Application Note

Application Note No.8 (Food)

Food

Dietary Supplements Analysis

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1. Introduction

The CFR (Code of Federal Regulations) 21 Part 111 "cGMP (current Good Manufacturing Practice) in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplement" by the U.S. Food and Drug Administration (hereinafter referred to as "FDA") came into effect on August 24, 2007. The regulation states that dietary supplement manufacturers must perform one or more tests to ensure content uniformity in the supplement, specifically, purity, composition and content quantitation.
The regulation is also required to apply to supplements imported into the United States from overseas. Dates have been set for dietary supplement manufacturers, based on company size, to comply with the regulation. Companies with 500 employees or more were obliged to put this regulation into effect starting June 24, 2008. Companies with 20 to 499 employees started on June 24, 2009. Those with less than 20 employees started on June 24, 2010.
This application note introduces examples of analysis of dietary supplements using analysis methods that conform with those of the United States Pharmacopeia (hereinafter referred to as "USP").

2. USP Dietary Supplement Test Methods

The USP is a book of pharmacopeial standards. It lists the standards for medicines, dietary supplements, food composition and so on that are distributed in the United States. The official version, United States Pharmacopeia-National Formulary, United States Pharmacopeial Convention (USP-NF) lists the testing methods for dietary supplements of every kind of product. Supplements consisting of multiple components such as multivitamins are categorized in Table 1. The categories within this table use nearly identical analysis methods. However, the analysis methods for all categories are based on the analysis method used in "Oil- and Water-Soluble Vitamins with Minerals Tablets."

In the case of "Oil- and Water-Soluble Vitamins with Minerals Tablets," one to three methods are listed for each component and, any one of the methods can be used to measure the component. However, if "Method 2" or "Method 3" is used, the advantages of doing so should be described on the product label. Minerals are analyzed using the atomic absorption method and vitamins are analyzed predominately with the HPLC method (Table 2).

Commercially available "Multivitamin & Mineral" tablets were used as samples. In the case of "Oil- and Water-Soluble Vitamins with Minerals Tablets," the vitamins and minerals within this sample are analyzed using the HPLC and atomic absorption testing methods specified in USP32-NF27. The testing methods listed in the USP-NF have been validated and are the official methods. Tests that deviate from these official methods must be revalidated. However, if the tests fall within a given limit, the analytical conditions can be changed without revalidating. (Analytical condition changes are listed in the HPLC "SYSTEM SUITABILITY" section of the USP32-NF27, <621> Chromatography/ Physical Tests.)

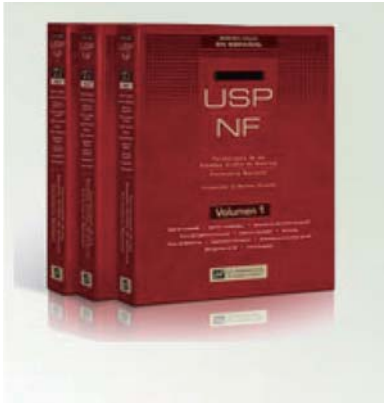
Table 1 USP-NF Classification of Dietary Supplements Containing Multiple Constituents

1	Calcium with Vitamin D Tablets
2	Calcium and Vitamin D with Minerals Tablets
3	Oil-Soluble Vitamins Capsules
4	Oil-Soluble Vitamins Tablets
5	Oil- and Water-Soluble Vitamins Capsules
6	Oil- and Water-Soluble Vitamins Oral Solution
7	Oil- and Water-Soluble Vitamins Tablets
8	Oil- and Water-Soluble Vitamins with Minerals Capsules
9	Oil- and Water-Soluble Vitamins with Minerals Oral Solution
10	**Oil- and Water-Soluble Vitamins with Minerals Tablets**
11	Water-Soluble Vitamins Capsules
12	Water-Soluble Vitamins Tablets
13	Water-Soluble Vitamins with Minerals Capsules
14	Water-Soluble Vitamins with Minerals Oral Solution
15	Water-Soluble Vitamins with Minerals Tablets

*From USP32-NF27 (Issued in 2008)

Table 2 Analysis Methods for "Oil- and Water-Soluble Vitamins with Minerals Tablets"

Constituent	Method 1	Method 2	Method 3
Vitamin A	HPLC	HPLC	HPLC
Cholecalciferol or Ergocalciferol (Vitamin D)	HPLC	HPLC	HPLC
Vitamin E	HPLC	HPLC	HPLC
Phytonadione (Vitamin K_1)	HPLC	Post-column HPLC	
Beta Carotene	Absorbance (UV)		
Ascorbic Acid	Titration	Automation method	
Biotin	HPLC	Microorganism quantitation	HPLC
Cyanocobalamin	HPLC	Microorganism quantitation	
Folic Acid	HPLC	HPLC	
Calcium Pantothenate	HPLC	Microorganism quantitation	HPLC
Niacin or Niacinamide	HPLC (batch analysis)	HPLC	HPLC (batch analysis)
Pyridoxine Hydrochloride	HPLC (batch analysis)	HPLC	HPLC (batch analysis)
Riboflavin	HPLC (batch analysis)	HPLC	HPLC (batch analysis)
Thiamine	HPLC(batch analysis)	HPLC	HPLC (batch analysis)
Calcium	Atomic Absorption		
Chromium	Atomic Absorption		
Copper	Atomic Absorption		
Fluoride	Ion electrode method	HPLC	
Iodide	Titration	Automation method	
Iron	Atomic Absorption		
Magnesium	Atomic Absorption		
Manganese	Atomic Absorption		
Molybdenum	Atomic Absorption	Absorbance (UV)	
Phosphorous	Absorbance (UV)		
Potassium	Atomic Absorption		
Selenium	Atomic Absorption	Absorbance (UV)	
Zinc	Atomic Absorption		



3. Vitamin Analysis

Vitamins are classified as either water-soluble (easily dissolved in water) or fat-soluble (not easily dissolved in water). Water-soluble vitamins include the vitamin B group and vitamin C. Fat-soluble vitamins include vitamins A, D, E, and K.
The Shimadzu Prominence HPLC system and the LC-2010CHT integrated HPLC were used to analyze the vitamins, and the Shimadzu SPD-M20A Photodiode Array Detector was used as the detector.

3-1. Vitamin A

Fat-soluble vitamins (including vitamin A) can be analyzed using either reversed-phase chromatography or normal-phase (adsorption) chromatography. According to "Method 1" of "Oil- and Water-Soluble Vitamins with Minerals Tablets" in the USP32-NF28, vitamin A is to be analyzed using normal-phase chromatography for separation, and UV-VIS absorption for detection. The normal-phase chromatography involves using a modified separation column with aminopropyl functional groups on the packing material surface to adsorb the separated substances. Trans-retinyl acetate is used as the standard solution, and the vitamin A in the sample is calculated based on the peak area ratio of the vitamin A peaks in sample and standard solutions. For the system suitability test, a mixed solution of retinyl acetate and retinyl palmitate is measured, and the resolution between the two must be 10 or greater. Further, peak area repeatability must be 3 % RSD or less. The chromatogram obtained from measurement of retinyl acetate and retinyl palmitate mixed solution is shown in Fig. 1. The analytical conditions and the results of the system suitability test are shown in Tables 3 and 4, respectively.
The analysis results of the commercially available "Multivitamin & Mineral" tablets and the preparation methods are shown in Fig. 2 and 3, respectively. The analysis results show that the value for vitamin A is higher than the value indicated on the product label. This is because beta carotene in the tablet is included and reported as vitamin A on the label. When the recovery test using the standard solution was conducted to check sample preparation, excellent results were obtained, with a recovery rate of 94 %.

Shimadzu Prominence HPLC System

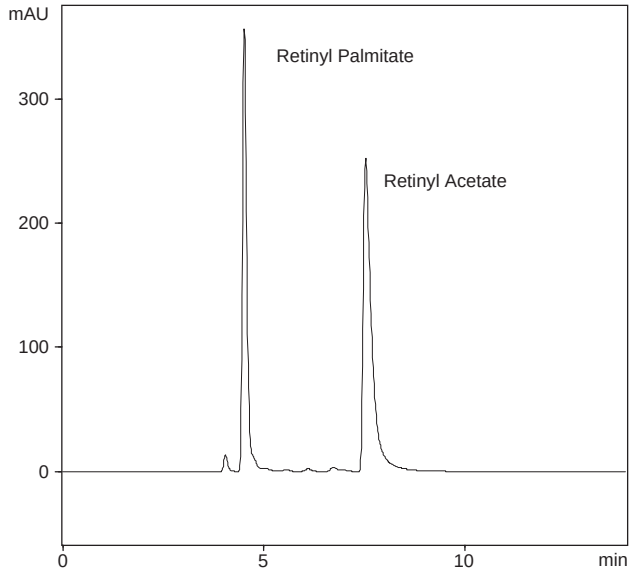


Fig. 1 Chromatogram of Retinyl Acetate / Retinyl Palmitate Mixed Solution
(Retinyl acetate: 9.27 mg/L, Retinyl palmitate: 10.66 mg/L)

Table 3 Analytical Conditions for Vitamin A

Instrument	: Shimadzu HPLC Prominence Series
Column	: Luna 3 µm NH2 100 Å (150 mm L. × 4.6 mm I.D.)
Mobile phase	: n-hexane
Flow rate	: 0.8 mL/min
Column temp.	: 30 °C
Detection	: SPD-M20A 325 nm (190 to 600 nm) (40 °C)
Injection vol.	: 40 µL

Table 4 System Suitability Test Results of Vitamin A

	Resolution *1	Relative Standard Deviation (%) *2	
		Retention Time	Peak Area
Retinyl Palmitate	—	0.056	0.203
Retinyl Acetate	10.8	0.066	0.163

*1: Resolution with respect to retinyl palmitate (Standard of USP32-NF27 is 10 or greater.)
*2: Standard of USP32-NF27 is 3 % or less.

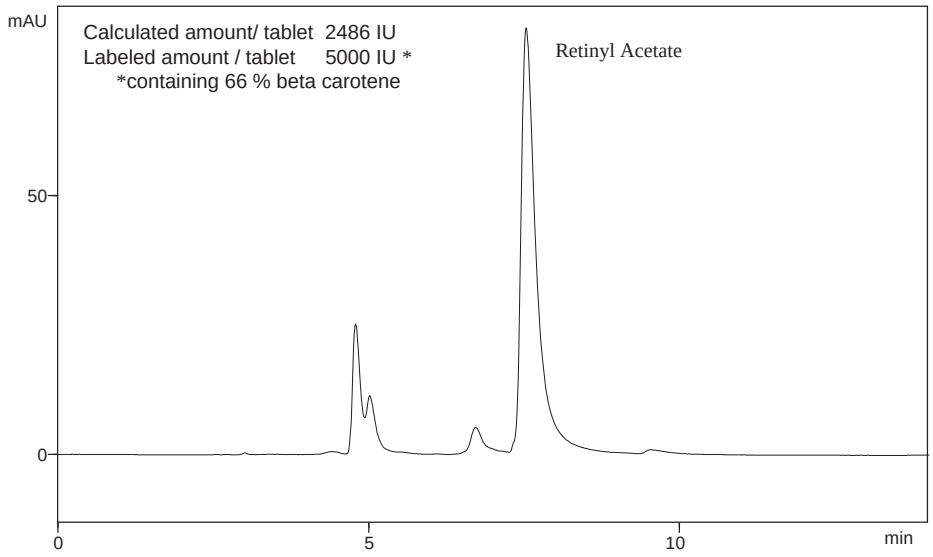


Fig. 2 Analysis Results for Commercially Available Supplement (Tablets)

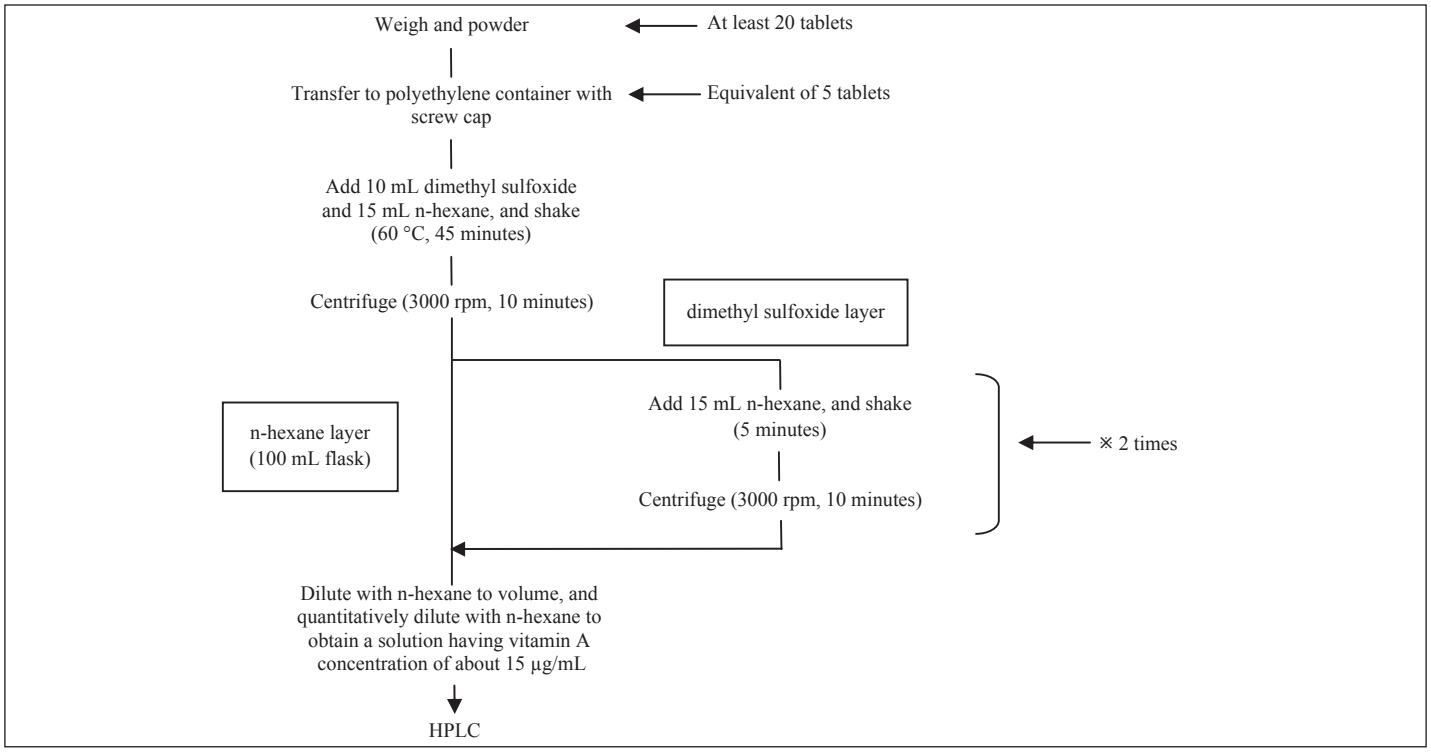


Fig. 3 Sample Preparation Flow for Vitamin A Measurement

3-2. Cholecalciferol and Ergocalciferol (Vitamin D)

Fat-soluble vitamin D can be analyzed under virtually the same separation conditions as fat-soluble vitamin A. The analytical conditions are shown in Table 5. This analysis was conducted using the Shimadzu LC-2010CHT integrated HPLC. Vitamin D includes cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), and separating these two substances is difficult using normal-phase (adsorption) chromatography. Although not mentioned in the USP-NF, it is best to check which vitamin D is present in the sample first, and then conduct analysis using a standard solution. For vitamin D analysis, a volume of the standard solution should be heated for one hour at 60 °C to partially isomerize the pre vitamin D, which can be used as the system suitability test solution. The resolution of the vitamin D precursor obtained by isomerization must satisfy the specified value.

The chromatograms of the cholecalciferol standard solution and the system suitability test solution are shown in Fig. 4. The system suitability test results are shown in Table 6. The analysis results for commercially available "Multivitamin & Mineral" tablets are shown in Fig. 5. The preparation method is the same as for vitamin A. Adjust the final concentration of vitamin D to 2 µg/mL. Since some peaks are observed after elution of vitamin D, the analysis time should set to 60 minutes.

Table 5 Analytical Conditions for Vitamin D

Instrument	: Shimadzu LC-2010CHT
Column	: Luna 3 µm NH2 100 Å (150 mm L. × 4.6 mm I.D.)
Mobile phase	: n-hexane/isopropyl alcohol = 99/1
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Detection	: 265 nm (40 °C)
Injection vol.	: 100 µL

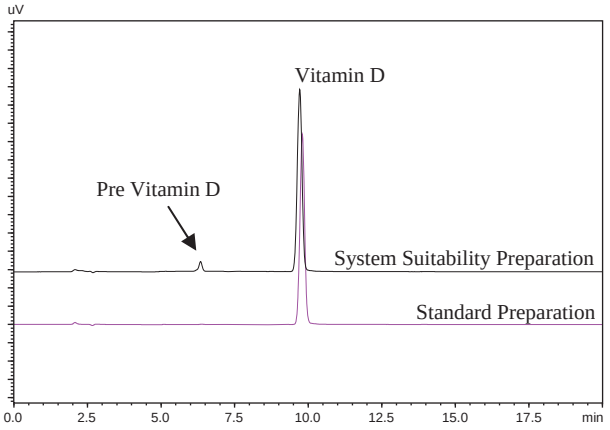


Fig. 4 Chromatograms of System Suitability Test Solutions for Cholecalciferol Analysis
(Cholecalciferol: 2 µg/mL)

Table 6 System Suitability Test Results For Vitamin D*

Resolution	Relative Standard Deviation (%)	
	Retention Time	Peak Area
13.0	0.164	0.690

*Standard of USP32-NF27: Resolution: 10 or greater, Relative standard deviation: 3 % or less

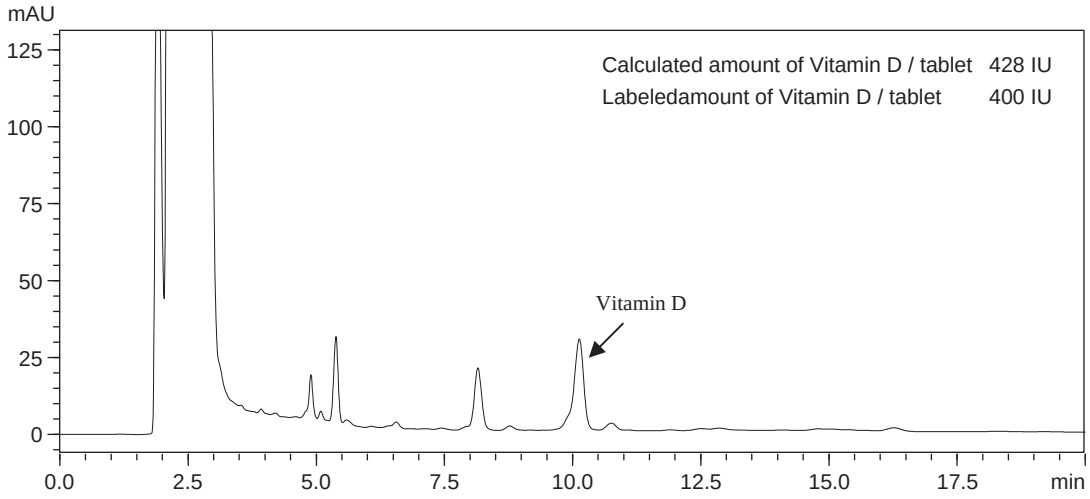


Fig. 5 Analysis Results for Commercially Available Supplement (Tablet)

3-3. Vitamin E

Vitamin E is another common fat-soluble vitamin. It, as well as vitamins A and D, can be analyzed using normal-phase (adsorption) chromatography. However, according to USP32-NF27, the separation method used is reversed-phase chromatography. Vitamin E has four different isomers, including α, β, γ, and δ-tocopherols depending on the location of the methyl group. According to USP32-NF27, α-tocopherol (or, α-tocopherol acetate ester, α-tocopherol succinate ester) is analyzed as a standard solution. For this analysis, since the actual sample of vitamin E is α-tocopherol acetate ester, α-tocopherol acetate ester was used as the standard solution. For the system suitability test, a mixed solution of ergocalciferol (vitamin D) and α-tocopherol acetate ester is separately prepared, and the relative retention time with respect to ergocalciferol, the resolution, and the tailing factor must satisfy the specified criteria values. The chromatogram of the system suitability test solution measured repeatedly is shown in Fig. 6. The analytical conditions and results of the system suitability testing are shown in Tables 7 and 8, respectively. The column flow capacity is being changed from the conditions in the USP-NF to conform to the column inside diameter.

The analysis results for commercially available "Multivitamin & Mineral" tablets are shown in Fig. 7. The preparation method is the same as that for vitamin A, however, reconstitution of the sample matrix in methanol rather than hexane is required at the last step in order for it to be injected into a reversed-phase separation system.

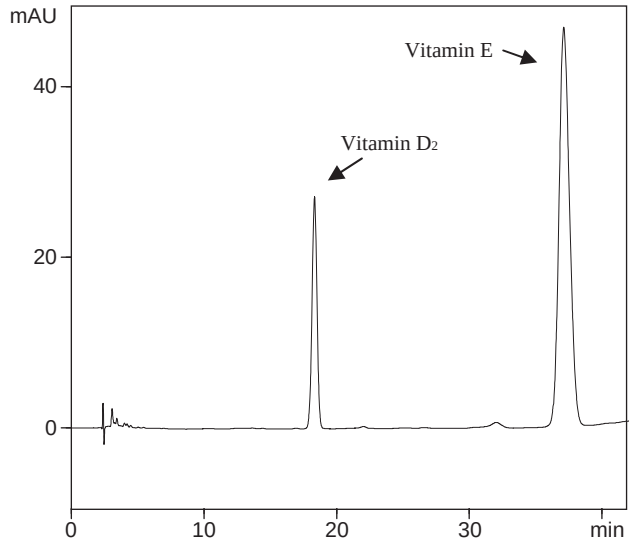


Fig. 6 Chromatogram of System Suitability Test Solution (Ergocalciferol: 0.0065 mg/mL, α-tocopherol acetate ester: 1.013 mg/mL)

Table 8 System Suitability Test Results for Vitamin E

		Results	Standard
Resolution		12.7	12 min.
Tailing Factor		1.16	0.8 to 1.2
Relative Retention Time*		0.49	Approx. 0.5
%RSD	(Retention Time)	0.213	3 % max.
	(Peak Area Value)	0.611	

*Relative retention time with respect to ergocalciferol

Table 7 Analytical Conditions for Vitamin E

Instrument	: Shimadzu HPLC Prominence Series
Column	: Shim-pack VP-ODS (150 mm L. × 6.0 mm I.D.)
Mobile phase	: 1 % phosphoric acid/methanol = 5/95
Flow rate	: 1.2 mL/min
Column temp.	: 40 °C
Detection	: SPD-M20A 254 nm (190 to 600 nm) (40 °C)
Injection vol.	: 50 µL

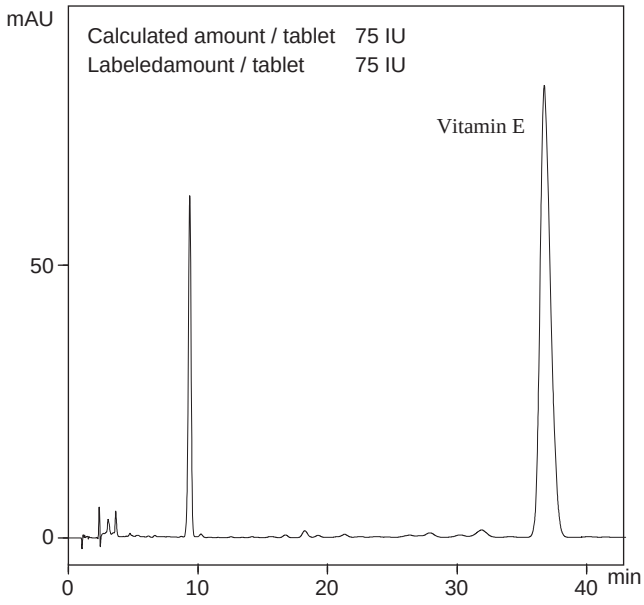


Fig. 7 Analysis Results for Commercially Available Supplement (Tablet)

3.4 Niacin or Niacinamide, Pyridoxine, Riboflavin, and Thiamine

The water-soluble vitamin B group is usually analyzed by reversed-phase chromatography. Batch analysis is possible depending on the sample. The batch analysis procedure of the four constituents (niacin (or niacinamide) and pyridoxine, riboflavin, and thiamine) is described in USP32-NF27. Since thiamine and pyridoxine display relatively weak retention on the separation column, reversed-phase ion pair chromatography with a mobile phase (with sodium 1-hexanesulfonate as a reagent) is specified in the conditions for batch analysis.

In the USP-NF, the elution positions of the various substances are shown as relative retention times with respect to thiamine as a guideline. The standard solution chromatogram is shown in Fig. 8. The analytical conditions for the four constituents are shown in Table 9, and the relative retention times as well as the repeatability for the four constituents are shown in Table 10. Since overlapping of the thiamine peak was evident in the actual sample analysis, the mobile phase composition was changed to within the specified range to delay the elution time of thiamine. Therefore, the relative retention times with respect to thiamine for constituents other than thiamine are smaller than the standard values indicated in USP-NF.

The analysis results for commercially available "Multivitamin & Mineral" tablets are shown in Fig. 9 and the preparation methods are shown in Fig. 10. Care must be taken with respect to adsorption to the vial surface and solubility during sample preparation. Use of the laboratory glassware listed in the USP-NF for preparing the sample is not absolutely necessarily, however, the diluent (water/acetonitrile/acetic acid = 94/5/1) specified in the USP-NF must be used.

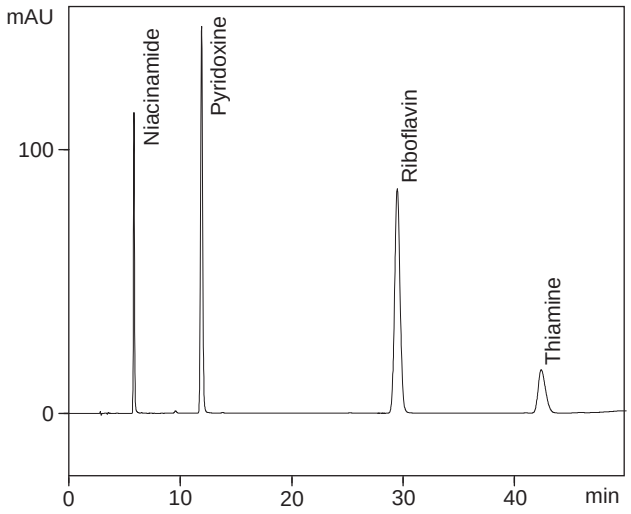


Fig. 8 Chromatogram of Standard Solution
(Niacinamide 400 mg/L, pyridoxine hydrochloride 100 mg/L, riboflavin 100 mg/mL, thiamine hydrochloride 100 mg/L)

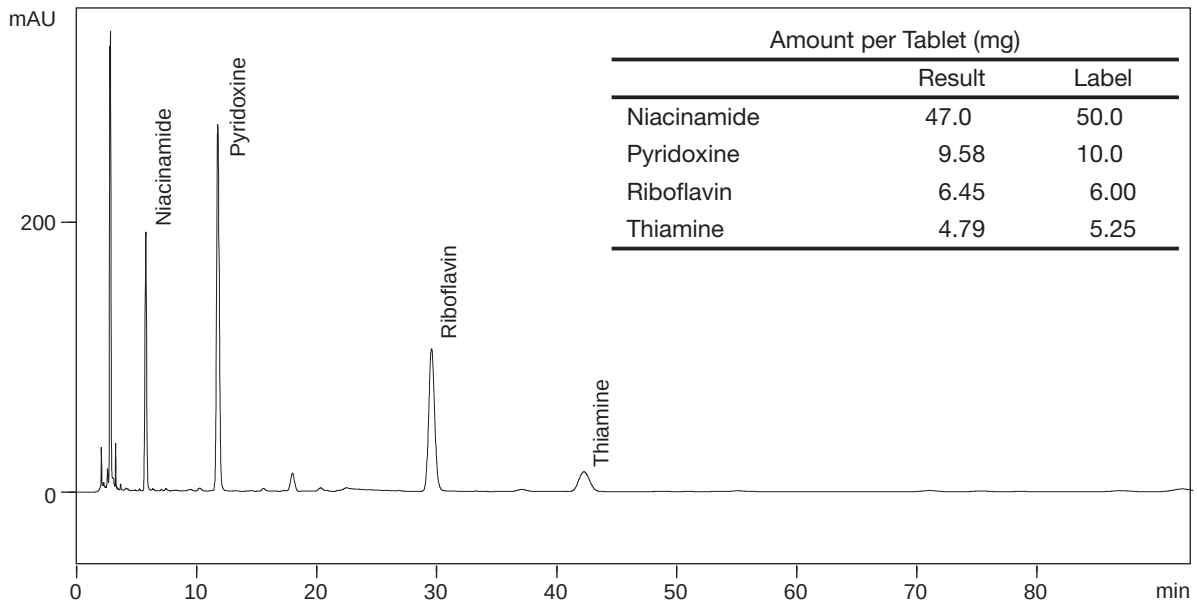
Table 9 Batch Analysis Conditions for Niacinamide, Pyridoxine, Riboflavin and Thiamine

Instrument	: Shimadzu HPLC Prominence Series
Column	: Luna 5 µm C18(2) (250 mm L. × 4.6 mm I.D.)
Mobile phase	: water/methanol/acetic acid = 83/17/1 Containing sodium 1-hexanesulfonate 126 mg/100 mL
Flow rate	: 1.0 mL/min
Column temp.	: 40 °C
Detection	: SPD-M20A 280 nm (190 to 600 nm) (40 °C)
Injection vol.	: 10 µL

Table 10 Relative Retention Times and Injection Repeatability in Analysis of Niacinamide, Pyridoxine, Riboflavin and Thiamine

	Relative Retention Time *1	Relative Standard Deviation (%) *2	
		Retention Time	Peak Area
Niacinamide	0.14 (0.3)	0.054	0.107
Pyridoxine	0.28 (0.5)	0.059	0.079
Riboflavin	0.69 (0.8)	0.133	0.097
Thiamine	1.00 (1.0)	0.052	0.160

*1: Relative retention time for thiamine (values in parentheses are approximate standard value according to USP-NF)
*2: Standard of USP32-NF27 is 3 % or less.



Amount per Tablet (mg)

	Result	Label
Niacinamide	47.0	50.0
Pyridoxine	9.58	10.0
Riboflavin	6.45	6.00
Thiamine	4.79	5.25

Fig. 9 Analysis Results for Commercially Available Supplement (Tablets)

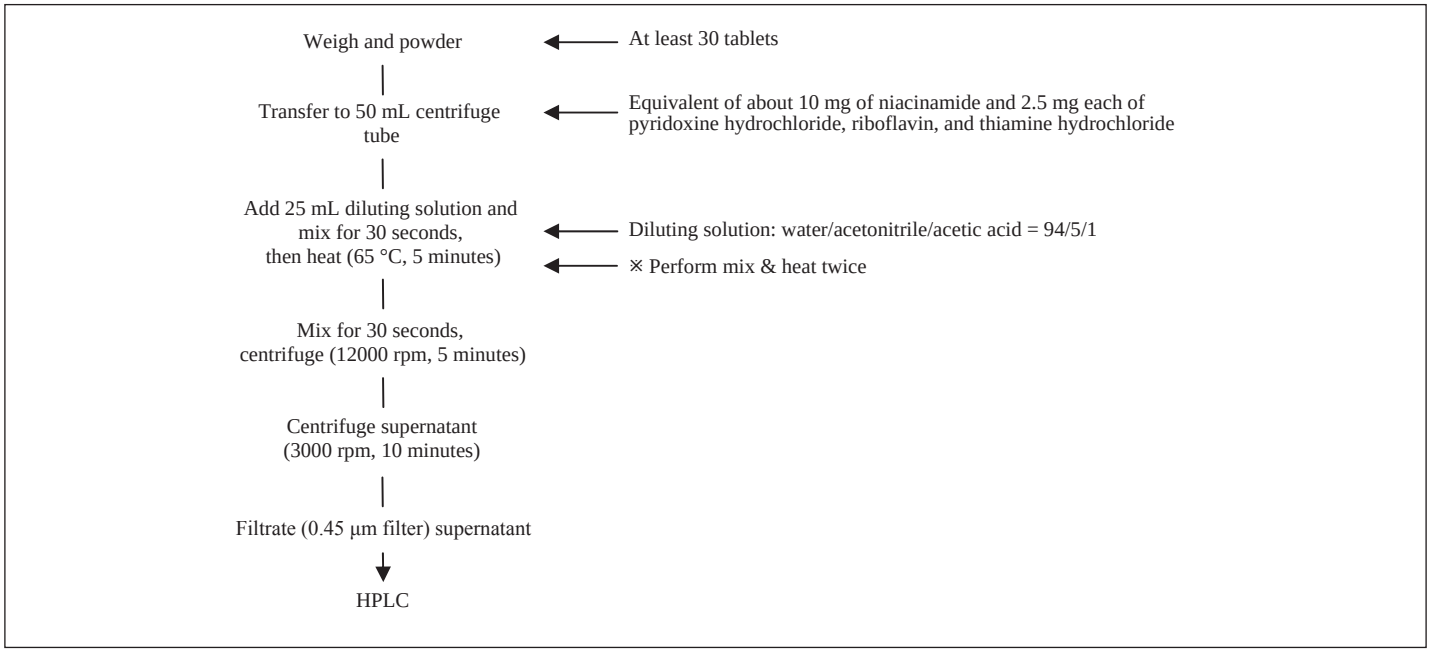


Fig. 10 Sample Preparation Flow for Analysis of Niacinamide, Pyridoxine, Riboflavin and Thiamine

4. Mineral Analysis

The test methods specified for the 13 mineral elements (Ca, Cr, Cu, F, I, Fe, Mg, Mn, Mo, P, K, Se, Zn) in USP32-NF27 "Oil- and Water-Soluble Vitamins with Minerals Tablets" are listed in Table 2. Flame atomic absorption is used as the test method for 10 of the 13 elements (not including elements F, I, P). For this analysis, ten mineral elements were analyzed using the Shimadzu AA-7000 Series Atomic Absorption Spectrophotometer.

4-1. Analytical Conditions

The measurement wavelength of each element, and type of flame and matrix modifier used, are shown in Table 11. The N_2O-C_2H_2 flame was used to measure Ca and Mo, and the air-C_2H_2 flame was used to measure all other elements. For measurement of Ca and Mg, La was added as a matrix modifier to the standard solution and sample solution before analysis. For measurement of Mo and Se, ammonium chloride was added to the standard solution and sample solution prior to analysis.

Table 11 Analytical Conditions

Element	Wavelength	Flame	Matrix Modifier
Ca	422.7 nm	N_2O-C_2H_2	0.1 % La
Cr	357.9 nm	Air-C_2H_2	
Cu	324.7 nm	Air-C_2H_2	
Fe	248.3 nm	Air-C_2H_2	
Mg	285.2 nm	Air-C_2H_2	0.1 % La
Mn	279.5 nm	Air-C_2H_2	
Mo	313.0 nm	N_2O-C_2H_2	2 % Ammonium Chloride
K	766.5 nm	Air-C_2H_2	
Se	196.0 nm	Air-C_2H_2	2 % Ammonium Chloride
Zn	213.8 nm	Air-C_2H_2	

4-2. Constructing a Calibration Curve

According to the USP32-NF27, calibration curves are to be created based on linear approximation using a standard solution having the element concentrations shown in Table 13. Quantitation is to be conducted using the analysis results from the test solution prepared for the concentrations indicated in red in the table. Examples of the calibration curves are shown in Fig. 14 to 23 on the following page. Calibration curves are generated with the concentration range of the standard solution. In the case of Zn, since the high concentration of the standard solution causes curvature of the calibration curve under normal conditions, the burner angle was changed for measurement to improve the linearity.

Table 12 Calibration Curve Concentrations

Element	STD (µg/mL)						
Ca		1.0	1.5	**2.0**	2.5	3.0	
Cr		**1.0**		2.0		3.0	4.0
Cu	0.5	1.0		**2.0**		3.0	4.0
Fe		2.0		4.0	**5.0**	6.0	8.0
Mg		0.2	0.3	**0.4**	0.5	0.6	
Mn		0.5	0.75	**1.0**		1.5	2.0
Mo	5.0	**10.0**			25.0		
K	0.5	**1.0**	1.5	2.0	2.5		
Se	5.0	**10.0**			25.0		
Zn	0.5	1.0	1.5	**2.0**	2.5		

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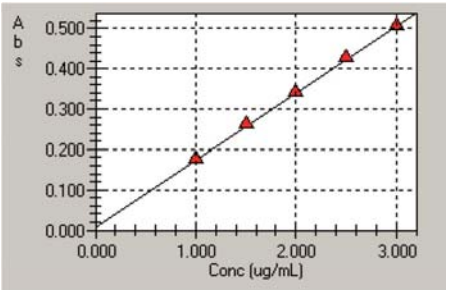

Fig. 11 Calibration Curve for Ca

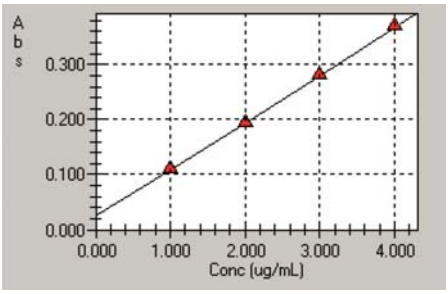

Fig. 12 Calibration Curve for Cr

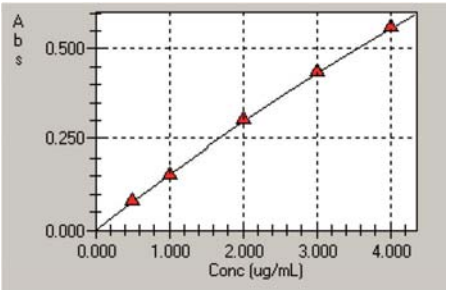

Fig. 13 Calibration Curve for Cu

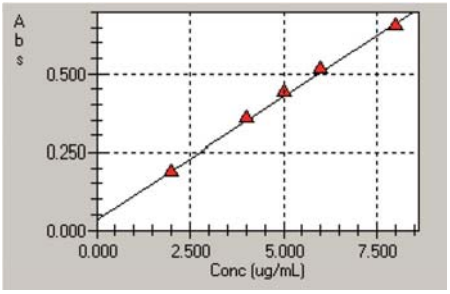

Fig. 14 Calibration Curve for Fe

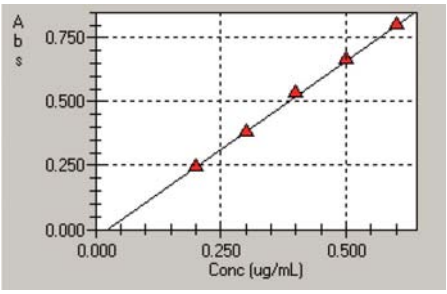

Fig. 15 Calibration Curve for Mg

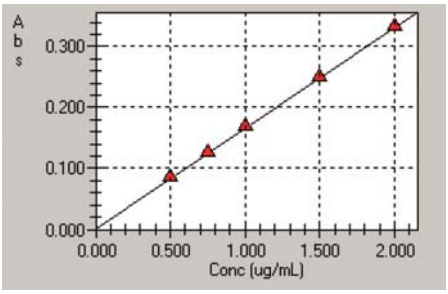

Fig. 16 Calibration Curve for Mn

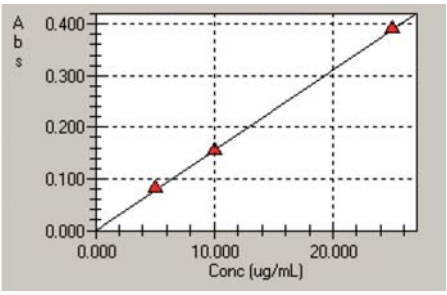

Fig. 17 Calibration Curve for Mo

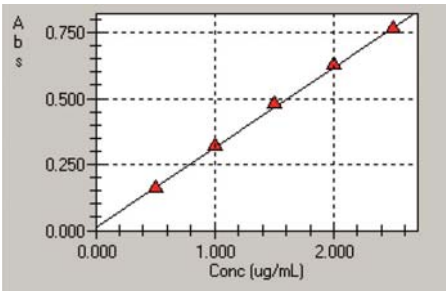

Fig. 18 Calibration Curve for K

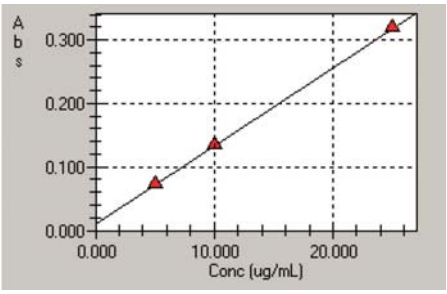

Fig. 19 Calibration Curve for Se

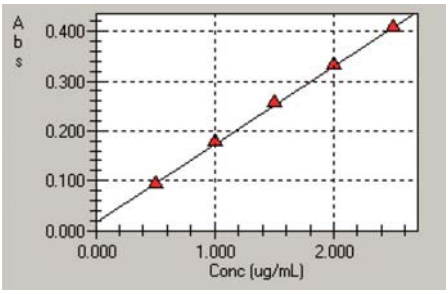

Fig. 20 Calibration Curve for Zn

4-3. Analysis of Mineral in Actual Sample

For actual sample analysis, elements Mo and Se have a different sample preparation method than the other elements. For elements other than Mo and Se, first the tablets are ashed in a muffle furnace at 550 °C, and then hydrochloric acid added, and finally brought to boiling. Mo and Se undergo wet decomposition by reactions with nitric and perchloric acids. Preparation methods for both cases are shown in Fig. 21 and 22. The analysis results for actual commercially available "Multivitamin & Mineral" tablets are shown in Table 13. Mo was not detected in this sample.

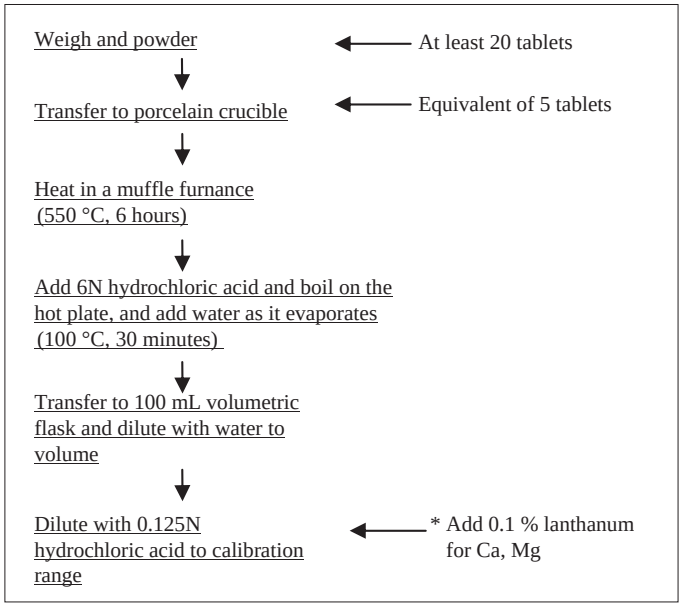

Fig. 21 Sample Preparation Flow for Elements Other Than Mo and Se

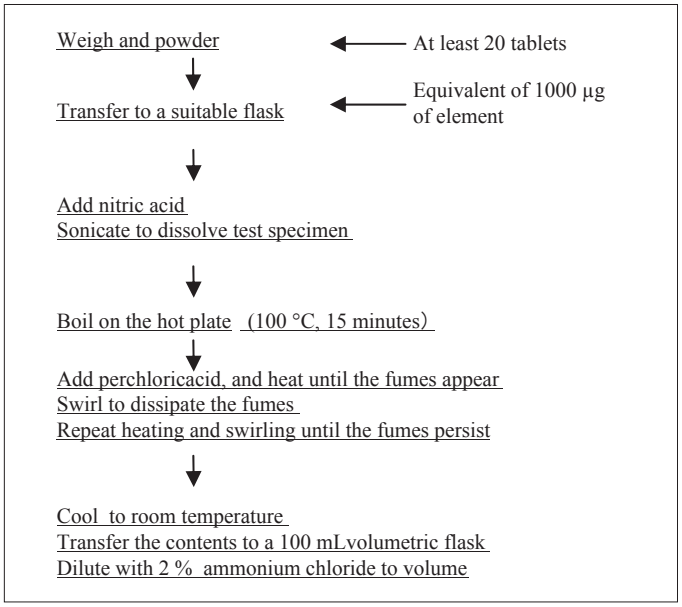

Fig. 22 Sample Preparation Flow for Elements Mo and Se

Table 13 Analysis Results for Commercially Available Supplement (Tablets)

	Ca	Cr	Cu	Fe	Mg	Mn	Mo	K	Se	Zn
Quantitation Results (µg/mL)	1.67	1.07	1.91	4.67	0.50	0.72	-	1.05	9.40	1.56
Dilution Factor	5000	10	100	100	5000	500		5000	1	500
Calculated Results (mg/tablet)	167	210*	3.8	9.3	50	7.2		105	188*	16
Labeled Values (mg/tablet)	165	200*	3.5	9.0	50	7.5	-	100	200*	15

*Unit: µg/tablet (mg/tablet for others)

5. Summary

The dietary supplement market has increased dramatically due to rising public concern with health issues. In an advanced country like the United States, the supplement market now stands at about three trillion yen (about 35.5 billion dollar). Currently in Japan there are no laws exclusively for supplements. However, it is presumed that the current mandatory "Proof of Correct Product Labeling" in the cGMP regulation by the United States FDA will in some way influence how Japan will handle its position on supplements in the future. Here we introduced examples of multivitamin & mineral tablet analysis based on methods in the USP. In Japan, methods for the analysis of elements in vitamins and minerals in food are outlined in food-hygienic test guidelines, etc. However, partial separation modes for fat-soluble vitamins, etc. differ from the USP methods described in this Application Note. We believe that the USP test methods are interesting examples for study.

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