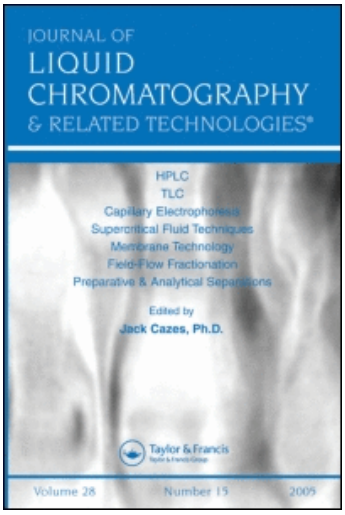

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G. W. Chase[a]; R. R. Eitenmiller[ab]; A. R. Long[a]
[a] U. S. Food and Dru Administration, Atlanta, GA [b] Department of Food Science and Technology, University of Georgia, Athens

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LIQUID CHROMATOGRAPHIC ANALYSIS OF ALL-RAC-α-TOCOPHERYL ACETATE, TOCOPHEROLS, AND RETINYL PALMITATE IN SRM 1846

G. W. Chase, Jr., R. R. Eitenmiller,[1] A. R. Long

U. S. Food and Drug Administration
60 Eighth Street
Atlanta, GA

[1] Department of Food Science and Technology
University of Georgia
Athens, GA

ABSTRACT

A liquid chromatographic method is described for the analysis of all-rac-α-tocopheryl acetate, tocopherols, and retinyl palmitate in the infant formula standard reference material 1846. The vitamins are extracted in isopropanol and hexane/ethyl acetate without saponification and quantitated by normal phase chromatography with fluorescence detection. Retinyl palmitate, all-rac-α- tocopheryl acetate, and naturally occurring tocopherols are quantitated isocratically with a mobile phase of 0.5% isopropanol in hexane. The results were within the certified ranges for all-rac-α-tocopheryl acetate and retinyl palmitate. Recoveries averaged 97.5% for retinyl palmitate and 101% for all-rac-α- tocopheryl acetate (n = 20).

The method provides a rapid, specific, and easily controlled assay approach to the analysis of vitamin A and vitamin E in fortified infant formula. Additionally, the method eliminates the use of chlorinated solvents.

INTRODUCTION

Since the passage of the Infant Formula Act in 1980 by Congress, much interest has been generated in the area of nutrient analysis of formulas and other infant foods. Many methods have been developed for fat soluble vitamins. Most methods for analysis of vitamins A and E use saponification in the extraction procedure.[1-6] In fact, some type of saponification technique was used by each of the laboratories involved in the certification of the National Institute of Standards and Testing (NIST) milk based infant formula Standard Reference Material (SRM 1846). The current AOAC Official Methods for vitamins A and E in milk based infant formula (methods 50.1.02, 50.1.03, and 50.1.04) utilize saponification followed by hexane/ethyl ether or hexane/methylene chloride extraction.[7] The AOAC methods, however, do not adequately describe precautionary steps for saponification. Failure to blanket the sample with nitrogen prior to saponification, or use of a condenser on top of the saponification tube, can lead to vitamin loss. Furthermore, a saponification technique usually requires parititioning of the analyte into an organic solvent which may result in emulsions that are difficult to break and, thus, increasing the possibility of low analyte recoveries. The organic solvent, methylene chloride, used in the AOAC methods 50.1.03 and 50.1.04[7] can have a negative environmental impact.

In addition, saponification converts the all-rac-α-tocopherol acetate to all-rac-α-tocopherol which cannot be resolved from naturally occurring RRR-I-tocopherol. On a biological activity basis, all-rac-α-tocopherol has only 74% of the activity of RRR-α-tocopherol.[7] Therefore, coelution of the synthetic all-rac-isomer, together with the naturally occurring RRR-isomer, decreases the overall accuracy of the assay when vitamin E activity is expressed in α-tocopherol equivalents (α-TE where 1 α-TE = 1 mg RRR-α-tocopherol).[9]

Other methods have used direct solvent extraction with various solvent mixtures and LC with UV or fluorescence detection.[10-14] All-rac-α-tocopheryl acetate fluoresces weakly compared to free tocopherols. In the past, this property was a hindrance to quantitation of the ester by fluorescence. Fortunately, present day fluorescence detectors are sufficiently sensitive to allow for routine detection of α-tocopheryl acetate.[14,15]

An alternative technique eliminating the saponification step was developed by Landen[11] for the analysis of vitamins A and E in infant formula. Samples were dehydrated with magnesium sulfate and extracted with isopropanol and methylene chloride. The fat was removed via gel permeation chromatography followed by quantitation on reverse phase LC with UV detection. This method was used extensively in this laboratory; however, due to environmental concerns with methylene chloride usage, the technique has all but been abandoned.

The objective of this study was to develop a simplified, specific assay procedure for vitamin A and vitamin E in fortified infant formulas that does not require saponification. The newly developed NIST Infant Formula SRM (SRM 1846) was used as the test matrix for this study. An extraction scheme derived from the procedure of Landen[11] was developed, however, it eliminates the use of methylene chloride, a gel permeation chromatographic clean up, reverse phase chromatography, and UV detection. The extraction procedure in this study, uses sample dehydration with magnesium sulfate and extraction with isopropanol and hexane/ethyl acetate (85:15 v/v). After evaporation and filtration, the sample extract is injected directly into a normal phase LC system using more specific and sensitive fluorescence detection.

MATERIALS

Reagents

Hexane: LC grade (Baxter Healthcare Corp., Muskegon, MI, USA); Isopropanol: LC grade (EM Science, Gibbstown, NJ, USA); Ethyl Acetate: LC grade (Baxter Healthcare Corp.); Magnesium Sulfate: Anhydrous (Fisher Chemical, Fairlawn, NJ, USA); Mobile Phase: Isopropanol @ 0.5% in hexane. Vitamin A Standard: approximately, 50 mg of retinyl palmitate (Fluka Bio Chemika, Switzerland) was accurately weighed and dissolved in 50.0 mL of hexane. The exact concentration was determined by the $E^{1\%}$ value of 975. Appropriate dilutions were made with the mobile phase to give five working standard concentrations ranging from 0.5 to 6.0 μg/mL. Vitamin E Standard: approximately 200 mg of all-rac-α-tocopheryl acetate (Fluka Bio Chemika) was accurately weighed and dissolved in 50.0 mL of hexane. Gamma and δ-tocopherol were obtained from the University of Georgia, Athens, GA, USA. The tocopherols were purified by preparatory LC. RRR-α-tocopherol was obtained from Fluka. The concentration of each tocopherol was calculated from their respective $E^{1\%}$ values of 42, 75.8, 91.4, and 87.3 for all-rac-α-

tocopheryl acetate, RRR-α, γ and δ-tocopherol, respectively. The appropriate dilutions were made with the mobile phase to give five working standards, each containing the four combined tocopherols, with the following range of concentrations: RRR-α-tocopherol (0.8 to 10 μg/mL), γ-tocopherol (2.6 to 33 μg/mL), δ-tocopherol (1.3 to 13 μg/mL) and all-rac-α-tocopheryl acetate (8.0 to 106 μg/mL). Butylated hydroxytoluene (BHT): approximately 9 mg of BHT (Sigma, St. Louis, MO, USA) was dissolved in 25.0 mL of hexane to give a concentration of 360 μg/mL.

Apparatus

Analytical LC: retinyl palmitate, all-rac-α-tocopheryl acetate, and the tocopherols were quantitated separately. One 50 μL injection was made for vitamin E and a separate 50 μL injection was made for retinyl palmitate on a Lichrosorb Si 60 (5μm, 4.6mm x 25 cm) column (E. Merck, Darmstadt, Germany). The LC consisted of a LDC Analytical Constametric 3200 pump (Thermo Separation Products, Riviera Beach, FL, USA), Waters 715 auto injector (Waters Inc., Milford, MA, USA), a Hewlett Packard (Avondale, PA, USA) 1046A fluorescence detector (Ex=285 nm, Em=310 nm for all-rac-α-tocopheryl acetate and the tocopherols, and an Ex=325 nm, Em=470 nm for retinyl palmitate), and a Hewlett Packard Model 3396 integrator. Flow rates were 1.0 mL/min and 0.5 mL/min, respectively, for the analysis of vitamin E and retinyl palmitate. Polytron^{R} Homogenizer: Kinematica PT10-35 (Brinkman Instruments, Westbury, NY, USA). Rotary Evaporator: Buchi Rotavapor EL 130 (Brinkman Instruments). Turboevaporator: TurboVap II (Zymark, Hopkinton, MA, USA).

METHODS

The standard reference material for milk based infant formula, (SRM 1846) was obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). The contents of each packet (approximately 28 g) were reconstituted with approximately 145 g of hot water (70-80°C) and thoroughly mixed.

Approximately 6.5 g of the reconstituted infant formula was accurately weighed into a 100 mL wide mouth graduated cylinder. Isopropanol was heated to near boiling before adding 15 mL to the sample and mixing thoroughly with a spatula.

A 7.5 g portion of magnesium sulfate was added, followed by 30 mL of the extraction solution (hexane/ethyl acetate, 85:15, v\v) and 1.0 mL of the BHT solution, with mixing by spatula after each addition.

The mixture from above was homogenized with a Polytron Homogenizer for 1 min at medium speed prior to filtering the mixture through a coarse porosity fritted glass filter into a 125 mL Philips beaker using a vacuum bell jar filtration apparatus. The vacuum was then released, the magnesium sulfate cake was broken up with the aid of a spatula and washed twice with 15 mL of the extracting solution.

The extraction process was repeated by transferring the magnesium sulfate cake back to the original graduated cylinder and adding 20 mL of the extracting solution plus 5 mL of isopropanol. The procedure was then repeated for the once extracted mixture beginning with "homogenized with a Polytron Homogenizer..."

The combined filtrate was transferred to a 500 mL round bottom flask containing 1 g of magnesium sulfate and evaporated to dryness using the rotary evaporator. The residue was immediately dissolved in 10 mL of hexane and filtered through a 0.45 μm Nylon 66 filter (Alltech Associates, Deerfield, IL, USA) into a 125 mL Philips beaker using a bell jar filtration apparatus. The flask was washed with three 7 mL portions of hexane. The hexane wash was filtered into the flask containing the dissolved extract. The combined filtrate was evaporated to a volume of less than 5 mL using a turboevaporator at 45°C with a nitrogen pressure of 10 psi. The evaporated filtrate was diluted to 10 mL with the mobile phase and injected.

RESULTS AND DISCUSSION

Figure 1a illustrates the LC chromatogram of all-rac-α-tocopheryl acetate and the natural vitamin E homologs in a sample extract of SRM 1846. The all-rac-α-tocopheryl acetate is resolved from the γ and δ-tocopherol. Since the SRM 1846 does not contain oils that contain appreciable amounts of naturally occuring levels of RRR-α-tocopherol, it was not necessary to be able to quantitate both the RRR-α-tocopherol and all-rac-α-tocopheryl acetate. However, the all-rac-α-tocopheryl acetate is also easily separated from the RRR-α-tocopherol as illustrated in the standard chromatogram of Figure 1b, thereby making this method capable of routine quantitation of all vitamin E homologs.

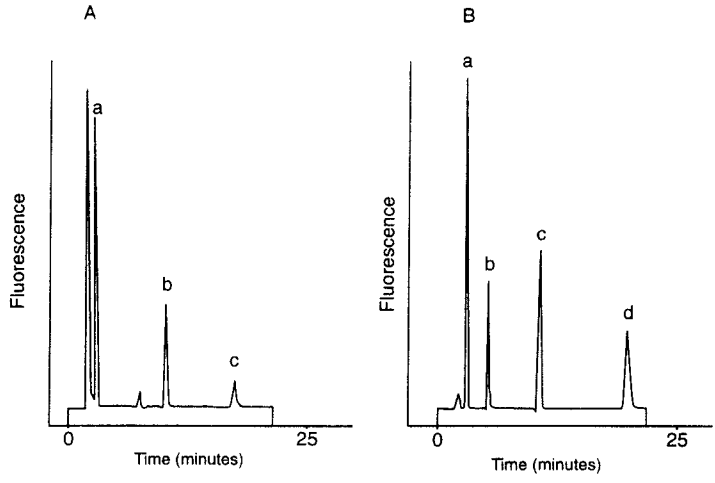

Figure 1. LC chromatogram of all-rac-α-tocopheryl acetate using fluorescence detection (Ex 285 nm, Em 310 nm), flow rate of 1.0 mL/min, injection volume of 50 μL, and mobile phase of 0.5% isopropanol in hexane. 1A is the extract of an infant formula where (a) is all-rac-α-tocopheryl acetate, (b) is γ tocopherol and (c) is delta tocopherol. 1B is the vitamin E standard where (a) is all-rac-α-tocopheryl acetate (42.4μg/mL), (b) is RRR-α-tocopherol (4.02 μg/mL), (c) is γ-tocopherol (13.3 μg/mL) and (d) is δ-tocopherol (6.54 μg/mL).

Fluorescence response for all-rac-α-tocopherol acetate, RRR-α-tocopherol, γ-tocopherol and δ-tocopherol were linear (r=0.999) for the 8.0-106 μg/mL, 0.8-10 μg/mL, 2.6-33 μg/mL, and 1.3-13 μg/mL ranges, respectively. The limit of detection was calculated to be 3.14 μg/mL for all-rac-alpha-tocopheryl acetate. The calculation was based on the equation, $y-a=3(s_{y/x})$, where a is the y-intercept in the linear expression $y=bx+a$ and $s_{y/x}$ is equal to, $\Sigma\{(y_i-y_i)^2/n-2\}^{1/2}$, where y_i is the actual peak response and y_i is the peak response as calculated from the linear expression of $y=bx+a$.[16] Once y is determined, x, which is then the estimated value of the limit of detection, can be calculated.

Figure 2a and 2b illustrate the LC chromatogram for retinyl palmitate in the sample and standard, respectively. The flow rate is reduced for the analysis of the vitamin A ester resulting in a run time of 8 min. The retinyl palmitate differs in its fortified concentration as compared to the tocopherols, thus, the gain was increased to aid in the detection of vitamin A. Two injections from

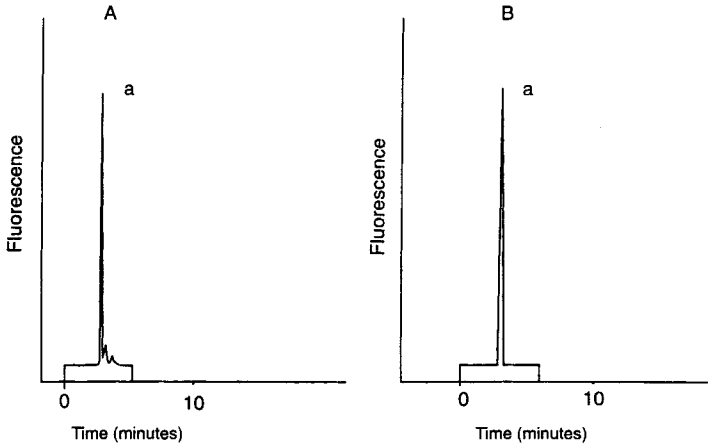

Figure 2. LC chromatogram of retinyl palmitate using fluorescence detection (Ex 325 nm, Em 470 nm), flow rate of 0.5 mL/min, injection volume of 50 μL, and a mobile phase of 0.5% isopropanol in hexane. 2A is the extract of an infant formula where (a) is the retinyl palmitate. 2B is the retinyl palmitate (a) standard (1.25 μg/mL).

the same extract were used to simplify the overall method. For example, one injection was for the vitamin E determination and, a second injection was for the vitamin A determination. The vitamins of interest can easily be quantitated from a single injection by using a programmable pump in conjunction with the programmable fluorescence detector. The retinyl palmitate fluorescence response was linear (r=0.999) from 8.0 to 106 μg/mL. The limit of detection, calculated in a manner as previously described for all-rac-α-tocopheryl acetate, was 0.187 μg/mL.

Ten replicates of SRM 1846 were assayed for retinyl palmitate, all-rac-α-tocopheryl acetate and natural tocopherol content. Since vitamins A and E are expressed as the alcohol form in the SRM 1846 certificate, ester forms of the vitamins were converted to the alcohol forms. Table 1 illustrates the NIST ranges for all rac-α, γ and δ-tocopherol and vitamin A, as well as the results obtained in this study. The all-rac-α-tocopherol and retinol values are certified values; whereas, the γ and δ-tocopherol values are non-certified. The all-rac-αl-tocopherol and retinol data obtained utilizing our method approximate those of the SRM certified values. The γ-tocopherol level meets the non-certified specifications, but the δ-tocopherol level exceeds the non-certified specification

Table 1

Assay Values of SRM 1846

	NIST Value (mg/kg)	Found (mg/kg) (n = 10)
α tocopherol*	246 - 296	281 ± 6.15 (cv = 2.19)
γ tocopherol	70.5 - 76.5	76.2 ± 4.62 (cv = 6.06)
δ tocopherol	17.53 - 18.45	19.1 ± 0.81 (cv = 4.25)
retinol*	5.16 - 6.52	5.37 ± 0.26 (cv = 4.79)

* certified values.

slightly. NIST used saponification techniques to quantitate the γ and δ-tocopherols; whereas, this study does not. The higher δ-tocopherol level found with the present method could indicate higher tocopherol stability and/or better extraction efficiency compared to the saponification method.

Initial attempts using hexane as the extraction solvent resulted in low recoveries for retinyl palmitate. Substitution of 100% hexane with hexane:ethyl acetate (85:15 v/v); to increase the polarity of the solvent; resulted in increased recoveries for all-rac-α-tocopheryl acetate and retinyl palmitate. Studies by Ueda and Igarashi[17] indicated the usefulness of ethyl acetate for vitamin E extractions. Using the hexane:ethyl acetate mixture, recoveries were run in five replicates at four different levels. The mean certified value plus approximately 50, 100, 150, and 300% of the certified value was studied. The levels of 50, 100, 150, and 300 % were obtained by using the process of standard additions to SRM 1846. For example, since α-tocopherol is certified at a mean level of 271 mg/kg, 50% of that value would be the standard addition level of 135.5 mg/kg, thus giving a total of 406.5 mg/kg.

Since infant formulas are fortified with all-rac-α-tocopheryl acetate and retinyl palmitate, the ester forms were used for the recovery studies. Alpha, γ and δ-tocopherols vary in concentration according to the type of oil used in the formulation. Table 2 illustrates the recoveries obtained in this study. The recoveries were fairly constant for the 50-150% levels for both the retinyl palmitate and all-rac-α-tocopheryl acetate, however, at 300% the recoveries decreased to 91.8 and 94.2%, respectively. The 300% level for retinyl palmitate corresponds to almost twice the legal limit allowed (750 IU/100 kcal).

Table 2

Recovery Results of all Rac-α-Tocopheryl Acetate and Retinyl Palmitate

Level (%)*	% Recovered Vitamin A	% Recovered Vitamin E
50 (n = 5)	98.6 ± 5.7 (cv = 5.8)	103 ± 4.3 (cv = 4.2)
100 (n = 5)	100 ± 4.0 (cv = 4.0)	103 ± 3.5 (cv = 3.4)
150 (n = 5)	99.4 ± 4.6 (cv = 4.6)	103 ± 1.1 (cv = 1.1)
300 (n = 5)	91.8 ± 3.3 (cv = 3.6)	94.2 + 2.6 (cv = 2.7)

* Corresponds to percentage of mean certified value added to the sample.

Recovery studies utilizing the method of standard additions for a matrix already containing a known certified amount of analyte, cannot show how the analytes would behave at levels less than the certified value. Since a true sample blank does not exist for infant formula, recoveries in this study are only for values exceeding the certified value. Work is currently being conducted in this laboratory to develop a "zero control" infant formula or a true matrix blank. A "zero control" reference material would enable one to determine the method's performance as the analyte of interest is reduced in quantity to near zero.

Peak purity was established using a peak ratioing technique described by Haroon et al.[18] The emission wavelength was kept constant for the analytes while the fluorescence was measured at three different excitation wavelengths. The fluorescence emission of retinyl palmitate at 470 nm was determined at excitation wavelengths of 315, 325, and 335 nm. Ratios were calculated for 315/325 and 335/325. For all-rac-α-tocopheryl acetate, the emission wavelength was held at 310 nm and the excitation was at 275 and 295 nm. Ratios were calculated for 275/285 and 295/285. These ratios were compared for the standard and sample as illustrated in Table 3. Good agreement was obtained for the ratios of the standard and sample for both retinyl palmitate and all-rac-α-tocopheryl acetate, indicating the purity of the peaks.

This method presents an alternative technique to saponification for analysis of infant formulas and provides data in agreement with SRM certified values. Elimination of saponification permits quantitation of the added ester forms and natural vitamin E homologs. Fat removal from the extract is not necessary, since normal phase chromatography on silica can accomodate up to 2 mg of fat per injection.[10] Analysis of a milk based formula with the

Table 3

Peak Purity Evaluation[a]

Nutrient	Excitation Wavelength	Peak Response Ratios Standard	Sample
retinyl palmitate	315/325	1.15	1.13
	335/325	0.86	0.82
all-rac-α-tocopherol acetate	275/285	0.66	0.69
	295/285	0.52	0.47

[a] Emission wavelengths were constant for vitamins A (470 nm) and E 3310 nm) (n = 2).

procedure will load approximately 1.6 mg of fat per 50 μL injection on to the column. Stability of the analytes is increased when working with the ester forms of vitamins A and E. A true quantitation of vitamin E content can be established to include natural tocopherols and added all-rac-α-tocopheryl acetate. The use of chlorinated solvents is eliminated. The method is simple and rapid as an experienced analyst can assay up to 10 samples per day.

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