Agilent 1290 Infinity LC
The ideal partner for MS – Part 5

Improved mass accuracy by enhanced separation of compounds of isobaric mass using Agilent 1290 Infinity LC technology

Application Note

Pharmaceutical and Chemical

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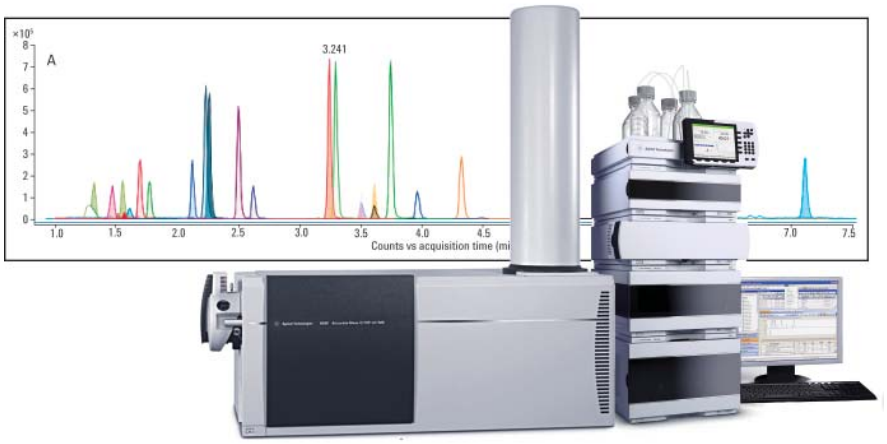

Abstract

This Application Note demonstrates the advantage of using an Agilent 1290 Infinity LC with 1.8 µm columns as the front-end of an Agilent 6500 Series Accurate Mass Quadrupole Time-of-Flight (Q TOF) mass spectrometer to achieve better separation of the analyte compound from other compounds compared to conventional HPLC separation on a 5 µm column. Coelution can compromise the identification of compounds by accurate mass measurement if coeluting compounds have isobaric mass. The presented data show that there is a lower probability for coeluting compounds by improved separation on an Agilent 1290 Infinity LC used with 1.8 µm particle size columns.

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Introduction

For an analysis, target compounds can be classified in three categories:

1) **Known knowns**, which could be present in the sample and therefore will be quantified with a triple quadrupole approach by previous adjustment of the mass spectrometer to the particular compounds.

2) **Known unknowns**, which are chemically characterized and inherent in a database but possibly not expected in the sample.

3) **Unknown unknowns**, which are compounds inherent in the sample but are chemically not characterized and not identified so far.

The first approach is called targeted analysis, and the second, a non-targeted screening.

A typical application for accurate mass measurement instruments like qadrupole time-of-flight (QTOF) mass spectrometers is the screening of samples for "known unknowns". Typical samples for screening are toxicological samples for drugs of abuse or food samples for pesticides. For the final search of the compounds in the acquired data accurate mass retention time (AMRT) data bases are available.

This is a clear contrast to the use of triple quadrupole (QQQ) mass spectrometers which are used for quantification of "known knowns" in a sample where unit mass resolution and accuracy is sufficient. For the application of QTOF instruments in compound screening, high resolution and high mass accuracy is required. A problem for the non-targeted analysis occurs when compounds of isobaric mass are coeluting where the mass difference between the compounds cannot be resolved by the mass spectrometer.

This problem can only be partially resolved by increasing the resolution of the instrument. Compounds with the same integer mass and containing only C-, H-, N-, and O-atoms are always in the same mass defect range as their natural matrix compounds and could be isobaric. It is possible that even mass spectrometric resolution above 100,000 could not be enough. This is where chromatography comes into play. The Agilent 1290 Infinity LC system with its capability to run high resolving 1.8 µm particle size columns can help to resolve these compounds chromatographically, to avoid coelution.

This Application Note demonstrates the advantage of using an Agilent 1290 Infinity LC system with 1.8 µm columns as the front end of an Agilent 6500 Series Accurate Mass Quadrupole Time-of-Flight (Q TOF) mass spectrometer to achieve better separation of the analyte compound from other compounds, compared to conventional HPLC separation on a 5 µm column. Coelution can compromise the identification of compounds by accurate mass measurement if coeluting compounds have isobaric mass. The presented data show that there is a lower probability for coeluting compounds by improved separation on an Agilent 1290 Infinity LC used with 1.8 µm particle size columns.

Experimental

Equipment:

- Agilent 1290 Infinity LC system including:
- Agilent 1290 Infinity Binary Pump,
- Agilent 1290 Infinity High Performance Autosampler,
- Agilent 1290 Infinity Thermostatted Column Compartment
- Agilent 6530 Accurate Mass Quadrupole Time-of-Flight (Q TOF) Mass Spectrometer

Columns:	• Agilent ZORBAX Eclipse Plus, RRHD, C18, 2.1 ×150 mm, 1.8 µm • Agilent ZORBAX Eclipse Plus C18, 2.1 ×150 mm, 5 µm
Software for data acquisition and data analysis:	• MassHunter data acquisition software for QTOF • MassHunter qualitative data analysis software • MassHunter Personal Compound Database Manager and Database (ForensicsTox_Testmix_AM_PCDL.cdb)

HPLC method:

Solvent A:	Water + 0.1% formic acid
Solvent B:	Acetonitrile + 0.1% formic acid
Flow rate:	0.8 mL/min
Gradient:	0 min 5% B – 7.0 min 95% B – 8.0 min 95% B
Stop time:	8.0 min.
Post time:	3 min
Injection volume:	1 µL
Needle wash:	6 s in MeOH
Column temperature:	40 °C

MS method:

Source:	Sheath gas temperature:	350 °C
	Sheath gas flow:	11 L/min
	Capillary gas temperature:	300 °C
	Capillary gas flow:	5 L/min
	Nebulizer pressure:	50 psi
	Capillary:	4,000 V
	Nozzle Voltage:	500 V
	Polarity:	positive
TOF:	MS only, 100-1,700 *m/z*, 2 scan/sec., 2 GHz data rate	
Samples:	Stock solutions (100 µg/mL)of: Mianserin ($C_{18}H_{20}N_2$, MW: 264.1626 ,$[M+H]^+$=265.1699), Tetracaine ($C_{15}H_{24}N_2O_2$, MW: 264.1838 ,$[M+H]^+$=265.1911) Agilent LC/MS Toxicology Test Mixture, (p/n 5190-0470), Table 1	

Results and Discussion

The mass spectrometric resolution is defined as the measure of separation between two peaks expressed as m/Δm. The resolving power of a TOF instrument is defined as the quotient of *m/z* and the peak width at half height. The resolving power defines a threshold for accurate mass measurement. If two compounds of isobaric mass below that threshold are approaching the detector at the same time, they cannot be resolved, and the measured accurate mass will be wrong. This will lead to false calculation results for the chemical formula[1].

Typically, this problem is avoided in most cases by chromatographic separation in the front end HPLC system. However, there are a few sets of isobaric compounds which undergo colelution under standard HPLC conditions and cause problems for their identification.

As an example for a set of coeluting, isobaric compounds tetracaine and mianserin were chosen. They would require a mass resolution of about 12,500 to be resolved while the used time-of-flight mass spectrometer delivers a resolution of about 7,500 at *m/z* 322. For the chromatographic separation, a conventional Agilent ZORBAX Eclipse Plus 2.1 × 150 mm, 5 µm column was used. Under the given conditions, both compounds could not be separated (Figure 1) and the accurate mass measured was off by a mass error of 34 ppm and 45 ppm from the masses of the test compounds tetracaine and mianserin, respectively. This will prevent the correct identification of both compounds and finally lead to a wrong formula calculation.

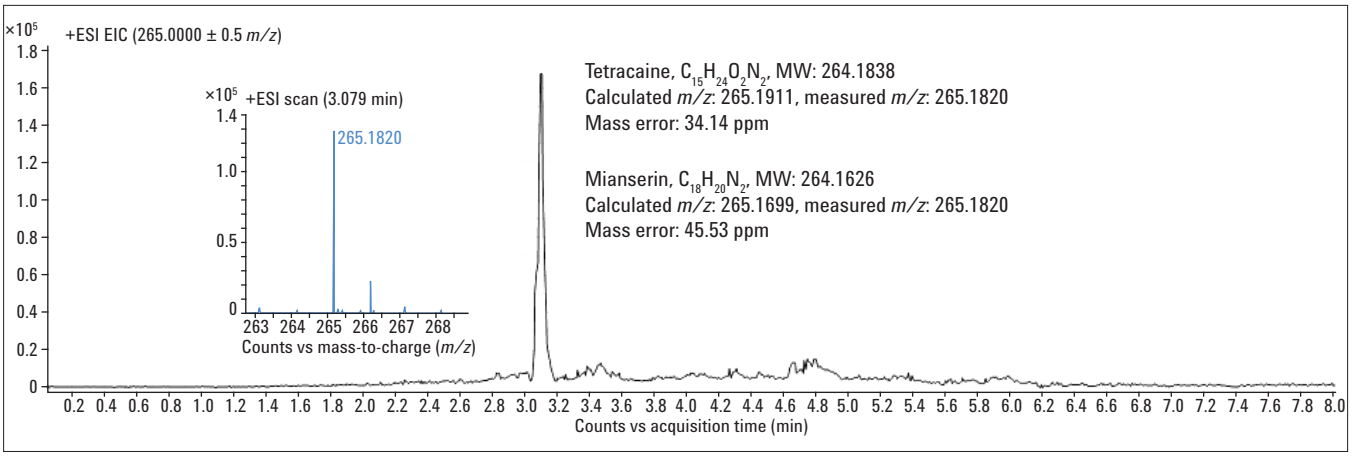

Figure 1

Coeluting compounds Tetracaine and Mianserin of isobaric mass at insufficient mass resolution of about 7,500 while a mass resolution of 12,500 would be required. The chromatographic separation was done on an Eclipse Plus 2.1 × 150 mm, 5 µm column.

The situation completely changes if the column is changed to a 1.8 µm particle size high resolving column run with an Agilent 1290 Infinity LC system as front end. Now, both compounds are chromatographically resolved peaks and can be identified as separated compounds (Figure 2).

Tetracaine and mianserin are identified with low mass errors of 2.81 ppm and 1.98 ppm, respectively. In a window of 10 ppm and a chemical space of $C_{3\text{-}50}H_{2\text{-}100}O_{0\text{-}10}N_{0\text{-}5}$, their formulae were calculated uniquely, which gives a reliable identification.

The high impact of chromatographic resolution for the identification of compounds is demonstrated in the following example on the identification of toxicologically important compounds. The mixture of compounds includes the toxicological test mixture (Table 1) and at the same concentration both the local anesthetic tetracaine and the tetracyclic antidepressant mianserin.

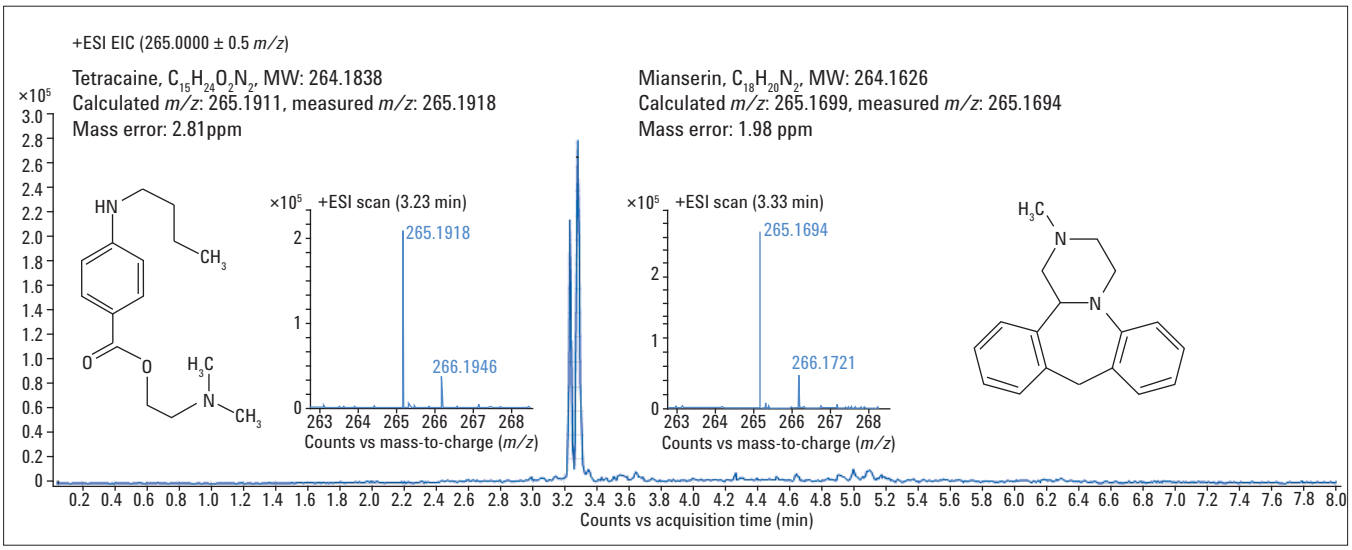

Figure 2

Chromatographically resolved isobaric compounds Tetracaine and Mianserin, column Eclipse Plus, RRHD, 2.1 × 150 mm, 1.8 µm both formulas were uniquely identified in $C_{3\text{-}50}H_{2\text{-}100}O_{0\text{-}10}N_{0\text{-}5}$ in a 10 ppm window.

Compound Name	Formula	Mass	RT (min)	CAS	IUPAC name
Amphetamine	$C_9H_{13}N$	135.10480	1.430	300-62-9	1-Phenyl-2-propanamine
Methamphetamine	$C_{10}H_15N$	149.12045	1.550	537-46-2	(2S)-N-Methyl-1-phenyl-2-propanamine
Phentermine	$C_{10}H_15N$	149.12045	1.660	122-09-8	2-Methyl-1-phenyl-2-propanamine
3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463	1.500	4764-17-4	1-(1,3-Benzodioxol-5-yl)-2-propanamine
Methylendioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028	1.600	69610-10-2	1-(1,3-Benzodioxol-5-yl)-N-methyl-2-propanamine
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593	1.770	14089-52-2	1-(1,3-Benzodioxol-5-yl)-N-ethyl-2-propanamine
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.19870	2.630	77-10-1	1-(1-Phenylcyclohexyl)piperidine
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723	2.260	57-42-1	Ethyl 1-methyl-4-phenyl-4-piperidinecarboxylate
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004	3.358	146-22-5	7-Nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Diazepam	$C_{16}H_{13}ClN_2O$	284.07164	4.382	439-14-5	7-Chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091	3.591	604-75-1	7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Morphin-D3	$C_{17}H_{16}D_3NO_3$	288.15532	5.594		
Codeine	$C_{18}H_{21}NO_3$	299.15214	1.579	76-57-3	(5alpha,6alpha)-3-Methoxy-17-methyl-7,8-didehydro-4,5-epoxymorphinan-6-ol
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214	1.340	125-29-1	(5alpha)-3-Methoxy-17-methyl-4,5-epoxymorphinan-6-one
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656	4.040	846-50-4	7-Chloro-3-hydroxy-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Cocaine	$C_{17}H_{21}NO_4$	303.14706	2.240	50-36-2	Methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287	3.730	28981-97-7	8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine
Methadone	$C_{21}H_{27}NO$	309.20926	3.300	76-99-3	6-(Dimethylamino)-4,4-diphenyl-3-heptanone
Delta9-tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458	7.060	8/3/1972	(6aR,10aR)-6,6,9-Trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107	3.740	1622-61-3	5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Oxycodone	$C_{18}H_{21}NO_4$	315.14706	1.489	76-42-6	(5alpha)-14-Hydroxy-3-methoxy-17-methyl-4,5-epoxymorphinan-6-one
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193	3.698	846-49-1	7-Chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-2H-1,4-benzodiazepin-2-one
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813	1.730	57-24-9	Strychnidin-10-one
Proadifen	$C_{23}H_{31}NO_2$	353.23548	3.747	302-33-0	2-(Diethylamino)ethyl 2,2-diphenylpentanoate
Heroin	$C_{21}H_{23}NO_5$	369.15762	2.140	561-27-3	(5alpha,6alpha)-17-Methyl-7,8-didehydro-4,5-epoxymorphinan-3,6-diyl diacetate
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129	2.520	19794-93-5	2-{3-[4-(3-Chlorophenyl)-1-piperazinyl]propyl}[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316	3.270	52-53-9	2-(3,4-Dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino}-2-isopropylpentanenitrile

Table 1
Content of the Agilent LC/MS Toxicology Test Mixture (p/n: 5190-0470), 1 µg/mL each, retention times refer to Figure 3.

In the first experiment, the mixture was separated under conventional HPLC conditions on a 2.1 × 150 mm, 5 µm column (Figure 3A). For the identification of the toxicological compounds in the mixture, a database search was done with the identification criteria settings: mass accuracy ± 10 ppm, retention time ± 0.2 min, EIC window ± 0.5 min. With this data base search, 27 compounds were identified by accurate mass and retention time. A large number of compounds coeluted between one and three minutes with differences in intensities by more than an order of magnitude. They were easily identified by their accurate mass and retention time. The compounds tetracaine and mianserin coeluting under this conditions were not identified because the mass resolution is not sufficient and the resulting mass is far of the settings window of the database search.

Changing to a 2.1 × 150 mm, 1.8 µm column, which can be operated by the Agilent 1290 Infinity LC system, changes the result especially for the compounds of isobaric mass tetracaine and mianserin (Figure 3B). Here, both compounds, coleuting under standard HPLC conditions are separated. This separation of the isobaric compounds removes the disturbing influence on their accurate mass measurement and enables their identification by the database search. Tertracaine was identified at a retention time of 2.749 minutes with a mass error of 2.34 ppm and mianserin was identified at a retention time of 2.807 minutes with a mass error of 4.05 ppm.

These measurements were done with an Agilent 6530 Accurate Mass QTOF in Dynamic Range Mode where a mass resolution of about 7,500 at 322 *m/z* can be achieved. The same instrument is able to deliver a resolution of about 13,500 at 322 *m/z* in high Resolution Mode. The Agilent 6540 Ultra High Definition Accurate Mass QTOF can even deliver a resolution of > 25,000 at 322 *m/z*. This means that the example here could be resolved by increasing the mass resolution in

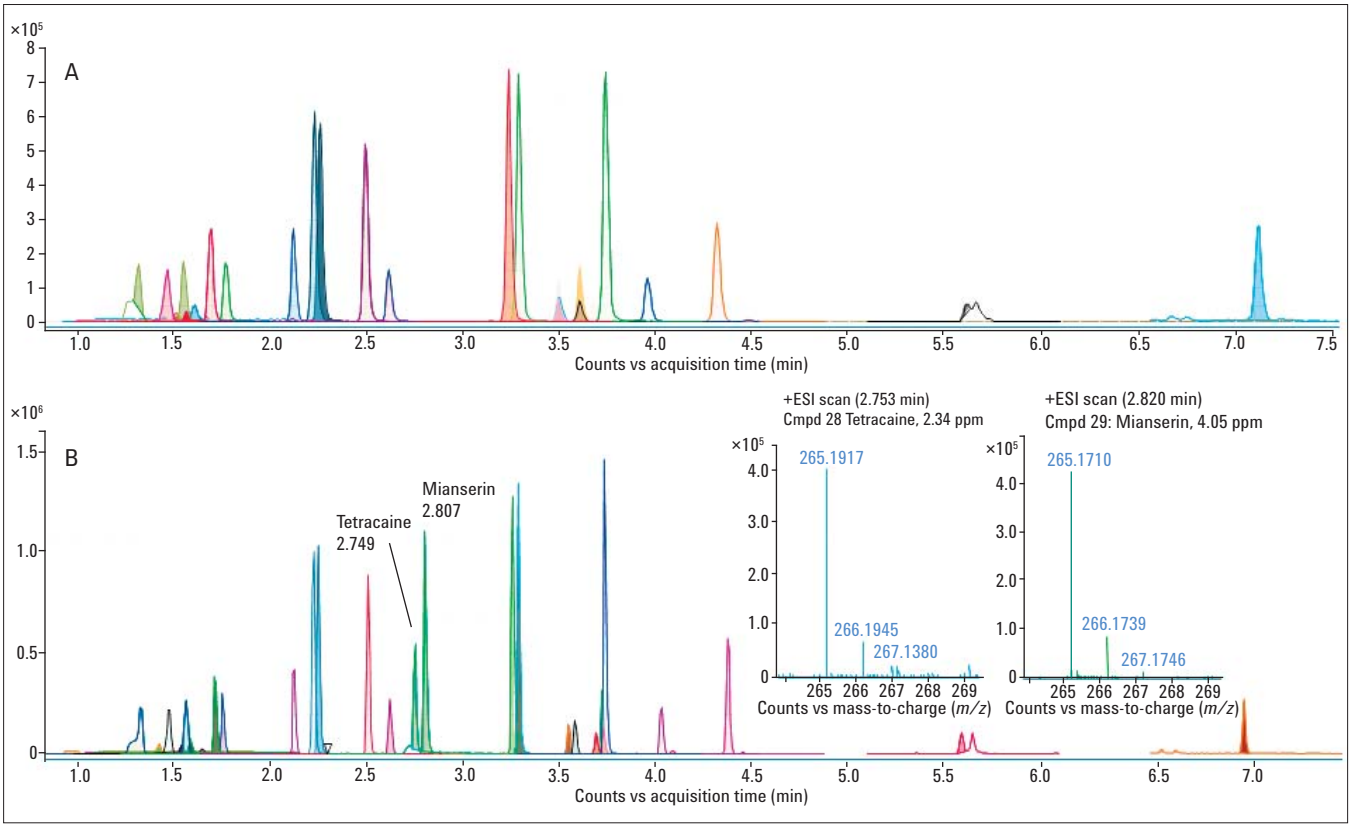

Figure 3

Identification of compounds in a Toxicology Test Mix of 29 compounds (see experimental, Table 1 + tetracaine and mianserin, 100 pg/mL).

A) Resolved on an Agilent ZORBAX Eclipse Plus 2.1 × 150 mm, 5.0 µm, 27 compounds identified.

B) Resolved on an Agilent ZORBAX Eclipse Plus, RRHD, 2.1 × 150 mm, 1.8 µm, 29 compounds identified. Chromatographic conditions: Gradient 5-95% AcN in 7 min. Search in Personal Compound Database: Threshold 10 ppm, retention time +/- 0.2 min, EIC extraction +/- 0.50 min.

case of coelution. This does not mean that chromatographic performance can be neglected. There may always be another coeluting compound of interest, a metabolite coeluting with the compound of interest, a coeluting standard or, most probably, a coeluting isobaric matrix compound where the achieved mass spectrometric resolution is not sufficient[1].

Conclusion

This Application Note demonstrates the use of the Agilent 1290 Infinity LC system as a valuable front end for high end mass spectrometers such as the Agilent 6500 QTOF series mass spectrometers. An example is shown where the mass spectrometric resolution of the accurate mass measurement instrument or the chosen data acquisition mode is not sufficient to resolve compounds of isobaric accurate mass coeltuing under conventional HPLC conditions. The Agilent 1290 Infinity LC system with its capability to run long 1.8 µm columns for high chromatographic resolution is able to resolve those compounds which enable the mass spectrometer to deliver the correct accurate mass for reliable formula calculation, database search and herewith compound identification.

References

1.
Pleander A., Decker P., Baessmann C., Ojanperä I., „Evaluation of a high resolving power time-of-flight mass spectrometer for drug analysis in terms of resolving power and acquisition rate.“ *J. Am. Mass Spectrom.* 22 (2011) 379-385.

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