A liquid chromatographic method for vitamin K₁ in milk-based infant formula is described. The vitamins are extracted from infant formula by matrix solid-phase dispersion and quantitated by reversed-phase chromatography with fluorescence detection. Vitamin K₁ is converted to the fluorescent hydroquinone with a postcolumn zinc reductive reactor. The limit of detection is 12 pg, and the limit of quantitation is 38 pg on-column. Linear responses were obtained in the range 0.55–22.1 ng/mL ($r^2 = 0.9998$). Recoveries of vitamin K₁ from an analyte-fortified blank material for milk-based infant formula averaged 91.7% ($n = 25$). The method provides a rapid, specific, and easily controlled assay for vitamin K₁ in fortified infant formula.

Unlike other fat-soluble vitamins, vitamin K₁ is unstable to alkaline conditions and cannot withstand saponification. For this reason, innovative extraction procedures have been developed to quantitate vitamin K in foods and other biological matrixes. Vitamin K₁ methods recently were reviewed in detail by Eitenmiller and Landen (1). Information on the vitamin K content of the food supply was greatly expanded through the work of Sadowski’s research group at the U.S. Department of Agriculture Human Nutrition Research Center on Aging, Tufts University (2–12). Procedural aspects of the methodology were recently reviewed (13).

Use of postcolumn chemical reduction of the quinone to the fluorescent hydroquinone allows selective and sensitive quantitation of vitamin K₁ after solid-phase extraction on silica and/or C₁₈ and reversed-phase chromatography.

Development of reliable fluorescence-based detection systems for converting the quinone (vitamin K) to the fluorescent hydroquinone followed the original work of Lagenberg and coworkers (14–16) and Haroon et al. (17), who used electrochemical and chemical reduction, respectively, to determine vitamin K₁ in plasma and other biological samples.

In the United States, infant formula is fortified at 4 μg vitamin K₁ per serving (18). The current AOAC INTERNATIONAL method (Method 992.27) for vitamin K₁ in infant formula (19) is based on studies by Hwang (20) that involve pretreatment of the formula with ammonium hydroxide and methanol followed by extraction with dichloromethane–isooctane, 2:1. Open-column silica chromatography is required for cleanup of the extract prior to normal-phase liquid chromatography (LC) on silica. Advantages of the AOAC INTERNATIONAL method are the avoidance of saponification and the ability to resolve vitamin K₁ from the biologically inactive cis isomer present in synthetic vitamin K₁ preparations. Problems with the AOAC method include hard-to-interpret chromatograms due to lipid interferences, inability to measure cis-vitamin K₁ in matrixes containing corn oil, and an overall high repeatability relative standard deviation (RSDr) of 20.9% (1).

Because of these deficiencies, investigators have explored many analytical avenues to improve methods for analysis of vitamin K₁ in infant formula. Indyk et al. (21) assayed total vitamin K₁ (trans and cis isomers) in infant formula by incorporating lipase digestion (22), extraction of the digest with hexane after addition of ethanol–methanol (95 + 5) and potassium carbonate, and semipreparative LC fractionation on silica. The purified extract was quantitated for vitamin K₁ content by reversed-phase chromatography on C₁₈ with detection at 269 nm. Because of the inability of reversed-phase chromatography to resolve trans- and cis-vitamin K₁, the method provided a measure of total vitamin K₁. However, the authors commented that the procedure was acceptable for production compliance because samples are monitored with reference to total vitamin K₁.

In more recent work, Indyk and Woolard (23) provided a simplified approach to the analysis of total vitamin K₁ in milk and infant formula. The method involves lipase digestion, extraction of the digest as previously noted (21), and centrifugation. Semipreparative LC was eliminated, and the hexane extract was evaporated with the residue redissolved in methanol. LC analysis incorporated postcolumn zinc reduction to induce formation of fluorescence and reversed-phase
LC for quantitation of total vitamin K₁. Recently, the method was subjected to an AOAC INTERNATIONAL collaborative study.

Ware et al. (24) used lipase and α-amylase digestion, extraction with 1% sodium bicarbonate–isopropyl alcohol (1 + 1), and C₁₈ solid-phase cleanup to prepare a variety of medical foods for vitamin K₁ analysis. Their determinative step used zinc reduction (postcolumn) and reversed-phase LC to measure total vitamin K₁. The method was applicable to a wide selection of medical foods of varying protein and fat contents. Zinc postcolumn reduction was used by Jakob and Elmadfa (25) in a study of the vitamin K₁ content of animal and plant foods. Electrochemical detection efficiently was used by Piironen et al. (26) and Koivu et al. (27) in determining vitamin K₁ in oils, margarine, butter, and cereals, respectively. The method relied on semipreparative LC for cleanup of extracts and use of MK-4 as an internal standard.

Researchers at the Atlanta Center for Nutrient Analysis, of the U.S. Food and Drug Administration, recently have established matrix solid-phase dispersion (MSPD) as a rapid and simple technique to extract retinyl palmitate, all-rac-α-tocopheryl acetate, vitamin E, and β-carotene from infant formula and medical foods (28–31). MSPD is a nonaggressive extraction approach that not only saves time and solvents but also decreases the chance for manual errors and analyte degradation. The objective of this research was to use MSPD to extract vitamin K₁ from infant formula and couple the analysis to zinc postcolumn reduction to utilize fluorescence as the detection mode.

**METHOD**

**Apparatus**

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

(b) Column.—Alltech C₈, 3 µm, 4.6 × 150 mm, part No. 287133 (Alltech Associates, Deerfield, IL).

(c) Integrator.—Waters Millenium Data System. A stand-alone integrator also can be used.

(d) Fluorescence detector.—Model 1046A programmable fluorescence detector (Hewlett Packard, Avondale, PA) or equivalent.

(e) Reservoirs with frits.—Varian 15 mL size, part No. 1213-1016 (Varian, Harbor City, CA).

(f) Turboevaporator.—Turbo Vap II (Zymark, Hopkinton, MA) or a suitable technique to evaporate the extracts.

(g) Vortex mixer.—Maxi-Mix I (Thermolyne, Dubuque, IA).

(h) Reduction reactor.—2.5 cm × 3.2 mm stainless steel, packed with zinc powder, 100 mesh (Aldrich Chemical Co., Milwaukee, WI).

(i) Ultrasonic cleaner.—5.2 gallon ultrasonic cleaner (Thomas Scientific, Swedesboro, NJ).

**Reagents**

(a) Hexane.—LC grade (Burdick and Jackson, Muskegon, MI).
Prepare this solution by weighing 68.1 g zinc chloride and sodium acetate, and 1.0 M acetic acid per liter of methanol.

Filter the mobile phase through a 0.45 µm filter prior to use.

The diluting solution containing 10% hexane. Filter the mobile phase through a 0.45 µm filter prior to use.

Vitamin K₁ standard.—Accurately weigh 25 mg vitamin K₁ (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 the 5 final working standards ranged from 0.553 to 2.48 µg/mL.

Prepare this solution by weighing 68.1 g zinc chloride and sodium acetate, and 1.0 M acetic acid per liter of methanol.

Filter the mobile phase through a 0.45 µm filter prior to use.

The diluting solution containing 10% hexane. Filter the mobile phase through a 0.45 µm filter prior to use.

Mobile phase.—The diluting solution containing 10% hexane. Filter the mobile phase through a 0.45 µm filter prior to use.

Bondesil.—C₁₈ preparative grade, part No. 1221-3013 (Varian).

Isopropyl palmitate.—Cat. No. 29,178-1, tech 90%, (Aldrich Chemical Co.).

Spiking solutions.—Appropriate dilutions were made from the vitamin K₁ stock standard in hexane so that an aliquot (Aldrich Chemical Co.).

Vitamin K₁ as listed in the Code of Federal Regulations (18).

Blankc 100 ± 6.7 (6.7)

1/2× 101 ± 6.7 (6.7)

× 95.7 ± 2.1 (2.2)

2× 88.8 ± 1.7 (1.9)

4× 86.4 ± 0.6 (0.7)

8× 86.6 ± 0.4 (0.5)

Table 1. Recovery of vitamin K₁ from a fortified milk-based infant formula blank

<table>
<thead>
<tr>
<th>Fortification level</th>
<th>Average recovery, % (CV, %)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>8×</td>
<td>86.6 ± 0.4 (0.5)</td>
</tr>
<tr>
<td>4×</td>
<td>86.4 ± 0.6 (0.7)</td>
</tr>
<tr>
<td>2×</td>
<td>88.8 ± 1.7 (1.9)</td>
</tr>
<tr>
<td>×</td>
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¹ Values are means ± standard deviations.

Table 2. Evaluation of vitamin K₁ peak purity⁵

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<tr>
<th>Peak ratio wavelength, nm</th>
<th>Standard</th>
<th>Sample</th>
</tr>
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<tr>
<td>238/248</td>
<td>0.66</td>
<td>0.64</td>
</tr>
<tr>
<td>258/248</td>
<td>0.14</td>
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</tr>
</tbody>
</table>

⁵ Ratios are based on the average of triplicate injections.

Sample Description and Preparation

A zero control reference material (ZRM; 32) originally used for fat-soluble method development studies was used for recovery studies. National Institute of Standards and Technology (NIST; Gaithersburg, MD) SRM 1846, milk-based infant formula, also was used to validate the method. The infant formula ZRM was prepared by weighing ca 10 g of the ZRM, combining it with 50 g boiling water, and thoroughly mixing. For SRM 1846, the entire packet contents (ca 30 g) was weighed, combined with 150 g water (90°–100°C), and thoroughly mixed.

Sample Extraction

Weigh 2 g Bondesil C₁₈ into a mortar. Add 100 µL isopropyl palmitate and gently blend the isopropyl palmitate onto the C₁₈ with a pestle. Accurately weigh 0.50 g reconstituted sample into the C₁₈–isopropyl palmitate mixture and add the spike solution. Use the pestle to gently blend the reconstituted sample and the C₁₈–isopropyl palmitate into a fluffy, slightly sticky powder. Accurately transfer the C₁₈–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. Evaporate the combined eluates at 45°C in the Turbo Vap under 5 psi of nitrogen to near dryness. Dilute residue to 1 mL with hexane. Agitate the Turbo Vap vessel on a Vortex mixer for 15 s and then sonicate

Table 2. Evaluation of vitamin K₁ peak purity⁶

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⁶ Emission wavelengths were constant for vitamin K₁ (418 nm).
for 15 s. Quantitatively transfer hexane extract to a 10.0 mL volumetric flask and bring to volume with the diluting solution.

Calculation

The concentrations (ng/mL) of vitamin K1 in the sample extract are calculated by linear regression analysis.

Results and Discussion

Figure 1A shows a chromatogram of naturally occurring vitamin K1 in the blank. Vitamin K1 is native to many oils used in infant formula and is present at a level (100 ng/g) corresponding to less than 2 μg/100 kcal of infant formula, which is less than half the minimum level of 4 μg/100 kcal required by law (18). Because by definition a ZRM is devoid of the nutrient of interest (32), this low level found for vitamin K1 makes the ZRM suitable for use as a blank for spiking purposes, provided that each spiking level is corrected for the natural occurring vitamin K1. Figure 1B shows the chromatogram of a vitamin K1 standard overlaid with the extract of a commercial infant formula at a concentration of 277 pg on-column. Fluorescence responses for vitamin K1 hydroquinone were linear from 0.55 to 22.1 ng vitamin K1/mL ($r^2 = 0.9998$). A standard set consisting of 5 standards in the concentration range 0.55–22.1 ng/mL injected in duplicate on 9 occasions over a 26-day period gave a standard deviation of ±732 (coefficient of variation, CV = 5.9%). The vitamin K1 hydroquinone gave a limit of detection of 0.20 ng vitamin K1/mL, corresponding to about 27 ng/g, or an on-column concentration equivalent to 12 pg. The limit of quantitation was 0.64 ng vitamin K1/mL, corresponding to 80 ng/g or an on-column concentration equivalent to 38 pg.

Table 1 shows recoveries from milk-based infant formula blanks spiked at the 1/2×, ×, 2×, 4×, and 8× levels, where × is the minimum level for vitamin K1 as listed in the Code of Federal Regulations (18). Each spiking level was assayed 5 times. The 100 ng/g level found in 5 replicates of the blank served as a baseline value and was subtracted from each subsequent spiking level.

Vitamin K1 recoveries were acceptable over the spiking range studied, given that spiking levels are in the parts-per-billion range. Our laboratory has observed that most infant formulas are fortified at either 8 or 15 mg vitamin K1/100 kcal. Usually, even with an overage of fortification, the method is more than adequate to assay vitamin K1 at levels of 150% of label declaration.

Additionally, the method was tested on 10 replicates of NIST milk-based infant formula SRM 1846. For this study, the entire packet of SRM 1846 was mixed with 150 mL water (90°–100°C). Total vitamin K1 in SRM 1846 was 0.944 ± 0.026 mg/kg (CV = 2.8%). This amount agrees with the NIST certificate for SRM 1846, which lists vitamin K1 as a mass fraction of 1 mg/kg but does not provide a certified value for vitamin K1. Our laboratory routinely assays SRM 1846 by the AOAC INTERNATIONAL method (1) and has observed values ranging from 0.628 to 1.23 mg/kg. Recently, Ware et al. (8) observed a mean value of 0.95 ± 0.088 mg/kg (range, 0.807–1.123 mg/kg; CV = 9.3%) for 31 separate analyses. In
that study, as well as in our regulatory laboratory, 2.5 g from each packet of SRM 1846 was dissolved in hot water (50°–60°C). According to the new certificate released by NIST, the SRM 1846 sample is to be mixed with water at 90°–100°C to avoid problems with homogeneity.

Analysis of an in-house control consisting of a commercially available milk-based infant formula gave 11.4 ± 0.57 µg/100 kcal (CV = 4.9%; n = 10). This value corresponds to 143% of the label declaration and is consistent with the values that are routinely observed in our laboratory with the AOAC method (19) and with the expected overages used in fortification.

As an integral part of any method development work, the peak purity of the vitamin K₁ was established by peak ratios (17). The emission wavelength was kept constant for analytes while fluorescense was measured at 3 excitation wavelengths. The fluorescence emission of vitamin K₁ at 418 nm was determined at excitation wavelengths of 238, 248, and 258 nm. Ratios were calculated for 238/248 and 258/248 and compared for the standard and the commercial infant formula extract (Table 2). Good agreement was obtained for ratios of standard and sample for vitamin K₁, indicating purity of the vitamin K₁ peaks.

Addition of hexane to the mobile phase, standards, and samples is essential to maintain low column backpressure and good recoveries. When hexane was not used, column pressure became excessive from fat buildup on the column. Use of 10% hexane, which is miscible with methanol, kept the fat in solution and allowed maintenance of a lower column pressure. Hexane also increased recoveries by solubilizing vitamin K₁, which is only sparingly soluble in methanol.

Previously, Chase et al. (28–31) used isopropyl palmitate as a modifier for the efficient elution of retinyl palmitate. But because retinyl palmitate was not quantitated in that study, we tried to eliminate it from the method. Initial studies without the retinyl palmitate resulted in recoveries as low as 70%. As discussed in an earlier work (28), the isopropyl palmitate acts as a keeper solvent for the vitamin K₁.

Figure 2 represents 90 injections of the extract of the in-house control. Because MSPD does not remove the fat, we determined whether the fat would have an effect on the zinc reduction column. Initially, 10 injections were made of the extract of the in-house control to serve as a baseline from which to measure the change for the remaining 90 injections. When the hexane was omitted from the mobile phase, the response for the vitamin K₁ in the in-house control extract changed by >20% after 90 injections. However, inclusion of hexane in the mobile phase, standards, and sample extracts resulted in a change of <10% for 90 injections. The peak response and retention time of the standard injected after every 20 sample injections remained unchanged. No problems were observed with miscibility with 10% hexane in methanol.

This method provides a simple and rapid technique to assay vitamin K₁ in milk-based infant formula and requires only 18 mL 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. A competent analyst can easily extract 20 samples in 1 working day with overnight injections.

References


