

Simultaneous Determination of Vitamin A and E in Infant Formula by HPLC with Photodiode Array Detection

Hong-Min Lee, Byung-Man Kwak, Jang-Hyuk Ahn*, Seung-Hwan Jeong, Sung-Lye Shim¹,
Kyong-Su Kim¹, Taehyung Yoon², Dong-gil Leem², and Jayoung Jeong²

Food Safety Center, Research and Development Institute, Namyang Dairy Co., Ltd., Gongju 314-914, Korea

¹*Department of Food and Nutrition, Chosun University, Kwangju 501-759, Korea*

²*Nutrition and Functional Food Research Team, Korea Food and Drug Administration, Seoul 122-704, Korea*

Abstract

The objective of this study was to develop a method to simultaneously quantify vitamins A and E in infant formula. To determine the vitamin A and E content, vitamin A and four different vitamin E isomers (α -, β -, γ -, and δ -tocopherol) were separated by high performance liquid chromatography with a photodiode array detector using a Develosil RPAQUEOUS RP-C₃₀ column (4.6×250 mm, 5 μ m). The vitamin A and E contents in the certified reference material determined using this method were within the certified range of standard values. The limits of detection (LODs) and limits of quantitation (LOQs) for vitamin A were 0.02 and 0.06 μ g/L, respectively. LODs and LOQs for the vitamin E isomers ranged from 0.20 to 0.55 and from 0.67 to 1.81 μ g/L, respectively. Linear analyses indicated that the square of the correlation coefficient for the vitamin A and E isomers was 0.9997–0.9999. The recovery of vitamins ranged from 96.69 to 97.79%. The results demonstrate that this novel method could be used to reliably analyze vitamin A and E content in infant formula.

Key words: vitamin A, vitamin E, high performance liquid chromatography, photodiode array detector, normal phase, reverse phase

Introduction

Infant formula is manufactured to support adequate growth of infants through the addition of ingredients and nutrients. The most commonly used infant formulas contain purified cow's milk as the primary ingredients. The CODEX Standard defined infant formula as a breast-milk substitute that must be manufactured to satisfy, by itself, the nutritional requirement of infants during the first months of life up to the introduction of appropriate complementary food (Codex, 2007). Since the composition of the nutrients in infant formula should be accurately formulated, precise analytical methods are needed for quality control.

Various methods have been described in the CODEX (CEN, 2000a, 2000b) and the KFDA (2009a) to determine the suitability of nutrients in infant formula. Several studies are currently being conducted to develop methods

to analyze the complex matrix of infant formula after sample preparation. In particular, because vitamins are unstable chemical compounds that can easily be oxidized and destroyed by oxygen, heat and ultraviolet, methods to rapidly analyze the vitamin content in infant formula are needed. The methods should be simple and highly accurate, reproducible, and stable results should be easily obtained.

Determination of fat soluble vitamins has been previously accomplished using HPLC with UV, fluorescence (FLD), electrochemical (ED), or evaporative light scattering (ELSD) detection methods (Chase *et al.*, 1994; Podda *et al.*, 1996; Speek *et al.*, 1985; Wang and Wang, 2001; Warner and Mounts, 1990).

Among them the most widely used method to detect and quantify vitamin A and E is FLD which is significantly more sensitive than UV. The method has been used RP-HPLC for analyzing vitamin A and vitamin E has been detected by NP-HPLC. In other words, to measure and quantify vitamins A and E levels, two different injections and mobile phases would be needed. Therefore, PDA has been used for the simultaneous determination of vitamins A and E in the same injection at multiple UV

*Corresponding author: Jang-Hyuk Ahn, Food Safety Center, Research and Development Institute, Namyang Dairy Co., Ltd., Gongju 314-914, Korea. Tel: 82-41-856-0381; Fax: 82-41-857-7933, E-mail: ppori5470@hotmail.com

wavelengths in these days. Daood *et al.* (2008) simultaneously measured the levels of carotenes and α -tocopherol in foods by RP-HPLC-PDA.

Qian and Sheng (1998) used RP-HPLC coupled with UV detection for the simultaneous determination of fat-soluble vitamin A (retinol acetate), D and E (α -tocopherol) in animal feeds.

In order to determine the fat soluble vitamin content in infant formula, it is necessary to remove interfering substances from the complex matrix. Since the infant formula contains a large amount of fat and many other compounds, sample preparation requires saponification, protein precipitation and liquid-liquid extraction prior to instrumental analysis for fat-soluble vitamin content (Jonathan and Karlene, 2002; Soledad *et al.*, 1997).

Several studies have simultaneously quantified fat-soluble vitamins in infant formula by NP-HPLC-PDA and NP-HPLC-MS (Chase and Long, 1998; Jorge *et al.*, 2006, 2008; Kamal-Eldin *et al.*, 2000; Olivier *et al.*, 2004; Syvaioja *et al.*, 1985; Tuan *et al.*, 1989). However, vitamin A and only 3 kinds vitamin E isomers (α -, γ -, δ -tocopherol) could be separated with those methods.

The biological activity of Vitamin E which attracts the most interest is the prevention of lipid peroxidation. The α -tocopherol is the most active tocopherol against peroxyl radicals. The β -, γ -tocopherols have the 0.5 and 0.1 activity of a natural α -tocopherol respectively (KNS, 2001). As the tocopherols have a different activity, it is important that β -, γ -tocopherols should be separated to quantify the vitamin E content in infant formula.

The KFDA (2009b) and AOAC (2005) methods have introduced only the NP-HPLC methods for the determination of vitamin E. Some studies have been conducted with the aim of separating the 4 different vitamin E isomers. Until now there have been no studies that successfully separated β -, γ -tocopherols in infant formulas by RP-HPLC. The separation of tocopherols, especially of the vitamin E isomers β -, γ -tocopherol by conventional RP-HPLC systems, has proved difficulties in the past.

The objective of this study was to develop and to validate a HPLC-PDA method for the simultaneous determination of vitamin A (retinol) and E (α -, β -, γ -, δ -tocopherol) in milk-based infant formula using a single injection by NP and RP-HPLC-PDA.

Materials and Methods

Samples

In this study, Infant/Adult Nutritional Formula SRM

(Standard Reference Material) 1849 (National Institute of Standard & Technology, USA) contained vitamin A (retinol) 1037.32 ± 82.23 IU/100 kcal and vitamin E 12.20 ± 0.61 IU/100 kcal was used as a standard to verify the presence of vitamin A and E. 16 kinds of infant and baby powder in domestic circulation were purchased from local hypermarkets in South Korea. The samples were maintained at 0-4°C until analysis.

Standard and Reagents

Retinol-acetate was used as a standard for vitamin A and was purchased from U.S.Pharmacopeia (USA). α -, β -, γ - and δ -tocopherol vitamin E standards were purchased from Supelco (USA). All of the solvents used were of analytical and chromatographic grade. Water was purchased from J, T. Baker Inc. (USA), and ethanol, ethyl acetate, isopropanol and hexane were purchased from Fisher (Hampton, NH, USA). KOH and pyrogallol were purchased from Junsei Chemical (Japan). The Ultrapure water used in all experiments had a resistance over 18.0 M Ω and was purified using the EASY pure system (Barnstead, USA).

Instruments

A Waters Alliance HPLC apparatus (Waters, USA) equipped with a Model 2695 separation module, a Model 996 photodiode array detector (PDA) and 474 fluorescence detector (FLD) was used. The system was operated by Empower software. A Develosil RPAQUEOUS C₃₀ (4.6×250 mm, 5 μ m, Phenomenex, Japan) Sunfire RP-C₁₈ (4.6×150, 3.5 μ m, waters, USA) column was used as the reverse phase column. The NP-NH₂ (4.6×250 mm, 5 μ m, Phenomenex, Japan) column was used as the normal phase column. A Vortex mixer Maxi mix 37900 mixer (Thermolyne, USA), VS-202DR Horizontal shaker (Vision scientific, Korea), WB-2000 Rotary evaporator (Heidolph, Germany), devices that filter's Advantec Dismic-13_{JP} (PTFE, 0.20 μ m, Advantec, Japan) were used for analyses.

Preparation of standard solution

A hundred milligram each of vitamin A (retinol-acetate) and isomers of vitamin E (α -, β -, γ - and δ -tocopherol) were dissolved in 100 mL of organic solvent (1000 mg/L) as mixed stock solution. The mixed stock solution of vitamin A and E was further diluted to create the working solutions. The organic solvent consisted of ethanol in RP and hexane in NP, respectively.

Table 1. Chromatographic separation conditions from different columns

	C ₃₀ column/PDA	C ₁₈ column/PDA	NH ₂ column/PDA
Column	Develosil RPAQUEOUS C ₃₀ (4.6×250 mm, 5 μm)	Sunfire RP-C ₁₈ (4.6×150 mm, 3.5 μm)	NP-NH ₂ (4.6×250mm, 5 μm)
Mobile phase	(A) methanol (95%) (B) water (5%)	(A) methanol (95%) (B) water (5%)	(A) hexane (93%) (B) ethyl acetate (7%)
Flow rate	1.2 mL/min	1.0 mL/min	1.2 mL/min.
Injection Volume	10 uL	10 uL	10 uL
PDA wavelength range	325 nm for retinol 298 nm for vitamin E isomers	325 nm for retinol 298 nm for vitamin E isomers	325 nm for retinol 298 nm for vitamin E isomers

Sample preparation

Because fat-soluble vitamins are light sensitive, precautions were taken to minimize exposure of the extracted solutions to daylight by using amber glassware. Sample preparation was performed according to the KFDA method (KFDA, 2009b). Hexane was used as the extraction solvent instead of petroleum ether. One gram of the sample was weighed and placed into a 250 mL flask. The sample was dissolved by adding H₂O 3 mL and adding 30 mL of ethanol, 1 mL of 10% pyrogallol-ethanol and 3 mL of 18.9 N solution of potassium hydroxide. The sample solution was mixed well and saponification was allowed to occur for 30 min in a water bath (90°C). After rapid cooling to room temperature, the sample solution was transferred to a brown separate funnel containing 30 mL of water. A separate funnel containing 30 mL of hexane was added and shaken for 10 min. The separate funnel was then left to stand for 30 min. The bottom layer was transferred to another funnel and extracted 2 times with 30 mL hexane. All extracted hexane layers were added to a separate funnel and washed with 50 mL water, repetitively, until the red color of the phenolphthalein indicator disappeared. The water was removed from the funnel and anhydrous sodium sulfate was placed in the hexane layer for dehydration. The hexane layer was transferred to a brown flask. The hexane solution was dried at 40°C using a vacuum evaporator. The dried residue was dissolved in 10 mL of ethanol (In case of normal phase dissolved in 10 mL of hexane). Samples were filtered through a membrane filter (0.2 μm) prior to analysis.

Separation conditions

HPLC separation was performed using 3 kinds of columns. The mobile phase on a reverse phase was used consisting of a mixture of methanol and water (95:5, v/v) at a flow rate of 1 mL/min (RP-C₁₈) and 1.2 mL/min (RP-C₃₀), respectively. The mobile phase on a normal phase

was used consisting of a mixture of hexane and ethyl acetate (93:7, v/v) at a flow rate of 1.2 mL/min. Table 1 shows the different chromatographic separation conditions.

Results and Discussion

Comparison for separation condition

Vitamin A and vitamin E isomers were separated by a Sunfire RP-C₁₈ (4.6×150 mm, 5 μm) column using a PDA detector; however, the β-tocopherol and γ-tocopherol peaks of vitamin E were not separated and appeared as a single overlapping peak. The 5 vitamin peaks were separated in the following order: retinol, δ-tocopherol, (γ-tocopherol+β-tocopherol) and α-tocopherol. The PDA spectra of each standard material are shown in Fig. 1. When the normal phase NP-NH₂ (4.6×250 mm, 5 μm) column was used all peaks could be separated. The 5 vitamin peaks were separated in the following order: α-tocopherol, β-tocopherol, γ-tocopherol, retinol and δ-tocopherol. The PDA spectra of each standard material are shown in Fig. 2. Vitamin A and the 4 different vitamin E isomers could be separated using a Develosil C₃₀ column. The 5 vitamin peaks were separated in the following order: retinol, δ-tocopherol, γ-tocopherol, β-tocopherol and α-tocopherol. The PDA spectra of each standard material are shown in Fig. 3.

Method validation

The method validation procedures were performed according to ICH harmonized tripartite guideline, IUPAC technical report and AOAC guideline. The method was evaluated in terms of LOD, LOQ, recovery, linearity, accuracy and precision (ICH, 2005; Michael *et al.*, 2002). The limits of detection (LODs) and quantitation (LOQs) of vitamin A and each vitamin E isomers were determined based on the signal to noise ratio of 3 and 10,

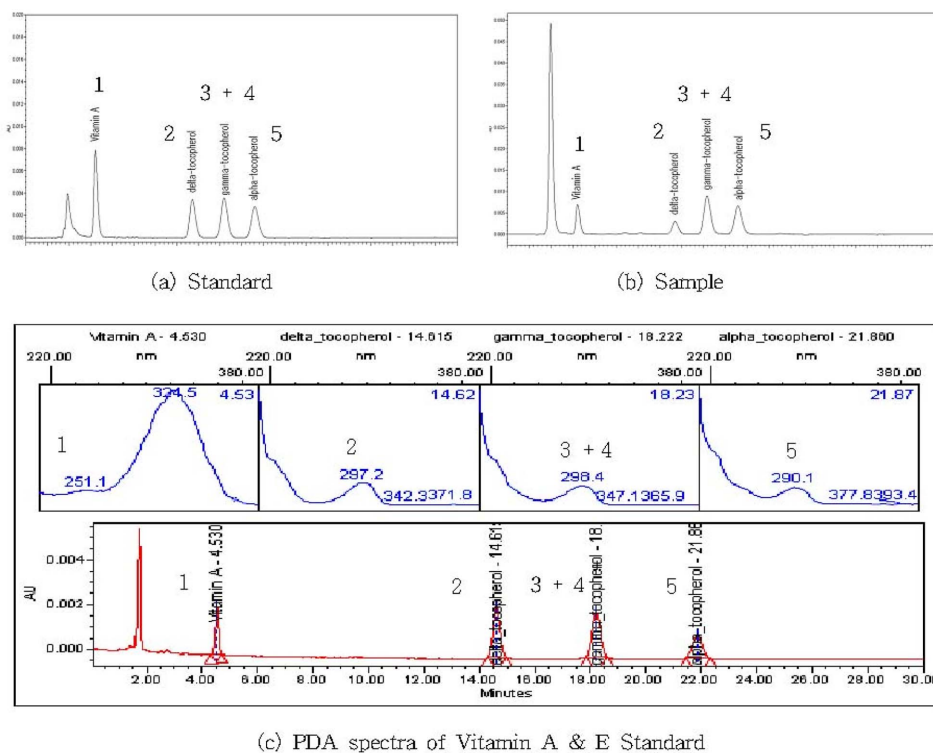


Fig. 1. RP-C₁₈ column/PDA chromatograms of vitamin A and E (1, retinol; 2, δ-tocopherol; 3, γ-tocopherol; 4, β-tocopherol; 5, α-tocopherol).

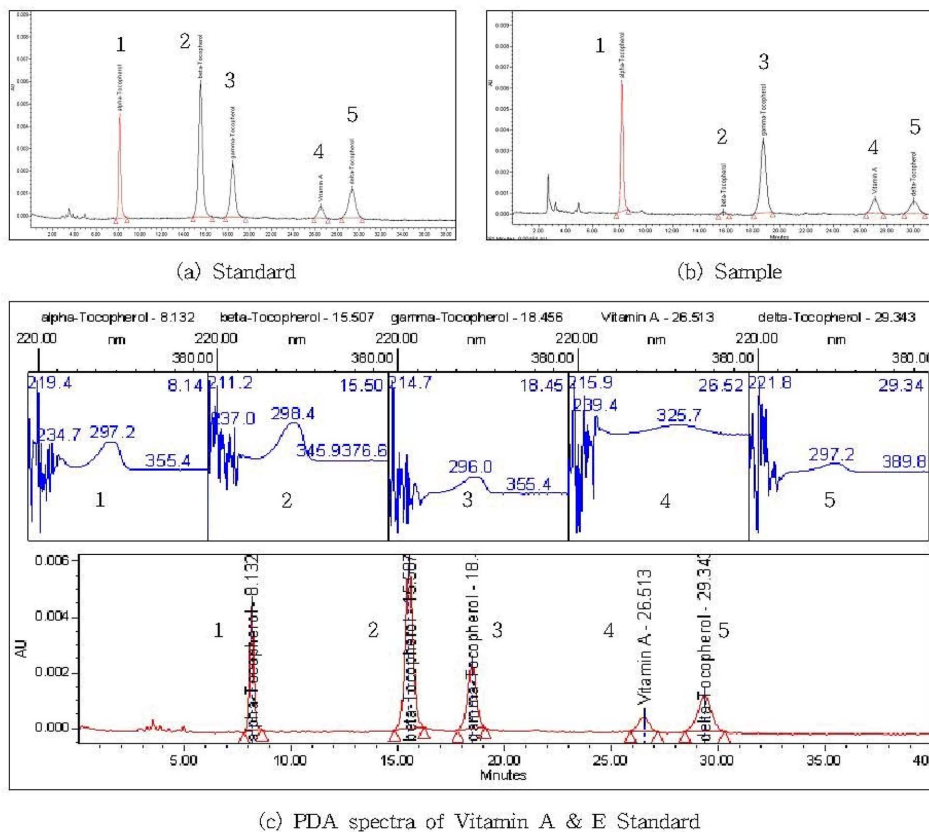


Fig. 2. NP-NH₂ column/PDA chromatograms of vitamin A and E (1, α-tocopherol; 2, β-tocopherol; 3, γ-tocopherol; 4, retinol; 5, δ-tocopherol).

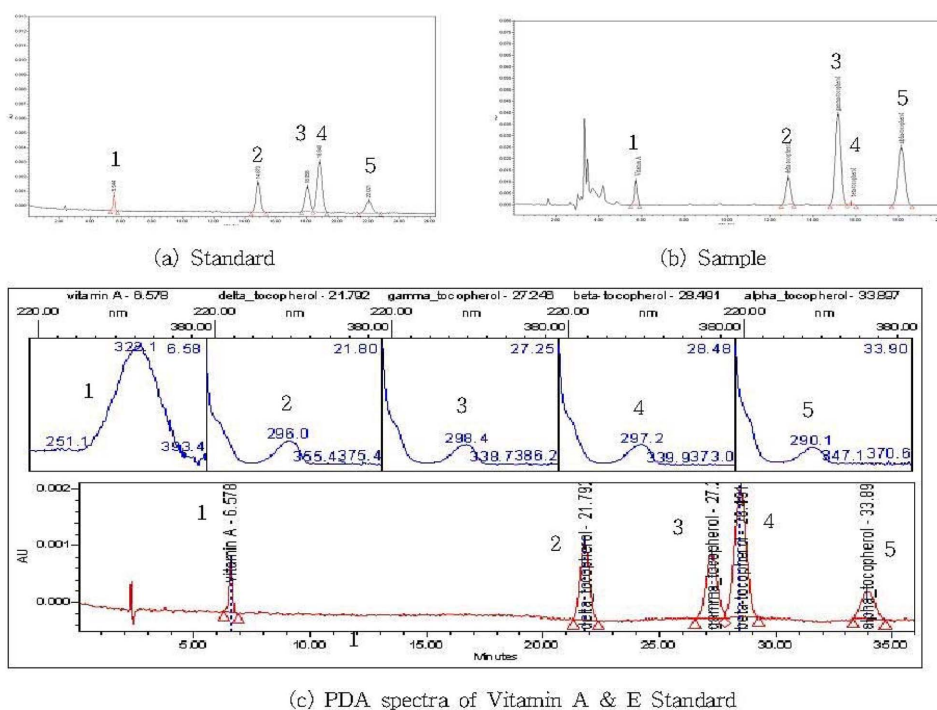


Fig. 3. Develosil RPAQUEOUS C₃₀ column/PDA chromatograms of vitamin A and E (1, retinol; 2, δ -tocopherol; 3, γ -tocopherol; 4, β -tocopherol; 5, α -tocopherol).

Table 2. Limits of detection and quantitation in vitamin A and E isomers by different columns

Compound	Method	LOD ¹⁾ ($\mu\text{g}/\text{kg}$)				LOQ ²⁾ ($\mu\text{g}/\text{kg}$)			
Vitamin A	C ₃₀ column/PDA	0.06				0.20			
	C ₁₈ column/PDA	0.01				0.03			
	NH ₂ column/PDA	0.02				0.08			
Vitamin E	Method	α	β	γ	δ	α	β	γ	δ
	C ₃₀ column/PDA	0.20	0.55	0.46	0.43	0.67	1.81	1.52	1.43
	C ₁₈ column/PDA	0.63	-	0.50	0.45	2.06	-	1.65	1.48
	NH ₂ column/PDA	0.64	0.61	1.21	2.18	2.12	2.00	4.00	7.20

¹⁾LOD: limits of detection

²⁾LOQ: limits of quantitation

respectively. LODs and LOQs are presented in Table 2. In the case of the reverse phase Sunfire RP-C₁₈ column, the LODs and LOQs for vitamin A and E ranged from 0.01 to 0.63 $\mu\text{g}/\text{kg}$ and from 0.03 to 2.06 $\mu\text{g}/\text{kg}$, respectively. However, β -tocopherol was not separated when this column was used. In the case of the normal phase NP-NH₂ column, the LODs and LOQs for vitamin A and E ranged between 0.02 and 2.18 $\mu\text{g}/\text{kg}$ and between 0.08 and 7.20 $\mu\text{g}/\text{kg}$, respectively. In the case of the reverse phase Develosil RPAQUEOUS C₃₀ column, the LODs and LOQs for vitamin A and E ranged between 0.06 and 0.46 $\mu\text{g}/\text{kg}$ and between 0.20 and 1.81 $\mu\text{g}/\text{kg}$, respectively.

Recovery tests were repeatedly performed 3 times with

Infant / Adult Nutritional Formula SRM 1849. This formula contained $1,037.32 \pm 82.23$ IU/100 kcal of vitamin A and 12.20 ± 0.61 IU/100 kcal of vitamin E. The recovery was calculated by dividing the quantitative concentration determined using the calibration curve by the concentration of SRM. The recoveries for vitamin A and E were 97.76 ± 3.13 and $97.57 \pm 1.66\%$, respectively, with the reverse phase column (RP-C₁₈)/PDA. The recoveries for vitamin A and E were 96.95 ± 3.23 and $98.97 \pm 2.12\%$, respectively, with Normal phase column (NP-NH₂)/PDA. The recoveries for vitamin A and E were 97.97 ± 3.47 and $96.69 \pm 1.19\%$, respectively, with Develosil RPAQUEOUS C₃₀/PDA. Table 3 shows the recovery rates of all tests.

Table 3. Recovery rates of vitamin A and E by different columns

Compound	Method	Content (IU/100 kcal)	Recovery (%)	Standard content (IU/100 kcal)
Vitamin A	C ₃₀ column/PDA	1016.45±36.05	97.97±3.47	1037.32±82.23
	C ₁₈ column/PDA	1013.92±32.26	97.76±3.13	
	NH ₂ column/PDA	1005.69±33.52	96.95±3.23	
Vitamin E	C ₃₀ column/PDA	11.80±0.14	96.69±1.19	12.20±0.61
	C ₁₈ column/PDA	11.90±0.20	97.57±1.66	
	NH ₂ column/PDA	12.07±0.26	98.97±2.12	

Considering the results of the recovery test, this method can be considered to be accurate.

The linearity, accuracy and precision tests were performed only with Develosil RPAQUEOUS C₃₀/PDA, since the β -tocopherol and γ -tocopherol peaks of vitamin E were not separated by Sunfire RP-C₁₈ (4.6×150 mm, 5 μ m) column, and analysis using a normal phase column has rarely been performed in these days and NP-PDA method for determination vitamin A and E in infant formula has been already studied (Jorge. *et al.*, 2006).

The linearity was calculated for five consecutive standard curves. We checked the linearity of the method using five vitamin A working solutions (5, 10, 20, 30 and 40 μ g/L) and vitamin E isomer working solutions (2.5, 5, 10, 15 and 20 μ g/L). The regression equation and coefficient of determination (r^2) for vitamin A was expressed as $Y=2,247X+2,343.4$ (X , concentration; Y , area) was calculated to be 0.9997. The regression equations and coefficients of determination (r^2) for vitamin E isomers (α -, β -, γ -, and δ -

Table 4. Accuracy and precision of vitamin A

Content (μ g/kg)	Vitamin A			
	Intraday (n=3)		Interday (n=3)	
	Precision (CV ¹), (%)	Accuracy (%)	Precision (CV ¹), (%)	Accuracy (%)
10	1.90±0.04	99±2.08	1.52±0.03	98±1.21
15	1.76±0.03	98±1.02	1.80±0.04	99±1.25
30	0.87±0.01	99±0.33	1.73±0.03	99±0.48

¹)CV: Coefficient of variation

tocopherol) were expressed as $Y=6,031X+3,586.5$, $Y=2,639X+718.3$, $Y=4,244X+3,841.9$, $Y=4,704X+3,465.5$ and were calculated to be 0.9997, 0.9997, 0.9997, 0.9999, and 0.9998, respectively.

Intra-day accuracy and precision were analyzed in 3 replicates within 24 h and inter-day accuracy and precision were analyzed in 3 replicates for three consecutive days. Samples spiked with four concentrations of vitamin

Table 5. Accuracy and precision of vitamin E

Content (μ g/kg)	Vitamin E							
	Intraday (n=3)							
	Precision (CV ¹), (%)				Accuracy (%)			
	α	β	γ	δ	α	β	γ	δ
5	1.27±0.04	0.92±0.03	0.77±0.01	0.83±0.03	97±1.15	98±1.74	98±1.35	98±1.02
8	1.12±0.01	1.04±0.22	1.04±0.62	0.74±0.01	98±1.44	99±1.58	99±1.42	99±1.28
15	0.55±0.01	0.36±0.04	0.33±0.01	0.36±0.01	99±1.54	99±1.35	98±1.44	99±1.75
Content (μ g/kg)	Interday (n=3)							
	Precision (CV ¹), (%)				Accuracy (%)			
	α	β	γ	δ	α	β	γ	δ
	5	1.58±0.06	1.57±0.06	1.26±0.04	1.29±0.03	98±1.64	97±1.36	97±1.29
8	1.35±0.02	1.48±0.36	1.25±0.76	1.05±0.02	97±1.86	98±1.75	98±1.09	98±1.38
15	0.86±0.03	0.99±0.12	0.89±0.02	0.75±0.01	99±1.49	98±1.25	99±1.37	99±1.58

¹)CV: Coefficient of variation

A (retinol-acetate - 10, 20, 30, and 40 µg/L) and vitamin E isomer (α -, β -, γ -, and δ -tocopherol - 5, 8, 15, and 20 µg/L) were prepared for the determination of evaluation accuracy. This was expressed as a percentage of the mean of the quantitative values of the reference samples, was calculated as (content determined in spiked sample–content determined in unspiked sample)/spiked content \times 100. The accuracy of intra-day and inter-day values for vitamin A and E were in the range of 98 \pm 1.02 to 99 \pm 2.08% and 97 \pm 1.15 to 99 \pm 1.75%, respectively. The detailed data for vitamin A is shown in Table 4 and for vitamin E is shown in Table 5. The low values of RSD (%) and the high accuracy values show that the results have high accuracy and precision.

To determine precision, four concentrations of the standard solutions vitamin A (retinol-acetate - 10, 20, 30 and 40 µg/L) and vitamin E isomer (α -, β -, γ -, and δ -tocopherol-5, 8, 15, and 20 µg/L) in the calibration range were evaluated. This was expressed as RSD (%), and calculated as (standard deviation/mean) \times 100. Precision of the intra- and inter-day values for vitamin A were less than 1.90% as shown in Table 4 and those of the vitamin E were less than 1.58% as shown in Table 5. As the results of the test, the developed method could be proper for analyses of the vitamin A and E in infant formula.

Quantitation of vitamin A and E in infant formula by HPLC-PDA

The vitamin A and E content in 16 types of infant formula that are commercially available in the market were analyzed using the established analysis method with the Develosil RPAQUEOUS C₃₀ column/PDA. The results of these experiments were in the range from 464 \pm 3.23 to 555 \pm 6.12 IU/100 kcal for vitamin A and from 0.99 \pm 0.06 to 1.20 \pm 0.06 IU/100 kcal for vitamin E (Table 6). Infant formula prepared ready for consumption should contain a

range from 250 to 750 IU/100 kcal and a minimum of 0.7 IU/100 kcal to provide a safe and adequate vitamin A and E content, respectively (KFDA, 2009a). All our data showed good agreement with the KFDA recommendation

Some of studies were performed to develop a method to determine 4 different tocopherols and simultaneously fat-soluble vitamin A and E in infant formula. NP-HPLC with fluorescence detection has been applied to the determination of vitamin E (α -, β -, γ - and δ -tocopherols) in infant formulas (Syvaaja *et al.*, 1985; Tuan *et al.*, 1989) and vegetable oils (Chase *et al.*, 1994; Kamal-Eldin *et al.*, 2000). Several studies for simultaneous quantitation vitamin A and E in milk-based infant formula have been conducted using NP-UV (Soledad *et al.*, 1997; Chase and Long, 1998), NP-PDA (Jorge *et al.*, 2006), LC-MS (Olivier *et al.*, 2004) and RP-PDA (Rodas *et al.*, 2003) until recently. However, all of the methods have determined only retinol or retinyl-palmitate as a vitamin A. For vitamin E, only α -tocopherol or α -, γ - and δ -tocopherols were determined. There have been no studies that vitamin A (retinol) and 4 different vitamin E isomers (α -, β -, γ - and δ -tocopherols) were determined simultaneously for vitamin A and E quantitation in infant formula.

This study was performed to develop a novel method to simultaneously determine the presence of fat-soluble vitamin A and E. A PDA detector was used for the simultaneous detection of these vitamins on both a reverse phase and a normal phase column. Complete separation of vitamin A (retinol) and the 4 vitamin E isomers (α -, β -, γ -, and δ -tocopherol) was demonstrated on a reverse phase Develosil RPAQUEOUS C₃₀ column and a normal phase NP-NH₂ column with a PDA detector. Above all things, we tried to separate all 5 peaks on a reverse phase because there were not lots of studies about complete separation of β - and γ -tocopherol peaks on a reverse phase and the published certified methods for vitamin E in Food

Table 6. Determination of vitamin A and E in 16 infant formula samples circulated in the market (n=16)

Sample No.	Content (IU/100 kcal)		Sample No.	Content (IU/100 kcal)	
	vitamin A	vitamin E		vitamin A	vitamin E
1	487 \pm 5.21	1.00 \pm 0.07	9	466 \pm 3.23	1.06 \pm 0.06
2	509 \pm 2.01	1.15 \pm 0.08	10	522 \pm 4.66	1.11 \pm 0.05
3	474 \pm 5.81	1.05 \pm 0.04	11	490 \pm 3.33	1.05 \pm 0.07
4	509 \pm 3.33	1.11 \pm 0.05	12	493 \pm 2.70	1.20 \pm 0.06
5	464 \pm 3.23	1.03 \pm 0.06	13	450 \pm 4.48	1.07 \pm 0.06
6	484 \pm 5.37	1.15 \pm 0.05	14	535 \pm 5.33	1.09 \pm 0.07
7	458 \pm 5.82	0.99 \pm 0.06	15	483 \pm 3.26	1.11 \pm 0.05
8	534 \pm 4.70	1.14 \pm 0.06	16	555 \pm 6.12	1.13 \pm 0.07

(AOAC, 2005; CEN, 2000b; KFDA 2009b) have adopted only the NP-HPLC method. However, we could separate the 5 peaks using a Develosil RPAQUEOUS C₃₀ column by RP-HPLC-PD. Moreover, in this study, both RP- and NP-condition were developed for simultaneous determination method of fat-soluble vitamin A and E in infant formula. The proposed method allows for the simultaneous detection of vitamin A (retinol) and the 4 vitamin E isomers (α -, β -, γ -, and δ -tocopherol) in a single injection. Although the β - and γ -tocopherol peaks could not be separated with the commonly used RP-C₁₈ column, the content of β -tocopherol in circulative infant formula was very low and was within the noise of the system. Thus, this method can be used to detect vitamin E in samples that do not include β -tocopherol. The HPLC-PDA method was used for simultaneous determination of vitamin A and E in reverse phase and normal phase.

Acknowledgements

The work performed in this study was supported the by Korea Food and Drug Administration research project (KFDA 09081KFDA088) in 2009. We would like to thank Dr. Ja-Young Jeong, Dr. Dong-Gil Leem and Mr. Tae-Hyung Yoon of KFDA nutrition and functional food research team. We also thank Prof. Kyong-Su Kim of Chosun university, for helping as project partner.

References

1. AOAC (2005) Official Methods of Analysis. 18th ed, Association of Official Analytical Chemists, Gaithersburg, MA, USA, Ch 50, pp. 4-5.
2. CEN (European Committee for Standardization) (2000a) Foodstuffs-Determination of vitamin A by HPLC. Part I. Measurement of all-trans-retinol and 13-cis-retinol. BS EN 12823-1.
3. CEN (European Committee for Standardization) (2000b) Foodstuffs-Determination of vitamin E by HPLC. Measurement of alpha-, beta-, gamma-, and delta-tocopherol. BS EN 12822.
4. Chase, G. W., Akoh, C. C., and Eitenmiller, R. R. (1994) Analysis of tocopherols in vegetable oils by high-performance liquid chromatography: comparison of fluorescence and evaporative light-scattering detection. *J. Am. Oil Chem. Soc.* **71**, 877-880.
5. Chase, G. W. and Long, A. R. (1998) Liquid chromatographic method for analysis of all-rac-alpha-tocopheryl acetate and retinyl palmitate in milk-based infant formula using matrix solid-phase dispersion. *J. AOAC Int.* **81**, 582-586.
6. Codex (2007) Standard for infant formula and formulas for special medical purposes intended for infants. Codex Stan. 72-1981.
7. Daood, H. G., Korbasz, M., and Hamdan, S. (2008) Simultaneous HPLC determination of Ergosterol, Tocopherols and Cartenoids in foods. *Chromatographia* **68**, S137-S140.
8. ICH (International Conference on Harmonization of Technical) Expert Working Group (2005) ICH Harmonized tripartite guideline validation of analytical procedures : text and methodology Q2(R1). Step 4:1-18.
9. Jonathan, W. D. and Karlene, R. S. (2002) Determination of vitamin A (Retinol) and vitamin E (alpha-tocopherol) in foods by liquid chromatography. Collaborative. *J. AOAC Int.* **85**, 424-434.
10. Jorge, L., Chavez, S., Ana, I. C., and M. Carmen, L. S. (2006) Simultaneous analysis of vitamin A and E in infant milk-based formulae by normal-phase high performance chromatography-diode array detection using a short narrow-bore column. *J. Chromatogr. A* **1122**, 138-143.
11. Jorge, L., Chavez, S., Ana, I. C., and M. Carmen, L. S. (2008) Vitamins A and E content in infant milk-based powdered formulae after opening the packet. *Food Chem.* **106**, 299-309.
12. Kamal-Eldin, A., Gorgen, S., Pettersson, J., and Lampi, A. M. (2000) Normal-phase high-performance liquid chromatography of tocopherols and tocotrienols. Comparison of different chromatographic columns. *J. Chromatogr. A* **881**, 217-227.
13. KFDA (Korea Food and Drug Administration) (2009a) Food Standards Codes. Korean Foods Industry Association, Seoul, Korea.
14. KFDA (Korea Food and Drug Administration) (2009b) Food Standards Codes. Korean Foods Industry Association, Seoul, Korea, pp. 742-744.
15. KNS (The Korean Nutrition Society) (2001) Recommended dietary allowances for Koreans. 7th rev. The Korea Nutrition Society, Seoul, Korea. pp. 94-98.
16. Michael, T., Stephen, L. R. E., and Roger, W. (2002) Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* **74**, 835-855.
17. Olivier, H., Marie, J. T., and Christopher, J. B. (2004) Simultaneous quantification of Vitamin A, D3 and E in fortified infant formulae by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **1022**, 115-123.
18. Podda, M., Weber, C., Traber, M. G., and Packer, L. (1996) Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinol, and ubiquinones. *J. Lipid Res.* **37**, 893-901.
19. Qian, H. and Sheng, M. (1998) Simultaneous determination of fat-soluble vitamin A, D and E and pro-vitamin D2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis. *J. Chromatogr. A* **825**, 127-133.
20. Rodas, M. B., Morera, P. S., Castellote B. A. I., and Lopez-Sabater, M. C. (2003) Rapid determination by reversed-phase high-performance liquid chromatography of vitamins A and E in infant formulas. *J. Chromatogr. A* **1018**, 197-202.
21. Soledad, A. H., Sonia, N. R., M Teresa, V. N., and Abel, M.

- F. (1997) Determination of vitamins A and E in infant milk formula by high-performance liquid chromatography. *J. Chromatogr. A* **778**, 243-247.
22. Speek, A. J., Schrijver, J., and Schreurs, W. H. P. (1985) Vitamin E composition of some seed oils as determined by high-performance liquid chromatography. *J. Food Sci.* **50**, 121-124.
23. Syvaöja, E. L., Piironen, V., Varo, P., Koivistoinen, P., and Salminen, K. (1985) Tocopherols and tocotrienols in Finnish food: human milk and infant formulas. *Int. J. Vitam. Nutr. Res.* **55**, 159-166.
24. Tuan, S., Lee, T. F., Chou, C. C., and Wei, Q. K. (1989) Determination of vitamin E homologues in infant formulas by HPLC using fluorometric detection. *J. Micronutr. Anal.* **6**, 35-45.
25. Wang, L. H., and Wang, J. F. (2001) Determination of retinoids in human serum, tocopherol and retinyl acetate in pharmaceuticals by RP-HPLC with electrochemical detection. *J. Pharm. Biomed. Anal.* **25**, 785-793.
26. Warner, K., and Mounts, T. L. (1990) Analysis of tocopherols and phytosterols in vegetable oils by HPLC with evaporative light-scattering detection. *J. Am. Oil Chem. Soc.* **67**, 827-831.

(Received 2010.9.20/Revised 2010.11.29/Accepted 2011.3.18)