Liquid Chromatographic Analysis of *all-trans*-Retinyl Palmitate, *β*-Carotene, and Vitamin E in Fortified Foods and the Extraction of Encapsulated and Nonencapsulated Retinyl Palmitate

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A liquid chromatographic method is described for the analysis of natural vitamin E homologues, *all-rac*-α-tocopheryl acetate, retinyl palmitate (encapsulated and nonencapsulated), and *β*-carotene in various fortified foods. The vitamins are extracted in 2-propanol and hexane without saponification and quantitated by normal phase chromatography with fluorescence and visible detection. The sample components were identified using an on-line three-dimensional photodiode array detector, which permitted profiling of the 190−800 nm absorption spectrum of any chromatographic peak. The method showed linearity for the analytes in their respective calibration ranges. The percent recoveries for retinyl palmitate using starch- and gelatin-encapsulated standards were 101.0 ± 1.0 and 100.1 ± 0.9, respectively. The method measures six or more analytes in a single injection and differentiates between natural and synthetic forms of vitamin E.

Keywords: *Direct extraction; vitamin E; encapsulated retinyl palmitate; fortified foods; LC*

INTRODUCTION

Fortification of foods that were not previously fortified by law is becoming more common. Examples include peanut butter and margarine with the addition of all-rac-α-tocopheryl acetate (α-TAC), retinyl palmitate (RP), and *β*-carotene (margarine). The industry can add vitamins to a food in a pure or encapsulated form to increase retention during processing and storage. Analytically, the use of encapsulated vitamins places greater demands upon the sample extraction procedure because the encapsulation matrix must be destroyed for complete vitamin extraction. Encapsulated retinyl palmitate is commonly available to the food industry and used extensively (Eitenmiller and Landen, 1999).

Prior to quantification of fat-soluble vitamins by LC, extraction can be completed by direct solvent extraction (Thompson and Hatina, 1979; Landen, 1982; Chase et al., 1997) or more vigorously by saponification (Kramer et al., 1997; Hewavitharana and van Brakel, 1998). Direct solvent extraction is often selected because of time, cost, and better ability to limit formation of artifacts. In addition, saponification converts the α-TAC to all-rac-α-tocopherol that cannot be resolved chromatographically from naturally occurring (*R*,*R*,*R*)-α-tocopherol. On a biological activity basis, all-rac-α-tocopherol has only 74% of the activity of (*R*,*R*,*R*)-α-tocopherol (Combs, 1992; Eitenmiller and Landen, 1999); on a nutritional basis, unlike α-TAC, α-tocopherol possesses antioxidant properties (Hewavitharana et al., 1996). Therefore, coelution of the synthetic all-rac-isomer, together with the naturally occurring (*R*,*R*,*R*)-isomer, decreases the overall accuracy of the assay. If encapsulated vitamins are present, the analyst must be assured that the solvents efficiently extract the vitamins.

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High-performance liquid chromatography (HPLC) is currently the most accepted technique for the separation and quantitation of vitamin E, retinyl palmitate, and *β*-carotene from foods (Lang et al., 1992; Tee and Lim, 1991; Rizzolo and Polesello, 1992; Eitenmiller and Landen, 1999). RP and vitamin E are most commonly detected using a fluorescence detector, while *β*-carotene is detected using a UV/visible detector at its inherent yellow absorption wavelength of 450 nm.

The objective of this study was to develop a simple, rapid and highly efficient procedure for the simultaneous assay of total vitamin A and vitamin E activity in fortified, plant-based foods using direct solvent extraction suitable for application to foods containing encapsulated retinyl palmitate. Validation parameters for the overall method were determined to ensure the method's validity

MATERIALS AND METHODS

Apparatus. The normal-phase HPLC system was equipped with Waters 2690 separations module, a Waters 996 photodiode array detector, and a Shimadzu RF-10A_{XL} programmable fluorescence detector attached to a Waters Millennium 2010, ver. 3.01, chromatography manager. The system was monitored by a compatible IBM computer.

The column was a 25 cm × 4.6 mm 5 μm LiChrosorb Si60 (Hibar Fertigsaube RT. Darmstadt, F. R. Germany) equipped with a precolumn packed with Perisorb A 30−40 μm (Darmstadt, F. R. Germany). The wavelengths were time-programmed: time 0 min, λ_{ex} 325 nm, λ_{em} 470 nm; time 4 min, λ_{ex} 285 nm, λ_{em} 310 nm; time 6 min, λ_{ex} 290 nm, λ_{em} 330 nm. Required equipment apparatus include a sonicator, FS30 (Fisher Scientific) or equivalent, a Polytron homogenizer (Pro Scientific Inc.) or equivalent, and a bell-jar filtration apparatus, Knotes (Vineland, NJ) or equivalent.

Reagents. All reagents are of LC grade or analytical purity. Butylated hydroxytoluene (BHT), *all-rac-γ*-tocopherol (*γ*-T), *all-rac-δ*-tocopherol (*δ*-T), gelatin-encapsulated RP material (250,000 USP unit/g), and starch-encapsulated RP material (500 000 USP unit/g) were purchased from Sigma (St. Louis,

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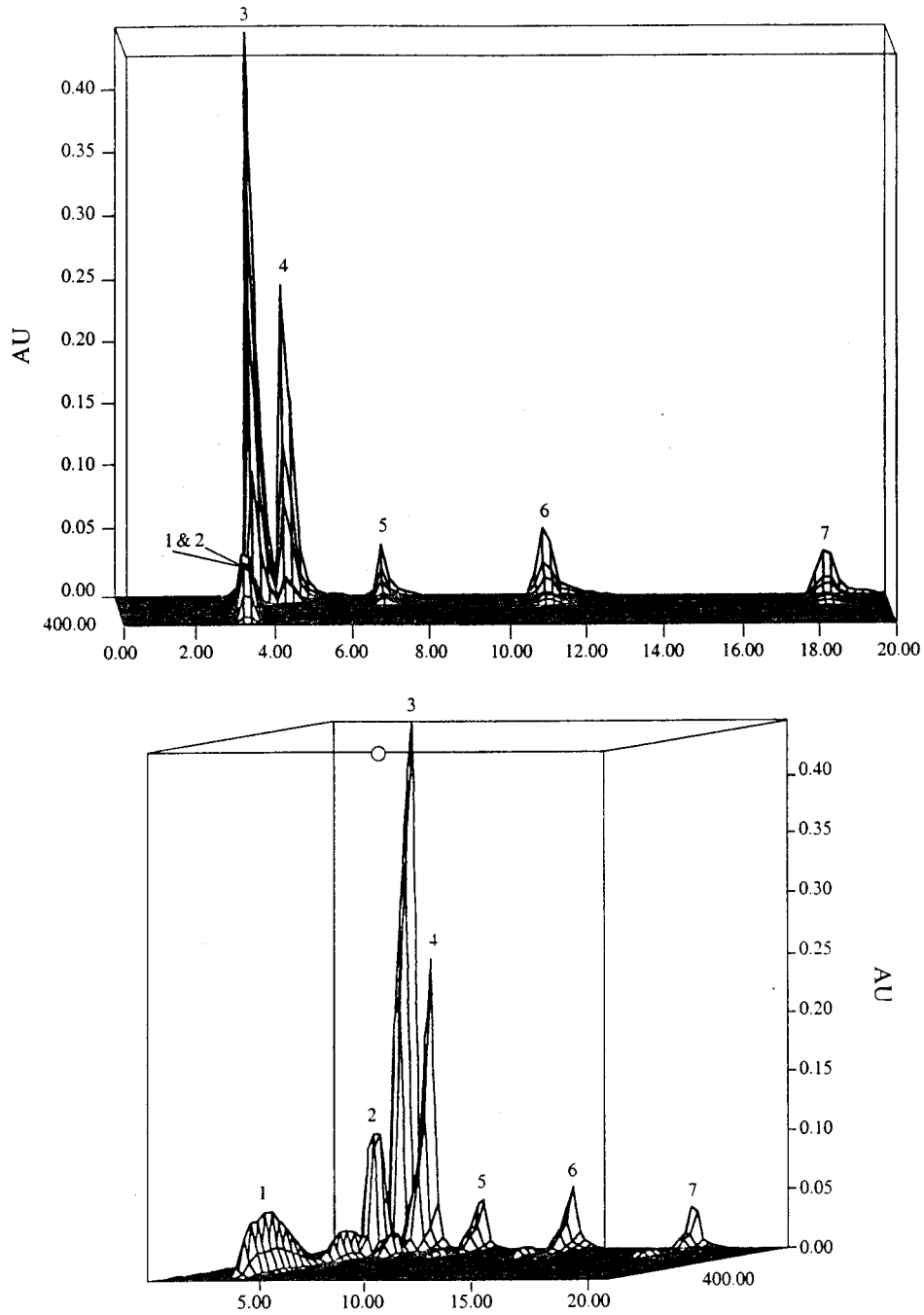

Figure 1. 3-D graphic profile of β-carotene (1), RP (2), α-TAC (4), α-T (5), γ-T (6) and δ-T (7) on normal-phase column with mobile phase containing 0.5% IPA in hexane at gradient flow rate. The absorbance was monitored from 210 to 550 nm at a resolution of 1.2 nm. Top: front view. Bottom: side view.

Table 1.

time (min)	flow rate (mL/min)
0	0.85
1.00	0.90
5.50	0.90
5.55	1.10
5.70	1.30

MO); α-TAC, β-carotene, and RP were purchased from Fluka Bio Chemika (St. Louis, MO). *all-rac*-α-Tocopherol (α-T) was purchased from BASF Corporation (Parsippany, NJ).

The isocratic mobile phase contained 0.5% 2-propanol in *n*-hexane with gradient flow rate programmed from 0.9 to 1.3 mL/min shown in Table 1.The mobile phase was filtered using a 0.45 μm nylon membrane filter (MSI Inc., Westboro, MA) and de-gassed using a FS30 sonicator. Extracting solvent was 0.003% BHT in *n*-hexane.

Preparation of Standard Solutions. The standard solutions were prepared as described by Ye et al. (1998). The reference $E^{1\%}_{1\,cm}$ data are listed in Table 2. Appropriate dilutions were made with the respective mobile phase to give three working standard concentrations ranging from 0.0653 to 1.632 μg/mL for RP, 0.926−23.14 μg/mL for α-TAC, 0.314−7.857 μg/mL for α-T, 0.310−7.758 μg/mL for γ-T, 0.309−7.735 μg/mL for δ-T, and 0.011−1.11 μg/mL for β-carotene.

Sample Preparation. Samples used in this study were commercially fortified breakfast cereal, peanut butter, and margarine. A zero control reference material (ZRM) (Chase et al., 1997), unfortified peanut butter, was used for recovery studies. Cereal samples were ground, and margarine and peanut butter were well mixed by stirring with a stainless steel spatula before sampling.

Direct Solvent Extraction. In a preliminary study, we found that a direct solvent extraction employing 2-propanol

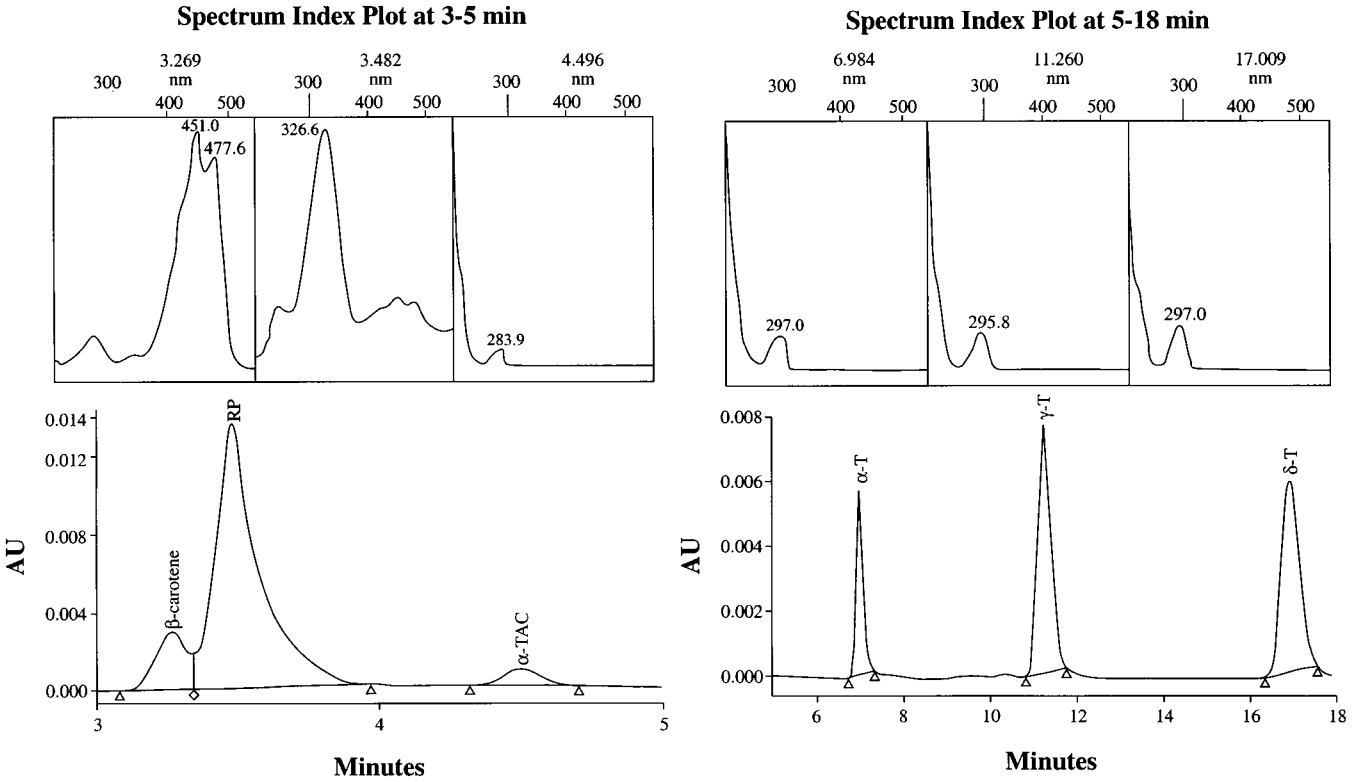

Figure 2. Spectrum index plot showing part of elution profile of the HPLC separation and the spectra of the eluting components of the standards. Top: spectra, values at top right corner of each box are retention times (T_R) of respective peak. Bottom: chromatogram, peak 1 (T_R = 3.27) β-carotene; peak 2 (T_R = 3.48) RP; peak 3 (T_R = 4.50) α-TAC; peak 4 (T_R = 6.98) α-T; peak 5 (T_R = 11.27) γ-T; peak 6 (T_R = 17.00) δ-T.

Table 2. Specific Absorption Coefficients ($E^{1\%}_{1\,cm}$) and Maximum Wavelengths (λ_{max}) for Tocopherols, Retinyl Palmitate, β-Carotene, and α-Tocopheryl Acetate[a]

analytes	λ_{max} nm	$E^{1\%}_{1\,cm}$[b]
α-T	292	71
γ-T	298	92.8
δ-T	298	91.2
α-TAC	286	42
β-Carotene	**453**	**2592**
RP	325	940

[a] Scott, 1978; Bauernfeind, 1981; Furr et al., 1992. [b] In ethanol, bold values in hexane.

and hexane fails to efficiently extract encapsulated RP, but simple addition of hot water (80 °C) and sonication allows the procedure to be used for quantification of natural tocopherols, α-TAC, and RP from most fortified foods when starch- or gelatin-encapsulated RP is present.

For cereal and peanut butter, accurately weigh 3.0 g into a 125 mL centrifuge tube. Add hot deionized water (80 °C, 2 mL) to the sample, sonicate for 5 min to facilitate dissolution, and then add 5 mL of 2-propanol. Add approximately 5 g of anhydrous magnesium sulfate, mix with a stainless steel spatula, and add 20 mL of extracting solvent. Homogenize the mixture with a Polytron homogenizer for 1 min at medium speed. Filter the extract through a 60 mL coarse porosity fritted glass filter into a 125 mL Philips beaker using a vacuum bell jar filtration apparatus. Wash twice with 5 mL of extraction solvent. Repeat the extraction by transferring the material on the fritted glass filter to the original centrifuge tube and extract with 5 mL of 2-propanol and 20 mL of extracting solvent. Transfer combined filtrates to a 100 mL volumetric flask and dilute to volume with *n*-hexane. Pipet 2 mL of the extract and evaporate under nitrogen until dry, dilute with 2 mL of mobile phase, and inject 20 μL. For recoveries, add 1.0 mL of each spike solution or 10.0 mg of encapsulated RP material (starch or gelatin) to 3.0 g of ZRM and assay as outlined above.

For margarine, the extraction method of Ye et al. (1998) was modified by using a mixture of ethyl acetate/hexane (10:90, v/v) containing 0.01% BHT in place of hexane. This solvent provides a recovery value of 100% for β-carotene in margarine. Briefly, add 50 mL of ethyl acetate/hexane (10:90) to 5.0 g of sample and sonicate until the sample material has dissolved. Add 3 g of anhydrous $MgSO_4$, mix, and then let stand for 2 h. Filter the extract. Pipet 2 mL of the extract, evaporate under nitrogen until dry, dilute with 2 mL of mobile phase, and inject 20 μL. All steps were carried out under yellow light.

Saponification. Samples were saponified by using the method of McMurray et al. (1980) with little modification. Add 10 mL of ethanol containing pyrogallol (6% w/v) to each sample (1.5 g) in a saponification vessel and agitate to avoid agglomeration. After sonication of the solution for 10 min, add 2 mL of 60% potassium hydroxide in deionized water (freshly prepared) and flush the vessel with nitrogen for 1 min. After attachment of an air condenser, digest the contents at 70 °C for 30 min in a shaker water bath. Following 5 min of sonication of the digest and cooling the digest in an ice bath, add 20 mL of 2% sodium chloride in deionized water and extract the mixture three times with 10 mL of extracting solvent. Collect combined extracts into a 50 mL tube containing 3 g of anhydrous magnesium sulfate, decant into a 50 mL volumetric flask, and dilute to volume with extracting solvent. Filter (0.45 μm) and inject 20 μL.

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

RESULTS AND DISCUSSION

The postrun analysis by the chromatogram manager allows detailed analysis of each peak in the chromatogram for comparison with stored reference spectra. The 3-D graphic profile of the HPLC run of a mixture of standards containing *all-trans*-β-carotene, RP, α-TAC,

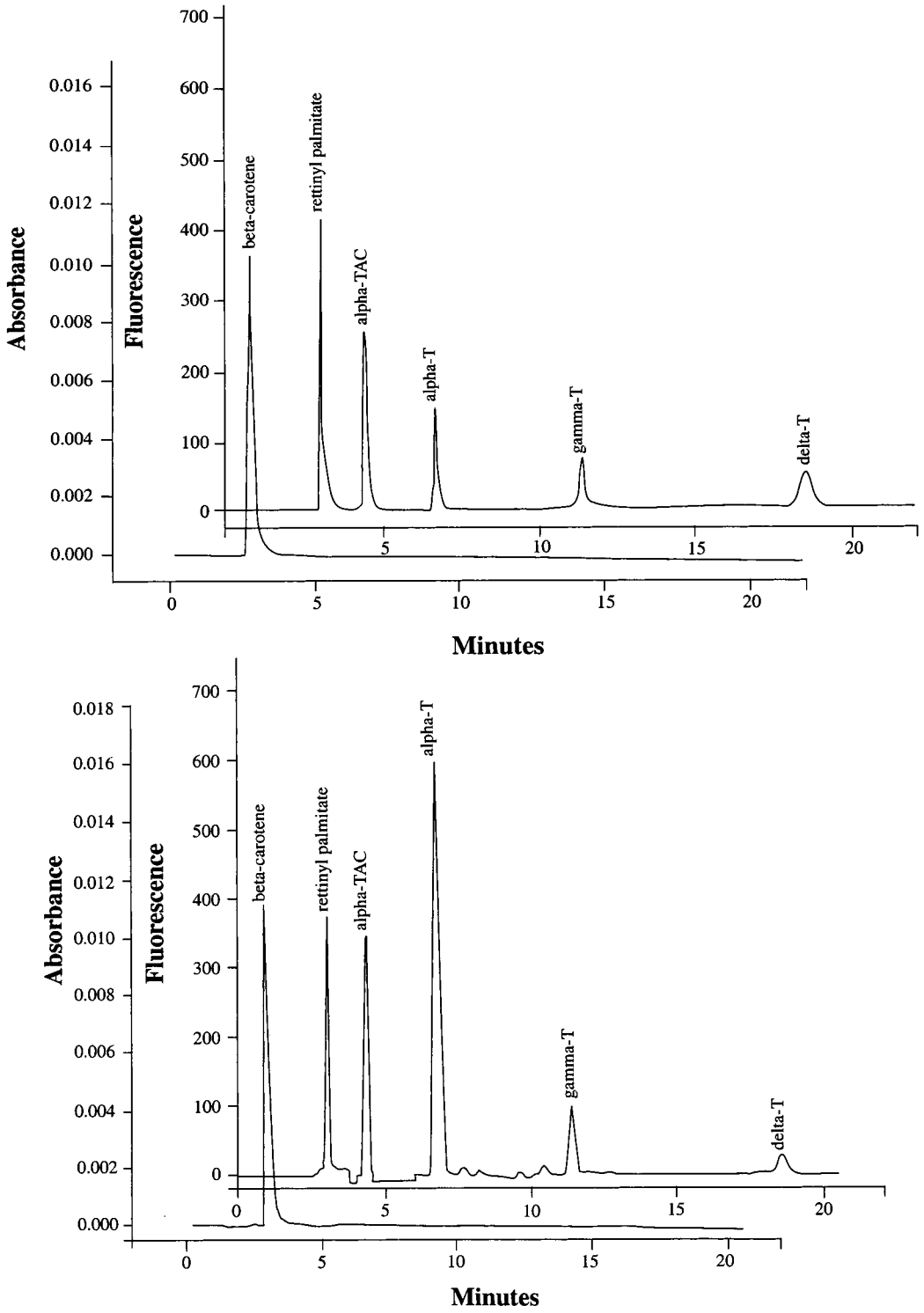

Figure 3. Chromatograms of standard mixture (top) and margarine extract (bottom). Tocopherols, λ_{ex} 290 nm, λ_{em} 330 nm; α-TAC, λ_{ex} 285 nm, λ_{em} 310 nm; RP, λ_{ex} 325 nm, λ_{em} 470 nm; β-carotene, λ 450 nm on LiChrosorb Si60 (5 μm, 4.6 × 250 mm). Mobile phase is 0.5% 2-propanol in hexane with gradient flow rate.

α-T, γ-T, and δ-T is illustrated in Figure 1 covering spectra from 210 to 550 nm at 1.2 nm resolution. The 3-D cube can be rotated in any direction and angle to allow a direct view of the spectrum from any side with a clear side or topographic view of the different components and their absorption intensity. Postrun analysis can be used to identify the different compounds on the time axis at any selected derivation wavelength channel. A typical spectrum index plot is shown in Figure 2 for β-carotene, RP, α-TAC, α-T, γ-T, and δ-T.

Identification was achieved by comparing the retention times with those of the standards and obtaining UV/visible spectra to confirm peak identity. Identification using only spectral maxima or peak retention time can lead to inaccurate assignments, since the spectral accuracy of ±1 nm cannot, in all cases, distinguish between the compound of interest and other unknown interferences. Additionally, retention time can shift from run to run. Therefore, using both retention time and full spectrum matching greatly increases the accuracy

Table 3. Accuracy of Assay[a]

analyte	amt. added (mg/100 g)	% recovery (mean±S. D.)[b]	RSD (%)
RP (nonencaps.)	1.568	99.6 ± 1.3	1.3
	3.192	96.2 ± 3.0	3.1
	6.384	101 ± 2.5	2.5
α -TAC	8.164	103 ± 1.7	1.6
	14.40	100 ± 2.6	2.6
	28.81	103 ± 2.7	2.6

[a] Unfortified peanut butter was used as a zero control reference material (ZRM). [b] n=5.

of the identification process. However, this technique cannot be used when impurity compounds are present and overlap analyte peaks that exhibit absorption at range of 210 to 550 nm but do not fluoresce. In this case, the confirmation of peak purity of the compound can be done by varying the excitation wavelength at a constant emission wavelength (Ye et al., 1998). Comparison of the ratios of peak heights between standards and samples provides an indication of peak homogeneity.

Since mobile-phase gradient elution leads to baseline drift in normal-phase chromatography on silica (Balz et al, 1993), a flow rate gradient was used to speed up the running time. Typical chromatograms obtained from the standard mixture (α-T, γ-T, δ-T, α-TAC, RP, and β-carotene), and margarine extracts are shown in Figure 3. Fluorescence responses for α-T, γ-T, δ-T, α-TAC, RP, and visible absorbance responses for β-carotene were linear (r^2=0.999) for the ranges 6.28−157, 6.2−155, 6.18−155, 18.5−463, 1.3−32.6, and 0.22−2.22 ng/20 μL injected, respectively.

By use of direct solvent extraction and normal-phase chromatography, analytical method validation parameters including accuracy, precision, limit of detection, and limit of quantitation were calculated to validate the complete procedure for analysis of various fortified foods for α-TAC and nonencapsulated and encapsulated RP. Internal standard was not used in this study since the analytes behave differently during extraction and can have varying extraction deficiencies. The ZRM has the same matrix as the real sample; therefore, the recovery results from the spike ZRM justify the accuracy of the method. On the basis of five trials at each of three spike levels for nonencapsulated RP and α-TAC using unfortified peanut butter as a ZRM, the percent mean recoveries were greater than 95 for both RP and α-TAC. These results show the accuracy of the proposed method (Table 3). Due to the presence of natural tocopherols in the ZMR, this product would not be suitable for use as a ZMR for tocopherols. Therefore, the recoveries of tocopherols were done by standard addition. The percent recovery (mean ± SD) for α-, γ-, and δ-T were 99.0 ± 1.6, 101.1 ± 1.9, and 101.9 ± 2.1 (n = 5), respectively.

The encapsulated RP standard material (10.0 mg) was added to 3 g of ZRM. The percent recoveries for starch- and gelatin-encapsulated RP standards were 101.0 ± 1.0 and 100.1 ± 0.9 (n = 5), respectively. The intra- and interday assay results for each analyte were determined using one brand of margarine and are reported in Table 4 as the precision of the assay. The relative standard deviation (n = 5) ranged from 1.2 to 5.0% and 1.6 to 5.2% for intra- and interday, respectively. These results validate the precision of the method.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the method of Ye et al. (1998). The LOD in nanogram per 20 μL is 0.018, 0.42, 0.13, 0.062, 0.036, and 0.052 for RP, α-TAC, α-T, γ-T, δ-T, and β-carotene, respectively. The LOQ in nanogram per 20 μL is 0.048, 1.18, 0.38, 0.16, 0.096, and 0.13 for RP, α-TAC, α-T, γ-T, δ-T, and β-carotene, respectively. These values are well below extract levels of each analyte from fortified foods. Table 5 shows vitamin E, retinyl palmitate, and β-carotene content in the commercially fortified samples.

Table 4. Precision of Assay[a]

analyte	mean ± S. D. (mg/100 g)[b]	RSD (%)
	Intraday	
RP	2.381 ± 0.03	1.2
α-TAC	12.62 ± 0.50	3.9
α − T	6.251 ± 0.10	1.6
γ − T	14.13 ± 0.42	3.0
δ − T	2.874 ± 0.14	5.0
β-carotene	0.248 ± 0.01	4.2
	Interday	
RP	2.198 ± 0.09	4.2
α-TAC	12.39 ± 0.44	3.5
α -T	5.912 ± 0.31	5.2
γ − T	14.72 ± 0.64	4.3
δ − T	3.188 ± 0.15	4.8
β-carotene	0.246 ± 0.004	1.6

[a] Sample: margarine (brand C lot # 1, 5.0 g). [b] n = 5.

Table 5. Determination of Retinyl Palmitate (RP), β-Carotene, Vitamin E Homologues (α-, γ-, and δ-T), and α-Tocopheryl Acetate (α-TAC) Content of Various Food Products

	mg/100 g[b]						
product[a]	RP	β-carotene	α-TAC	α-T	γ-T	δ-T	α-TE[c]
cereal A	1.26		95.22	1.71	0.57		65.56
marg. A	1.38	0.49		19.42	8.89	3.21	20.41
marg. B	1.19	0.57	12.73	14.38	4.47	1.58	23.40
marg. C	2.11	0.28	14.98	7.21	20.08	4.27	19.38
p. butter A	2.64		15.79	9.99	9.48		21.52

[a] Fat content for cereal, A = 3%; for margarine, A = 71%, B = 43%, C = 64%; for peanut butter, A = 50%. [b] Mean result from three independent samples of each product. [c] Given per 100 g. α-TE = RRR α-T equivalents, one α-TE = 1 mg of α-T, or 0.1 mg of γ-T, or 0.03 mg of δ-T, or 0.67 mg of α-TAC (Ball, 1988).

Table 6. Assay Comparison between Direct Extraction and Saponification

		amount found (per g) mean ± SD, RSD% (n = 5)	
sample	analyte	direct extract.	saponification
starch encaps. stand.	RP	157.0 ± 1.3 mg, 0.8	154.0 ± 1.5 mg, 1.0
gelatin encaps. stand.	RP	206.2 ± 0.9 mg, 0.4	203.0 ± 3.6 mg, 1.8
cereal	RP	12.50 ± 0.19 μg, 1.5[a]	11.73 ± 0.09 μg, 0.8[a]

[a] n = 3.

Both encapsulated standards (starch and gelatin forms) for RP and one of the fortified products (cereal) were analyzed by both direct solvent extraction and saponification. The analytical values of RP obtained from the direct extraction method were significantly higher than that from saponification (P < 0.01) (Table 6). These results further confirmed that the direct solvent extraction procedure can be used to extract encapsulated RP in fortified foods.

CONCLUSION

Direct solvent extraction presents an alternative technique to saponification for analysis of total vitamin E, RP, and β-carotene in various fortified foods. Elimi-

nation of saponification permits quantitation of the synthetic and natural forms of vitamin E simultaneously. The use of on-line photodiode array detection gives complete absorption spectrum information which permits the simultaneous identification and quantification of each analyte with greatly increased efficiency compared to conventional assays. The single extraction and single HPLC analysis used for the six or more analytes make the method well suited for routine work with significant cost savings.

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