Application Note

Forensic Toxicology

Agilent

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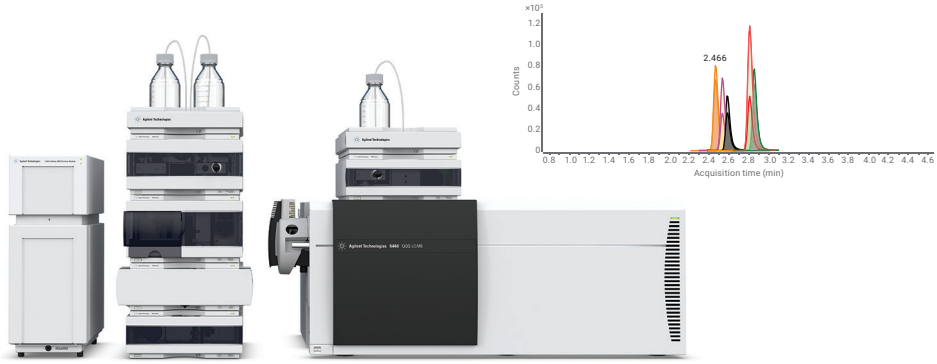
Quantitative Determination of Drugs Using Supercritical Fluid Chromatography with Triple Quadrupole Mass Spectrometry



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Abstract

This application note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System in combination with triple quadrupole mass spectrometry for the fast separation of 25 drugs, and quantitative determination down to a limit of detection of 30 pg/mL. For all compounds, calibration curves showed excellent linear correlation. The statistical evaluation of replicate measurements showed highest precision and accuracy for all 25 compounds. Finally, the determination of amphetamines in a urine sample is described.

Introduction

A broad range of compounds of forensic interest are screened and quantified for several application areas in forensic toxicology. These fields range, for example, from doping control, postmortem forensic toxicology, drug testing, and even to the determination of explosive residues.

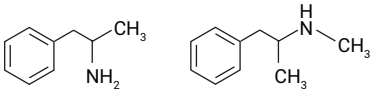
The group of drugs itself is also diverse regarding chemical properties, which are important for separation and detection. Chemical structures range from simple aromatic amines and polycyclic aromatic benzodiazepines to complex morphine-like structures, and even hydrophobic compounds such as tetrahydro cannabinol (THC). So far, the challenging separation for quantitative screening of all compound classes at-a-glance was done by reversed-phase HPLC/MS.[1]

This application note demonstrates the separation of different classes of drugs in a single quantitative screening run by supercritical fluid chromatography (SFC). Quantitative screening by SFC can be done in a short run time of only a few minutes, and can achieve highest sensitivity when combined with triple quadrupole mass spectrometry. The test suite used for this application note comprised 25 compounds of amphetamines, benzodiazepines, morphines, morphine analogs, and THC. After the creation of calibration curves and a statistical evaluation, a spiked biological sample was analyzed with a focus on the class of amphetamines. In this study, the following compounds of interest were screened:

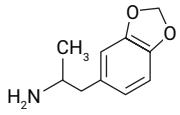
- Amphetamine
- Methamphetamine
- 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA) and 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA)

Figure 1 shows the chemical formulas. Related chemical and toxicological information are publicly available.

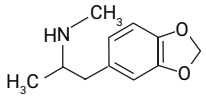
Experimental



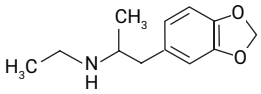
Amphetamine Methamphetamine



3,4-methylenedioxy-amphetamine (MDA)



3,4-methylenedioxy-methamphetamine (MDMA)



3,4-methylenedioxy-N-ethyl-amphetamine (MDEA)

Figure 1. Chemical structures of amphetamines used in this study.

Instrumentation

Agilent 1260 Infinity Analytical SFC System (G4309A):

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS system (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent Splitter kit (G4309-68715)

Instrumental setup

The recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS system has been described in a previous study.

Column

Agilent ZORBAX SB-C8, 4.6 × 100 mm, 1.8 µm (part number 828975-906)

Software

- 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

Connection of the SFC to the MS by splitting and make-up flow:

- Make up composition: Methanol/Water (95/5) + 0.2% formic acid
- Make-up flow: 0.3 mL/min

Standards

The Agilent LC/MS Forensic Toxicology Test Mixture was used as a standard stock solution. This mixture comprises 25 compounds at a concentration of 1.00 µg/mL, each in methanol. A 1:10 dilution in methanol was used as stock solution for the generation of the calibration curve (100 ng/mL).

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Sample preparation

A urine sample was spiked with the complete suite of compounds inherent to the Agilent LC/MS Toxicology Test Mixture (100 ng/mL), diluted 1:5 with methanol, vortexed, then centrifuged at 14,000 g for 5 minutes. The supernatant was filtered; the filtrate was used directly for injection.

SFC method

Parameter	Description
SFC Flow	2 mL/min
SFC Gradient	0 minutes – 2% B, 5 minutes – 25% B
Stop Time	5 minutes
Post Time	2 minutes
Modifier	Methanol + 0.2% formic acid (FA) + 10 mM ammonium formate
BPR Temperature	60 °C
BPR Pressure	200 bar
Column Temperature	60 °C
Injection Volume	1 µL, 3 times loop overfill

MS method

Parameter	Description
Ionization Mode	Positive
Capillary Voltage	3,000 V
Nozzle Voltage	500 V
Gas Flow	8 L/min
Gas Temperature	220 °C
Sheath Gas Flow	12 L/min
Sheath Gas Temperature	380 °C
Nebulizer Pressure	25 psi
MRM Conditions	See Table 1, showing precursor ions, fragment ions, fragmentor voltage, and collision energy details. The system was used in dynamic MRM mode to ensure best sensitivity.

Table 1. MRM conditions: Precursor ions, fragment ions, fragmentor voltage, and collision energy (sorted by retention time, see Table 2). The final DMRM method was created from the MRM method.

Compound	Precursor Ion	Fragmentor (V)	Quantifier Ion	CE	Qualifier Ion	CE
THC	315.2	150	193.2	20	123.3	30
Temazepam	301.1	117	255.1	29	177	45
Clonazepam	316.1	110	270	24	214	40
Diazepam	285.1	169	193	45	154	25
Lorazepam	321	102	275	21	194	49
Nitrazepam	282.1	148	236.1	25	180	41
Proadifen	354.2	153	167	29	91.1	45
Oxazepam	287	150	269	12	241	20
Cocaine	304.2	138	182.1	17	77	61
Verapamil	455.3	158	165	37	150	45
Trazodone	372.2	159	176	25	148	37
Oxycodone	316.2	143	298.1	17	256.1	25
Meperidine	248.2	128	220.1	21	174.1	17
MDEA	208.1	107	163	9	105	25
Heroin	370.2	149	268.1	37	165	61
PCP	244.1	86	91	41	86.1	9
Amphetamine	136.1	66	119.1	5	91	17
MDA	180.1	61	163	5	105	21
Methamphetamine	150.1	92	119	5	91	17
MDMA	194.1	97	163	9	105	25
Methadone	310.2	112	265.1	9	105	29
Alprolazame	309.1	179	281	25	205	49
Codeine	300.2	158	165.1	45	58.1	29
Hydrocodone	300.2	159	199	29	128	65
Strychnine	335.2	195	184	41	156	53

Abbreviations: tertrahydro cannabinol (THC), 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), phencyclidine (PCP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethyl-amphetamine (MDMA).

Results and discussion

The chromatographic method for the separation of the 25 drugs was developed using the 100 ng/mL dilution. This solution was also used to optimize the conditions for make-up flow, Agilent Jet Stream, and MS conditions by means of the MRM optimizer software and the source optimizer software.

The final SFC method separated the 25 compounds in a run time of 5 minutes in a gradient from 2 to 25% methanol comprising formic acid and ammonium formate (Figure 2). The first compound that eluted from the column was THC at 0.99 minutes, and the last eluting compound was strychnine at 4.05 minutes. The compound that showed the highest intensity was methadone, eluting at 2.95 minutes

The 100 ng/mL solution was used to create individual calibration curves for the inherent compounds by a dilution pattern of 1:5:2 with methanol. The dilution series was measured down to a concentration of 0.01 ng/mL for all compounds to identify the individual limit of quantification (LOQ) and limit of detection (LOD). The compounds were detected with highest sensitivity showing LOQs below 100 pg/mL, and LODs below 30 pg/mL, all at good linearity correlations (Table 2). For a statistical evaluation, the 10 ng/mL calibration solution was injected 15 times. The calculated relative standard deviation (RSD) of the retention times was typically below 0.3%, and the area RSDs were in a good range, below 4%. The calculated concentration precision was below 3.5%, and the corresponding concentration accuracies were between 95 and 105%.

Table 2. Retention times of the 25 drugs, retention time and area RSDs, concentration precision, and accuracy of the 10 ng/mL concentration level. LOD and LOQ, linearity from individual calibration curves from 100 ng/mL down to the individual LOQ.

Compound	RT	RT RSD (%)	Area RSD (%)	LOD (pg/mL)	LOQ (pg/mL)	Linearity Correlation R^2	Concentration Precision (%)	Concentration Accuracy (%)
THC	0.997	0.44	4.34	60	200	0.9994	3.78	101.7
Temazepam	1.498	0.44	2.59	40	130	0.9951	2.42	105.5
Clonazepam	1.642	0.39	2.66	100	300	0.9982	4.25	102.4
Diazepam	1.668	0.41	3.81	30	100	0.9997	3.79	101.2
Lorazepam	1.742	0.32	4.78	300	1000	0.9975	5.15	106.9
Nitrazepam	1.768	0.37	1.64	20	65	0.9993	3.91	110.9
Proadifen	1.771	0.27	2.43	15	40	0.9996	1.61	106.9
Oxazepam	1.862	0.23	2.04	150	500	0.9952	2.15	105.8
Cocaine	1.994	0.39	1.42	10	40	0.9998	1.27	98.5
Verapamil	2.147	0.29	3.09	<5	10	0.9998	1.99	105.6
Trazodone	2.370	0.25	4.04	<5	10	0.9993	3.61	112.1
Oxycodone	2.478	0.29	3.65	40	130	0.9951	5.34	105.8
Meperidine	2.494	0.26	4.53	6	20	0.9951	2.42	105.5
MDEA	2.506	0.18	3.48	<5	10	0.9956	3.31	104.1
Heroin	2.518	0.27	3.53	40	150	0.9983	3.18	106.3
PCP	2.550	0.22	2.73	15	55	0.9991	2.34	110.1
Amphetamine	2.592	0.17	3.34	20	70	0.9943	2.29	93.1
MDA	2.631	0.16	4.34	60	200	0.9995	2.86	95.2
Methamphetamine	2.839	0.15	4.67	<5	10	0.9983	4.24	105.5
MDMA	2.900	0.16	3.13	10	30	0.9991	2.69	105.6
Methadone	2.947	0.15	2.86	10	30	0.9998	2.43	102.4
Alprolazame	3.228	0.13	2.13	10	30	0.9995	2.89	105.8
Codeine	3.290	0.19	4.39	20	50	0.9931	3.83	111.8
Hydrocodone	3.631	0.21	2.91	25	80	0.9931	2.73	112.3
Strychnine	4.055	0.13	1.15	50	150	0.9992	1.28	100.3

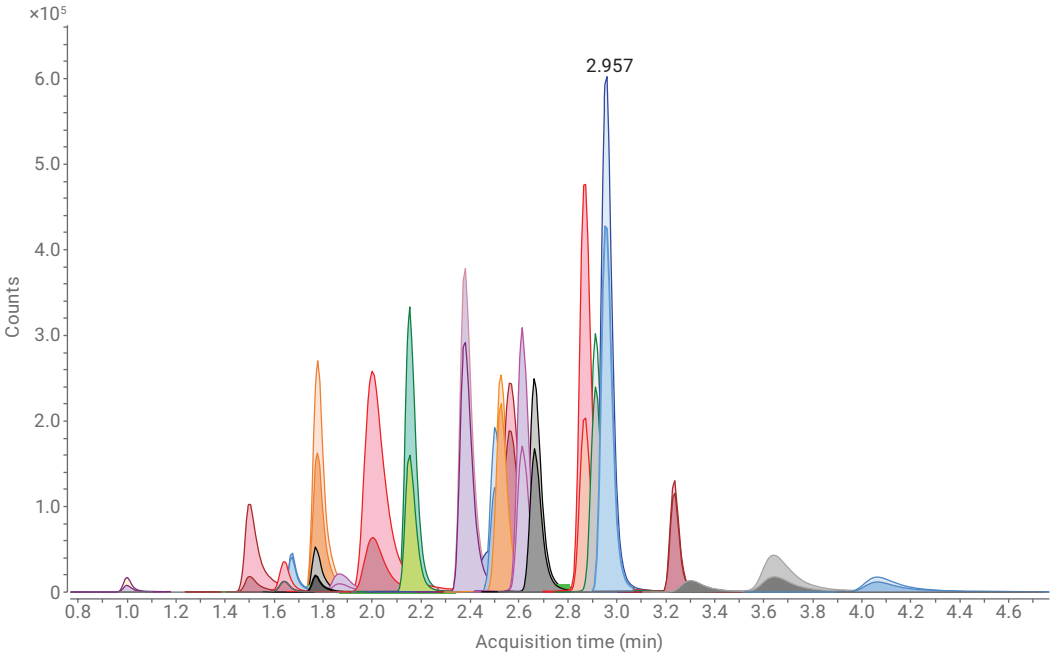


Figure 2. Separation of the mixture comprising 25 drugs by SFC separation in a run time of 5 minutes and detection by DMRM.

As an example, the compounds belonging to the class of amphetamines were examined more closely in a spiked urine sample. The sample was spiked at a level of 100 ng/mL, diluted 1:5 with methanol, yielding a final concentration of 20 ng/mL and used for injection as described in the experimental section. The five amphetamine compounds eluted between 2.4 and 3.1 minutes in the short gradient, ranging within 5 minutes from 2 to 25% methanol (Figure 3). For a more precise evaluation, the sample was injected 10 times. The RSDs for retention time and concentration, calculated from the replicate injections, were below 0.4% and below 3%, respectively. The concentration accuracy was in the range of 82 to 101%, which is excellent for quantification (Table 3).

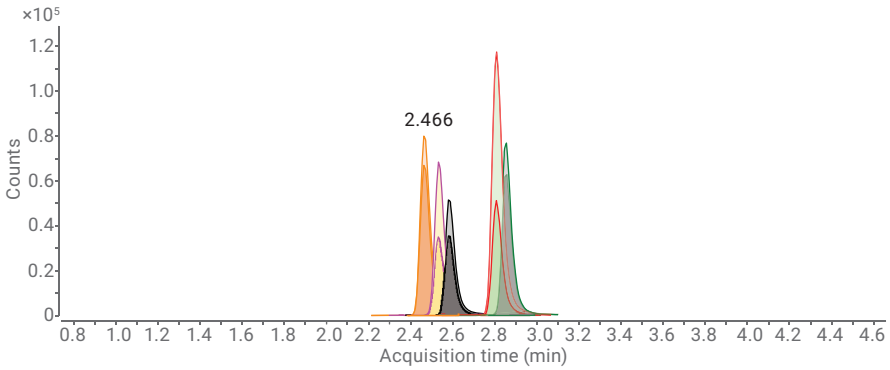


Figure 3. Sample of 20 ng/mL amphetamines (amphetamine, methamphetamine, MDA, MDMA, and MDEA) in spiked urine (100 ng/mL), diluted 1:5 with methanol.

Table 3. Results for the quantitative measurement of amphetamine compounds by SFC/triple quadrupole in a spiked and diluted urine sample.

Compound	RT (min)	RT RSD (%)	Measured Concentration (ng/mL)	Concentration Precision RSD (%)	Concentration Accuracy (%)
MDEA	2.466	0.42	19.61	2.75	97.98
Amphetamine	2.554	0.42	16.41	3.03	82.05
MDA	2.595	0.37	20.19	1.37	100.95
Methamphetamine	2.813	0.21	17.27	1.75	86.35
MDMA	2.860	0.17	17.71	2.16	88.55

As an example, the calibration curve obtained for MDA, from 0.2 ng/mL up to 100 ng/mL, showed an excellent linearity coefficient of 0.9995. The quantifier and qualifier ions obtained from the measured sample at a concentration level of 20 ng/mL showed good peak shape, and their ratio was in the expected range (Figure 4).

Conclusion

This application note demonstrates the use of the Agilent 1260 Infinity Analytical SFC System for the fast separation of a large number of drugs. The combination of the SFC system with the Agilent 6460 triple quadrupole MS enabled rapid screening and quantification. All compounds were eluted and separated in a short 5-minute gradient with high retention time and area precision of 0.3% and 4%, respectively. All calibration curves showed excellent linearity, and the LODs were below 30 pg/mL, which gives evidence of the high sensitivity achievable. The concentration precision was below 3.5%, and the accuracy between 95 and 105%. The analysis of a forensic toxicology sample was demonstrated by the quantification of amphetamines in a spiked urine sample. The concentration of spiked compounds was determined with excellent concentration precision and accuracy.

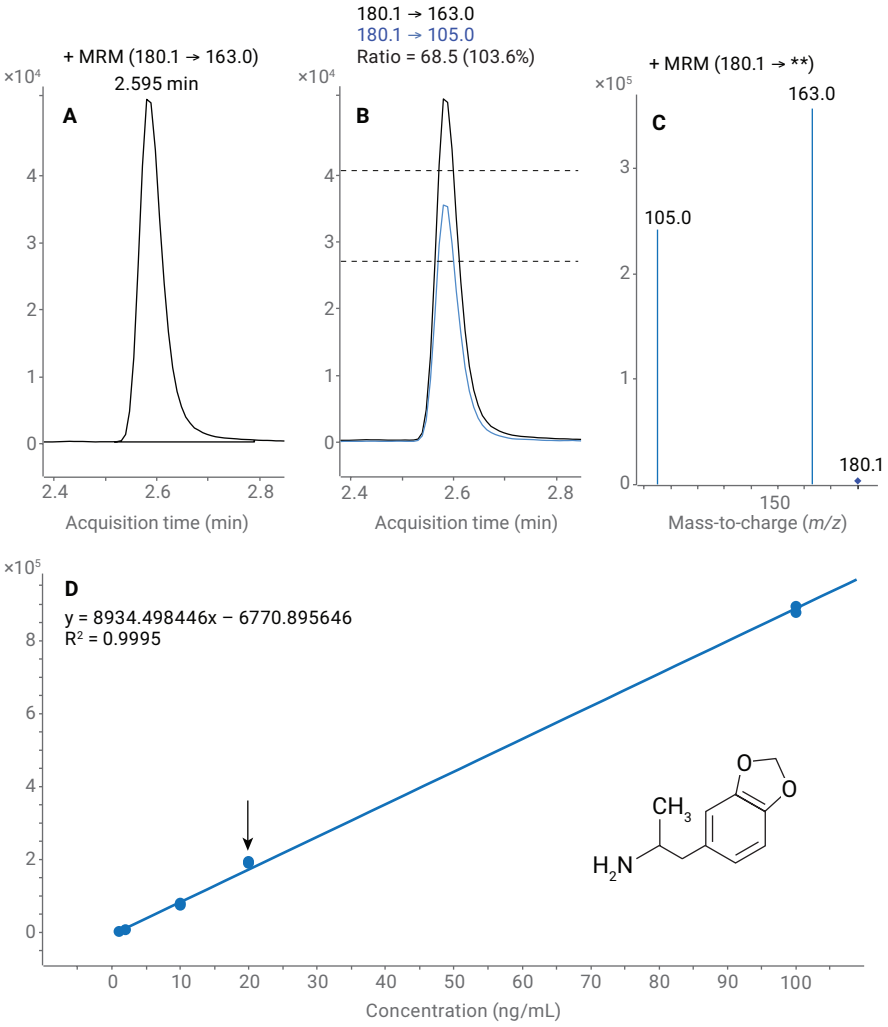


Figure 4. Qualitative measurement of MDA in a spiked urine sample. (A) Quantifier ion of MDA at a concentration level of 20 ng/mL. (B) Quantifier ion, qualifier ion and their ratio. (C) MS/MS spectrum of MDA. (D) Calibration curve of MDA between 0.2 ng/mL and 100 ng/mL with linearity correlation 0.9995. The measured concentration is indicated by the arrow.

Reference

1. Stone, P. J. W. An Application Kit for the Screening of Samples for Analytes of Forensic and Toxicological Interest using LC/QQQ MS/MS with a Dynamic MRM Transition Database, *Agilent Technologies application note*, publication number 5990-4254EN, **2016**.

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