

Research Article

A Novel Approach for Microencapsulation of Nanoemulsions to Overcome the Oxidation of Bioactives in Aqueous Phase

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Abstract: Microencapsulation is a promising technique to retain the physical attributes of nanoemulsions and to overcome the oxidation of bioactives that become more available to aqueous phase during emulsification. Purity Gum Ultra (PGU) and Hi-CAP 100 (HiCap) emulsified nanoemulsions of Clove Oil (CO) co-encapsulated with Canola oil (CA) and Medium Chain Triglyceride (MCT) (5:5% v/v CO:CA and CO:MCT) were prepared through high pressure homogenization. Microencapsulation of nanoemulsions was performed using Whey Protein Isolates (WPI) under vacuum using freeze drying, which is considered as appropriate method for heat sensitive compounds. The reconstituted emulsions of microencapsulated powder had similar particle sizes as that of fresh nanoemulsions while uncoated showed a big increase (<400 nm). Oxidation of bioactives with and without CO, before and after freeze drying was investigated at different intervals during 60 days of storage at 4 and 25°C by Thiobarbituric Acid Reactive Substances (TBARS) assay. Microencapsulated PGU-CO:CA showed minimum TBARS values compared to other emulsions. Gas chromatography analysis of microcapsules also showed higher retention of CO and lower content on the interphase for aqueous interaction. Conclusively, this study proposes a novel strategy using a freeze drying process to microencapsulate nanoemulsion.

Keywords: Canola oil, clove oil, freeze drying, medium chain triglycerides, TBARS, whey protein isolates

INTRODUCTION

Oxidative rancidity causes food quality deterioration that leads to the formation of unhealthy compounds with undesirable off-flavors. In lipids, atmospheric oxygen oxidizes the fatty acid or acylglycerol radicals that are formed during three progressive stages (initiation, propagation and termination) (Parkin and Damodaran, 2003; Choe and Min, 2006; Decker *et al.*, 2010). Lipid oxidation rate in oil in water emulsions is influenced by types of lipids, prooxidant and antioxidant. One of the most common mechanisms of lipid oxidation in emulsions is the interaction between lipid hydroperoxides exposed at the surface of droplet and transition metals originating in the aqueous phase which causes deterioration of bioactive compounds that become more available to water phase during emulsification (McClements and Decker, 2000).

Okuda *et al.* (2005) prepared oil in water emulsion encapsulating methyl linolenate with liquid and solid octadecane and suggested that in liquid state the methyl linolenate migrate to interfacial surface and get oxidized by reacting with iron ion present in aqueous phase. In another study, beta carotene emulsion was stabilized by addition of plant oils resulted in

significant oxidation of beta carotene in aqueous phase of emulsion (Szterk *et al.*, 2013). Particle size has a great influence on the oxidation. As the size decreases, surface area of particle increases thus allow more oxygen to diffuse in (Lethuaut *et al.*, 2002). To overcome this, it is necessary to use some agents that can avoid oxidation in aqueous phase of emulsion with small particle size and enhance bioavailability of bioactive.

Moreover, co-encapsulation of bioactive with antioxidants in emulsion system and further microencapsulation of emulsion using different wall material has been utilized to overcome the oxidation of bioactives in emulsion aqueous phase. Mei *et al.* (1999) used galloyl derivatives as antioxidant to prevent oxidation of salmon fish oil emulsion prepared with SDS and Brij. Similarly Xu *et al.* (2013) increased the stability of beta carotene emulsion by co-encapsulation with EDTA and α -tocopherol. There are different methods reported for microencapsulation but freeze drying or lyophilization is appropriate technique for microencapsulation in addition to dehydration of temperature susceptible materials i.e., essential oils and aromas (Desai and Park, 2005).

Clove Oil (CO) being well known for its antioxidant and pro-oxidant properties is considered as

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a good mean to extend the shelf life of products by reducing the oxidation process (Farak *et al.*, 1989; Dorman *et al.*, 2000; Atsumi *et al.*, 2005). In this research we prepared nanoemulsion of bioactives (CA and MCT) co-encapsulated with CO using two modified starches (PGU and HiCap) followed by microencapsulation with Whey Protein Isolates (WPI) using freeze drying. The main objective was to increase oxidative stability of bioactive that is induced by the aqueous phase in emulsion. As well as conserving the size of nanoparticles after reconstituting dried microparticles in water and evaluating effect of freeze drying on the surface and encapsulated oil content in dried microparticles using Gas Chromatography (GC).

MATERIALS AND METHODS

Clove oil was bought from jishui county man herbal medicinal oil refinery Co., Ltd. (Jiangxi, China). Canola oil was purchased from local market and used without further purification. Medium-chain triacylglycerol (MCT; Neobee 1053) obtained from Stepan Co. (Maywood, NJ), Purity Gum Ultra and HI-CAP 100 from National Starch (Bridgewater, NJ, USA) and whey protein isolates from Hilmar, USA. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Formation of nanoemulsion: The nanoemulsions were prepared using PGU and HiCap as emulsifiers. Briefly, CA and MCT blended with CO at ratio of 5:5 (v/v) were coated with two emulsifiers PGU and HiCap 2-3% (w/w). The mixture was stirred at 13,500 rpm for 3 min using Ultra-Turrax (T25 IKA Janke and Kunkle, GmbH and CO KG, Germany) followed by 5 processing cycles of homogenization (IKA-Labor Pilot 2000/4, IKA-Werke GmbH and Co. Staufen, Germany) at 150 MPa.

Particle size distribution: Size of nanoemulsions were measured using dynamic light scattering and phase analysis light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, U.K.). The dilution factor for samples was 1:100 in deionized water to avoid multiple light scattering effects. Measurements were performed at time of preparation and after second and fourth week of storage. Particle size data was calculated as Z-average mean diameter and Polydispersity Index (PDI) for dynamic light scattering.

Microencapsulation of nanoemulsion: Nanoemulsions were encapsulated with 15% (w/w) WPI solution at ratio of 1:1 as a wall material. Followed by mixing at 1700 rpm for 5 min and then increased to 2000 rpm for next 2 min using overhead stirrer (RW 20 digital IKA, Germany). After this the encapsulated and unencapsulated nanoemulsions were transferred to vacuum freeze dryer (LG-5, Centrifugal Machinery Research Institute, Shanghai). Furthermore these powders were analyzed for particle size distribution and

stored at two different temperatures (4 and 25°C) for oxidation study.

Determination of moisture content: The moisture content of encapsulated and unencapsulated powders was measured gravimetrically according to Ezhilarasi *et al.* (2013) with few modifications. Briefly, 0.5 g powder were accurately placed into the aluminum dish and heated at 105°C in hot air oven until stable weight was obtained. Each measurement was carried out in triplicates.

Antioxidant activity by TBARS assay: Thiobarbituric Acid Reactive Substances (TBARS) analysis was carried out as reported by Jirovertz *et al.* (2006). Samples were analyzed after 1, 15, 30 and 60 days of preparation. Standard curve of malonaldehyde was prepared using 1, 1, 3, 3-tetramethoxypropane and TBARS were expressed as $\mu\text{mol/g}$ of dry matter.

Determination of surface oil by GC: Eugenol is the major content present in the clove oil. The amount of eugenol on the surface of powder particles was determined as a measure for the oil released from microcapsule. One hundred milligram of powder was washed with 10 mL of hexane in a capped glass bottle and agitated on a magnetic stirrer for 5 min at ambient temperature and filtered. One micro metre of transparent filtrate was then injected in the Gas Chromatograph (GC) fitted with HP-INNOWAX capillary column (0.30 m \times 0.32 mm \times 0.25 μm) and a Flame Ionization Detector (FID). The oven temperature was set initially at 40°C for 2 min and increased to 120°C at the rate of 8°C/min, then to 230°C at the rate of 10°C/min. A standard curve was drawn by running standard eugenol solution of different concentrations to quantify the eugenol content in sample.

Determination of encapsulated clove oil: CO encapsulated in microcapsule was extracted from the powder washed with hexane. Powder was first stirred in 20 mL dist. water for 10 min to dissolve the WPI. Followed by addition of 20 mL hexane in the above solution and sonicated at 45°C for 10 min to extract the encapsulated CO. After cooling to room temperature the samples were centrifuged at 4000 rpm for 15 min. GC was used to measure the encapsulated eugenol content.

Statistical analysis: Data was analyzed using Microsoft Excel 2007. All graphs were created using Origin Pro 8G (OriginLab Corporation, Northampton, MA-USA).

RESULTS AND DISCUSSION

Preparation of CO nanoemulsions: Stable CO nanoemulsions blended with CA and MCT separately at a ratio of 5:5% (v/v) using 2% (w/w) modified starch PGU, as emulsifier were prepared by following the

Table 1: Size distribution of raw nanoemulsion, before and after mixing with whey protein isolates and freeze dried particles before and after mixing with whey protein isolates when dried particles are reconstituted in the same water volume^a

Composition	Raw nanoemulsion		Nanoemulsion+WPI before FD	
	Size (nm) (mean±S.D.)	PDI (mean±S.D.)	Size (nm) (mean±S.D.)	PDI (mean±S.D.)
PGU-CO:CA	150.660±0.235	0.09800±0.00040	160.900±0.572	0.18100±0.006
PGU-CO:MCT	153.600±1.558	0.12900±0.03471	161.967±1.826	0.12467±0.027
HiCap-CO:CA	222.333±5.070	0.20667±0.00800	259.433±1.806	0.36633±0.010
HiCap-CO:MCT	235.733±4.403	0.22167±0.00700	260.567±3.090	0.37600±0.010
Composition	Nanoemulsion without WPI after FD		Nanoemulsion with WPI after FD	
	Size (nm) (mean±S.D.)	PDI (mean±S.D.)	Size (nm) (mean±S.D.)	PDI (mean±S.D.)
PGU-CO:CA	422.367±3.412	0.43633±0.039	190.200±4.414	0.17900±0.017
PGU-CO:MCT	439.667±3.913	0.21367±0.009	194.333±1.307	0.21800±0.021
HiCap-CO:CA	453.433±3.091	0.21700±0.020	336.267±1.484	0.38267±0.027
HiCap-CO:MCT	497.933±6.316	0.26033±0.019	349.567±1.389	0.39167±0.010

WPI: Whey protein isolates; FD: Freeze drying; PDI: Polydispersity index; PGU: Purity gum ultra; HiCap: HI-CAP 100; CO: Clove oil; CA: Canola oil; MCT: Medium chain triglyceride; ^a: Data expressed as mean±standard deviation (n = 3)

Table 2: Size distribution of different concentration of whey protein isolates

Concentration of WPI (%)	PGU-CO:CA		PGU-CO:MCT	
	Size (nm) (mean±S.D.)	PDI (mean±S.D.) ^a	Size (nm) (mean±S.D.)	PDI (mean±S.D.)
5	177.733±0.418	0.269±0.020	180.60±2.315	0.2640±0.007
10	167.433±1.880	0.215±0.006	167.70±1.651	0.2117±0.010
15	160.900±0.571	0.181±0.005	161.96±1.826	0.1247±0.026
Concentration of WPI (%)	HiCap-CO:CA		HiCap-CO:MCT	
	Size (nm) (mean±S.D.)	PDI (mean±S.D.)	Size (nm) (mean±S.D.)	PDI (mean±S.D.)
5	418.13±5.279	0.410±0.013	421.10±11.038	0.4650±0.020
10	381.50±9.899	0.376±0.009	388.20±5.940	0.3957±0.011
15	259.43±1.806	0.366±0.009	260.57±3.087	0.3760±0.010

WPI: Whey protein isolates; FD: Freeze drying; PDI: Polydispersity index; PGU: Purity gum ultra; HiCap: HI-CAP 100; CO: Clove oil; CA: Canola oil; MCT: Medium chain triglyceride; ^a: Data express as mean±standard deviation (n = 3)

same procedure as described in our previous study (Majeed *et al.*, Unpublished data). The Z-average diameter along with PDI of each formulation is presented in Table 1. The nanoemulsification process is characterized by studying the influence of the surfactant on particle size and PDI of nanoemulsions. PGU based CO:CA and CO:MCT emulsions showed almost similar particle size i.e., 150 and 153 nm, respectively as shown in Table 1. On the other hand HiCap based CO:CA and CO:MCT emulsions showed quite bigger particle sizes even at high emulsifier concentrations 3% (w/w) compared to PGU as shown in Table 1. The nanoemulsions prepared with 3% (w/w) HiCap showed monodispersed droplets (PDI>0.3), with an average diameter of 237 nm. However, nanoemulsions prepared with 2 and 2.5% (w/w) HiCap had bigger particle sizes i.e., 494, 358 nm, respectively. Therefore, 3% (w/w) HiCap was chosen on behalf of narrow droplet size and PDI. PGU emulsified particles showed smaller particle size at lower concentration compared to HiCap. This may be due to lower molecular weight of PGU as compared to HiCap. However regarding the type of oil phase, no significant difference was observed.

Influence of wall material concentration: To adjust the polymer concentration for microencapsulation of CO nanoemulsions, variable concentrations of Whey Protein Isolates (WPI) 5, 10 and 15 wt% were used.

The optimum polymer concentration was chosen on the basis of particle size distribution and PDI values. As shown in Table 2, the size and PDI of nanoemulsions increased with 5 and 10 wt% WPI i.e., in case of PGU based CO:CA nanoemulsions the particle size decreased to 177,169 nm, respectively and PDI values were greater than >0.2. Whereas, particle size (160 nm) and PDI (0.187) decreased considerably when 15 wt% WPI was coated on PGU CO:CA nanoemulsion template. Therefore, 15 wt% WPI concentration was chosen for the rest of the experiments. Almost similar trend was observed in all nanoemulsion formulation coated with 15 wt% WPI as represented in Table 2.

Microencapsulation of CO nanoemulsions: The production of dried microparticles to entrap nanoemulsion droplets was performed by freeze drying the suspension in an aqueous bulk phase, thereby solubilizing the 15 wt% WPI as wall material. To evaluate the efficiency of microencapsulation, nanoemulsions coated with and without wall material were subjected to freeze drying. The impact of wall material on the microparticle size and PDI distributions was evaluated after reconstituted in water. The reconstituted nanoemulsions particle sizes along with PDI are presented in Table 1. PGU based CO:CA and CO:MCT nanoemulsions coated with 15% WPI showed almost the same particle sizes (d<200 nm) as that of the

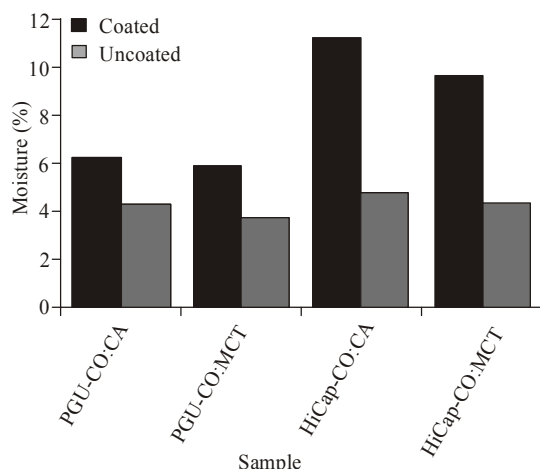


Fig. 1: Moisture content in coated and uncoated freeze dried microparticles

raw nanoemulsions. These results were in accordance with the findings of Li *et al.* (2011) as they reported similar size and individual nature of nanoemulsion when dried microparticles were dispersed in water. Similarly, Liang *et al.* (2013) acquired same particle size of fresh and spray dried reconstituted beta-carotene nanoemulsion. However, in case of HiCap based freeze dried microparticles showed increased particle sizes when reconstituted in water (336 and 369 nm) but yet remained within nanometric range ($r < 200$ nm) with good dispersity ($PDI < 0.04$). PGU nanoemulsions were more uniform this may be due to better interaction between the PGU and WPI. HiCap proved to be less stable because of less affinity with wall material. On other hand there was a rapid increase in the freeze dried unencapsulated nanoparticles where the size increased beyond the nanometric range (Table 1). There was no significant difference observed in the diameter and PDI of the coated nanoemulsions indicating freeze drying has not affect the characteristics of nanoemulsions. As well as when the freeze dried powders were re-suspended in water, encapsulated powders showed better dissolution than unencapsulated. So freeze drying microencapsulation proved to be a better approach as did not affect the characteristics of nanoemulsions (Abdelwahed *et al.*, 2006; El and Simsek, 2012).

Moisture content of freeze dried microparticles:

Moisture content of coated and uncoated powders is shown in Fig. 1. These data clearly revealed that the moisture content of coated microcapsules is higher as compared to uncoated. This may be due to more affinity of WPI of retaining water molecules after freeze drying, also reported by Najafi *et al.* (2011) that the type of coating material significantly influences the moisture content of dried powder. During freeze drying PGU microcapsules performed better and dried quickly that resulted in higher loss of water content compared to HiCap which lead to less moisture in final powder. The moisture content of WPI coated PGU-CO: CA and

PGU-CO:MCT was 6.22 and 5.89% while for uncoated was 4.17 and 3.73% for uncoated. On other hand HiCap these values were 11.23 and 9.81% for coated while 4.85 and 4.34% for uncoated. Najafi *et al.* (2011) explained that powder with higher moisture content favored diffusion of bioactives to surface resulting decrease of encapsulated content. Similar variation was observed in case of HiCap microparticles showed higher surface oil content and oxidation compared to PGU microparticles. While in uncoated, freeze drying shattered the structure of nanoparticles resulted in higher loss of moisture. The results of this study also showed that variation in the type of oil did not influenced moisture content in both coated and uncoated powders.

Determination of TBARS:

In antioxidant activity assay, control (without CO) emulsion showed higher TBARS values as compared to clove oil containing emulsion (Fig. 2). This indicated that oxidation of bioactive compounds was lowered by co-encapsulation of clove oil. Similar results were reported by Mei *et al.* (1999) as they increased the oxidative stability of Brij stabilized salmon oil emulsion using phenolic antioxidant. Types of emulsifier also play an important role in oxidative stability. PGU came up to be more efficient in controlling the stability of encapsulated lipid phase as compared to HiCap. After microencapsulation of nanoemulsion with WPI, coated freeze dried emulsions showed lower oxidation values compared to uncoated ones. Freeze drying beside of being considered a gentle process for heat liable compounds also affects the antioxidants and their activity (Chang *et al.*, 2006). It is also observed during this study that direct freeze drying conditions damaged nanoemulsions, increase of particle size as well as release of bioactives and CO in the surrounding aqueous phase thus resulting in higher oxidation. While after microencapsulation WPI formed a protective covering and thus protect the inner core from the outer aqueous phase as well as from freeze drying conditions. Similar conclusion was drawn by Dzondo-Gadet *et al.* (2005) on Safou pulp oil encapsulated in 6DE maltodextrins using spray and freeze drying and suggested that later showed better efficiency against oil oxidation. On other hand the TBARS values for emulsions containing MCT were comparatively lower as compared to CA. This stability study was conducted for 60 days of storage by keeping the samples at 4 and 25°C. Both of these factors showed increasing trend in TBARS values with respect to time. But no significant increase was observed among samples stored at different temperature.

Comparison between surface and encapsulated eugenol content:

It is undesirable that entrapped oil diffuses to the surface of capsules and get oxidized which in turns give off-flavors and decreasing the shelf stability (Anandaraman and Reineccius, 1986). In this study, the highest encapsulated conc. of eugenol

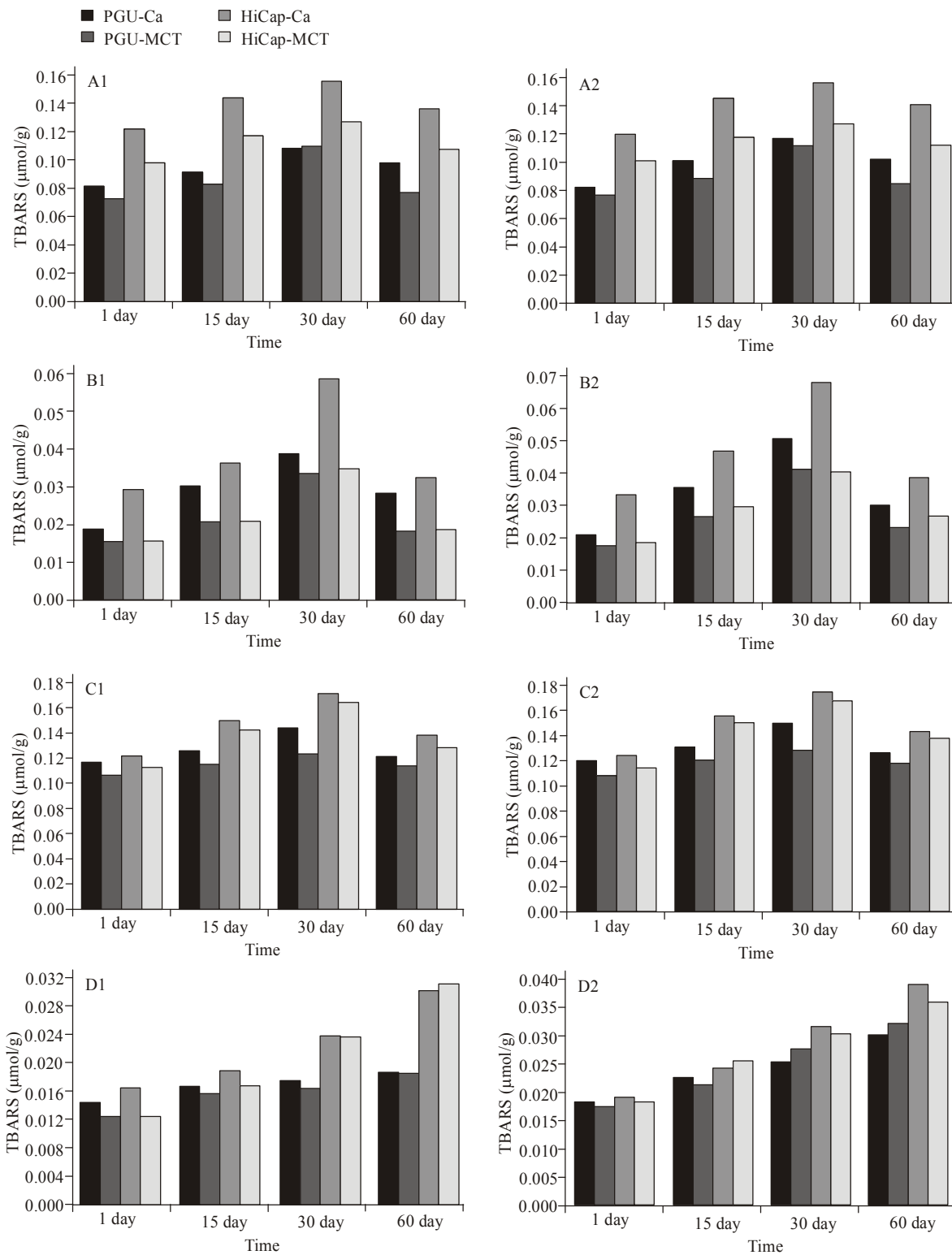


Fig. 2: Formation of Thiobarbituric Acid Reactive Substances (TBARS) in control (A) clove oil co-encapsulated nanoemulsions (B), uncoated (C) and WPI coated (D) freeze dried clove oil co-encapsulated nanoemulsion at different interval (from 1-60 days) and temperature of storage 4°C (1) and 25°C (2)

(4.968707 ppm) with minimum surface oil (1.192096 ppm) refers to WPI-PGU microcapsules encapsulating blend of CO-CA. Table 3 showed the concentration

(ppm) of eugenol on the surface and in the core of nanocapsules. Microcapsules loaded with CO-MCT showed almost the same conc. 5.085629 ppm and

Table 3: Quantitative determination of surface and encapsulated oil in WPI coated microcapsules

Composition	Concentration of surface oil (ppm)	Concentration of encapsulated oil (ppm)	Encapsulated oil content (%)
PGU-CO:CA	1.192096	4.968707	80.65032
PGU-CO:MCT	1.510958	5.085629	77.09485
HiCap-CO:CA	2.342874	4.747545	66.95718
HiCap-CO:MCT	2.494503	4.578048	64.72980

PGU: Purity gum ultra; HiCap: HI-CAP 100; CO: Clove oil; CA: Canola oil; MCT: Medium chain triglyceride; ppm: Parts per million

1.510958 ppm. Regarding WPI-HiCap encapsulating CA and MCT, the core content was lower as compared to surface i.e., 4.747545 and 4.578048 ppm encapsulated and 2.342874 and 2.494503 ppm on the surface. Thus PGU nanocapsules showed higher encapsulated oil content (80 and 77%) than HiCap nanocapsules (67 and 64%). Najafi *et al.* (2011) showed that powder with higher moisture content favored diffusion of bioactives to surface resulting decrease of encapsulated content. Similar variation was observed in case of HiCap microparticles showed higher surface oil content and oxidation compared to PGU microparticles. Similarly surface oil and entrapped oil was also influenced by the particle size and interaction between nanoparticles and wall material. The lower particle size of PGU nanoemulsion enveloped with WPI resulted in compact arrangement having smaller the pores available for the volatile content (eugenol) to diffuse out to the surface. So microencapsulation hindered the effects of freeze drying.

CONCLUSION

This study proposes a novel technique using a freeze drying process to microencapsulate CO co-encapsulated nanoemulsions with wall material (WPI) for reducing oxidation of bioactive due to aqueous phase. The present study also demonstrates microencapsulation not only conserved the nanoemulsions in nanometric range but also retained high volatile (eugenol) content effectively. On other hand the freeze dried uncoated nanoemulsions could not tolerate the freeze drying conditions and resulted increase in particle size with higher TBARS values. This study can be exploited to design new strategies for improving the stability of bioactives within food system.

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