



Determination of α -tocopherol and α -tocopheryl acetate in diets of experimental animals Study of stability in the diets[☆]

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Abstract

A simple method is described which permits, avoiding saponification, α -tocopherol and α -tocopheryl acetate measurement in semi-synthetic diets for experimental animals by HPLC, with both UV and fluorescence detection. Phenylododecane was chosen as internal standard with remarkable performances, and EDTA and BHT were added to prevent oxidation in aqueous and non-aqueous phases respectively. The mobile phase was methanol–water (94:6, v/v) at a flow-rate of 2 ml/min. Samples were homogenized and extracted twice with *n*-hexane by probe sonication. Extracts were evaporated to dryness and redissolved with chloroform–methanol (1:1, v/v). Validation parameters were studied between 25 ng and 6 μ g for α -tocopherol and between 3 and 24.2 μ g for α -tocopheryl acetate, which corresponds to the range of values in the existing diets. Results had correlation coefficients >0.99 ; recoveries $>85\%$; R.S.D. $<6\%$, so the method is adequate to control vitamin E intake in animals as well as vitamin E stability in food during storage. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The methods followed for tocopherol analysis in foods and feeds are, overall, similar to those used for tissues. Foods or feeds need to be either grounded and extracted directly [1–3] or saponified and extracted [4–10].

Semi-synthetic animal diets used on biochemical and metabolic studies, usually contain free metal

ions, such as copper and manganese, that accelerate oxidation of tocopherols when the diet gets in contact with water in the homogenization step.

Vitamin E is usually present in these diets as α -tocopherol and α -tocopheryl acetate, and contents must be carefully measured when studying vitamin E concentration in plasma and tissues.

Tocopherols are separated by HPLC on normal-phase [1–3,6,8,9,11–20] or reversed-phase columns [4,7,10,11,21–27] and generally detected by fluorescence, which is more sensitive and selective than UV.

Normal-phase separates all forms of tocopherols, but since reversed-phase is more reproducible and easy to work with and only the α -isomer was

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measured, because it is the most abundant in animals and has the greatest biological activity [28], reversed-phase was selected.

Simultaneous detection with both fluorescence and UV is used since α -tocopherol is better detected by fluorescence, and α -tocopheryl acetate has no fluorescence, therefore internal standard with both responses is needed. Moreover, the wide range of α -tocopherol content found in diets, depending of the type and percentage of fat, sometimes saturated the fluorescence detector, due to the high α -tocopherol content, and then samples can be measured in less sensitive UV detector, avoiding new dilution and analysis. Other internal standards, such as vitamin A acetate [29] or vitamin K₁ [27] cannot be used because they are also present in the samples.

This paper describes a simple method avoiding saponification, which permits α -tocopherol and α -tocopheryl acetate detection, by UV and fluorescence. Phenylododecane was chosen as internal standard with a remarkable performance. Ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) were added to prevent oxidation in aqueous and non-aqueous phases, respectively.

Validation parameters: linearity, intra- and inter-assay precision and accuracy have been determined and stabilities of α -tocopherol and α -tocopheryl acetate in storage conditions have been evaluated.

2. Experimental

2.1. Instrumentation

A Beckman HPLC system provided with a 126 pump, an automatic injector (507e), a 168 diode array detector, a GOLD System data processor and a Waters 474 fluorimetric detector were used. The chromatographic analysis was performed on a 5- μ m particle C₁₈ Nucleosil 120 column (15×0.46 cm) kept in a Bio-Rad column oven at 40°C.

2.2. Reagents

All solvents were HPLC grade quality purchased from Scharlau (Barcelona, Spain). α -Tocopherol and α -tocopheryl acetate were from Fluka (Madrid, Spain), phenylododecane was from Merck (Madrid,

Spain). EDTA and BHT were from Sigma (St. Louis, MO, USA) and all other reagents were analytical grade from Merck.

2.3. Methods

The semi-synthetic diet used in the experiment is based in the one previously used in our laboratory [30] and the composition appears in Table 1. Olive oil diet was selected for method development and validation for the intermediate vitamin E content compared to other oils in the diet, as sunflower seed or fish oils.

2.4. Procedure

2.4.1. Stock and working standards

Individual stock solutions of α -tocopherol and α -tocopheryl acetate as external standard, and phenylododecane as internal standard, were prepared at a concentration of 8.0 mg/ml in ethanol. These solutions were stored in aluminium foil-covered containers and kept at -20°C for a period under 15 days. On the day of the assay, the α -tocopherol solution was diluted 1:200 (v/v) and the α -tocopheryl acetate 1:33 (v/v), both in ethanol, and a 1:1 (v/v) mixture of both dilutions was used as the working standard. Vitamin concentration in respective dilutions before mixing was determined at 294 nm for α -tocopherol ($\epsilon_{1\%}^{1\text{cm}}$ 71) and 284 nm for α -tocopheryl acetate ($\epsilon_{1\%}^{1\text{cm}}$ 43.5). The phenylododecane stock solution was diluted 1:20 (v/v) in ethanol.

2.4.2. Working standards treatment

In 80×12 mm low-actinic glass tubes, the following reagents were mixed: 50 μ l of the working standard of α -tocopherol (0.02 mg/ml) plus α -tocopheryl acetate (0.12 mg/ml), 50 μ l of BHT 40 mM (in ethanol), 50 μ l of ethanol, 50 μ l of the working solution of phenylododecane (0.4 mg/ml), 200 μ l of methanol, 100 μ l of water and 200 μ l of EDTA 0.1 mM (in water). Tubes were shaken for good homogenization. BHT and EDTA concentrations, and the ethanol-methanol-water (1:1:1.5, v/v/v) proportion was optimized in order to (a) avoid precipitation of BHT in water and of EDTA in ethanol, (b) to ensure a good dissolution of every

Table 1
Composition of diets

Compound	Mass/4 kg diet
CuSO ₄	0.4 g
ZnSO ₄	0.364 g
MgSO ₄	13.513 g
MnSO ₄	4.5 g
(NH ₄) ₂ Fe(SO ₄) ₂ ·6H ₂ O	9.352 g
NH ₄ MoO ₄	0.106 g
CaHPO ₄	0.870 g
KH ₂ PO ₄	59.02 g
NaIO ₃	0.0012 g
K ₂ CrO ₄	0.114 g
NaCl	16 g
CaCO ₃	39.56 g
Corn starch ^a	2.52 or 2.32 kg
Casein	0.68 kg
Oil (fat) ^b	200 or 400 g
Cellulose	400 g
Complex B+C (Becozyme):	2 tablets
Thiamine (B ₁)	15 mg/tablet
Riboflavin (B ₂)	15 mg/tablet
Nicotinamide (PP)	50 mg/tablet
Piridoxin (B ₆)	10 mg/tablet
Calcium pantotenat (B ₅)	25 mg/tablet
Biotin (H)	150 µg/tablet
Cyanocobalamin (B ₁₂)	10 µg/tablet
Ascorbic Acid (C)	200 mg/tablet
Biotin	0.002 g
Folic acid (Acfol)	0.02 g
Vitamin K:	
Fitomenadione (Konaktion)	0.096 g
Menadione	0.003 g
Choline clorohydrate	16 g
Vitamin A:	
Retinol (Auxina A)	50 000 I.U.
Vitamin D ₃ :	
Cholecalciferol	0.1312 g
Vitamin E:	
α-Tocopheryl acetate (Auxina E)	400 I.U.

^a 2.52 kg for 5%-fat diet and 2.32 kg for 10%-fat diet.

^b The kind of oil or fat is different depending on the experimental group: palm fat (Gracom, Spain), sunflower oil (Koipe, Spain), olive oil 1° (Koipe, Spain) or fish oil (Cofares, Spain); 200 g for 5%-fat diet and 400 g for 10%-fat diet.

component prior to extraction, and (c) to get a good separation of phases after extraction.

Extraction of vitamins was performed twice by probe sonication with 1 ml of *n*-hexane, followed by centrifugation at 2000 g for 5 min, at room temperature. Supernatants were pooled and evaporated to dryness in a centrifuge concentrator (Virtix, USA).

The residue was redissolved in 200 µl of chloroform-methanol (1:1, v/v).

2.4.3. Sample treatment

Approximately 100 mg of the non-desiccated diet were exactly weighed, and 50 µl of BHT 40 mM (in ethanol), 100 µl of ethanol, 50 µl of the working solution of phenyldecane, 200 µl of methanol, and 200 µl of EDTA 0.1 mM (in water) were added. Samples were homogenized by probe sonication to make a 'sample homogenate', with ethanol-methanol-water (1:1:1.5, v/v/v) from which vitamins were extracted as described above for working standards.

2.5. Chromatographic analysis

The reversed-phase HPLC analyses were carried out with methanol-water (94:6, v/v) as eluent, at a flow-rate of 2 ml/min.

Fluorescence detection was performed with excitation at 260 nm and emission at 600 nm in the first 8 min for phenyldecane, and at 295 nm and 350 nm until the end of run (min 18) for α-tocopherol. Simultaneous UV detection was at 260 nm in the first 8 min (phenyldecane), at 294 nm until min 11 (α-tocopherol), and 284 nm until the end of run (α-tocopheryl acetate). A 30-min run was allowed for diets containing fish oil, in order to permit elution of vitamin A palmitate, abundant in this type of oil.

Peak identification was performed with retention times as compared with standards and confirmed with characteristic spectra using the photodiode array detector.

2.6. Validation

Standards linearity was verified by the analysis of duplicates of thirteen points in the range of 25.0 ng to 6.0 µg of α-tocopherol and 3.0 to 24.2 µg of α-tocopheryl acetate in order to cover the whole range of concentrations in samples. Solutions were processed as described above for working standards.

Sample linearity for both forms of vitamin E was tested in duplicates of 160, 325, 650, 975 and 1300 µl of 'sample homogenate'. Volumes of all tubes were brought to 1400 µl with the appropriate amount of BHT, ethanol, internal standard, methanol, water

and EDTA to achieve the ethanol-methanol-water (1:1:1.5, v/v/v) proportion in every tube. Subsequent processing was as described above for samples.

Intra- and inter-assay precision was determined by processing two six-sample series of 650 μl of the same homogenate plus internal standard on different days (the homogenate was kept at 4°C and under nitrogen between days). Standards for quantification were prepared from 50 μl of the working solution and treated simultaneously.

For recovery evaluation of α -tocopherol and α -tocopheryl acetate, an homogenate was prepared with solid diet, BHT, methanol, EDTA and water, arranged in such a way that 550 μl of the homogenate had 50% of the amount of diet and so, 50% of the vitamin E present in a normal sample, and the rest of vitamin E was added as a standard (from 12.5 ng to 3 μg of α -tocopherol and from 1.5 to 12.1 μg of α -tocopheryl acetate). The total volumes and proportions were equalled and samples were processed as described above, including the corresponding standards for quantification.

2.7. Stability

To test stability, freshly manufactured diets were prepared and divided into 300-g portions, covered with aluminium foil, and kept at -20°C for 15 days. On the evening of the 14th day, portions were placed at 4°C until the morning, when six aliquots were tested. The remaining sample was kept for the 2 following days before analysis in a room at $22 \pm 3^\circ\text{C}$, $55 \pm 10\%$ humidity, 1 atm, corresponding to normal animal quarter storage room conditions (1 atm = 101 325 Pa).

To avoid the influence of differences in the water content among samples, results are expressed as micrograms of vitamin per gram of dry mass.

3. Results and discussion

Fig. 1 shows some chromatograms from standards and samples with UV and fluorescence detection, evidencing adequate separation of analytes. As there can be seen, a number of unidentified peaks appear in standards chromatograms they correspond to

BHT, EDTA and a minor peak of phenyldodecane in UV and solvent impurities with fluorescence emission plus a minor peak of phenyldodecane in fluorescence.

Validation results appear in Table 2. Both standards and samples show a good linearity, with correlation coefficients over 0.99, except for α -tocopherol measured with UV in samples, indicating that low levels of α -tocopherol are better detected with fluorescence.

Intra-assay precision shows R.S.D. ranging between 2.7 and 3.9% and inter-assay R.S.D. ranges between 2.3 and 4.8% which are considered appropriate values for the amount of analyte to be measured. Results are expressed as micrograms of vitamin per gram of dry mass.

Recoveries range between 88 ± 9 and $92 \pm 9\%$ (Table 2), indicating an adequate accuracy of the method. Previous studies in our laboratory following the same procedure but without adding EDTA or BHT showed recoveries ranging from 0 to 20% for α -tocopherol, and near 80% for α -tocopheryl acetate (unpublished data). This finding shows the need to protect α -tocopherol from oxidation, which may occur directly in the aqueous interphase in the presence of metals or as a result of the free radical generation and the promotion of lipid peroxidation process started by these metals. According to the present results, these effects seem to be prevented by the presence of EDTA, which is a water soluble metal complexing agent, and BHT, which is soluble in organic solvents and a free radical quencher.

To evaluate the homogeneity of vitamin distribution in the manufactured diets, six aliquots of fresh diet were measured using the proposed method. The R.S.D. values were 7.9% for α -tocopherol and 6.9% for α -tocopheryl acetate, which is, as expected, a bit higher than R.S.D. in inter-assay precision, but permits confirmation of a good distribution of both vitamins in the diet.

The content of α -tocopherol decreased in the diet from day 0 to day 15 of storage [29.3 ± 0.9 to 15 ± 2 $\mu\text{g/g}$ (dry mass), $P < 0.001$], whereas the amount of α -tocopherol found at day 17 [13.0 ± 0.5 $\mu\text{g/g}$ (dry mass)] as compared to day 15 was within the limits of confidence for the method.

The content of α -tocopheryl acetate found in the samples stored for different days was stable

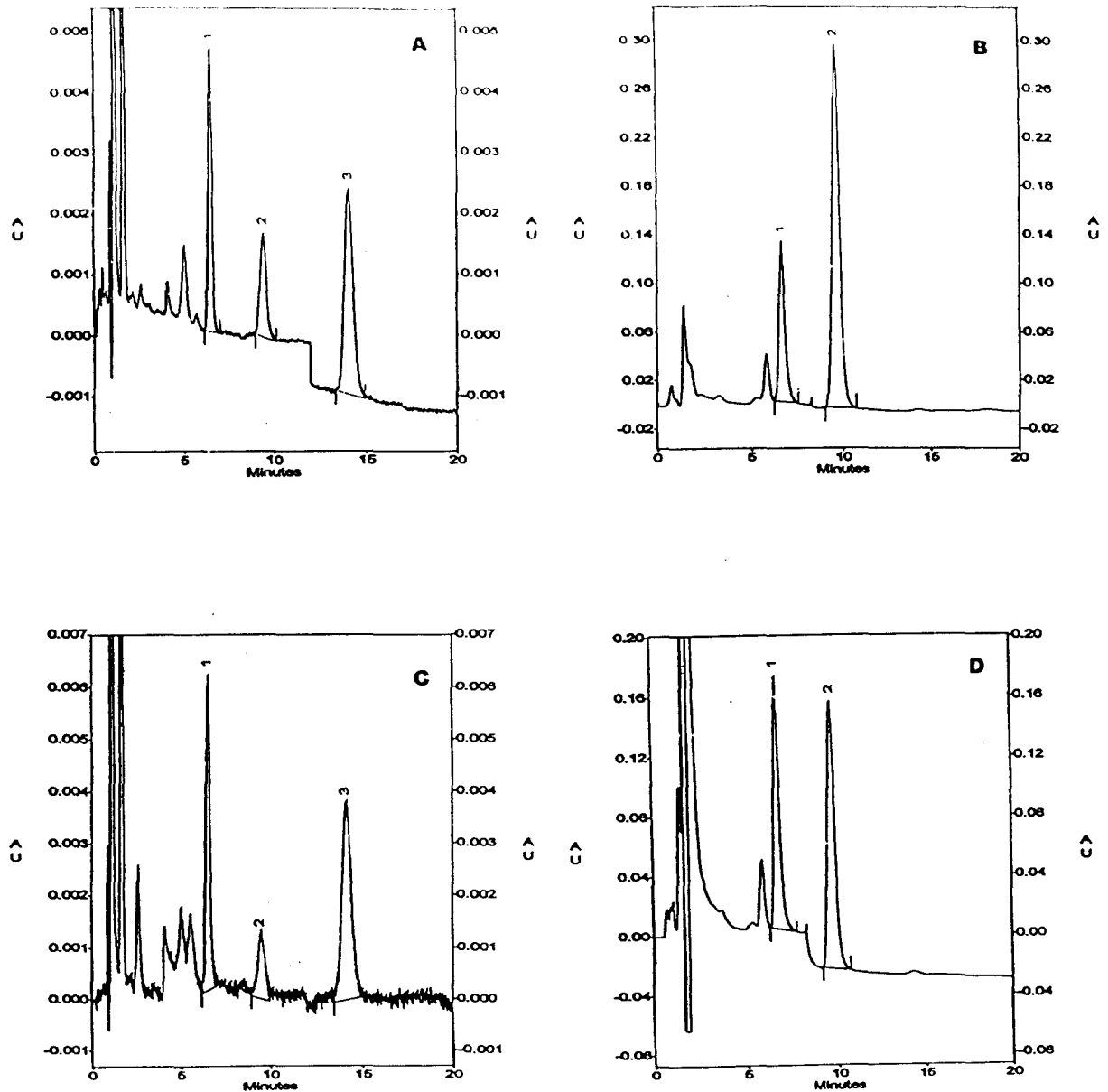


Fig. 1. Chromatograms of (A) standards UV; (B) standards fluorescence; (C) sample UV; (D) sample fluorescence. 1=Phenyldodecane; 2= α -tocopherol; 3= α -tocopheryl acetate. HPLC conditions: 40 μ l volume injected, methanol–water (94:6, v/v) as eluent, at a flow-rate of 2 ml/min. Fluorescence detection: excitation at 260 nm and emission at 600 nm ($t=0$ –8 min) and at 295 nm and 350 nm ($t=8$ –18 min). Simultaneous UV detection was at 260 nm ($t=0$ –8 min), at 294 nm ($t=8$ –11 min) and 284 nm till the end of run.

(86.8 ± 2.4 μ g/g at day 0, 87.6 ± 1.3 at day 15 and 81.3 ± 2.4 at day 17) and no significant differences were detected as expected, due to the known resistance of α -tocopheryl acetate to oxidation.

Table 3 shows the α -tocopherol and α -tocopheryl acetate content in diets containing 5 or 10% of either palm fat, sunflower, olive and fish oils. Results may vary depending on the brand of oil, since some of

Table 2
Validation parameters

		α -Tocopherol		α -Tocopheryl acetate	
		Fluorescence	Ultraviolet		
Standards linearity	Intercept	0.04 \pm 0.02	-0.009 \pm 0.009	0.01 \pm 0.02	
	Slope	0.627 \pm 0.007	0.164 \pm 0.001	0.112 \pm 0.001	
	<i>r</i>	0.999	0.999	0.995	
	Range (μ g/tube):	0.025–3.0	0.1–6.0	3.0–24.2	
Samples linearity	Intercept:	0.02 \pm 0.03	0.005 \pm 0.04	0.02 \pm 0.05	
	Slope	0.0181 \pm 0.0003	0.0400 \pm 0.0003	0.0136 \pm 0.0003	
	<i>r</i>	0.999	0.982	0.998	
Precision (μ g/dry g)					
	Intra-assay	Mean	17.0	15.9	89.1
		R.S.D. (%)	3.6	3.9	2.7
Inter-assay		Mean	16.5	15.9	89.5
		R.S.D. (%)	4.8	4.1	2.3
Accuracy		Recovery (%)	88	92	89
		R.S.D. (%)	8.8	9.2	6.9

Table 3
Vitamin E content in diets^a

	α -Tocopherol (μ g/dry g)	α -Tocopheryl acetate (μ g/dry g)	Vitamin E (I.U./dry g)
Palm 5%	0.57 \pm 0.03	75.4 \pm 4.3	76.2 \pm 4.3
Olive 5%	18.0 \pm 1.9	45.5 \pm 0.5	72.3 \pm 2.6
Olive 10%	25.8 \pm 0.7	53.3 \pm 5.7	91.7 \pm 5.7
Sunflower 5%	63.3 \pm 7.1	38.6 \pm 5.7	132.9 \pm 16.3
Sunflower 10%	177.7 \pm 8.3	27.6 \pm 2.5	292.3 \pm 14.9
Fish 10%	3.2 \pm 0.6	55.9 \pm 3.9	60.7 \pm 5.4

^a Results are expressed as mean \pm S.D.

them have α -tocopheryl acetate added, and with the degree of rancidity of the oil. These differences must be considered when studying the effect of diets on vitamin E stability in animals. Results shown in Table 3 are also expressed as total vitamin E activity, considering that 1 I.U. is referred to as 1 mg of the synthetic form, racemic α -tocopheryl acetate, and 1 mg of the natural form of D- α -tocopherol has a biopotency of vitamin E equal to 1.49 I.U. [28].

4. Conclusion

A method to measure α -tocopherol and α -tocopheryl acetate in semi-synthetic diets of experimental animals in presence of metallic ions and

other vitamins has been developed and validated, involving EDTA, as a water soluble metal quellant, and BHT, as a free radical quencher, in order of preventing α -tocopherol oxidation, and phenyldodecane as the internal standard which responds both to UV and fluorescence detectors. This method has been used to determine these two forms of vitamin E in diets prepared with different levels and types of fat or oil and to study their stability in experimental conditions.

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