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Rapid determination by reversed-phase high-performance liquid chromatography of Vitamins A and E in infant formulas☆

B. Rodas Mendoza, S. Morera Pons, A.I. Castellote Bargalló, M.C. López-Sabater*

Facultat de Farmàcia, Dpto. Nutrició i Bromatologia, Centre de Referència en Tecnología dels Aliments (CeRTA), Universitat de Barcelona, Avda. Joan XXIII s/n, Barcelona 08028, Spain

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Abstract

A rapid, sensitive method has been developed for the simultaneous determination of retinol acetate, δ-, γ-, α-tocopherol and α-tocopherol acetate. We compare two experimental procedures for simultaneous direct solvent extraction of these vitamins without previous saponification. Method I: the fat milk sample was extracted with ethanol-hexane and injected directly into the chromatographic column. Method II: the power milk sample was extracted with ethanol-hexane and also injected directly into the column. Under optimum conditions the limits of detection for retinol acetate, δ-, γ-, α-tocopherol and α-tocopherol acetate were 0.33, 21.2, 32.9, 32.5 and 3.2 ng and the limits of quantification were 0.42, 25.3, 37.9, 36.8 and 6.3 ng, respectively. The precision results showed that the relative standard deviations of repeatability and reproducibility were between 0.74 and 5.7%.

Keywords: Infant formulas; Food analysis; Vitamins; Tocopherols; Retinol acetate

1. Introduction

Vitamin E is the main physiological fat-soluble antioxidant. It prevents oxidative damage to neonates suddenly exposed to higher oxygen levels than those in the intrauterine environment [1–3].

In addition to their Vitamin E function, tocopherols are fat-soluble substances widely distributed in nature, they are natural antioxidants in foods and are important for stability of infant formulas because of the increased production of reactive oxygen radicals can occur in stored infant formula feeds due to their iron content and high polyunsaturated fatty acids with insufficient antioxidant protection [4,5].

The antioxidant activities of δ-, γ-, α-tocopherol, the forms commonly found in vegetable oils, have been studied extensively. RRR-α-tocopherol contributes more biologically active Vitamin E to diets than any other tocopherol isomer; however,

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* Corresponding author. Tel.: +34-93-402-45-12; fax: +34-93-403-59-31.
E-mail address: mclop@farmacia.far.ub.es (M.C. López-Sabater).

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α-tocopherol is unstable in many food products during storage [6].

Vitamin A is an essential nutrient for all animal species for normal vision, growth and cellular differentiation. These roles are critical during periods of proliferative growth and tissue development, such as infancy [7,8].

Foods such as milk or infant formula are most commonly fortified with Vitamin A in the form of retinol acetate or retinol palmitate and Vitamin E in the form of α-tocopherol acetate [9]. These molecules are more stable and less susceptible to oxidation. The concentration of these vitamins after storage and manufacture needs to be checked in order to ensure correct intake and the accuracy of the label statements.

Methods to extract and measure Vitamins A and E in milk and milk products are time consuming and tedious. Traditionally, fat-soluble vitamin analysis is performed by alkaline saponification of the entire sample matrix, or of an isolated lipid fraction, followed by liquid extraction with organic solvents like diethyl ether or *n*-hexane [10–13]. A few methods analyse these vitamins directly without saponification in various matrices [14–19], only two in Infant Formulas [20,21].

Several analytical methods are used for the determination and quantification of Vitamins A and E by normal-phase high-performance liquid chromatography (NP-HPLC) [22–24] and more often by reversed-phase high-performance liquid chromatography (RP-HPLC) [25–29], gas chromatography [30] and recently by supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) [31–34].

The aim of the present study was to develop and validate for routine analysis two rapid methods for the direct and simultaneous determination of Vitamins A and E in infant formulas by RP-HPLC without saponification.

2. Experimental

2.1. Reagents and standards

All chemicals used were of analytical-reagent grade. Retinol, retinol acetate, retinol palmitate, δ-, γ-, α-tocopherol and α-tocopherol acetate were purchased from Sigma (St. Louis, MO, USA), ethanol absolute for HPLC from Sharlau (Barcelona, Spain), *n*-hexane and methanol for HPLC from SDS (Peypin, France) and dichlorometane from Panreak (Barcelona, Spain).

2.2. Preparation of standards

Stock standard solutions of all *trans*-retinol, retinol palmitate, retinol acetate, δ-, γ-, α-tocopherol and α-tocopherol acetate containing 1 mg/ml were prepared in 100% ethanol and stored at −20 °C in dark bottles for up to a month. Working standard solutions were prepared from these solutions and diluted with ethanol prior to analysis. Since standards are highly susceptible to oxidation and degradation, the purity and the standard concentrations were analysed by HPLC with photodiode-array detection (DAD) every week.

2.3. Sample preparation

A commercial powdered infant formula for term infants, purchased from Spain market, was used to validate the method. The formula was stored in the dark at room temperature (18–20 °C) to maintain the stability of the vitamins, and was analysed prior to their expiry date.

2.3.1. Method I

2.3.1.1. Fat extraction. The infant formula was extracted with dichloromethane-methanol (2:1) following a modification of the Folch method as described by Chen et al. [36]. Dichloromethane was used instead of chloroform due to its lower toxicity and equal extracting capacity. Approximately 25 g of infant formula was weighed into a 250 ml Erlenmeyer flask flushed with nitrogen and with a glass stopper. An amount 150 ml of dichloromethane and 75 ml of methanol were added. The sample was shaken with magnetic stirring for 30 min at room temperature. The contents of the flask were filtered and transferred to a 500 ml separator funnel. An amount of 40 ml of water was added to the separator funnel, and then shaken with occasional venting, and the layers were allowed to separate. The dichloromethane phase was filtered through anhydrous sodium sulphate into a round-bottomed flask. The filter was rinsed with two portions of 10 ml of dichloromethane. The organic phase was evaporated and the residue was dissolved

in diethyl ether in order to eliminate non-lipid substances retained by methanol, and then filtered. The solvent was evaporated to dryness, first on a rotary evaporator and then under a stream of nitrogen. The lipid residue was kept in dark vials, which were flushed with nitrogen, capped tightly, and stored at −20 °C until analysis. To prevent the loss of vitamins, exposure to high temperatures and bright light were avoided throughout the entire process.

Lipid extract of 0.5 g was weighed to the nearest mg, placed in a centrifuge tube and dissolved in 1 ml of absolute ethanol. The mixture was stirred for 1 min and 200 μl of *n*-hexane was added. The mixture was again stirred for 1 min in a vortex mixer followed by centrifugation (2500 × *g* for 5 min) to aid solvent separation. The clear organic top layer was removed and passed through a 0.45 μm pore size nylon filter before injection into HPLC.

2.3.2. Method II

Infant formula sample of 1 g of was weighed to the nearest mg, placed in a centrifuge tube and dissolved in 4 ml of absolute ethanol. The mixture was stirred for 2 min and 400 μl of *n*-hexane was added. The mixture was again stirred for 1 min in a vortex mixer followed by centrifugation (2500 × *g* for 5 min) to aid solvent separation. The clear organic top layer was removed and passed through a 0.45 μm pore size nylon filter before injection into HPLC.

2.4. Chromatographic analysis

Separation by HPLC was carried out using a Hewlett-Packard liquid chromatographic system (Waldbronn, Germany) with and HP-1050 pump system and a HP-1040 M photodiode-array detector and a Waters 717 Plus Autosampler (Milford, MA, USA). The data were stored and processed by an HPLC Chemstation (Dos Series) (Hewlett-Packard). The column was a Tracer Spherisorb ODS2 C_{18}, 250 × 4.6 mm i.d., 5 μm particle size (Tracer Analítica, Barcelona, Spain) protected with a guard cartridge (Tracer, C_{18}, 5 μm) system. The mobile phase was 100% methanol and the elution was performed at a flow-rate of 1 ml/min. The analytical column was kept at 50 °C. The working standard solutions were always analysed together with the samples. Detection was performed at 292 mn for δ-, γ-, α-tocopherol and α-tocopherol acetate and 325 nm for all *trans*-retinol, retinol acetate and retinol palmitate. The total run time required was less than 20 min. Chromatographic peaks were identified by comparing retention times of samples with those of standard compounds. Quantitation was carried out by external standardization.

3. Results and discussion

Fig. 1 shows the chromatograms corresponding to standards and Fig. 2 shows the chromatograms obtained for an infant formula after application of the two proposed methods. The retinol acetate, δ-, γ-, α-tocopherol and α-tocopherol acetate were satisfactorily separated without interferences from the lipid extract matrix or the powder matrix. Peaks monitored by DAD were identified by their retention times in comparison with external standards and by standard additions. Total vitamin content and the composition of milk formula used in this study are showed in Tables 1 and 2, respectively. Although the separation obtained by both methods was satisfactory, with

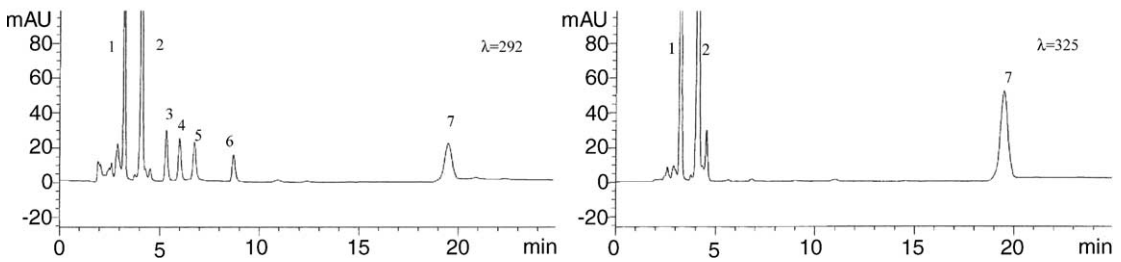

Fig. 1. Chromatograms corresponding to standards. Peak identification: (1) retinol; (2) retinol acetate; (3) δ-tocopherol; (4) γ-tocopherol; (5) α-tocopherol; (6) α-tocopherol acetate; (7) retinol palmitate. See conditions under Section 2.

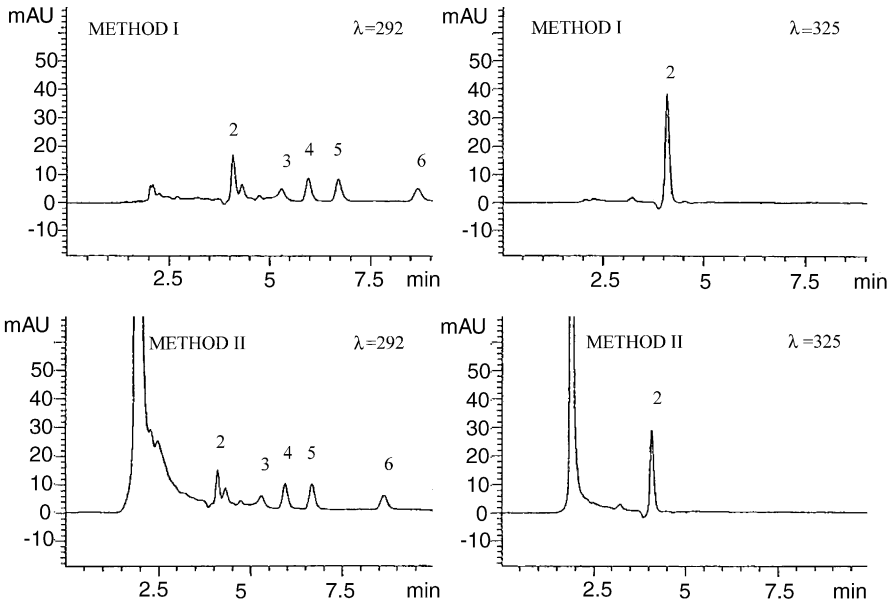

Fig. 2. Chromatograms corresponding to an infant formula after application on the two proposed methods. Peak identification: (2) retinol acetate; (3) δ-tocopherol; (4) γ-tocopherol; (5) α-tocopherol; (6) α-tocopherol acetate. See conditions under Section 2.

method II we gave the best results when Vitamins A and E were determined. The treatment of the sample during fat extraction when method I was developed, cause a minor loss of vitamins that not occurs when the matrix powder was used directly.

3.1. Precision of the methods

For each method, a total of six replicate determinations of a samples were performed under optimum conditions to determine repeatability and, for each method, six replicate analyses of the same sample were made on different days to determine reproducibility. The precision results (Table 3) showed that the rel-

Table 2
Composition of the full-term infant formula

	100 ml	100 g
Energy (kcal)	70	514
Proteins (g)	1.6	12.0
Carbohydrates (g)	8.0	58.0
Fat (g)	3.5	26.0
Minerals (g)	0.3	2.0
Vitamin A (μg)	62.0	450.0
Vitamin E (mg)	3.4	25.0

1 cal = 4.184 J.

Table 1
Determination of Vitamins A and E isomers using the two methods

	α-Tocopherol[a]	γ-Tocopherol[a]	δ-Tocopherol[a]	α-Tocopherol acetate[a]	VE[b]	VA[c]
Method I[d]	2.63	2.00	0.93	11.37	15.90	201.3
Method II[d]	3.75	3.40	1.50	10.90	18.09	330.4

[a] Results expresed as mg/100 g.
[b] VE = total Vitamin E content expressed as mg of α-tocopherol equivalents (mg α-TE).
[c] VA = total Vitamin A content expressed as μg of retinol acetate/100 g.
[d] $n = 6$ replicates.

Table 3
Precision of the proposed methods (R.S.D. %)

Compound	Repeatability		Reproducibility	
	Method I	Method II	Method I	Method II
Retinol acetate	0.74	1.13	1.38	2.73
δ-Tocopherol	2.15	2.20	3.84	5.70
γ-Tocopherol	1.42	2.09	1.26	2.63
α-Tocopherol	0.77	1.97	1.25	3.42
α-Tocopherol acetate	1.77	0.79	2.93	2.29

Table 4
Linearity of the HPLC method

Compound	Range (μg)	Equation	r^2
Retinol acetate	5–100	$y = 4644.2x - 142.49$	0.999
δ-Tocopherol	5–50	$y = 251.14x - 38.271$	0.999
γ-Tocopherol	5–50	$y = 298.54x - 69.54$	0.999
α-Tocopherol	5–50	$y = 249.68x - 43.806$	0.999
α-Tocopherol acetate	5–100	$y = 42.804x - 0.0827$	0.999

x, amount (μg); y, peak area; r^2, determination coefficient.

ative standard deviations (R.S.D.s) of concentrations determined by external calibration of the repeatability and reproducibility were between 0.74 and 5.7%.

3.2. Linearity

The linearity of standard curves (Table 4) was expressed in terms of the determination coefficient (r^2) from plots of the integrated peak area versus concentration of the standard (μg/ml). These equations were obtained over a wide concentration range, in accordance with the levels of these compounds found in infant formulas. Linear equations were found, with satisfactory linearity ($r^2 > 0.99$).

3.3. Sensitivity

The detection (LOD) and quantification (LOQ) limits were calculated according to the USP criteria [35]. These results are shown in Table 5.

3.4. Accuracy

Accuracy was estimated by means of recovery assays. Two spiking levels were assayed in triplicate.

Table 5
Sensitivity of the HPLC method

Compound	LOD (ng)	LOQ (ng)
Retinol acetate	0.33	0.42
δ-Tocopherol	21.2	25.3
γ-Tocopherol	32.9	37.9
α-Tocopherol	32.5	36.8
α-Tocopherol acetate	3.2	6.3

Table 6
Recoveries of vitamins added to the samples before applying the proposed methods

Compound	Recovery (%)	
	Method I[a]	Method II[a]
Retinol acetate	100.7 ± 0.70	96.8 ± 0.99
δ-Tocopherol	103.1 ± 0.93	100.5 ± 1.30
γ-Tocopherol	92.8 ± 0.22	97.5 ± 0.28
α-Tocopherol	99.6 ± 0.34	98.3 ± 0.85
α-Tocopherol acetate	106.3 ± 1.43	99.4 ± 0.92

[a] Mean ± S.D.

Lipid extract of 0.5 g and powdered infant formula of 1 g were spiked with 5 and 50 μg of retinol acetate, δ-, γ-, α-tocopherol, and α-tocopherol acetate, respectively. The samples were subjected to the entire extraction and determination process. Table 6 shows the recovery percentages obtained.

4. Conclusions

The present results show the applicability of these two methods for the routine analysis of infant formulas. We report here two simplified methods, which use a direct extraction. Both methods are simple and eliminate the saponification step required by other methods. Such methods are ideal for a large numbers of samples. Performing sample preparation and analyte extraction in a single reaction tube gives the methods the advantage of minimising manipulation, thereby reducing sample loss and allowing for optimum analyte recovery.

Method I can be used to monitor the lipidic sources in the manufacture of infant formulas. It could also be applied in the study of lipid fraction stability when using oxidative stability tests in order to predict the shelf

life of an infant formula. Method II provided an accurate and fast technique, eliminates the fat extraction step, and is the method of choice when the fat fraction is not required for further analyses. The method is especially recommended for routine analytical work.

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References

[1] O.D. Saugstad, Acta Paediatr. Scand. 79 (1990) 881.
[2] D. Van Zoeren-Grobben, J.H.N. Lindeman, E. Houdkamp, R. Brand, J. Schrijver, H. Berger, Am. J. Clin. Nutr. 60 (1994) 900.
[3] M.A. Crawford, K. Ghebremeskel, A. Phylactos, Br. J. Clin. Pract. 49 (1995) 3.
[4] C.P. Speer, M. Gahr, M.J. Pabst, Acta Paediatr. Scand. 75 (1986) 444.
[5] H. Yoshida, M. Tatsumi, G. Kajimoto, J. Am. Oil Chem. Soc. 68 (1991) 566.
[6] R. Brigelius-Flohé, M.G. Traber, FASEB J. 13 (1999) 1145.
[7] B.A. Underwood, Am. J. Clin. Nutr. 59 (1994) 517S.
[8] C.D. Berdanier, Advanced Nutrition Micronutrients, CRC Press, New York, 1998.
[9] M.J. Gonzalez-Corbella, M. Tortras-Biosca, A.I. Castellote-Bargalló, M.C. López-Sabater, Food Chem. 64 (1999) 1.
[10] S. Albalá-Hurtado, S. Novella-Rodriguez, M.T. Veciana-Nogués, A. Mariné-Font, J. Chromatogr. A 778 (1997) 243.
[11] L. Gámiz-Gracia, A. Velasco-Arjona, M.D. Luque de Castro, Analyst 124 (1999) 801.
[12] C. Turner, L. Mathiasson, J. Chromatogr. A 874 (2000) 275.
[13] A. Escrivá, M.J. Esteve, R. Farré, A. Frígola, J. Chromatogr. A 947 (2002) 313.
[14] C. Barbas, M. Castro, B. Bonet, M. Viana, E. Herrera, J. Chromatogr. A 778 (1997) 415.
[15] H. Qian, M. Sheng, J. Chromatogr. A 825 (1998) 127.
[16] F.J. Ruperez, C. Barbas, M. Castro, E. Herrera, J. Chromatogr. A 839 (1999) 93.
[17] J.Z. Huo, H.J. Nelis, P. Lavens, P. Sorgeloos, A.P. De Leenheer, J. Chromatogr. B 724 (1999) 249.
[18] E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventós, M.C. de la Torre, M.C. López-Sabater, J. Chromatogr. A 881 (2000) 251.
[19] J.K. Lodge, M.G. Traaber, A. Elsner, R. Brigelius-Flohé, J. Lipid Res. 41 (2000) 148.
[20] G.W. Chase, A.R. Long, J. AOAC Int. 81 (1998) 577.
[21] K.E. Sharpless, M. Arce-Osuna, J. Brown Thomas, L.M. Gill, J. AOAC Int. 82 (1999) 288.
[22] T.S. Chin, J.S. Godber, J. Am. Oil Chem. Soc. 70 (1993) 1289.
[23] A. de Winne, P. Dirinck, J Agric. Food Chem. 44 (1996) 1691.
[24] G.W. Chase, R.R. Eitenmiller, A.R. Long, J. AOAC Int. 82 (1999) 107.
[25] J.Z. Huo, H.J. Nelis, P. Lavens, P. Sorgeloos, A.P. De Leenheer, Anal. Biochem. 242 (1996) 123.
[26] F.J. Ruperez, C. Barbas, M. Castro, S. Martínez, E. Herrera, J. Chromatogr. A 823 (1998) 483.
[27] M.M. Delgado-Zamarreño, M. Bustamante-Rangel, A. Sánchez-Pérez, J. Hernández-Méndez, J. Chromatogr. A 935 (2001) 77.
[28] D.I. Sánchez-Machado, J. López-Hernández, P. Paseiro-Losada, J. Chromatogr. A 976 (2002) 277.
[29] S.A. Tanumihardjo, K.L. Penniston, J. Lipid Res. 43 (2002) 350.
[30] F.J. Ruperez, D. Martín, E. Herrera, C. Barbas, J. Chromatogr. A 935 (2001) 45.
[31] S.L. Abidi, J. Chromatogr. A 881 (2000) 197.
[32] G. Carlucci, P. Mazzeo, S. Del Governatore, G. Di Giacomo, G. Del Re, J. Chromatogr. A 935 (2001) 87.
[33] J.L. Luque-García, M.D. Luque de Castro, J. Chromatogr. A 935, 3.
[34] C. Turner, J.W. King, L. Mathiasson, J. Chromatogr. A 936 (2001) 215.
[35] The United States Pharmacopeia (USP XXIII), Mack Printing, Easton, 1989, p. 1711.
[36] I.S. Chen, C.S.J. Shen, A.J. Sheppard, J. Am. Oil. Chem. Soc. 58 (1981) 599.