

Quantitation and optimization of β -carotene and α -tocopherol in emulsion prototype with reversed-phase chromatography

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Abstract

Emulsion products that are rich in pro-vitamin A, vitamin E and antioxidants have been successfully formulated from red palm oil (FO-MSM), pumpkin (SLK), and dragon fruit (SBN). This study was aimed to determine the concentrations of β -carotene and α -tocopherol in the prototype products of FO-MSM, SLK, and SBN by reverse phase chromatography and to determine the optimal deodorization time for FO-MSM on β -carotene and α -tocopherol levels. Optimal conditions for analysis of samples was with the mobile phase methyl dichloride: methanol: acetonitrile (2: 1: 3). The injection volume was 10 μ L for β -carotene and 20 μ L for α -tocopherol. Isocratic elution was carried out at a flow rate of 1.0 mL/min at room temperature ($25\pm 2^\circ\text{C}$). The mobile phase was first eliminated for 45 mins. The β -carotene and α -tocopherol peaks were identified by matching the peak retention times (Rt) of β -carotene and α -tocopherol in the sample with the standard Rt of β -carotene and α -tocopherol. The reverse-phase chromatography successfully determined β -carotene and α -tocopherol in prototype products of FO-MSM, SLK, and SBN. The developed emulsion prototype had 2044.5 ± 196.1 ppm of β -carotene and 38.8 ± 0.4 ppm of α -tocopherol. The content of β -carotene in the developed emulsion prototype was more than tenfold increase from the original prototype without sacrificing the product's taste. β -carotene showed no significant difference in reducing the deodorization time from 5 hrs to 1 hr, while the 2.5-fold increase of the α -tocopherol content was observed. Therefore, the optimal deodorization time for FO-MSM was determined based on the value of α -tocopherol and not by β -carotene. RP-18 was successfully used to determine β -carotene and α -tocopherol in FO-MSM, SLK, and SBN emulsion prototype products.

1. Introduction

Emulsion products rich in pro-vitamin A, vitamin E, and antioxidants have been successfully formulated from local raw materials, namely red palm oil, the flesh of yellow pumpkin (*Cucurbita moschata*), and the flesh of dragon fruit (*Hylocereus costaricensis*). Carotenoids, tocopherols, and tocotrienols were the main feature of the olein fraction of red palm oil (FO-MSM) that are beneficial to humans' growth and reduce the risk of degenerative diseases (Catanzaro *et al.*, 2016; Musa *et al.*, 2017). Yellow pumpkin juice (SLK) is a recognized

carotenoid source that specifically contains lutein and cucurbitaxanthin, and β -carotene (Matsuno *et al.*, 1986). β -carotene, lutein, and xanthins are the characteristics of carotenoid-based functional food emulsions formulated. The prototype contained 141.65 ± 0.47 ppm of β -carotene (Rahmadi *et al.*, 2015).

New formulas were developed to address the off-taste of FO-MSM and the earthy-green flavor of the yellow pumpkin. Dragon fruit juice (SBN) successfully masked the off-taste (Rahmadi *et al.*, 2017). In contrast to the previous formulas (Rahmadi *et al.*, 2015; Rahmadi

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et al., 2017), the currently developed formula contains 300% higher of FO-MSM without sacrificing the taste of the product.

β -carotene and α -tocopherol are potent antioxidants. Hence they are prone to light and heat exposure. The loss of both compounds from raw material due to industrial processing was observed (Kreps *et al.*, 2014). The FO-MSM was heat processed to remove the off odor and taste, i.e., five-hour deodorization at 100°C and 80-90 mmHg (Rahmadi *et al.*, 2017). Therefore, it is essential to have an insight into the degree of β -carotene and α -tocopherol degradations in the deodorization stage. Maintaining natural β -carotene and α -tocopherol contents can be achieved by reducing heat exposure of the developed prototypes, which, in our case, is to perform deodorization at the optimum time.

The spectrophotometry method had limited ability to measure β -carotene content in the developed prototypes due to pectin in SBN (Rahmadi *et al.*, 2017). The pectin content was also a concern for measuring α -tocopherol content in the emulsions. Therefore, we opted to use a more sensitive method, i.e., HPLC, to measure β -carotene and α -tocopherol contents of the developed prototype.

In the preliminary β -carotene and α -tocopherol analysis, it was noted that the AOAC method (Schierle *et al.*, 2004; AOAC, 2006) required a degree of adjustment in order to quantitate β -carotene and α -tocopherol in the emulsion optimally. The use of high polarity and high hydrophobicity RP-18 column was part of the main reason. In comparison to normal-phase chromatography, reverse-phase chromatography (RP-HPLC) uses resins with the characteristic of containing a hydrophobic stationary phase that covalently bonds alkyl chains (Shire, 2015). The RP-HPLC separates molecules based on surface hydrophobicity. Therefore, non-polar compounds are retained longer, and their separations are easily occurring (Ligor *et al.*, 2014). Organic solvents are commonly added to decrease the water concentration in the mobile phase. The decrease of water concentration causes the weakening of the hydrophobic attraction of the hydrophobic groups on the chromatography column (Shire, 2015). The more polar compounds are eluted first following the non-polar compound i.e., β -carotene and α -tocopherol.

RP-18 column performed efficient separation of the pro-vitamin A compounds (Mitrowska *et al.*, 2012). Carotenoid content in vegetables was investigated using reverse-phase HPLC with an isocratic elution system at a wavelength of 450-475 nm (Kidmose *et al.*, 2006). In addition to β -carotene quantitation, reverse-phase

chromatography was employed to measure α -tocopherol and all-trans-retinol levels simultaneously. The compounds were detected with UV at wavelengths of 325 and 295 nm, respectively. The complete separation in the RP-18 column was achieved for both compounds (Khan *et al.*, 2010).

The main aim of this study was to quantitate β -carotene and α -tocopherol in prototype products of red palm oleic fraction emulsion (FO-MSM), yellow pumpkin (SLK), and dragon fruit (SBN) with reverse-phase chromatography. The research's secondary aim was to find the optimum deodorization time for FO-MSM concerning β -carotene and α -tocopherol levels.

2. Materials and methods

2.1 FO-MSM preparation

Crude palm oil (CPO) was obtained from PT. Teladan Prima Group Plantations with the specification of less than 3% of free fatty acids (FFA). To remove gum and FFA, warm water (80-90°C) was added to CPO, and the mixture was shaken in a separating funnel before the addition of 200-400 mL of 10% NaOH (Sigma, Singapore). Flushing was conducted with warm water repeatedly until gum and Na-FFA were eluted entirely. The sample was kept overnight at room temperature (25±2°C), then the olein fraction was collected. Subsequently, the liquid fraction (olein) was deodorized in a rotary evaporator (Buchi R-200, Switzerland) for the specified times at 100°C, 60 RPM, 80-90 mmHg. About 2% (w/v) of pharmacy grade active carbon (Eagle Indo Pharma, Indonesia) was added to each deodorization batch.

2.2 Emulsion product preparation

The deodorized FO-MSM, SLK, and SBN were mixed under the specified formulations. Food-grade carboxymethyl cellulose (CMC), fructose syrup, cinnamon powder, and xanthan gum were added to form the emulsion at specified concentrations into the mixture. The specified concentrations are patent protected (Indonesian Intellectual Property Rights granted number: S00201708790). Flavors were added to the emulsion prototype, namely raspberry (0.7% v/v), and citric acid (0.25% w/v).

2.3 Sample preparation and detection

About 40-50 g of samples were carefully weighed, and each sample was placed into Erlenmeyer. The sample was dissolved in chloroform (Merck, USA) and methanol (Merck, USA) at a ratio of 2:1. The samples were mixed for 1 hr at room temperature (25±2°C), after which the solvents were added at approximately 65 mL.

The procedure was repeated four times. The solution was then filtered (Whatman No. 4) and salted with 14 mL of 0.88% NaCl (Merck, USA) solution and then mixed. The mixture formed two layers, in which the top layer was removed, and the bottom layer was filtered and collected. Nitrogen gas was blown to evaporate the remaining solvent. Each oil extract was placed in a dark bottle and stored in the refrigerator (-8 to -5°C) until further analysis.

The oil extract was removed from the storage area and kept at room temperature (25±2°C) until reaching the ambient temperature. About 0.1 to 0.25 g of sample was carefully weighed and saponified with 10 mL of absolute ethanol (Merck, USA) and 2.5 mL of 50% KOH (Merck, USA) in double-distilled water (ddH₂O) (w/v) (Central Lab, Padjajaran University, Indonesia). Subsequently, the mixture was heated on a water bath at 80°C for 45 min. The mixture was cooled, and 2.5 mL of glacial acetic acid (Merck, USA) was added. The mixture was transferred into a 25 mL measuring flask, and the volume was adjusted with ethanol: tetrahydrofuran (THF) (Merck, USA) at a ratio of 1:1 (v/v). Samples were filtered with 0.2 µm polyvinylidene fluoride (PVDF) pore syringe filter (Millipore, USA).

To quantitate α-tocopherol, the fresh sample of FO-MSM was carefully weighed at 0.25 g and then dissolved in 10 mL ethanol. The volume was fixed to 25 mL by adding ethanol: THF at a ratio of 1:1. Samples were filtered with 0.2 µm PVDF.

2.4 Chromatographic condition

HPLC instrument (Waters series 1500 systems) was equipped with Isocratic HPLC Pump, Photometric Diode Array (Waters Acquity PDA), and Lichrospher® RP-18 HPLC Column (150 × 4.6 mm × 5 µm). Each sample was injected into HPLC at ten µL of volume. The isocratic elution was carried out at a flow rate of 1.0 mL/min at room temperature (25±2°C) with the mobile phase of methyl dichloride (Merck)/methanol (Merck)/acetonitrile (Merck) at a ratio of 2:1:3. The mobile phase had been firstly degassed for 45 mins. The β-carotene peaks in the samples were identified by matching the peak retention time (R_t) of β-carotene in the sample with the R_t of β-carotene standard (Sigma Aldrich, USA).

For α-tocopherol quantitation, each sample was injected into HPLC at 20 µL of volume. The isocratic elution was carried out at a flow rate of 1.0 mL/min at room temperature (25±2°C) with the mobile phase of methanol (Merck) that was first degassed for 45 mins. The α-tocopherol peaks in the samples were identified according to the R_t of α-tocopherol standard (Sigma Aldrich, USA).

2.5 Linearity test and calibration curve

Standard solutions were arranged at a stepwise increase in concentration from 3 to 30 µg/mL for β-carotene and from 0.5 to 10 µg/mL for α-tocopherol. The solutions were prepared in the same manner with the procedures for sample quantitation. The standards were injected into the HPLC system. Calibration curve was produced by linear regression equation of [peak area] = a + b * [standard concentration]. Multiple correlation coefficient (R²) was calculated as an indication of the linearity of the calibration curve.

2.6 Recovery of β-carotene

The recovery was calculated with the formula (Rathore, 2007):

$$\% \text{Recovery} = \frac{a-b}{*c} \times 100\%$$

Where a = concentration in β-carotene fortified into the sample, b = concentration in the sample without β-carotene standard and *c = concentration of the β-carotene standard added

3. Results and discussion

3.1 β-carotene extraction from the emulsion

The stages of preparation of carotenoid samples from foodstuffs comprise deproteination, extraction and dissolution, precipitation and concentration, and redilution (Amorim *et al.*, 2014). For FO-MSM, SLK, and SBN emulsion products, the most crucial step was extraction and dissolution, because carotenoids were bound to pectin and gum complexes. This condition was similar to research conducted by (Mahato *et al.*, 2019). To optimally obtain the carotenoid content from the emulsion matrices, the extraction and dissolution process was repeated four times. Repetition of extraction and dissolution of carotenoid from the matrices was optimized during the sample preparation stage, and this stage depends on the sample matrices (Saini and Keum, 2017)

The preparation of carotenoid extraction in food products may use a wide variety of organic solvents (Ha *et al.*, 2010). Chloroform and methanol were selected carefully based on previous research (Gupta *et al.*, 2015), however, the solvent ratio is sample-specific (Harlen *et al.*, 2018). Chloroform and methanol were used in this study with a 2:1 ratio because they could maintain β-carotene stability during the preparation stage (San and Yildirim, 2010). To improve the stability of β-carotene and α-tocopherol against light and heat oxidations, it is common to include a tiny amount of butylated hydroxytoluene (BHT) or vitamin C during the sample preparation stage (Khan *et al.*, 2010). The emulsion

contained 19.32 ± 0.62 mg/100 mL of vitamin C that may stabilize β -carotene and α -tocopherol (Rahmadi *et al.*, 2017).

3.2 Calibration curve for β -carotene

To ensure that the analytical procedures with RP-18 column to produce high repeatability, the standard β -carotene curve was produced based on a linearization of the standard peak area of β -carotene with ten concentration variations, step-wisely increased from 3 ppm to 30 ppm. Figure 1 shows the Rt value of β -carotene observed in the range of 4 and 4.3 mins. Figure 2 shows the calibration curve with the equation of [peak area] = $65415 \times$ [concentration of β -carotene] - 33971 with the value of multi correlation (R²) obtained was 0.9998.

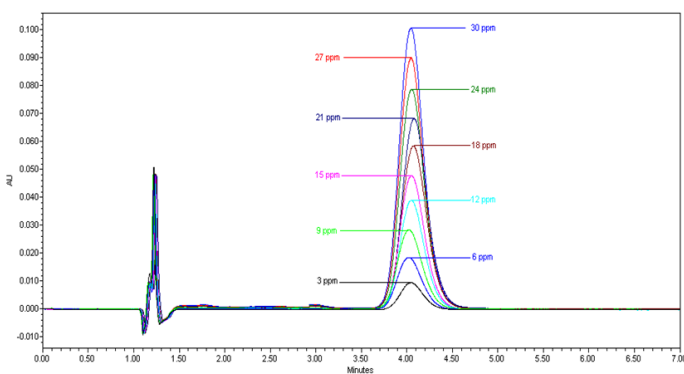


Figure 1. Peaks of the β -carotene standard with RP-18 column

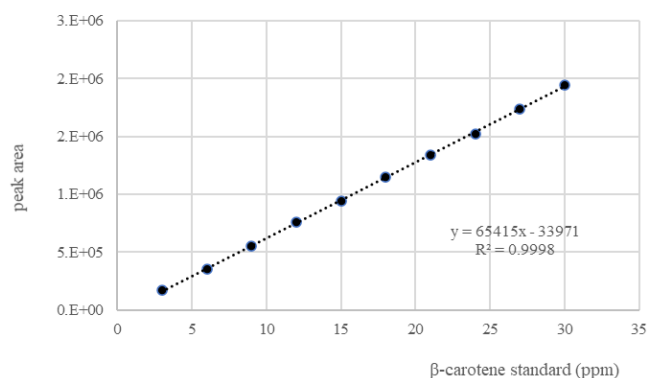


Figure 2. Standard curve of β -carotene

During the analysis, we experience a slight shift in Rt between the β -carotene standard, the emulsion samples, and β -carotene recovery samples due to the composition and physical condition of the mobile phase sample interaction. Retention time (Rt) may slightly differ due to a slight difference in mobile phase mixture concentration; regardless, the chromatographic conditions were similar between analysis. The slight difference in the mobile phase mixture was considered as the reason for Rt shifting in the sample analysis. The difference was more pronounced if the observed material has Rt of more than 5 mins (Khan *et al.*, 2010).

The first minutes of detection indicated that lutein

and xanthin were co-elute with the mobile phase. Therefore, the peaks were observed before the β -carotene peak emerged in the minute of 3 to 5 of the detection. The saponification process and the solvent used to provide a selective system for beta-carotene analysis so that beta-carotene levels can be detected at 3-5 mins. The condition was similar to our previous publication (Rahmadi *et al.*, 2015), where inseparable lutein and xanthin peak were observed with Rt around 1 to 2 mins. It was also noted that carotene isomers, i.e., α - and β -carotene, were not easily separated and may produce a wide peak (Khoo *et al.*, 2011). A mobile gradient phase may be employed to separate better closely similar compounds or isomers (Berhow *et al.*, 2013; Melendez-Martinez *et al.*, 2013).

3.3 β -carotene determination in the emulsion

The levels of β -carotene emulsion products and their constituent ingredients were successfully measured (Figure 3). The components of the emulsion product comprising SBN, food additives (BTP), FO-MSM, and SLK had β -carotene levels at 48.4 ± 17.3 , 1039.0 ± 258.5 , 1409.8 ± 31.0 , and 1516.7 ± 25.8 ppm, respectively. The five hours deodorized FO-MSM and SLK had almost similar β -carotene levels. It was noted that the commercial food additive contained β -carotene in a reasonably high concentration, although still lower than the β -carotene levels in FO-MSM and SLK.

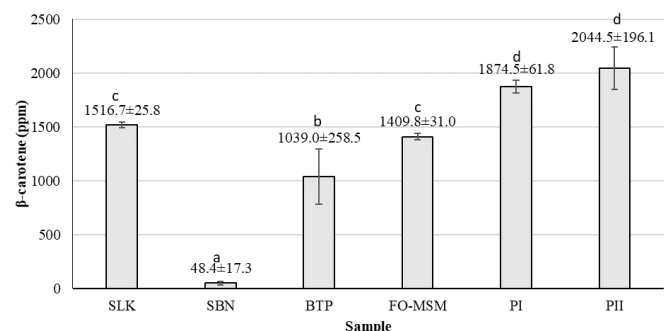


Figure 3. β -carotene content of the emulsions. Prototype containing: SLK = yellow pumpkin juice only, SBN = dragon fruit juice only, BTP = food additive only, FO-MSM = olein fraction of red palm oil only, PI = all, but without SBN, PII = all. $\bar{\pm}$ indicates Standard deviation (SD); letters above the numbers indicating the least significant difference at $\alpha = 5\%$

SBN had no significant contribution to β -carotene content. The non-SBN and SBN emulsion prototypes had β -carotene content of 1874.5 ± 61.8 and 2044.5 ± 196.1 ppm, respectively. It was confirmed that the role of SBN in prototype II was limited to coat the pronounced off-taste of FO-MSM, as observed in the previous prototype (Rahmadi *et al.*, 2017). Compared to the formulas that contained only FO-MSM or SLK, the emulsion prototypes from a combination of both materials produced better β -carotene concentrations. The emulsion

contains better β -carotene stability than FO-MSM alone.

The stability of β -carotene in emulsion products depends on the type of emulsion and carrier oil (Boon *et al.*, 2010; Qian *et al.*, 2012a). Other factors that may affect the physical and chemical stability of β -carotene in emulsion products are pH, ionic strength, and temperature (Qian *et al.*, 2012b). Furthermore, the food matrix influences the higher stability of β -carotene (O'Sullivan *et al.*, 2010). Although not significant, β -carotene levels in emulsion product with SBN addition tended to be higher than β -carotene levels in the prototype without SBN. The presence of pectin, i.e., in dragon fruit juice, improved the stability of β -carotene. The stability of β -carotene resulted in better bio-accessibility through *in vitro* digestive model (Xu *et al.*, 2014).

3.4 Recovery of β -carotene

Compared to the measurement α -tocopherol, the detection of β -carotene was relatively more complicated for the emulsion prototypes. Therefore the non-SBN and SBN samples were fortified with three ppm of the β -carotene standard to measure the recovery of β -carotene (Table 1). In general, %Recoveries for β -carotene for the non-SBN and SBN samples were 104.2% with a standard deviation (SD) of 7.8% and 71.9% with SD of 10.1%, respectively. The %Recovery is a simple procedure of verifying analyte determination because it minimizes errors due to the sample solution's composition and physical properties (Rathore, 2007).

Table 1. Recovery of β -carotene

Sample	Repetition	%Recovery	Average %R	Standard Deviation
Prototype without SBN	1	109.88	104.2	7.8
	2	95.32		
	3	107.45		
Prototype with SBN	1	81.12	71.9	10.1
	2	73.38		
	3	61.12		

3.5 Calibration curve and α -tocopherol determination

The α -tocopherol calibration curve was linearly regressed from five observation points resulting in the equation of [peak area] = 16773 * [concentration of α -tocopherol standard] - 3393.5 with the value of R² was at 0.9991 (Figure 4). The emulsion prototype made with SBN had 38.8±0.4 ppm of α -tocopherol. Low α -tocopherol concentration was due to the lack of α -tocopherol rich material in the prototype. It was noted that the main α -tocopherol content was from FO-MSM. Most of the α -tocopherol from FO-MSM was suspected to be degraded during deodorization. In light of this hypothesis, the optimal deodorization time for FO-MSM was determined based on the value of α -tocopherol.

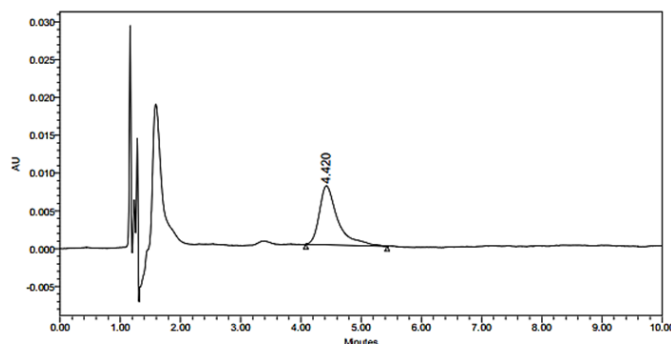


Figure 4. Standard curve α -tocopherol standard and chromatogram of α -tocopherol at a concentration of 10 ppm

3.6 Optimal deodorization time for FO-MSM based on the level of α -tocopherol

The application of heat is required for deodorization; therefore β -carotene and α -tocopherol contents may be degraded. Our observation found that α -tocopherol compound has lesser stability than β -carotene during heating, therefore the duration of deodorization is a crucial factor in α -tocopherol loss in the formula's olein fraction (Figure 5). The α -tocopherol content obtained in the sample of red palm oil was 77.0±0.7, 65.8±2.1, 55.2±1.4, and 30.5±0.1 ppm for the deodorized FO-MSM for 1, 2, 3, and 5 hours, respectively. The decrease in α -tocopherol levels in red palm oil was in linear proportion to the deodorization time.

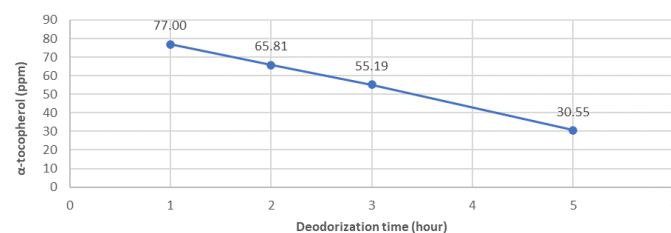


Figure 5. The decrease of α -tocopherol content during deodorization

This result is similar to previous research; α -tocopherol decomposed easier than other tocopherol isomers or β -carotene at any processing temperature above 60°C. The longer a product was heat processed, the lower α -tocopherol content was retained (Seppanen *et al.*, 2010). In the sunflower oil processing, α -tocopherol was wasted up to 50%. Deodorization and neutralization stages caused a higher loss of α -tocopherol. Each processing stage contributed to 17±0.5% (w/w) to the loss of α -tocopherol compared to the bleaching stage (9% loss w/w). Deodorization caused α -tocopherol to oxidize to form tocopherol-esters (Naz *et al.*, 2011). Figure 6 indicates that the optimum deodorization time for FO-MSM to be used in the emulsion was one hour, as it would conserve more α -tocopherol while β -carotene content remains the same.

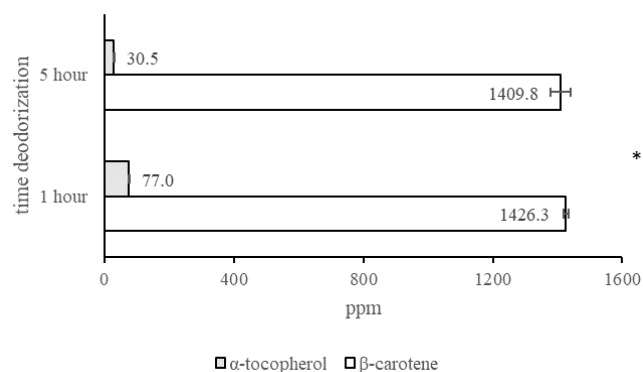


Figure 6. The comparison of β -carotene and α -tocopherol contents of the FO-MSM. \top indicates Standard Deviation (SD); *Indicates significantly different based on Multiple T-test.

4. Conclusion

The RP-18 was successfully utilized to determine β -carotene and α -tocopherol in prototype products of red palm olein fraction emulsion (FO-MSM), yellow pumpkin (SLK), and dragon fruit (SBN). We can increase the content of the β -carotene content of the emulsion prototype from 141.65 ± 196.1 ppm by more than ten folds without sacrificing the product's taste. The currently developed emulsion prototype had 2044.5 ± 196.1 ppm of β -carotene and 38.8 ± 0.4 ppm of α -tocopherol. The optimal deodorization time for FO-MSM was determined based on the value of α -tocopherol and not by β -carotene since 2.5 folds increase of the α -tocopherol content was observed as a result of reducing the deodorization time from 5 hours to 1 hour. There is room for improvement to further increase the α -tocopherol content of the emulsion by applying the optimum deodorization time for FO-MSM.

Conflict of interest

The authors declare no conflict of interest.

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