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Analysis of Organic Acids using a Mixed-Mode LC Column and an ACQUITY QDa Mass Detector

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Abstract

Organic acids (OA) are an important group of compounds for many industries including food and beverages, animal feeds, and human health. The separation of fifteen organic acids on a mixed-mode LC column, the Atlantis Premier BEH C_{18} AX Column, has been studied using an ACQUITY UPLC H-Class System coupled with an ACQUITY QDa Mass Detector. The effects of the chromatographic conditions, such as organic solvent content, ionic strength and pH of the mobile phase, on the retention and selectivity of organic acids were studied. An analytical method for organic acids has been developed and applied to fruit juices. The performance characteristics of the analytical method, including the limit of quantitation (LOQ), the relationship between the chromatographic peak area and concentration, the precision and the accuracy have been evaluated. This analytical approach has good retention and resolution of OA, the run time is short, and the detection is sensitive and selective. This solution is suitable for the determination of organic acids in fruit juices and beverages as well as other application areas.

Benefits

Coupling the Atlantis Premier BEH C_{18} AX Column with the ACQUITY UPLC H-Class and ACQUITY QDa Mass Detector for the analysis of organic acids offers:

- Greater retention with improved chromatographic resolution
- Highly sensitive detection that can benefit authenticity testing of fruit juices and beverages
- Highly selective detection that is less prone to interference from co-eluting compounds in the sample matrix
- Fast analysis with run time less than 8 minutes

Introduction

Organic acids (OA) are an important group of compounds for many industries including food and beverages, animal feeds, and human health. They affect the flavor and aroma, the stability, and the microbiological control of juices and beverages.[1] They often form characteristic profiles for different fruits and beverages, which can be used as markers or fingerprints for authenticity testing.[2] Figure 1 shows the structures and pK_a values of OA studied in this work.

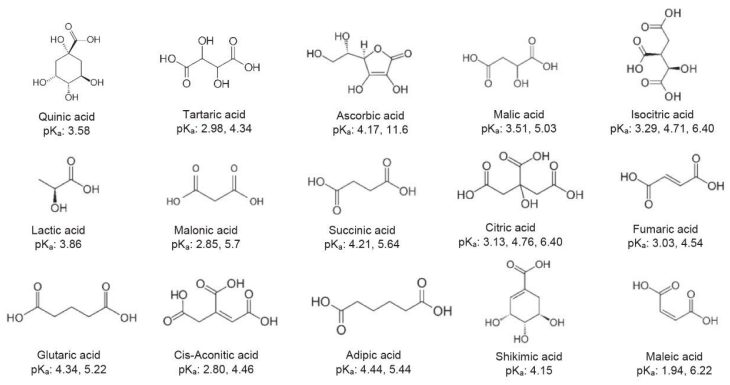

Figure 1. Structures of organic acids and their pK_a values.

The analysis of OA is commonly carried out by liquid chromatography (LC) either by anion-exchange, reversed-phase or mixed-mode separation. Anion-exchange chromatography of OA has acceptable resolution, but run times are usually long. The reversed-phase separation has limited resolution for OA. Mixed-mode separations have greater resolution for OA than the reversed-phase LC, and the run times are often shorter than those in anion-exchange chromatography. The detection techniques that are often used in the analysis of OA include conductivity, UV/Vis, and differential refractive index (DRI). These detection techniques are not selective for OA, which makes OA analysis susceptible to interference from co-eluting compounds. It is highly desired to have a solution that can address the resolution and interference issues for OA analysis.

In this application note we describe the development of an analytical solution based on Waters Atlantis Premier C_{18} AX Column and Waters ACQUITY QDa Mass Detector to address the major issues in OA analysis. The Atlantis BEH C_{18} AX Column offers excellent retention and resolution for OA analysis. The ACQUITY QDa Mass Detector is a highly selective detector for OA. The combination of these two technologies provides an excellent solution to the issues in the challenging OA analysis.

Experimental

LC Conditions

LC system:	ACQUITY UPLC H-Class System
Software:	Empower 3 CDS
Column:	Atlantis Premier BEH C_{18} AX 1.7 µm, 2.1 x 150 mm

Column temp.:	30 °C
Flow rate:	0.35 mL/min
Injection volume:	1.0 µL
Runtime:	8.0 min
Mobile phases:	A: Water with 50 mM ammonium formate and 0.9% formic acid (pH=2.9) B: Acetonitrile with 0.9% FA C: Water with 0.9% FA

Gradient elution program:

Time (min)	%A	%B	%C	Curve
0.0	0	0	100	6
1.4	0	0	100	6
1.5	60	0	40	6
5.0	60	40	0	6
7.0	60	40	0	6
7.1	0	0	100	6
8.0	0	0	100	6

MS Conditions

MS system:	ACQUITY QDa Mass Detector (Performance)
Ionization mode:	ESI-
Capillary voltage:	0.8 V
Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	1 Hz
SIR masses:	$[M-H]^-$ (Table 1)

Item	Organic acid	Formula	Monoisotopic mass (Da)	$[M-H]^-$ (*m/z*)
1	Quinic acid	$C_7H_{12}O_6$	192.06	191
2	Tartaric acid	$C_4H_6O_6$	150.02	149
3	Ascorbic acid	$C_6H_8O_6$	176.03	175
4	Malic acid	$C_4H_6O_5$	134.02	133
5	Isocitric acid	$C_6H_8O_7$	192.03	191
6	Lactic acid	$C_3H_6O_3$	90.03	89
7	Malonic acid	$C_3H_4O_4$	104.01	103
8	Succinic acid	$C_4H_6O_4$	118.03	117
9	Citric acid	$C_6H_8O_7$	192.03	191
10	Fumaric acid	$C_4H_4O_4$	116.01	115
11	Glutaric acid	$C_5H_8O_4$	132.04	131
12	*cis*-Aconitic acid	$C_6H_6O_6$	174.02	173
13	Adipic acid	$C_6H_{10}O_4$	146.06	145
14	Shikimic acid	$C_7H_{10}O_5$	174.05	173
15	Maleic acid	$C_4H_4O_4$	116.01	115

Table 1. The formula, monoisotopic mass, and molecular ion mass to charge ratio (m/z) of organic acids.

Standard Preparation

Individual OAs were dissolved in MilliQ water at 1.0% (w/v) to make stock solutions. Fumaric acid was dissolved in absolute ethanol at 1.0% (w/v). A solution containing all OA, each at 600 µg/mL (record 3 significant figures) was prepared by mixing individual OA stock solutions together and diluting with MilliQ water. Lower concentration solutions were prepared by serial dilution with water from this OA mix stock solution.

Sample Preparation

Samples of fruit juices, including apple, grape, and pomegranate, were purchased from local stores. These juices were diluted with MilliQ water at a 1 to 10 ratio (1 mL juice mixed with 9 mL water) and filtered with a 0.45 µm glass microfiber membrane filter (GMF). The filtered juice solutions were further diluted with water as needed.

Results and Discussion

Optimization of Chromatography

Mobile phase composition

The Atlantis Premier BEH C_{18} AX Column is a mixed-mode column. Its stationary phase contains not only C_{18} groups, but also tertiary alkylamine moieties, which become positively charged below approximately pH 8.[3] It was found that the mobile phase composition (organic solvent content) had a significant impact on the retention of OA. Figure 2 shows capacity factors (k′) of OA in isocratic elution with different mobile phase compositions. In water with 50 mM ammonium formate and 0.9% formic acid, the organic acids had k′ values from 0.4 to 2.5. When the organic content (mobile phase B, acetonitrile with 0.9% formic acid) was increased to about 50%, these acids exhibited the least retention on the column and all k′ values were less than 0.5. When the organic content was further increased to high organic content, the OA had high k′ values, again. The elution order of OA was also altered in different mobile phase compositions.

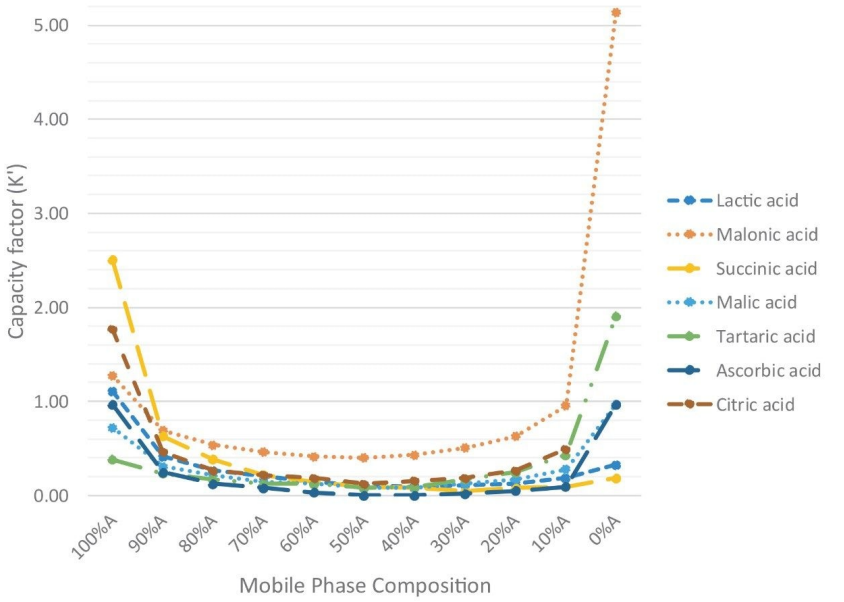

Figure 2. Capacity factors (k') of organic acids in isocratic elution with various mobile phase compositions for an Atlantis Premier BEH C_{18} AX Column. Mobile phase A: water with 50 mM ammonium formate and 0.9% formic acid (pH=2.9); B: acetonitrile with 0.9% formic acid.

Mobile phase buffer concentration

The buffer concentration is another major factor in method optimization for OA analysis. As shown in Figure 3, it affects both the retention and the selectivity of organic acids. When the concentration of ammonium formate was decreased from 50 to 0 mM, the k′ of citric acid and malonic acid were significantly increased, while other OA had moderate increases in k′. This significant increase in k′ for citric and malonic acids is related to their low pK_a values compared to other OA's in Fig. 3. The elution order of OA was also altered when the buffer concentration was changed.

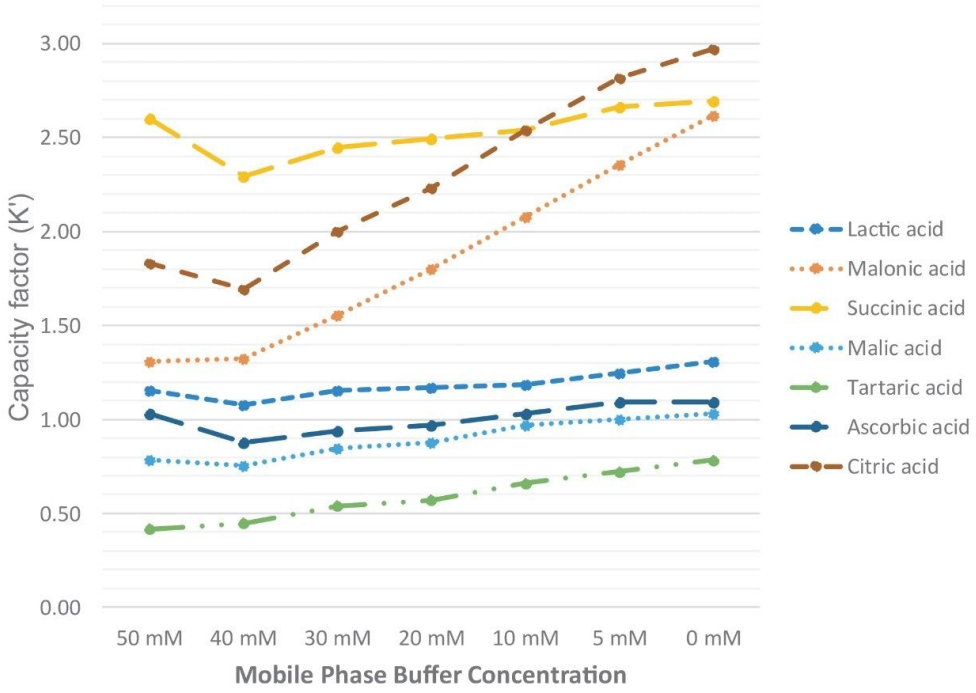

Figure 3. Capacity factors (k') of organic acids in isocratic elution with different buffer concentrations in water (with 0.9% formic acid, pH=2.9) for an Atlantis Premier BEH C $_{18}$ AX Column. Buffer: ammonium formate.

pH of mobile phase

The pH of the mobile phase is also an important factor in method development. The pH of the mobile phase affects the acid dissociation equilibrium of OA, which affects their retention and peak shape. pH 2.9 was used in this study to obtain good peak shape.

Analysis of organic acids

A gradient elution program has been developed and used for the OA analysis (see the Experimental section). It is essentially a binary solvent gradient with an additional segment of isocratic elution with water containing no buffer (low ionic strength). With the isocratic elution with water containing no buffer followed by a gradient elution, we obtained greater retention for the early eluting OA and good resolution for the important quinic acid, tartaric acid, and malic acid.

Chromatograms of 15 organic acids were collected in 11 Single Ion Recording (SIR) channels (Figure 4). Most molecular ions $[M-H]^-$ of these OA have different mass to charge ratios (*m/z*). They were detected in separate SIR channels, which eliminated the possible interference from each other. The RT, limit of quantification (LOQ), and calibration results of 15 organic acids are in Table 2. The LOQ of organic acids in solvent solution were in the range of 0.2 to 6 ppm (µg/mL in solution).

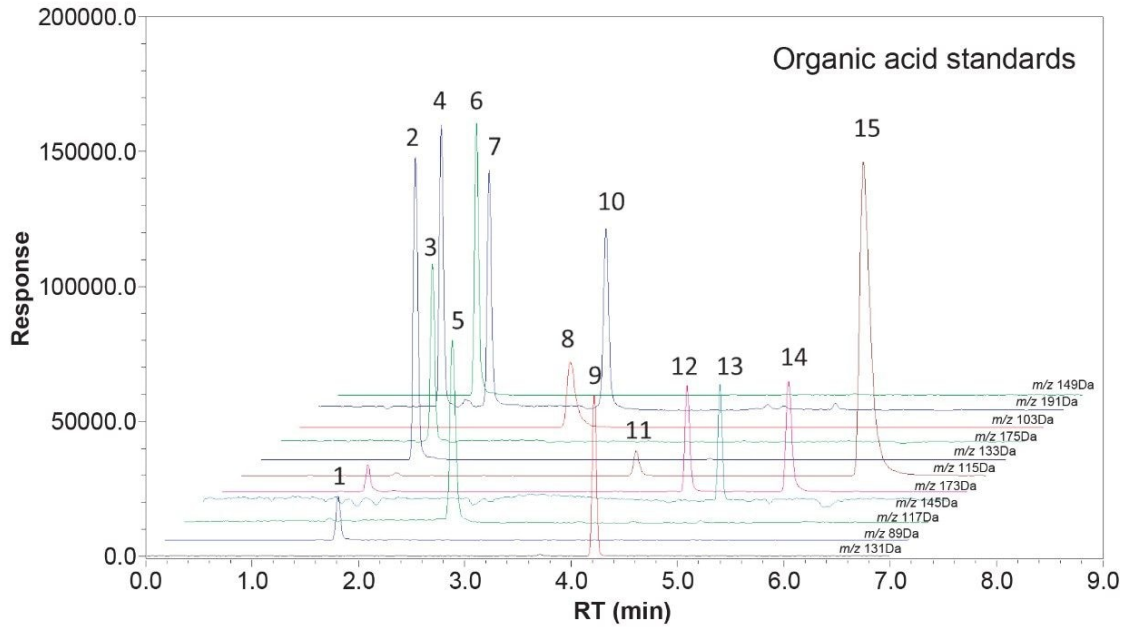

Figure 4. Chromatograms of 15 organic acid standards in 11 SIR channels. Organic acid concentration: 20 ppm. Column: Atlantis Premier BEH C_{18} AX, 1.7 µm, 2.1 x 100 mm. Peaks ID: 1. Lactic acid; 2. Malic acid; 3. Ascorbic acid; 4. Quinic acid; 5. Succinic acid; 6. Tartaric acid; 7. Isocitric acid; 8. Malonic acid; 9. Glutaric acid; 10. Citric acid; 11. Fumaric acid; 12. cis-Aconitic acid; 13. Adipic acid; 14. Shikimic acid; 15. Maleic acid.

	Acids	RT (min)	Equation	R^2	LOQ (ppm)	Range (ppm)	$[M-H]^-$ (*m/z*)	Peak area RSD (%)	
								200 ppm	60 ppm
1	Quinic	1.16	$Y = -1.10e+001 X^2 + 1.48e+004 X - 3.53e+002$	0.996	2	2–600	191	4.5	8.6
2	Tartaric	1.31	$Y = -1.09e+001 X^2 + 1.63e+004 X - 6.05e+003$	0.995	0.6	0.6–600	149	4.1	9.6
3	Ascorbic	1.46	$Y = -5.83e+000 X^2 + 8.038e+003 X - 4.26e+003$	0.992	2	2–600	175	5.3	7.9
4	Malic	1.48	$Y = -3.54e+001 X^2 + 1.79e+004 X - 3.17e+003$	0.995	0.2	0.2–200	133	1	4.3
5	Isocitric	1.63	$Y = -7.58e+000 X^2 + 1.37e+004 X - 9.84e+003$	0.995	6	6–600	191	5.7	10.5
6	Lactic	1.65	$Y = -1.70e+000 X^2 + 2.34e+003 X + 1.93e+002$	0.995	2	2–600	89	5.1	3.7
7	Malonic	2.60	$Y = -4.96e+000 X^2 + 7.68e+003 X - 2.76e+003$	0.995	2	2–600	103	5.6	9
8	Succinic	2.64	$Y = -7.84e+001 X^2 + 1.38e+004 X - 3.15e+002$	0.980	2	2–60	117	–	15.1
9	Citric	2.80	$Y = -5.76e+000 X^2 + 1.30e+004 X - 3.81e+004$	0.984	6	6–600	191	14.1	16.7
10	Fumaric	3.78	$Y = -9.29e+001 X^2 + 2.48e+003 X - 5.72e+003$	0.998	4	6–600	115	1.1	7.3
11	Glutaric	4.26	$Y = -5.16e+000 X^2 + 7.67e+003 X - 2.10e+003$	0.997	2	2–600	131	5.4	5.3
12	*cis*-Aconitic	4.39	$Y = -4.52e+000 X^2 + 6.16e+003 X - 8.59e+003$	0.995	1	2–600	173	6.3	9.7
13	Adipic	4.86	$Y = -3.63e+000 X^2 + 6.23e+003 X + 1.15e+002$	0.998	6	6–600	145	3	2.5
14	Shikimic	5.33	$Y = -4.79e+000 X^2 + 6.61e+003 X - 1.98e+003$	0.987	0.6	0.6–600	173	4.5	7.7
15	Maleic	5.88	$Y = -4.29e+002 X^2 + 5.76e+004 X + 3.663e+003$	0.996	0.2	0.2–60	115	–	2.4

Table 2. Organic acid retention times, calibration results, LOQ, and peak area repeatability.

The accuracy of quantitation of organic acids was evaluated in a spiking study. Four important organic acids, quinic acid, tartaric acid, malic acid, and citric acid, were spiked at two levels, 0.1 mg/mL and 4 mg/mL, in apple, grape, and pomegranate juices. The recoveries, following AOAC guidelines ,[4] were calculated and shown in Table 3. The majority of the recoveries were within the 80-120% range. External calibration was used in the spiking study. However, it is recommended to use internal standards if more accurate results are needed. Using internal standard is a common practice to improve the accuracy and precision in analyses, especially when mass spectrometry is involved.

	Apple juice			Grape juice			Pomegranate juice		
	Original level (mg/mL in juice)	Spiking level (mg/mL in juice)		Original level (mg/mL in juice)	Spiking level (mg/mL in juice)		Original level (mg/mL in juice)	Spiking level (mg/mL in juice)	
		0.1 mg/mL	4 mg/mL		0.1 mg/mL	4 mg/mL		0.1 mg/mL	4 mg/mL
Quinic acid	0.24	109%	88%	0	85%	84%	0.02	118%	92%
Tartaric acid	0	135%	89%	0.75	82%	82%	0.007	92%	88%
Malic acid	3.36	N/A*	86%	1.64	N/A*	94%	0.57	99%	95%
Citric acid	0.06	N/A*	119%	0.12	105%	116%	7.45	N/A*	163%

*Recovery is not valid due to low spiking level.

Table 3. The recovery of common organic acids in fruit juices.

The main benefits of this method for OA analysis can be highlighted in pomegranate fruit juice analysis. Figure 5 shows an overlay of pomegranate juice chromatograms with an insert of a chromatogram overlay at enlarged scale. A tartaric acid content as low as 0.007 mg/mL in pomegranate juice was determined (see Table 3). Please note the flat baseline in the tartaric acid SIR chromatogram, and how many peaks are present near the tartaric acid peak (RT 1.5 min) in Figure 5. For non-selective detectors, it would be very difficult to quantify the tartaric acid at such a low level with many peaks eluting so closely to it. Tartaric acid was used as a marker for adulterated pomegranate juice. The level of tartaric acid in adulterated pomegranate juice was found to be 0.07 mg/mL or higher.[5] Sensitive detection of OA, such as tartaric acid for pomegranate juice in this case, is necessary for fruit juice authenticity testing.

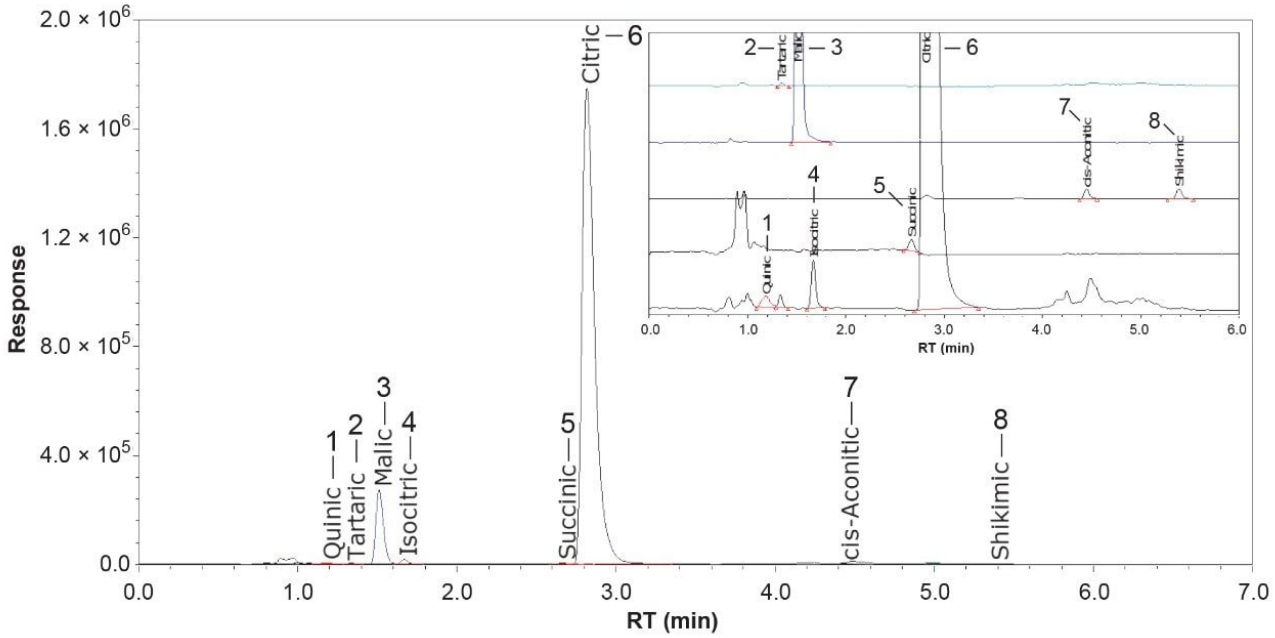

Figure 5. SIR chromatogram overlay of a pomegranate juice. The juice was diluted 10 times with deionized water before it was analyzed. The insert shows the detected OA chromatograms at enlarged scale with baseline offset.

Conclusion

The analysis of OA can be a challenging task given the difficulty of separating the large number of naturally occurring OA that could be present in samples. Waters UPLC/QDa System with an Atlantis Premier BEH C_{18} AX Column offers an excellent approach for OA analysis. The key advantages of this OA analysis approach include:

- Greater retention and improved resolution
- Highly sensitive detection that is beneficial in certain applications
- Highly selective detection that makes the analysis less prone to interference from co-eluting compounds in the sample matrix
- Fast analysis with run time less than 8 minutes

References

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