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Determination of E vitamers in microalgae using high-performance liquid chromatography with fluorescence detection

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Abstract

No validated high-performance liquid chromatography (HPLC) methods for the determination of vitamin E in algae have been reported. We developed and evaluated a new procedure for the quantitation of microalgal α- and γ-tocopherol (α-T and γ-T) using reversed-phase HPLC with fluorescence detection. The compounds of interest were extracted with a mixture of petroleum ether–diisopropyl ether (3:1, v/v) after saponification in the presence of pyrogallol. δ-Tocopherol (δ-T) or tocol was used as an internal standard. Linearity was achieved ($r>0.999$) over the range of 0.11~2.32 μg/ml for α-T and 0.02~0.23 μg/ml for γ-T. The within-run coefficients of variation for repetitive analysis were 1.9%, 1.7% and 1.0% ($n=10$) for α-T at concentrations of 0.09, 1.24 and 1.55 μg/ml, and 1.9%, 0.8% and 0.3% ($n=10$) for γ-T at concentrations of 0.02, 0.08 and 0.23 μg/ml, respectively. The recoveries at low concentration levels (0.11 and 0.02 μg/ml) were 82.4% and 96.2% ($n=5$) for α-T and γ-T, respectively. For medium concentrations (0.78 and 0.12 μg/ml) and high concentrations (2.32 and 0.20 μg/ml), the recoveries were 94.3~98.7% for the two tocopherols. The limits of quantitation of α-T and γ-T were 20 and 5 μg/g dry mass, respectively. This method has been routinely used as part of nutritional studies on fish and shrimp larvae fed on microalgae. © 1997 Elsevier Science B.V.

Keywords: Algae; Vitamins; Tocopherol

1. Introduction

Microalgae are extensively used in hatcheries as a food source for fish and shrimp [1]. Larval feed should not only provide macronutrients but also micronutrients, including vitamins. Among the latter, vitamin E is considered an essential constituent because of its well documented ability to protect membrane lipids from oxidative damage [2]. However, previously published comparative studies on the biochemical composition of microalgae reveal substantial differences in their vitamin E content [3–6]. This variability is not only inherent in the species but also depends on environmental conditions, e.g., light and temperature [1].

On-going studies in our laboratories on the nutritional requirements of fish and shrimp larvae have involved the extensive use of microalgae as a food source. A primary goal has been to investigate the effect of micronutrients such as vitamin E on pro-

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duction characteristics and stress resistance of larvae and juveniles. To this end, an analytical method for the quantitation of tocopherols in algae was needed. Before the advent of high-performance liquid chromatography (HPLC), this analysis was mainly carried out by paper and thin layer chromatography [3,4,6–8]. More recently, some HPLC methods have been used to determine vitamin E in various algal species, but none of them has been validated with regard to reproducibility, recovery, specificity and analyte stability [5,9–11].

This paper reports a new validated reversed-phase (RP) HPLC method for the determination of α- and γ-tocopherol (α-T and γ-T) in algae, with particular emphasis on the maximization of analyte recovery and stability in the course of the sample pretreatment.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Varian 5020 pump (Varian, Palo Alto, CA, USA), a Valco injector fitted with a 100-μl loop (Valco, Houston, TX, USA) and a Perkin-Elmer LS-4 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA) set at 296 nm (excitation) and 340 nm (emission), respectively. A 5 μm Hypersil ODS column (150×4.6 mm, Shandon, Runcorn, UK), preceded by a RP guard column (50×3 mm, Chrompack, Middelburg, Netherlands) was eluted with methanol–water (96:4, v/v) at a flow-rate of 1 ml/min.

2.2. Chemicals and reagents

α-T and γ-T were purchased from Acros Organic (Geel, Belgium) and Eastman Kodak (Rochester, NY, USA), respectively. δ-T and tocol came from Eisai (Tokyo, Japan). Methanol of HPLC-grade was obtained from Romil (Loughborough, UK). Butylated hydroxytoluene (BHT) (analytical-grade) and pyrogallol were from Merck (Darmstadt, Germany) and chlorophyll A from Sigma (St. Louis, MO, USA).

Aqueous KOH solution was prepared in a concentration of 60% (w/v). Pyrogallol was dissolved in methanol in a concentration of 5% (w/v) and this solution was stored at −20°C.

The extraction solvent was a mixture of petroleum ether (40–60°C) and diisopropyl ether (3:1, v/v).

2.3. Biological samples

A 1-ml volume of algal suspension (*Chlorella* sp.) was inoculated in a test tube containing 20 ml of sea water (25 ppt salinity and filtered over a 0.45-μm filter), 50 μl of Walne medium and 1 drop of vitamin mixture. After 14 days of growth under illumination at 18°C, the mixture was transferred to an Erlenmeyer flask containing 400 ml of sea water, 400 μl of Walne medium and 3 drops of vitamin mixture and cultured for 14 days. The algae were then transferred to a carboy containing 4 l of sea water, 4 ml of Walne medium and 400 μl of vitamin mixture and culturing was continued at 25°C for one week. Finally, the 4 l of algal suspension were mixed in 20 l of sea water with 20 ml of Walne medium and 2 ml of vitamin mixture for further culturing. The algae were harvested just prior to the initiation of the stationary phase by using continuous centrifugation at 4000 rpm. After centrifugation, the cell mass was rinsed twice with 0.5 l of 0.5 *M* ammonium formate to remove salt and the cells were separated by centrifugation at 3000 rpm for 5 min. Algal samples were stored at −20°C until use.

2.4. Stock and working solutions

The stock solutions were prepared by dissolving the tocopherols in methanol to yield concentrations of 3660 μg/ml, 1905 μg/ml, 1042 μg/ml and 927 μg/ml for α-T, γ-T, δ-T and tocol, respectively. The concentrations were determined based on their UV absorption [12]. Working solutions were made by diluting these stock solutions and were stored at −20°C. δ-T, which was absent in *Chlorella* sp., was used as the internal standard (I.S.) for method validation. Alternatively, tocol was used as the I.S. for samples containing δ-T.

2.5. Sample preparation

Algae (100–200 mg wet mass) were homogenised in 2 ml of pyrogallol-methanol (PM) containing the

I.S. using a Potter–Elvehjem tube. The supernatant, obtained after centrifugation (2000 rpm, 2 min), was transferred to an extracting tube. The residue was rehomogenised with 2 ml of PM. 1 ml of PM was used to rinse the Potter tube. After the addition of 1.5 ml of aqueous 60% KOH to the combined supernatants, the mixture was vortex-mixed for 1 min and left at room temperature for 20 min to complete the saponification. 5 ml of petroleum ether–diisopropyl ether (3:1, v/v) was used to extract the mixture by vortexing for 2 min. The supernatant obtained after centrifugation (2000 rpm, 10 min) was evaporated to dryness under nitrogen at room temperature. The residue was redissolved in 5 ml of the chromatographic solvent and a 100-μl aliquot was injected on the HPLC column.

2.6. Standardisation and quantitation

Standardisation was performed by analysing algal homogenates supplemented with the analytes in concentrations of 0.11, 0.31, 0.62, 1.24 and 2.32 μg/ml for α-T and 0.02, 0.04, 0.08, 0.16 and 0.23 μg/ml for γ-T, and four replicates per concentration of each tocopherol, according to the NCCLS proposed guideline [13]. Units were μg/ml of extract (homogenate) as they related to method validation, whereas results of the analysis of algae were expressed as μg/g dry mass.

2.7. Recovery

The recovery of α-T and γ-T was evaluated by analysing five samples at three concentration levels (0.11, 0.78 and 2.32 μg/ml for α-T and 0.02, 0.12 and 0.20 μg/ml for γ-T). The concentration ranges were chosen based on the expected tocopherol contents in the algae. The homogenised samples supplemented with each analyte were analysed in the same way as described above, except that the I.S. was added at the end. Two unsupplemented samples were also analysed and the mean tocopherol concentrations were subtracted from those of the spiked samples. The recovery was calculated by comparing peak height ratios of the spiked samples with those of the corresponding standards in methanol.

2.8. Reproducibility

Reproducibility was evaluated by analysing ten samples taken from the same homogenate at three concentration levels (0.09, 1.24 and 1.55 μg/ml for α-T and 0.02, 0.08 and 0.23 μg/ml for γ-T, respectively). The low and middle concentrations of α-T were obtained in extracts of algae (22 mg and 122 mg wet mass per sample, respectively), whereas the others were from spiked samples.

2.9. Stability

The stability of tocopherols in solution, including algal extracts was assessed by repetitively injecting aliquots of them at different times and measuring the absolute peak heights. To study the effect of chlorophyll, tocol, α-, γ- and δ-T were dissolved in methanol–water (96:4, v/v) containing BHT (1 mg/ml) and this solution was divided in two parts. One part was supplemented with 1 mg of chorophyll A to obtain a green color corresponding in intensity to that of an extract of approximately 150 mg (wet mass) of algae, whereas the other part was kept as the control. The change in peak heights was monitored in both solutions as a function of time.

2.10. Limits of detection and quantitation

The detection limits (absolute quantities injected) were determined using pure standard dilutions. The limits of quantitation for the different tocopherols were estimated from the analysis of spiked samples. Each dilution was injected three times and the mean value was used in the calculation. A peak height corresponding to three times the noise level was set as the limit of detectability.

3. Results and discussion

In a previous paper we compared different strategies for the isolation of tocopherols from whole aquatic animals [14]. A monophasic extraction with methanol in conjunction with mechanical homogenisation proved to be a rapid, efficient and reliable approach. However, two complications prevented its application to algae. First, their high lipid content

could possibly lead to extraction problems and/or contamination of the HPLC column. Second, the stability of tocopherols in methanolic extracts of algae was poor, as illustrated in Table 1A. Upon storage of these solutions at room temperature, approximately 48% of α-T was lost over a time period of 7 h. The presumably oxidative degradation of α-T was thought to be due to the presence of chlorophyll in the extracts, as haem compounds are known to act as sensitizers to the oxidation of vitamin E [15]. As shown in Fig. 1, the presence of chlorophyll was indeed found to promote the degradation of α-T and, to a lesser extent that of γ-T, while δ-T and tocol remained unaffected. This observation prompted us to include an alkaline saponification step in the sample preparation to destroy the chlorophyll and at the same time hydrolyse the lipids. Initially, the procedure consisted of homogenisation using 10% potassium hydroxide in methanol in the presence of BHT (1 mg/ml), followed by a double phase extraction with hexane. The stability of α-T in methanol–water was excellent (Table 1B), but the recoveries of tocopherols from spiked algae were low, i.e., 44% (0.5 μg added per ml of extract), 35% (0.3 μg/ml) and 26% (0.2 μg/ml) (n=3) for α-T, γ-T and δ-T, respectively. Similar low extraction yields were obtained in the absence of algae indicating that the loss of the analytes was not due to matrix effects but probably to insufficient antioxidative protection. BHT was subsequently replaced by pyrogallol, which proved superior as a protective agent. A disadvantage of

Table 1
Comparison of peak heights of α-T in algal extracts injected on different days and using two extraction methods

Algae	Peak height (mm)		
	Day 1	Day 2	Day 3
(A) Monophasic extraction with methanol			
Rotifers+*Chlorella*	64	28	
Chaetoceros	7	5	
Tetraselmis	104	33	
Isochrysis	124	75	
(B) Saponification followed by double phase extraction[a]			
Chlorella α-T	100	96	95
Chlorella γ-T	23	24	24

[a] Followed by evaporation under nitrogen and reconstitution with methanol–water (96:4, v/v)+BHT.

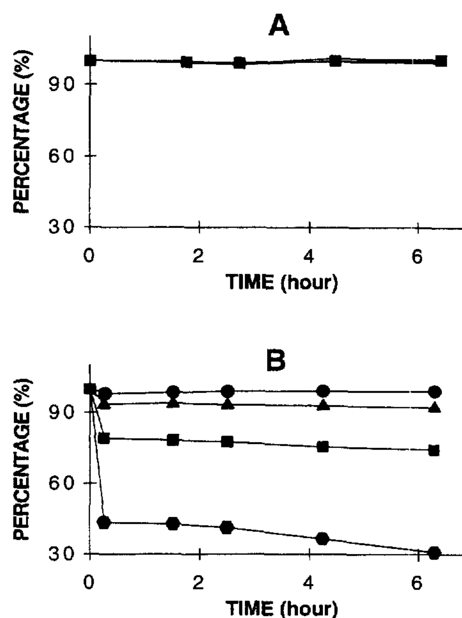

Fig. 1. Effect of chlorophyll on the peak height (% relative to the peak height at time zero) of four tocopherols as a function of time, (A) methanol–water (96:4, v/v)+BHT and (B) methanol–water (96:4, v/v)+BHT supplemented with chlorophyll A. (●) α-T; (■) γ-T; (▲) δ-T; (●) tocol. The plots for the four tocopherols overlap in (A).

pyrogallol was that it could not be combined with KOH in the initial homogenisation step because a dark brown sticky precipitate prevented grinding of the cells. Hence, the initial extraction was carried out with PM and the alkali was added afterwards. Recoveries of 88% (0.3 μg/ml), 94% (0.3 μg/ml) and 94% (0.2 μg/ml) (n=3) were obtained for α-T, γ-T and δ-T, respectively. However, despite the alkaline treatment and double phase extraction, some degradation of α-T still occurred in the final extract after evaporation of the hexane and reconstitution of the residue, the loss being about 9% over a period of 4 h. This remaining problem was overcome by increasing the concentration of KOH in the homogenate (from 4.6 to 13.8%) and extending the saponification time from 2 to 20 min. Optimal conditions were established based on a visual scoring of the disappearance of the green color in the extract and maximum peak height of α-T. Representative chromatograms of extracts of *Chlorella* are depicted in Fig. 2.

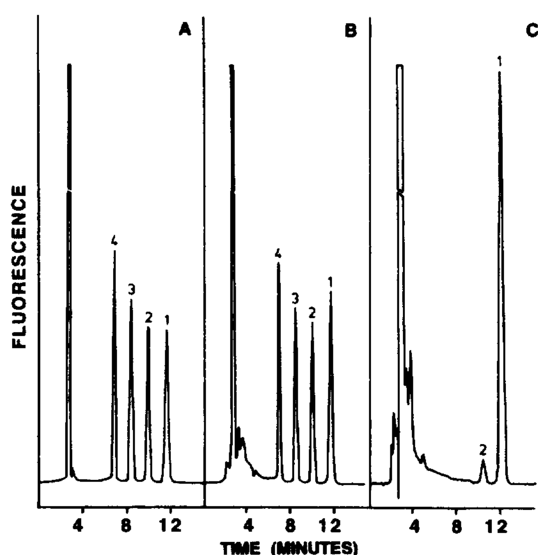

Fig. 2. Chromatograms of extracts of *Chlorella* sp. (A) Pure standards. (B) *Chlorella* supplemented with tocopherols and treated with saponification followed by double phase extraction using petroleum ether–diisopropyl ether (3:1, v/v). (C) Extract of *Chlorella* obtained by saponification and double phase extraction with a mixture of petroleum ether–diisopropyl ether (3:1, v/v). Peak identification: (1) α-T; (2) γ-T; (3) δ-T; (4) tocol (internal standard). Chromatographic conditions: as described in Section 2.

The recovery of α-T after extraction with hexane was satisfactory, unlike that of the more polar tocopherols, tocol and δ-T, two candidate internal standards (Table 2). Improved extraction of the latter two compounds was achieved with a mixture of petroleum ether–diisopropyl ether, as illustrated in Table 2. With hexane, three extractions were required for a near quantitative recovery of tocol and δ-T, whereas the mixture gave approximately 95% yield in one step.

The overall recoveries obtained at three levels were 82.4±3.4%, 96.0±1.5% and 94.3±0.4% for α-T and 96.2±0.7%, 98.5±0.4% and 98.7±0.6% for γ-T (mean±S.D., $n=5$).

Linearity ($r>0.9991$ and 0.9998 for α-T and γ-T, respectively) was achieved in the concentration ranges 0.11~2.32 μg/ml for α-T and 0.02~0.23 μg/ml for γ-T and the results met the requirements of the NCCLS guideline [13].

Table 3 shows the within-run reproducibility of the tocopherol determination in algae at three concentration levels.

Table 2
Comparison of the recoveries of tocopherols after saponification and double phase extraction with petroleum ether–diisopropyl ether (3:1, v/v) and hexane, without and with mechanical homogenisation

Tocopherol	Recovery (%)			
	1st extract	2nd extract	3rd extract	Total
Hexane extraction				
	without homogenisation			
α-T	99.1	0.9	0.0	100.0
γ-T	94.0	5.6	0.4	100.0
δ-T	63.3	19.8	13.8	97.0
Tocol	42.2	20.4	10.6	73.2
	with homogenisation			
α-T	99.4	0.6	0.0	100.0
γ-T	98.3	1.7	0.0	100.0
δ-T	86.0	8.9	1.3	96.2
Tocol	72.8	16.3	4.5	93.5
Mixture extraction				
	without homogenisation			
α-T	98.8	1.2	0.0	100.0
γ-T	99.0	1.0	0.0	100.0
δ-T	94.6	1.6	0.0	96.2
Tocol	94.5	4.4	0.2	99.1
	with homogenisation			
α-T	99.0	1.0	0.0	100.0
γ-T	98.8	1.2	0.0	100.0
δ-T	94.6	1.4	0.0	96.0
Tocol	94.5	2.9	0.2	97.6

The recoveries of α-T and γ-T are relative recoveries (the sum of all quantities recovered was taken as 100%). The recovery of δ-T and tocol was determined on spiked samples.

The limits of detection (absolute quantities injected) were 0.8 and 0.3 ng for α-T and γ-T, respectively. The corresponding limits of quantita-

Table 3
Within-run reproducibility of the determination of α- and γ-T in *Chlorella* sp. ($n=10$)

Tocopherol	Concentration (μg/ml)	C.V. (%)
α-T	0.09	1.9
γ-T	0.02	1.9
α-T	1.24	1.7
γ-T	0.08	0.8
α-T	1.55	0.9
γ-T	0.23	0.3

Table 4
Contents of tocopherols in different algae obtained with different extraction methods

Algal species	Concentration of tocopherols (μg/g dry mass)			Extraction method
	α-T	γ-T	δ-T	
Chlorella sp.	7.4			Methanol
Chaetoceros	6.4			Methanol
Tetraselmis	49			Methanol
Isochrysis	101			Methanol
Chaetoceros	163	57		Sapo+hexane[a]
Tetraselmis	259	21		Sapo+hexane
Dunaliella	228	35		Sapo+hexane
Chlorella sp.	222			Sapo+mixture[b]
Chaetoceros	692	46	6	Sapo+mixture
Tetraselmis	289	3	1	Sapo+mixture
Isochrysis	302	5	2	Sapo+mixture

[a] Saponification followed by double phase extraction with hexane.
[b] Saponification followed by double phase extraction with petroleum ether–diisopropyl ether (3:1, v/v).

tion in algal extracts (per g dry mass) were approximately 20 μg/g and 5 μg/g.

The method has been routinely applied to quantitate tocopherols in several species of algae that are used as food for aquatic organisms (Table 4). These data again illustrate the importance of the extraction method.

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