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Sample preparation for routine high-performance liquid chromatographic determination of retinol palmitate in emulsified nutritional supplements by solid-phase extraction using monosodium L-glutamate as dissolving agent

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Abstract

Retinol palmitate (vitamin A, 73.3 μg/g) in an emulsified nutritional supplement was determined by solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with fluorescence detection (excitation 350 nm, emission 480 nm) using monosodium L-glutamate as a dissolving agent to obtain higher recovery of vitamin A from the emulsified sample solution. A Bond Elut C_2 cartridge (500 mg) was chosen for SPE after comparison with 16 other types. A sample solution was applied to a conditioned Bond Elut C_2 cartridge and then vitamin A was eluted with ethanol followed by HPLC. The proposed method was simple, rapid (sample preparation time by SPE: ca. 8 min, retention time: ca. 8 min), sensitive [detection limit: ca. 0.1 pg/injection (100 μl) at a signal-to-noise ratio of 3:1], highly selective and reproducible (relative standard deviation (RSD): ca. 2.9% ($n=5$), between-day RSD ca. 3.7 (5 days). The recovery of vitamin A was over 90% by the standard addition method.

Keywords: Food analysis; Retinol palmitate

1. Introduction

Sample preparation is one of the most important steps to ensure that subsequent high-performance liquid chromatography (HPLC) analysis is effective. Solid-phase extraction (SPE) is a simple and rapid technique and has been applied to complex sample matrices for extraction of, among others, nutrients from foods, drugs and their metabolites from biological fluids [1,2].

Usually, it is difficult to separate both fat-soluble vitamins and the oily particles in emulsified samples. It is crucial to remove oily particles before HPLC analysis for the optimal determination of fat-soluble vitamins in the samples. We demonstrated that fat-soluble vitamins and oily particles could be separated on an SPE C_{18} cartridge after addition of aqueous sodium sulfate solution to the emulsified nutritional supplements and these vitamins could be determined using non-aqueous HPLC mobile phases [3–6]. However, there are two pitfalls. First, it is necessary to use large volumes of eluent (10 ml) for the complete elution of fat-soluble vitamins. Second, after injecting the extract (100 μl) more than 100

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times onto the same analytical column, the column inlet pressure increased by 250 kg/cm^2 than the initial use of the same analytical column. In order to retain the fat-soluble vitamins onto the packing material in the SPE cartridge, we added some salt like sodium sulfate solution to the sample prior to its loading to the SPE cartridge. Normally, various kinds of foods, food products and foodstuffs contain calcium. Consequently, we suspected calcium sulfate was yielded in the SPE cartridge. Calcium sulfate has very low solubility to the water. Calcium sulfate seems to flow into the SPE extract. After injecting the extract more than 100 times onto the same column, the small quantity of the precipitated calcium sulfate of each injection, we suspected that calcium sulfate has been accumulated from the inlet tube to the column through the frit. The precipitated calcium sulfate increased the column inlet pressure, causing the pump to stop. In order to prevent this problem, we tested some other kinds of salts instead sodium sulfate.

Trace amounts of vitamin A [7–12] in biological fluids and foods have been determined by HPLC. However, extraction of trace amounts of retinol palmitate (vitamin A) in nutritional supplements, containing emulsifying agents, has not previously been studied by HPLC after SPE. Such a method would be invaluable in process and quality controls, but to be of use in these areas the method must be very rugged, so, careful optimization of retention and elution parameters for SPE is essential.

To improve the previously published sample preparation [3–6], this paper deals with the testing/selection of an SPE cartridge after comparison with 16 other types (non-polar, polar and mix-phases) to extract vitamin A in samples as small as possible, SPE parameters, choice of salt, which does not precipitate with calcium ion present in samples, and salt concentration to optimize the sample preparation. We also demonstrate the validity of the determination of vitamin A in emulsified nutritional supplements and drugs by HPLC after SPE.

2. Experimental

2.1. Reagents and materials

Retinol palmitate used here was of Japanese Pharmacopeia Standard (Japan). Monosodium L-glutamate (MSG) was obtained from Ajinomoto (Tokyo, Japan). Other reagents were all of analytical or HPLC grade. Emulsified nutritional supplements were commercially available. Bond Elut C_2 cartridges (500 mg, 3 ml) were purchased from Varian (Harbor City, CA, USA).

2.2. Standard vitamin A preparation

Standard vitamin A (35 mg) was freshly diluted with ethanol in a 100 ml brown volumetric flask prior to use. Vitamin A in this stock solution (5 ml) was further diluted with ethanol in a second 50 ml brown volumetric flask. This 50 μl of the resulting solution was diluted to 5 ml with ethanol containing small amounts of water to mimic the composition of a sample, which has been extracted on a conditioned SPE cartridge, to yield a final concentration of 70 μg/100 ml.

2.3. Sample preparation

The Bond Elut C_2 cartridges were conditioned by washing with 3 ml of methanol and then with 5 ml of deionized water prior to use.

In a brown volumetric flask (100 ml) the emulsified nutritional supplements (5 g) was dissolved in 20 m*M* MSG aqueous solution. This solution (1 ml) was applied to the conditioned Bond Elut C_2 cartridge. The cartridge was washed with deionized water (5 ml) followed by elution with ethanol (eluate volume: 5 ml).

2.4. Apparatus and conditions

A Model 655 A-12 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model F-1080 fluorescence detector (Hitachi), set at excitation wavelength 350 nm and emission wavelength 480 nm, was used. A Model C0 630 column oven (GL Sciences, Tokyo, Japan) was used. The samples were applied by a Rheodyne Model 7125 sample-loop injector with an effective volume of 100 μl. Separation took place by non-aqueous reversed-phase HPLC on a 15×0.3 cm I.D. column of an Inertsil ODS 80A (5 μm) (GL Sciences) with guard column (1×0.3 cm I.D. column of an Inertsil ODS 80A (5 μm)) using methanol–ethanol (50:50,

v/v) as the mobile phase. The flow-rate was 0.4 ml/min at 40 °C.

3. Results and discussion

3.1. Effect of dissolving agent on the retention of standard vitamin A onto SPE cartridge

Usually, a hexane extraction method is used for the separation of oily particles in a sample, after the addition of an inorganic salt such as sodium chloride to the sample in a separatory funnel. However, this method takes a long time, because the sample often forms an emulsion, which may take as long as 1 h to break up. In addition, this procedure is tedious. Furthermore, it is not possible to separate the oily particles and fat-soluble vitamins by the hexane extraction method. So, a hexane extraction method is not suitable sample preparation for the vitamin A in emulsified foods.

SPE method also required some salt for the sample preparation of fat-soluble vitamins in emulsified samples to break up [3–5]. From the above, it is essential to look for the suitable dissolving agent containing some salt for the present development of routine work.

Previously [3–6], it was demonstrated that dissolving agents containing salt like sodium sulfate played an important role on the complete retention of fat-soluble vitamins onto sorbent.

Food samples often contain calcium, which forms very slightly soluble calcium sulfate. As described above, sodium sulfate aqueous solution was not suitable dissolving agent for the routine work.

First effort was focused on effect of dissolving agent on the retention of standard vitamin A onto SPE cartridge. Eight kinds of dissolving agents were examined (Table 1). Water was not suitable, because recovery of vitamin A was 73.9%. Seven other salts were suitable dissolving agent, because of high recovery of vitamin A. However, an aqueous sodium sulfate solution was not suitable, because it formed calcium sulfate. In the present work, alcohol-soluble compounds and *umami* substance were suitable[1].

Potassium iodide and *umami* substances like MSG were often contained in certain foods. The next effort was focused on the suitable concentration of KI and MSG (Table 2).

We examined KI and MSG, which do not precipitate with calcium ion. Using either of them, fat-soluble vitamins are successfully retained onto the SPE packing materials and the column inlet pressure no longer increased, even after over 100 injections.

[1]The term *umami* (derived from the Japanese word *umai* = delicious) is used to indicate the taste of glutamic acid. Detailed information on the term and on the underlying investigations can be found in Refs. [13–15].

Table 1
Effect of salt on retention of vitamin A onto Bond Elut C_2 cartridge (500 mg) and suitability for dissolving agent

Dissolving agent	Recovery (%)	Suitability for dissolving agent
Water	74	No[a]
Sodium sulfate (0.5 *M*)	100	No[b]
(Alcohol–soluble compound, 0.5 *M*)		
Potassium iodide	100	Yes
Sodium perchlorate	100	Yes
Ammonium acetate	100	Yes
Umami substance, 0.5 M		
MSG	100	Yes
IMP[c]		
GMP[d]	100	Yes

[a] Vitamin A was not retained onto the cartridge completely.
[b] Calcium sulfate might be formed in the eluate.
[c] Sodium inosine-5′-monophosphate.
[d] Sodium guanosine-5′-monophosphate.

Table 2
Effect of concentration of MSG and KI on recovery of vitamin A

Concentration (*M*)	Recovery (%)	
	MSG	KI
0	81.9	80.6
0.01	98.7	97.8
0.015	100	100
0.02	100	100
0.05	100	100
0.1	100	100

The optimum concentration of both dissolving agent was over 15 m*M*. In the present work, we chose MSG, because it chelate with metal ions in food samples. Twenty m*M* MSG was used. Good results were obtained employing the method. It was found that MSG was the suitable salt for the present purpose of sample preparation for vitamin A in emulsified samples by SPE, because of good results (see Tables 6 and 7).

3.2. Choice of SPE choice

Two parameters—vitamin A peak shape on the chromatogram, and extraction efficiency from emulsified samples—were the criteria for selection of SPE cartridge. Previous works [3–5] indicated that a bed mass of 500 mg of sorbents would be needed for the emulsified nutritional supplements, because larger amounts of oily particles in samples were retained onto the SPE cartridge strongly and trace amounts of fat-soluble vitamins could not be retained onto the sorbent completely if smaller mass of sorbents like 100 or 200 mg were used.

Again, attention was paid to the suitable choice of the cartridge vitamin A extracted from the cartridge with the smaller volume of eluent. Non-polar, polar and mix-phase SPE sorbents were examined in this study. A Bond Elut C_2 (500 mg) cartridge was chosen for SPE after comparison with 16 other cartridges (Table 3), because it gave the excellent recovery of vitamin A from the sorbent with smaller volume of eluent (eluate volume: 5 ml) than the other types examined here and previous reported Bond Elut C_{18} cartridge (eluate volume: 10 ml) [3–5].

Table 3
Comparison of sorbent with recovery of vitamin A (eluent:ethanol, eluate volume: 5 ml)

Cartridge	Recovery (%)	RSD (%) ($n=3$)
Non-polar phases		
Bond Elut (500 mg)	83.7	3.3
C_{18}		
C_8	90.5	3.1
C_2	100	2.9
CH	95.9	3.1
PH	93.8	3.2
LMS	16.6	4.5
Sep-Pak (tC_{18}(500 mg))	89.1	3.2
PLS-2 (270 mg)	34.9	4.1
Nexsps (60 mg)	29.4	4.5
Oasis HLB 60 (60 mg)	58.0	4.3
Oasis HLB 200 (200 mg)	34.8	4.4
Empore disk C_{18} (7 mm)	72.1	3.8
Polar phase		
Bond Elut CN (500 mg)	17.7	4.5
NH_2 (500 mg)	64.6	3.8
Mixed phases		
Certify (130 mg)	8.9	4.5
Oasis MCX (60 mg)	27.8	3.9

3.3. SPE (elution factors)

3.3.1. Effect of eluent on elution of vitamin A

SPE method development requires optimization of retention and elution of vitamin A standard. Standard was injected into the cartridge followed by elution with different eluents. The optimum elution of vitamin A was provided by ethanol and 2-propanol (Table 4). Consequently, we used ethanol as the eluent, because the mobile phase contains ethanol.

Table 4
Effect of eluents on the elution of vitamin A using Bond Elut C_2 cartridge (500 mg) (eluate volume: 5 ml)

E1uent	Recovery (%)	
	Mean±	SD ($n=3$)
Methanol	21.8	0.65
Ethanol	100	2.97
2–Propanol	100	2.95
Acetonitrile	11.1	0.45
Mobile phase (methano1–ethanol, 1:1)	93.6	3.15

3.3.2. Effect of ethanol volume on the elution of vitamin A from the cartridge

An elution profile of the content (%) of vitamin A in successive 1 ml aliquots of eluate was developed after standard vitamin A was injected into the cartridge. Vitamin A in each 1 ml fraction (eluate) 1 to 6 was analyzed (Table 5). Recovery (%) of vitamin A in the fractions 1 to 3 was over 95. It was found that complete elution of vitamin A from the cartridge was obtained with 5 ml of eluate (fractions 1 to 5)

Eluate volume for the complete elution of previously reported fat-soluble vitamins [3–5] needs 10 ml using Bond Elut C_{18} cartridge (500 mg). Thus, the present method (eluate volume: 5 ml) is beneficial for the determination of trace amounts of vitamin A, because the proposed method increased sensitivity.

3.4. Chromatography

First, we examined the effect of dissolving agents on the sharpness of the standard vitamin A peak on the chromatogram. When standard vitamin A was diluted with water-free ethanol (5 ml) followed by injection, broad peak was obtained. On the other hand, when standard vitamin A was diluted with ethanol containing small amounts of water, which was obtained after that eluent (ethanol) was passed through the conditioned Bond Elut C_2 cartridge, sharp peak was obtained on the chromatogram (Fig. 1). This effect is almost certainly caused by sample enrichment focusing of analysis at the top of the column due to the water content [3–6]. Whatever the cause, it was necessary to prepare both the standards and samples in the same way.

Table 5
Effect of ethanol volume on the elution of vitamin A from the C_2 cartridge

Fraction (eluate volume: 1 ml)	Recovery (%)	
	Mean	SD ($n=3$)
1	72.6	2.7
2	20.6	0.8
3	4.8	0.3
4	1.7	0.1
5	0.3	0.1
6	0	

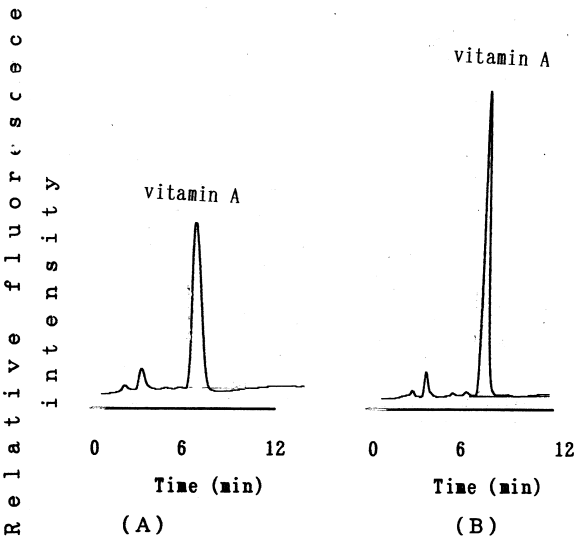

Fig. 1. Chromatograms of (A) standard vitamin A (2.65 ng) diluted in water-free ethanol and (B) standard vitamin A (2.65 ng) diluted in ethanol containing small amounts of water, which passed through the conditioned Bond Elut C_2 cartridge (500 mg). HPLC was carried out on a 15×0.3 cm I.D. column of Inertsil ODS80A (5 μm) with guard column of Inertsil ODS80A (5 μm) (1×0.3 cm I.D.) using methanol–ethanol (50:50, v/v) as a mobile phase at a flow-rate of 0.4 ml/min at 40 °C.

The vitamin A peak in the sample after SPE by HPLC is shown in Fig. 2 (fluorescence detection at excitation wavelength 35 nm, emission wavelength 480 nm). Large volume injection (100 μl) gave peak of sufficient intensity for our detection needs. Retention time was about 8 min. The limit of detection according to Fig. 2 (supplement A) (73.1 ng injected), was ca. 0.1 ng per injection at a signal-to-noise ratio of 3:1).

3.5. Determination of vitamin A

The calibration graph for vitamin A was constructed by plotting the peak height of vitamin A against the amount of vitamin A added. Satisfactory linearity was obtained in the range of 0.1 to 140 ng on column ($y = 0.063x + 0.047$, y = peak height, x = amount of vitamin A in ng, $r^2 = 0.999$),

A known amount of vitamin A was added to the nutritional supplement, and overall recoveries were

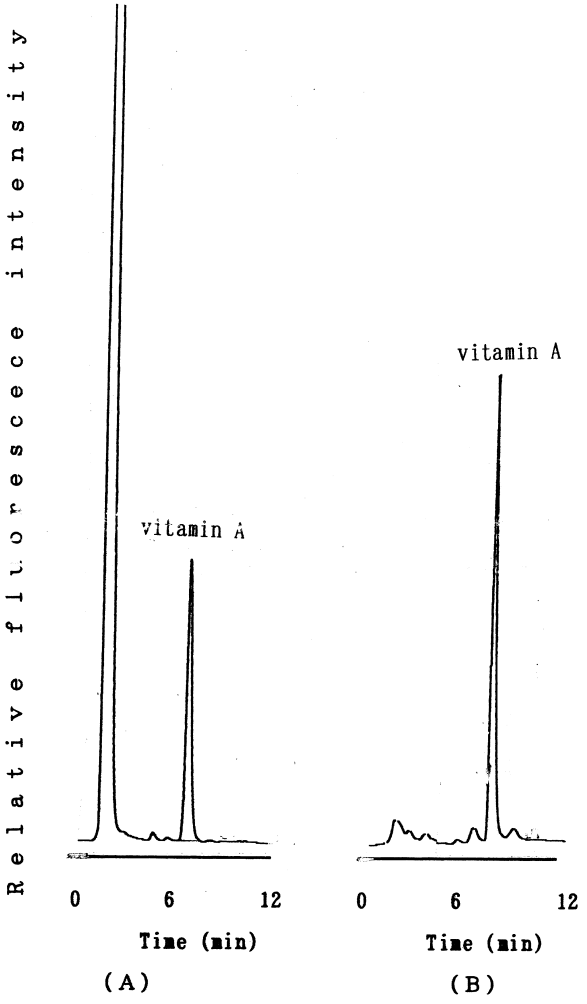

Fig. 2. Chromatograms of (A) vitamin A in an emulsified nutritional supplement and (B) drug by HPLC with fluorescence detection (excitation wavelength 350 nm, emission wavelength 80 nm) after SPE. HPLC conditions as in Fig. 1.

estimated by the standard addition method (Table 6). As listed in Table 6, vitamin A was recovered over 90%. The relative standard deviation (RSD) was 2.9% ($n=5$) with no addition of vitamin A. Between-day RSD (5 days) was 3.7%.

Table 7 summarizes the analytical data for vitamin A in emulsified nutritional supplements and drugs by the final method. There was good agreement for vitamin A between concentration indicated and concentration found.

In the present research, we determined vitamin A with no use of an internal standard, giving the linear calibration graphs, high recoveries (over 90%) and low RSD (2.9%) for vitamin A.

Table 6
Recoveries of vitamin A added to an emulsified nutritiona1 supplement

Added (μg/g)	Found (μg/g)	Recovery	
		(μg/g)	(%)
0	73.1	–	–
17.5	89.7	16.6	94.7
35	106.3	33.2	98.7
70	140.3	67.2	97.5

RSD: 2.9% ($n=5$) with no addition of vitamin A.

4. Conclusion

This paper deals with the development of a method using optimized SPE followed by non-aqueous HPLC to determine trace amounts of vitamin A in emulsified nutritional supplements. The choice of the Bond Elut C_2 cartridge was made after investigating the recovery of vitamin A from 16 different types of SPE cartridges, because it was beneficial that vitamin A was eluted from this SPE cartridge which had a smaller volume (5 ml) than C_{18} cartridge (10 ml).

MSG was chosen as the dissolving agent for sample preparation, because it did not form calcium sulfate precipitates in the eluate and did not increase the inlet pressure. These effect was the more suitable in the routine work.

The result is a simple, rapid, selective method for estimating vitamin A in emulsified food and drug

Table 7
Analytical data for vitamin A in an emulsified nutritional supplements and drugs by the proposed method

	Concentration indicated	Concentration determined	
		Mean	RSD (%)
Supplement			
A	73.3 μg/g	73.1 μg/g	2.9 ($n=5$)
B	250	255	2.8 ($n=3$)
Drug			
C	35.0 μg/g	33.5 μg/g	2.8 ($n=3$)
D	5.5 μg/tablet	5.2 μg/tablet	3.1 ($n=3$)

samples using Bond Elut C_2 and an HPLC Inertsil ODS 80A column with fluorescence detection (excitation wavelength 350 nm, emission wavelength 480 nm) using MSG as dissolving agent to obtain higher recovery of vitamin A. The proposed method was simple, rapid (sample preparation time by SPE: ca. 2 min, retention time: ca. 8 min), sensitive (detection limit: ca. 0.1 pg per injection (100 μl) at a signal-to-noise ratio of 3:1), highly selective and reproducible (RSD): ca. 2.9% ($n=5$), between-day RSD ca. 3.7% (5days). Recovery of vitamin A was over 90% by the standard addition method.

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