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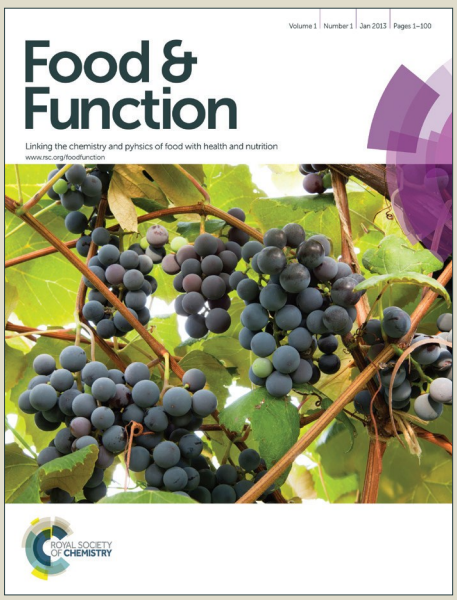

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Title: Formulation and Characterization of Nanoencapsulated Curcumin using Sodium Caseinate and its Incorporation in Ice cream

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In the present investigation, preparation and characterization of curcumin nanoemulsion with milk protein (sodium caseinate) and further its incorporation into the ice cream was undertaken. Among the different combinations, most stable formulation was observed with milk fat (8%), medium chain triglycerides (2%), curcumin (0.24%) and sodium caseinate (6%) with a mean particle size (333.8 ± 7.18 nm), zeta potential (-44.1 ± 0.72 mV) and encapsulation efficiency (96.9 ± 0.28%), respectively. The effect of different processing conditions (heating, pH and ionic strength) on particle size distribution and zeta potential of nanoemulsion was evaluated. During heat treatment, the particle size of nanoemulsion was increased from 333.8 ± 7.18 to 351.1 ± 4.04 nm.The nanoemulsion was destabilized at pH 4.6 and particle size increased above and below pH 5.0. However, there was a slight increase in the particle size with change in ionic concentration. Release kinetics data suggested that in simulated gastro-intestinal digestion, nanoemulsion was stable to pepsin digestion (5.25% release of curcumin), while pancreatic action led to 16.12% release of curcumin from the nanoemulsion. Finally, our formulation was successfully incorporated into the ice cream and sensory attributes were evaluated. No significant difference was observed in the scores of sensory attributes between control and ice cream prepared with curcumin nanoemulsion. Moreover, the encapsulation efficiency of curcumin incorporated into the ice cream was 93.7%, which indicates that it can withstand the processing conditions. The findings suggest that the ice cream is a suitable dairy product for the delivery of lipophilic bioactive component which can be used for therapeutic purposes.

Keywords: Curcumin, Nanoemulsion, Sodium Caseinate, Milk Protein

1. Introduction

Now-a-days, treatments available for human diseases (obesity, diabetes and cancer) are very cost effective. Alternatively, people are looking towards naturally occurring bioactive components in plants. The bioactive components(*e.g.* curcumin) can be incorporated into food systems for the development of health promoting functional foods and it has been growing recently.[1] Curcumin (diferuloylmethane) is a natural hydrophobic yellow colour pigment extracted from turmeric (*Curcuma longa*) and widely used as dietary spice or colouring agent in India and Asian countries.[2] *Curcuma longa* contains three different types of curcuminoids *viz*., curcumin (80%), demethoxycurcumin (15%) and bisdemethoxycurcumin (5%), respectively.[3,4] It is also being used as a remedy since ancient times because of several pharmacological properties such as anti-oxidant, anti-inflammatory, anti-parasitic, anti-mutagenic, anticancer, chemoprotective, hepatoprotective, antimicrobial and antiviral activities.[5-8] At high dose (8.0 g per day) of curcumin used in phase I human clinical trials associated with limiting toxicity.[9] But its application as a functional ingredient is currently limited because of its poor water-solubility, extremely low absorption & bioavailability and rapid degradation at neutral and alkaline pH conditions.[4] To improve bioavailability of curcumin, several researchers have been investigated with different carriers like albumin,[10,11] β-lactoglobulin,[12] phospholipids,[13] chitosan,[14] cyclodextrins,[15] polyvinyl alcohol/polyvinyl alcohol hydrogel,[16] bovine whole casein micelles[17] and whey protein concentrate[18], respectively.

Different methods (amorphous solid form, solid dispersions, melt extrusion, liposomes and nanocarriers) are available to overcome the difficulties with hydrophobic compounds.[19,20] Micro/nano encapsulation of bioactive molecules helps easily incorporation into the food and beverage systems to improve their poor solubility, sensitivity to processing conditions, masking of colour and adverse effect on the sensory attributes. However, there is currently limited restriction of food grade surfactants because of synthetic in nature, economic or sensory issues and are not acceptable in all countries.[21] Nanoemulsions are transparent heterogeneous mixture of oil in water (O/W) and stabilized by emulsifiers. As compared to conventional microemulsions, nanoemulsions are thermodynamically stable due to their nano sized structures.[22] These are predominately produced by either high energy emulsification (high pressure homogenization) or by low energy emulsification.[23]

Milk proteins such as casein are used in the stabilization ofemulsions as emulsifier and encapsulation of lipophilic components. Casein can form thicker interfacial layer (10 nm)

around the lipid droplets compared to whey proteins (1-2 nm). In addition, they are easily degraded in the gastric enzymes because of open tertiary structure (due to high proline content) to release encapsulated materials.[24] Moreover, in food industry sodium caseinate (NaCas) is widely used as emulsifier because of their emulsification, water & fat binding, thickening, gelation, increase of viscosity and stability properties.[25] Pharmaceutical and food sectors are gained much attention towards developing a suitable delivery systems for the incorporation of curcumin O/W emulsions and need to be stable in the food products. In view of above, the present investigation an attempt has been made to formulate and characterize O/W curcumin nanoemulsion with NaCas and subsequently incorporated in the ice cream because it is an ideal food system, nutritious and widely consumed dessert by the all age-groups.

2. Materials and methods

2.1. Materials

Curcumin (purity ~97%, from *Curcuma longa L.*) was obtained from Plant Lipids Pvt. Ltd. (Kerala, India). Medium chain triglycerides-60 (MCT-60) was procured from Kamani Oil Industries Pvt. Ltd. (Mumbai, India). Sodium caseinate was the product of Thomas Baker Pvt. Ltd. (Mumbai, India). Pepsin and pancreatin were purchased from Sisco Research Laboratories Pvt. Ltd. and Hi Media laboratories Pvt. Ltd. (Mumbai, India), respectively. All other reagents and chemicals were of analytical grade.

2.2. Solubility of curcumin in different oils

The solubility studies of curcumin at room temperature, five different oils such as butter, palm, olive, sunflower and MCT-60 were used. Different concentrations (0.12-0.6%) of curcumin was added to 5 mL of each oil in test tubes and mixed using vortex mixer to dissolve curcumin properly. The mixture was then centrifuged at 1,300 x g for 10 min in order to check visible undissolved particles. The maximum concentration at which no undissolved sediments of curcumin appeared on centrifugation was termed as solubility (mg/mL).

2.3. Preparation of O/W curcumin nanoemulsion

A two-step nanoemulsion was prepared according to Jafari *et al*., (2007)[26] with slight modification. Among the different formulations we tried, the most stable O/W curcumin emulsion was observed with curcumin (0.12-0.6%), MCT-60 (1-5%), milk fat (5-9%) with NaCas (1-7%) alone and used further in this study (Fig. 1). To formulate O/W emulsion, an aqueous phase containing emulsifier NaCas (6%) was dissolved completely in milk fat (8%)

by stirring at 60 °C and curcumin (0.24%) which is previously solubilized in MCT-60 (2%) was added into it and termedas oil phase. The entire formula was magnetically stirred for 15 minutes to obtain a coarse emulsion and further it was passed throughthe homogenizer (200 kg cm^{-2}, GOMA Engineering Pvt. Ltd., Mumbai) with two stage cycles. The final emulsion was lyophilized (-80 °C and <10 mm mercury pressure, LYPHLOCK, Labconco, Kansas City, MO) to get the freeze dried powder.

2.4. Characterization of the nanoemulsion

2.4.1. Particle size and zeta potential measurement

The particle size distribution and polydispersity index (PDI) of the nanoemulsion was determined using dynamic light scattering (Malvern Instruments, Worcestershire, UK) measurement at ambient temperature using a scattering angle of 90° with an average of 12 runs. To avoid multiple scattering, the emulsion was diluted ten times with deionized water. Zeta potential was also measured in a similar way. All the measurements were recorded in triplicates.

2.4.2. Stability and solubility of nanoemulsion

The stability of curcumin nanoemulsion was measured by the method of Dalev and Simenova (1995)[27]. Briefly, emulsion (1 mL) was centrifuged at 1300 x g for 10 minutes in order to check the visible undissolved particles. For the solubility, equal quantity (1 mg/mL) of curcumin alone and freeze dried nanoencapsulated curcumin were dissolved in phosphate buffered saline (PBS-0.01 M, pH 7.4).

2.4.3. Effect of processing conditions on physic-chemical characteristics

Different processing conditions such as heat treatment, pH and ionic strength were performed for O/W curcumin nanoemulsion. The emulsion was subjected to different heat treatments *i.e.* pasteurization (63 °C for 30 minutes), boiling (95°C for 10 minutes) and sterilization (121°C for 15 minutes). To measure the pH of nanoemulsion, a pH range (3-7) was used. In case of ionic strength, different salt concentrations (0.1-1MNaCl) were added to the emulsion. For above all the processing conditions, mean particle size, PDI and zeta potential were evaluated.

2.4.4. Entrapment efficiency

The encapsulation efficiency was determined and calculated by method of Surassmo *et al*., (2010)[28] with modifications. Our formulation was passed through the Amicon® Ultra-15, sample concentrators (molecular weight cut off, 100000 Da; Merck Life science Pvt. Ltd.,

Mumbai, India) and centrifuged at 1300 x g for 30 minutes. After centrifugation, permeate was collected to calculate encapsulation efficiency by measuring its total phenolic content by Folin-Ciocalteu's method given by Zheng andWang (2001).[29]

2.4.5. *In vitro* release of curcumin under simulated digestion

Release of curcumin nanoemulsion under the gastro-intestinal conditions was carried out by simulated gastric fluid (SGF)[30] and simulated intestinal fluid (SIF)[31] methods, respectively.

Simulated gastric fluid: The SGF mixture (NaCl-125mM, KCl-7 mM, $NaHCO_3$-45 mM and pepsin-0.32%) was added to the curcumin emulsion in the ratio of 3:1 and adjusted to pH 1.5. The sample was kept in a shaking water bath at 37 °C for 2 hours. At every 1 hour, sample was removed from the water bath and deactivated the enzyme at 90 °C for 10 minutes.

Simulated intestinal fluid: SIF was prepared by adding pancreatin (4 mg/mL) and bile salts (25 mg/mL) and adjusted to pH 7.5. After 2 hours, the SGF mixture was added to SIF and further incubated at 37 °C in a water bath for another 2.5 hours. At each hour, sample was removed and inactivated the enzyme at 90 °C for 10 minutes. Gallic acid phenolic compound was used as a standard curve. For release (%) of curcumin after SGF and SIF method, total phenolic content was calculated according to the calibration curve and mentioned in the section 2.4.4.

2.4.6. Morphological characterization using scanning electron microscopy

The curcumin nanoemulsion morphology was evaluated using scanning electron microscopy (SEM). The powder was sprinkled on a double-sided adhesive tape mounted on SEM stubs and coated with gold in a vacuumevaporator. Then examined under SEM with 20KX magnification (Electron Microscopy Ltd., Cambridge,UK).

2.5. Incorporation of the nanoemulsion into ice cream

The suitable delivery of nanoencapsulated curcumin, dairy product *i.e*. ice cream was prepared and presented in Fig. 2. All the ingredients (skim milk powder, stabilizer, emulsifier, milk and cream) were mixed together by using plunger and further heated to 70-72 °C. After that, the prepared emulsion with or without curcumin was added into the ice cream mix and subjected to homogenization at a two stage cycles. Further, the entire mixture was pasteurized at 80 ± 2 °C for 5 minutes and cooled by placing 5 ± 2 °C under cold condition for overnight. On the other day, flavour (mango) was added to ice cream mix and transferred to batch freezer (GOMA Engineering Pvt. Ltd. Mumbai, India) to collect the ice cream product in sterile plastic cups. The samples were stored in freezer at -18 ± 2 °C and used for further analysis.

2.5.1. Sensory attributes of curcumin encapsulated into ice cream

The samples of control (without curcumin) and encapsulated curcumin are incorporated in ice cream are studied for their sensory evaluation (colour & appearance, body & texture, melting quality and flavour) using 9-point hedonic scale.[32] The samples were drawn from the refrigerator just before serving score cards to the panel. Finally, encapsulation efficiency of curcumin nanoemulsion in the product was determined. To ensure impartiality and minimize subjective bias the samples were labelled as A (without curcumin) and B (with curcumin), respectively.

2.6. Statistical analysis

All the data in this study were represented as mean ± standard errors of mean (S.E.M). Statistical analysis was performed by Student's t-test and oneway ANOVA by using Tukey's test (GraphPad Software, version 5.01) and p value <0.05 was considered to be significant difference.

3. Results and discussion

3.1. Curcumin solubility in oil phase

Different concentrations of curcumin (0.12-0.6%) were dissolved in butter, palm, olive, MCT-60 and sunflower oils. We found that curcumin showed maximum solubility in MCT-60 (0.40 ± 0.01 mg/mL, mean ± SEM) followed by palm (0.35 ± 0.06), butter (0.30 ± 0.04), olive (0.20 ± 0.08) and sunflower oil (0.2 ± 0.03 mg/mL), respectively. Our results were corroborated by Ahmed *et al*., (2012)[33] who reported that maximum solubility of curcumin was found in MCT as compared to long chain triglycerides.The higher solubility in MCT-60 may be due to the molecular characteristics (molecular weight, polarity and interactions) of the oil. Based on these results, MCT was used as lipid phase in this study for the preparation of curcumin nanoemulsion.

3.2. Physico-chemical characterization of O/W curcumin nanoemulsion

3.2.1. Effect of NaCas on droplet size and zeta potential

The mean particle size (*Z*-average diameter) and PDI of our formulation was found to be 333.8 ± 7.28 nm and 0.15 ± 0.01, respectively. Though, zeta potential was recorded as -44.1 ± 0.72 mV. To improve the storage stability, freeze dried nanoemulsion is used widely and easily incorporate into the products. As shown in Table 1, the freeze dried nanoemulsion was reconstituted based on total solids and further analysed for their mean particle size, PDI and zeta potential. After reconstitution, the curcumin nanoemulsion mean particle size was significantly ($p<0.05$) increased due to the particle aggregation, whereas, zeta potential was

decreased. Using MCT, small particle size with narrow distributions was observed as compared to short chain triglycerides as the lipid phase.[33] The increased in the NaCas concentration (0.5 to 5 wt.%) in the emulsion results decreased in the average droplet diameter due to the wide coverage of protein concentration on oil droplet to prevent its aggregation which is agreement with our results.[34,35]

3.2.2. Stability and solubility of nanoemulsion

For stability, we observed that no phase separation after centrifugation. As mentioned above, at physiological pH (7.2 to 7.4) curcumin was unstable and degraded immediately. To challenge as a drug delivery of curcumin nanoemulsion in pharmacological therapy, we solubilize curcumin alone or nanoemulsion (1 mg/mL) in PBS (pH 7.4) and observed that complete solubilization of freeze dried curcumin nanoemulsion as compared to native curcumin which is less soluble with undissolved flakes (Fig. 2).

3.2.3. Effect of processing conditions on physic-chemical characteristics

Our formulation was tested by changing various processing parameters like temperature, pH and ionic strength (Table 2). The curcumin nanoemulsion prepared with NaCaswas analysed with respect to its mean particle size and zeta potential on different temperature conditions. Particle size distribution of the nanoemulsion was increased from 340.0 ± 7.18 to 351.1 ± 4.04 nm with increasing temperature (63 °C to 121 °C). The zeta potential of the nanoemulsion was increased towards negative charge with change in temperature from -45.5 ± 0.38 mV at 63 °C to -47.8 ± 0.35 mV at 95°C but it was further decreased to -42.5 ± 0.62 mV at 121 °C. There was not a linear relation between temperature and mean particle size of the nanoemulsion and these were stable to physical separation/aggregation at all temperature ranges. The stability of heated emulsions at 121°C for 15 minutes increased almost linearly with an increase in caseinate concentration (1 to 4%) and no further increase in particle size at higher concentrations as compared to the unheated emulsions.[36]

Determination of pH value is very important factor for the emulsion stability because of chemical reactions compromise the quality of the product. The pH of emulsions prepared with oils was decreased due to hydrolysis of fatty acid esters into free fatty acids.[37]As presented in Table 2, the particle size was increased from 292 to 328 nm, yet the zeta potential was decreased from 28 to -43 mV with the increase of pH value from 5 to 7 while, the particle size was decreased from 340 to 338 nm and zeta potential was increased from -44to 40 mV with pH 3 to 4 values, respectively. Liu and co-workers (2008)[38] reported that size of casein molecules wereincreased with increasing pH values from 6.0 to 12.0. The

individual casein molecules are become more negatively charged and separated from each other due to electrostatic repulsion which results looser casein molecules contribute towards the increase of the droplet size of casein with alkaline pH. In addition, size of the particle was decreased towards acidic pH may results compact structure of casein molecules. In case of zeta potential, particles at pH 3 has positive net charge as compared to pH 7, due to the fact that the surface charges on the NaCas shifts to positive side at acidic condition.

In food and cosmetic industry salts are used as additives. Salts are also present in the form of bile salts in the gastro intestinal tract. So, they affect the functional properties of emulsion. In this regard, different salt concentration (0.1 to 1.0M NaCl) was used in the present study (Table 2). We observed that the mean particle size of curcumin nanoemulsion was slightly increased with increasing the ionic concentration without any significant difference. The zeta potential of the prepared nanoemulsion was increased with change in ionic strength from -39.7 ± 0.30 to -31.6 ± 0.68 mV at 0.1 to 1.0M. Srinivasan *et al*., (2000)[39] found that emulsions prepared with 1 and 3% NaCas before or after emulsification, no difference in the particle size with 0 to 1M NaCl concentration. On contrary, the droplet diameter was decreased with addition of 0 to 20 mM concentration of NaCl to the prepared emulsion with calcium caseinate.[40]

3.2.4. Encapsulation efficiency

The encapsulation efficiency of curcumin nanoemulsion was determined by means of total phenolic content and found to be 96.9 ± 0.28%. The high encapsulation of curcumin may be due to better entrapment in the hydrophobic core and NaCas as hydrophilic environment. Several researchers reported that encapsulation efficiency was found to be >85%, 83% and 97% with curcumin zein, casein and poly lactide-co-glycolide (PLGA) nanoemulsions, respectively.[41-43]

3.2.5. *In vitro* release of curcumin under simulated digestion

The release of curcuminfrom oil in water curcumin nanoemulsions was determined by SGF and SIF method (Table 3). In SGF, after 2 hours, only 5% release of curcumin was observed. This is due to that resistance of NaCas by the action of pepsin action and followed by 16% release in intestinal fluid after 2.5 hours digestion.This finding was in agreement with studies showing biphasic release of encapsulated curcumin with glycerol monooleate and found 46% release over 24 hours and sustained release of about 66% after 10 days.[44] Similarly, PLGA loaded curcumin nanoparticles are also exhibited biphasic releasing pattern and 59% release occurred after 12 hours and increased to 89% at end of 6 days.[45] The slow release of

lipophilic curcumin upon digestion in the intestine is incorporated into the bile salts or phospholipids (mixed micelles). These mixed micelles along with the curcumin then entered into the systemic circulation through enterocytes present in intestinal cell membrane.[46]

3.2.6. Morphological characterization using scanning electron microscopy

As shown in Fig. 3, the surface morphology of freeze dried O/W curcumin nanoemulsion was confirmed by SEM observation. It is evident from SEM, that the particles are spherical and smooth structure. The nanoemulsions which are spherical in shape will easily enter into the cells as compared the rod shape structures.

3.3. Sensory attributes of curcumin encapsulated into ice cream

Our formulation was successfully incorporated into the ice cream mix and the sensory parameters *viz*., colour & appearance, body &texture, melting quality and flavour were evaluated. No significant difference was observed between control (without curcumin) and encapsulated curcumin nanoemulsion for all the sensory attributes (Table 4). However, a significant difference ($p<0.05$) was observed in encapsulation efficiency before and after preparation of ice cream and found to be 96% and 93%, respectively.

4. Conclusion

Curcumin is a natural polyphenolic compound with wide therapeutic potential. The bioactive component (curcumin) can be incorporated into food systems for the development of health promoting functional foods. In the present study, nanoencapsulation of lipophilic curcumin molecule by using NaCas as emulsifier was found to be stable under different processing conditions such as temperature, pH and ionic strength. Release kinetics data also suggest that our formulation was stable in simulated gastro-intestinal conditions. Further, ice cream was selected as ideal food system for the delivery of curcumin nanoemulsion and found to be stable under different processing conditions. In future, more studies are warrant in this filed to study their functional attributes and used as therapeutic purposes.

Conflict of interest

All authors declare that no conflict of interest

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Table 1 Mean particle size, zeta potential and PDI of fresh and reconstituted curcumin nanoemulsion

Characteristics	Fresh	Reconstituted
Mean particle size (nm)	333.8 ± 7.8^{a}	492.3 ± 12.4^{b}
Zeta potential (mV)	-44.1 ± 0.72^{a}	-42.4 ± 0.2^{a}
PDI	0.15 ± 0.01^{a}	0.21 ± 0.09^{a}

Values mentioned above is mean ± SEM (n=3).Mean values within a row with unlike superscript letters were differ significantly ($p<0.05$). PDI, polydispersity index

Table 2 Effect of NaCas on different processing conditions

	Particle size (nm)	Zeta potential (mV)
Heat treatment		
63 °C/30 min	340.0 ± 7.18^{a}	-45.5 ± 0.38^{a}
80 °C/30 min	349.4 ± 3.26^{b}	-46.3 ± 0.41^{a}
95 °C/10 min	351.1 ± 4.04^{b}	-47.8 ± 0.35^{a}
121 °C/15 min	336.2 ± 5.90^{a}	-42.5 ± 0.62^{a}
pH		
3	340.9 ± 8.04^{a}	40.9 ± 0.46^{a}
4	338.5 ± 7.89^{a}	28.6 ± 0.57^{b}
5	292.9 ± 1.61^{b}	-28.1 ± 0.31^{c}
6	311.8 ± 9.43^{a}	-35.4 ± 0.47^{d}
7	328.6 ± 7.01^{ab}	-43.4 ± 0.60^{e}
Ionic strength		
0.1 M	346.0 ± 6.46^{a}	-39.7 ± 0.30^{a}
0.5 M	354.0 ± 5.56^{a}	-34.1 ± 0.33^{b}
1.0 M	357.9 ± 9.60^{a}	-31.6 ± 0.68^{b}

Value mentioned above is mean ± S.E.M (n=3). Mean values within a column with unlike superscript letters were differ significantly ($p<0.05$). NaCas, sodium caseinate

Table 3 Release of curcumin from nanoemulsion under simulated gastro-intestinal digestion

Time (h)	Gastric digest (% Release)	Intestinal digest (%Release)
0	3.46± 0.12	10.94± 0.21
1	4.69± 0.43	13.36± 0.25
2	5.25± 0.34	15.33± 0.35
2.5	-	16.12± 0.42

Value mentioned above is mean ± S.E.M (n=3).

Table 4 Sensory acceptability of ice cream incorporated with nanoemulsion of curcumin

Characteristics	Control (A)	Encapsulated curcumin (B)
Colour and appearance	7.6±0.31[a]	7.6±0.20[a]
Body and texture	8.1±0.18[a]	7.4±0.26[a]
Melting quality	7.7±0.30[a]	7.6±0.35[a]
Flavour	7.7±0.38[a]	7.4±0.43[a]

Values mentioned above is mean ± S.E.M (n=8). Mean values within a row with unlike superscript letters were differ significantly ($p<0.05$).

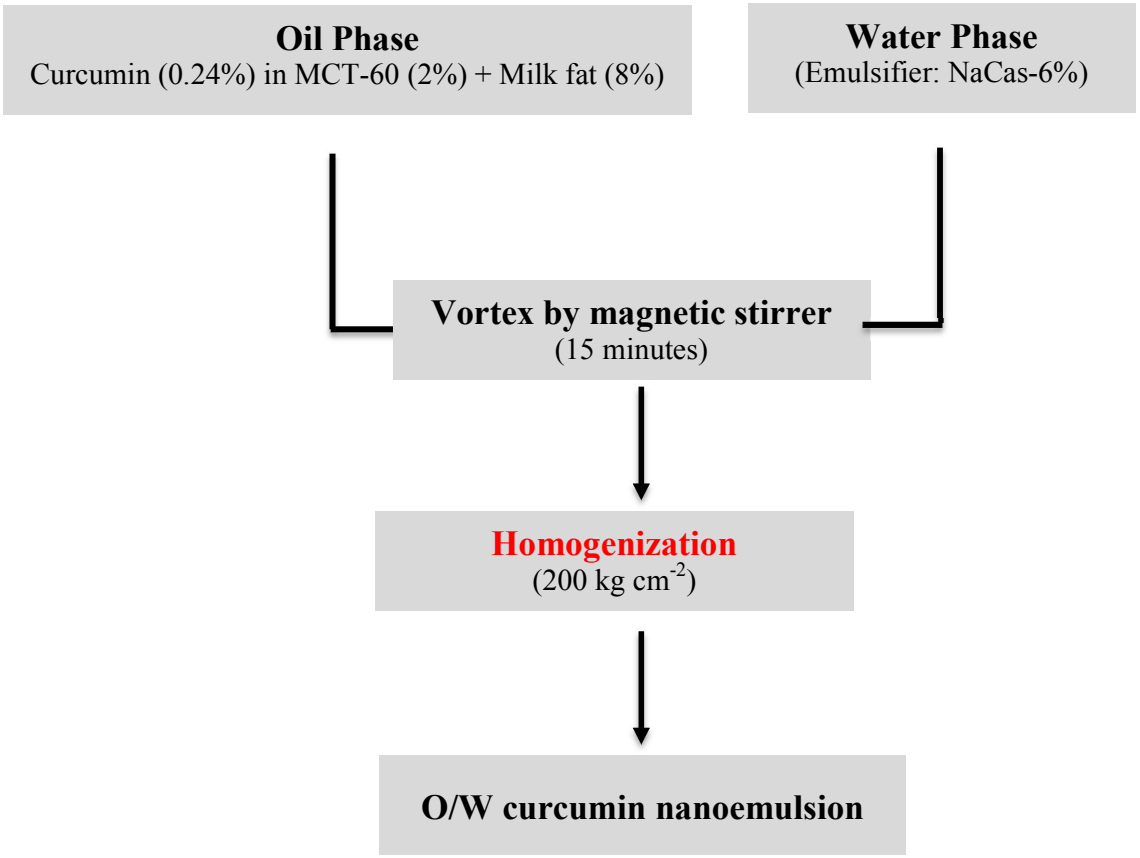

Fig. 1 Preparation of oil in water curcumin nanoemulsion. MCT, medium chain triglycerides; NaCas, sodium caseinate; O/W, oil in water

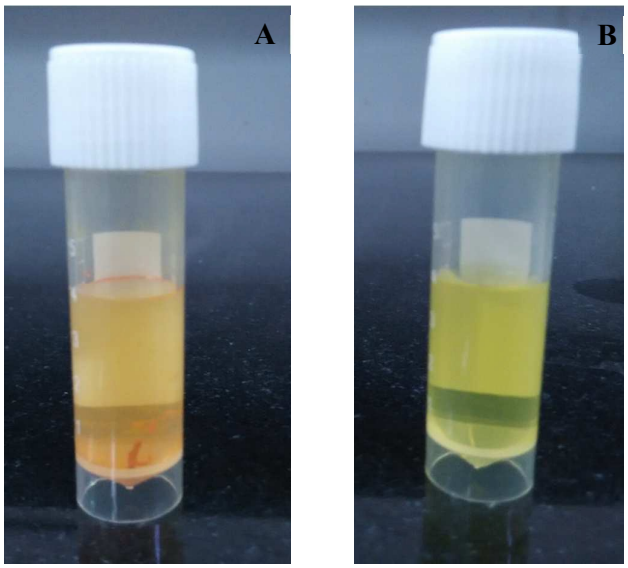

Fig. 2 Solubility of curcumin alone (A) and nanoencapsulated curcumin (B) in phosphate buffered saline

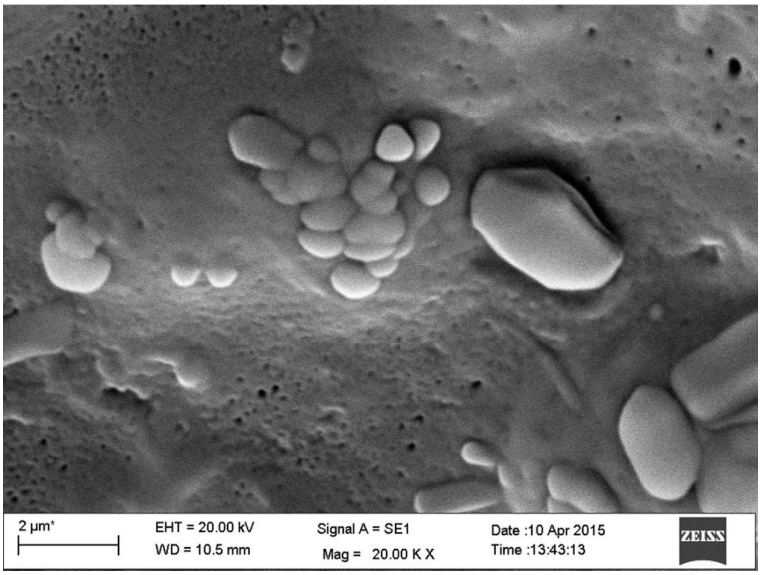

Fig. 3 Scanning electron micrograph of curcumin nanoemulsion

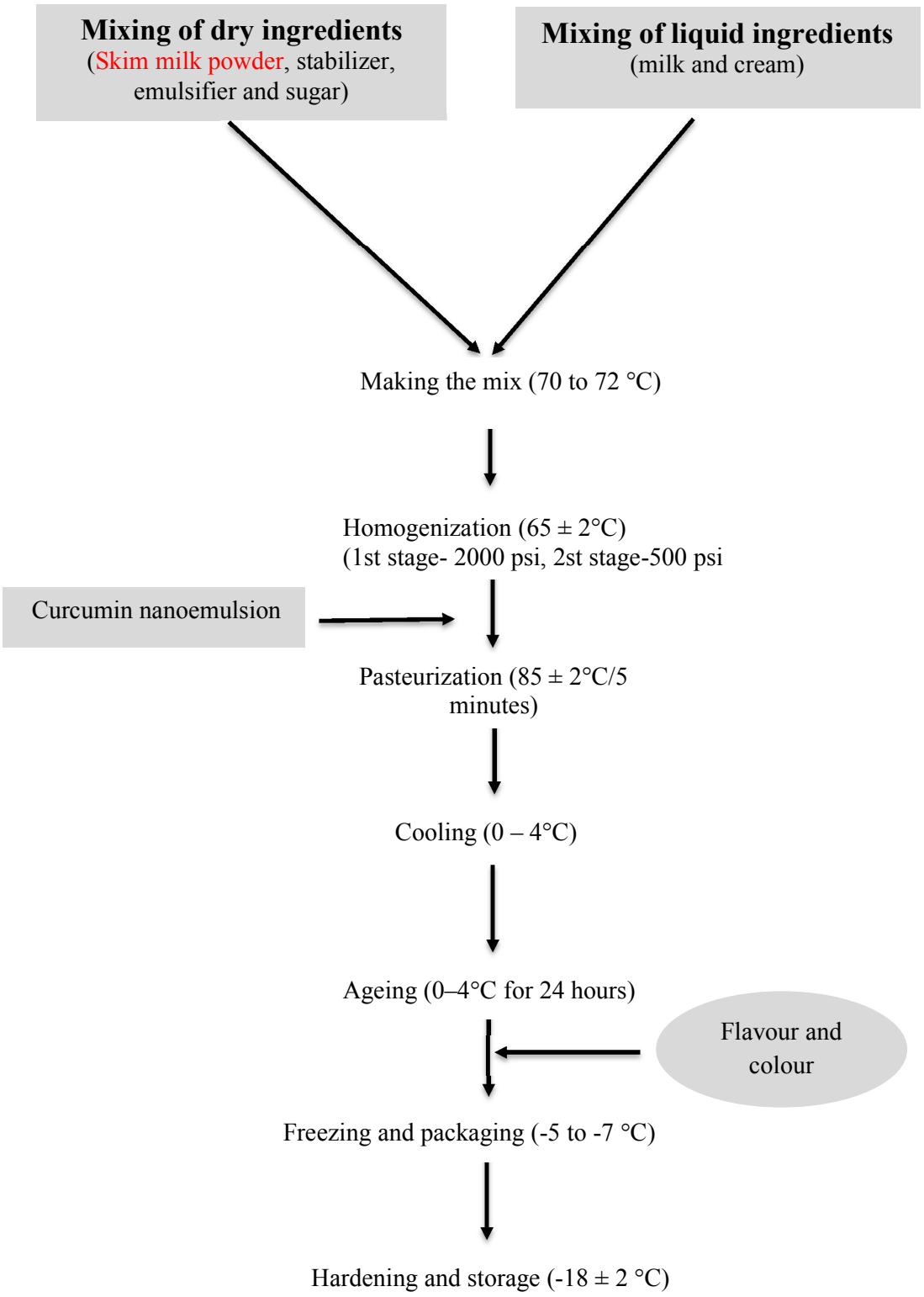

Fig. 4 Flow diagram for the incorporation of the nanoemulsion into ice cream