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Selective encapsulation of quercetin from dry onion peel crude extract in reassembled casein particles

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ABSTRACT

Quercetin, a lipophilic dietary flavonoid, used as a therapeutic agent and a food component was encapsulated with casein particles to improve its water solubility as well as bioavailability in the food and pharmaceutical formulations. The nano-structured casein particles were reassembled with pure quercetin from the synthetic solution and the encapsulation yield was assessed by studying the effect of pH and the concentration of casein and additives like salts and Cetyl trimethylammonium bromide (CTAB). A maximum encapsulation yield of 97% was obtained for the reassembled casein particles formed with the addition of 0.5% (w/v) sodium caseinate, 0.1 M of calcium chloride, 0.5 M of di potassium hydrogen phosphate, 0.1 mM CTAB and 1 M of sodium citrate at a pH of 7. The identified process condition was further used to encapsulate the quercetin from the aqueous crude extract of dried onion peels. The microwave-assisted extraction was able to produce the crude extract with maximum quercetin content of 39.37 μM per ml of the extract. Further the response surface methodology (RSM) was used to optimize the significant encapsulation variables for the maximum encapsulation yield of quercetin from aqueous crude extract. A maximum encapsulation yield of 96% was achieved at the pH 7.09 by using 2.1% (w/v) of sodium caseinate.

1. Introduction

Quercetin (3,5,7,3′,4′-pentahydroxy flavone), a major dietary flavanol, has been reported to be one of the most potent antioxidant apart from exhibiting anti-viral, anti-carcinogenic, anti-bacterial and anti-inflammatory effects (Materska, 2008; Nathiya et al., 2014). The major dietary sources of quercetin include onions, apples, tomatoes, tea, brassica vegetables. Quercetin has been extracted from onions and Raphanus sativus L. by following various extraction strategies like solvent extraction (Horbowicz, 2002), ultrasound-assisted extraction (Sharifi et al., 2017), microwave-assisted extraction (Jin et al., 2011), sub-critical water extraction (Lee et al., 2014), maceration, digestion and soxhlet extraction (Sharifi et al., 2017). Subsequently, the crude extracts are subjected to the purification of quercetin using any one of the methods including HPLC, thin layer chromatography and capillary electrophoresis (Dmitrienko et al., 2012). Upon successful purification, quercetin was used in the food and pharmaceutical formulations. However, the applications of quercetin in therapeutics are limited due to the lower solubility in water and their instability in the physiological medium, which restricts its use in oral administration (Pool et al., 2013).

Despite the presence of five hydroxyl groups, quercetin is known to be lipophilic, conferring a low bioavailability (Pool et al. 2013). However, it was reported that the glycosylation of at least one of the hydroxyl groups of quercetin improves its hydrophilicity (Materska, 2008; Nathiya et al., 2014). There is a greater demand for developing formulations of quercetin that would enhance its water solubility as well as its bioavailability. Studies suggest, encapsulation of quercetin in a potent carrier would serve as a remedy to this problem, providing protection against oxidation, isomerization, and degradation (Pool et al., 2013). Quercetin encapsulation will thus increases its shelf life and would also lead to controlled and sustainable delivery of it when ingested in the body (Nathiya et al. 2014; Tavares et al. 2014). The encapsulation of quercetin has been documented in literature using the micellar systems like oil nano-emulsions (Pool et al., 2013), elastic

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liposomes (Cadena et al., 2013) and the nano particles like chitosan-alginate nano-particles (Aluani et al., 2017). However, the encapsulation of quercetin in the micelles like particles formed by food proteins is the emerging technology to improve their bioavailability.

Food proteins are the natural nano-vehicles for the delivery of the bioactive compounds by holding the promising advantages like high nutritional value, absorbability, negligible toxicity and excellent functional properties like emulsification, gelation, etc. as well as their applications in the food industries. In addition, the food proteins can be easily metabolized and hydrolyzed by the digestive system (Elzoghby et al., 2011). The milk proteins are ideal candidates as nano-vehicle for the delivery of bioactive compounds, as milk is a natural, nutrient-rich beverage consumed worldwide (Sáiz-Abajo et al., 2013; Tavares et al., 2014). Casein, a major milk protein (about 80% of the total protein), has been reported as an excellent candidate for the encapsulation of bioactive compounds due to; (i) their ability to self- assemble as micelle and bind ions and small molecules, (ii) their excellent emulsifying and stabilizing properties, (iii) their surface-activity and the water-binding capacity. The four different types of caseins namely 'α_{s1}', 'α_{s2}', 'β' and 'κ' are present in the ratio of 4:1:4:1, respectively. Being a dietary protein, caseins possess biocompatibility and biodegradability on oral administration (Boratyn, 2017; Elzoghby et al., 2011; Sáiz-Abajo et al., 2013; Semo et al., 2007). Caseins are capable of crossing the plasma membrane in an energy independent manner, which enhances the cellular uptake of the encapsulated bioactive compound once orally administered (Boratyn, 2017). Caseins are amphiphilic molecules and naturally occur as spherical colloidal nano-forms termed as micelles, which are capable of binding hydrophobic compounds in their hydrophobic domains via hydrophobic interactions(Boratyn, 2017; Elzoghby et al., 2011; Tavares et al., 2014). Casein micelles have been exploited as a potent encapsulating device for the encapsulation of various hydrophobic compounds such as Vitamin D2 (Semo et al., 2007), β-Carotene (Sáiz-Abajo et al., 2013), Vitamin A and D_3 (Loewen et al., 2018). However, the encapsulation of quercetin in casein particles was not attempted so far.

Accordingly, the objective of the present study is formulated to extract the hydrophobic quercetin from the dry onion peels, a no-cost bio-waste, which contains high quercetin content and selectively encapsulate it in the hydrophobic domain of the reassembled casein particle by eliminating the purification steps. The encapsulation capacity of the casein particle and effect of various factors were studied. The results obtained were extended to determine the optimal process condition with the help of response surface methodology (RSM).

2. Materials and methods

2.1. Materials

Quercetin dihydrate extra pure powder, DPPH, sodium caseinate were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai. Ethanol (analytical grade 99.9%), tri- potassium citrate, calcium chloride, ammonium molybdate, Sodium carbonate, sodium nitrite and di-potassium hydrogen phosphate anhydrous were obtained from Merck, India. Cetyl trimethylammonium bromide (CTAB) extra pure, aluminum chloride anhydrous, sodium hydroxide pellets, HPLC grade water, acetonitrile, folin and ciocalteu's phenol reagent and trifluoroacetic acid were purchased from Loba Chemie Pvt. Ltd. Gallic acid was obtained from Sigma Aldrich. The crude quercetin was extracted in the laboratory from dried peels of red onions bought from Surathkal market, Mangalore. Room temperature was maintained and deionised water was used for the experiments, unless and otherwise mentioned.

2.2. Crude extract of quercetin from dried onion peels

The dry onion peels were washed thoroughly and allowed to dry for 10 h at 60 °C (Lee et al., 2014). The dried peel of onion was grounded as fine powder using a mixer grinder and stored at 4 °C until further use. 1 gm of the grounded powder was soaked in 40 ml of 60% ethanol (v/v) for one hour (Horbowicz, 2002; Jin et al., 2011) and further subjected to any one of the extraction methods, namely conventional solvent extraction (CSE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) to prepare the crude extract. The mixture was stirred on a heated magnetic stirrer at 60 °C for 1 h for the CSE. However, the ethanol-soaked powder was subjected to 60 °C for 10 min using a commercial microwave oven (Model-MC28H5033CK, Samsung) during MAE and subjected to ultrasonication at 50% amplitude for 10 min (Sharifi et al., 2017) using Sonics Vibra Cell, Model-VCX 130 during UAE. The samples derived from each of the extraction procedure was centrifuged at 1600 rpm for 30 min (Jin et al., 2011). The supernatant collected from each sample was subjected to different phytochemical analyses after filtered through Whatman filter paper 42 and syringe filters of 0.22 micron. The quercetin content in the extract was determined using ammonium molybdate (Mir et al., 2013). 1 ml of 0.01 M ammonium molybdate and 1 ml of deionized water were added to 1 ml of extract and incubated for 20–30 min. The absorbance of the complex was measured at 405 nm. A standard curve was prepared using the standard quercetin at different concentrations.

2.3. Reassembly of casein particles and encapsulation

The casein particles were reassembled by the method described by Sáiz-Abajo et al. (2013) and Semo et al. (2007). Standard casein stock solution (5% w/v), salts (tri-potassium citrate, calcium chloride, and di-potassium hydrogen phosphate), 12 μM of quercetin in ethanol were mixed together and made up to 40 ml at the physiological pH of milk 6.7. The mixture was continuously stirred for an hour to attain the equilibrium encapsulation and centrifuged at 13,000 rpm at 25 °C for 2 h using KUBOTA 6930 centrifuge. The pelleted down encapsulated casein particles were separated. The unbound standard quercetin content was analyzed in the supernatant using total flavonoid content assay (Sultana et al., 2012) and encapsulation yield was estimated (Eq. (1)).

$$\text{Encapsulation yield}\,(\%) = \frac{\text{Total Quercetin added initially} - \text{Unencapsulated Quercetin}}{\text{Total Quercetin added initially}} \times 100 \quad (1)$$

The effect of sodium caseinate concentration (0.5–5% w/v), tri-potassium citrate salt (0–2 M), calcium chloride (0–1 M) and di-potassium hydrogen phosphate (0–1 M) were studied by changing their respective concentrations for the better encapsulation yield. The effect of pH on the encapsulation yield was studied between pH of 6 to 8, apart from the physiological pH of milk. The effect of surfactant CTAB as additive in the micellar system was studied by incorporating the concentration between 0.1 to 0.8 mM, which was below its critical micelle concentration (0.92–1 mM at 25 °C in water). The encapsulation capacity of the reassembled casein particles was studied by increasing the quercetin concentration between 14–22 μM in the aqueous solution. The size distribution of the non-encapsulated and encapsulated casein particles at increasing concentration of sodium caseinate

Table 1 – Independent variables and their coded values .

Independent variables	Symbols	Low level (−1)	Mid level (0)	High level (+1)
Sodium caseinate concentration (%w/v)	A	0.5	2	3.5
pH	B	6	7	8
Quercetin concentration (μM)	C	10	15	20

was analyzed by dynamic light scattering (DLS) using SZ-100 Nanopartica (Horiba scientific). Further the change in casein particle size at different conditions like, sodium caseinate + salts, sodium caseinate + salts + quercetin and sodium caseinate + salts + quercetin + CTAB were also analyzed.

2.4. *Encapsulation from crude extract*

The optimal condition obtained for the encapsulation of standard quercetin was extended to the crude extract of onion peels. The encapsulation yield was further improved by optimizing the three independent variables, sodium caseinate concentration (A), pH (B) and quercetin concentration (C) using RSM. The Box-Behnken design was employed to generate the experimental conditions at two levels (−1, 0, +1) for the variables (Table 1). The encapsulation experiments at 17 design points were performed in quadruplicate and the response, encapsulation yield (Y %), was obtained. The generalized quadratic equation (Eq. (2)) was considered to represent the encapsulation process and further used to predict the optimal encapsulation condition.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i<j}^{3} \beta_{ij} x_i x_j \quad (2)$$

where, Y – encapsulation yield %, β_0 – constant, β_i, β_{ii} and β_{ij} are linear, quadratic and interactive coefficients, respectively. x_i and x_j are the independent variables. The experimental design and RSM was performed by using Design- Expert 11 (Stat- Ease, Inc., Minneapolis, MN).

2.5. *Phytochemical analyses*

Antioxidant activity of encapsulated and pure quercetin was determined by DPPH assay according to Vaisali et al. (2016) with slight modifications. The pellets obtained from encapsulation process after centrifugation was re-suspended in equal volumes of water by stirring for 8 h. Isoelectric precipitation of the resuspended casein particles was carried out at pH 4.6 (Sinaga et al., 2017; Ye & Harte, 2013) and centrifuged at 13,000 rpm for 20 min at 25 °C. The supernatant collected was subjected to the DPPH assay. 2 ml of quercetin sample was added to 2 ml of 0.1 mM DPPH in ethanol. The mixture was mixed thoroughly and was allowed to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm by using LABINDIA analytical (UV 3000+) UV/Vis spectrophotometer. Ethanol was used as a blank and DPPH without quercetin as a control. The potency of quercetin to scavenge DPPH radical was determined in percentage as (Eq. (3)),

$$\%\ \text{DPPH scaveng ing activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad (3)$$

The total phenolic content determination was carried out by the method described by Pękal and Pyrzynska (2014). 1 ml of the onion extract, 0.1 ml of Folin-Ciocalteu's reagent and 0.9 ml of deionised water were added and the solution was allowed to stand for 5 min. 1 ml of Sodium carbonate (7% w/v) and 0.4 ml of deionised water were added with the solution and the blue colored complex was formed. The solution was kept for 30 min to stabilize the complex. The absorbance was then measured at 765 nm in a spectrophotometer. The data obtained was expressed in terms of gallic acid equivalent.

Table 2 – Program method for HPLC analysis of different concentrations of standard quercetin.

Factors	Values
Solvent (A: B)	90% Acetonitrile: 0.1% Trifluoroacetic acid
Solvent ratio	50:50
Flow rate	0.3 ml/min
Column temperature	30 °C
Wavelength	370 nm
Detector	PDA (Photodiode array detector)
Runtime	25 min

The total flavonoid content estimation of the extract was performed by aluminum chloride colorimetric method (Sultana et al., 2012). The solution was prepared by mixing 1 ml sample, 4 ml of water and 0.3 ml of sodium nitrite solution (10% w/v) and allowed to stand for 5 min. Further 0.3 ml of aluminum chloride (10% w/v) was added and incubated for next 5 min. 2 ml of sodium hydroxide (1% w/v) was then added, mixed thoroughly and immediately the absorbance was measured at 510 nm in spectrophotometer. A standard curve was prepared at different concentrations of standard quercetin (1–24 μM) and used to evaluate the total flavonoid content in the sample.

2.6. *Chromatographic analysis of quercetin*

The unbound quercetin in the aqueous solution at different condition was analyzed in the HPLC (SHIMADZU LC-20AD) using the C18 column. The total flavonoid content assay, which was used for total flavonoid content estimation, was validated initially with standard quercetin solution. A binary gradient elution method was followed with a program method shown in Table 2. A standard curve was developed for the estimation of quercetin in various samples. The unbound quercetin analysis was made before and after the encapsulation at different conditions.

3. Results and discussion

The experiments were conducted to study the effect of various factors on the quercetin encapsulation yield using the synthetic aqueous solution of standard quercetin. The assumption behind the study was that, the factors which stabilize the reassembled casein micellar like particles are supposed to portray a higher encapsulation yield. The total flavonoid content assay was used to determine the unbound quercetin in the aqueous solution and accordingly the encapsulation yield was calculated based on the material balance,

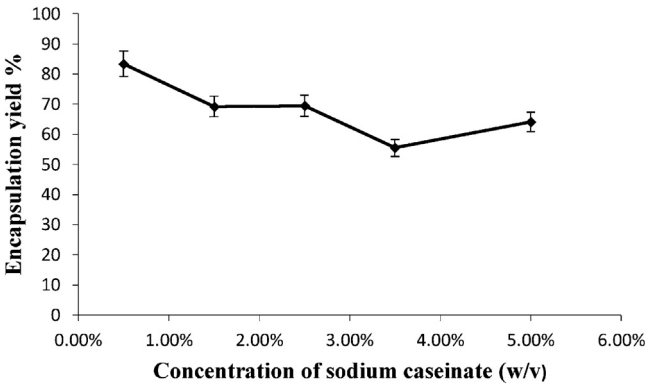

Fig. 1 – Effect of concentration of sodium caseinate on the quercetin encapsulation yield in the reassembled casein particles.

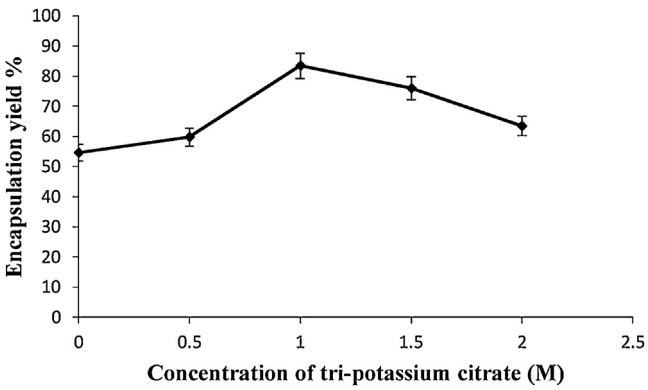

Fig. 2 – Effect of tri-potassium citrate on the encapsulation yield in the reassembled casein particles.

since the casein particles interferes with the assay. However, the quercetin concentration in the crude extract and the samples with crude extract from the experiments were analyzed in the HPLC. The micellar like particle formation and their stabilization were studied with standard quercetin at different conditions and the obtained optimum conditions were extended to improve the selective encapsulation of quercetin from crude extract of onion peel.

3.1. *Effect of sodium caseinate concentration on encapsulation yield*

The sodium caseinate concentration was varied from 0.5 to 5% (w/v) and the maximum encapsulation yield was observed at 0.5% (w/v) as shown in Fig. 1, which was fixed for further studies. The encapsulation of quercetin was found to decrease with increasing sodium caseinate concentration (Fig. 1). Initially, the increasing sodium caseinate concentration helps to form a greater number of smaller casein particle aggregates in the system. The casein particles behave as solid spheres at lower sodium caseinate concentration and the hydrophobic interactions between the casein particles and quercetin was utilized for the encapsulation. However, the encapsulation yield was found to be less at higher sodium caseinate concentration, since the hydrophobic attractive force between the casein particles was utilized to form bigger size aggregates rather than to encapsulate the quercetin. Further these sphere-shaped bigger aggregations are found to be soft in nature and easily get deformed in the presence of shear stress (Vasina, 2016). The deformation caused at higher concentrations make the casein particles less potent to encapsulate the quercetin, thus reducing its encapsulation yield. The aggregation and formation of the micelles are well studied and different theories or models are proposed in the literature. The models are generally fitted under three major categories namely, coat-core structure, models of sub-micelles, and models of internal structure, which were discussed in length by Phadungath (2005). An in-depth analysis may able to reveal the type of aggregation employed for the sodium caseinate particles at different concentration.

3.2. *Effect of salts on encapsulation yield*

The experiments were conducted to improve the stability of the bigger casein particles aggregation with higher surface hydrophobic force so that the bigger aggregations with higher attractive force may able to encapsulate larger quantity of quercetin. The citrate ions are known to have a stabilizing impact on the casein particle structure because of its binding capacity to the Ca^{2+} ions (Tsioulpas et al., 2007). Hence the effect of citrate salt concentration on the quercetin encapsulation yield was studied by considering the tri-potassium citrate as a citrate salt. The encapsulation yield was observed to increase up to the tri-potassium citrate concentration of 1 M and then decreased on further increasing the concentration (Fig. 2). The maximum encapsulation yield of 83% was obtained with the addition of 1 M tri-potassium citrate. It is deduced that the casein particles formed at this condition could be stable and entrap maximum quercetin into the casein particle due the calcium phosphate complex formation with the casein proteins in the presence of citrate ions. In the colloidal state, calcium is present as a complex with phosphoester, carboxyl groups of micellar caseins or with colloidal phosphates and citrates associated with casein micelles (Gebhardt et al., 2007). Even though citrate act as the chelatants on the casein micelles by chelating the ionic calcium present in the aqueous phase and indirectly act on the casein micelles (Broyard & Gaucheron, 2015), the citrates are also known to allow more caseins to cross link and growth of colloidal particle mass (Loewen et al., 2018; Tsioulpas et al., 2007). The extent of micellar demineralization depends on the association constants between calcium and chelatants like citrate (Broyard & Gaucheron, 2015). Hence, the addition of lower concentration of tri-potassium citrate (up to 1 M) increases the encapsulation and the encapsulation efficiency was found to decrease at higher citrate salt concentration due to their chelating action and diminishing calcium phosphate nanoclusters. Further, experiments were performed with the addition of 1 M tri-potassium citrate to study the effect of other variables and improve the encapsulation yield.

Presence of the calcium ions are known to play an important role in stabilizing the internal structure of the casein particle aggregations by either forming a part of colloidal calcium phosphate (CCP) or by directly binding to the caseins (Sarode et al., 2015; Tsioulpas et al., 2007). The effect of calcium ion was studied by adding the calcium chloride salt up to the concentration of 1 M (Fig. 3). The maximum encapsulation yield of 87% was obtained at a concentration of 0.5 M. However, higher concentration of calcium ions cause the aggregation of casein particles and finally precipitated out from the solution (Thomar & Nicolai, 2015; Ye & Harte, 2013). The calcium precipitation was observed beyond the concentration of 0.5 M and the presence of other salts like di-potassium hydrogen phosphate in the solution leads to the precipitation at lower

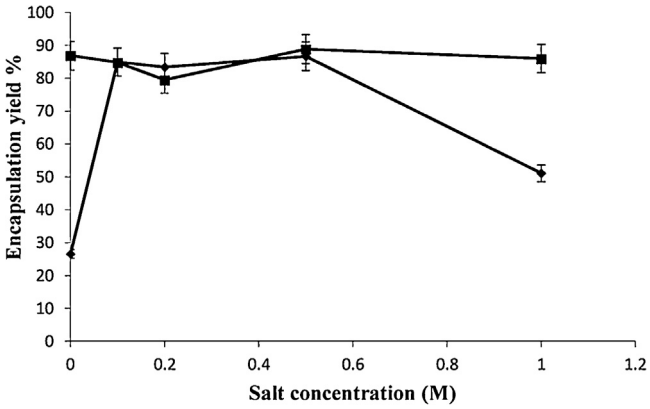

Fig. 3 – The effect of calcium chloride (♦) and di-potassium hydrogen phosphate (■) on the encapsulation yield.

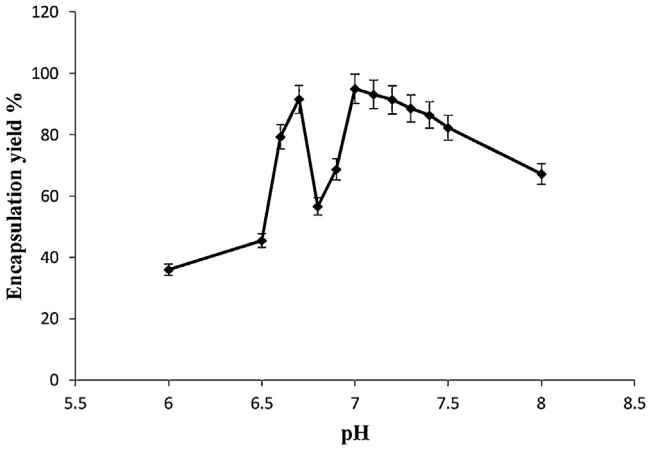

Fig. 4 – The effect of the pH on the encapsulation yield of the reassembled casein particles.

concentration itself. Hence, 0.1 M of calcium chloride concentration, which was giving the second-best yield of 85%, was considered for the encapsulation. Phosphate ions also play an important role in stabilizing the micellar structure by forming the colloidal calcium phosphate nano-clusters. Experiments were conducted by adding different concentration of di-potassium hydrogen phosphate salt in the presence of 0.1 M calcium chloride. The maximum encapsulation yield of 89% was obtained on using 0.5 M of di-potassium hydrogen phosphate, which was fixed for the further studies. Even though the stabilization was observed, which was felt through the improvement of encapsulation yield, the impact of this salt on the particles was following an irregular pattern which demands further studies (Fig. 3).

3.3. *Effect of pH on encapsulation yield*

The physiological pH of milk, i.e., 6.7 was maintained to study the effect of all the variables on the encapsulation yield. Casein, being a protein, is affected by the change of pH, hence the effect of pH on the encapsulation yield was studied by varying the solution pH from 6 to 8. The highest encapsulation yield of 95% was obtained at pH 7. The encapsulation yield was observed to decrease at both acidic and alkaline condition as shown in Fig. 4. At the acidic pH lesser than 6.7, the calcium ions associated with the reassembled casein particles in the form of calcium phosphate complex get solubilized and form individual calcium ions and phosphate ions. These ions were further displaced from the reassembled casein particles

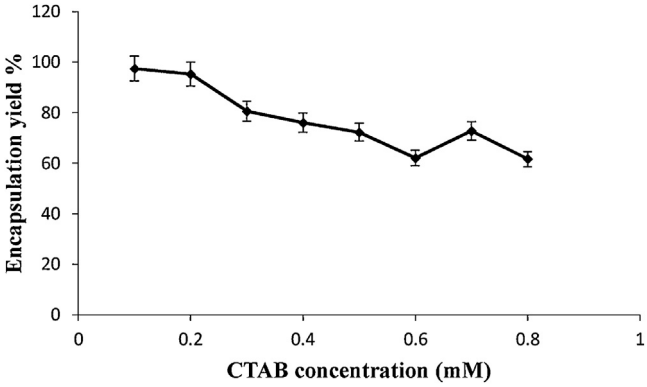

Fig. 5 – The effect of CTAB surfactant on the encapsulation yield.

by the hydronium ions (H_3O^+) and resulted to the destabilization of the casein particles (Sarode et al., 2015; Vasina, 2016). The decreased pH also leads to aggregation of the casein particles and shrinking. At the extreme acidic pH the casein particle aggregations collapsed due to the lack of electrostatic repulsion between the κ-caseins (Vasina, 2016; Ye & Harte, 2013). However, the disassembly and disruption of the casein particle aggregations happen due to deprotonation of the phosphate and increased chelating potential of calcium at the alkaline pH (Vasina, 2016; Ye & Harte, 2013). The destabilization of the micellar structure leads to decrease in the encapsulation yield. When the pH approaches to 6, the reassembled casein particles start to precipitate and complete precipitation was observed at the isoelectric pH 4.6. Similarly, a gradual decrease in the encapsulation yield was observed with increasing pH beyond 7. A sharp decrease in the encapsulation yield was observed at the pH 6.8 and 6.9, which need to be further investigated and analyzed with the characterization of the casein particles.

3.4. *Effect of CTAB concentration on encapsulation yield*

Caseins are known to act as emulsifiers which can reduce the interfacial tension and resulted to an unstable aggregations (Elzoghby et al., 2011; Sáiz-Abajo et al., 2013; Tavares et al., 2014). The addition of ionic surfactants as co-surfactant was used to stabilize the casein particles by utilizing the electrostatic force between the casein and surfactant molecule. The impact of cationic surfactant CTAB on the micellar stability, which would further affect the encapsulation yield, was studied at different concentration of CTAB (Fig. 5). The highest encapsulation yield was obtained at the lowest concentration of 0.1 mM CTAB, which was much lower than the critical micelle concentration of CTAB. As the CTAB concentration increases, the encapsulation yield was found to reduce due to the hydrophobic precipitation of the casein protein. The casein micellar structures have a negative charge on their surface, which was thought to be stabilized by the presence of the cationic surfactants like CTAB in between the reassembled casein particles. The negatively charged amino acid sites of the caseins may bind to the cationic head group of the surfactants due to the electrostatic attraction and stabilizing the micellar structure (Bordbar & Haertle, 2013; Chakraborty & Basak, 2008; Liu & Guo, 2007; Vinceković et al., 2014). The incorporation of CTAB in the casein particle structure also increases the size of the particles and encapsulate up to 97% of the

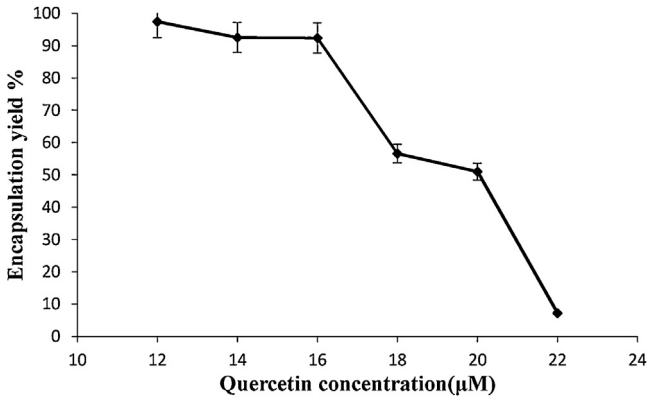

Fig. 6 – The effect of quercetin concentration on the encapsulation yield.

quercetin. However, the CTAB molecules reassembled at the higher concentration of CTAB beyond the critical micellar concentration (i.e., 0.92 mM at 25 °C in water) and tend to form the independent micelles on its own due to the inter CTAB attraction instead of stabilizing the casein particles. The roll of CTAB concentration on the casein particles further justified after understanding the basic structure of the aggregation.

3.5. *Effect of quercetin concentration on encapsulation yield*

The maximum encapsulation capacity of the reassembled casein particle may be obtained by studying the increased concentration of quercetin in the aqueous phase. The loading capacity was studied by increasing the quercetin concentration from 14 μM to 22 μM, as shown in Fig. 6. The encapsulation yield was kept on decreasing with the increase in the quercetin concentration. Thus, 12 μM equivalent to 4.06×10^{-3} gm/L was found to be an optimum concentration to encapsulate 97% of the quercetin present in the mixture. The obtained results indicated that the available attractive sites of casein particles for the encapsulation got saturated by quercetin at the concentration of 12 μM and hence the increased numbers of quercetin molecules with increasing concentration beyond 12 μM are not encapsulated. Accordingly, the encapsulation yield was found to decrease with increasing quercetin concentration. Quercetin is known as a potent anti-cancer and anti-inflammation agent because of its antioxidant property. Studies have shown that the tumor cell growth was inhibited by 50% inhibitory concentration (IC_{50}) ranged from 7 nM to 100 μM concentration (Baghel et al., 2016; Son & Anh, 2013). Hence, the 12 μM of quercetin encapsulated in the reassembled casein particle can be considered for oral administration for the anticancer therapy apart from the application of quercetin as antioxidant in food formulations.

3.6. *Size distribution of reassembled casein particles*

To investigate the casein particle size at different samples obtained during the experimentation was subjected to dynamic light scattering (DLS), in which the sample particles in the cell experience Brownian motion. The system automatically selects the optimum scattering angle and the cell position to collect the scattered light depending on the sample concentration and scattered light intensity. Initially, the casein particles formed at various sodium caseinate concentrations were analyzed without the addition of additives and quercetin.

Table 3 – Mean size of the casein particles with different concentration of sodium caseinate .

Concentration of sodium caseinate (% w/v)	Mean particle size (nm)	
	Before encapsulation (nm)	After encapsulation (nm)
0.5	182.2	261.5
1.5	235.9	261.7
2.5	232.4	262.4
3.5	262.6	300.4
5	296.9	334.4

Table 4 – Particle size analysis of casein particles at different combination of variables.

Sample combinations	Mean particle size (nm)
Quercetin (12 μM)	114.3
Sodium caseinate (0.5% w/v) + salts (1 M tri potassium citrate, 0.1 M calcium chloride, 0.5 M di potassium hydrogen phosphate)	113.8
Sodium caseinate (0.5% w/v) + salts (1 M tri potassium citrate, 0.1 M calcium chloride, 0.5 M di potassium hydrogen phosphate) + Quercetin(12 μM)	292.2
Sodium caseinate (0.5% w/v) + salts (1 M tri potassium citrate, 0.1 M calcium chloride, 0.5 M di potassium hydrogen phosphate) + Quercetin (12 μM) + CTAB (0.1 mM)	482.1

The particle size analysis confirmed the formation of the casein particles and shown the effect of sodium caseinate concentration on the size of the particles. The mean size of the casein particle aggregation was found to increase with increasing sodium caseinate concentration due to the aggregates of smaller casein particles (Vasina, 2016). The particle size of the encapsulated reassembled casein particle is further increased after encapsulation when compared to its initial size, which confirms the encapsulation of quercetin (Table 3). The drastic increase in the size was observed beyond the sodium caseinate concentration of 2.5% indicates the higher degree of aggregation of submicelles. However, the highest encapsulation was showed at lower concentration of sodium caseinate 0.5% (w/v) (Fig. 1).

The samples obtained by the addition of salts, quercetin and CTAB with sodium caseinate (0.5% w/v) was analyzed and the particle sizes were reported in Table 4. The sodium caseinate molecules were kept intact in the aggregation form by the addition of tri-potassium citrate and di-potassium hydrogen phosphate salts and stabilizes the casein particles without increasing the size. However, the encapsulation of quercetin in to these casein particles increases the mean size from 114 to 292 nm. The mean particle size is further found to increase to 482 nm by the addition of cationic surfactant CTAB (0.1 mM) with a particle size distribution of 20% smaller particles under 110 nm and 15% particles above 800 nm. The addition of cationic surfactant not only increased the encapsulation yield via electrostatic attraction but also increases the size of the aggregation due to the insertion of CTAB between the casein particle aggregations. Hence the particle size was found to increase from 292 to 482 nm. Maximum size of the reassembled casein particles containing CTAB confirmed the highest encapsulation yield.

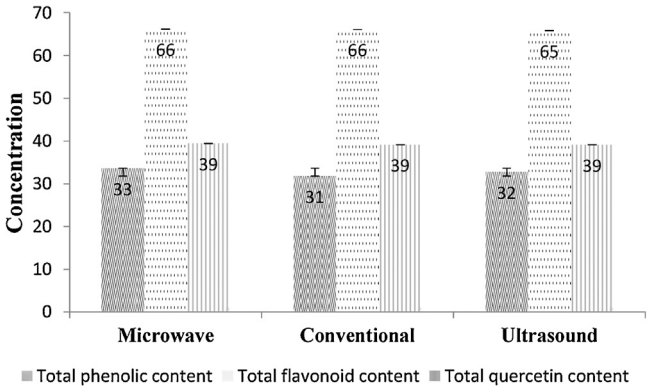

Fig. 7 – The phytochemical analysis of the aqueous extracts obtained by following CSE, MAE and UAE.

3.7. *DPPH radical scavenging assay*

Quercetin is a potent antioxidant that donates two hydrogen atoms to DPPH radical, which transforms it to a quinone intermediate (Materska, 2008). The potency of quercetin to scavenge the stable 2′-diphenyl-2-picrylhydrazyl (DPPH) free radical is measured using this assay. Reduction of DPPH to hydrazine is witnessed by the change in color from purple to yellow which was quantified by the decrease in the absorbance at 517 nm (Formagio et al., 2014; Kalita et al., 2013). The degree of discoloration indicates the capability of the antioxidant to scavenge the free radical. High antioxidant activity of quercetin is attributed to the presence of 3,4-dihydroxy substitution in the B-ring (Vaisali et al., 2016). The antioxidant capacity of the quercetin was analyzed at different concentrations (2–14 μM). The highest antioxidant activity of ~85% was achieved at a very low concentration of 12 μM and after which it was constant. Therefore, this concentration was considered for all the previous experiments.

The antioxidant activity was analyzed for the sample which shown the highest encapsulation yield of 97% equivalent to 11.7 μM of quercetin out of the 12 μM added. However, the antioxidant activity of 11.7 μM of encapsulated quercetin was observed to be 82% which is equivalent to 11.1 μM of free quercetin. The obtained result shows a marginal decrease in the antioxidant activity due to encapsulation. The smaller reduction in the antioxidant activity may be due to the non-availability of all the active sites for the free radical scavenging, which were utilized for the micellar interaction or shielded by the sodium caseinate molecules.

3.8. *Extraction and encapsulation of quercetin from onion peels*

The crude extracts obtained from dried onion peels using aqueous ethanol (60% v/v) as solvent by following CSE, MAE, and UAE was analyzed for total phenolic, total flavonoid and total quercetin contents (Fig. 7). It was observed from Fig. 7 that the extracts obtained from all the three extraction methods were resulting into a similar concentration of phenolic, flavonoid and quercetin contents. However, the MAE yielded little higher concentration of all the components as shown in Fig. 7. Jin et al. (2011) reported that the MAE was the most effective method for quercetin extraction. The MAE produces higher yield at lesser time due to the bulk heating characteristics of microwave irradiation that leads to expansion and rupture of the cell wall and releases the quercetin into the aqueous ethanol solvent (Jin et al., 2011). The combination of the microwave irradiation with traditional solvent extraction made the process more effective than the other two processes. The crude obtained in the MAE process was further used for the selective encapsulation of quercetin using reassembled casein particles.

Table 5 – Box- Behnken design of experiments and their responses for the encapsulation of quercetin.

Run	Variables			Encapsulation yield, Y (%)	
	A	B	C	Predicted	Actual
1	3.5	7	10	50.3	48.7
2	2	7	15	94.7	96.3
3	0.5	8	15	67.7	64.5
4	3.5	8	15	76.4	75.6
5	0.5	6	15	66.7	67.5
6	2	6	20	71.9	69.5
7	2	7	15	94.7	97.1
8	2	8	20	71.3	73.0
9	2	7	15	94.7	96.4
10	2	6	10	42.0	40.4
11	3.5	7	20	72.4	71.6
12	3.5	6	15	64.9	68.1
13	0.5	7	10	45.9	46.7
14	0.5	7	20	69.9	71.5
15	2	7	15	94.7	87.3
16	2	8	10	55.1	57.6
17	2	7	15	94.7	96.4

3.9. *Response surface methodology*

The response surface methodology was used to study the effect of important independent variables and their interactive effect on the encapsulation yield and to obtain an optimized process condition for the maximum encapsulation yield of quercetin from the crude extract of onion peel. The encapsulation studies with the aqueous solution of pure quercetin demonstrated that the concentration of sodium caseinate and quercetin in the mixture and the pH of the solution had a significant effect on the encapsulation. The different salts and CTAB at optimum concentration in the solution significantly improves the stability and size of the casein particles there by increasing the quercetin encapsulation yield. Hence, the individual and interactive effect of operating variables, like sodium caseinate (A), the pH of the solution (B) and quercetin concentration (C) on the selective encapsulation of quercetin from the crude extract of onion peel was studied by designing the experiments using Box- Behnken method. Totally 17 experimental runs were performed as shown in Table 5, which represents the range of independent variables. The whole design of experiments was carried out randomly and the corresponding responses were obtained (Table 5) to optimize the encapsulation process. The encapsulation yield varied from 40.41% as the lowest to 97.065% as the highest.

Multiple regression analysis was conducted to obtain the best fit model for the analysis of the significant factors affecting the encapsulation yield. The quadratic model having highest F-value (77.55) with p-value of <0.0001 was considered as the significant model as compared to the linear (F-values of 1.27), 2F1 (0.06) and cubic (0.88) model equations. The regression analysis implies that the quadratic model (Eq. (4)) is the best fit model to represent the effect of all the independent variables on the encapsulation yield, while the other models showed a significant lack of fit. The best fit model showed an

Table 6 – ANOVA for quadratic model for determining the encapsulation yield.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-Value
Model	5034.17	9	559.35	34.29	<0.0001
A-Casein	24.29	1	24.29	1.49	0.2619
B-PH	78.12	1	78.12	4.79	0.0648
C-Quercetin	1061.18	1	1061.18	65.05	<0.0001
AB	27.47	1	27.47	1.68	0.2356
AC	0.8902	1	0.8902	0.0546	0.8220
BC	46.78	1	46.78	2.87	0.1342
A^2	726.37	1	726.37	44.52	0.0003
B^2	675.10	1	675.10	41.38	0.0004
C^2	2029.04	1	2029.04	124.37	<0.0001
Residual	114.20	7	16.31		
Lack of fit	45.39	3	15.13	0.8797	0.5229
Pure error	68.80	4	17.20		
Corrected total	5148.37	16			

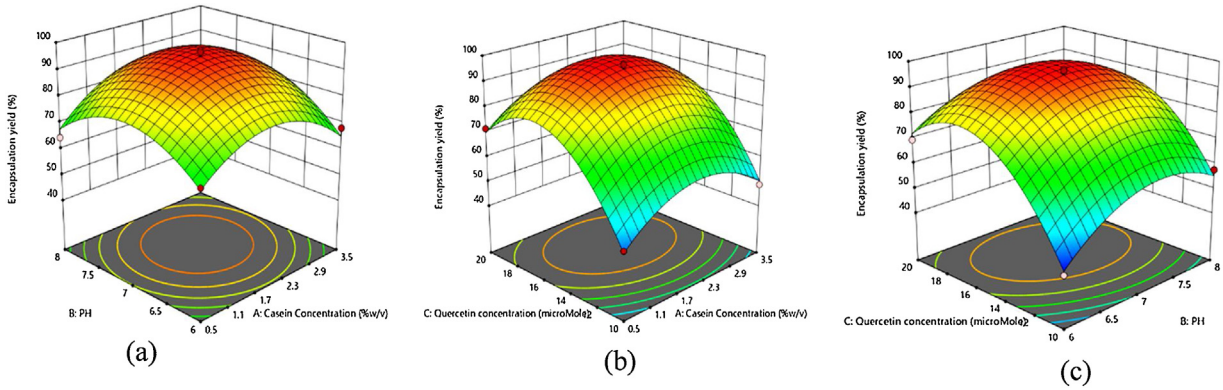

Fig. 8 – Contour and surface plots showing the interactive effect of pH and sodium caseinate concentration (a); quercetin concentration and pH (b); quercetin and sodium caseinate concentrations (c) on the encapsulation yield.

adjusted R^2 value of 0.95 compared to the predicted R^2 value of 0.84.

$$\text{Encapsulation yield, } Y(\%) = -854.65 + 13.226(A) + 187.164(B) + 33.56(C) + 1.747(AxB) - 0.063(AxC) - 0.684(BxC) - 5.837A^2 - 12.662B^2 - 0.878C^2 \quad (4)$$

The analysis of variance (ANOVA) was performed for the quadratic model equation fitted for the encapsulation yield (Table 6). The F-value (34.29) for the quadratic model implied that the model was significant. The model terms of the quadratic model namely, C, A^2, B^2 and C^2 were found to be significant as their p-values were less than 0.05, while the other terms of the equation were insignificant (p-values greater than 0.1). The coefficient of quercetin and the squared term of quercetin were positive and negative respectively. This result indicates that at low concentration of quercetin, encapsulation yield increased with the increase in quercetin concentration, while with additional increase in quercetin concentration past the mid-point of the concentration limit resulted in the decrease of the encapsulation yield. Further, the negative impact of quercetin concentration on the casein concentration and pH was observed based on the coefficient of the term's AC and BC.

The contour and surface plots were developed by utilizing the quadratic model equation to visualize the interactions effects between two independent variables, where the other variable was kept constant (Fig. 8). The surface plots and the contour plots were analyzed in order to deduce the optimum condition for the maximum encapsulation yield (Jin et al., 2011). Fig. 8a represents the encapsulation yield as the function of pH and casein concentration while quercetin concentration was kept constant. At the optimal point the encapsulation yield was 97.4% at pH 7 and 2% (w/v) of sodium caseinate concentration. Fig. 8b represents the encapsulation yield as the function of quercetin concentration and pH, where the optimal encapsulation yield of 96.4% was attained at pH 7 and 15 μM quercetin concentration and the casein concentration was kept constant at 2% (w/v). Similarly, Fig. 8c represents the encapsulation yield as the function of quercetin and casein concentration where the optimal encapsulation yield of 96.4% was attained at 15 μM quercetin concentration and 2% (w/v) casein concentration. Further, the desirability-based optimization was performed using the quadratic model by specifying the goal of optimization as the maximum response, i.e., encapsulation yield. The maximum encapsulation yield of 96.4% was predicted at the variable combination of 2.1% (w/v) of casein and 16.27 μM quercetin concentration in the crude at a pH of 7.09 with a desirability of 0.998. To check the accuracy of the prediction, the encapsulation experiment was performed at the predicted variable condition by maintaining the sodium caseinate and quercetin concentrations as 2.1% (w/v) and 16.27 μM, respectively at pH of 7.09. The experimental encapsulation yield of 96.2% was achieved against the predicted yield of 96.4%. This result confirmed the predictability of the quadratic model and applicability to explain the interactive effect of the independent variables on the encapsulation yield.

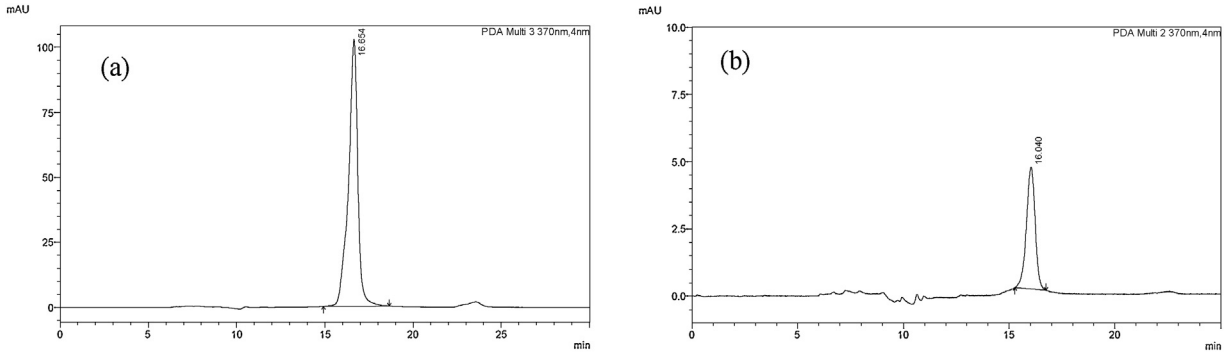

Fig. 9 – HPLC chromatogram of (a) standard quercetin and (b) unbound quercetin using a PDA detector at 370 nm.

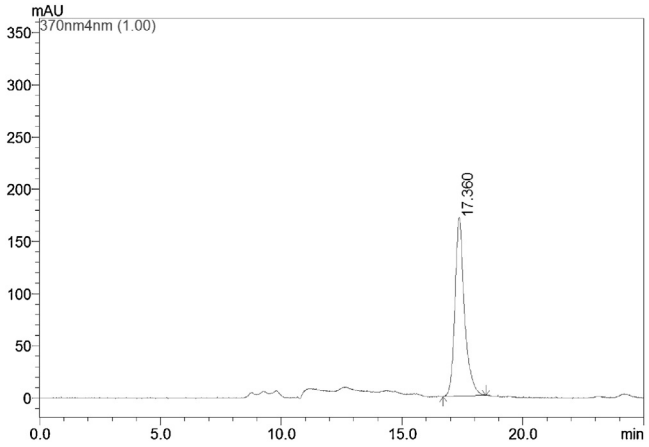

Fig. 10 – HPLC chromatogram of the encapsulated crude quercetin at the optimized condition.

3.10. *HPLC analysis*

The encapsulation yield from the synthetic solution was also confirmed by analyzing the unbound quercetin present in the aqueous medium using HPLC. The area of the elution peak was considered for determining the unbound quercetin concentration from the standard curve. Fig. 9a shows the chromatogram obtained for the standard quercetin and Fig. 9b shows the chromatogram of unbound quercetin after encapsulation. The unbound quercetin was eluted at 16.04 min to that of the standard quercetin elution time of 16.65 min. The encapsulation yield derived from HPLC analysis and total flavonoid content assay was 95% and 97% respectively. This result also authenticates the total flavonoid content assay which was used to study the effect of various parameters on the encapsulation yield. Further the chromatogram was obtained for the sample obtained by performing the encapsulation at optimized condition (Fig. 10). The encapsulated quercetin from the crude extract was eluted at 17.36 min. The variation in the elution time from the standard quercetin may be due to the presence of minute concentrations of other flavonoids which may be encapsulated with the quercetin in the reassembled casein particles. The absence of other peaks in the chromatogram also confirms the selective encapsulation of quercetin in to the casein particles by leaving all the other contaminants in the aqueous medium.

4. Conclusions

The possibility of encapsulation of quercetin into reassembled casein particles was analyzed and maximum encapsulation was achieved by preparing the casein particles with 0.5% (w/v) sodium caseinate, 1 M sodium citrate, 0.1 M calcium chloride and 0.5 M di potassium hydrogen phosphate as stabilizing agents and 0.1 mM CTAB as additives at a pH of 7. The current study also demonstrated the ability of reassembled casein particles for the selective encapsulation of quercetin from the crude extract obtained from dried onion peel by performing microwave assisted extraction. The highest encapsulation yield of 96% was achieved at the process condition as casein concentration of 2.1% (w/v) and pH 7.09 for the crude containing the quercetin concentration of 16.27 μM, which was obtained by performing the RSM. The HPLC chromatogram confirmed that the quercetin was encapsulated at higher purity without any additional purification step. The intensified process by integrating the purification and encapsulation together in a single step and excluding the explicit purification steps may reduce the process cost and provides the advantage of pure quercetin when it was used in different application. Further, the water solubility of the quercetin may get improved by encapsulating the quercetin into the nutritive value casein particles and making it suitable for various applications in food and pharmaceutical industries.

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