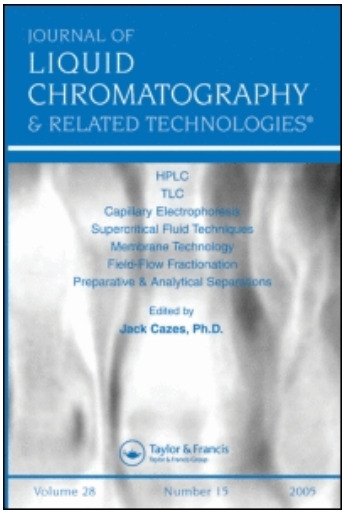

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A LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF RETINYL ACETATE IN SOY BASED INFANT FORMULA USING MATRIX SOLID PHASE DISPERSION

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A LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF RETINYL ACETATE IN SOY BASED INFANT FORMULA USING MATRIX SOLID PHASE DISPERSION

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ABSTRACT

Retinyl acetate in soy based infant formula was extracted by matrix solid phase dispersion (MSPD) and chromatographed by isocratic normal phase chromatography on a Si 60 column with a mobile phase of 0.28% (v/v) isopropanol in hexane. Detection was by fluorescence (Ex λ=325, Em λ=470). Fluorescence response was linear (r^2=0.999) from 0.10 - 2.5 μg/mL. Recoveries determined on a soy based infant formula zero control reference material (ZRM) containing added analyte at five levels averaged 94.7% (n=25) with CV's from 0.57-3.53%. The method expands the use of MSPD extraction to the analysis of retinyl acetate in soy based infant formulas.

INTRODUCTION

Retinyl acetate is sometimes used as the vitamin A source in formulated products. Although used less frequently than retinyl palmitate by the food and pharmaceutical industries, analysts need to be aware of its occasional use. Most infant formulas are formulated with retinyl palmitate; however, we have examined soy based infant formula and other products containing retinyl acetate. AOAC International[1] does not provide methodology for the analysis of vitamin A in soy based infant formulas. Methods available for analysis of vitamin A in milk based infant formula[2] are often applied, however, matrix differences exist, and the methodology has not been validated for analysis of soy based infant formula.

We have recently presented a method for the analysis of all-rac-α-tocopherol acetate and retinyl palmitate in soy based infant formula using matrix solid phase dispersion (MSPD) as the primary extraction step.[3] MSPD eliminates saponification, uses small solvent volumes, speeds the analysis time, and allows quantitation of specific ester forms of the analytes.[3-5]

The objective of the present study was to apply the MSPD technique[6] to the analysis of retinyl acetate from soy based infant formula. A zero control reference material (ZRM) was used to validate the method at analyte concentrations below the declared value.[7]

EXPERIMENTAL

Apparatus

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

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

Data handling system: Waters Millennium Software (Waters Inc.) or equivalent. An integrator is also suitable.

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

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

Turboevaporator: Turbo Vap II (Zymark, Hopkinton, MA, 01748).

Reagents

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

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

Methylene Chloride: LC grade (EM Science).

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

Mobile phase: Hexane containing isopropanol @ 0.28% v/v.

Retinyl acetate stock standard solution: Accurately weigh the contents of the USP Reference Standard capsule of retinyl acetate (U.S. Pharmacopeial Convention, Rockville, MD, 20852) into a 10.0 mL volumetric flask and dilute to volume with hexane. Determine the exact concentration from the $E^{1\%}_{1cm}$ value of 1550.[8] Make the appropriate dilutions with the mobile phase to give five working standard concentrations ranging from 0.10 to 2.5 μg/mL.

Isopropyl palmitate: (K & K Laboratories, Plainview, NY)

Chromatographic Conditions

Instrument parameters: injection volume of 50 μL; flow rate of 1.0 mL/min; fluorescence detector parameters for retinyl acetate (Ex λ = 325 nm, Em λ = 470 nm, gain = 10).

LC configuration: Inject the sample and standards for retinyl acetate. Use a run time of 10 min to allow any extraneous peaks to elute.

Sample Description and Preparation

A zero control reference material (ZRM) soy based infant formula powder was used for recovery studies.[3-5,7] Approximately 10 g of the ZRM powder was

sampled as previously discussed in earlier work[3-5,7] 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 Bondasil C_{18} into a mortar. Add 100 µL of isopropyl palmitate and gently blend the isopropyl palmitate onto the C_{18} with a pestle. 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 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 all 14 mL into a 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 retinyl acetate in the sample extract is determined from a linear regression analysis.

RESULTS AND DISCUSSION

Figure 1a is the LC chromatogram of retinyl palmitate and retinyl acetate standard. Figure 1b is the chromatogram of an extract of a commercial soy based infant formula fortified with retinyl acetate. No other peaks were observed to interfere with the retinyl acetate which elutes at about 9 min. The fluorescence response was linear with a coefficient of determination (r^2) of 0.999 for retinyl acetate for the 0.10 to 2.5 µg/mL range. Standard retinyl acetate at five concentrations in the range of 0.10 to 2.5 µg/mL were injected in duplicate on three different occasions to determine the reproducibility of the slope as determined from the linear regression. A mean slope (n=3) of 75530 $\pm$ 1980 (CV=2.60%) was observed. The limit of quantitation for retinyl acetate was found to be 0.15 µg/mL and the limit of detection was 0.08 µg/mL.

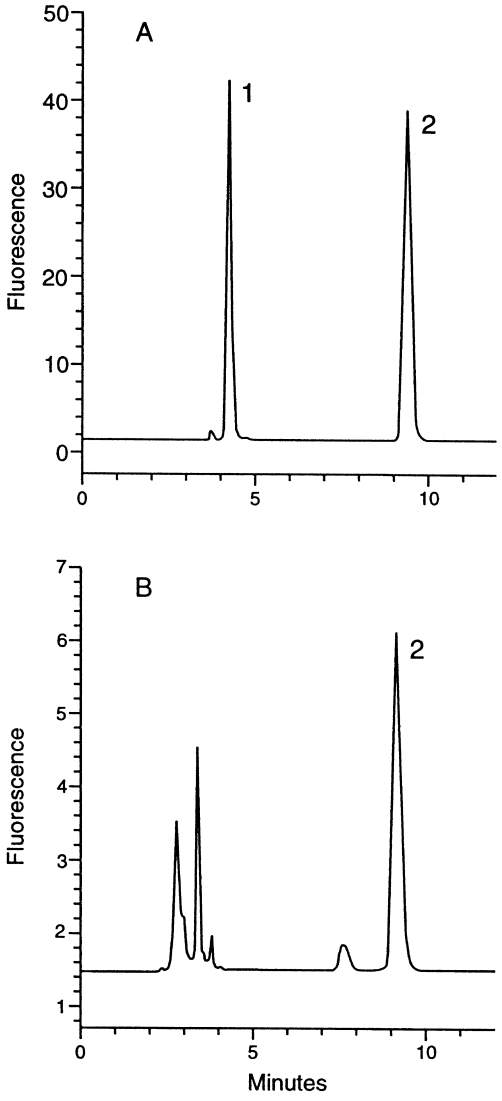

Figure 1. LC chromatogram of retinyl acetate using fluorescence detection (ex λ = 325 nm, em λ = 470 nm), flow rate of 1.0 mL/min, injection volume of 50 μL and a mobile phase of hexane containing 0.28% (v/v) isopropanol. "A" is a chromatogram of the retinyl palmitate (1) and retinyl acetate (2) standard and "B" is the chromatogram of an extract of a commercial infant formula.

Table 1

Recovery of Retinyl Acetate

Level	Retinyl Acetate[a] (% Recovered)
8x[b]	96.1 ± 1.5 (1.52)
4x	90.8 ± 0.5 (0.57)
2x	98.5 ± 3.5 (3.53)
1x	90.5 ± 0.9 (4.22)
1/2x	106 ± 1.1 (1.08)
blank[c]	not applicable

[a] Mean ± SD of five replicate assays. %CV are in parenthesis.
[b] Five replicates were assayed at each spiking level and blank where "x" is equivalent to 250 IU/100 kcal.
[c] No peaks were observed above the baseline noise in the ZRM chromatogram.

A total of 30 analyses were performed on the soy based infant formula ZRM. The ZRM was run five times without fortification. No peaks were observed in the unfortified extract that would interfere with the retinyl acetate determination. Five spiking levels were also run in replicates of five to include 1/2x, x, 2x, 4x, and 8x, where "x" is equivalent to 250 IU/100 kcal for vitamin A. Such a wide range of fortification levels was studied in order to determine at what point the method would not function. Table 1 lists the average recoveries for each spiking level. The recoveries were constant for the 1/2 x to 8x range.

Replicate (n=10) analysis of a commercial soy based infant formula labeled to contain 5.39 μg/g of retinyl acetate resulted in analyte determinations of 5.37 $\pm$ 0.15 (CV=2.81%) μg/g. This level approximates values observed in this laboratory for similar products using AOAC International milk based infant formula methods.[2]

The peak purity of the retinyl acetate in the commercial soy based infant formula was determined by a peak rationing technique. The emission wavelength was kept constant for the analytes while the fluorescence was measured at three different excitation wavelengths. The fluorescence emission of retinyl acetate at 470 nm was determined at excitation wavelengths of 315, 325 and 335 nm. Ratios were calculated for 315/325 and 335/325.

Table 2

Peak Purity Evaluation[a]

Nutrient	Excitation Wavelength	Peak Response Ratos Standard[b]	Sample
Retinyl Acetate	315/325 35/325	1.17 0.89	1.17 0.88

[a] Emission wavelengths were constant for retinyl acetate (470 nm) (n=2).
[b] Ratios are based on the average of duplicate injections.

Table 2 illustrates the agreement between the peak ratios of the sample and standard extracts. In addition, to further indicate the peak purity, the column eluent was collected for the retinyl acetate standard and sample extract peaks and subjected to thin layer chromatography. The R_f for the retinyl acetate in the standard and sample extracts were similar.

The method as described differs from earlier work done for retinyl palmitate[3-5,7] due to the fact that the mobile phase is modified slightly to increase the isopropanol content from 0.125 to 0.28%. This method provides an accurate and fast technique to quantitate retinyl acetate in soy based infant formula without saponification. The use of a ZRM confirmed that the method is valid for use with products containing less than the label declaration amount.

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