

Selective Extraction and Isolation of Vitamin B_{12} Using Homogeneous Liquid–Liquid Extraction with Perfluoro Surfactant

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Cyanocobalamin (vitamin B_{12}) was selectively extracted from a biological sample by the homogeneous liquid–liquid extraction based on a pH-dependent phase separation with perfluorooctanoate ion ($PFOA^-$). When the experimental conditions were $[PFOA^-]_T = 2.86 \times 10^{-3}$ mol L^{-1}, $[HCl]_T = 0.66$ mol L^{-1}, and $[Acetone]_T = 2.86$ vol %, 8.46×10^{-7} mol L^{-1} (1.14 ppm) of vitamin B_{12} was selectively extracted at 95.2% (sample solution; 35 mL → a liquid sedimented phase; 30 μL). This liquid sedimented phase consisted of large amounts of HPFOA. When microliter levels of THF and water were added to this sedimented phase, vitamin B_{12} is completely and rapidly back-extracted from the sedimented phase to the water phase. Vitamin B_{12} was then extracted into the water phase at 96.2% and all HPFOA remained in the sedimented phase. The rest of the sedimented phase after the back-extraction was dried and completely recovered as a powder of HPFOA. Consequently, this method was able to recycle HPFOA as a separation medium. Vitamin B_{12} in a water phase was also crystallized by the addition of excess THF. Moreover, this method was applied to the extraction from rare oysters. As a result, a few micrograms of vitamin B_{12} were recovered.

It is very important and beneficial in several industries, such as chemistry, medicine and food, to selectively extract and separate an effective species from a biological sample.

Cyanocobalamin (vitamin B_{12}) is an essential species for human physiological functions and it is also known[1] to prevent pernicious anemia such as megaloblastic anemia. Humans cannot synthesize vitamin B_{12} in the body; therefore, one have to assimilate it from foods, medicines, and supplement foods. However, vitamin B_{12} is not in plants and vegetables, and is only slightly present in several biological foods such as oysters and livers. Therefore, vitamin B_{12} is industrially produced by a fermentation method using a vitamin B_{12} production bacteria such as *Propionibacterium shermanii*[2] and *Pseudomonas dentrificans*.[2] However, there is serious problem in the vitamin B_{12} production regarding the purification and separation procedures. It is difficult to directly purify and isolate trace amounts of vitamin B_{12}. Especially, since the oyster extracts or culture mediums after bacteria fermentation include many coexisting chemicals, a direct purification and separation are difficult. So far, the separation methods of vitamin B_{12} include the solid extraction methods such as column chromatography,[3] affinity chromatography,[4–8] membrane separation,[9] and gel filtration.[9] However, the problems with these solid extraction methods include (i) it takes a long time to pretreat a large quantity of the sample solution, (ii) the extracted material clogs the texture of the filler, (iii) disposable fillers are needed to adsorb the proteins and they are expensive, and (iv) a process is necessary to elute the vitamin B_{12} from the solid phase. On the other hand, the separation technique based on liquid–liquid distribution such as solvent extraction has great advantages over the solid extraction with respect to process simplicity, run time, resources, and cost. However, vitamin B_{12} is a hydrophilic compound, therefore, it is difficult to extract it from water using an organic solvent.

On the other hand, a homogeneous liquid–liquid extraction method based on the pH dependent phase separation phenomenon with perfluorooctanoate ion ($PFOA^-$: $CF_3(CF_2)_6COO^-$) was reported by Igarashi et al.[10] such $PFOA^-$ is well known as a perfluoro surfactant. This homogeneous liquid–liquid extraction utilizes the phase separation phenomenon from a homogeneous solution and the target solutes are extracted into the separated phase. Similar phase separation phenomena such as the salting effect,[11–13] cloud point extraction,[14,15] and aqueous two-phase extraction[16,17] are known as types of homogeneous liquid–liquid extractions. Among them, the homogeneous liquid–liquid extraction with $PFOA^-$ has significant advantages such as (i) the possibility of concentrating more than 10000-fold (volume ratio), (ii) a run time of approx. 10 min, and (iii) optional control of the volume of the sedimented phase at microliter levels. These features could not be done using the salting effect, cloud point extraction, and aqueous two-phase extraction procedures. Therefore, a homogeneous liquid–liquid extraction with $PFOA^-$ has been applied to the preconcentration method for several instrumental analyses.[18–21]

In this study, vitamin B_{12} was selectively extracted by this pH dependent phase separation phenomenon. Moreover, this procedure was able to isolate pure vitamin B_{12} from an organic phase consisting of a perfluoro surfactant. The authors developed the separation and recovery system for vitamin B_{12} from a biological sample.

Experimental

Reagent. Vitamin B_{12} was from Nakarai Tesk Co., Ltd. (Kyoto, Japan). Perfluoroocatanoic acid (HPFOA) was from Daikin Co., Ltd. (Osaka, Japan) and $PFOA^-$ was prepared by neutraliza-

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tion with lithium hydroxide. All other chemicals were of analytical reagent grade from the Kanto Chemical Co. (Tokyo, Japan), unless otherwise noted. The real sample was from commercial oysters. These were collected from the sea near Miyagi Pref. Japan.

Apparatus. A Nippon Bunko V-570 double beam spectrophotometer and a 1-mL quartz cell (light path length: 1 cm) were used for the spectrophotometry. CAPI-3200 (Otsuka Electronics, Osaka) was used as the capillary electrophoresis (CE) system. A 75-μm I.D. fused-silica capillary (Otsuka Electronics) with a 50-cm total length was used. A Nippon Bunko FT/IR-470 was used for the infrared spectrometer. A Hitachi R-1200 was used for the F-NMR.

Procedure. Twenty-nine mL of a sample solution containing vitamin B_{12}, 1 mL of 0.1 mol L^{-1} $PFOA^-$, and 1 mL of acetone was placed in a 50-mL cylindrical glass vial. When 4 mL of 6 mol L^{-1} HCl was added, 30 μL of a water-immiscible liquid phase appeared from the homogeneous solution. The sedimented liquid phase was removed by a micro-syringe and placed in another vial. A 25 μL aliquot of THF and 10 μL of distilled water were then added to this vial and it was centrifuged. After the centrifugation, the water phase containing vitamin B_{12} was removed by a micro-syringe and placed in another vial. The entire scheme of this procedure is shown in Fig. 1. Finally, excess THF was added to the vial; thereby, vitamin B_{12} was crystallized.

Pretreatment of Oyster as a Biological Sample. A 180 g sample of oysters was ground in a mortar and the materials were placed in a beaker. Fifty mL of distilled water was added to the beaker and then the mixture was homogenized for one hour. The solution was filtered by an exclusive paper and further filtered using a glass filter. A 0.2 mL aliquot of 10^{-2} mol L^{-1} potassium cyanide was added to this filtrate (approx. 150 mL) and this solution was agitated for two hours. The solution was divided into four vials and these was added to a ten mL of chloroform. After shaking (200 rpm for 20 min), they were centrifuged at 1700 rpm for 10 min. The supernatants were placed in other vials. This chloroform extraction procedure was repeated until the supernatant became clear. This treated supernatant was filtered using a membrane cartridge. The treated solutions were used as the sample solutions for the homogeneous liquid–liquid extraction.

The Analytical Conditions and Procedures for the Instrumental Measurement. Regarding the spectrophotometric measurement and CE measurement, the sedimented phase and the isolated crystals were diluted with 0.1 mol L^{-1} sodium tetraborate solution (Borax; pH 9.4) and these solutions were measured. The running buffer condition of CE was $[SDS]_T = 0.1$ mol L^{-1} and $[Borax]_T = 20$ mmol L^{-1}.

Results and Discussion

Extraction of Vitamin B_{12} Using Liquid–Liquid Distribution. So far, vitamin B_{12} has been thought to be difficult to extract using organic solvents by the liquid–liquid distribution procedure such as the solvent extraction method, because it is a hydrophilic compound (12.5 mg/mL H_2O[22]). The authors attempted to extract vitamin B_{12} by several extraction methods based on the phase separation phenomenon, because the formed organic phases include large amounts of moisture. Extraction methods that were examined included not only a general solvent extraction method with a nonpolar solvent, but also a pH-dependent phase separation, an ion-pair phase separation, a salting effect, and a cloud point extraction. These results are shown in Table 1. Vitamin B_{12} was only extracted by the homogeneous liquid–liquid extraction method based on a pH-dependent phase separation using $PFOA^-$/acetone/acid. The pH-independent phase separation showed satisfactory percentage in case of using acetone. Therefore, though the acetone was also examined in the salting extraction, vitamin B_{12} was not extracted. The red pigment was also extracted by cloud point extraction with Triton X-100. However, when this extract was measured by a spectrophotometric analysis, there was no Soret band (361 nm) of cobaramide in the

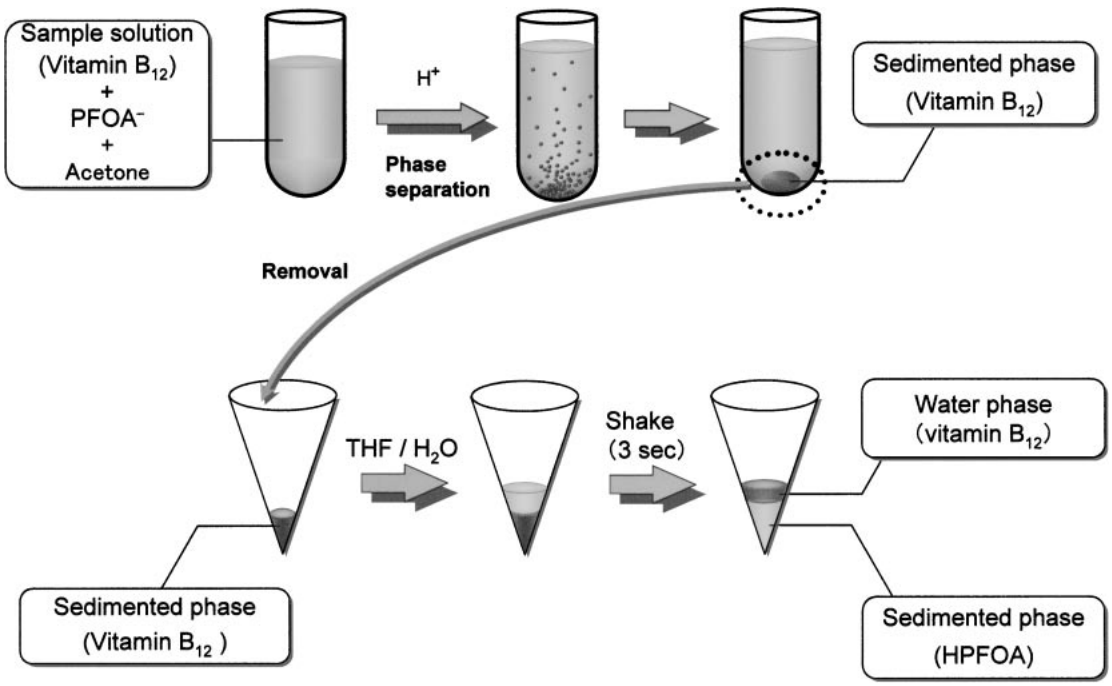

Fig. 1. Scheme of the extraction and the back-extraction technique for vitamin B_{12}.

Table 1. Extraction of Vitamin B_{12} by Several Methods

Method	Reagent	Extraction[a)]/%
Solvent extraction	Water/1-octanol[b)]	0.4
pH-dependent phase separation	$PFOA^-$/Acetone/H^+ (pH < 1)[c)]	95.2
	Zonyl FSA®/THF/Acetic acid (pH = 5)[d)]	2.0
Ion-pair phase separation	$PFOA^-/TBA^+$[e)]	2.0
Salting effect	Water/Acetone/NaCl[f)]	0.8
Cloud point extraction	Water/Triton X-100[g)]	0.0

a) [Vitamin $B_{12}]_T = 8.46 \times 10^{-7}$ mol/L (1.14 ppm). b) $V_W/V_O = 30$ mL/5 mL = 6-fold. c) $V_W/V_O = 35$ mL/30 μL = 1167-fold. d) Ref. 24. e) TBA^+; Tetrabutylammonioum ion. f) $V_W/V_O = 16$ mL/8 mL = 2-fold, [NaCl] = saturated concentration. g) Temperature > 90 °C.

absorbance spectra. Accordingly, this red pigment was considered as thermal decomposition matter in the phase separation. Since vitamin B_{12} structurally has some amino groups, the ion-pair solvent extractions and ion-pair salting extraction were examined at pH < 1. The water/1-octanol/ClO_4^- and water/1-octanol/$PFOA^-$ system as an ion-pair solvent extractions and water/acetone/NaCl/$PFOA^-$ as ion-pair salting extraction, were examined. However, vitamin B_{12} as a counter ion was not extracted by these methods.

Homogeneous Liquid–Liquid Extraction Based on pH-Dependent Phase Separation with Perfluoro Surfactant. Microliter quantities of a water-immiscible liquid phase are obtained from the homogeneous solution that includes $PFOA^-$ and small amounts of a water-miscible organic solvent, when the pH was adjusted to below 1. The phase separation phenomenon depends on the pH of acid dissociation of a perfluoroalkanecarboxylic acid. The acidic dissociation constant (pK_a) of $PFOA^-$ was reported to be 1.01.[10] The reaction formula as follows:

$$PFOA^- + H^+ \rightarrow HPFOA$$

When the protonation occurred, HPFOA simultaneously formed microliter quantities (μL) of the liquid phase by inclusion around the water-miscible organic solvent and water. With this phase separation, analytical targets in the sample solution were simultaneously extracted into this sedimented phase. The phase volume is then proportional to the added $PFOA^-$ concentration, as shown in Fig. 2. Accordingly, the microliter levels of a constant phase volume can be freely established. Based on the ease of handling of the sedimented phase, the total $PFOA^-$ concentration was determined to be 2.86×10^{-3} mol L^{-1}; therefore, 30 μL of the sedimented phase was obtained from 35 mL of the sample solution. The volume ratio (V_W/V_S) between the water phase (V_W) and the sedimented phase (V_S) was 1167-fold. In this phase separation condition, vitamin B_{12} was extracted by a homogeneous liquid–liquid extraction using several water-miscible organic solvents. These results are shown in Table 2. Though vitamin B_{12} is insoluble in organic solvents such as acetone, vitamin B_{12} was well extracted. In case of water-miscible alcohols such as ethanol and methanol, a large volume of alcohol was necessary for the phase separation phenomenon; these were therefore not appropriate for the vitamin B_{12} extraction. The influence of the acetone volume on the extraction is shown in Fig. 3. In the low range of acetone volume, the extraction was satisfactory. Especially, acetone and dimethyl sulfoxide

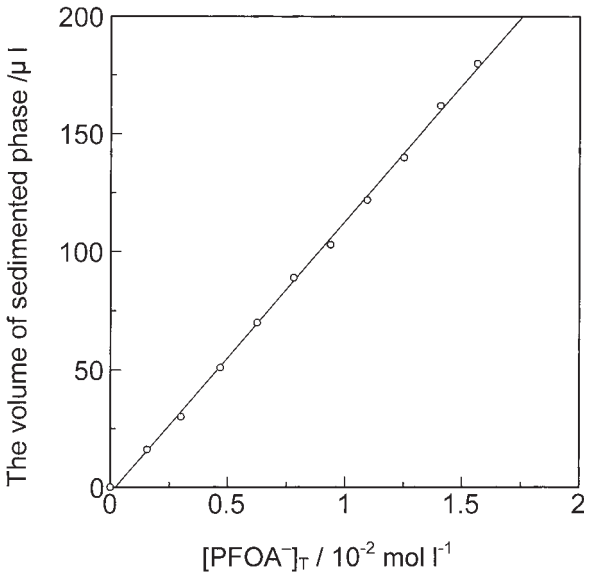

Fig. 2. Relationship between the volume of sedimented phase and the added $PFOA^-$. Total volume; 35 mL at 25 °C, $[Acetone]_T = 2.86$ vol %, $[HCl]_T = 0.66$ mol L^{-1}.

Table 2. Influence of Water-Miscible Organic Solvent on Vitamin B_{12} Extraction

Water miscible organic solvent	Extraction/%
Acetone	95.2
DMSO	76.8
Dioxane	68.7
Acetonitrile	64.8
DMF	49.8
Methanol*	48.2
THF	7.3
Ethanol*	4.2

Experimental condition; [Vitamin $B_{12}]_T = 8.46 \times 10^{-7}$ mol/L (1.14 ppm), $[PFOA^-]_T = 2.86 \times 10^{-3}$ mol/L, [Organic solvent]$_T$ = 2.86% (except the alcohol group), $[HCl]_T = 0.66$ mol/L. Concentration factor; 1167-fold (Water phase; 35 mL → Sedimented phase; 30 μL).
* $[Alcohol]_T = 28.6\%$.

(DMSO) are known to form a high moisture content in the sedimented phase (In case of acetone, HPFOA:H_2O:Acetone = 1.0:6.6:4.0[10]). Thus, vitamin B_{12} is considered to have an affinity for the sedimented phase which contains a large

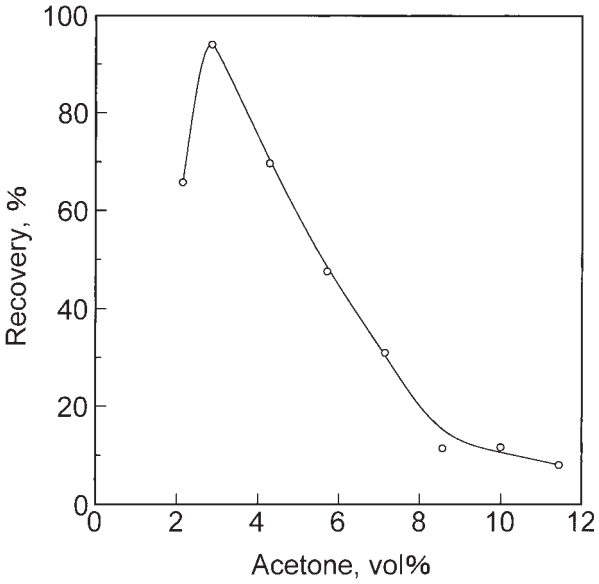

Fig. 3. Influence of acetone volume on the vitamin B_{12} extraction. $[\text{Vitamin } B_{12}]_T = 8.5 \times 10^{-7}$ mol L^{-1} (1.14 ppm), $[PFOA^-]_T = 2.86 \times 10^{-3}$ mol L^{-1}, $[HCl]_T = 0.66$ mol L^{-1}.

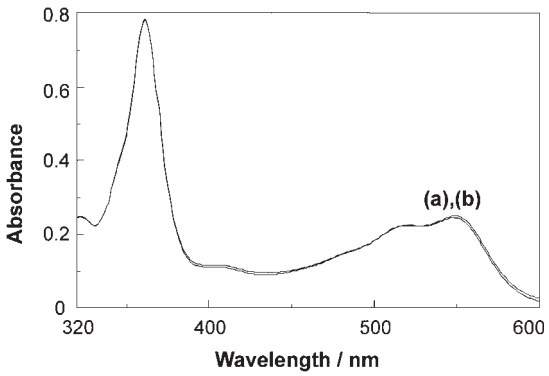

Fig. 4. Absorbance spectra of the extract and the standard substance. (a) Extract, (b) Standard substance, pH = 9.4.

amount of moisture.

Influence of an Acidic Condition on the Vitamin B_{12} Extraction. In the phase separation phenomenon, the pH was made to be less than 1 with hydrochloric acid. The influence of the decomposition by acid was then examined. The absorbance spectra are shown in Fig. 4 (the extract is in Fig. 4(a) and the standard substance in Fig. 4(b)). During the measurement, the sedimented phase including vitamin B_{12} was diluted with 0.1 mol L^{-1} sodium tetraborate solution. The absorption of vitamin B_{12} was observed at 361 nm and 551 nm. There was no difference between the spectra of the extract and that of the standard substance. An extract was stable for at least 150 min after the addition of 10 mL of 6.0 mol L^{-1} HCl. Moreover, the extract and standard substance were measured by capillary electrophoresis (CE), which is a high-performance separation analysis. These two peaks were completely corresponded. The IR was also measured; there was no difference between their IR spectra. Consequently, the hydrochloric acid did not influence the decomposition. The concentration of hydrochloric acid was examined. The maximum percentage (95.2%) was obtained at using a 0.66 mol L^{-1} HCl solution. When the HCl concentration increased, this percentage gradually decreased (in the case of $[HCl]_T = 2.0$ mol L^{-1}, the extraction percentage was 50%). This is considered to decrease the sedimented phase volume by increasing the chloride ion, and the distribution of vitamin B_{12} was simultaneously and gradually reduced.[23]

Table 3. Extraction Percentages of Several Chemicals

Group	Chemicals	Extraction/%
Vitamin	Thiamin (VB_1)	0.9
	Riboflavin phosphate (VB_2)	1.2
	Pyridoxine (VB_6)	9.4
	Cyanocobalamin (VB_{12})	95.2
	Ascorbic acid (VC)	0.0
Amino acid (L-form)	Alanine	0.0
	Arginine	0.1
	Asparagine	0.0
	Aspartic acid	0.1
	Cysteine	0.1
	Cystine	0.0
	Glutamic acid	0.1
	Glutamine	0.0
	Glycine	0.0
	Histidine	0.1
	Isoleucine	0.3
	Leucine	0.2
	Lysine	0.1
	Methionine	0.1
	Phenylalanine	0.2
	Serine	0.1
	Threonine	0.1
	Tryptophan	2.0
	Valinealanine	0.1
Base	Adenine	0.0
	Guanine	0.0
	Thymine	0.0
	Cytosine	0.0
Saccharide	D-(+)-Glucose	0.0
	D-(−)-Fructose	0.0
	D-(+)-Galactose	0.0
	ATP	0.0
Metal ion	Fe^{2+}	0.0
	Co^{2+}	0.0

Selectivity and Influence of Protein. The selectivity was examined. Water-miscible components that contained the biological sample were also examined. The vitamin group extraction using the ion-pair phase separation phenomenon was reported by Oshite et al.;[24] however, the selectivity was poor toward specific vitamins. As shown in Table 3, vitamin B_{12} was selectively extracted by the proposed method. However, in the biological sample, most substance was proteins. An albumin, which is a simple protein, horseradish peroxidase (HRP) and hemoglobin, which are the conjugated proteins were examined as model proteins. These proteins interfered with the phase separation phenomenon. That is because the intermolecular cation of the proteins prevented a phase separa-

Table 4. Separation and Recovery from Sedimented Phase

	Recovery/%	RSD/%	Phase
Vitamin B_{12}	96.2	3.3	Water phase
HPFOA	101.5	8.0	Sedimented phase

tion by associating with $PFOA^-$. Therefore, the protein removal as a pretreatment was examined in this study. Three protein removal techniques were examined. These include the salting effect, chloroform solvent extraction, and membrane filtration. A 10 ppm HRP concentration was used as the model protein. The chloroform extraction removed the HRP at 98.6% and a membrane filtration achieved 94.4%. The salting effect (H_2O/acetone/NaCl system) removed very little. Regarding the chloroform extraction, a microvolume of chloroform is soluble in the sample solution. The influence of this solubized chloroform on the homogeneous liquid–liquid extraction of vitamin B_{12} was examined. There was no difference found in the extraction percentages between the use of chloroform before (95.2%) and after (95.6%).

Separation of Vitamin B_{12} from Sedimented Phase. The sedimented phase consisted of HPFOA/H_2O/acetone. Accordingly, vitamin B_{12} must be isolated from the sedimented phase which included large amounts of HPFOA. In this study, a back-extraction technique for vitamin B_{12} was found, in which a micro-volume of THF was added to the sedimented phase. As shown in Table 4, HPFOA and vitamin B_{12} were completely separated into two phases; the sedimented phase and the water phase. This was caused by the fact that vitamin B_{12} was not distributed in the sedimented phase including THF (Table 2). The component ratio in the sedimented phase changed with the addition of the micro-volume of THF. By this back-extraction, vitamin B_{12} was isolated in the water phase. The relationship between the added THF volume and the extraction of vitamin B_{12} is shown in Fig. 5. When 25 μL of THF was added to 30 μL of the sedimented phase, the maximum percentage (96.2%) was obtained. The addition of a micro-volume of THF could maintain the liquid–liquid two-phase condition (water/sedimented phase); however any excess THF was soluble in the sedimented phase and it was homogenized. On the other hand, the water phase including vitamin B_{12} was separated using a micro-syringe and placed in another vial. Excess amounts of THF were added to this vial and thereby vitamin B_{12} was crystallized. The isolated extract was measured by ^{19}F-NMR. The fluorine was completely non-detected from the isolated one.

On the other hand, the sedimented phase after a back-extraction was dried and recovered as a HPFOA powder. The HPFOA was recycled for the vitamin B_{12} extraction without any loss (Fig. 6).

Extraction of Vitamin B_{12} from Biological Samples. Vitamin B_{12} was extracted from oyster as a real sample. The procedure is described in the experimental procedure section. There are many chemical species for vitamin B_{12} derivatives in a biological sample, such as a cobamide coenzyme with protein, alkylcobalamin, and hydroxycobalamin. However, these species are labile; therefore, they are generally transferred to the cyanocobalamin which is most stable species of the cobalamin derivatives. Cyanidation of the cobalamin derivative

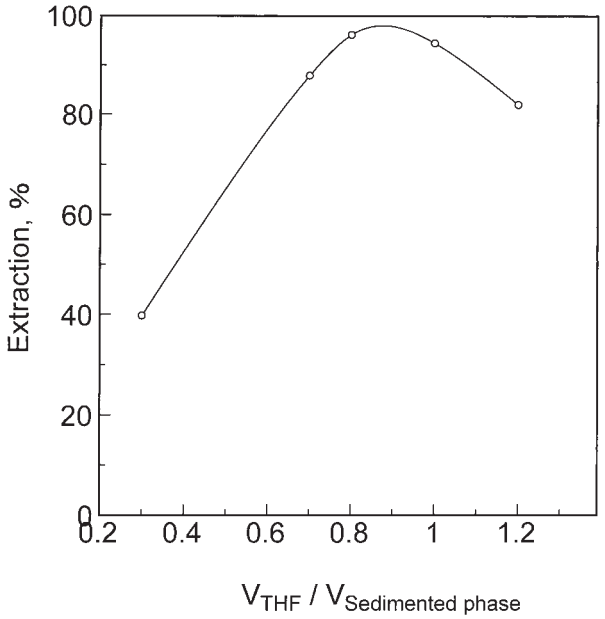

Fig. 5. Relationship between the added THF volume and the extraction of vitamin B_{12}.

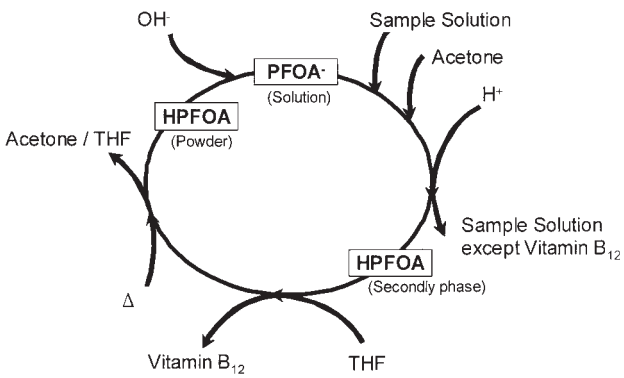

Fig. 6. Scheme of recycle system of HPFOA.

easily carried out with a potassium cyanide and is based on the ligand exchange reaction. This formation constant was reported to be 10^{12} M^{-1} [25,26] which is the largest number for all the ligands. In this study, the ligand exchange reaction with potassium cyanide was used. Ten micrograms of cyanocobalamin was recovered as crystals.

Conclusion

A selective separation and recovery system for vitamin B_{12} was developed. The perfluoro surfactant as a separation medium was completely recycled; thereby the extraction could be done without the loss of the separation medium. The pH-dependent phase separation phenomenon using the perfluoro surfactant was determined as a new procedure for a separation and recovery system from biological samples.

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