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Technical Note 89

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Determination of Water- and Fat-Soluble Vitamins by HPLC

INTRODUCTION

Vitamins are a well-known group of compounds that are essential for human health and are classified into two main groups, water-soluble and fat-soluble. Water-soluble vitamins include B group vitamins (thiamine/B_1, riboflavin/B_2, nicotinamide/nicotinic acid/B_3, pantothenic acid/B_5, pyridoxine/pyridoxal hydrochloride/B_6, folic acid/B_9, and cyanocobalamine/B_{12}) and ascorbic acid (vitamin C). Fat-soluble vitamins include mainly retinol (vitamin A), tocopherol (vitamin E), radiostol (vitamin D), and antihemorrhagic vitamins (vitamin K). These vitamins play specific and vital functions in metabolism, and their lack or excess can cause health problems. The supply of vitamins 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 multivitamin tablets and/or consume milk powder and vitamin-fortified beverages to supplement their diet. To ensure that these foods and tablets contain the labeled amounts of vitamins, there needs to be a quality control assay for them.

Water-soluble vitamins are added selectively based on the average minimum daily requirement. For example, most B group vitamins and vitamin C can be found on the label of milk powders for pregnant women and infants; also, a large amount of vitamin C is found in sports drinks.

Commonly added fat-soluble vitamins are vitamins A, E, D, K, and ß-carotene. Vitamins A and E are usually added in their acetate form and sometimes, vitamin A is added in the palmitate form. Vitamins A and E are rarely added directly. Vitamin D is added either as D_3 (cholecalciferol) or D_2 (ergocalciferol). Both forms are rarely added to the same product.

HPLC Methods for Water- and Fat-Soluble Vitamin Analysis

Traditional HPLC Method

Reversed-phase HPLC is a well-suited technique for vitamin analysis.[1] In typical regulated HPLC methods[2,3] and commonly reported HPLC methods,[4–7] water-soluble vitamins are determined using an aqueous mobile phase with low-organic solvent content, whereas fat-soluble vitamins are determined using organic solvent mobile phases. This is due to their different solubility and reversed-phase retention properties. Commonly used buffers for the separation of water-soluble vitamins are phosphate, formic acid, and acetic acid. Non-aqueous reversed-phase (NARP) retention is commonly used for fat-soluble vitamins so that the vitamins are soluble throughout the analysis. A typical NARP mobile phase consists of a polar solvent (acetonitrile), a solvent with lower polarity (e.g., dichloromethane) to act as a solubilizer and to control retention by adjusting the solvent strength, and a third solvent with hydrogen-bonding capacity (e.g., methanol) to optimize selectivity.[1]

Reported HPLC Method

There are numerous methods for the simultaneous determination of water- and fat-soluble vitamins. Dionex has reported an HPLC method for the analysis of functional waters in the simultaneous determination of water- and fat-soluble vitamins.[8] The vitamins were separated on the Acclaim® PolarAdvantage (PA) II column with a single injection using an aqueous-to-nonaqueous mobile phase gradient; however, due to large differences in sample preparation methods, this method is inefficient in the analysis of solid samples, such as multivitamin tablets. The sample preparation requires more than one solvent to extract both water- or fat-soluble vitamins efficiently; therefore, a single injection from the sample is not possible.

HPLC Method Developed in the Present Work

Based on the Dionex HPLC method for the analysis of vitamins in a dry syrup, the authors tested a method with two injections during the same analysis (injecting the extracts for water- and fat-soluble vitamins, respectively).[9] This double-injection method can resolve the problem of inefficient analyses of multivitamin tablet samples; however, some strongly retained compounds from the first injection can interfere with the fat-soluble vitamin analysis in the second injection. For example, some fat-soluble vitamins, such as ß-carotene and acetate of vitamin A and vitamin E, were found in the extract of water-soluble vitamins. This problem was not observed for the vitamins determined in the dry syrup. To avoid possible interferences with fat-soluble vitamin determination, the authors developed an integrated and efficient dual-mode tandem solution for the simultaneous determination of water- and fat-soluble vitamins in different types of samples, such as multivitamin tablets and beverages.

In the analysis presented here, water and a mixture of dichloromethane and methanol were used for extracting water- and fat-soluble vitamins, respectively. These samples were analyzed using an UltiMate® dual-pump HPLC system consisting of a DGP 3600 pump, WPS 3000TSL autosampler, and VWD-3400RS UV-vis detector. The simultaneous determination was completed in one sequence using the column-switching mode, including valve-switching and a second injection. This process was controlled by the Chromeleon® 6.80 SR7 Chromatography Data System (CDS) software. All the analytes were seen in one chromatogram using the wavelength-switching mode. Reversed-phase HPLC columns—Acclaim PA, PA2, and C18—were used for the separations with an aqueous mobile phase (phosphate buffer-CH_3CN) for water-soluble vitamins and a nonaqueous mobile phase (CH_3OH-CH_3CN-methyl tert-butyl ether) for fat-soluble vitamins. Detection wavelength-switching mode was applied for sensitivity optimization. The proposed solution has the following advantages:

- The simultaneous separation of 21 water- and fat-soluble vitamins can be completed within 25 min.
- Any interference from the first injection is eliminated.
- It is flexible and convenient to select suitable columns for different assay requirements.

PHYSICAL AND CHEMICAL PROPERTIES OF WATER- AND FAT-SOLUBLE VITAMINS AND THEIR CHROMATOGRAPHY

Solubility and Stability of Water- and Fat-Soluble Vitamins

The physical properties of water- and fat-soluble vitamins, such as solubility and stability in different solvents, are summarized in Table 1. Knowledge of these properties is important for sample preparation and analysis.

Riboflavin (vitamin B_2) is easily dissolved in a basic solution but is unstable,[1] so its stock solution must be prepared at the time of use. The freshly prepared stock solution is diluted with DI water to yield a series of riboflavin standard solutions for making the calibration curve. The stability of the standard solutions was investigated. As shown in Figure 1, all three standard

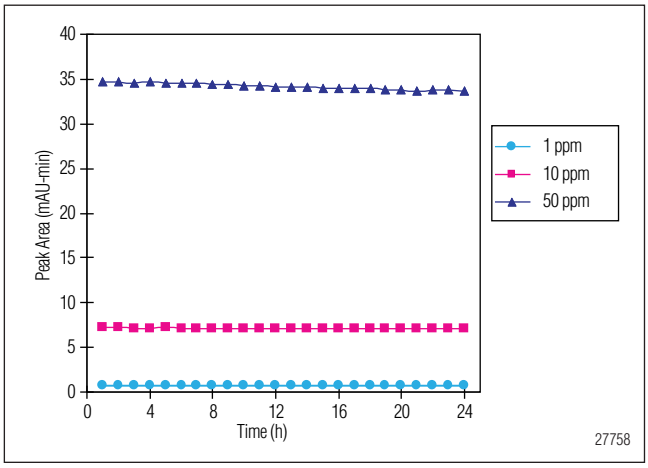

Figure 1. Stability of riboflavin solutions obtained from diluting the stock standard solution with DI water.

solutions with different concentrations, 50, 5, and 1 µg/mL, had sufficient stability over 24 h. There is a small loss in peak area for the most concentrated solution. Peak area RSDs were 0.27% for 5 µg/mL and 0.26% for 1 µg/mL. The peak area RSD for the 50 ug/mL solution is 1.3% but that includes some downward trending. These results demonstrate that the riboflavin standard solutions were sufficiently stable for preparing the calibration curve.

Table 1. Solubility and Stability of Water- and Fat-Soluble Vitamins

Water-Soluble Vitamins	Solubility	Stability	Fat-Soluble Vitamins	Solubility	Stability
Thiamine (vitamin B_1)	Soluble in water; slightly soluble in ethanol; insoluble in ether and benzene.	Stable in acidic solution, unstable in light or being heated.	**Retinol (vitamin A)**	Soluble in ethanol, methanol, chloroform, ethyl-ether, and oil; insoluble in water and glycerol.	Easy oxidation and moisture absorption in the air; easy metamorphism in light; stable in oil.
Riboflavin (vitamin B_2)	Soluble in basic aqueous solution; slightly soluble in water and ethanol; insoluble in chloroform and ether.	Unstable in light, and heating; slightly unstable in basic solution.	**Retinol acetate (vitamin A acetate)/ retinol palmitate (vitamin A palmitate)**	Soluble in chloroform, ethyl ether, cyclohexane, and petroleum ether; slightly soluble in ethanol; insoluble in water.	Easily oxidized in the air; metamorphism in light.
Nicotinamide (vitamin B_3)	Soluble in water, ethanol, and glycerol.	Stable in acidic and basic solutions; stable when exposed to air.	**β-Carotene**	Soluble in chloroform and benzene; insoluble in water, glycerin, propylene glycol, acid, and alkali solutions, ethanol, acetone, and ether.	Unstable when exposed to air and light
Nicotinic acid (vitamin B_3)	Soluble in water.				
Pantothenic acid (vitamin B_5)	Soluble in water, ethanol, alkali carbonate hydroxide solution and alkali solution; insoluble in ether.	Unstable in acidic and basic solutions; unstable when heated; calcium salt is stable.	**Ergocalciferol (vitamin D_2)**	Soluble in alcohol, ether, and chloroform; insoluble in water.	Unstable when exposed to air, light, heating, inorganic acids, and aldehydes.
Pyridoxine/ pyridoxal hydrochloride (vitamin B_6)	Soluble in water, ethanol, methanol, and acetone; insoluble in ether and chloroform.	Stable in acid solution; unstable in alkali solution.	**Cholecalciferol (vitamin D_3)**	Soluble in alcohol, ether, acetone, chloroform, and vegetable oil; insoluble in water.	Normally, vitamin D_3 is more stable than vitamin D_2. Stable stored in a vacuum brown ampoule at 4 °C.
Folic acid (vitamin B_9)	Soluble in alkali solution; slightly soluble in methanol; insoluble in water and ethanol.	Stable when exposed to air; unstable when exposed to light.	**Tocopherol (vitamin E)/tocopherol acetate (vitamin E acetate)**	Soluble in alcohol, ether, acetone, chloroform, and oil; insoluble in water.	Stable in alkali solution and upon heating; slight oxidation in the air; unstable in UV.
Ascorbic acid (vitamin C)	Soluble in water; slightly soluble in ethanol; insoluble in ether.	Unstable when exposed to air.	**Phylloquinone (vitamin K_1)**	Soluble in ether, acetone, and chloroform; slightly soluble in oil and methanol; insoluble in water.	Unstable when exposed to light, acid, oxidizers, and halogen.
Cyanocobalamine (vitamin B_{12})	Soluble in water and ethanol; insoluble in ether, acetone, and chloroform.	Unstable in alkali and strong acid solutions.			

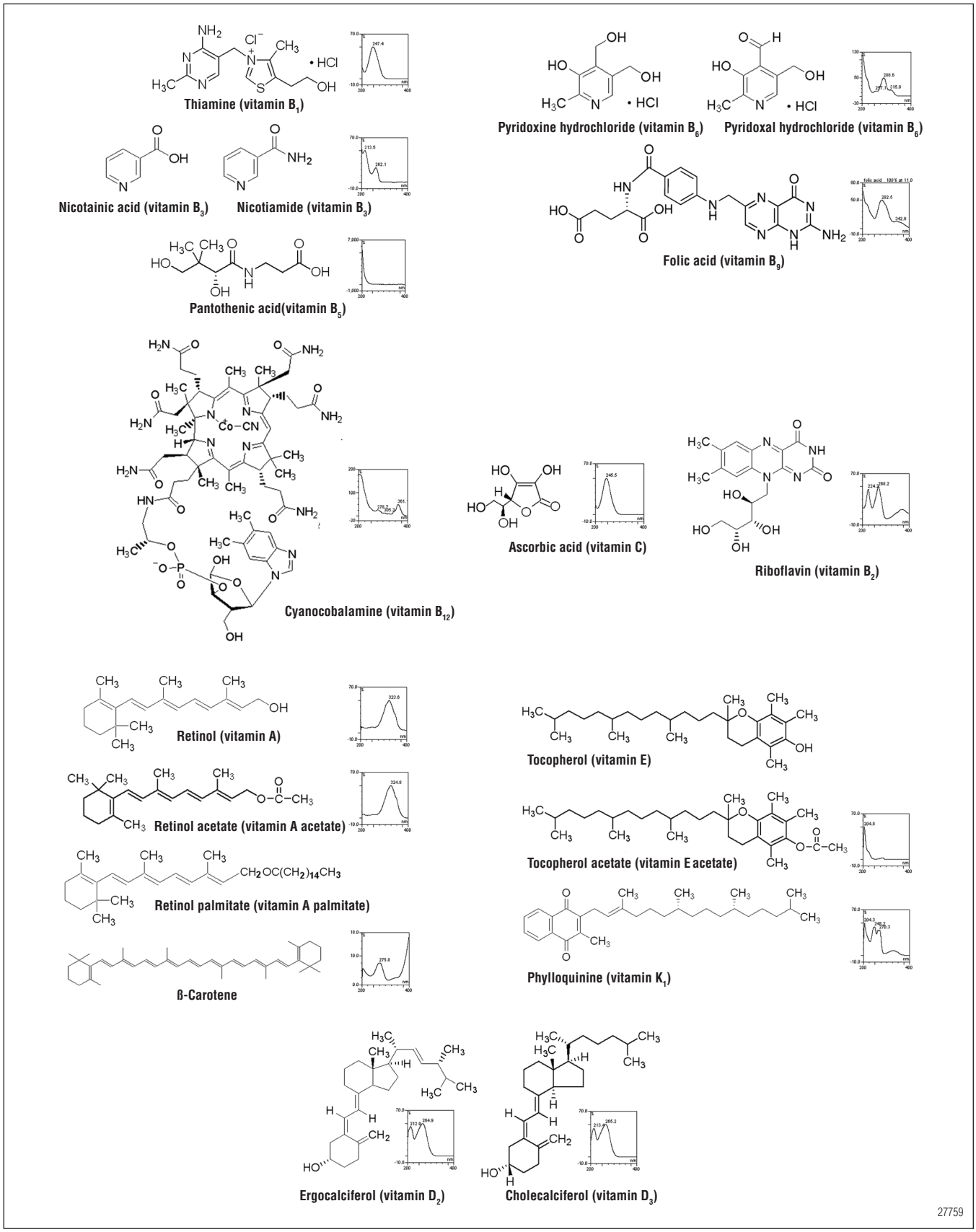

Figure 2. Structures and UV spectra (obtained with the DAD-3000 detector) of water-and fat-soluble vitamins.

Chemical Structures, UV Spectra, and Detection Wavelengths

The UV spectra of water-and fat-soluble vitamins vary significantly due to their multiple structures (Figure 2) and therefore, multiwavelength detection is required for achieving the best sensitivity. Usually, the maximum absorbance is the best choice, but the wavelength selected can be different because at certain wavelengths, impurities may interfere with analyte detection. For example, as shown in Figure 3, impurities may interfere with the detection of vitamin B_6 (peak 1) at 210 nm. Although it has more absorption at 210 nm, vitamin B_6 is best detected at 280 nm where the interferences are eliminated. Another example is the detection of vitamin C. Its maximum absorption is at approximately 245 nm; however, a large amount of vitamin C is usually added to some functional waters (e.g., sports drinks), which may result in the concentration being outside the linear range of calibration. Therefore, detection at other wavelengths (i.e., 254 or 265 nm) may place its concentration in a linear calibration range. Table 2 lists some reported detection wavelengths for water- and fat-soluble vitamins[1] and the detection wavelengths used in the analysis presented here.

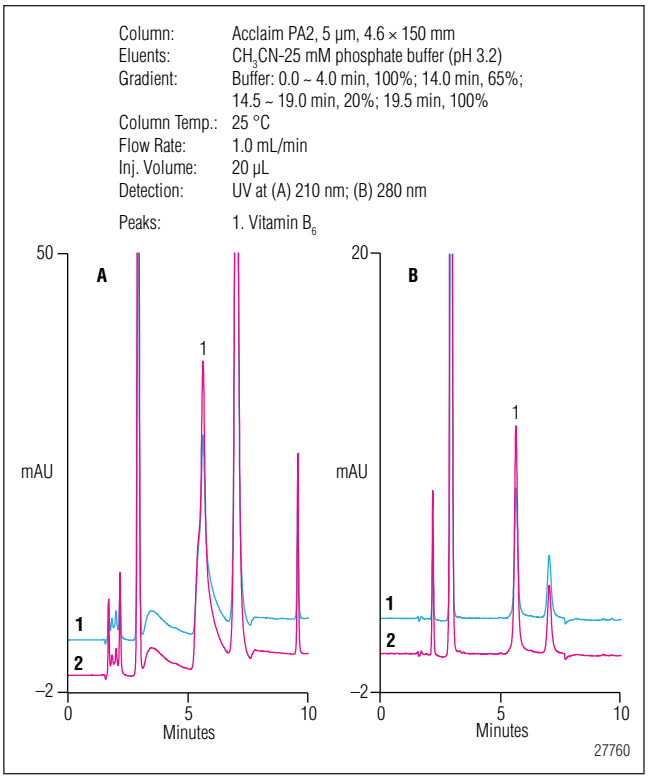

Figure 3. Chromatograms of vitamin B_6 collected at (A) 210 and (B) 280 nm in a multivitamin and mineral supplement tablet. Chromatograms: (1) sample, (2) spiked sample.

Table 2. Detection Wavelengths Reported[1] and Used in This TN

Water-Soluble Vitamin	Detection Wavelength (nm)		Fat-Soluble Vitamin	Detection Wavelength (nm)	
	Reported	Used in This TN		Reported	Used in This TN
Thiamine (vitamin B_1)	248, 254	270	Retinol (vitamin A)	313, 325, 328, 340	325
Riboflavin (vitamin B_2)	254, 268, 270	270	Retinol acetate (vitamin A acetate)	325	325
Nicotinamide (vitamin B_3)	254	260	Retinol palmitate (vitamin A palmitate)	325	325
Nicotinic acid (vitamin B_3)	254	270	β-Carotene	410, 436,450, 453, 458, 470	450
Pantothenic acid (vitamin B_5)	197, 210, 220	210	Ergocalciferol (vitamin D_2)	254, 265, 280, 301	265
Pyridoxal/pyridoxine hydrochloride (vitamin B_6)	210, 280	290	Cholecalciferol (vitamin D_3)	254, 265, 280, 301	265
Folic acid (vitamin B_9)	254, 258, 290, 345, 350	280	Tocopherol (vitamin E)	265, 280, 300	265
Ascorbic acid (vitamin C)	225, 245, 254, 260, 265	270	Tocopherol acetate (vitamin E acetate)	284, 290	265
Cyanocobalamine (vitamin B_{12})	254	360	Phylloquinone (vitamin K_1)	247, 254, 270, 277	265
			Lutein	450	450
			Lycopene	450	450

Vitamin Retention Behaviors on the Acclaim HPLC Columns

Water- and fat-soluble vitamins are a structurally diverse groups of compounds, resulting in different behaviors on HPLC columns. In the work presented here, the retention behaviors of water- and fat-soluble vitamins are investigated on three types of reversed-phase columns—the Acclaim PA, PA2, and C18 columns.

The Acclaim PA, PA2, and C18 are silica-based columns designed for high-efficiency separations and manufactured using ultrahigh-purity silica.[10] The structures of their stationary phases are seen in Figure 4. The Acclaim 120 C18 is a typical high-performance, reversed-phase column, and features a densely bonded monolayer of octadecyldimethylsiloxane (ODS) on a highly pure, spherical, silica substrate with 120 Å pore structure. It is recommended for general-purpose reversed-phase applications that require high-surface coverage (i.e., high carbon load), low silanol activity, and excellent peak efficiency.

The Acclaim PA column is a reversed-phase silica column with an embedded sulfonamide polar group to enhance the stationary phase. This column has selectivity similar to a C18 column for analytes of low polarity, with the added advantage of compatibility with aqueous-only mobile phases. Some classes of compounds (e.g., nitroaromatics) show significantly different selectivity patterns on this bonded phase. The high-density bonding provides good retention of hydrophilic analytes. The Acclaim PA column exhibits some normal-phase HPLC characteristics above 90% organic solvent composition of the mobile phase.

The Acclaim PA2 column, like the Acclaim PA column, is a high-efficiency, silica-based, reversed-phase column but with a different embedded polar group. This stationary phase has an embedded amide. The PA2 column has all the advantages of conventional polar-embedded phases, but its multidentate binding has enhanced hydrolytic stability at both low and high pH (pH 1.5–10). The Acclaim PA2 column provides selectivity that is complementary to conventional C18 columns and the Acclaim PA column for method development.

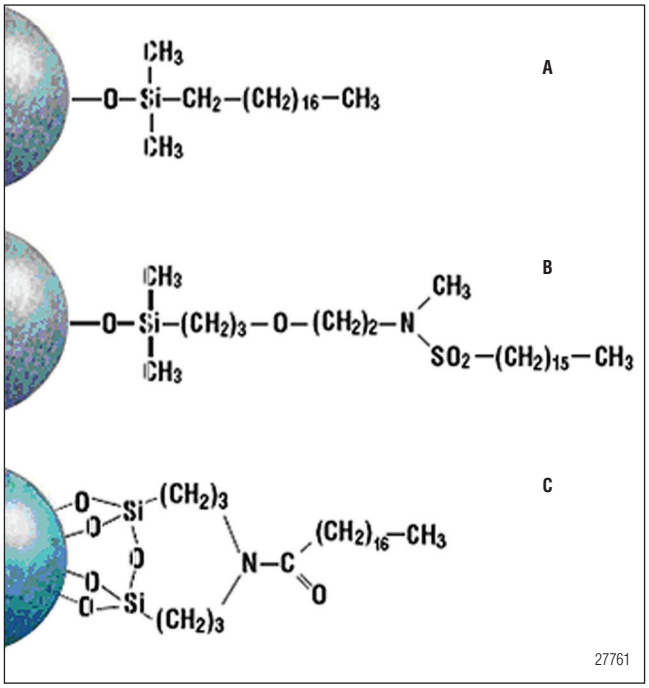

Figure 4. Structures of Acclaim (A) 120 C18, (B) PA, and (C) PA2 stationary phases.

Retention Behaviors of Water-Soluble Vitamins

The pH value of the mobile phase buffer may significantly affect the retention of water-soluble vitamins. In the study presented here, a phosphate buffer was used to avoid the baseline absorbance shift that occurs at 210 nm when using some acids (e.g., formic and acetic acid) during a gradient. This is because the proportion of these acids in the mobile phase changes. The phosphate buffer was also used to retain vitamin B_1 because its retention is inadequate when using formic acid without the addition of an ion-pairing reagent to the mobile phase. Figure 5 shows the retention time changes of water-soluble vitamins on the Acclaim PA, PA II, and C18 columns with changes in the pH value of the phosphate buffer.

When the Acclaim PA, PA II, and C18 columns were used, the retention times of water-soluble vitamins, except for nicotinic acid, exhibited similar trends with the buffer pH value.

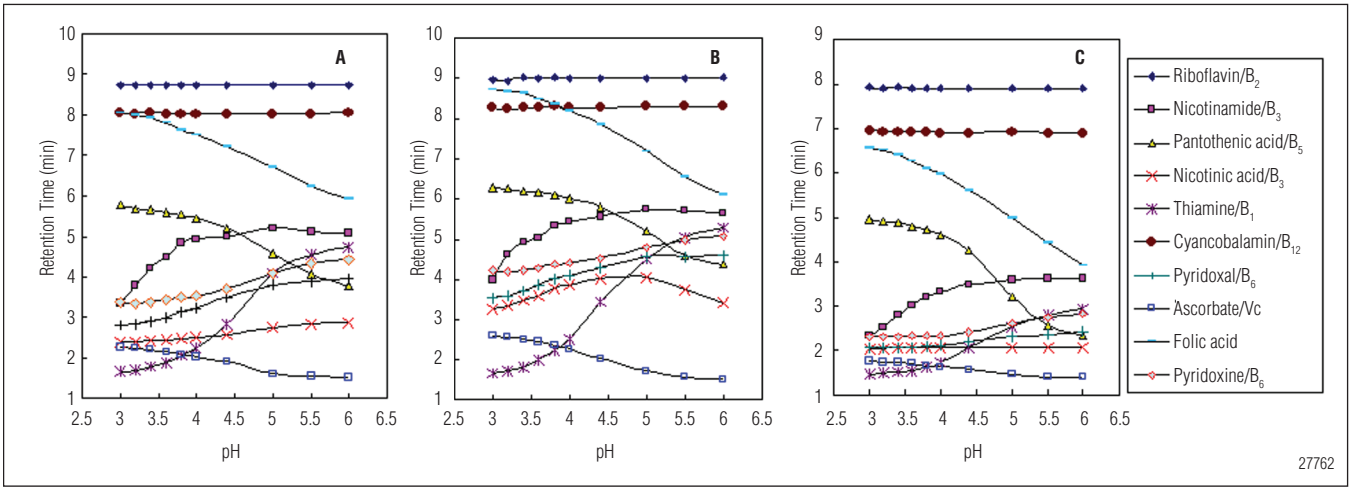

Figure 5. Retention time changes of water-soluble vitamins on the Acclaim (A) PA, (B) PA2, and (C) C18 columns with buffer pH value.

On the PA2 column, the retention time of nicotinic acid increased when the buffer pH value increased to pH 5, then began to decrease (Figure 5B). Conversely, it kept increasing on the PA column (Figure 5A) and exhibited very little increase on the C18 column. Compared to the C18 column, the PA and PA2 columns demonstrated better selectivity for compounds with high polarity (i.e., thiamine, ascorbic acid, nicotinic acid, pyridoxine, and pyridoxal) in the range from pH 3.0 to 4.0. This can be attributed to the embedded polar groups in the stationary phase, thus demonstrating the suitability of PA and PA2 columns for the separation of water-soluble vitamins. Additionally, PA and PA2 columns are compatible with aqueous-only mobile phases.

The Acclaim PA and PA2 columns exhibited different selectivity for water-soluble vitamins. For example, tailing peaks of nicotinic acid were observed on the PA2 column, but nicotinic acid had good peak symmetry on the PA column in the range of pH 3.0 ~ 6.0. Figure 6 presents the overlay of chromatograms obtained at pH 3.6. The PA column is therefore recommended for the separation of nicotinic acid. If there is no nicotinic acid in the samples, the PA2 column is recommended due to the more rugged separation of vitamin B_{12} and folic acid as they require control of the pH value for separation on the PA column.

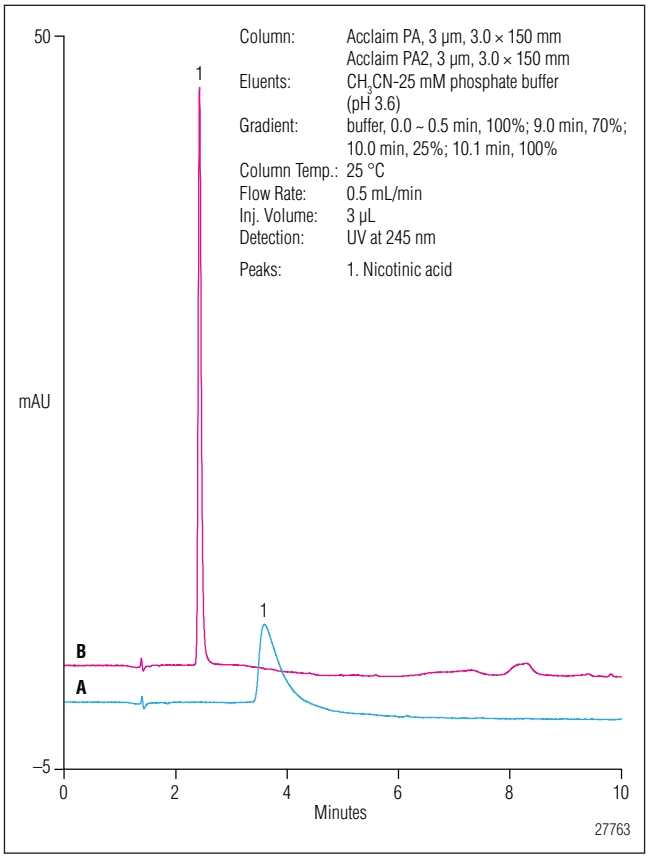

Figure 6. Chromatograms of nicotinic acid on (A) Acclaim PA2 and (B) Acclaim PA columns at pH 3.6.

Retention Behaviors of Fat-Soluble Vitamins

The NARP mobile phase used in this analysis consisted of methanol, acetonitrile, and methyl tert-butyl ether (MTBE). On a low-pressure gradient pump, the use of MTBE may yield better reproducibility than dichloromethane because the density of MTBE (0.74 g/mL) is similar to methanol (0.79 g/mL) and acetonitrile (0.78 g/mL), whereas the density of dichloromethane is 1.33 g/mL. Solvents with similar density are more easily mixed, especially when using a low-pressure gradient

pump, thereby yielding better reproducibility. As these fat-soluble vitamins are all low-polarity compounds, the Acclaim C18 column is a good choice for their separation. Their retention is mainly affected by the proportion of the solvents in the mobile phase. The separation of fat-soluble vitamins on the C18 stationary phase is usually not difficult, except for ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) due to their similar structures. The resolution (R_S) between them may be improved by careful selection of the proportion of acetonitrile.

DUAL-MODE TANDEM SOLUTION FOR THE SIMULTANEOUS SEPARATION OF WATER- AND FAT-SOLUBLE VITAMINS

Configurations and Principle

Valve-Switching

The different solubilities of water- and fat-soluble vitamins make it difficult to choose a solvent to dissolve them completely. Therefore, water- and fat-soluble vitamins are commonly determined by reversed-phase HPLC (RP-HPLC) and nonaqueous reversed-phase HPLC (NARP-HPLC), respectively. The UltiMate 3000 dual-pump HPLC system provides an ideal platform for the efficient combination of RP- and NARP-HPLC on one HPLC system for fulfilling the requirement of simultaneous determination. The valve-switching in parallel-HPLC on the Dionex UltiMate 3000 ×2 Dual HPLC system[11] cannot be applied to the simultaneous separation of water- and fat-soluble vitamins because similar mobile phases are required. Here, the authors present a new valve-switching technique that combines RP- to NARP-HPLC efficiently on this system with dual pumps, a UV detector, an autosampler, and a Chromeleon time base.

Under optimized chromatographic conditions, the total analysis time of 21 water- and fat-soluble vitamins was found to be less than 25 min. During the analysis of water-soluble vitamins on the PA column, the C18 column was equilibrated for the separation of fat-soluble vitamins. When the analysis of water-soluble vitamins was complete, the analysis of fat-soluble vitamins was begun while the PA column was equilibrated for the next separation of water-soluble vitamins. See Figure 7 for details on valve-switching.

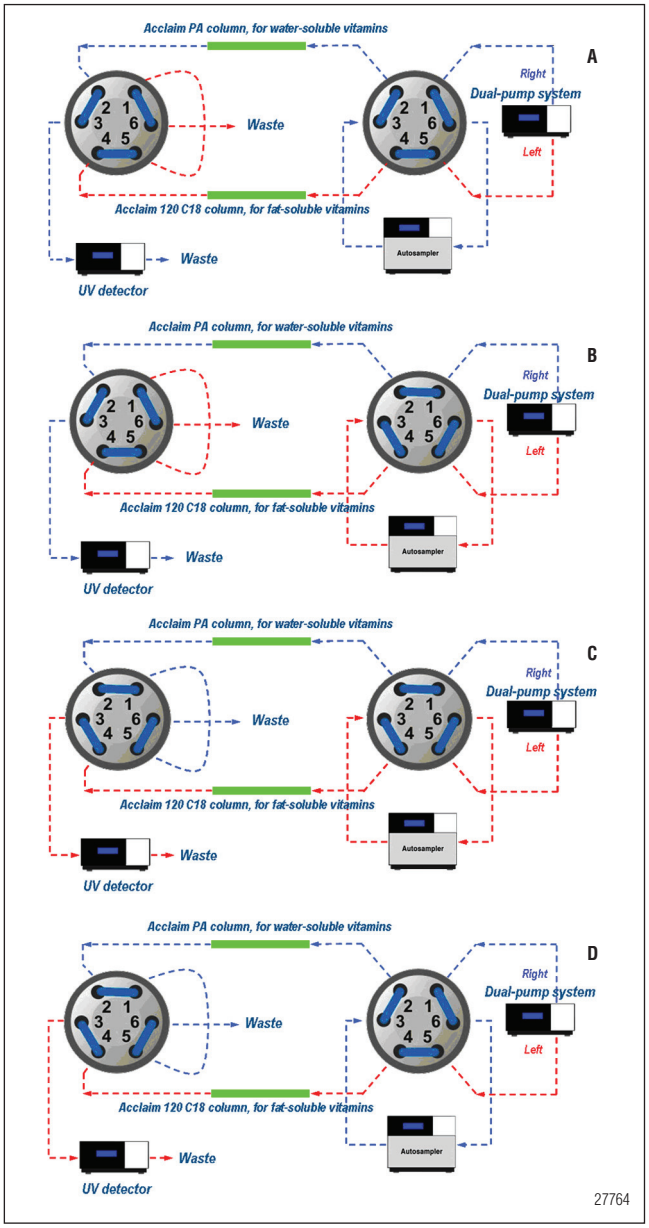

Figure 7. Schematic of valve-switching for the simultaneous determination of water- and fat-soluble vitamins.

Description: (A) At 0.00 min, connect the right pump to the autosampler and prepare for water-soluble vitamin analysis on the Acclaim PA column. (B) At 0.50 min, complete the injection for water-soluble vitamins analysis (first injection). Switch the right valve to position 1_2 and connect the left pump to the autosampler. Equilibrate the Acclaim 120 C18 column while running the analysis of water-soluble vitamins. (C) At 10.00 min, switch the left valve to position 1_2 and start the injection for fat-soluble vitamin analysis (second injection); meanwhile, the left pump and autosampler are connected to the UV detector. The analysis of fat-soluble vitamins on the Acclaim 120 C18 column is running. (D) At 10.5 min, complete the second injection; switch the right valve to position 6_1 and connect the right pump to the autosampler again. At this time, equilibrate the Acclaim PA column while running the analysis of fat-soluble vitamins on the Acclaim 120 C18 column. At 20.00 min, return the valves to their initial positions (i.e., schematic A).

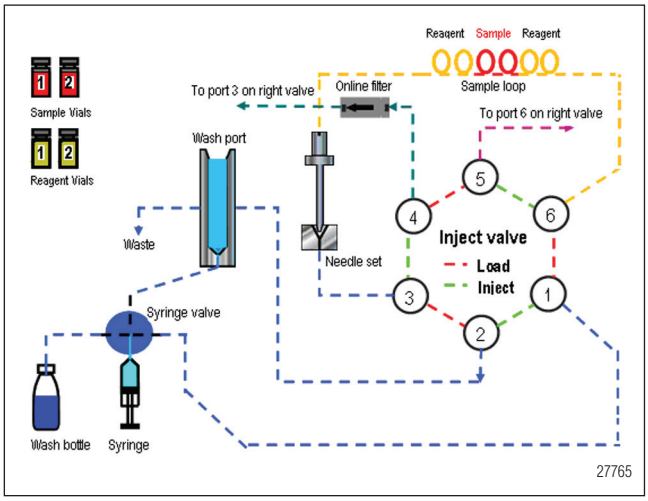

Figure 8. Schematic of envelop-injection mode running on the WPS 3000TSL autosampler.

Double-Injection Mode

Because the water- and fat-soluble vitamins are prepared using different solvents and placed in two vials, the double-injection mode was needed. This function was controlled by the Chromeleon CDS system, using an additional injection command in the program file (Table 4).

Envelop-Injection Mode for Fat-Soluble Vitamin Analysis

The significant difference in the polarity of the mobile phase and solvents used for dissolving samples may result in peak broadening when using the traditional injection mode. The typical method for resolving this problem is to concentrate the sample solution, and then dilute the concentrated sample solution with the mobile phase or solvents with similar polarity to the mobile phase. This method is not appropriate for some fat-soluble vitamins due to their instability. An injection mode named envelop-injection, which may improve the peak shape, is recommended instead of the traditional injection mode.

Table 3. Comparison of Peak Width Using Envelop and Traditional Injection Modes

Fat-Soluble Vitamin	Injection Mode	
	Envelope	Traditional
Retinol (vitamin A)	0.10	0.22
Retinol acetate (vitamin A acetate)	0.12	0.20
β-carotene	0.14	0.14
Ergocalciferol (vitamin D_2)	0.12	0.11
Cholecalciferol (vitamin D_3)	0.11	0.21
Tocopherol (vitamin E)	0.08	0.11
Phylloquinone (vitamin K_1)	0.12	0.13

Note: The peak width (min) collected at baseline level by the Chromeleon CDS system.

The envelop-injection mode consists of three steps: 1) draw a certain solvent from the reagent vials into the sample loop; 2) draw the sample solution from the sample vials into the sample loop; and 3) repeat the first step again. The schematic of envelop-injection mode running on the WPS 3000TSL autosampler is shown in Figure 8. The envelop-injection mode can be performed on the Dionex UltiMate 3000 ×2 Dual HPLC system controlled by the Chromeleon CDS system.

It is possible to select a suitable solvent that can adjust the polarity of sample solvent similar to that of the mobile phase. In this analysis, 75% acetonitrile was used. Table 3 compares the peak widths of some fat-soluble vitamins obtained using the envelope- and traditional-injection modes.

Programs

The details of the programs, including valve-switching, double-injection mode, envelop-injection mode, and wavelength-switching, for the simultaneous separation of water- and fat-soluble vitamins are presented in Table 4.

Table 4. Gradients and Programs for the Simultaneous Separation of Water- and Fat-Soluble Vitamins

```
DispSpeed =                                30.000 [µl/s]
WashSpeed =                                30.000 [µl/s]
PumpLeft_Pressure.Step =                   Auto
PumpLeft_Pressure.Average =                On
Data_Collection_Rate =                     10.0 [Hz]
TimeConstant =                             0.50 [s]
UV_VIS_1.Wavelength =                      270 [nm]
ValveLeft =                                6_1
ValveRight =                               6_1
PumpRight_Pressure.Average =               On
ColumnOven.TempCtrl =                      On
ColumnOven.Temperature.Nominal =           25.0 [°C]
ColumnOven.Temperature.LowerLimit =        5.0 [°C]
ColumnOven.Temperature.UpperLimit =        85.0 [°C]
EquilibrationTime =                        0.5 [min]
ColumnOven.ReadyTempDelta =                0.5 [°C]
Sampler.TempCtrl =                         On
Sampler.Temperature.Nominal =              15.0 [°C]
Sampler.Temperature.LowerLimit =           4.0 [°C]
Sampler.Temperature.UpperLimit =           45.0 [°C]
Sampler.ReadyTempDelta =                   5.0 [°C]
PumpLeft.Pressure.LowerLimit =             0 [bar]
PumpLeft.Pressure.UpperLimit =             400 [bar]
PumpLeft.MaximumFlowRampDown =             0.500 [ml/min_]
PumpLeft.MaximumFlowRampUp =               0.500 [ml/min_]
PumpLeft.%A.Equate =                       "ACN/MeOH 1:4"
PumpLeft.%B.Equate =                       "MTBE"
PumpLeft.%C.Equate =                       "MeOH/H2O 1:1"
PumpRight.Pressure.LowerLimit =            0 [bar]
PumpRight.Pressure.UpperLimit =            400 [bar]
PumpRight.MaximumFlowRampDown =            0.500 [ml/min_]
PumpRight.MaximumFlowRampUp =              0.500 [ml/min_]
PumpRight.%A.Equate =                      "25mM KH2PO4_pH3.6"
PumpRight.%B.Equate =                      "ACN/25mMKH2PO4 pH3.6 7:3"
PumpRight.%C.Equate =                      "MeOH/MTBE 7:3"
DrawSpeed =                                4.000 [µl/s]
DrawDelay =                                3000 [ms]
DispenseDelay =                            3000 [ms]
WasteSpeed =                               32.000 [µl/s]
SampleHeight =                             2.000 [mm]
InjectWash =                               BeforeInj
WashVolume =                               300.000 [µl]
PunctureOffset =                           0.0 [mm]
SyncWithPump =                             On
PumpDevice =                               "PumpRight" ;*1
```

; Note: *1. Defined which pump is synchronized with autosampler at this time.

Table 4 Continued

```
;The User Defined Program Start:
InjectMode =                              UserProg
ReagentAVial=                             B5    ;RegentA = 75%ACN,25%H2O
UdpSyringeValve                           Position=Needle
UdpInjectValve                            Position=Load
UdpDraw From=ReagentAVial, Volume=60.000, SyringeSpeed=GlobalSpeed, SampleHeight=1.000
UdpMixWait                                Duration=1
UdpDraw From=sampleVial, Volume=10.000, SyringeSpeed=GlobalSpeed, SampleHeight=1.000
UdpMixWait                                Duration=1
UdpSyringeValve                           Position=Waste
UdpDispense To=Waste, Volume=70.000, SyringeSpeed=30.000, SampleHeight=GlobalHeight
UdpMixNeedleWash                          Volume=600.000
UdpSyringeValve                           Position=Needle
UdpDraw From=ReagentAVial, Volume=60.000, SyringeSpeed=GlobalSpeed, SampleHeight=1.000
UdpMixWait                                Duration=1
UdpSyringeValve                           Position=Waste
UdpDispense To=Waste, Volume=60.000, SyringeSpeed=30.000, SampleHeight=GlobalHeight
UdpSyringeValve                           Position=Needle
UdpWaitStrokeSync
UdpInjectValve                            Position=Inject
UdpInjectMarker

;The User Defined Program End

0.000                                     Autozero
                                          InjectMode =                        Normal ;*2
                                          PumpRight.Flow =                    0.550 [ml/min]
                                          PumpRight.%B =                      0.0 [%]
                                          PumpRight.%C =                      0.0 [%]
```

; Note: *2. To apply an injection mode regulated by UDP (User Defined Program) to the first and/or second injections, the ; protocol must be defined at the start time. In this TN, there is no need to use the envelop ; injection mode for the first injection for water-soluble vitamins; therefore, the injection mode at 0.00 min must be defined as normal; otherwise, it will use the UDP mode as defined ; before.

```
                                          PumpLeft.Flow =                     0.550 [ml/min]
                                          PumpLeft.%B =                       1.0 [%]
                                          PumpLeft.%C =                       0.0 [%]
                                          Wait     UV.Ready and ColumnOven.
                                          Ready and Sampler.Ready
                                          Inject
                                          PumpLeft_Pressure.AcqOn
                                          PumpRight_Pressure.AcqOn
                                          UV_VIS_1.AcqOn
                                          PumpRight.Flow =                    0.550 [ml/min]
                                          PumpRight.%B =                      0.0 [%]
                                          PumpRight.%C =                      0.0 [%]
                                          PumpLeft.Flow =                     0.550 [ml/min]
                                          PumpLeft.%B =                       1.0 [%]
                                          PumpLeft.%C =                       0.0 [%]
0.400                                     WashSampleLoop                      Volume=300 ;*3 *4
```

Table 4 Continued

```
; Notes: *3. This time is based on that the time multiplied by the flow rate on the right pump must be bigger than the injection ; volume. The status of the autosampler must be in ready status at this time.

; Notes *4. After the first injection, the sample loop is full of solvent A (25 mM KH2PO4) delivered by the right pump. Using the ; solvent C drawn by the syringe of the autosampler, wash the sample loop to prevent precipitation of KH2PO4 on the column used ; for fat-; soluble vitamins. On-line degas wash kit (P/N 6820.2450) is used for washing solvent from channel C of the left ; pump.

0.500                                   ValveRight =                    1_2
                                        PumpRight.Flow =                0.550 [ml/min]
                                        PumpRight.%B =                  0.0 [%]
                                        PumpRight.%C =                  0.0 [%]

1.200                                   injectvalvetoinject  ;*5

; Note: *5. When sampler loop wash is completed, the inject valve switches back to the inject position; and then the mobile phase ; from the left pump flows through the autosampler.

2.45                                    UV_VIS_1.Wavelength =           290 [nm] ;*6
                                        Autozero

; Note: *6. The user may select another suitable detection wavelength.

3.90                                    UV_VIS_1.Wavelength =           260 [nm]
                                        Autozero
5.300                                   UV_VIS_1.Wavelength =           210 [nm]
                                        Autozero
5.750                                   UV_VIS_1.Wavelength =           280 [nm]
                                        Autozero
8.390                                   UV_VIS_1.Wavelength =           360 [nm]
                                        Autozero
9.000                                   PumpRight.Flow =                0.550 [ml/min]
                                        PumpRight.%B =                  36.0 [%]
                                        PumpRight.%C =                  0.0 [%]
                                        UV_VIS_1.Wavelength =           270 [nm]
                                        Autozero
9.450                                   PumpRight.Flow =                0.550 [ml/min]
                                        PumpRight.%B =                  36.0 [%]
                                        PumpRight.%C =                  0.0 [%]
9.500                                   PumpRight.Flow =                0.550 [ml/min]
                                        PumpRight.%B =                  0.0 [%]
                                        PumpRight.%C =                  100.0 [%] ;*7
```

Table 4 Continued

```
; Note: *7 Use solvent C from the right pump to flush all the strongly retained compounds (usually, fat-soluble vitamins may partially ; dissolve in water) out of the column when the separation of water-soluble vitamins is complete. When the flush time is set to 0.5 ; min, the actual flush time is 0.5 min plus the time used for the second injection (more than 3 min when using the envelop ; injection mode).

10.000                          UV_VIS_1.Wavelength =       325 [nm] ;*8
                                Autozero
                                ValveLeft =                 1_2
                                injectmode =                userprog ;*9
                                PumpDevice =                "Pumpleft" ;*1
                                SyncWithPump =              On
                                Position=                   Position+1 ;*10
                                Inject
                                Autozero
                                PumpRight.Flow =            0.475 [ml/min]
                                PumpRight.%B =              0.0 [%]
                                PumpRight.%C =              100.0 [%]

; Note: *8. The second injection is envelop injection mode regulated by UDP for fat-soluble vitamin analysis. It starts at 10 min and ; will take more than 3 min, which can be adjusted by users if necessary. This time also relates to the time using pure organic ; solvent to flush the column for water-soluble vitamins.

; Note: *9. In this TN, the envelop injection mode was applied to the fat-soluble analysis to improve the separation performance; the injection mode is changed to UDP mode.

* Note *10. Define the vial position for the fat-soluble sample (e.g., if position = BA1, the position +1 = BA2)

10.010                          PumpRight.Flow =            0.475 [ml/min]
                                PumpRight.%B =              0.0 [%]
                                PumpRight.%C =              70.0 [%]

10.400                          WashSampleLoop              Volume=300

10.500                          PumpLeft.Flow =             0.550 [ml/min]
                                PumpLeft.%B =               1.0 [%]
                                PumpLeft.%C =               0.0 [%]
                                ValveRight =                6_1

11.000                          PumpRight.Flow =            0.475 [ml/min]
                                PumpRight.%B =              0.0 [%]
                                PumpRight.%C =              70.0 [%]

11.010                          PumpRight.Flow =            0.475 [ml/min]
                                PumpRight.%B =              0.0 [%]
                                PumpRight.%C =              0.0 [%]
```

Table 4 Continued

```
11.200                          Injectvalvetoinject ;*5

13.070                          UV_VIS_1.Wavelength =           450 [nm]
                                Autozero
13.400                          UV_VIS_1.Wavelength =           325 [nm]
                                Autozero
13.900                          UV_VIS_1.Wavelength =           265 [nm]
                                Autozero
14.000                          PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   15.0 [%]
                                PumpLeft.%C =                   0.0 [%]
14.500                          PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   37.0 [%]
                                PumpLeft.%C =                   0.0 [%]
16.000                          PumpRight.Flow =                0.475 [ml/min]
16.500                          PumpRight.Flow =                0.750 [ml/min]
17.900                          UV_VIS_1.Wavelength =           470 [nm]
                                Autozero
18.380                          UV_VIS_1.Wavelength =           325 [nm]
                                autozero
18.750                          UV_VIS_1.Wavelength =           450 [nm]
                                Autozero
19.000                          PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   37.0 [%]
                                PumpLeft.%C =                   0.0 [%]
20.000                          ValveLeft =                     6_1 ;*11
                                PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   60.0 [%]
                                WashBufferLoop                  Volume=300.000
                                UV_VIS_1.AcqOff

; Note: *11. The left valve switches back to 6_1, and waits for the next sample.

21.000                          PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   60.0 [%]
                                PumpLeft.%C =                   0.0 [%]
21.100                          PumpLeft.Flow =                 0.550 [ml/min]
                                PumpLeft.%B =                   1.0 [%]
                                PumpLeft.%C =                   0.0 [%]
23.500                          PumpRight.Flow =                0.750 [ml/min]
24.000                          PumpRight.Flow =                0.550 [ml/min]
25.000                          PumpRight.Flow =                0.550 [ml/min]
                                PumpLeft_Pressure.AcqOff
                                PumpRight_Pressure.AcqOff
                                PumpRight.%B =                  0.0 [%]
                                PumpRight.%C =                  0.0 [%]
                                End
```

Equipment

Dionex UltiMate 3000 HPLC system consisting of:

- DGP 3600A Pump with SRD 3600 air solvent rack
- WPS 3000TSL Autosampler
- TCC-3200 Thermostated Column Compartment, with two 2p–6p valves
- VWD-3400RS UV-vis Detector (DAD-3000 for UV spectrum)

Chromeleon 6.80 SR7 CDS software

Kudos® SK3200LH Ultrasonic generator, Kudos Ultrasonic Instrumental Co., China

Orion 420A+ pH meter, Thermo

Reagents

Deionized water, from Milli-Q® Gradient A10

Acetonitrile (CH_3CN), methanol (CH_3OH), methyl tert-butyl ether (MTBE) and dichloromethane (CH_2Cl_2), HPLC-grade, Fisher

Potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), and potassium bicarbonate ($KHCO_3$), analytical-grade, SCRC, China

Standards

Water-and Fat-Soluble Vitamin Standards

Folic acid (vitamin B_9), ascorbic acid (vitamin C), phylloquinone (vitamin K_1) tocopherol (vitamin E), tocopherol acetate (vitamin E acetate), ß-carotene, retinol (vitamin A), and retinol acetate (vitamin A acetate); ≥ 98%, Sigma-Aldrich

Retinol palmitate (vitamin A palmitate), neat, Supelco

Thiamine (vitamin B_1), riboflavin (vitamin B_2), nicotinamide (vitamin B_3), nicotinic acid (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxine hydrochloride (vitamin B_6), pyridoxal hydrochloride (vitamin B_6), cyanocobalamin (vitamin B_{12}), ergocalciferol (vitamin D_2), and cholecalciferol (vitamin D_3), lutein, lycopene; ≥ 97%, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), China

Preparation of Standard Solutions

Water-Soluble Vitamins

Prepare standards of vitamin B_1, B_3 (nicotinamide and nicotinic acid), B_5, B_6 (pyridoxine hydrochloride and pyridoxal hydrochloride), B_{12}, and vitamin C by accurately weighing 10–20 mg of the vitamin powder and adding DI water to 10–20 g to form stock solutions of 1.0 mg/mL for each vitamin, respectively. Due to the limited solubility of vitamin B_2 and vitamin B_9 in water, prepare the concentration of the stock solution of vitamin B_2 using 5 mM KOH. Prepare 0.5 mg/mL of vitamin B_9 using 20 mM $KHCO_3$ instead of DI water. Due to the limited stability of vitamin C and vitamin B_2, prepare them at the time of use.

Fat-Soluble Vitamins

Prepare standards of vitamin A and its acetate, and palmitate, D_2, D_3, and vitamin E and its acetate by accurately weighing 10–20 mg of each and adding CH_3OH to 10–20 g to form stock solutions of 1.0 mg/mL for each vitamin, respectively. Prepare the standard of vitamin K_1 using acetone instead of CH_3OH; and prepare the standards of ß-carotene, lutein, and lycopene using CH_2Cl_2 instead of CH_3OH. Due to the limited stability of ß-carotene, prepare a stock solution of 0.5 mg/mL at the time of use.

Store the stock standard solutions at 4 °C when not in use; also, store the stock standards of fat-soluble vitamins in the dark. Prepare water-soluble vitamin working standards from the stock standards on the day of use by dilution with DI water. Use a mixture of CH_3OH-CH_2Cl_2 (1:1, v/v) for preparing fat-soluble vitamin working standards.

Sample and Sample Preparation

Samples

Two beverages were purchased from a supermarket; two multivitamin and mineral supplement tablets for women and pregnant women were purchased from a pharmacy; and two animal feeds, one for chicken and one for swine were provided by a customer.

Sample Preparation

Vitamins are added to the samples to be analyzed as supplements that are different from those naturally existing in meat and plants. Therefore, prepare these samples by direct solvent extraction, not enzymatic-, alkaline-, or acid-hydrolysis. Dilute the beverage samples (e.g., vitamin drink) if needed, and analyze directly.

1) Extraction of water-soluble vitamins from vitamin and mineral supplement tablets and animal feed: Grind the tablets with a mortar and pestle. Put accurately weighed 0.100 g of ground powder into 100 mL volumetric flasks and add 80 mL of water. After 15 min of ultrasonic extraction, add water to the mark.
2) Extraction of fat-soluble vitamins: Add accurately weighed 0.125 g of ground powder of Brands 1, 3, 4, and 5 into 10 mL volumetric flasks and add 8 mL of CH_3OH-CH_2Cl_2 (1:1, v/v) to each flask. After 15 min of ultrasonic extraction, add CH_3OH-CH_2Cl_2 (1:1, v/v) to the mark.

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

Optimized Chromatographic Conditions for the Simultaneous Separation of 21 Water- and Fat-Soluble Vitamins

As shown in Figure 9, 21 water- and fat-soluble vitamins were separated simultaneously under the following optimized chromatographic conditions combined with valve-switching, double-injection, envelope-injection, and wavelength-switching.

Columns: Acclaim PA, 3 µm, 120 Å, 3.0 × 150 mm (P/N 063693) for water-soluble vitamins
Acclaim C18, 3 µm, 120 Å, 3.0 × 150 mm (P/N 063691) for fat-soluble vitamins

Column Temp.: 25 °C

Mobile Phases: For water-soluble vitamin determination:
A) 25 mM Phosphate buffer (dissolve ~3.4 g KH_2PO_4 in 1000 mL water, and adjust pH to 3.6 with H_3PO_4)
B) CH_3CN-Mobile Phase A (7:3, v/v)

For fat-soluble vitamin determination:
A) CH_3OH-CH_3CN (8:2, v/v)
B) Methyl tert-butyl ether (MTBE)
Both in gradient (Table 3)

Flow Rate/
UV Detection: Table 3

Inj. Volume: 10 µL

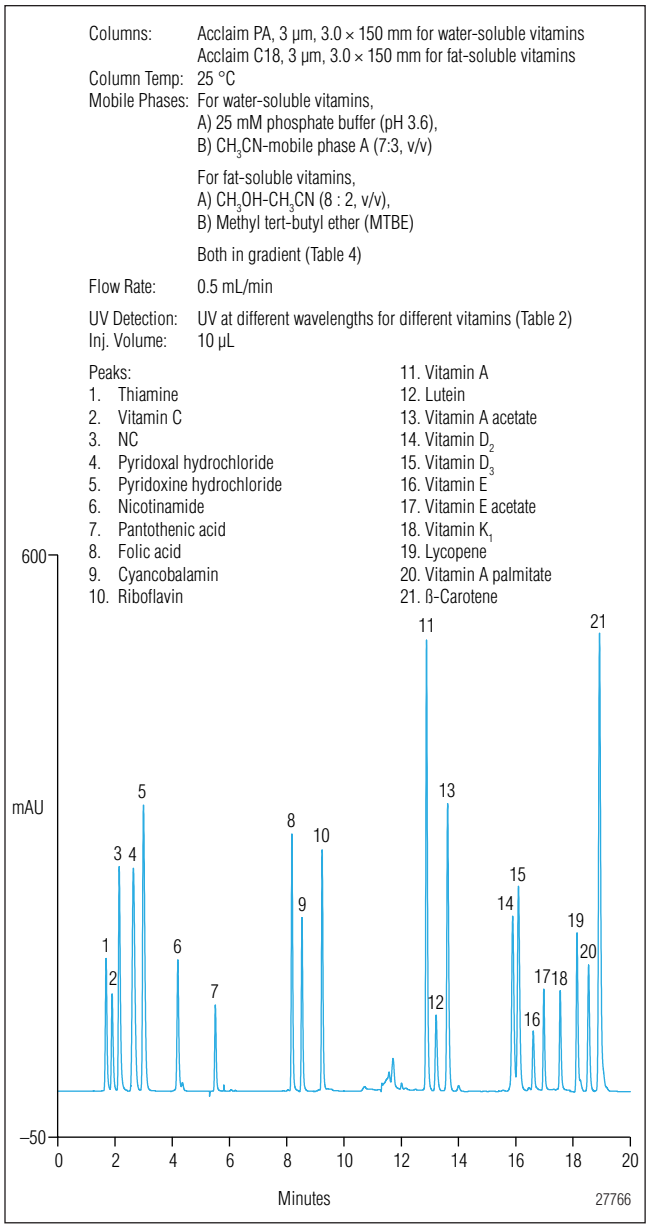

Figure 9. Chromatograms of the simultaneous separation of 21 water- and fat-soluble vitamins.

Table 5. RSDs for Retention Time and Peak Area of Water- and Fat-Soluble Vitamins*							
Water-Soluble Vitamin	**Retention Time RSD**	**Peak Area RSD**	**Concentration of Spiked Standards (µg/mL)**	**Fat-Soluble Vitamin**	**Retention Time RSD**	**Peak Area RSD**	**Concentration of Spiked Standards (µg/mL)**
Thiamine (B_1)	0.056	0.487	0.5	Retinol (A)	0.025	0.316	0.5
Riboflavin (B_2)	0.030	0.139	0.5	Retinol acetate (A acetate)	0.034	0.310	0.5
Nicotinamide (B_3)	0.032	0.591	0.75	Retinol palmitate (A palmitate)	0.033	0.836	0.5
Nicotinic acid (B_3)	0.033	3.884	0.75	Ergocalciferol (D_2)	0.049	3.624	0.25
Pantothenic acid (B_5)	0.037	2.515	1.0	Cholecalciferol (D_3)	0.047	4.266	0.25
Pyridoxine hydrochloride (B_6)	0.057	0.113	0.5	Tocopherol (E)	0.037	0.360	10
Pyridoxal hydrochloride (B_6)	0.065	3.944	0.5	Tocopherol acetate (E acetate)	0.027	0.637	10
Folic acid (B_9)	0.055	1.804	0.5	Phylloquinone (K_1)	0.025	0.605	0.25
Cyanocobalamin (B_{12})	0.056	3.369	0.25	β-Carotene	0.035	0.400	0.25
	0.060**	2.680**	0.0005	Lutein	0.058	2.810	0.5
Ascorbic acid (C)	0.047	9.419	0.5	Lycopene	0.030	3.410	0.5

*Ten consecutive injections for water- and fat-soluble vitamins.
**Seven consecutive injections for vitamin B_{12} using on-line SPE with a dual function and UDP injection mode.

Method Performance (Reproducibility, Linearity, and Detection Limits)

The method reproducibility was estimated by making consecutive injections of a multivitamin and mineral supplement tablet sample mixed with water- and fat-soluble vitamin standards, respectively. Excellent RSDs for retention time and peak area were obtained, as shown in Table 5.

Table 6. Calibration Data and MDLs for Water- and Fat-Soluble Vitamins*					
Water-Soluble Vitamin	**Regression Equations**	**r (× 100%)**	**Concentration Range of Standards (µg/mL)**	**RSD for Calibration Curve**	**MDL* (µg/mL)**
Thiamine (B_1)	A = 0.5584 c - 0.0369	99.992	0.05–20	1.9396	0.005
Riboflavin (B_2)	A = 1.5503 c - 0.0240	99.998	0.05–20	0.7268	0.002
Nicotinamide (B_3)	A = 0.5247 c + 0.0274	99.994	0.075–30	1.5054	0.015
Nicotinic acid (B_3)	A = 0.4007 c + 0.0010	99.999	0.075–30	0.6595	0.005
Pantothenic acid (B_5)	A = 0.0947 c - 0.0035	99.995	0.1–40	1.3506	0.021
Pyridoxine hydrochloride (B_6)	A = 0.7450 c - 0.0075	100.00	0.05–20	0.168	0.002
Pyridoxal hydrochloride (B_6)	A = 0.7170 c - 0.0062	99.999	0.05–20	0.4713	0.002
Folic acid (B_9)	A = 0.8112 c + 0.0112	99.999	0.05–20	0.4937	0.003
Cyanocobalamine (B_{12})	A = 0.0990 c - 0.0034	99.985	0.025–10	2.2583	0.007
	A = 0.0518 c + 0.0138 **	99.995**	0.0002–0.02	1.1511**	0.00005**
Ascorbic acid (C)	A = 0.4104 c – 0.0580	99.997	0.2–80	1.0358	0.14
Fat-Soluble Vitamin	**Regression Equations**	**r (× 100%)**	**Range of Standards (µg/mL)**	**RSD**	**MDL* (mg/L)**
Retinol (A)	A = 2.2185 c + 0.1250	99.974	0.05–20	2.8906	0.005
Retinol acetate (A acetate)	A = 2.0224 c + 0.1034	99.985	0.05–20	2.1889	0.005
Retinol palmitate (A palmitate)	A = 0.5081 c + 0.0288	99.970	0.05–20	3.1294	0.007
Ergocalciferol (D_2)	A = 0.7142 c + 0.0277	99.984	0.025–10	2.2682	0.005
Cholecalciferol (D_3)	A = 0.7080 c + 0.0483	99.971	0.025–10	3.0302	0.005
Tocopherol (E)	A = 0.0192 c + 0.0208	99.974	1–400	2.8936	0.121
Tocopherol acetate (E acetate)	A = 0.0292 c + 0.0448	99.975	1–400	2.869	0.223
Phylloquinone (K_1)	A = 0.7499 c - 0.0104	99.999	0.025–10	0.6564	0.009
β-Carotene	A = 4.3342 c + 0.0522	99.988	0.025–10	1.9871	0.003
Lutein	A = 0.1348 c - 0.0026	99.995	0.05–20	1.2969	0.015
Lycopene	A = 0.7292 c - 0.0304	99.968	0.05–20	3.3230	0.022

*The single-sided Student's *t* test method (at the 99% confidence limit) was used to determine MDL, where the standard deviation (SD) of the peak area of 10 injections is multiplied by 3.25 to yield the MDL.

**Obtained using on-line SPE with a dual function and UDP injection mode for vitamin B_{12} analysis.

Calibration linearity for the water- and fat-soluble vitamins was investigated by making five consecutive injections of a mixed standard prepared at six different concentrations. The external standard method was used to establish the calibration curve and to quantify these vitamins in samples. Table 6 reports the data from the calibration as calculated by Chromeleon software.

The detection limit was calculated using the equation:

$$\textbf{Detection limit} = S t_{(n-1,\ 1-\alpha = 0.99)}$$

where, S represents standard deviation (SD) of replicate analyses, n represents number of replicates, $t_{(n-1,\ 1-\alpha = 0.99)}$ represents Student's *t* value for the 99% confidence level with n–1 degrees of freedom. Using 10 consecutive injections of a multivitamin and mineral supplement tablet sample mixed with vitamin standards, the authors determined the S value and calculated method detection limits (MDL), which are also reported in Table 6.

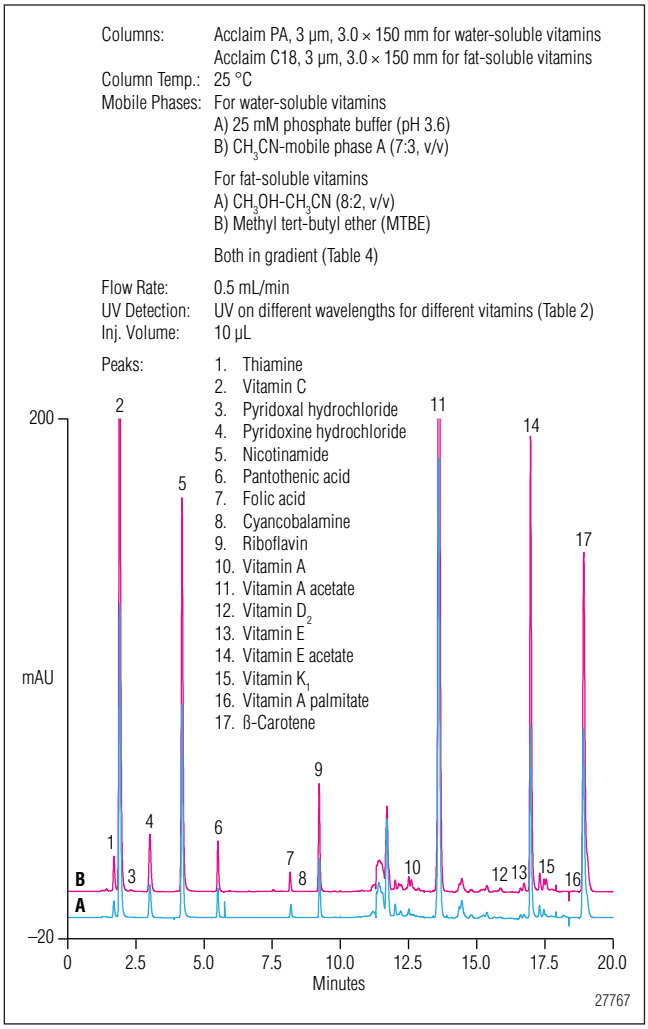

Figure 10. Chromatograms of (A) vitamin and mineral supplement tablet (for women) and (B) the same sample spiked with standards. There was a 1000-fold sample dilution for water-soluble vitamins, and a 100-fold dilution for fat-soluble vitamins.

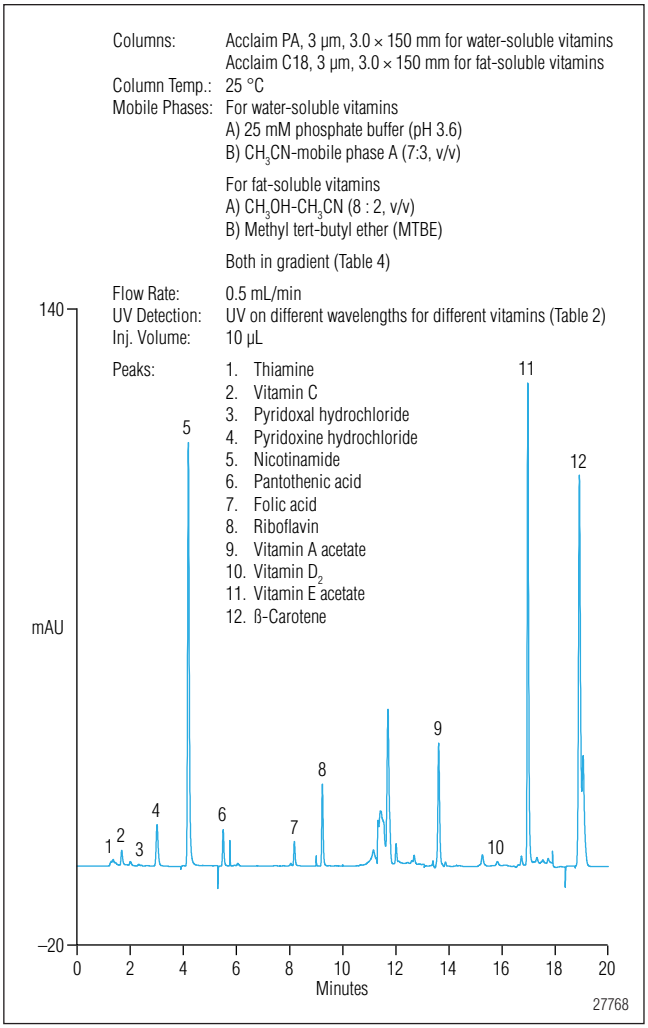

Figure 11. Chromatogram of a vitamin and mineral supplement tablet for pregnant women. There was a 1000-fold sample dilution for water-soluble vitamins, and a 100-fold dilution for fat-soluble vitamins.

Results

Figure 10 shows the chromatograms of the multi-vitamin and mineral supplement tablet sample for women and the same sample spiked with a mixed standard.

Figures 11–14 show the chromatograms of the other samples. Analysis results are summarized in Table 7 and both the comparison to the labeled values and the recovery experiments suggest that the method is accurate.

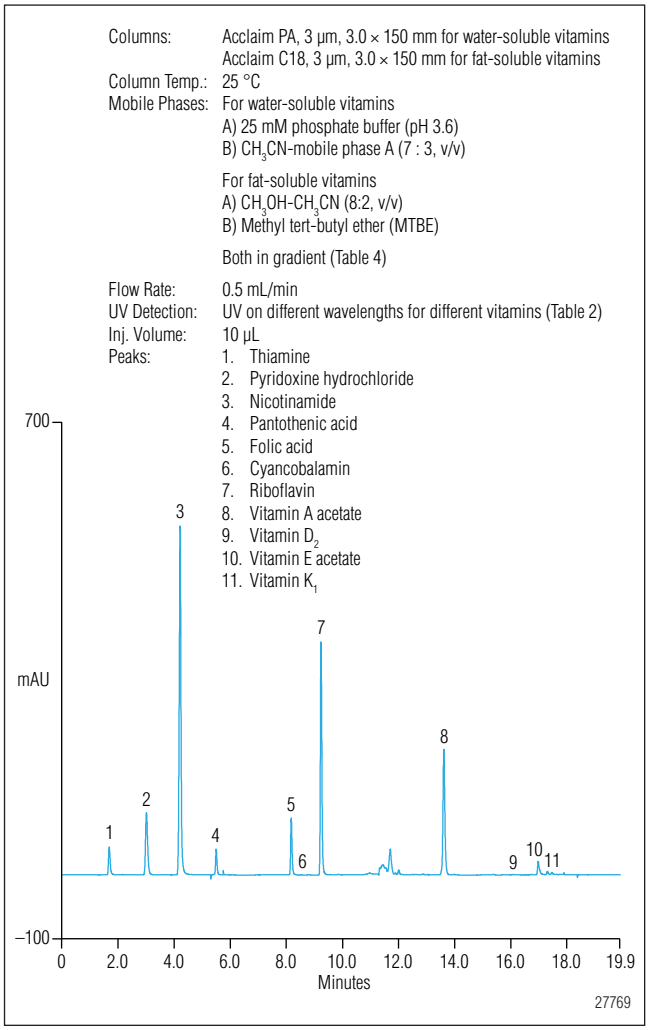

Figure 12. Chromatogram of chicken feed. There was a 1000-fold sample dilution for water-soluble vitamins, and a 100-fold dilution for fat-soluble vitamins.

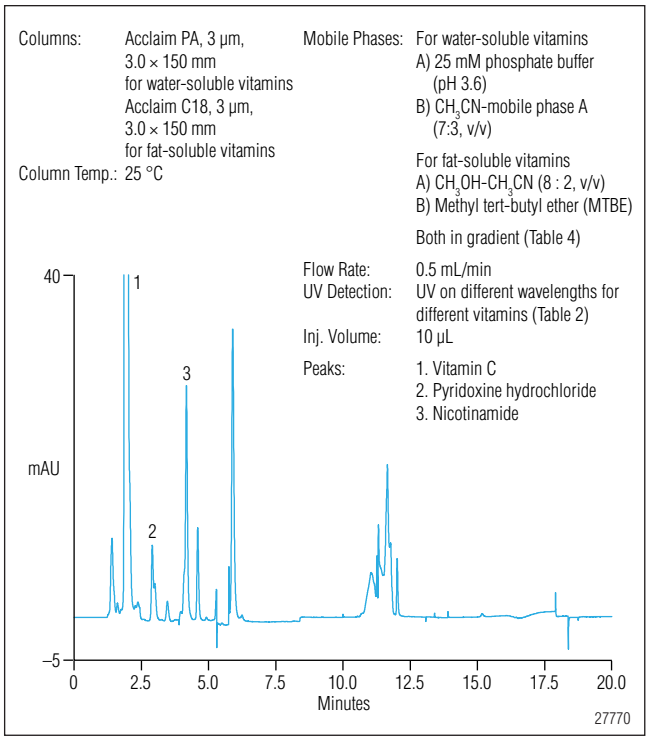

Figure 13. Chromatogram of beverage #1. There was a 2-fold sample dilution for both water- and fat-soluble vitamins analysis.

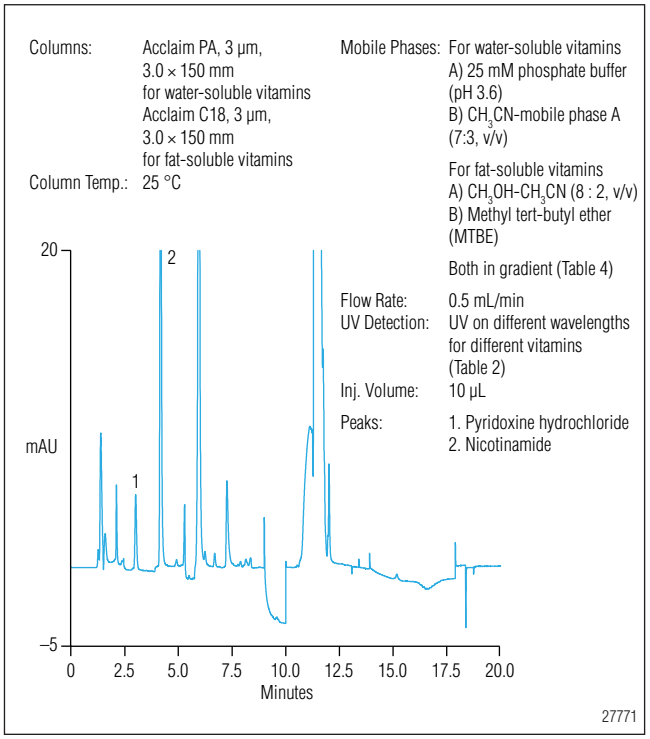

Figure 14. Chromatogram of beverage #2. There was a 2-fold sample dilution for both water- and fat-soluble vitamins analysis.

Table 7. Analysis of Water- and Fat-Soluble Vitamins in the Samples

Sample/Vitamin			Vitamin and Mineral Supplement Tablet						
			For Women					For Pregnant Women	
			Labeled (mg/g)	Detected (mg/g)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	Labeled (mg/g)	Detected (mg/g)
Water-Soluble	B_1		1.00	0.84	1.00	0.88	88	1.00	0.52
	B_2		1.13	0.98	1.00	0.78	78	1.00	1.10
	B_3	Nicotinamide	13.3	12.4	10.0	10.7	107	12	14.6
		Nicotinic acid	—	—	—	—	—	—	—
	B_5		6.67	7.89	6.00	6.01	100	4.0	4.82
	B_6	Pyridoxine	1.33	1.62	1.50	1.25	83	1.27	1.46
		Pyridoxal	—	—	—	—	—		0.03
	B_9		0.27	0.38	0.20	0.20	100	0.67	0.60
	B_{12}		0.004	0.018**	0.02	0.018**	90	0.0017	ND*
	C		40	23	20	23.2	116	57	0.15
Fat-Soluble	A		—	—	—	—	—	—	—
	A acetate		0.80	0.76	1.00	0.87	87	0.20	0.12
	A palmitate		—	—	—	—	—	—	—
	D	D_2	0.0067	0.0057	0.020	0.017	85	0.0067	0.011
		D_3		—	—	—	—		—
	E		—	—	—	—	—	—	—
	E acetate		20.0	18.3	25.0	26.9	108	25.0	25.9
	K_1		0.017	0.019	0.025	0.028	112	—	—
	β-carotene		0.22	0.18	0.15	0.14	93	0.50	0.24

Sample/Vitamin			Beverage				Animal Feed			
			#1		#2		Chicken Feed		Swine Feed	
			Labeled (μg/mL)	Detected (μg/mL)	Labeled (μg/mL)	Detected (μg/mL)	Labeled (mg/g)	Detected (mg/g)	Labeled (mg/g)	Detected (mg/g)
Water-Soluble	B_1		—	0.37	—	—	5	5.46	4.4	3.71
	B_2		—	0.035	—	—	14	14.1	11	11.2
	B_3	Nicotinamide	3.3 ~ 10	9.23	≥ 6	12.1	75	74.0	45	48.2
		Nicotinic acid	—	—	—	—	—	—	—	—
	B_5		—	0.41	—	—	25	24.6	20	20.9
	B_6	Pyridoxine	0.4 ~ 1.2	1.14	≥ 0.8	1.09	10	11.3	6.6	6.80
		Pyridoxal		0.06		—		—		—
	B_9		—	—	—	—	4	6.30	2	1.94
	B_{12}		0.0006 ~ 0.0018	ND/ 0.0017***	—	—	0.042	0.16	0.05	0.15
	C		250 ~ 500	351	—	—	—	—	—	—
Fat-Soluble	A		—	—	—	—	7.5	—	6	—
	A acetate							7.51		7.20
	A palmitate							—		—
	D	D_2					—	—	—	—
		D_3					0.015	0.015	0.01	0.017
	E						65	—	72	—
	E acetate							71.7		54.8
	K_1						—	0.40	—	0.32

*ND, not detected.
**Estimated value, which is lower than the MDL.
***Detected by using on-line SPE and UDP injection mode; 2.5 mL (100 μL, 25 times) sample injected.

Determination of Vitamin B_{12} Using On-Line Solid-Phase Extraction and UDP Injection Mode

The excessive consumption of cyanocobalamine (vitamin B_{12}) may cause asthma and folic acid deficiency; therefore, the added amount of vitamin B_{12} is usually at a very low level (e.g., ng/g). Thus, a traditional HPLC method is insufficient for determining vitamin B_{12} in most samples. In this study, the authors reported an on-line SPE mode to determine vitamin B_{12} at ng/g level on the Dionex UltiMate 3000 ×2 Dual HPLC system equipped with a large-sample loop (2500 µL).[12] The developed SPE mode was different from the traditional one. The bound analyte on the SPE column was selectively eluted from the SPE column using a mobile phase gradient, similar to the first dimension of a two-dimensional chromatography system. This reduced the number of interferences for sample analysis. As the SPE process was running, the analytical column was equilibrating. Before the front portion of the analyte peak eluted from the SPE column, the SPE column was switched into the analytical flow path. When the analyte completely eluted from the SPE column, the SPE column was switched out of the analytical flow path and back to the SPE flow path. Therefore, only those interferences co-eluting with the analytes entered the analytical column, so more interferences were removed. The volume of analyte cut from the SPE column was separated on the analytical column and detected by a UV detector. This on-line SPE mode with dual function (i.e., analyte capture and partial separation) was automatically controlled by the Chromeleon CDS. For details on the method, see Reference 12.

Here, the principle of on-line SPE determination of vitamin B_{12} was based on the method reported in Reference 12. The configuration had a small alteration to allow convenient switching between the two applications—simultaneous determination of water- and fat-soluble vitamins and on-line SPE for vitamin B_{12} analysis. The schematic of on-line SPE for VB_{12} analysis is shown in Figure 15. Consecutive injections (e.g., 25 times of a 100 µL injection, 2.5 mL total) controlled by using a user-defined program (UDP) may be employed instead of large-volume injection for on-line SPE.

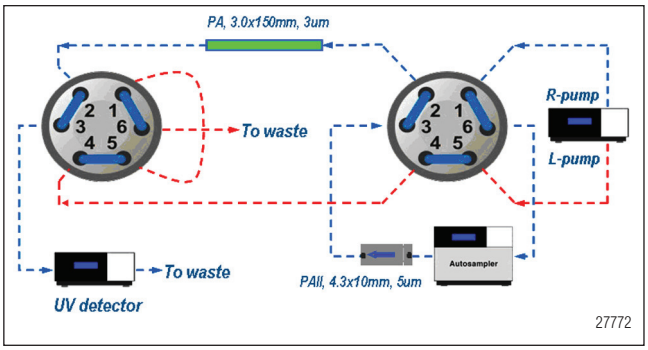

Figure 15. Schematic of on-line SPE for vitamin B_{12} analysis.

The method reproducibility was estimated by making seven consecutive injections of the 0.5 ng/mL vitamin B_{12} standard. The results are shown in Table 5. Calibration linearity for vitamin B_{12} was investigated using five standard concentrations (0.2, 0.5, 1.0, 5.0, and 20 ng/mL). The external standard method was used to establish the calibration curve and to quantify vitamin B_{12} in samples. Excellent linearity was observed from 0.2 to 20 ng/mL when plotting the concentration vs the peak area, and the results are shown in Table 6. The method detection limit (MDL) for vitamin B_{12} was calculated using the single-sided Student's *t* test method (at the 99% confidence limit), where the standard deviation of the peak area of seven injections of 0.2 ng/mL vitamin B_{12} standard was multiplied by 3.71 to yield the MDL, and the estimated value was 0.002 ng/mL.

Figure 16 shows the chromatogram of vitamin B_{12} in beverage #1 sample. The detected amount (Table 7) is in concordance with the labeled amount.

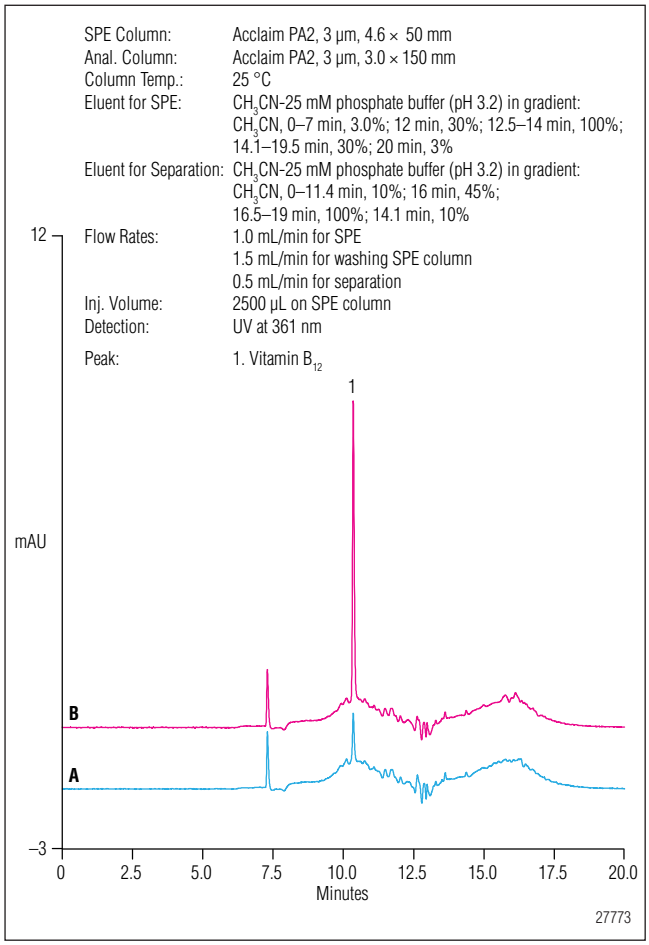

Figure 16. Chromatograms of vitamin B_{12} in (A) beverage #1 and (B) the same sample spiked with 0.45 ng/mL of vitamin B_{12} standard using on-line SPE with a dual-function and UDP injection mode.

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