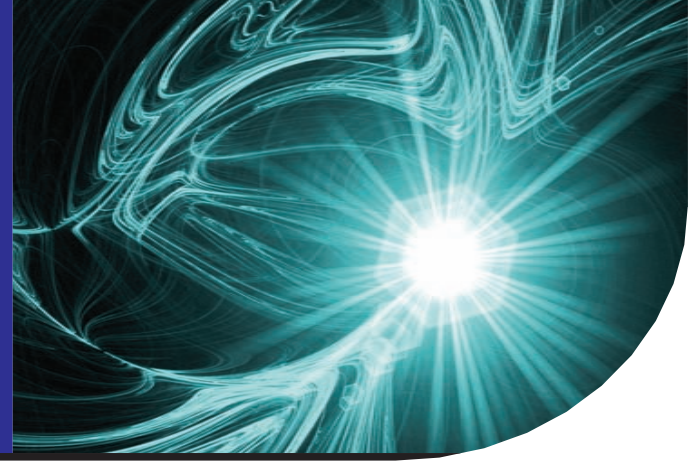

HPLC Assay of Water-Soluble Vitamins, Fat-Soluble Vitamins, and a Preservative in Dry Syrup Multivitamin Formulation

INTRODUCTION

Vitamins are vital to human development and long-term health; therefore, infants are usually prescribed a vitamin supplement to ensure they receive the recommended daily allowance of each vitamin. Children under one year of age are usually given this supplement in liquid form. This supplement can be produced as a dry syrup using a powdered preparation to which the pharmacist adds liquid to produce the dosage form for the patient. The work shown here describes an HPLC method to quantify water- and fat-soluble vitamins in a dry syrup.

> ***"This application also can be run by UHPLC using a 2.2 µm Acclaim PA2 column in 2.1 × 100mm format to save time, reduce mobile phase consumption, and reduce waste"***

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Vitamins were extracted from the dry syrup prior to analysis. The water soluble vitamins (WSV) were extracted with water and a pH adjustment with KOH to dissolve folic acid. The fat soluble vitamins (FSV) were extracted with either DMSO or ethyl acetate. To include all vitamins in the same chromatogram, the authors used a Chromeleon® Chromatography Data System (CDS) software feature that allows more than one injection for the same analysis. The WSV sample was injected first, then after elution of all WSV, the FSV sample was injected. This application also can be run by UHPLC using a 2.2 µm Acclaim PA2 column in 2.1 × 100mm format to save time, reduce mobile phase consumption, and reduce waste. This document shows that the UltiMate® 3000 system with an Acclaim PA2 column is an excellent solution for vitamin determinations.

Equipment

Dionex UltiMate 3000 system including:

Equipment	Conventional LC	UHPLC
Integrated vacuum degasser solvent rack	SRD-3600	SRD-3600
Pump	DGP-3600A	HPG-3400RS
Split-loop sampler	WPS-3000TSL	WPS-3000TRS
Column compartment	TCC-3200	TCC-3000RS
Diode array detector	PDA-3000	DAD-3000RS
Sample loop size*	100 µL	100 µL
Mixer	Standard	200 µL Static mixer kit
Flow cell	13 µL SST	2.5 µL SST
Chromeleon software version	6.80 SP 6	6.80 SR 7

**The work was done with 100 µL loop but the authors recommend using a 10 µL loop.*

Reagents and Standards

Deionised water (DI), Type I reagent-grade, 18 MΩ-cm resistivity or better

Acetonitrile (CH3CN), HPLC grade (LAB-SCAN)

Methanesulfonic acid (MSA), puriss. ≥ 99% grade (Fluka)

Ammonium di-hydrogen orthophosphate, AR grade (Ajax)

Ethyl acetate, AR grade (Ajax)

Dimethyl sulfoxide (DMSO), AR grade (Sigma-Aldridge)

Thiamine*

Nicotinamide*

Ascorbic acid*

Pyridoxine hydrochloride*

Calcium pantothenate*

Cyanocobalamine*

Folic acid*

Riboflavin*

Sodium benzoate*

Retinol acetate*

α-Tocopherol acetate*

** These standards were provided by the customer but are available from a number of companies that supply laboratory chemicals.*

Conditions

Conventional HPLC

Column: Acclaim PA2, 3µm, 4.6 × 150mm (P/N 063191), Acclaim PA2 Guard, 5 µm, 4.3 × 10mm (P/N 063195) Acclaim Guard Kit (P/N 059526)

Mobile Phase: A: 0.05% MSA
B: CH3CN
C: 10 mM NH4H2PO4 pH 2.5 with MSA

Sampler Temp.: 10°C

Column Temp.: 35°C

Injection Volume: 30 µL for water-soluble vitamins at 0.00 min, and 30 µL for fat-soluble vitamins at 18.00 min

Detection: UV-vis at 210nm, 285nm, wavelength scanning 200–800nm, data collection rate 5 Hz, rise time 0.5 sec

Gradient: Table 1

UHPLC

Column: Acclaim RSLC PA2, 2.2 µm, 2.1 × 100mm (P/N 068990)

Mobile Phase: A: 0.05% MSA
B: CH3CN
C: 5 mM NH4H2PO4 pH 3.0 with MSA

Sampler Temp.: 10°C

Column Temp.: 35°C

Injection Volume: 4 µL for WSV at 0.00 min, 0.5 µL for FSV at 7.5 min

Detection: UV-vis at 210 nm, 285nm, data collection rate 10 Hz, response time 0.5 sec

Gradient: Table 1

Preparation of Solutions and Reagents

Mobile Phases

Mobile Phase A (0.05% MSA)

Weigh 999.5g water, transfer 0.5 mL MSA to the same bottle, and mix well.

Mobile Phase C (10 mM $NH_4H_2PO_4$ pH 2.5)

Weigh 1.15g ammonium di-hydrogen orthophos-phate into a 250mL beaker, add 100mL water, stir until dissolved, transfer to a 1 L volumetric flask, and bring to volume with water. Adjust to pH 2.5 with MSA (350 µL).

Standard Solutions and Sample Preparation

1000 mg/L Stock Standard Solutions

WSV standard solutions

Weigh 0.01g of each vitamin into separate 10mL volumetric flasks, add 5mL water, and swirl the flask until dissolved. Prepare the preservative (sodium benzoate) in the same manner. To dissolve folic acid, add 10µL of 8 M KOH. Bring to volume with water.

FSV standard solutions in ethyl acetate

Weigh 0.0g (0.02g for α-tocopherol) of standard in separate 50mL glass bottles, add 2mL water, add 10mL ethyl acetate, quickly cap the bottle, place in an ultrasonic bath for 10 to 15 min, shake, and wait until the layers are completely separated. Use the top ethyl acetate layer as the stock standard solution.

Table 1.

Table 1. Gradient Program, Flow Program, Sample Injection Times, and Wavelength Switching Times

Chromatographic Condition	Time (min)	Flow (mL/min)	% A	% B	% C	Remark	UV_VIS_1
Conventional HPLC	-7.00	1.00	100.0	0.0	0.0		210
	0.00	1.00	100.0	0.0	0.0	Inject WSV (position in the sequence)	
	3.00	1.00	100.0	0.0	0.0		
	3.10	1.00	0.0	0.0	100.0		
	9.00	1.00	0.0	30.0	70.0		
	9.50	1.00	0.0	45.0	55.0		
	13.00	1.00	0.0	45.0	55.0		
	13.10	1.00	55.0	45.0	0.0		
	15.00	1.00	55.0	45.0	0.0		
	16.00	1.50	5.0	95.0	0.0		
	17.00	1.50	5.0	95.0	0.0		*285
	18.00	1.50	5.0	95.0	0.0	*Inject FSV (position in the sequence+1)	
	21.00	1.50	5.0	95.0	0.0		
	22.00	1.50	0.0	100.0	0.0		
	27.00	1.50	0.0	100.0	0.0		
	28.00	1.00	100.0	0.0	0.0		
UHPLC	-5.00	0.40	100.0	0.0	0.0		210
	0.00	0.40	100.0	0.0	0.0	Inject WSV (position in the sequence)	
	1.00	0.40	100.0	0.0	0.0		
	1.00	0.40	0.0	0.0	100.0		
	1.10	0.40	0.0	4.0	96.0		
	2.00	0.40	0.0	4.0	96.0		
	4.70	0.40	0.0	45.0	55.0		
	5.50	0.40	0.0	45.0	55.0		
	5.50	0.40	55.0	45.0	0.0		
	6.50	0.40	55.0	45.0	0.0		
	6.60	0.60	5.0	95.0	0.0		
	7.50	0.60	5.0	95.0	0.0	*Inject FSV (position in the sequence+1)	
	7.60	0.60	5.0	95.0	0.0		*285
	8.00	0.60	5.0	95.0	0.0		
	8.10	0.60	0.0	100.0	0.0		
	11.0	0.60	0.0	100.0	0.0		

**Manually insert the command in the program file.*

Table 2.

Table 2. Summary of Calibration Results (DMSO Extraction)

Vitamin	Standard Conc. (mg/L)			Cal.Type	Points	Coeff.Det. (× 100%)	Offset	Slope
	L1	L2	L3					
Thiamine	1.5	2.0	3.0	LOff	3	99.9872	-0.0310	0.6078
Nicotinamide	15.0	20.0	30.0	LOff	3	99.9977	0.4961	1.6711
Ascorbic acid	60.0	80.0	120.0	LOff	3	99.9862	-0.5339	0.3030
Pyridoxine hydrochloride	1.5	2.0	3.0	LOff	3	99.9995	-0.0489	1.8413
Calcium Pantothenate	6.0	12.0	18.0	LOff	3	99.9989	0.0153	0.1595
Cyanocobalamin	0.1	0.5	1.0	LOff	3	99.9929	-0.0027	1.3739
Folic acid	0.1	0.2	0.3	LOff	3	99.9626	-0.0096	1.6568
Riboflavin	1.5	3.5	5.0	LOff	3	99.8216	-0.0692	0.8223
Benzoate	5.0	10.0	15.0	LOff	3	99.9974	0.1505	0.6664
Retinol acetate	25.0	35.0	50.0	LOff	3	99.9759	0.0151	0.0948
α-Tocopherol acetate	25.0	35.0	50.0	LOff	3	99.9152	-0.0814	0.0905

FSV standard solutions in DMSO

Weigh 0.01g (0.02g for α-tocopherol) of standard in separate 50mL glass bottles, add 10mL DMSO, and place in an ultrasonic bath for 10 to 15 min.

Working Standards Preparation

For concentrations of working standard solutions, see *Table 2*. *Table 3* shows an example of the volumes of stock standards required to make the level 2 working standard. The WSV and FSV standards were prepared separately.

The WSV working standards (each containing the preservative sodium benzoate) were diluted with mobile phase A and the FSV working standards were diluted with mobile phase B.

Sample Preparation

A dry syrup containing a mixture of vitamins is provided in small bottles with a mark to indicate how much liquid to add to prepare the syrup. Add water to this mark (45mL) and shake for few minutes. The sample is now ready for further preparation. A placebo consisting of the dry syrup without added vitamins is also used.

Table 3.

Table 3. Preparation of the Level 2 Working Standard

Vitamin	Concentration (mg/L)	Volume of 1000 mg/L Stock Standard Solution in Final 25 mL for WSV and 10 mL for FSV (µL)
Thiamine	2.0	50
Nicotinamide	20.0	500
Ascorbic acid	80.0	2000
Pyridoxine hydrochloride	2.0	50
Pantothenic acid	12.0	300
Cyanocobalamine	0.5	12.5
Folic acid	0.2	5.0
Riboflavin	3.5	87.5
Sodium benzoate	10.0	250
Retinol acetate	35.0	350
α-Tocopherol acetate	35.0	350

Note: Prepare stock standard and working standard solutions and samples just prior to the analysis. Store these solutions in brown bottles and use brown vials for analysis.

Sample Preparation for WSV Analysis

Shake the sample bottle and pipet 0.25mL of sample, wipe the outside of the pipette, dispense into a 25mL volumetric flask, rinse the inside of the pipette with 0.25mL water, add 10 µL of 8 M KOH, swirl the flask, and bring to volume with mobile phase A.

Sample Preparation for FSV Analysis (Ethyl Acetate Extraction)

Shake the sample bottle and pipet 0.5mL of sample, wipe the outside of the pipette, dispense into a 50mL glass bottle, rinse the inside of the pipette with 0.5mL water, add 5mL ethyl acetate, and then cap the bottle. Place the capped bottle in an ultrasonic bath for 10 min, shake for few minutes, and wait until the layers are completely separated. Pipet 1mL of the top layer and dispense into 3mL CH_3CN.

Sample Preparation for FSV Analysis (DMSO Extraction)

Shake the sample bottle and pipet 0.25mL of sample, wipe the outside of the pipette, dispense into a 10mL volumetric flask, rinse the inside of the pipette with 0.25mL water, add 2mL DMSO, and place in an ultrasonic bath for 10 min. Bring to volume with CH_3CN.

Table 4 shows the composition of 5mL of a correctly prepared sample.

Table 4.

Table 4. Comparison of Sample Results between DMSO and Ethyl Acetate Extractions

Vitamin	Labeled Content for Each 5 mL (mg)	DMSO Extraction			Ethyl Acetate Extraction		
		Average Found Concentration of 3 Preparations (mg per 5 mL)	RSD	Assay (%)	Average Found Concentration of 3 Preparations (mg per 5 mL)	RSD	Assay (%)
Thiamine	1	1.1	0.62	110	1.1	1.18	110
Nicotinamide	10	10.3	1.19	103	10.3	1.23	103
Ascorbic acid	35	36.1	0.86	103.1	36.6	1.05	105
Pyridoxine hydrochloride	1	1.2	0.86	120	1.2	1.04	120
Calcium pantothenate	5	6.7	1.55	134	6.7	0.66	134
Cyanocobalamin	0.0025	n.a.	—	—	n.a.	—	—
Folic acid	0.1	0.1	2.70	100	0.1	1.40	100
Riboflavin	1	1.1	2.01	110	1.1	0.36	110
Benzoate	—	4.7	1.32	—	4.9	1.15	—
Retinol acetate	0.05 (1990IU)	6.6	2.46	13200	6.9	1.23	13800
α-Tocopherol acetate	7.5	7.0	2.96	93.3	7.4	3.78	98.7

Table 5.

Table 5. Standard Amounts for Preparation of the Spiked Placebo Sample

Vitamin	Amount Added (mg)
Thiamine	12
Nicotinamide	120
Ascorbic acid	420
Pyridoxine hydrochloride	12
Pantothenic acid	60
Cyanocobalamine	—
Folic acid	—
Riboflavin	12
Sodium benzoate	60
Retinol acetate	100
α-Tocopherol acetate	200

Table 6.

Table 6. Resolution and Peak Purity Results

Vitamin	Resolution* (USP)	Match	% RSD Match	PPI (nm)	% RSD PPI
Thiamine	8.07	999	0.53	229.5	0.21
Nicotinamide	5.97	1000	0.06	214.9	0.03
Ascorbic acid	6.84	1000	0.03	221.8	0.01
Pyridoxine hydrochloride	43.68	999	0.35	240.5	0.14
Calcium pantothenate	20.73	997	2.27	194.3	1.08
Cyanocobalamin	2.78	997	2.61	235.7	1.02
Folic acid	4.02	987	7.75	251.5	2.61
Riboflavin	31.30	1000	1.00	274.0	0.36
Benzoate	72.44	1000	0.03	208.5	0.01
Retinol acetate	36.22	1000	0.65	302.8	0.21
α-Tocopherol acetate	n.a.	999	0.42	196.5	0.20

** All values were calculated by Chromeleon software.*

Spiked Placebo Sample Preparation

Weigh 24g of placebo into an empty bottle and add accurately weighed vitamin standards (except vitamin B_{12} and folic acid, which are added later using the 1000mg/L stock standard solutions). Add water to reach the mark on the side of the bottle, shake for few minutes, and continue the sample preparation either for WSV or FSV. The amounts of added standards are listed in *Table 5*. For folic acid and vitamin B_{12}, 5µL and 2.5µL of the 1000mg/L standards, respectively, were added to the 25mL volumetric flask during the WSV sample preparation.

Results and Discussion

Separation and Detection

This application uses the Acclaim PA2 column to separate water- and fat-soluble vitamins and features of the Dionex UltiMate 3000 system and Chromeleon software that allow multiple injections during a single separation. The WSV, FSV, and benzoate were separated on Acclaim PA2 column in 28 min using a CH_3CN/MSA/$NH_4H_2PO_4$ mobile phase. The WSV standard

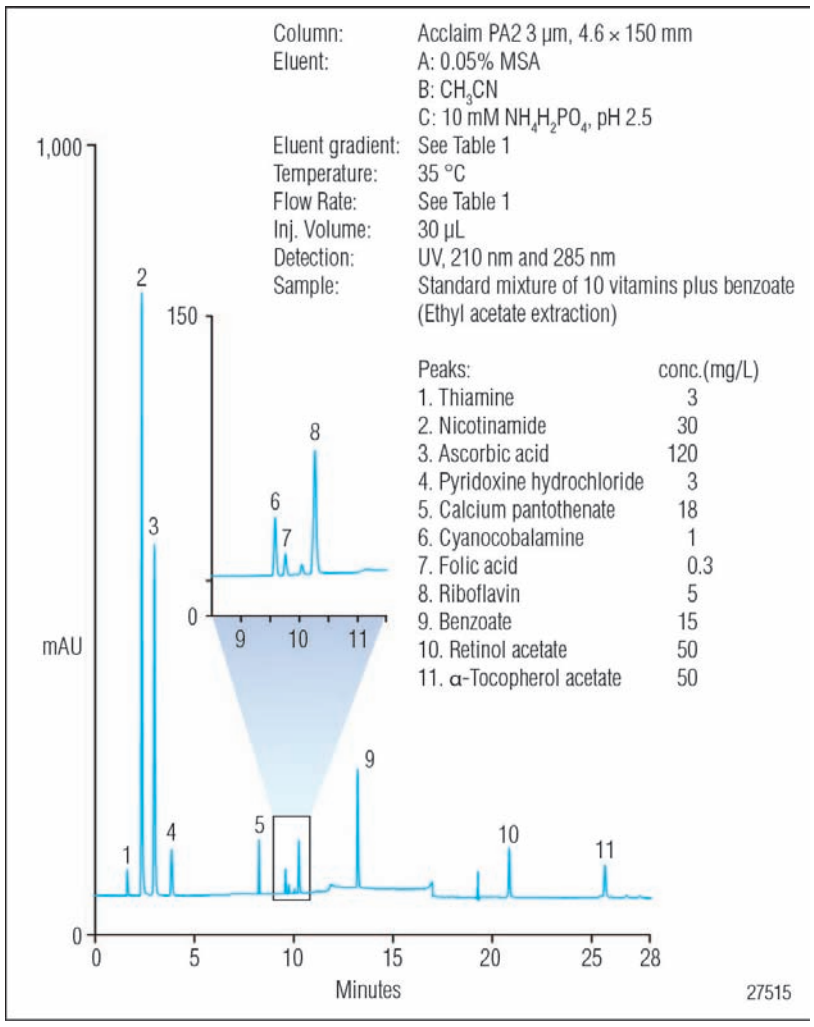

Figure 1. Chromatogram of a standard mixture of 10 vitamins plus benzoate (ethyl acetate extraction).

Table 7.

Table 7. Summary of Calibration Results (Ethyl Acetate Extraction)

Vitamin	Standard Conc. (mg/L)			Cal.Type	Points	Coeff.Det. (× 100%)	Offset	Slope
	L1	L2	L3					
Thiamine	1.5	2.0	3.0	LOff	3	99.9993	-0.0439	0.6165
Nicotinamide	15.0	20.0	30.0	LOff	3	100.0000	0.3913	1.6799
Ascorbic acid	60.0	80.0	120.0	LOff	3	99.9983	-0.8858	0.3027
Pyridoxine hydrochloride	1.5	2.0	3.0	LOff	3	99.9858	-0.0636	1.8455
Calcium Pantothenate	6.0	12.0	18.0	LOff	3	99.9994	0.0076	0.1599
Cyanocobalamin	0.1	0.5	1.0	LOff	3	99.9865	0.0010	1.3293
Folic acid	0.1	0.2	0.3	LOff	3	99.9998	-0.0150	1.6203
Riboflavin	1.5	3.5	5.0	LOff	3	99.8485	-0.0868	0.8248
Benzoate	5.0	10.0	15.0	LOff	3	100.0000	0.0928	0.6667
Retinol acetate	25.0	35.0	50.0	LOff	3	99.9989	0.0687	0.0949
α-Tocopherol acetate	25.0	35.0	50.0	LOff	3	99.9154	0.1184	0.0813

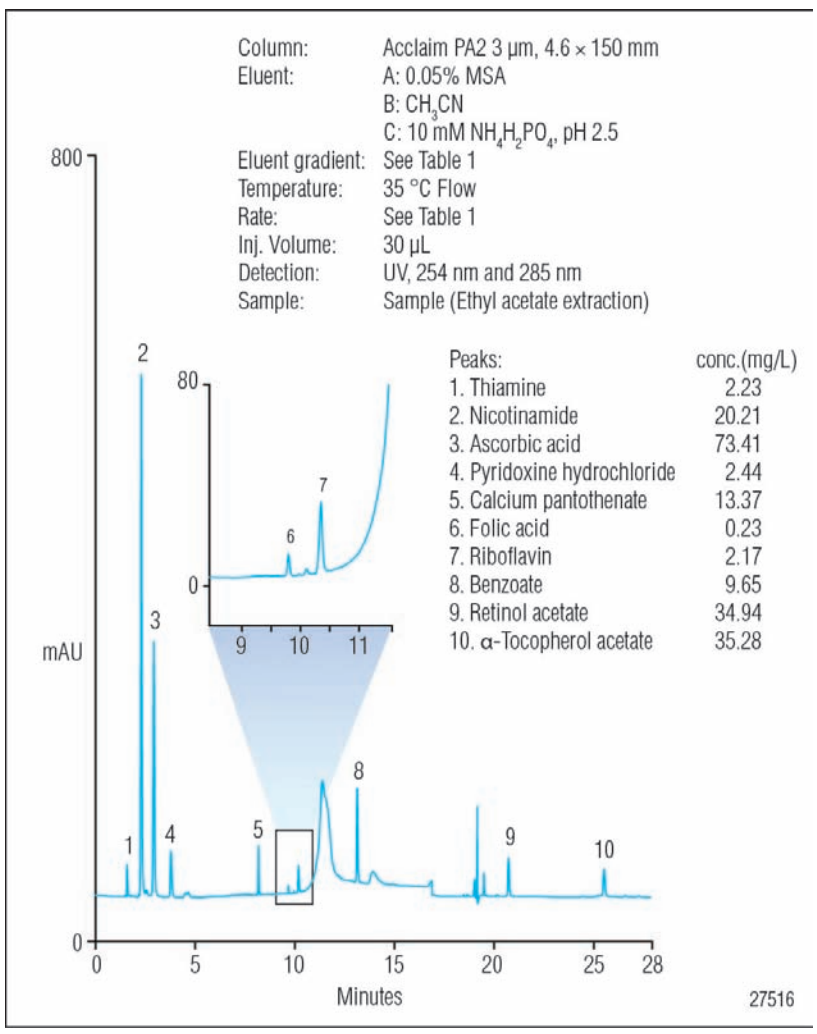

Figure 2. Chromatogram of the dry syrup sample (ethyl acetate extraction).

containing benzoate was injected at 0.0 minute. After separation, the flow rate was increased to 1.5mL/min and CH_3CN was increased to 95% for several minutes, then the FSV were injected.

Table 6 shows that the resolution of all compounds was greater than 2.78. Spectral-matching data in the same table suggest that each peak represents one compound. *Figure 1* shows the separation of both sets of vitamins and benzoate using ethyl acetate for extracting the FSV from the level 3 working standard.

Method Calibration

Before sample analysis, a three-point calibration was prepared for each vitamin and each extraction method. The calibration data in *Tables 2* and *7* show linear peak area response for each vitamin in the specified concentration range using either extraction method.

Sample Analysis

The multivitamin dry syrup sample and the same product without added vitamins (the placebo) were provided by a customer. Both samples were prepared as described on the label before using the sample preparation described here. The product label values were used to judge the success of the assay. The authors also compared the extraction of FSV using either DMSO or ethyl acetate. The original work was performed with DMSO, but there was concern that DMSO could damage the column, so extraction with ethyl acetate was evaluated. *Figure 2* shows the chromatogram of the sample extracted with

Table 8.

Table 8. Vitamin Recovery from the Placebo: Comparison of DMSO and Ethyl Acetate Extractions

Vitamin	Spiked Concentration (mg/L)	DMSO Extraction			Ethyl Acetate Extraction		
		Average Found Concentration of 3 Preparations (mg/L)	RSD	Recovery (%)	Average Found Concentration of 3 Preparations (mg/L)	RSD	Recovery (%)
Thiamine	2.0	2.0	1.10	100	2.0	0.63	100
Nicotinamide	20.0	18.5	0.17	92.5	18.5	0.32	92.5
Ascorbic acid	70.0	70.0	0.49	100	71.6	0.29	102
Pyridoxine hydrochloride	2.0	2.1	0.45	105	2.1	0.39	105
Calcium pantothenate	10.0	10.6	0.27	106	10.6	0.42	106
Cyanocobalamin	0.1	0.1	1.99	100	0.1	1.29	100
Folic acid	0.2	0.2	3.58	100	0.2	2.84	100
Riboflavin	2.0	2.0	1.40	100	2.0	1.58	100
Benzoate	10.0	9.8	0.39	98.0	9.9	0.25	99.0
Retinol acetate	41.7	31.1	1.62	74.6	38.1	0.89	91.4
α-Tocopherol acetate	41.7	34.1	2.99	81.8	35.3	2.09	84.7

Table 9.

Table 9. Sample Peak Purity Result and Spectral Matching with the Spectral Library

Vitamin	DMSO Extraction					Ethyl Acetate Extraction				
	Match	% RSD Match	PPI	% RSD PPI	Match with Library	Match	% RSD Match	PPI	% RSD PPI	Match with Library
Thiamine	999	1.34	232.6	0.56	999.87	1000	0.24	229.5	0.10	999.87
Nicotinamide	1000	1.02	215.9	0.47	999.71	1000	0.67	215.4	0.31	991.74
Ascorbic acid	1000	0.05	231.1	0.02	999.93	1000	0.02	221.8	0.01	999.94
Pyridoxine hydrochlo-ride	1000	0.56	219.4	0.25	999.95	999	0.63	240.9	0.26	999.97
Calcium pantothenate	1000	0.11	192.9	0.05	999.97	998	1.14	194.0	0.55	999.98
Cyanocobalamin	992	3.76	248.2	1.21	995.95	993	3.84	251.5	1.35	996.59
Folic acid	1000	0.92	281.8	0.32	999.93	1000	1.01	274.0	0.37	999.93
Riboflavin	999	0.37	208.7	0.17	997.13	1000	0.03	208.5	0.01	999.09
Benzoate	999	0.43	312.8	0.12	999.95	999	1.15	302.4	0.37	999.98
Retinol acetate	1000	0.10	196.3	0.05	999.91	999	0.38	196.5	0.18	998.48
α-Tocopherol acetate	999	1.34	232.6	0.56	999.87	1000	0.24	229.5	0.10	999.87

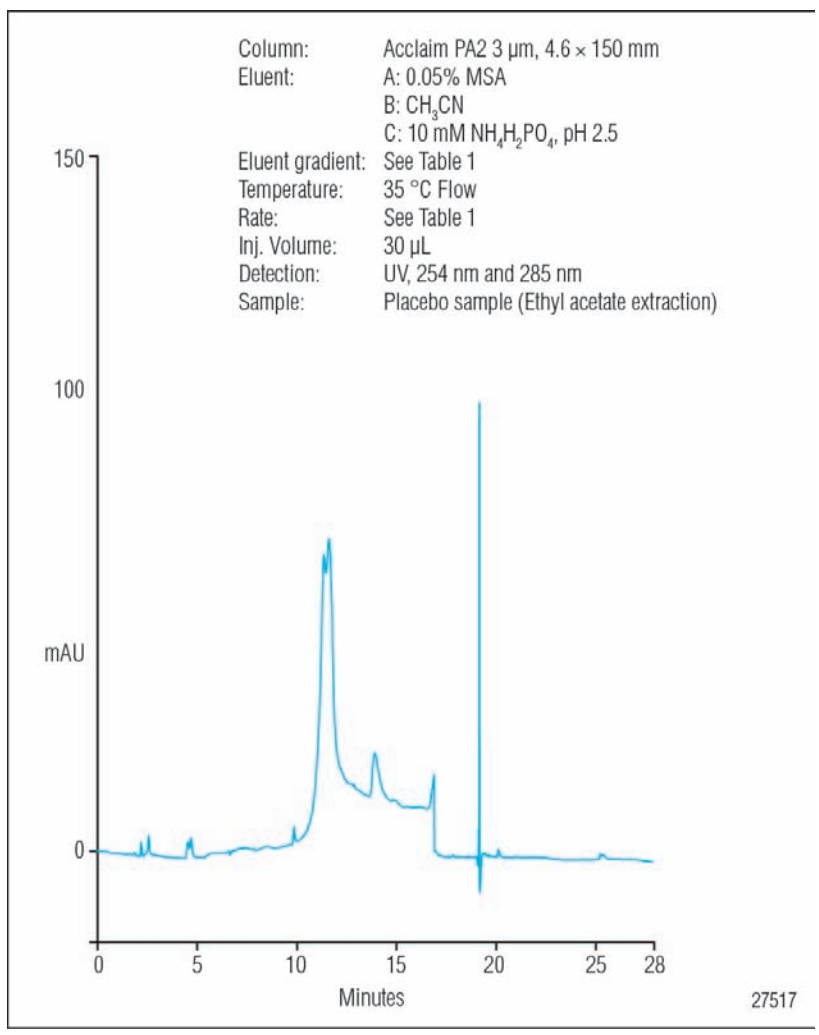

Figure 3. Chromatogram of the placebo sample (ethyl acetate extraction).

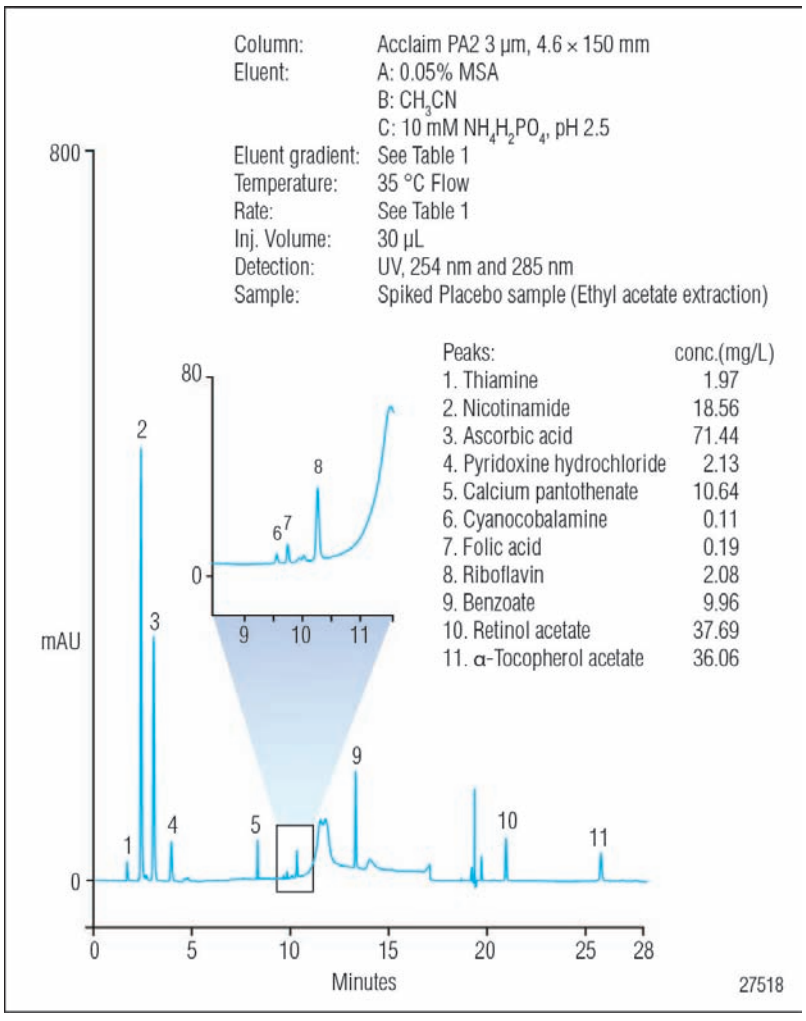

Figure 4. Chromatogram of the spiked placebo sample (ethyl acetate extraction)

ethyl acetate (chromatograms from the DMSO extraction are equivalent and, therefore, not presented). The amounts of WSV determined ranged from 100 to 134%. These values suggest the assay is accurate due to over-fortification. For the FSV, the assay measured 93.3% and 98.7% of the labeled value for vitamin E using DMSO and ethyl acetate extractions, respectively.

A very large amount of vitamin A was found in this FSV sample, compared to the label value. There were no anomalies in the recovery and peak purity results (*Tables 8* and *9*), so perhaps a mistake was made during preparation of the original sample. Each sample was prepared three times to evaluate reproducibility. Reproducibility and assay results are shown in *Table 4*.

To evaluate recovery, individual vitamins were added to the placebo sample prior to sample preparation in order to achieve a final concentration equivalent to the amount expected in the sample (see Spiked Placebo Sample Preparation). Recoveries for both extraction methods ranged from 74.6 to 106%. The recoveries of FSV by DMSO and

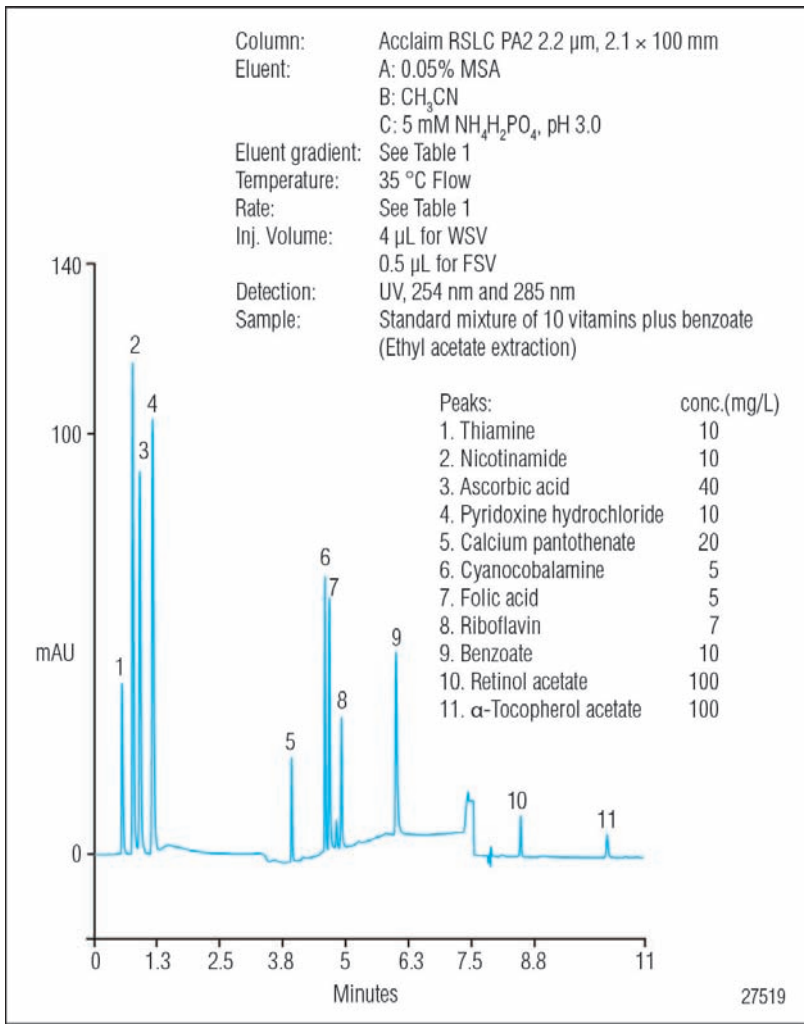

Figure 5. Chromatogram of a mixture of 10 vitamins plus benzoate (ethyl acetate extraction)

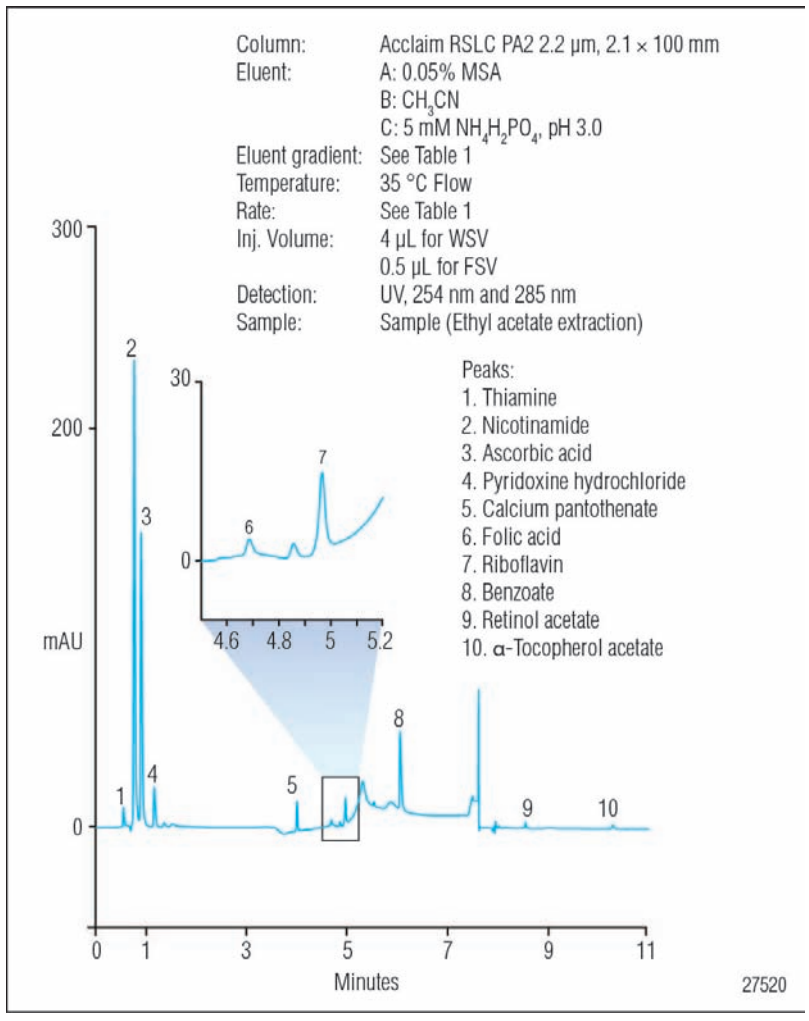

Figure 6. Chromatogram of the dry syrup sample (ethyl acetate extraction)

ethyl acetate extractions were evaluated in triplicate, and the recovery results were between 74.6 to 81.8% and 87.7 to 91.4%, respectively.

Recoveries and reproducibility results are reported in *Table 8*. *Figure 3* shows chromatography of the placebo sample after ethyl acetate extraction, and *Figure 4* shows chromatography of the placebo spiked with the mixed vitamin standard. Although results from the two extraction techniques are similar, ethyl acetate is recommended because injecting DMSO may shorten column lifetime.

Faster Analysis

The Acclaim PA2 column is available in a 2.2µm particle size and a 2.1 × 100mm format. Therefore, it is possible to accelerate the vitamin separation on an UltiMate 3000 Rapid Separation LC (RSLC) system, saving both analysis time and solvent usage. *Figure 5* shows the result of the method acceleration. Run time was reduced from 28 to 11 min, and flow was reduced 60%. The RSLC method uses 5.3mL of mobile phase over the 11 min run time, compared to 34mL for the conventional method. This represents a significant savings in solvent use and reduction in waste production. *Figure 6* demonstrates that the faster method is also successful for analysing the dry syrup sample.

Conclusion

The Acclaim PA2 column can successfully analyse a sample from 100% aqueous to 100% organic solvent, thereby allowing water- and fat-soluble vitamins to be separated in a single analysis. This method is judged accurate, based on analysis of multivitamin dry syrup and a spiked placebo product. The Acclaim PA2 column, combined with an UltiMate 3000 system, is an excellent solution for vitamin determinations.