Biological Forum – An International Journal **8**(2): 73-87(2016)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Preparation and Evaluation of Nanoliposomes Containing green tea Extract and Investigating its Efficacy in Extending the Shelf Life of Fresh Orange and Pomegranate Juices

S. Naghavi*, S.H. Peighambardoust**, S. Azadmard-Damirchi and M. Sowti Khiabani*******
**Ph.D. Student, Department of Food Science, College of Agriculture, University of Tabriz, Tabriz, IRAN*
***Professor of Food Technology, Department of Food Science, College of Agriculture, University of Tabriz, Tabriz, IRAN*
****Professor of Food Chemistry, Department of Food Science, College of Agriculture, University of Tabriz, Tabriz, IRAN*
*****Associate Prof. of Food Microbiology, Department of Food Science, College of Agriculture, University of Tabriz, Tabriz, IRAN*

(Corresponding author: S.H. Peighambardoust)
(Received 08 June, 2016, Accepted 27 July, 2016)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Green tea leaves have high contents of phenolic compounds which has strong antioxidant properties but can have bitter taste which makes it less useful in food applications. Encapsulation in nanoliposomes could mask undesirable taste of green teas components and protect them against processing and storage. The objective of this study was to: 1) produce and evaluate physical properties of green tea extract (GTE) nanoliposomes using ethanol injection method; and 2) to incorporate them in different concentrations to fresh orange and pomegranate juices to preserve their quality upon storage. Particle size distribution and average diameter of GTE nanoliposomes were determined using dynamic light scattering method. Zeta potential was determined and encapsulation efficiency was evaluated using determination of total phenolic compounds of GTE in nanoliposomes. By incorporating more concentration of GTE in nanoliposomes their particle size increased from 67.7 nm for lower concentration to 370 nm for higher concentration. The average particle size of larger nanoliposomes (>100 nm) was increased upon storage time. Zeta potential data ranged from -20 to -25 mV indicating that all GTE nanoliposomes possess negative electrostatic charges promoting their stability by preventing them from coalescence and flocculation. Encapsulation efficiency was significantly ($p<0.05$) reduced by increasing in GTE concentrations in nanoliposomes. The nanoliposome system could preserve GTE compounds from possible oxidation and deterioration during processing and storage when used in formulation of orange and pomegranate juices. It was shown that the nanoliposome encapsulation of GTE improved its beneficial properties including antioxidant activities. Variations in GTE concentration as well as type of capsulation were critical in some of the parameters studied, i.e. pH, acidity, ascorbic acid and anthocyanins content. Higher GTE concentrations maintained the quality of fresh orange and pomegranate juices better than lower concentrations. Different concentrations of GTE in both non-capsulated and capsulated forms did not influence pH and acidity of orange and pomegranate juices. Acid ascorbic contents of both orange and pomegranate juices was significantly and negatively influenced by storage time. However, by incorporating GTE in nanoliposomal form to both orange and pomegranate juice, destruction of acid ascorbic was reduced during storage compared to control samples. In pomegranate juice the same trend was seen for anthocyanin content. By incorporation GTE nanoliposomes at concentrations of 300 ppm more anthocianins was preserved upon a long storage days compared to control. Concluding, the methodology used in this study to produce GTE nanoliposomes and incorporating them into orange and pomegranate juices could meaningfully preserve vit. C and anthocyanin contents of the juices during long storage days, while keeping their other physicochemical properties constant. Overall, this paper showed a possible use of GTE as a natural preservative ingredient to extend the shelf-life of fresh orange and pomegranate juices.

Keywords: Green tea; Orange; Pomegranate; Shelf life; encapsulation; Nanoliposome, Phenolic compounds

INTRODUCTION

The increasing public demand for natural additives has led to the growing use of these substances in food industries as antimicrobial, antioxidant and preservative agents. Worldwide interest has been arisen for the recovery and utilization of antioxidants from natural sources. Green tea is one of the most favorable natural compounds. It contains high quantities of catechins that are chemically defined as flavan-3-ols (Balentine *et al.* 1997). The major polyphenol in green tea is catechins and among them Epigallocatechin-3-gallate (EGCG) is the main and biologically most-active compound (Yang and Koo, 1997). The antioxidant activity of GTE has been well documented (Chan *et al.,* 2007; Wiseman *et al.,* 1997). This effect is due to the polyphenol content which has potent free radical scavenger activity through donating hydrogen atoms, interrupting chain oxidation reaction and accepting free radical (Noudoost *et al.*, 2015). One of the major problems which greatly limit application of natural compounds in food preparation and pharmaceutical industries is low efficiency resulting from sensitivity to adverse environmental conditions and decrease of stability and bioavailability during processing and storage (Lu *et al*., 2011). These compounds likely cause unpleasant taste, such as astringency, when added directly into food. Limited stability, conditioned solubility (Fang and Bhandari, 2010), and poor bioavailability (Mukhtar and Ahmad, 2000) have limited their use as additives in food, as well. They may also associate with food components, such as proteins, causing significant aggregation and precipitation, and quantity and/or functional loss of the polyphenols (Rashidinejad *et al.* 2014). Therefore, search for a new method in order to selectively protect natural compounds during processing and storage is important. For this purpose, various methods have been suggested including encapsulating main compounds, such as nanoparticles (Liang *et al.,* 2011), coprecipitation (Sosa *et al.,* 2011), coacervation of core micelles (Zhou *et al.,* 2012) microparticles (Elabbadi *et al*., 2011) and liposomes (Lu *et al.,* 2011; Gülseren *et al.,* 2012; Gulseren and Corredig, 2013). Encapsulation of phenolic compounds can provide an approach to solve these drawbacks (Sun-Waterhouse *et al.,* 2013) and to improve the antioxidant properties and shelf-life of foods. Liposomes and nanoliposomes could be attractive encapsulation systems for delivery and controlled release of phenolic compounds in food matrices. Conventional liposomes contain lecithin and cholesterol, which have hydrophilic and lipophilic groups to form a lipid bilayer that encapsulates an aqueous phase. They have also been widely used for delivery of vaccines, hormones, enzymes and vitamins in to the body (Gregoriadis 1984). They possess a number of benefits, including possibility of large-scale production, targetability (Mozafari *et al*. 2008), possibility of manufacturing by using safe natural ingredients, such as egg, soy, or milk (Thompson *et al.* 2007), biocompatibility, small size, ability to carry a wide variety of bioactive compounds (De Leeuw *et al*. 2009), as well as health benefits of liposomal ingredients such as phospholipids and sphingolipids for health (Koopman *et al.,* 1985).

An increasing number of studies have aimed at designing liposomal formulations to stabilize and protect phenolic compounds, to improve their aqueous solubility and bioavailability, and to achieve targeted and/or sustained release (Caddeo *et al.* 2008; Isailovic *et al.* 2013; Takahashi *et al.* 2009). To our best knowledge, there is no published report on the application of liposomal structures containing GTE in stabilizing and extending the shelf life of fruit juices. Thus, the objective of this study was to evaluate the effect of nanoliposome encapsulation, using ethanol injection method, on the antioxidant activity of GTE in extending the shelf life of orange and pomegranate juices.

MATERIALS AND METHODS

A. Preparations of GTE

Green tea leaves were collected from Gilan, Iran. Green tea aqueous extract solution was prepared by mixing ground tea leaves in distilled water (100°C, 1: 10 w/v), brewingfor 10 min at 80°C with stirring, and removing solid matter by filtration. The extract solution was dried at 60°C and stored at 4°C until used (Molan *et al.,* 2008).

B. Preparations of nanoliposomal GTE

To prepare nanoliposomal GTE, the ethanol injection method was used. Soy phosphatydilecholine (SPC) (Nanocs, USA) in concentration of 15µmol lipid/mL, cholesterol (SPC /cholesterol ratio 4:1) and GTE were dissolved in 5 mL of ethanol (Carlo erba, France). Then, 10 mL of ethanolic solution were slowly (during 10 min) injected into 70 mL of distilled water under homogenizer mixing at 20,000 rpm of homogenizer speed (Heidolph, Germany) (Zou *et al.* 2014).

C. Physicochemical characterization and morphology of nanoliposomal GTE

Physicochemical characterization of nanoliposomal GTE, including determination of encapsulation efficiency, particle size, size distribution, and zeta potential were determined as follows:

D. Encapsulation efficiency

The liposome encapsulation efficiency was determined using following equation:

$$EE\ (\%) = [(C_{total} – C_{free})/C_{total}] \times 100$$

Where, C total is the total concentration of phenolic compounds measured after liposome disruption by methanol and C free is the unentrapped phenolic compounds measured in the lower chamber of Millipore Amicon® after centrifugation. The content of phenolic compounds in samples was determined with the Folin-Ciocalteu reagent test (Sigma-Aldrich, St Louis, MO).

E. Particle size and zeta potential

The particle size and polydispersity index of nanoliposomal GTE were determined by DLS (Dynamic laser light scattering) (Brookhaven Instruments Corporation, Holtsville, NY, USA). The zeta potential of the liposomes were measured according to fang 2005 by a laser scattering method using Zeta sizer Nano- ZS (Malvern Instruments, England, UK) equipped with a helium-neon laser with a λ of 630 nm. Liposomal suspensions were diluted 100-fold with double-distilled water before the measurement of both particle size and zeta potential. The determination was repeated three times for each sample. The morphology of the nanoliposomal GTE was observed by TEM (Transmission Electron Microscopy). One drop of nanoliposomal GTE solution was 10- fold diluted with deionized buffer, dripped to the copper mesh surface, allowed to air dry for 5 min. The samples were then negatively stained with 1% phosphotungstic acid and observed by a high-voltage transmission electron microscope (Zeiss - EM10C - 80 KV, Germany).

F. Total phenolic contents (TPC)

Determination of phenolic compounds in GTE solution, before and after liposomal encapsulation was accomplished as suggested Noudoost *et al.* (2015). In order to estimate total phenolics, 1 ml of extracts (5 mg mL^{-1}) was combined with 1 ml Folin and Ciocalteu's phenol reagent (Merck, Germany). 1 ml saturated Na2Co3 (7.5%, w/v) solution (Merck, Germany) was then added to the mixture after 3 min and total volume of mixture was adjusted to 10 mL with distilled water. This reaction mixture was then kept in dark place for 90 min. The absorbance was read measured by spectrophotometer (CECIL, Aquaris 1100, England) at 725 nm according to Capannesi *et al.* (2000). TPC was determined by calibration curve which achieved from defined concentration of caffeic acid absorbance and the final results reported as mg/kg.

G. Fruit juice experiments

Sample collection. Oranges (*Citrus sinensis*) of the cv. Valencia purchased from local market and stored unwrapped in bulk. Oranges with less than 75 mm or more than 90 mm of diameter were discarded. The oranges were brought to the laboratory and stored at 4 °C until processing. Freshly harvested pomegranates (*Punica granatum*) were obtained from Taft (Yazd, Iran) local market. Only average sized (d=75-90 mm) fruits were used for juice extraction.

Orange juice extraction. Unpeeled oranges were manually washed with tap water, cut in halves and squeezed using an manual orange juicer on the same day of purchase (Fig. 1). Freshly squeezed juice was passed through a strainer (twice) to remove pulp and seeds. The filtrate was homogenised using a lab-scale homogenizer for 5 min at 4°C. The juices were enriched with both non-capsulated and nanoliposome capsulated GTE at concentrations of 100, 200 and 300 ppm. The juice was stored at 4°C in sterilized and opaque polyethylene containers (with a 50-mm thickness and 500 cc capacity). Conventionally treated (pasteurized) juice with no GTE for its use as a control in subsequent analysis. A sample with 100 ppm of empty nanoliposomes was also prepared as control for GTE liposomal samples. Samples were pasteurized at 98 °C for 15 min. Afterwards, the orange juice was immediately cooled in ice water for 2 min. The juices were stored in refrigeration and darkness at 4°C (±1 °C) without controlled humidity. Samples were analyzed by triplicate immediately after processing.

Pomegranate juice extraction. Pomegranates were cut in halves and arils were hand-separated from the pith. Juice was immediately obtained by applying pressure on arils inside a nylon mesh with a manual press. The extracted cloudy juice contained 2% pulp. For obtaining clarified juice, the cloudy juice was passed through a strainer (twice) to remove pulp and seeds. Pasteurization and subsequent handling and storage was performed similar to those of orange juice.

Physico-chemical analyses. The pH of 20 mL samples were determined at room temperature and constant agitation using a pH-meter (model 420A, Orion, USA). It was expressed as the negative logarithm of the hydrogen ion concentration in a solution (Martin-Diana *et al.*, 2009).

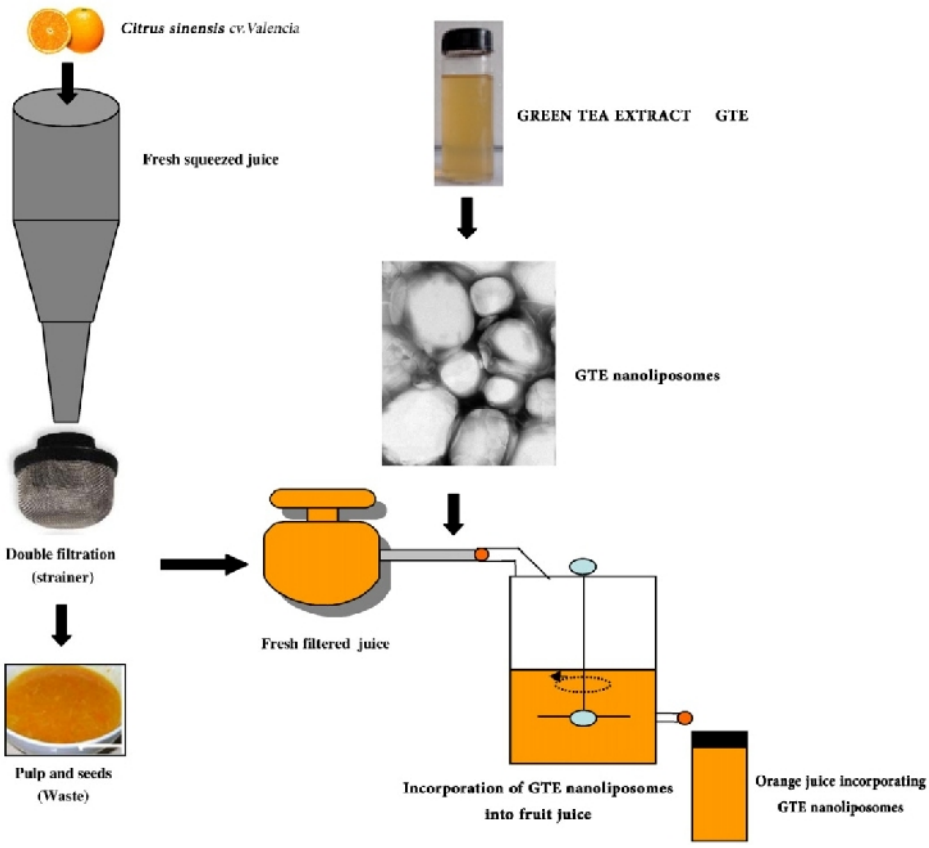

Fig. 1. Fruit juice processing chart.

Titratable acid content (TAC) was determined according to the official method (AOAC 1984). Briefly, a 20 mL juice sample was titrated with NaOH standard titration solution (0.1 mol/L) to pH 8.2 ± 0.05 using a calibrated pH meter to monitor the pH. Results were expressed as g citric acid per liter of sample.

The turbidity of each sample of juice was measured using a direct reading spectrophotometer (model DR/2000, Hach, USA). The wavelength of the instrument was brought to 810 nm and deionized water used as a blank. The measurements of the samples of fruit juice were done in triplicate with a solution of 1:25 (juice/water), to work with in the detectable range. The results were given in mg of suspended solids per litre of solution(Martin-Diana *et al.*, 2009).

Vitamin C (reduced ascorbic acid) was measured following the AOAC (2006, chap. 45) Official Method 967.21. Ascorbic acid was estimated by titration with the colored oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA was added as a chelating agent to reduce interference from copper and iron (Letaief *et al.*, 2016).

Total anthocyanin concentration was measured by pH differential method (Giusti&Wrolstad, 2001). Briefly, 0.5 mL of each sample was brought to pH1.0 by 4.5 mL of KCl-HCl solution (0.025 mol/L) and to pH4.5 by 4.5 mL of NaAc-HAc solution (0.4 mol/L), respectively. Absorbance of equilibrated reaction mixture solutions (▫ 25°C, 15 min standing) were measured at 520 nm and at 700 nm, respectively using a UV-VIS spectrophotometer at ambient temperature with distilled water as blank. Results were expressed as mg CyE (cyanidin-3-glucoside equivalent)/mL juice. Each juice sample was tested in triplicate (Li *et al.*, 2015)

Statistical Analysis

The data collected in this study were expressed as mean and subjected to one-way analysis of variance (ANOVA). Multiple comparisons were performed by Tukey's test. Statistical significance was set at $p<0.05$. All analyses were performed using SPSS Version 16.0.1 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analyses and contour plots were analyzed using Statgraphics software 2.1 (Statistical Graphics Co., Rockville, USA).

RESULTS AND DISCUSSION

A. Physical properties of GTE nanoliposomes

Particle size distribution. Particle size distribution is an important factor in nanoliposome preparation, because it would affect the stability of nanoliposomes and also bioactive compounds release from encapsulation. Table 1 demonstrates particle size of nanoliposomes containing different GTE concentration upon storage time for 7 days. In the first day of production, the size of the nanoliposomes was in the range of 67.7 to 370 nm.

Table 1: Particle size of liposomes containing different concentrations of green tea extract.

	GTE concentration in nanoliposomes (cm^3)	Particle size (nm)	
		Day 1	Day 7
F1	**5**	67.7 Ad	68.1 Ad
F2	**10**	100.1 Ac	99.2 Ac
F3	**15**	190.4 Bb	253 Ab
F4	**20**	370 Ba	489 Aa

Each cm^3 of GTE contained 20 mg phenolic compounds. Data are mean of triplicates. Small and capital different letters show significant ($p<0.05$) differences between means for formulations and days, respectively.

Increasing extract concentration in nanoliposomes caused an increase in nanoliposome size. The smallest and largest particles in day 1 were belong to nanoliposomes containing 5 and 20 cm^3 GTE, respectively. Nanoliposomes incorporating GTE showed smaller size than those of empty liposomes. Particle size obtained in this study for nanoliposomes incorporating GTE was similar to the results obtained by Zou *et al.* (2014) who reported particle size 66.8 nm for nanoliposomes entrapping tea polyphenols prepared by a combination of ethanol injection and high pressure microfluidization. Gulseren and Corredig (2013) also reported nanoliposomes of about 90 nm (pH = 7) entrapping tea polyphenols using high pressure homogenization. Smaller size of GTE nanoliposomes comparing to the size of empty nanoliposomes confirms the higher cohesion and packing among the apolar chains in the vesicular membrane. Sinico *et al.* (2005) and Valenti *et al.* (2001) also reported smaller size of essential oil loading liposomes compared to that of empty liposomes. By increasing GTE concentration, nanoliposomes particle size increased (Fig. 2).

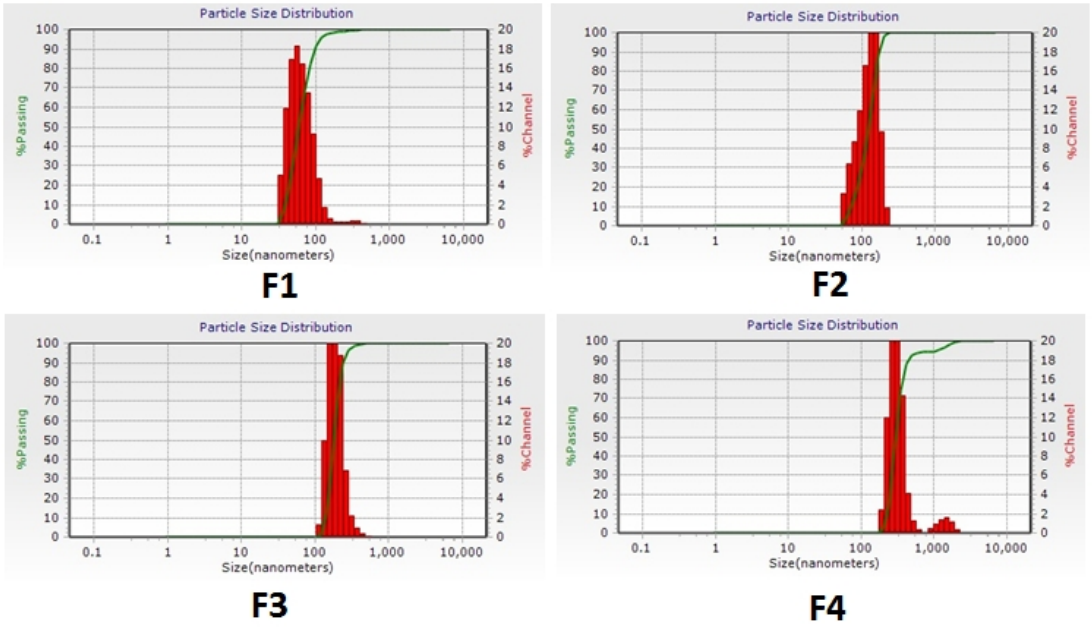

Fig. 2. Average volumes of nanoliposomes particles containing different concentrations of GTE in Day 1. F1 to F4 treatments are described in Table 1.

This might be explained by the fact that phenolic compounds in GTE are not solely encapsulated inside the liposomes, but part of them entrapped between nanoliposomal bilayers or adsorbed by the surface of liposomes. Evaluating size of nanoliposomes after one week storage at 4°C (Table 1) showed no change in size of GTE liposomes containing 5 and 10 cm^3 extracts, while an increase in size nanoliposomes was observed for higher concentrations of GTE after one week storage. It can be concluded that initial size of liposomes affects size increase upon storage. Larger liposomes show size increase during storage. This might be related to negative charges of phenolic compounds in liposomes causing a repulsive force leading to the expansion of layers and size increase in nanoliposomes.

Zeta Potential. The zeta potential is an index of the magnitude of the repulsive interaction between colloidal particles and is used to assess the stability of a vesicular suspension. In particles with low zeta potential, there is only a little repulsion force and the particles will eventually aggregate, resulting in suspension instability (Caddeo *et al.* 2008). Generally, if the absolute zeta potential shows a value greater than 30 mV, the system is stable. However, it was also deemed in some studies that even at low zeta potential values (i.e., close to 0), liposomes might remain stable, and therefore the zeta potential value might not be a direct indicator of the stability of liposome (Zou *et al.* 2014).

Zeta potential for nanoliposome with different concentrations of GTE are shown in Table 2.

Table 2: Zeta potential for nanoliposome with different concentrations of GTE.

Formulation	GTE contentration in nanoliposomes (cm^3)	Zeta potential (mV)
F1	**5**	-23 b
F2	**10**	-25 a
F3	**15**	-26 a
F4	**20**	-30 c

Data are mean of triplicates. Different letters show significant ($p<0.05$) differences between means.

All the formulations carried the anionic negative charge. Incorporating GTE to liposomes increased the zeta potential of particles, which was probably due to the polyphenols being negatively charged by partial dissociation under this pH, considering the anionic charge of extract solution. Increasing GTE concentration led to more negative charge of nanoliposomal system. As mentioned before, this might be related to negative charges of phenolic compounds in large liposomes which cause more repulsive forces leading to the expansion of layers and size increase in nanoliposomes which possess more negative charges.

It has also been shown that in small unilamellar liposomes, phenolic compounds could mainly incorporate or absorb to the liposomal membrane, rather than the interior (Gibis *et al.*, 2012), which could explain the decrease in anionic charge of liposomal formulations with higher concentrations of extract. In other studies zeta potential of -67.2 mV (pH=6.62) (Lu *et al.*, 2011), -6.16 mV (pH=6.0) (Zou *et al.* 2014) and -12 mV (pH=5.0) (Gulseren and Corredig, 2013) have been reported for nanoliposomes entrapping tea polyphenols. The different value of zeta potential of liposomes entrapping phenolic compounds could be probably attributed to variations in competence and property of phospholipids.

Encapsulation Efficiency. Encapsulation efficiency (EE) is an important characteristic of encapsulation technique showing loading capacity of the liposomes. EEof nanoliposomes entrapping GTE in different concentrations has been shown in Table 3. EE% for all the formulations was in the range of 65-95%. Storage of samples at 4°C for one week did not decrease EE% (data not shown). Increasing in GTE concentration led to a decrease in EE.

Table 3. Encapsulation efficiency of nanoliposome with different concentrations of GTE.

Formulation	GTE contentration in nanoliposomes (cm^3)	Encapsulation efficiency (%)
F1	**5**	95 a
F2	**10**	93 b
F3	**15**	87 c
F4	**20**	65 d

Data are mean of triplicates. Different letters show significant ($p<0.05$) differences between means.

As discussed before, more negative charge of higher concentrations of GTE in nanoliposomes (Table 2) makes them unstable in terms of size (Fig. 2) and repulsive forces, thereby reduces the encapsulation efficiency.

Similarly, Gulseren and Corredig (2013) reported a decrease in EE of nanoliposomes encapsulating tea polyphenols from 60% to 40% by increasing extract concentration. Nakayama *et al.* (2000) demonstrated that at low concentrations EGCG and ECG prevent leakage of calcein from liposomes, possibly due to their gallic acid ester contents, which is responsible for their bilayer affinity, while at high concentrations, the polyphenols may disrupt the membrane structure. Encapsulation efficiencies ranging from 20 to above 90% have been reported for liposomes entrapping phenolic compounds, depending on the physico-chemical properties of the phenolic compound and variations in preparing method. Relatively low EE% of about 20-57% for salidroside (concentration of 5 to 10%) entrapping liposomes prepared from different methods was reported by Fan et al.(2007), which was the highest (28-57%) through the freeze-thawing method, followed by thin film evaporation, reverse phase evaporation, melting and sonication. Fang *et al.* (2006) indicated much higher EE of 84-99% for EGCG compared to only 39-57% for (+)-catechin and 31-64% for (-)-epicatechin, which was attributed to the presence of a galloyl group in EGCG, causing greater lipophilicity of EGCG. Also, Zou *et al.* (2014) reported EE of 78.5% for nanoliposomes entrapping tea polyphenols prepared by a combination of ethanol injection method and high pressure microfludization, which was in consistent with our results. In another study, Kosaraju *et al.* (2006) in an effort for encapsulating olive leaf extract by spray drying, reported a low EE of only 27.5% which was much lower than our results. These results indicate that nanoliosomes could be potential carriers for delivery of olive leaf extract.

B. Fruit juice experiments

Orange juice

Ph. Effect of GTE concentration and storage days on pH of orange juice enriched with non-capsulated (Fig. 3-A) and nanoliposomal GTE (Fig. 3B) indicates a slight but not significant ($p<0.05$) drop in pH during 15 days of storage. Different concentrations of GTE in both non-capsulated and capsulated forms did not influence pH of orange juice.

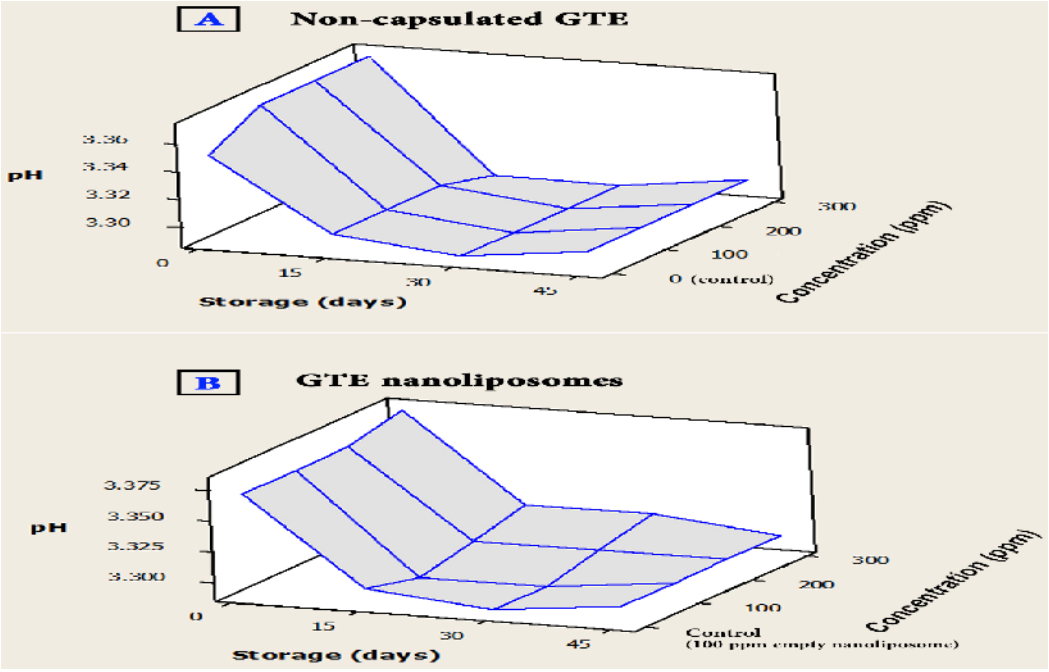

Fig. 3. Effect of concentration and storage days on pH of orange juice enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B).

This is in contradictory with results reported by Martin-Diana *et al*. (2009) about the effect of chitosan nanoliposomes on orange juice pH. They reported an increasing trend for pH of orange juice as function of both liposome concentrations and storage days. In their study chitosan reduced fruit juice acidity based on its acid-binding properties. They explained that when the pH is lower than 6.5, chitosan carries a positive charge along its backbone (Einbuand Varum, 2003). Such effect was not seen in case of GTE nanoliposmes. Our results are in parallel with those reported by Kelebek *et al* (2008) in fresh orange juice who found pH values between 3.05 and 3.35 in *C. sinensis* (variant Sanguinello and Moro).

Titratable acidity content (TAC). TAC results of orange juices enriched with non-capsulated GTE (Fig. 4) also paralleled pH results as explained before. No significant ($p<0.05$) difference was seen for TAC results as function of different GTE concentrations and storage time. However, samples enriched with 100 ppm GTE nanoliposomes (Fig. 4-B) had significantly higher TAC than those of other concentrations. Storage days did not significantly ($p<0.05$) influence TA of samples enriched with GTE nanoliposomes.

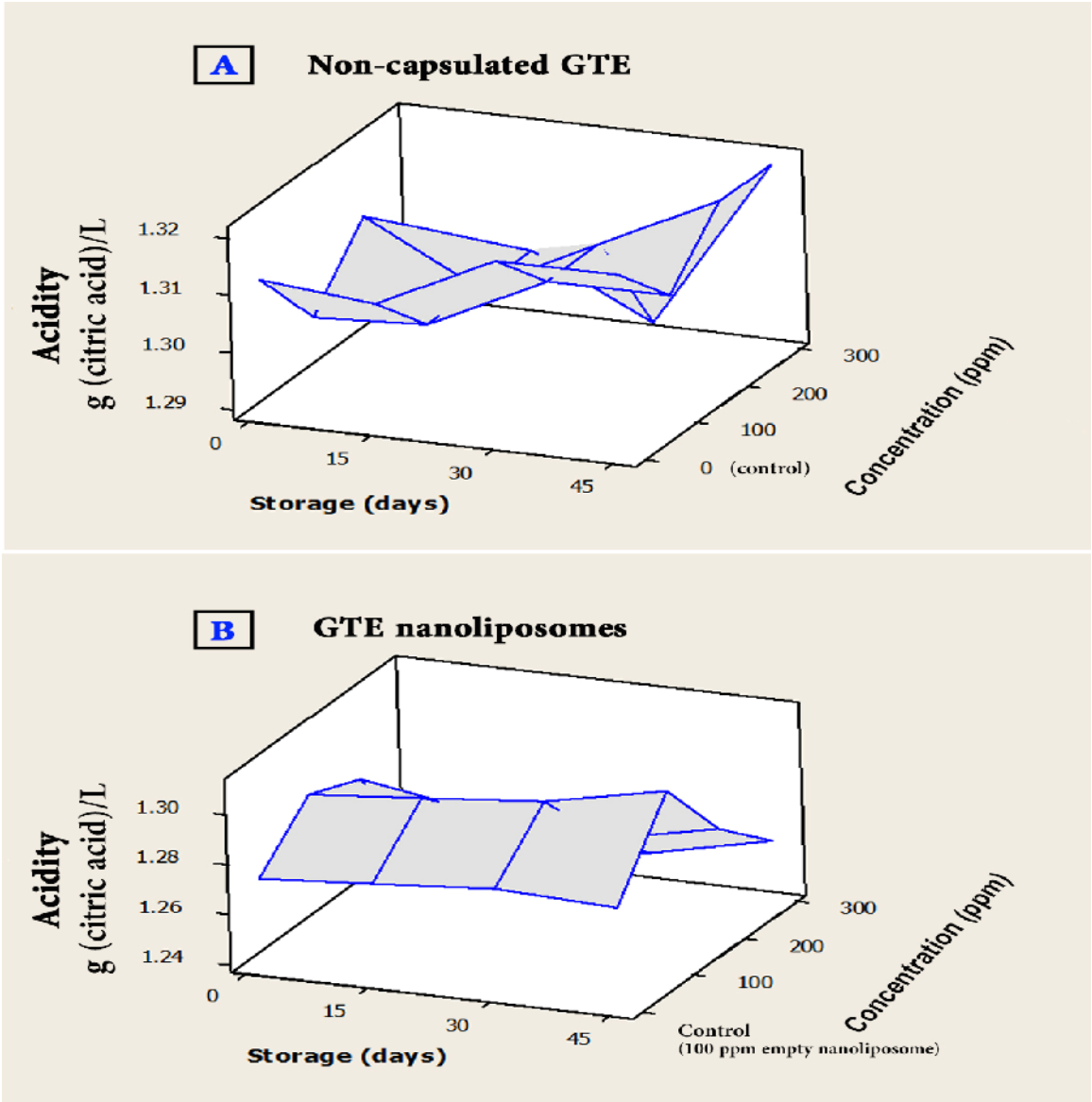

Fig. 4. Effect of concentration and storage days on titratable acidity content (TAC) of orange juice enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B).

Acid ascorbic content. Effect of GTE concentration and storage days on acid ascorbic (AA) content of orange juice enriched with non-capsulated (Fig. 5-A) and nanoliposomal GTE (Fig. 5-B) indicates a significant ($p<0.05$)linear effect of the storage time on AA content. AA content has beendescribed as an indicator of quality in juices and orange juice is consider one of the best sources of vitamin C by the consumers (Shaw & Moshonas, 1991). In control sample, where no GTE added to fruit juice, destruction of AA is considerable. However, incorporating GTE especially in liposomal form could eliminate the destruction of vit. C upon long storage times (over 30 days). Antioxidant effect of GTE is responsible for preventing the loss of AA in fruit juice. This effect is more enhanced when capsulated form incorporated to the orange juice. Nevertheless, increasing GTE concentration in both free and liposomal form did not enhance antioxidant effect of green tea extract. Out results are in line with those reported by by Martin-Diana (2009) on the effect of chitosan nanoliposomes on AA content of orange juice. They reported a decreasing trend of AA storage days. In their study chitosan reduced AA content of orange juice. They explained that acid sequestration effect of chitosan described by Imeri & Knorr (1988) might have caused this ascorbic acid reduction. Low chitosan concentrations did not affect the AA content, which decreased in samples with higher chitosan concentration.

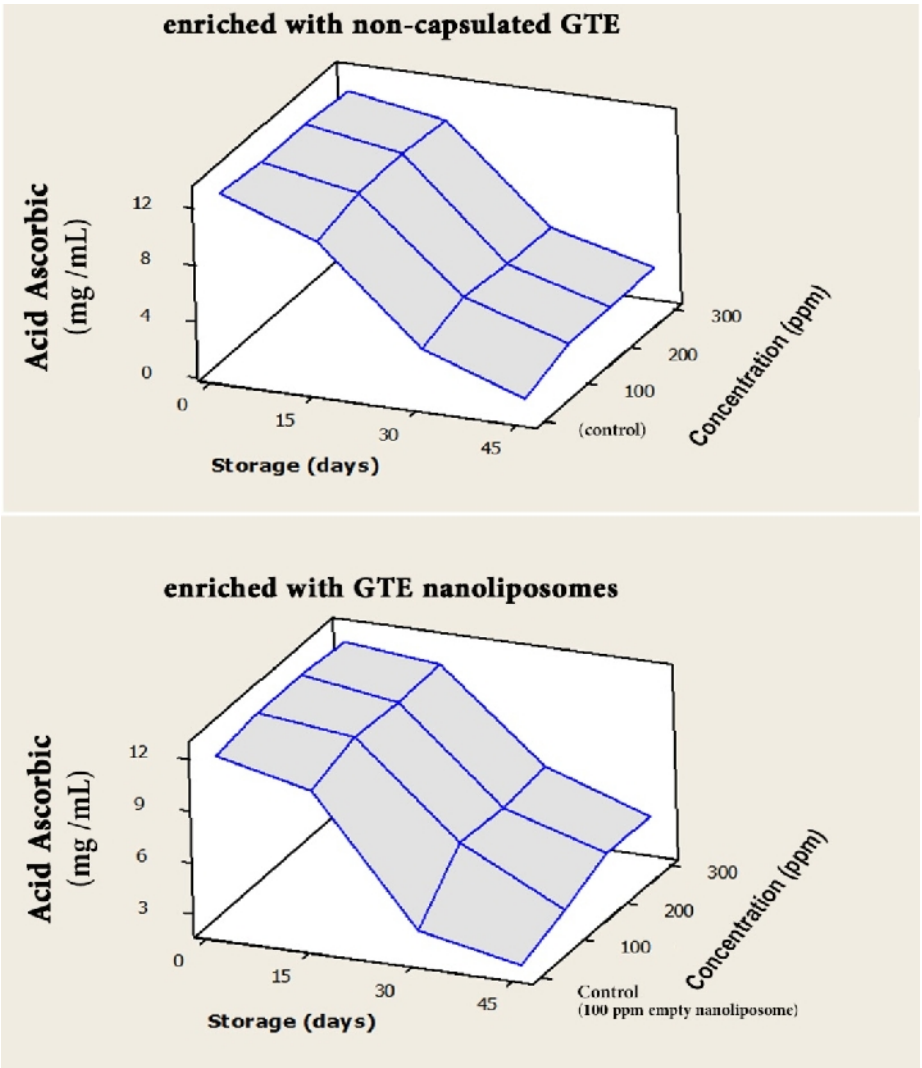

Fig. 5. Effect of concentration and storage days on acid ascorbic content of orange juice enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B).

Increasing the concentration of GTE nanoliposomes significantly ($p<0.05$) increased AA content of orange juice at long storage times (over 30 days). In our study, GTE nanoliposomes (at a concentration of 100 ppm) retained an acceptable level of AA during storage days (95% after 15 d, 50% after 30 d and 26% after 45 d).

Turbidity. Turbidity data of orange juice incorporated with non-capsulated GTE is shown in Fig. 6-A. By increasing GTE concentration and prolonging storage time turbidity increased. It appears that GTE in non-capsulated form affect the clarity of orange juice and reduces its appearance acceptability. When GTE is added in nanoliposomal capsulated form there was no significant difference for turbidity data of different concentrations and storage time. Fig. 6-B shows the variation of turbidity at different GTE-nanoliposome concentrations and over storage time; significant ($p<0.05$) linear effects of the storage time and chitosan concentration were observed. Fresh orange juices are usually cloudy and this is an acceptable and desirable component (Sin *et al.*, 2006). The turbidity in the juice is mainly caused by the polysaccharides present in the juice (Grassin and Fauquembergue, 1996). Concentration of GTE-nanoliposome controlled the turbidity of the orange juice. These results were in agreement to Chatterjee *et al.* (2004) and Martin-Diana *et al.* (2009) who reported a decrease in turbidity due to chitosan addition. Chitosan is different from GTE because it is reported that the chitosan has been used for juice clarification (Chatterjee *et al.*, 2004). In our study, perhaps, small size of nanocapsuls and capsulated nature of the core material (GTE) account for the different turbidity results compared to those of non-capsulated (free) forms.

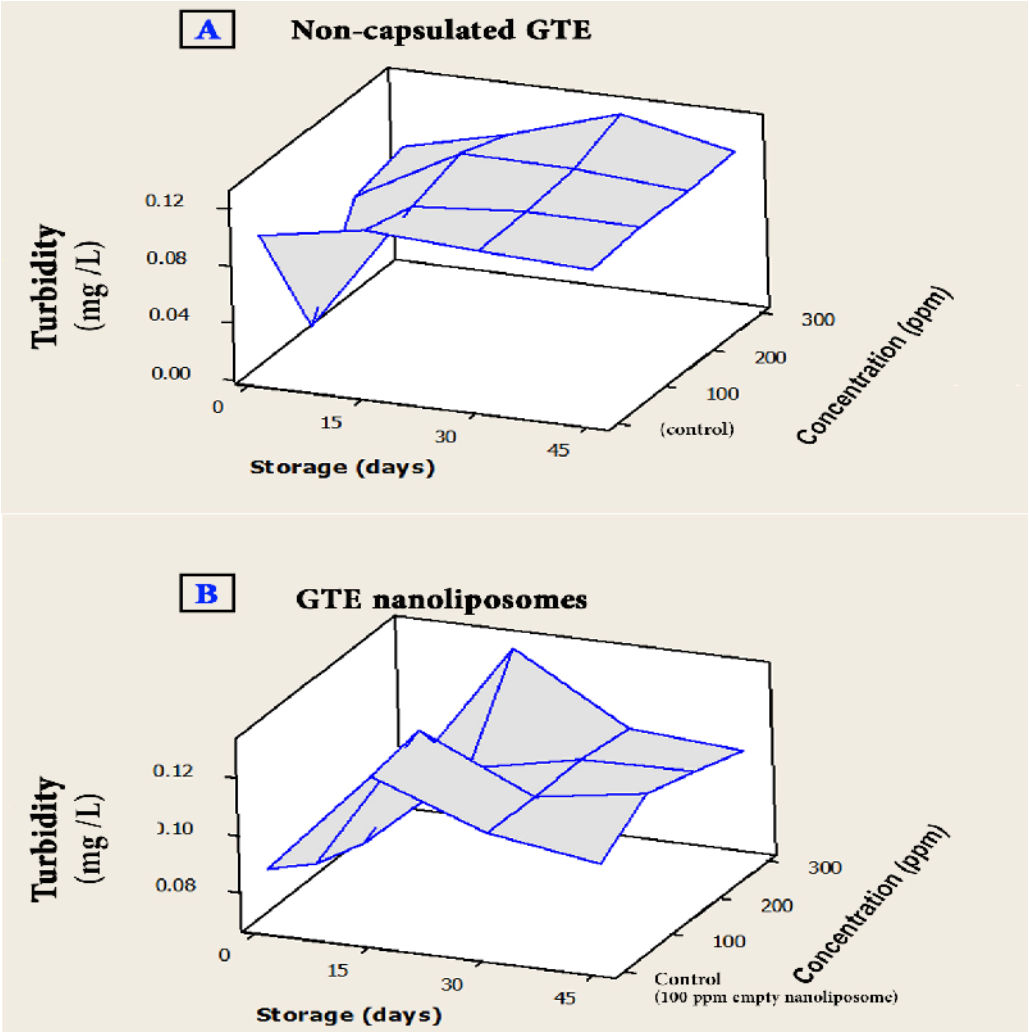

Fig. 6. Effect of concentration and storage days on turbidity of orange juice enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B).

Pomegranate juice.

Acid ascorbic content. Effect of GTE concentration and storage days on acid ascorbic (AA) content of pomegranate juice enriched with non-capsulated (Fig. 7-A) and nanoliposomal GTE (Fig. 7-B) indicates a significant ($p<0.05$) linear effect of the storage time on AA content. AA content has been described as an indicator of quality in juices (Shaw & Moshonas, 1991). In control sample, where no GTE added to fruit juice, destruction of AA is considerable. However, incorporating GTE especially in liposomal form could eliminate the destruction of vit. C upon long storage times (over 30 days). Antioxidant effect of GTE is responsible for preventing the loss of AA in fruit juice. This effect is more enhanced when capsulated form incorporated to the pomegranate juice. Nevertheless, increasing GTE concentration in both free and liposomal form did not enhance antioxidant effect of green tea extract. AA results of pomegranate juice paralleled those for orange juice discussed before.

Anthocyanin content. Fig 8 shows variations of anthocyanin content of pomegranate juice as functions of concentrations of GTE and storage time for juices enriched with non-capsulated (Fig. 8-A) and nanoliposomal GTE (Fig. 8-B) forms. As can be seen in both figures (A and B), there is a significant ($p<0.05$) linear effect of the storage time on anthocyanin content of pomegranate juice. A rapid and considerable decrease in anthocyanin content of pomegranate juice was seen for control sample, where there is no GTE in formulation. Although anthocyanin content of pomegranate juice also decreases over storage time when GTE is added in both non-capsulated and liposomal forms, however, this effect is less enhanced compared to control. It can be concluded that green tea has antioxidant effect preventing oxidation of anthocyanins in pomegranate juice, and by increasing its concentration, this protective effect is increased. Antioxidant effect of GTE is responsible for preventing the loss of anthocyanins in fruit juice.

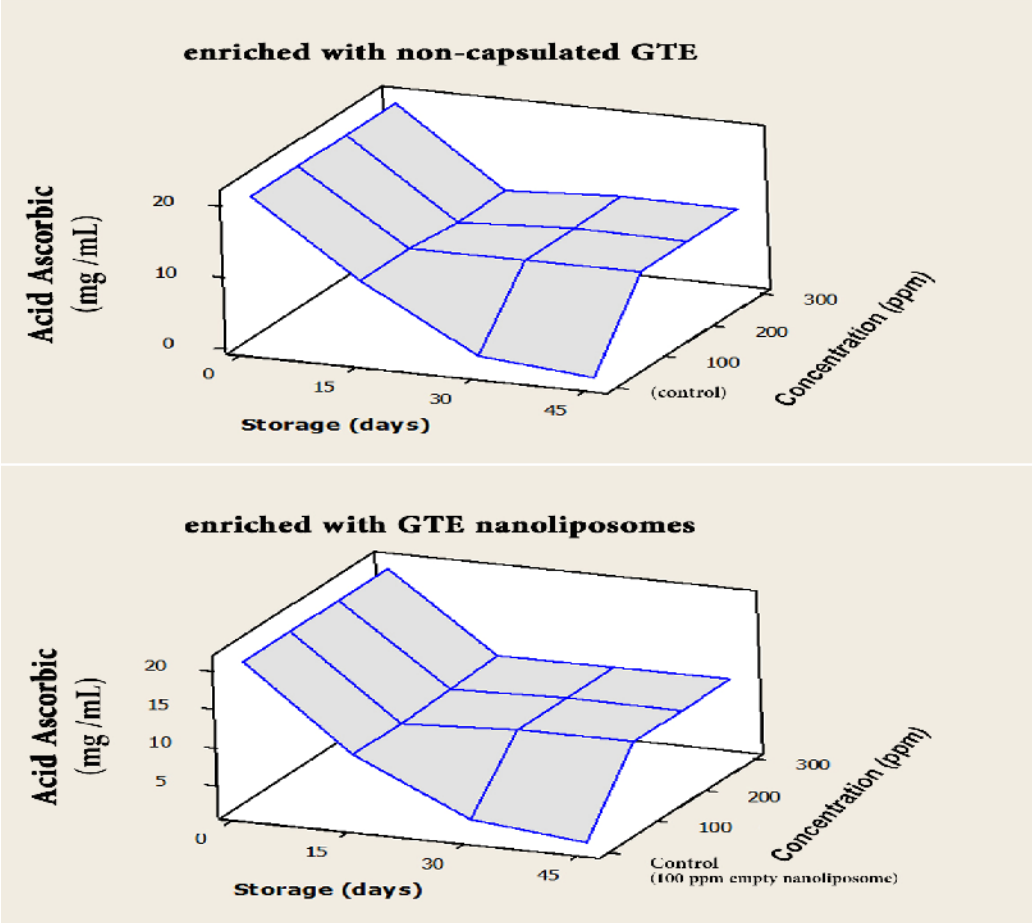

Fig. 7. Effect of concentration and storage days on acid ascorbic of pomegranate enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B)

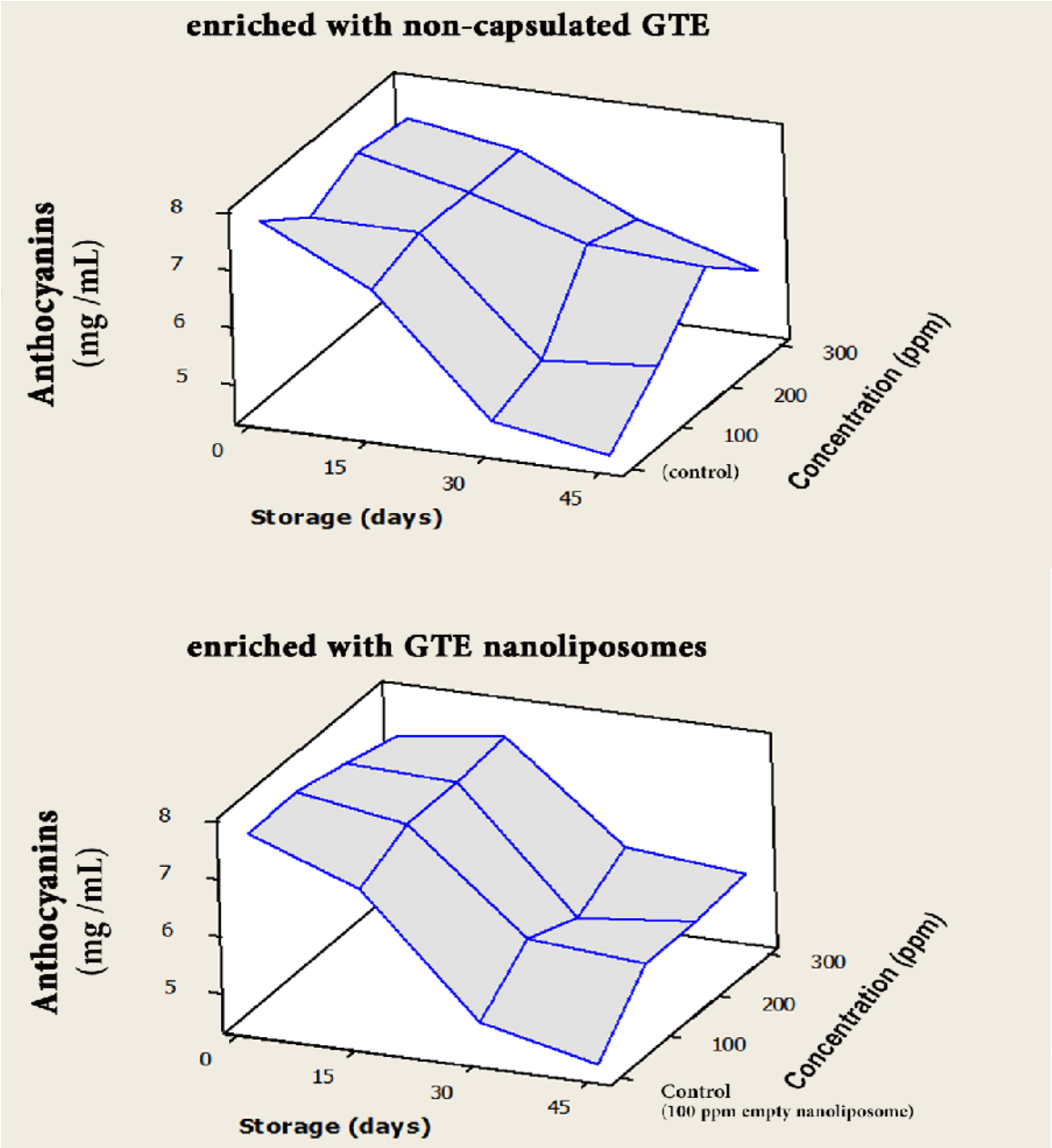

Fig. 8. Effect of concentration and storage days on anthocyanin content of pomegranate enriched with naon-capsulated GTE (A) and GTE nanoliposomes (B)

Turbidity. Turbidity results of pomegranate juice incorporated with non-capsulated and capsulated GTE was similar to those reported for orange juice. Hence, the data are not shown here. In case of pomegranate juice GTE in non-capsulated form affect the clarity of juice and reduces its appearance acceptability. When GTE is added in nanoliposomal capsulated form there was no significant difference for turbidity data of different concentrations and storage time.

pH and acidity. Effect of GTE concentration and storage days on pH and total acidity content (TAC) of pomegranate juice enriched with non-capsulated (Table 4) and nanoliposomal GTE (Table 5) indicates no significant ($p<0.05$) change in pH and TAC of juice upon storage. Different concentrations of GTE in both non-capsulated and capsulated forms did not influence pH and TAC of juice.

Table 4: Effect of GTE concentration and storage days on pH and total acidity content of pomegranate juice enriched with non-capsulated GTE.

	Day 1		Day 15		Day 30		Day 45	
	pH	Acidity	pH	Acidity	pH	Acidity	pH	Acidity
Ctrl	3.46	1.13	3.47	1.06	3.49	1.07	3.45	1.08
100 ppm	3.45	1.11	3.47	1.05	3.48	1.05	3.46	1.07
200 ppm	3.46	1.15	3.48	1.05	3.47	1.05	3.46	1.07
300 ppm	3.47	1.15	3.49	1.04	3.47	1.05	3.46	1.06

Data are mean of triplicates and are not significantly ($p<0.05$) different.

Table 5: Effect of concentration and storage days on pH and total acidity content of pomegranate juice enriched with GTE nanoliposomes.

	Day 1		Day 15		Day 30		Day 45	
	pH	Acidity	pH	Acidity	pH	Acidity	pH	Acidity
Ctrl (100 ppm empty nanoliposome)	3.46	1.16	3.49	1.08	3.50	1.04	3.46	1.04
100 ppm	3.46	1.11	3.49	1.04	3.48	1.04	3.47	1.05
200 ppm	3.46	1.15	3.49	1.02	3.48	1.02	3.47	1.04
300 ppm	3.48	1.15	3.50	1.00	3.50	0.95	3.49	0.96

Data are mean of triplicates and are not significantly ($p<0.05$) different.

CONCLUSIONS

Ethanol injection method used in this study successfully produced GTE nanoliposomes with desired physical properties. The size of the nanoliposomes was in an acceptable range. However, increasing GTE concentration in nanoliposomes caused an increase in nanoliposome size. Incorporating GTE to liposomes increased the zeta potential of particles, which was probably due to the polyphenols being negatively charged by partial dissociation, considering the anionic charge of extract solution. Encapsulation efficiency for all the formulations was in the range of 65-95%. This paper showed a possible use of GTE as a natural preservative ingredient to extend the shelf-life of fresh orange and pomegranate juices. The nanoliposome system could preserve GTE compounds from possible oxidation and deterioration during processing and storage when used in formulation of orange and pomegranate juices. The results of this study demonstrated that the nanoliposome encapsulation of GTE improved its beneficial properties including antioxidant activities. Variations in GTE concentration as well as type of capsulation were critical in some of the parameters studied, i.e. pH, acidity, ascorbic acid and anthocyanins content. Higher GTE concentrations maintained the quality better than lower concentrations from a quality point of view. Different concentrations of GTE in both non-capsulated and capsulated forms did not influence pH and acidity of orange and pomegranate juices. Acid ascorbic contents of both orange and pomegranate juices was significantly and negatively influenced by storage time. However, by incorporating GTE in nanoliposomal form to bothorange and pomegranate juice, destruction of acid ascorbic was reduced during storage compared to control samples. In pomegranate juice the same trend was seen for anthocyanin content of the juice. By incorporation GTE nanoliposomes at concentrations of 300 ppm more anthocianins was preserved upon a long storage days compared to control, where no GTE was added to the juice. Concluding, the methodology used in this study to produce GTE nanoliposomes and incorporating them into orange and pomegranate juices could meaningfully preserve vit. C and anthocyanin contents of the juices during long storage days, while keeping their other physicochemical properties constant.

REFERENCES

Balentine DA, Wiseman SA and BouwensLC. 1997. The chemistry of tea flavonoids. Critical Reviews in *Food Science and Nutrition;* **37**(8): 693-704.

Caddeo C, Teskac K, Sinico C and Kristl J. 2008. Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells. *International Journal of Pharmaceuticals*; **36,** 183-191.

Capannesi C, Palchetti I, Mascini M, and Parenti A, 2000. Electrochemical sensor and biosensor for polyphenols detection in olive oils. *Food Chemistry* **71**: 553-562.

Chan E, Lim Y and Chew Y. 2007. Antioxidant activity of *Camellia sinensis* leaves and teafrom a lowland plantation in Malaysia. *Food Chemistry*; **102**(4): 1214-1222.

Chatterjee S, Chatterjee S., Chatterjee BP and Guha AK. 2004. Clarification of fruit juice with chitosan. *Process Biochemistry*; **39**: 2229-2232.

De Leeuw J, De Vijlder HC, Bjerring P and Newmann, HA. 2009. Liposomes in dermatology today. Journal of the *European Academy of Dermatology and Venereology;* **23**, 505-516.

Einbu A. andVarum KM. 2003. Structure-property relationship in chitosan. In T. Priotr (Ed.), Chemical and functional properties of food saccharides (pp. 223).

Elabbadi A, Jeckelmann N, Haefliger OP and Ouali L. 2011. Complexation/encapsulation of green tea polyphenols in mixed calciumcarbonate and phosphate micro-particles. *Journal of Microencapsulation*; **28**(1): 1-9.

Fan M, Xu S., Xia S and Zhang X. 2007 Effect of different preparation methods onphysicochemical properties of salidroside liposomes. *Journal of Agricultural and Food Chemistry*; **55**: 3089-3095.

Fang Z and Bhandari B. 2010. Encapsulation of polyphenols - a review. Trends in Food Science and Technology; 21: 510-523.

Gibis M, Vogt E. and Weiss J. 2012. Encapsulation of polyphenolic grape seed extract inpolymer-coated Liposomes. Food Function; 3: 246-254.

Grassin C andFauquembergue P. 1996. Application of pectinases in beverages. In J. Visser &A.G.J. Voragen (Eds.), Pectin and pectinases (pp. 453-462). *Elsevier Science* B.V.

Gregoriadis G. 1984. Liposome technology. Volume II: Incorporation of drugs, proteins and genetic material. Boca Raton: CRC press.

Gulseren I and Corredig M. 2013. Storage stability and physical characteristics of tea-polyphenol bearing nanoliposomes prepared with milk fat globule membrane phospholipids. Journal of Agricultural and FoodChemistry; **61**(13): 3242-3251.

Gülseren I, Guri A and Corredig M. 2012. Encapsulation of tea polyphenols in nanoliposomes prepared with milkphospholipids and their effect on the viability of HT-29 human carcinoma cells. *Food Digestion;* **3**(1-3): 36-45.

Imeri AG and Knorr D. 1988. Effect of chitosan on yield and compositional data ofcarrot and apple juice. Journal of Food Science; 53:1707-1709.

Isailovic BD, Kostic IT, Zvonar A, Dordevic VB, Gasperlin M, Nedovic VA and Bugarski BM 2013. Resveratrol loaded liposomes produced by different techniques. *Innovative Food Science and Emerging Technology*, **19**; 181-189.

Kelebek H, Canbas A and Selli S. 2008. Determination of phenolic composition and antioxidant capacity of blood orange juices obtained from cvs. Moro and Sanguimello (*Citrus sinensis* (L.) Osbeck) grown in Turkey. *Food Chemistry;* **107**: 1710-1716.

Koopman, JS, Turkish, VJ and Monto, AS. 1985. Infant formulas and gastrointestinal illness. *American Journal of Public Health*; **75**, 477-480.

Kosaraju SL, D'ath L. and Lawrence A. 2006. Preparation and characterisation of chitosan microspheres for antioxidant delivery. *Carbohydrate Polymer*; **64**: 163-167.

Letaief H, Zemni H, Mliki A, Chebil S. 2016. Composition of *Citrus sinensis* (L.) Osbeckcv "Maltaise demi-sanguine"juice. A comparison between organic and conventional farming. *Food Chemistry*; **194**: 290-295.

Li X, Wasila H, LiuL, Yuan T, Gao Z, Zhao B, Ahmad I. 2015. Physicochemical characteristics, polyphenol compositions andantioxidant potential of pomegranate juices from 10 Chinese cultivars and the environmental factors analysis. *Food Chemistry*; **175**: 575-584.

Liang J, Li F, Fang Y, Yang W, An X,Zhao L. 2011. Synthesis, characterization andcytotoxicity studies of chitosan-coated tea polyphenols nanoparticles. *Colloids Surf. B. Biointerfaces*; **82**(2): 297-301.

Lu Q, Li D.C. and Jiang JG. 2011. Preparation of a tea polyphenol nanoliposome system andits physicochemical properties. *Journal of Agricultural and Food Chemistry*; **59**(24): 13004-13011.

Martin-Diana AB, Rico D, Barat JM, Barry-Ryan C. 2009. Orange juices enriched with chitosan: Optimisation for extending the shelf-life. *Innovative Food Science and Emerging Technologies* **10**: 590-600.

Molan AL, De S and Meagher L. 2008. Antioxidant activity and polyphenol content ofgreen tea flavan-3-ols and oligomericproanthocyanidins. *International Journal of Food Science and Nutrtion*; **60**(6): 497-506.

Mozafari MR, Johnson C., Hatziantoniou S. and Demetzos C. 2008. Nanoliposomes and their applications in food nanotechnology. *Journal of Liposome Research*; **18**, 309-327.

Mukhtar H and Ahmad N. 2000. Tea polyphenols: Prevention of cancer and optimizing health. *American Journal of Clinical Nutrition;* **71**: 1698-1702.

Nakayama T, Hashimoto T, Kajiya K. and Kumazawa S. 2000. Affinity of polyphenols for lipid bilayers. *BioFactors*; **13**: 147-151.

Noudoost B, Noori N, AmoAbedini Gh, Gandomi H, Akhondzadeh Basti A, JebeliJavan A, Ghadami F. 2015. Encapsulation of green tea extract in nanoliposomes and evaluation of its antibacterial, antioxidant and prebiotic properties. *Journal of Medicinal Plants*; **14**(55): 66-78.

Rashidinejad A, Birch EJ, Sun-Waterhouse D and Everett DW. 2014. Delivery of green tea catechin and epigallocatechingallate in liposomes incorporated into low-fat hard cheese. Food chemistry; 156: 176-183.

Shaw PE and Moshonas MG. 1991. Ascorbic acid retention in orange juice storedunder simulated consumer home conditions. *Journal of Food Science*; **56**: 867-868.

Sin HN, Yusof S, Sheikh Abdul Hamid N and Rahman RA. 2006. Optimization of enzymatic clarification of sapodilla juice using response surface methodology. *Journal of Food Engineering*, **73**, 313-319.

Sinico C, De Logu A, Lai F, Valenti D, Manconi M, Loy G, Bonsignore L and Fadda, AM. 2005. Liposomal incorporation of Artemisia arborescens L. essential oil and in vitro antiviral activity. *European Journal of Pharmaceutics and Biopharmaceutics;* **59**: 161-168.

Sosa M, Rodríguez-Rojo S, Mattea F, Cismondi M and Cocero M. 2011. Green tea encapsulation by means of high pressure antisolventco precipitation. *Journal of Supercritical Fluids*; **56**(3): 304-311.

Sun-Waterhouse D, Wadhwa SS, and Waterhouse GI. 2013. Spray-drying microencapsulation of polyphenol bioactives: a comparative study using different natural fibre polymers as encapsulants. *Food Bioprocessing Technology;* **6**: 2376-2388.

Takahashi M., Uechi S, Takara K, Asikin Y and Wada K. 2009. Evaluation of an oral carrier system in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. *Journal of Agricultural and Food Chemistry*; **57**; 9141-9146

Thompson AK, Mozafari MR and Singh H. 2007. The properties of liposomes produced from milk fat globule membrane material using different techniques. *Le Lait;* **87,** 349-60.

Yang T and Koo M. 1997. Hypocholesterolemic effects of Chinese tea. *Pharmacology Research;* **35**(6): 505-512.

Valenti D, DeLogu A, Loy G, Sinico C, Bonsignore L, Cottiglia F, Garau D, Fadda AM. 2001. Liposome-incorporated Santolinainsularis essential oil: preparation, characterization and in vitro antiviral activity, *Journal of Liposome Research;* **11**: 73-90.

Wiseman SA, Balentine DA and Frei B. 1997. Antioxidants in tea. *Critical Reviews in Food Science and Nutrition*; **37**(8): 705- 718.

Zhou H, Sun X, Zhang L, Zhang P, Li Jand Liu Y-N. 2012. Fabrication of biopolymeric complex coacervation core micelles for efficient tea polyphenol delivery via a green process. *Langmuir*; **28**(41): 14553-14561..

Zou LQ, Liu W, Liu WL, Liang RH, Li T, Liu CM, Cao YL, Niu J and Liu Z. 2014.Characterization and bioavailability of tea polyphenol nanoliposome prepared by combining an ethanol injection method with dynamic high-pressure microfluidization. *Journal of Agricultural and Food Chemistry;* **62,** 934-941.