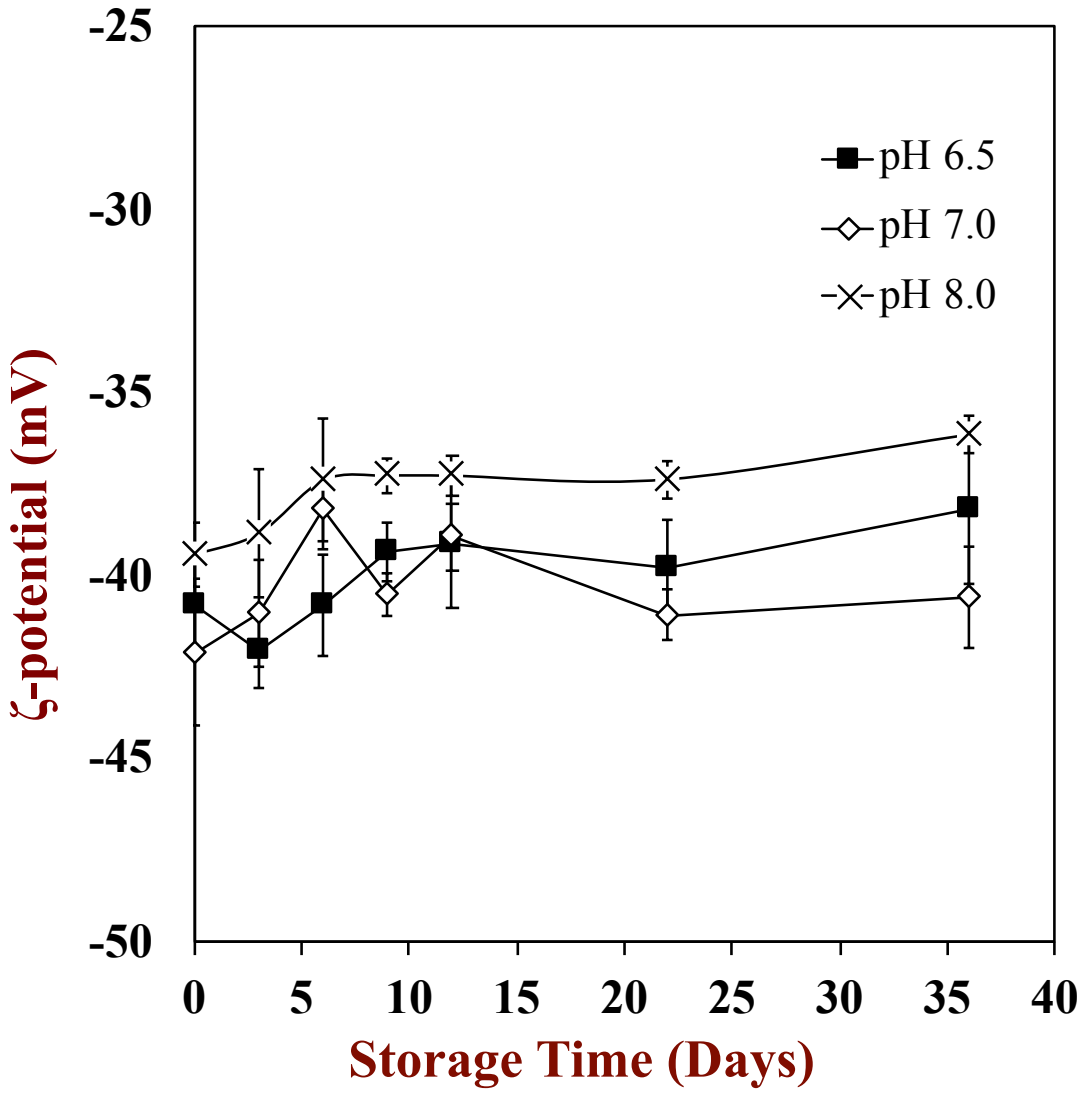
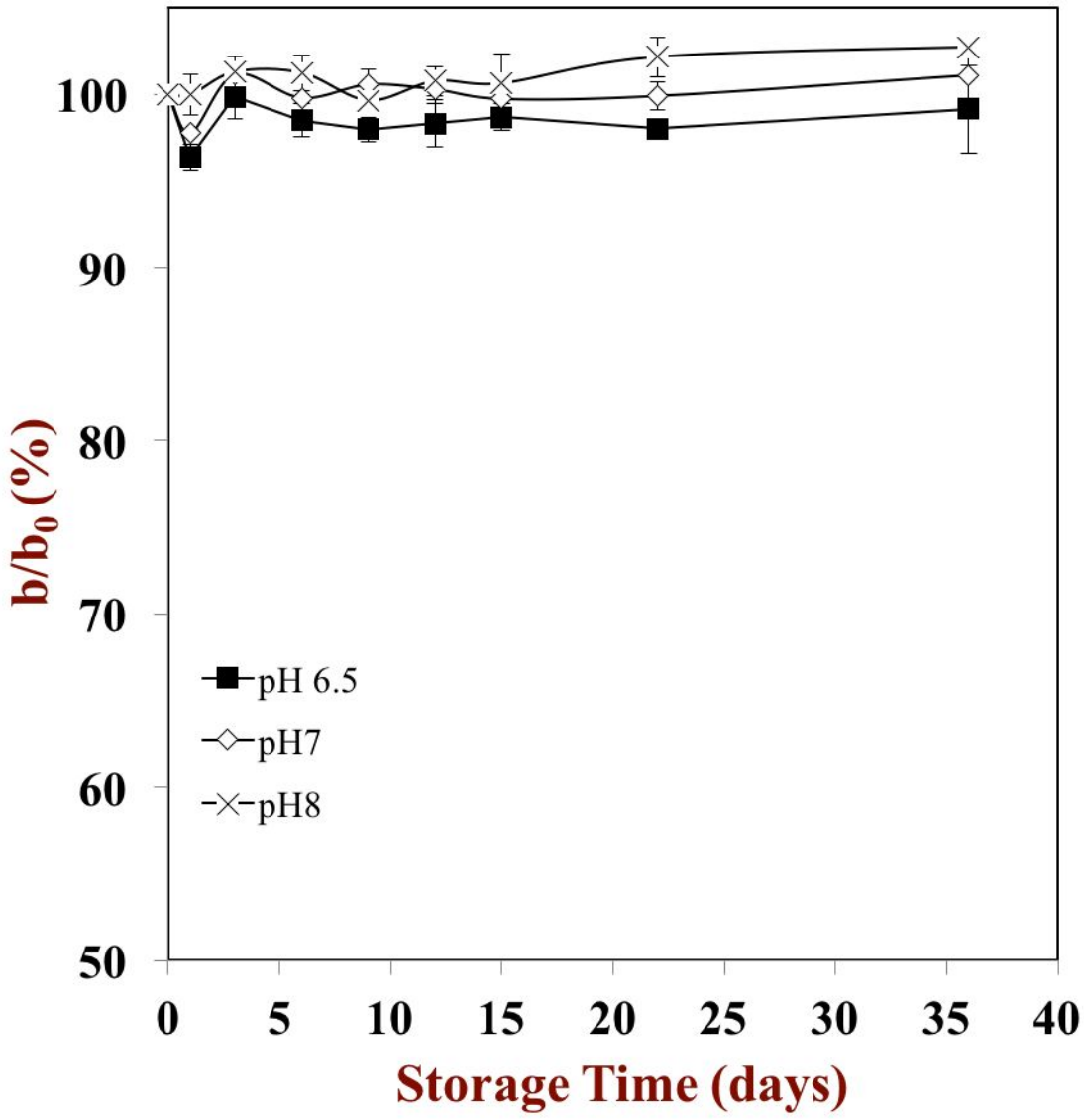


Fig. 4. Influence of pH on the a) mean particle diameters (D_{32}) and b) electrical characteristics (ζ- potential) of curcumin-loaded soymilk during 4 ºC storage for 36 days.

Fig. 5

5 a


100
90
80
70
60
50
b/b0 (%)
pH 6.5
pH7
pH8
0
5
10
15
20
25
30
35
40
Storage Time (days)

5 b

Fig. 5 Influence of pH on a) the yellow color (*b**) and b) curcumin concentration within the soymilks during 4 ºC storage for 36 days.

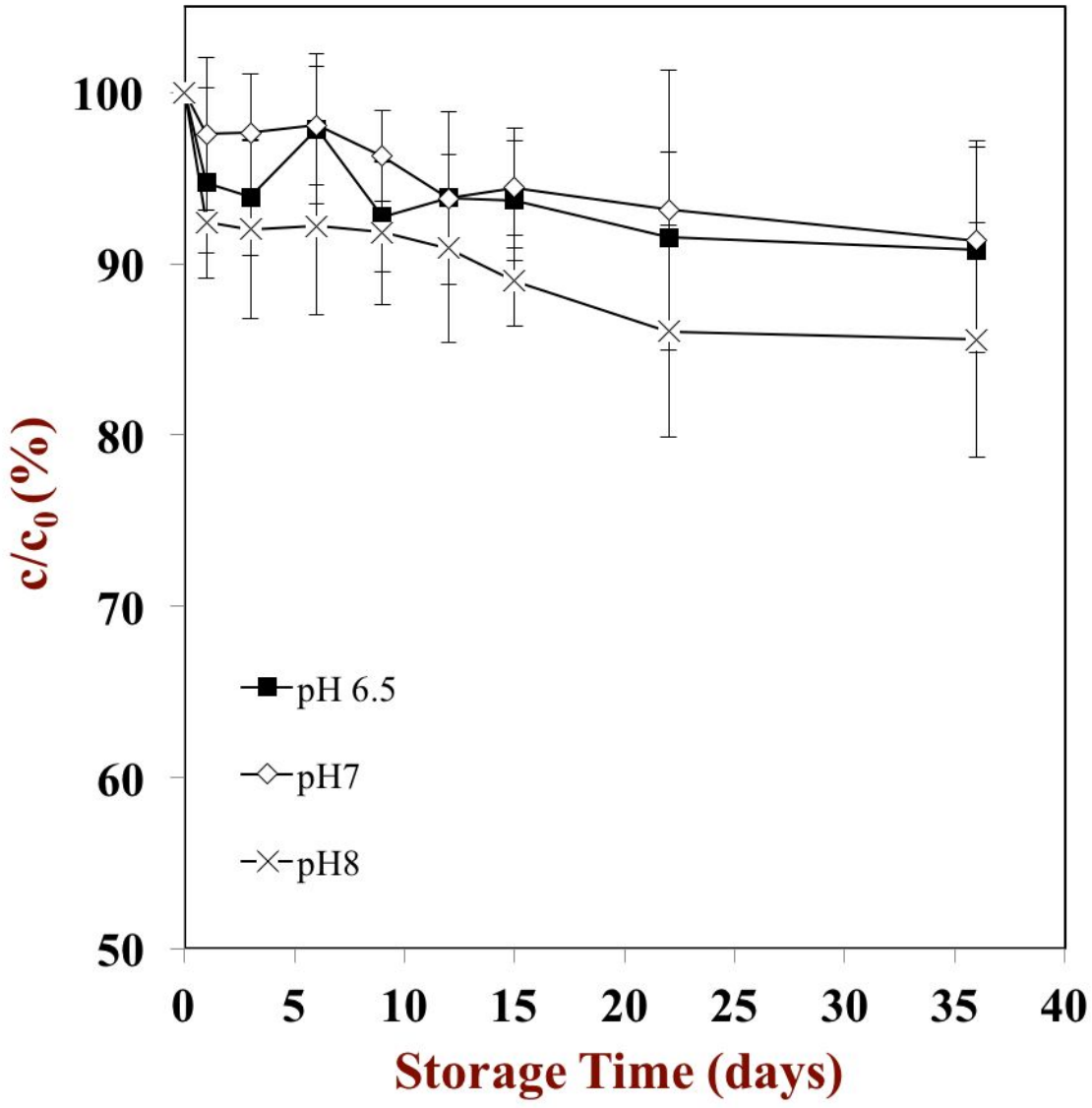
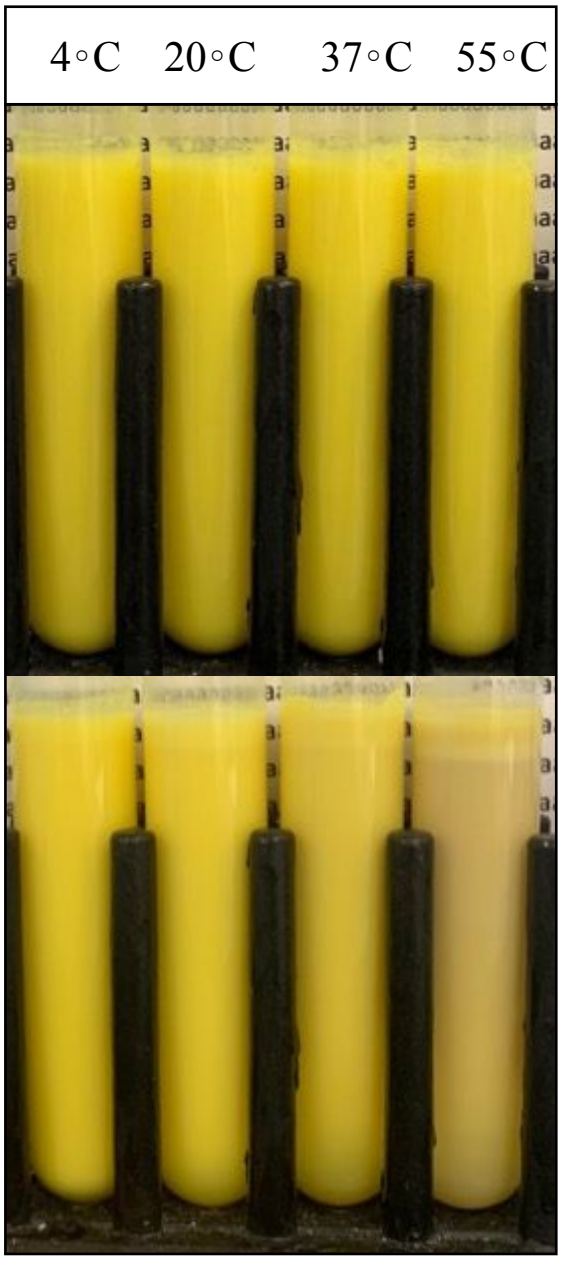
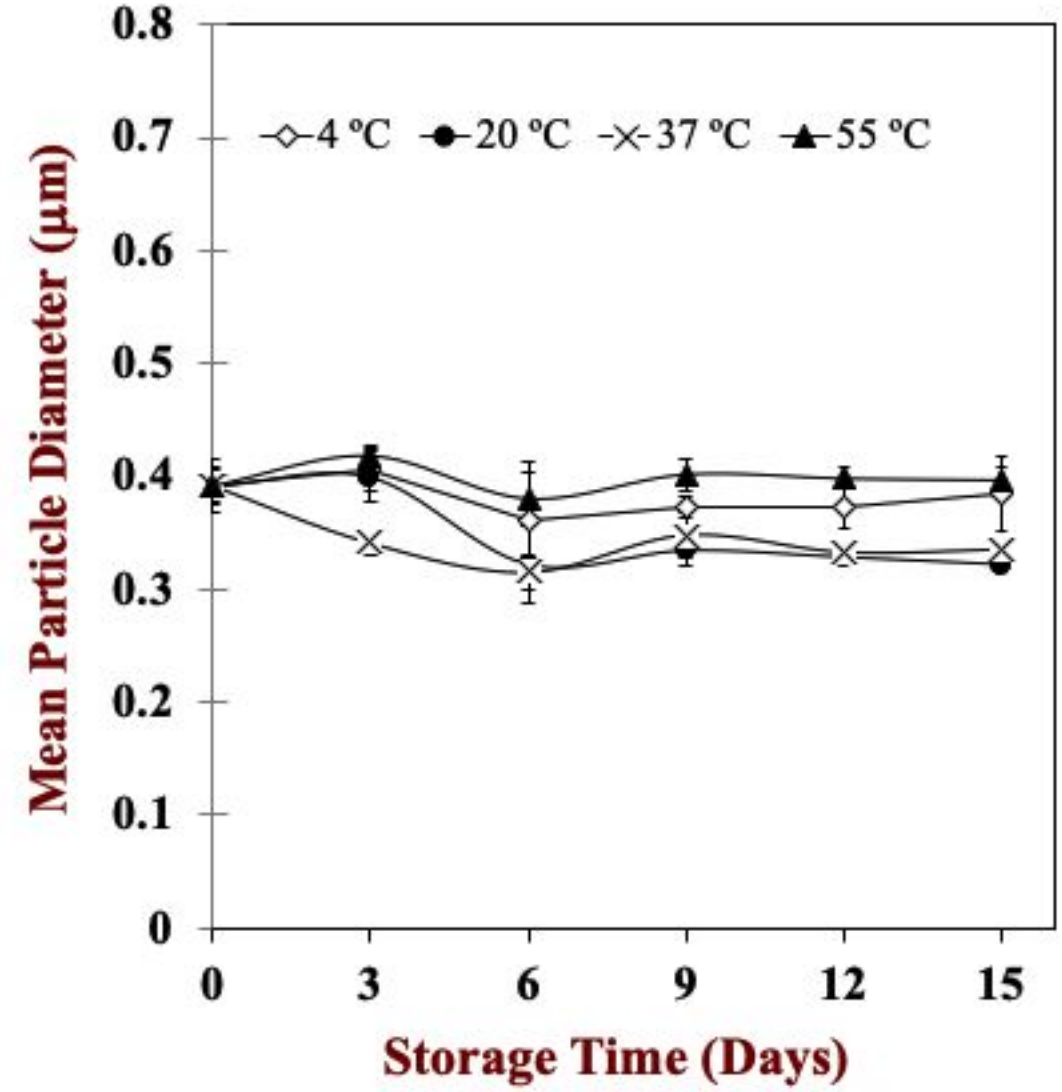


Fig. 6

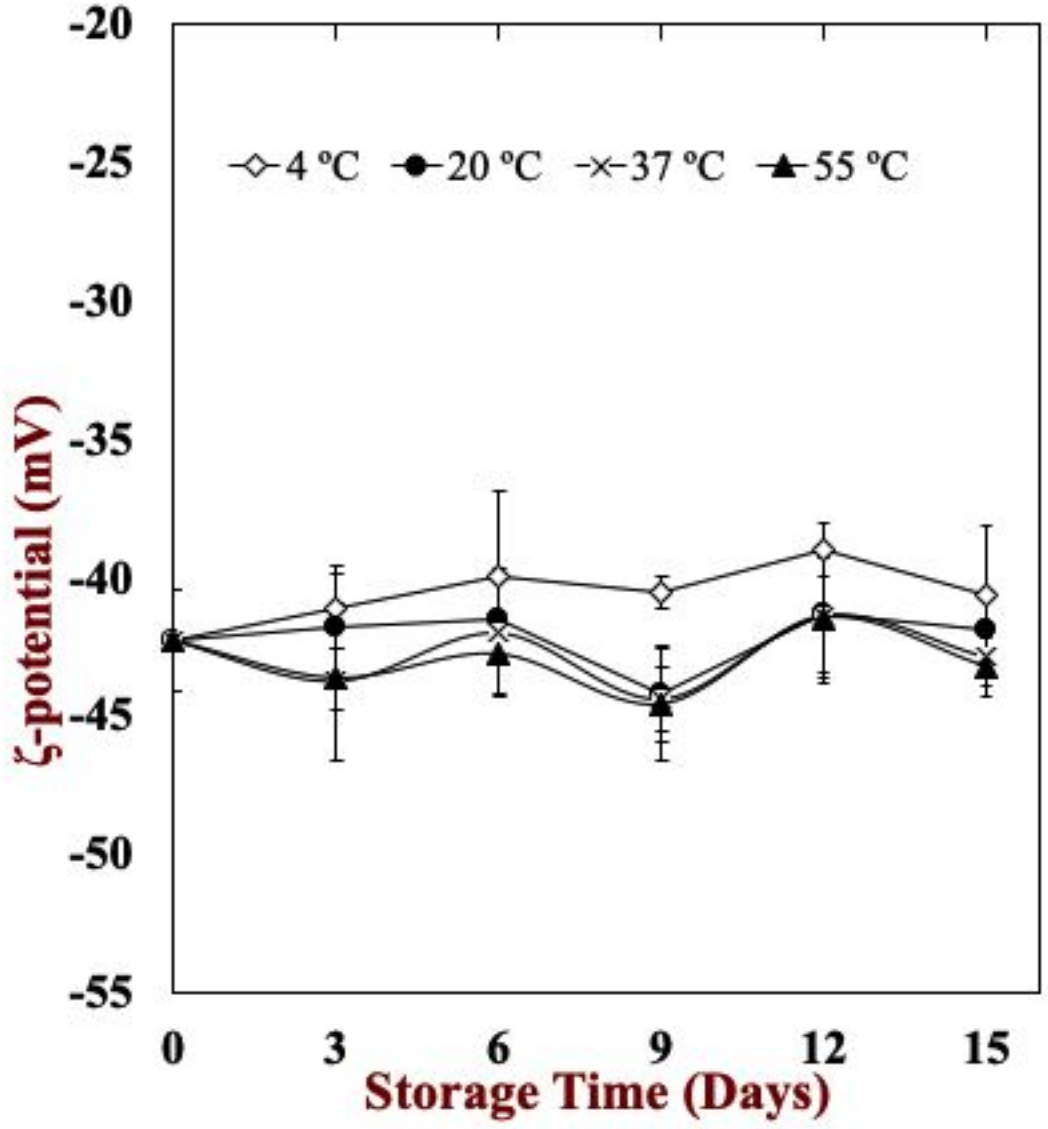
6 a



6 b


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4 ºC
20 ºC
37 ºC
55 ºC
Mean Particle Diameter (µm)
0
3
6
9
12
15
Storage Time (Days)

6 c


-20
-25
-30
-35
-40
-45
-50
-55
4 ºC
20 ºC
37 ºC
55 ºC
ζ-potential (mV)
0
3
6
9
12
15
Storage Time (Days)

6d

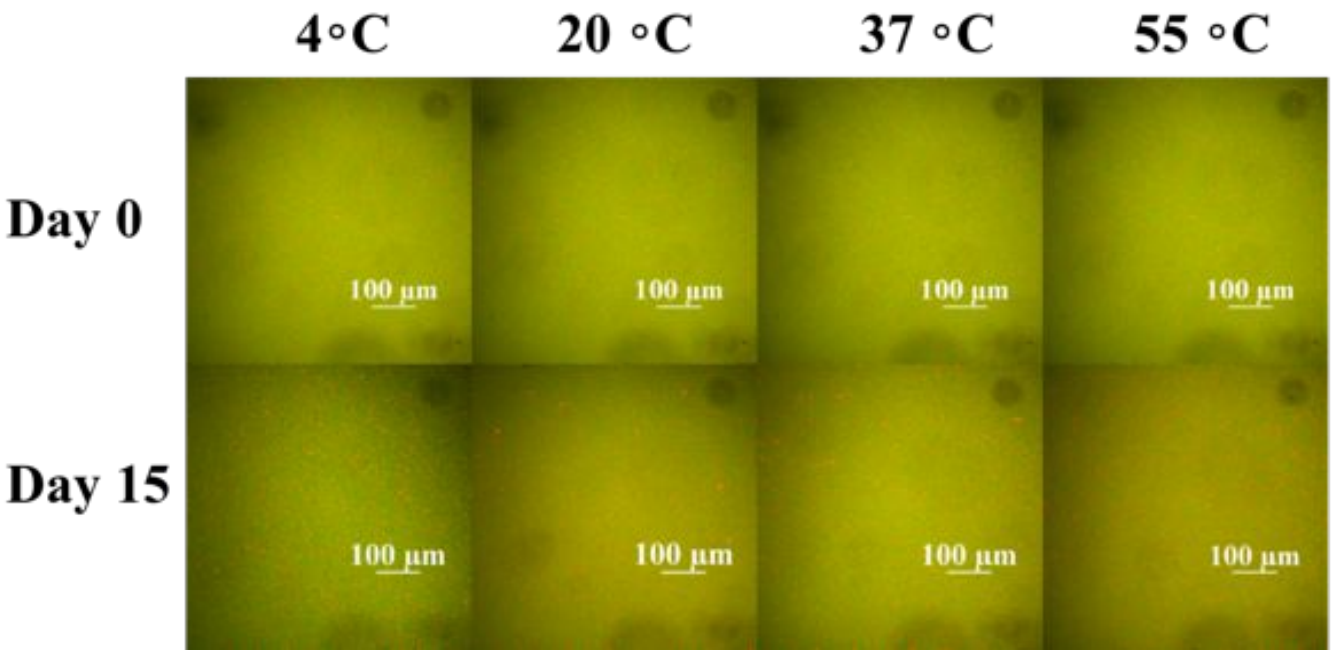


Fig. 6 Influence of temperatures on the a) appearances of curcumin-loaded soymilk before and after 15-days storage. The impact of temperature on b) mean particle diameters (D_{32}) and c) electrical characteristics (ζ- potential) of curcumin-loaded soymilks during 15-days storage. d) the microstructure of curcumin-loaded soymilks before and after incubated under four different temperatures and a length of 100 um scale bars were applied.

Fig 7a

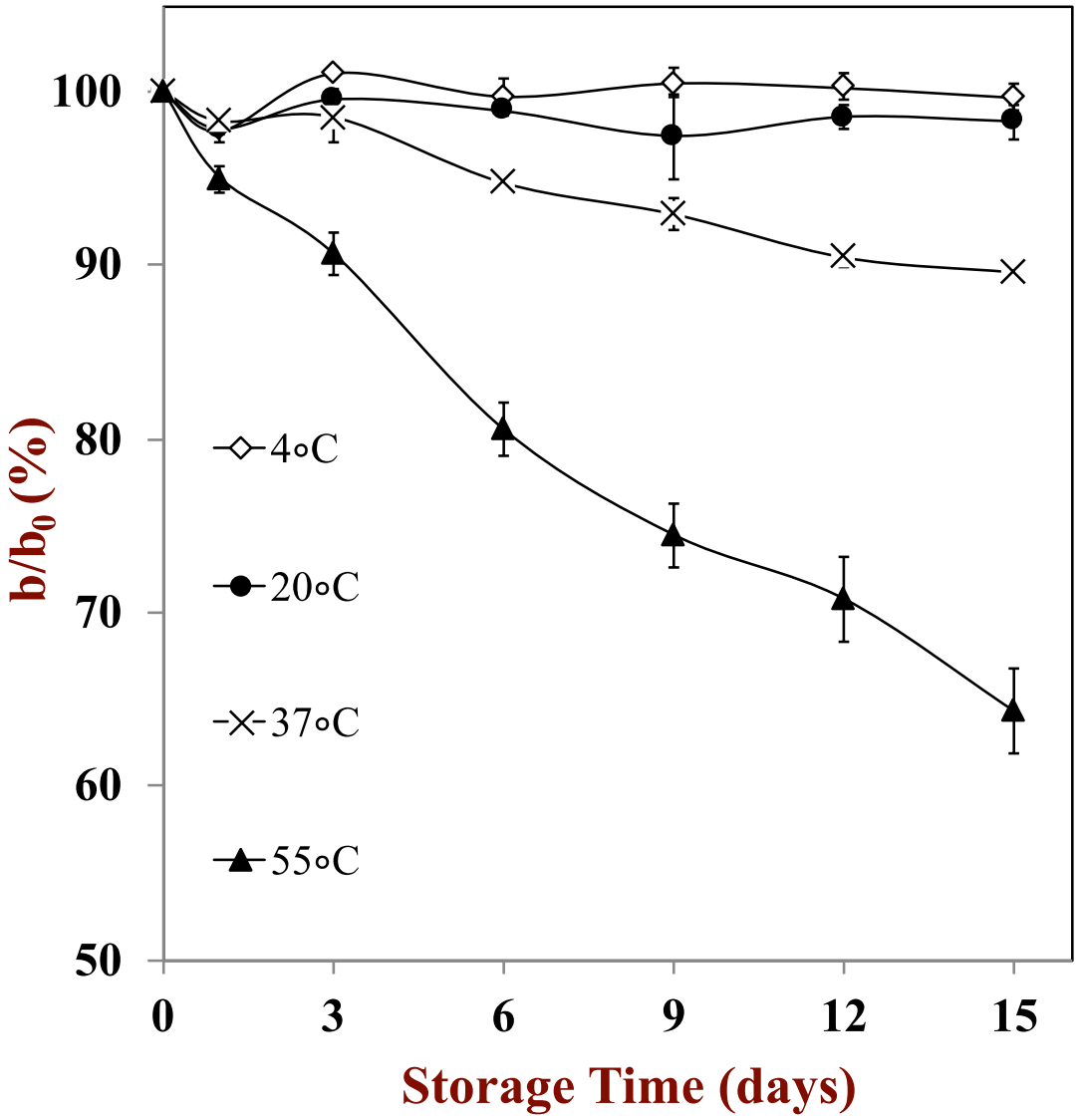

100
90
80
70
60
50
b/b0 (%)
4◦C
20◦C
37◦C
55◦C
0
3
6
9
12
15
Storage Time (days)

Fig. 7b

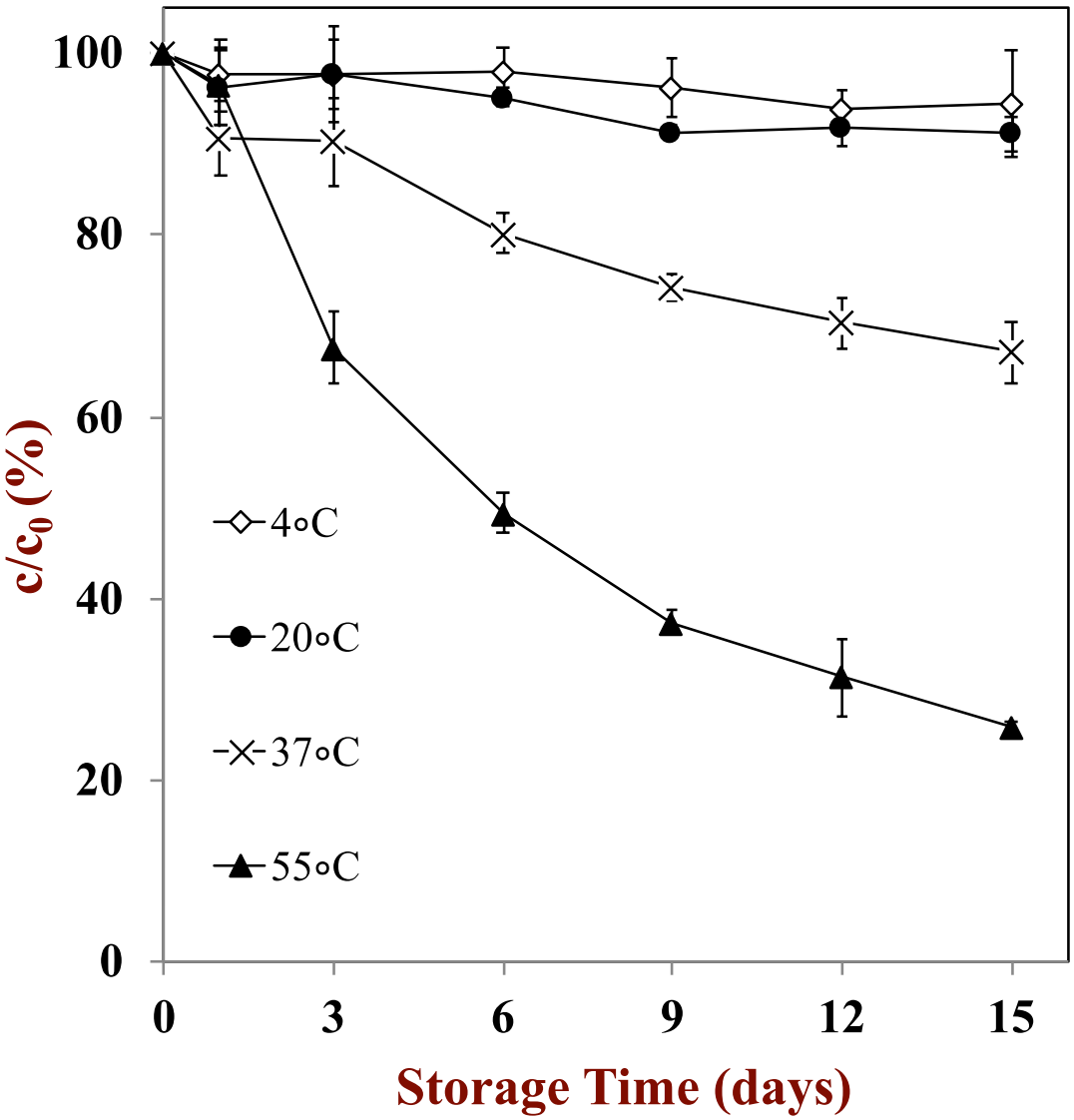


Fig. 7 Influence of temperature on a) yellow color (*b**) and b) curcumin concentration within soymilks during 15-days storage.

Fig. 8

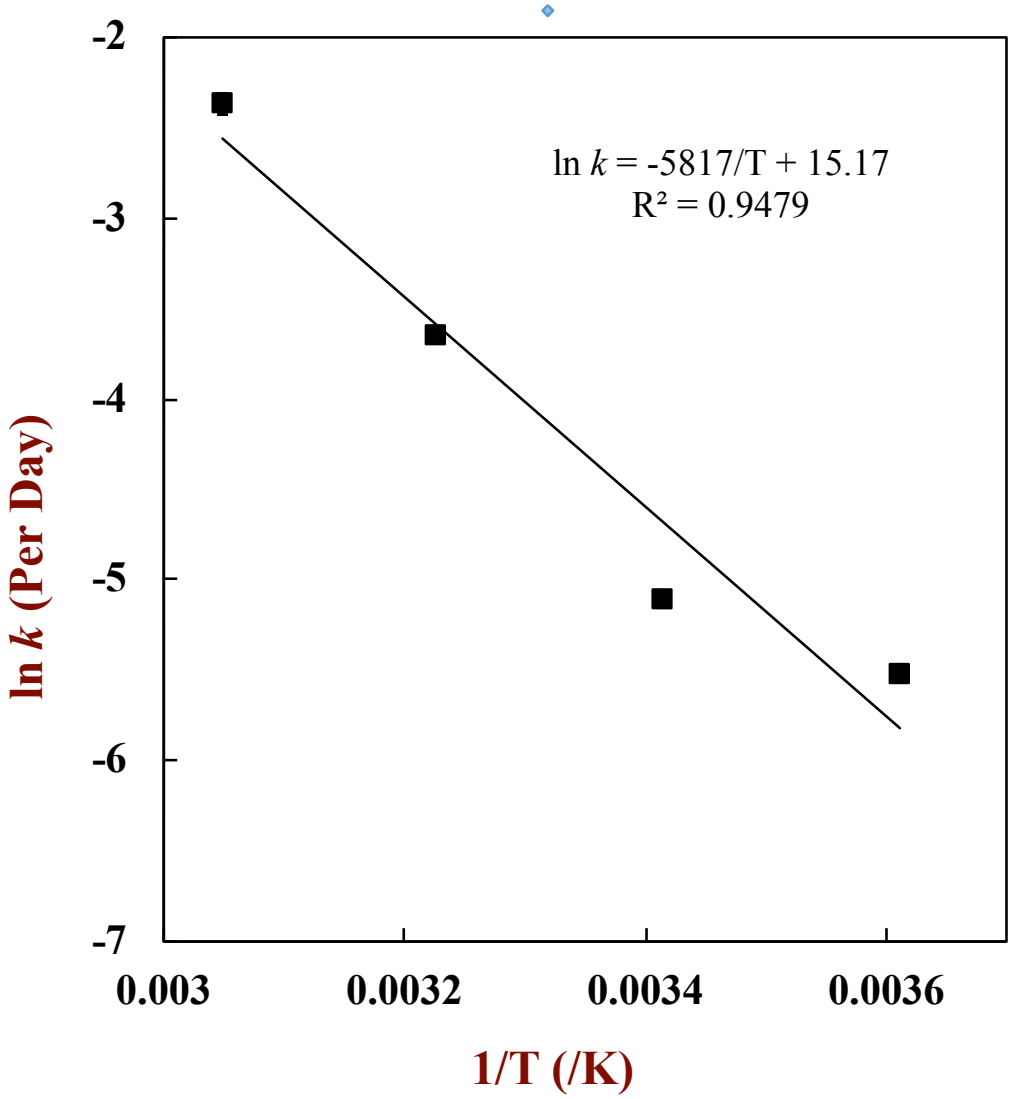
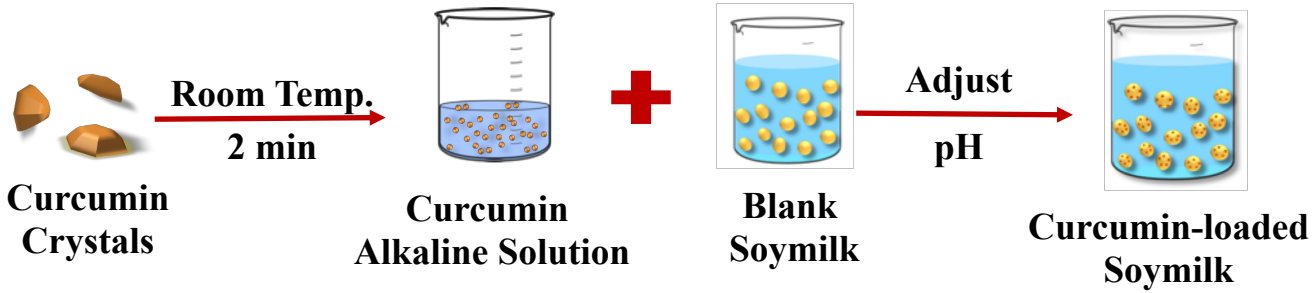


Fig. 8 Arrhenius equation graph of curcumin within soymilks, which represents the temperature dependence of the chemical reaction rate.


Room Temp.
2 min
Adjust
pH
Curcumin
Crystals
Curcumin
Alkaline Solution
Blank
Soymilk
Curcumin-loaded
Soymilk