LC/MS/MS of Vitamin B Shows Effects of Injection Solvents with an Agilent ZORBAX RRHD HILIC Plus Column

Application Note

Food Testing & Agriculture

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Abstract

A rapid LC/MS/MS analysis of vitamin B related compounds (4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid) is optimized on an Agilent ZORBAX Rapid Resolution High Definition HILIC Plus column using an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. The method uses isocratic elution with an acetonitrile rich mobile phase and an ammonium acetate buffer. All analytes have good peak shape with this hydrophilic interaction chromatography (HILIC) application. The method is then used to demonstrate the importance of choosing an appropriate injection solvent when performing LC analyses in HILIC mode. Various injection solvents are investigated, including water, acetonitrile, methanol, and combinations. Of the solvents explored, it was determined that pure acetonitrile yields the best retention and peak shape for all compounds, with water being the worst solvent for analytical performance and peak shape.

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Introduction

HILIC is gaining popularity in liquid chromatography, particularly for its ability to retain and separate small polar analytes – an area where common reversed-phase liquid chromatography (RPLC) methods often fail. This novel mode of chromatography results in unique retention mechanisms, because water is used as the strong eluting solvent and can have distinct advantages over traditional RPLC in both sample preparation and LC/MS sensitivity, due to the use of highly organic mobile phases. The highly organic mobile phases do not require samples to be dried prior to injection, and their higher volatility than traditional RPLC mobile phases makes this technique well suited for applications with mass spectrometers [1].

HILIC retention on a silica-based column is believed to involve a combination of mechanisms. First, a water layer must be adsorbed onto the polar silica surface, creating a liquid/liquid extraction system. The polar analytes can then partition into and out of this adsorbed water layer, with more polar analytes having a stronger interaction with this immobilized water layer. Charged polar analytes can also undergo ion exchange with the charged silica. Elution is typically from least to most polar, the opposite of RPLC. For HILIC method development, it is important to remember that the solvent strengths are different than in RPLC. For HILIC mode, solvent strength is tetrahydrofuran < acetone < acetonitrile < isopropanol < ethanol < methanol < water, with water being the strongest solvent [2,3,4].

HILIC is used extensively to analyze polar molecules. In this application note, an analysis of 4 vitamin B related compounds is optimized by LC/MS/MS using an Agilent ZORBAX Rapid Resolution High Definition HILIC column. The compounds of interest are 4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid, and they are shown in Figure 1.

Additionally, a major consideration for HILIC performance is its sensitivity to injection solvent strength. This is evaluated using the 4 vitamin B related compounds and a variety of injection solvents, ranging in strength from 100% acetonitrile to 100% water. Solvent effects on peak shape and retention are discussed.

Experimental

An Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer was used. The setup was optimized for lowest possible extra-column volume with short 0.075 mm id capillaries found in the Agilent Ultra Low Dispersion Kit (p/n 5067-5189) and with an Agilent LC System Rack (p/n 5001-3726) [5].

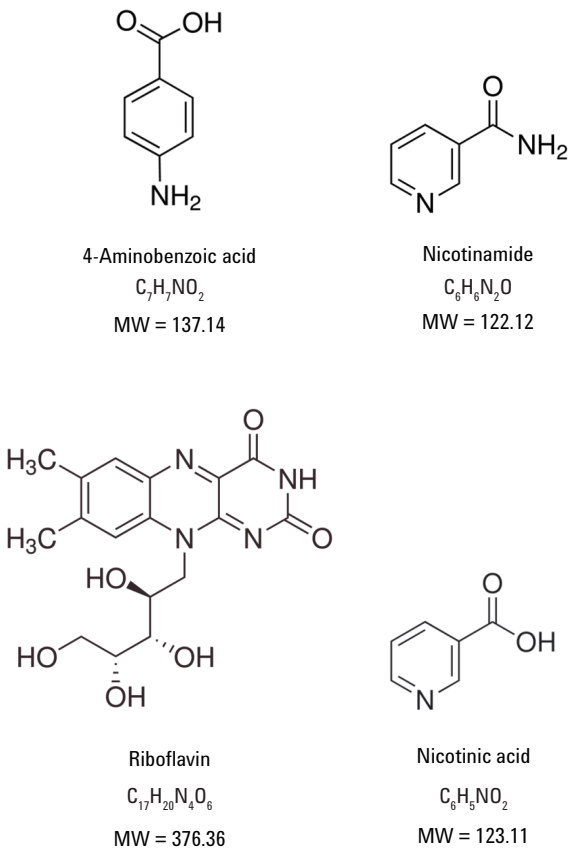

Figure 1. Vitamin B related compounds of interest.

Conditions

Columns:	Agilent ZORBAX Rapid Resolution High Definition (RRHD) HILIC Plus, 2.1 × 50 mm 1.8 µm (p/n 959757-901) and 2.1 × 100 mm, 1.8 µm (p/n 959758-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Flow rate:	0.4, 0.7 or 1.0 mL/min, isocratic
Temperature:	25 °C
Sample:	For method optimization: 0.1 µL injection of 12.5 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in acetonitrile (minimal amount of water, <5%) For injection solvent comparison: 1 µL injection of 5.7 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents (including H_2O, CH_3CN, CH_3OH and combinations thereof); analytes are illustrated in Figure 1
MS source:	Positive ESI, capillary, 4000 V, drying gas temperature, flow rate and nebulizer pressure vary with mobile phase flow rate and are specified in Table 1
MS acquisition:	For method optimization: dynamic MRM (dMRM), delta EMV 200 V, MS cycle time varies with mobile phase flow rate and is specified in Table 1, compound MRM transitions are detailed in Table 2 For injection solvent comparison: selected ion mode (MS2SIM), delta EMV 200 V, dwell time 15 ms, compounds were identified by their precursor ions listed in Table 2, and were generated using their respective fragmentor voltages in Table 2
Software:	Agilent MassHunter versions B.03.01, B.02.00, and B.03.01 were used for data acquisition, qualitative, and quantitative analyses, respectively.

All 4 analytes were purchased as powders from Sigma Aldrich and prepared to desired concentrations in various solvent systems. Acetonitrile and methanol were purchased from Honeywell. Ammonium formate and formic acid were purchased from Sigma Aldrich. Water used was 18 MΩ Milli-Q water.

Table 1. Mass spectrometer parameters for optimized vitamin analyses at various flow rates.

	0.4 mL/min	0.7 mL/min	1.0 mL/min
Source	ESI+	ESI+	ESI+
Delta EMV	200 V	200 V	200 V
MS Dwell Time	70 ms	50 ms	30 ms
Drying Gas Temperature	200 °C	200 °C	300 °C
Drying Gas Flow Rate	10 L/min	11 L/min	11 L/min
Nebulizer Pressure	30 psi	35 psi	55 psi
Capillary Voltage	4000 V	4000 V	4000 V

Table 2. Mass spectrometer MRM transitions for optimized vitamin analyses.

	Precursor ion	Fragmentor voltage	Product ion	Collision energy
4-Aminobenzoic acid	138	110	120	15
	138	110	94	15
Nicotinamide	123	130	80	25
	123	130	53	35
Riboflavin	377	160	243	30
	377	160	172	40
Nicotinic acid	124	130	80	20
	124	130	53	40

Results and Discussion

Optimized LC/MS/MS analyses of 4-aminobenzoic acid, nicotinamide, riboflavin, and nicotinic acid are shown in Figure 2 at various flow rates. The ZORBAX RRHD HILIC Plus column produces good peak shape and efficiency for all compounds regardless of the mobile phase flow rate.

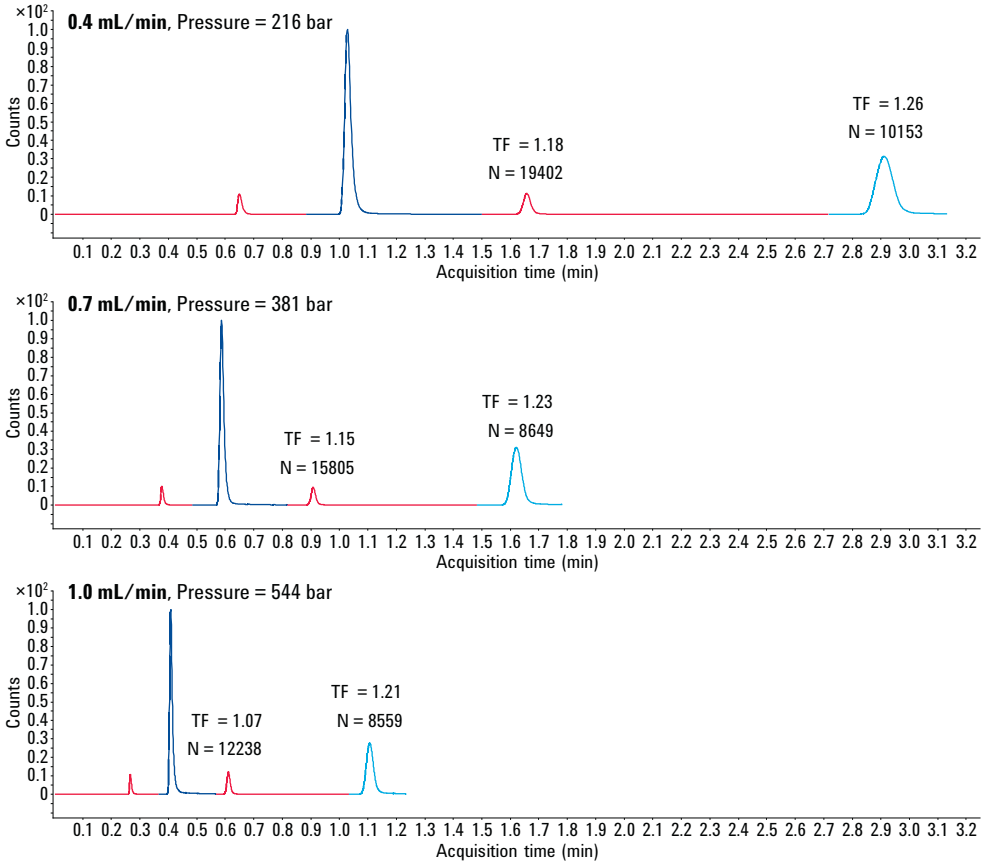

Figure 2. Optimized analyses of vitamin B related compounds at various flow rates by LC/MS/MS using a 2.1 x 100 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm (p/n 959758-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4, 0.7, or 1.0 mL/min
Injection:	0.1 µL of 12.5 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in CH_3CN
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V; Drying gas temperatures, Flow rates, Nebulizer pressures, and Dwell times are found in Table 1
MS acquisition:	dMRM; Compound transitions are found in Table 2

Figures 3A and 3B show the effects of injecting strong solvents in HILIC mode. Each sample was prepared to the same concentration in various solvents. A stock solution of the vitamin B related compounds in acetonitrile was diluted 1:10 with the different solvent combinations. Injecting water, the strongest solvent, severely distorts peak shape, particularly for nicotinamide and riboflavin. Because nicotinic acid is more retained, it is less affected than the earlier eluting peaks, however, there is still a shift in retention time. While methanol is not as strong a solvent as water in HILIC mode, it still has an effect on chromatography. Injecting between 100% and 50% methanol produces broader peaks than injecting the sample in pure acetonitrile. There is also a loss of retention for riboflavin and nicotinic acid when methanol is injected with HILIC mode. From these comparisons of injection solvents, it is apparent that the sample solvent in HILIC mode should be as weak as possible, which for HILIC means as high of a percentage of acetonitrile as possible (depending on analyte solubility).

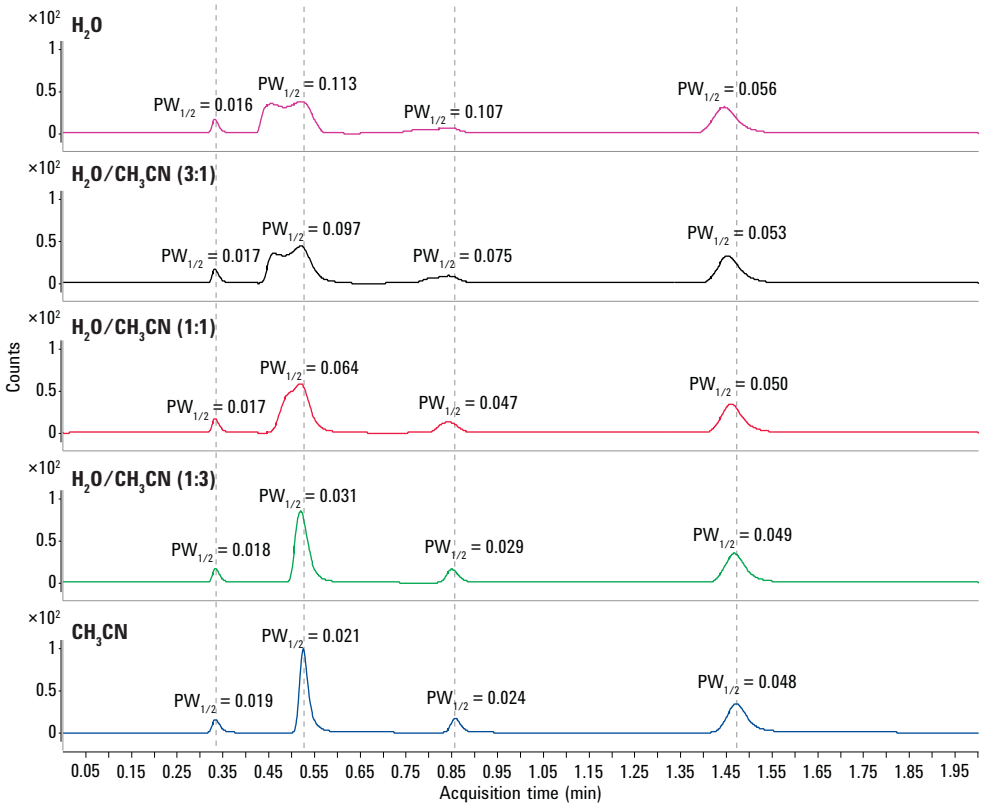

Figure 3A. The impact of injection solvent on HILIC/LC/MS performance using a 2.1 × 50 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Water + acetonitrile.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 50 mm, 1.8 µm (p/n 959757-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4 mL/min, Pressure = 135 bar
Injection:	1 µL of 5.7 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V, Drying gas: 200 °C, 10 L/min, Nebulizer: 30 psi, Dwell: 15 ms
MS acquisition:	MS2SIM: *m/z* 138 (Frag 110 V), *m/z* 123 (Frag 130 V), *m/z* 377 (Frag 160 V), *m/z* 124 (Frag 130 V)
Sample preparation:	100 µL stock solution in CH_3CN was diluted into 1 mL of the solvents labeled on the chromatograms to the left; final concentration was 5.7 µg/mL of each compound

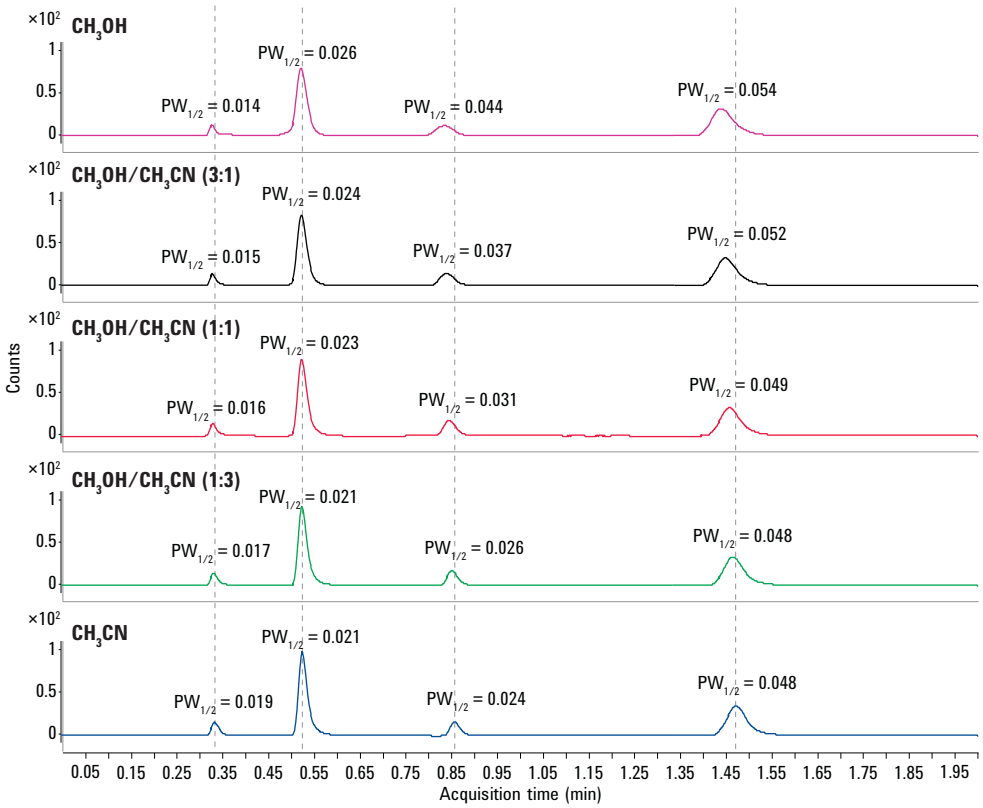

Figure 3B. The impact of injection solvent on HILIC/LC/MS performance using a 2.1 × 50 mm, 1.8 µm Agilent ZORBAX RRHD HILIC Plus column and an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Methanol + acetonitrile.

System:	Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer
Column:	Agilent ZORBAX RRHD HILIC Plus, 2.1 × 50 mm, 1.8 µm (p/n 959757-901)
Mobile phase:	CH_3CN/100 mM NH_4HCO_2 pH 3.2 (9:1)
Isocratic:	0.4 mL/min, Pressure = 135 bar
Injection:	1 µL of 5.7 µg/mL each of: 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid in various solvents
Thermostatted column compartment:	25 °C
MS source:	ESI+, Capillary: 4000 V, Drying gas: 200 °C, 10 L/min, Nebulizer: 30 psi, Dwell: 15 ms
MS acquisition:	MS2SIM: *m/z* 138 (Frag 110 V), *m/z* 123 (Frag 130 V), *m/z* 377 (Frag 160 V), *m/z* 124 (Frag 130 V)
Sample preparation:	100 µL stock solution in CH_3CN was diluted into 1 mL of the solvents labeled on the chromatograms to the left; final concentration was 5.7 µg/mL of each compound

Conclusions

Four vitamin B related compounds are successfully analyzed by LC/MS/MS in HILIC mode. The Agilent ZORBAX HILIC Plus column delivers good peak shape and efficiency for these polar analytes. Additionally, it is shown that water, the strongest solvent in HILIC mode, should never be used as the sample solvent, as it results in significant peak distortion for early eluting peaks. Methanol is also a strong solvent in HILIC mode, though not as strong as water, and it also affects peak shape and retention, though not as drastically as water.

References

1. E. S. Grumbach, D. M. Wagrowski-Diehl, J. R. Mazzeo, B. Alden, P. C. Iraneta. *LC/GC Magazine*, **22**, 1011 (2004).

2. D. McCalley. *J. Chrom. A*. **1217**, 3408. (2010).

3. P. Hemström, K. Irgum. *J. Sep. Sci.* **29**, 1784 (2006).

4. D. McCalley. *J. Chrom. A.* **1171**, 46 (2007).

5. A. Mack. *Optimizing Performance of an Agilent ZORBAX RRHD Eclipse Plus C18 Column by Enhancing an Agilent 1290 Infinity LC System for Ultra-Low Dispersion*. Agilent Technologies, Inc. Publication number 5990-9502EN (2011).

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Printed in the USA
April 12, 2012
5991-0209EN

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