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In-vitro release Properties of Squalene Stabilized by Chitosan-whey Protein isolate

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Abstract

The present study was carried out to investigate the *in-vitro* release characteristics of encapsulated squalene as affected by the wall material composition. Squalene was encapsulated with chitosan-whey protein isolate as the wall material. The encapsulation efficiency of the spray dried powder was found to be 75.40 ± 0.22%. The *in-vitro* release profile of microcapsules indicated maximum release of squalene at the 4th h of digestion (intestinal phase). Particle size analysis showed that with the exception in 3rd h, the size of the digested emulsions exhibited a decreasing trend. Zeta potential values also exhibited a decreasing trend after the 1st h of digestion. The results of the study showed that chitosan-whey protein isolate can be very well utilised as a wall material for lipophilic compounds as it confers the final product a higher encapsulation efficiency and aids in the release of about 59.17% of squalene from the encapsulated preparation.

Keywords: Particle size, zeta potential, poly dispersity Index (PDI)

Introduction

Oil-in-water emulsions are being employed to improve the solubility, bioavailability and functionality of lipophilic compounds (Liu et al., 2016). In addition to this, they also help in the targeted and sustained release of the compounds at the desired site. However, the release of a compound depends upon several factors such as colloidal changes happened to the emulsions during the process of digestion, ionic strength, pH etc. The type of wall materials/emulsifiers are reported to play a significant role in the *in-vitro* release of bioactive compounds (Betz et al., 2012). Proteins, carbohydrates, and certain polar lipids are the most commonly employed wall materials for the delivery of lipophilic compounds. It has been reported that a combination of wall materials such as protein and carbohydrate are known to perform better as emulsifiers/stabilizers than the individual wall materials (Guzey & McClements, 2006). Proteins adsorb at the oil-water interface and thereby help to prevent the occurrence of emulsion destabilizing mechanisms such as flocculation and coalescence by virtue of its electrostatic repulsion force (Jain & Anal, 2018). Similarly, polysaccharides stabilize emulsion by enhancing the viscosity of emulsion and thereby retarding the droplet movement (Ozturk & McClements, 2016).

Chitosan, a linear polysaccharide obtained by the deacetylation of chitin are reported to have wide range of applications. Its non-toxicity along with its biodegradability and biocompatibility have even broadened its applications (Muxika et al., 2017). It is being widely utilized as a stabilizer for oil-in-water emulsions because of its viscosity enhancing property and ability to form a strong network of oil droplets. However, certain studies have suggested that combining chitosan with a suitable protein can have enhanced positive attributes on the encapsulated powder properties (De Queiroz et al., 2018; Liu et al., 2020; Hu et al., 2020). Whey protein, a byproduct from the dairy industry is reported to have excellent functionalities. It is finding wider applications in the food industry due to its properties such as gelatinization, emulsification, foaming properties, water solubility etc. (Wijaya, Van der Meeren & Patel, 2017). It consists of

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β-lactoglobulin and α-lactalbumin as the major fractions (Norwood et al., 2016).

Chitosan and whey protein isolate complexes are being used for encapsulation of many biologically active compounds (da Silva Bastos et al., 2012; Tavares & Noreña, 2019). Though they are reported to improve the encapsulation attributes, it is also essential to understand how they behave during the digestion process. The release behaviour of encapsulated samples has to be established, especially if they are meant for food applications. Employing *in-vitro* digestion studies seems to be more feasible in analysing the release behaviour of bioactives as they are less time-consuming methods and have no ethical issues. The encapsulation of squalene using chitosan-whey protein as wall material and its characterization has already been studied previously (Lekshmi et al., 2019). However, the influence of wall material composition on the *in-vitro* release characteristics of squalene has not been studied. Hence, the objective of the present study was to analyse the *in-vitro* release kinetics of squalene as influenced by the wall material composition.

Materials and Methods

Chitosan (degree of deacetylation - 85%) and whey protein (obtained from sattvic foods, Goa) were taken in a ratio of 1:7 and dissolved separately in acetate buffer (pH 5.5) and distilled water respectively. After complete dissolution, they were mixed together to get a solid content of 20% (w/v). Squalene was added to the solution at the rate of 30% of total solid concentration and then subjected to high speed homogenization of 10, 000 rpm for 10 min. This was followed by spray drying of the emulsions using a Tall type spray drier (SM Scientech, SMST) at operating conditions of: inlet temperature 170 ± 2°C, outlet 90 ± 2°C, feed flow 17 rpm and blower speed 3600 rpm. The encapsulated squalene produced by spray drying was stored at -20°C till analysis.

The effect of different wall materials on the squalene release pattern was studied with the help of an *in-vitro* static model that simulates digestion in the mouth, stomach and intestines (Hur et al., 2009). Briefly, 3 g of the encapsulated powder were taken in a 100 ml flask and incubated at 37°C in a shaking water bath with an agitation of 200 rpm. The in-vitro digestion of the samples was carried as per the method of Flores et al. (2014). Briefly, 6 ml of salivary juice was added to the sample and kept mixing for 5 min. This was followed by the addition of 12 ml of gastric juice to simulate the digestion in stomach and mixing for 2 h. After 2 h, 12 ml of duodenal juice and 6 ml of bile juice was added to simulate intestinal digestion and again mixing for 2 h. Throughout the process, aliquots (2.5 ml) were collected at regular intervals of 1 h for a total of 4 h. Aliquots thus collected were stored at -20°C until further analysis.

The frozen sample was thawed before analysis. An aliquot of the sample was subjected to particle size, zeta potential and PDI analysis using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, England). The sample was adequately diluted before measurement. The readings were taken in triplicates. The microstructure of sample was captured using optical microscope (Leica ICC50 HD) equipped with digital camera of objective magnification 40X. An image of the emulsion was acquired using digital image processing software (image-pro plusTM, version 6).

Squalene was extracted from the digested sample using the following method. Aliquot of the sample (1.5 ml) was mixed with 20 ml of chloroform – methanol mixture (2:1 on v/v basis) and 3 ml of water. This was then transferred to a separating funnel and the lower layer was collected by passing through anhydrous sodium sulphite. Solvent (chloroform) was then evaporated and made up to 1 ml using HPLC grade acetone. The squalene thus extracted was quantified using HPLC (Shimadzu high pressure gradient system).

Squalene was quantified using a reverse phase HPLC (Shimadzu high pressure gradient system) with a C•stationary phase (150 × 4.6 mm, 3 μ, Purospher® STAR) and diode array detector. The flow rate of 1 mL min^{-1} was held constant throughout the total run time of 20 min. Isocratic elution was carried out using acetonitrile – acetone (60:40) Squalene was detected and quantified at 208 nm wavelength. An external standard (Squalene, 98% purity, sigma) was (concentration of 5 mg ml^{-1}) was used as standard.

Results and Discussion

The efficiency of chitosan-whey protein isolate (CS-WPI) as wall material for encapsulation of squalene has already been studied and reported (Lekshmi et al., 2019). The encapsulation efficiency of the encapsulated squalene was found to be 75.40 ± 0.22% with

a total oil content of 73.055 ± 0.24 mg g^{-1} of encapsulated powder.

It is important to evaluate the release behaviour of encapsulated squalene to establish its potential as a food supplement and its targeted delivery. Release of squalene from the encapsulated formulation were studied by employing an *in-vitro* digestion model and results are given in Table 1. It was clear from the *in-vitro* release profile that the maximum squalene release has happened at the 4th h of digestion. The *in-vitro* digestion results showed that the release exhibited a slow and sustained pattern. Flores et al. (2014) have reported similar results stating the use of whey protein-based microcapsules in providing a sustained release. Release of oil from encapsulated formulations (micro or nano) can be due to any of the following mechanisms: surface erosion, disintegration, diffusion and desorption (Hariharan et al., 2006).

Table 1. In-vitro release profile of squalene from different encapsulated preparations

Duration	Release percentage (%)
1st h	27.13
2nd h	44.21
3rd h	57.52
4th h	59.17

A gradual and sustained release of squalene was observed as the digestion progressed. The initial release can be due to the squalene deposited at the surface as well as near to the surface (Anitha et al., 2011). It was found that the maximum release occurred at the intestinal phase of digestion. Chatterjea & Shinde (2012) reported that the digestion and absorption of lipids mainly happens in the small intestine. Through encapsulation, oil release is controlled to prevent any degradation before it enters into the small intestine. A significant finding of the study is that encapsulation of squalene has succeeded in achieving its sustained and controlled release to the targeted site, the small intestine.

The changes in particle size, zeta potential and PDI of encapsulated powder when exposed to the in-vitro digestion is given in Table 2. There was a significant difference in the size distribution among the samples taken at intervals when exposed to in-vitro digestion studies. The particle size of chitosan-whey protein isolate coated squalene showed a decreasing trend with the exception in the 3rd h. The change in particle size when the emulsions moved from gastric to intestinal digestion can be due to the action of specific enzymes along with the incorporation of digested products. Hydrolytic action of pancreatin on the emulsifiers such as whey protein and chitosan might be the reason for an increased particle size in the first hour of intestinal digestion. Further, bile salts might have displaced the emulsifiers that were initially adsorbed on the lipid droplets. During the process of displacements, voids might appear on the oil droplets which have the tendency to combine with another void area through the mechanism of coalescence resulting in larger particles (Cheong et al., 2016). Similar behaviour has been reported by Hur et al. (2009) who observed an increase in particle size when the particles moved from simulated stomach to small intestine.

The PDI of the digested emulsions were also found to vary significantly with the digestion process. The PDI of the digested emulsions showed a decreasing trend with the digestion time. PDI values of emulsions at all stages of digestion were above 0.3, indicating they all had a broader size distribution. The inconsistency in PDI showed that some had smaller sized and some possess larger sized

Table 2. Particle size, zeta potential and PDI of encapsulated squalene at various stages of digestion

Duration	Particle Size (μm)	Zeta potential (mV)	Polydispersity Index (PDI)
1st h	4.47 ± 0.25 b	+1.68 ± 0.29 a	0.339 ± 0.13 ab
2nd h	3.82 ± 0.27 a	+3.79 ± 0.32 c	0.42 ± 0.01 b
3rd h	5.19 ± 0.33 a	-3.71 ± 0.25 bc	0.45 ± 0.03 b
4th h	3.72 ± 0.16 a	-3.10 ± 0.45 b	0.27 ± 0.05 c

(All data are given as mean ± SD, n=3; a–c Different letters with mean value indicate differences (pd≤0.05) between treatments)

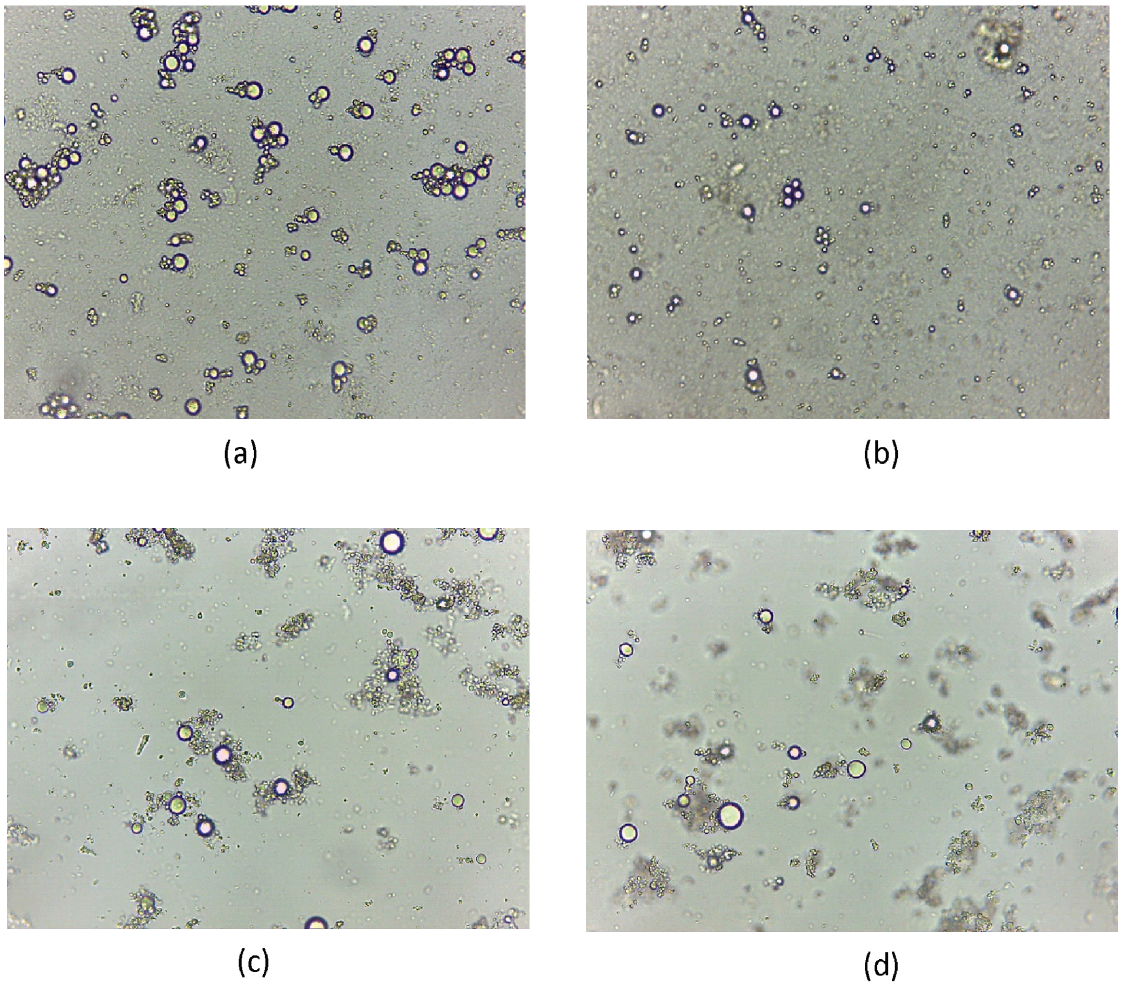

Fig. 1. Microstructure of encapsulated squalene at various stages of digestion

[Microstructure of digested emulsions (a, b,c, d represents the digested squalene emulsions stabilized by CS-WPI at 1st, 2nd, 3rd, 4th h of digestion respectively]

droplets. This was evident from the microstructure of emulsions too. Cheong et al. (2016) have reported similar results of increasing PDI with increasing digestion time in case of kenaf seed oil-based nano emulsions.

Similarly, zeta potential of the sample also showed a significant difference. There was a decrease in zeta potential values after the 1st h of digestion. However, the digested emulsions exhibited a positive value up to gastric digestion stage (2 h). Following the gastric digestion its positive charge has changed to negative with an increase in magnitude. In general, the negative charge of emulsions might be due to the preferential adsorption of OH- ions from the water by the oil droplets (McClements, 2015). The positive charge in CS-WPI can be due to the fact that less displacement of whey proteins might have happened up to gastric digestion phase. The globular proteins in whey proteins is reported to form a covalent crosslinked interfacial layer which is very difficult to displace unlike the other wall materials. Furthermore, they are also reported to resistant against the action of acid and enzymes (Chen & Subirade, 2005). The combined effect of crosslinked layer formed along with its resistance to acid may be the reason for the positive charge. However, at a later stage it might have been displaced effectively by the cation of bile salts and phospholipids present in the digestive fluids. The microstructure of the emulsions at different stages of digestion is shown in Fig. 1. The microstructure of the emulsions shows the particle morphology and size during various stages of digestion.

The present study was aimed at investigating the release behaviour of encapsulated squalene as affected by the wall material composition. The significant findings of the study showed that use of chitosan-whey protein isolate helped in attaining the sustained release of squalene. The maximum release of squalene has occurred at the 4th h of *in-vitro* digestion process. This was well supported by the results of particle size and zeta potential.

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