Development and Single-Lab Validation of an UHPLC-APCI-MS/MS Method for Vitamin K_1 in Infant Formulas and Other Nutritional Formulas

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Abstract

Accurate analysis of vitamins is essential to help the public maintain adequate intakes of vitamins. Currently in Atlanta Center for Nutrient Analysis (ACNA), AOAC Method 999.15 with fluorescence detection is utilized for the analysis of vitamin K in infant formulas, dietary supplements and other medical foods. An UHPLC-(+)-APCI-MS/MS method for vitamin K_1 (phylloquinone) was developed to improve the accuracy, selectivity and efficiency of the analysis. SRM1849a and infant formula samples were used to demonstrate that vitamin K_1 data by LC-MS/MS analysis matched well with those from the AOAC Method 999.15. A single-laboratory validation of an UHPLC-MS/MS method for vitamin K_1 analysis in SRM1849a showed good accuracy with a mean value of 99.6% of the certified value (n = 8). Recoveries of vitamin K_1 at two different spike levels were 99.6 and 103.7% from SRM1849a. Mean recovery of vitamin K_1 from four different infant formula samples was 102.4% ranging from 95.6 to 115.5% with %RSD of 7.8 ~15.6. Precision, measured as repeatability (%RSDr), was 8.7 for SRM1849a and ranged from 3.7 to 13.4 for four infant formula samples. Application of this method will help ACNA facilitate the accurate analysis of vitamin K in infant formulas and other samples.

Keywords:
weight loss drugs, sibutramine, LC-MS/MS

1. Introduction

Infant formulas and dietary supplements containing various vitamins are widely consumed by the general public to maintain adequate intakes of vitamins. Vitamin deficiencies due to insufficient intake tend to occur in developing countries or from medical conditions that interfere with the absorption or metabolism of vitamins[1]. Toxicity due to higher intakes of certain vitamins, such as vitamins A and D from supplements and fortified foods also poses a safety concern as well [2]. Vitamin K exists in nature as vitamin K_1 (phylloquinone) from plants and algae and vitamin K_2 [collectively referred to as menaquinones (MK), including MK-4 and MK-7] from bacteria with a variable number ($4 \sim 10$) of unsaturated isoprenoids attached to the 1,4-naphthoquinone moiety [3]. The most important physiological function of vitamin K is to serve as a cofactor in the conversion of vitamin K-dependent proteins to their active forms, including prothrombin in blood coagulation[4]. The major dietary source of vitamin K is phylloquinone from plant sources, and the highest concentrations of vitamin K_1 are found in green leafy vegetables. Significant concentrations are also present in other vegetables, vegetable oils, fruits, grains and dairy products [5]. Vitamin analysis in infant formulas and related products is inherently difficult due to different chemical structures and characteristics of water- and fat-soluble vitamins, disparate concentrations, and complex matrices. Thus, analytical methods for the fat soluble vitamins traditionally required separate and multiple sample extraction steps with different detection techniques for specific vitamins. Recently, several liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods for vitamin analysis of infant formulas have been officially given Official Action status by AOAC INTERNATIONAL. These methods include vitamin D analysis in infant formula and adult/pediatric nutritional formula [AOAC Methods 2011.11][6], analysis of total folates in infant formula and adult nutritionals [AOAC Method 2011.06] [7], and pantothenic acid analysis in infant formula and other nutritional formula [AOAC Method 2012.16] [8]. At present, no official methods for the analysis of other vitamins utilizing LC-MS/MS have been approved by AOAC INTERNATIONAL.

Current official methods utilized in ACNA for vitamin K

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analysis in infant formulas and other food matrices are AOAC Methods 992.27 [trans-Vitamin K_1 (Phylloquinone) in Ready-To-Feed Milk-Based Infant Formula: Liquid Chromatographic Method] and 999.15 [Vitamin K in Milk and Infant Formulas: Liquid Chromatographic Method] [9]. AOAC Method 999.15, which utilizes fluorescence detection with the post-column derivatization, has been used for the analysis of vitamin K in many different matrixes due to its robustness, though it has been applicable mainly to the determination of total vitamin K_1 in samples with >1 μg vitamin K_1/100 g. These methods cannot, however, distinguish *trans*-vitamin K_1 from *cis*-vitamin K_1, the biologically inactive isomer.

Application of LC-MS in the analysis of vitamin K and other thermally labile fat-soluble vitamins in foods was limited until soft ionization methods, especially atmospheric pressure chemical ionization (APCI), were widely adopted [10]. Suhara et. al [11] showed that LC-APCI-MS/MS was applicable to the assay of vitamin K analogues in human plasma. Later work by Ducros et. al [12] also demonstrated that APCI was more sensitive than ESI for vitamin K analysis in plasma. Phinney et. al [13] developed isotope dilution LC-MS methods for the analysis of fat and water soluble vitamins in a number of Standard Reference Materials (SRM). Furthermore, a number of LC-MS/MS applications for the simultaneous determination of multiple vitamins in infant formulas and other foods have been reported [14, 15]. Schimpf et. al [16] also reported the chromatographic separation of *trans*-vitamin K_1 from its *cis*-isomer on a C_{30} column. LC-MS methods for the analysis of vitamin K in foods were extensively reviewed by Eitenmiller et. al [17], Ahmed et.al [18], and Gentili [19].

This study describes the single laboratory validation of a HPLC-MS/MS method for vitamin K_1 in infant formulas utilizing multiple reaction monitoring (MRM) under the positive APCI mode. While the same extraction method in AOAC Method 999.15 was used for this study, the new method with MRM quantitation renders the post-column derivatization step with zinc unnecessary. Application of this method will help to ensure the accurate assessment of vitamin K in infant formulas, medical foods and other nutritional formulas.

2. Experimental

2.1. Chemicals and reagents

Reference standard and samples with declared amount of vitamin K_1 (Table 1): (a) Standard reference material. Infant/Adult Nutritional Formula (Catalog No.: SRM1849a) from National Institute of Standards and Technology [NIST] (Gaithersburg, MD). (b) Infant formula and medical food samples. Commercially available milk-based infant formulas and medical foods.

Other Chemicals and Reagents: Vitamin K_1 analytical standard (Phylloquinone, $C_{31}H_{46}O_2$, CAS: 84-80-0) from SigmaAldrich and USP, Acetonitrile (MeCN) and methanol (MeOH): LC-MS grade or equivalent, Hexane: HPLC grade or equivalent. Lipase: Type VII from Candida rugosa, Sigma-Aldrich. Potassium carbonate (K_2CO_3, anhydrous): ACS grade or equivalent,

EMD. Monobasic potassium phosphate (KH_2PO_4) and potassium hydroxide (KOH), ACS Reagent grade or equivalent. Syringe filter: 4 mm, 0.22 μm PTFE membrane. Ammonium formate (NH_4HCO_2): Mass spectrometry grade.

2.2. Equipment

LC-MS/MS system QTRAP-4000 mass spectrometer with Analyst (Version 1.5.2) and MultiQuant (Version 2.1) software for data acquisition and analysis (AB SCIEX, Framingham, MA), interfaced with Agilent 1290 Infinity LC system with autosampler, binary pumps and a thermostatted column compartment (Agilent Technologies, Inc. Santa Clara, CA). Agilent 1200 Series HPLC system with a fluorescence detector (FLD) was used to generate LC-FLD data from regulatory samples to compare LC-MS and LC-FLD data.

2.3. Vitamin K_1 Extraction

The extraction of vitamin K_1 followed AOAC Method 999.15. Briefly, about 1 g of powdered sample was accurately weighed and dissolved in 15 mL of warm water (<40 ^{o}C) with vortexing. Phosphate buffer (0.8 mM, 5 mL) and lipase powder (1 g) were subsequently added. The sample was incubated at 37 ± 2°C for 2 h and cooled to ambient temperature. Reagent alcohol (10 mL) and K_2CO_3 (1 g) were added and mixed. The solution was extracted with hexane (30 mL) and an aliquot (1.0 mL or less depending on the vitamin K_1 amount in samples) was transferred into LC vials. Solvent was evaporated under a gentle stream of N_2, and dried extracts were reconstituted with MeOH, passed through the syringe filter (0.22 μ PTFE) into low volume glass inserts in LC vials for the analysis.

2.4. Chromatographic Separation

An Accucore C18 column (2.6 μm particle size, 2.1 mm i.d., X 50 mm, Thermo Scientific Inc., Gibbstown, NJ), connected to the Accucore C18 guard column (2.6 μm particle size, 2.1 mm i.d., X 10 mm), was used. The column compartment was maintained at 40 ^{o}C and the injection volume was set at 5 μL. Chromatographic separation of vitamin K_1 was achieved under the gradient flow (0.6 mL/min) of eluents, initially at 50/50 mix of [A] water and MeCN (50/50, v/v with 0.1% formic acid) and [B] MeCN and MeOH (75/25, v/v with 2.5 mM ammonium formate). The gradient was then changed at 0.25 min to 100% of [B] at 1.3 min, maintained there until 3.9 min, then switched back to 50% of [B] at 4.0 min. The total run time was 4.2 min per injection including the time for re-equilibration (Table 2). The retention time of vitamin K_1 was at 2.59 min.

3. Results and Discussion

3.1. Optimization of Chromatography

A solid core HPLC C18 column (2.6 μm particle size, 2.1 mm i.d.) was selected over a fully porous counterpart during the initial evaluation of the different columns. Under the same LC condition, the retention time for vitamin K_1 was four times longer when a fully porous C18 column (5 μm particle size, 4.5 mm i.d.) was used. In addition, the back pressure of

Table 1. Description of the reference standard material, infant formula and medical food samples

Sample Descriptors	Declared amount of vitamin K_1/serving (μg, unless specified otherwise)	Serving size (g)	Sample Types
SRM1849a	1.06 ± 0.17 mg/kg		SRM
1	8	19.6	Powder infant formulas
2	8	21.75	
3	9	19.7	
4	9	19.7	
5	8	78.43	Liquid Infant formulas
6	8	150.6	
7	11	78.45	
8	12	101.4	
9	16	253	Liquid medical foods
10	19	108.34	
11	30	100	Powder medical foods
12	61	1072	Liquid medical foods
13	85	1040	

Table 2. HPLC gradient conditions

Time (min)	Flow rate (μL/min)	A (%)	B (%)
0.00	600	50	50
0.25	600	50	50
1.30	600	0	100
3.90	600	0	100
4.00	600	50	50
4.10	600	100	0
4.20	600	50	50

LC was significantly higher when a fully porous column with similar length was used. Extraction of vitamin K_1 only with ethyl acetate without addition of lipase, similar to the ones described by Phinney et. al [13] for the analysis of fat soluble vitamins in SRM1849a, provided minimal extraction efficiency in infant formulas (less than 20% compared with the extraction utilizing lipase, data not shown). Extraction conditions of AOAC Method 999.15 results in the degradation of other fat soluble vitamins present in infant formulas, including vitamin D, retinyl esters and α-tocopherol acetate. Therefore, the optimized final positive-APCI-MRM method is useful for only vitamin K_1 quantitation, although other fat soluble vitamins were chromatographically resolved.

Representative total ion chromatograms (TIC) and extracted ion chromatograms (XIC) of vitamin K_1 by LC-MS/MS in standards, sample extracts from SRM1849a and milk-based formulas are presented in Figure 1. No interfering peaks were present under the optimized conditions. The ratio of the amount of vitamin K_1 between AOAC Method 999.15 using fluorescence detection (FLD) and those from LC-MS/MS are presented in Table 4, where the same extracts from the samples were used for the comparison. Overall, the values for vitamin K_1 by LC-MS/MS were, although slightly higher, not significantly different than those obtained by LC-FLD. For example, Vitamin K_1 values in SRM1849a by LC-MS/MS analysis (n = 5) were 12.7% greater than those determined by LC-FLD. Again, LC-MS/MS values of vitamin K_1 from 10 infant formula and medical food samples were 8.9% higher in average (not statistically

significant with p = 0.438 from one-sample Students t-test) than those analyzed by LC-FLD. Higher values obtained by LC-MS/MS are most likely due to the incomplete reaction of vitamin K_1 during the post-column derivatization step in AOAC Method 999.15, where the reduction of vitamin K_1 to the corresponding fluorophore is required for quantitation. For most infant formulas and medical food samples in Table 4, LC-MS/MS analysis gave higher values of vitamin K_1 compared to the LC-FLD quantitation. Furthermore, LC-MS/MS analysis provides much shorter run time and the confirmation of the target compound as well. It is also noteworthy that the measured values of vitamin K_1 were significantly higher (up to 2.7-fold) than the declared amount in many samples. *Trans-* and *cis-*vitamin K_1 were baseline separated with a solid-core Accucore C30 column (2.6 μm particle size, 2.1 mm i.d. X 100 mm), which recently became available. The retention time of trans- and cis-vitamin K_1 were 6.99 and 7.34 min with an Accucore C30 guard column (2.6 μm particle size, 2.1 mm i.d., X 10 mm), respectively, when the temperature of the column compartment and the solvent flow were maintained at 15 ^{0}C and 0.35 mL/min of MeCN and MeOH (75/25, v/v, with 2.5 mM ammonium formate), respectively [Figure 2].

3.2. Validation

3.2.1. Linearity

The overlay chromatogram of five levels of vitamin K_1 standards by LC-MS/MS MRM analysis is presented in Figure 3. Response peak areas of the quantitation transition of vitamin

Table 3. Mass spectrometry parameters for measurement of vitamin K_1

Analyte	Parent ion (m/z)	Product ion (m/z)	DP[a]	EP[a]	CE[a]	CXP[a]
vitamin K_1	451.3	187.0	75	45	39	11
	451.3	197.0	75	45	39	11
	451.3	128.1	75	45	110	11

[a]DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Cell Exit Potential (units, V)

K_1 standards were plotted against the different concentrations. Correlation coefficients (r) were 0.9995 or better. The calibration curve could be extended up to 300 ng/mL of vitamin K_1 concentration, if necessary.

3.2.2. Accuracy

LC-MS/MS assay of SRM1849a resulted in a mean value of 1.056 mg/kg (n = 8, with%RSD of 8.7) [Table 5]. This is similar to the certified value of 1.06 ± 0.17 mg/kg by NIST (not significantly different after Students t-test, p = 0.909). This demonstrates the excellent accuracy of the LC-APCI-MS/MS method, when coupled with the extraction procedures from AOAC Method 999.15. It should be noted that the certified value was obtained by LC-MS with ethyl acetate extraction, followed by resolution on a C18 column with an isocratic mobile phase of MeOH and MeCN (60/40, v/v, 5 mM ammonium acetate) [13]. Values of vitamin K_1 found by LC-MS/MS analysis are listed in Table 4 for the selected infant formula samples 1 to 4. Spike recovery studies were carried out with these samples. Recovery data for SRM1849a and the milk-based infant formulas are summarized in Table 6. The spike recovery of vitamin K_1 in SRM1849a was evaluated at levels of 10 and 100% of the certified values. Mean recoveries at least 4 replicates were 99.6 and 103.7% with RSDs of 8.3 and 10.0%, respectively. In selected infant formula samples, vitamin K_1 was spiked at 30 to 300% of the declared amount with recoveries ranging from 95.6 to 115.5% (with RSDs of 7.8 to 15.6%).

3.2.3. Precision

Quantitation by LC-MS/MS method showed good precision for vitamin K_1 concentrations in the range studied. Repeatability (%RSDr) for SRM1849a was 8.7% (Table 5), and the values of %RSDr for different infant formula samples ranged from 3.7 to 13.4% (Table 7).

3.2.4. Carryover

The issue of carryover was evaluated by measuring the signal of vitamin K_1 in MeOH as a solvent blank, immediately after a run of the intermediate working standard (5 μL injection, 2.75 μg/mL). No carryover was observed (Figure 4).

4. Conclusion

Quantitation of vitamin K_1 in infant formulas via LC-MS/MS analysis, following the extraction procedures in AOAC Method 999.15, compared closely with values obtained by the traditional LC-FLD analysis. The UHPLC-APCI-MS/MS method

afforded additional confirmation of vitamin K_1 and rendered the post-column derivatization using zinc powder unnecessary. A C30 column can be utilized when quantitation of both trans- and cis-vitamin K_1 are necessary. Studies are underway to expand the LC-MS/MS method to other fat soluble vitamins and other matrices, including medical foods and other dietary supplements.

4.1. Mass Spectrometer Parameters

Vitamin K_1 solution in MeOH (ca. 10 μg/mL) was infused (at a flow rate of 10μL/min) under the positive APCI mode for the initial compound optimization. Solvent flow of 0.6 mL/min produced the best ionization efficiency of vitamin K_1 under APCI. The parameters for curtain gas (CUR), collision gas, nebulizer current, temperature (TEM), ion source gas 1 were optimized at 10, High, 3, 500 ^{o}C and 30, respectively. Declustering potential (DP), entrance potential (EP), cell exit potential (CXP) were set 75, 45 and 11 V, respectively (Table 3). The transitions from a molecular ion (m/z = 451.3 for $[M+H]^+$) to three product ions were selected for the MRM analysis of vitamin K1.The transition of 451.3 → 187.0 was chosen as a quantitative transition, while two others (451.3→ 197.0 and 451.3 → 128.1) were used as qualification transitions [20]. The values for collision energy (CE) for the transitions were optimized at 39, 110 and 39 V, respectively. The retention time for vitamin K_1 was 2.59 min with a 50 mm Accucore C18 column. The scheduled MRM (sMRM) feature was utilized for the sample analysis. Target retention time for vitamin K_1, MRM detection window, target scan time were set at 2.6 min, 60 sec and 0.5 sec, respectively. When confirmation of vitamin K_1 was necessary, enhanced product ion (EPI) scan (positive polarity, 4000 Da/sec scan rate) was triggered by information dependent acquisition (IDA). Triggering condition of the single most intensive signal in sMRM window of vitamin K_1 was set at > 4000 cps, to verify the identity of a peak.

4.2. Single Laboratory Validation

Guidelines provided by FDA Foods Program Science and Research Steering Committee (SRSC) were followed [21]. The experimental results obtained were expressed as means SD with %RSD, where necessary.

Accuracy Accuracy was evaluated by analyzing vitamin K_1 in SRM1849a (n = 8) with comparison to the certified value, and by spiking SRM1849a and selected infant formula samples. Based on the Guidelines mentioned above[21], samples were spiked with vitamin K_1 at different levels. Recovery was calculated by the following equation.

Table 4. Comparison of vitamin K_1 analysis data between LC-FLD and LC-MS/MS in SRM1849a, infant formula and medical food samples

SRM	NIST value[a] mean (mg/kg) ± U	LC-FLD[b] mean (mg/kg) ± S.D.	LC-MS/MS	Ratio between MS/MS vs. FLD mean ± S.D.	n
SRM1849a	1.06 ± 0.17	0.998 ± 0.03	1.124 ± 0.05	1.127 ± 0.06	5
SRM1849[c]	2.20 ± 0.18	2.23	2.17	0.973	2

Sample	Declared (µg/serving)	LC-FLD (µg/serving)	LC-MS/MS	Ratio between MS/MS vs. FLD
1	8	20.6	18.8	0.913
5	8	15.7	17.5	1.115
6	8	14.2	17.9	1.261
7	11	17.4	20.7	1.190
8	12	9.4	10.7	1.138
9	16	46.0	44.1	0.959
10	19	27.4	29.9	1.091
11	30	30.3	29.6	0.977
12	61	76.2	89.5	1.175
13	85	157.4	167.8	1.066
Mean ± S.D.				1.089 ± 0.111[d]

[a]Value listed in the certificate of analysis (COA) by NIST, where U stands for the expanded uncertainty
[b]LC-FLD: AOAC Method **999.15**
[c]Superseded by SRM1849a
[d]Values from LC-FLD and LC-MS/MS are not significantly different

Table 5. Accuracy of vitamin K_1 analysis by LC-MS/MS of SRM1849a

Sample	NIST value[a] mean (mg/kg) ± U	Vitamin K_1 amount found mean (mg/kg)	%RSD$_r$	Number of replicates
SRM1849a[b]	1.06 ± 0.17	1.056	8.7	8

[a]Value listed in the certificate of analysis (COA) by NIST, where U stands for the expanded uncertainty
[d]Values of vitamin K_1 in SRM1849a are not significantly different from the NIST certified value.

Table 6. Spike recoveries of vitamin K_1 from SRM1849a and infant formula samples

Sample[a]	Declared amount of vitamin K_1	Spiked amount of vitamin K_1	Unit	Spike recovery mean (%)	RSD (%)	Number of replicates
SRM1849a	1.06	0.14	mg/kg	99.6	8.3	4
		1.37		103.7	10.0	6
1	8	26.8	µg/serving	102.5	15.4	4
2	8	3.0		115.5	13.8	4
3	9	2.7		95.8	7.8	4
4	9	2.7		95.6	15.6	5

[a]Same sample descriptors in Table 1.

Table 7. Precision (repeatability, %RSD$_r$) for the analysis of infant formula by LC-MS/MS

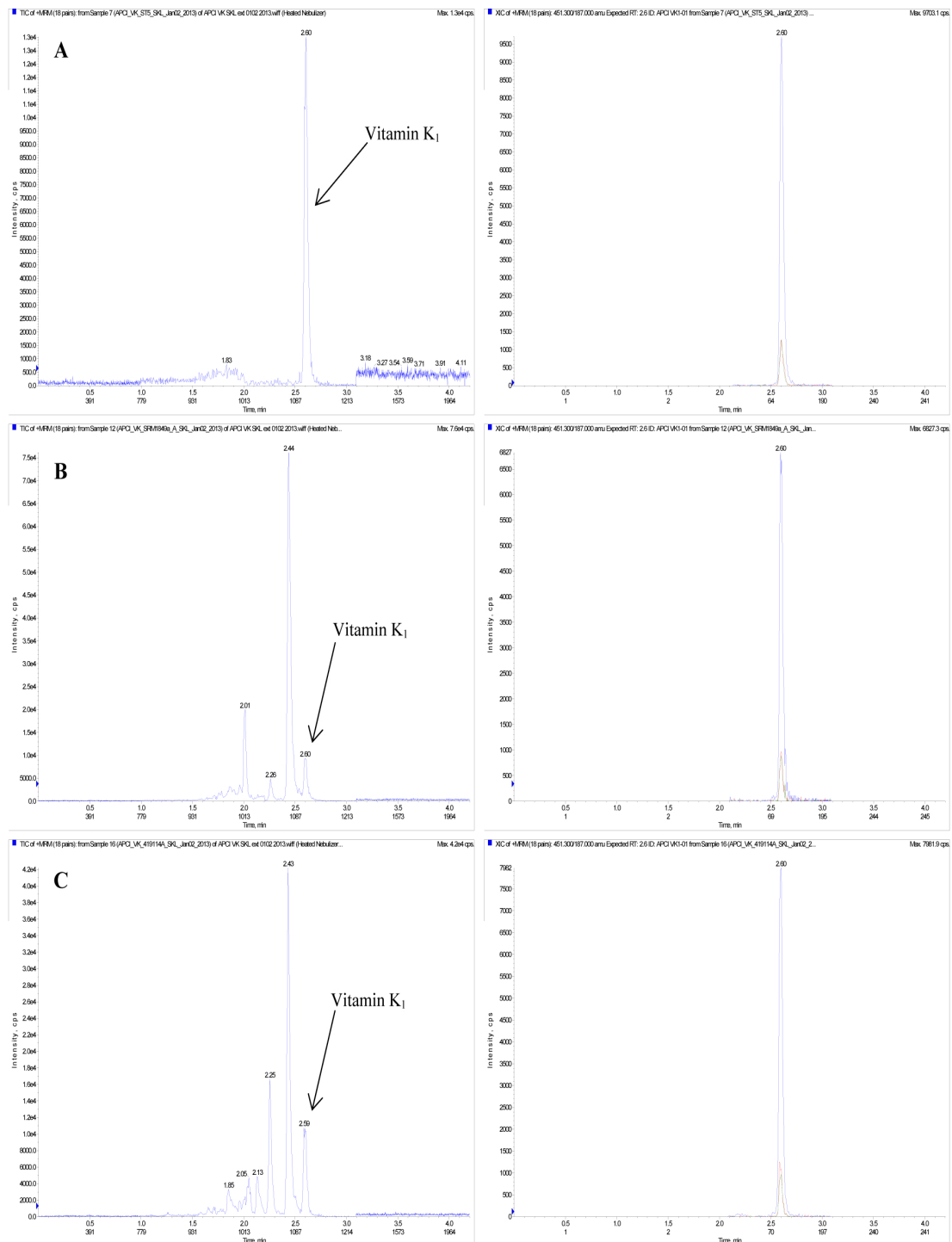| Sample[a]	Declared amount of vitamin K_1	Unit	Vitamin K_1 mean amount found	%RSD$_r$	Number of replicates
1	8	µg/serving	20.0	7.5	4
2	8		15.2	13.4	4
3	9		10.3	9.7	6
4	9		11.8	3.7	5

[a]Same sample descriptors in Table 1.

Figure 1. Representative total ion chromatograms (TIC, left panes) and extracted ion chromatograms (XIC, right panes) of vitamin K_1 in A) standards, B) sample extracts from SRM1849a and C) sample extracts from a milk-based infant formula by LC-APCI-MS/MS.
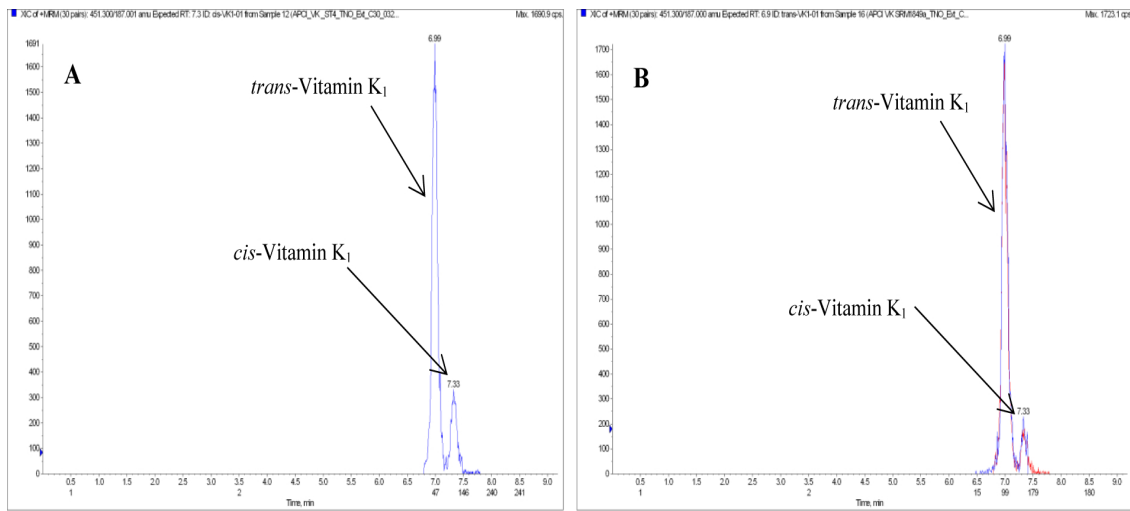
Figure 2. Chromatographic separation of *trans-* and *cis-*vitamin K_1 (XIC) in A) standards and B) sample extracts from SRM1849a by LC-APCI-MS/MS.
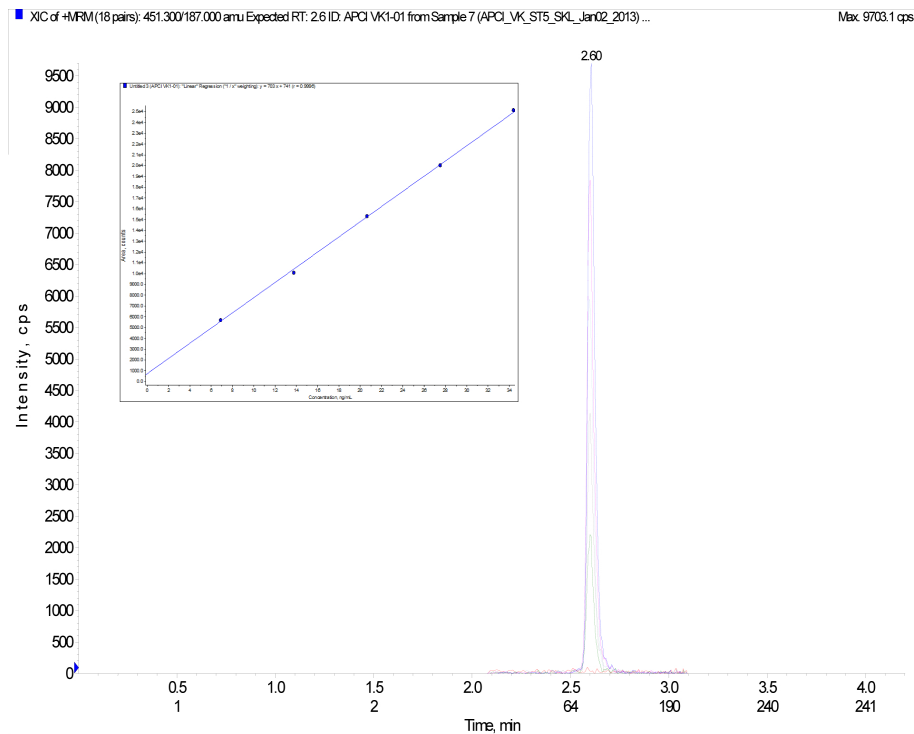

Figure 3. The overlay of the quantitation transition peaks of vitamin K_1 (5 different levels standards and the solvent). Typical calibration curve of vitamin K_1 is presented in an inset.
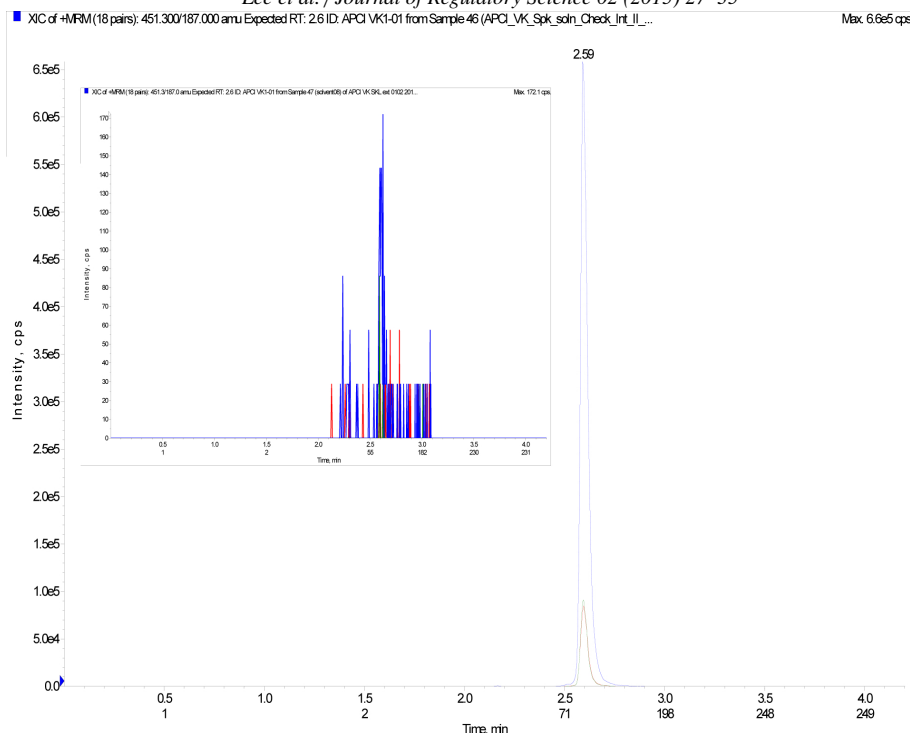
Figure 4. Verification of the absence of vitamin K$_1$ carryover with an Accucore C18 column by monitoring vitamin K$_1$ peaks from the subsequent injection of solvent, preceded by the injection (5 μL) of vitamin K$_1$ solution (2.75 μg/mL). Different scale on y-axis for the solvent blank is noted in the inset.

Spike recovery of vitamin K$_1$ (%) = [Amount recovered (μg/serving)] X 100 / [amount spiked (g/serving)]

Precision Repeatability relative standard deviation (%RSDr) in LC-MS/MS analysis and spike recovery of vitamin K$_1$ was calculated with SRM1849a and four different infant formula samples.

Linearity For the ease of comparison between two different methods, concentrations of vitamin K$_1$ standards (5 points) for calibration from AOAC Method 999.15 were also used for LC-MS/MS method. The intermediate working standard of about 2.75 μg/mL of vitamin K$_1$ in MeOH was prepared on the day of the analysis by diluting a stock standard solution (ca. 1.0 mg/mL in MeOH, accurately weighed). From the intermediate working standard, 5 levels were prepared to yield vitamin K$_1$ standard solutions with the concentrations of about 5   35 ng/mL.

Statistical analysis Students t-test was performed using the Prism 6.02 (GraphPad Software Inc., La Jolla, CA) to test the statistically significant differences between vitamin K1 data measured by LC-MS/MS in SRM1849a and the certified value of vitamin K$_1$ by NIST. The same method was also used to test the statistical significance between the measured values of vitamin K$_1$ by LC-MS/MS and those by LC-FLD in infant formulas.

Calculations 1) Amount of vitamin K$_1$/serving size (μg/g) = [Conc. found (ng/mL) X dilution factor X serving size (g)] /[sample weight (g) X 1000 ng/μg]

2) Vitamin K$_1$ found vs. declared (%) = [Amount found (μg/serving)] X 100 / [amount declared (μg/serving)]

5. Declaration of Conflicting Interest

The authors declare that there is no conflict of interest. Research was funded by U.S. Food and Drug Administration.

6. Disclaimer

The views expressed are those of the authors and should not be construed to represent the views or policies of the U.S. Food and Drug Administration. Any reference to a specific commercial product, manufacturer, or otherwise, is for the information and convenience of the public and does not constitute an endorsement, recommendation or favoring by the U.S. Food and Drug Administration.

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8. Article information

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