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Fortification of Yoghurt with Conjugated Linoleic Acid (CLA) Encapsulated in Reassembled Casein

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Abstract

This study aimed to fortify yoghurt with conjugated linoleic acid (CLA) to enhance the product's nutritional value. Direct use of free CLA in yoghurt fortification is difficult due to its hydrophobic nature, low oxidative stability, and oily taste. Therefore CLA was encapsulated in reassembled casein micelles (r-CM) to overcome these difficulties. Different ratios of CLA (0.5, 0.75, and 1.0 g/g protein) were efficiently entrapped in r-CM forming nano-sized particles that exhibited high stability against UV-induced oxidation for the entrapped CLA. The CLA-loaded r-CM particles (one g/100 ml) were added to buffalo milk used in the Manufacture of yoghurt, which increased the CLA content up to 10 times the control. The adding CLA loaded r-CM had no adverse effect on the acid development and sensory properties of yoghurt during storage; however, it did increase the values of its textural parameters. Scanning electron microscopy revealed that encapsulated CLA was evenly distributed in the yoghurt matrix. CLA loaded r-CM is recommended to produce high CLA content and acceptable quality yoghurt.

Keywords: Yoghurt, buffalo milk, Conjugated linoleic acid, reassembled casein micelles, nanoencapsulation.

1. Introduction

Yoghurt achieves continuous consumer acceptability and market growth among dairy products due to its favourable sensory and nutritional attributes. Yoghurt can be considered a good vehicle to provide consumers with an additional functional ingredient. However, these ingredients should have no adverse effects on the normal growth of the used starter and the quality of the obtained yoghurt [1]. Several nutrients have been used in the fortification of yoghurt including fibre from different sources [2,3], minerals such as calcium [4] and iron [5], vitamin D [6], folate [7] and multiple micronutrients [8].

Conjugated linoleic acid (CLA) is a generic name for many linoleic acid isomers that don't have methylene (CH_2) group in-between the two double bonds. CLA has gained much attention since 1980 for its health-promoting activities [9]. Originally CLA was discovered as an anticancer component [10], but subsequent studies showed that it can prevent the development of atherosclerosis, modulate immune and/or inflammatory responses, reduce body fat, and improve lean body mass [11]. *Cis*-9,*trans*-11, and *trans*-10, *cis*-12 are the two main CLA isomers with documented biological activities and their mixture (50:50) has been approved for food as GRAS (generally recognized as safe) in the United States since 2008.

The levels of CLA in healthy diets are too low to exhibit its beneficial effects [12] and CLA is recommended to be incorporated in foods as a supplement. Fortification of foods with CLA represents a challenge to the food industry particularly in the case of using free CLA due to its hydrophobicity, low oxidative stability, and oily taste. In order to overcome these difficulties CLA have been encapsulated in different wall materials. Whey protein concentrate (WPC) was found as very effective coating material to prevent the oxidative deterioration of CLA [13, 14]. This study revealed that the application of WPC-coated CLA did not cause any objectionable change in the sensorial properties of the fortified food. Encapsulation of CLA in a mixture of agar and waxy corn starch was reported to significantly improve the oxidative stability of CLA [15]. Glycated whey protein with maltodextrin was reported to be more efficient in microencapsulation of CLA than WPC [16]. The mixture of pea protein concentrates and maltodextrin (3:1) was used efficiently to encapsulate CLA [17]. Zhuang et al. [18] spray-dried CLA emulsions prepared with milk protein concentrates and the mixture of whey protein isolates and sodium caseinates. The obtained CLA microcapsules had low encapsulation efficiency and oxidative stability.

Therefore, this study aimed to evaluate the use of encapsulated CLA with reassembled casein micelles to produce CLA-rich yoghurt.

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2. Materials and Methods.

2.1. Materials

Bovine sodium caseinate (NaCas) contains 90% protein as declared by the supplier (Acros, NJ, USA). Conjugated linoleic acid (CLA) was purchased from Vitamin World, Inc. (Ronkonkoma, NY, USA). Buffalo milk was obtained from the Faculty of Agriculture, Cairo University, Giza, Egypt. Yoghurt starter (mixture of *Lactobacillus delbreukii* subsp *bulgaricus* and *streptococcus thermophilus*) was obtained from Ch. Hansen (Denmark) and activated before use in sterilized reconstituted skim milk (10% total solids).

2.2. Methods

2.2.1. Entrapment of CLA within reassembled casein micelles (r-CM).

CLA was encapsulated in reassembled casein micelles (r-CM) as Zimet et al. [19] described. Sodium caseinate solution (2%) was pre-equilibrated overnight at 4°C. Aliquots of 1.00, 1.50, and 2.00 g CLA dissolved in absolute ethanol (up to 6 ml) were slowly added separately to 100 ml portions of the sodium caseinate solution while vigorously stirred for 5 min at 4°C. Then 2 ml of 0.4 M tripotassium citrate, 12 ml of 0.08 M K_2HPO_4, and 10 ml of 0.08 M $CaCl_2$ were added. Eight portions of 1.25 ml 0.08 M K_2HPO_4 and 2.5 ml 0.08 M $CaCl_2$ were successfully added to the CLA-caseinate solution at 15 min intervals while stirring at 4 ℃ and maintaining pH between 6.7 and 7 with 0.1 N HCl or 1 N NaOH. The final volume was adjusted to 200 ml with distilled water (DW) and adjusted to pH 6.7 with 0.1 N HCl and further stirred for 1 h at 4 ℃. The CLA-loaded r-CM from different treatments were freeze-dried using a freeze dryer (LABCONCO, USA).

2.2.2. Encapsulation efficiency (EE) of CLA.

Ten mg of the freeze-dried CLA-rCM nanoparticles from different treatments were accurately weighed and extracted with 10 ml ethanol by ultrasonication (model SONICS Vibra Cell) for 10 min. The extract was then filtered through the 0.45 μm membrane filter. The absorbance of the filtrate was measured at 233 nm using UV–Vis spectrophotometer (spectrum, Taiwan) according to [20]. The CLA was determined from a standard curve prepared by plotting the absorbance of CLA solutions (0.002 mg/ml to 0.014 mg/ml) against concentration. The encapsulation efficiency (EE %) was calculated using the following equation:

$$EE\% = \frac{\text{Extracted CLA content}}{\text{CLA weight used in nanoencapsulation}} \times 100$$

Also, CLA was determined in all yoghurt samples fresh and after cooling storage using gas chromatography-mass, according to Hurst et al. [21].

Residual CLA

Non-encapsulated CLA was determined according to Sankarikutty et al. [22].

2.2.3. Dynamic Light Scattering (DLS) parameters for CLA loaded r-CM Nanoparticles.

The average diameter, the size distribution, and zeta potential of CLA-loaded r-CM Nanoparticles were measured using a particle size analyzer (Nano-ZS, Malvern Instruments Ltd., UK).

2.2.4. Oxidative stability of CLA-loaded r-CM Nanoparticles.

Aliquots (10 ml) of the CLA-loaded r-CM from the different treatments and control were placed in closed vials to occupy half the vial volume. The vials were heated at 85 ℃/60 s and then subjected to UV light exposure for 16 hrs. The oxidative stability of CLA-free and loaded r-CM was measured by determining the residual CLA in solution and peroxide value. CLA was extracted from 2 ml of the irradiated samples using 4 ml ethanol+2 ml DW+2 ml n-heptane, the solvent was removed by evaporation, and the residual CLA was methylated as described by [13]. The CLA content was then determined by gas chromatographic analysis [23] using Hewlett Packard HP 6890 gas chromatograph, operated under the following conditions: Detector, flame ionization (FID); column, capillary, 30.0 m X 530 μm, 1.0 μm thickness, polyethylene glycol phase(INNO Wax); N2 with flow rate, 15 ml/ min with average velocity 89 cm/s (8.2 psi); H2 flow rate, 30 ml/min; air flow rate, 300 ml/min; split ratio, 8:1, split flow, 120 ml/min; gas saver, 20 ml/min. Detector temperature, 280 °C; column temperature, 240 °C; injection temperature, 280 °C. Peroxide value (PV) of UV irradiated samples were determined according to AOAC [23] methods.

2.2.5. Manufacture of yoghurt fortified with CLA-loaded r-CM Nanoparticles.

Yoghurt was made according to the method described by Lee and Lucey [24]. Fresh buffalo's milk was standardized to 6% fat and supplemented with the freeze-dried CLA-rCM preparations to provide 0.5, 0.75 and 1.00 g CLA/100 ml milk. CLA fortified milk from different treatments was heated at 85° C for 5 minutes and then cooled to 44°C. Yoghurt starter culture was added milk at 3%, stirred, distributed into 100 mL plastic containers, and incubated at 44° C until complete coagulation. All yoghurt samples were stored for 14 days in the refrigerator at 4 ± 1° C.

2.2.6. Physicochemical analysis of yoghurt.

Acidity and total solids, protein, fat, and ash content of yoghurt samples were determined according to the methods described by AOAC [23]. The pH values of yoghurt samples were measured using a digital pH meter (Persica pH 900, Switzerland).

2.2.7. Texture analysis of yoghurt.

Texture profile analysis (TPA) of yoghurt was carried out using a Texture Analyzer (Mult- test 1*d* Mecmesin, Food Technology Corporation, Slinfold, W. Sussex, UK) as described by El-Kholy et al. [25] . Measurements were carried out at 20°C by double compression that generate a plot of force (N) versus time using a 25 mm diameter perplex conical shaped probe. The samples were compressed by 30% of their actual depth at a speed of 1mm/s. Reaching a depth of 20 mm during compression and relaxation of the sample. All measurements were carried out at a temperature of 20 °C.

2.2.**8. Microstructural characterization**

To prepare CLA yoghurt samples, cubes (3 ± 0.5mm3) were cut from different areas of the yoghurt cup and fixed in 3% glutaraldehyde in 0.05 M phosphate buffer pH 7 for 2 h at 48˚C. The fixed cubes were rinsed with 0.05 M phosphate buffer. The fixed cubes were dehydrated by consecutive soaking in 30, 50, 70, and 95% ethanol each for 20 min, and finally was rinsed successively twice by absolute ethanol (100%) at 48˚C and 58˚C. Cubes were immediately dried in the critical point drier (Samdri PVT-3B, Tousimis, Rockville, MD) for 5 h, according to Vardhanabhuti, et al. [26]. The yoghurt samples were analyzed using a scanning electron microscope (SEMJoel Jsm 6360LA, Japan) after the surfaces were vacuum coated with gold [25].

2.2.9. Sensory Evaluation of yoghurt.

The yoghurt samples were sensory evaluated by a taste panel of 6 members from the staff of the dairy science department, National Research Center, Egypt. They assessed each yoghurt sample and used a quality rating score card for evaluation of flavor (60 points), body and texture (30 points), and appearance (10 points), according to [27].

2.2.10. Statistical analyses

The analyses of prepared samples were conducted at least in triplicates; one-way ANOVA and Tukey's tests completed comparisons of the treatments by SPSS, ver. 16.0 statistics programs. A 95% minimum confidence level was taken for all statistical analyses [28].

3. Results and discussion.

3.1. *Encapsulation efficiency of CLA in r-CM*

Previous studies [19, 29] showed the ability of r-CM to bind and entrapment of hydrophobic nutraceuticals, including essential fatty acids such as docosahexaenoic acid (DHA). Also, the binding of hydrophobic compounds to caseins enhanced their tendency to aggregate and induced micellar nanoparticles' formation [30]. Similarly, the present results showed that r-CM was efficiently entrapped CLA (Table 1). The encapsulation efficiency was a little decreased with the increase of the percentage of the loaded CLA indicating the high efficiency of CLA to bind to r-CM.

Table 1: Particle size (nm), Zeta potential and particle dispersity index (PDI) of caseinate, r-CM, and CLA loaded r-CM

Sample	Size (nm)	Calculated PDI	Zeta potential	EE.
NaCas	174.20 ± 19	0.453	-12.72	-
Control r-CM	56.78 ± 12	0.433	-14.95	-
T1	67.91 ± 7	0.567	-11.53	96.55 ± 0.95[a]
T2	124.7 ± 28	0.594	-6.92	92.75 ± 1.25[b]
T3	197.4 ± 23	0.685	-4.67	89.95 ± 0.75[c]

* Means with different superscript are significantly (P<0.05) different

NaCas; Sodium caseinate, EE; encapsulation efficiency, Control r-CM; control reassembly casein, T1; 0.50 % conjugated linoleic acid loaded into reassembly casein, T2; 0.75 % conjugated linoleic acid loaded into reassembly casein, T3; 1.00% conjugated linoleic acid loaded into reassembly casein

3.2. *Mean particle size and Zeta potential of CLA loaded r-CM.*

The applied method for preparing r-CM yielded particles of sizes comparable to those reported before [19]. Encapsulation of 0.5% CLA increased the mean particle size of the loaded CLA r-CM, increasing the entrapped CLA percentage markedly. The present results differ from that reported [19] that the particle size exhibited no changes with DHA loading. This may be attributed to differences in the ratio of loaded material to casein micelles in the two studies. The r-CM had a negative charge that decreased with the entrapment of the CLA.

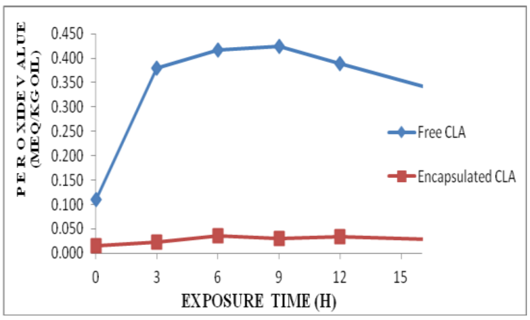

Fig. 1: Changes in the peroxide value of free conjugated linoleic acid (CLA) and CLA loaded in r-CM after UV exposure up to 16 h

3.3. Protective *effect of r-CM on UV-induced oxidation of CLA*

Exposure of free CLA to UV irradiation resulted in ~ 25% loss of its initial concentration (Table 2). Marked protection for the stability of CLA was evident by encapsulation in r-CM. The increased loaded CLA slightly decreased the free CLA indicating the high efficiency of the r-CM to protect CLA from UV-induced oxidation. Measurement of the peroxide value (Fig 1) revealed a rapid increase in PV of free CLA after 3 h of exposure to UV irradiation and almost level on further exposure time. On the other hand, the PV of encapsulated CLA remained almost unchanged during UV exposure confirming the stability of CLA to UV-induced oxidation. This suggests the r-CM either limits the penetration of the UV and/or isolate the entrapped CLA from the atmospheric oxygen. Soliman et al. [31] found that the peroxide value increased for free wheat germ oil through exposure to UV light, while the encapsulation by casein preserves the oxidative stability until 18 h of UV exposure.

Table 2: Stability of free CLA and CLA loaded r-CM after UV light exposure*

Sample	Residual of CLA %
Free CLA	73.70 ± 2.3^{d}
T1	96.25 ± 1.8^{a}
T2	94.95 ± 1.1^{b}
T3	93.75± 0.9^{c}

* Means with different superscripts are significantly (P<0.05) different

Free CLA; free conjugated linoleic acid, T1; 0.50 % conjugated linoleic acid loaded into reassembly casein, T2; 0.75 % conjugated linoleic acid loaded into reassembly casein, T3; 1.00% conjugated linoleic acid loaded into reassembly casein

3.4. *Gross composition and CLA content of yoghurt.*

The total solids, fat, protein, and ash content of yoghurt increased significantly (P <0.05) by fortification with the CLA-loaded r-CM preparations (Table 3). The CLA-loaded r-CM contained proteins (casein), lipids (CLA) and ash (colloidal calcium phosphate) which add to the corresponding constituents in the control yoghurt. The yoghurt content of CLA increased by almost 5, 7, and 10 times by adding 0.5%, 0.75%, and 1%% CLA loaded r-CM, respectively, which would increase the nutritional value of yoghurt markedly. These results agree with those found by Hamed et al. [27].

Table 3: Gross composition of yoghurt fortified with CLA-loaded r-CM*

Samples	TS	fat	Ash	protein
Control	**16.06 ± 0.06^{d}**	**6.00 ± 0.1^{d}**	**0.58 ± 0.02^{b}**	**4.55 ± 0.06^{c}**
T1	**17.47 ± 0.08^{c}**	**6.47 ± 0.06^{c}**	**0.75 ± 0.02^{a}**	**5.42 ± 0.13^{b}**
T2	**17.78 ± 0.04^{b}**	**6.73 ± 0.06^{b}**	**0.75 ± 0.02^{a}**	**5.44 ± 0.06^{b}**
T3	**18.07 ± 0.06^{a}**	**7.03 ± 0.15^{a}**	**0.75 ± 0.02^{a}**	**5.46 ± 0.18^{a}**

*see footnote table 2

The addition of CLA-loaded r-CM preparation had no probable effect on the growth and activity of the starter microorganisms, as apparent from the normal acid development in yoghurt during the storage (Table 4). The acidity of fresh and stored yoghurt containing CLA-loaded r-CM was significantly (P< 0.05) higher than that of the control, which can be attributed to the acidity of the additive. The results agree with Hamed et al. [27].

Table 4: Changes in pH and acidity of yoghurt fortified with CLA-loaded r-CM*

Treatments	Fresh		15 days	
	pH	Acidity	pH	Acidity
Control	4.85 ± 0.05^{a}	0.80 ± 0.02^{c}	4.56 ± 0.02^{c}	0.90 ± 0.03^{c}
T1	4.85 ± 0.04^{a}	0.90 ± 0.05^{b}	4.71 ± 0.03^{a}	1.14 ± 0.02^{b}
T2	4.80 ± 0.06^{a}	1.06 ± 0.02^{a}	4.65 ± 0.03^{b}	1.19 ± 0.01^{a}
T3	4.82 ± 0.03^{a}	1.07 ± 0.02^{a}	4.65 ± 0.03^{b}	1.16 ± 0.02^{ab}

*see footnote table 2

Table 5 shows the total CLA extracted from control yoghurt and fortified yoghurt with different ratios of 0.5, 0.75, and 1.00 % of CLA. This study demonstrated that the CLA content in control yoghurt was decreased with an increased period time cold storage from 14.19 to 10.35 mg CLA/ gm fat yoghurt after 15 days. But, the yoghurt fortification with different ratios from CLA encapsulated has almost retained its content of CLA. (Paszczyk et al. [32] found that the storage of yoghurt made from cow milk at 8±1 °C for 21 days causes changes in the fatty acid profile. Storage resulted in a significant decrease of CLA and trans C18:1 isomers in cow milk yoghurts.

Table 5: Changes in CLA of yoghurt fortified with CLA-loaded r-CM*

Samples	Residual mg CLA/ gm fat in yoghurt		
	Fresh	After 7days	After 15 days
Control	**14.19 ± 0.7^{d}**	**14.05 ± 0.5^{d}**	**10.35 ± 0.9^{d}**
T1	**70.33 ± 1.2^{c}**	**52.49 ± 1.4^{c}**	**52.02 ± 1.7^{c}**
T2	**96.57 ± 1.9^{b}**	**76.52 ± 1.7^{b}**	**76.19 ± 2.7^{b}**
T3	**121.03 ± 3.1^{a}**	**97.33 ± 2.5^{a}**	**96.57 ± 3.4^{a}**

*see foot note table 2

3.5. ***Texture parameters of yoghurt***

It's well-known that food structure significantly impacts various attributes (such as texture, functioning, and appearance). The protein network's microstructure and structure for fermented dairy products influence rheological and textural qualities [33].

Due to a higher acidification rate, the release of colloidal calcium phosphates from casein-micelles is expedited, resulting in the early release of individual caseins from the micelles, allowing for the early creation of the casein network. As a consequence of this condition, quick protein aggregation produces a limited number of protein-protein bonds and substantial particle/cluster rearrangement, resulting in a weak gel with big pores and increased whey separation [34].

Table 7: Texture profile analysis for control yoghurt and fortified with CLA loaded r-CM

Sample	Storage days	Hardness (g)	Springiness (mm)	Cohesiveness	Gumminess (g)	Chewiness (g*mm)
Control	Fresh	50.00	0.43	0.54	26.83	11.58
	15 days	12.00	0.56	0.46	55.60	31.27
T1	Fresh	70.00	0.68	0.71	49.78	34.05
	15 days	150.00	0.69	0.52	77.47	53.58
T2	Fresh	90.00	0.47	0.49	44.02	20.50
	15 days	160.00	0.58	0.62	99.01	57.45
T3	Fresh	110.00	0.54	0.50	54.83	29.80
	15 days	190.00	0.79	0.81	154.40	121.56

*see foot note table 2

Fortification of yoghurt with CLA-loaded r-CM increased its hardness. This increase was related to the additive and casein micelles percentage, possibly due to an increase in acidity (Table 7). This increase can be attributed to the increase in the total solids brought by the added nanoparticles and that these particles were included in the formed yoghurt matrix. After two weeks of cold storage, the hardness of yoghurt from different treatments and control. A similar increase in the hardness of yoghurt during storage was reported [35], which can be attributed to the slight losses in moisture content during storage. The effects of added CLA-loaded r-CM on springiness, cohesiveness, gumminess and chewiness of yoghurt were consistent with the percentage of the additive. However, all these parameters increased during storage, but the most pronounced increases were found in the gumminess and chewiness.

3.6. Scanning Electron Microscopy for control yoghurt and fortified with CLA loaded r-CM

Figure 2 illustrates the microstructure of the gels control plain yoghurt and CLA fortified yoghurt using SEM (20µm, 6000X, 20kV). Our microstructural observations are in agreement with the textural results. Nano-encapsulated CLA fortified yoghurt (Fig 2B) illustrated that the smoother than control (Fig 2A) and the caves in the casein network of control yoghurt were less may be due to the consistency of r-CM inside the caves of the casein network. Kalap [36] found that the casein micelles acquire a globular appearance after removing the superficial material by acidification.

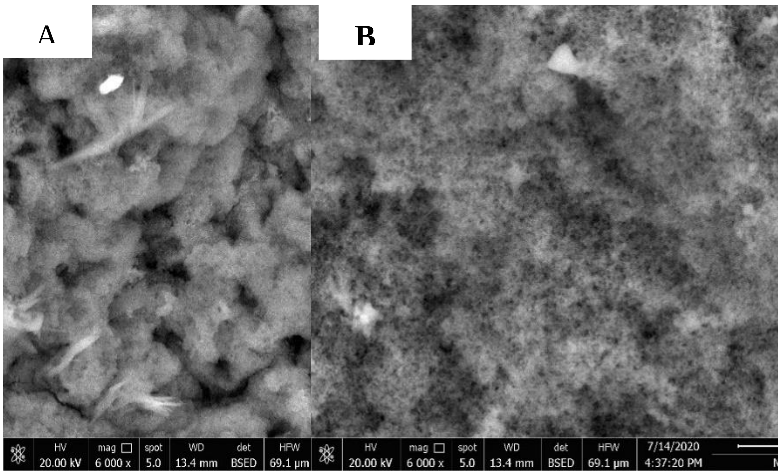

Fig. 2 Scanning Electron Microscopy for (A) control yoghurt and (B) fortified with CLA loaded r-CM

3.7. Sensory properties of yoghurt.

No significant differences (P<0.05) were found in scores for acceptability of yoghurt as affected by the added CLA-loaded r-CM. This indicates the efficiency of the r-CM in masking the oily taste of CLA. The scores for the different sensory attributes decreased after 15 days of storage, but no significant differences (P<0.05) were found between treatments and control. These results agree with those found El-Kholy, et al. [25]. Slightly increased in sensory acceptability of T2, which contains 0.75 CLA-loaded r-CM.

Table 8: Sensory properties of plain yoghurt and fortification yoghurt at 5±2°C*.

Sample	Flavor (60)		Body &Texture(30)		Color &Appearance(10)	
	Fresh	21 days	Fresh	21 days	Fresh	21 days
Control	53.00 ± 2.34^{a}	48.12 ± 1.75^{a}	24.95 ± 1.61^{a}	23.85 ± 1.70^{a}	8.65 ± 0.45^{a}	7.50 ± 0.70^{b}
T1	52.57 ± 4.27^{a}	47.24 ± 3.45^{a}	26.50 ± 2.19^{a}	24.75 ± 1.95^{a}	8.80 ± 0.44^{a}	7.85± 0.55^{ab}
T2	53.00 ± 3.29^{a}	50.15 ± 4.15^{a}	27.50 ± 0.58^{a}	25.75 ± 2.25^{a}	9.50 ± 0.44^{a}	9.00±0.73^{a}
T3	50.25 ± 2.27^{a}	48.37 ± 1.95^{a}	26.89 ± 2.75^{a}	23.95 ± 1.95^{a}	9.01 ± 1.84^{a}	8.05 ± 0.85^{a}

*see foot note table 2

4. Conclusions

Reassembled casein micelles efficiently entrapped CLA forming particles the nano-sizes and protected CLA from UV-induced oxidation. The addition of the CLA-loaded r-CM nanoparticles in buffalo milk yoghurt increased the CLA content of the product up to 10 times its original CLA content without any adverse effect on its sensory properties. Also, the study recommended using 0.75 CLA-loaded r-CM, which is so economical, and its have slightly increased sensory acceptable evaluation.

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