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Determination of tocopherol acetate in emulsified nutritional supplements by solid-phase extraction and high-performance liquid chromatography with fluorescence detection

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Abstract

The present paper deals with a method of solid-phase extraction of tocopherol acetate (TA, 49.6 μg/g) from emulsified nutritional supplements, which contains 50 kinds of compounds, followed by high-performance liquid chromatography (HPLC) with fluorescence detection The TA concentration is 5~100 000 times lower than that of other compounds in the samples. Measuring the loading capacity of the larger amounts of vegetable oil onto the Bond Elut C_{18} cartridge was examined for the complete retention of smaller level of TA. A sample solution was applied to a solid-phase extraction cartridge and then TA was eluted by acetonitrile followed by HPLC. This method was suitable for the determination of TA in emulsified nutritional supplements. The proposed method was simple, rapid (analysis time: ca. 15 min), sensitive [detection limit: ca. 0.1 ng per injection (100 μl) at a signal-to-noise ratio of 3:1], and reproducible (relative standard deviation: ca. 2.5% (n=5)). The calibration graph of TA was linear in the range of 0.1 to 100 ng per injection (100 μl). Recovery of TA was over 90% by the standard addition method.

Keywords: Food analysis; Tocopherol acetate; Vitamins

1. Introduction

Several recent publications have demonstrated simple and fast solid-phase extraction (SPE)–high-performance liquid chromatography (HPLC) analysis of vitamins in foods [1–4]. Continuing that research, this paper demonstrates a simple and rapid determination of tocopherol acetate (TA, 49.6 μg) in emulsified nutritional supplements by SPE and HPLC with fluorescence detection (FLD). TA is usually used as a food supplement and it has a GRAS status. It is necessary to inspect the intermediate and finished products for process control and quality control following good manufacturing practice. Thus, a simple, rapid and reproducible analytical method of TA in the presence of many kinds of compounds in foods, including emulsified foods with a high fat content, is required.

As described previously [1–4], it is necessary to remove the oily particles in samples with high fat content to optimize the retention of trace amounts of compounds before the SPE step, because large amounts of lipophilic species, which retain strongly on the reversed-phase SPE cartridge such as Bond Elut C_{18}, reduce the capacity of the cartridge for these compounds. The use of SPE is necessary, however, because the analytes present in trace amounts, must be concentrated to permit detection, and oily components in these samples must be removed.

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Tocopherol in samples has been determined by HPLC [5–10]. However, SPE conditions to optimize the retention and elution of tocopherol and TA in samples with high fat content, where emulsions are often problems when using liquid–liquid extraction as a sample preparation method, has not been studied.

The emulsified nutritional supplement samples used here are in powdered form and had to be dissolved prior to use. They are complex sample types containing 50 different components, for example, amino acids, vitamins, organic acids, plant oil, sugars and minerals at concentrations at least 5–100 000 times higher than that of TA.

Previous papers [2–4] showed that the traditional hexane extraction method was not suitable for the sample preparation of emulsified food samples and SPE method was suitable sample preparation for fat-soluble vitamins analysis in emulsified food samples. But SPE conditions which optimize retention and elution from high fat-content samples in emulsion form have not been previously determined.

Here we describe the development of an analytical procedure that utilizes optimized SPE combined with a HPLC analysis to obtain the TA peak using a water-free mobile phase. We further explore this method's applicability to clean-up and concentration of TA in dried emulsified nutritional supplements. In addition, this paper deals with measuring the loading capacity of the larger amounts of vegetable oil onto the SPE cartridge was examined for the complete retention of smaller level of fat-soluble vitamins. This paper also deals with the stability of TA in both emulsified nutritional supplement sample solutions and eluate obtained by SPE under different storage conditions and for different periods of storage.

2. Experimental

2.1. Reagents and materials

TA used in this study was of Japanese Pharmacopeia Standard. Acetonitrile, methanol, ethanol, sodium sulfate and ethylenediaminetetraacetic acid disodium dihydrate ($EDTA{\cdot}2Na{\cdot}2H_2O$) purchased from Wako (Osaka, Japan) were all of analytical or HPLC grade. Emulsified nutritional supplement samples were obtained from Ajinomoto (Tokyo, Japan). Light-resistant brown volumetric flasks and glassware [6,11] were used in this study. The membrane filters (0.5 μm) were purchased from GL Sciences (Tokyo). Bond Elut C_{18} cartridges (500 mg, 3 ml) were purchased from Varian (Harbor City, CA, USA). The Bond Elut C_{18} cartridges were conditioned by washing with 5 ml of methanol and then with 10 ml of deionized water prior to use.

2.2. Standard TA preparation

Standard TA (25 mg) diluted with acetonitrile was freshly prepared in a 50 ml brown volumetric flask prior to use. TA in this stock solution was then diluted with acetonitrile 100 times in a brown volumetric flask. A 2 ml aliquot of the resulting solution was diluted to 20 ml with acetonitrile, which was obtained by passing through the conditioned Bond Elut C_{18} cartridges (to mimic the conditions of an extracted sample where the eluent will acquire traces of water as it passes through a conditioned SPE cartridge). This was found in previous work to be beneficial, giving a sharp TA peak on the chromatogram. The final concentration of TA in this solution was 50 μg/100 ml.

2.3. Sample preparation

After the nutritional supplement (10 g) was dissolved in a brown volumetric flask (100 ml) with 5% sodium sulfate aqueous solution with 1 m*M* $EDTA{\cdot}2Na{\cdot}2H_2O$, this solution (1 ml) was applied to the conditioned Bond Elut C_{18} cartridge. The cartridge was washed with deionized water (10 ml) and then each with 5, 25 and 50% aqueous acetonitrile solution (5 ml) sequentially, followed by elution with acetonitrile. The eluate (10 ml) was further filtered with a membrane filter. An aliquot (100 μl) of filtrate was injected into the chromatograph.

2.4. Apparatus and conditions

A Model 655 A-12 high-performance liquid chromatograph (Hitachi, Tokyo) equipped with a Model F-1000 fluorescence detector (Hitachi) set at excitation wavelength 295 nm and emission wavelength 325 nm was used. The samples were applied

by a Rheodyne Model 7125 sample loop injector with an effective volume of 100 μl. Separation took place on a 15×0.46 cm I.D. column of Inertsil ODS-2 (5 μm) (GL Sciences, using methanol–ethanol (50:50) as the mobile phase. The flow-rate was 0.4 ml/min at 40°C.

3. Results and discussion

3.1. Standard TA preparation

Firstly, we focused on the effect of solvent composition on the sharpness of the TA peak on the HPLC chromatograms of standards. When standard TA was diluted with water-free acetonitrile, broad TA peaks were obtained on HPLC analysis. On the other hand, when TA was diluted with acetonitrile, containing trace amounts of water, which was obtained after that eluent (acetonitrile) was passed through the conditioned cartridge, sharp TA peaks were obtained. The addition of water was not needed when emulsion samples were analyzed because about 350 mg of water ($n=5$), found to be retained on the conditioned cartridge, dissolves into the elution solvent and contributes to sharpen the peak on the HPLC. This effect may be caused by standard enrichment at the head of the HPLC column. Whatever the cause, it is fortuitous that the optimum conditions coincide with those that result from use of SPE. Thus, it was desirable to prepare both the standard and sample TA the same way as described previously [2,3].

From the previous reports [2,3], it might be considered that standard fat-soluble compounds must be prepared in the same way as sample preparation, when a water-free mobile phase is used, because trace amounts of water effects the peak sharpness.

Table 1
T (%) value of washings and eluate obtained by using each C_{18} SPE cartridge at 650 nm

Sample[a]	*T* (%)	
	Washings	Eluate
Nutritional supplement	98.5	99.5
Soybean oil	98.1	9.5

[a] Sample preparation; see Sections 2.3 and 3.2.

3.2. SPE

3.2.1. Measuring the loading capacity of the plant oil onto the Bond Elut C_{18} cartridge

The second attention was paid to the measuring of the loading capacity of the plant oil onto the Bond Elut C_{18} cartridge for the complete retention of trace levels of fat-soluble analytes in the matrices with larger amounts of oily particles like emulsified nutritional supplements. This examination has not been studied until now.

In order to exploit a method for the determination of trace concentrations of the supplement TA contained in foodstuffs, which exist as emulsion status, we prepared the samples employing the SPE cartridges C_{18} (500 mg).

One of the critically important aspects in the experiment was how much of the oily samples can be loaded onto the SPE cartridges without sacrificing the recovery of the target compounds. The reason for this is that if oil in the matrices is not completely retained on the SPE solbent, the trace quantity of the target fat-soluble vitamin E compounds (TA) is not quantitatively retained on the SPE sorbent since some portion of it may co-elute with the oil in the matrices or oily particles.

3.2.2. Different behavior of oily particles from the emulsified nutritional supplement sample and the imitation trial soybean oil

The label on the emulsified nutritional supplement did not mention what kinds of plant oil were used. Only the concentration of the plant oil was described. We used soybean oil as the plant oil for the experiment. We examined how much quantity (mg) of soybean oil can be loaded on the C_{18} SPE cartridge (500 mg), without elution during loading.

When the emulsified nutritional supplement sample (10 g) was mixed with water (100 ml), the mixture formed an emulsion. This solution (1 ml) was applied onto a C_{18} SPE sorbent and then the cartridge was washed with 10 ml of water. Vitamin K_1 [3] or TA was retained on the cartridge. Vitamin K_1 [3] and TA were eluted using 10 ml of eluent, like ethanol or acetonitrile. The eluate was prepared for the HPLC sample.

In the above washing and eluate, oily particles were not observed visually (Table 1). Therefore, we

concluded that oily particles were still retained on the cartridge. Vitamin K_1 or TA retained on the cartridge had been already eluted ($T>98\%$). Consequently TA and the oil were separated by SPE. However, when the imitation trial sample of 2 mg of soybean oil, which corresponded to the oil content of the nutritional supplement sample, was loaded on the conditioned C_{18} cartridge followed by washing with 10 ml of water, the oil was not observed in the water eluate. The oil was retained at that time. Then 10 ml of acetonitrile was passed through the cartridge for eluting the oil. The eluate was cloudy (Table 1). The oil was eluted. Therefore, the results of the imitation trial consisting of the soybean oil and those of the actual emulsified nutritional supplement sample were quite different.

The reason for this difference is considered to be that the emulsified nutritional supplement is manufactured by adding the appropriate concentration of the emulsifying agents at high pressure (we do not know the emulsifying agent). We suppose that the soybean oil seems to be retained on the SPE sorbent in a different way comparing the nutrient matrices oil. We could not succeed in preparing the similar imitation sample using soybean oil. Therefore, we could not measure the loading limit of the vegetable oil using the soybean oil due to the above described reasons.

The washings and eluate obtained from the real sample solution (1 ml), which contains oily particles (2 mg) according to the label, showed clear ($T>$ 98%). When the real sample solution (10 ml) was applied onto the SPE cartridge, the washings and eluate also showed clear ($T>98\%$). So, oily particles (20 mg) are considered to be retained onto the C_{18} SPE cartridge (500 mg).

3.3. Retention and elution factors

We reported in previous papers [1–4] the determination of trace amounts of cyanocobalamin, β-carotene, vitamin K_1 and vitamin D_2 in foods by HPLC after SPE using Bond Elut C_{18} cartridges. It was found that three factors; the pH value in sample solutions, choice of eluent and eluent be investigated for efficient retention and elution of these compounds, Consequently, these factors were also explored for the analysis of TA in nutrients with high fat content.

3.3.1. Effect of eluent on elution of TA

Standard TA (400 μg) was injected into the cartridge followed by elution with four eluents (methanol, ethanol, isopropanol and acetonitrile). TA was completely eluted with these four eluents (each eluate volume 10 ml). In the present study, acetonitrile was used as the eluent (see Section 3.4). The retention was tested by examining the cartridge eluent for TA.

3.3.2. Effect of acetonitrile volume on the elution of TA from the cartridge

An elution profile of the content (%) of TA in successive 2 ml aliquots of elution solvent was developed, after standard TA (400 μg) had been injected into the cartridge. TA in each 2 ml fractions one to five was analyzed. Recoveries (%) of TA in fractions one to three were over 90% with acetonitrile. It was found that complete elution of TA from the cartridge was obtained with 10 ml of eluent (acetonitrile). When acetonitrile–water (99:1, 98:2, 97:3, 95:5, 90:10) was used as the eluent (each eluate volume 10 ml), recovery of TA was 75.5, 48.5, 5.5 0, 0%, respectively. So, we used HPLC grade acetonitrile without the addition of water.

3.3.3. Effect of pH value in the sample solution on the retention of TA

Standard TA (400 μg) was diluted in 2 ml of 10 m*M* potassium phosphate buffer (pH 3 to 8.5) and was applied to the cartridge followed by elution with 10 ml of acetonitrile. Each 10 ml of eluate volume was analyzed for TA.

Optimal recovery of TA on the Bond Elut C_{18} cartridge was found over the sample pH range 3 to 7.5. In other words, TA retention is not very sensitive to loading pH.

A summary of the optimized sample preparation method is thus TA (400 μg) diluted in 10 m*M* potassium phosphate buffer (pH 3 to 7.5, 2 ml) was applied on the conditioned Bond Elut C_{18} cartridge followed by elution with acetonitrile (eluate volume 10 ml). The pH value in the nutrient sample solution

(see Section 2) was about 6, so the sample can be applied to the SPE cartridge without further pH adjustment (a benefit in routine sample preparation). This proves that vitamin K_1 and TA in the nutrient sample solution (pH ca. 6) could be extracted by SPE simultaneously (i.e. using the same method).

3.4. Chromatography

Glossy TA in the emulsified nutritional supplement with high fat content treated with Bond Elut C_{18} cartridge can be observed by HPLC–FLD on the Inertsil ODS-2 column with sufficient intensity (Fig. 1), and analysis time was about 15 min. Both the standard and sample TA preparations are performed the same way, as explained in Section 3.1.

Previous papers [3,4] indicate that ethanol and methanol were suitable eluents for vitamin K_1 and vitamin D_2, respectively.

In the present paper, both ethanol and methanol were also suitable eluents for TA. However, TA could not be identified using either eluents, because unknown co-eluting peaks were observed on the chromatograms. Therefore, we employed acetonitrile, because TA could be identified on the chromatogram. According to the label of contents, nutrient samples contained folic acid, pyridoxine, riboflavin, tyrosine and tryptophan, all of which are detected with FLD. Therefore, the cartridge was washed with water and then aqueous acetonitrile (5 to 50%) to remove these compounds before elution with acetonitrile.

A concentration at the limit of detection from Fig. 1 was ca. 0.1 ng per injection (100 μl) at a signal-to-noise ratio 3:1. The on-column focusing of TA by the acetonitrile–water injection conditions improves detection. A similar technique has previously been used [1–4,12,13]. Despite the large injection volume (100 μl), TA was concentrated on an Inertsil ODS-2 column, separated and then detected without interference from other compounds.

The present method has a great benefit in that both vitamin K_1 and TA can be extracted by the same SPE method, because the loading pH was the same, and HPLC conditions (analytical column, mobile phase, column temperature, and detector) were similar.

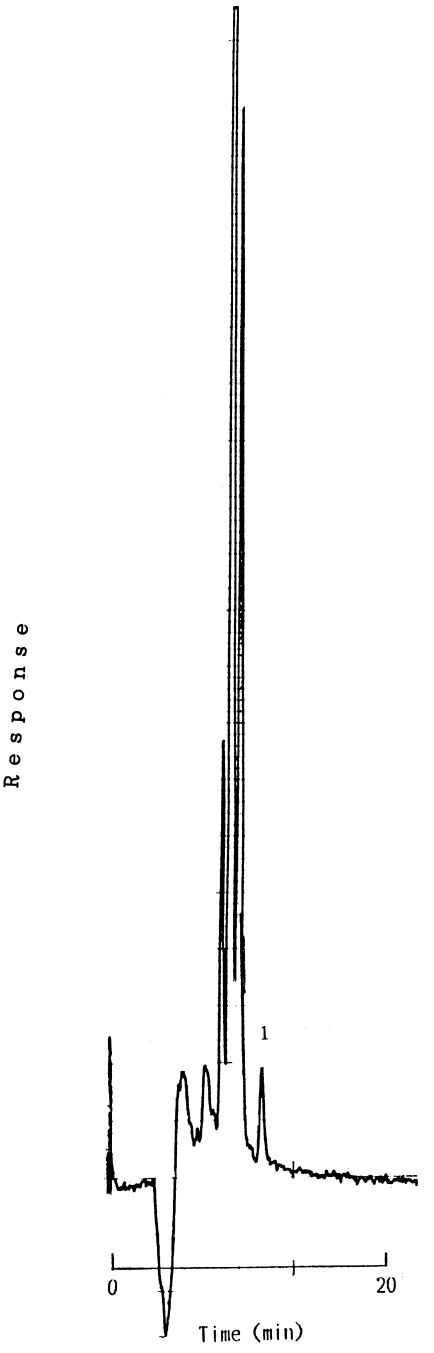

Fig. 1. Chromatograms of TA in an emulsified nutritional supplement by HPLC with FLD (excitation 295 nm, emission 325 nm) after SPE. HPLC was carried out on a 15×0.46 cm I.D. column of Inertsil ODS-2 (5 μm) using methanol–ethanol (50:50) as the mobile phase at a flow-rate of 0.4 ml/min at 40°C. Peak 1=TA (47.1 ng injected).

3.5. Determination of TA

The calibration graph for TA was constructed by plotting the peak height of TA against the amount of TA added. Satisfactory linearity was obtained in the range of 0.1 to 100 ng on the column ($y=4.8354x-0.061$, where y=peak height and x=amount of TA)

A known amount of TA was added to the emulsified nutritional supplement and overall recoveries were estimated by the standard addition method (spiking method as shown in Table 2. As listed in Table 2, TA was recovered over 90% by the standard addition method. The relative standard deviation (RSD) was 2.6% ($n=5$) with no addition of TA.

The proposed method shows a great advantage that vitamin K_1 and TA in nutrient sample solution can be directly applied to SPE simultaneously without further pH adjustment, because the pH value in the sample solution was neutral and TA in the eluate (10 ml) permits direct analysis without further concentration.

There was good agreement in the TA value in the nutrient between the concentration indicated and that found in the extracted nutrient samples, by this method.

3.6. Stability of TA

Finally, we examined the stability (%) of TA in the nutrient. The periodic change of TA concentration in both nutrient sample solutions and acetonitrile eluates obtained from SPE, stored at both 5°C and room temperature for 24 h were studied to determine how long TA in each sample was stable.

It was clear that TA was stable for 24 h in both conditions for the acetonitrile eluates. On the other hand, TA was only stable for 5 h in both conditions for the nutrient sample solutions. Peak height was constant for 5 h, and then the peak height decreased periodically. When the above sample was stored for 24 h at both 5°C and room temperature, the TA content (%) was about 100 and 87.7%, respectively. Since TA prepared by the proposed method was very stable, it was possible to analyze TA in acetonitrile eluates stored overnight, for a routine work. α-Tocopherol (the hydrolysis product of TA) peak was not observed on any chromatograms. This appeared to be a general benefit from using SPE for vitamin analysis since several other similar findings have been reported [1–4].

Table 2
Recoveries of TA added to emulsified nutritional supplement[a]

(mg per 100 g)		Recovery	
Added	Found	(mg per 100 g)	(%)
0	4.71	–	–
2.5	7.02	2.31	92.4
5.0	8.36	3.65	93.0
10.0	14.16	9.45	94.5

[a] RSD: 2.6% ($n=5$) with no addition of TA.

4. Conclusion

A simple and rapid analytical method for TA in an emulsified nutritional supplement has been demonstrated that uses SPE and HPLC with FLD (excitation wavelength 295 nm, emission wavelength 325 nm) on the Inertsil ODS-2 column. The dissolving agent (acetonitrile) with trace amounts of water effected a sharpening of the TA peak on the chromatogram. The effects of pH value in sample solution and eluate volume on the retention and elution of TA on the Bond Elut C_{18} cartridge were examined. Measuring the loading capacity of the soybean oil onto the Bond Elut C_{18} cartridge was examined for the complete retention of trace amounts of fat-soluble vitamins.

The stability of TA in the nutrient sample solution by the proposed method was also examined. This method established here seems to be applicable to the analysis of TA in the nutrient, because of its simplicity, speed (analysis time of TA: ca. 15 min, reproducibility (RSD: 2.6%) and selectivity. Furthermore, it provides recoveries of over 90%, obtained by the standard addition method. Application of the proposed method to the determination of TA in other foods and biological fluids is being studied.

A summary of sample dissolving agents, SPE conditions. HPLC conditions, recovery (%) and RSD for fat-soluble vitamins is shown in Table 3.

Although dissolving agents, SPE and HPLC conditions were different, it was concluded that SPE was the most suitable sample preparation technique for the fat-soluble vitamins in emulsified food samples,

Table 3
A summary of sample dissolving agents, SPE conditions, HPLC conditions, recoveries and RSDs for fat-soluble vitamins in emulsified foods

Vitamin	Concentration indicated ratio (o/v)[a]	Dissolving agent[b]	SPE		HPLC				Analysis time (min)	Recovery (%) (RSD, %, n=5)
			Loading pH	Eluent[c]	Mobile phase	Injection volume (μl)	Column	Detection wavelength (nm)		
β-Ca[d]	1110	Deionized water	2~7.5	Ethanol	Acetonitrile–ethanol (70:30)	200	Inertsil ODS 80 A	UV 450	8	>90 (1.3%)
K_1 [3]	25 000	5% Na_2SO_4	5.5~7.5	Ethanol	Methanol–ethanol (50:50)	100	Inertsil ODS-2[e]	FLD (excitation 320 nm, emission 430 nm)	12	>90 (2.3%)
D_2 [4]	105 000	0.2 *M* K_2HPO_4	7.5~8.5	Methanol	Methanol Acetonitrile–methanol (75:25)	200	Hitachigel 30111-0[f] Inertsil ODS-2[g]	UV 265	20	ca. 80 (6.2%)
TA	500	5% Na_2SO_4	3~7.5	Acetonitrile	Methanol–ethanol (50:50)	100	Inertsil ODS-2	FLD (excitation 295 nm, emission 325 nm)	15	>90 (2.5%)

[a] Oily particles/vitamin.
[b] For sample preparation.
[c] Each eluate volume 10 ml.
[d] β-Carotene.
[e] RC-10 platinum oxide catalyst reduction postcolumn (3×0.40 cm I.D.).
[f] Precolumn (10×0.46 cm I.D.).
[g] Analytical column (15×0.46 cm I.D.).

giving recoveries of over 80%, RSDs of 1.3 to 6.2%, and analysis times of 8 to 20 min).

The simultaneous determination of four fat-soluble vitamins could not be achieved.

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