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Determination of vitamin D_2 in emulsified nutritional supplements by solid-phase extraction and column-switching high-performance liquid chromatography with UV detection

Hiroshi Iwase*

Ajinomoto Co., Inc., Central Research Laboratories, 1-1 Suzuki-cho Kawasaki-ku, Kawasaki 210-8681, Japan

Abstract

This paper deals with a method for solid-phase extraction of trace amounts of vitamin D_2 (VD_2, 19 ng/g) from emulsified nutritional supplements, which contain 50 kinds of compounds, followed by column-switching high-performance liquid chromatography (HPLC) with UV detection at 265 nm. VD_2 is present at 1000–20 000 000 times lower concentration than other components. Bond Elut C_{18} cartridge was chosen as for the emulsified nutritional supplements after comparison with eight other types. A sample solution was applied to the solid-phase extraction cartridge and VD_2 was eluted by methanol followed by HPLC. The effects of sample pH, eluent composition and eluate volume on the retention and elution of VD_2 on Bond Elut C_{18} cartridge were examined. The resulting method was simple, rapid (analysis time: ~20 min), sensitive (detection limit: ~0.1 ng per injection (200 μl) at a signal-to-noise ratio 3:1), and reproducible (relative standard deviation: ~6.2%, $n=5$). The calibration graph for VD_2 was linear in the range of 0.1–3 ng per injection (200 μl). Recovery of VD_2 was ~80% by the standard addition method.

Keywords: Food analysis; Column switching; Vitamins

1. Introduction

Numerous techniques have been developed to extend the application of high-performance liquid chromatography (HPLC), including pre- and post-column labelling, column-switching and solid-phase extraction (SPE) [1]. Sample preparation is crucial in the analysis of trace amounts of compounds like vitamin D_2 (ergocalciferol, VD_2) in such complex matrices as high fat content nutrient samples, which form an emulsion. It is important to optimize this step to ensure that the subsequent HPLC analysis is effective. A simple and rapid sample preparation method is very desirable for the analysis of trace amounts of VD_2 in emulsified nutritional supplements.

Numerous methods have been developed for the analysis of trace levels of vitamin D by HPLC [2–6]. Previous papers have shown [7–9] that the traditional hexane extraction method was not suitable for the sample preparation of emulsified food samples. Refs. [8,9] demonstrated that the SPE method was suitable for 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 emulsions have not been previously determined.

It is necessary to use SPE both to concentrate analytes for detection and to remove interferences by non-polar compounds. Thus, these must be removed from samples, since lipophilic species adsorb strong-

*Corresponding author.

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ly onto reversed-phase cartridges like Bond Elut C_{18} reducing capacity for low levels of analytes as previously described [7–9].

Because Bond Elut cartridges have given satisfactory sample preparation for β-carotene from soup (fat content 5%) and vitamin K_1 (VK_1) from nutrient (fat content 2%) [8,9], we used it once again to extract trace amounts (18 ng/g) of VD_2 from nutrient (fat content 2%). The method development required a speedy and simple sample stabilization.

The emulsified nutritional supplement samples used here are in powdered form and had to be dissolved in water prior to use. They are complex sample types containing 50 compounds including amino acids, vitamins, organic acids, plant oils, dextran and minerals at concentrations at least 1000–20 000 000 times higher than that of VD_2.

In nutrition studies, it is important to know how stable VD_2 is in aqueous solution. Similarly, its stability in eluate from SPE must be examined in analytical chemistry.

This paper demonstrates a simple and rapid analytical method for the trace detection of VD_2 (19 ng/g) in nutrient with high fat content (2%) by SPE and column-switching HPLC with UV detection at 265 nm. In order to develop a reliable method, the choice of polar and non-polar sorbents for the emulsified nutritional supplement samples and the effect of two other variables were studied: (1) the effect of large amounts of other compounds on SPE and (2) the effect of potassium phosphate concentration on recovery of VD_2 at low levels.

Here we describe the development of an analytical procedure that employed optimized SPE combined with HPLC to obtain a sharp VD_2 peak. This was further applied to clean-up and concentration of VD_2 in dried prepared nutrient. In addition, this paper deals with the stability of VD_2 in both nutrient sample solutions and methanol eluates obtained by SPE under different storage conditions and for different periods of storage.

2. Experimental

2.1. Reagents and materials

The VD_2 used in this study was from the Japanese Pharmacopeia Standard. Other reagents were all of analytical or HPLC grade. Nutritional supplement samples were commercially available. Light-resistant brown volumetric flasks and glassware [10,11] were used. The membrane filters (0.5 μm) were purchased from GL Sciences (Tokyo, Japan). 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 VD_2 preparation.

To a brown 50-ml volumetric flask, 75 mg of solid standard was added and then dissolved in methanol which was then made up to the mark. From this solution, 5 ml was taken and diluted with methanol in a second brown 50-ml volumetric flask. Five more serial dilutions were carried out in the same way. Finally 2 ml was diluted in a 20-ml flask, giving a concentration of 15 ng/100 ml methanol.

2.3. Sample preparation

The nutritional supplement (10 g) was dissolved in a brown volumetric flask (100 ml) in 0.2 *M* K_2HPO_4 aqueous solution with 1 m*M* ethylenediaminetetraacetic acid disodium dihydrate (EDTA·$2Na·2H_2O$). This solution (4 ml) was applied to the conditioned Bond Elut C_{18} cartridge. The cartridge was washed with deionized water (10 ml) and then 10% aqueous methanol solution (5 ml) followed by elution with methanol (10 ml). This eluate was concentrated to dryness in vacuo followed by dilution in exactly 0.5 ml with methanol and further filtered with a membrane filter. An aliquot (200 μl) was injected into the chromatograph.

2.4. Apparatus and conditions

Two Model 655 A-12 high-performance liquid chromatographs (Hitachi, Tokyo) equipped with a Model L-4000 variable-wavelength detector (Hitachi) set at 265 nm and a Model PT-8000 column switching device (Toyosoda, Tokyo) were used. A Hitachigel 3011-0 precolumn (5 μm) (10× 0.46 cm I.D., Hitachi) and analytical column of

Inertsil ODS-2 (5 μm) (15×0.46 cm I.D., GL Sciences) were used. The samples were applied by a Rheodyne Model 7125 sample loop injector with an effective volume of 200 μl.

2.5. Chromatographic conditions

After injecting 200 μl of the extracted sample on to the pre-column, which has been previously equilibrated with methanol, the column was washed for 4 min with methanol at a flow-rate of 0.8 ml/min at room temperature. The substances adsorbed on the precolumn were flushed on to the analytical column with acetonitrile–methanol (75:25) for 15 s at a flow-rate of 0.6 ml/min at 40°C by switching the six-port valve. After introducing the substances on to the analytical column the six-port valve was returned to the original position. The precolumn was washed with methanol for the next injection.

3. Results and discussion

3.1. Transmittance (T%) of eluate obtained from cartridges

The previous paper [8] showed that non-polar sorbents provided superior to polar sorbents for the extraction of fat-soluble vitamins in emulsified food samples diluted with water (neutral pH).

Again, uptake of the oily fraction by the SPE phase was gauged by absorbance of the both aqueous fraction (unbound) and eluate at 650 nm for the choice of suitable sorbent of VD_2 in an emulsified nutritional supplement, which was diluted in aqueous K_2HPO_4 solution (weak alkaline pH).

We often encounter emulsified nutrient and soup samples with high fat contents. It is not easy to carry out the sample preparation of these samples to ensure the subsequent HPLC analysis is effective.

The first effort was focused on the possibility of the separation of oily particles in nutrient samples by using SPE cartridges. Various SPE cartridges were tested. Evaluation was made by measuring the transmittance (*T* %) value in the both effluent and eluate obtained from each cartridge at 650 nm (Table 1). *T* (%) value of both effluent and eluate obtained by using various cartridges at 650 nm was suitable for the removal of oily particles [7].

Table 1
T (%) value of effluent and eluate obtained by using various cartridges at 650 nm[a]

Bond Elut cartridge (500 mg)	*T* (%)	
	Effluent	Eluate
Sample before SPE		4.7
Sample after SPE		
C_{18}	98.3	99.1
C_8	97.7	99.2
C_2	98.1	99.4
CH	98.5	98.8
PH	98.1	99.1
Polar phase		
CN	6.8	35.5
CN–N	7.1	34.9
NH_2	6.7	37.7

[a] Sample preparation; see Section 2.3.

A comparison of the former (non-polar phase) and the later (polar phase) showed that the former gave the higher *T* (%) values in both aqueous fraction (unbound) and eluates. When the sample solutions were applied to each cartridge, fat-soluble analytes like VD_2 could be retained completely on the formers and both aqueous fraction and eluates obtained from the cartridges were clear (*T* %; over 98%). On the other hand, both the aqueous fraction and eluates obtained from the latter cartridges were cloudy (*T*%; below 50%) and VD_2 were not retained completely, because the oily particles and VD_2 passed through the cartridges together.

From the above results, we chose the Bond Elut C_{18} cartridge for the present work.

3.2. SPE (retention and elution factors)

We reported in previous papers [7–9], the determination of trace amounts of cyanocobalamin, β-carotene and VK_1 in foods carried out 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 volume — are significant for efficient retention and elution of these vitamins. Consequently, these factors were also explored for the analysis of VD_2 in emulsified nutritional supplement.

Table 2
Effect of eluents on the elution of VD_2[a]

Eluent	Recovery (%)
Methanol	100
Ethanol	99.8
Isopropanol	63.1
Acetonitrile	39.2

[a] Standard VD_2 (3.6 μg) was injected into the cartridge followed by elution with each eluent (eluate volume; 10 ml).

3.2.1. Effect of eluent on elution of VD_2

SPE method development requires optimization of retention and elution of a VD_2 standard. Standard VD_2 (3.6 μg) was injected into the cartridge followed by one of four eluents (Table 2, methanol, ethanol, isopropanol and acetonitrile).

The optimal elution of VD_2 is obtained with methanol (Table 2). Retention was tested by examining the aqueous fraction (unbound) for traces of VD_2. As VD_2 was not detected in aqueous fraction it was concluded that VD_2 was completely retained after application in aqueous solution.

A previous paper [8] indicated that ethanol was a suitable eluent for VK_1 and therefore this was investigated as an eluent for VD_2. However, we found that VD_2 could not be extracted with ethanol.

3.2.2. Effect of methanol volume on the elution of VD_2 from the cartridge

An elution profile showing the content (%) of VD_2 in successive 2 ml aliquots of eluate was developed, after standard VD_2 (3.6 μg) had been injected into the cartridge. VD_2 in each 2 ml fraction 1 to 5 was analyzed (Table 3).

Table 3
Effect of methanol volume on the elution of VD_2 from the cartridge[a]

Fraction (eluate volume; 2 ml)	Recovery (%)
1	46.7
2	35.3
3	13.2
4	3.3
5	1.5

[a] Standard D_2 (3.6 μg) was injected into the cartridge followed by elution with successive 2 ml aliquots of methanol VD_2 in the fractions 1 to 5 (each 2 ml) was analyzed

Recoveries (%) of VD_2 in the fractions 1 to 3 were over 90% with methanol. It was found that complete elution of VD_2 from the cartridge was obtained with methanol (eluate volume; 10 ml).

When methanol–water (99:1, 98:2, 97:3, 95:5, 90:10) was used as the eluent (each eluate volume; 10 ml), recovery of VD_2 was 85.5, 68.5, 18.5 0 and 0%), respectively. So, we use HPLC grade methanol without the addition of water.

3.2.3. Effect of pH value in sample solution on retention of VD_2

Standard VD_2 (3.6 μg), diluted in 2 ml of 10 m*M* potassium phosphate buffer (pH 2 to 8.5), was applied to the cartridge, followed by elution with 10 ml of methanol. The VD_2 in the each eluate (each 10 ml) was analyzed.

The optimal recovery of VD_2 on the Bond Elut C_{18} cartridge was found over the sample pH range of 7.5–8.5, demonstrating that VD_2 retention is very sensitive to loading pH. Ref. [8] indicated that the suitable pH range in sample solution for loading VK_1 was 5.5–7.5. Thus, it is clear that in order to obtain optimal recovery of VK_1 and VD_2 different sample preparation methods are required.

The pH value in the nutrient sample solution (see Experimental) was about 7.7. Thus, sample can be applied to SPE without further pH adjustment. This saves time in routine sample preparation. A large volume (10 ml) of methanol is needed to elute VD_2, because VD_2 is very strongly absorbed on a 500 mg C_{18} cartridge. To improve the method, the use of a smaller size (100 mg C_{18}) cartridges might be considered, because it will require smaller elution volumes to give good recovery. However, there is a possibility that trace amounts of VD_2 in the nutrient with high fat content could not be retained completely on a smaller size cartridge, because large amounts of lipophilic species, which retain strongly on the Bond Elut C_{18}, reduce the sorbent's available capacity for VD_2. To avoid this possibility, we used the 500-mg cartridge in this study.

3.3. Nutritional supplement sample solution preparation

The SPE data quoted above indicate that it was necessary to adjust the pH of the nutrient solution to

7.5–8.5 — a different range from the VK_1 sample solution previously reported (pH 5.5–7.5). So, a study was made of the preparation of sample solution with a suitable pH value for analysis of VD_2.

The primary purpose of this work was to determine the effect of solvent on recovery of VD_2. The nutrient often forms an emulsion, requiring addition of inorganic salt such as sodium chloride to break it up [7].

The effect of dipotassium hydrogenphosphate concentration (0.1–0.5 *M*) on the recovery (%) of VD_2 was examined for the SPE sample preparation. In excess of 0.1 *M* dipotassium hydrogenphosphate gave a suitable recovery (%) of VD_2. To make sure, 0.2 *M* was used as the dissolving agent in this study.

A previous paper [9] indicated that the addition of 1 m*M* EDTA·2Na·$2H_2O$ to the solvent was effective in stabilising VK_1, so, 0.2 *M* dipotassium hydrogenphosphate with 1 m*M* EDTA·2Na·$2H_2O$ was used. The pH value in sample solution was ~7.8. So, this solution could be applied to SPE without further pH adjustment. Previous research [9] also indicated that nutrient sample solution (1 ml) could be applied to the cartridge followed by elution (10 ml) without further concentration. Using VD_2 over the desired concentration range with 4-ml cartridges, it was found that 80 ml of nutrient solution could be applied while allowing direct injection of the eluate without need for concentration. There are two possible pitfalls to using such a large sample however, (1) there is a possibility that large amounts of non-polar compounds, which are strongly retained on the cartridge will reduce the capacity of the Bond Elut for trace amounts of VD_2 and (2) accurate loading of larger volume of 80 ml to the cartridge (3 ml) is not convenient in routine work. It was found it easier to reduce a 10-ml eluent to 0.5 ml, than to pass 80 ml of food sample through a small SPE cartridge.

3.4. Chromatography

Attention was then turned to the analysis of VD_2 in nutrient, without using column-switching. VD_2 could not be identified due to interference peaks, possibly from unknown fat-soluble compounds, so column-switching proved necessary. It would not be needed with a better SPE method, producing a cleaner sample. The full run took over 2 h.

At the beginning of the work, retention times of standard VD_2 and column connection times from precolumn to analytical column were investigated in an effort to eliminate interference with VD_2 estimation from the complex sample matrix.

A chromatogram of nutrient sample alone using HPLC with UV detection at 265 nm using a precolumn is shown in Fig. 1. The peaks of VD_2 are observed at a retention time of ~4 min. After washing for 4 min for various periods to examine the connection time.

When connection times were set shorter than 15 s, the peaks of VD_2 were smaller, and when connection times was longer than for 15 s other many unknown co-eluting peaks were observed and VD_2 could not be identified. Thus, the 15-s column connection time was adopted as optimum.

Chromatograms of VD_2 in nutrient are shown in Fig. 2. These were produced by using column-switching HPLC with UV detection at 265 nm. The VD_2 peak was separated completely. The limit of detection from Fig. 2 was ~0.1 ng per injection (200 μl) at a signal-to-noise ratio 3:1 and analysis time was about 20 min.

3.5. Determination of VD_2

The calibration graph for VD_2 was constructed by plotting the peak height of VD_2 against the amount of VD_2. Satisfactory linearity was obtained in the range of 0–2 pg on column ($y=1.1124x-0.060$, y=peak height, x=amount of VD_2 in ng, $r^2=0.998$). A known amount of VD_2 was added to nutrient and overall recoveries were estimated by the standard addition method, as shown in Table 4. VD_2 was recovered at 80% by the standard addition method. The relative standard deviation (RSD) was 6.2% ($n=5$) with no addition of VD_2.

The proposed method has the great advantage that VD_2 in nutrient sample solution can be directly applied to SPE without further pH adjustment from 7.8: thus, VD_2 can be analysed directly.

There was good agreement between actual and estimated values for VD_2 in nutrient.

Table 5 shows analytical data for VD_2 in nutrients with high fat-content. There was good agreement in VD_2 value in nutrients between the concentration indicated and that found by this method, irrespective

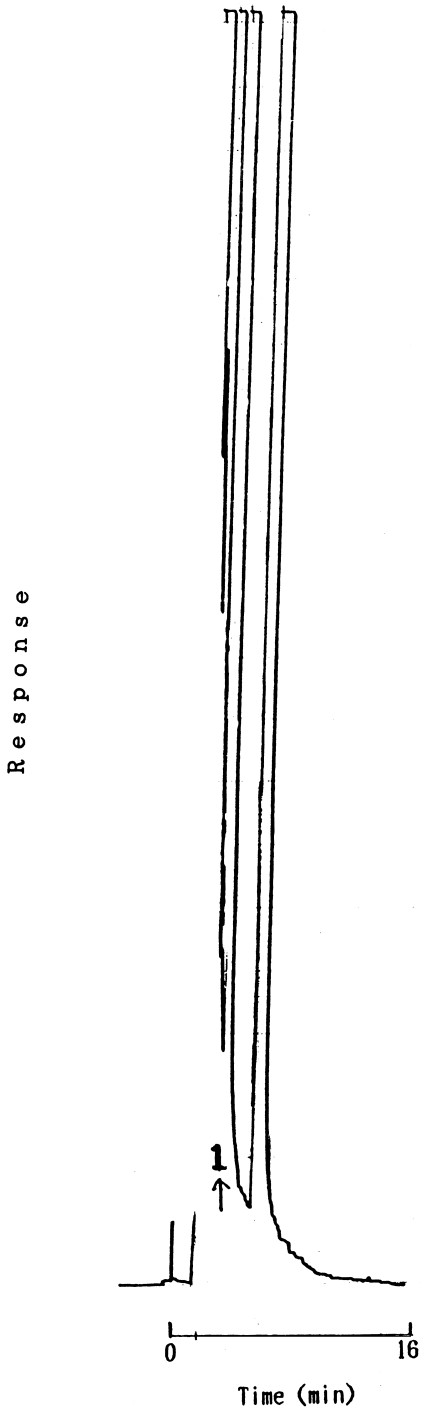

Fig. 1. Elution of VD_2 in nutrient on a precolumn of Hitachigel 3011-0 (5 μm) (10×0.46 cm I.D.) with detection at 265 nm. Mobile phase: methanol at a flow-rate of 0.8 ml/min. 1=VD_2.

of the concentration indicated ratio of oily particles and VD_2 was over ~300 000 times.

3.6. Stability of VD_2

Finally we looked at the stability (%) of VD_2 in nutrient samples, looking first at the changes over the period of 24 h in both nutrient sample solutions and methanol eluates stored at 5°C or room temperature.

At first, the periodic changes of VD_2 levels in both nutrient sample solutions and ethanol eluates obtained by SPE were examined. These were stored at both 5°C and room temperature for 24 h to examine how long VD_2 in each sample was stable. It was clear that VD_2 was stable for 24 h in both conditions for the methanol eluates. On the other hand, VD_2 was stable for only 5 h in both conditions for the nutrient sample solutions. Peak height was constant for 5 h, and then decreased. When the above sample was stored for 24 h at both 5°C and room temperature, the VD_2 content (%) was about 93.2 and 88.6%, respectively. It was found that VD_2 prepared by the proposed method was very stable. An advantage of this method is that once a sample has been extracted, it does not need to be analysed the same day as the SPE is stable with VD_2 suffering no decline overnight.

4. Conclusion

A simple and rapid analytical method for VD_2 in emulsified nutritional supplements with a high fat content has been demonstrated that uses SPE and column-switching HPLC with UV detection at 265 nm. The possibility of the complete retention and separation of trace amounts of VD_2 in nutrient with large amounts of oily particles on the cartridge was demonstrated. The effects of pH value in sample solution, eluent composition and eluate volume on the retention and elution of VD_2 on Bond Elut C_{18} cartridges were examined. The stability of VD_2 in nutrient sample solution by the proposed method was also examined.

The method established here seems to be applicable to the analysis of VD_2 in nutrient, because of its simplicity, speed (analysis time of VD_2: ~20 min) and reproducibility (RSD: 6.2%). It provides re-

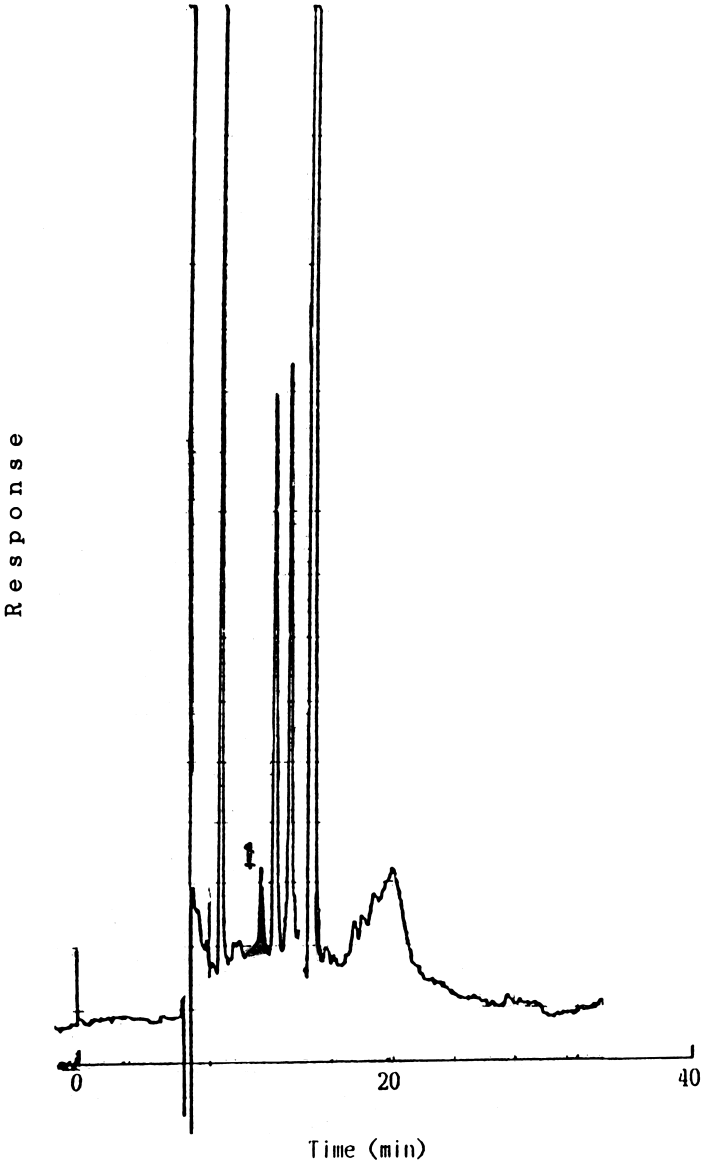

Fig. 2. Chromatogram of VD_2 in emulsified nutritional supplement by column-switching HPLC with UV detection at 265 nm. After injection 200 μl of sample solution (methanol, prepared after SPE) onto the precolumn, the column was washed for 4 min with the mobile phase (methanol) at a flow-rate of 0.8 ml/min at room temperature. The substances adsorbed on the precolumn were introduced on to the analytical column with acetonitrile–methanol (75:25) for 15 s at a flow-rate of 0.6 ml/min at 40°C by switching the six-port valve. After introducing the substances on to the analytical column the six-port valve was returned to the original position. 1=VD_2 (3.2 ng injected).

coveries of 80% using the standard addition method. Application of the proposed method to the determination of VD_2 in other foods is being studied.

A summary of sample dissolving agents, SPE conditions, recovery (%) and RSD for fat-soluble vitamins [7–9] is shown in Table 6. Although dissolving agents and SPE conditions were different, it was concluded that SPE sorbent was the suitable sample preparation technique for the emulsified food samples (concentration indicated ratio of oily par-

Table 4
Recoveries of VD_2 added to nutritional supplement[a]

Added (μg/100 g)	Found (μg/100 g)	Recovery	
		(μg/100 g)	(%)
0	2.0	–	–
1.0	2.8	0.8	80
2.0	3.6	1.6	80
4.0	5.4	3.4	85

[a] R.SD: 6.2% (n=5) with no addition of VD_2.

ticles/vitamin (1,10–105 000), giving recovery (over 80%) and RSD (1.3–6.2%).

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Table 5
Analytical data for VD_2 in nutritional supplement

Nutrients	Concentration indicated ratio (oily particles/VD_2)	Concentration indicated (μg/100 g)	Concentration determined (μg/100 g)	RSD (%) (n=5)
A	~105 000	1.9	2.0	6.2
B	~300 000	1.6	1.6	7.2
C	~180 000	2.0	2.5	6.5

Table 6
Summary of sample dissolving agents, SPE conditions, recovery (%) and RSD for fat-soluble vitamins

Vitamin	Concentration		SPE		Recovery (%) (RSD)[d]
	Indicated ratio (o/v)[a]	Dissolving agent[b]	Loading pH	Eluent[c]	
β-Carotene [8]	1110	Deionized water	2–7.5	Ethanol	>90 (1.3%)
VK_1 [9]	25 000	5% Na_2SO_4	5.5–7.5	Ethanol	>90 (2.3%) (1.3%)
VD_2	105 000	0.2 M K_2HPO_4	7.5–8.5	Methanol	~80 (6.2%)

[a] Oily particles/vitamin.
[b] For sample preparation.
[c] Each eluate volume; 10 ml.
[d] n=5