



## Colloidal approach to prepare colour blends from colourants with different solubility profiles



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### ABSTRACT

Food colouring plays a vital and a determining role in the processing and the manufacturing of food products because the appearance of products is critical for attracting consumers and influencing their food choices. However, factors such as legislative restrictions, limited number of approved colourants and the processing, formulation and stability issues of the natural colourants severely limits the application of food colouring in actual product formats. Hence, finding alternatives to the currently utilised formulation practises, represents an important area of research. Here, we report a simple colloidal approach to prepare colour blends by co-incorporating colourants with contrasting aqueous solubility profiles in composite colloidal particles. Curcumin and indigocarmine were selected as water insoluble and water soluble food-grade colourants respectively and incorporated in the colloidal particles prepared from food protein-zein. Composite particles obtained by loading of curcumin and indigocarmine (at different ratios) had mean particle size ranging from 76 to 300 nm. The spherical shape of the colloidal particles was confirmed using transmission electron microscopy and the colloidal dispersions were further characterised using UV–Vis and fluorescence spectroscopy. The incorporation of colourants in colloidal particles led to the generation of different shade of colour in yellow–green–blue range. The encapsulation also led to the stabilization of individual pigments against photodegradation. Such composite colloidal particles could potentially serve as an approach for developing tuneable colouring system for food and nutraceutical applications.

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### 1. Introduction

Coloring in consumer products often rely on the use of chemicals that belong to the class of synthetic, nature-identical or natural colourants (Mortensen, 2006). Natural and nature-identical (produced by chemical synthesis with structures similar to ones found in the nature) colourants are often more desirable alternatives to the synthetic ones due to their comparatively better safety profiles (Wissgott & Bortlik, 1996). However, these colourants are susceptible to degradation in presence of heat, light or pH changes in aqueous environment (Astete, Sabliov, Watanabe, & Biris, 2009). Furthermore, due to the strict regulations in the food industry, there are only limited number of approved colourants and hence to obtain the desired colour shade, formulators often have to depend on the blending of different colours together to get the right shade.

However, these colourants differ in their solubility characteristics, with natural colourants being mostly hydrophobic (except for anthocyanins) in nature whereas the colourants prepared by chemical synthesis have comparatively better aqueous solubility (Henry & Houghton, 1996; Wissgott & Bortlik, 1996). Therefore, generating water dispersible blends of colourants with contrasting aqueous solubility could potentially lead to a better utilisation of natural and nature-identical colourants for obtaining newer and much more tuneable colour shades using approved colourants.

Curcumin, Cur (E100) and indigocarmine, IC (E132) (Fig. S1) are approved food colourants and are commonly used to obtain primary colour shades of yellow and blue respectively (Henry & Houghton, 1996). Apart from the primary colours (these are colours that cannot be created through the mixing of other colours), secondary colours are often required to make food products more appealing to the consumers. Green is one of most important secondary colour as far as consumer goods is concerned because as a colour, it can be associated with being fresh and 'natural'. Currently, chlorophyll and copper complexes of chlorophylls and chlorophyllins are the only known natural and nature-identical approved colourants for food applications. However, their use in food products is often limited due to their susceptibility to

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photodegradation and instability in the acidic pH (3.5–5) of most food beverages such as teas and juices (Henry & Houghton, 1996). Thus, creating an acid pH stable green colour from approved colourants represents an important research problem among the researchers working in the field of food colouring.

To address this issue, we introduce a new concept based on the use of approved colourants with different solubility profiles. A range of acid pH stable shades of green colour were generated by incorporating a natural yellow pigment and a nature-identical blue dye in protein-based nanoscale colloidal particles. It is well-known that many colourants interact with proteins via different non-covalent interactions including hydrogen bonding, ionic and hydrophobic interactions (Colyer, 2003; Gelmza, 1990; McCorquodale, Piper, & Colyer, 2002; Sereikaite, Bumeliene, & Bumelis, 2005). Both Cur and IC are also reported to interact with proteins via hydrophobic and ionic interactions respectively (Fathima, Devi, Rekha, & Dathathreyan, 2009; McCorquodale et al., 2002; Pulla Reddy, Sudharshan, Appu Rao, & Lokesh, 1999; Zhang, Zhao, & Li, 2001). Thus, the hypothesis of the current work was to co-incorporate both Cur and IC in common colloidal particles based on dye-protein interactions to obtain different shades of green colour which is stable at acidic pH and against photodegradation.

Zein, a proline-rich protein obtained from corn has been extensively studied to generate colloidal particles due to its hydrophobic nature (Patel, Bouwens, & Velikov, 2010; Zhong & Jin, 2009; Zhong, Tian, & Zivanovic, 2009). The fact that zein, Cur and IC all show good solubility in ethanol, was exploited to generate composite colloidal particles using well-established anti-solvent precipitation method (Patel, Hu, Tiwari, & Velikov, 2010; Patel, Bouwens, et al., 2010; Zhong & Jin, 2009; Zhong et al., 2009). The interaction was studied by X-ray diffraction and spectroscopic measurements (UV and fluorescence spectroscopy). Further, the stability of pigments in colloidal form was evaluated through UV irradiation studies.

## 2. Experimental

### 2.1. Materials

Curcumin (minimum 90% as per supplier's claim) was purchased from Sanjivani Phytopharma Pvt. Ltd., India. Zein and Indigocarmine were purchased from Sigma Aldrich Inc., USA. Absolute ethanol was obtained from VWR BDH Chemicals, UK and water purified by the MilliQ system was used for all the experiments.

### 2.2. Preparation of colloidal particles

Stocks of zein:Cur; zein:IC and zein:Cur:IC mixtures were prepared by dissolving accurately weighed quantities of components in 100 ml of ethanol:water (80:20 wt/wt). Colloidal dispersions were prepared by adding these stock solutions to 300 ml water with 0.02 %wt Tween 80 under continuous stirring (1000 rpm) using magnetic stirrer (Model EM3300T, Labotech Inc, Germany). To keep the final pH of dispersion at around the acidic range (pH 3.5) where zein colloidal particles are most stable, the pH of water used to prepare stock solutions and the dilution was adjusted to 3.5 using 0.1 M HCl. Samples for solid state characterization including XRD were obtained by subjecting the colloidal dispersion to freeze drying at  $-85^{\circ}\text{C}$  using a vacuum of  $-0.040$  mbar (Lab-conco Freezone 6 plus, Labconco Corporation, USA).

### 2.3. Analysis of particle size and surface potential

The particle size (volume weighted mean,  $d_{4,3}$  and  $\zeta$ -potential of dispersions were measured using Zetasizer Nano ZS (Malvern

Instruments Ltd., UK) after appropriate dilution (with water at pH 3.5). All measurements were carried out at  $20^{\circ}\text{C}$  and the results reported are averages of three readings.

### 2.4. Transmission electron microscopy

The shape of colloidal complexes was analysed by taking TEM photographs using Technai transmission electron microscope (FEI Company, The Netherlands). The dispersion was diluted in MilliQ water and one drop of the diluted dispersion was placed on a 200-mesh carbon coated copper grid. The photographs were taken at various magnifications and 100 kV voltage.

### 2.5. Spectroscopy measurements

Absorbance spectra of the samples were obtained after appropriate dilution (with water adjusted to pH 3.5) using UVIKON XL UV-Vis spectrophotometer (SECOMAM, France). The scanning range was 200–800 nm at a rate of 200 nm/min. Colloidal dispersions were diluted 50 times to get within the linearity range of colourants. Fluorescence emission spectral scans were obtained using Microplate Spectrofluorometer SpectraMax M2 Microplate Reader (Molecular devices Corp., USA). The samples were excited at 494 nm (with a cut off filter at 515 nm), the emission spectral scan was recorded from 510 to 600 nm.

### 2.6. X-ray diffraction

Diffraction lines of the samples were obtained with a Bruker AXS (Karlsruhe, Germany) D8 Discover diffractometer. The instrument was equipped with a copper anode that produces Cu K $\alpha$  X-rays with an accelerating voltage 40 kV and a tube current 40 mA. The diffractogram was collected with a monochromator of 0.3 mm during 300 s. An angular range of  $\theta_1$  at  $4.5^{\circ}$  and  $\theta_2$  at  $10^{\circ}$ ,  $25^{\circ}$  and  $40^{\circ}$ , were used with a  $\theta$  rocking of  $1^{\circ}$  and XY amplitude of 2 mm resulted in a 2 $\theta$  between  $4^{\circ}$  and  $55^{\circ}$  after merging the separate recordings. Rocking and amplitude oscillation were used to obtain an average diffractogram of the sample and minimize a preferred orientation of crystals.

### 2.7. Photostability studies

Molecular solutions of Cur, IC and colloidal dispersions (zein:Cur and zein:IC) with concentrations of pigments equivalent to the solutions were subjected to ultraviolet light (302 nm) using a Gel Doc™ 1000 single wavelength minitransilluminator (Bio-Rad Laboratories, The Netherlands) for 70 min. Sample concentrations used for each samples was 500  $\mu\text{g}/\text{ml}$  of the colourant(s); the samples were stored in disposable cuvettes (path length 1 cm); distance from illuminant 10 cm and source of illumination was a standard 302 nm UV lamp. The sampling was carried out every 5 min and the amount of Cur and IC in the samples was analysed spectrophotometrically at 424 and 620 nm respectively.

## 3. Results and discussion

### 3.1. Preparation and characterization of colloidal particles

The preferential solubility of zein in binary solvents comprising of lower aliphatic alcohols and water is well documented (Dill, 1927; Evan & Manley, 1941; Wilson, 1987) and has also been utilised by us in the past to generate discrete spherical colloidal particles of zein using anti-solvent precipitation method (Patel, Heussen, Hazekamp, Drost, & Velikov, 2012; Patel, Bouwens, et al., 2010; Patel, Hu, et al., 2010). This method is based on the

**Table 1**

Average particle size and  $\zeta$ -potential values for dispersions containing zein:Cur (20:1); zein:Cur:IC (20:1:0.25; 20:1:0.5; 20:1:1; 20:0.5:1 and 20:0.25:1) and zein:IC (20:1).

Samples (Zein:Cur:IC)	Cur ( $\mu\text{M}$ )	IC ( $\mu\text{M}$ )	Mean particle size (nm)	$\zeta$ -potential (mV)
20:1:0	27	0.0	176.2	26.0
20:1:0.25	27	5.25	216.2	25.1
20:1:0.5	27	10.5	299.4	17.5
20:1:1	27	21	214.8	15.7
20:0.5:1	13.5	21	278.3	14.7
20:0.25:1	6.75	21	197.2	14.3

principle of spontaneous precipitation of zein from the primary solvent (ethanol:water) when mixed with a second fluid phase (water) which is miscible with the primary solvent but not a good solvent (anti-solvent) for zein (Patel, Heussen, et al., 2012).

Both Cur and IC could be dissolved in the ethanol:water mixtures and hence composite colloidal particles were prepared by simultaneously precipitating zein along with Cur and IC from aqueous ethanolic solution. The resultant colloidal dispersions containing zein:Cur:IC in different ratios (Table 1) on weight basis are shown in Fig 1. Different shades of green could be obtained by varying the Cur:IC ratio. The resultant dispersion had a pH of 3.5 and the other parameters including average particle size and  $\zeta$ -potential values are reported in Table 1. The average volume weighted particle size of dispersion varied from 76 to 300 nm (Fig. S2) with polydispersity index of less than 0.25. Polydispersity index is the ratio of standard deviation and average particle size, hence a lower polydispersity index indicates a narrow size distribution and also suggests effectiveness of the method to prepare particles. The particle size values did not show any particular trend, but it was noticed that the colloidal particles with single pigment (zein:Cur and zein:IC) showed lower sizes as compared to the particles with co-incorporated pigments (Zein:Cur:IC). In case of the  $\zeta$ -potential values, zein colloidal particles usually shows a high positive charge due to the ionisation of the amino acids in aqueous environment (Patel, Bouwens, et al., 2010), Cur is a neutral molecule and interacts with zein based on hydrogen bonding and hydrophobic interaction thus the surface charge of zein:Cur

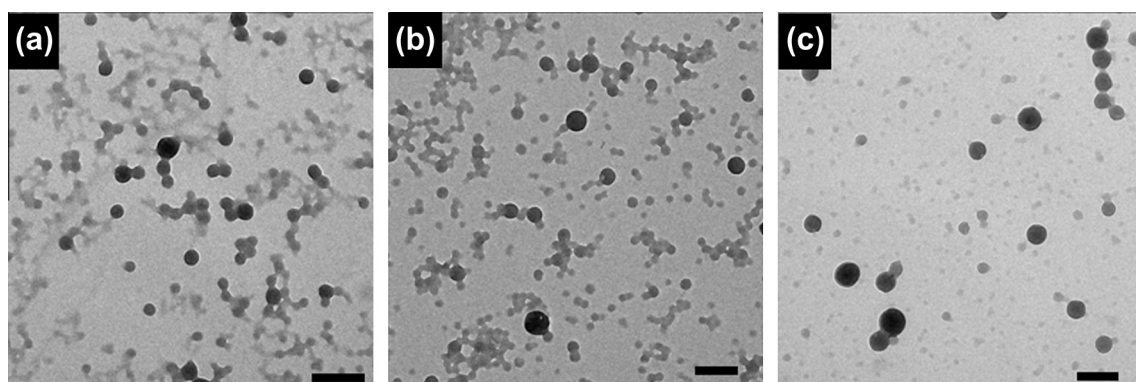
composite colloidal particles remains in the same range as that of blank zein particles as reported in our earlier study (Patel, Hu, et al., 2010). IC on the other hand is anionic in nature and we observed that there was a decrease in the  $\zeta$ -potential values of colloidal dispersion due to the presence of IC. The lowering of positive surface charge also indicates the electrostatic nature of the interaction between zein and IC. In all cases, the  $\zeta$ -potential value was less than 30 mV, thus Tween 80 was included at 0.02% wt level in the precipitating aqueous medium to provide steric stabilization. In general, there are two contrasting forces that determines the stability of colloidal particles: van der Waals attraction and electrostatic repulsion. When particles collide and approach too close to each other (due to Brownian motion), the relative strength of attractive or repulsive forces determine if the particles just “bounce-off” each other or aggregate after collision. Hence, the colloidal particles can be stabilized against aggregation by either electrostatic repulsion (due to the high surface charge of particles) or by forming a barrier around the particles (steric stabilization) that prevents close approach of particles and hence opposing van der Waals attractive forces which are a function of the closeness of the particles to each other. The values of  $\zeta$ -potential (<30 mV) indicates that the electrostatic repulsion is not enough to provide stability. Tween 80 was used to form a barrier layer around particles which prevents the approach of particles to a distance that is too close to each other. The particle size and  $\zeta$ -potential of particles formed without Tween 80 were in similar ranges but the samples showed aggregation on storage due to the lack of stabilization.

### 3.2. Morphology studies

TEM images shown in Fig. 2 confirms the spherical morphology of the colloidal particles formed using zein:Cur (20:1 wt/wt); zein:Cur:IC (20:1:1 wt/wt/wt) and zein:IC (20:1 wt/wt). As reported by us earlier, precipitating zein from aqueous ethanol in water results in the formation of spherical shaped particles (Patel, Bouwens, et al., 2010; Patel, Hu, et al., 2010). The formation of spherical shaped polymeric particles obtained by anti-solvent precipitation method has been studied extensively (Beck-Broichsitter, Rytting, Lehardt, Wang, & Kissel, 2010; Quintanar-Guerreiro, Allemann, Fessi, & Doelker, 1998; Sternling & Scriven, 1959).



**Fig. 1.** Colloidal dispersions with a tuneable yellow–green–blue colour containing (from left to right) zein:Cur:IC (20:1:0; 20:1:0.25; 20:1:0.5; 20:1:1; 20:0.5:1; 20:0.25:1 and 20:0:1). The ratios are expressed on weight basis and the total concentration of zein in all cases was 1 wt.%.



**Fig. 2.** Representative TEM images of colloidal particles made from (a) zein:Cur (20:1); (b) zein:IC (20:1) and (c) zein:Cur:IC (20:1:1), scale bars = 500 nm.



The particle formation and growth is influenced by nucleation and diffusion controlled Ostwald ripening (Sitnikova, Sprik, & Wegdam, 2005; Vitale & Katz, 2003).

### 3.3. Spectral studies

Both Cur and IC absorb light strongly in the visible range at around 426 and 620 nm respectively which is in agreement with the reported literature (Roberts, Burguieres, & Warner, 1998; Tonnesen, 1992). On comparing the spectra of Cur and IC with that of colloidal dispersions (Fig. S3), we observed a prominent hypochromic effect at 424 nm and bathochromic shift (from 611 to 619 nm) for Cur and IC respectively indicating interactions of dyes with protein zein.

Fig. 3a shows the experimental spectra of colloidal dispersions with different ratios of Cur and IC. The absorbance of a dilute colloidal dispersion containing particles with both scattering and absorption properties is given by the Beer–Lambert law  $I = I_0 \exp(-NC_{\text{Ext}}(\lambda)L)$ , where  $C_{\text{Ext}} = C_{\text{Abs}} + C_{\text{Sca}}$  is the extinction cross-section which equals to a sum of the absorption ( $C_{\text{Abs}}$ ) and scattering cross-sections ( $C_{\text{Sca}}$ ),  $N$  is the number particle concentration and  $L$  is the optical path length. If we assume that the scattering cross-section and particle concentration is equal in all cases, for same optical path length, the absorbance spectra will be determined by the concentration and absorption properties of the two colourants embedded in the zein particles. In this case, we can express the measured wavelength dependent absorbance  $A_{\text{tot}} = -\log(I/I_0)$ , as a sum of the absorbance of the two colourants:  $A_{\text{tot}}(\lambda) = L\varepsilon_1(\lambda)c_1 + L\varepsilon_2(\lambda)c_2$ , where  $c_i$  is the total concentration of the colourant and  $\varepsilon_i$  is the molar absorptivity with units of  $\text{mol}^{-1} \text{cm}^{-1}$ . In order to understand the spectra evolution of the colloidal dispersion of zein composite particles containing different concen-

tration of the two colourants, we calculated the spectra of the dispersions using the assumption that the absorption properties are linear combination of the absorption properties of the two colourants which ignores the contribution of the scattering of the colloidal carriers (i.e. zein particles). The theoretically calculated spectra using the spectra of zein:Cur and zein:IC colloidal particles and the corresponding relative concentrations of Cur and IC are shown in Fig. 3b and were found to resemble the theoretical spectra very well. In the follow-up section we further describe the optical properties at 510 nm which also further correlates well with the experimental values and thus confirms the suitability of model used. Fig. S4a shows the plots of absorbance at 510 nm (corresponding to the mid  $\lambda$  for spectra of green colour) versus relative concentration of one of the colourants. The intensity of absorbance was maximum as the shade of colour approached green at near equivalent proportion of both colourants. When the absorbance is extracted from the calculated spectra of the linear combination of the spectra of particles containing the individual colourants, very similar dependence is observed (Fig. S4b). This is a confirmation that the colour shade in the composite particles is indeed dominated by the absorption spectra of the embedded individual colourants. Due to small particle size of the protein particles the contribution of light scattering is small. This allows flexibility and tuneability in design of both the degree of shades and the colour intensity.

Cur is strongly fluorescent when excited at 494 nm (Chignell et al., 1994) whereas IC is very weakly fluorescent (Zhang et al., 2001) and likewise fluorescence of IC could not be detected at the concentrations used in preparing colloidal dispersions. In the case of Cur, the incorporation of pigment in colloidal particles resulted in a blue shift by 10 nm and a prominent hypochromic effect (Fig. 4). Cur molecule has two aryl buten-2-one (feruloyl) chromophores joined by a methylene group and its spectral properties are probably due to the interaction of two chromophores via the conjugation of their  $\pi$ -electrons (Chignell et al., 1994). Striking effect was observed on the fluorescence of Cur, with a progressive hypochromic effect with increasing proportion of IC. The quenching of fluorescence due to the presence of IC indicates an interaction between the phenolic hydroxyl groups of the chromophore with the amino group of IC.

### 3.4. X-ray diffraction studies

Crystalline compounds are converted to amorphous forms when loaded into the colloidal particles (Patel, Drost, den Adel, Hazekamp, & Velikov, 2012; Patel, Heussen, Hazekamp, & Velikov, 2011; Patel, Hu, et al., 2010; Patel et al., 2012). The X-ray diffractogram (Fig. S5), showed sharp peaks at diffraction angles (2 theta) of 8.9, 12.1, 14.4, 17.1, 18.0, 21.0, 23.2, 24.3, 25.3, 27.1 and 28.8 for Cur powder and at diffraction angles (2 theta) of 15.5, 17.7, 23.2, 24.8, 27.0, 30.8 and 33.6 for IC powder, suggesting highly crystalline nature of these pigments. In contrast, the composite particles (Z:Cur:IC = 20:1:1) showed absence of all the peaks, suggesting formation of amorphous Cur and IC due to the suppression of their crystallisation in the nanoscale confinement and possibly due to the formation of an amorphous complex with protein within the particle matrix. Similar effects were observed when crystalline polyphenols were co-precipitated with zein to form amorphous particles (Patel, Heussen, et al., 2012; Patel, Hu, et al., 2010).

### 3.5. Photostability studies

Cur and IC are both photolabile and their colour solutions are known to fade under light illumination (Galindo, Jacques, & Kalt, 2001; Singh et al., 2010). The major photodegradation products formed are vanillin and isatin respectively (Song, Zhang, & Gao,

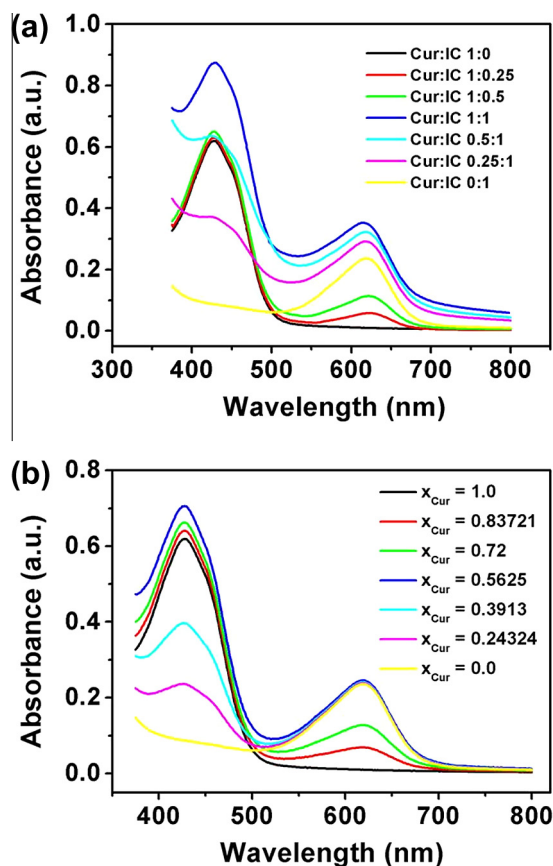
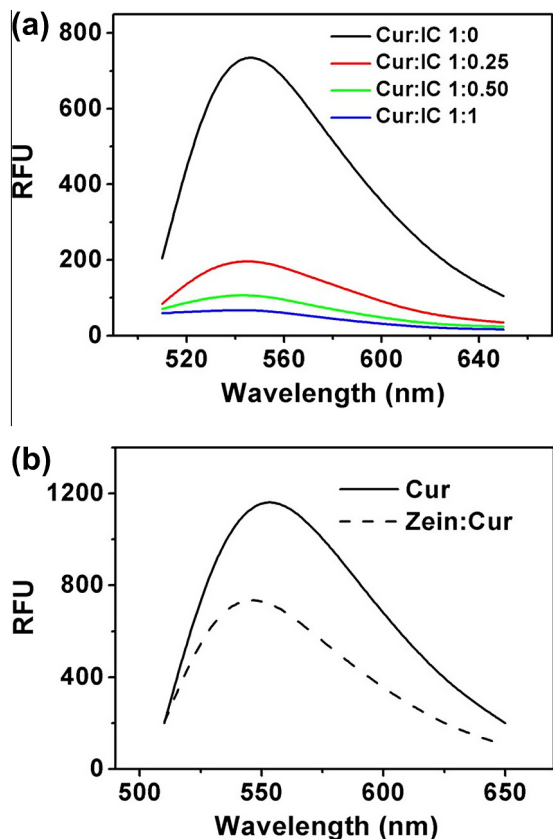
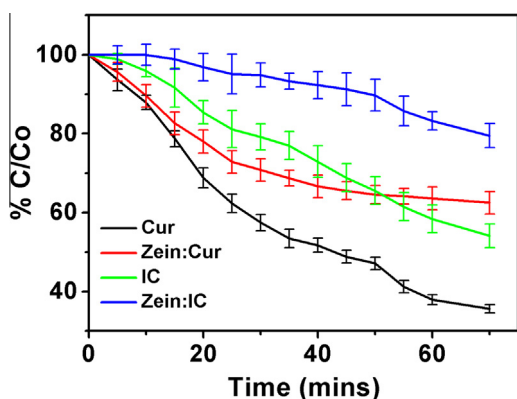


Fig. 3. Experimental (a) and calculated (b) spectra of normalised absorbance of composite colloidal dispersions prepared at varying ratios of Cur:IC.



**Fig. 4.** (a) Fluorescence spectra of colloidal dispersions containing different ratios of Cur:IC (1:0.25; 1:0.5; 1:1); (b) Comparative fluorescence spectra of Cur (molecular solution) and Cur loaded in colloidal particles with zein:Cur ratio of 20:1. Excitation wavelength = 494 nm (with a cut off filter at 515 nm).



**Fig. 5.** Comparative graph showing the percent of unchanged Cur and IC against UV irradiation time (at 302 nm) for molecular solutions of Cur, IC and colloidal dispersions containing zein:Cur (20:1) and zein:IC (20:1).

2009; Sousa et al., 2008). Formulating a reactive species into colloidal form can significantly change its physical characteristics such as aqueous solubility which in turn determines its stability against light degradation in solution. In our case, since a small amount of colourants are loaded in the particles, these small molecules are embedded in the polymeric matrix of the particles that minimizes the degradation of colourants caused by light in aqueous environment. Based on the results of UV irradiation study, both Cur and IC showed enhanced photostability in colloidal form. Fig. 5 shows a comparison graph of percent of unchanged Cur and IC against UV

irradiation time for molecular solutions of Cur, IC and colloidal dispersions prepared using zein:Cur (20:1) and zein:IC (20:1). As seen from the graph, molecular solutions of both Cur and IC degraded quite rapidly under UV light with concentration dropping to 35% and 55% of their initial concentration respectively at the end of 70 min. On the other hand, entrapment in the biopolymeric matrix of zein resulted in decreasing their photodegradation as observed from the final values of 63% and 79% of their initial concentrations at the end of 70 min. It can be suggested that the presence of a hydrophobic zein matrix around photo labile molecules of Cur and IC provides protection against the damaging effects of UV radiations by minimizing the interaction of light rays with the entrapped molecules. This is in agreement with our previous study where we reported that the loading of a photolabile molecule in colloidal particles resulted in significant enhancement of its photostability (Patel, Hu, et al., 2010).

#### 4. Conclusion

In conclusion, a simple concept of obtaining colour blends from a mixture of two colourants with different solubility profiles was successfully demonstrated. The colloidal approach was based on the incorporation of both curcumin (water insoluble) and indigocarmine (water soluble) in composite colloidal particles of zein. Different shades of colour in yellow–green–blue range were generated by simply loading different ratios of curcumin and indigocarmine in the composite particles with a mean particle size ranging from 76–300 nm. The absorption properties of the composite particles are dominated by the absorption spectra of the embedded individual colourants. Further, the entrapment of pigments in colloidal particles resulted in the enhancement of the stability of both curcumin and indigocarmine towards photodegradation. This finding can be viewed as an important step towards the promising research of generating other colour blends of choice from natural and nature-identical colours which are approved for food applications.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.03.082>.

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