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Determination of Water- and Fat-Soluble Vitamins in Nutritional Supplements by HPLC with UV Detection

INTRODUCTION

Vitamins are a well-known a group of compounds that are essential for human health. These compounds can be classified in two main groups, water- and fat-soluble. Water-soluble vitamins include B group vitamins (V_{B1} , V_{B2} , V_{B3} , V_{B5} , V_{B6} , V_{B9} and V_{B12}) and ascorbic acid (V_C), while the fat-soluble vitamins primarily include retinol (V_A), tocopherol (V_E), radiostol (V_D), and antihemorrhagic vitamin (V_K). These vitamins play different specific and vital functions in metabolism, and can cause health problems when they are either lacking or in excess. The supply of vitamins for a healthy life depends on diet; however, even foods that contain the necessary vitamins can have reduced vitamin content after storage, processing, or cooking. Therefore, many people take a multi-vitamin tablet to supplement their diet. To ensure that these tablets contain the labeled amounts of vitamins, there must be a quality control assay for these tablets.

Reversed-phase HPLC is a technique well suited for vitamin analysis. Water-soluble vitamins are often determined using an aqueous mobile phase, while the fat-soluble vitamins use organic-solvent mobile phases, based on their very different solubility properties.¹ Although simultaneous separation of water- and fat-soluble vitamins has been reported,² the differences in sample preparation and separation mode result in longer separation time for the detection of fat-soluble vitamins.

Therefore, the simultaneous separation method is inefficient for many samples.

In this application note (AN 251), integrated and efficient methods for determining water- and fat-soluble vitamins in nutritional supplements were developed. Efficient and simple methods for the extraction and determination of the water- and fat-soluble vitamins were established, and the separations were performed on the UltiMate® HPLC system with Acclaim® columns. Most regulated methods^{3,4} and commonly reported HPLC methods⁵⁻⁸ use different mobile phases and columns for water- and fat-soluble vitamins. The water-soluble vitamins, including seven B group vitamins and V_C , were well-resolved on the Acclaim Polar Advantage (PA) II column using a phosphate buffer in the mobile phase. The silica-based column contains a polar-embedded stationary phase⁹ on which the highly polar V_C can be well-retained without using an ion-pairing reagent in the mobile phase.^{4,10} The fat-soluble vitamins, including V_A , V_A acetate, V_E acetate, V_{D2} , V_{D3} , V_{K1} and β -carotene, were well-resolved on the Acclaim 120 C18 column using an organic-solvent mobile phase. Method performance, including linearity, detection limits, reproducibility, and analyte recovery, was evaluated for both methods. Five nutritional supplement samples were analyzed, and the results were equivalent to the labeled values.

EQUIPMENT

Dionex UltiMate 3000 HPLC system consisting of:
DGP 3600A Pump with SRD 3600 solvent rack with degasser
WPS 3000TSL Autosampler
TCC-3200 Thermostatted column compartment
VWD-3400RS UV-vis Detector
Chromleon® Chromatography Data System 6.80 SR7
Kudos® SK3200LH Ultrasonic generator, Kudos Ultrasonic Instrumental Co., Shanghai, China
IKA® MS1 Minishaker, IKA Works, Guangzhou, China
Thermo Orion 420A+ pH Meter

REAGENTS AND STANDARDS

Deionized water, from Milli-Q® Gradient A10
Acetonitrile (CH₃CN), methanol (CH₃OH), methyl tert-butyl ether (MTBE) and dichloromethane (CH₂Cl₂), HPLC grade, Fisher
Potassium dihydrogen phosphate (KH₂PO₄), phosphoric acid (H₃PO₄), and potassium bicarbonate (KHCO₃), analytical grade, SCRC, China
Folic acid (V_{B9}), ascorbic acid (V_C), phyloquinone (VK1), tocopherol acetate (V_E acetate), β-carotene, retinol (V_A), and retinol acetate (V_A acetate), ≥ 98%, Sigma-Aldrich
Thiamine (V_{B1}), riboflavin (V_{B2}), nicotinamide (V_{B3}), pantothenic acid (V_{B5}), pyridoxine (V_{B6}), cyanocobalamin (V_{B12}), ergocalciferol (V_{D1}), and cholecalciferol (V_{D2}), ≥ 97%, National Institute for the Control of Pharmaceutical and Biological Products (NICBPB), China.

SAMPLES

Five vitamin supplement samples (Brands 1 to 5) were analyzed. The ingredients are listed in Table 1. Brands 1 and 2 were from two different pharmaceutical companies, and Brands 3, 4, and 5 were from a third company that produces special vitamin tablets for women, children, and the elderly.

STANDARD PREPARATION

Water-Soluble Vitamin Standards

Water-soluble vitamin standards of V_{B1}, V_{B3}, V_{B5}, V_{B6}, V_{B12}, and V_C are prepared by accurately weighing 10 to 20 mg of the vitamin powder and adding 10 to 20 g of DI water to make stock solutions of 1.0 mg/mL for each vitamin. Because of the limited stability of V_C, it should be freshly prepared. Because of the limited solubility in water of V_{B2} and folic acid (V_{B9}), the concentration of

stock solution of V_{B2} is decreased to 0.25 mg/mL in DI water; while that of V_{B9} is prepared using 20 mM of KHCO₃ instead of DI water to make a solution of 0.5 mg/mL.

Fat-Soluble Vitamin Standards

Fat-soluble vitamin standards of V_A acetate, V_{D2}, V_{D3}, and V_E acetate were prepared by accurately weighing 10 to 20 mg of each and adding CH₃OH to 10 to 20 g to form stock solutions of 1.0 mg/mL for each vitamin. The standards for V_{K1} and β-carotene were prepared using acetone and CH₂Cl₂ instead of CH₃OH. Because of the limited stability of β-carotene, a stock solution of 1.0 mg/mL was freshly prepared every 3 days.

The well-prepared stock standards were stored at 4 °C when not in use, and the stock standards of fat-soluble vitamins were stored in the dark. Water-soluble vitamin working standards were prepared from the stock standards on the day of use by dilution with DI water. A mixture of CH₃OH-CH₂Cl₂ (1:1, v/v) was used for fat-soluble vitamins.

Table 1. Ingredients in Nutritional Supplement Samples, Brands 1 to 5

	Brand 1	Brand 2 (B-complex)	Brand 3 (for women)	Brand 4 (for children)	Brand 5 (for the elderly)
Ca	V _{B1}	V _{B1}	V _{B1}	V _{B1}	V _{B1}
P	V _{B2}	V _{B2}	V _{B2}	V _{B2}	V _{B2}
K	V _{B3}	V _{B3}	V _{B6}	V _{B6}	V _{B6}
Cl	V _{B5}	V _{B5}	V _{B9}	V _{B9}	V _{B9}
Mg	V _{B6}	V _{B6}	V _C	V _C	V _C
Fe	V _{B12}	V _{B12}	V _A acetate	V _A acetate	V _A acetate
Cu	V _{B9}		V _E *		V _E *
Zn	V _C			V _D	V _D
Mn	V _A acetate		β-carotene		
I	V _D		Ca	Ca	Ca
Cr	Biotin		Fe	Fe	Fe
Mo	V _E *		Zn	Zn	Zn
Se	V _{K1}		Sn	Sn	Sn
Ni	β-carotene				
Sn					
Si					
V					

Note: * V_E acetate was detected in these samples, but it is labeled as V_E.

SAMPLE PREPARATION

Extraction of Water-Soluble Vitamins

Grind the tablets with a mortar and pestle. Place 0.100 g of accurately weighed ground powder for Brands 1 and 2, and 0.2 g of accurately weighed ground powder for Brands 3 to 5, respectively, into 100 mL volumetric flasks; then add 80 mL of water to each flask. After 15 min of ultrasonic extraction, add water to the mark.

Extraction of Fat-Soluble Vitamins

Place 0.125 g of accurately weighed ground powder of Brands 1, 3, 4, and 5 into 10 mL volumetric flasks, respectively, and add 8 mL of CH₃OH-CH₂Cl₂ (1:1, v/v) to each flask. After 15 min of ultrasonic extraction, add CH₃OH-CH₂Cl₂ (1:1, v/v) to the mark.

The well-prepared sample solutions should be stored in the dark and diluted, if necessary. Prior to injection, filter the solutions through a 0.2 µm filter (Millex®-GN).

CONDITIONS

For Determination of Water-Soluble Vitamins

Column: Acclaim PA2, 5 µm, 120 Å, 4.6 × 150 mm (P/N 063197)
Temperature: 25 °C
Mobile Phase: A: CH₃CN
B: 25 mM Phosphate buffer (dissolve about 3.4 g KH₂PO₄ in 1 L water, and adjust pH to 3.2 with H₃PO₄)
In gradient (Table 2)
Flow Rate: 1.0 mL/min
Injection Vol: 20 µL
UV Detection: Absorbance at 210, 245, 265, and 280 nm (Table 3)

For Determination of Fat-Soluble Vitamins

Column: Acclaim C18, 5 µm, 120 Å, 4.6 × 150 mm (P/N 059133)
Temperature: 25 °C
Mobile Phase: A: CH₃OH-CH₃CN (8:2, v/v)
B: MTBE
In gradient (Table 2)
Flow Rate: 1.0 mL/min
Injection Vol: 20 µL
UV Detection: Wavelength-switching absorbance at 265, 325, and 450 nm (Table 3)

Table 2. Gradients for Water- and Fat-Soluble Vitamin Separations

Water-Soluble Vitamins			Fat-Soluble Vitamins		
Retention Time (min)	A (%) CH ₃ CN	B (%) 25 mM Phosphate buffer (pH 3.2)	Retention Time (min)	A (%) CH ₃ OH-CH ₃ CN (8:2,v/v)	B (%) MTBE
0.0	0	100	0.0	95	5
4.0	0	100	3.0	95	5
14.0	35	65	4.5	80	20
14.5	80	20	5.0	65	35
19.0	80	20	10.0	65	35
19.5	0	100	10.1	95	5
20.0	0	100	15	95	5

Table 3. Detection Wavelengths for Water- and Fat-Soluble Vitamins

Water-Soluble Vitamins		Fat-Soluble Vitamins		
Detection Wavelength (nm)	Analytes	Switching Time (min)	Detection Wavelength (nm)	Analytes
210	V _{B3*} , V _{B5*} , V _{B6*} , V _{B12}	0.0	325	V _{A*} , V _A acetate
245	V _{B1*} , V _{C*} , V _{B12*}	4.2	265	V _{D2*} , V _{D3*} , V _{K1*} , V _E acetate
265	V _{B2}	9.0	450	β-carotene
280	V _{B6*} , V _{B9}			

Note. * V_{B6} and V_{B12} are detected at 280 and 245 nm, respectively, when analyzing Brand 1 (details shown in *Sample Analysis – Water-Soluble Vitamins*).

RESULTS AND DISCUSSION

Separation and Detection

Water-Soluble Vitamins

Some smaller acids, such as formic and acetic, are commonly used as mobile phase buffers and absorb at 210 nm. For this reason, the baseline absorbance shifts during the gradient as the amount of these acids in the mobile phase changes. Additionally, initial experiments demonstrated that the retention of V_{B1} is inadequate when using formic acid unless an ion-pairing reagent is added to the mobile phase. Therefore, a phosphate buffer was used to avoid the baseline shift and to retain V_{B1}.

The buffer's pH value may significantly affect the retention of water-soluble vitamins on the polar group embedded Acclaim PA2 column, which is suitable for separation of compounds with high polarity. This is especially important for the separations of V_{B1} , V_C , V_{B12} , and V_{B9} . As shown in Figure 1, the eight water-soluble vitamins are well separated at pH 3.2. The water-soluble vitamins are a structurally diverse group of compounds with different absorbance maxima. Therefore, as shown in Table 3, four detection wavelengths were chosen for their detection.

Fat-Soluble Vitamins

Non-aqueous reversed-phase (NARP) is usually used for determining fat-soluble vitamins by HPLC so that the vitamins are soluble throughout the analysis. A typical NARP mobile phase consists of a polar solvent (usually acetonitrile), a solvent with lower polarity (e.g., dichloromethane) to act as a solubilizer and to control retention by adjusting the solvent strength, and an amount of a third solvent with hydrogen-bonding capacity (e.g., methanol) to optimize selectivity. Therefore, an acetonitrile, methanol, and MTBE mobile phase was used to separate fat-soluble vitamins using the Acclaim 120 C18 column. Because these are all low-polarity compounds, a C18 stationary phase is a good choice for their separation. Figure 2 shows good resolution of seven fat-soluble vitamins: V_A , V_A acetate, V_{D2} , V_{D3} , V_E acetate, V_{K1} , and β -carotene. The fat-soluble vitamins also are a structurally diverse group of compounds with different absorbance maxima. Therefore, as shown in Table 3, three detection wavelengths were chosen to determine them, and wavelength-switching was employed in order to obtain a single chromatogram and increase detection sensitivity.

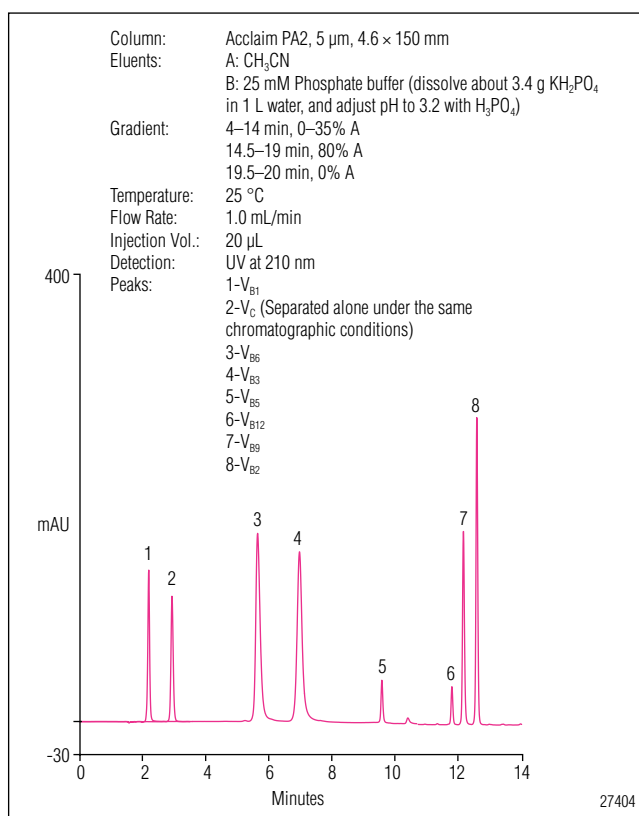


Figure 1. Overlay of chromatograms of V_C and V_B group vitamins.

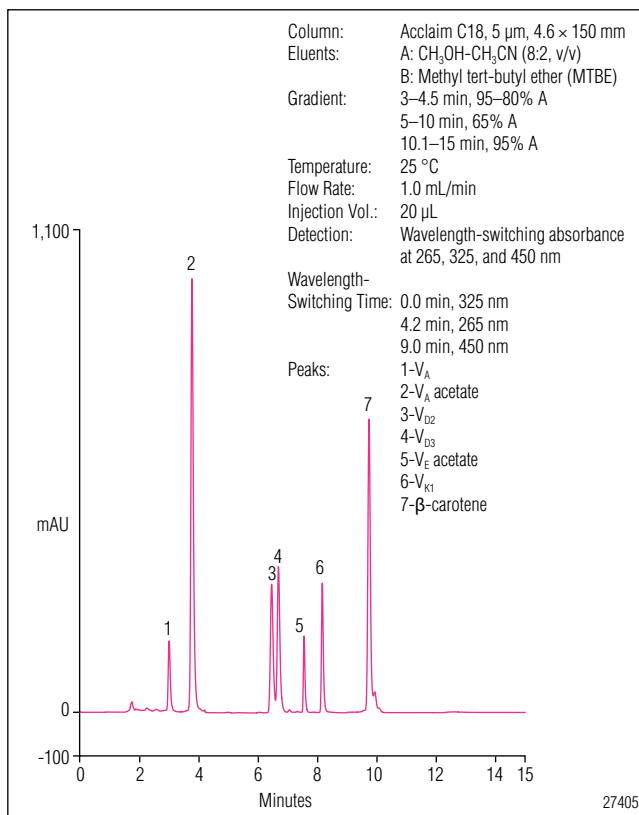


Figure 2. Chromatogram of fat-soluble vitamins and β -carotene.

Reproducibility, Linearity, and Detection Limits

Prior to sample analysis, the reproducibility was estimated by making six replicate injections for water-soluble vitamins and nine replicate injections for fat-soluble vitamins. Excellent RSDs for retention time and peak area are shown in Table 4.

Calibration linearity for the water- and fat-soluble vitamins was investigated, respectively, by making three replicate injections of a mixed standard prepared at five or six different concentrations. The external standard method was used to calculate the calibration curve and to quantify these compounds in samples. Table 5 reports the data from the calibration as calculated by the Chromeleon software. We found linear calibration curves for each vitamin over the ranges that were evaluated. The single-sided Student's *t* test method was used for estimating method detection limits (MDL). These data are also reported in Table 5.

Table 4. Reproducibility of Retention Time and Peak Area for Water*- and Fat-Soluble Vitamins**

Water-Soluble Vitamin	Retention Time RSD	Peak Area RSD	Fat-Soluble Vitamin	Retention Time RSD	Peak Area RSD
V _{B1}	0.000	0.184	V _A	0.063	1.309
V _{B2}	0.028	0.216	V _A acetate	0.069	0.159
V _{B3}	0.181	0.059	V _{D2}	0.057	0.744
V _{B5}	0.078	0.428	V _{D3}	0.051	0.609
V _{B6}	0.181	0.085	V _E acetate	0.029	2.049
V _{B9}	0.030	0.513	V _{K1}	0.027	0.375
V _{B12}	0.043	0.181	β-carotene	0.064	0.569
V _C	0.000	1.100			

Note. * Seven consecutive injections for water-soluble vitamins.

** Nine consecutive injections for fat-soluble vitamins.

Table 5. Calibration Data, as Reported by Chromeleon Software, and MDLs for the Water- and Fat-Soluble Vitamins

Water-Soluble Vitamin	Detection Wavelength (nm)	Regression Equation	r (%)	RSD	Range (µg/mL)	MDL* (µg/mL)
V _{B1}	245	A = 0.8006 c - 0.0506	99.998	0.708	0.1–50	0.07
V _{B2}	265	A = 1.6092 c + 0.0028	99.999	0.498	0.1–25	0.01
V _{B3}	210	A = 1.1735 c - 0.0398	99.996	0.893	0.1–50	0.04
V _{B5}	210	A = 0.1137 c - 0.0165	99.865	5.834	0.1–50	0.19
V _{B6}	210	A = 1.1556 c - 0.0296	99.988	0.719	0.1–50	0.03
	280	A = 0.5652 c - 0.0460	99.996	1.316		0.10
V _{B9}	280	A = 1.1232 c - 0.0323	99.982	2.131	0.05–25	0.02
V _{B12}	210	A = 0.9677 c - 0.0227	99.923	4.442	0.01–5	0.03
	245	A = 0.2475 c - 0.0107	99.934	3.794		0.06
V _C	245	A = 1.0048 c - 0.2599	99.993	1.346	0.1–100	0.27
Fat-soluble Vitamin		Regression Equation	r (%)	RSD	Range (µg/mL)	MDL* (µg/mL)
V _A acetate		A = 2.3497 c - 0.0142	99.998	1.112	0.05–40	0.01
V _{D2}		A = 0.7995 c + 0.0012	99.997	1.288	0.05–40	0.02
V _{D3}		A = 0.8520 c + 0.0242	99.997	1.367	0.05–40	0.01
V _E acetate		A = 0.0281 c + 0.0117	99.998	1.134	0.5–400	0.14
V _{K1}		A = 0.4456 c + 0.0021	99.997	1.191	0.125–10	0.01
β-carotene		A = 5.3657 c - 0.1324	99.972	4.043	0.05–40	0.03

Note. * The single-sided Student's *t* test method (at the 99% confidence limit) was used for determining MDL, where the standard deviation (SD) of the peak area of seven injections is multiplied by 3.50 to yield the MDL.

Table 6. Analysis Results of Water- and Fat-Soluble Vitamins in Nutritional Supplement Samples*

Analyte		Brand 1					Brand 2					
		Labeled (mg/g)	Detected (mg/g)	Added (mg/g)	Found (mg/g)	Recovery (%)	Labeled (mg/g)	Detected (mg/g)	Added (mg/g)	Found (mg/g)	Recovery (%)	
Water-Soluble	V _{B1}	1.00	0.94	1.00	0.97	97	5.59	5.64	2.80	2.74	96	
	V _{B2}	1.13	1.01	1.14	1.12	98	5.59	5.49	2.80	2.88	103	
	V _{B3}	13.3	12.7	13.4	12.9	96	42.0	42.8	20.8	20.4	98	
	V _{B5}	6.67	6.58	6.70	6.76	101	14.0	14.2	7.00	6.66	95	
	V _{B6}	1.33	1.31	1.35	1.33	99	5.59	5.65	2.80	2.67	95	
	V _{B9}	0.27	0.26	0.27	0.26	99	/	/	/	/	/	
	V _{B12}	0.004	0.012	0.04	0.034	85	0.0056	0.005	0.056	0.056	100	
	V _C	40	37.0	40.0	38.5	96	/	/	/	/	/	
Fat-Soluble	V _A acetate	0.80	0.71	5.00	4.21	84						
	V _D	V _{D2}	0.0067	0.0088	0.01	0.0103						103
		V _{D3}		/	/	/						/
	V _E acetate	20.0	24.0	4.00	3.68	92						
	V _{K1}	0.017	0.0026	0.04	0.043	108						
	β-carotene	0.22	0.18	0.25	0.246	98						
Analyte		Brand 3					Brand 4		Brand 5			
		Labeled (mg/g)	Detected (mg/g)	Added (mg/g)	Found (mg/g)	Recovery (%)	Labeled (mg/g)	Detected (mg/g)	Labeled (mg/g)	Detected (mg/g)		
Water-Soluble	V _{B1}	0.50	0.49	1.00	0.95	95	0.50	0.49	0.50	0.47		
	V _{B2}	0.50	0.46	1.00	0.94	94	0.50	0.50	0.50	0.48		
	V _{B3}	/	/	/	/	/	/	/	/	/		
	V _{B5}	/	/	/	/	/	/	/	/	/		
	V _{B6}	0.50	0.47	1.00	1.01	101	0.50	0.47	0.50	0.49		
	V _{B9}	0.10	0.10	0.20	0.19	95	0.05	0.05	0.075	0.068		
	V _{B12}	/	/	/	/	/	/	/	/	/		
	V _C	25.0	24.5	50.0	47.7	95	25.0	24.5	50.0	49.9		
Fat-Soluble		Labeled (mg/g)		Detected (mg/g)			Labeled (mg/g)	Detected (mg/g)	Labeled (mg/g)	Detected (mg/g)		
	V _A acetate	0.15		0.14			0.20	0.30	no amount **			
	V _D	V _{D2}	/		/			0.0025	/	/		
		V _{D3}	/		/				0.002	0.0025	0.002	
	V _E acetate	5.00		5.32			/	0.66	6.5	6.9		
β-carotene	0.05		0.04			/	0.05	/	0.01			

Note. * Three injections for each (n = 3)

** V_A acetate is labeled in Brand 5, but its amount is not declared.

Sample Analysis

Water-Soluble Vitamins

Water-soluble vitamins can be extracted from the nutritional supplement samples without causing chemical change by using water with ultrasonic extraction. Although V_{B9} (folic acid) and V_{B12} have limited solubility in water, they still can be extracted effectively because of their low-levels in the samples. Good recoveries of water-soluble vitamins in the spiked sample (Table 6) verified

that water extraction is suitable; however, some fat-soluble vitamins, such as V_E acetate, V_A, and β-carotene, can be partially extracted, and their retention is very strong under the chromatographic conditions used for water-soluble vitamins analysis. Therefore, after sample analysis, the column should be washed using a mobile phase with a high-percentage of organic solvent. The frequency of this cleaning will vary with the sample, the sample injection volume, and the number of injections.

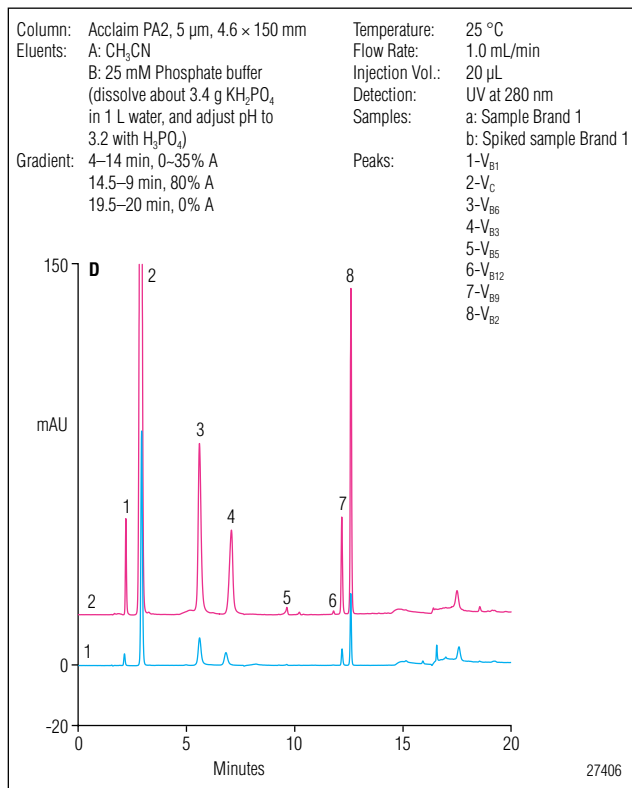
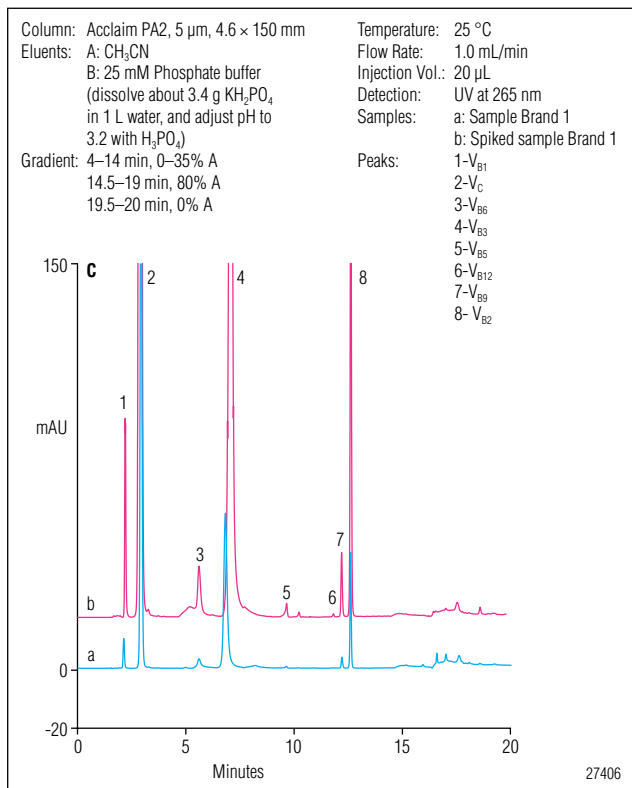
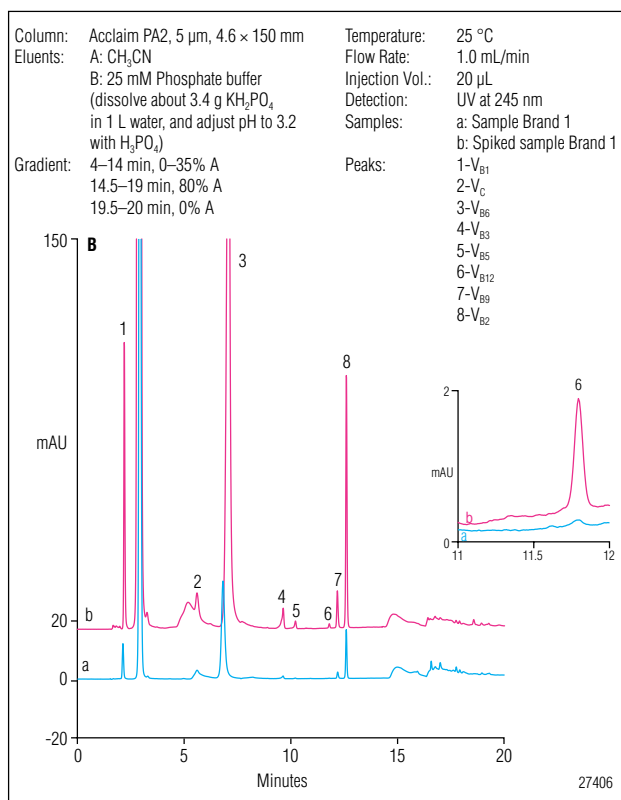
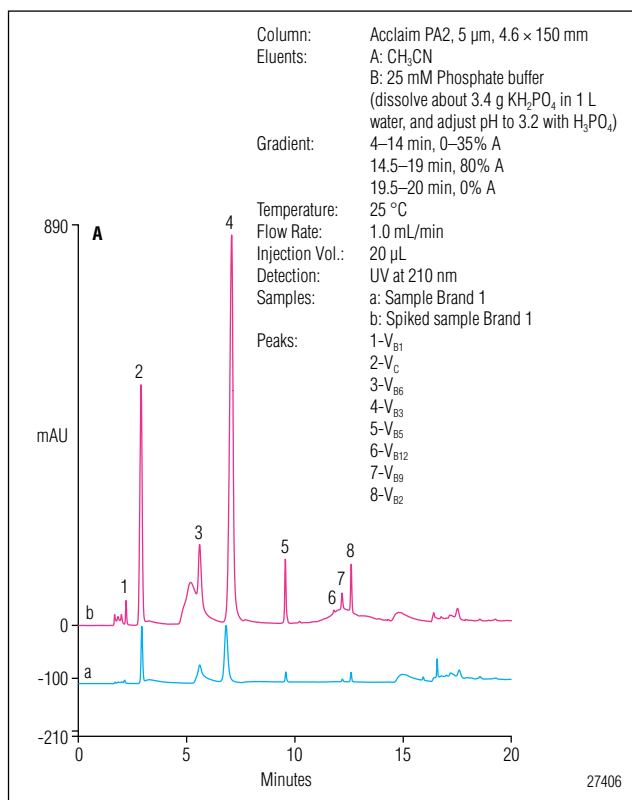


Figure 3. Chromatograms of water-soluble vitamins in Brand 1 collected at A) 210 nm, B) 245 nm, C) 265 nm, and D) 280 nm.

Five nutritional supplement samples were analyzed. Figure 3 shows chromatograms of Brand 1, which were collected at different detection wavelengths, and the same

sample spiked with standards. For Brand 1, impurities may interfere with the detection of V_{B6} (peak 3) and V_{B12} (peak 6) at 210 nm. Although these samples have

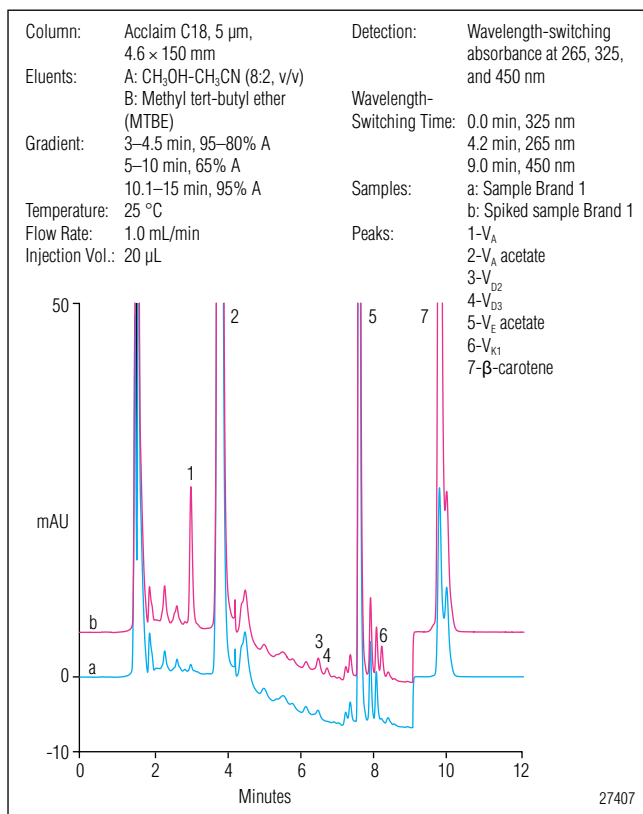


Figure 4. Chromatograms of fat-soluble vitamins in Brand 1.

greater absorption at 210 nm, they are best detected at 280 and 245 nm, respectively, in order to eliminate the interference. In the other samples, no impurities interfered with the detection of V_{B6} and V_{B12} at 210 nm. Analysis results of the five samples (i.e., recovery of analytes added to the samples and amounts of the water-soluble vitamins in each sample) are summarized in Table 6. The recoveries of the water-soluble vitamins ranged from 94% to 103%, and the detected amounts were in agreement with the labeled values, suggesting that the extraction and determination are accurate.

Fat-Soluble Vitamins

The fat-soluble vitamins were extracted from the nutritional supplement samples without causing chemical change. This was achieved through use of a solvent system (a mixture of dichloromethane and methanol) that was capable of effectively penetrating the sample matrix and was used in conjunction with ultrasonic extraction.

Four nutritional supplement samples (Brands 1, 3, 4, and 5) were analyzed. Figure 4 shows chromatograms of Brand 1 and the same sample spiked with standards,

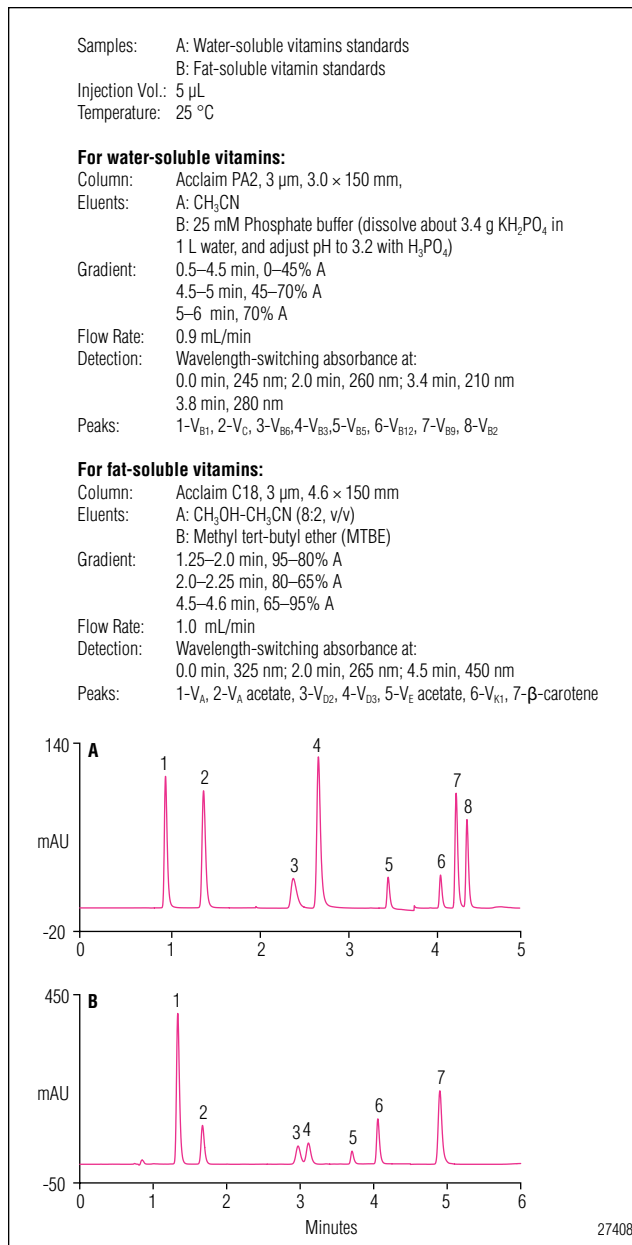


Figure 5. Fast separation of A) water- and B) fat-soluble vitamins on the UltiMate 3000 system using the Acclaim HPLC columns packed with resins with smaller particle diameter.

which were collected at three different detection wavelengths using wavelength-switching. Analysis results of the four samples are summarized in Table 6. The detected amounts of fat-soluble vitamins are in agreement with the labeled value, except for the amount of V_{K1} in Brand 1, which was determined as 152% of the labeled value. Although the amounts of β -carotene and V_E are not labeled, they were found in Brand 4. The same was true for β -carotene in Brands 1 and 5.

CONCLUSION

HPLC is an efficient method for determining the vitamin content of nutritional supplements. After the appropriate sample preparation, water-soluble vitamins are determined in less than 20 min using an Acclaim Polar Advantage II column starting with a 100% aqueous mobile phase with no ion-pairing reagent. Fat-soluble vitamins are determined in under 15 min using the Acclaim 120 C18 column and a highly organic mobile phase.

APPENDIX

Faster separations of water- and fat-soluble vitamins may be completed on the UltiMate 3000A system using the Acclaim HPLC columns packed with resins with smaller particle diameter (3 μm , 3.0 \times 150 mm, PA2 [P/N 063705] for water-soluble vitamins and C18 [P/N 063691] for fat-soluble vitamins). As shown in Figure 5, good separations are completed within 4.6 min for water-soluble vitamins and within 5.2 min for fat-soluble vitamins. See Tables 7 and 8 for the gradient and detector parameters for faster separations.

Table 7. Gradients for Faster Water- and Fat-Soluble Vitamin Separations

Water-Soluble Vitamins			Fat-Soluble Vitamins		
Retention Time (min)	A (%) CH ₃ CN	B (%) 25 mM Phosphate buffer (pH 3.2)	Retention Time (min)	A (%) CH ₃ OH-CH ₃ CN (8:2,v/v)	B (%) MTBE
0.0	0	100	0.0	95	5
0.5	0	100	1.25	95	5
4.5	45	55	2.0	80	20
5.0	70	30	2.25	65	35
6.0	70	30	4.5	65	35
			4.6	95	5

Table 8. Wavelength Switching Time for Faster Water- and Fat-Soluble Vitamin Separations

Water-Soluble Vitamins		Fat-Soluble Vitamins	
Switching Time (min)	Detection Wavelength	Switching Time (min)	Detection Wavelength
0.0	245	0	325
2.0	260	2.0	265
3.4	210	4.5	450
3.8	280		

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Dionex Corporation

1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America

U.S./Canada (847) 295-7500

South America

Brazil (55) 11 3731 5140

Europe

Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
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Asia Pacific

Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

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