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Optimization of Conjugated Linoleic Acid (CLA) Synthesis Conditions Applying it in Milk Enrichment

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Abstract

In present study, linoleic acid from sunflower oil and propylene glycol as solvent were used to synthesize conjugated linoleic acid (CLA), and the response surface methodology (RSM) was applied to optimize the process. The results showed that the propylene glycol as solvent and reaction time were found to have positive influence on the response, but the high reaction temperature especially at high solvent levels and reaction time, has led to a reduction in the production of CLA. Solvent content of 6.25 mL, a temperature of 100 °C and a time of 140 min were found to be the optimal conditions in the isomerization for the production of cis9-trans11 and trans10-cis12 CLA isomers. In the next step, the produced CLA were concentrated and purified and used for the enrichment of skim milk. Then, qualitative characteristics of milk including pH, peroxide value, sensory properties and CLA retention during storage of enriched milk in the 5 °C were investigated for 90 days. There was no significant (P>0.05) difference between the control and treated samples for one month after storage (P>0.05), but with increasing storage time up to 60 and then 90 days the differences increased (P<0.05). According to the results, the level of remaining CLA in milk, as well as pH decreased over time, but the peroxide value of the samples was increased.

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Keywords
Alkali isomerization
Conjugated linoleic acid
Milk enrichment
Propylene glycol
Response surface methodology

Introduction

Conjugated linoleic acid (CLA) refers to a group of conjugated fatty acid isomers of octadecadienoate ($C_{18:2}$) with unsaturated bonds with different positional and geometric arrangements (Khaskheli, Talpur, Demir, Cebeci, & Jawaid, 2013). There is enhancing interest in producing CLA as a food additive and functional supplement because of its possible health profits associated with its absorption (Yurawecz, Kramer, Gudmundsen, Pariza, & Banni, 2006). CLA has been shown to prevent tumor promotion in tentative animals, to decrease atherosclerotic plaque and to lower blood cholesterol (Yeung, Yang, Huang, Wang, & Chen, 2000). CLA may increase immune functions and reduce fat supply while it develops muscle and bone density (Li & Watkins, 1998). However, CLA as an antioxidant is found ineffective (Yang, Huang, Wang, & Chen, 2002).

It is found, 28 isomers out of a total of 56 isomers theoretically possible have been identified, including 6,8; 7,9; 8,10; 9,11; 10,12; 11,13; and 12,14 carbon locations in all the possible geometric arrangements cis-cis, cis-trans, trans-cis, and trans-trans (Roach, Mossoba, Yurawecz, & Kramer, 2002). However, only cis9-trans11 CLA and trans10-cis12 CLA Isomers have shown significant biological activity (Pariza, Park, & Cook, 2001). The principal isomer in the natural food products is the cis9-trans11 CLA. The sources containing this fatty acid are milk, dairy products, meat and fat of the ruminants such as beef, goat and sheep, as this isomer is produced by the bacteria of the tripe microorganisms (Salamon, Visi, András, Kiss, & Csapo, 2012).

Industrial production of CLA includes: (1) alkali isomerization of linoleic acid; (2) dehydration of ricinoleic acid methyl ester; and (3) microbial synthesis of c9-t11 -18:2 from linoleic acid using different microorganisms (Jiang, Björck, & Fonden, 1998). Each of these methods produces a different level of CLA isomers. Among these methods, alkali conversion of linoleic acid is the most commonly process for synthesize of CLA because it is financially viable (Yang *et al.*, 2002). In contrast to the chemical catalysis, microbiological methods have a high specificity results and argument toward the production of bioactive CLA isomers, mainly toward the cis9-trans11-CLA isomer (Khaskheli *et al.*, 2013). Unfortunately, the low transformation of linoleic acids (LA) to CLA and the long reaction times using by the microbiological procedures make them uncompetitive against alkali isomerization (Salamon *et al.*, 2012).

In alkali isomerization, the positions of protons were transformed to conjugated locations of the double bonds during the hydrocarbon chain of LA, that is, without methylene carbons between them (Lundberg, 1958). For this purpose, vegetable oils with a high LA level are heated at 180 °C with a strong alkali as sodium or potassium hydroxide and ethylene glycol for appropriate time (Salamon *et al.*, 2012; Yang & Liu, 2004).

Silva-Ramírez, Rocha-Uribe, González-Chávez, & González (2017) found that the selective synthesis of bioactive CLA is produced at the optimal reaction conditions even when oils rich in LA are employed as substrate.

After finding beneficial effects of CLA, different kinds of foods were evaluated in order to determine, which can be the best source of CLA. Food products such as milk, cheese, yogurt, butter, cream and meat were found to contain the highest level of CLA (0.2-2 g CLA/100 g fat). But based on the results of surveys with different animals, the daily intake doses of CLA, which can effectively contribute to the prevention of cancer in humans 3.5 g CLA/day, is significantly higher than the average true CLA consumption (0.5- 1.5 g CLA/day). With the increase of consumption of commercial dairy product the effective intake level cannot be achieved (Jiang *et al.*, 1998). The solution could be the formulation of food with increased CLA-level. There are three different approaches to receiving higher level of CLA in functional foods. The first one is synthetic CLA addition to the food product (Garcia, Keough, Arcos, & Hill Jr, 2000). The second is extraction of meat fat fraction enriched in CLA and addition it, to the food products (Romero, Rizvi, Kelly, & Bauman, 2000). And at last CLA content of dairy products can be enhanced with different feeding techniques (Donovan, Schingoethe, Baer, Ryali, Hippen, & Franklin, 2000).

Several studies have been conducted during the two recent decades in an effort to enhance the CLA content of milk and meat. For example, Bauman, Baumgard, Corl, & Griinari (1999) published an excellent article describing the biosynthesis of CLA in ruminants. Other articles have focused on the potential health benefits of CLA (MacDonald, 2000; Pariza, Park, & Cook, 2000). In other

studies Martínez-Monteagudo, Saldaña, Torres, & Kennelly (2012) evaluated the effect of pressure-assisted thermal sterilization (PATS) conditions on CLA residue in enriched milk. Their results showed after 14 min of treatment at 100 MPa, at least 80% of CLA was retained, regardless of temperature used. CLA was not stable up to 14 min of treatment at 600 MPa and 120 °C with a retention value of approximately 3%. Under the same PATS conditions, CLA was lost to a greater extent in enriched milk than in enriched AMF. Possibly, CLA is lost through an oxidation reaction that is catalyzed by free metal ions released when applying pressure. The addition of catechin at 1 g/kg of milk effectively enhanced CLA retention (>90%) at any PATS condition in both milk and anhydrous milk fat (AMF). Shantha, Decker, & Ustunol (1992) studied the effective factors on the formation of CLA in dairy products. These researchers reported that addition of whey protein and skim milk powder increased the amount of this fatty acid in cheese and fatless yogurt.

In this study, we investigated the production of CLA from sunflower oil by alkali isomerization and its application in the enrichment of low-fat milk. Therefore present research was carried out in two stages; (1) Optimization of the conditions for CLA production by alkaline isomerization and use of propylene glycol as solvent. (2) Concentration and purification of produced CLA and its use in skim milk enrichment.

Materials and methods

Materials

Refined sunflower oil was purchased from Nina Corporation (company), which contained about 55% linoleic acid. Boron trifluoride-metanol complex, H_2SO_4, HCl, n-Hexane, Sodium methoxide, and Potassium methoxide purchased from Merck Company. Propylene glycol purchased from Sigma-Aldrich Company and NaOH purchased from Applichem Company.

Chemical isomerization

Production of CLA with LA isomerization was performed as described by Chin, Liu, Storkson, Ha, & Pariza (1992). Accordingly, 1.2 g of the desired alkali was dissolved in 5 mL of the propylene glycol under nitrogen gas blast conditions for 20 min at the desired temperature. Then 2.5 mL of oil was added to the mixture with 100, 120, 140, 160 and 180 °C temperatures. The temperature and time required to perform isomerization reaction were kept constant. After the reaction mixture was completed, the mixture was cooled at room temperature and then neutralized by 40 mL of phosphoric acid (H_3PO_4) phosphoric acid (H_3PO_4) because phosphoric acid prevents the possible degradation of CLA fatty acids (Liu, Wang, Xu, Ding, & Guo, 2017). The pH was reduced to less than 4 (about 3) and then distilled water was added and mixed for 5 min. After that the extraction procedure was performed 3 times with 20 mL hexane. To remove hexane, mixture was rinsed by NaCl 5% solution and finally, hexane remaining was removed by vacuum rotary evaporation (BUCHI, Model R215-B491) and the purification and dewatering step was performed by sodium sulfate (Chin *et al.*, 1992; Silva-Ramírez *et al.*, 2017).

Methylation of oil samples

About 10 drops of oil were poured into test tubes. Then, 7 mL of special chromatography hexane solvent and 2 mL of methanol potassium 2 M were added to the test tubes, respectively. The test tubes were sealed, well stirred and afterward were placed in a serology water bath with temperature of 50-55 °C for 15 min. The water bath temperature was regularly controlled by the thermometer to remain constant at the desired temperature and the contents of the test tubes were stirred every 5 min. After 15 min, the test tubes remained stationary for 5 min in water bath at 50-55 °C. Afterward, the test tubes were removed from the water bath without shaking and the supernatant was used for

injection into the gas chromatography (ISO, 2000).

CLA and fatty acid determination

The fatty acid methyl esters according to the ISO standard were measured by the gas chromatography (Model 6890 N, AGILENT company, China) mobilized with FID detector and HP-5 capillary column (30 m length, 0.320 mm width, 0.25 μm thickness) under the following conditions: The injector temperature was 250 °C and the oven temperature was 160-215 °C (The column temperature is same oven temperature). The air pressure and nitrogen were set to be 6 bars. The chromatograms were analyzed with the Galaxi software V1.19 and the peaks were appointed with comparison with a GC reference FAME standard (463-Nu Check Prep., Inc., Elysian, MN) (Cruz-Hernandez, Deng, Zhou, Hill, Yurawecz, Delmonte, Mossoba, Dugan, & Kramer, 2004).

Purification of CLA mixture by urea-inclusion crystallization

The crude mixture of CLA was gradually added to urea and dissolved in methanol (70 °C) in a ratio of 1:5:2 (oil, urea, methanol). Then it was cooled at room temperature for 4 h and its pH decreased to 3 by HCl. CLA was recovered in the primary liquor by vacuum filtration with 45 μm diameter. Using this process, the CLA concentration increased up to 90% (Yang & Liu, 2004).

Enrich the milk with CLA

For this purpose, 3.3 g synthesized and purified CLA (Cruz-Hernandez *et al.*, 2004) added to 675 mL skim-milk with fat content of 0.5% and then the sterilization process was performed. Enriched skim-milk was stored in a refrigerator at 5 °C for 3 months. During this period, the quality parameters of the product including pH, peroxide value, sensory properties and also CLA durability were investigated.

pH

To determine the pH of milk, pH meter with an appropriate electrode model Metrohm 744 was used.

Peroxide Value (PV)

The peroxide value of milk samples was calculated based on the measurement of iodine produced by reaction with peroxides. 5 g of milk was intermixed by 10 mL of chloroform and 15 mL of acetic acid (3:2 ratio), and then 1 mL of saturated iodine potassium solution increased. After 1 min stirring and keeping for 5 min in a dark place, 1 mL starch glue and 30 mL distilled water were added. The final mixture was titrated with sodium thiosulfate 0.02 N and the peroxide value was calculated based on the following equation (Jacobsen, 2010).

$$PV = (meg O_2/kg) = \frac{(S - B) \times N \times 1000}{m} \quad (1)$$

In this equation, S is the amount of thiosulfate sodium used for milk samples (mL), B volume of thiosulfate consumed for the control sample (mL), N is the sodium thiosulfate solution normality and m is the sample weight of the milk.

Sensory properties

Sensory evaluation was performed by 10 trained panelists. The panelists were selected on the basis of their accuracy and interest, and were trained about milk score test in the form of hedonic 5-point scale. The scores of this test are in the range of 1 to 5, so that the number 5 is the best and the number 1 is the worst (Meilgaard, Carr, & Civille, 1999).

Experimental planning and statistical analysis

The present study was carry out in two separate stages. In the first stage response surface methodology (RSM) was employed to assess the effect of independent changeable (Solvent content, x1; heat of the process, x2; reaction time, x3) on the production of c9-t11 and t10-c12 isomers. Face central composite

design was used to plan the experimental data. Tentative data was modeled employing the three-dimensional representations of the response surface generated by the model and Design Expert software version 6.01 (States Inc., Minneapolis, USA). For this purpose second-order polynomial fitted on the data (Eq. 2). For data analysis in the milk enrichment step, a completely randomized one-way design by Minitab 16 software were used. Differences were considered significant at (P<0.05) with the Duncan's multiple range experiment.

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (2)$$

In this equation Y is the predicted reaction, b_0 is the constant coefficient, b_i is linear effects, b_{ii} is quadratic effects and b_{ij} is interaction effects, and x_i, x_j are independent coded changeable.

Results and discussion

The effect of variables on the c9-t11 isomer production

The effect of variables of temperatures (100, 120, 140, 160, and 180 °C), times (20, 50, 80, 110, and 140 min) and solvent content (1.25, 3.75, 6.25, 8.75, and 11.25 mL) on production conditions of c9-t11 isomer was presented in Fig. (1). According to the results of the analysis variance and coefficients of the assorted model on the response data, it was found that among the variables, the most impact belonged to the amount of propylene glycol solvent, and then it was reaction temperature of isomerization. As can be seen, with increasing the temperature to 180 °C and increasing the solvent ratio to 11.25 mL the amount of c9-t11 isomers increased (Fig. 1a).

It should be noted that in a constant amount of solvent, by decreasing the temperature to 100 °C, the c9-t11 isomer production was partially reduced, but these changes were not significant (P>0.05), so that, the highest production of c9-t11 isomer was observed at temperature of 180 °C and 1.25 mL solvent. Using the temperature of 100 °C with increasing solvent content to 6.25 mL, the production of c9-t11 isomer was increased, but by increasing the solvent content up to 6.25 mL, the rate of this isomer declined.

According to the results, it was found that solvent concentrations lower than or greater than 6.25 mL had an undesirable effect on isomerization and the production of c9-t11 isomer. Therefore, by decreasing and increasing the solvent as the reaction medium, the amount of catalyst and substrate availability is disturbed, and finally isomerization and production of c9-t11 isomer decreased. In other words, in more quantities of propylene glycol, the ability of catalyst in bonding to the substrate is reduced due to the high dilution of the reaction medium, and in concentrations less than 6.25 mL, due to the concentration of the reaction medium, the ability of catalyst in connect to the substrate decreased, as a result, the isomerization reaction and the production of c9-t11 isomer decreased.

The interaction impression of time and temperature process on the production of c9-t11 isomer is presented in Fig. (1b). As can be seen, the rate of isomerization reaction first slowly increased for 80-90 min, but in the final 40 min the reaction speed increased significantly (P<0.05). In the fixed amount of solvent (6.25 mL), the lowest quantity of isomerization and production of c9-t11 isomer was obtained at 180 °C during 80 min, also the highest isomerization of LA and production of c9-t11 isomer was performed for 140 min at 100 °C. Therefore, it can be concluded that more time and especially high temperature have no positive effect on the increase of isomer production and due to the conversion of c9-t11 isomer to other isomers (which are mainly trans-isomers), its amount in the reaction mixture is reduced.

The results of this study are consistent with other researchers' reports. For example; Yang *et al.* (2002) considered the conversion efficiency in the alkaline

isomerization method more than any factor dependent on temperature and reported that the profile of conjugated linoleic acid is a responsibility of temperature. When used at 50 °C, only the c9-t11 and c9-c11 isomers were produced, but with increasing temperature to 120 °C, two other isomers (t10-t12 and t9-t11) were also produced. More trans-isomers including t10-t12, t9-t11 and t8-t10 appeared at 160 °C. Yang & Liu (2004) also reported that temperatures below 160 °C are suitable for the maximum production of trans10-cis12 and cis9-trans11 isomers. They announced that, when high temperatures and long times are used, the amount of these isomers were reduced and the c10-c12 isomer increased, and when the temperature is set at 160 °C and time factor increases, both of the major isomers were reduced and the trans-linoleic acid form of the CLA, including t10-t12, is further enhanced. Iwata, Kamegai, Sato, Watanabe, & Kasai (1999) showed that with the use of propylene glycol as a solvent, a higher conversion rate of LA to CLA was observed compared to the ethylene glycol, while the CLA products obtained by this method had a much lower coloration than other conventional methods and more solubility than other solvents.

Response function to estimate the amount of c9-t11 isomer according to the calculated regression coefficients was obtained as the following equation (Eq. 3). As the coefficients of the equation show the changes in the amount of isomers has inverse relationship whit changing the studied variables, so that the quadratic effect of the temperature and the linear effect of the solvent had the least and the most effect on the amount of isomer production, respectively.

$$Y(C9,T11) = 12.13 - 0.422x_1 - 0.048x_2 - 0.006x_3 - 0.026x_1^2 + 0.00006x_2^2 + 0.00015x_3^2 + 0.0051x_1x_2 + 0.0016x_1x_3 - 0.00016x_2x_3 \quad (3)$$

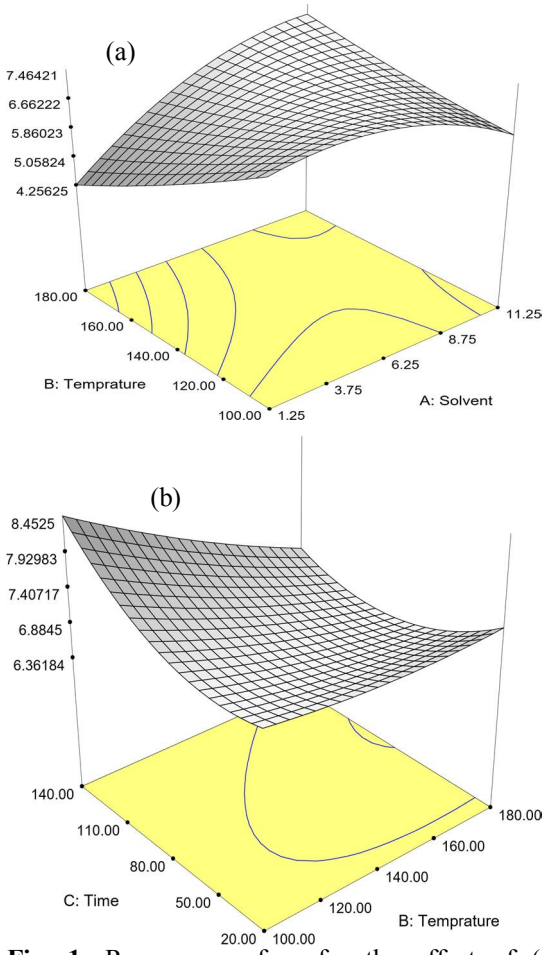

Fig. 1. Response surface for the effect of (a) solvent content and temperature (time; 80 min) (b) temperature and time (solvent; 6.25 mL) on the production of c9-t11 isomer

The effect of variables on the t10-c12 isomer production

The changes in synthesize of trans10-cis12 isomer in the reaction mixture was similar to the c9-t11 isomers. The latter fitted model indicating the synthesize yield of the t10-c12 isomers as a function of the independent changeable is established as Eq. (4). According to the results of the analysis variance and coefficients of the fitted model, the linear effect of the temperature and solvent and also the interaction effect of these two variables on the amount of t10-c12 isomers were more than the other studied variables effect.

As shown in Fig. (2a), with increasing process temperature and solvent content, the production of t10-c12 isomer increased non-linearly. Decreasing the solvent to 1.25 mL and also raising the temperature to 180 °C resulted in a sharp drop in the

production of isomer t10-c12. While at 100 °C, with increasing solvent content to 6.25 mL, the production of t10-c12 isomer increased. However, with an increase in the amount of solvent to values higher than 6.25 mL, the isomerization reaction reduced again, although these changes were not significant. Therefore, similar to those observed in c9-t11 isomer, more and less than 6.25 mL of solvent had an adverse effect on the isomerization and production of t10-c12 isomer.

The reaction time had less effect on the changes in the production of isomers than the temperature and solvent.

As shown in Fig. (2b), with increasing the reaction time, the amount of t10-c12 increased, but these changes were not noticeable at higher temperatures and lower solvent content. Therefore, it can be said that the effect of time in lower temperature (100-140 °C) conditions and average solvent content (6.25 mL) has a greater impact on the production of CLA isomers.

These results were in with the investigation highlights conducted by (Yang & Liu, 2004), who gathering that the linear or the quadratic term of the time have no significant effect on the isomerization (P>0.05). High temperature over a long time leads to a reduction in trans-trans isomers and cis-cis isomers increases, and so the presence of impurities such as iron, copper and other metals enhances the trans-transit isomers production (Bernas, Kumar, Mäki-Arvela, Kul'kova, Holmbom, Salmi, & Murzin, 2003; Cardó, Bergadà, Cesteros, & Salagre, 2012). According to the results obtained by Sæbø (2003) the proper temperature for production of CLA was reported between 130 and 180 °C, and the production of very small amount of sub-isomers in the product during the reaction is inevitable.

In another similar research, Koohikamali (2017) used temperature of 100 to 140 °C to produce the highest concentrations of CLA, and stated that the opposite is observed at temperatures above 140 °C, because the production of the main CLA isomers is reduced. Studies show that at temperature of 140 °C the amount of isomerization reaction and conversion of linoleic acid to CLA is almost completed, and the amount of useful isomers reaches its maximum production (Bauer, Horlacher, & Claus, 2009; Khanal & Olson, 2004; Kritchevsky, 2000).

(4)

$$Y(T10,C12) = 12.07 - 0.421x_1 - 0.053x_3 - 0.0059x_3 - 0.012x_1^2 + 0.00009x_2^2 + 0.00006x_3^2 + 0.004x_1x_2 + 0.0015x_1x_3 - 0.00008x_2x_3$$

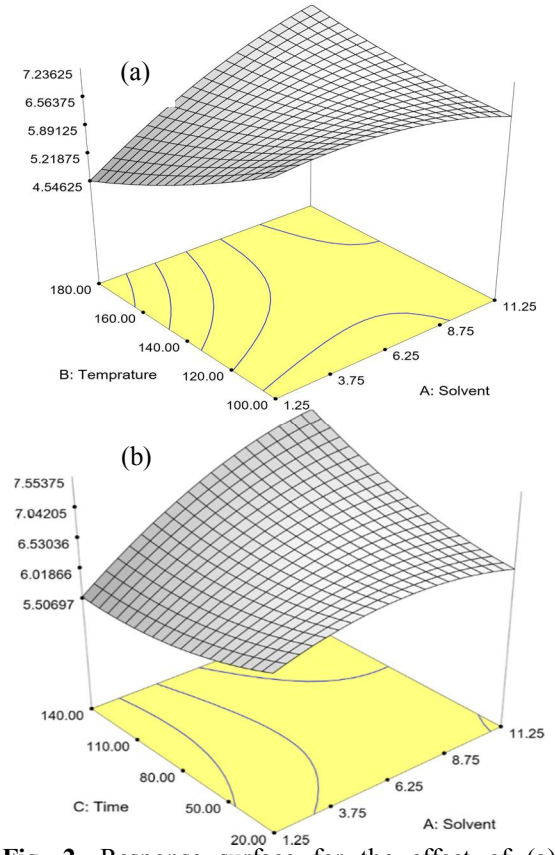

Fig. 2. Response surface for the effect of (a) solvent content and time (temperature; 140 ºC) (b) solvent content and temperature (time; 80 min) on the production of t10-c12 isomer

Optimization

Optimum condition for the production of CLA isomers, with the highest production efficiency, is determined using numerical optimization of plan Expert software. This optimum situation with a desirability of 77.5% is appendix in Table (1) that provides the highest value of trans10-cis12

and cis9-trans11 isomers. This showed that the optimum conditions for production of CLA isomers process were: Solvent concentration of 6.74 mL, reaction temperature of 100 °C and reaction time of 140 min. In optimal condition the amount of trans10-cis12 and cis9-trans11 isomers were equal till 8.44 and 7.62%, respectively.

Table 1. Optimization of the reaction process to produce maximum c9-t11 and t10-c12 isomers

Independent variables	minimum	maximum	optimum value
Solvent (mL)	1.25	11.25	6.74
Temperature (°C)	100	180	100
Time (min)	20	140	140
Desirability		77.5 %	

Quality evaluation of enriched milk

pH

Analysis of variance showed that milk enrichment had a significant (P<0.05) impression on its pH changes during storage time. Means comparison of the pH values of the control and enriched samples with CLA is presented in Fig. (3). As can be seen, in the production day and the first month, there was no statistically significant (P>0.05) difference in the pH of both samples, but in the second and third months of storage, the pH value of the control sample was significantly (P<0.05) higher than the enriched milk.

Storage time also had a significant effect on the pH of milk samples, so that with the passing of time pH values of milk samples decreased, but this reduces was higher in the enriched milk by CLA. Therefore, according to the results, the lowest amount of PH belonged to the sample of enriched milk after 3 months of storage that equal to 6.4 and compared to the production day with pH value of 6.7, decreased significantly (Fig. 3).

Therefore, according to the results, it was found that during the storage time pH of milk samples are leached to acidification due to oxidation of the fatty acids, and since CLA are more susceptible to oxidation because of their binary bonding, the amount of pH in the enriched milk samples more decreased (Koohikamali, 2017). In a similar study Farmani, Hesari, Bodbodak, & Pashaei Bahram (2017) by evaluating the qualitative characteristics of functional milk-carrot drink reported that the pH of this product was significantly reduced during the 13 days of storage, so that the product had a suitable quality until the tenth day, but it was unacceptable on the 13th day. Similar results were obtained by Georgantelis, Ambrosiadis, Katikou, Blekas, & Georgakis (2007) with examining the changes in the pH of meat products during storage. It should be noted that in the present study after 3 months of storage, all of sterilized milk samples in terms of pH were of good quality and had no problems with consumption.

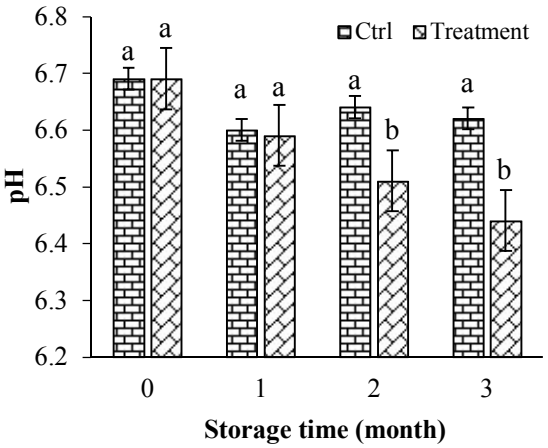

Fig. 3. PH values of control and enriched milk samples during storage

[a-b] different letters within the storage time indicate statistically significant differences at (P<0.05).

Peroxide value

Measuring the peroxide value (PV) is performed to determine the primary oxidation products in dairy products. Despite peroxides lack of flavor and taste, it can be used as an indicator for the quality changes created by light for each food product (Mortensen, Sørensen, & Stapelfeldt, 2002). The results of analysis of variance demonstrated that the effect of CLA addition and storage time on the changes in the PV of milk samples were significant (P<0.05). Means comparison of the PVs of the control and enriched

samples with CLA is presented in Fig. (4). As can be seen, there was no statistically significant differences in the peroxide value of both samples on production day until first month (P>0.05), but in other times (second and third months), the PV of the enriched samples were significantly higher than the control sample.

In both samples, from the production day until the third month of storage, the PV increased, so that the highest amount of PV on the 90th day was attributed to the enriched sample. It should be noted that although the PV of milk samples, especially in the enriched milk, during the three months of storage has reached 5-6 times the initial value, but both samples were useable for the consumer. The reason for increase in PV of milk samples during storage is oxidation of fatty acids especially unsaturated fatty acids, and in enriched milk samples due to presence of CLA, the oxidation reaction rate has been higher, which leads to an increase in the PV.

Oxidation of fatty acids is one of the main causes of food spoilage and it can resulted the texture, taste, aroma, color and shelf-life of the foods, and this limits the employ of sources containing unsaturated fatty acids in enrichment of food products (Jacobsen, 2010). The main substrates for the reaction of fat oxidation are unsaturated fatty acids with one or more double bond (Ottaway, 2008). Based on the findings, it appears that proper foods for enrichment with CLA and other unsaturated fatty acids are those that are consumed at a high rate, kept in a short time and at low temperature and sealed in non-penetrating packages to air and light (Kolanowski & Weißbrodt, 2007). In similar research, Ghorbni Hassan Sarai, Shahidi, Ghodoosi, Motamed Zadegan, & Varidi (2015) used omega-3 oil for enriching yogurt and reported that peroxide value of the enriched yogurt increased significantly during storage. Georgantelis *et al.* (2007) also reported a develop in the PV of meat products containing CLA during the shelf life. Campbell, Webster, Koziol-McLain, Block, Campbell, Curry, Gary, Glass, McFarlane, Sachs, Sharps, Ulrich, Wilt, Manganello, Xu, Schollenberger, Frye, & Laughon (2003) and Martínez-Monteagudo *et al.* (2012) stated that the conjugated double bond system (e.g. CLA) is more susceptible to autoxidation than the non-conjugated double bond system (e.g. linolenic acid).

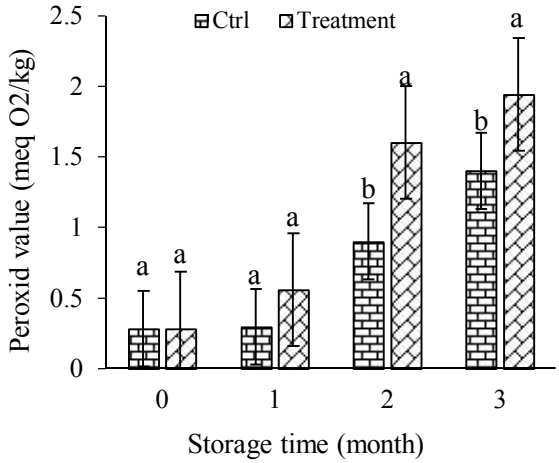

Fig. 4. Peroxide values of control and enriched milk samples during storage
[a-b] different letters within the storage time indicate statistically significant differences at (P<0.05).

Sensory Properties

The results showed that storage time and CLA had a significant (P<0.05) impression on the overall acceptance of milk samples.

According to the results of the means comparison, during 3 months of storage, the acceptance of milks were reduced and these changes were significantly higher in the enriched samples (Fig. 5). As can be seen, there was no significant (P>0.05) difference in the sensory properties of the control and the enriched samples during 30 days of storage, and the score of these samples was in the range of 3.5-4, which is acceptable in terms of consumption. While in the second and third months, although the overall acceptance of both milk samples decreased, this decrease was higher in fortified milk so that the lowest score of overall acceptance belonged to enriched milk on the 90th day, which equaled to 2.1 (Fig. 5). Therefore, based

on panelists score the enriched milk had no problem with consumption until 60-75 days after storage, but with an increase in storage time until the 90th day, its sensory properties is unacceptable. While the control sample received an acceptable consumer score after three months of maintenance, so that the average score of panelists on the 90th day was more than 3 (Fig. 5).

The reason for the reduction of the overall acceptance score of enriched milk compared to the control sample in the first month of storage can be due to the slight different odor which was caused by the addition of CLA to milk, and the increase in the negative response to this product in the last months of storage, which has a significant growth, can be due to the increased oxidation of the product during storage and the production of metallic taste in it, which makes the taste unpleasant for the consumers (El-Nor & Khattab, 2012).

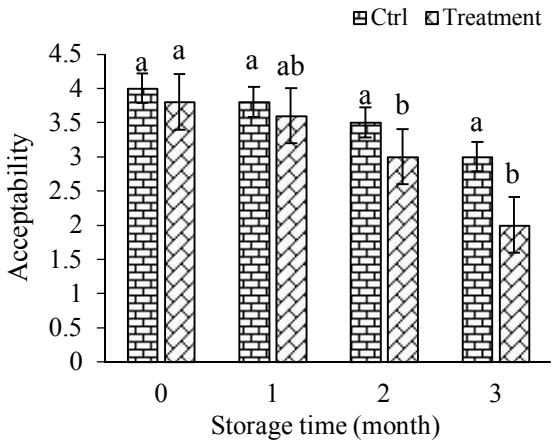

Fig. 5. Sensory properties score of control and enriched milk samples during storage
[a-b] different letters within the storage time indicate statistically significant differences at (P<0.05).

Constancy of CLA in enriched milk

Alteration of the CLA content of the enrichment milk samples during the 90 days storage are shown in Fig. (6). The amount of CLA measured in enriched milk samples on the production day was 51.75% of the total milk fatty acids, which was reduced by 51.2% during the first month of storage at refrigerator temperature, and these changes were not statistically significant (P>0.05). The amount of this fatty acids decreased in the second and third months of storage, by 50.6 and 49.4%, respectively, which was significant (P<0.05) compared to the first day of production.

As noted earlier, the reason for the reduction of CLA is the presence of double bands in their structure and, as a result, more sensitive to oxidation reactions. Another factor is probably the high temperature of milk sterilization that can increase the oxidation reaction rate and leads to structural degradation of these fatty acids (Zhang & Chen, 1997). Our findings are in conformity with the conclusions of Martínez-Monteagudo *et al.* (2012) that examined the stability of CLA added to milk. They reported that the amount of CLA decreases rapidly during product storage, and pressure and temperature can affect the reaction steps and accelerate the CLA oxidation. In another study, it was shown that with the storage of soybean oil for up to 60 days due to the sensitivity of unsaturated fatty acids to oxidation, the amount of unsaturated fatty acids such as linoleic acid, decreased from 61.76 to 57.83% (Khaneghah, Sh, & Ameri, 2012). Zhang & Chen (1997) also showed that the oxidation rate of conjugated linoleic acid as the free fatty acid was similar to that of free docosahexaenoic acid in air at 90 °C and considerably greater than those of free linoleic acid, linolenic acid, and arachidonic acid. They analyzed the remaining fatty acids by GC after esterification with BF_3. However, CLA in the triacylglycerol form was more stable, although it still oxidized more readily than linoleic acid, linolenic acid, or arachidonic acid. The impression of processing situation, dairy cultures, and storage situation on conjugated linoleic acid amount of dairy products have been studied. For example, pasteurizing raw milk for 30 min at 68.3 °C did not change the conjugated linoleic acid amount of milk (Baer, Ryali, Schingoethe, Kasperson, Donovan, Hippen, & Franklin,

2001). Producing milk under normal conditions (for 30 min up to 85 °C) into dairy products such as, cheese, sour cream , yogurt and ice cream had no influence on the conjugated linoleic acid amount (Dave, Ramaswamy, & Baer, 2002; Shantha *et al.*, 1992). Various studies have shown that the conjugated linoleic acid amount of dairy products when milk is processed at higher temperatures. For instance, clarification of ghee (butter oil) at 110 and 120 °C the CLA amount to 0.9 and 2.1% of fat, respectively, compared to 0.6% in raw milk (Aneja & Murthi, 1990). In another study, the processing of Cheddar cheese at 80 to 90 °C under atmospheric conditions increased CLA from 0.40 to 0.51% of fat; however, processing at 70 to 85 °C under nitrogen did not alter the conjugated linoleic acid amount (Shantha *et al.*, 1992).

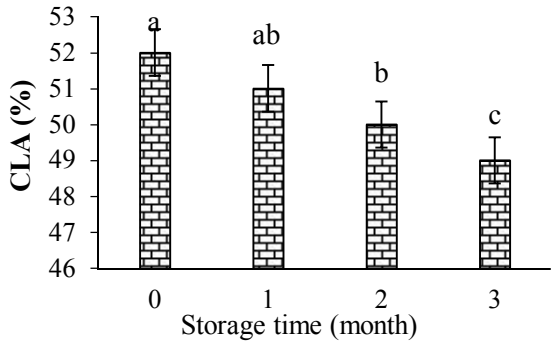

Fig. 6. Changes of CLA content in enriched milk samples during storage
[a-c] different letters within the storage time indicate statistically significant differences at (P<0.05).

Conclusions

In this study first, the effect of propylene glycol content and temperature and reaction time on the production yield of conjugated linoleic acid from sunflower oil were optimized and then the produced CLA was used to enrich the low-fat milk. According to the results, it was found that by increasing the solvent concentration to 6.25 mL and time up to 140 min, the linoleic acid isomerization and CLA production increased, but the reaction temperature had a negative impression on the trans10-cis12 and cis9-trans11 conjugated linoleic acid isomers production efficiency. The optimum condition that provided the maximum value of conjugated linoleic acid isomers were: process temperature of 100 °C, solvent content of 6.25 mL and reaction time of 140 min. Conjugated linoleic acid in dairy products were shown to be a stable compound under normal cooking and storage conditions. In milk and dairy products total of CLA content is about 0.34 to 1.07% of total fat. The results of this study showed that CLA-enriched milk had an acceptable stability during the 90 days refrigerated storage, so that at the end of the maintenance cycle, only about 4% of the conjugated linoleic acid isomers were reduced. The enriched samples in terms of pH and peroxide value also had a good quality compared to the control sample; but organoleptic assessment showed that the taste of the enriched milk samples after 3 months of storage was unpleasant for consumers due to the high oxidation of unsaturated fatty acids spatially conjugated linoleic acid isomers and the creation of a metallic taste. Therefore, based on chemical and sensory evaluations, the shelf life of the enriched milk in this study conditions is recommended for 75 days.

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بهینه‌سازی شرایط تولید اسید لینولئیک کونژوگه (CLA) و استفاده از آن در غنی‌سازی شیر

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چکیده

اسید لینولئیک کونژوگه (CLA) اخیراً به‌دلیل پتانسیل آن در محافظت در برابر سرطان، آتروژنز و دیابت موردتوجه زیادی قرار گرفته است. در مطالعهٔ حاضر، از اسید لینولئیک حاصل از روغن آفتاب‌گردان برای سنتز CLA استفاده شد و از روش سطح پاسخ (RSM) برای بهینه‌سازی فرایند استفاده شد. نتایج نشان داد که پروپیلن گلیکول به‌عنوان حلال و زمان واکنش تأثیر مثبت بر پاسخ می‌گذارد، اما دمای واکنش بالا به‌ویژه در مقادیر بالای حلال و زمان واکنش، منجربه‌کاهش تولید CLA می‌شود. محتوای حلال ۶/۲۵ میلی‌لیتر، دمای ۱۰۰ درجهٔ سانتی‌گراد و زمان ۱۴۰ دقیقه به‌عنوان شرایط بهینه در ایزومریزاسیون برای تولید ایزومرهای c9-t11 و t10-c12 اسید لینولئیک کونژوگه شناخته شد. در مرحلهٔ بعد، CLA تولیدشده متمرکز و تصفیه‌شده و برای غنی‌سازی شیر بدون چربی استفاده می‌شود. سپس، خصوصیات کیفی شیر شامل pH، مقدار پراکسید، خواص حسی و مقدار CLA در طی ۳ ماه از ذخیرهٔ شیر غنی‌شده در دمای ۵ درجهٔ سانتی‌گراد موردبررسی قرار گرفت. اختلاف معنی‌داری ($P<۰/۰۵$) بین نمونه‌های شاهد و تیمارشده به مدت ۱ ماه پس از ذخیره‌سازی مشاهده نشد، اما با افزایش مدت زمان نگهداری تا ۶۰ و بعد ۹۰ روز اختلاف بین این دو نمونه افزایش یافت ($P>۰/۰۵$). باتوجه‌به نتایج، مقدار CLA باقی‌مانده در شیر و همچنین pH با گذشت زمان کاهش‌یافته، اما مقدار پراکسید نمونه‌ها افزایش یافته است.

واژه‌های کلیدی: اسید لینولئیک کونژوگه، ایزومریزاسیون قلیایی، پروپیلن گلیکول، روش سطح پاسخ، غنی‌سازی شیر