Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 92 (2005) 737–744
Food Chemistry
www.elsevier.com/locate/foodchem

Random centroid optimization of phosphatidylglycerol stabilized lutein-enriched oil-in-water emulsions at acidic pH

Jack N. Losso [a,*], Armen Khachatryan [a], Masahiro Ogawa [a], J. Sam Godber [a], Fred Shih [b]

[a] *Food Protein Biotechnology Laboratory, Department of Food Science, Louisiana State University Agricultural Center, 111 Food Science Building, Baton Rouge, LA 70803, USA*
[b] *USDA-SRRC, New Orleans, LA, USA*

Received 7 June 2004; received in revised form 16 December 2004; accepted 16 December 2004

Abstract

Lutein has been identified by various studies as a dietary compound that may help delay the onset of macular degeneration. Random centroid optimization was applied to design, prepare and characterize lutein-enriched oil-in-water (o/w) emulsions containing corn oil (15–25%), whey proteins (1.5–3.5%), phosphatidylglycerol (0.03–0.3%), KC1 (80–100 mM), lutein (0.025–0.031%), and pH at 3.8–4.8. After nine experiments in the first random cycle and four experiments in the centroid cycle, optimal conditions for the preparation of stable lutein-enriched oil-in-water emulsion were: corn oil, 20%; whey proteins, 2%; phosphatidylglycerol, 0.157%; KCl, 93.7 mM; lutein, 0.0282%, and pH 4.55. The half life stability, stability index, and zeta potential values for freshly prepared emulsions were higher when stabilized with phosphatidylglycerol than with phosphatidylcholine. Phosphatidylcholine-stabilized emulsions collapsed after heating at 90 °C for 5 min. Emulsion parameters for phosphatidylglycerol-stabilized emulsions heated at 90 °C for 5 min or stored at 4 °C for 24 h were not significantly different from values obtained with freshly prepared emulsions. Lutein remained stable in fresh and heat-treated emulsions. The potential of phosphatidylglycerol and lutein as health enhancing bioactive compounds is discussed.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Lutein; Macular degeneration; Phosphatidylglycerol; Emulsions; Salad dressings; Functional foods; Diabetes

1. Introduction

Age-related macular degeneration (AMD) is a pathological process associated with irreversible photoreceptors loss whose cause is not exactly known. AMD affects about 50% of people at age 75 ± 5, and diabetic patients. However, risk factors associated with AMD have been identified to include age, smoking, family history, race, gender, iris colour, cumulative light damage in the macular photoreceptor by oxidative process, reactive oxygen intermediates (free radicals, H_2O_2, and singlet oxygen), diabetes and all its complications (retinopathy, nephropathy), atherosclerosis, low antioxidant intake, inflammation, and nutrition (AREDS, 2001; Beatty, Koh, Phil, Henson, & Boulton, 2000; Bernstein, 2002; Khan & Chakrabarti, 2003; Krinsky, Landrum, & Bone, 2003; Mozaffarieh, Sacu, & Wedrich, 2003; Zhao, Wintch, Ermakov, Gellermann, & Bernstein, 2003). There are two types of AMD: the "dry form" and "wet form". The dry form represents about 90% of the AMD, is atrophic, and consists of yellow pigmentation spots in the macula. The wet form consists of formation of new blood vessels or angiogenesis beneath the macula and usually progresses to complete loss of central vision (Bernstein, 2002). There are few effective treatments for AMD. Treatment options available today for AMD consist of slowing down the angiogenic process of AMD by

* Corresponding author. Tel.: +1 225 578 3883; fax: +1 225 578 5300.
E-mail address: jlosso@lsu.edu (J.N. Losso).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.12.029

either laser or photodynamic therapies. Laser treatment carries the possibility of damaging the remaining vision. Photodynamic therapy is a FDA approved and commonly performed therapy, but certainly not a definitive treatment. However, most AMD patients progress to legal blindness. The lack of standard therapies for AMD, in vitro studies, epidemiological survey, and recent quantitative Raman spectroscopic data, that showed the prevalence of xanthophylls in the human eye and the inverse relationship between xanthophyll concentration in the eye and macular degeneration, indicate that nutritional intervention using antioxidants and vitamins to enhance vision health may provide an effective way to prevent or treat AMD (Bernstein, 2002; Zhao et al., 2003).

Lutein (3,3′-dihydroxy-α-carotene) and zeaxanthin (3,3′-dihydroxy-β-carotene) have been identified as the only quantifiable carotenoids, i.e., 100 ng, in the human retina (Bernstein, 2002). Other carotenoids, such as lycopene and β-carotene, are present in the ocular tissues other than the retina. Higher levels of lutein and zeaxanthin were measured in the retina of individuals with no known clinical history of AMD while lower levels of both xanthophylls were measured in the eyes of individuals with known clinical history of AMD (Bone et al., 2001). Carotenoids are excellent antioxidants by virtue of their ability to utilize the long carbon and double bond chain to dissipate or absorb the damaging effects of free radicals. Lutein and zeaxanthin may be considered as conditionally essentially nutrients if these bioactive compounds provide clinical evidence of their effectiveness against AMD (Semba & Dagnelie, 2003).

Seddon, Ajani, and Sperduto (1994) suggested that 6 mg of lutein a day may reduce the risk of AMD by 43%. This concentration is equivalent to consuming 850 g of corn, 6800 g of tomatoes, 2 salad bowls of spinach or one salad bowl of kale a day. Tomato and spinach are high oxalate-containing foods that may cause a significant increase in urinary oxalate excretion and kidney stones (Hesse, Siener, Heynck, & Jahnen, 1993; Massey, Raman-Smith, & Sutton, 1993). Kale is a low-oxalate vegetable but is not normally found in sufficient quantities at any local food market for all the households in the community. Very few people can have the discipline and consistency to consume 2 lbs of corn or 16 lbs of tomatoes daily for lutein intake. As a consequence, food fortification with lutein extract is convenient and appealing to health-conscious consumers.

Salad dressings have become popular with health conscious people because salad dressings make salad tasty and appealing. Most salad dressings are prepared either with gums or enzyme-modified lecithin as emulsion stabilizers in order to preserve the stability of the emulsions during heat sterilization. Gums add viscosity to emulsions to prevent emulsion droplet coalescence or separation. Shaban, Borras, Vina, and Richter (2002) reported that phosphatidylglycerol protects human retinal pigment epithelial cells against apoptosis induced by A2E, a compound suspected to cause age-related macular degeneration. Phosphatidylglycerol is a product of phospholipase D-catalyzed hydrolysis of phosphatidylcholine in the presence of glycerol. We hypothesized that food fortification with lutein would allow ingestion of the suggested minimum daily intake of lutein. The objective of this study was to find the optimal conditions for the formulation of a phosphatidylglycerol-stabilized lutein-enriched oil-in-water emulsion at acidic pH.

2. Materials and methods

2.1. Materials

Whey protein isolate powder (WPI) containing 97.5% protein was supplied by Davisco International (Le Sueur, MN). L-α-Phosphatidyl-DL-glycerol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Corn oil was obtained from a local store and used without further purification. A lutein sample (FloraGlo, 75% lutein) was obtained from Kemin Foods (Des Moines, IA). Lutein standard and phosphatidylcholine were products of Sigma Chemical Co. (St. Louis, MO). The HPLC column, YMC C_{30}, was a product of Waters (Milford, MA). All reagents were either HPLC grade or reagent grade.

2.2. Methods

2.2.1. Preliminary emulsion preparation

All procedures were carried out at ambient temperature (23 °C). A coarse emulsion was prepared as follows. Whey protein isolates were dissolved in water, adjusted to the desired pH with 1 M NaOH or HC1 and adjusted to the desired volume. The whey protein solution was added to a measured volume of corn oil (20% v/v), lutein (0.15%), KC1 (100 mM), and phosphatidylglycerol (0.083%) in a high-speed blender (Warring Commercial, Model 33BL79, New Hartword, Connecticut) and the mixture was homogenized for 2 min at the highest speed setting. The emulsion was transferred to a high-pressure valve homogenizer (Fort Wayne, Indiana) and passed two times for 10 min each at 1725 rpm and 2000 psi to reduce the droplet size. The droplet-size reduced emulsion was transferred to a container and stirred for 30 min before further use.

2.2.2. Random centroid optimization technique

Four variables including pH (3.5–4.8), lutein concentration (0.025–0.031%), KC1 concentration (80–100 mM) and phosphatidylglycerol concentration (0.03–0.3%) were used for the emulsion designs. Nine sets of experimental conditions were given by random search using the Random centroid optimization (RCO)

programme of Nakai, Dou, Lo, and Scaman (1998). Emulsions were prepared and emulsion stability, under these experimental conditions, as described below, was evaluated according to Pearce and Kinsella (1978). The values of emulsion half-life times, obtained after the random search, were entered into the program as response. Four sets of experimental conditions were given by the centroid search that was calculated based on the random search results. The emulsion stability values of the four experiments were determined as well; the results were entered in to the program as response. Maps were drawn using the thirteen experimental results from the random and centroid searches. Thus, the mapping results directed the optimum formulation for the most stable emulsion. The most stable emulsion was prepared in triplicates following the mapping results. The stability of the prepared emulsion was evaluated by analyzing the emulsion's half life stability, stability index (ESI), droplet size distribution, droplet surface charge or zeta potential, stability to heat treatment, and lutein stability.

2.2.3. Emulsion preparation using RCO parameters

Oil (20%) was mixed with whey proteins (2%) in 93.7 mM KC1 solution at pH 4.55 at constant speed in a blender (Waring Commercial, Model 33BL79, New Hartword, CT) for 2 min, followed by addition of lutein (0.015%), and phosphatidylglycerol (0.018%). Emulsion samples were prepared in triplicates. Another set of emulsion samples in triplicates was prepared, under the same conditions, with the addition of phosphatidylcholine. The coarse emulsion, thus obtained, was passed through a high pressure valve homogenizer (Fort Wayne, Indiana) at 2000 psi for 10 min, twice. The resultant fine emulsion was transferred to containers for heat treatment and/or storage until further use.

2.2.4. Emulsifying properties

Emulsifying properties were measured according to the method of Pearce and Kinsella (1978). Thirty microlitre portions of the emulsions were pipetted, in triplicates, from the bottom of the container at 0 min and different times after homogenization. Each portion was immediately diluted to 5 ml with 0.1% of sodium dodecyl sulfate (SDS). The absorbance of the diluted emulsion was measured at 500 nm in a spectrophotometer (Spectramax Plus, Sunnyvale, CA). The values of absorbance measured immediately (A_0), and the absorbance measured after 24 h (A_{24}) following emulsion formation, were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI). EAI (m^2/g) = $2T[(A_0 \times \text{dilution factor})/(c \times \varnothing \times 1000)]$, where T is the turbidity and $T = 2.303$; and $\varnothing$ is the oil volume fraction of the emulsion.

ESI(min) = $A_0 \times \Delta t/\Delta A$, where Δt is the change in turbidity T occurring during the time interval, i.e., 24 h in this case and ΔA is the $A_0 - A_{24}$.

2.2.5. Surface properties of emulsions and emulsion stability to heat treatment

The zeta potential of emulsion droplets, diluted 1:400 with deionized distilled water, was determined by measuring the particle electrophoretic mobility using a Zetasizer (Malvern Instruments Ltd., Malvern, UK), which provides an absolute determination of zeta potential with no calibration required.

Twenty ml of emulsions, in a closed glass bottle and in triplicates, were placed in a water bath at 90 °C for 5 min. The samples were cooled to room temperature, visualized for oil separation, and analyzed for droplet size distribution, zeta potential values, and emulsion stability index (ESI) as described above. Droplet size distribution was analyzed using the Zetasizer (Malvern Instruments Ltd., Malvern, UK).

2.2.6. Stability and recovery of lutein in the emulsions

Two grams of emulsion were extracted with 80 ml of acetone, in screw-capped containers, at room temperature in the dark for 3 days. The three day extraction was carried out to ensure complete extraction of lutein. The acetone extracts were filtered through Whatman No. 4 filter paper and evaporated under vacuum. The residues were saponified with 200 ml of 10% KOH with gentle shaking overnight. The saponified solution was extracted with ether:hexane (1:1) mixture, using a separatory funnel. The upper phase, containing lutein in ether/hexane solution, was recovered and evaporated using a Buchi Rotavapor R-200 evaporator (Brinkman Instruments, Inc., Westbury, NY). The residue was dissolved in 20 ml of MTBE/methanol (5:95) mixture, filtered through 0.45 μm filter and 20 μl were separated by HPLC using a YMC C_{30} carotenoid S 3μ, 4.6 × 250 mm column. The HPLC separation was carried using a Waters Model 600E solvent delivery system fitted with a model 717A plus autosampler, a Model 486 tunable absorbance detector and Millenium 32 chromatography manager with data processor (Milford, MA). The flow rate was 1 ml/min, detection was at 450 nm, and the separation was isocratic using methyl-*tert*-butyl-ether (MTBE): methanol (5:95) as the mobile phase, with a total separation time of 35 min. Peaks on a chromatogram were identified by comparing their retention times with those of lutein standards (all-trans) separated under the same conditions. Lutein was quantified using a standard curve constructed with lutein obtained from Sigma.

3. Results and discussion

3.1. Random centroid optimization of emulsion preparation

The random and centroid search conditions are shown in Table 1. The pH range was chosen between

Table 1
Random and centroid search conditions for finding optimal conditions for emulsion preparations

Variables	Time (mean)[a]	Standard deviation
Cycle 1, random search		
(1) pH (4.12) Lutein (0.0288%) KC1 (97.5 mM) PGL (0.126%)	3.45	±0.47
(2) pH (4.78) Lutein (0.0293%) KC1 (91.1 mM) PGL (0.189%)	**12.64**	±2.98
(3) pH (4.56) Lutein (0.0262%) KC1 (92.4 mM) PGL (0.189%)	**9.15**	±1.93
(4) pH (3.95) Lutein (0.0311%) KC1 (91.1 mM) PGL (0.252%)	2.12	±0.77
(5) pH (4.77) Lutein (0.0307%) KC1 (94.9 mM) PGL (0.283%)	2.32	±1.24
(6) pH (4.59) Lutein (0.0300%) KC1 (94.9 mM) PGL (0.031%)	**7.31**	±0.86
(7) pH (3.86) Lutein (0.0259%) KC1 (85.9 mM) PGL (0.252%)	1.61	±0.00
(8) pH (4.06) Lutein (0.0256%) KC1(91.1 mM) PGL (0.126%)	2.17	±0.29
(9) pH (4.72) Lutein (0.0283%) KCI (91.1 mM) PGL (0.189%)	**12.77**	±2.83
Cycle 1, centroid search		
(1) pH (4.66) Lutein (0.0284%) KCI (92.4 mM) PGL (0.157%)	17.03	±2.05
(2) pH (4.55) Lutein (0.0282%) KCI (93.7 mM) PGL (0.157%)	**17.56**	**±2.37**
(3) pH (4.55) Lutein (0.0291%) KCI (93.7 mM) PGL (0.126%)	16.10	±1.30
(4) pH (4.50) Lutein (0.0283%) KCI (93.7 mM) PGL (0.126%)	16.20	±6.23

[a] Half-life stability of the emulsions (in minutes).

pH 3.8 and pH 4.8 because most salad dressing emulsions have a pH value between pH 3.5 and 4.5. Lutein concentration was chosen between 0.0252% and 0.0314%, based on the suggestion made by Seddon et al. (1994). Ionic strenth was kept between 80 and 100 mM pottassium chloride to provide good stablity to the emulisions (Demetriades & McClements, 1999). Phosphatidylglycerol was chosen in the range of 0.03–0.3%, based on data from phosphatidylglycerol-stabilized parenteral drug formulations (Chansiri, Lyons, Patel, & Hem, 1999). The random search suggested formulations 2, 3, 6, and 9 to provide the most stable emulsions in terms of half-life stability. The centroid search identified formulation number 2 (pH 4.55; lutein concentration = 0.015%; KC1 concentration = 93.7 mM, and phosphatidylglycerol concentration = 0.018%) as the most stable emulsion. The mapping results, for phosphatidylglycerol-stabilized

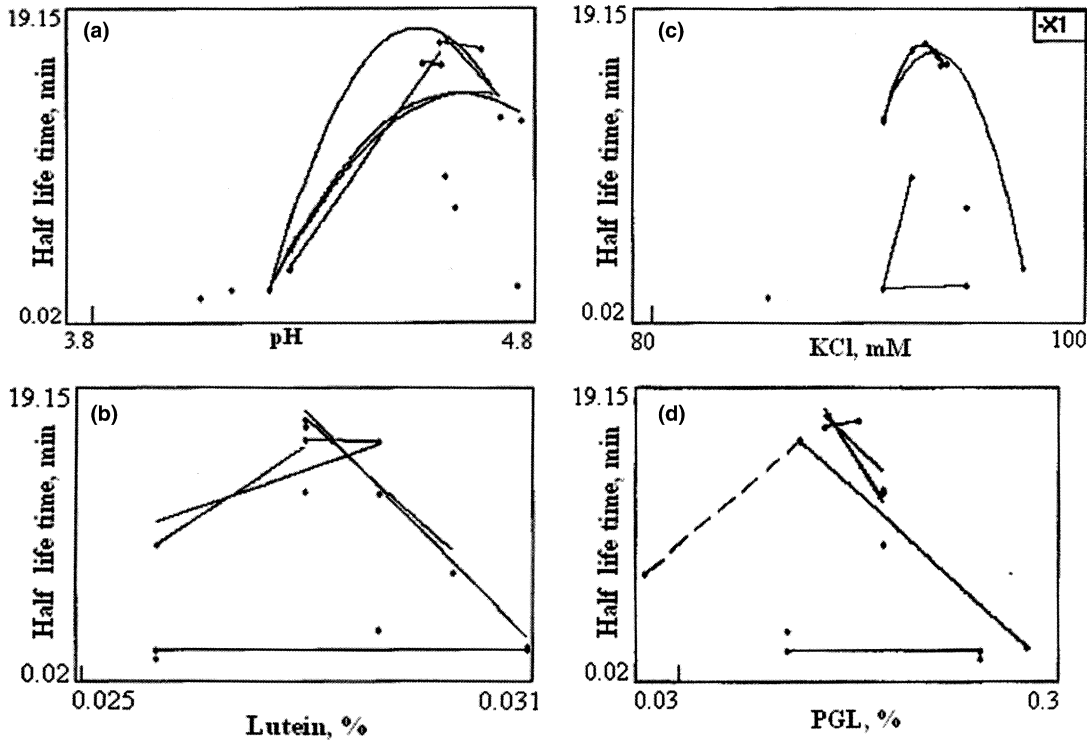

Fig. 1. Mapping results of emulsion stability drawn by RCO programme. (a) An illustration of turbidity measurement as half-life time of emulsion as a function of pH. (b) The half-life time of emulsion as a function of lutein concentration. (c) The turbidity measurement as half-life time of emulsion as a function of KCl concentration. (d) The stability of emulsion as half-life time of emulsion as a function of phosphatidylglycerol concentration.

emulsions, in Fig. 1, show the interaction of emulsion half life and pH (Fig. 1(a)); emulsion half life and lutein (Fig. 1(b)), the interaction of emulsion half life and KCl (Fig. 1(c)); and emulsion half-life and phosphatidylglycerol (Fig. 1(d)). All together, these Figures show that optimal emulsion half life was obtained at pH 4.55, 0.0282% lutein, 0.157% phosphatidylglycerol, 93.7 mM KC1, 20% corn oil, and 2% whey protein. These experimental conditions, which were suggested by the centroid search, provided the highest optimal time among the different experimental conditions analyzed (Table 1).

3.2. Surface properties of emulsions and emulsion stability

The surface properties of the most stable emulsion suggested by the centroid search are presented in Table 2. The half life stability of phosphatidylcholine-stabilized emulsions was shorter than the half life stability of phosphatidylglycerol-stabilized emulsions. The ESI values were higher for phosphatidylglycerol-stabilized emulsions than for phosphatidylcholine-stabilized emulsions. There was a significant difference in values of zeta potential between the two emulsions (−11 mV for phosphatidylcholine-stabilized emulsions vs −53 mV for phosphatidylglycerol-stabilized emulsions). Emulsions with zeta potential values of −11 to −20 mV are considered at the threshold of agglomeration; emulsions with zeta potential values of −41 to −50 mV have good stability, and emulsions with zeta potential values of −51 to −60 have good stability (van Nieuwenhuyzen & Szuhaj, 1998). Emulsion droplets were 0.73 μm and, following heat treatment and storage, the emulsion droplet size increased to 1.19 μm for the phosphatidylglycerol-stabilized while phosphatidylcholine-stabilized emulsions collapsed.

Emulsion prepared without phosphatidylglycerol was stable following formulation but collapsed after heat treatment at 90 °C for 5 min. Emulsion destabilization was associated with oil separation and formation of a distinct and visible oil phase in the emulsion. Phosphatidylglycerol is an anionic surfactant which imparts negative charges around emulsion droplets. As a result of phosphatidylglycerol, increased electrostatic repulsive force between emulsions and droplet–droplet repulsion prevented droplets coalescence and promoted emulsion stability (Chansiri et al., 1999; Washington, Chawla, Christy, & Davis, 1989). In commerce, o/w emulsions stabilized by egg yolk phospholipid are autoclaved at alkaline pH (pH 8–9) in order to preserve an adequate negative charge distribution around emulsion droplets and maintain emulsion droplet stability since emulsions prepared at acidic pH show an increased phosphatidylcholine hydrolysis and droplet size upon autoclaving (Chaturvedi, Patel, & Lodhi, 1992). In most cases, food emulsions are customarily prepared at pH below 4.5. However, the hydrolysis of phosphatidylcholine as a function of pH may explain the inability of phosphatidylcholine to stabilize emulsions at acidic pH during heat treatment (Chaturvedi et al., 1992). Phosphatidylglycerol, which is formed upon phospholipase D-catalyzed hydrolysis of phosphatidylcholine in the presence of glycerol under acidic conditions, is resistant to hydrolysis. Since most food emulsions are prepared in the acidic pH range, identifying an emulsion stabilizer that is efficient at acidic pH is desirable, hence the use of phosphatidylglycerol in this study.

N-retinyl-*N*-retinylidene ethanolamine (A2E) is an endogenous lipophilic and cationic compound suspected to cause the dry form of macular degeneration, which currently cannot be treated (Shaban et al., 2002). Shaban et al. (2002) reported phosphatidylglycerol to protect against *N*-retinylidene ethanolamine (A2E)-induced macular degeneration by inhibiting A2E-induced RPE cells apoptosis.

3.3. Lutein stability

The chemical and physical stabilities of food emulsions are key product quality issues. Lutein was isolated from freshly prepared emulsions and its HPLC profile was compared to the HPLC profile of standard lutein (Fig. 2(a) and (b), respectively). Fig. 2(a) provides the HPLC profile of standard lutein and Fig. 2(b) shows

Table 2
The surface properties of the most stable emulsion/suggested by the centroid search

	Fresh emulsions		Emulsions at 90 °C, 5 min		Emulsions stored 4 °C, 24 h[a]
	Phosphatidylcholine	Phosphatidylglycerol	Phosphatidylcholine	Phosphatidylglycerol	Phosphatidylglycerol
Half-life stability (h)	84.0 ± 2.0	92.0 ± 4.0	Collapsed	89.0 ± 3.0	61.0 ± 1.0
ESI	61.2 ± 1.2	77. 5 ± 3.5	Collapsed	76.2 ± 2.2	64.7 ± 3.5
Zeta potential (mV)	−11 ± 0.0	−53.0 ± 0.4	Not calculated[b]	−52.1 ± 0.8	−47.2 ± 0.0
Emulsion droplet size (μm)	0.73	0.73 ± 0.03	Not calculated[b]	0.75 ± 0.02	1.19 ± 0.03
Lutein recovery (%)	100	100	Not calculated[b]	83.4 ± 0.00	76.8 ± 5.6

[a] Emulsions prepared with lecithin (phosphatidylcholine) collapsed minutes after preparation and were not evaluated after 24 h storage.
[b] Not calculated because the emulsions collapsed.

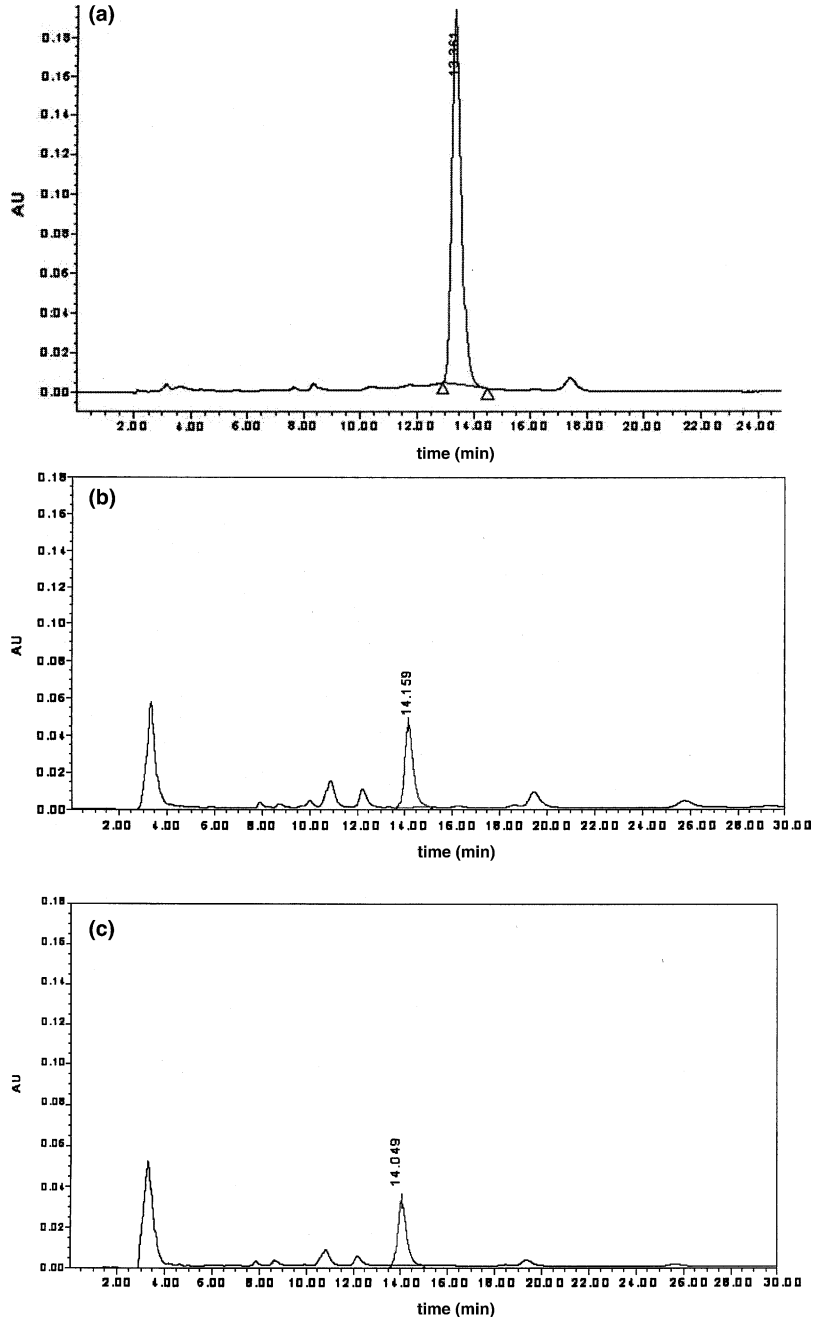

Fig. 2. HPLC chromatogram of lutein recovered from emulsions. (a) Lutein standard; (b) freshly made emulsion; (c), heat-treated emulsion; (d), one day old emulsion, and (e) the HPLC chromatogram of saponified corn oil used for the preparation of lutein-enriched emulsions. Elution was performed with MTBE/Methanol (5:95 v/vl) solution using a YMC C_{30} carotenoid column at flow rate of 1 ml/min. The injection volume was 20 μl, the detection was at 450 nm, and total running time was 30 min.

that lutein was quantitatively recovered from freshly prepared emulsion. Comparison was also made between the HPLC profile of isolated lutein and the HPLC profile of the unsaponifiable compounds in corn oil used for emulsion preparation. Fig. 2(c) shows that the unsaponifiable compounds in corn oil contributed the peaks that eluted in less than 10 min and these peaks were not degradation products of lutein. The stability of lutein in freshly prepared emulsions was compared to the stability of lutein from heat-treated emulsions and the results in Fig. 2(d) show that, as mentioned above, any peak eluted in less than 10 min was associated with the unsaponifiable fraction of the corn oil. Lutein degradation did not occur, as shown by the HPLC profile of lutein recovered from fresh emulsion, heat-treated emulsion, and emulsion stored for 24 h (Fig. 2(b)–(d)). A closer look at the HPLC profiles of lutein in fresh emulsions, emulsions heated at 90 °C for 5 min, emulsions heated

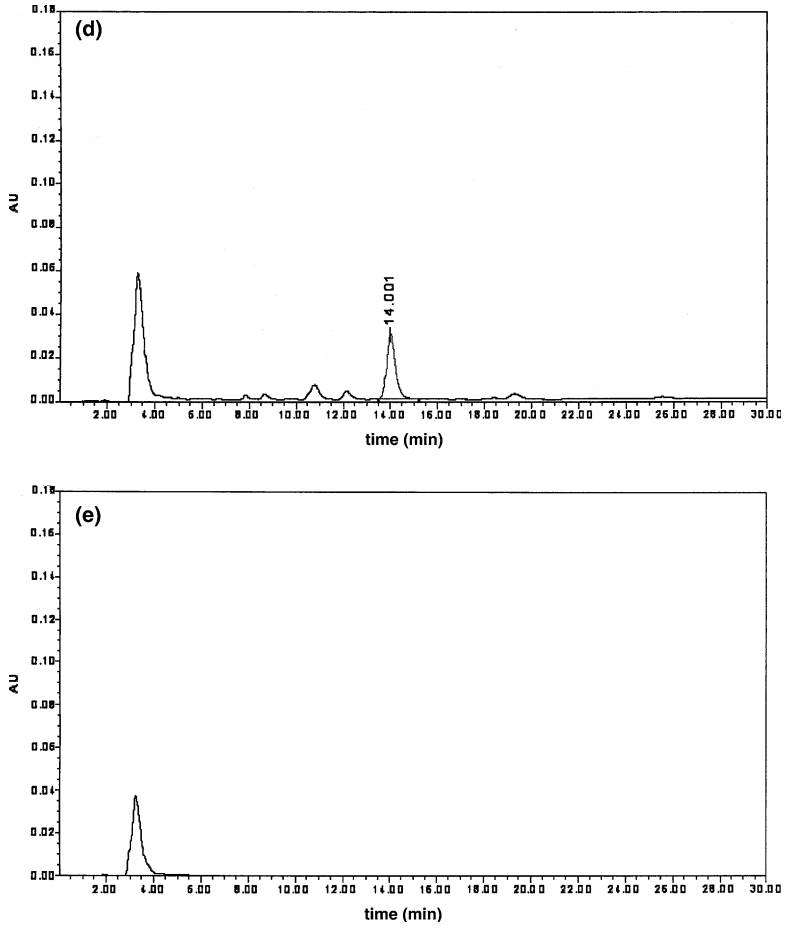

Fig. 2 (*continued*)

and stored at 4 °C for 24 h, showed that no additional peaks were present in heat treated lutein samples. Also, the concentration of recovered lutein was close to the concentration spiked in the emulsion (Table 2).

4. Conclusion

A phosphatidylglycerol-stabilized lutein-enriched o/w emulsion was prepared using RCO. The stability of the emulsion was evaluated by heat treatment at 90 °C for 5 min. Emulsions prepared with phosphatidylcholine collapsed after heat treatment. Emulsions prepared with phosphatidylglycerol were stable before and after heat treatment, as shown by the zeta potential values of the emulsion droplets. Phosphatidylglycerol imparted negative charges around emulsion droplets and enhanced emulsion droplet stability during and after heat treatment. Based on the HPLC profile of lutein, no lutein degradation products were observed at heat treatment at 90 °C for 5 min. Phosphatidylglycerol-stabilized lutein- enriched oil-in water emulsions have the advantage of containing two bioactive compounds, namely lutein and phosphatidylglycerol, reported to provide protection against age-related macular degeneration.

Acknowledgements

The financial support of the Louisiana Soybean and Grain Research and Promotion Board is greatly acknowledged. The authors gratefully acknowledge Dr. Shuryo Nakai, Professor Emeritus at the Department of Food, Nutrition, and Health at the University of British Columbia, Vancouver, BC, Canada for his critical review of the manuscript.

References

Age-Related Eye Disease Study Research Group (AREDS). (2001). Risk factors associated with age-related nuclear and cortical cataract: A case-control study in the Age-Related Eye Disease Study, AREDS Report No. 5. *Opthalmology*, *108*, 1400–1408.

Beatty, S., Koh, H., Phil, M., Henson, D., & Boulton, M. (2000). The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Survey of Ophthalmology, 45*, 115–134.

Bernstein, P. S. (2002). New insights into the role of the macular carotenoids in age-related macular degeneration. Resonance raman studies. *Pure and Applied Chemistry, 74*, 1419–1425.

Bone, R. A., Landrum, J. T., Mayne, ST., Gomez, C. M., Tibor, S. E., & Twaroska, E. E. (2001). Macular pigment in donoreyes with and without AMD: a case-control study. *Investigative Ophthalmology and Visual Science, 42*, 235–240.

Chansiri, G., Lyons, R. T., Patel, M. V., & Hem, S. L. (1999). Effect of surface charge on the stability of oil/water emulsions during steam sterilization. *Journal of Pharmaceutical Sciences, 88*, 454–458.

Chaturvedi, P. R., Patel, N. M., & Lodhi, S. A. (1992). Effect of terminal heat sterilization 10 on the stability of phospholipid-stabilized submicron emulsions. *Acta Pharmceutica Nordica, 4*, 51–55.

Demetriades, K., & McClements, D. (1999). Flocculation of whey protein stabilized emulsions as influenced by dextran sulfate and electrolyte. *Journal of Food Science, 64*, 206–210.

Hesse, A., Siener, R., Heynck, H., & Jahnen, A. (1993). The influence of dietary factors on the risk of urinary stone formation. *Scanning Microscopy, 7*, 1119–1128.

Khan, Z. A., & Chakrabarti, S. (2003). Growth factors in proliferative diabetic retinopathy. *Experimental Diabesity Research, 4*, 287–301.

Krinsky, N. I., Landrum, J. T., & Bone, R. A. (2003). Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annual Review of Nutrition, 23*, 171–201.

Massey, L. K., Raman-Smith, H., & Sutton, R. A. (1993). Effect of dietary oxalate and calcium on urinary oxalate and risk of formation of calcium oxalate kidney stones. *Journal of the American Dietetic Association, 93*, 901–906.

Mozaffarieh, M., Sacu, S., & Wedrich, A. (2003). The role of the carotenoids, lutein and zeaxanthin, in protecting against age-related macular degeneration: A review based on controversial evidence. *Nutrition Journal, 2*(1), 20.

Nakai, S., Dou, J., Lo, K. V., & Scaman, H. C. (1998). Optimization of site-directed mutagenesis. 1. New Random-Centroid Optimization Program for Windows Useful in Research and Development. *Journal of Agricultural and Food Chemistry, 46*, 1642–1654.

Pearce, K. M., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbimetric technique. *Journal of Agricultural and Food Chemistry, 26*, 716–723.

Seddon, J. M., Ajani, U. A., & Sperduto, R. D. (1994). Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. *JAMA, 272*, 1413–1420.

Semba, R. D., & Dagnelie, G. (2003). Are lutein and zeaxanthin conditionally essential nutrients for eye health?. *Medical Hypotheses, 61*(4), 465–472.

Shaban, H., Borras, C., Vina, J., & Richter, C. (2002). Phosphatidylglycerol potently protects human retinal pigment epithelial cells against apoptosis induced by A2E, a compound suspected to cause age-related macula degeneration. *Experimental Eye Research, 75*, 99–108.

van Nieuwenhuyzen, W., & Szuhaj, B. F. (1998). Effects of lecithins and proteins on the stability of emulsions. *Fett/Lipid, 100*, 282–291.

Washington, C., Chawla, A., Christy, N., & Davis, S. S. (1989). The electrokinetic properties of phospholipid-stabilized fat emulsions. *International Journal of Pharmaceutics, 54*, 191–197.

Zhao, D. Y., Wintch, S. W., Ermakov, I. V., Gellermann, W., & Bernstein, P. S. (2003). Resonance Raman measurement of macular carotenoids in retinal, choroidal, and macular dystrophies. *Archives of Ophthalmology, 121*, 967–972.