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Simultaneous determination of α-tocopheryl acetate and tocopherols in aquatic organisms and fish feed

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Abstract

In aquaculture, α-tocopheryl acetate (α-TA) is the main source of vitamin E used to fortify fish feed. α-TA in fish is often determined indirectly, i.e., by alkaline hydrolysis, followed by quantitation of ''total α-tocopherol'' (α-T) and subtraction of the natively present α-T. The aim of this study was to develop an HPLC method for the simultaneous quantitative determination of α-TA and free tocopherols in aquatic organisms and fish feed. The assay consists of a simple extraction with methanol containing butylhydroxytoluene (BHT) as an antioxidant, followed by reversed-phase chromatography with consecutive UV and fluorescence detection of α-TA and tocopherols, respectively. The peak of the internal standard tocol in the fluorescence trace was used for quantitation. Linearity was achieved over the range of 0.2 to 4.2 μg α-TA per ml extract of *Artemia* nauplii, which would correspond to 30.7 to 614.4 μg/g dry mass. The within-run coefficient of variation was 1.9% at a level of 310 μg/g dry mass. The recovery of α-TA ranged from 97.7 to 100.8% (concentration=2.1 and 20.5 μg/ml, $n=6$). The detection limit was about 7 ng and the quantification limit on spiked samples was 0.2 μg/ml. This method was routinely applied to determine α-TA and α-, γ- and δ-tocopherol (α-T, γ-T, δ-T) simultaneously in *Artemia*, fish feed, shrimp eggs and various other aquatic organisms.

Keywords: Vitamins; α-Tocopheryl acetate; Tocopherol

1. Introduction

α-Tocopheryl acetate (α-TA) is the main vitamin E (VE) source used for the fortification of feed in aquaculture, with the aim to improve the growth, resistance to stress and disease as well as survival of fish and shrimp [1–4]. In general, VE enhances the oxidative stability of the organisms owing to its ability to protect polyunsaturated fatty acids from peroxidation and to scavenge free radicals [5].

α-TA in fish is often determined indirectly, i.e., by alkaline hydrolysis followed by quantitation of the total α-tocopherol (α-T), consisting of the sum of the released α-T and the ''free'' α-T, natively present in the sample [3,6–9]. The concentration of α-TA is obtained by subtraction of the ''free'' from the ''total'' tocopherol level, both expressed in SI units.

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In vivo, α-TA performs its VE function only after hydrolysis to the free form, i.e., α-T. In addition, the acetate is not totally resorbed so that a substantial part of it remains in the gut. Consequently, the ''total'' tocopherol concentration of the fish does not reflect the functional VE status.

In our research we used as main study object the brine shrimp *Artemia*. This organism is the most applied live food source in larval aquaculture and can be easily enriched with α-TA . Part of the α-TA was found to be transformed by *Artemia* nauplii (larvae) to α-T before their ingestion by the fish. Hence, for quality control purposes, it was important to determine the concentrations of α-T and α-TA separately.

Also, we evaluated the VE status in various artificial shrimp diets differing in the formulated content of VE, and in shrimp postlarvae of an important commerical species (*Penaeus vannamei*, white-legged shrimp) fed on those diets. Finally, the uptake of VE from artificial diets and transfer to the offspring was analytically verified in reproducing giant freshwater prawn (*Macrobrachium rosenbergii*). Few high-performance liquid chromatography (HPLC) methods for the simultaneous determination of α-TA and tocopherols in other matrices have been reported, because, apart from foods and infant formulas there is rarely a need to do so. Moreover, differences in polarity and detectability between both compounds complicate this analysis. α-TA has a relatively strong UV absorption (typical detection limits are of the order of 1–2 ng) [11] and, because of its low polarity, elutes in a region of the chromatogram where usually few endogenous peaks from biological extracts occur. In contrast, tocopherols have weaker UV absorption and are more polar, thus making them more amenable to interference from other biological constituents. On the other hand, they are strongly fluorescent, whereas the esters fluoresce weakly. Nevertheless, some authors used fluorescence detection for the simultaneous determination of α-TA and tocopherols in wheat products, juices and infant formulas, allegedly achieving detection limits of 2–20 ng of α-TA [12,13]. However, using a Kontron SFM 25 or a Perkin-Elmer LS-4 fluorescence detector we were unable to reproduce these results. Lammi-Keefe [14] and Viñas et al. [15] determined α-TA and tocopherols in milk and in paprika with UV detection, but the selectivity of this method was insufficient for application to whole animals. No HPLC methods specifically designed for the simultaneous determination of α-TA and tocopherols in aquatic organisms have been reported. In view of the favourable UV absorption and fluorescence properties of α-TA and tocopherols, respectively, we opted for the consecutive use of UV and fluorescence detection.

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), a UV HPLC detector Model 430 (Kontron Instruments, Rotkreuz, Switzerland) set at 284 nm and a Kontron SFM 25 fluorescence detector with excitation at 296 nm and emission at 340 nm in sequence. In some experiments, a programmable Perkin-Elmer LS-4 detector (Perkin-Elmer, Norwalk, CT, USA) was also used. A 15×0.46 cm 5 μm Hypersil ODS column (Shandon, Runcorn, UK) preceded by a 50×3 mm Chromguard reversed-phase guard column (Chrompack, Middelburg, The Netherlands) and a 25×0.46 cm, 50 μm pre-sat silica saturation column (Alltech, Deerfield, IL, USA) was eluted with a mixture of methanol–water (96:4, v/v) at a flow-rate of 1 ml/min. The chromatograms were recorded by a Philips two-line recorder PM 8252A (Pye Unicam, Cambridge, UK) at a chart speed of 5 mm/min.

2.2. Chemicals and reagents

DL α-TA, α-T and γ-tocopherol (γ-T) were obtained from Sigma (St. Louis, MO, USA), Eastman Kodak (Rochester, NY, USA) and Acros Organic (Geel, Belgium), respectively. δ-Tocopherol (δ-T) and the internal standard (I.S.) tocol came from Eisai (Tokyo, Japan). Methanol (MeOH) and hexane were HPLC-grade and purchased from Romil (Loughborough, UK). Analytical grade butylhydroxytoluene (BHT) was obtained from Merck (Darmstadt, Germany).

2.3. Stock and working solutions

Stock and working solutions of α-TA contained approximately 10 mg/ml and 100 μg/ml in MeOH, respectively. The stock solutions of α-T, γ-T, δ-T and tocol, containing approximately 1–3 mg/ml as well as their working solutions were prepared in MeOH and stored in brown tubes at −20°C. The exact concentrations were determined spectrophotometrically based on the $E^{1\%}_{1cm}$ values from the literature [16].

2.4. Samples

2.4.1. Artemia

Nauplii of *Artemia franciscana* (Great Salt Lake strain) were produced from cysts and enriched with α-TA using standard procedures [17].

After hatching for 24 h, *Artemia* nauplii in four separate tanks were enriched twice with a fish oil emulsion (ICES 50/0,6/C, Artemia Reference Center, Gent, Belgium) (0.3 g/l) containing different concentrations (0.2, 2, 10 and 20%) of α-TA, i.e., at time zero and after 12 h. Twenty-four h after the start of the enrichment, the nauplii were harvested and subsequently stored at 4°C and 28°C for 24 h. Samples (three replicates) were taken at 12 and 24 h after the enrichment, as well as after 24-h storage at 4°C and 28°C.

2.4.2. Shrimp diet

The shrimp diet containing 22 ingredients including α-TA was prepared in the Artemia Reference Center, according to Teshima et al. [18].

To examine the relationship between the VE concentrations in the diet and the corresponding levels in shrimp, the feed was fortified with α-TA in 5 concentrations (0, 20, 100, 200, 2000 mg/kg) prior to its administration to the shrimp tanks.

2.4.3. Shrimp postlarvae

Penaeus vannamei postlarvae at the age of 18 days, post metamorphosis were stocked in 30-l aquaria at a density of 5 animals per l. Water temperature was kept at 28°C, and salinity at 30 g/l. The level of NH_4^+ and NO_2^- never exceeded 0.2 mg/l. The postlarvae were fed three times during the day on five different diets for a period of 29 days [19].

2.4.4. Giant freshwater prawn eggs

Adults of the giant freshwater prawn (*Macrobrachium rosenbergii*) were kept under standard maturation conditions for several months and fed an artificial diet containing 2000 mg α-TA/kg [20]. Eggs were obtained after spawning of the females.

2.5. Sample preparation

The sample preparation has been described previously [10]. Basically, samples were homogenized in methanol containing BHT (1 mg/ml), the homogenates were clarified by centrifugation and aliquots of the supernatant were injected.

2.6. Method validation

The recovery of α-TA was determined by supplementation of homogenized *Artemia* with the analyte and repetitive analysis using the standard procedure, except that the I.S. was added at the end. Extracts of the same sample to which both the analyte and the I.S. had been added just before the injection served as a reference (=100% recovery).

To evaluate the linearity of the method, an *Artemia* homogenate in methanol was supplemented with α-TA and the I.S. at five concentration levels (0.2, 0.4, 1.1, 2.1 and 4.2 μg/ml) and four replicates per concentration, according to the NCCLS proposed guideline [21].

The within-day reproducibility was evaluated by analyzing 10 replicate spiked samples and calculating the mean concentration, the standard deviation and the coefficient of variation (CV).

The limit of quantitation was estimated from the analysis of spiked samples. The absolute detection limit (quantity injected) was determined using pure standard solutions. A peak height corresponding to three-times the noise level was set as the detection limit.

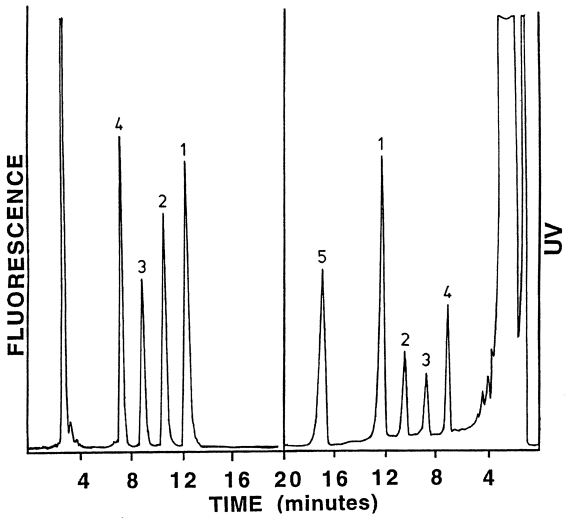

Fig. 1. Chromatograms of tocopherol standards in methanol obtained with consecutive UV and fluorescence detection. The amounts injected were 165, 45, 28 and 209 ng for α-T, γ-T, δ-T and α-TA, respectively. Chromatographic conditions: see text. Peak identification: 1=α-T; 2=γ-T; 3=δ-T; 4=tocol (I.S.); 5=α-TA.

2.7. Quantitation

Quantitation was based on peak height ratios (analyte versus tocol). Peak heights of α-, γ- and δ-T were measured in the UV trace, whereas that of tocol was determined in the fluorescence trace. Standardization for the determination of α-TA and tocopherols was performed using *Artemia* supplemented with known quantities of the analytes (α-TA and tocopherols) and tocol or, in daily routine, using MeOH–BHT–water instead of *Artemia* homogenates.

3. Results and discussion

3.1. Detection of α-TA

Balz et al. [13] reported that sufficient fluorescence intensity for the detection of α-TA could be achieved using Shimadzu RF 540, Perkin-Elmer 3000 and Hitachi F 1000 detectors at λ_{ex} 280–290 and λ_{em} 330–335 nm, yielding a detection limit of approximately 2 ng. With the Perkin-Elmer LS-4 fluorescence detector, the wavelengths of excitation and emission for α-TA were experimentally found to be 284 nm and 310 nm, respectively. Accordingly, the detector was initially set at 296 nm (λ_{ex}) and 340 nm (λ_{em}) for the first 14 min of the chromatographic run for the detection of tocopherols, then programmed to change the wavelengths to 284 nm (excitation) and 310 nm (emission) for the detection of α-TA. However, detection of low levels (on the order of 1.2 μg/ml) of extract of α-TA in *Artemia*

Table 1
Concentration of α-TA (μg/g DM[a]) in enriched *Artemia* (three replicates per sample) calculated on the basis of fluorescence (FL) or UV absorption of tocol as an I.S. (the two groups of data were compared in a paired *t*-test)

Sample	Concentration (mean±SD) (μg/g DM) of α-TA		CV (%) (n=3)	
	FL	UV	FL	UV
1	136±43	113±45	31.7	39.2
2	1007±77	999±86	7.6	8.6
3	58418±2823	61665±3102	4.8	5.0
4	6522±355	6781±370	5.5	5.5
5	381±14	356±23	3.7	6.3
6	3127±37	2940±17	1.2	0.6
7	34679±7297	32138±6900	21.0	21.5
8	12418±932	11172±880	7.5	7.9
9	7777±397	6854±355	5.1	5.2
10	6738±992	5747±1159	14.7	20.2
11	5307±464	4597±528	8.7	11.5
12	2269±334	1903±278	14.7	14.6
t-test			P=0.192	

[a] DM=dry mass.

samples proved impossible using this detector. A chromatogram of standard tocopherols and α-TA obtained with both UV and fluorescence detection is depicted in Fig. 1.

3.2. Internal standard

A crucial point in this method was how to detect the I.S. Tocol has not been used so far as an I.S. for the quantification of α-TA, partly because its weak UV absorbance and its polarity often lead to interferences at its elution position with other UV absorbing compounds in biological extracts. As a solution, it appeared convenient to use the tocol peak in the fluorescence trace for the quantitation of α-TA and to measure the peak of the latter in the UV trace. However, whether this hybrid procedure was appropriate was initially questioned. Arguments in favour of this approach were the low CV value (1.9%, $n=10$) in the reproducibility test and the absence of a significant difference ($P=0.52$, paired t-test, $n=10$) between the results obtained on spiked *Artemia* (concentration=316 μg/g) using either the UV or the fluorescent tocol peak. Remarkably, the CV value related to the use of the fluorescent peak (1.9%) was even lower than that resulting from the use of the UV peak (3.2%). For the repeated injection of a standard mixture the performance was in both cases equivalent (CVs of 1.0 and 0.9% for fluorescence and UV absorption, respectively).

Also, when comparing the levels of α-TA obtained on enriched *Artemia*, we found no significant difference in CVs depending on the choice of fluorescence or UV absorption for the measurement of the tocol peak ($P>0.05$, paired t-test, $n=36$) (Table 1), even though fluorescence appears to yield somewhat higher values. In view of the early elution position of tocol, fluorescence detection will also be advantageous to avoid possible matrix interferences. In summary, dual detection afforded maximal selectivity, optimal detectability of low concentrations of tocopherols and α-TA and good precision.

3.3. Method validation

The recovery of α-TA was 100.8±2.5% and 97.7±0.9% ($n=6$) for concentrations of 2.1 and 20.5 μg/ml, respectively.

Linearity was achieved over the concentration

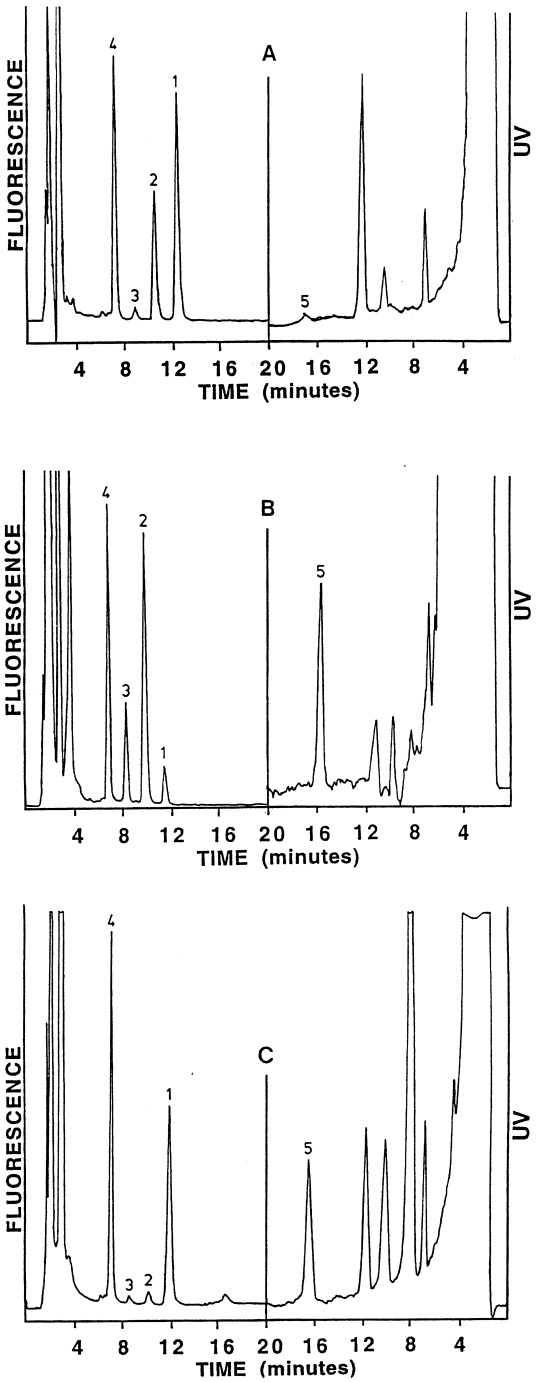

Fig. 2. Chromatograms of extracts of shrimp postlarvae (A), shrimp diet (B) and enriched *Artemia* nauplii (C) obtained using a monophasic extraction with methanol, and UV and fluorescence detection. Conditions and peak identities as in Fig. 1.

Table 2
Concentration of α-TA and tocopherols in *Artemia* enriched with α-TA at different levels and for different time periods

Enrichment (%)	Concentration (μg/g DM) (mean±SD, n=3)			
	α-T	γ-T	δ-T	α-TA
12-h				
0.2	142±3	6.6±0.2	4.6±0.1	60±8
2.0	219±6	8.0±0.7	4.9±0.3	585±72
10	269±13	8.6±0.5	4.5±0.3	2577±85
20	686±5	11.5±0.7	4.1±0.4	5360±106
24-h				
0.2	140±2	24.6±0.4	13.7±0.3	89±18
2.0	231±5	28.5±0.9	14.8±0.6	1401±102
10	278±16	25.9±0.7	12.9±0.8	6535±308
20	687±23	25.4±0.7	10.2±0.3	13 340±916
24-h+24-h storage at 4°C				
10	344±14	27.7±0.1	14.5±0.6	2214±146
20	733±10	25.6±0.4	11.7±0.9	3801±223
24-h+24-h storage at 28°C				
10	452±15	45.8±0.6	22.3±1.3	9326±383
20	1104±94	47.6±2.5	20.3±1.8	22 448±1421

range of 0.2 to 4.2 μg/ml (corresponding to 30.7 to 614.4 μg/g dry mass) for α-TA with correlation coefficients exceeding 0.9995.

Reproducibility data are given in Section 3.2.

The detection limit of pure α-TA (absolute quantity) was about 7 ng or, expressed as a concentration, 70 ng/ml. The limit of quantitation was estimated as 0.2 μg/ml.

3.4. Applications

Representative chromatograms of extracts of *Artemia*, shrimp postlarvae and a shrimp diet are shown in Fig. 2. Table 2 lists the concentrations of tocopherols and α-TA in enriched *Artemia* nauplii as a function of the levels of α-TA in the enrichment formulation. *Artemia* metabolizes α-TA to α-T but still retains high quantities of the former, that can be transferred to fish or shrimp larvae fed on it. The relationship between the dietary α-TA levels and those of α-T and residual α-TA in shrimp postlarvae is illustrated in Table 3. The boost in α-T resulting from dietary α-TA supplementation is obvious. Finally, when eggs of *Macrobrachium rosenbergii*, which had received a diet rich in α-TA, were analyzed, low quantities of the latter were unexpectedly detected intact (Fig. 3A). Although an artefact was initially suspected, the identity of the presumed α-TA peak was supported by subjecting samples to saponification in order to convert the α-TA to α-T.

Table 3
Concentrations of α-TA and tocopherols in shrimp postlarvae fed on different diets for 29 days

Dietary α-TA level (mg/kg)	Concentration (μg/g DM) (mean±SD, n=8)			
	α-T	γ-T	δ-T	α-TA
0	36.5±10.3	45.0±20.3	4.9±3.1	ND[a]
20	72.8±19.4	63.9±5.3	6.1±1.1	ND
100	73.4±18.6	48.8±13.2	4.6±1.6	7.3±2.7
200	109.9±41.7	46.7±18.5	3.9±1.7	8.4±3.9
2000	235.6±38.9	46.9±8.2	4.1±1.1	18.6±4.1

[a] ND=Not detected.

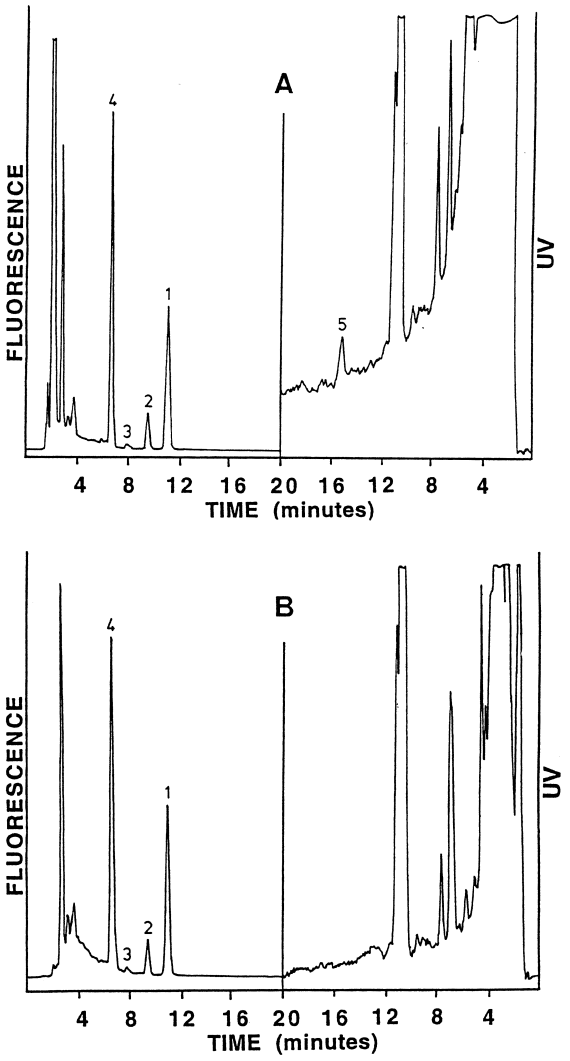

Fig. 3. Chromatograms of extracts of eggs of *Macrobrachium rosenbergii* obtained using a monophasic extraction with methanol (A) and a double phase extraction after saponification of the methanolic extract (B), and consecutive UV and fluorescence detection. (A) Monophasic extraction with methanol, (B) double phase extraction with light petroleum–diisopropylether (3:1, v/v) after saponification. Conditions and peak identities as in Fig. 1. Peak 5 is α-TA.

While the peak heights of γ-T, δ-T and tocol remained unaffected, that of α-T was found to increase by 20% after saponification, with a concomitant disappearance of the α-TA peak (Fig. 3B). However, as this finding should still be confirmed by mass spectrometric analysis, any speculation about an underlying biological phenomenon is premature at present.

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