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Journal of Chromatography A, 947 (2002) 97–102

JOURNAL OF CHROMATOGRAPHY A

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High-performance liquid chromatographic determination of tocopherols in infant formulas☆

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Received 12 June 2001; received in revised form 6 December 2001; accepted 6 December 2001

Abstract

A method for the simultaneous determination of α-tocopherol acetate and α-, δ-, and γ-tocopherols by normal-phase high-performance liquid chromatography (HPLC) with a fluorescent detector in infant formula is proposed. The values obtained in the determination of the analytical parameters: linearity, precision, limit of detection and accuracy (analysis of a standard reference material, SRM 1846), confirm the quality of the method. The proposed method is useful for the determination of α-, δ-, and γ-tocopherols and α-tocopherol acetate in infant formulas at a low cost and in a total time of 2 h.

Keywords: Infant formulas; Food analysis; Tocopherols; Vitamins

1. Introduction

Vitamin E is a generic term that includes, in decreasing order of physiological activity α-, β-, γ- and δ-tocopherols (α-T, β-T, γ-T and δ-T). Tocopherols are added during the manufacture of infant formulas either to improve the vitamin E supply or to prevent lipid oxidation, and the use of α-T (D and DL) and α- tocopherol acetate (α-TAc) (D and DL) for this purpose is permitted by law [1]. However, infant formulas can also contain tocopherols coming from the oils used in their manufacture, and therefore it is not sufficient to determine just α-T or both α-T and supplemented α-TAc to assess the vitamin E value of infant formulas.

High-performance liquid chromatography (HPLC) is the technique of choice for determining the different tocopherols with vitamin E and/or antioxidant activity [2]

In milk, dairy products and infant formulas, reversed-phase HPLC (RP-HPLC) after saponification is the most widely used technique to determine vitamin E. Different detectors have been used: electrochemical [3,4], UV [5,6], or UV and mass spectrometry [7], RP-HPLC with fluorescence detection was also used to measure different tocopherols in dairy products [8].

☆Presented at the 30th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques/1st Meeting of the Spanish Society of Chromatography and Related Techniques, Valencia, 18–20 April, 2001.

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01596-5

On the other hand, normal-phase HPLC (NP-HPLC) with fluorescence detection has been applied to the determination of α-, β-, γ- and δ-tocopherols in infant formulas [9–11].

Saponification converts the α-TAc to α-T, which cannot be differentiated from the naturally occurring α-T [12]. Therefore, the methods that include saponification of the sample prior to the chromatographic separation obtain total α-T (the sum of α-T and α-TAc).

Direct lipid extraction without saponification using NP-HPLC with detection by fluorescence has been utilized to determine α-TAc, α-, γ- and δ-T [12–15] and all these plus α-TAc, β-T and tocotrienols [16] in infant formulas. The same chromatographic system but with ultraviolet detection has been used to determine α-TAc, α- and γ-T in human milk [17].

A comparison between α-, β-, γ- and δ-T determination in dairy products and infant formulas (supplemented with α-TAc) by RP-HPLC and NP-HPLC with saponification and by NP-HPLC without saponification was carried out [18], using in both cases, fluorescence detection. Although the author proposed a simplified approach involving saponification, he concluded that when facilitated by the matrix, a non-saponification, total lipid dilution approach must be considered preferable to the saponification procedure because better accuracy is obtained.

The elimination of the saponification step permits quantification of the added ester forms and of natural vitamin E homologs. The use of NP-HPLC makes fat removal unnecessary in the case of infant formulas, since NP-HPLC can accommodate up to 2 mg of fat per injection. On the other hand, the stability of the analytes is increased when working with the ester forms of vitamins A and E [12]. Therefore, the aim of our study was to set up and validate an NP-HPLC method for measuring simultaneously α-TAc and α-T, δ-T and γ-T contents in infant formulas, that would have a simple sample preparation and be relatively inexpensive.

2. Experimental

2.1. Samples

Two adapted infant formulas supplemented with DL-α-T (formula A) or DL-α-TAc (formula B), respectively were used. The samples were maintained in their air-tight containers at 0–4 °C until analysis.

2.2. Apparatus

The HPLC system (Waters, Milford, MA, USA) consisted of a Model W600 quaternary pump. A Model 7725i manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 50 μl sample loop and a Model M474 fluorescence detector were used. Data were collected and analysed using the Millenium^{32} Chromatography Manager Simple System software package.

Solvents were filtered using a Millipore (Milford, MA, USA) system with 0.20 μm membrane filters (47 mm), and samples were filtered using a Millipore system with 0.20 μm membrane filters (13 mm).

2.3. Reagents

Tocopherols: (+)-α-TAc, (±)-α-T, (+)-δ-T and (+)-γ-T were obtained from Sigma (St. Louis, MO, USA). Chloroform Multisolvent, *n*-hexane 96% Multisolvent and ethyl acetate Multisolvent were obtained from Scharlau (Barcelona, Spain). Methanol was obtained from J.T. Baker (Deventer, The Netherlands).

High-purity water was produced with a Millipore system.

Standard solutions of α-TAc (430 mg/l), α-T (202 mg/l), δ-T (302 mg/l) and γ-T (62.5 mg/l) were prepared in *n*-hexane. These standard solutions were stable for at least 1 month at 4 °C in an argon atmosphere.

Fresh working standard solutions were prepared daily by appropriate dilutions of standards in *n*-hexane.

2.4. Sample preparation

In a 10 ml amber/light protected glassware screw-capped Pyrex tube, 1 ml of reconstituted (10%, w/w) powdered infant formula was mixed with 5 ml of chloroform–methanol (2:1, v/v) and mechanically stirred for 3 min. The mixture was let to stand for 5 min, then 1 ml of water was added and it was

manually shaken by inversion (two or three times). It was then centrifuged (1500 *g*, 10 min, 15 °C). The chloroform phase was dried under nitrogen, reconstituted with 1 ml of *n*-hexane and filtered through a 0.20 μm membrane filter. Dried samples were stable for at least 1 month and after being dissolved for 1 week.

2.5. Chromatographic procedure

The chromatographic separation was performed at room temperature on an Nova-Pak silica column (150×3.9 mm, 5 μm particle size, 60 Å nominal pore size). The mobile phase was a mixture of *n*-hexane–ethyl acetate (98:2, v/v) at a flow-rate of 1 ml/min. An aliquot of 50 μl of sample was injected. The fluorescence was measured at λ_{295} nm excitation and λ_{330} nm emission wavelengths. The quantification was made using external standard calibration.

3. Results and discussion

3.1. Optimization of the chromatographic conditions for tocopherol determination in infant formulas

Two different volume ratios of *n*-hexane–ethyl acetate (96:4 and 98:2) as mobile phase were tested. Both were adequate to obtain good resolution in the case of standard solutions. Applied to samples, only the 98:2 (v/v) ratio permitted separation of an interference with a retention time near to that of α-TAc.

With the chromatographic conditions summarized in the Experimental section it was possible to resolve the mixture of α-TAc, α-T, γ-T and δ-T in 18 min. The obtained retention times for the mentioned tocopherols showed repeatabilities of 0.8, 0.6, 0.8 and 0.6%, respectively, and reproducibilities of 4.9, 4.5, 3 and 3.8%, respectively.

The structures of tocopherols and the chromatograms corresponding to (a) standard, (b) infant formula A (supplemented with α-T) and (c) infant formula B (supplemented with α-TAc) are included in Fig. 1

3.2. Sample preparation/optimization of the method

In order to obtain the lipid fraction of the sample three different procedures were assayed: (a) a fat extraction according to Folch et al. [19]; (b) the extraction described by Chappell et al. [17]) for simultaneous HPLC analysis of retinal ester and tocopherol isomers in human milk; and (c) the procedure proposed by Balz et al. [16] for simultaneous determination of all tocopherols, tocotrienols and supplemented α-TAc by HPLC in food.

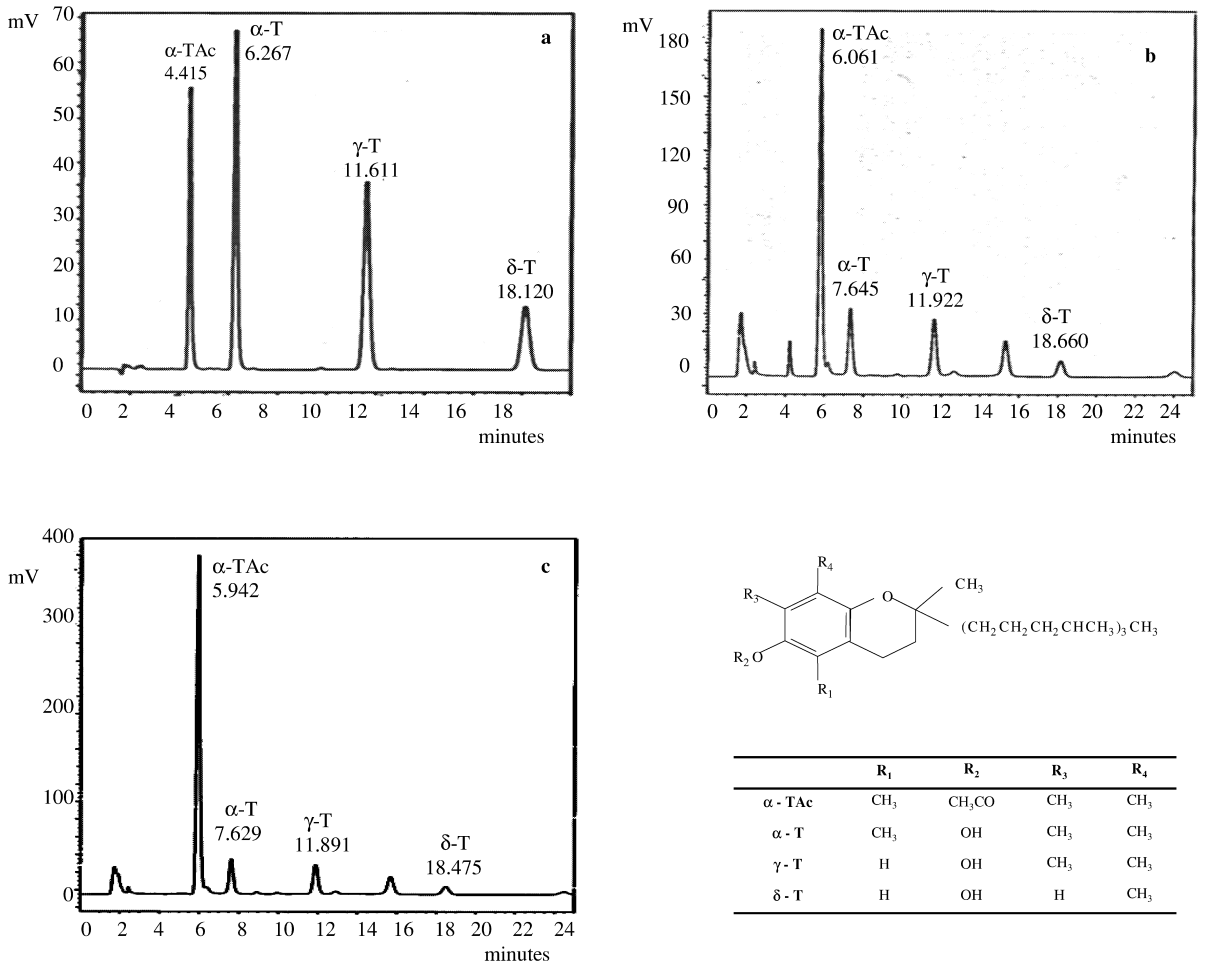
The assays were carried out in formula A and the results obtained are reported in Table 1. The lipid extraction procedure proposed by Balz et al. [16] is more complex, more time consuming and requires a larger consumption of reagents than the other two methods, and this contributes to the high variability of the results obtained with this procedure. Given that the results obtained (relative standard deviation, RSD) with procedures a and b were similar, that removing chloroform–methanol was easier than removing ethanol and that the Folch et al. method [19] is usually applied in our laboratory to extract fat from infant formulas, this was our method of choice.

However, in order to improve the quality of the procedure, the following points were taken into account.

(i) Reduction of sample size and volume extractant. The sample was a 10% (w/v) reconstituted infant formula. The sample:extractant ratios (v/v) assayed were: 1/5, 1/8, 0.5/5 and 0.5/8. In all cases the mixture was stirred for 3 min. No statistically significant differences ($P<0.05$) were detected between the results obtained with the different ratios. Therefore, in order to minimize the volume of solvents to be used, a 1:5 sample:extractant ratio was used.

(ii) Stirring time: once the sample: extractant ratio had been selected, two stirring times (3 and 6 min) were assayed. Given that no increase in the tocopherol contents was observed with the increase of the stirring time, the 3 min time was chosen.

(iii) Antioxidant use: the addition, in order to prevent the oxidation of tocopherols, of an antioxidant (methylated hydroxytoluene), at a 0.01% (w/v) final content, to the extractant solution and to the hexane used to prepare tocopherols standards proved to be unnecessary.



	R_1	R_2	R_3	R_4
α - TAc	CH_3	CH_3CO	CH_3	CH_3
α - T	CH_3	OH	CH_3	CH_3
γ - T	H	OH	CH_3	CH_3
δ - T	H	OH	H	CH_3

Fig. 1. The structure of tocopherols and the chromatograms corresponding: (a) standard, (b) infant formula supplemented with α-TAc, (c) infant formula supplemented with α-T.

(iv) Reextraction of the sample: a reextraction of the sample did not increase the tocopherol contents.

(v) No differences were detected between blowing a gentle stream of nitrogen and vacuum drying in the removal of hexane.

(vi) Temperature applied to reconstitute the infant

Table 1
Tocopherol contents (expressed as mg/100 g) obtained with different sample treatments

Refs.	Content					
	α-T		γ-T		δ-T	
	Mean±SD (mg/100 g)	RSD (%)	Mean±SD (mg/100 g)	RSD (%)	Mean±SD (mg/100 g)	RSD (%)
[18]	9.94±0.25	2.50	2.63±0.09	3.42	1.07±0.05	4.42
[16]	9.19±0.19	2.03	1.71±0.13	7.52	0.81±0.02	2.57
[15]	12.09±2.79	23.00	3.41±9.12	26.70	1.60±0.47	29.17

formula: the two temperatures assayed were 22 °C (room temperature) and 100 °C. The assay was carried out in a formula containing α-TAc. The increase in the water temperature did not improve either the sample dissolution or the tocopherols yield.

On the basis of the results of all these assays the method for determining α-TAc, α-T, γ-T and δ-T in infant formulas was optimised and is the one described in the Experimental section.

3.3. Stability of lipid extracts of the samples

To check the stability of the dried and *n*-hexane dissolved lipid extracts of the samples, (a) several dried extracts of the same sample were frozen and stored at −18 °C (the results obtained revealed that they were stable for at least 1 month) and (b) dried lipid extracts of the same sample were dissolved in hexane and stored at 4 °C, and then injected by triplicate on 5 consecutive days (during this time the tocopherol contents of the extracts were stable).

3.4. Analytical parameters

To check the quality of the proposed method the analytical parameters were determined.

3.4.1. Linearity

Linearity was tested by analysing two sets of standards, containing α-TAc and α-T (2–16 mg/l), γ-T (1–5 mg/l) and δ-T (0.4–2 mg/l) in hexane. The whole procedure including sample treatment was applied to one set, while the other set of standards were directly injected. The simple linear regression and correlation coefficient obtained are shown in Table 2.

The application of covariance analysis did not reveal statistically significant differences ($P<0.05$) between the values obtained in the two sets.

3.4.2. Detection limit

The detection limit (signal-to-noise ratio 3) for α-TAc, α-T, γ-T and δ-T were: 3.46, 1.60, 0.16 and 0.06 ng/50 μl injection volume, respectively.

3.4.3. Precision

The precision of the method was calculated from the analysis of four homogeneous aliquots of an infant formula (B), whereas the instrumental precision was checked from four consecutive injections of the same extract. Values expressed as RSD are reported in Table 2.

3.4.4. Accuracy

The accuracy of the method was checked by analysing a Standard Reference Material (SRM 1846): Milk-based Powdered Infant Formula. Vitamin E was added to the infant formulas as RRR-α-tocopheryl acetate and expressed as vitamin E (as α-T).

Given that the studied method makes it possible to quantify α-TAc and α-T independently, in order to compare the values obtained with those of the

Table 2
Linearity and precision of α-tocopherol acetate (α-TAC), α-tocopherol (α-T), γ-tocopherol (γ-T) and δ-tocopherol (δ-T) determination

	Linearity		Precision[b] ($n=4$)[c]	
	Standard[a]	Standard	Instrumental	Method
α-TAc	$y = 17998x + 1228$ $r = 0.9988$	$y = 21364x + 10943$ $r = 0.9902$	4.6	8.2
α-T	$y = 300106x - 123382$ $r = 0.9963$	$y = 333048x - 49300$ $r = 0.9992$	2.7	7.0
γ-T	$y = 149373x + 2289$ $r = 0.9996$	$y = 159295x + 11757$ $r = 0.9986$	1.4	9.1
δ-T	$y = 185310x - 17950$ $r = 0.9997$	$y = 194763x - 10387$ $r = 0.9977$	3.7	8.0

[a] Standard subjected to the sample treatment.
[b] RSD (%).
[c] n = number of samples.

Table 3
Accuracy of vitamin E as α-tocopherol (α-T), γ-tocopherol (γ-T) and δ-tocopherol (δ-T)

	Certified value[a] (mg/kg)	Reference value[a] (mg/kg)	Found[a] (mg/kg) ($n=3$)[b]
Vitamin E(as α-T)	271±25		275±19
γ-T		73.5±3.0	74.3±2.4
δ-T		17.99±0.46	18.00±0.76

[a] Mean±SD.
[b] n = number of samples.

reference material SRM 1846, the amount found of α-TAc was converted in α-T and added to the obtained α-T content. The values obtained are reported in Table 3.

4. Conclusions

The proposed method allows the simultaneous determination of α-TAc and α-T, δ-T and γ-T in infant formulas in a total time of 2 h, including the sample preparation and the NP-HPLC determination. The method does not require the use of HPLC grade solvents, this results in lower cost.

The values of the analytical parameters proved that the proposed method is useful for the determination of the different free tocopherols and α-TAc in infant formulas.

Acknowledgements

This study is part of a project (IFD97-0284) financially supported by CICYT (Spain) and FEDER/EU. We thank the Generalitat Valenciana for the financial support given to the ''Bionutest'' group.

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