DIONEX

Application Note 252

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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.

Vitamins are a chemically diverse set of compounds varying in size, structure, and other properties. They are generally classified by their water solubility, with the classifications of water-soluble and fat-soluble (water-insoluble). Differences in chemical properties, water solubility, and sample concentrations make it difficult to analyze all vitamins in all samples using a single chromatography method.

In AN 216, both water-soluble vitamins (WSV) and fat-soluble vitamins (FSV) were determined in bottled waters fortified with vitamins.[1] In these products, the FSV stay in solution as a result of other additives. AN 216 showed that the Acclaim® PA2 column, which features a polar-embedded phase, is ideal for vitamin determinations. The Acclaim PA2 column is compatible with fully aqueous eluents (making it ideal for retaining the more polar vitamins such as vitamin B_6) and fully organic mobile phases (ideal for retaining FSV). The column is also compatible with a low-pH mobile phase that allows suppression or partial suppression of ionization, depending on the pH, for vitamins that are anionic at neutral pH (e.g., vitamin C).

AN 216 covers determination of vitamins B_3 (the nicotinamide and nicotinic acid forms), B_5 (pantothenic acid), B_6 (pyridoxine), B_9 (folic acid), B_{12} (cyanocobalamine), A (retinol), C (ascorbic acid), and E (α-tocopherol) in vitamin-fortified bottled waters. This newer work covers determination of the same vitamins studied in AN 216, plus vitamins B_1 (thiamine) and B_2 (riboflavin) in a dry syrup. This determination also uses the Acclaim PA2 column, albeit with a different mobile phase; rather than the formic acid/methanol/acetonitrile mobile phase used in AN 216, the separation reported here uses a methanesulfonic acid/ammonium phosphate/acetonitrile mobile phase.

Vitamins were extracted from the dry syrup prior to analysis. The WSV were extracted with water and a pH adjustment with KOH to dissolve folic acid. The 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 × 100 mm format to save time, reduce mobile phase consumption, and reduce waste. Like AN 216, 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

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

Acetonitrile (CH_3CN), 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, 3 µm, 4.6 × 150 mm (P/N 063191), Acclaim PA2 Guard, 5 µm, 4.3 × 10 mm (P/N 063195) Acclaim Guard Kit (P/N 059526)

Mobile Phase: A: 0.05% MSA
B: CH_3CN
C: 10 mM $NH_4H_2PO_4$ 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 210 nm, 285 nm, wavelength scanning 200–800 nm, data collection rate 5 Hz, rise time 0.5 sec

Gradient: Table 1

UHPLC

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

Mobile Phase: A: 0.05% MSA
B: CH_3CN
C: 5 mM $NH_4H_2PO_4$ 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, 285 nm, data collection rate 10 Hz, response time 0.5 sec

Gradient: 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; for example, see the commands in red at 17 min in the program in Appendix A.

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

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phases

Mobile Phase A (0.05% MSA)

Weigh 999.5 g 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 1.15 g ammonium di-hydrogen orthophosphate into a 250 mL beaker, add 100 mL water, stir until completely 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 0.01 g of each vitamin into separate 10 mL volumetric flasks, add 5 mL water, and swirl the flask until dissolved. Prepare the preservative (sodium benzoate) in the same manner. To dissolve folic acid, add 10 µL of 8 M KOH. Bring to volume with water.

FSV standard solutions in ethyl acetate

Weigh 0.01 g (0.02 g for α-tocopherol) of standard in separate 50 mL glass bottles, add 2 mL water, add 10 mL 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.

FSV standard solutions in DMSO

Weigh 0.01 g (0.02 g for α-tocopherol) of standard in separate 50 mL glass bottles, add 10 mL DMSO, and place in an ultrasonic bath for 10 to15 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.

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 just prior to the analysis. Store these solutions in brown bottles and use brown vials for analysis.

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

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 (45 mL) 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.

Sample Preparation for WSV Analysis

Shake the sample bottle and pipet 0.25 mL of sample, wipe the outside of the pipette, dispense into a 25 mL volumetric flask, rinse the inside of the pipette with 0.25 mL 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 0.5 mL of sample, wipe the outside of the pipette, dispense into a 50 mL glass bottle, rinse the inside of the pipette with 0.5 mL water, add 5 mL ethyl acetate, and then cap the bottle. Place the capped bottle in an ultrasonic bath for 10 min, shake for few minutes, and then wait until the layers are completely separated. Pipet 1 mL of the top layer and dispense into 3 mL CH_3CN.

Sample Preparation for FSV Analysis (DMSO Extraction)

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

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

The label states “Add water, shake, and then continue to add water to reach the mark on the side of the bottle.” Table 4 shows the composition of 5 mL of a correctly prepared sample.

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. 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 24 g of placebo into an empty bottle and add accurately weighed vitamin standards to the same bottle (except vitamin B_{12} and folic acid, which are added later using the 1000 mg/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}, 5 µL and 2.5 µL of the 1000 mg/L standards, respectively, were added to the 25 mL volumetric flask during the WSV sample preparation.

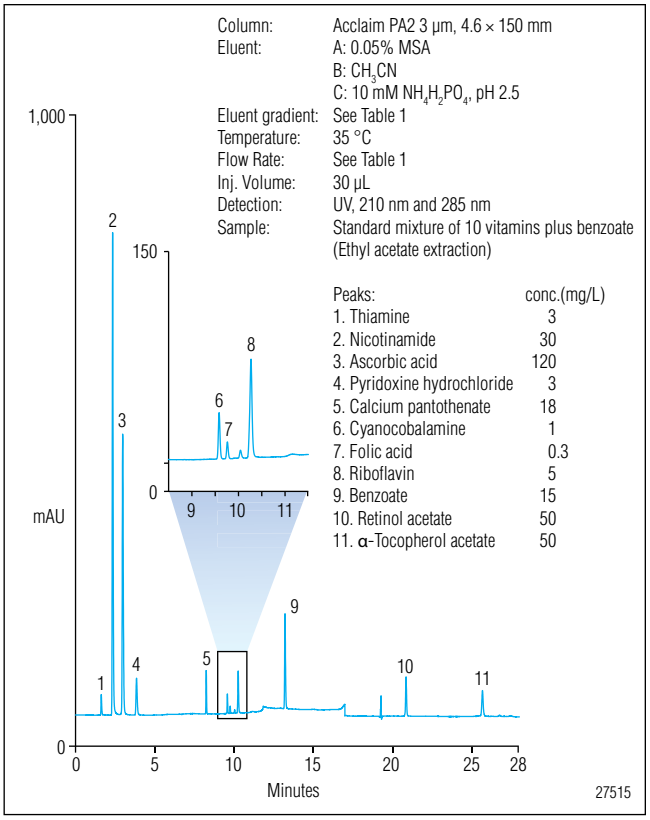


Figure 1. Chromatogram of a standard mixture of 10 vitamins plus benzoate (ethyl acetate extraction).

RESULTS AND DISCUSSION

Separation and Detection

This application uses the Acclaim PA2 column to separate water- and fat-soluble vitamins[1] 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 containing benzoate was injected at 0.0 minute. After separation, the flow rate was increased to 1.5 mL/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.

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

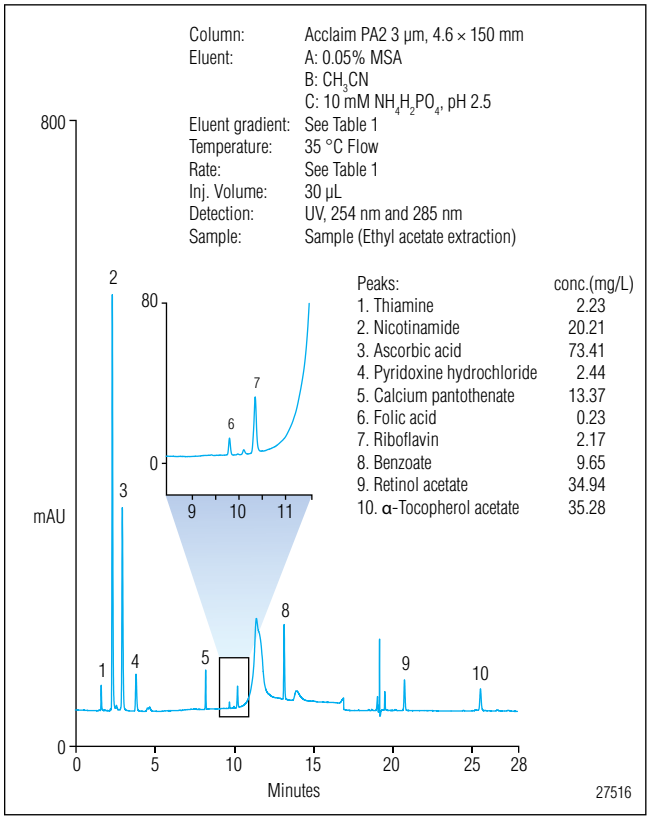


Figure 2. Chromatogram of the dry syrup sample (ethyl acetate extraction).

Method Calibration

Before sample analysis, a three-point calibration was prepared for each vitamin and each extraction method. The concentration range of each vitamin was chosen so that the sample concentration would fall in the middle of that range. 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 showed the amount of each vitamin in 5 mL, and the authors used those values 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 samples extracted using DMSO could damage the column, so extraction with ethyl acetate was also evaluated. Figure 2 shows the chromatogram of the sample extracted with ethyl acetate (chromatograms from the DMSO extraction are equivalent to those obtained for ethyl acetate extraction and, therefore, are not presented). The amounts of WSV determined ranged between 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.

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. 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 hydrochloride	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

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.

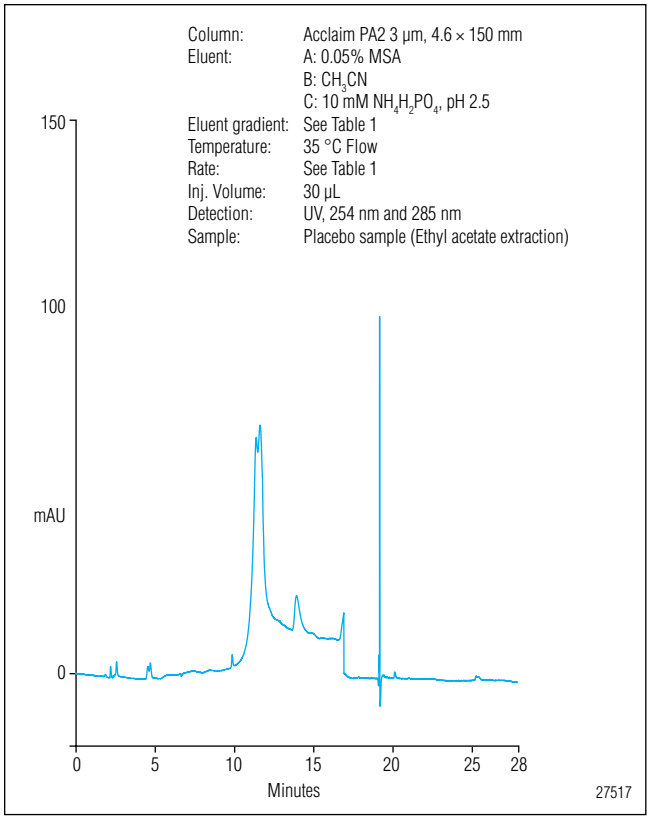


Figure 3. Chromatogram of the placebo sample (ethyl acetate extraction).

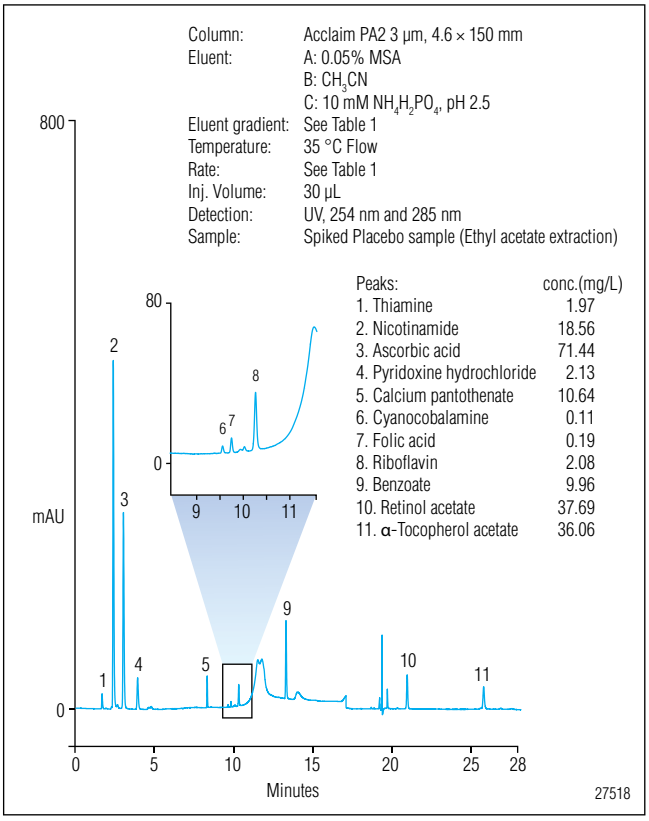


Figure 4. Chromatogram of the spiked placebo sample (ethyl acetate extraction).

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 level 2 calibration standard, or 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 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 on the column may shorten column lifetime, compared to ethyl acetate.

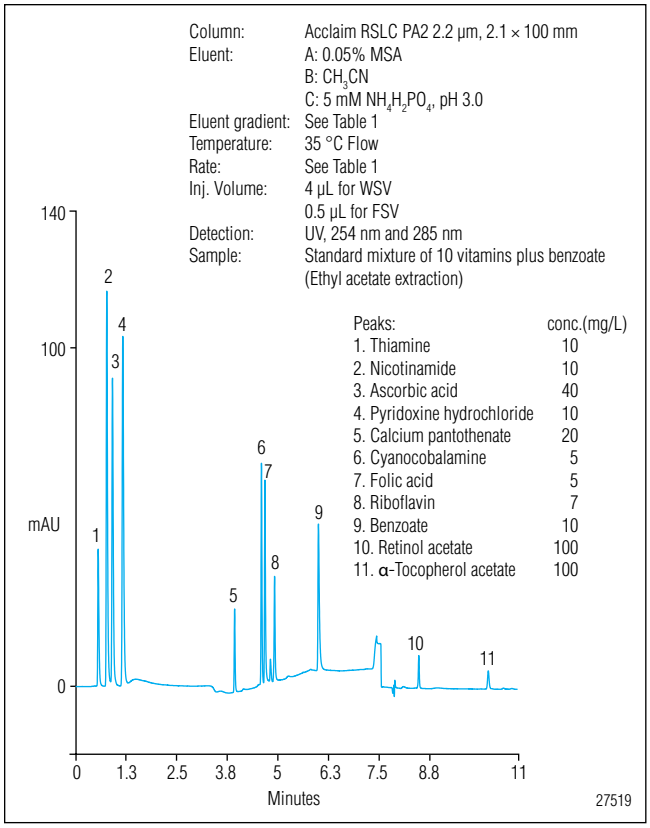


Figure 5. Chromatogram of a mixture of 10 vitamins plus benzoate (ethyl acetate extraction).

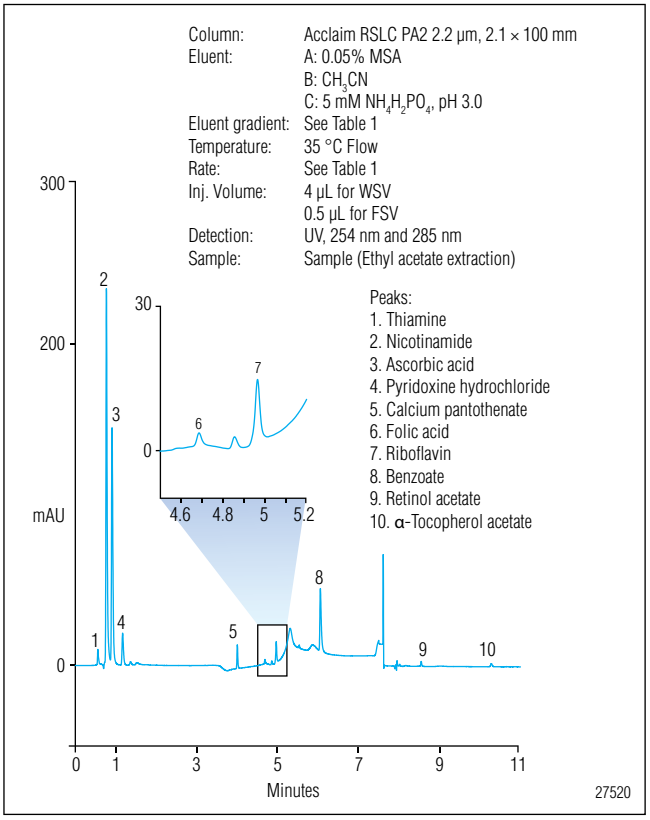


Figure 6. Chromatogram of the dry syrup sample (ethyl acetate extraction).

Faster Analysis

The Acclaim PA2 column is available in a 2.2 µm particle size and a 2.1 × 100 mm 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 using the standard extracted with ethyl acetate. Run time was reduced from 28 to 11 min, and flow was reduced 60%. The RSLC method uses 5.3 mL of mobile phase over the 11 min run time, compared to 34 mL 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 analyzing the dry syrup sample. Because the authors used a smaller column and had more efficient peaks, the sample size was reduced from 30 to 4 µL for WSV and from 30 to 0.5 µL for FSV.

CONCLUSION

The Acclaim PA2 column can successfully analyze a sample from 100% aqueous to 100% organic solvent, thereby allowing water- and fat-soluble vitamins to be separated in a single analysis. The Dionex UltiMate 3000 system and Chromeleon software facilitate this analysis by allowing multiple injections during the same run. 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.

REFERENCE

1. Dionex Corporation, *Determination of Water- and Fat-Soluble Vitamins in Functional Waters by HPLC with UV-PDA Detection.* Application Note 216, LPN 2145, 2009, Sunnyvale, CA.

APPENDIX A:

Example program file

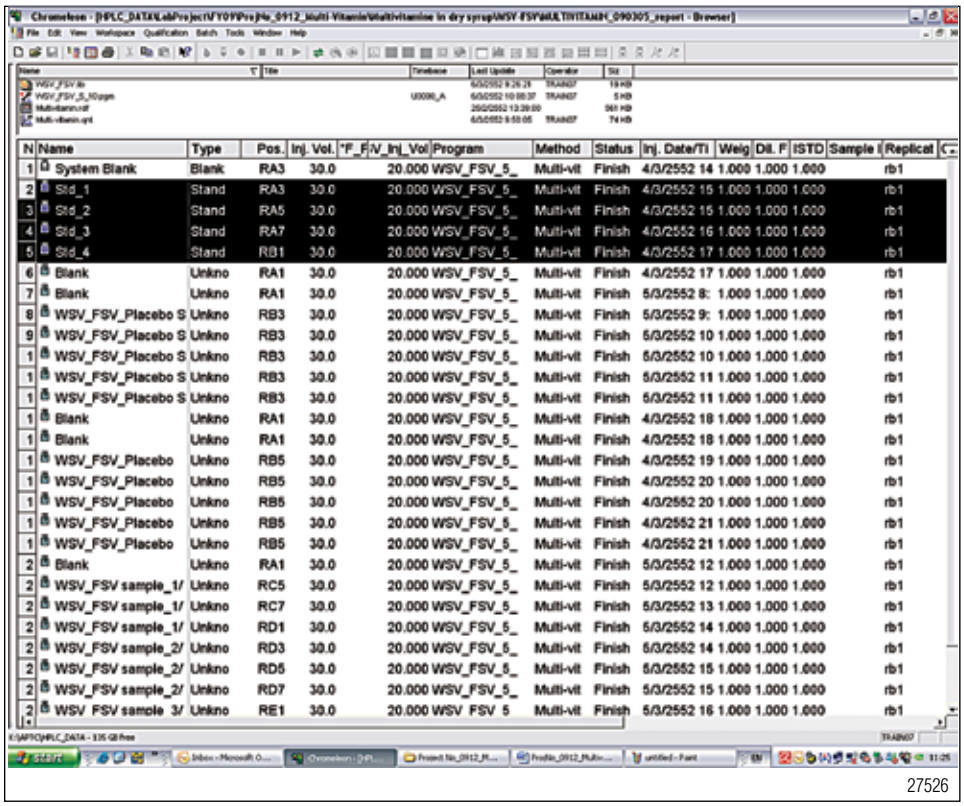
```
Sampler.TempCtrl =                  On
Sampler.Temperature.Nominal =       10.0 [°C]
Sampler.Temperature.LowerLimit =    4.0 [°C]
Sampler.Temperature.UpperLimit =    45.0 [°C]
Sampler.ReadyTempDelta =            1.0 [°C]
ColumnOven.TempCtrl =               On
ColumnOven.Temperature.Nominal =    35.0 [°C]
ColumnOven.Temperature.LowerLimit = 5.0 [°C]
ColumnOven.Temperature.UpperLimit = 85.0 [°C]
EquilibrationTime =                 0.5 [min]
ColumnOven.ReadyTempDelta =         0.5 [°C]
Column_A.ActiveColumn =             No
Column_B.ActiveColumn =             Yes
Column_B.SystemPressure =           "PumpRight"
Column_C.ActiveColumn =             No
Column_D.ActiveColumn =             No
PumpLeft.Pressure.LowerLimit =      0 [bar]
PumpLeft.Pressure.UpperLimit =      345 [bar]
PumpLeft.MaximumFlowRampDown =      3.000 [ml/min²]
PumpLeft.MaximumFlowRampUp =        3.000 [ml/min²]
PumpLeft.%A.Equate =                "%A"
PumpLeft.%B.Equate =                "%B"
PumpLeft.%C.Equate =                "%C"
PumpRight.Pressure.LowerLimit =     0 [psi]
PumpRight.Pressure.UpperLimit =     4000 [psi]
PumpRight.MaximumFlowRampDown =     3.000 [ml/min²]
PumpRight.MaximumFlowRampUp =       3.000 [ml/min²]
PumpRight.%A.Equate =               "0.05%MSA"
PumpRight.%B.Equate =               "ACN"
PumpRight.%C.Equate =               "10mM NH4H2PO4_pH2.5 with MSA"
DrawSpeed =                         3.000 [µl/s]
DrawDelay =                         3000 [ms]
DispSpeed =                         20.000 [µl/s]
DispenseDelay =                     0 [ms]
WasteSpeed =                        20.000 [µl/s]
SampleHeight =                      0.100 [mm]
InjectWash =                        AfterDraw
WashVolume =                        100.000 [µl]
WashSpeed =                         20.000 [µl/s]
PunctureOffset =                    0.0 [mm]
PumpDevice =                        "PumpRight"
InjectMode =                        Normal
SyncWithPump =                      On
PumpRight_Pressure.Step =           Auto
PumpRight_Pressure.Average =        On
Data_Collection_Rate =              5.00 [Hz]
Rise_Time =                         0.50 [s]
UV_VIS_1.Wavelength =               210 [nm]
UV_VIS_1.Bandwidth =                2 [nm]
UV_VIS_1.RefWavelength =            Off
UV_VIS_1.RefBandwidth =             1 [nm]
UV_VIS_2.Wavelength =               270 [nm]
UV_VIS_2.Bandwidth =                2 [nm]
```

```
            UV_VIS_2.RefWavelength =            Off
            UV_VIS_2.RefBandwidth =             1 [nm]
            UV_VIS_3.Wavelength =               280 [nm]
            UV_VIS_3.Bandwidth =                2 [nm]
            UV_VIS_3.RefWavelength =            Off
            UV_VIS_3.RefBandwidth =             1 [nm]
            UV_VIS_4.Wavelength =               360 [nm]
            UV_VIS_4.Bandwidth =                2 [nm]
            UV_VIS_4.RefWavelength =            Off
            UV_VIS_4.RefBandwidth =             50 [nm]
            UV_VIS_5.Wavelength =               380 [nm]
            UV_VIS_5.Bandwidth =                2 [nm]
            UV_VIS_5.RefWavelength =            Off
            UV_VIS_5.RefBandwidth =             50 [nm]
            3DFIELD.RefWavelength =             750 [nm]
            3DFIELD.RefBandwidth =              2 [nm]
            PumpLeft.Flow =                     0.000 [ml/min]
            PumpLeft.%B =                       100.0 [%]
            PumpLeft.%C =                       0.0 [%]
            PumpLeft.Curve =                    5
            3DFIELD.MinWavelength =             190 [nm]
            3DFIELD.MaxWavelength =             800 [nm]
            3DFIELD.BunchWidth =                2 [nm]

-7.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]

 0.000      Autozero
            Wait                                AZ_Done
            Wait                                ColumnOven.Ready and Sampler.Ready
            Inject
            PumpRight_Pressure.AcqOn
            UV_VIS_1.AcqOn
            UV_VIS_2.AcqOn
            UV_VIS_3.AcqOn
            UV_VIS_4.AcqOn
            UV_VIS_5.AcqOn
            3DFIELD.AcqOn

 3.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]

 3.100      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      100.0 [%]

 9.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      30.0 [%]
            PumpRight.%C =                      70.0 [%]
```

```
 9.500      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      55.0 [%]

13.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      55.0 [%]

13.100      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      40.0 [%]
            PumpRight.%C =                      0.0 [%]

15.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      45.0 [%]
            PumpRight.%C =                      0.0 [%]

16.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      95.0 [%]
            PumpRight.%C =                      0.0 [%]

17.000      UV_VIS_1.Wavelength =               285 [nm]

18.000      Position =                          Position+1
            Volume =                            30
            Inject

21.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      95.0 [%]
            PumpRight.%C =                      0.0 [%]

22.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      100.0 [%]
            PumpRight.%C =                      0.0 [%]

27.000      PumpRight.Flow =                    1.500 [ml/min]
            PumpRight.%B =                      100.0 [%]
            PumpRight.%C =                      0.0 [%]

28.000      PumpRight.Flow =                    1.000 [ml/min]
            PumpRight.%B =                      0.0 [%]
            PumpRight.%C =                      0.0 [%]
            PumpRight_Pressure.AcqOff
            UV_VIS_1.AcqOff
            UV_VIS_2.AcqOff
            UV_VIS_3.AcqOff
            UV_VIS_4.AcqOff
            UV_VIS_5.AcqOff
            3DFIELD.AcqOff
            End
```

Note: The second injection comes from Position=Position+1, Volume=30 and Inject commands. In the command Position=Position+1, Position is the current position in the autosampler (water-soluble vitamins position), so the Position+1 is the next position (fat-soluble vitamins position).

For example, in the figure below, the sequence lines for standard injections are 2 through 5; autosampler positions RA3, RA5, RA7, and RB1 have water-soluble vitamin standards; RA4, RA6, RA8, and RB2 have fat-soluble vitamin standards.



N	Name	Type	Pos.	Inj. Vol.	*F_F	V_Inj_Vol	Program	Method	Status	Inj. Date/Ti	Weig	Dil. F	ISTD	Sample I	Replicat
1	System Blank	Blank	RA3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 14	1.000	1.000	1.000		rb1
2	Std_1	Stand	RA3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 15	1.000	1.000	1.000		rb1
3	Std_2	Stand	RA5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 15	1.000	1.000	1.000		rb1
4	Std_3	Stand	RA7	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 16	1.000	1.000	1.000		rb1
5	Std_4	Stand	RB1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 17	1.000	1.000	1.000		rb1
6	Blank	Unkno	RA1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 17	1.000	1.000	1.000		rb1
7	Blank	Unkno	RA1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 8:	1.000	1.000	1.000		rb1
8	WSV_FSV_Placebo S	Unkno	RB3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 9:	1.000	1.000	1.000		rb1
9	WSV_FSV_Placebo S	Unkno	RB3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 10	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo S	Unkno	RB3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 10	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo S	Unkno	RB3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 11	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo S	Unkno	RB3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 11	1.000	1.000	1.000		rb1
1	Blank	Unkno	RA1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 18	1.000	1.000	1.000		rb1
1	Blank	Unkno	RA1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 18	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo	Unkno	RB5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 19	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo	Unkno	RB5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 20	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo	Unkno	RB5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 20	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo	Unkno	RB5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 21	1.000	1.000	1.000		rb1
1	WSV_FSV_Placebo	Unkno	RB5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	4/3/2552 21	1.000	1.000	1.000		rb1
2	Blank	Unkno	RA1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 12	1.000	1.000	1.000		rb1
2	WSV_FSV sample_1/	Unkno	RC5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 12	1.000	1.000	1.000		rb1
2	WSV_FSV sample_1/	Unkno	RC7	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 13	1.000	1.000	1.000		rb1
2	WSV_FSV sample_1/	Unkno	RD1	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 14	1.000	1.000	1.000		rb1
2	WSV_FSV sample_2/	Unkno	RD3	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 14	1.000	1.000	1.000		rb1
2	WSV_FSV sample_2/	Unkno	RD5	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 15	1.000	1.000	1.000		rb1
2	WSV_FSV sample_2/	Unkno	RD7	30.0		20.000	WSV_FSV_5_	Multi-vit	Finish	5/3/2552 15	1.000	1.000	1.000		rb1
2	WSV_FSV sample_3/	Unkno	RE1	30.0		20.000	WSV_FSV_5	Multi-vit	Finish	5/3/2552 16	1.000	1.000	1.000		rb1

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