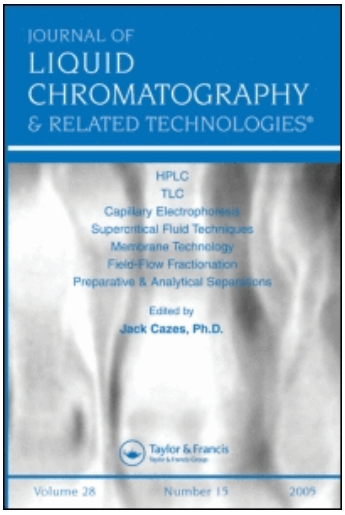

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 18 January 2000

To cite this Article Chase Jr., G. William , Eitenmiller, R. R. and Long, A. R.(2000) 'THE LIQUID CHROMATOGRAPHIC ANALYSIS OF VITAMIN K_1 IN SOY BASED INFANT FORMULA USING MATRIX SOLID PHASE DISPERSION', Journal of Liquid Chromatography & Related Technologies, 23: 3, 423 — 432
To link to this Article: DOI: 10.1081/JLC-100101461
URL: http://dx.doi.org/10.1081/JLC-100101461

J. LIQ. CHROM. & REL. TECHNOL., 23(3), 423–432 (2000)

THE LIQUID CHROMATOGRAPHIC ANALYSIS OF VITAMIN K_1 IN SOY BASED INFANT FORMULA USING MATRIX SOLID PHASE DISPERSION

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ABSTRACT

A liquid chromatographic method is described for vitamin K_1 in soy based infant formula. The vitamins are extracted from infant formula by matrix solid phase dispersion (MSPD) and quantitated by reversed phase chromatography with fluorescence detection. Vitamin K_1 is converted to the fluorescent hydroquinone with a post column zinc reductive reactor. The limit of detection is 12 pg and the limit of quantitation is 38 pg on- column. Linear response ranged from 0.70 – 11.0 ng/mL (r^2 = 0.998). Recoveries were determined on an analyte-fortified blank material for soy based infant formula and averaged 92.5% (n = 25) for vitamin K_1.

The method provides a rapid, specific, and easily controlled assay for the analysis of vitamin K_1 in fortified soy based infant formula.

INTRODUCTION

Recently, Chase et al.[1] developed a method to quantitate vitamin K_1 in fortified milk based infant formula. The extraction protocol utilized matrix solid phase dispersion (MSPD) followed by a concentration step, and injection of the extract directly onto a C8 LC column connected in series with a zinc post column reduction column. The zinc reduction column induced formation of the fluorescent hydroquinone for the quantification of total vitamin K_1 by fluorescence detection. This technique and others currently used for vitamin K_1 analysis resulted from work of Sadowski's research group at the United States Department of Agriculture Human Research Center on Aging, Tufts University.[2-12] The method of Chase et al[1] provides methodology advantages of speed, low solvent requirements, and improved repeatability when compared to the AOAC International method for vitamin K_1 in milk based infant formula (Method 992.27, 50.106).[13] The AOAC method uses an ammonium hydroxide and methanol pretreatment, extraction with 2:1 mixture of dichloromethane and isooctane, clean up by open column silica chromatography, and quantitation by LC with UV detection. The method suffers from high CVs and cannot be used for infant formula samples containing corn oil. The MSPD technique has been used extensively for isolating drugs from milk and tissue, and has been patented.[14]

Analytical results from the MSPD technique agreed with the certified values of the National Institute of Standards and Technology (NIST) Infant Formula Standard Reference Material (SRM) 1846. Recoveries utilizing a blank for vitamin K_1 averaged 91.7% (n=25).[1]

The objective of the present work is to expand the use of the MSPD method to the analysis of vitamin K_1 in soy based infant formula since no "official" method presently exists.[13]

EXPERIMENTAL

Apparatus

Liquid Chromatograph-LDC Analytical Constametric 4100 pump (Thermo Separation Products, Riviera Beach, FL) and Waters 715 autoinjector (Waters, Inc., Milford, MA); Column-Alltech C8, 3 μm, 4.6 x 150 mm, part number

287133 (Alltech Associates, Deerfield, IL); Integrator-Waters Millenium Data System (Waters, Inc.). A stand-alone integrator can also be utilized. Fluorescence detector-Model 1046A programmable fluorescence detector (Hewlett Packard, Avondale, PA) or equivalent; Reservoirs with frits.- Varian 15 mL size, part number 1213-1016 (Varian, Harbor City, CA); Turboevaporator-Turbo Vap II (Zymark, Hopkinton, MA) or a suitable technique to evaporate the extracts; Vortex mixer-Maxi-Mix I, (Thermolyne, Dubuque, IA); Reduction reactor-2.5 cm x 3.2 mm stainless steel, packed with zinc powder, 100 mesh (Aldrich Chemical Co., Milwaukee, WI); Ultrasonic cleaner-5.2 gallon ultrasonic cleaner (Thomas Scientific, Swedesboro, NJ).

Reagents

Hexane - LC grade (Burdick and Jackson, Muskegon, MI); Isopropyl alcohol - LC grade (EM Science, Gibbstown, NJ); Ethyl Acetate - LC grade (Burdick and Jackson); Methyl Alcohol - LC grade (Burdick and Jackson); Acetic Acid - glacial, reagent grade (GR) (EM Science); Zinc Chloride - $ZnCl_2$, catalog Z-33 (Fisher Scientific, Fairlawn, NJ); Sodium Acetate - anhydrous, catalog S-8750 (Sigma Chemical Co., St. Louis, MO).

Reductive Ionic Solution - 2.0 M zinc chloride, 1.0 M sodium acetate and 1.0 M acetic acid per liter of methanol. Prepare this solution by weighing 68.1 g of zinc chloride, 20.5 g sodium acetate into a 250 mL volumetric flask and adding about 200 mL of methanol and 15 mL of acetic acid. Dissolve by stirring and then dilute with methanol to volume.

Diluting Solution - One liter of methanol containing 5.0 mL of the reductive ionic solution; Mobile phase - The diluting solution containing 10% hexane. Filter the mobile phase through a 0.45 µm filter prior to use; Vitamin K_1 standard - Accurately weigh ca 25 mg vitamin K_1 (USP reference standard Phytonadione Lot L) into 50.0 mL volumetric flask and dilute to volume with hexane. Determine the exact concentration from the $E^{1\%}_{1cm} = 419$ at 248 nm.

Appropriate dilutions were made with hexane such that five final working standards ranged from 0.70 ng/mL to 11.0 ng/mL. Each working standard should contain 10% hexane with the remaining volume being diluting solution.

Bondesil - C_{18} preparative grade, part number 1221-3013 (Varian); Isopropyl palmitate.-catalog# 29,178-1, tech 90%, (Aldrich Chemical Co.,Milwaukee, WI.). Spiking solutions - The appropriate dilutions were made from the vitamin K_1 stock standard in hexane so that an aliquot not exceeding 125 µL would contain 1/2x, x, 2x, 4x, and 8x levels where x corresponds to the minimum level of 4 µg/100 kcal for vitamin K_1 of as listed in the Code of Federal Regulations.[16]

Chromatographic Conditions

Instrument Parameters

Injection volume, 60 µL; flow rate 1.0 mL/min. Fluorescence detector parameters: excitation wavelength (exλ = 248), emission wavelength (emλ = 418), gain = 12. The zinc reduction column is placed after the analytical column. In addition, a scrubber column was installed after the pump but before the injector. This was comprised of a 15 cm stainless steel column filled with zinc metal (cat. no. Z15, Fisher Scientific) to aid in reducing background noise.

LC Configuration

First, inject the working standard to establish linearity (r=0.999) allowing for a 10 min run time per injection. Upon completion of the standard injections, inject the samples while interspersing with alternate standard injections.

Sample Description and Preparation

A zero control reference material (ZRM) for infant formula that was used in our laboratory for development of a vitamin A and E method[15] was used for recovery studies. Preparation of the infant formula ZRM and the commercial infant formula was accomplished by weighing ca 10 g of powder, combining it with 50 g of boiling water, followed by thorough mixing on a stir plate. Sonicating the mixture of water and powder infant formula for 15 min followed by Polytron^{R} homogenization further ensured sample homogeneity.

Sample Extraction

Weigh 2 g Bondesil C_{18} into a mortar. Add 100 µL isopropyl palmitate and gently blend the isopropyl palmitate onto the C_{18} with a pestle. Accurately weigh ca 0.50 g reconstituted sample into the C_{18}-isopropyl palmitate mixture, followed by the addition of the spike solution. 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 and then insert the top frit on the powdery mix. Tightly compress the reservoir contents with a 10 cc syringe plunger. Pass 9 mL 0.5% isopropyl alcohol in hexane followed by 9 mL ethyl acetate through the reservoir. Collect both eluates into a 50 mL Turbo Vap vessel. The combined eluates are evaporated at 45°C in the Turbo Vap under 5 psi of nitrogen to near dryness. The residue is then diluted to 1 mL with hexane. The Turbo Vap vessel is vortexed for 15 sec and then sonicated for 15 sec. The hexane extract is brought to volume in a 10.0 mL volumetric flask with the diluting solution.

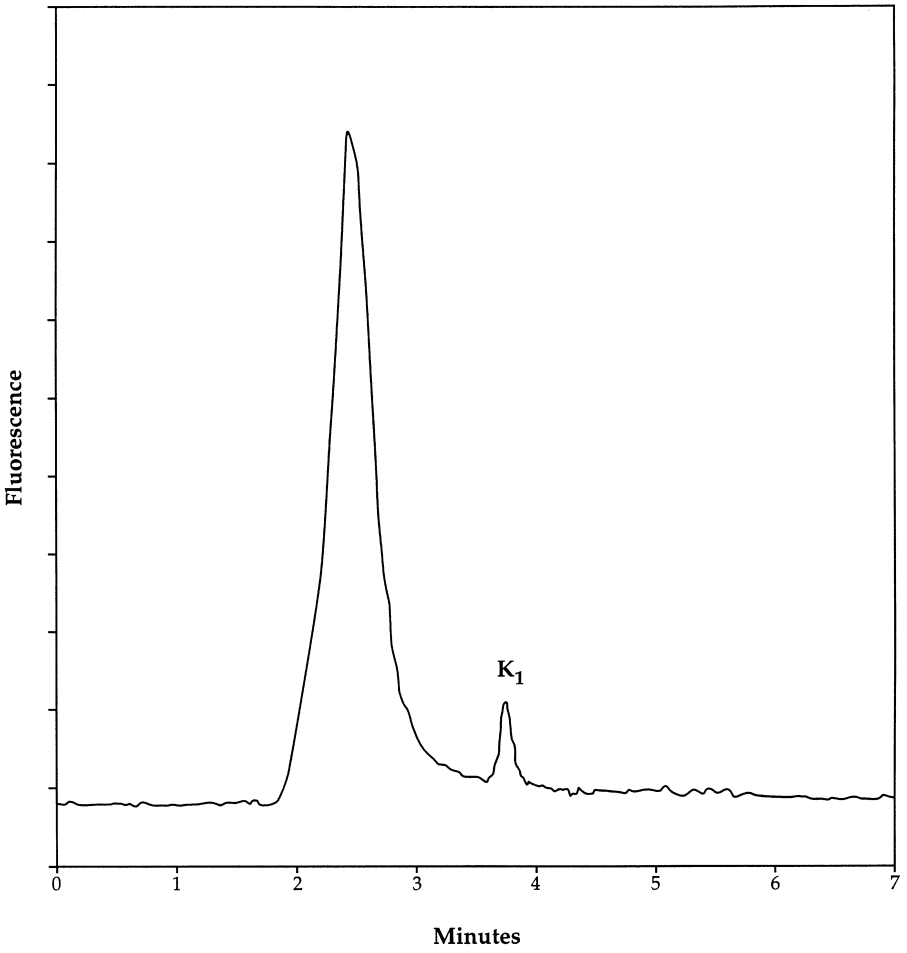

Figure 1. The chromatogram of the naturally occurring vitamin K_1 present in the soy based infant formula ZRM at a level of 133 ng/g. Flow rate of 1.0 mL/min, injection volume of 60 µL, excitation wavelength of 248 nm and emission wavelength of 418 nm and a gain of 12.

Calculation

The concentrations (ng/mL) of vitamin K_1 in the sample extract are calculated by linear regression analysis.

RESULTS AND DISCUSSION

Figure 1 illustrates the chromatogram of the naturally occurring vitamin K_1 present in the blank. Although the chromatogram is similar to that observed in earlier work,[1] a different product matrix justified the need for a method validation since this study is no longer identical to earlier work.[1] Furthermore, in a regulatory setting, it is generally required and it is advisable to validate any changes to a method, which has been developed for a different matrix. Since milk based infant formula and soy based infant formula formulations are diverse and the resulting matrices dissimilar, issues of solvent polarity, miscibility, partitioning characteristics, density characteristics and other physiochemical parameters become critically important in the validation process.

Vitamin K_1 is native to many oils used in the preparation of infant formula and was observed to be present at a level of 133 ng/g corresponding to 2.6 μg/100 kcal of infant formula which is slightly more than half the minimum level of 4 μg/100kcal required by law.[16] Since by definition, a ZRM, is that substance that is devoid of the nutrient of interest,[15] this low level found for vitamin K_1 makes the ZRM suitable for use as a blank for spiking purposes, provided that each spiking level is corrected for the naturally occurring vit. K_1.

The naturally occurring level of 133 ng/g was used as a baseline value for the method validation process. Each spiking level was corrected for the natural occurring amount of vitamin K_1. The level of 133 ng/g is slightly more than the baseline level observed in earlier work[1] for a milk based infant formula blank and can be attributed to the fact that both the milk and soy infant formula blanks differ in compositional components. Figure 2 represents the chromatogram of a vitamin K_1 standard overlaid with the extract of a commercial infant formula at a concentration of 377 pg on-column. Fluorescence responses for vitamin K_1 hydroquinone were linear from 0.70-11.0 ng vitamin K_1/mL ($r^2 = 0.998$).

In order to illustrate the stability of the LC system over time, the standard deviation of a standard set consisting of 5 standards at concentrations of 0.70 to 11.0 ng/mL injected in duplicate on 4 occasions over a 3 week period was found to be $\pm$ 487 (CV = 3.4%, n=4. The limit of detection was 0.20 ng vitamin K_1/mL (detected as the hydroquinone). This corresponds to about 27 ng/g or an on-column concentration equivalent to 12 pg. The limit of quantitation was 0.62 ng vitamin K_1/mL corresponding to 80 ng/g equivalent to 38 pg on-column.

Table 1 shows the recoveries obtained when the blank was spiked at the 1/2x, x, 2x, 4x, and 8x levels, where x is 4 μg/100 kcal as listed in the Code of Federal Regulations.[16] Each spiking level was assayed 5 times. The 133 ng/g level found in five replicates of the blank served as a baseline value and was subtracted from each subsequent spiking level.

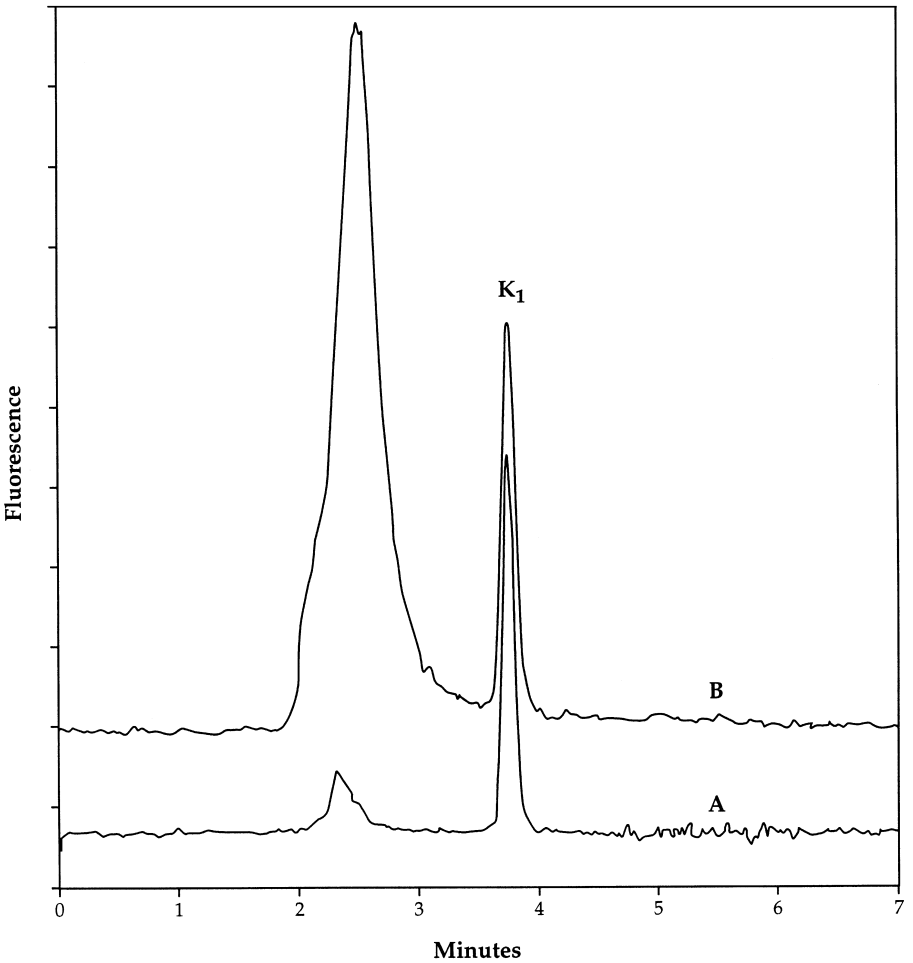

Figure 2. The chromatogram of a vitamin K_1 standard (A) overlaid with the extract of a commercial soy based infant formula (B) with a vitamin K_1 concentration of 377 pg on column. Flow rate was 1.0 mL/min, injection volume of 60 mL, excitation wavelength of 248 nm and emission wavelength of 418 nm and a gain of 12.

Vitamin K_1 recoveries were deemed acceptable over the spiking range studied when consideration is given to the fact that these spiking levels are in the parts per billion range. Our laboratory has observed that most infant formulas are fortified at either 8 or 15 μg vitamin K_1/100 Kcal.

Table 1

Recovery of Vitamin K_1 from Fortified Soy-Based Infant Formula Blank

Fortification Level Vitamin K_1	Average Recovery (%)[a]
8x[b]	95.0 ± 3.1 (3.3)
4x	102 ± 4.1 (4.0)
2x	90.2 ± 3.3 (3.7)
x	91.0 ± 3.0 (3.3)
1/2x	84.3 ± 3.0 (3.6)
Blank[c]	[found: 133 ± 4.7 ng/g (3.5)]

[a] Values are mean ± standard deviation. Percent CVs are given in parenthesis. [b] Five replicates were assayed at each spiking level and blank. x is equivalent t0 4 μg/100 Kcal. [c] Values for the blank correspond to naturally occurring levels for vitamin K.

Usually, even with an overage of fortification, the method is more than adequate to assay vitamin K_1 at levels of 150% of label declaration. Analysis of a commercially available soy based infant formula labeled to contain corn maltodextrin, palm olein, soybean oil, coconut oil, safflower oil, and soy protein isolate assayed at 11.4 ± 0.57 μg/100 Kcal (CV = 4.9%) (n=10). This value corresponds to 232% of the label declaration, which is consistent with the values that are routinely observed in our laboratory and with the expected overages added by infant formula manufacturers. AOAC International Method (992.27, 50.1.06)(13) is designed solely for milk based infant formula and was not compared to the present method. However, an earlier method developed by Landen[17] has been previously used routinely in this laboratory and consistently gave vitamin K_1 values greater that 200% of label declaration for soy based infant formula.

The purity of the vitamin K_1 peak was established by a rationing technique developed by Haroon et al.[18] The emission wavelength was kept constant for the analytes while fluorescence was measured at 3 excitation wavelengths. The fluorescence emission of vitamin K_1 at 418 nm was determined at excitation wavelengths of 238, 248, and 258 nm. Ratios were calculated for 238/248 and 258/248. The ratios were compared for the standard and the commercial infant formula extract (Table 2). Excellent agreement was obtained for ratios of standard and sample for vitamin K_1, indicating purity of the vitamin K_1 peaks. The addition of hexane to the mobile phase, standards and samples is essential in order to maintain low column back pressure and good recoveries.[1] When hexane is not utilized the column pressure will continue to climb as fat builds up on the column.

Table 2

Evaluation of Peak Purity[a]

Nutrient	Peak Ratio Wavelength (nm)	Peak Response Ratio[b] Standard	Sample
Vitamin K_1	238/248	0.59	0.59
	258/248	0.10	0.10

[a] Emission wavelengths were constant for vitamin K_1 (418 nm). [b] Ratios are based on the average of triplicate injections.

In addition, the hexane increases the recoveries by aiding in the solubility of vitamin K_1, since the nutrient is sparingly soluble in methanol. Previous studies observed that when hexane is omitted the recoveries drop into the 70% range.[1] Initial studies by Chase et al[14] using MSPD had utilized isopropyl palmitate as a modifier for the efficient elution of retinyl palmitate. Even though retinyl palmitate was not quantitated in this study, the isopropyl palmitate could not be eliminated from the method. Without the retinyl palmitate, recoveries were as low as 70%. The isopropyl palmitate acts as a keeper solvent for the vitamin K_1 protecting it from decomposition and somehow inhibits the vitamin K_1 from binding to the C_{18}.[14] This method provides a simple and rapid technique to assay vitamin K_1 in soy based infant formula using only 18 mL of solvent per sample. The small sample size of 0.5 g is not detrimental because properly prepared liquid samples are homogenous and the small sample size does not significantly influence the data. An experienced analyst in one working day with overnight injections can easily extract twenty samples.

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Received May 4, 1999
Accepted May 19, 1999
Manuscript 5059