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Application Note 148

Separating Water-Soluble Vitamins by Reversed Phase HPLC Using a Discovery C18 Column

Manufacturers routinely analyze vitamins in their products to determine quantitative levels and to monitor changes that have occurred in processing. In our study, we separated water-soluble vitamins by reversed-phase HPLC on a Discovery C18 column. This column yielded excellent resolution.

Key Words

- water-soluble vitamins
- Discovery C18 column
- reversed phase HPLC

Vitamins are an extremely diverse range of organic compounds present in minute amounts in natural foodstuffs. They are vital in the enzyme reactions that are necessary for carbohydrate, fat, and protein metabolism. Vitamins are classified into two groups: water-soluble and fat-soluble. Vitamins are relatively unstable, affected by factors such as heat, light, air, other food components, and food processing conditions (1,2).

Because of the critical role of vitamins in nutrition and their relative instability, qualitative and quantitative analyses are an important issue as well as a challenging task for food manufacturers. HPLC is the preferred technique for vitamin separation because of its high selectivity (3). Recent studies show various applications in determining vitamins in different sample sources (4-6).

We analyzed a standard mixture of water-soluble vitamins using a Discovery® C18 reversed-phase HPLC column, 15cm x 4.6mm ID, 5µm particles. Because each vitamin has its own maximum absorbance at a different wavelength, we used 220nm as a compromise to detect all vitamins in the sample. In the mobile phase, we attempted to use simple buffers without adding ion-pairing agents or competing amine modifiers.

Water-soluble vitamins are very hydrophilic. They can be eluted from reversed-phase columns with low concentrations of aqueous methanol or acetonitrile. A mixture of ten water-soluble vitamin standards, including ascorbic acid, niacin, pantothenic acid, pyridoxine, niacinamide, thiamine, folic acid, biotin, cyanocobalamin, and riboflavin, was analyzed by reversed-phase HPLC on a Discovery C18 column. Figure A shows the chromatogram of gradient elution of the ten vitamins at neutral pH. Excellent resolution and peak shape were achieved. Under the same condition, a multivitamin supplement – Centrum Multivitamin liquid - was separated and the peaks were identified (Figure B) by comparing to Figure A.

From the above example, it can be seen that these ten water-soluble vitamins can be well resolved within 25 minutes in one single run by an appropriate gradient elution. However, sometimes isocratic elution may be the preferred method, especially in QA/QC laboratories. Efforts have been made to develop a universal isocratic elution method to separate these vitamins on Discovery columns. However, cyanocobalamin and riboflavin are relatively hydrophobic, while the other eight water-soluble vitamins are extremely hydrophilic. A mobile phase condition that can elute cyanocobalamin and riboflavin will cause other vitamins to co-elute. Therefore, a set of two mobile phase conditions with different organic compositions would be a practical solution. Figure C shows the chromatogram of eight well-resolved water-soluble vitamins on Discovery C18 column using 1% methanol in 50 mM K_2HPO_4 buffer (pH 7). The eight vitamins are ascorbic acid, niacin, pantothenic acid, pyridoxine, niacinamide, thiamine, biotin, and folic acid, respectively. Under this condition, cyanocobalamin and riboflavin are strongly retained on the column. Further study has confirmed that the presence of cyanocobalamin and riboflavin has no effect on elution of the other eight vitamins even with multiple injections. Cyanocobalamin and riboflavin can be eluted from a Discovery C18 column using a mobile phase containing 20 % methanol in 50 mM K_2HPO_4 buffer (pH 7). The chromatogram is illustrated in Figure D. These two conditions combined can be a valuable method in separation of all water-soluble vitamins for those who prefer an isocratic elution method.

Figure A. Gradient Elution of Ten Water-Soluble Vitamins

Column: **Discovery C18, 15cm x 4.6mm ID, 5µm particles**
Cat. No.: **504955**
Mobile Phase: A: 50 mM K_2HPO_4, pH 7; B: Methanol; 1%B for 5 min, 1-30%B (linear gradient) over 15 min, 30%B for 5 min.
Flow Rate: 1mL/min
Pressure: <1500psi
Temp.: 35°C
Det.: UV, 220nm
Inj.: 10µL

1. Ascorbic acid (Vitamin C), 30 µg/mL
2. Niacin, 6 µg/mL
3. Pantothenic acid (Vitamin B_5), 100 µg/mL
4. Pyridoxine (Vitamin B_6), 3 µg/mL
5. Niacinamide, 6 µg/mL
6. Thiamine (Vitamin B_1), 12 µg/mL
7. Folic acid, 10 µg/mL
8. Biotin, 120 µg/mL
9. Cyanocobalamin (Vitamin B_{12}), 12 µg/mL
10. Riboflavin (Vitamin B_2), 30 µg/mL

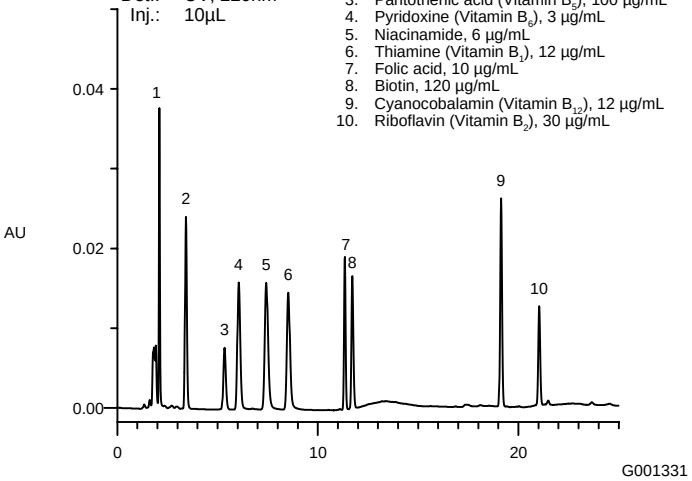


Figure B. Water-Soluble Vitamins in Centrum Multivitamin Liquid

Column: **Discovery C18, 15cm x 4.6mm ID, 5µm particles**
Cat. No.: **504955**
Mobile Phase: A: 50mM K_2HPO_4, pH 7: methanol; 1%B for 5 min, 1-30%B (linear gradient) over 15 min, 30%B for 5 min.
Flow Rate: 1mL/min
Pressure: <1500psi
Temp.: 35°C
Det.: UV, 220nm
Inj.: 10µL

1. Ascorbic acid (Vitamin C)
2. Niacin
3. Pantothenic acid (Vitamin B_5)
4. Pyridoxine (Vitamin B_6)
5. Niacinamide
6. Thiamine (Vitamin B_1)
7. Folic acid
8. Riboflavin (Vitamin B_2)

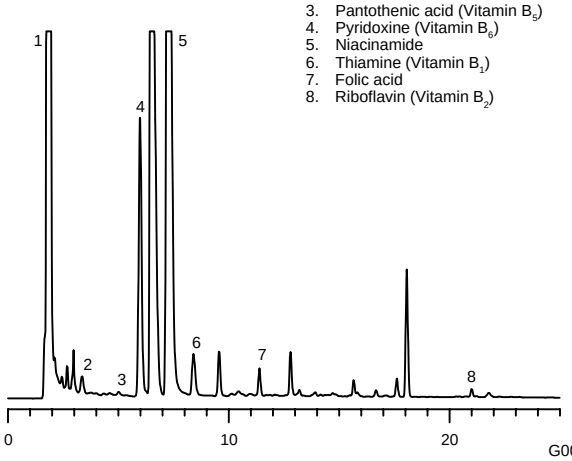


Figure C. Isocratic Elution of Water-Soluble Vitamins (I)

Column: **Discovery C18, 15cm x 4.6mm ID, 5µm particles**
Cat. No.: **504955**
Mobile Phase: A = 50mM K_2HPO_4, pH 7; B: Methanol; 99%A/1%B isocratically
Flow Rate: 1mL/min
Pressure: 1300psi
Temp.: 35°C
Det.: UV, 220nm
Inj.: 10µL

1. Ascorbic acid (Vitamin C), 30 µg/mL
2. Niacin, 6 µg/mL
3. Pantothenic acid (Vitamin B_5), 100 µg/mL
4. Pyridoxine (Vitamin B_6), 3 µg/mL
5. Niacinamide, 6 µg/mL
6. Thiamine (Vitamin B_1), 12 µg/mL
7. Biotin, 120 µg/mL
8. Folic acid, 10 µg/mL

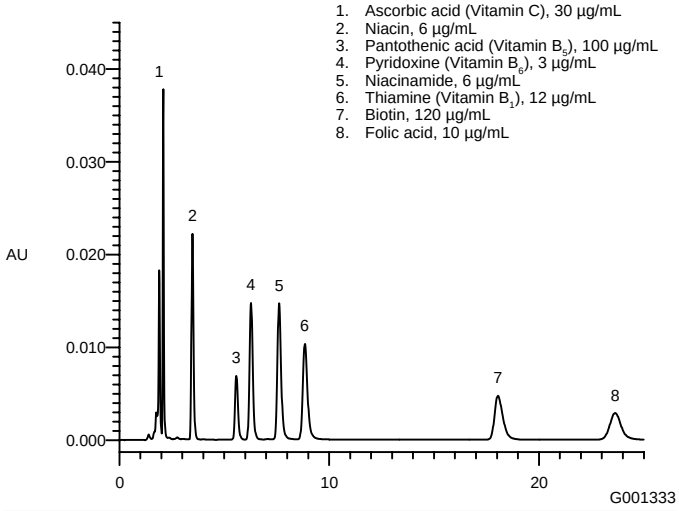
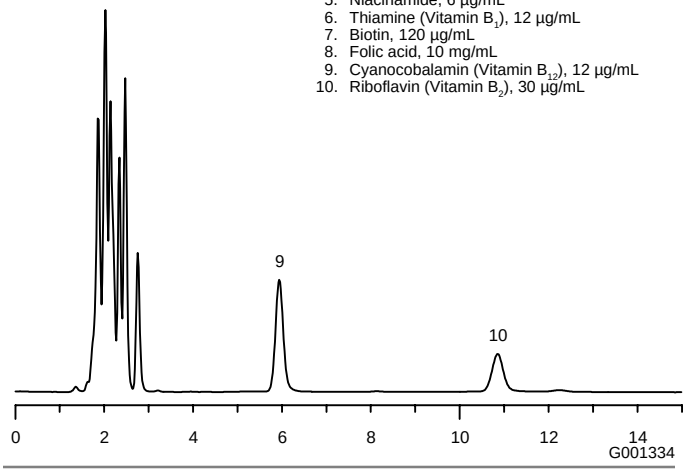


Figure D. Isocratic Elution of Water-Soluble Vitamins (II)

Column: **Discovery C18, 15cm x 4.6mm ID, 5µm particles**
Cat. No.: **504955**
Mobile Phase: A = 50mM K_2HPO_4, pH 7 B: Methanol; 80%A/20%B isocratically
Flow Rate: 1mL/min
Pressure: 1400psi
Temp.: 35°C
Det.: UV, 220nm
Inj.: 10µL

Compounds 1-8

1. Ascorbic acid (Vitamin C), 30 µg/mL
2. Niacin, 6 µg/mL
3. Pantothenic acid (Vitamin B_5), 100 µg/mL
4. Pyridoxine (Vitamin B_6), 3 µg/mL
5. Niacinamide, 6 µg/mL
6. Thiamine (Vitamin B_1), 12 µg/mL
7. Biotin, 120 µg/mL
8. Folic acid, 10 mg/mL
9. Cyanocobalamin (Vitamin B_{12}), 12 µg/mL
10. Riboflavin (Vitamin B_2), 30 µg/mL



Ordering Information:

Description	Cat. No.
Discovery C18 Column	
15cm x 4.6mm ID, 5µm particles	**504955**

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Trademarks

Discovery – Sigma-Aldrich Co.
Centrum – American Home Products Corp.

For additional information about this application, contact our Applications Laboratory at aplab@sial.com

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