Application Note

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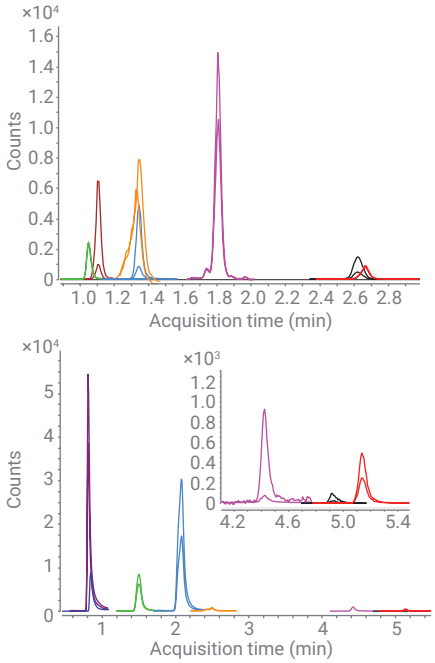
Analysis of Vitamins Using an SFC/UHPLC Hybrid System with a Triple Quadrupole LC/MS for Quantification

Suitable for Agilent 1260 Infinity III LC



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Abstract

This Application Note describes the quantitative determination of fat- and water-soluble vitamins using an Agilent 1260 Infinity II SFC/UHPLC Hybrid System with an Agilent 6470A Triple Quadrupole LC/MS System. A selection of fat-soluble vitamins are separated in SFC mode, and a selection of water-soluble vitamins are separated in UHPLC mode. All vitamins are detected and quantified using the LC/MS system. This study demonstrates that the 1260 Infinity II SFC/UHPLC Hybrid System can determine both fat- and water-soluble vitamins with comparable performance as standalone SFC and UHPLC systems.

Introduction

Performing both an SFC-based separation and a UHPLC-based separation of a given complex sample delivers complementary information about the sample content[1]. These separations are truly orthogonal due to their different separation mechanisms, which are based on the interaction of the analytes with completely different fluid media and stationary phases. Conversely, it is also possible to analyze samples for analytes, which either perform better under SFC separation conditions or under UHPLC separation conditions to obtain complementary information[2]. For example, a group of fat-soluble vitamins can easily be separated under SFC conditions, whereas a group of water-soluble B-vitamins can be separated under reversed-phase conditions.

This Application Note describes the analysis of fat-soluble and water-soluble vitamins on the 1260 Infinity II SFC/UHPLC Hybrid System in SFC mode and UHPLC mode with triple quadrupole mass spectrometry for their quantification.

Experimental

Instrumentation

Agilent 1260 Infinity II SFC/UHPLC Hybrid System:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II Diode Array Detector (G7115A) with high-pressure SFC flow cell
- Agilent 1260 Infinity II Multicolumn Thermostat (MCT) (G7116A) with Agilent InfinityLab Quick Change 4-position/10-port four-column selection valve (G4237A)
- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/10-port valve (G4232C)
- Agilent 1260 Infinity II Isocratic Pump (G7110B) and SFC/MS splitter kit (G4309-68715)
- Agilent 6470A Triple Quadrupole LC/MS with Agilent Jet Stream technology

Instrumental setup

For the conversion of an SFC system to an SFC/UHPLC hybrid system, a quaternary or binary UHPLC pump is added and connected by a 2-positon/10-port valve, which allows direct switching between SFC mode and UHPLC mode (Figure 1). The MCT is equipped with a 4-position/10-port four-column selection valve for switching, for example, from a typical SFC column to a typical analytical UHPLC column (not shown in Figure 1). The central point, outlined in Figure 1, is the plumbing at the 2-positon/10-port valve. The position of the valve shown in Figure 1A connects the SFC pump and the SFC control module to the shared modules of the instrument. After flowing through the autosampler, the column oven with the SFC column, and the detector, the CO_2 stream is connected back to the SFC control module for backpressure regulation. In this position, the quaternary UHPLC pump is connected to waste.

After switching the 2-positon/10-port valve to the UHPLC position, the quaternary pump is connected to the shared modules (Figure 1B). To maintain backpressure, the SFC pump is directly connected to the SFC control module.

An isocratic pump serves as a make-up pump to connect the SFC flowpath with the LC/MS by a flow splitter. In SFC mode, the CO_2 stream is connected to the first splitter for the introduction of the make-up flow after passing the shared modules. The second splitter divides the flow between backpressure regulation at the SFC control module and the MS (Figure 1A). In UHPLC mode, the quaternary pump is connected to the shared modules, and the full flow is diverted to the LC/MS (Figure1B).

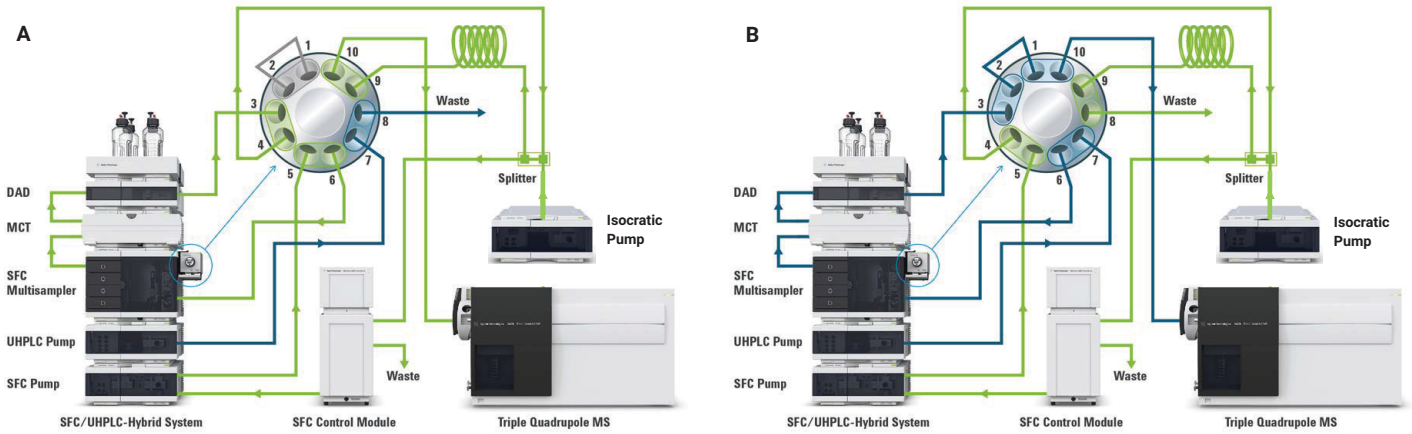

Figure 1. Instrumental setup of the SFC/UHPLC Hybrid system with connection to MS. A) SFC mode with connection to the make-up pump and flow splitting to the MS. B) UHPLC mode with direct flow connection to the MS.

Columns

- **SFC mode:** Agilent ZORBAX SB-C18, 3.0 × 100, 1.8 µm (p/n 828975-302)
- **UHPLC mode:** Agilent ZORBAX SB-Aq, 3.0 × 100 mm, 1.8 µm (p/n 828975-314)

Software

- Agilent OpenLab CDS ChemStation Edition for LC and LC/MS Systems, revision C.01.07 SR3
- Agilent MassHunter Data Acquisition software for triple quadrupole mass spectrometer, version 07.01.
- Agilent MassHunter Qualitative software, version 07.00
- Agilent MassHunter Quantitative software, version 07.00
- Agilent MassHunter MRM and Source optimizer software, version 07.00

SFC method for separation of fat-soluble vitamins

Parameter	Value
Solvent A	CO_2
Modifier B	Methanol
SFC flow	1.5 mL/min
Gradient	0 minutes–1 %B 2.5 minutes–3 %B 4 minutes–15 %B
Backpressure regulator (BPR) temperature	60 °C
BPR pressure	200 bar
Column temperature	40 °C
Injection volume	5.0 µL
Feed solvent	Methanol
Over feed volume	3 µL
Feed speed	1,000 µL/min
Needle wash	3 seconds in methanol
MS Make up	0.3 mL/min, methanol + 5 % water + 0.1 % formic acid

UHPLC method for separation of water-soluble vitamins

Parameter	Value
Solvent A	Water + 5 mM ammonium formate + 0.1 % formic acid
Solvent B	Methanol + 0.1 % formic acid
Flow rate	0.7 mL/min
Gradient	0 minutes–1 %B 7 minutes–70 %B
Stop time	7 minutes
Post time	3 minutes
Column temperature	40 °C
Injection volume	5.0 µL
Needle wash	3 seconds in methanol

MS method for SFC

- **Source parameters for SFC mode:** See Table 1.
- **MRM conditions:** See Table 2 for the creation of the dynamic MRM method for the determination of seven fat-soluble vitamins in SFC mode.

MS methods for UHPLC mode

- **Source parameters for UHPLC mode:** See Table 1.
- **MRM conditions:** See Table 3 for the creation of the dynamic MRM method for the determination of eight water-soluble vitamins in UHPLC mode.

Table 1. MS source conditions for SFC and UHPLC modes.

MS Source conditions	SFC Method	UHPLC Method
Gas temperature	220 °C	300 °C
Gas flow	9 L/min	9 L/min
Sheath gas temperature	350 °C	400 °C
Sheath gas flow	11 L/min	11 L/min
Nebulizer	50 psi	35 psi
Capillary voltage	4,000 V	3,000 V
Nozzle voltage	1,000 V	0 V
EMV	+ 150 V	+ 200 V
Polarity	Positive	Positive

Table 2. Precursor ions, product ions, fragmentor voltage, and collision energy used for the setup of the dynamic MRM triple quadrupole method for the determination of the seven fat-soluble vitamins separated in SFC mode using the 1260 Infinity II SFC/UHPLC Hybrid System.

Compound	RT (min)	Precursor ion (*m/z*)	Fragmentor (V)	Product ion 1 (*m/z*)	Collision energy (V)	Cell acceleration (V)	Product ion 2 (*m/z*)	Collision energy (V)	Cell acceleration (V)
Menaquinone (K_2)	1.048	445	145	187	20	4	81	31	2
Phylloquinone (K_1)	1.102	451.7	155	187	22	4	171	36	4
α-Tocopherol	1.336	431	120	165	18	4	137	46	3
Retinol (A)	1.357	269	100	93	21	1	81	18	1
Retinyl palmitate	1.795	269	120	93	21	1	81	23	1
Ergocalciferol (D_2)	2.621	397	120	107	20	4	69	24	2
Cholecalciferol (D_3)	2.659	385	120	25	8	4	107	28	2

Table 3. Precursor ions, product ions, fragmentor voltage, and collision energy used for the setup of the dynamic MRM triple quadrupole method for the determination of the eight water-soluble vitamins separated in UHPLC mode using the 1260 Infinity II SFC/UHPLC Hybrid System.

Compound	RT (min)	Precursor ion (m/z)	Fragmentor (V)	Product ion 1 (m/z)	Collision energy (V)	Cell acceleration (V)	Product ion 2 (*m/z*)	Collision Energy (V)	Cell acceleration (V)
Pyridoxamine (B_6)	0.817	169.1	87	152	8	2	134	20	2
Thiamine (B_1)	0.854	265.1	90	144	8	2	122	12	2
Nicotinic acid (B_3)	1.501	124	117	80.1	20	2	53.2	32	2
Nicotinamide (B_3)	2.084	123	112	80.1	20	2	53.2	32	2
Pantothenic acid (B_5)	2.500	220.1	80	202	8	2	90.1	80	2
Biotin (B_7)	4.425	245	85	227.1	10	2	123	28	2
Folic acid (B_{11})	4.925	442	102	295.1	12	2	176	40	2
Cyanocobalamin (B_{12})	5.139	678.6	170	359	20	2	147	40	2

Samples

Seven fat-soluble vitamins: Menaquinone (K_2), phylloquinone (K_1), α-tocopherol (E), retinol (A_1), retinyl palmitate, ergocalciferol (D_2), and cholecalciferol (D_3) (the final concentrations in the mixture were 1 ppm each in MeOH, which has been used for the creation of calibration curves by further dilution, as described below).

Eight-water soluble vitamins: Thiamine (B_1), pyridoxamine (B_6), nicotinic acid (B_3), nicotinamide (B_3), biotin (B_7), pantothenic acid (B_5), folic acid (B_{11}), and cyanocobalamin (B_{12}) (the final concentrations in the mixture was 1 ppm each in MeOH, which has been used for the creation of calibration curves by further dilution, as described below),

Chemicals

All solvents were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). Chemicals were purchased from Sigma-Aldrich (Germany).

Results and Discussion

Analysis of fat-soluble vitamins in SFC mode

As an example of the analysis of fat-soluble vitamins in SFC mode, a mixture of seven fat-soluble vitamins was used (Figure 2). This mixture was separated in a short four-minute gradient using a C18 column with methanol as a modifier for CO_2. All seven compounds eluted in a time window between 1.0 and 2.8 minutes, and were detected by a triple quadrupole LC/MS in dynamic MRM mode (Figure 3). Vitamins K_1 and K_2 eluted early, but separated between 1.0 and 1.2 minutes. Vitamins D_2 and D_3 eluted at the end of the gradient, between 2.55 and 2.72 minutes.

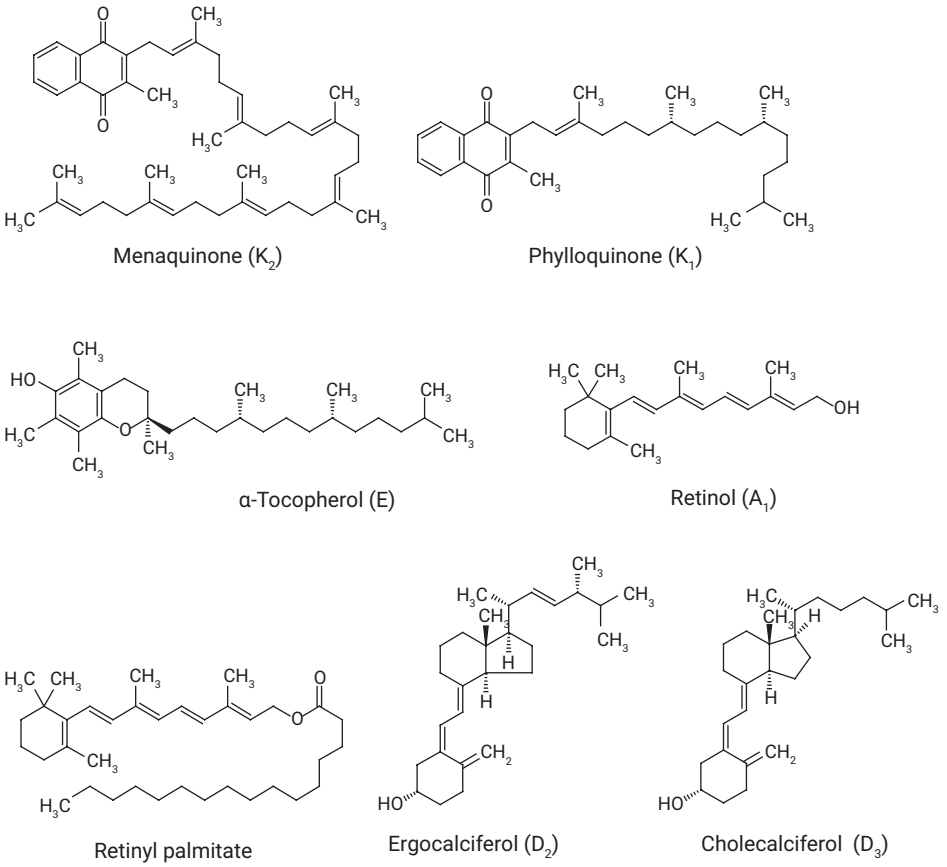

Figure 2. Structures of the seven fat-soluble vitamins, which were separated under SFC conditions using the 1260 Infinity II SFC/UHPLC Hybrid system.

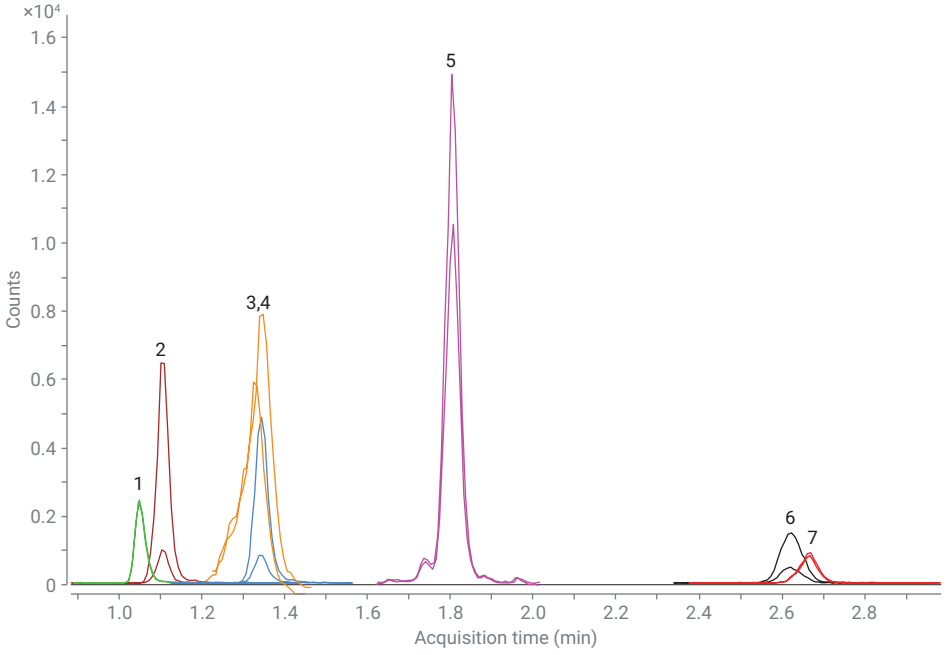

Figure 3. Separation of seven fat-soluble vitamins (100 ppb each) in a short gradient under SFC conditions with detection by triple quadrupole MS in dynamic MRM mode (see Table 2).

For all compounds, calibration curves were created between 1,000 and 1 ppb (1,000, 200, 100, 20, 10, 2, and 1 ppb). The limit-of-quantification (LOQ) was calculated for a signal-to-noise (S/N) ratio of 10, and the limit-of-detection (LOD) for an S/N ratio of 3. For instance, the LOQ for the early eluting vitamin K_2 was 5 ppb, and for the late eluting vitamin D_2 the LOQ was 10 ppb, both with excellent linearities (Figure 4).

The typically observed LOQs were at or below 5 ppb, the typical retention time precisions were at or below 0.15 %, and the typical area precisions below 2.7 % (Table 4).

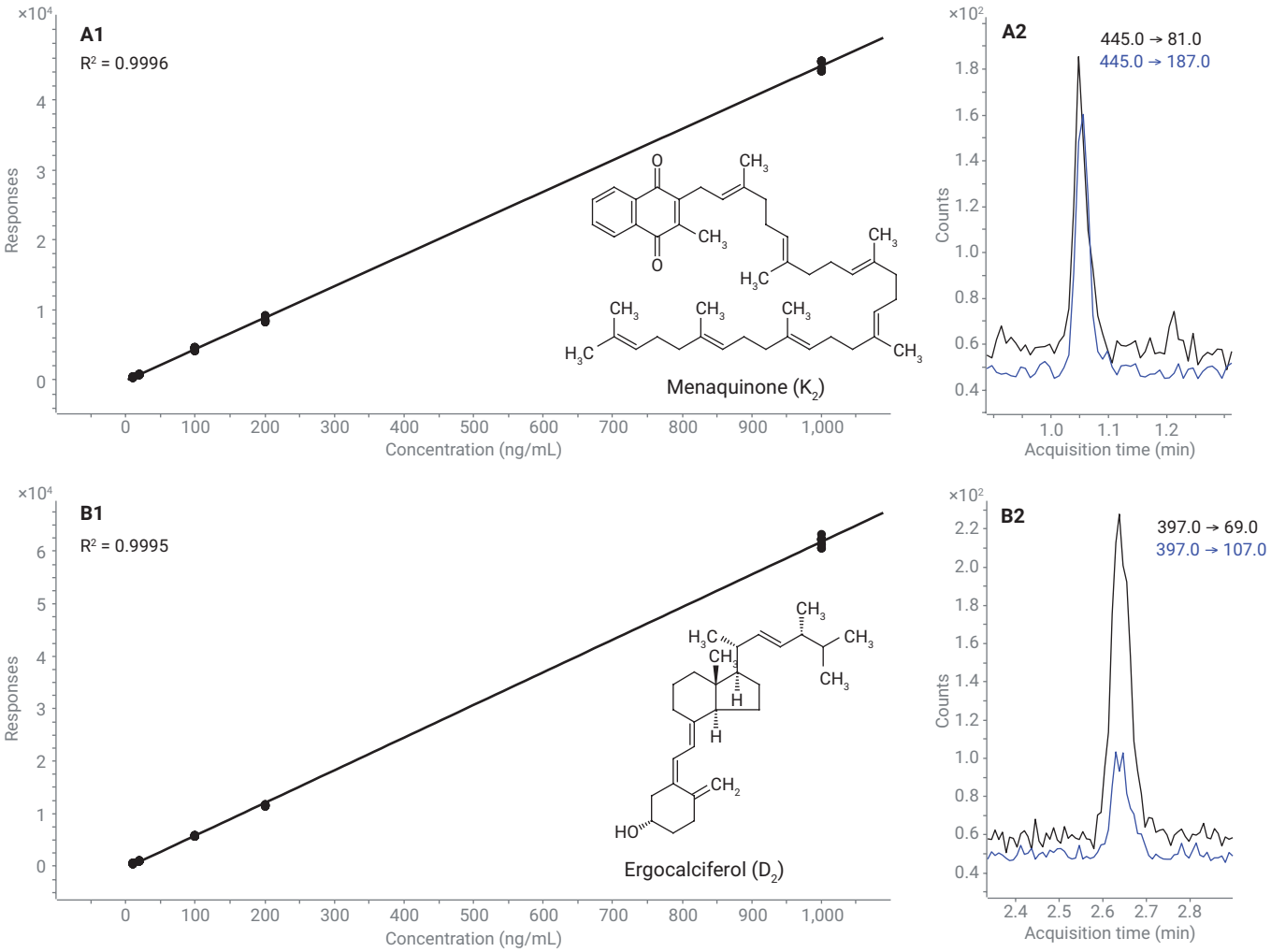

Figure 4. Calibration curves of: A1) Menaquinone (K_2) from 1,000 to 5 ppb; A2) Quantifier and qualifier ions at the LOQ of 5 ppb (S/N = 10); B1) Ergocalciferol (D_2) from 1,000 to 10 ppb; B2) Quantifier and qualifier ions at the LOQ of 10 ppb (S/N = 10).

Table 4. Results obtained from the seven fat-soluble vitamins separated under SFC conditions for retention time and area precision, LOD, LOQ, and linearity (n = 10).

No.	Compound	RT (min)	RT RSD (%)	Area RSD	LOD (ppb)	LOQ (ppb)	Linearity
1	Menaquinone (K_2)	1.048	0.13	2.79	2	5	0.9996
2	Phylloquinone (K_1)	1.102	0.11	3.81	0.6	2	0.9995
3	α-Tocopherol	1.336	0.25	2.27	0.7	2.5	0.9991
4	Retinol (A)	1.357	0.17	2.3	5	10	0.9995
5	Retinyl palmitate	1.795	0.09	2.32	2	5	0.9991
6	Ergocalciferol (D_2)	2.621	0.15	1.84	3	10	0.9995
7	Cholecalciferol (D_3)	2.659	0.15	2.73	5	15	0.9997

Analysis of water-soluble vitamins in UHPLC mode

In UHPLC mode, a mixture of eight water-soluble vitamins contained in the B-complex were analyzed (Figure 5). The eight compounds inherent in the mixture were separated in a short seven-minute gradient by a C18 SB-Aq reversed-phase column with acetonitrile/water as mobile phase (Figure 6). The elution started at 0.81 and 0.85 minutes with pyridoxamine and thiamine, respectively. All eight compounds eluted in a time window between 0.8 and 5.2 minutes, with cyanocobalamin as the last eluting compound.

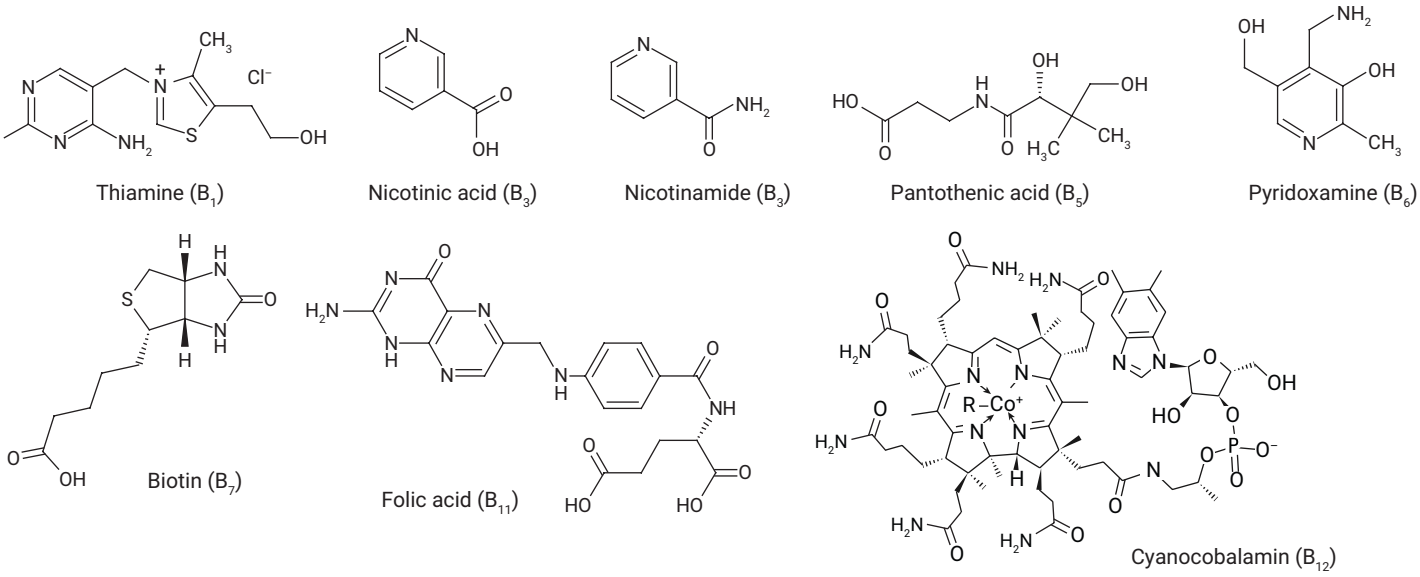

Figure 5. Structures of the eight water-soluble vitamins, which were separated under UHPLC conditions using the 1260 Infinity II SFC/UHPLC Hybrid System.

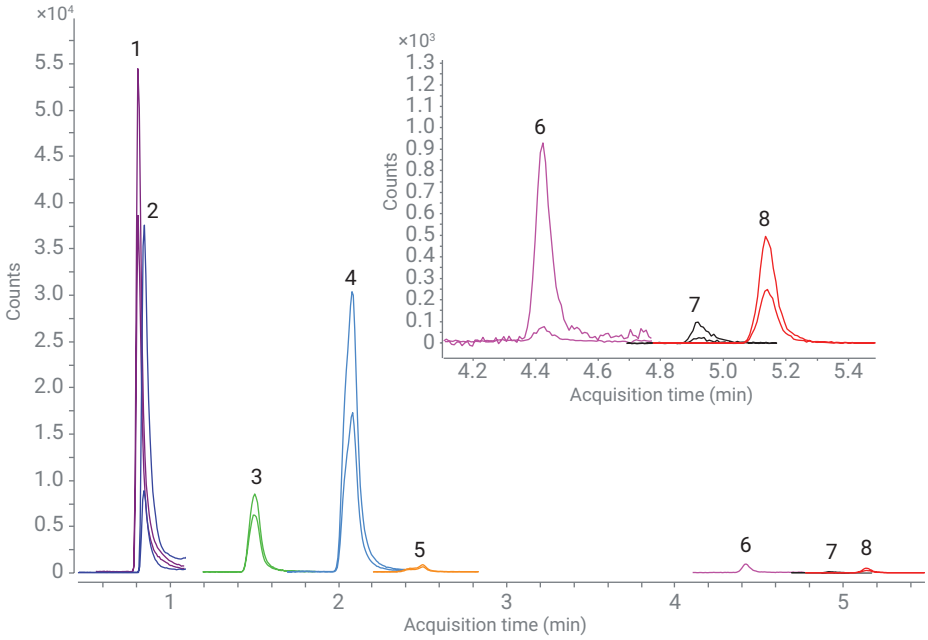

Figure 6. Separation of eight water-soluble vitamins (100 ppb each) in a short gradient under UHPLC conditions with detection by triple quadrupole MS in dynamic MRM mode (see Table 3). The inset shows the last three lower abundant ions.

Calibration curves between 1,000 and 1 ppb were created for all eight compounds. The LOQ was calculated for an S/N ratio of 10, and the LOD for an S/N ratio of 3. For instance, the calculated LOQ for nicotinic acid was 0.6 ppb, and for the late eluting vitamin B_{12} the LOQ was 5 ppb, both with excellent linearities (Figure 7).

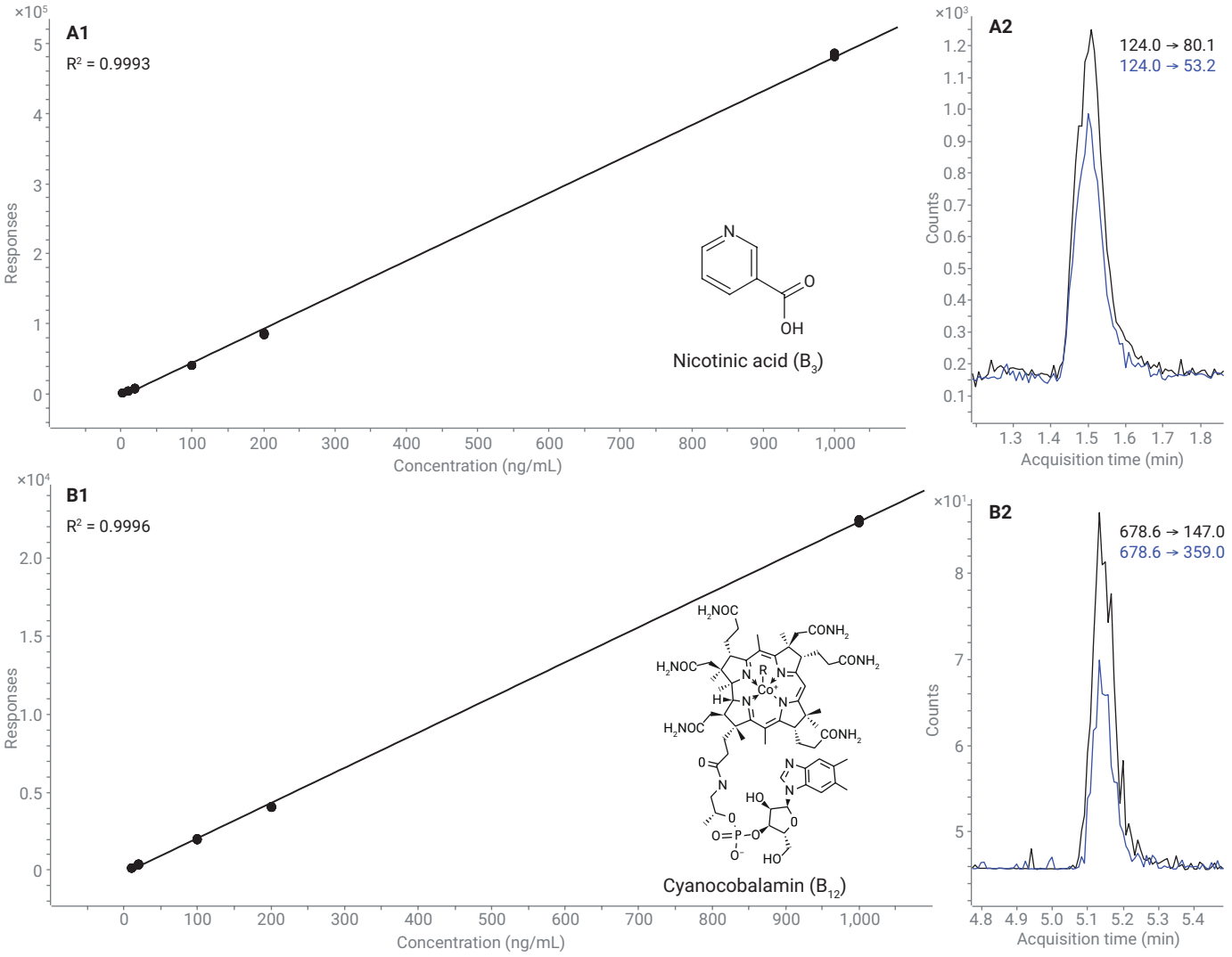

Figure 7. Calibration curves of: A1) Nicotinic acid (B_3) from 1,000 to 1 ppb; A2) Quantifier and qualifier ions at 1 ppb (S/N = 15); B1) Cyanocobalamin (B_{12}) from 1,000 to 10 ppb; B2) Quantifier and qualifier ions 10 ppb (S/N = 20).

Some of the observed LOQs were at or below 1 ppb, the typical retention time precisions were at or below 0.2 %, and the typical area precisions below 2.0 % (Table 5).

Conclusions

This Application Note demonstrates the advantage of the 1260 Infinity II SFC/UHPLC Hybrid System for the analysis of compounds that are preferably analyzed under SFC or UHPLC conditions, on a single system. Analytical results were delivered in both SFC and UHPLC modes, which are comparable with results from standalone instruments regarding LODs, LOQs, and precision and linearity of peak areas and retention times. The values determined using the 1260 Infinity II SFC/UHPLC Hybrid System with the Agilent 6470 Triple Quadrupole LC/MS for peak area RSD, retention time RSD, linearity, and LODs and LOQs demonstrate the excellent performance of the combined system.

Table 5. Results obtained from the eight water-soluble vitamins separated under UHPLC conditions for retention time and area precision, LOD, LOQ, and linearity (n = 10).

No.	Compound	RT (min)	RT RSD (%)	Area RSD	LOD (ppb)	LOQ (ppb)	Linearity
1	Pyridoxamine (B_6)	0.817	0.22	0.76	0.2	0.6	0.9990
2	Thiamine(B_1)	0.854	0.24	1.44	0.1	0.3	0.9991
3	Nicotinic acid (B_3)	1.501	0.16	1.06	0.2	0.6	0.9993
4	Nicotinamide (B_3)	2.084	0.14	0.92	0.3	1	0.9994
5	Pantothenic acid (B_5)	2.500	0.16	1.32	3	10	0.9993
6	Biotin (B_7)	4.425	0.09	2.69	2	5	0.9993
7	Folic acid (B_{11})	4.925	0.13	2.93	3	10	0.9997
8	Cyanocobalamin (B_{12})	5.139	0.18	1.22	2	5	0.9996

References

1. Naegele, E. Orthogonal Chromatographic Separations using the Agilent 1260 Infinity II SFC/UHPLC Hybrid System, *Agilent Technologies Application Note*, publication number 5991-8276EN, **2017**.
2. Maria Rambla-Alegre, M.; Dunkle, M. N.; Vanhoenacker, G.; David, F.; Sandra, P.; Vollmer, M. Analysis of Antioxidants in vegetable oils using the Agilent 1260 Infinity SFC/UHPLC Hybrid System with MS detection, *Agilent Technologies Application Note*, publication number 5991-1546EN, **2017**.

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