Simplified Extraction Procedure and HPLC Determination for Total Vitamin E and β-Carotene of Reduced-Fat Mayonnaise

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ABSTRACT: A simple, rapid procedure using direct solvent extraction and liquid chromatography was developed for the simultaneous determination and identification of α-tocopheryl acetate, β-carotene and tocopherols in reduced-fat mayonnaise. The method used a zero-control reference material (ZRM) (made in-house from olive oil and eggs) for accuracy determination. The vitamins and β-carotene were quantified by fluorescence and photodiode array detectors, respectively. The overall % recoveries (mean±S.D.) (n=5) for β-carotene, α-tocopheryl acetate, α-tocopherol, γ-tocopherol and δ-tocopherol were 101.4±1.4, 99.0±4.2, 102.0±3.6, 101.3±4.4 and 101.9±6.2, respectively. The method differentiates between natural and synthetic forms of vitamin E for accurate assessment of vitamin E biological activity. Comparative assays were performed using both direct solvent extraction and saponification.

Keywords: direct solvent extraction, vitamin E, β-carotene, reduced-fat mayonnaise, HPLC

Introduction

HIGH-PERFORMANCE LIQUID CHROmatography (HPLC) is the currently most accepted technique for the separation and quantification of β-carotene and tocopherols in foods (Rizzolo and Polesello 1992; Barua and others 1993; Hewavitharana and others 1996; Eitenmiller and Landen 1999). Most published procedures employ one detector. Simultaneous detection of β-carotene and tocopherols by a single detector is difficult because of differences in absorption and fluorescence properties of the analytes. β-Carotene does not fluoresce but exhibits strong absorption at 450 nm, whereas the tocopherols weakly absorb in the UV region (292 to 298 nm, in ethanol) (Kasparek 1980). However, tocopherols exhibit strong native fluorescence due to the chroman ring structure (Duggan and others 1957). Since these analytes can be extracted together from foods such as margarine, mayonnaise and vegetables, use of detectors in series makes simultaneous assay possible. In this paper, we describe the use of fluorescence and photodiode array detectors to simultaneously measure five or more analytes extracted from reduced-fat mayonnaise in a single injection.

Mayonnaise, a major dietary source of vitamin E, provides about 4% of the vitamin E daily intake in the United States (Eitenmiller 1997). Reduced-fat mayonnaise is often fortified with β-carotene and all rac-α-tocopheryl acetate (α-TAC). These analytes can be easily extracted simultaneously from the mayonnaise matrix. In this study, a direct solvent extraction procedure (Landen 1982) was used with little modification which results in reduced vitamin degradation and speed of extraction compared to saponification. The method avoids saponification and since the chemical forms of the vitamins are not altered, α-TAC can be quantified. Since all-rac-α-tocopherol has only 74% of the biological activity of RRR α-tocopherol (Pryor 1995; Eitenmiller and Landen 1999), saponification procedures that convert α-TAC into all-rac-α-tocopherol do not accurately quantify vitamin E. AOAC International (1995) does not provide official methods for the analysis of vitamin E and β-carotene in mayonnaise and reduced-fat mayonnaise.

The objective of this study was to develop a simple, rapid method to simultaneously assay the β-carotene and total vitamin E activity in reduced-fat mayonnaise. Validation parameters for the overall method were determined to ensure the method's validity.

Materials and Methods

Chromatography

The normal-phase HPLC system was equipped with a Waters 2690 Separations Module, a Waters 996 Photodiode Array Detector attached to a Waters Millennium 2010, Version 3.01 (Waters Corp., Milford, Mass., U.S.A.) Chromatography Manager, on a compatible IBM computer, and a Shimadzu RF-10A_{XL} programmable fluorescence detector (Shimadzu Corp., Columbia, Md., U.S.A.). The wavelengths were time programmed: time 0 min, λ_{ex} 285 nm, λ_{em} 310 nm; time 7 min, λ_{ex} 290 nm, λ_{em} 330 nm. Photodiode array detector monitored at 220 to 500 nm. The column was a 25cm x 4.6mm 5 μm LiChrosorb Si60 (Hibar Fertigsaule, Darmstadt, Germany) equipped with a guard column packed with Perisorb A 30-40 μm. The isocratic mobile phase contained 0.27% isopropanol in n-hexane with a gradient flow rate from 0.9 to 1.5 mL/min:

time (min)	flow rate (mL/min)
0	0.90
4.50	0.90
5.05	1.35
5.30	1.50

The mobile phase was filtered using a 0.45-μm nylon membrane filter (MSI Inc., Westboro, Mass., U.S.A.) and degassed by stirring under vacuum. Other required equipment include a sonicator FS30 (Fisher Scientific, Atlanta, Ga, U.S.A.), a Polytron® homogenizer (Pro Scientific Inc., Monroe, Conn., U.S.A.), and a bell-jar filtration apparatus (Knotes, Vineland, N.J., U.S.A.).

Chemicals and standards

All chemicals were of LC grade or analytical purity. Butylated hydroxytoluene (BHT), all-rac-γ-tocopherol (γ-T) and all-rac-δ-tocopherol (δ-T) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), all-rac-α-tocopherol (α-T) was purchased from BASF Corporation (Parsippany, N.J., U.S.A.), all-rac-α-tocopheryl acetate (α-TAC) and β-carotene were purchased from Fluka Bio Chemika (St. Louis, Mo., U.S.A.).

Preparation of standard solutions

The standard solutions were prepared as described by Ye and others (1998). The reference $E^{1\%}_{1cm}$ data are listed in Table 1. The appropriate dilutions were made with the respective mobile phase to give three working standard concentrations ranging from 0.62 to 15.43 μg/mL for α-TAC, 0.314 to 7.857 μg/mL for α-T, 0.83 to 20.67 μg/mL for γ-T, 1.24 to 30.94 μg/mL for δ-T and 0.011 to 1.288 μg/mL for β-carotene, respectively.

Preparation of mayonnaise zero-control reference material (ZRM)

Mayonnaise was prepared according to the CFR (1995) description of mayonnaise with little modification. The product contained 65% olive oil, 2% acetic acid and liquid whole egg. The mixture was homogenized with a Polytron® homogenizer for 5 min and stored at 24 ± 2 °C for not longer than 3 days. This ZRM was used for validation of the extraction of all analytes except α-T. The ZRM was used for recovery studies. Use of a ZRM for method validation studies is described by Chase and others (1997).

Samples

Samples used in this study were commercial fortified reduced-fat mayonnaise labeled to contain 33% and 13% fat. Mayonnaise samples were well mixed by stirring with a stainless steel spatula before sampling.

Extraction solvents

Two extracting solvents were used in the study. Extracting solvent I used for extraction of the mayonnaise consisted of 0.003% BHT in hexane-ethyl acetate (90:10). Initial studies by authors showed that this solvent mixture extracted β-carotene more efficiently than pure hexane. Extracting solvent II, 0.003% BHT in hexane, was used as the extractant for saponified mayonnaise digests.

Table 1—Specific absorption coefficients ($E^{1\%}_{1cm}$) and maximum wavelengths (λ_{max}) for three tocopherol homologs, α-tocopheryl acetate and β-carotene [a]

Analytes	λ_{max} nm	$E^{1\%}_{1cm}$ [b]
α-T	292	71
γ-T	298	92.8
δ-T	298	91.2
α-TAC	286	42
β-Carotene	**453**	**2592**

[a] Scott 1978; Bauernfeind 1981.
[b] In ethanol, bold value in hexane.

Direct solvent extraction method

The direct solvent extraction method used in this study is the method developed by Landen (1982). The sample (3.0 g) was accurately weighed into a 125 mL centrifuge tube, hot deionized water (80 °C, 2 mL) was added to the sample which was then sonicated for 5 min to facilitate dissolution of all vitamin fortification forms. Then 5 mL isopropanol was added. Approximately 5 g anhydrous magnesium sulfate was added and mixed with a stainless steel spatula plus 20 mL extracting solvent I. The mixture was homogenized with a Polytron® homogenizer for 1 min at medium speed. The generator tip of the homogenizer was rinsed with isopropanol and the extract was filtered through a 60-mL coarse-porosity fritted glass filter into a 125-mL Philips beaker using a vacuum bell jar filtration apparatus. After release of the vacuum, the material was broken up on a fritted glass filter and washed twice with 5 mL extraction solvent I. The extraction was repeated by transferring the material on the fritted glass filter to the original centrifuge tube with 5 mL isopropanol and 20 mL extracting solvent I added prior to homogenizing the mixture for 1 min and filtration. The combined filtrates were transferred to a 100-mL volumetric flask and diluted to volume with n-hexane. Then 4 mL of the extract was pipetted into a 10-mL test tube and evaporated under nitrogen until dry. After adding 1 mL of mobile phase 10 μL was injected. For recoveries, 1.0 mL of each spiked solution was added to 3.0 g ZRM and assayed as outlined above. The spike additions represented concentrations of β-carotene, α-TAC, γ-T and δ-T and ranged from 2.13 - 19.32, 107.1 - 857.1, 107.7 - 862.2 and 107.4 - 859.5 μg/mL, respectively.

Saponification

Mayonnaise was saponified by the method of McMurray and others (1980). Ethanol (10 mL) containing pyrogallol (6% w/v) was added to each sample (1.5 g) in a saponification vessel and agitated to avoid agglomeration. The vessel was flushed with nitrogen for 1 min and sonicated for 10 min. Then, 2 mL 60% potassium hydroxide in deionized water (freshly prepared) was added and the vessel was flushed with nitrogen for 1 min. After attachment of an air condenser, the contents were digested at 70 °C for 30 min in a shaking water bath. After 5 min of sonication and cooling in an ice bath, 20 mL of 2% NaCl in deionized water was added and the mixture was extracted three times with 10 mL of extracting solvent II. The combined extracts were collected into a 50-mL tube containing 3 g anhydrous magnesium sulfate, decanted into a 50-mL volumetric flask, and diluted to volume with extracting solvent II. After passing through a filter (0.45 μm), 20 μL was injected.

Statistical analysis

Statistical analysis (two-way ANOVA) was performed with the Statistical Analysis System (SAS 1990). Means were compared by the least significant difference (LSD) test at $\alpha = 0.05$.

Calculation of RRR α-T equivalent (α-TE)

In order to assess the vitamin E activity of mayonnaise, the level of the individual homologs must be converted to RRR α-T equivalents (α-TE). One α-TE is equal to 1 mg of α-T (Pryor 1995).The factors to convert mg of the other tocopherols to mg of RRR α-tocopherol to calculate α-TE units are β-T, 0.5; γ-T, 0.1 and δ-T, 0.03 (Pryor 1995).

Peak identification

Peak identification was performed by one of the following methods:

1. Comparing the retention times with those of the standards and by comparison of the UV/visible spectra with standard spectra.

2. The fluorescence emission wavelength was set at 330 and 310 nm for tocopherols and α-TAC, respectively. The peak responses were determined at excitation wavelengths of 280, 290 and 300 nm for tocopherols, 265, 275 and 285 nm for α-TAC, respectively. Peak response ratios were calculated for tocopherols at 290/300, 290/280, and 300/280 nm, α-TAC at 275/285, 275/265 and 285/265 nm, respectively (Ye and others 1998).

Results and Discussion

TYPICAL CHROMATOGRAMS OBTAINED from a standard mixture and from mayonnaise are shown in Figures 1 and 2. No interfering peaks were observed in the chromatograms of reagent blanks. Initially, 0.5% isopropanol (IPA) in hexane was used as the mobile phase. Running time was 30 min; however, an impurity peak co-eluted with α-TAC that appeared as a shoulder on the α-TAC peak. In order to resolve the impurity, several different ratios of IPA and hexane were tested. Based on the resolution between the α-TAC peak and the impurity peak, 0.27% IPA in hexane was the best solvent choice with a running time near 40 min. Unfortunately, gradient elution is a poor alternative for silica columns due to a drifting baseline (Balz and others 1993). To shorten the running time, a flow rate gradient was applied to reduce the running time to 30 min.

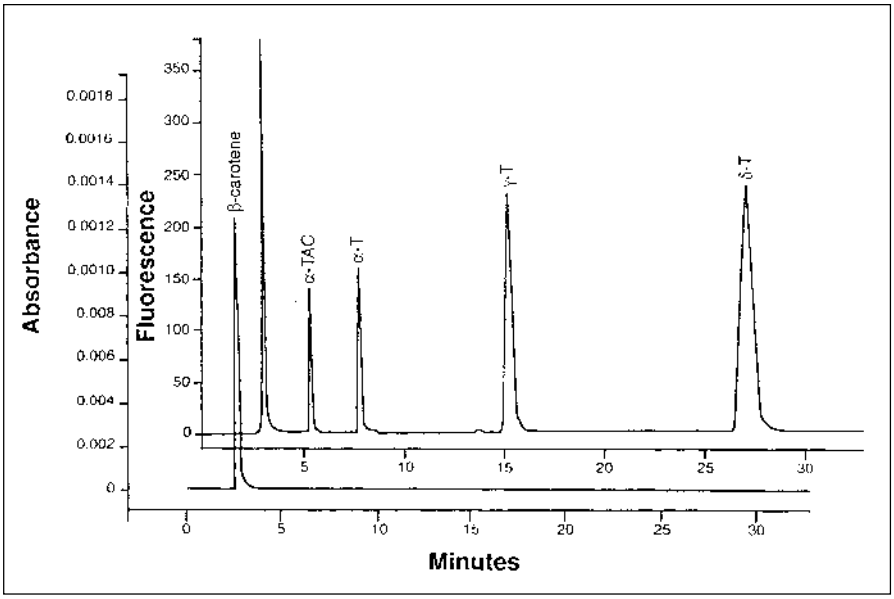

Figure 1—Chromatogram of standard mixture, tocopherols, λ_{ex} 290 nm, λ_{em} 330 nm; α-TAC, λ_{ex} 285nm, λ_{em} 310 nm; β-carotene, λ 450 nm on LiChrosorb Si60 (5 mm, 4.6x250 mm). Mobile phase: 0.27% isopropanol in hexane with gradient flow rate

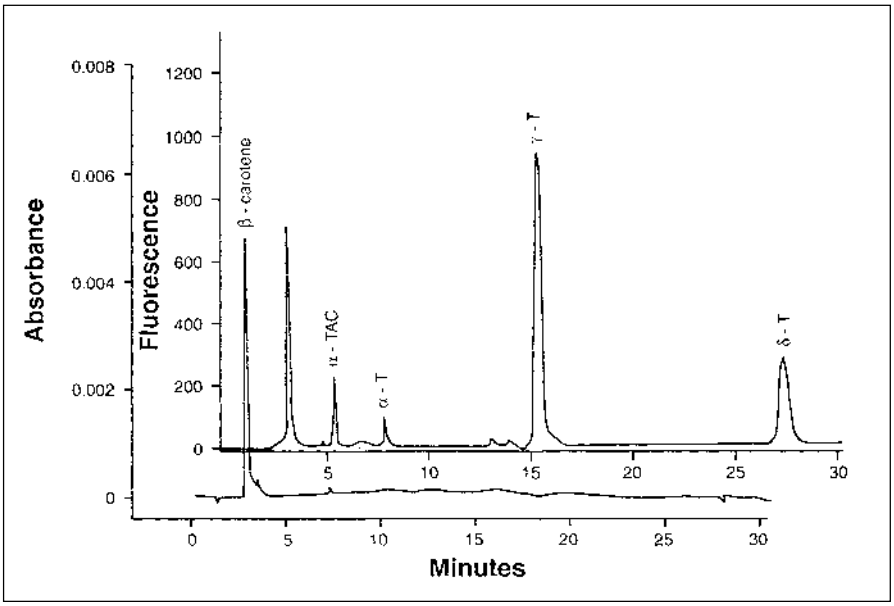

Figure 2—Chromatogram of mayonnaise extract, tocopherols, λ_{ex} 290 nm, λ_{em} 330 nm; α-TAC, λ_{ex} 285 nm, λ_{em} 310 nm; β-carotene, λ 450 nm on LiChrosorb Si60 (5 μm, 4.6x250 mm). Mobile phase: 0.27% isopropanol in hexane with gradient flow rate

Fluorescence responses for α-TAC, α-T, γ-T, δ-T and visible absorbance response for β-carotene were linear (r^2 = 0.999) for the range 6.17 to 154.3, 3.14 to 78.57, 8.28 to 206.9, 12.38 to 309.4, and 0.11 to 12.88 ng/injection, respectively. The LC suitability data are included in Table 2 as analytical figures of merit for each analyte.

Identification using only spectral maxima or peak retention time can lead to inaccurate assignments, since the spectral accuracy of ±1 nm does not, in all cases, distinguish between the compound of interest and other unknown interferences. Also, retention time can be shifted from run to run (such as due to an air bubble in the system or fluctuating temperatures which could slightly change composition of the mobile phase). Therefore, use of both retention time and full spectrum matching greatly increases the accuracy of the identification process. For fluorescence detection, it is impossible to get a full UV/visible spectrum (220 - 500 nm) for identification when a fluorescent analyte coelutes with an impurity peak that does not fluoresce. In this case, the identification and proof of lack of fluorescence interference of the compound can be done by comparing the fluorescence ratio of the height of the peak corresponding to its respective standard at 3 specific excitation wavelengths while keeping the emission wavelength constant (Ye and others 1998). Table 3 illustrates the ratio comparison between the standard and the peak in the position of δ-T. Other analytes provided peak ratios identical to the standard. Good agreement was obtained for the ratio for δ-T of the standard and the peak of sample A indicating good peak purity. However, the ratio for sample C does not agree with that of the standard indicating an unknown impurity. Because of the potential for the presence of interferences in the region of δ-T in all types of mayonnaise, efforts should be made to ensure peak purity before reporting quantitative values for δ-T.

Using direct solvent extraction and normal-phase chromatography, analytical method validation parameters such as accuracy, precision, limit of detection and limit of quantification werecalculated to prove the validity of the complete procedure for analysis of reduced-fat mayonnaise for α-TAC, β-carotene, γ-T and δ-T. To study the accuracy of the method, recoveries were determined for α-TAC, β-carotene, γ-T

Table 2—Analytical figures of merit for the chromatography of three vitamin E homologs (α-T,γ-T, δ-T), α-tocopheryl acetate (α-TAC), and β-carotene

Analytes	Linearity[a] r^2	Theoretical plates[b] N	Tailing factor[c] T	System suitability[d] S	Resolution[e] RS
α-TAC	0.999	β17,929	1.0	1.2	6.0
α-T	0.999	15,974	1.0	1.0	18.1
γ-T	0.999	11,026	1.0	0.8	14.5
δ-T	0.999	10,574	1.0	1.7	
β-Carotene	0.999	3,185	1.0	0.5	

[a]Range from 6.17 to 154.3, 3.14 to 78.57, 8.28 to 206.9, 12.38 to 309.4 and 0.11 to 12.88 ng/injection for α-TAC, α-T, γ-T, δ-T and β-carotene (n = 5), respectively.
[b]Calculated as $N = 16(t/w)^2$, where t is the retention time of the analyte and w is the width of the peak at its base.
[c]Calculated at 5% peak height, $T = w_{0.05}/2f$, where f is the distance from the peak (USP 1995).
[d]RSD% of 5 replicate injections at 30.85, 15.7, 41.4, 61.9, and 1.09 ng/injection for α-TAC, α-T, γ-T, δ-T and β-carotene, respectively.
[e]$RS = 2(t_2 - t_1)/(w_1 + w_2)$.

Table 3—Chromatographic peak purity test for δ-T[a, b]

	δ-T		
Excitation wavelengths (nm)	Standard	Sample A	Sample C
290/300	1.34	1.39	0.74
290/280	1.93	1.90	2.38
300/280	1.45	1.44	3.22

[a]Constant emission wavelength 330 nm.
[b]Fluorescence ratios shown were calculated by dividing the values for the 2 peak heights for the analyte (δ - T) obtained from separate chromatographic runs at two different excitation wavelengths, with the emission wavelength constant at 330 nm.

Table 5—Method precision based on repetitive analyte analyses[a]

Analyte	Mean±S.D. (mg/100g)[b]	RSD (%)
Intra-day		
α-TAC	3.80±0.09	2.5
α-T	1.07±0.08	7.2
γ-T	15.40±0.25	1.6
δ-T	6.14±0.15	2.5
β-carotene	0.073±0.002	2.3
Inter-day		
α-TAC	3.91±0.16	4.0
α-T	1.00±0.10	9.8
γ-T	15.12±0.62	4.1
δ-T	6.09±0.18	3.0
β-carotene	0.076±0.003	4.4

[a]Sample: reduced-fat mayonnaise (Brand A, 3.0g).
[b]n = 5.

Table 4—Method accuracy based on analyte recovery from mayonnaise zero-control reference material (ZRM)[a]

	Amount added	% Recovery	
Analyte	(mg/100g)	(mean±S.D.)[b]	RSD(%)[c]
β-Carotene	0.071	100.7±2.1	2.1
	0.166	103.1±4.8	4.7
	0.644	100.5±1.1	1.0
α-TAC	3.57	103.7±5.8	5.6
	14.28	95.3±1.9	2.0
	28.57	98.0±2.5	2.6
γ-T	3.59	106.4±2.2	2.1
	14.37	98.2±2.1	2.1
	28.74	99.3±1.4	1.4
δ-T	3.58	106.3±2.2	2.1
	14.32	104.7±1.8	1.7
	28.65	94.8±2.3	2.5

[a]Mayonnaise ZRM made from olive oil and liquid whole egg.
[b]n = 5.
[c]RSD = relative standard deviation.

Table 6—Assay values of β-carotene, α-T, γ-T and δ-T in three reduced-fat mayonnaise products using different extraction methods: direct solvent extraction vs. saponification[1, 2]

		Product A						
		β-carotene	α-TAC	α-T	total α-T	α-TE[3]	γ-T	δ-T
Extraction method	Direct extraction	0.074 a[4]	3.81	1.13	4.61 a[5]	6.33	15.37 a	6.09 a
	Saponification	0.046 b	—	4.66	4.66 a	6.07	13.10 a	3.56 b
		Product B						
		β-carotene	α-TAC	α-T	total α-T	α-TE[3]	γ-T	δ-T
Extraction method	Direct extraction	0.047 a	—	2.15	2.15 a[5]	4.28	19.58 a	5.77 a
	Saponification	—[7] b	—	2.31	2.31 a	4.09	16.74 a	3.42 b
		Product C						
		β-carotene	α-TAC	α-T	total α-T	α-TE[3]	γ-T	δ-T
Extraction method	Direct extraction	0.150 a	5.97	—	5.44 a[5]	5.87	4.30 a	—[6]
	Saponification	0.113 b	—	4.22	4.22 b	4.45	2.35 b	—[6]

[1]Fat content for product A= 33%, B=33 %, C=13%
[2]Values in the same column and in the same sample that are followed by different letters are significantly different ($P < 0.05$)
[3]α-TE = RRR α-T equivalents, one α-TE = 1 mg of α-T, 0.1mg of γ-T, 0.03 mg of δ-T, 0.67 mg of α-TAC. (Pryor 1995), per 100 g
[4]Concentration expressed as mg/100g sample (mean, n = 2)
[5]Natural α-T and synthetic α-TAC
[6]δ-T was not quantifiable either due to its absence or to the presence of a substantial overlapping, unresolved component.
[7]Below detection limit.

and δ-T using the mayonnaise ZRM. The % mean recoveries were between 95 and 106 for α-TAC, β-carotene, γ-T and δ-T at three spike levels, which proves the accuracy of the method (Table 4). The chromatogram of the ZRM extract showed that the product has nondetectable levels of α-TAC, β-carotene, γ-T and δ-T, justifying the use of the ZRM in this study. Due to the presence of natural α-T in the ZRM, this product was not suitable for use as a ZRM for α-T. Therefore, the recovery of α-T was done by standard addition. The percent recovery (mean ± S.D.) for α-T

was 102.0±3.6 (n = 5).

The intra-day and inter-day assay results for each analyte were determined, using one brand of reduced-fat mayonnaise, and reported as the precision of the assay (Table 5). The relative standard deviation (n = 5) ranged from 1.6 to 7.2% and 3.0 to 9.8% for intra-day and inter-day, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the method described by Ye and others (1998). The LOD values in ng/injection were 0.88, 0.26, 0.09, 0.08 and 0.01 for α-TAC, α-T, γ-T, δ-T and β-carotene, respectively. The LOQ values in ng/injection were 2.49, 0.65, 0.21, 0.19, and 0.03 for α-TAC, α-T, γ-T, δ-T and β-carotene, respectively.

Three brands of reduced-fat mayonnaise were analyzed by both direct solvent extraction and saponification. The analytical values obtained from the direct solvent extraction method were significantly higher than that from saponification for γ-T and δ-T($P < 0.01$) (Table 6). The amount of α-T (total α-T) derived from α-T and the α-TAC converted to α-T were similar to the amount obtained after saponification for two of the three margarine samples. For sample C, direct solvent extraction gave a higher value compared to saponification. β-Carotene contents were significantly higher when direct solvent extraction was used. This observation is probably due to the effect of soap precipitation during the extraction, causing loss of β-carotene through physical entrapment (Lietz and Henry 1997). The β-carotene content ranged from 0.05 - 0.15 mg/100g in the three mayonnaise samples. Vitamin E content expressed as RRR α-T equivalents (α-TE) ranged from 4.28 - 6.33 mg α-TE/100g.

Conclusion

THE METHOD DESCRIBED PRESENTS AN alternative technique to saponification for analysis of total vitamin E and β-carotene in reduced-fat mayonnaise. Elimination of saponification permits quantification of the synthetic and natural forms of α-T simultaneously. The simultaneous approach used for the analytes (β-carotene, α-TAC, α-T, γ-T, δ-T or β-T) makes the method well suited for routine work.

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