Technical Overview

Simplify Your Method Development Using the Agilent 1260 Infinity II Prime LC System and InfinityLab LC/MSD iQ

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Abstract

This technical overview demonstrates how a method development workflow including hyphenation with MS can be dramatically simplified using the latest Agilent instrumentation and features. This overview presents an example of this process using an Agilent 1260 Infinity II Prime LC System for the analysis of the Agilent LC/MS 7-analyte system suitability kit. This kit has been designed for chromatographic system suitability tests on reversed-phase LC and LC/MS systems. By effortless variation of column chemistries, mobile phases, buffer concentrations, and temperatures, the best chromatographic performance could be achieved with the Agilent InfinityLab Poroshell 120 EC-C18 column. For mass spectral detection, the Agilent InfinityLab LC/MSD iQ could be easily coupled to the LC-UV system without the need for tedious method development using the Auto Acquire mode.

Introduction

Chromatographic system suitability tests are needed to ensure a minimum of compliance for chromatographic and instrumental performance. For this reason, Mutton *et al.* have developed a seven-component test mixture designed for applications using a generic gradient and a reversed-phase LC/MS system.[1] The test mix (Figure 1) consists of 8-bromoguanosine (8-BG), amitriptyline (Ami), 4-chlorocinnamic acid (4-CCA), diethyl phthalate (DEP), diamyl phthalate (DAP), di-n-hexyl phthalate (DHP), and dioctyl phthalate (DOP). It provides structural diversity containing neutrals, an acidic (4-CCA), and a basic (Ami) compound, which can show secondary interactions with reversed-phase columns at low pH. Due to its broad lipophilicity range, with 8-BG having a logP of −2.27 and DOP a logP of 8.97, a gradient covering a broad range of elution strength must be precisely delivered to elute all compounds. Additionally, DHP has a lower concentration and can be used to check the system sensitivity. Following Mutton *et al.*[1] and aiming towards a system suitability standard mixture that is readily available and consistent, Agilent has released the LC/MS 7-analyte system suitability kit as a commercial product.

This technical overview demonstrates a method development workflow for analysis of the LC/MS 7-analyte system suitability kit using the 1260 Infinity II Prime LC System. The most influential parameters affecting chromatographic separations are stationary phase (column chemistry), mobile phase composition (pH, buffer type/molarity, organic modifier), and temperature. As variation of these parameters can be a time-consuming task, several features for automation of method development are available: The Agilent 1260 Infinity II Multicolumn Thermostat, equipped with a four-column selection valve, can be used to reduce user interaction during column screening and the 1290 Infinity Valve Drive equipped with a 12-position/13-port solvent selector valve head can dramatically simplify solvent screening. The Agilent 1260 Infinity II Flexible Pump alone can utilize up to four different solvent channels without compromising on precision and accuracy.[2] Using the 12-position/13-port solvent selector valve head enables one pump channel to access up to 12 additional solvent channels, which provides even more flexibility in a method development process. Another helpful feature for buffer molarity screening is the BlendAssist functionality of the 1260 Infinity II Flexible Pump, which is capable of varying buffer or modifier concentration using ternary or quaternary gradients.[3]

Agilent InfinityLab Poroshell 120 columns are based on superficially porous particle technology and provide the speed and resolution of a sub-2 µm column with reduced backpressure, making them exceptionally well suited to the 1260 Infinity II Prime LC System, which has a maximum operating pressure of 800 bar. InfinityLab Poroshell 120 columns are available with different bonding chemistries, including those of the well-established ZORBAX column portfolio, making them an excellent choice for successful method development.

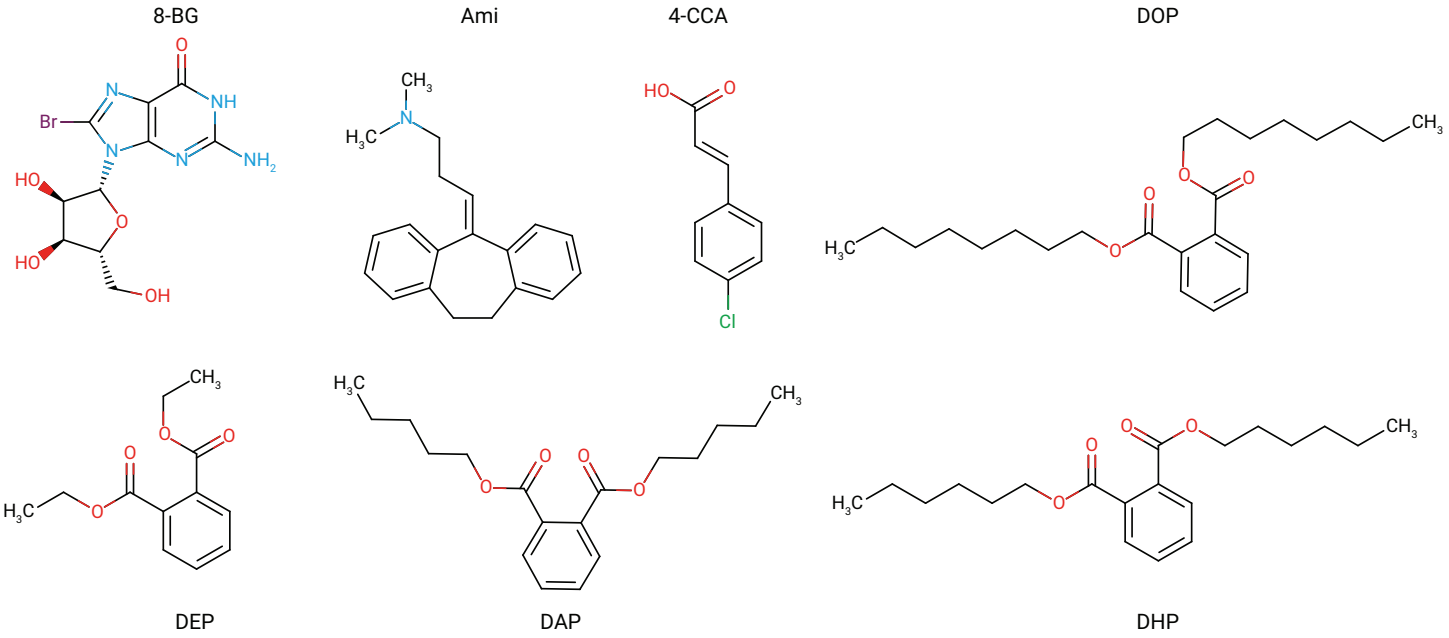

Figure 1. Chemical structures of the analytes in the Agilent LC/MS 7-analyte system suitability kit.

The LC/MSD iQ provides an orthogonal detection principle to UV detection, enabling compound identification by molecular mass detection and sometimes higher sensitivity and specificity compared to UV detection. The Auto Acquire mode automatically adjusts optimal settings for the electrospray ionization (ESI) source, enabling short method setup times and ease of use. Due to its compact design, the MSD can be coupled with 1260 and 1290 Infinity II LC Series in a single stack using the Agilent InfinityLab Flex Bench system. Consequently, the MS can be easily added to an existing HPLC system for improved specificity and confidence into UV results.

Experimental

Instrument

- Agilent 1260 Infinity II Flexible Pump (G7104C), No mixer equipped
- Agilent 1260 Infinity II Multisampler (G7167A), 20 µL loop (G4267-60311), 0.12 mm seat assembly (G4267-87012)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) equipped with four-column selector 800 bar (G4237A), standard heat exchanger (G7116-60015)
- Agilent 1260 Infinity II Diode Array Detector HS (G7117C), Agilent InfinityLab Max-Light cartridge cell (G2112-60008)
- Agilent 1290 Infinity Valve Drive (G1170A) equipped with 12-position/13-port solvent selector valve head (5067-4159)
- Agilent InfinityLab LC/MSD iQ (G6160AA)

Software

Agilent OpenLab CDS (Version 2.4)

Sample

Agilent LC/MS 7-analyte system suitability kit (p/n 5191-4546)

Solvents

- Mobile phase A
  - Water + 0.1% formic acid
  - Water + 0.1% trifluoroacetic acid
  - Water + 10 mM ammonium formate, pH 3
  - Water + 10 mM ammonium acetate, pH 3.8
  - Water + 10 mM ammonium acetate, pH 4.5
  - Water + 10 mM ammonium acetate, pH 5.4
  - Water + 10 mM ammonium bicarbonate, pH 6.2
  - Water + 10 mM ammonium bicarbonate, pH 6.6
- Mobile phase B
  - Acetonitrile
  - Acetonitrile/methanol (1:1) (v/v)
- All solvents were purchased from Merck, Honeywell, or VWR.
- 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, Merck-Millipore).

Columns

- Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm (p/n 699675-902)
- Agilent InfinityLab Poroshell 120 EC-C8, 2.1 × 50 mm, 1.9 µm (p/n 699675-906)
- Agilent InfinityLab Poroshell 120 Bonus-RP, 2.1 × 50 mm, 2.7 µm (p/n 699768-901)
- Agilent InfinityLab Poroshell 120 SB-C8, 2.1 × 100 mm, 2.7 µm (p/n 685775-906)
- Agilent InfinityLab Poroshell 120 Sb-Aq, 2.1 × 100 mm, 2.7 µm (p/n 685775-914)
- Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 µm (p/n 699675-912)
- Agilent InfinityLab Poroshell 120 PFP, 2.1 × 50 mm, 1.9 µm (p/n 699675-408)

Methods

For column and solvent screening, instrument parameters were fixed (Table 1) while different mobile phases and columns were used in every possible combination.

Table 1. Generic parameters for method screening experiments using LC-UV.

Parameter	Value
Pump	
Flow Rate	1 mL/min
Gradient	0 to 1.5 minutes: 2 to 100% B 1.5 to 2.3 minutes: 100% B 2.4 to 3.8 minutes: 2% B
Multisampler	
Injection Volume	0.3 µL
Thermostat	Off
Column compartment	
Temperature	40 °C
DAD	
Peak Width	>0.0031 minutes (80 Hz)
Wavelength	Signal: 254/6 nm Reference: 360/100
Spectra Acquisition	On, 190 to 400 nm

The 1260 Infinity II Multicolumn Thermostat equipped with an Agilent InfinityLab Quick Change four-column selection valve (p/n 5067-4279) was used for screening of several chromatographic columns in a single sequence. For column storage, a specific method for flushing with pure organic solvent has been added to the end of each sequence. The 1290 Infinity Valve Drive equipped with a 12-position/13-port selector valve head (p/n 5067-4159) enabled testing of different solvent combinations in a single sequence without manual exchange of solvents. If several solvents were used in a single sequence, system flushing was performed using a wait time after a new method load. Each combination of column and solvent was measured in triplicate. For evaluation of chromatographic data from column and solvent screening, the third sample injection of every combination was considered. Integration start time was set to 0.5 minutes to exclude the sample solvent peak and peaks with height and/or area below 10 were excluded. Chromatographic data showing fewer than seven peaks, a resolution value below 3 or peak symmetry outside of 0.5 to 1.5 were excluded. The following criteria were used to decide which combination of column and solvent is the most beneficial of the remaining data: minimum resolution, resolution sum, and minimum symmetry. For the most beneficial combination, buffer molarity and temperature screening were conducted. Buffer molarity screening was carried out using molarities between 2 and 10 mM using 2 mM increments and temperature screening was performed between 40 and 60 °C using 5 °C increments.

Table 2 shows the final LC-UV-MS method parameters.

Table 2. Final LC-UV-MS method.

Parameter	Value
Column	InfinityLab Poroshell 120 EC-C18 2.1 × 50 mm, 1.9 µm (p/n 699675-902)
Solvent	A: Water + 10 mM ammonium bicarbonate, pH 6.6 B: Acetonitrile
Gradient	0 minutes: 2% B 1.5 minutes: 100% B 2.3 minutes: 100% B 2.4 minutes: 2% B
Stop Time	3.8 min
Flow Rate	1 mL/min
Injection Volume	0.3 µL
Column Temperature	45 °C
Sample Temperature	Ambient
DAD	254/6 nm, reference 360/100, 80 Hz
MS Scan Type	SIM
Gas Temperature/Flow	325 °C/13 L/min
Nebulizer/Capillary Voltage	55 psi/~3,500 V
Compound/Mass (*m/z*)/ Polarity/Fragmentor (V)	4-CCA/181/negative/100 8-BG/230/positive/100 Ami/278.2/positive/100 DAP/307.2/positive/100 DEP/223.1/positive/100 DHP/335.2/positive/100 DOP/391.3/positive/110

Results and discussion

Column and solvent screening

To develop a method for chromatographic separation of the Agilent LC/MS 7-analyte system suitability kit, a total number of 112 combinations of columns and solvents were tested using a generic gradient elution method (Table 1). For this first step of method development, columns and solvents were selected to cover a wide range of reversed-phase column chemistries as well as different pH values. Application of the column selection and solvent selection valve enabled testing of different solvent and column combinations without the need of changing solvents and columns in between. Therefore, less system interaction and manual work were needed, and a large number of screening runs could be performed with minimal effort. As coupling to the LC/MSD iQ was planned as a further step, MS-compatible solvents were preferred and pH values were adjusted in accordance with the buffering range of additives. The ten most beneficial column and solvent combinations are summarized in Table 3. These 10 combinations were exclusively achieved with EC-C8 and EC-C18 bonding chemistry. Minimum symmetry values showed very similar values for all 10 combinations, while resolution sum ranged between 77.6 and 96.1 (Table 3). Minimum resolution values decreased with low pH solvents and increased with solvents with higher pH.

The InfinityLab Poroshell 120 EC-C18 (2.1 × 50 mm, 1.9 µm) showed excellent results in terms of resolution, symmetry, and peak width with several different mobile phases. As shown in Figure 2, different buffers affected the elution order of Ami (peak no. 2), 4-CCA (peak no. 3), and DEP (peak no. 4). The peak shape of Ami was particularly

impacted by buffers with different pH values. Overall, the combination of an InfinityLab Poroshell 120 EC-C18 column with an aqueous 10 mM ammonium bicarbonate buffer at pH 6.6 and acetonitrile as solvents showed the best peak shape for Ami (Figure 2A) as well as the highest values for minimum resolution, resolution sum, and minimum symmetry (Table 3).

For this combination, retention time precision was calculated using the last 7 of 10 injections showing excellent results below the pump specification of 0.15% relative standard deviation and 0.02 minutes standard deviation (Table 4). For further optimization, this setup was chosen for buffer molarity and temperature screening.

Table 3. Combinations of column and solvent showing all seven analytes separated with a minimum resolution of 3 and peak symmetries between 0.5 and 1.5 (bold: best combination; AmBic = ammonium bicarbonate; AmFor = ammonium formate; AmAc = ammonium acetate; ACN = acetonitrile; MeOH = methanol).

Minimum Resolution	Resolution Sum	Minimum Symmetry	Bonding Chemistry	Solvent A	Solvent B
5.4	82.3	0.54	EC-C8	Water + 10 mM AmBic pH 6.6	ACN/MeOH (1/1)
6.7	80.8	0.52	EC-C18	Water + 10 mM AmBic pH 6.6	ACN/MeOH (1/1)
6.8	95.5	0.57	EC-C8	Water + 10 mM AmBic pH 6.6	ACN
8.4	96.1	0.58	EC-C18	Water + 10 mM AmBic pH 6.6	ACN
6.8	77.6	0.54	EC-C18	Water + 10 mM AmBic pH 6.2	ACN/MeOH (1/1)
7.9	90.2	0.57	EC-C18	Water + 10 mM AmBic pH 6.2	ACN
3.2	89.8	0.51	EC-C18	Water + 10 mM AmFor pH 3	ACN
3.1	88.3	0.51	EC-C18	Water + 10 mM AmAc pH 3.8	ACN
3.3	95.9	0.51	EC-C8	Water + 10 mM AmAc pH 4.5	ACN/MeOH (1/1)
3.6	90.9	0.53	EC-C18	Water + 10 mM AmAc pH 4.5	ACN/MeOH (1/1)

Table 4. Retention time precision using an Agilent InfinityLab Poroshell 120 EC-C18 column (solvent A: 10 mM ammonium bicarbonate buffer at pH 6.6, solvent B: acetonitrile).

Compound	8-BG	4-CCA	DEP	Ami	DAP	DHP	DOP
Average Retention Time (Minutes)	0.767	1.000	1.529	1.655	2.029	2.149	2.361
Standard Deviation (Minutes)	0.001	0.001	0.002	0.002	0.002	0.002	0.002
Relative Standard Deviation (%)	0.132	0.116	0.100	0.120	0.076	0.073	0.080

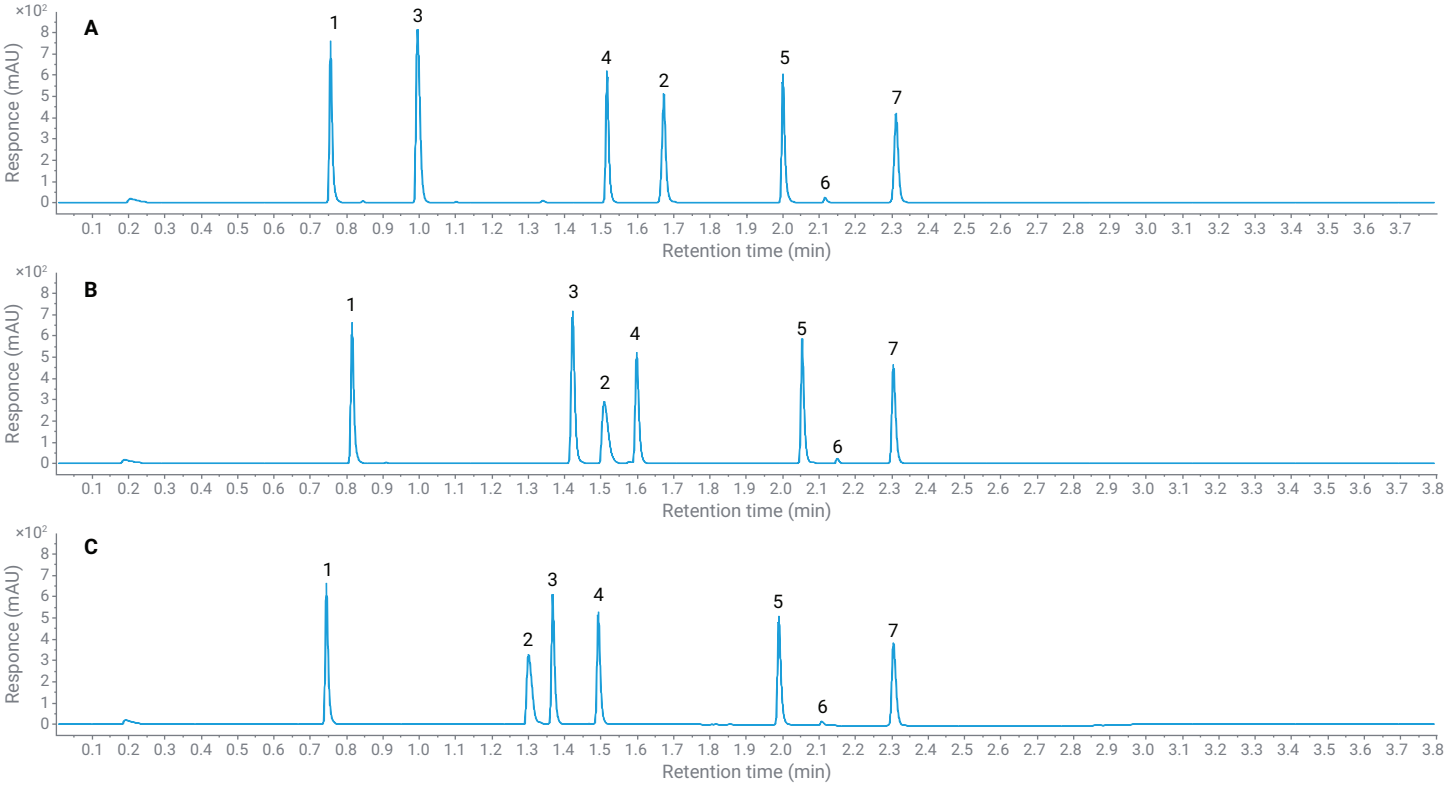

Figure 2. LC-UV analysis of the LC/MS 7-analyte system suitability kit using an Agilent InfinityLab Poroshell 120 EC-C18 column and different solvents: A) A: water + 10 mM AmBic pH 6.6, B: ACN; B) A: water + 10 mM AmAc pH 4.5, B: ACN/MeOH (1:1); C) A: water + 10 mM AmFor pH 3, B: ACN (1 = 8-BG, 2 = Ami, 3 = 4-CCA, 4 = DEP, 5 = DAP, 6 = DHP, 7 = DOP).

Buffer molarity screening

Aiming for a preferably MS-friendly method, buffer molarity was automatically decreased from 10 to 2 mM using the BlendAssist software feature. Using the quaternary mixing capability of the 1260 Infinity II Flexible Pump, the 10 mM buffer was automatically diluted with water to lower buffer concentrations gradually to 2 mM. Thereby, the time-consuming task of preparing different buffer concentrations could be avoided and several concentrations could be assessed in a single sequence run without any manual interaction. As reduction of buffer molarity showed a negative influence on peak height and width for Ami and 4-CCA (Figure 3), 10 mM buffer was chosen for the final method.

Temperature screening

For optimization of column temperature, five different methods with temperatures between 40 and 60 °C were tested using a wait time of 30 min for temperature adjustment if a new method was loaded. Higher temperatures (55, 60 °C) had a negative impact on the peak width of 8-BG (peak no. 1, Figure 4). Lower temperatures (40, 45, and 50 °C) showed feasible results, while 45 °C seemed to be the best trade-off regarding chromatographic performance, and ran at lower backpressure compared to the initial temperature setup at 40 °C.

Hyphenation with MS

MS analysis was conducted using Auto Acquire for automated definition of ESI parameters and SIM for selective detection of target compounds. Due to high responses of several target substances, the LC/MS 7-analyte system suitability kit was diluted with the sample solvent dimethylformamide (1:10). 8-BG was detected using its main fragment (*m/z* 230), because its labile N-glycosidic bond leads to in-source fragmentation that results in a more abundant fragment ion compared to its precursor ion. While peak heights of target compounds were very comparable with UV detection, the ionization efficiency showed a high variability. The signal for 4-CCA showed the lowest abundance with a peak height of less than 0.1% compared to Ami, which showed the highest intensity. An overlay of the normalized mass traces resulting from SIM is depicted in Figure 5.

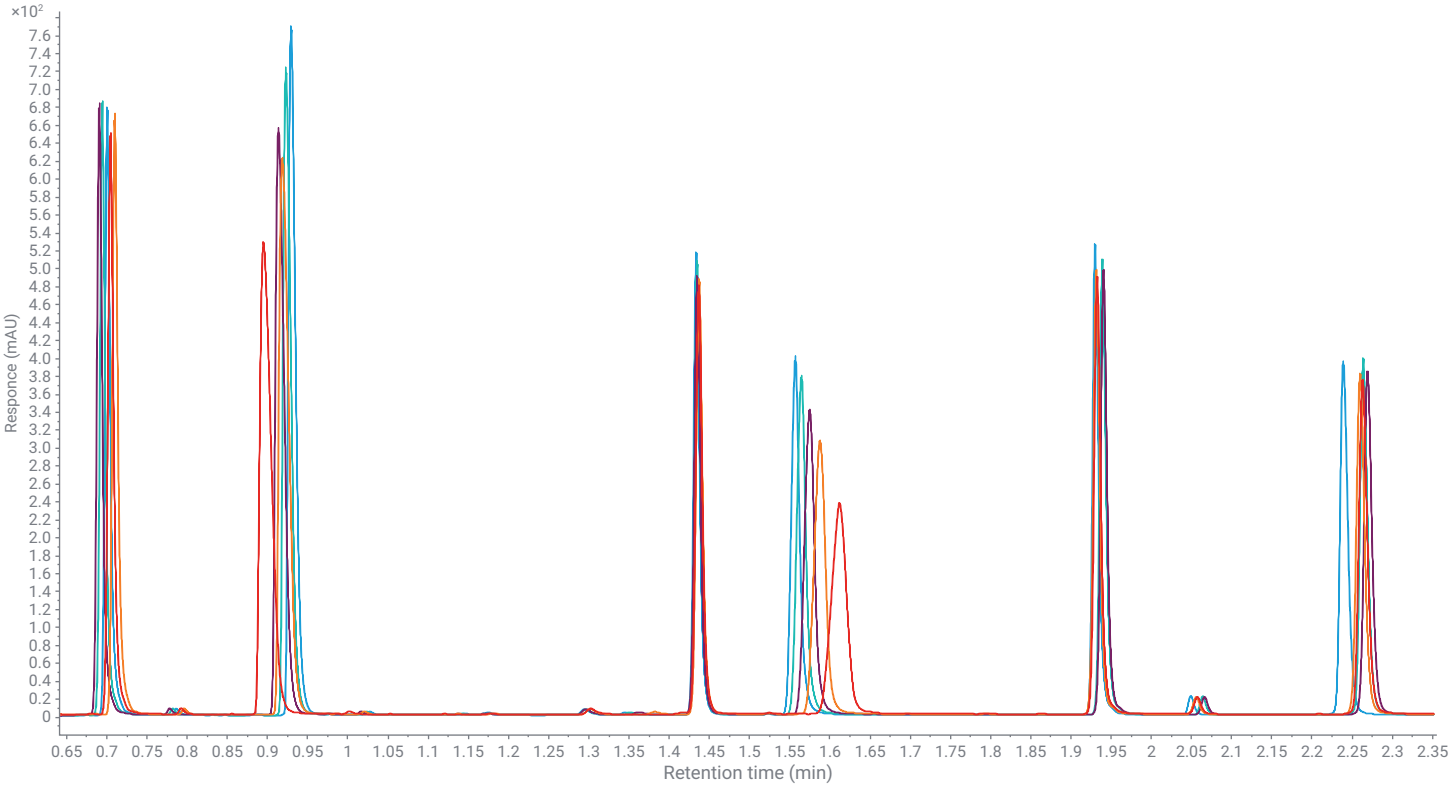

Figure 3. Buffer molarity screening using Agilent BlendAssist software (2, 4, 6, 8, 10 mM).

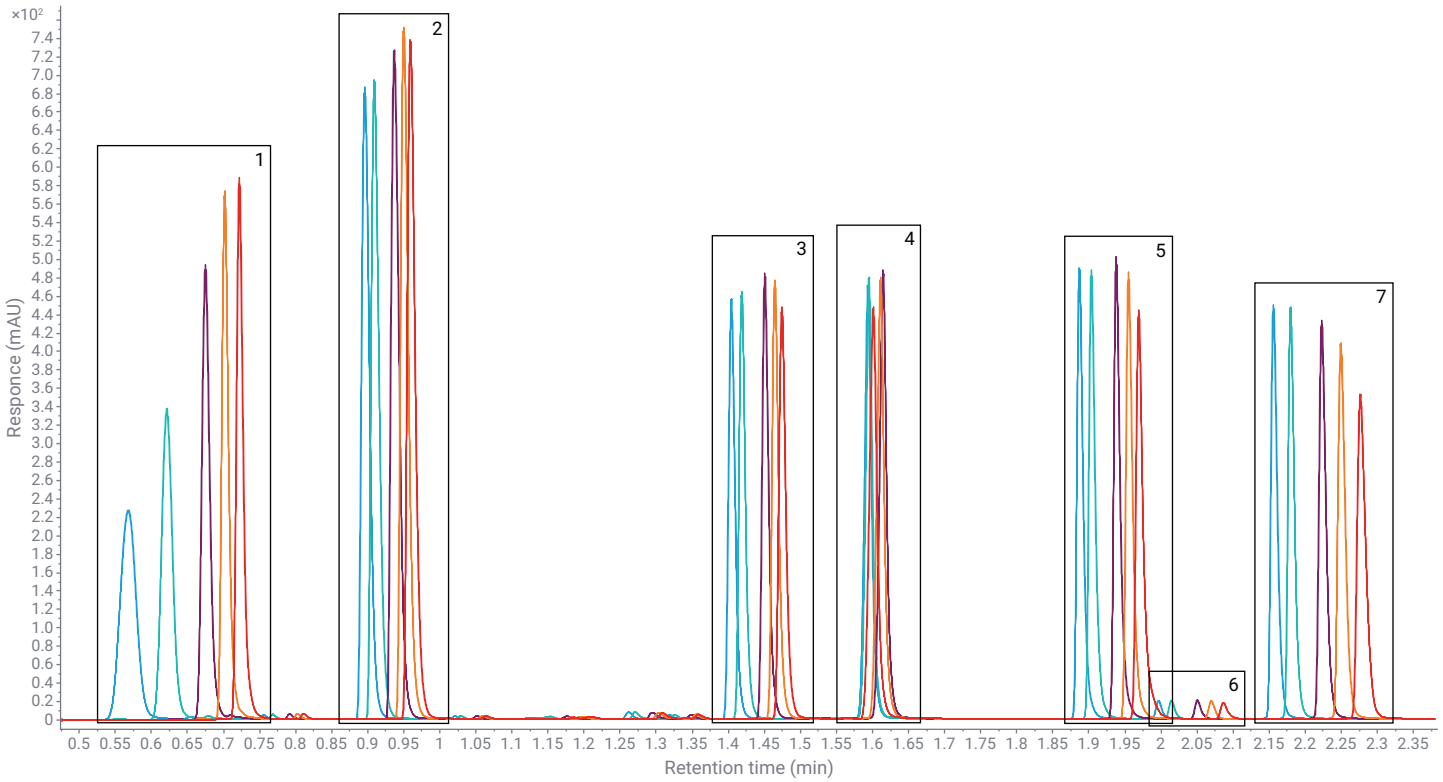

Figure 4. Temperature screening (40 ,45, 50, 55, 60 °C), elution order: 1 = 8-BG, 2 = 4-CCA, 3 = DEP, 4 = Ami, 5 = DAP, 6 = DHP, 7 = DOP).

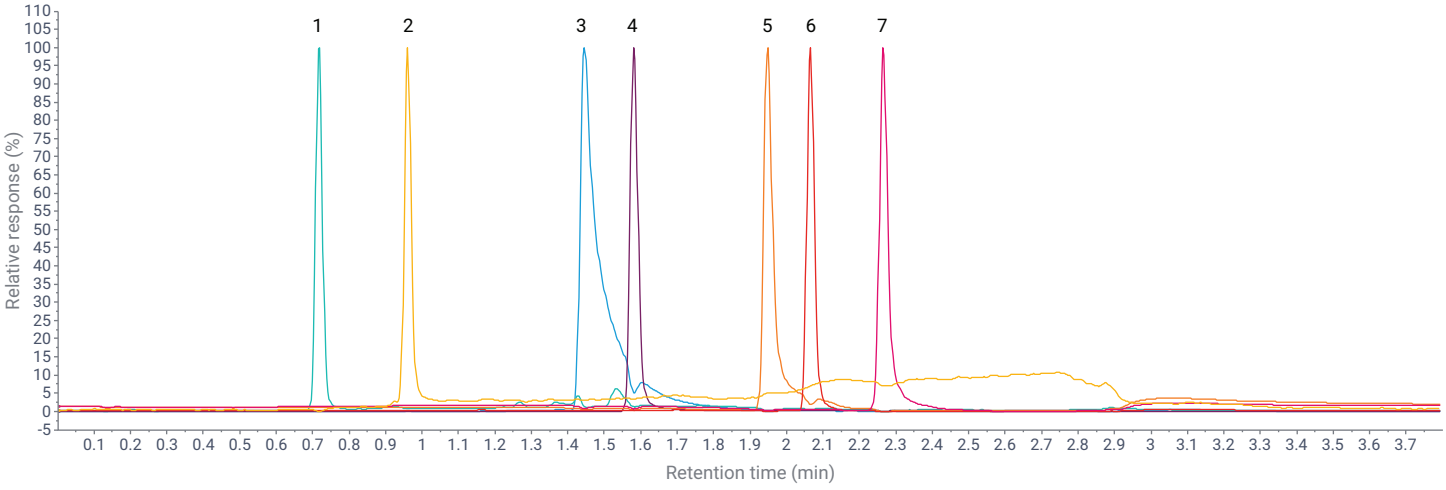

Figure 5. LC/MS (SIM) analysis of the LC/MS 7-analyte system suitability kit using an Agilent InfinityLab Poroshell 120 EC-C18 column (solvent A: 10 mM ammonium bicarbonate buffer at pH 6.6, solvent B: acetonitrile). Elution order: 8-BG (1), 4-CCA (2), DEP (3), Ami (4), DAP (5), DHP (6), DOP (7).

Conclusion

This technical overview shows a method development workflow for analysis of the LC/MS 7-analyte system suitability kit using the 1260 Infinity II Prime LC System coupled to the LC/MSD iQ. With this setup, excellent resolution, peak symmetries, and retention time precision could be easily achieved. An InfinityLab Poroshell 120 EC-C18 column in combination with an aqueous 10 mM ammonium bicarbonate buffer at pH 6.6 and acetonitrile as solvents showed the best chromatographic performance. Mass spectral detection of target compounds could be easily achieved using the Auto Acquire mode of the LC/MSD iQ. Due to characteristics of the test mixture, like its responsiveness to temperature and pH changes or its broad lipophilicity range, the LC/MS 7-analyte system suitability kit is perfectly suited to assess the LC/MS system condition. By using available technical features of the 1260 Infinity II Prime LC system such as column selectors, solvent selection valves, and BlendAssist software for automation of method development, the manual interaction and consequently the time required for method development could be significantly reduced.

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