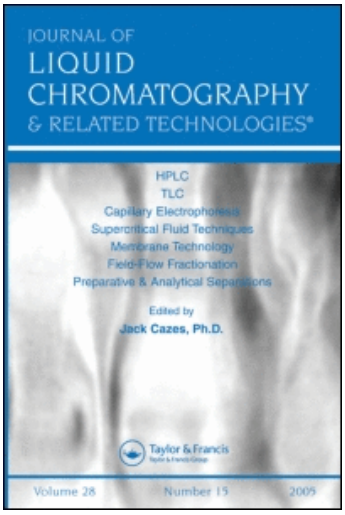

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LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF ALL-RAC-α-TOCOPHERYL ACETATE AND RETINYL PALMITATE IN SOY BASED INFANT FORMULA USING MATRIX SOLID PHASE DISPERSION

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ABSTRACT

A liquid chromatographic method is described for the analysis of all-rac-α-tocopheryl acetate and retinyl palmitate in soy based infant formula. The vitamins are extracted from infant formula without saponification by matrix solid phase dispersion (MSPD) and quantitated by normal phase chromatography with fluorescence detection. Retinyl palmitate and all-rac-α-tocopheryl acetate are quantitated isocratically with a mobile phase of hexane containing isopropanol @ 0.125% (v/v) and 0.5% (v/v), respectively. Results compared favorably to the label declaration on a retail infant formula product.

Recoveries were determined on a soy based zero control reference material (ZRM) fortified with the analytes for soy based infant formula and averaged 99.0% (n=30) for retinyl palmitate and 97.1% (n=25) for all-rac-α-tocopheryl acetate. Five concentrations were examined for each analyte and the results were linear (r^2=0.998) over the concentration examined with CV's from 1.24-5.44%.

The method provides a rapid, specific and easily controlled assay approach for the analysis of retinyl palmitate and all-rac-α-tocopheryl acetate in fortified soy based infant formula. The cost per analysis at present market value of expendables when using MSPD is between $3-4.00. Considering the escalating cost of manpower, solvents, and hazardous chemical disposal fees, the MSPD technique is efficient and highly cost effective.

INTRODUCTION

Recently, Chase et al.[1] developed a method to quantitate vitamin A and vitamin E in fortified milk based infant formula. The extraction protocol eliminated saponification through the use of matrix solid phase dispersion (MSPD). As previously discussed[1] the MSPD technique has been used extensively for isolating drugs from milk and tissue, and has been patented.

In each application, extremely small amounts (7mL) of selectively chosen solvents were used to extract the analyte(s) of interest and sample throughput was maximized. After a concentration step, the extract is injected directly into a normal phase LC system with quantitation by fluorescence detection.

Analytical results agreed with the certified values of the National Institute of Standards and Technology (NIST) infant formula Standard Reference Material (SRM) 1846. Recoveries utilizing a zero control reference material (ZRM) for retinyl palmitate averaged 96.8% and 91.5% for all-rac-α-tocopheryl acetate (n=25).[1]

The objective of this present work is to expand the aforementioned method and develop a method for the analysis of retinyl palmitate and all-rac-α-tocopheryl acetate in soy based infant formula as no "official" method presently exists.[2] Furthermore, a ZRM was utilized as a tool to evaluate the analytical protocol.

EXPERIMENTAL

Apparatus

(a) Liquid Chromatograph.- LDC Analytical Constametric 4100 pump (Thermo Separation Products, Riviera Beach, FL, 33404) and a Waters 715 autoinjector (Waters Inc., Milford, MA, 01757).

(b) Column.- Lichrosorb Si 60, 5 μm, 4.6 mm x 25 cm (E. Merck, Darmstadt, Germany).

(c) Integrator.- Model 3396 or equivalent (Hewlett Packard, Atlanta, GA, 30339). A computerized data system is best.

(d) Fluorescence detector.- Model 1046A programmable fluorescence detector (Hewlett Packard) or equivalent.

(e) Reservoirs with frits. - Varian 15 mL size, part number 1213-1016 (Varian, Harbor City, CA, 90710).

(f) Turboevaporator.- Turbo Vap II (Zymark, Hopkinton, MA, 01748). Other evaporation techniques are acceptable provided that a quantitative transfer is possible to a 1.0 mL final volume.

Reagents

(a) Hexane.- LC grade (Burdick and Jackson, Muskegon, MI, 49442). Dry the hexane over molecular sieves before use.

(b) Isopropanol.- LC grade (EM Science, Gibbstown, NJ, 08027).

(c) Methylene Chloride. - LC grade (EM Science).

(d) Bondesil. - C_{18}, preparative grade, part number 1221-3013 (Analytichem International, Harbor City, CA, 90710).

(e) Mobile phase.- Hexane containing isopropanol @ 0.5% v/v for vitamin E analysis and hexane containing isopropanol @ 0.125% v/v for retinyl palmitate analysis.

(f) Retinyl palmitate stock standard solution.- Accurately weigh approximately 50 mg of retinyl palmitate (Fluka Bio Chemika, Switzerland) into a 50.0 mL volumetric flask and dilute to volume with hexane. Determine the exact

concentration from the $E^{1\%}_{1cm}$ value of 975. Make the appropriate dilutions with the respective mobile phase to give five working standard concentrations ranging from 0.20 to 2.5 μg/mL.

(g) Vitamin E stock standard solution.- Accurately weigh approximately 200 mg of all-rac-α-tocopheryl acetate (Fluka Bio Chemika) into a 50.0 mL volumetric flask and dilute to volume with hexane. Determine the exact concentration from the $E^{1\%}_{1cm}$ value of 42. The appropriate dilutions were made with the mobile phase to give five working standard concentrations ranging from 1.0 to 25 μg/mL.

(h) Isopropyl palmitate. - (K & K Laboratories, Plainview, NY)

Chromatographic Conditions

(a) Instrument parameters.- injection volume of 50 μL; flow rate of 1.0 mL/min; fluorescence detector parameters for vitamin E (exλ = 285 nm, emλ = 310 nm, gain = 8); fluorescence detector parameters for retinyl palmitate (exλ = 325 nm, emλ = 470 nm, gain = 9).

(b) LC configuration.- Inject the sample and standards for vitamin E analysis first. Use a run time of 20 min to allow all the tocopherols to elute. Upon completion of the vitamin E analysis, change to the mobile phase for retinyl palmitate and allow 30 minutes to equilibrate. Inject the standards and samples for retinyl palmitate analysis using a run time of 10 min.

Sample Description and Preparation

A zero control reference material (ZRM) soy based infant formula powder was used for recovery studies.[3] Approximately 10 g of the ZRM powder was sampled as previously discussed in earlier work[1,4] and combined with 50 g of boiling water and thoroughly mixed. A commercially available infant formula was also assayed in 10 replicates. Twelve cans of the powdered commercial formula were thoroughly mixed to homogeneity prior to taking a representative sample. Approximately 5 g of commercial formula was combined with 25 g of boiling water and thoroughly mixed.

Sample Extraction

Weigh 2 g of the Bondesil C_{18} into a mortar. Add 100 μL of isopropyl palmitate and gently blend the isopropyl palmitate onto the C_{18} with a pestle.

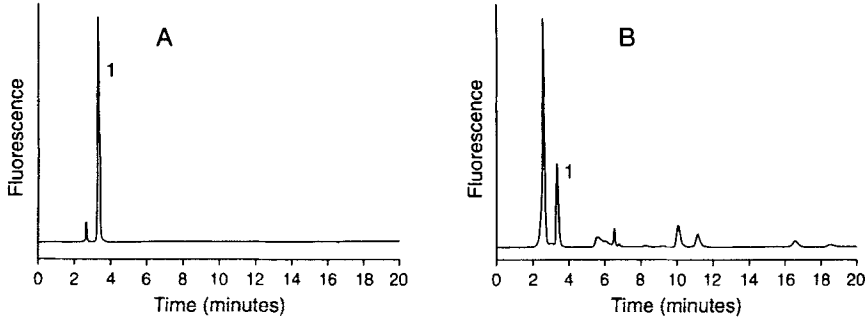

Figure 1. LC chromatograms of vitamin E using fluorescence detection (ex λ = 285 nm, em λ = 310 nm), flow rate of 1.0 mL/min, injection volume of 50 μL and a mobile phase of 0.5% isopropanol in hexane. "A" is a chromatogram of the standard, and "B" is the chromatogram of an extract of a commercially available soy based infant formula. All-rac-α-tocopheryl acetate is identified as peak .[1]

Accurately weigh approximately 0.50 g of reconstituted sample and the spike solution into the C_{18} / isopropyl palmitate mixture. Use the pestle to gently blend, not grind, the reconstituted sample and the C_{18} / isopropyl palmitate into a fluffy, slightly sticky powder. Accurately transfer the C_{18} /matrix blend into a 15 mL reservoir tube with a frit at the bottom, followed by inserting the top frit on the powdery mix. Tightly compress the reservoir contents with a 10 cc syringe plunger. Pass 7 mL of hexane containing 0.5% isopropanol (v/v) followed by 7 mL of methylene chloride through the reservoir, collecting both eluents into one 50 mL turbovap vessel. The combined eluents are dried at 45°C in the turbovap under 5 psi of nitrogen to near dryness. The residue is then diluted to 1.0 mL with hexane, transferred to an injection vial and injected onto the LC.

Calculation

The concentration in μg/mL of all-rac-α-tocopheryl acetate and retinyl palmitate in the sample extract is determined from a linear regression analysis.

RESULTS AND DISCUSSION

Figure 1a is the LC chromatogram of a standard of all-rac-α-tocopheryl acetate which elutes at about 3.6 min. Figure 1b is the chromatogram of an extract of a commercially available soy based infant formula fortified with all-rac-α-tocopheryl acetate having a similar retention time. The peaks observed

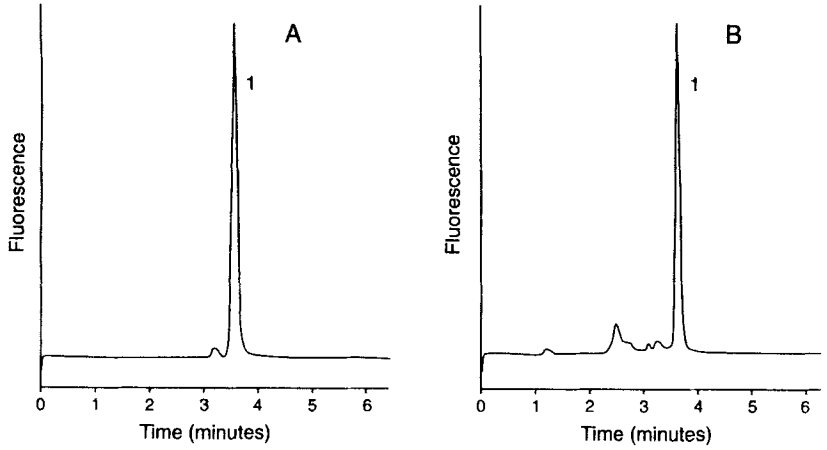

Figure 2. LC chromatogram of retinyl palmitate using fluorescence detection (ex λ = 325 nm, em λ = 470 nm), flow rate of 1.0 mL/min, injection volume of 50 μL and a mobile phase of 0.125% isopropanol in hexane. "A" is the chromatogram of the retinyl palmitate standard and "B" is the chromatogram of an extract of a commercially available soy based infant formula. Retinyl palmitate is identified as (1) in the chromatograms.

after the all-rac-α-tocopheryl acetate peak are the naturally occuring tocopherols as previously discussed.[1] The coefficient of determination (r^2) of the fluorescence response for all-rac-α-tocopherol acetate was 0.998 for the 1.0-25 μg/mL. The five standards ranging from 1.0 -25 μg/mL were injected in duplicate on two separate occasions, two weeks apart. The average slope (n=2) was 4368 ± 173 (CV=3.97%) for all-rac-α-tocopheryl acetate. The limit of quantitation was 1.77 μg/mL for all-rac-α-tocopheryl acetate as previously discussed.[1]

Figure 2a is the LC chromatogram of the retinyl palmitate standard, which elutes at about 3.6 min. Figure 2b is the chromatogram of an extract of a commercially available soy based infant formula fortified with retinyl palmitate having a similar retention time. The coefficient of determination (r^2) of the fluorescence response for retinyl palmitate was 0.998 from 0.2 to 2.5 μg/mL.

The five standards ranging from 0.2 to 2.5 μg/mL were injected in duplicate on two separate occasions, two weeks apart. The average slope (n=2) was 24356 ± 419 (CV=1.70) for retinyl palmitate. The limit of quantitation for retinyl palmitate was 0.26 μg/mL.

Table 1

Recovery of All-Rac-α-Tocopheryl Acetate and Retinyl Palmitate from Fortified ZRM Soy Based Infant Formula

Level	Retinyl Palmitate[1] (Average % Rec.)	All-Rac-α-Tocopheryl Acetate[1] (Average % Rec.)
16x[2]	83.8 ± 2.2 (2.61)	94.9 ± 1.2 (1.24)
8x	95.2 ± 2.2 (2.26)	93.7 ± 2.00 (2.09)
4x	91.7 ± 2.0 (2.20)	99.1 ± 2.8 (2.86)
2x	97.1 ± 5.1 (5.22)	89.8 ± 4.9 (5.44)
x	107 ± 4.7 (4.40)	108 ± 3.6 (3.37)
1/2x	104 ± 1.9 (1.80)	below limit detection
Blank[3]	not applicable	not applicable

[1] "±" pertains to the standard deviaton and CV% is in parenthesis.
[2] Five replicates were assayed at each spiking level and blank where x is equivalent to 250 IV/100 kcal and 0.7 IV/100 kcal for vitamin A and E, respectively.
[3] No peaks were observed above the baseline noise in the ZRM chromatograms.

A series of recovery studies were performed on fortified soy based ZRM. The manufacture of infant formula ZRM's and their potential for nutrient methods development and validation has been published.[1,3,4] Table 1 shows the recovery data obtained when the ZRM was spiked at the 1/2x, x, 2x, 4x, 8x and 16x levels, where x is the minimum level for retinyl palmitate and all-rac-α-tocopheryl acetate as listed in the Code of Federal Regulations.[5] Each spiking level was assayed five times. The retinyl palmitate recoveries were acceptable up to the 8x level but decreased to 83.8% at the 16x level.

A similar phenomena was noticed previously[1] for retinyl palmitate in a milk based ZRM. Since all the solvent is collected off the column, evaporated, and diluted to volume the high retinyl palmitate spike is not completely removed from the C_{18}. Nevertheless, the method is more than adequate for retinyl palmitate assay since a 16x level for retinyl palmitate in infant formula corresponds to 4000 IU/100 kcal which greatly exceeds normal fortification levels of 310 IU/100 kcal. Also, all-rac-α-tocopheryl acetate recoveries were acceptable over a range of x to 16x. Method performance was excellent having high absolute recoveries (Table 1), linear standard curves (r=0.999), and low variation (CV's from 1.24 to 5.44%).

Table 2

Peak Purity Evaluation

Nutrient	Excitation Wavelength*	Peak Response Ratios Standard	Sample
Retinyl Palmitate	315/325	1.21	1.26
	335/325	0.82	0.86
All-rac-α-tocopheryl acetate	275/285	0.71	0.71
	295/285	0.31	0.29

* Emission wavelengths were constant for retinyl palmitate (470 nm) and all-rac-α-tocopheryl acetate (310 nm) (n=2).

Replicate (n=10) analysis of a commercial soy based infant formula labeled to contain 8.47 μg/g of retinyl palmitate and 103 μg/g of all-rac-α-tocopheryl acetate resulted in analyte determinations of 11.9 ± 0.47 (cv=3.97%) μg/g (retinyl palmitate) and 165 ± 5.6 (cv=3.38%) μg/g (all-rac-α-tocopheryl acetate). These levels approximate values observed in this laboratory for similar products using AOAC milk based infant formula methods and coincide with the overages that are normally found in fortified products.

As an integral part of the methods development process, the peak purity of the analytes from the commercial soy based formula was established using a previously published peak ratioing technique.[1,4] These ratios were compared for the standard and commercial soy based formula as illustrated in Table 2. 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. In addition, to further indicate the peak purity and identity of observed peaks, the column eluent was collected at retention times corresponding to the retinyl palmitate and all-rac-α-tocopheryl acetate in the standard and sample extract. These collected eluents were enriched and subjected to thin layer chromatography. The R_f for the retinyl palmitate and all-rac-α-tocopheryl acetate from the standard and sample extracts were similar.

This method provides an alternative to the classical saponification techniques that are currently employed for the retinyl palmitate and all-rac-α-tocopheryl acetate assay of commercial soy infant formula. Previous work[1] has illustrated that the quantitation of all-rac-α-tocopheryl acetate and the native tocopherols allows precise calculation of α-tocopherol equivalents (α-TE) for

assessment of biological activity. Hydrolysis of all-rac-α-tocopheryl acetate by saponification prevents the independent quantitation of all-rac-α-tocopheryl acetate since all-rac-α-tocopherol isomers cannot be resolved from naturally occurring RRR-α-tocopherol. The technique is extremely rapid, simple, and only uses 14 mL of solvent per sample. Twenty samples can be easily analyzed by a competent analyst in one working day, thereby, greatly increasing sample throughput compared to existing infant formula methods. Plans are underway to collaborate the method and extend the technique to analyze other nutrients in infant formula and medical foods.

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