Application Note

Metabolomics

Analysis of Water-Soluble Vitamins and their Metabolites

Performance Gains in Hydrophilic Interaction Chromatography (HILIC) with LC/MS/MS

Authors

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Abstract

Water-soluble vitamins and their metabolites were analyzed by LC/MS/MS with Agilent InfinityLab Poroshell 120 HILIC-Z columns. The effect of varying mobile phase additives with different pH values was explored. The best overall performance was achieved with a mid-pH additive using 10 mM ammonium acetate and acetonitrile as the mobile phase. Performance gains for phosphorylated compounds could be obtained using peek-lined column hardware and InfinityLab deactivator additive.

Introduction

There are increased demands for comprehensive analysis of water-soluble vitamins and their metabolites. Most analytes are small, highly polar compounds that are not well retained on a reversed-phase column. An InfinityLab Poroshell 120 HILIC-Z column with superficially porous particle technology is ideal for the retention of this class of compounds. This Application Note separated 24 vitamins and their metabolites using the InfinityLab Poroshell 120 HILIC-Z column with an Agilent 6460 triple quadrupole LC/MS system. The influence of different mobile phase additives was explored when using the Poroshell 120 HILIC-Z column. In addition, the effect of peek-lined hardware in combination with the InfinityLab deactivator additive, a mobile phase additive to reduce interactions with active metal surfaces, was examined. These effects were compared with standard phosphoric acid wash protocols and conventional chromatography that does not use any passivation strategy.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. HPLC grade acetonitrile was purchased from J. T. Baker (Center Valley, PA, USA.). Water was purified using an ELGA PURELAB Chorus system (High Wycombe, UK). Formic acid, acetic acid, ammonium acetate, and ammonium hydroxide were purchased from J&K Scientific (Beijing, China). All vitamins and the metabolite standards (Table 1) were obtained through Anpel (Shanghai, China). The standards stock solutions were prepared with concentrations and solvent listed in Table 1.

The mixture solution was prepared by mixing the individual stock solutions and diluted with acetonitrile. Table 1 shows the individual concentration of all the components.

Equipment and materials

- Column inlet: Agilent InfinityLab Quick Connect LC fitting (p/n 5067-5965)
- Column outlet: Agilent InfinityLab Quick Turn LC fitting (p/n 5067-5966)
- Agilent Captiva Econofilter, PTFE membrane, 13 mm diameter, 0.2 µm pore size (p/n 5190-5265)
- Agilent vial, screw top, amber, write on spot, certified, 2 mL (p/n 5182-0716)
- Agilent bonded screw cap, bonded blue, PTFE/red silicone septa (p/n 5190-7024)
- Agilent InfinityLab solvent bottle, amber, 1,000 mL (p/n 9301-6526)
- Agilent InfinityLab Stay Safe cap, GL45, three ports, one vent valve (p/n 5043-1219)
- Eppendorf pipettes and repeater
- Sonicator (VWR, Radnor, PA, USA)

Table 1. Stock solutions for all the analytes.

Compound Name	Concentration (mg/mL)	Solvent	Concentration in Mixture (µg/mL)
Thiamine	1.0	ACN:water (9:1)	0.16
Thiamin diphosphate	1.0	ACN:water (1:1)	3.4
Thiamin monophosphate	1.0	Water	3.4
Riboflavin	0.125	Ethanol:1% NH_4OH in water (9:1)	0.43
Riboflavin phosphate	1.0	Water	3.4
Flavin adenine dinucleotide (FAD)	1.0	Water	3.4
Nicotinamide	1.0	ACN:water (9:1)	0.04
Niacin	1.0	ACN:water (9:1)	3.4
NAD	1.0	ACN:water (7:3)	1.7
NADH	1.0	ACN:water (7:3)	6.9
NADP	1.4	ACN:water (7:3)	10.8
NADPH	1.0	ACN:water (7:3)	6.9
D-Pantothenic acid	1.0	ACN:water (7:3)	3.4
Pyridoxine	1.0	ACN:water (9:1)	0.04
Pyridoxal	1.0	ACN:water (9:1)	0.02
Pyridoxamine	1.0	ACN:water (9:1)	0.12
Pyridoxal 5'-phosphate	1.0	Water	3.4
Biotin	0.5	ACN:water (1:1)	1.7
Folic acid (FA)	0.5	ACN:1% NH_4OH in water (1:1)	1.7
Folinic acid/Folinate	0.5	ACN:water (1:1)	1.7
5-Methytetrahydrofolate	0.5	ACN:water (1:1)	1.7
Tetrahydrofolate (THFA)	0.5	ACN:water (1:1)	8.6
Dihydrofolate (DHFA)	0.5	ACN/0.1% NH_4OH in water (1:1)	1.7
Cyanocobalamin (VB12)	0.5	ACN:water (1:1)	5.1

Instrumentation

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6460 triple quadrupole LC/MS (G6460A)

Software

- Agilent MassHunter software for LC/MS data acquisition, version B.08.02
- Agilent MassHunter workstation for qualitative analysis software, version 10.0

Table 2. LC/MS method parameters.

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 685775-924) InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100, 2.7 µm, PEEK-lined (p/n 675775-924)
Stock solution	A) 100 mM ammonium formate in water adjusted pH to 3.0 with formic acid B) 100 mM ammonium acetate in water C) 100 mM ammonium acetate in water adjusted pH to 9.0 with ammonium hydroxide
Mobile Phase 1	A) 100 mL of stock solution a with 900 mL of water B) 100 mL of stock solution a with 900 mL of acetonitrile
Mobile Phase 2	A) 100 mL of stock solution b with 900 mL of water B) 100 mL of stock solution b with 900 mL of acetonitrile
Mobile Phase 3	A) 100 mL of stock solution c with 900 mL of water B) 100 mL of stock solution c with 900 mL of acetonitrile
Mobile Phase 4	A) 100 mL of stock solution c with 900 mL of water, added with 1.0 mL deactivator additives B) 100 mL of stock solution c with 900 mL of acetonitrile, added with 1.0 mL deactivator additives
Gradient	0 to 1 minute, 100% B; 1 to 8 minutes, 100 to 50% B; Stop time: 10 minutes
Flow Rate	0.30 mL/min
Column Temperature	40 °C
Injection Volume	0.5 µL
MS Conditions	
Ion Mode	ESI/Jet Stream ESI, Positive/ Negative
Drying Gas Temperature	250 °C
Drying Gas Flow	6 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	Positive Negative 3,500 V 2,500 V
Nozzle Voltage	Positive Negative 500 V 1,000 V
ΔEMV	0 V

Results and discussion

Three kinds of mobile phase additives with different pH values were explored. With an increase in mobile phase pH from pH 3.0 to pH 7.0, most compounds were retained on the InfinityLab Poroshell 120 HILIC-Z column, and achieved better resolution using mid-pH mobile phase. When comparing pH 7.0 and pH 9.0 mobile phases, retention of most compounds changed very little. However, the mid pH mobile phase provided higher signals in basic conditions under positive mode. Therefore, the mid-pH mobile phase addition provides the best overall separation of all compounds shown in Figure 1.

During the investigation, the phosphorylated molecules including pyridoxal 5'-phosphate, riboflavin phosphate, thiamine diphosphate, and thiamine monophosphate had poor peak shape. When these compounds interact with the steel pathway, including the pump and steel tubing, they often cause peak tailing tail at high concentrations and disappear completely at low concentrations (Figure 2A).

A simple solution was to deactivate the metal sites on the steel surface. This deactivation was done using a mild phosphoric acid wash (0.5% phosphoric acid in 90:10 acetonitrile:water).[1] The phosphoric acid strongly bonds to the active sites on the system, enabling satisfactory analysis of sticky compounds. The peak shapes of four phosphorylated molecules were all improved after washing with 0.5% phosphoric acid as shown in Figures 2A and 2B.

Table 3. Masses monitored by multiple-reaction monitoring.

Compound	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	Polarity
Nicotinamide	123.1	80.1	100	21	Positive
Pyridoxal	168.1	150	75	9	Positive
Pyridoxine	170.1	152	80	13	Positive
Riboflavin	377.2	243	135	25	Positive
Pyridoxamine	169.1	152	170	25	Positive
Niacin	124	80.1	110	21	Positive
Thiamine	265.1	122	70	13	Positive
Biotin	245.1	227	105	13	Positive
D-Pantothenic acid	220.1	90.1	80	13	Positive
D-Pantothenic acid	218.1	87.9	145	9	Negative
VB12	678.3	147.1	165	40	Positive
FAD	786.2	348	180	21	Positive
Riboflavin phosphate	457.1	439	140	13	Positive
Pyridoxal 5'-phosphate	248	150	135	13	Positive
NADH	666.1	136.4	180	60	Positive
NAD	664.1	136.3	175	60	Positive
Folinate	474.2	327	110	17	Positive
5M-TFH	460.2	313	120	17	Positive
Thiamine monophosphate	345.1	122	85	17	Positive
FA	442.2	295	95	9	Positive
DHFA	444.2	178	110	9	Positive
Thiamine diphosphate	425.1	122	95	21	Positive
THFA	446.2	299	115	17	Positive
NADP	744	136.4	180	60	Positive
NADPH	746	136.4	180	60	Positive

Another solution is to add deactivator additive (1 mL of activator, part number 5191-3940 or 5191-4506 in 1 L mobile phase),[2] which chelates free metals and covers exposed active metal sites in the sample flowpath. This additive reduces metal-analyte interaction. Before adding deactivator additive to the mobile phase, perform the same phosphoric acid wash of the system. The results demonstrate a continued improved peak shape shown in the third chromatograms of Figure 2C. As an alternative solution to improve peak shape of these compounds, use the PEEK-lined column with activator additives added. The peak shapes of some compounds were also improved in the chromatograms of Figure 2D.

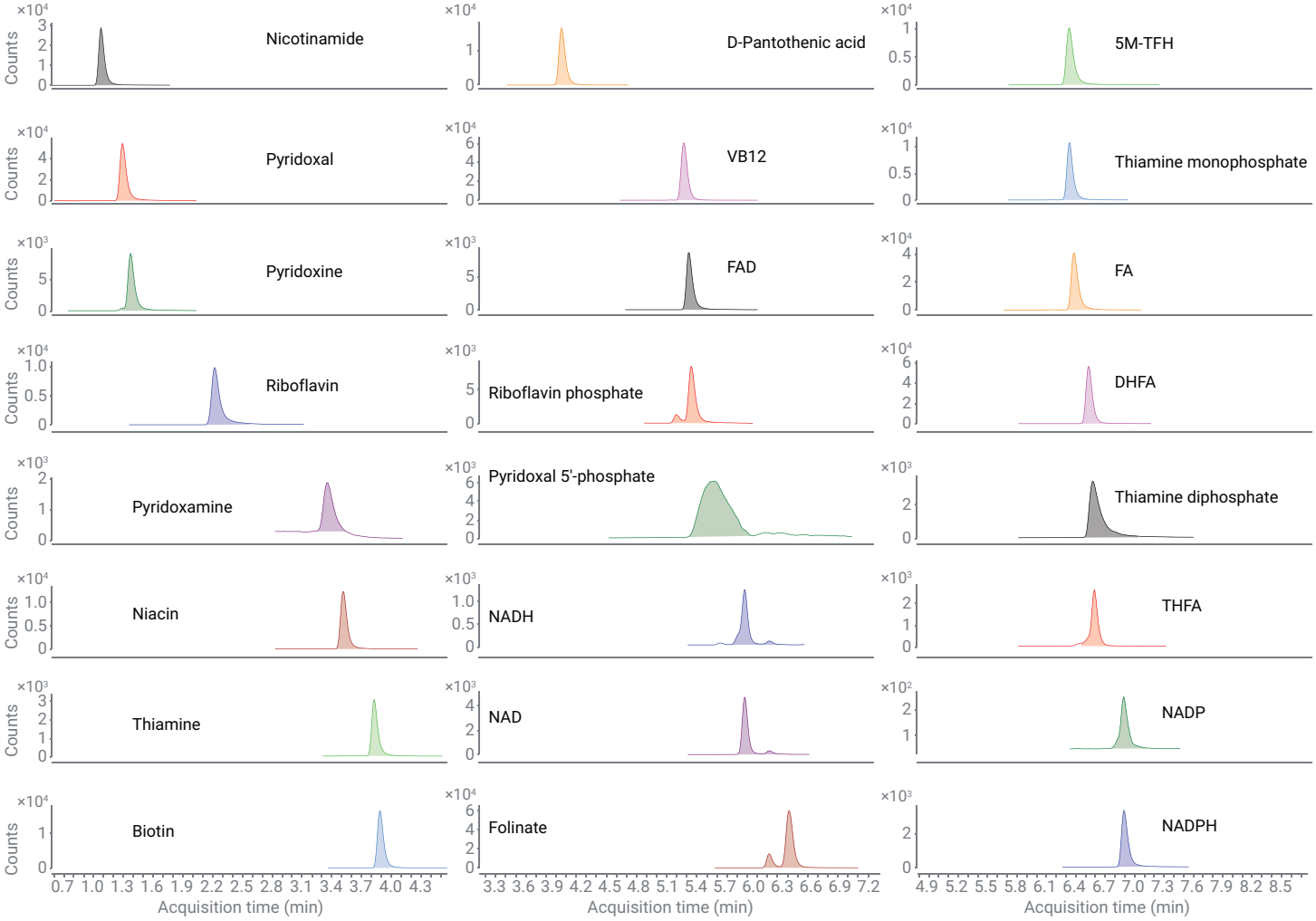

Figure 1. Selected product ions chromatograms for the analytes.

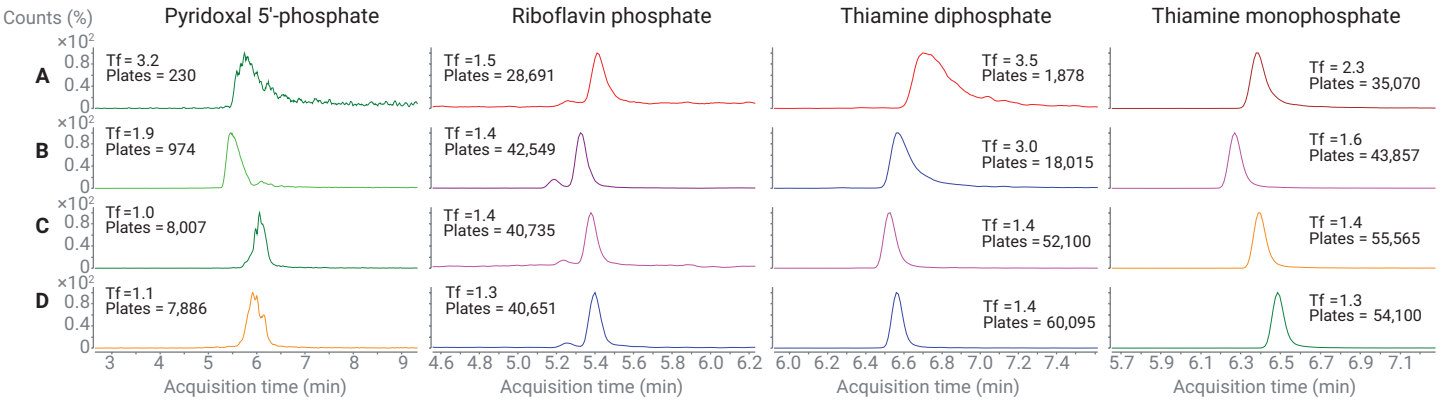

Figure 2. Interactions of phosphorylated metabolites with steel: before and after wash. A) Before system wash; B) After system wash, HILIC-Z column; C) After system wash, HILIC-Z column, with deactivator; D) After system wash, HILIC-Z PEEK-lined column, with deactivator.

Conclusion

The best overall performance of these water-soluble vitamins and their metabolites analysis was achieved using an ammonium acetate-supplemented mobile phase at mid pH with the Agilent InfintyLab Poroshell 120 HILIC-Z column. The peak shape of some phosphorylated metabolites could be significantly improved by flushing the instrument with 0.5% phosphoric acid in 90:10 acetonitrile:water and InfinityLab deactivator additives. The peek-lined InfintyLab Poroshell 120 HILIC-Z column further improved the peak shape of phosphorylated metabolites. The method described in this Application Note is well suited for the analysis of water-soluble vitamins and their metabolites.

References

1. Hydrophilic Interaction Chromatography Method Development and Troubleshooting, *Agilent Technologies Technical Overview*, publication number 5991-9271EN, **2018**.
2. InfinityLab Deactivator Additive User Guide, *Agilent Technologies*, publication number 5991-9516EN, **2018**.

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Printed in the USA, November 12, 2019
5994-1553EN
DE.4472800926

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