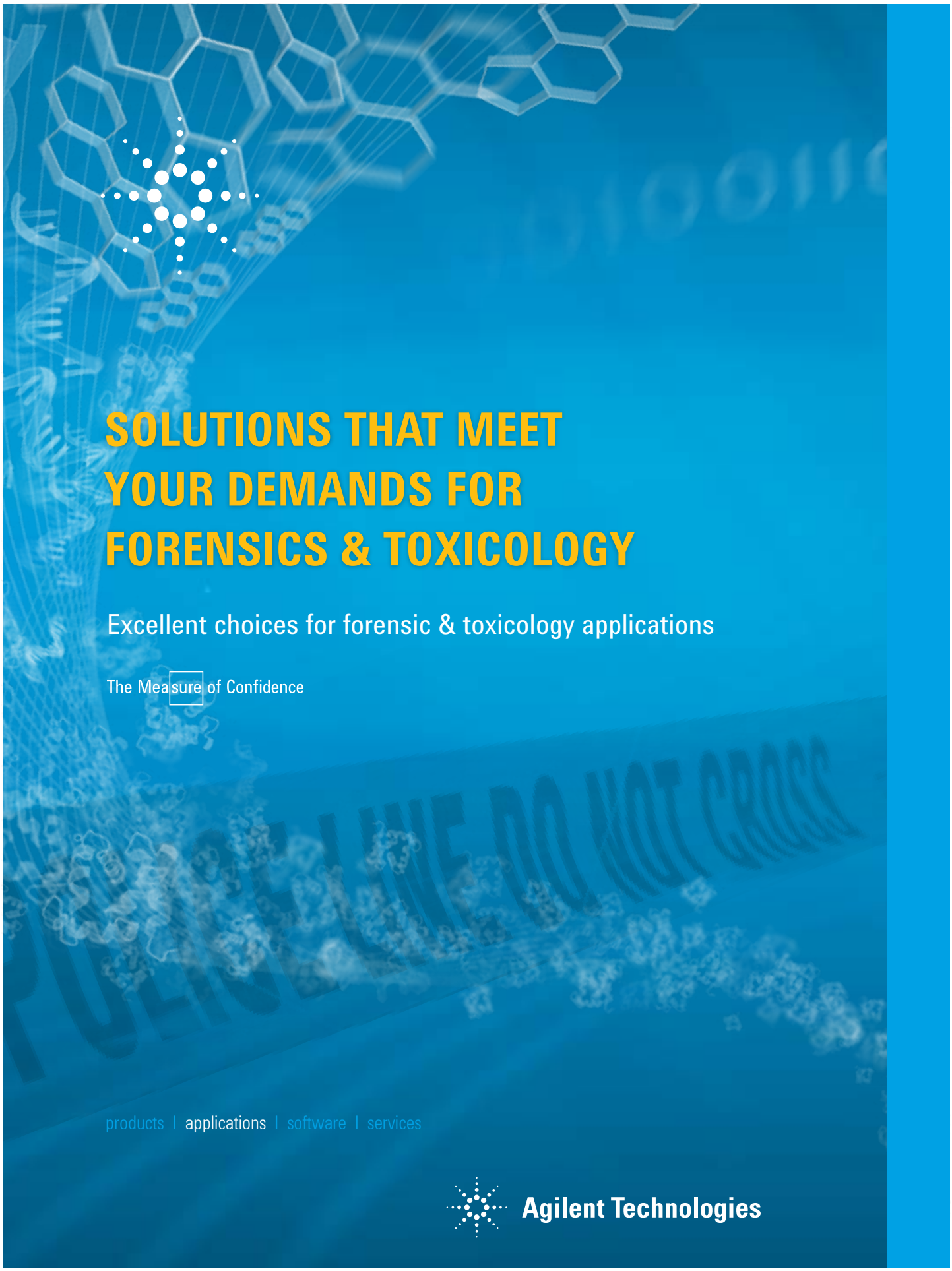

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- Rapid Forensic Toxicology Screening Using an Agilent 7890A/ NPD/5975C/DRS GC/MSD System
- Clenbuterol and Norandrosterone Analysis by Positive Chemical Ionization with the 5973N MSD

Applications by Technique

GC/MS

Implementation of Deconvolution Reporting Software (DRS) in Doping Control

Application Note

Forensic Toxicology

Authors

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Abstract

The deconvolution reporting software (DRS) combines quantitation and identification based upon self-created libraries and the NIST library. This study shows that the DRS software can be implemented in routine doping control and is very reliable. For the review of full scan data, it was found that one reviewer and DRS can replace the current approach of two reviewers.

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Introduction

Several screening methods currently used in doping control rely on GC-MS. Using this technique, stimulants, anabolic androgenic steroids, aromatase inhibitors, narcotics, and other substances can be detected in the urine of athletes [1, 2]. Except for anabolic steroids, which have to be detected at a minimum required performance level (MRPL) of 10 ng/mL according to the World Anti-Doping Agency (WADA) guidelines, these substances can be detected using the GC-MS instrument in the full-scan mode.

At present the GC-MS data generated is interpreted by two independent reviewers by looking at two ion traces of diagnostic ions in a retention time window typical for the component. In case of a suspicious sample, the full-scan spectrum aids in the decision to start a confirmation procedure. This process is time consuming and requires trained and concentrated staff to avoid reporting a false negative result.

The deconvolution reporting software (DRS), introduced in 2004, is a tool to produce an easy-to-read report based upon four different aspects. These are retention time, mass selective detector (MSD) ChemStation software, an automated mass spectral deconvolution and identification system (AMDIS), and the National Institute of Standards and Technology (NIST) library.

The goal of this study was to evaluate the possibilities of DRS in routine screening methods in doping control by implementing it in the GC-MS screening method for narcotics and stimulants.

Materials and Methods

All urine samples were extracted according to the procedure previously described by Van Thuyne et al. [2]. Analysis was carried out on an Agilent 5973 MSD directly coupled to an Agilent 6890 GC. Interpretation of the results was performed by two scientists, after which each sample was processed using the DRS present in the MSD ChemStation software (Revision D.03.00).

The sensitivity of the DRS software was examined by spiking nine different components to negative urine in the range 10 to 500 ng/mL. These substances were: mephentermine, amphetamine, methoxyphenamine, crotethamide, benzylpiperazine, MDEA, methylphenidate, pipradrol, morphine, and fenethylline.

Afterward, 1,366 routine samples were interpreted by both operators, followed by the DRS software.

Results and Discussion

The goal of deconvolution is to extract one signal from a complex mixture. In this way, the mass spectrum of coeluting peaks can be purified, allowing a better identification and confirmation of its structure. The tool to perform this process in the DRS software is AMDIS. AMDIS works by grouping all extracted ions having the same peak apex and a similar rise and fall of the ion trace. AMDIS has no correlation with peak integration.

In order to use the DRS software, several steps have to be taken. The first one is to develop or reform an existing GC method to a retention time-locked method. As the identification is based upon both retention time and mass spectral criteria, it is of utmost importance to keep the retention times constant. After locking the GC method, a library has to be created and calibrated (that is, adding retention time data). The development of the library is done by injecting one or more reference mixtures and can be performed in two different ways. The first one relies on the MSD ChemStation software. Using this procedure, all information present in the spectrum of the component is stored in the library. The second way is to use the deconvoluted spectra obtained by AMDIS to create a library. Both methods have their advantages. Using the ChemStation software is much faster than using AMDIS. However, using this approach, all monitored ions, including those that are part of the background, are present in the reference spectrum. Using the deconvoluted spectrum results in a limited number of ions, which can be attributed with a certainty of 100 percent to the reference component, which are then transferred in the library. Using amphetamine as an example, 333 different m/z values were entered in the library using the ChemStation software, while this number was restricted to 12 ions using the AMDIS approach. As a result, analysis of a spiked sample with MDA (500 ng/mL) results in a match factor of only 36 percent using the ChemStation software, while using the AMDIS database resulted in a match factor of 98 percent. Every small ion present in the spectrum in the ChemStation library is taken into account to determine the match factor. The absence of these ions in the deconvoluted spectra (because they are not present or belong to the background and therefore are filtered out) results in the low match factor.

Table 1. DRS Output of MDA Using the ChemStation Library (A) and the AMDIS Library (B)

A							
R.T.	CAS number	Compound name	Agilent ChemStation amount (ng)	AMDIS Match	R.T. Diff sec.	NIST Reverse match	Hit number
8.9707	4764174	MDA-TMS		**36**	–4.0		
B							
R.T.	CAS number	Compound name	Agilent ChemStation amount (ng)	AMDIS Match	R.T. Diff sec.	NIST Reverse match	Hit number
8.9749	4764174	MDA-TMS		**98**	0.0		

After the library is optimized, several options can be selected to customize the layout of the output, including a quantification using the ChemStation software and a reconfirmation of the detected substances using a comparison with the NIST library. However, not all substances are present as their TMS derivatives in the NIST library and not all substances are added in the NIST with their common names. Therefore, the reconfirmation using NIST was skipped from the processing method. However, for unknown samples this can be a helpful tool.

In order to optimize the cutoff match value used by the DRS negative urines samples were spiked with nine different components in a concentration range between 10 and 500 ng/mL. The selection of the components was made based upon their full-scan spectrum. TMS-derivatized morphine shows a good spectrum for deconvolution because it possesses numerous ions over its complete mass spectrum with medium to high relative intensities. The other selected components, however, only have a poor mass spectrum. These substances are stimulants, showing only one abundant fragment ion with a low specificity and a small molecular ion. The goal of selecting these substances was to investigate the potential influence of a (small) matrix interference on the DRS match value obtained. This value should be optimized in such a way that all substances can be detected at a concentration as low as possible without the risk of a false positive result. This was the case when applying a cutoff value of 45 percent at a concentration of 50 ng/mL, where a false positive result for methoxyphenamine was obtained (Table 2).

Setting the cutoff value to 65 percent would result in false negative results for MDEA and morphine. At a concentration of 100 ng/mL, which is below the MRPL of all substances in this screening method, all nine components are detected using a cutoff of 65 percent. Therefore, this value of 65 percent was used. In addition, a maximal deviation in retention time of 6 seconds compared to the reference library was applied. This makes retention time locking of utmost importance.

Table 2. DRS Output of Negative Urine Spiked with Nine Components at 50 ng/mL Using a Cutoff of 45 Percent

R.T.	Compound Name	Agilent ChemStation Amount (ng)	AMDIS Match	R.T. Diff sec.	NIST Reverse match	Hit number
3.6341	Mephentermine		91	–4.1		
4.7247	Amphetamine-TMS		88	–2.3		
8.4520	**Methoxypenamine-TMS**		**45**	**2.9**		
9.2010	Crotethamide		79	–1.7		
9.2239	Benzylpiperazine-TMS		91	–1.5		
10.0936	MDEA-TMS		49	–1.3		
10.5285	Methylphenidate-TMS		73	–1.0		
11.8508	Pipradrol-TMS		79	2.0		
13.4639	Morphine-bis-TMS		56	–1.2		
14.4412	Fenethylline		86	–0.6		

In order to test the capabilities of the DRS, a library containing more than 100 components was created to implement in the screening method for stimulants and narcotic agents. The DRS interpretation of the data was performed after both operators had reviewed the macros of the samples in order to avoid influence of the DRS output on the operators' conclusion. The DRS did not produce any false negative results.

All positive results observed by the operators were also picked out by the DRS. In total, 105 samples contained one or more substances identified in both ways. However, the DRS picked out three additional positive samples, which remained unnoticed by the analysts. The first case was a sample positive for amphetamine. Normally amphetamine elutes at a retention time of 4.78 minutes and the ions *m/z* 116 and *m/z* 192 are monitored. Figure 1A shows the ion traces in a QC sample. As can be seen, a systematic interference elutes at the end of this retention time window that can also be observed in a negative urine sample (Figure 1B). With this knowledge, both operators interpreted Figure 1C as negative. However, due to a large interference at the beginning of the chromatogram, retention times were shifted with approximately 0.1 minute. Because the peak apex was found within an interval of 0.1 minute from the expected retention time, the DRS recognized this sample as an amphetamine-positive sample, which was confirmed during a consecutive B-analysis.

The second case was a sample positive for methylphenidate. Figure 2 shows the ion traces as printed in the macro and the corresponding mass spectrum. The lack of the detection of *m/z* 91, diagnostic for methylphenidate, and differences between the obtained spectrum and a reference spectrum lead to the conclusion that this sample was negative. However, DRS identified methylphenidate, which was later confirmed by the detection of ritalinic acid in the diuretic screening method.

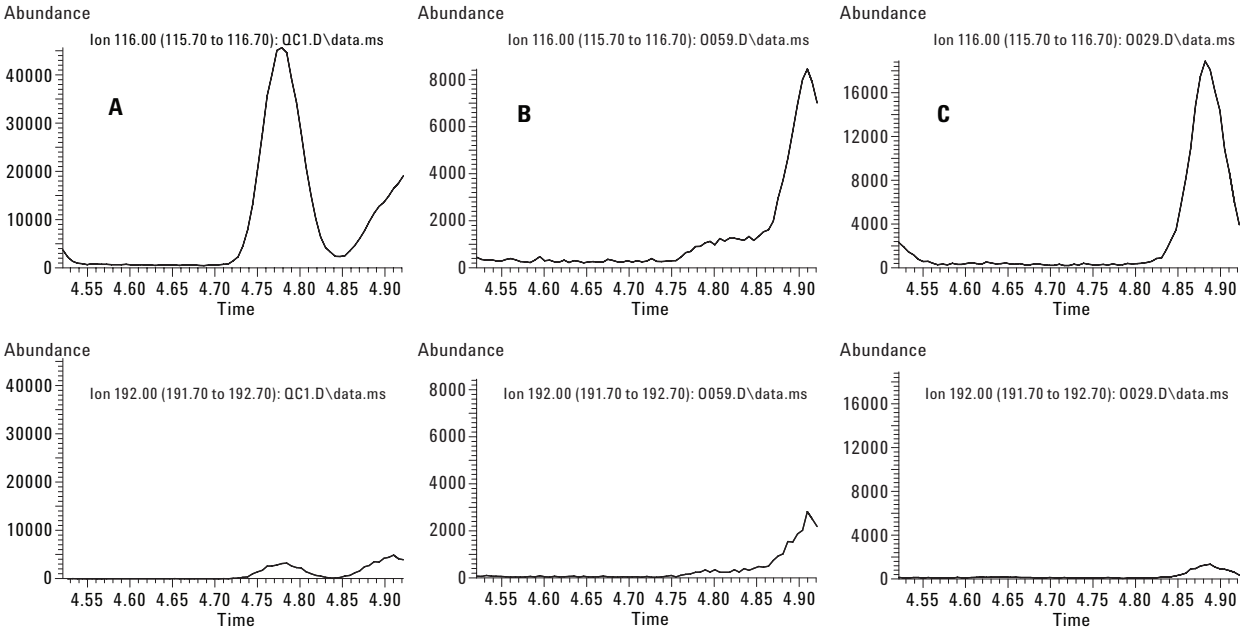

Figure 1. Ion traces for amphetamine in a QC sample (A), negative sample (B), and DRS-positive sample (C).

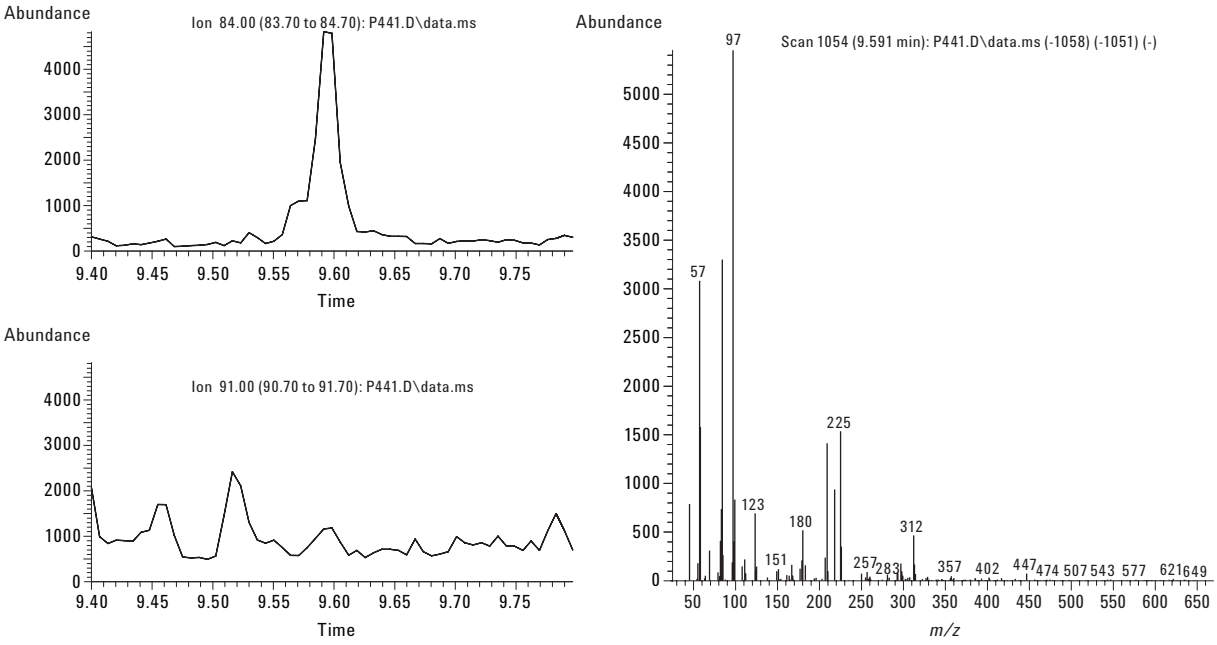

Figure 2. Ion traces for methylphenidate and corresponding spectrum for the peak at RT 9.59 min.

The third sample was positive for morphine. Because of the structural resemblance between morphine and hydromorphone, both substances elute within a small time interval and have the same diagnostic ions monitored in the screening method. At MRPL level, both substances are separated by 0.06 minute as can be seen in Figure 3B.

Obviously, the routine sample shown in Figure 3A contains morphine. A confirmation procedure pointed out that the concentration was higher than 50 µg/mL. According to the DRS software, this sample also contains hydromorphone. Because the ion traces in the macro are scaled to the highest peak detected in a retention time window, small amounts of a substance closely eluting to a highly concentrated peak remain

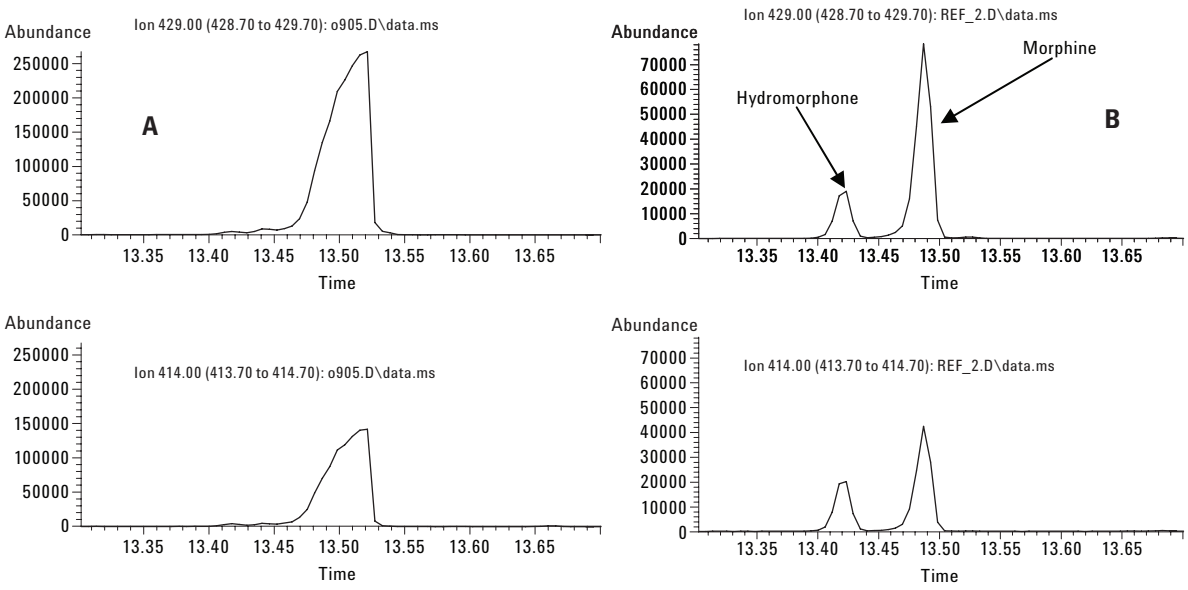

Figure 3. Positive screening result for morphine (A) and QC sample for morphine/hydromorphone (B).

unnoticed. The presence of hydromorphone in this sample could be confirmed. The presence of hydromorphone in the urine of people taking high amounts of morphine has been described in the past [3].

Besides the presence of other doping substances, matrix interferences can also hamper the detection of substances of interest. As described previously, the use of ethyl acetate as extraction solvent for a comprehensive method for the detection of anabolic steroids, narcotic agents, and stimulants was hampered because of a large interference at the retention time of several stimulants, including amphetamine [4] (Figure 4). This interference has been identified as glycerol and can also be detected in smaller amounts using diethyl ether (cfr Figure 1A). Applying the DRS on a sample spiked at 500 ng/mL and using ethyl acetate as extraction solvent results in a match factor of 89 percent, proving the applicability of the DRS to identify target substances in complex matrices.

The deconvolution software is developed to operate in the full-scan mode. However, in doping control, anabolic androgenic steroids have to be detected using selected ion monitoring because of the low level excreted in urine. At present, analytical equipment offers the possibility of combining SIM and scan runs in one analytical run due to improved electronics, allowing a faster data transfer. However, the DRS is designed to operate using only one dataset, making its use in SIM/scan impossible.[1] Its use in a method using only SIM, however, is possible. For this purpose, at least three diagnostic ions have to be selected for each component to avoid numerous false positive results. Because only a selected number of ions are used, the resulting match factors are also higher. This requires different settings compared to a processing method for scan data. This optimization has been performed for approximately 20 different anabolic steroids using a match factor cutoff value of 85 percent. Using this processing method, identification of oral-turinabol in a WADA PT-sample was possible.

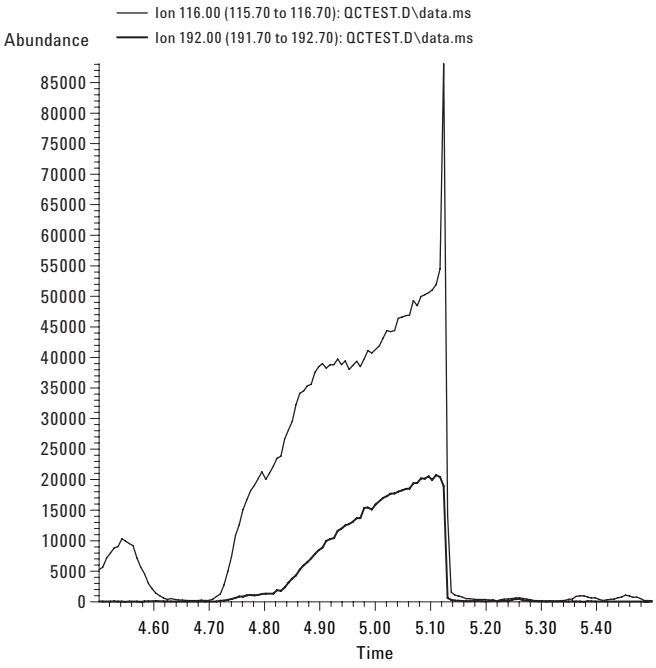

Figure 4. Extracted ion chromatogram for amphetamine using ethyl acetate as extraction solvent.

Conclusions

The DRS software is a tool that combines quantitation and identification based upon self-created libraries and the NIST library. During this study it was proven that DRS software can be implemented in routine doping control and is very reliable. Therefore, the current system of reviewing obtained data, for example, two analysts looking at macros, can be reduced to one person and the DRS software. However, in order to use the DRS software in SIM/scan methodology, several improvements have to be made, including the incorporation of more diagnostic ions for anabolic androgenic steroids monitored in the SIM mode.

Acknowledgements

The authors wish to acknowledge Agilent Technologies for providing the DRS software and the Ministry of Culture, Sport, Youth and Media of the Flemish Community for its financial support.

[1]The DRS software can process both the SIM and scan datafiles from a single SIM/scan run, but requires extra steps. Running the file as acquired will result in the scan data being processed by DRS. The datafile can then be copied to a new name and the Scan file is renamed from DATA.MS to DATASCAN.MS. The SIM file (DATASIM.MS) is then renamed to DATA.MS. The SIM part of the SIM/scan datafile can now be processed with a DRS method constructed for the SIM data.

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Analysis of Drugs of Abuse by GC/MS using the Ultra Inert Inlet Liners with Wool

Application Note

Forensic Toxicology

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Abstract

With efficient deactivation on glass wool, the Ultra Inert liners with wool provide excellent inertness, homogeneous sample mixing and evaporation, non-volatile residue trapping, and column and detector protection for drugs of abuse screening.

Introduction

GC inlet liners are the centerpiece of the inlet system where the sample is vaporized, mixed with the carrier gas, and introduced to the capillary column. Inlet liners with wool are widely used because the wool promotes homogenous sample mixing and better quantitation. Wool provides a large surface area which aids the vaporization of liquid samples. It also acts as a trap to collect non-volatile residue in the sample, thus protecting the GC column from the negative impact of sample matrix. Wool liners also reduce sample loss by preventing sample droplets from reaching the bottom of the inlet before vaporization. Agilent MS certified liners with glass wool provide excellent performance for general application purposes. However, for specific applications of active compounds analysis, liners with superior inertness are required to achieve the most reliable results.

GC/MS screening methods are important in toxicology laboratories. With the continuing emergence of new drugs and toxins, the list of target compounds to be screened can number in the hundreds. For those compounds that are compatible with GC, using GC/MS in full-scan mode with electron impact ionization (EI) is well suited for the task [1]. Samples for screening usually require minimal sample preparation, or even no clean-up, to preserve target analytes. However, heavy-matrix samples, such as plasma or urine extracts, deteriorate the performance of the analytical column and detector, resulting in short column life and frequent MS source maintenance. Therefore, it is beneficial to use inlet liners with wool to protect the entire GC/MS system.

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However, if liners with wool are poorly deactivated, they can cause the adsorption or decomposition of target analytes for basic drugs of abuse. As shown in Figure 1, those drugs usually contain hetero atoms, which strongly interact with the free silanol groups (Si-OH) in glass [2]. The resulting compound adsorption and decomposition causes chromatographic problems such as broad or distorted peaks, tailing peaks, ghosting phenomena, and low responses. Liners with glass wool magnify these negative effects due to the large surface area of glass wool and difficulty of complete deactivation. A properly and efficiently deactivated inlet liner with glass wool is imperative for satisfactory chromatography with accurate and reproducible responses for these forensic/toxicology applications.

Agilent's Ultra Inert liner deactivation process significantly improves the efficacy and robustness of glass wool deactivation. The surface area is deactivated thoroughly. For the first time, liners with glass wool can analyze basic drugs of abuse using GC/MS.

The liners with wool were evaluated using Agilent Forensic/Toxicology analyzer checkout standards, including 28 popular and difficult basic drug compounds. These compounds cover the retention range from early to late eluting compounds, and contain different categories of drugs including amphetamins, alkaloids, and benzodiazpines. Figure 1 shows the chemical structures for some of the analytes. All liner tests were conducted using a GC/MS system with simultaneous collection of scan and SIM data. A 5 µg/mL standard was used for chromatographic evaluation. A 500 ng/mL standard (10× dilution) was used to assess the repeatability of injections over 50 injections.

Experimental

Chemicals and Reagents

The Agilent GC/MS Forensic/Toxicology analyzer checkout mixture standard (p/n 5190-0471) was used to evaluate the performance of Ultra Inert liners with glass wool. HPLC grade Toluene and Methanol was purchased from Honeywell B&J (Muskegon, MI, USA), and Acetonitrile (AcN) was purchased from Sigma-Aldrich (St Louis, MO, USA). An Internal Standard (IS) was purchased from AccuStandard (New Haven, CT, USA), containing 0.5 mg/mL of Acenaphthene-D_{10}, Phenanthrene-D_{10}, Triphenylphosphate, Chrysene-D_{12}, and Perylene-D_{12} in Acetone.

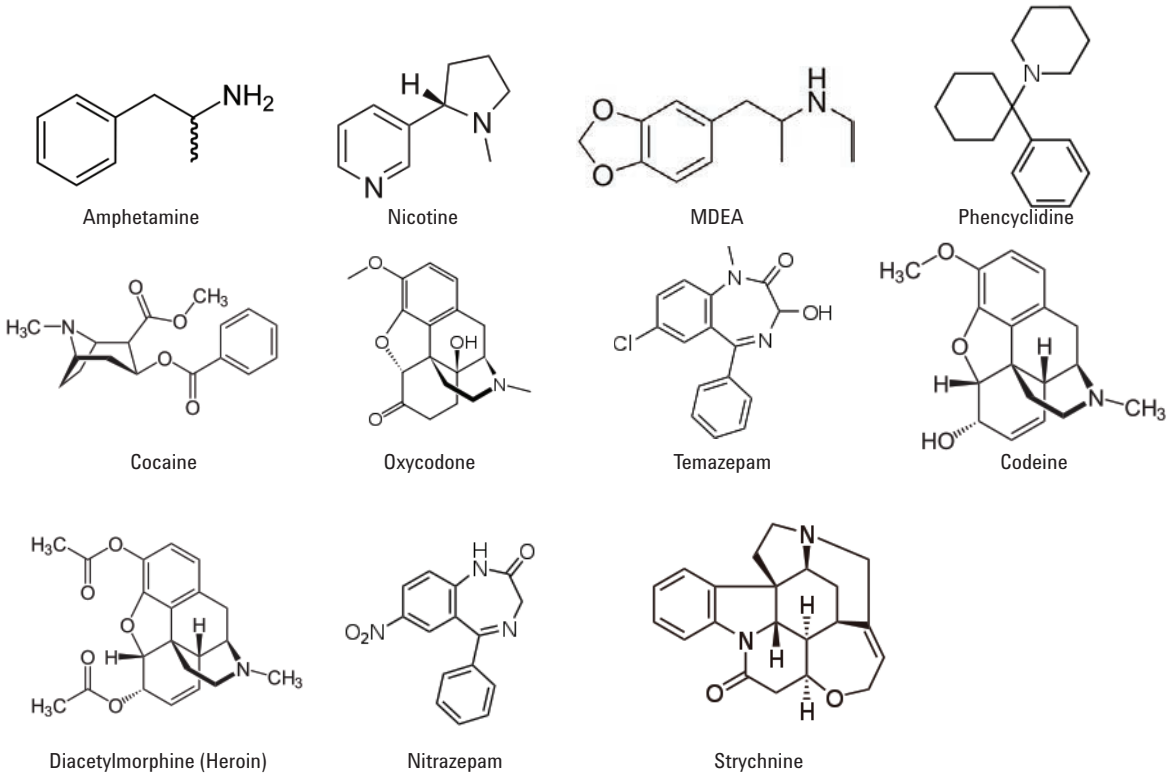

Figure 1. Chemical structure of selected basic drugs.

Solutions and Standards

The original checkout standard sample was made in a 90/5/5 Toluene/MeOH/AcN solution. A 90/5/5 Toluene/MeOH/AcN blank solvent mixture was prepared by combining 90 mL of Toluene, 5 mL of MeOH and 5 mL of AcN, and was used as reagent blank. The 5 µg/mL original standards were directly used for injection, and were diluted 10 times with blank solvent to 500 ng/mL solution. 4 µL of IS stock solution was spiked to 1 mL of standard solution, when necessary, to generate a concentration of 2 µg/mL for IS in the sample.

Instrumentation

All testing was done on an Agilent 7890A GC system equipped with a 7683B autosampler and a 5975C MSD.
Table 1 lists the instrument conditions. Table 2 lists flow path consumable supplies. Table 3 list the Selected Ion Monitoring (SIM) conditions for 28 target analytes.

Table 1. Instrumental conditions for Agilent GC/MS system used for basic drug compounds test

GC	Agilent 7890A Series
Autosampler	Agilent 7683B, 5 µL syringe (p/n 5181-5246), 1 µL injection volume
	Preinj solvent A (90/5/5 Toluene/MeOH/AcN) washes: 1 Sample pumps: 3 Postinj solvent B (90/5/5 Toluene/MeOH/AcN) washes: 3
Carrier gas	Helium, constant pressure
Inlet	Splitless mode: 280 °C
Purge flow	50 mL/min, switched mode, hold for 0.75 min
Inlet pressure	18.7 psi (RT locked) during run, 1.0 psi during back flush
RT locking	Proadifen (SKF-525a) @ 8.569 min
Oven profile	100 °C for 0.5 min, to 325 °C at 20 °C/min, hold 2.5 min
Post run	2 min at 325 °C
Capillary Flow Technology	Purged Ultimate Union (p/n G3182-61580) used for back flushing the analytical column and inlet
Aux EPC gas	Helium plumbed to Purge Ultimate Union
Aux pressure	4 psi during run, 75 psi during back-flushing
Analytical column	DB-5MSUI, 15 m × 0.25 mm, 0.25 µm (p/n 122-5512UI)
Connections	Inlet to Purged Ultimate Union (p/n G3182-61580)
Restrictor	Inert Fused Silica tubing, 0.65 m × 0.15 mm (p/n 160-7625-5)
Connections	Between Purged Ultimate Union and the MSD
MSD	Agilent 5975C inert with performance electronics
Vacuum pump	Performance turbo
Mode	Scan/SIM
Tune file	Atune.u
EM voltage	Atune voltage
Transfer line temp	300 °C
Source temp	300 °C
Quad temp	150 °C
Solvent delay	1.4 min
Scan mass range	40 – 570 amu

Table 2. Flow Path Supplies

Vials	Amber screw cap (p/n 5182-0716)
Vial caps	Blue screw cap (p/n 5182-0717)
Vial inserts	150 µL glass w/ polymer feet (p/n 5183-2088)
Septum	Advanced Green Non-Stick 11 mm (p/n 5183-4759)
Ferrules	0.4 mm id, 85/15 Vespel/graphite (p/n 5181-3323)
O-rings	Non-stick liner O-ring (p/n 5188-5365)
Capillary Flow Technology	Purged Ultimate Union (p/n G3182-61580) Internal nut (p/n G2855-20530) SilTite metal ferrules, 0.10-0.25 mm id (p/n 5188-5361)
Inlet seal	Gold plated inlet seal with washer (p/n 5188-5367)
Inlet liners	Agilent Ultra Inert deactivated single taper splitless liner with wool (p/n 5190-2293)

Table 3. SIM acquisition conditions used for 28 basic drug compounds by GC/MS

Analytes (Peak no. on chromatogram)	SIM *	RT (min)	Collection window (min)
Amphetamine (1)	**44**, 91	1.77	1.4 – 2.7
Phentermine (2)	**58**, 134	1.96	
Methamphetamine (3)	**58**, 91	2.08	
Nicotine (4)	**84**, 133	3.06	2.7 – 3.6
Methylenedioxyamphetamine (MDA) (5)	**44**, 135	3.92	3.6 – 5.0
Methylenedioxymethamphetamine (MDMA) (6)	**58**, 135	4.27	
Methylenedioxyethylamphetamine (MDEA) (7)	**72**, 135	4.57	
Meperidine (8)	**71**, 247	5.63	5.0 – 7.0
Phencyclidine (9)	**200**, 242	6.49	
Methadone (10)	**72**, 57	7.72	7.0 – 8.9
Cocaine (11)	**182**, 82	8.10	
Prodifen (SKF-525a) (12)**	**86**, 99	8.57	
Oxzepam (13)	**239**, 267	8.73	
Codeine (14)	**299**, 162	9.01	8.9 – 9.5
Lorazepam (15)	**239**, 274	9.08	
Diazepam (16)	**256**, 283	9.22	
Hydrocodone (17)	**299**, 242	9.29	
Tetrahydrocannabiol (18)	**231**, 314	9.36	
Oxycodone (19)	**315**, 230	9.63	9.5 – 10.4
Temazepam (20)	**271**, 273	9.87	
Flunitrazepam (21)	**312**, 286	9.96	
Diacetylmorphine (Heroin) (22)	**327**, 369	10.02	
Nitrazepam (23)	**253**, 206	10.62	10.4 – 11.6
Clonazepam (24)	**314**, 286	10.94	
Alprazolam (25)	**279**, 308	11.32	
Varapamil (26)	**303**, 304	12.03	11.6 – 14.0
Strychnine (27)	**334**, 335	12.18	
Trazodone (28)	**205**, 70	12.96	

* Ions in Bold were quantifiers, and the other ions were qualifiers.

** Prodifen was used for the RT locking.

A back-flushing system was used because it shortens analysis times for samples that contain high-boiling matrix interferences, reduces column head trimming, and reduces frequency of MSD source cleaning [3,4]. The instrument configuration is similar to the configuration shown in Figure 1B in the previous setup [4], except no retention gap was used for this application. Retention time locking (RTL) was used to eliminate recalibration of individual retention times and timed events such as SIM groups [5].

Results and Discussion

The purpose of these tests was to evaluate the Ultra Inert deactivated liners with wool for screening analysis of drugs of abuse by GC/MS. The Agilent Forensic/Toxicology analyzer checkout standard was used for the evaluation (Table 3). The feasibility of using Ultra Inert liners with wool was determined by chromatographic evaluation, liner to liner reproducibility, and multi-injections repeatability. In parallel, liners with wool from multiple sources were tested for comparison.

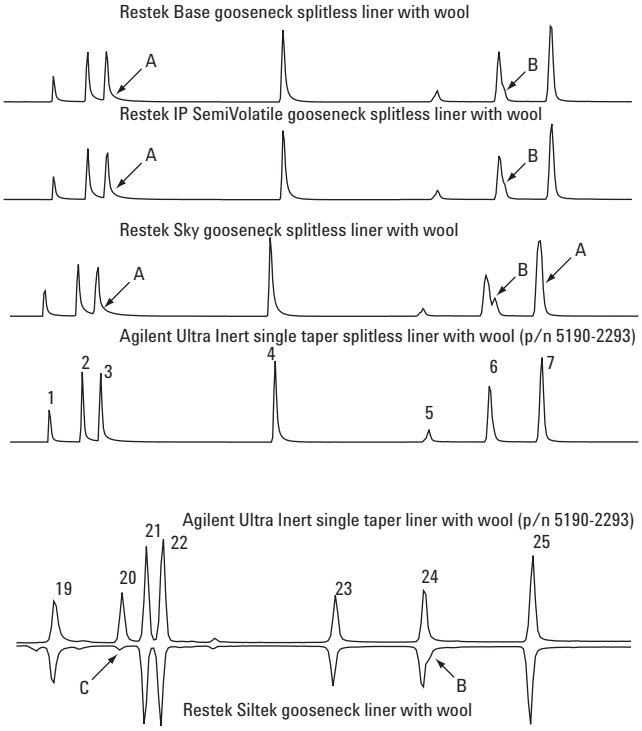

Figure 2. Chromatographic problems for drug of abuse compounds shown on GC/MS SIM chromatograms when using other equivalent liners and their comparison with chromatograms obtained by Ultra Inert liners with wool. See Table 3 for peaks identification and Table 1 for instrument conditions. 5 ng checkout standards on column. A) Broad or distorted peak, B) ghosting shoulder, C) poor sensitivity

Chromatographic performance

The adsorption or decomposition of basic drug compounds may cause various chromatographic problems including broad, distorted peaks, peak tailing, ghost peaks, and loss of sensitivity. All of these problems were observed in liners tests using the checkout standard. Peak shape problems usually occurred for early eluting compounds, such as Phentermine, Methamphetamine, MDA, and MDMA. The late eluting compounds, such as Temazepam, can disappear due to the loss of sensitivity. Figure 2 shows problematic chromatograms obtained using similar liners compared to chromatograms obtained using Ultra Inert liners with wool. As seen in Figure 2, with 5 ng on column, other liner deactivations cause chromatographic problems such as broad or distorted peaks and significant loss of response. However, the corresponding chromatograms with Agilent Ultra Inert deactivated liners show better peak shape and typically higher responses. Figure 3 shows a full chromatogram of 5 ng checkout standard on column using Agilent Ultra Inert splitless liner with wool by GC/MS. Figure 3 shows that Ultra Inert liners with wool provide the best chromatogram for all of analytes tested, even though there is small peak tailing or broadening observed for certain compounds. Six replicates of Ultra Inert liners were tested, each providing similar chromatographic performance, indicating excellent liner to liner reproducibility. The satisfactory chromatograms obtained by Ultra Inert liners demonstrate that the Ultra Inert liner deactivation process provides sufficient liner and glass wool inertness to prevent drugs of abuse from adsorption and decomposition.

1. Amphetamine, 2. Phentermine, 3. Methamphetamine, 4. Nicotine, 5. MDA, 6. MDMA, 7. MDEA, 8. Meperidine, 9. Phencyclidine, 10. Methadone, 11. Cocaine, 12. SKF-525a, 13. Oxazepam, 14. Codeine, 15. Lorazepam, 16. Diazepam, 17. Hydrocodone, 18. Tetrahydrocannabinol, 19. Oxycodone, 20. Temazepam, 21. Flunitrazepam, 22. Heroin, 23. Nitrazepam, 24. Clonazepam, 25. Alprazolam, 26. Verapamil, 27. Strychnine, 28. Trazodone.

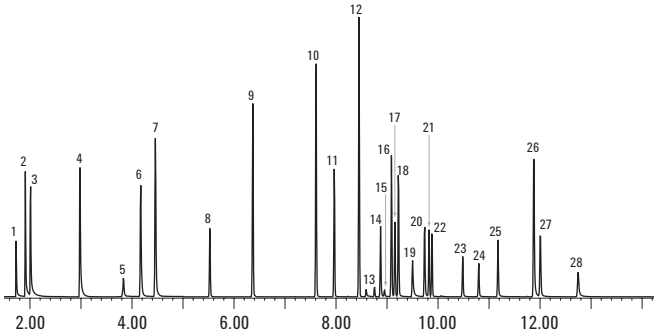

Figure 3. Chromatogram of forensic/toxicology analyzer checkout standard (5 ng checkout standards on column) using Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293) by GC/MS. See Table 1 for instrument condition. Satisfactory peaks shape achieved for all of analytes

Liner to liner reproducibility

To quantitatively evaluate the liner to liner reproducibility, six Ultra Inert liners from four different lots were tested. 5 µg/mL and 500 ng/mL samples spiked with 2 µg/mL IS were used. Twelve sensitive compounds were selected for evaluation. The Response Factors (RFs) were calculated for each concentration level. The average RF values were evaluation criteria for the liner to liner reproducibility test. See Table 4. The results show excellent liner to liner performance consistency with less than 7% RSD, except for Temazepam with 11.7%, across six liners from four different lots.

*Table 4. Liner to Liner Reproducibility: 12 sensitive basic drug compounds average RF (5 µg/mL and 500 ng/mL) and RSD values for six replicates of UI deactivated liners with wool (p/n 5190-2293) **

Compounds	Liner 1 (Lot 1)	Liner 2 (Lot 1)	Liner 3 (Lot 1)	Liner 4 (Lot 2)	Liner 5 (Lot 3)	Liner 6 (Lot 4)	Mean RF	RSD
Methamphetamine (3)	0.875	0.876	0.882	0.940	0.955	0.904	0.905	3.8
MDMA (6)	0.807	0.789	0.783	0.848	0.874	0.841	0.824	4.4
Phencyclidine (9)	0.494	0.510	0.494	0.488	0.509	0.521	0.503	2.5
Cocaine (11)	0.636	0.645	0.647	0.637	0.660	0.668	0.649	2.0
Oxazepam (13)	0.050	0.055	0.052	0.055	0.062	0.057	0.055	7.6
Codeine (14)	0.096	0.098	0.095	0.090	0.099	0.102	0.097	4.2
Oxycodone (19)	0.073	0.071	0.070	0.076	0.082	0.080	0.075	6.5
Temazepam (20)	0.101	0.121	0.115	0.088	0.096	0.104	0.104	11.7
Heroin (22)	0.097	0.099	0.096	0.095	0.100	0.102	0.098	2.7
Nitrazepam (23)	0.038	0.032	0.037	0.034	0.037	0.036	0.036	6.3
Clonazepam (24)	0.035	0.035	0.034	0.032	0.034	0.033	0.034	3.5
Trazodone (28)	0.061	0.065	0.064	0.058	0.060	0.064	0.062	4.4

$$^{*}\ RF = \frac{\text{Peak Area}_{\text{Analyte}} \times \text{Concentration}_{\text{Internal Standard}}}{\text{Peak Area}_{\text{Internal Standard}} \times \text{Concentration}_{\text{Analyte}}}$$

Injection repeatability and deactivation stability

Multi-injection repeatability and deactivation stability were tested by continuously injecting 1 µL of 0.5 µg/mL standard samples for 50 injections. Data was collected and RF values were calculated every 10 injections. RSD values were calculated over 50 injections. Table 5 shows the RSD value for all of the basic drug analytes with 0.5 ng on column.

A 0.5 ng on column concentration was used for this repeatability test since low level concentrations show greater deviation contributions than high concentration samples. Higher responses of analytes could hide some deviation impact and generate better repeatability. Twenty-two of 28 analytes have excellent repeatability for 50 injections of standard solution with less than 20% RSD. 5 of 28 analytes have relatively high RSD (between 20 – 25%), but still should be acceptable at the level of 0.5 ng on column. Temazepam is a very difficult compound and extremely sensitive to the liner inertness.

Table 5. Deactivation stability: 50 injections repeatability (%RSD) for Agilent Ultra Inert deactivated liners with wool (p/n 5190-2293) for all of tested basic drug compounds with 0.5 ng of standard on column. (n = 3)

Compound	RSD (%) over 50 injections	Compound	RSD (%) over 50 injections
Amphetamine	0.3	Lorazepam	20.9
Phentermine	1.1	Diazepam	3.7
Methamphetamine	1.5	Hydrocodone	3.7
Nicotine	2.3	Tetrahydrocannabinol	8.5
MDA	3.7	Oxycodone	22.2
MDMA	2.2	Temazepam	59.9
MDEA	2.0	Flunitrazepam	8.7
Meperidine	1.9	Heroin	10.7
Phencyclidine	15.6	Nitrazepam	11.2
Methadone	3.4	Clonazepam	12.0
Cocaine	7.8	Alprazolam	13.1
Prodifen	4.4	Verapamil	15.4
Oxazepam	20.4	Strychnine	11.0
Codeine	20.5	Trazodone	23.6

As shown in Figure 2, when an inefficient deactivated liner was used the response of Temazepam (5 ng on column) can almost disappear. Compared to other similar liners, Agilent Ultra Inert liner with wool generated highest RF for Tempazepam, which is clearly shown in Figure 4. This indicates that Agilent Ultra Inert liners with wool provide the best inertness compared to competitor's equivalent liners.

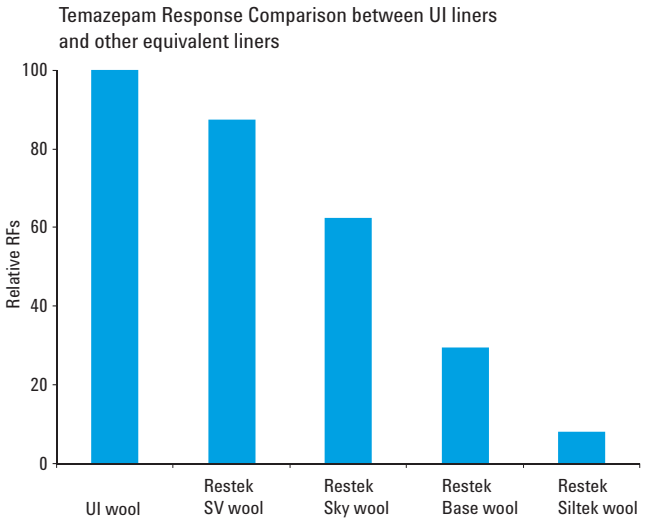

Figure 4. Sensitive compound response (Temazepam) comparison for Ultra Inert liner with wool (p/n 5190-2293) and other equivalent liners. RF calculation was based on the average RF of 0.5 ng and 5 ng standard on column. Ultra Inert liner average RF value was set to 100% and other liners average RF values were scaled.

The response of Temazepam decreased with more samples were injected, thus generated high RSD over injections. This phenomenon was observed for all of the liners tested, and the response decrease can be even worse for other liners. When Temazepam is a target analyte and the interested concentration is at ppb level, it is strongly recommend that an Ultra Inert liner with wool should be used for no more than 30 samples.

Real matrix sample analysis

Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/10th volume. Figure 5 shows the chromatogram of 2 ppm matrix spiked sample using Agilent Ultra Inert liner with wool by GC/MS, which is satisfactory for both early eluted compounds' peak shape and late eluted compounds' sensitivity. There are some minor interference peaks from matrix showing up.

Conclusion

Agilent Ultra Inert liners with wool have shown excellent inertness for the analysis of basic drugs of abuse. Ultra Inert liners with wool provide satisfactory chromatography for the selected popular and difficult basic drug compounds. The liner to liner performance shows excellent reproducibility with an average of 5% RSD for these active compound RF values. With efficient and robust deactivation of the wool, Agilent Ultra Inert liners with wool provide excellent inertness for forensic and toxicology screening. The benefits provided by liners with wool such as homogeneous sample mixing and evaporation, non-volatile residue trapping, and column and detector protection, are gained without compromise of chromatography or sensitivity of active analytes. Ultra Inert liners with wool are an excellent choice for screening analysis for drugs of abuse.

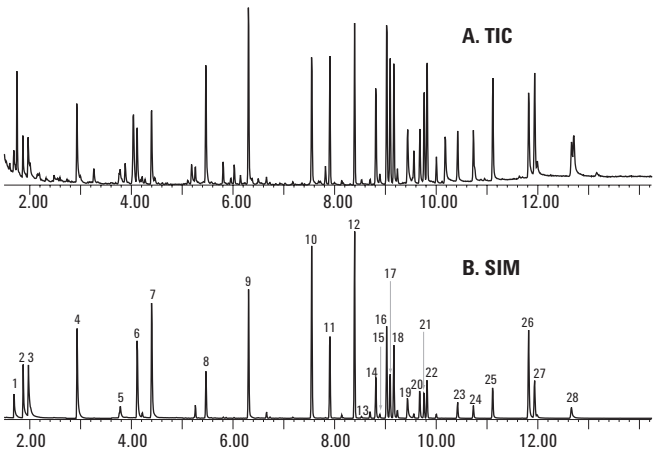

Figure 5 Chromatogram of forensic/toxicology analyzer checkout standard (2 ng on column) with whole blood matrix using Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293) by GC/MS. Refer to Table 1 for instrument condition, and Table 3 for peaks identification. A) Full scan chromatogram, B) SIM chromatogram. Satisfactory peaks shape and response achieved for all of analytes.

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Longterm Detection of Anabolic Steroid Metabolites in Urine

Application Note

Forensics/Doping Control

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Abstract

A method for the longterm detection of anabolic steroid metabolites in urine was developed using both the Agilent 6890N network GC system with an Agilent 5975B Series GC/MSD and the Agilent 7000 Series Triple Quadrupole GC/MS. Monitoring the sulfate conjugates of these metabolites, instead of the routine glucuronide metabolites allowed detection of methenolone for a post-administration period that was almost twice as long. This was achieved using GC/MS in SIM mode. Performing the analysis on the Triple Quadrupole GC/MS in SRM mode extends the detection period even further, to almost three times as long as the conventional glucuronide approach.

Agilent Technologies

Introduction

The nonmedical use of anabolic/androgenic steroids (AAS) is illegal in the US and banned by most sport organizations. However, AAS "doping" has long been a problem in some amateur and professional sports, and is becoming a growing problem in high school and collegiate athletics. The detection of stanozolol in a urine sample led to the disqualification of sprinter Ben Johnson from the 1988 Olympic Games, and the loss of his gold medal and two world records. The detection of an elevated testosterone/epitestosterone ratio in a screening test eventually led to loss of the 2006 Tour de France title by Floyd Landis, and a two-year ban.

Sophisticated violators of drug doping regulations know that AAS can be "safely" used in the "training" period, if their use is stopped long enough prior to a scheduled test to avoid detection. The timing between the last use of an AAS and urine sample collection for doping control is carefully adjusted by the violator for each drug to ensure negative test results. Retrospective detection of AAS is therefore essential to successful doping control, and doping laboratories around the world are looking for ways of detecting AAS for longer periods of time after cessation of use.

Typical doping control analysis for anabolic steroids and related substances in urine includes screening by gas chromatography/mass spectrometry (GC/MS). However, traditional analytical methods can typically detect AAS for only five days or so after administration. Detection of anabolic steroids for longer periods of time after drug use can be achieved by finding and monitoring longterm metabolites in urine [1-3], and using instrumentation capable of detecting very low concentrations of AAS.

This application note describes methods developed on both the Agilent 6890N network GC system with an Agilent 5975B Series GC/MSD and the Agilent 7000 Series Triple Quadrupole GC/MS that extend the time period over which some AAS compounds can be detected. This is made possible by analyzing for sulfate conjugates of the AAS metabolites in urine, which persist much longer than the metabolites that are routinely analyzed [4, 5]. Performing this analysis on the 6890N network GC system with Agilent 5795B GC/MS platform enables detection of some AAS metabolites almost twice as long as conventional GC/MS methods. However, the Agilent 7000 Series Triple Quadrupole GC/MS provides less interferences and high sensitivity to detect these metabolites for post-administration time periods that are almost three times longer than those obtained with the conventional GC/MS methods.

Experimental

Standards and Reagents

The standards and reagents used are listed in Table 1.

Instruments

The GC/MS experiments were performed on an Agilent 6890N gas chromatograph equipped with a split/splitless capillary inlet and an Agilent 5795B GC/MSD. The GC/MS/MS experiments were performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet and an Agilent 7000 Series Triple Quadrupole GC/MS. The instrument conditions are listed in Tables 2 and 3.

Table 1. Standards and Reagents

Internal Standard	**4-chloro-testosterone**	**Fluka**	**33755**
Reagents	N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSFTA)	Campbell Science	Cat. number DR100
	Methanol	Burdick & Jackson	GC230-4
	Acetonitrile	Burdick & Jackson	015-4
	Ammonium Iodide	Fluka	09874
	Mercaptoethanol	Sigma	63689

Table 2. Agilent 6890N Network GC and Agilent 5975B Series GC/MSD Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Custom HP-1ms 10 m × 0.15 mm, 0.12 µm film
Injection	1 µL
Carrier gas	Hydrogen, Constant Flow, 1.0 mL/min
Oral-Turinabol oven program	150 °C (0.4 min), 60 °C/min to 192 °C; 5.4 °C/min to 217 °C; 47 °C to 310 °C (0.29 min)
Methenolone oven program	180 °C (0.4 min), 62 °C/min to 235 °C; 42 °C/min to 290 °C; 40 °C to 310 °C (1.9 min)
Transfer line temp	280 °C
MS Conditions	
Tune	Autotune
EMV Offset	+400
Acquisition parameters	EI; selected ion monitoring
Solvent delay	1.5 min
MS temperatures	Source 230 °C; Quadrupole 150 °C

Table 3. Agilent 7000 Series Triple Quadrupole GC/MS Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Custom HP-1ms 10 m × 0.15 mm, 0.12 µm film
Methenolone injection	1 µL
Oral-Turinabol injection	0.2 µL
Carrier gas	Hydrogen, Constant Flow, 1.0 mL/min
Oven program	180 °C (0.4 min), 62 °C/min to 235 °C; 42 °C/min to 290 °C; 40 °C to 310 °C (1.9 min)
Transfer line temp	280 °C
MS Conditions	
Tune	Autotune
EMV Gain	20
Acquisition parameters	EI, selected reaction monitoring
Collision gas flows	N_2 Collision Gas: 1.5 mL/min
Solvent delay	1.5 min
MS temperatures	Source 230 °C; Quad 150 °C

Sample Preparation

After initial cleanup (removal of urine matrix) on solid phase extraction cartridges, the extract is incubated with b-glucuronidase to effectively cleave glucuronide conjugates and produce free steroids. The mixture is then applied to a C-18 solid phase extraction cartridge and the uncleaved steroid sulfates as well as any remaining contaminants are washed off with acetonitrile. Free steroids that were formerly bound as glucuronides remain on the cartridge and are then eluted with methanol and derivatized for GC/MS analysis.

The acetonitrile wash fraction, which contains steroid sulfates, is dried down and then incubated with b-Glucuronidase/Arylsulfatase to hydrolyze the sulfate conjugates. The hydrolyzed extract is then applied again to a C-18 solid phase extraction cartridge, which is rinsed with acetonitrile in order to wash off any remaining contaminants. The free steroids are then eluted with methanol, dried and derivatized for GC/MS analysis.

Derivatization was achieved by dissolving the dried sample in 100 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)-NH_4I-ethanethiol (100:2:3, v/w/v) and heating for 15 minutes at 65 °C [3].

Analysis Parameters

The GC/MS parameters used in the analysis of the various AAS metabolites are shown in Table 4, and the Triple Quadrupole GC/MS parameters used are shown in Table 5.

Results

Detection of Longterm Steroid Metabolites

Anabolic steroids and their metabolites are excreted in urine as conjugates with glucuronic acid or sulfate, and the steroids must first be deconjugated before analysis. b-Glucuronidase from *E.coli* is currently used for routine steroid analysis in doping control. However, this enzyme does not cleave the sulfate conjugates, which are then "invisible" during routine testing. The sulfate conjugates persist in urine much longer than the glucuronide conjugates, allowing longer-term detection if a method is used to "visualize" these sulfate conjugates.

The anabolic steroids and their metabolites can be released from the sulfate conjugates using b-Glucuronidase/ Arylsulfatase and a selective extraction procedure. Resultant chromatograms of the extracted urine show excellent separation of glucuronide and sulfate conjugated methenolone metabolites using conventional GC/MS in selected ion monitoring (SIM) mode (Figure 1). The lower chromatogram of the glucuronide fraction shows the presence of the 3a-hydroxy metabolite and the parent (17b-hydroxy) conjugate. These two are commonly targeted in routine screening and confirmation procedures in doping control.

The upper chromatogram in Figure 1, showing the sulfate fraction from the extraction, reveals a metabolite highlighted in red that matches the 16-hydroxy 3,17-dione structure. In this case, 18% of this hydroxydione metabolite is glucuronide bound (red peak at 6.9 minutes in the lower chromatogram). The majority, 82%, is excreted as the sulfate conjugate (upper chromatogram). The commonly targeted methenolone metabolites in the glucuronide fraction were detectable in urine for only five days after oral drug administration. However, the hydroxydione sulfate conjugated metabolite can be detected much longer, for at least nine days, by conventional GC/MS. Sulfate-conjugated 16-hydroxy-3,17-dione metabolites are major metabolites and are common for a group of anabolic steroids with a methyl group in the A-ring, including stenbolone, drostanolone and mesterolone (data not shown).

Table 4a. Agilent 6890N Network GC with Agilent 5975B Series GC/MSD Analysis Methenolone Parameters

Compound	RT (min)	SIM	Dwell Time (ms)
Methenolone Metabolite	2.48	517	40
(16 -hydroxy-1a-methyl-5a-androst-1-en-3,17-dione)		518	40
		532	40
4-Chlorotestosterone (ISTD)	2.46	466	40
		468	40

Table 4b. Agilent 6890N Network GC with Agilent 5975B Series GC/MSD Analysis Oral-Turinabol Parameters

Compound	RT (min)	SIM	Dwell Time (ms)
Oral-Turinabol (Dehydrochlormethyltestosterone) Metabolite	7.06	656	40
		658	40
4-Chlorotestosterone (ISTD)	6.82	466	40
		468	40

Table 5. Agilent 7000 Series Triple Quadrupole GC/MS Analysis Parameters

Compound	RT (min)	SRM	Dwell Time (ms)	Collision Energy (EV)
Methenolone Metabolite	2.60	532→517	40	10
(16b-hydroxy-1a-methyl-5a-androst-1-en-3,17-dione)		517→207	40	17
Oral-Turinabol Metabolite	2.42	656→244	40	10
(Dehydrochlormethyltestosterone Metabolite)		658→244	40	10
4-Chlorotestosterone (ISTD)	2.34	466→431	40	10

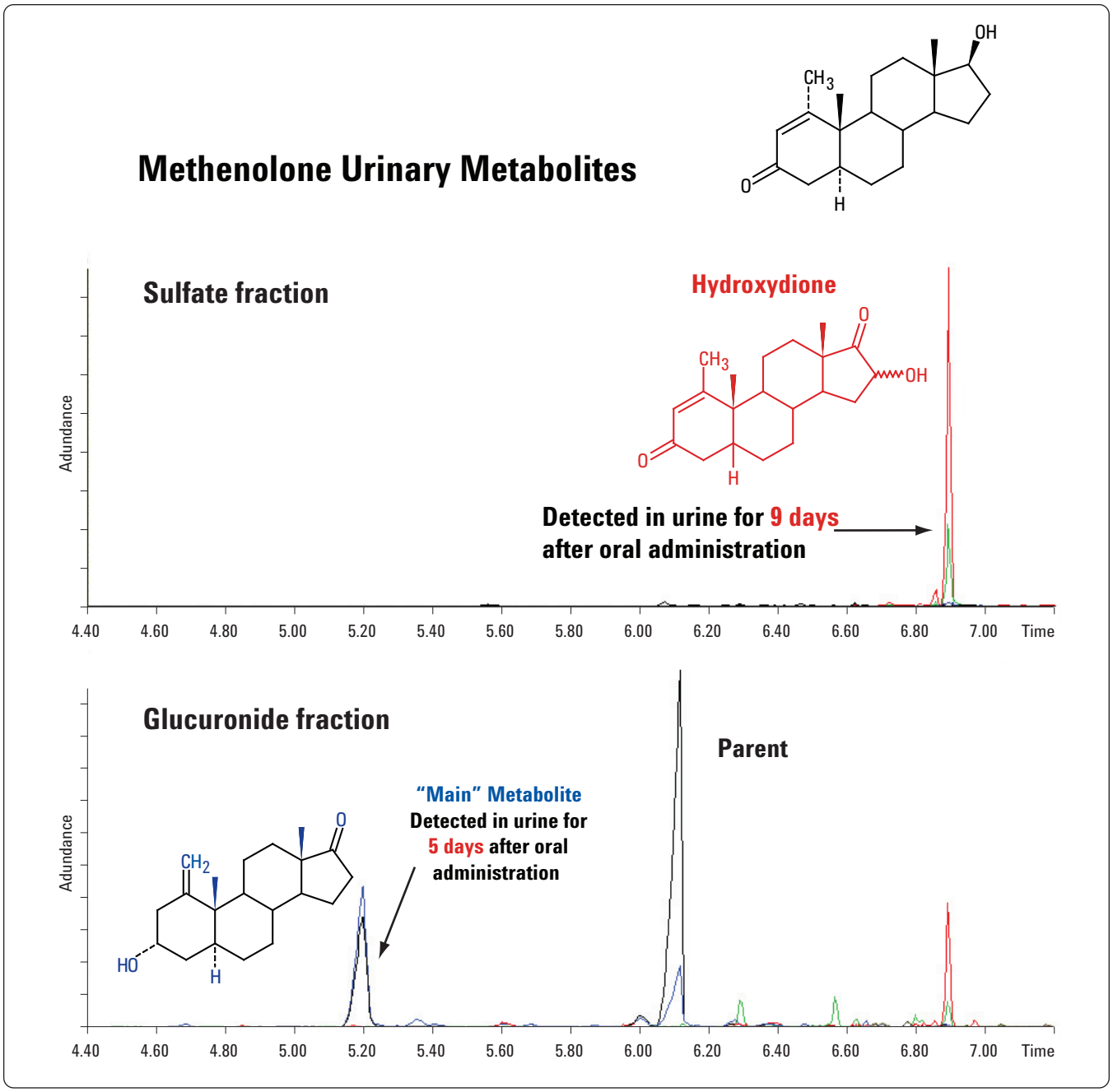

Figure 1. GC/MS SIM chromatograms of the glucuronide and sulfate fractions extracted from urine of a subject injected with methenolone. The lower chromatogram of the glucuronide fraction shows the presence of the 3α-hydroxy metabolite and the parent (17β-hydroxy) conjugate in the glucuronide fraction. The upper chromatogram shows the sulfate fraction from the extraction, revealing a peak highlighted in red that matches the 3,17-dione 16-hydroxy metabolite (hydroxydione), 82% of which is excreted as sulfate. The methenolone metabolites in the glucuronide fraction were detectable in urine for only five days after oral drug administration, while sulfate conjugated metabolite can be detected for at least nine days. The blue trace is the 431m/z ion indicative of the 3α-hydroxy metabolite, and the red trace is the 517 m/z ion indicative of the hydroxydione metabolite.

Triple Quadrupole GC/MS Extends the Detection Window

Selected reaction monitoring (SRM) analysis of urine performed on the Triple Quadrupole GC/MS instrument can extend the detection window for anabolic steroids even further. This is due to a lack of matrix interferences and increased sensitivity over conventional GC/MS SIM analysis. Methenolone detection is possible 14 days after steroid administration using SRM, versus nine days with GC/MS SIM (Figure 2). After 12 days, GC/MS SIM analysis cannot distinguish the methenolone metabolite peak from the background. Detection of oral turinabol in urine by GC/MS SIM analysis is problematic due to interferences that can be interpreted as the metabolite peak, even before administration. As a result, detection thresholds must be increased to a level at which chemical noise no longer interferes with the signal. Analysis of the same urine samples on the Triple Quadrupole GC/MS using SRM is very selective, revealing the absence of a metabolite peak before administration due to a flat baseline, and can detect the metabolite as much as nine days after administration of the steroid (Figure 3).

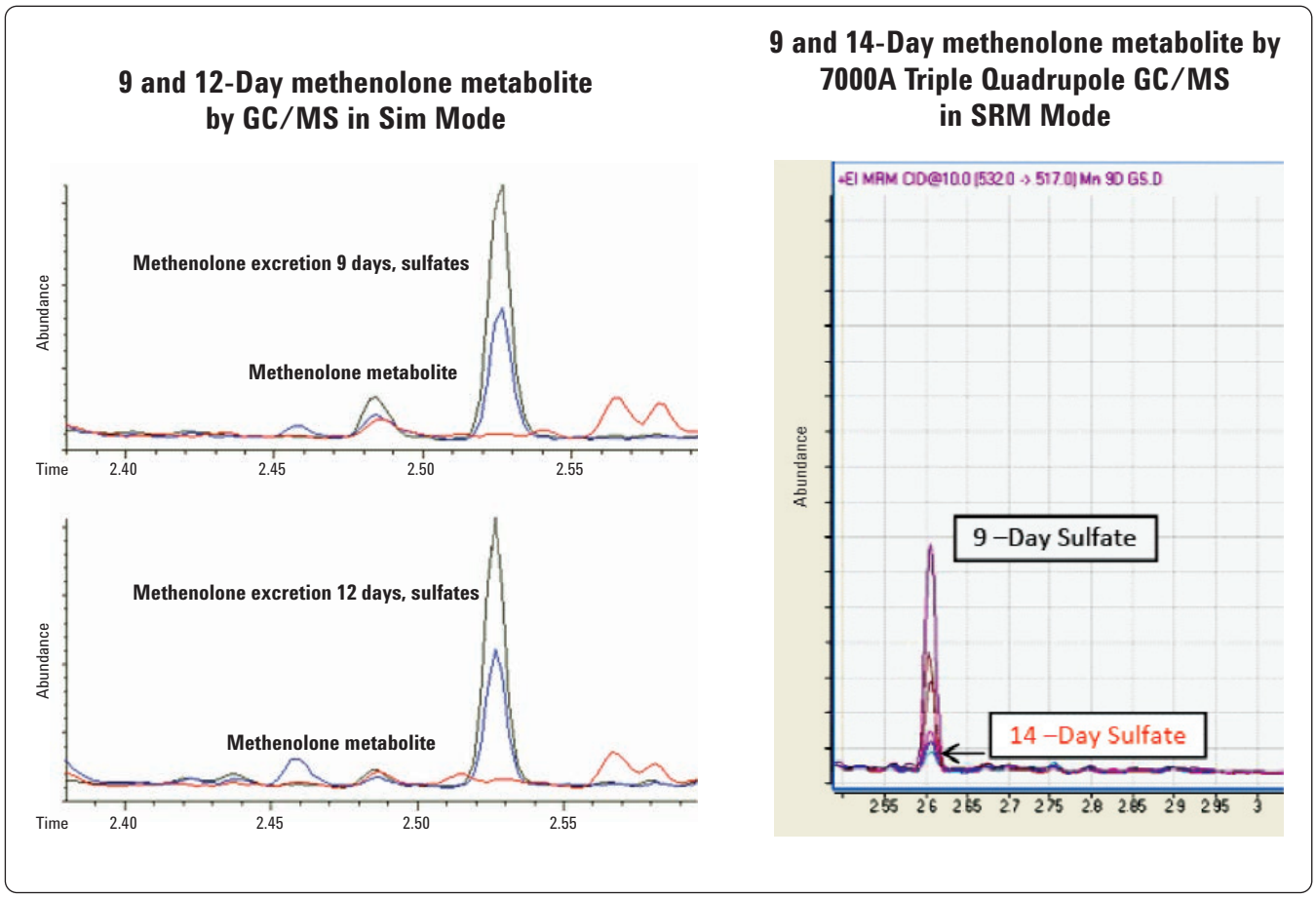

Figure 2. Comparison of analysis of the sulfate fraction of extracted urine for methenolone metabolite by GC/MS SIM and Triple Quadrupole GC/MS SRM. The GC/MS SIM analysis used the 532, 518, and 517 ions for detection, and the methenolone metabolite peak cannot be distinguished from the background after 12 days. In contrast, the Triple Quadrupole SRM analysis shows detectable metabolite peaks at 9, 10, 11, 12, 13 and 14 days, using the 532→517 transition.

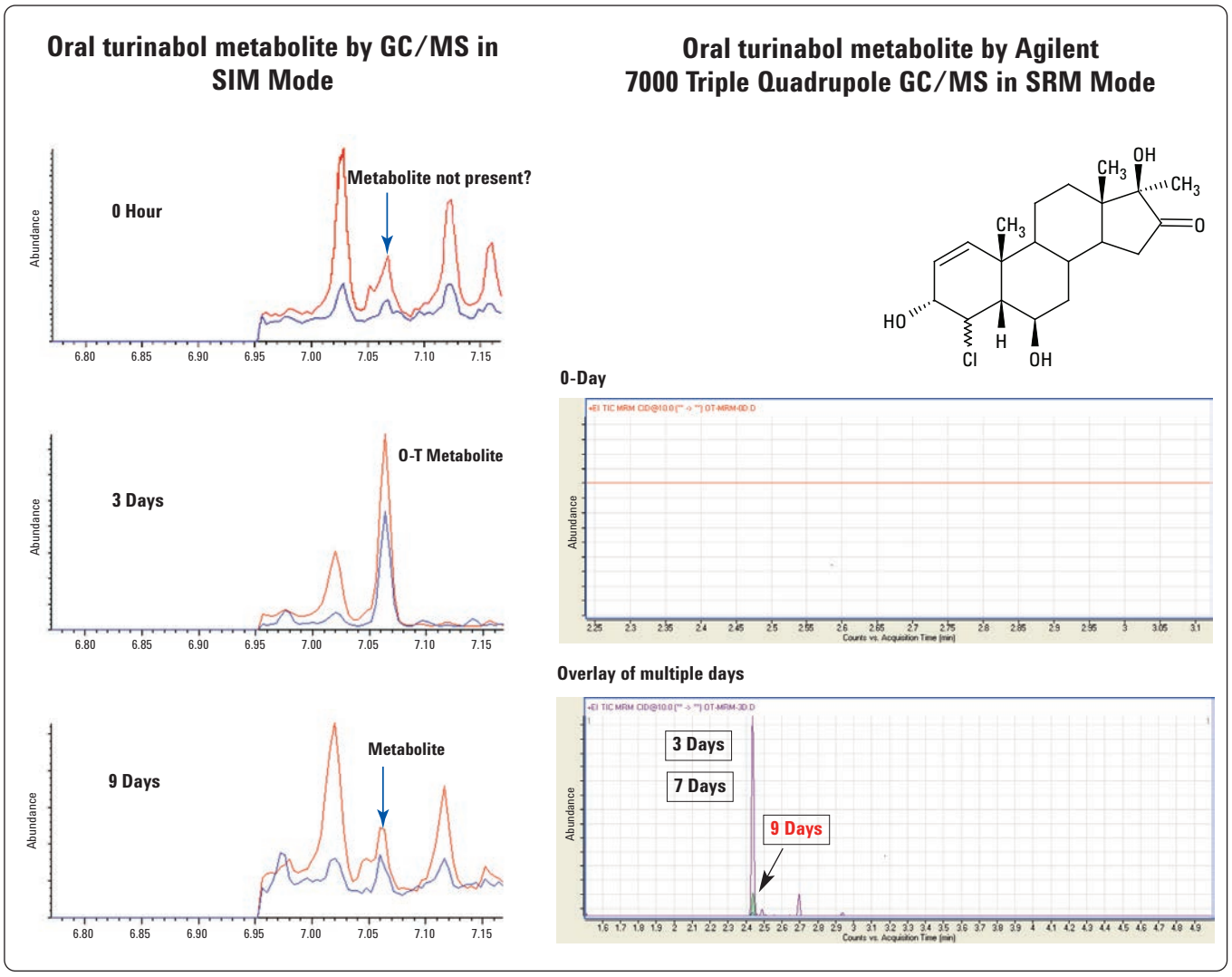

Figure 3. Comparison of analysis of the sulfate fraction ("Free Fraction") of extracted urine for oral-turinabol metabolites by GC/MS SIM and Triple Quadrupole GC/MS SRM. GC/MS SIM analysis is problematic due to interferences that can be interpreted as the metabolite peak, even before administration of the drug (0 hr). Analyzing the same urine samples on the Triple Quadrupole GC/MS using SRM reveals the absence of a metabolite peak before administration, and can detect the metabolite as much as nine days after administration of the steroid. The GC/MS SIM analysis utilized the 658 and 656 m/z ions for detection, while the Triple Quadrupole SRM analysis utilized the 656→244 m/z transition. The three day SRM trace is red, green is seven days, and blue is nine days.

Conclusion

The detection window for anabolic steroids can be expanded significantly by monitoring sulfate-conjugated metabolites rather than those conjugated as glucuronides. A unique selective extraction procedure using b-Glucuronidase/ Arylsulfatase provides metabolites cleaved from their sulfate conjugates and ready for derivatization and GC/MS analysis. This procedure can almost double the detection window for some anabolic steroids from five to nine days for methenolone, using GC/MS SIM analysis. Performing analysis on the Triple Quadrupole GC/MS using SRM can extend the detection time after administration from nine to 14 days for methenolone due to the elimination of matrix interferences. This instrument system also allows the monitoring of some anabolic steroids that are not easily analyzed by traditional GC/MS in the SIM mode. The Triple Quadrupole GC/MS is therefore a preferable alternative to GC/MS SIM for longterm monitoring of anabolic steroids.

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Rapid Forensic Toxicology Screening Using an Agilent 7890A/NPD/5975C/DRS GC/MSD System

Application Brief

Bruce Quimby

Laboratories that perform toxicology screens on forensic samples are challenged by the requirement to analyze large numbers of samples containing complex matrix interferences. The system described here addresses these demands by combining fast GC to reduce the run time; simultaneous collection of scan, SIM, and NPD data in one shorter run; backflushing to prevent heavy matrix components from fouling the detectors; and Deconvolution Reporting Software (DRS) to simplify data interpretation. The scan data is deconvoluted and used to identify any of 278 target compounds. SIM data is used to look for select low-level compounds not detectable in scan mode. The nitrogen response of the NPD is used to highlight nontarget compounds, identity confirmation, and can be used for quantitation if needed. Using an extract of a whole blood sample, the system finds all the molecules detected by the conventional method in significantly less time.

Highlights

- DRS simplifies data interpretation, especially in dirty samples.
- Simultaneous collection of SIM, scan, and NPD signals saves time.
- The 7890A GC/MS High Speed Oven Accessory provides high programming rates, even with 120 V service.
- Backflushing reduces ghost peaks in high matrix samples.

Experimental and Results

The Forensic Toxicology GC/MSD RTL Database of 277 compounds was downloaded from Agilent's Web site and converted for use with DRS. The method was scaled to precisely two times faster using Agilent's Method Translation software. Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single-step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/6th volume. Extracts were analyzed using the conditions in Table 1. The simultaneously acquired chromatograms for scan, SIM, and the NPD for one of the samples is shown in Figure 1. The 245 target ion for fentanyl shown is one of 13 SIM ions monitored. This example is particularly challenging because of the high levels of matrix interferences as seen in the scan TIC. The drug compounds present were identified using a combination of 1) full-spectrum searching of the deconvolved spectra against the target library (AMDIS), 2) target and qualifier ion ratios in the MSD ChemStation, and 3) response on the NPD.

Compounds identified by AMDIS deconvolution but not found by the MSD ChemStation because of out-of-range qualifiers were manually inspected in QEdit. Quantitation was forced if AMDIS indicated an acceptable spectral and retention time match and if there was a corresponding NPD response.

The SIM data was used to screen for several compounds (see Table 1) that are often at levels too low to be detected in scan mode. In this sample, fentanyl was found present at a low level in the scan data and confirmed with the SIM responses. The signal-to-noise ratio of the SIM target ion was 10 times greater than that of the scan.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC	Agilent Technologies 7890A		
Inlet	EPC split/splitless		
Mode	Splitless, 2 µL injected		
Inlet temp	280 °C		
Pressure	24.15 psig, retention time locked to oxycodone at 5.505 min		
Purge flow	50 mL/min		
Purge time	1 min		
Gas saver	Off		
Gas type	Helium		
Liner	Agilent splitless inlet liner, single-taper, Part # 5181-3316		
Oven	**240 V**		
Oven ramp	°C/min	Next °C	Hold min
Initial		120	1.00
Ramp 1	40	320	2.50
Total run time	8.5 min		
Equilibration time	0.5 min		
Backflush time	2.0 min		
Backflush temp	320 °C		
Column	Agilent Technologies DB-17 ms, Part # 123-4712		
Length	15.0 m		
Diameter	0.32 mm		
Film thickness	0.25 µm		
Mode	Constant pressure		
Outlet	2-way splitter with solvent vent		
Splitter pressure	3.8 psi during acqusition, 75 psi during backflush with inlet set to 1.0 psi during backflush		
Splitter restrictors	MSD:1.44 m × 0.18 mm id × 0.18 µm film DB-17 ms (Part # 121-4722). NPD:0.75 m of same		
Solvent venting	0 to 1.40 min		
NPD	Capillary NPD with EPC, option 251		
Gas flows	Hydrogen 3.0 mL/min, air 60 mL/min, nitrogen makeup 12 mL/min		
NPD temp	310 °C		
MSD	Agilent Technologies 5975C, Performance Turbo		
Solvent delay	None (solvent vented with splitter)		
EM voltage	Tune voltage		
Mode	SIM/scan		
Scan	42-550 amu, sampling: 2[1]		
SIM ions	Group 1 (PCP) 84, 186, 200, 242; Group 2 at 4.5 min, (norfentanyl butyl derivative, 6-acetylmorphine, heroin, fentanyl) 42, 82, 83, 146, 158,189, 231, 245, 268, 284, 310, 327, 369; Group 3 at 6.5 min (LSD) 221, 323, 181, 207; all dwell times 10 msec		
Quad temp	150 °C		
Source temp	280 °C		
Transfer line temp	280 °C		

Table 3 shows the DRS report for the sample in Figure 1. The report lists the compounds quantitated by the MSD ChemStation and identified by deconvolution. The quantitative results are rough approximations, as the response factors used here were only average responses for screening purposes. Note that there are several nondrug compounds in the target library that are detected as well.

The spectra of peaks found on the NPD that did not correspond to targets were searched against the NIST and Pfleger libraries for identification. The peak on the NPD in Figure 1 labeled with a question mark was not a target compound. Search results of the spectrum indicated it was cyheptamide (later found to be an internal standard added in sample preparation).

For comparison, the sample in Figure 1 was analyzed in the same way but with the 1x method for reference. All drugs found with the original 1x method were found with the 2x method.

The use of the two-way splitter with solvent venting allows the solvent peak (and any other unwanted peaks) to be vented before reaching the detectors. This helps extend the useful life of the NPD bead. The device also allows backflushing at the end of the run. As seen in Figure 1, there are large matrix peaks that elute after the last target compound. Backflushing quickly removes these compounds, saving time and reducing detector and column maintainence.

The significant time savings available with the method described here vs. the original method where three separate runs of scan, SIM, and NPD are needed to access the same information are shown in Table 2.

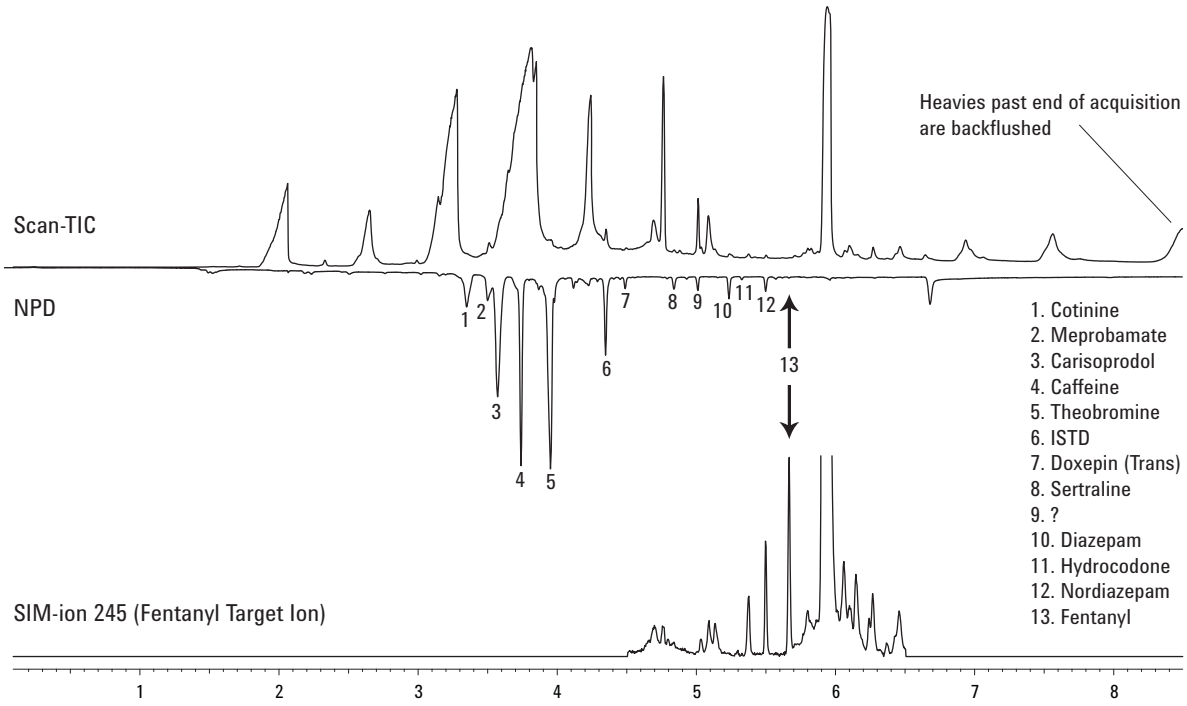

Figure 1. Chromatograms from screen of whole blood sample.

Table 2. Time Savings Using the Agilent 7890A-5975C

	Typical 6890 1X	7890A 2X	Minutes Saved
Run time without matrix bake-out, includes equib	17	8.5	8.5
Run time with matrix bake-out 6890 or Splitter 7890A	24	10.5	13.5
Cool down time from 320 to 120	2.3	1.6	0.7
Autosampler time, 7890A with overlap	1	0.1	0.9
Acquiring scan, SIM, and NPD signals separately vs. simultaneously	81.9	12.2	69.7

Time savings > 85%
Not including time saved using DRS

Table 3. DRS Report from Screen of Whole Blood Sample

			Agilent	AMDIS		NIST	
R.T.	Cas #	Compound Name	ChemStation Amount (~ng)	Match	R.T. Diff sec.	Reverse Match	Hit Num.
2.1124	nc06	DIETHYLPROPRION		61	-3.9		
2.6530	nc013	TETRADECANOIC ACID	44.17	99	-1.1		
3.3558	nc046	COTININE	1.94	78	0.9		
3.5188	nc060	MEPROBAMATE	1.79	74	-2.2		
3.5792	nc063	CARISOPRODOL	1.88	85	-0.1		
3.745	nc079	CAFFEINE	1.43				
3.958	nc089	THEOBROMINE	5.46	75	1.8		
4.3516	nc0123	10,11-DIHYDRO-DIBENZ[B,F] [1,4]OXAZEPIN-	9.25	98	-0.1		
4.494	nc0133	DOXEPIN(TRANS)	1.45				
4.4940	nc0131	DOXEPIN(CIS)		67	3.0		
4.7654	nc0155	BIS[2-ETHYLHEXYL] PHTHALATE	138.58	94	0.4		
4.8420	nc0158	SERTRALINE[2]	0.54	88	-0.1		
5.087	nc0178	DOXYLAMINE METABOLITE	0.21				
5.238	nc0187	DIAZEPAM	0.45	92	0.3		
5.3285	nc0191	HYDROCODONE	0.1	62	0.2		
5.5009	nc0198	NORDIAZEPAM	0.41	62	0.0		
5.6695	nc0210	FENTANYL	0.1	64	0.8		
5.7097	nc0218	GAMMA-TOCOPHEROL	0.94	81	0.5		
5.8750	nc0226	VITAMEN E	0.81	90	2.7		
5.930	nc0233	CHOLESTEROL	65.83	98	0.2		

Conclusions

Significant time savings can be realized in the screening of toxicology samples with the system described. The cycle time required per sample is reduced 85%. Data interpretation time is also reduced with the use of DRS.

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Agilent Technologies

Clenbuterol and Norandrosterone Analysis by Positive Chemical Ionization with the 5973N MSD

Application Note

Drug Testing

Bernhard Wuest, *Agilent Technologies*

Introduction

Anabolic steroids are among the most widely abused chemicals in sports. The structure and functionality of these banned compounds are similar to the male hormone testosterone. Athletes abuse anabolic steroids to increase muscle growth and to shorten the recovery time after intensive training activities.

After extraction and derivatization, anabolic steroid samples are usually analyzed with high-resolution capillary gas chromatography (HRCGC) and mass selective detection (MSD). Exclusive use of GC/MS in electron ionization (EI), selected ion monitoring (SIM) mode can result in misidentification and poor quantification due to interfering compounds in the matrix that have the same *m/z* value.

Using positive chemical ionization (PCI) with ammonia as reagent gas can improve both selectivity and sensitivity. The work described here with the 5973 GC/MSD demonstrates the advantages of positive chemical ionization (PCI) over electron ionization (EI) and as well as the stability, reliability and robustness of the complete GC/MSD system. System stability and reliability derive from precise control of the reagent gas with a digital mass flow controller, and from dedicated temperature control of the ion source. In this study, urine samples were analyzed to determine the presence of clenbuterol and norandrosterone (see Figure 1).

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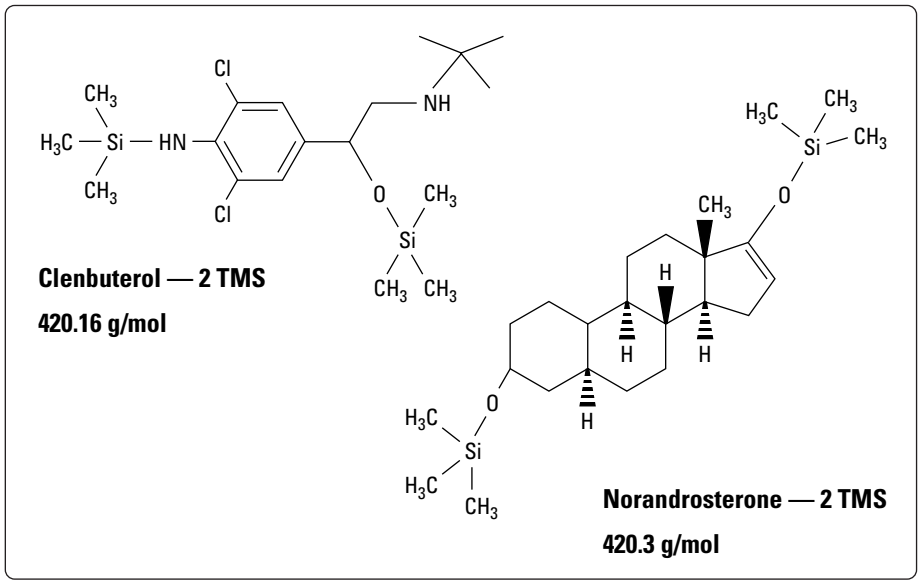

Figure 1. Structure of clenbuterol and norandrosterone derivatives.

Experimental

The instruments used for this analysis were a 6890 gas chromatograph with a 5973 mass spectrometer. A series of 120 injections of actual urine samples was made using chemical ionization (ammonia). Every sixth and seventh injection consisted of a 10- and 2-ng/ml standard, respectively.

Oven temperature program:	180°C .(1 min), 5°C/min, 300°C .(5 min)
Inlet liner:	Single-tapered deactivated with a small amount of glass wool (Agilent Part No: 5062-3587)
Injection volume:	2 µl
Split:	8:1
Column:	HP5 MS 30 m × 0.25 mm × 0.33 µm, 1.2 ml/min constant flow
MS mode:	Selected ion monitoring
EM offset:	400 V above tune
SIM mode:	Low resolution, 150 msec dwell time
Chemical ionization:	Ammonia, 1 ml/min

Results and Discussion

The EI and PCI spectra are shown in Figures 2 and 3. The PCI spectra show the molecular ion (M+1) for the TMS derivatives of the two compounds. The compounds are distinguished not only by their spectra, but also by their retention times. Positive chemical ionization provides a much cleaner total ion chromatogram than electron ionization (see Figure 4). Single-ion chromatograms were used to locate the compounds.

The short-term stability for a standard is shown in Figure 5 in which the single-ion chromatograms for eight runs are overlaid. The plot in Figure 6 provides an indication of the reproducibility of the analysis; a slight decrease in response is normal.

To demonstrate the stability with real samples, 120 injections of urine samples were run along with standards at two concentrations. The long-term stability of the system is shown in Figure 7 in which ion chromatograms for norandrosterone from eight runs are overlaid. Figure 8 shows the long-term stability with an excellent RSD of 8.5% during the run sequence. There is a slight increase for clenbuterol a result of better system inertness. The decrease in norandrosterone is due to normal liner degradation after 120 injections.

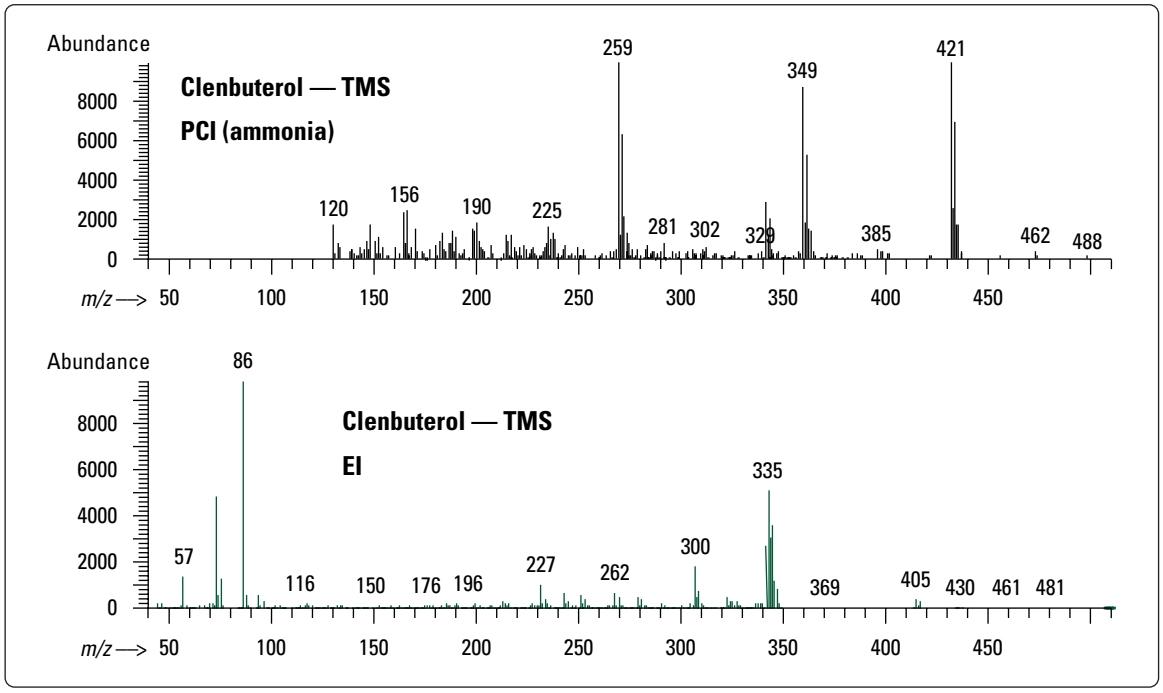

Figure 2. EI and PCI (ammonia) spectra for clenbuterol — TMS.

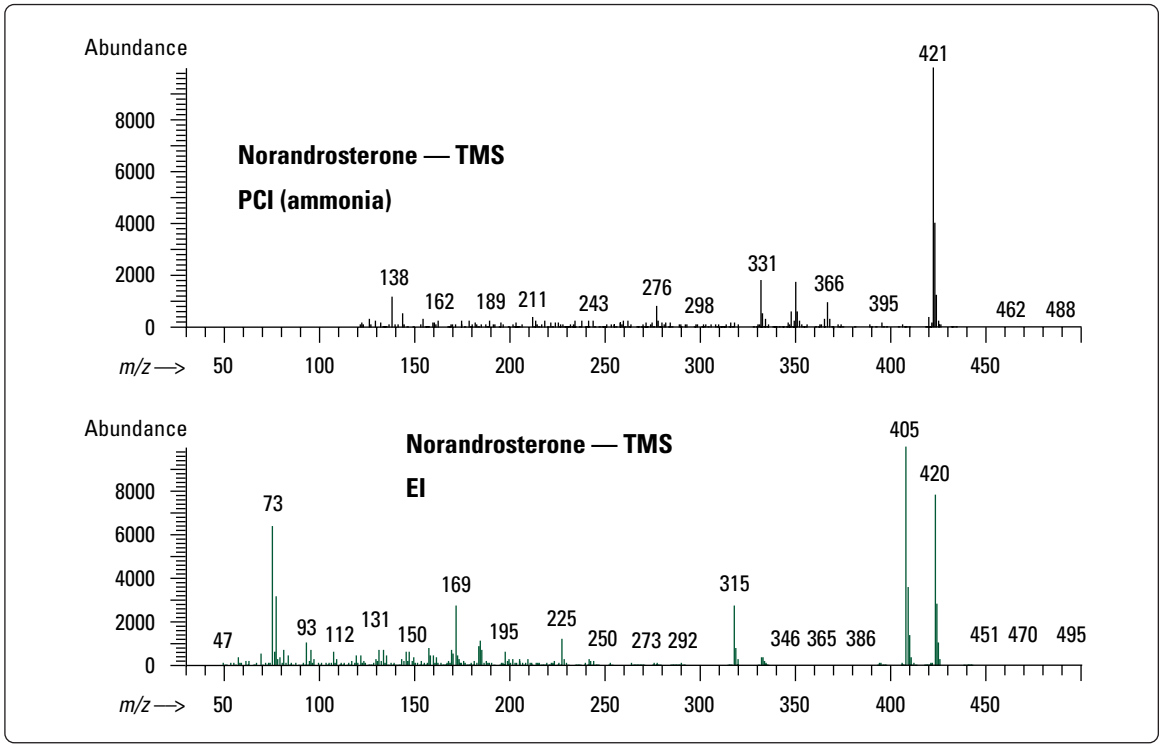

Figure 3. EI and PCI (ammonia) spectra for norandrosterone — TMS.

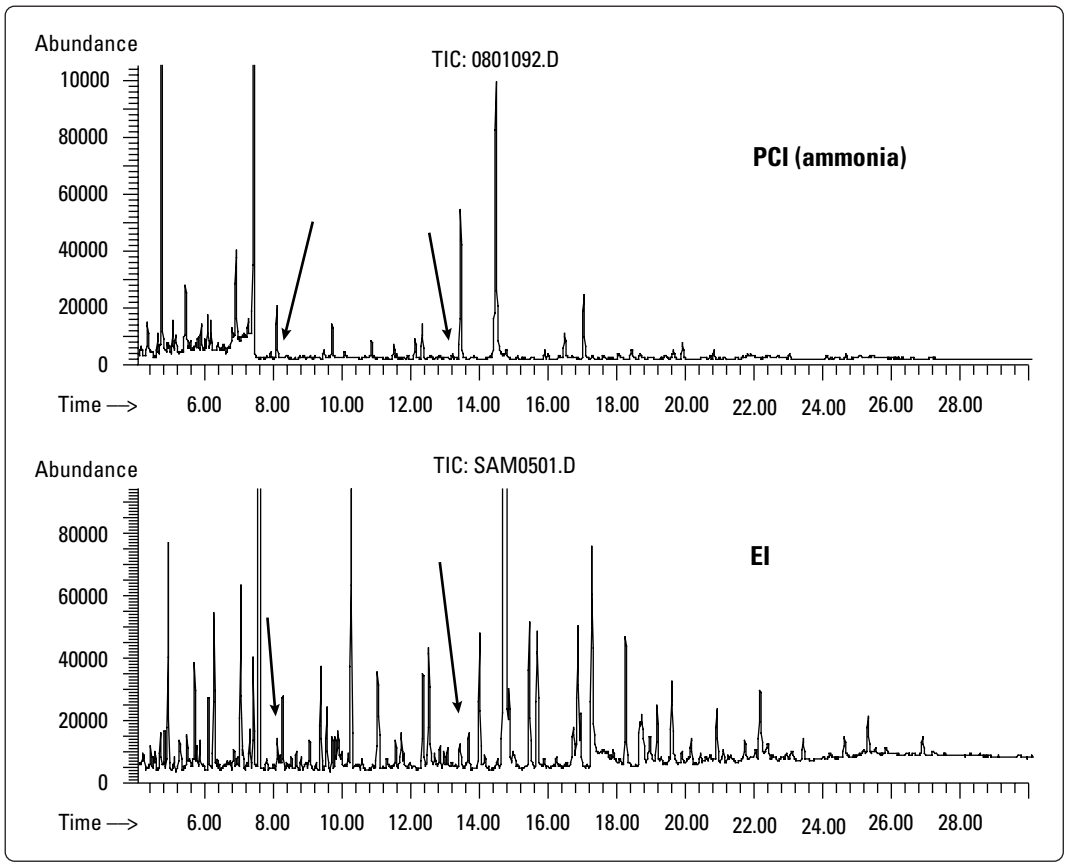

Figure 4. Total ion chromatograms for EI and PCI (ammonia).

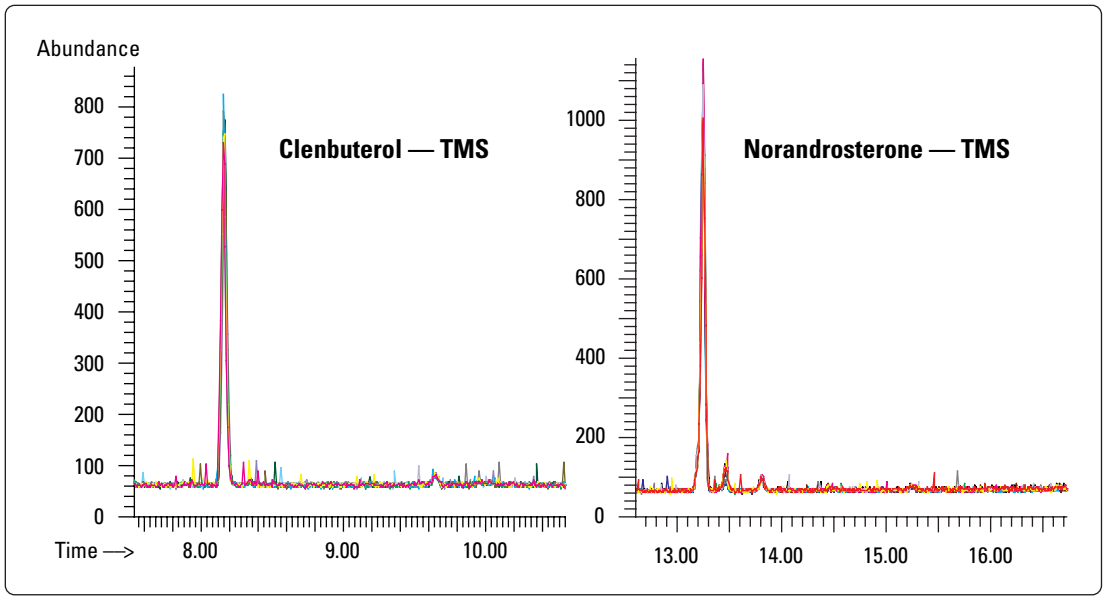

Figure 5. Short-term stability for standards.

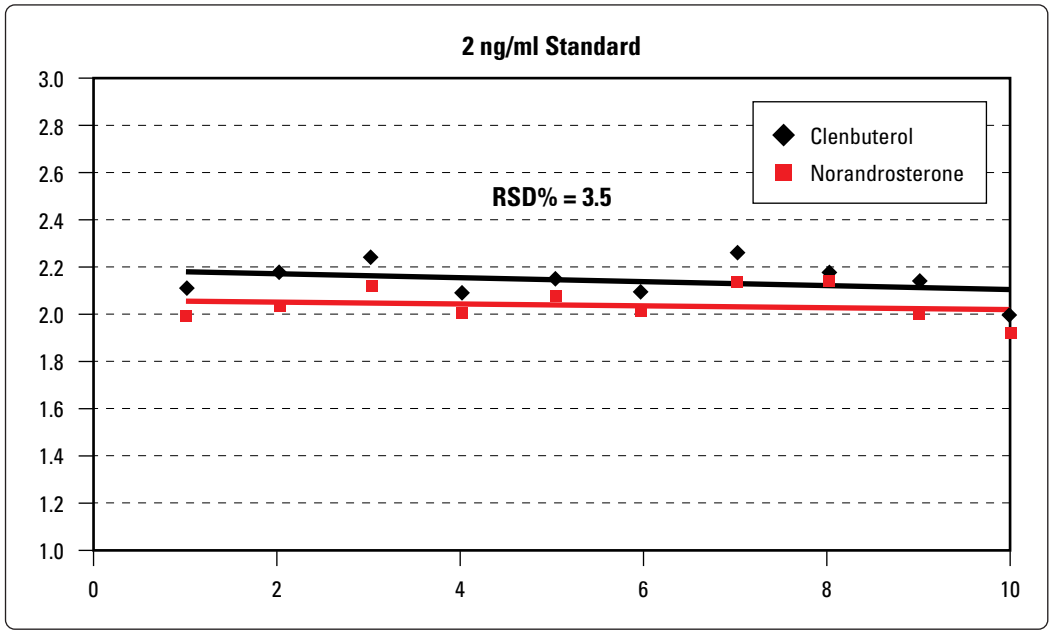

Figure 6. Short-term stability for standards.

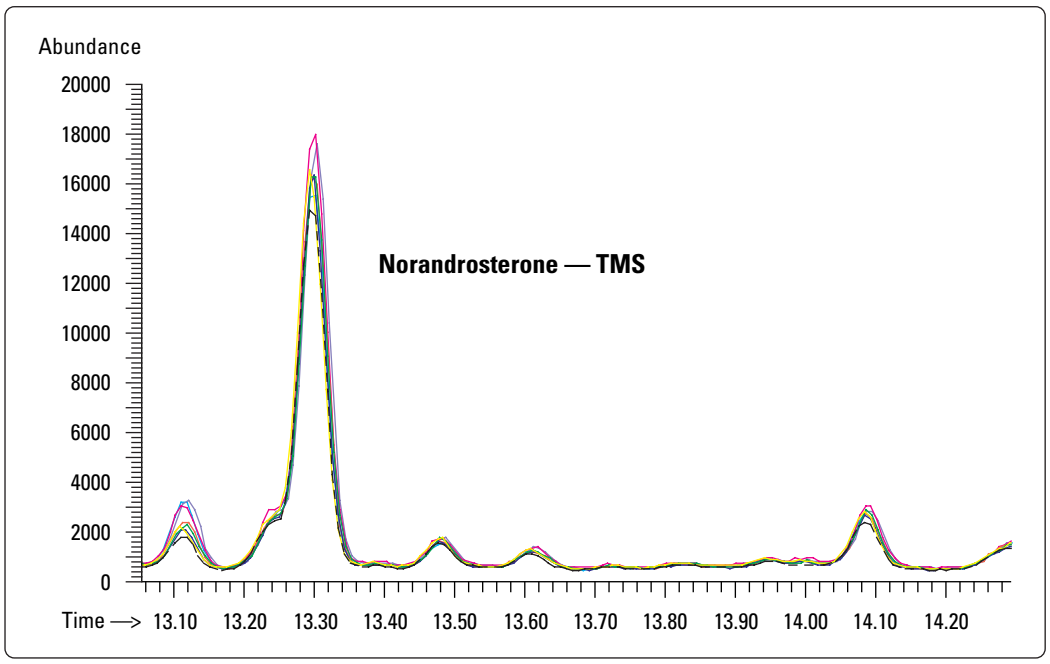

Figure 7. Long-term stability after injections of urine samples.

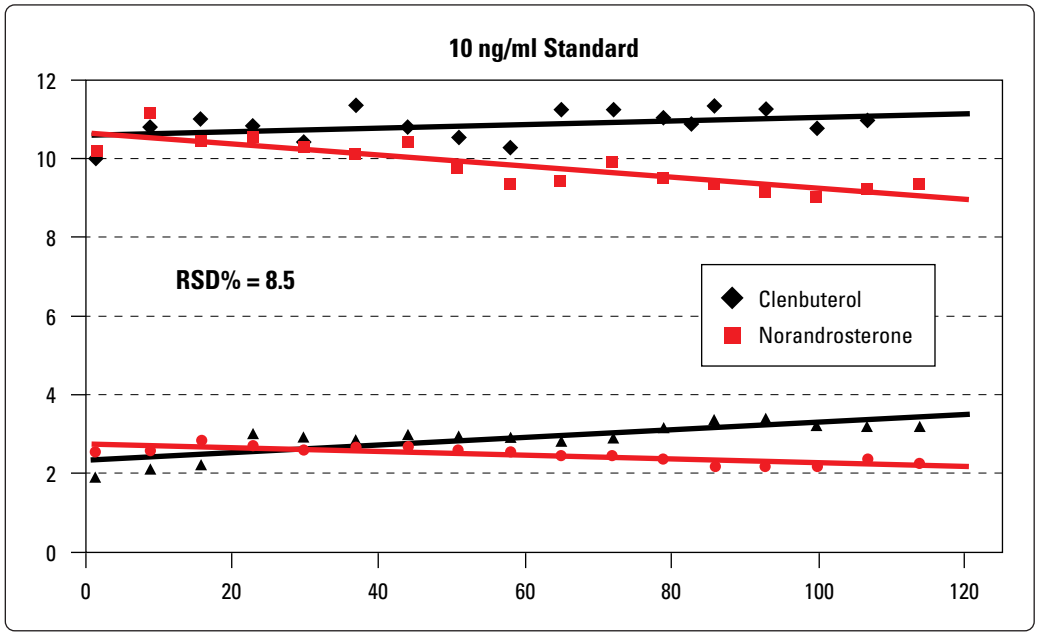

Figure 8. Long-term stability for clenbuterol and norandrosterone.

Conclusion

In the analysis of steroids by positive chemical ionization, it is necessary that the GC/MS system provide the following.

- Precise control of GC carrier gas
- Accurate, reproducible oven temperature ramping
- Stable and controllable ion-source and quadrupole temperatures
- Precise and stable CI reagent gas control

The 5973 GC/MSD uses a patented nonstainless-steel ion source that gives very stable results relative to those obtainable with stainless steel sources. Combined with a low-background flow system that uses ultraclean parts, the 5973 is capable of detecting compounds at low levels. It is concluded that the 6890 GC and 5973 GC/MSD provide robust, sensitive, and reliable detection of clenbuterol and norandrosterone in urine samples.

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DOPING CONTROL

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- Fast and Comprehensive Doping Agent Screening in Urine by Triple Quadrupole GC/MS
- Fast GC/MS/MS of Androgenic Anabolic Steroids in Urine Using a VF-5ms Column
- Improved Forensic Toxicology Screening Using A GC/MS/NPD System with a 725-Compound DRS Database

Applications by Technique

GC/MS/MS

Fast and Comprehensive Doping Agent Screening in Urine by Triple Quadrupole GC/MS

Application Note

Forensics/Doping Control

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Abstract

A rapid method was developed on the Agilent 7000 Series Triple Quadrupole GC/MS system to screen for more than 150 doping agents in seven classes of substances, at or below WADA MRPLs [1]. A short capillary column, rapid scan speed and hydrogen as carrier gas enable a run time of less than 8 minutes.

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Introduction

Drug "doping" (drug misuse and cheating in sports) is a growing global challenge, given the ongoing development of new therapeutics and "designer" drugs. Since its advent, in 2000 the World Anti-Doping Agency (WADA) has maintained and updated a list of prohibited substances, adherence to the list has been controlled by accredited doping control laboratories. WADA sets minimum required performance levels (MRPLs) for the detection of the substances on the list, which includes:

- Five categories of substances prohibited at all times (anabolic agents, hormones and related substances, beta-agonists, anti-estrogenic agents, and diuretics and other masking agents)
- Four categories of substances prohibited during competition (stimulants, narcotics, cannabinoids and glucocorticosteroids)

Although there is growing interest in samples such as blood (serum/plasma), saliva and hair, urine remains the most common sample type. In order to obtain the necessary selectivity for all of the different classes of prohibited substances at or below their MRPLs, hyphenated chromatographic mass spectrometric methods are preferred [2], and GC-MS and LC-MS are now used as complementary techniques in doping control. While several fast GC tandem mass spectrometric methods have been published, these methods normally lacked the combination of quantitative determination of the endogenous steroid profile and a qualitative analysis of a wide range of exogenous steroids and other doping agents.

This application note describes a method developed on the Agilent 7000A Triple Quadrupole GC/MS system for the detection of a wide range of endogenous and exogenous anabolic steroids and other doping agents, with a run time of less than 8 minutes.

Experimental

Standards and Reagents

The standards and reagents used were as described in reference 1.

Instruments

The method was developed on an Agilent 7890 gas chromatograph equipped with a split/splitless capillary inlet and an Agilent 7000A Triple Quadrupole GC/MS sytem, using a Gerstel MPS2 autosampler and PTV injector. The analysis parameters are listed in Tables 2–6.

Table 1. Agilent 7000A Triple Quadrupole GC/MS Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Analytical column	Agilent J&W HP-1 Ultra Inert 12.5 m × 0.2 mm id, 0.11 µm film (cut from a 50 m column, p/n 19091A-005)
Injection	5 µL; Injector conditions: 100 °C (0.15 min), 12 °C/sec to 280 °C
Carrier gas	Hydrogen, constant flow, 1.0 mL/min
Column temperature program	100 °C (0.4 min), 90 °C/min to 185 °C; 9 °C/min to 230 °C; 90 °C/min to 310 °C (0.95 min)
Transfer line temp	310 °C
MS conditions	
Tune	Autotune
EMV Gain	Autotune
Acquisition parameters	EI, Multiple Reaction Monitoring
Collision gas flows	N_2 Collision Gas: 1.5 mL/min
Quench gas flows	Helium, 2.25 mL/min
MS temperatures	Source 280 °C; Quad 180 °C

Sample Preparation

One mL of urine was incubated with β-Glucuronidase to effectively cleave glucuronide conjugates and produce free steroids. The urine was then extracted by liquid-liquid extraction with diethyl ether and the residue after evaporation was derivatized for GC/MS analysis.

Derivatization was achieved by dissolving the dried sample in 100 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)-NH_4I-ethanethiol (100:2:3, v/w/v) and heating for 60 minutes at 80 °C [3].

Analysis Parameters

The Agilent Triple Quadrupole GC/MS system parameters used in the analysis of several classes of prohibited substances are shown in Tables 2–6.

Results

Sample Preparation

Doping control laboratories need to be able to detect very low levels of a wide variety of prohibited substances in a relatively small volume of a complex, biological matrix (usually urine). From this small sample, the labs must screen for and eventually confirm (using a totally independent analysis) the presence of any prohibited substance. Due to the sensitivity and selectivity of MS/MS detection, this sample preparation method uses only 1 mL of urine for the screening of a wide range of doping agents, a volume that is 2–5 times lower than that routinely used for GC/MS anabolic steroid screening methods.

This method is also comprehensive, encompassing one or more metabolites of all prohibited narcotics, the most frequently used β2-agonists, hormone antagonists and modulators, and beta-blockers. In addition, a large number of stimulants and several substances from all other groups of prohibited substances are covered by this method (Tables 2–5). The only anabolic agents not covered are those for which GC-MS is not particularly suitable (for example tetrahydrogestrinone, methyltrienolone, stanozolol).

Several quality assurance measures are incorporated into the method to cover the three basic steps in sample preparation: hydrolysis, extraction and derivatization. Using a large excess of β-glucuronidase assures efficient hydrolysis after 1.5 h at 56 °C. The use of both glucuronidated and free steroids with similar structure (d4-A-glucuronide and d5-Et (free)) allows for an adequate evaluation of hydrolysis efficiency. The use of a diverse mixture of internal standards allows for differences in physicochemical properties that can cause differences in extraction efficiency. These internal standards also enable quantification of non-deuterated structural analogues. Finally, the inclusion of transitions for mono-TMS derivatized androsterone and etiocholanolone in the method facilitates evaluation of the derivatization efficiency. This integrated approach provides a comprehensive evaluation of the sample preparation efficiency per sample, rather than per batch or only at the time of validation, since all major sample preparation steps are monitored.

The levels of 5α-androstane-3,17-dione and 5β- androstane-3,17-dione are also monitored in this method, as elevated concentrations of these compounds can be indicative of microbial contamination, which can alter the endogenous steroid profile.

Gas Chromatography

The aim of this study was to develop a fast GC/MS method, capable of quantifying the endogenous steroids shown in Table 6 as well detecting a wide range of prohibited substances qualitatively. Sufficient resolution between compounds is a prerequisite for adequate quantification. In this method, the separation of the isomers androsterone and etiocholanolone, present at relatively high concentrations (Table 6), and to a minor extent the other isomers (11β-OH-A and 11β-OH-Et and 5aab and 5bab) put restrictions on chromatographic speed and injected volumes. This method enables injection of 5 µL of sample using a PTV-injector, which is substantially higher than previous methods using split/splitless injection.

Using a relatively short capillary column (12.5 meters) in combination with a high linear velocity of hydrogen as carrier gas, rather than helium, enabled a substantial reduction in the GC run time, to 7.98 minutes. However, even at high concentrations (4.8 µg/mL), androsterone and etiocholanolone are sufficiently separated to provide adequate quantification

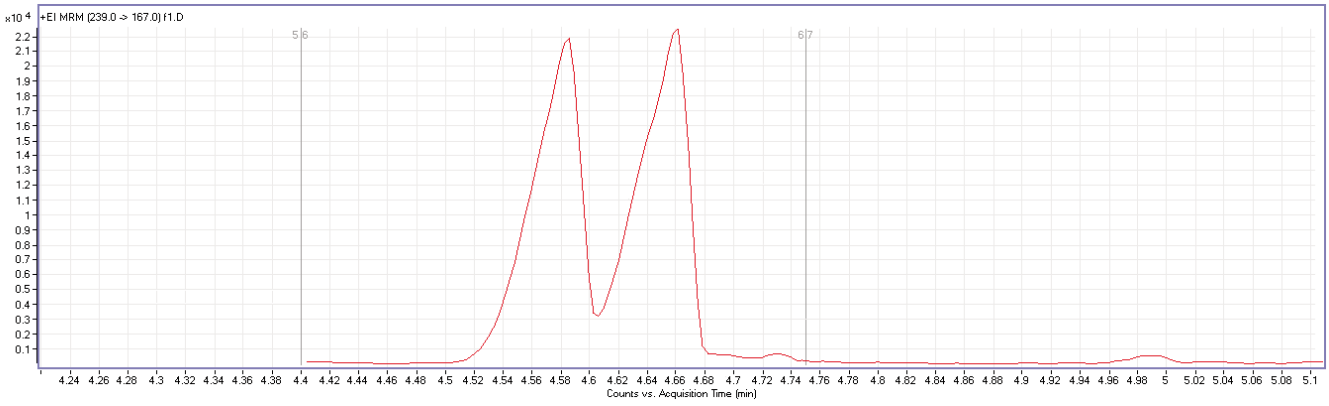

Figure 1. Extracted ion chromatogram (m/z 239 -> 167) for androsterone-bis-TMS and etiocholanolone-bis-TMS at the highest calibrator concentration (4.8 µg/mL).

(Figure 1). Shorter run time greatly improves sample turn around, which can be essential in those cases (for example Olympic competition) involving very short sample reporting times (24–48 h).

Mass Spectrometry

A multistep process was used to determine and optimize the mass spectrometric conditions. In the first step, full scan spectra were obtained for every derivatized compound. After selection of a suitable precursor ion, full product scan mass spectra were acquired at different collision energies (10 and 25 eV). Suitable product ions were then chosen and SRM transitions set up. Selection of the final product ions (at least two transitions per substance) and optimization of the collision energy (5, 10, 15, 20, 30, 35 eV) were then performed on both reference standards and extracts from spiked urine samples. The best signal-to-noise (S/N) ratio was used to determine the most appropriate transitions and collision energies for each analyte. Tables 2–5 list the final mass spectrometer settings for all of the analytes included in the method.

Quantitative Method Validation

The substances analyzed in the quantitative part of the method include those steroids traditionally used in doping control to establish the use of a prohibited substance (T, E, A, Et, DHT, DHEA, androstenedione, 5aab, 5bab. This method also monitors other endogenous steroids which are not affected by the intake of natural anabolics (11bOH-A and 11b-OH-Et), as well as markers of microbiological degradation (5α-androstanedione and 5β-androstanedione). The inclusion of these additional parameters can greatly assist in the evaluation process of atypical steroid profiles, due to elevated production of endogenous steroids or alteration by microbiological degradation. The method also quantifies salbutamol, the most widely used β2-agonist, norandrosterone and the major metabolite of cannabis (11-nor-Δ9- tetrahydrocannabinol.-9 carboxylic acid, THC-COOH).

Although large differences in calibration ranges exist between the monitored compounds, correlation coefficients of 6-point calibration curves (3 replicates per calibrator) made in steroid-stripped urine were acceptable. Additional analysis revealed that the residual standard deviations at every point of the calibration curves were lower than 2/3 of the maximum residual standard deviation as calculated by Horwitz (www.cipac.org/document/Guidance%20Documents/validat.pdf). Moreover, the bias at each of these points was below 15%, demonstrating acceptable accuracy as well. Therefore, in agreement with Eurachem guidelines [4], this method can be regarded as validated for quantitative purposes.

Qualitative Analysis

Method validation for the non-threshold substances was also performed in accordance with Eurachem guidelines. Selectivity was confirmed by the lack of matrix interferences in ten blank urine samples. These samples were then spiked at different concentration levels of all of the target analytes. The lowest concentration at which concurrent signals (S/N>3) for each monitored transition were obtained at the expected retention time (± 1%) in all samples was defined as the limit of detection (LOD). These LOD's for the exogenous substances are listed in Tables 2–6. The method includes 41 metabolites of anabolic steroids, 4 other anabolic agents, 6 β2-agonists, 11 hormone antagonists and modulators, 19 narcotics and 16 stimulants.

It should be noted that in some cases, the observed LOD for a metabolite exceeds WADA's MRPL (Minimum Required Performance Level). For these substances, the method was regarded as non-validated, although they remained part of the method. For all such cases, the method includes another metabolite of the same parent drug with an LOD at or below the MRPL. This is the case for fluoxymesterone for example: the LOD for 6β-hydroxyfluoxymesterone (Table 2) is 20 ng/mL, while WADA's MRPL is set at 10 ng/mL. However, the LOD of 9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one, another fluoxymesterone metabolite, is compliant with the MRPL. The WADA technical document does not specify which metabolites need to be monitored, with the exception of a few substances. Therefore, the method can be considered WADA compliant for the detection of fluoxymesterone.

Moreover, the use of multiple metabolites rather than a single metabolite to determine misuse of a doping agent has multiple advantages. Firstly, it can provide additional supporting evidence for misuse, since in most cases the concentration in a "positive" sample will be clearly above the MRPL. Additionally, the inclusion of multiple metabolites can assist in the detection of a prohibited substance at different time points after use. Indeed, it is widely known that the excretion profile of metabolites is time and inter-individual dependent. Therefore, a compound which is the major metabolite in one individual after a defined post-administration time might only be a minor metabolite in another individual which took the drug at the same time.

The current method is also capable of detecting all compounds from the class of "other anabolic agents," except for the group of selected androgen receptor modulators that are still in clinical phase trials and not included in this study.

Besides the anabolic agents, a wide variety of hormone antagonists and modulators can be detected at or below the MRPL. This list includes substances with a steroidal structure (formestane, 6α-OH androstenedione and the metabolite of exemestane: 17β-hydroxy-6-methylene-androsta-1, 4-diene-3-one) as well as non steroidal compounds (aminoglu-thetimide, anastrazole, letrozole metabolite, raloxiphene, toremiphene, 4-OH-cyclofenil, 4-OH-tamoxifen and the isomers of 4-OH-methoxytamoxifen). Moreover, as androsta-1,4,6-triene-3,17-dione also metabolizes to boldenone and its metabolites [3], the only substances from this class which are not included in the method are testolactone, clomiphene and fulvestrant, due to the lack of reference standards for their metabolites.

Most prohibited narcotics also undergo extensive Phase I and Phase II metabolism. Therefore, all WADA prohibited narcotics and/or their metabolites were included in the current method. Except for fentanyl, which shows superior detection by LC-MS, all LOD's were lower than the WADA MRPL, making the methodology very well suited for monitoring the misuse of narcotics. The method also screens for codeine, since use of codeine can be detected as morphine. When the detection of morphine can be attributed to the use of codeine, a laboratory should not report such cases [5].

In general, urine is not well suited to the determination of the post-administration time of sample collection. However, the current method offers the ability to determine post-administration time for several substances by monitoring metabolites for which the excretion profile is time-dependent. This is the case for heroin, for example, since the method monitors not only the parent substance but also morphine and 6-monoacetylmorphine (MAM).

The method is also capable of simultaneously quantifying 11-nor-Δ9-tetrahydrocannabinol.9 carboxylic acid (THC-COOH), the major metabolite of cannabis and one of the most detected doping agents world-wide. Thus, this method can also be used in forensic science, toxicology, drugs of abuse and work place testing laboratories.

In contrast to the narcotics, most stimulants are not excreted as conjugates, and the inclusion of these substances was not the focus of this research. Nevertheless, a wide range of stimulants (or their metabolites), including cocaine and its metabolite benzoylecgonine are included in the method.

The method covers the most frequently used β2-agonists in sports. Moreover, in the case of fenoterol both the parent drug (O-TMS tetrakis derivatized) and a degradation product, the C,N-methylene fenoterol-tetrakis- TMS derivative, were monitored [6]. Although the degradation product was not detected in the validation study, its inclusion in the method will increase the detection capability of the method for real samples, since fenoterol can be rapidly degraded.

Although beta blockers are only prohibited in particular sports, 15 beta blockers were included in the method since their inclusion can optimize laboratory efficiency when their detection is required.

The method uses an optimized derivatization protocol [7], but the effectiveness of the derivatization step is confirmed by monitoring for the presence of mono-TMS derivatized androsterone and etiocholanolone. The formation of multiple derivatives of several other compounds (for example celiprolol, pindolol) is still possible. While one of the derivatives usually gives a better signal than the other, the inclusion of the second derivative can be regarded as a safety precaution. Given the high speed of changing SRM transitions in the Agilent 7000 Series Triple Quadrupole GC/MS system (500 transitions/sec), this addition of transitions does not decrease the overall performance of the method.

Table 2. Agilent 7890/7000A GC/MS Analysis Parameters for Endogenous Anabolic Androgenic Steroids (AAS; Prohibited Class S1a)

Class	RT (min)	Substance	Transitions	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)
S1a	4.14	5β-androst-1-en-17β-ol-3-one	432.0 → 194.0 432.0 → 206.0	15 15	5	10
	5.32	Boldenone	430.0 → 206.0 430.0 → 191.0	10 30	10	10
	5.09	1-Androstenediol	434.0 → 195.0 434.0 → 127.0	20 20	5	10
	5.05	1-testosterone	432.0 → 194.0 432.0 → 206.0	5 10	10	10
	5.09	17α-methyl-5α-androstane-3α,17β-diol	435.0 → 255.0 435.0 → 213.0	20 20	2	2
	5.12	17α-methyl-5β-androstane-3α,17β-diol	435.0 → 255.0 435.0 → 213.0	20 20	5	2
	6.7	oxymesterone	534.0 → 389.0 534.0 → 444.0	20 20	10	10
	4.15	epimetendiol	358.0 → 301.0 358.0 → 196.0	15 5	2	2
	6.57	6β-hydroxymethandienone	517.0 → 229.0 517.0 → 337.0	20 15	5	10
	5.63	Metenolone PC	446.0 → 208.0 446.0 → 195.0	10 15	5	10
	4.92	1-Methylene-5α-androstan-3α-ol-17-one (metenolone metab)	446.0 → 341.0 446.0 → 195.0	15 5	20	10
	5.64	17α-Ethyl-5β-estrane-3α,17β-diol (norethandrolone major metab)	421.0 → 241.0 421.0 → 331.0	15 5	10	10
	5.4	17α-Ethyl-5α-estrane-3α,17β-diol (norethandrolone minor metab)	421.0 → 241.0 421.0 → 145.0	15 25	5	10
	4.77	2α-methyl-5α-androstan-3α-ol-17-one (drostanolone metab)	448.0 → 433.0 448.0 → 253.0	10 25	10	10
	6.05	Bolasterone PC	460.0 → 355.0 460.0 → 315.0	15 15	10	10
	5.62	7α,17α-dimethyl-5β-androstane-3α,17β-diol (bolasterone metab)	284.0 → 269.0 284.0 → 213.0	5 10	10	10
	6.13	Calusterone PC	460.0 → 355.0 460.0 → 315.0	15 15	10	10
	5.45	7β,17α-dimethyl-5β-androstane-3α,17β-diol (calusterone metab)	229.0 → 105.0 269.0 → 159.0	30 5	/	10
	5.07	1α-Methyl-5α-androstan-3α-ol-17- one (mesterolone metab)	448.0 → 433.0 448.0 → 253.0	10 20	5	10
	5.63	4-Chloro-4-androsten-3α-ol-17-one (clostebol metab)	466.0 → 181.0 466.0 → 431.0	20 15	10	10
	6.47	norclostebol	452.0 → 216.0 452.0 → 321.0	20 15	2	10
	6.67	fluoxymesterone PC	552.0 → 407.0 552.0 → 357.0 552.0 → 319.0	15 15 15	/	10
	6.93	6β-OH-fluoxymesterone	640.0 → 640.0 640.0 → 143.0	10 25	20	10
	5.04	9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one	462.0 → 208.0 462.0 → 337.0	15 15	5	10
	6.17	oxandrolone	363,0 → 161,0 308.0 → 117.0	15 15	10	10
	5.56	epioxandrolone	363,0 → 161,0 308.0 → 117.0	15 15	20	10
	6.68	dehydrochloromethyltestosterone PC	478.0 → 285.0 478.0 → 353.0	20 5	10	10
	6.82	6β-hydroxy-dehydrochloromethyltestosterone	315.0 → 227.0 315.0 → 241.0	20 15	20	10
	5.19	17α-trenbolone	307.0 → 291.0 307.0 → 275.0	10 20	10	10
	7.1	2-Hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one (formebolone metab)	444.0 → 356.0 367.0 → 257.0	25 25	/	10
	6.48	17α-methyl-4-androstene-11α,17β-diol-3-one (formebolone metab)	534.0 → 389.0 534.0 → 339.0	15 25	10	10
	5.85	mibolerone	446.0 → 431.0 446.0 → 341.0	15 20	10	10
	6.14	ethisterone	456.0 → 316.0 456.0 → 301.0	15 15	1	10
	4.76	3α,5α-tetrahydronorethisterone	431.0 → 167.0 431.0 → 193.0	20 20	2	10
	7.11	16-OH-furazabol	490.0 → 231.0 490.0 → 143.0	15 35	10	10
	5.94	methyldienolone	430.0 → 285.0 430.0 → 325.0	10 10	10	10
	5.97	13β,17α-diethyl-5α-gonane-3α, 17β-diol (norbolethone metab)	435.0 → 255.0 435.0 → 159.0	10 15	20	10
	6.14	13β,17α-diethyl-5β-gonane-3α, 17β-diol (norbolethone metab)	435.0 → 255.0 435.0 → 345.0	20 5	5	10
	3.68	madol	345.0 → 255.0 345.0 → 201.0	15 15	10	10
	6.11	2α,17α-dimethyl-17β-hydroxy-5α-androstane-3-one	462.0 → 141.0 462.0 → 143.0	15 15	10	10
	6.27	4-OH-nandrolone (oxabolone)	506.0 → 147.0 506.0 → 93.0 506.0 → 195.0	20 25 20	2	10
	6.48	4-OH-testosteron	520.0 → 225.0 520.0 → 431.0	15 15	2	10
	6.33	6-OH-androstenedione	518.0 → 319.0 518.0 → 413.0	15 15	1	10
	5.19	7β-OH-DHEA	430.0 → 325.0 430.0 → 220.0	10 10	20	10

PC = Parent Compound

Table 3. Agilent 7890/7000A Triple Quadrupole GC/MS System Analysis Parameters for Endogenous AAS when administered exogenously, Other Anabolic Agents, Beta-2 Agonists, Hormone Antagonists and Modulators, Diuretics and Other Masking Agents (Prohibited Classes S1b, S1c, S3, S4 and S5, respectively)

Class	RT (min)	Substance	Transitions	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)
S1b	4.04	19-norandrosterone	405.0 → 225.0 405.0 → 315.0	10 5	1	2
	4.12	5β-Androstane-3,17-dione	290.0 → 275.0 290.0 → 185.0	10 10		
	4.64	5α-androstane-3α,17β-diol	256.0 → 185.0 256.0 → 157.0	15 15		
	4.71	5β-androstane-3α,17β-diol	256.0 → 185.0 256.0 → 157.0	15 15		
	4.58	androsterone	239.0 → 167.0 239.0 → 117.0	35 35		
	4.63	etiocholanolone	239.0 → 167.0 239.0 → 117.0	35 35		
	5.09	5α-Androstan-3,17-dione	290.0 → 275.0 290.0 → 185.0	10 10		
	4.98	DHEA	432.0 → 327.0 432.0 → 237.0	10 10	EAAS	/
	5.14	epitestosterone	432.0 → 209.0 432.0 → 327.0	10 10		
	5.13	5α-androstane-3β,17β-diol	421.0 → 255.0 421.0 → 213.0	20 20		
	5.29	4-androstenedione	430.0 → 209.0 430.0 → 234.0	15 15		
	5.24	DHT	434.0 → 195.0 434.0 → 182.0	20 20		
	5.41	testosteron	432.0 → 209.0 432.0 → 327.0	10 10		
	5.52	11β-OH-androsterone	522.0 → 236.0 522.0 → 324.0	10 10		
	5.6	11β-OH-etiocholanolone	522.0 → 236.0 522.0 → 324.0	10 10		
	4.13	Mono TMS Androsterone	347.0 → 253.0	20	qas	/
S1c	3.37	zilpaterol	308.0 → 218.0 308.0 → 203.0 291.0 → 219.0	10 15 15	5	10
	6.43	zeranol	433.0 → 295.0 433.0 → 309.0	15 15	10	10
	2.42	clenbuterol	335.0 → 227.0 335.0 → 300.0	10 10	0.2	2
	5.37	3α-hydroxytibolone	443.0 → 193.0 443.0 → 167.0	35 30	5	10
S3	2.17	salbutamol	369.0 → 207.0 369.0 → 191.0	15 15	25	100
	1.96	terbutaline	356.0 → 267.0 356.0 → 355.0	25 25	50	100
	6.07	fenoterol	322.0 → 68.0 322.0 → 279.0	15 15	100	100
	6.6	fenoterol C,N-methylene	308.0 → 207.0 308.0 → 179.0	15 15	/	50
S4	6.73	formoterol	178.0 → 121.0 178.0 → 135.0	20 20	50	100
	7.82	salmeterol	311.0 → 149.0 311.0 → 121.0	15 25	100	100
	5.02	bambuterol	354.0 → 72.0 354.0 → 282.0	25 10	5	100
	3.63	aminogluthetimide deriv.1	361.0 → 206.0 361.0 → 221.0	30 10	5	50
	5.26	aminogluthetimide deriv.2	580.0 → 551.0 580.0 → 519.0	20 20	/	50
	3.16	anastrazole	293.0 → 70.0 293.0 → 209.0	10 15	50	50
	3.17	letrozole metabolite	291.0 → 160.0 291.0 → 217.0	15 20	2.5	50
	6.94	exemestane PC	441.0 → 307.0 441.0 → 193.0	20 20	/	50
	6.94	17β-hydroxy-6-methylene-androsta-1,4-diene-3-one	443.0 → 207.0 443.0 → 193.0	20 20	25	50
	6.43	4-OH-androstene-3,17-dione (formestane)	518.0 → 221.0 518.0 → 190.0	15 10	2	10
	6.57	toremiphene	405.0 → 58.0 405.0 → 72.0	15 5	25	50
	6.86	4-hydroxy-methoxytamoxifen 1	489.0 → 72.0 489.0 → 58.0	5 15	25	50
	7.02	4-hydroxy-methoxytamoxifen 2	489.0 → 72.0 489.0 → 58.0	5 15	25	50
	5.78	4-OH-tamoxifen	459.0 → 72.0 459.0 → 58.0	5 15	2.5	50
	7.74	raloxiphene	578.0 → 193.0 578.0 → 413.0	35 30	25	50
	6.57	4-OH-cyclofenil	512.0 → 422.0 512.0 → 343.0	10 5	2.5	50
S5	3.13	probenecid	328.0 → 103.0 328.0 → 193.0	25 15	12.5	250

PC = Parent Compound

Table 4. Agilent 7890/7000A kTriple Quadrupole GC/MS System Analysis Parameters for Stimulants, Narcotics and Cannabinoids (Prohibited Classes S6, S7 and S8, respectively)

Class	RT (min)	Substance	Transitions	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)
S6	2.16	carphedon	272.0 → 104.0 272.0 → 229.0	25 15	50	500
	4.98	6-OH-bromantan	395.0 → 91.0 393.0 → 91.0	30 30	2,5	500
	2.08	pemoline	178.0 → 104.0 392.0 → 178.0	10 10	5	500
	2.28	octopamine	174.0 → 866.0 426.0 → 206.0 426.0 → 179.0	5 15 15	100	500
	7.14	strychnine	316.0 → 144.0 316.0 → 220.0	15 10	100	200
	1.37	crotethamide	154.0 → 86.0 154.0 → 69.0	10 15	50	500
	1.97	ethamivan	295.0 → 223.0 295.0 → 265.0	25 20	50	500
	1.36	fencamfamine	215.0 → 186.0 215.0 → 98.0	5 15	50	500
	4.24	fenspiride	241.0 → 96.0 241.0 → 154.0	10 10	25	500
	2.57	3,3-dihenylpropylamine	174.0 → 86.0 174.0 → 100.0	15 15	50	500
	4.65	prenylamine	238.0 → 58.0 238.0 → 91.0	20 20	50	500
	1.94	clobenzorex	168.0 → 125.0 168.0 → 89.0	20 35	100	500
	2.51	cyclazodone	360.0 → 178.0 360.0 → 247.0	15 15	10	500
	6.57	famprofazone	286.0 → 72.0 286.0 → 214.0	20 15	50	500
	1.66	benzphetamine	148.0 → 91.0 148.0 → 65.0	20 35	10	500
	1.74	methylphenidate	156.0 → 45.0 156.0 → 84.0	35 10	100	500
	6.47	amineptine	193.0 → 115.0 193.0 → 178.0	15 15	10	500
	4.53	amineptine C5 metabolite	193.0 → 115.0 193.0 → 178.0	15 15	50	500
	2.7	cocaine	303.0 → 82.0 303.0 → 198.0	15 5	50	500
	3.07	benzoylecgonine	240.0 → 82.0 361.0 → 82.0	20 20	100	500
	3.56	prolintane metabolite14	322.0 → 293.0 322.0 → 205.0	20 20	excr	500
	2.28/2.34	prolintane metabolite 5a/b	304.0 → 142.0 304.0 → 75.0	20 20	excr	500
	2.67	prolintane metabolit e9	228.0 → 158.0 228.0 → 138.0	20 20	excr	500
	2.52	sibutramine metabolite 1	158.0 → 116.0 158.0 → 102.0	10 10	excr	500
	2.74/2.82	sibutramine metabolite 2/3	246.0 → 156.0 246.0 → 84.0	20 20	excr	500
S7	7.47	buprenorphine	554.0 → 522.0 554.0 → 450.0	15 20	0.5	10
	6.57	dextromoramide	265.0 → 166.0 265.0 → 98.0	15 10	20	200
	4.91	heroine	369.0 → 327.0 369.0 → 268.0	10 25	2.5	200
	4.66	MAM	399.0 → 287.0 399.0 → 340.0	15 10	20	200
	5.37	fentanyl	245.0 → 189.0 245.0 → 146.0	10 15	/	10
	2.19	norfentanyl	175.0 → 120.0 175.0 → 56.0	5 15	/	10
	4.32	hydromorphone	429.0 → 234.0 429.0 → 357.0	15 25	100	200
	2.73	methadon	296.0 → 191.0 296.0 → 281.0	20 10	10	200
	2.93	methadon 2	296.0 → 191.0 296.0 → 281.0	20 10	40	200
	2.37	normethadon 1	224.0 → 103.0 224.0 → 191.0	35 35	100	200
	2.73	normethadon 2	296.0 → 191.0 296.0 → 252.0	20 20	10	200
	2.14	EDDP	277.0 → 105.0 277.0 → 220.0	25 20	40	200
	4.42	morphine	429.0 → 287.0 429.0 → 220.0	20 35	10	200
	4.37	oxycodone	459.0 → 368.0 459.0 → 312.0	15 15	200	200
	4.76	oxymorphone	502.0 → 70.0 517.0 → 355.0	30 15	40	200
	3.12	pentazocine	357.0 → 246.0 357.0 → 289.0	15 15	100	200
	1.47	pethidine	247.0 → 71.0 247.0 → 173.0	5 5	4	200
	3.97	codeine	371.0 → 229.0 371.0 → 234.0	5 5	10	200
	4.21	ethylmorphine	385.0 → 214.0 385.0 → 234.0	35 10	10	200
	2.51	pipradrol	239.0 → 161.0 239.0 → 221.0	20 20	5	200
	5.25	fenbutrazate	261.0 → 103.0 261.0 → 175.0	35 15	50	200
S8	6.06	THC-COOH	371.0 → 289.0 371.0 → 265.0	15 15	<5	7,5

PC = Parent Compound

Table 5. Agilent 7890/7000A Triple Quadrupole GC/MS System Analysis Parameters for Beta Blockers Prohibited in Competition in Certain Sports (Prohibited Class P2) and the Internal Standards (ISTDs)

Class	RT (min)	Substance	Transitions	Collision energy (eV)	LOD (ng/mL)	MRPL (ng/mL)
P2	1.91	oxprenolol	150.0 → 109.0 221.0 → 72.0	15 15	50	500
	3.62	betaxolol	364.0 → 209.0 364.0 → 172.0	10 10	100	500
	2.94	bisoprolol	405.0 → 56.0 405.0 → 172.0	25 15	100	500
	3.07	pindolol 1	204.0 → 133.0 220.0 → 75.0	15 15	500	500
	3.65	pindolol 2	205.0 → 130.0 292.0 → 218.0	15 15	50	500
	3.05	esmolol	352.0 → 193.0 352.0 → 56.0	5 15	100	500
	3.02	metipranolol	366.0 → 281.0 366.0 → 239.0	5 15	25	500
	2.64	propanolol	316.0 → 231.0 316.0 → 75.0	5 15	25	500
	3.15	timolol	373.0 → 186.0 373.0 → 70.0	15 35	50	500
	4.12	carteolol	421.0 → 186.0 421.0 → 365.0	15 5	50	500
	4.12	levobunolol	234.0 → 233.0 234.0 → 217.0	5 10	25	500
	2	celiprolol 1	319.0 → 129.0 205.0 → 89.0 205.0 → 117.0	15 15 15	/	500
	3.45	celiprolol 2	200.0 → 128.0 200.0 → 144.0	15 15	500	500
	4.53	nadolol	510.0 → 70.0 510.0 → 186.0	35 20	250	500
	6.2	acebutolol 1 + 2	278.0 → 166.0 278.0 → 208.0	30 30	500	500
	1.72	alprenolol	321.0 → 72.0 306.0 → 203.0	15 15	250	500
	6.67	labetolol	383.0 → 265.0 383.0 → 251.0	15 15	100	500
ISTD	4.66	5β-Androstane-3a,17b-diol-d5	246.0 → 190.0 246.0 → 164.0	15 15		
	4.62	5α-Androstane-3a,17b-diol-d3	244.0 → 202.0 244.0 → 188.0	15 15		
	4.51	androsterone-d4	423.0 → 333.0 423.0 → 243.0	20 20		
	4.56	etiocholanolone-d5	424.0 → 334.0 424.0 → 244.0	20 20		
	5.12	epitestosterone-d3	435.0 → 330.0 435.0 → 209.0	5 20	ISTD	/
	5.38	testosterone-d3	435.0 → 330.0 435.0 → 209.0	20 20		
	5.17	DHT-d3	437.0 → 205.0 437.0 → 195.0	15 15		
	2.16	salbutamol-d3	372.0 → 210.0 372.0 → 193.0	20 20		
	5.97	17α-methyltestosterone	446.0 → 301.0 446.0 → 198.0	25 20		

PC = Parent Compound

Table 6. Target Substances for Quantitative Analysis

Substance	Internal standard	Calibrators (ng/mL)	Correlation coefficient (R^2)
Testosterone	d3-T	2-5-20-50-100-200	0.9918
Epitestosterone	d3-E	2-5-20-50-100-200	0.9933
Androsterone	d4-A	48-120-600-1200-2400-4800	0.9903
Etiocholanolone	d5-E	48-120-600-1200-2400-4800	0.9716
11β-OH-androsterone	d4-A	40-100-500-1000-2000-4000	0.9769
11β-OH-etiocholanolone	d5-E	40-100-500-1000-2000-4000	0.9877
Dihydrotestosterone	d3-DHT	4-10-40-100-200-400	0.9755
Dehydroepiandrosterone	d3-DHT	4-10-40-100-200-400	0.9927
4-androstene-3,17-dione	d3-DHT	4-10-40-100-200-400	0.9908
5α-androstane-3α,17β-diol	d3-aab	4-10-40-100-200-400	0.9841
5β-androstane-3α,17β-diol	d5-bab	4-10-40-100-200-400	0.9603
5α-androstane-3β,17β-diol	d3-aab	4-10-40-100-200-400	0.9933
5α-androstane-3,17-dione	MT	4-10-40-100-200-400	0.9975
5β-androstane-3,17-dione	MT	4-10-40-100-200-400	0.9853
19-norandrosterone	MT	1-3-5-10-15-20	0.9902
Salbutamol	d3-sal	100-300-500-1000-1500-2000	0.9807
THC-COOH	MT	5-15-25-50-75-100	0.9862

Conclusion

A fast GC-MS/MS method for the quantitative determination of the steroid profile, salbutamol, THC-COOH and norandrosterone as well as the qualitative detection of 142 doping agents (or their metabolites) was developed and validated. The use of a wide range of internal standards provides an evaluation of the sample preparation efficiency to assure accuracy of the results. Using hydrogen as a carrier gas and a short (12.5 m) capillary column with the Agilent 7000A Triple Quadrupole GC/MS system, all doping agents could be detected within a single run of less than 8 minutes.

Acknowledgments

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Application Note SI-02313

Fast GC/MS/MS of Androgenic Anabolic Steroids in Urine Using a VF-5ms Column

Cynthia Mongongu, Agence Française de Lutte contre le Dopage
Johan Kuipers, Varian, Inc.

Introduction

The use of anabolic steroids in sport is prohibited by the World Anti-Doping Agency. Athletes are therefore subject to continuous screening for these banned substances. The analysis of large numbers of samples in a short time with a high degree of specificity is an important requirement for any screening program. The key factor is the use of a rapid gas chromatographic method in combination with a sensitive detector. This note describes a fast and sensitive method to screen 13 anabolic androgenic steroids within 12 minutes, based on a short FactorFour™ VF-5ms GC column and multiple reaction monitor (MRM) detection. This method is approximately twice as fast than a classical steroids' method analysis.

Sample Preparation

Urine (2 mL) was prepared by adding 17a-methyltestosterone as an internal standard, and the 13 compounds at concentrations of 2, 5 ng/mL. The urine sample was then buffered to pH 6 and incubated at 55 °C for one hour after the addition of 50 µL of ß-glucuronidase. The hydrolyzed urine was passed through an SPE cartridge, which was conditioned successively with methanol and water. The column was rinsed with water, 10% methanol in water, and hexane. The steroids were then eluted with methylterbutyl ether. The eluate was evaporated to dryness and subsequently derivatized with 50 µL of MSTFA/NH_4I/dithioerythritol at 60 °C for 20 minutes.

Conditions

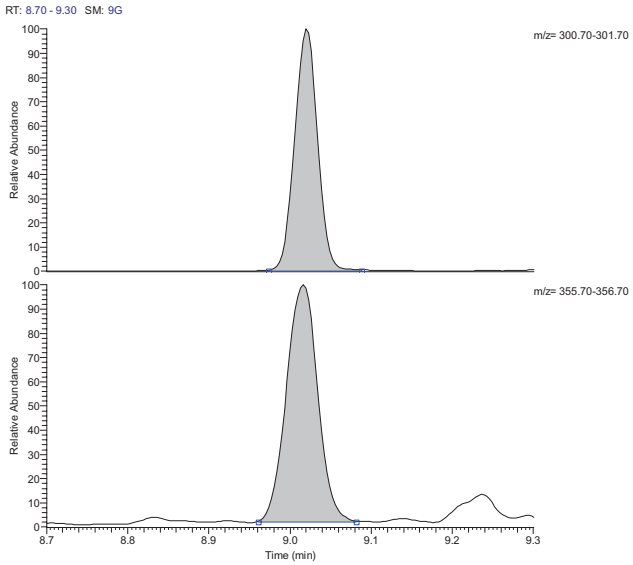
Column:	FactorFour VF-5ms, 10 m x 0.15 mm x 0.15 µm (Part no: CP9034)
Cartridge:	BondElut™ C18, 200 mg
Sample Vol:	3 µL
Carrier Gas:	0.5 mL/min Helium, constant flow
Injector:	250 °C, split ratio 1:10
Temp Gradient:	170 °C for 0.5 min, 10 °C/min to 260 °C, 50 °C/min to 320 °C (1 min)
Detector:	Triple quadripole GC, 70 eV EI Mode, ion source 250 °C

Results

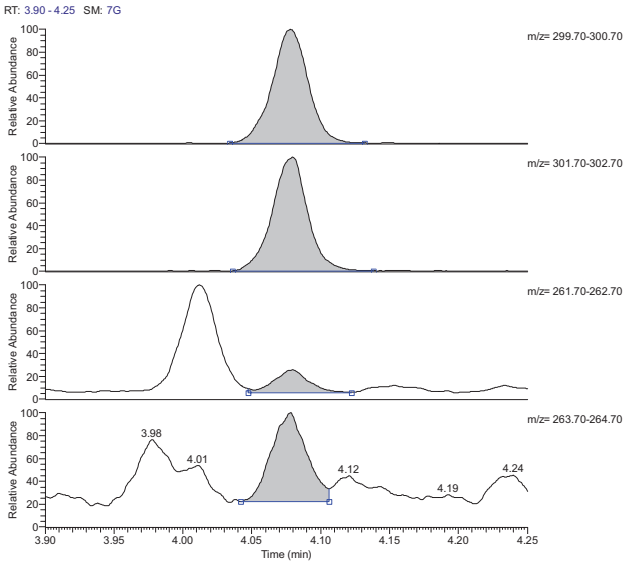
Table 1 shows the characteristics of the 13 steroids. Figure 1 shows the mass spectra obtained using the method described.

Table 1. Anabolic steroids, detection level in sample, retention time, associated precursors and daughter ions.

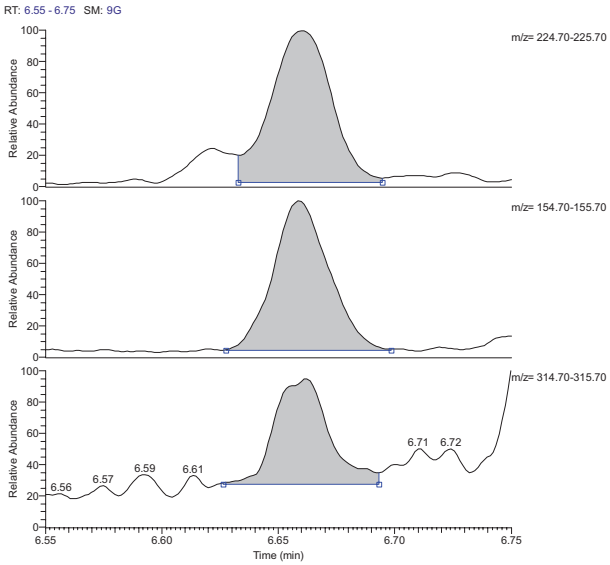
Compounds	Detection level (ng/mL)	Retention time (min)	Relative retention time	Precursor ion	Daughter ions		
17a-Methyltestosterone (ISTD)	200	9.02	-	446	301	356	
Clenbuterol	2	4.08	0.452	335	300	262	
				337	302	264	
19-Norandrosterone	2	6.66	0.738	405	225	155	315
Epimethenediol	2	6.92	0.767	358	301		
19-Noretiocholanolone	2	7.12	0.789	405	155	225	315
17-Epimethanedienone	2	8.26	0.916	444	206	339	
5a-Methyltestosterone	2	8.01	0.888	435	255	345	
5b-Methyltestostérone	2	8.07	0.895	435	255	345	
Norethandrolone metabolite	5	8.67	0.961	421	331	241	
Ethisterone	2	9.17	1.017	456	316	301	208
Bolasterone	5	9.05	1.003	460	445	355	315
Calusterone	5	9.14	1.013	460	445	355	315
6ß-Hydroxymethanedienone	2	9.74	1.080	517	229	317	281
Fluoxymesterone metabolite	5	9.33	1.034	552	495	319	
				462	337		



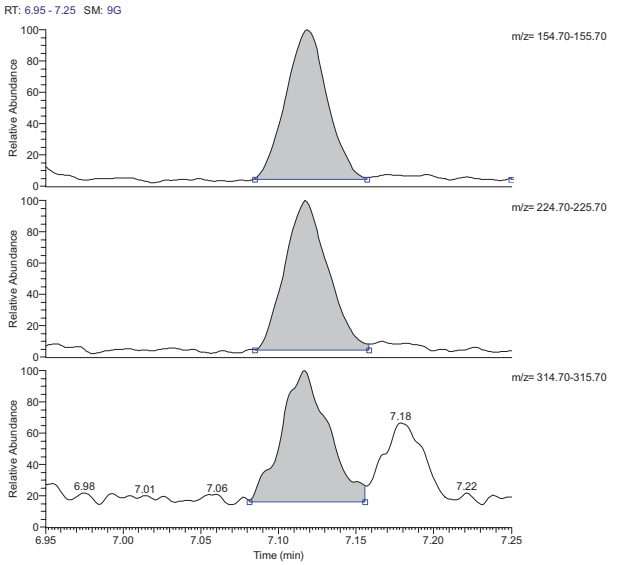
17a-Methyltestosterone (ISTD).



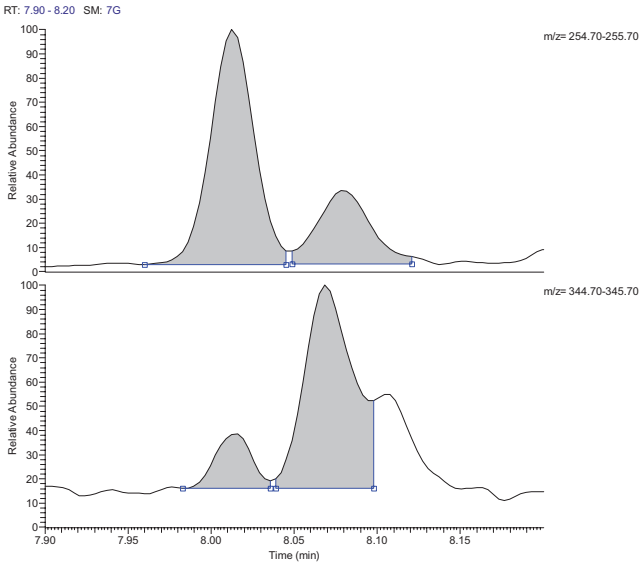
Clenbuterol.



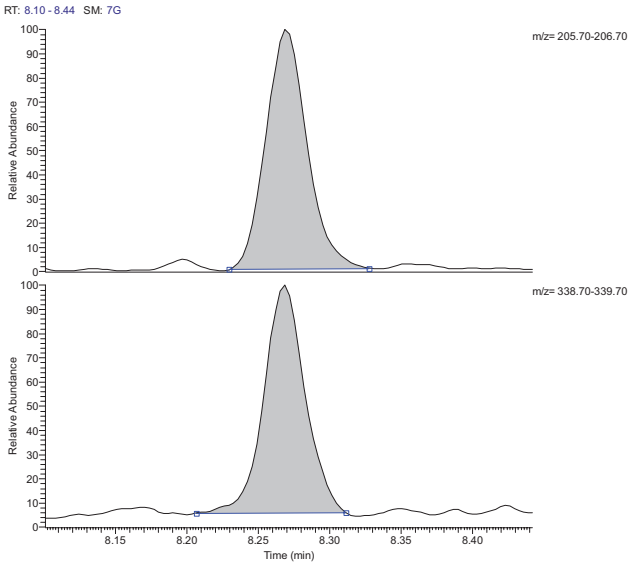
19-Norandrosterone.



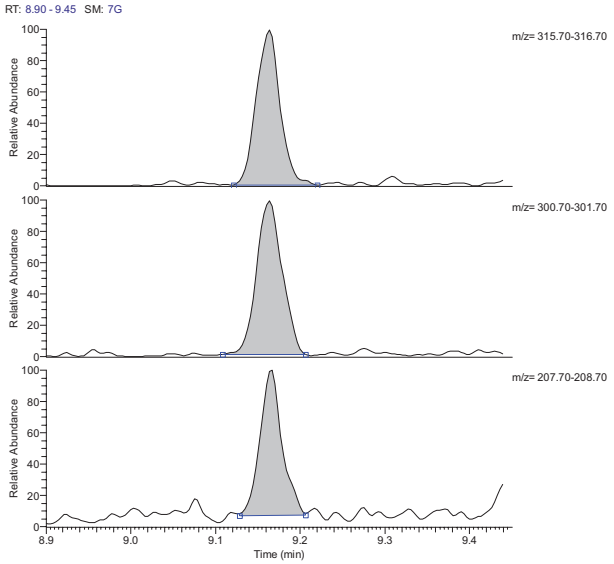
19-Noretiocholanolone.



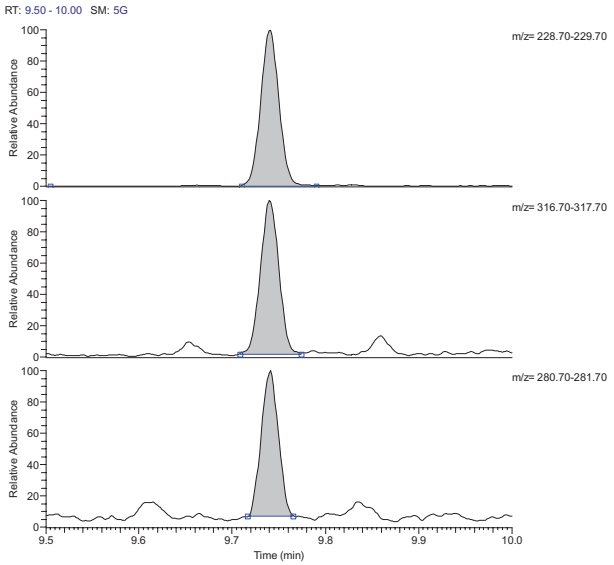
5a-Methyltestosterone, 5b-Methyltestosterone.



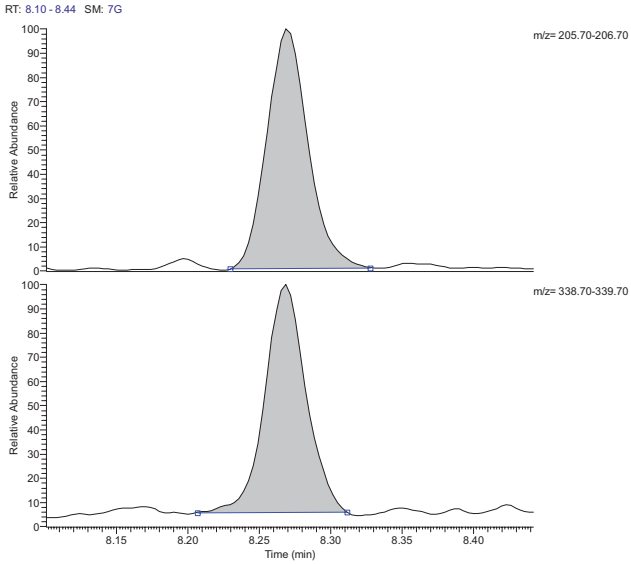
17-Epimethanedienone.



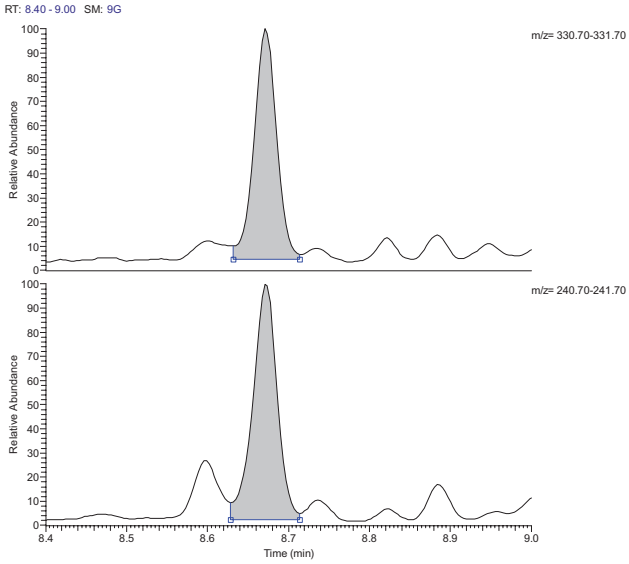
Ethisterone.



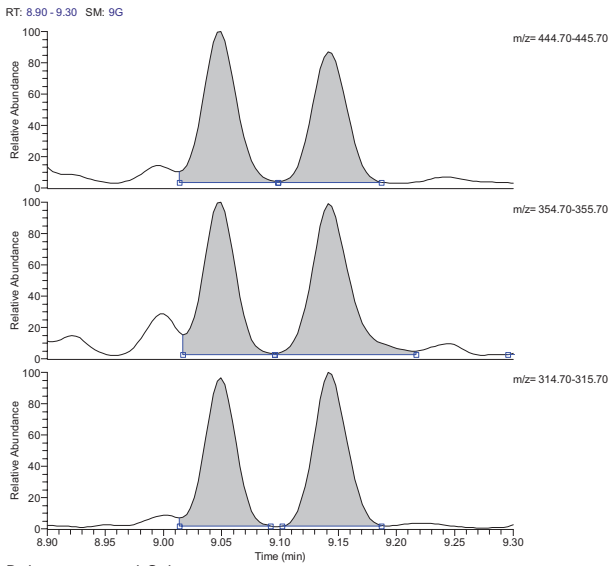
6ß-Hydroxymethanedienone.



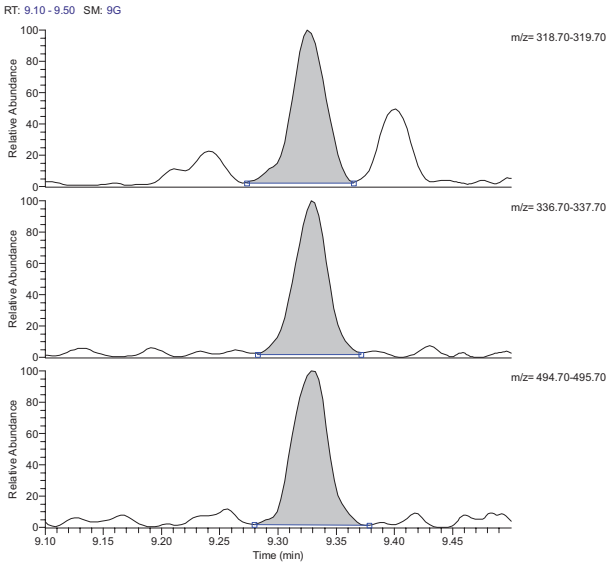
Epimethenediol.



Norethandrolone metabolite.



Bolasterone and Calusterone.



Fluoxymesterone metabolite.

Figure 1. Mass spectral information of the anabolic steroids.

Conclusion

The GC/MS/MS method described here detected ten anabolic steroids commonly tracked as banned substances using a VF-5ms capillary column. The method was optimized for a fast analysis speed, while maintaining important chromatographic separations of structurally related steroids that exhibited identical MRM fragmentation patterns. This approach permitted rapid detection of prohibited substances and delivered specific information on the compound detected.

These data represent typical results.
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Application Note SI-02313

Improved Forensic Toxicology Screening Using A GC/MS/NPD System with a 725-Compound DRS Database

Application

Forensic Toxicology

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Abstract

Laboratories that perform toxicology screens are challenged by the requirement to look for large numbers of target compounds in samples that contain complex matrix interferences. GC/MS methods are widely used and accepted for this analysis. Full-scan EI methods offer many advantages for broad-range screening, such as unlimited numbers of targets, full-spectrum identity confirmation, and library searching for identification of nontargets. With recent advances in GC/MS technology, there are several opportunities to substantially increase the number of targets screened for and simultaneously reduce the time required per sample.

With the system described here, samples are screened for 725 compounds using Agilent's G1674AA Forensic Toxicology DBL. Data review time is substantially reduced using Agilent Deconvolution Reporting Software. Post-run bakeout of heavy-matrix compounds is replaced with column backflushing, which is faster and reduces system maintenance. Run time is reduced by using a fast GC run (9.75 min injection to injection) and simultaneously collecting scan, SIM, and NPD data. The scan data is deconvoluted and used to identify any of the 725 target compounds. SIM data is used to look for select low-level compounds not detectable in scan mode. The nitrogen response of the NPD is used to highlight nontarget nitrogen compounds and identity confirmation and can be used for quantitation if needed. Using extracts of whole blood samples, the system finds all the compounds detected by the conventional method in significantly less time.

Introduction

GC/MS screening methods play an important role in the toxicology laboratory. With the continuing emergence of new drugs and toxins, the list of target compounds to be screened can easily number in the hundreds. For those compounds that are compatible with GC, GC/MS in full-scan mode with electron impact ionization (EI) is well suited for the task. The technique offers several advantages:

- It uses straightforward, reliable, and familiar instrumentation.
- Any number of targets can be monitored.
- The target list is not limited by the number of MRMs like MS/MS techniques.
- Years later, archived full-scan data can be examined for new targets.
- Identity confirmation is based on full spectra.
- Nontarget unknown compounds can be identified by searching spectra against NIST and other industry standard libraries.
- Ionization suppression due to matrix is much less of a problem than with LC/MS techniques.

While GC/MS methods offer the above advantages, there are limitations with the conventional approach. As the number of target compounds in the screen increases, the size of tasks involved in the development, maintenance, and application of the methods grows very rapidly. These considerations often limit the scope of screening methods used in toxicology labs.

GC/MS methods are typically developed to analyze between 10 and 100 individual compounds. A target compound is deemed to be present if the target ion and two or three qualifier ions with specific abundance ratios fall within a defined retention time window. The identity of the target may be further confirmed by comparison of the scan at the apex of the peak with a library reference spectrum.

Matrix interferences are usually minimized by optimizing a combination of the sample preparation, GC, and MS parameters. For methods that deal with only a few matrix types, the ions chosen for identification purposes can be selected such that they are minimized in the matrix. With a limited number of targets addressed by the method, recalibration of response factors, retention times, and qualifier ion abundance ratios can be accomplished with the injection of a few calibration mixtures.

Screening methods for very large numbers of targets in varying and complex matrices offer a new set of challenges for the method developer. When screening for hundreds of targets, several factors must be addressed:

- Use of sample preparation to reduce matrix interferences is now limited because rigorous cleanup steps may unintentionally remove targets. This reduced level of cleanup can result in significantly higher levels of matrix interferences to contend with.
- Recalibration of response factors, retention times, and qualifier abundance ratios is difficult because of the large number of targets.
- The methods may be deployed in multiple laboratories without ready access to standards for all of the targets.
- The time required for data review of hundreds of targets in complex matrices can become unmanageably large.
- Even with a very large database of targets, it is possible that important compounds not in the target list could be present in a sample.

In recent years, several techniques have become available to help address the above set of challenges. Retention time locking (RTL) produces retention times that precisely match from instrument to instrument and those in a database [1]. This eliminates the need for recalibration of the individual retention times and timed events like SIM groups. The introduction of reliable and inert Capillary Flow Technology (CFT) splitters allows for the simultaneous collection of mass spectral and nitrogen/phosphorus detector (NPD) data [2]. The NPD chromatogram highlights nitrogen-containing compounds, including those not in the MS target list. It is useful in confirming the presence of a nitrogen-containing target compound and can serve as an alternative means of quantitation.

The introduction of the synchronous SIM/Scan feature allows for the simultaneous acquisition of both full-scan and SIM data from the same injection [2, 3]. The scan data can be used for screening the full list of targets in the database, while the SIM data looks for a high-priority subset of compounds (like fentanyl) down to very low levels.

One of the most significant tools developed for reducing the time required for data review is Agilent's Deconvolution Reporting Software (DRS) [4]. It uses advanced computational techniques (deconvolution) to extract the spectra of targets from those of overlapped interference peaks. It then compares the extracted spectrum with a library to determine if the target is present. If desired, hits can be confirmed by also searching against the main NIST MS reference library. The entire process is automated and provides a major time savings in data interpretation. The use of DRS also substantially reduces the number of both false positives and false negatives.

Since DRS uses the entire spectrum instead of just four ions, DRS can often correctly identify a target in the presence of interferences where the typical approach would fail. Also, since it uses the entire spectrum for identification instead of precise target/qualifier ion ratios, frequent updating of the ratios is not necessary. This is useful for targets that are rarely encountered but are still screened for.

This application describes the combination of the above techniques with a new database of 725 compounds, the Agilent G1674AA Forensic Toxicology DBL, to be used for screening purposes. The DBL contains:

- RTL methods for DB-5MS and DB-35MS columns

- Spectral libraries for DRS and the MSD ChemStation
- Preconfigured RTL methods for multiple speeds with run times of 30, 15, 10, 7, or 5 minutes, depending on hardware configuration
- Methods for both MSD direct connection (vacuum) and Capillary Flow Technology splitters (3.8 psig).
- Three quant databases included for each method:
  - Target and qualifiers are the biggest four ions.
  - Ions are optimized to give the best signal-to-noise ratio versus column bleed and background.
  - Ions are optimized to give the best signal-to-noise ratio versus common fatty acids found in blood.

The names of all the compounds in the database are listed in the appendix at the end of this application. Compounds in the DBL include drugs and select breakdown products, TMS derivatives, and acetyl derivatives. For those compounds entered as derivatives, in general, primary and secondary amino (including aliphatic and aromatic) compounds are acetylated. Hydroxyl groups (alcohols/phenols/carboxylic acids, etc.) are converted to TMS derivatives with BSTFA. Compounds having multiple functionalities (for example, phenylpropanolamine, which has a primary aliphatic amine and an alcohol) were acetylated with no further derivatization.

Methods are provided for two stationary phases to allow two-column confirmation and the ability to run other methods that require the same column on the same hardware. In general, the DB-5MS methods are preferred because the final oven temperature is lower.

The chromatographic conditions chosen for development of the database are general in nature and are compatible with the analysis of other compounds beyond those in the table. Since no one target list, no matter how large, can satisfy every lab's needs, new compounds can be added to the screen.

The retention times for compounds in the database are provided for both the column connected directly to the MSD and for the column outlet pressure at 3.8 psig using a CFT splitter. This was done to ensure that the retention times observed during sample analysis would closely match those in the database regardless of the instrument configuration.

The chromatographic conditions for the database were chosen to be compatible with Agilent's method translation technique. Constant-pressure mode was used in the GC inlet so that method translation could be used to precisely time-scale the methods for faster operation [5]. Provided with the Agilent Forensic Toxicology DBL are the files to run the analysis at precisely twofold (2x), threefold (3x), fourfold (4x), and sixfold (6x) faster than the primary database (1x). The choice of speed is determined by the degree of chromatographic resolution desired and the hardware capabilities of the GC/MSD system to be used.

For systems with a 120 V GC oven, an MSD with diffusion pump, and the column connected directly into the MSD, only 1x or 2x methods can be used. The 3x, 4x, and 6x methods require the fast oven (240 V) and performance turbopump because column flow rates exceed 2 mL per minute. Performance electronics are also preferred for the same methods. The 6x methods require both a 240 V oven and the oven "pillow" accessory to attain the 60 °C/min ramp rate. Note that use of the pillow requires that the MSD, inlet, and NPD (if used) be located in the back GC positions.

Three different versions of each method set are provided based upon the choice of ions used in the quant database. A method using the largest four ions in a compound's spectrum is supplied. The target ion is the ion with the largest abundance. The three qualifiers are the next three largest ions assigned in order of decreasing abundance. These method sets are provided for legacy reasons, and are used in some more advanced approaches.

The drawback of the largest four-ion approach is that, in some cases, the signal-to-noise performance suffers. For example, if the biggest ion for a compound is 207 and the stationary phase has its largest bleed ion at 207, the signal-to-noise ratio at that mass can be significantly reduced. The same problem is seen with low masses such as 44, where CO_2 and other background gases can result in interferences and increased noise. To reduce this problem, a second method set is provided where ions chosen for the quant database are selected to give best signal-to-noise ratios relative to column bleed and background gases. These are the methods that would normally be used, as they typically give best overall performance.

A third method type is provided where the choice of ions has been optimized for samples having large amounts of fatty acids typically seen in blood samples. These methods give the best signal-to-noise

ratios in high fatty-acid matrices. They are not the best choice for samples having low levels of interfering fatty acids.

Experimental

System Configuration

The system configuration used is shown in Figure 1. The GC is an Agilent 7890A (G3440A).

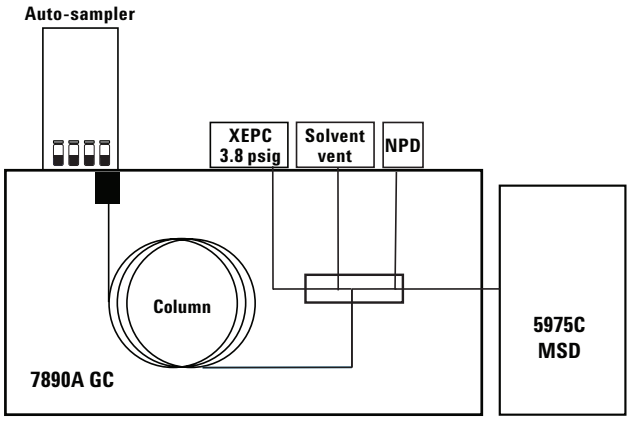

Figure 1. GC/MS/NPD system configuration used for screening blood extracts.

Key components are:

Fast Oven The primary 1x method uses a 30-m column with a 10 °C/min ramp rate and only requires the 120 V oven. With the 7890A 240 V oven (option 002), the screening method can be run up to 4 times faster using a 15-m column. If the 240 V GC is further equipped with options 199 and 202 (puts split/splitless injection port and MSD interface in the back of the oven) and uses the G2646-60500 oven insert accessory, the speed can be increased to 6 times faster (60 °C/min) with a custom length 10-m column. If an NPD is used with a splitter, option 299 places it in the back of the oven for use with the pillow.

NPD The 7890A Option 251 is a nitrogen phosphorus detector. The signal from the NPD is collected, stored, and processed by the MS ChemStation simultaneously with the MS data. NPD detectors are highly selective and exhibit very sensitive response to nitrogen and phosphorus compounds, with detection limits in the low picogram range. The NPD data can be used in several ways. Nontarget nitrogen (and phosphorus) compounds are highlighted for the data reviewer. The presence of a response at the retention time of an identified compound can be used to support confirmation of identity. The response on the NPD can be used for quantitative analysis, but only after calibration with a standard, as the response factors are compound dependent and can vary with compound class. The NPD bead is incompatible with halogenated solvents and excess silanizing reagents. If these are to be used with an NPD, the splitter setup should have solvent venting capability.

Capillary Flow Technology Splitters Agilent offers two different column effluent splitters that can be used with the 7890A for this application. Option 889 is a two-way splitter that divides the effluent of the column between the MSD and the NPD. The 7890A Option SP1 (7890-0363) does the same, but adds solvent venting capability as well. The devices are based on diffusion bonded plate technology combined with metal column ferrules to make inert, easy-to-use, leak-free, high-temperature splitters. The splitters use Auxillary EPC for constant pressure makeup (7890A Option 301). The Auxillary EPC makeup can be pressure programmed at the end of the run to higher pressure, while at the same time the inlet pressure is lowered to near ambient. This causes the flow in the column to reverse direction, backflushing heavy materials out the split vent of the inlet. Backflushing significantly reduces analysis times for samples that contain high-boiling matrix components and reduces both column head trimming and frequency of MSD source cleaning [6]. The Aux EPC also allows column changing and maintenance without venting the MSD.

For methods that use solvents compatible with the NPD and do not have silanizing reagent in the samples, the standard two-way splitter can be used. If halogenated (or other NPD incompatible) solvents or silanizing reagents are used, then the two-way splitter with solvent vent, 7890A Option SP1 (7890-0363), should be used to protect the NPD bead. This is the configuration used here.

MSD System The 5975C Inert MSD with performance turbo (G3243A) or 5973N Inert MSD with Performance Electronics and performance turbo (G2579A) EI MSD is used. These configurations provide faster full-scan rates while maintaining sensitivity. The scan rates are compatible with the narrower peaks generated by fast chromatography. The performance turbo pump is required to handle the higher flows associated with systems using splitters. It is also required for the faster versions (3x, 4x, and 6x) of the screening method with vacuum outlet (column connected directly to MSD). The standard turbo pump can be used for the slower 1x and 2x vacuum outlet versions of the method. Both the performance and standard turbos are compatible with backflushing. Backflushing cannot be done on systems with a diffusion pump.

Synchronous SIM/Scan The D.02.00 (or higher) revision of the Agilent MSD ChemStation is used because it supplies the synchronous SIM/Scan feature. SIM/Scan operates by collecting SIM data every other cycle and scan data on alternate cycles throughout the entire chromatogram. As with conventional SIM methods, not all 725 targets can be monitored in a single run due to the required time separation between SIM groups. In general, the acquisition of SIM data is set up to collect high-priority targets at very low levels. Examples would be fentanyl and phencyclidine.

DRS Software (G1716AA) Spectral deconvolution of the MS data enables identification of analytes in the presence of overlapped matrix peaks [4, 7]. This significantly reduces chromatographic resolution requirements, which allows detection of targets in higher levels of matrix or can be used with fast chromatography to shorten analysis times. DRS utilizes the AMDIS deconvolution program from NIST, originally developed for trace chemical weapons detection in complex samples. DRS presents the analyst with three distinct levels of compound identification: (1) ChemStation, based on retention time and four-ion agreement; (2) AMDIS, based on "cleaned spectra" full ion matching and locked retention time; and (3) NIST05a search using a 163,000-compound library.

G1674AA Forensic Toxicology DBL This supplies the mass spectral library, method, and DRS files for the 725 compound screening methods.

Table 1. Gas Chromatograph and Mass Spectrometer Conditions

GC	
Agilent Technologies 7890A with autoinjector and tray	
Inlet	EPC split/splitless
Mode	Constant pressure
Injection type	Splitless
Injection volume	1.0 µL
Inlet temperature	280 °C
Liner, Agilent dual-taper deactivated	P/N 5181-3315
Pressure, nominal	14.9 psig
RT locking compound	Proadifen (SKF-525a)
RT locking time	4.285 min
Purge flow	50 mL/min
Purge mode	Switched
Purge time	0.4 min
Gas type	Helium
Inlet backflush pressure	1 psig
Oven	
Voltage (VAC)	240*
Initial oven temperature	100 °C
Initial oven hold	0.25 min
Ramp rate	40 °C/min
Final temperature	325 °C
Final hold	1.25 min
Total run time	7.13 min
Equilibration time	0.5 min
Backflush time	0.5 min
Backflush temperature	325 °C
Column	
Type	DB-5MS
Agilent part number	Custom
Length	10 m
Diameter	0.25 mm
Film thickness	0.25 µm
Nominal initial flow	2.52 mL/min
Outlet pressure	3.8 psig
2-Way Splitter w/Solvent Vent	
7890A SP-1, num. 7890-0363	
MSD restictor length	0.69 m
MSD restictor diameter	0.15 mm
NPD restictor length	0.36 m
NPD restictor diameter	0.15 mm
Split ratio MSD:NPD	1.4:1
Solvent vent time range	0–0.75 min
Splitter pressure during run	3.8 psig
Splitter pressure during backflush	76 psig
NPD	
Hydrogen flow	3 mL/min
Air flow	60 mL/min
Nitrogen makeup flow	8 mL/min
Temperature	300 °C
MSD	
Agilent Technologies 5975 or 5973 inert with performance electronics	
Vacuum pump	Performance turbo
Tune file	Atune.U**
Mode	SIM/scan
Solvent delay	0.7 min
EM voltage	Atune voltage
Low mass	40 amu
High mass	570 amu
Threshold	0
TID	Off
Sampling	1
Quad temperature	180 °C
Source temperature	300 °C
Transfer line temperature	300 °C

*Injection port and MSD interface in back positions and G2646-60500 oven pillow

**Gain normalized, 1x

Instrument Operating Parameters

Data Acquisition

The instrument operating parameters used (unless noted otherwise) are listed in Table 1.

DB-5MS was chosen as the stationary phase for the current system. The final temperature required to elute the last compound in the screen is 325 °C instead of 345 °C as required with DB-35MS. This results in shorter run times and longer column life.

The method parameters were chosen to give the best trade-off between chromatographic resolution and sample throughput. For the blood samples analyzed here, the 4x method gave adequate resolution with an relatively short run time. Although the 4x method can be run on a standard 15-m column, a 10-m column was chosen because it gives very similar resolution with a lower column flow rate.

Time was also saved by using backflushing instead of post-run column baking to remove heavy sample matrix compounds. Backflushing is more effective, faster, and does not send the heavy materials and column bleed into the NPD and MSD source. With the current configuration, all heavy materials were removed from the column with a 0.5-minute backflush. The shorter column length (10 m) results in a reduced backflushing time compared to the 15-m column.

The 4x method can be run with a 240 V oven without the pillow accessory. The pillow was used here because it somewhat decreases the cooldown time of the oven and reduces the amount of electricity consumed by the instrument.

Further reduction in the cycle time of the instrument was achieved by using the overlapped injection setting in the autoinjector. With this feature, the autoinjector prepares the next sample for injection and has the syringe ready while the oven is cooling down from the current injection. This feature can save approximately 1 minute in cycle time, depending on the injection parameters used.

The simultaneous acquisition of SIM, scan, and NPD

Table 2. SIM Groups Used in SIM/Scan Mode

SIM Group (number)	Start Time (min)	Compound	RT (min)	Target (amu)	Q1 (amu)	Q2 (amu)
1	0	Amphetamine	0.900	44	91	65
2	0.97	Methamphetamine	1.050	58	91	65
3	1.5	Methylenedioxyamphetamine(MDA)	1.978	136	135	51
4	2.06	Methylenedioxymethamphetamine(MDMA)	2.147	58	135	77
4		Ecgonine methyl ester	2.222	94	82	96
4		Ethylecgonine	2.223	94	82	96
5	2.52	Meperidine	2.826	246	218	247
6	2.96	Ketamine	3.138	180	182	209
6		Phencyclidine	3.249	243	242	200
6		Tramadol	3.389	58	263	59
7	3.64	Methadone	3.866	72	57	165
7		Dextromethorphan	3.895	271	212	270
8	3.98	Cocaine	4.042	182	82	94
8		Cocaethylene	4.175	196	82	94
9	4.53	Diazepam	4.598	258	286	257
9		Tetrahydrocannabinol	4.666	299	300	231
9		6-Acetyl-morphine	4.773	268	327	328
10	4.85	Oxycodone	4.801	315	230	115
10		Temazepam	4.922	271	273	272
10		Diacetylmorphine	4.992	310	268	327
10		Fentanyl	5.177	245	146	189
11	5.25	Zolpidem	5.332	235	236	219
11		Clonazepam-M (amino-)	5.433	285	258	286
12	5.53	Alprazolam	5.630	308	279	280
12		Zaleplon	5.695	305	263	248
13	5.8	Zopiclone	5.905	112	99	139
13		Lysergide (LSD)	6.000	323	324	222

(all dwell times 5 msec)

data save a substantial amount of time compared to acquiring them in separate runs. The compounds and corresponding SIM groups monitored are listed in Table 2. Because the peaks in the 4x method are relatively narrow, the dwell times for SIM ions were set to 5 milliseconds.

By using the above time-saving steps, the cycle time from injection to injection is 9.6 minutes.

Data Analysis

Based on experience with analyzing 50 blood extracts, a data analysis scheme evolved that incorporated the DRS, SIM and NPD data.

The resulting data review scheme consisted of the following:

- Deconvolution results were generated with DRS and reviewed to determine compounds present. The AMDIS minimum match factor was set to 50. Any compounds with match factors less than 65 or retention time differences greater then 4 seconds were considered suspect (for example, not present unless other data like target/qualifier ratios supported presence). For suspect identifications, the NPD signal was inspected to see if there was a corresponding response of the same peak shape and retention time. If the suspect compound is nitrogen containing (as the vast majority of the compounds in the table are), NPD response provided evidence supporting the presence of the compound.
- Compounds identified by AMDIS but not found by the MSD ChemStation because of out-of-range qualifiers were manually inspected in QEdit. Quantitation was forced if AMDIS indicated an acceptable spectral and retention time match.
- A separate ChemStation data analysis method was used to review the SIM results for the 27 compounds listed in Table 2. Since SIM can detect compounds lower than can be confirmed with spectral data, identification relied on target/qualifier ion ratios and NPD data.
- The NPD trace was examined to find any larger peaks that did not correspond to identified targets. The deconvoluted spectra at the retention time of these peaks were searched against the NIST 05a library. As a practical matter, uncorrelated small NPD peaks were not pursued as they are numerous and the signal-to-noise ratio of the corresponding scan data is too small to be useful.

Except where otherwise indicated, the 4x method supplied with the ions optimized against column bleed was used for ChemStation data analysis . The approximate response factors supplied with the method were adjusted using a standard of 5 ng/uL of proadifen (the locking compound). The responses of all compounds in the quant database were multiplied by the factor required to make the calculated result for the proadifen standard equal 5 ng/µL. This allows the concentration of an identified target to be estimated if the compound has not been individually calibrated.

The approximate response factors supplied with the method are only intended to give a rough estimate of the concentration of uncalibrated analytes. Since valid quantitation requires recent recalibration of response factors on the specific instrument used for analysis, the estimated concentration should never be used to report quantitative results. The error in these values can easiliy be a factor of 10 or higher. The purpose of the estimated values is to give an approximate amount that can be used to guide standard preparation for quantitative calibration of the compound, if needed. Individual calibration should be used for all reported analytes.

The SIM data analysis method for the 27 compounds was constructed using the target and first two qualifier ions from the 4x fatty acid optimized method. This was to minimize interference from the matrix in the blood samples.

The peak recognition windows used in the MSD ChemStation were set to ± 0.150 minute for the scan data, ± 0.075 for the SIM data, and ± 6 seconds in AMDIS. These values were found to be sufficiently wide enough to allow for some retention time drift, yet narrow enough to minimize the number of false positives.

For comparison purposes, the data were also analyzed with two conventional data review approaches.

The first approach is the standard quantitation software, where the EIC of the target ion for each compound in the quant database is extracted and integrated. If a peak is detected within the peak recognition time window, the ratios of the qualifiers to the target are measured. Several optional forms of reporting are available. The reports used here were 1) report only compounds with a peak detected in the target ion EIC and that have all qualifiers within the acceptable range for ratios, and 2) report all compounds with a peak detected in the target ion EIC, regardless of qualifier status. The results of a report can then be reviewed in QEdit, where the EICs of the extracted target and qualifier

ions are overlayed for ease of inspection. The reference spectrum for the compound and the apex spectrum for the quant peak being examined are also displayed. Based on inspection of the EICs and spectra, the reviewer can include or exclude the compound from the report.

The second data review approach was to use the ChemStation Screener software. This is almost identical to QEdit, except that it also reports a cross-correlation value (XCR) of the apex spectrum for peak versus the reference library. The XCR value is an indication of spectral match quality and can be used as an additional parameter with which to locate targets. Screener has report options similar QEdit, and the same two types were used here. Note that Screener is a qualitative tool; compounds identified in Screener must then be quantified in QEdit.

Samples

Whole blood extracts prepared for GC/MS analysis were supplied by NMS Labs (Willow Grove, PA). The whole blood was prepared with a single step liquid/liquid extraction into a solvent, evaporated to dryness, and reconstituted in toluene at 1/10th volume.

Results and Discussion

Figure 2A shows the chromatographic results from one of the blood extracts, the simultaneously acquired scan, SIM, and NPD signals. The traces make the sample look deceptively simple. Figure 2B shows the same Scan TIC and NPD signals with the scales expanded. More than 400 individual compounds are in these chromatograms when low-level responses are included.

The data from the sample were reviewed with the conventional approaches. The first report with the standard quantitation software listed compounds where all qualifier-to-target ratios were within the rather generous 50% relative limits used here. Without manual review of the 28 compounds reported, 22 were false positives; that is, they were not really present. Of the 11 target compounds actually in the sample, this report only found six of them, leaving five as false negatives.

As this situation is not uncommon, it is usually necessary to have all compounds reported that have a response at the target ion, regardless of the qualifier ratio status. These “maybes” must then be manually reviewed in QEdit. Since the integrator must be set to capture very small peaks, there are large numbers of reponses due to integration of baseline

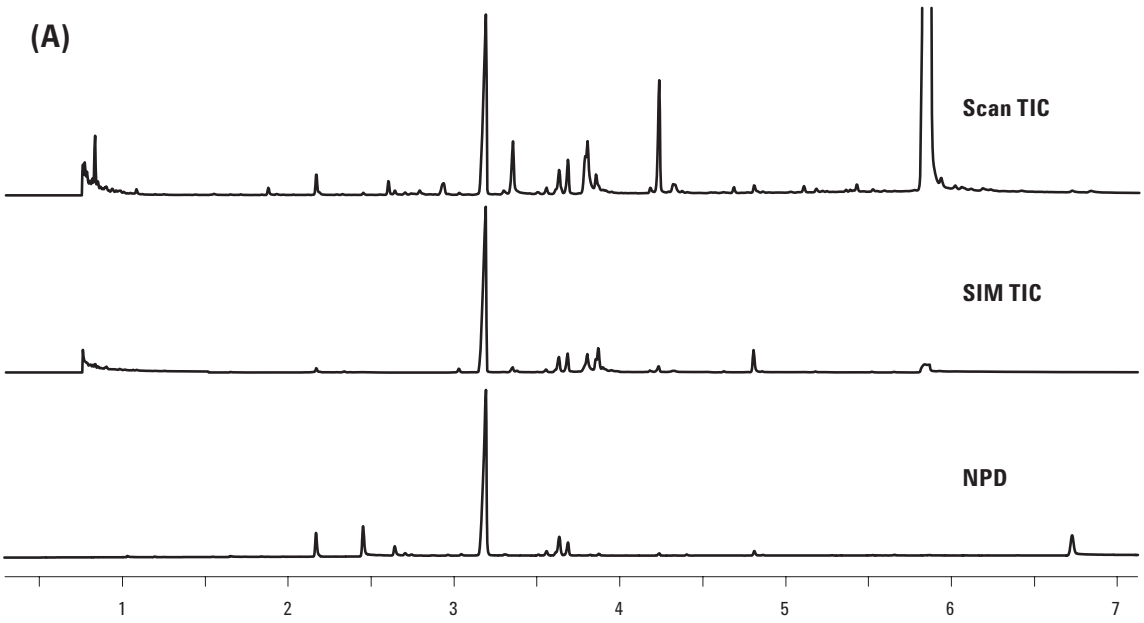

Figure 2A. Chromatograms of scan, SIM, and NPD signals from analysis of blood extract.

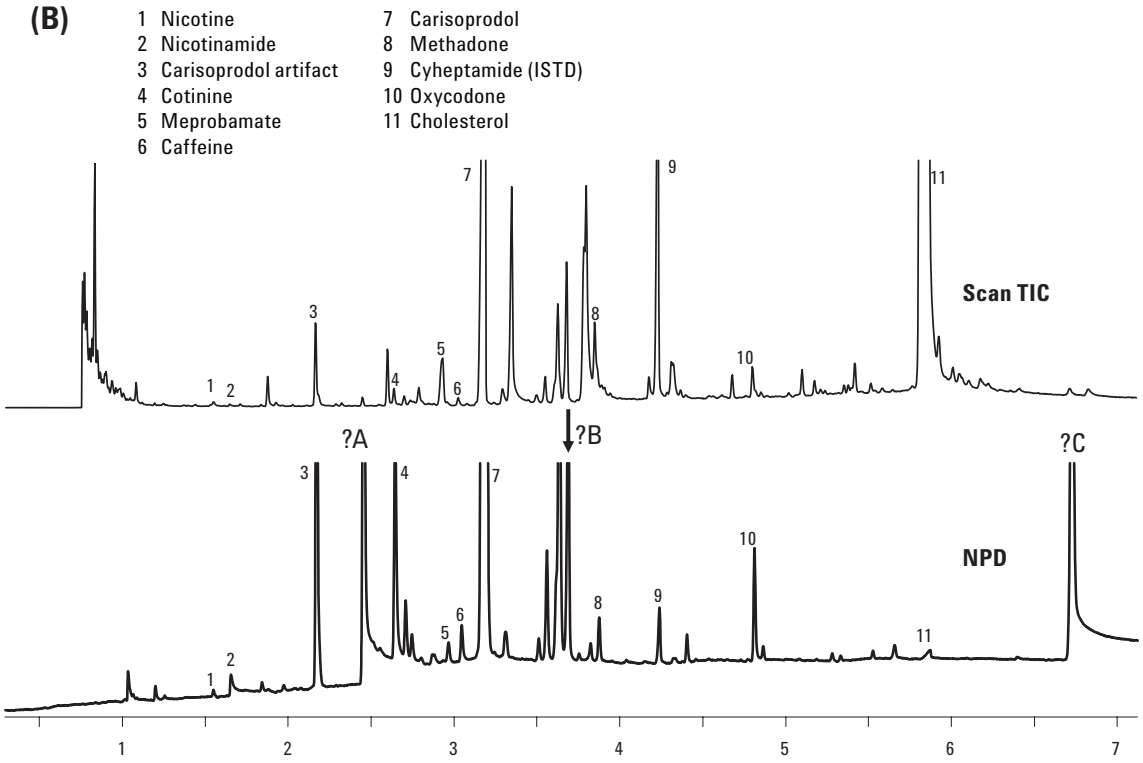

Figure 2B. Expanded scale chromatograms of scan TIC and NPD signals from analysis of blood extract. (continued)

noise. For the sample here, 367 compounds were reported found (that is, there was a response at the target ion). Of those, 356 were false positives. All 11 compounds actually present were found, so there were no false negatives. Thus, to avoid false negatives, the reviewer must manually evaluate 367 compounds to find the 11 present.

The data from the sample were then evaluated with the ChemStation Screener software. As expected, Screener reports based only on ion target/qualifier ion ratios gave very similar results to QEdit. The only way to avoid false negatives is to evalute hundreds of target ion responses to find the 11 actually present.

In an attempt to reduce the number of false positives requiring evaluation, the Screener report listing all 273 compounds with a target ion response was sorted by the XCR in descending order. Several of the compounds actually present were clustered near the top of the list. However, the target actually present with the lowest XCR value was the 162nd compound in the list. This result suggests that XCR improves the likelihood of correctly locating target compounds, but will still result in false negatives without close inspection of all of the compounds with a target ion response.

For the types of samples discussed here, correctly identifying the targets present with the conventional approach is one of the most time-consuming steps in the entire analytical process. This is why the use of deconvolution and DRS is so useful.

When this same sample was evaluated with the DRS software, 12 compounds were reported by AMDIS with a match factor for the deconvoluted spectrum greater than 50 and with retention times within 6 seconds of the locked retention time. After reviewing the 12 listed compounds, one was removed because its match factor was too low. All 11 compounds actually present were identified, with only one false positive included. The entire DRS and review process to correctly locate the targets actually present required about 5 minutes instead of more than an hour using either the QEdit target only or Screener approaches. With the compounds present in the sample identified by DRS, the final report was generated after using QEdit to quantify the targets.

MSD Deconvolution Report
Sample Name: CA5995
Date File: C:\msdchem\1\Appnote\FT5_4 x 10m_SamplesSimScan\CA5995_mss.D
Date/Time: 11:39 AM Wednesday, Apr 2 2008

The NIST library was not searched for the compounds that were found in the AMDIS target library.

			Agilent	AMDIS	
R.T.	CAS #	Compound Name	ChemStation Amount (~ng)	Match	R.T. Diff. sec.
1.539	54115	Nicotine	0.03	59	–0.5
1.6446	98920	Nicotinamide	0.27	93	–0.9
2.1631	999401024	Carisoprodol artifact	64.87	93	–0.5
2.6367	486566	Cotinine	1	96	–0.4
2.928	57534	Meprobamate	4.11	99	0.0
3.033	58082	Caffeine	0.04	82	–0.5
3.1832	78444	Carisoprodol	127.4	96	1.0
3.8653	76993	Methadone	0.39	74	–0.1
4.2279	7199293	Cyheptamide	22.5	98	0.1
4.8014	76426	Oxycodone	2.37	82	0.0
5.850	57885	Cholesterol	922.73	97	3.4

Figure 3. DRS report for the analysis in Figure 2.

Figure 3 shows the DRS report for the sample. For each compound identified, the retention time (R.T.), Chemical Abstracts number (CAS#), and compound name are listed. A line is generated in the report if a compound is found by the Agilent ChemStation, AMDIS, or both.

The report shows that a compound has been determined as present by the Agilent ChemStation if a value appears in the Agilent ChemStation Amount column. This means that the identification criteria set in the DATA ANALYSIS section of the method have been met. Typically the criteria are that the target ion is present (and integrated) and all three qualifier ions are present in ratios that fall within the percent uncertainty values for that compound. The compound would also appear here if the data reviewer manually forced integration of the target ion.

The match value listed under the AMDIS column is the degree to which the extracted (deconvoluted) spectrum of the peak at that RT matched the spectrum in the AMDIS target library. The higher this number (out of a possible 100), the better the spectra agree. The column "R.T. Diff. sec." lists the difference in seconds between the observed RT and that in the AMDIS target library. The lower this number, the better the RTs agree.

An optional third feature of the report is the NIST search column (not shown). The NIST column lists the reverse match quality of the extracted spectrum compared with the NIST main library spectrum with the same CAS#. With the present setup, there are a large number of compounds for which a CAS# is not available. The Forensic Toxicology DBL contains some contrived CAS#s that would not be found in the NIST library. In the present analysis, the NIST search feature is therefore turned off.

Also shown in the NPD trace in Figure 2B are three peaks labeled ?A, ?B, and ?C. These three relatively large peaks are not in the target list of 725 compounds. The deconvoluted spectra corresponding to each of the three NPD responses were found in AMDIS and searched against the main NIST library. Peak ?A was identified as tributyl phosphate, a phosphorus compound commonly found as a sample handling artifact. Peak ?B was identified as 10,11-dihydrodibenz(b,f)(1,4)oxazepin-11-one. It was later found to be a second internal standard added during sample preparation. Peak ?3 remains unidentified. It is not in the NIST 05a Library (the best hit was only a 38 match) and it appears in many samples.

It is instructive to go through the identification of some of the compounds in the report and look at

the details of the identifications made. Oxycodone was readily identified because it had a high match quality in the AMDIS column and a very small retention time difference. Figure 4A shows the extracted ion chromatograms (EIC) as seen in QEdit. All the ions are clearly visible without interference and the ratios of the qualifier ions to the target are within the acceptable range. Also shown are the SIM ion EICs. They also are clearly visible without interference and the ratios of the qualifier ions to the target are within the acceptable range. The bottom trace from the NPD in Figure 4A shows a response with the same shape and at the same time as the oxycodone response in the mass traces. Figure 4B compares the deconvoluted spectrum found at the oxycodone retention time with the target library reference spectrum of oxycodone. The match is very good, with a match factor of 82. Oxycodone was an easy identification with all parameters clearly pointing to its presence.

Figure 5 shows a situation that is a bit more challenging. The compound here is methadone, whose spectrum has one large ion at 72; the remaining ones are very small. The EICs in Figure 5A are from

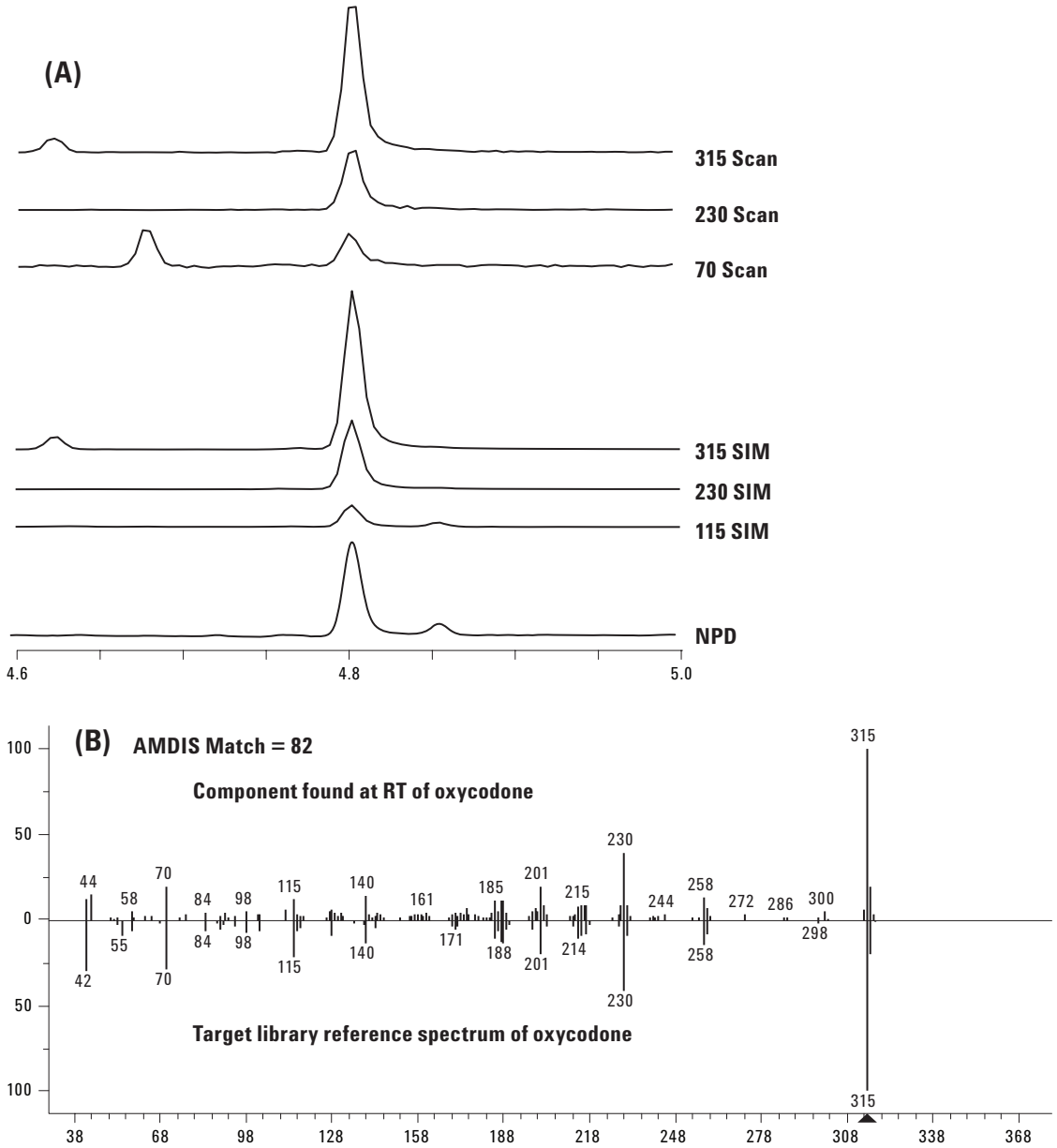

Figure 4. (A) Oxycodone response in SIM, scan, and NPD signals collected simultaneously. (B) Comparison of deconvoluted oxycodone spectrum with target library reference spectrum.

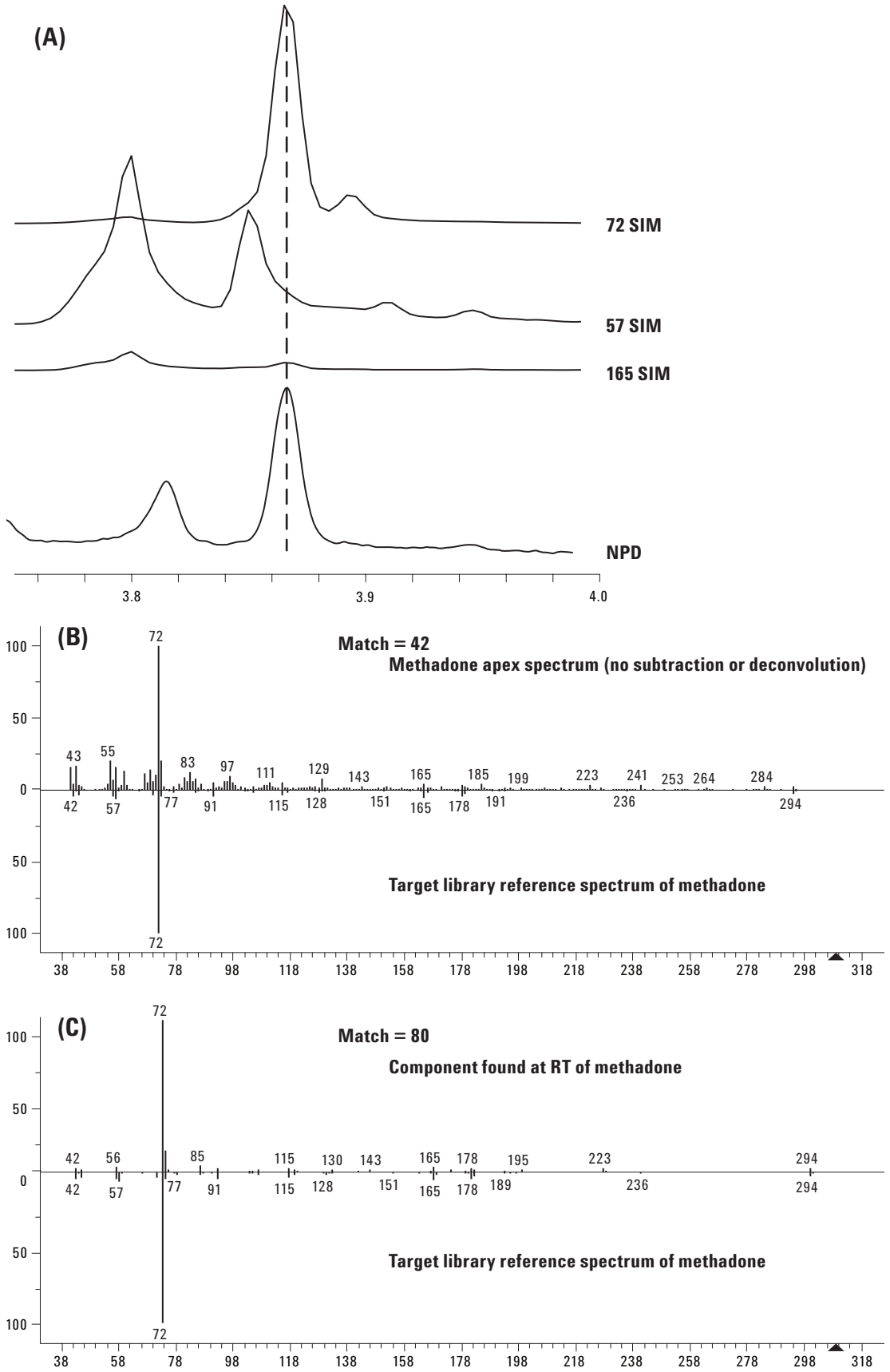

Figure 5. **(A) Methadone SIM and NPD chromatograms.**
(B) Comparison of reference spectrum with methadone spectrum without subtraction or deconvolution.
(C) Methadone deconvoluted spectrum searched against target library.

the SIM data. The traces from the scan data were identical (except of course with a lower signal-to-noise ratio). While there is a clear peak at the target ion, the middle qualifier (57) has a significant interference from the overlapping octadecanoic acid peak. With only the EIC data, the identification is questionable due to the loss of one of the qualifiers to interference. The NPD response shown below the SIM traces does support the fact that there is a nitrogen-containing compound at that retention time.

Figure 5B shows the apex spectrum at the methadone peak without subtraction or deconvolution compared with the target library reference spectrum. The match quality is unacceptably poor at 42 due to the interference of the octadecanoic acid peak. While the 72 ion is clearly visible, the other methadone ions are obscured. In Figure 5C the deconvoluted spectrum from the methadone retention time is compared with the reference. Deconvolution successfully removed the octadecanoic acid interference, and now the match quality is 80, clearly indicating the presence of methadone in the sample. The indication of methadone is also supported by two of the three ions being clearly present and in the correct ratio as well as an NPD response with the same retention time and peak shape.

Although caffeine is not a particularly high-priority target compound, the example shown in Figure 6 is

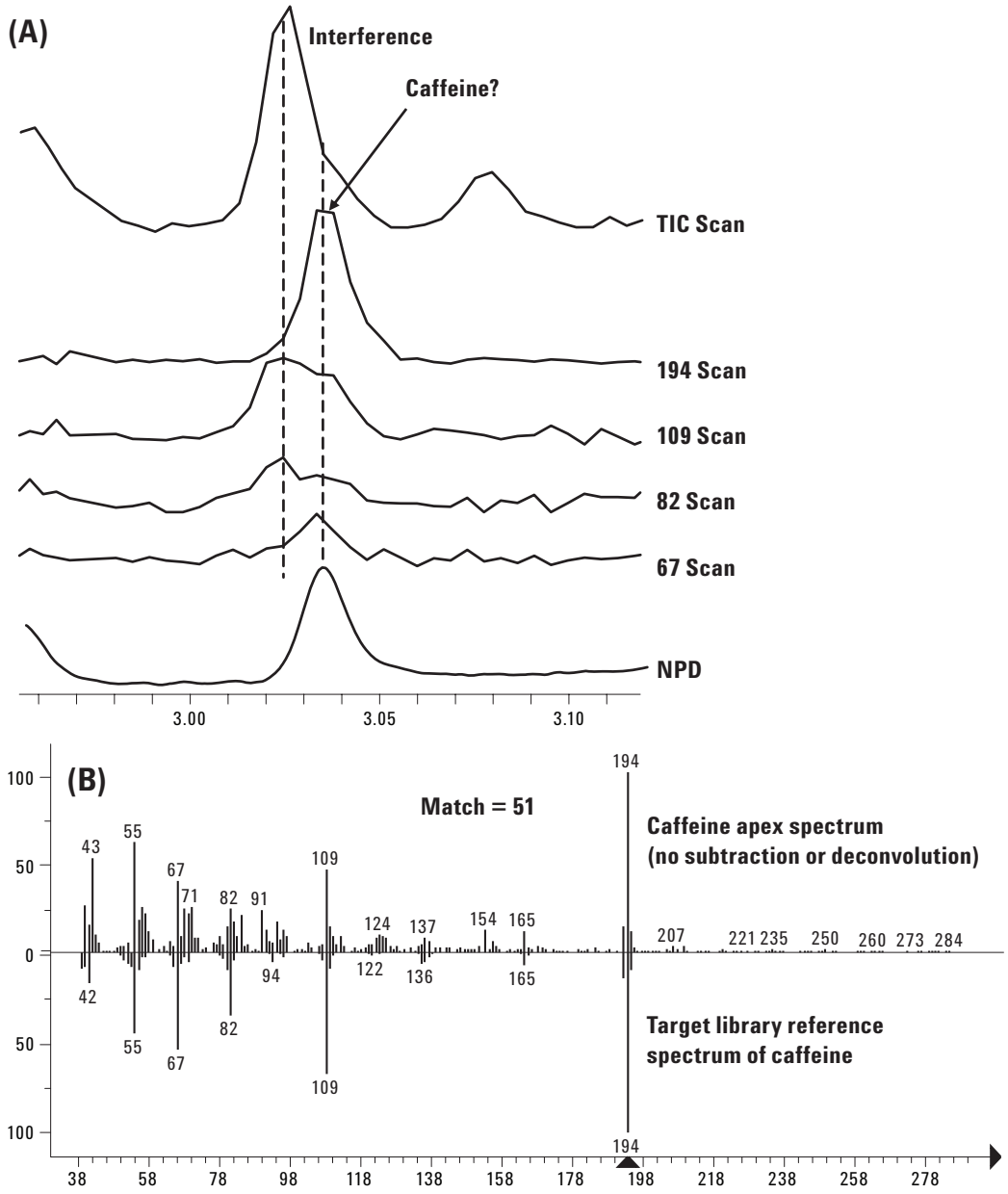

Figure 6. (A) TIC, scan EICs, and NPD signals for caffeine.
(B) Caffeine spectrum without subtraction or deconvolution shows interference from matrix compound.

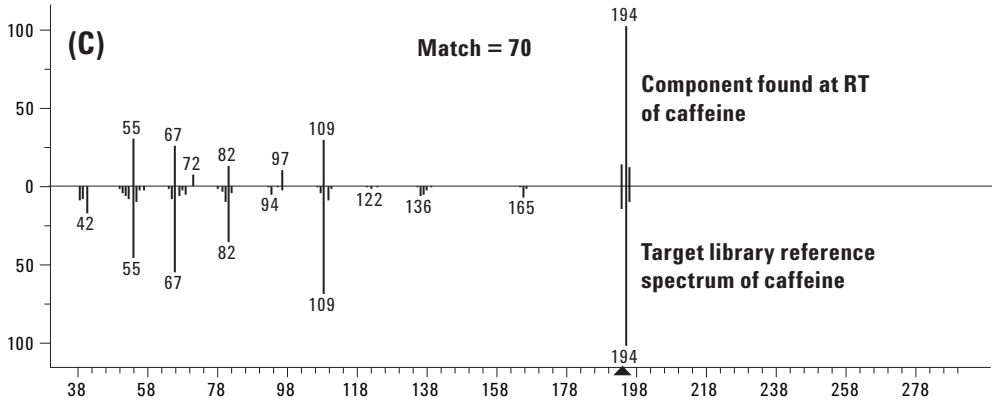

Figure 6C Caffeine deconvoluted spectrum searched against target library. (continued)

instructive. The caffeine, if present, is at a very low level as seen from the low signal-to-noise ratio of the four scan EICs shown in Figure 6A. Two ions, 109 and 82, also have interference problems from a large overlapping peak, as shown in the TIC trace at the top. The NPD trace does indicate a nitrogen-containing compound with the same peak shape and retention time as caffeine. The interfering peak was identified as 6,10,14-trimethyl-2-pentadecanone by searching the deconvoluted spectrum against the NIST main library. This compound also shares ions 109 and 82 with caffeine, resulting in the interference.

Figure 6B shows the apex spectrum of the caffeine peak without subtraction or deconvolution. When compared to the reference spectrum of caffeine, the match quality is poor, at only 51. Figure 6C shows the deconvoluted spectrum at the caffeine retention time compared to the reference spectrum and now the match quality is significantly improved to 70. This example demonstrates that the deconvolution process works even on small peaks with a low signal-to-noise ratio.

The example in Figure 7 is taken from a different sample and its purpose is to show the limits of deconvolution compared to the limits of the conventional approach. They are in fact similar because both approaches are limited by the same thing: signal-to-noise ratio. Figure 7A shows the scan and SIM EICs and the NPD trace for alprazolam. In the scan data, three of the four ions are barely visible and the fourth is lost in the noise. The SIM data clearly show a peak present at the alprazolam retention time and the ratios are in the correct range. The NPD also shows a response at the same retention time and with a similar shape. Figure 7B shows the deconvoluted spectrum compared to the NIST 05a library spectrum of alprazolam. The match factor is only 57.5. The match is marginal because AMDIS could only find a fraction of the alprazolam ions due to the extremely low level of the compound. This again illustrates that the target/qualifier approach using scan data and deconvolution begin to fail at about the same signal-to-noise ratio. In this example, the SIM data and NPD data are very helpful. If only the scan data were available for this sample, the identification of alprazolam would be doubtful and probably not reported. Taken with the SIM data in the correct ratios and the supporting evidence of the NPD response, a much stronger case can be made that alprazolam is indeed present, although at a very low level.

The last example is from a sample containing extraordinarily high levels of fatty acid interferences. These are clearly visible in Figure 8A. In QEdit, the presence of meprobamate was indicated with the peak shown at 3.007 minutes in Figure 8B. Although the ratios of the qualifiers to the target ion were within the relatively wide windows used here, the identification was doubtful. Examination of the EICs shows what looks like multiple peaks at the retention time that QEdit found. The retention time was also farther away (+ 0.080 minute) from the expected retention time of 2.928 minutes than is typically seen with the method. Also, there is no clear peak shape evident in the four traces at the 3.007 retention time. Based on these results alone, meprobamate looks like a false positive.

The EIC traces shown were from the column bleed optimized method. The use of 83 as the target ion clearly has interference problems with the high-level of fatty acids in this sample. When the method with

fatty acid optimized ions was used, the picture became a bit clearer. In this method, ion 62 is used as the target because of its significantly lower degree of interference. Looking at the trace for ion 62 in Figure 8, the peak now appears at 2.948 and is much closer to the expected retention time at 2.928 minutes. While the response at ion 62 looks a bit more like a real peak, the other ions in the fatty acid optimized method were still questionable due to the degree of interference, suggesting that it still may be a false positive. The NPD trace (not shown) did not resolve the question, as there were NPD peaks near 2.928 and 3.007 minutes.

The question was easily settled using the new A.04 release of DRS software. This version allows you to import into QEdit the AMDIS extracted peak profile from the deconvolution data and overlay it with the QEdit EICs. It also imports the deconvoluted spectrum for comparison with the QEdit-subtracted spectrum and the library reference spectrum. These capabilities simplify the review process by showing the deconvolution information inside of QEdit. Inspection of the AMDIS extracted peak profile relative to the EICs of the scan data shows that in fact the response at the target found with the fatty acid optimized method is indeed meprobamate. The

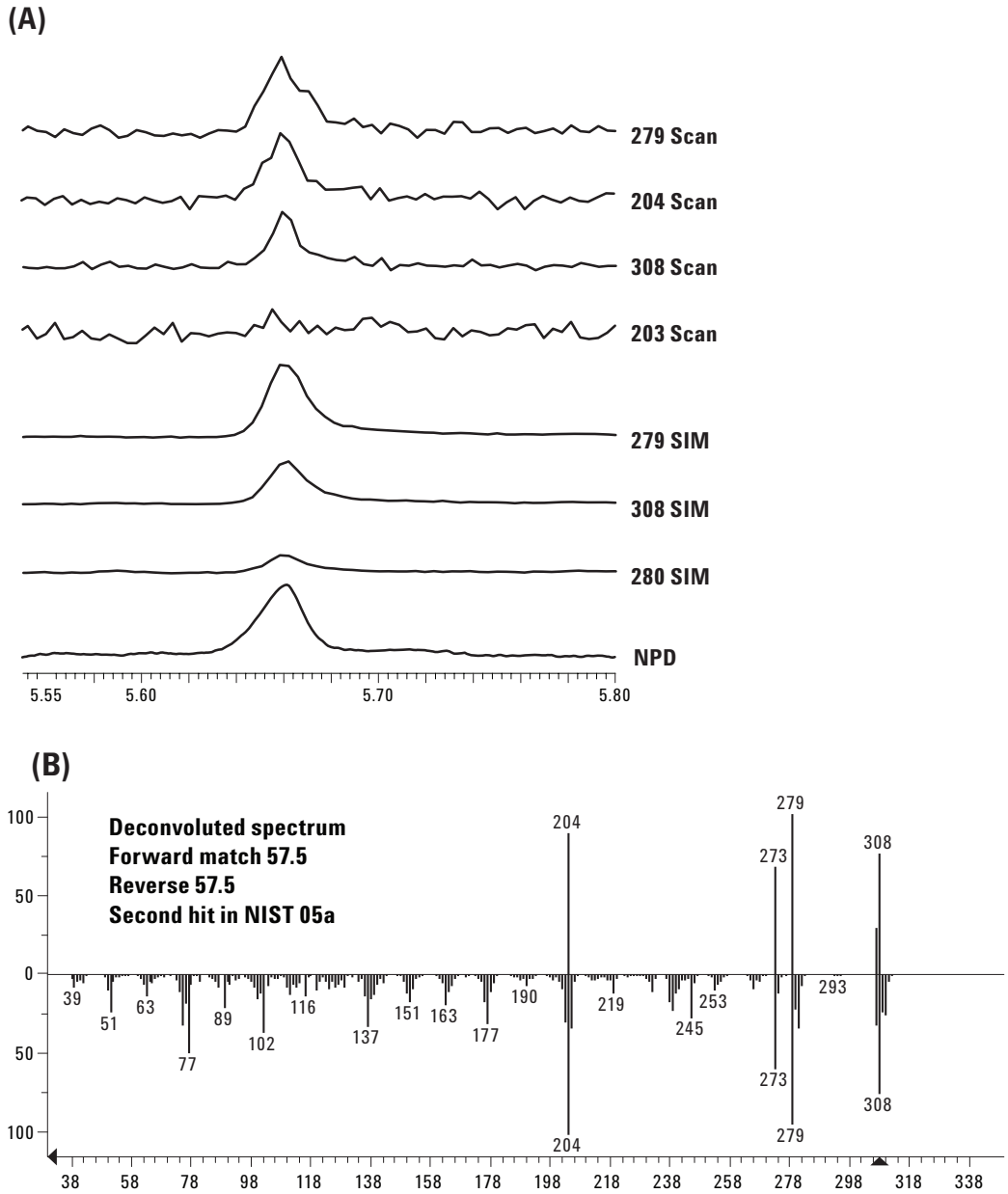

Figure 7. (A) Alprazolam response on SIM, scan, and NPD signals.
(B) Alprazolam deconvoluted spectrum searched against NIST 05a library.

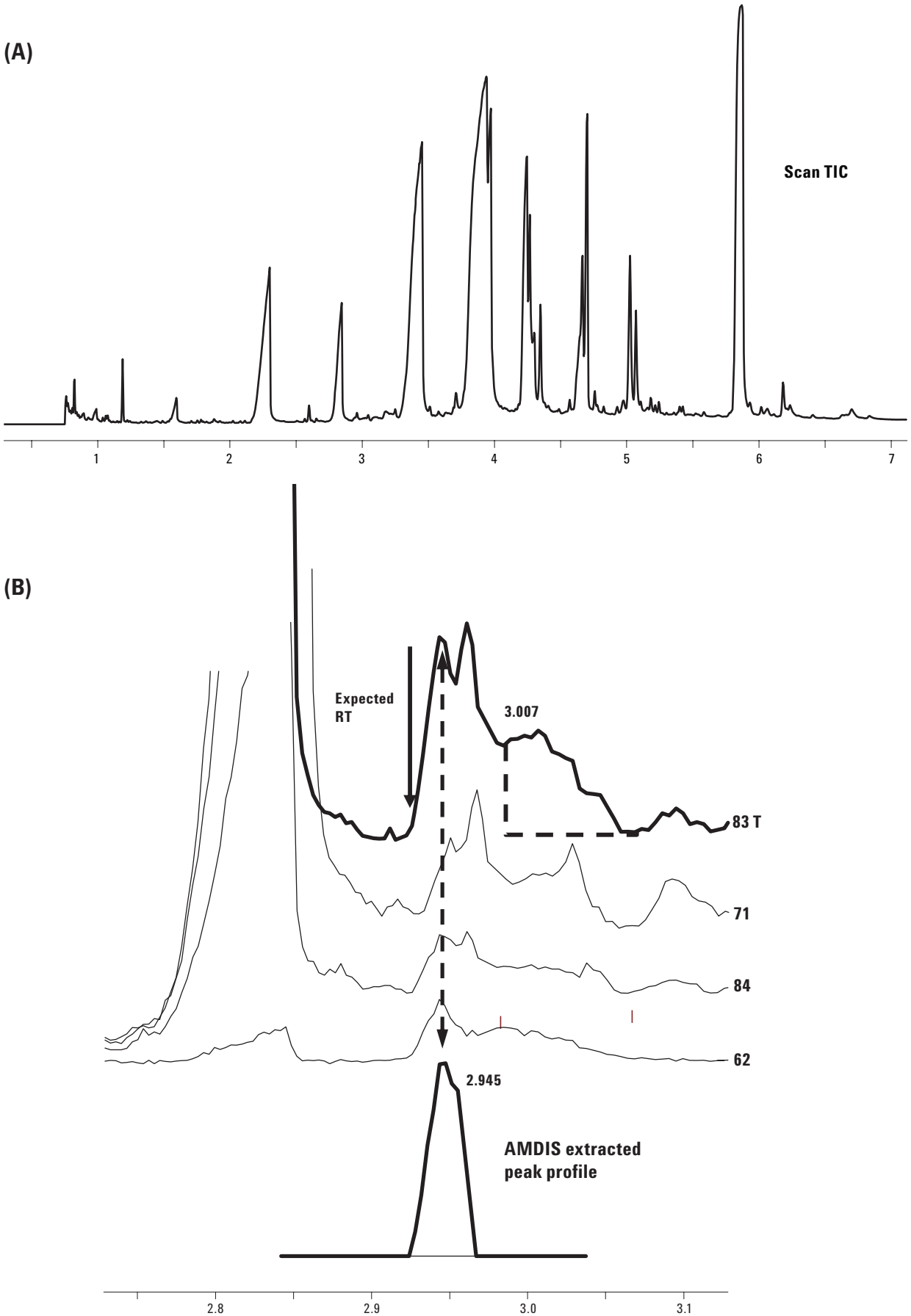

Figure 8. **(A) Scan TIC chromatogram of sample with high levels of fatty acids.**
(B) Scan EICs from bleed optimized method overlayed with AMDIS extracted peak profile.

(C)

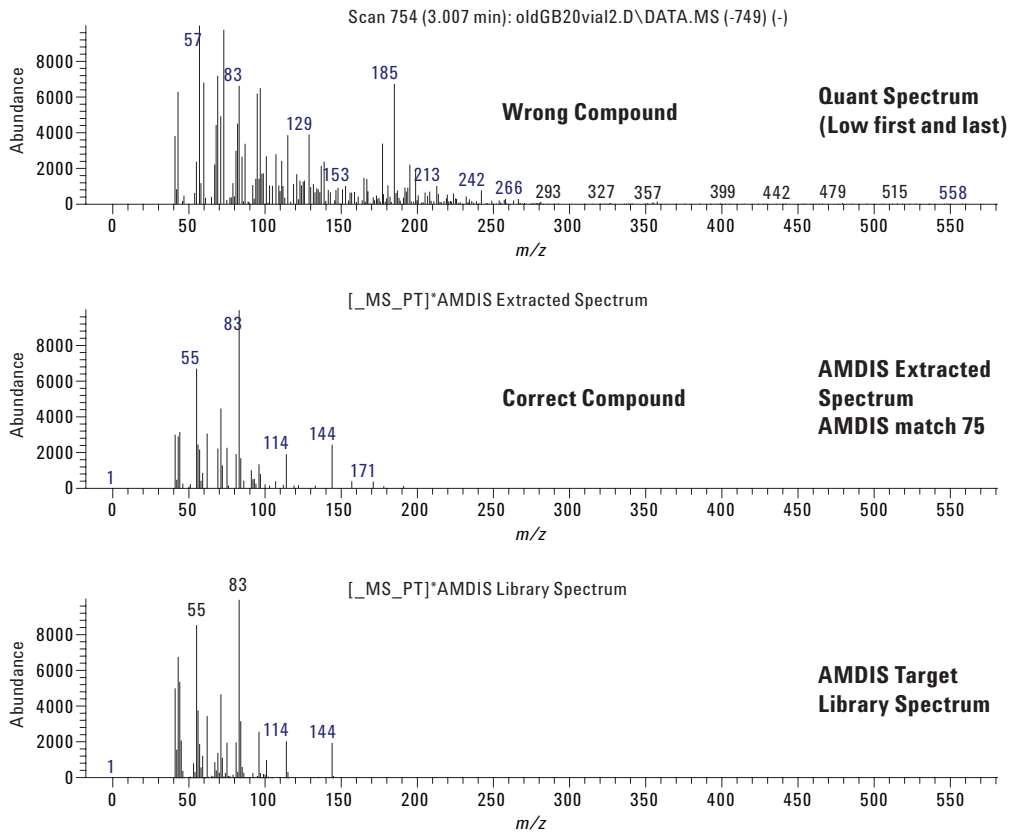

Figure 8C. Three meprobamate spectra presented in QEdit for comparison during data review using DRS A.04. (continued)

AMDIS extracted peak profile looks very similar to the peak profile in ion 62. If desired, the AMDIS extracted peak profile can be integrated for quantitation if the target ion has interference problems.

The best confirmation is provided by the deconvoluted spectrum. In Figure 8C are the three spectra presented in QEdit for comparison. The three spectra shown here were from the bleed optimized method. This method had incorrectly chosen the 3.007 peak as possibly being meprobamate, where the topmost spectrum is the spectrum at 3.007 minutes minus the spectrum five scans before, as the method uses “lowest first and last” as the subtraction method. Since the peak was found at the wrong retention time, the spectrum is of the wrong compound and of course does not match that of meprobamate. When searched against the NIST main library, meprobamate was not in the top 100 hits.

The middle spectrum is the deconvoluted component found by AMDIS. It has a match factor against the reference spectrum, shown in the bottom, of 75, confirming the presence of meprobamate. This example shows the utility of deconvolution in determining the presence of compounds that could easily be missed with the conventional approaches.

Conclusions

The system described here offers several advantages for screening toxicology samples. The advantages derive from a combination of techniques that result in both faster and more accurate screening results.

- Retention time locked target database of 725 compounds for screening with MS (G1674AA Forensic Toxicology DBL)

- CFT splitter – Use the NPD with MS data for added confirmation, find nontarget suspect compounds, and alternate quantitation
- SIM/Scan – Acquire SIM data on high-priority targets simultaneously with scan data. Saves time by eliminating need to run samples in both modes.
- DRS – Automated deconvolution increases accuracy of target identification, even in the most challenging matrices. The reduction of data interpretation from more than an hour to less than 10 minutes is especially useful.
- Fast chromatography using shorter columns, faster ovens, and backflushing to greatly reduce run times.

There is considerable advantage to using a single system that combines all of the techniques discussed. However, adding any of the above separately or in different combinations can also provide advantages. The most significant improvement can be gained by using DRS. The time savings in the data review step easily justifies the effort required to implement it.

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Appendix

Compound name	CAS number*
10,11-Dihydro-10-hydroxycarbazepine	999402-02-7
10,11-Dihydro-10-hydroxycarbazepine TMS	999423-02-8
10,11-Dihydrocarbamazepin	003564-73-6
5-Amino-2-chloropyridine	005350-93-6
5-Methoxy-dipropyltryptamine	999001-02-4
6-Acetyl-morphine	002784-73-8
6-Acetyl-morphine TMS	999155-02-1
7-Aminoflunitrazepam	034084-50-9
7-Aminoflunitrazepam TMS	999176-02-2
7-Hydroxyamoxapine	037081-76-8
8-Methoxyloxapine	070020-54-1
Acepromazine	000061-00-7
Acetaminophen	000103-90-2
Acetaminophen 2TMS	055530-61-5
Acetanilide	000103-84-4
Adiphenine	000064-95-9
Adiphenine-M/artifact (ME)	003469-00-9
Alfentanil	071195-58-9
Allobarbital	000052-43-7
Allopurinol TMS	999178-02-8
Alphaprodine	000077-20-3
Alphenal	000115-43-5
Alprazolam	028981-97-7
Alprenolol TMS	999381-02-1
Alverine	000150-59-4
Amantadine	000768-94-5
Amantadine AC	999127-02-5
Ambroxol	018683-91-5
Ambroxol 2AC	999341-02-5
Aminoglutethimide	000125-84-8
Aminopyrine	000058-15-1
Amitriptyline	000050-48-6
Amlodipine AC	999299-02-4
Amobarbital	000057-43-2
Amobarbital 2TMS	999179-02-1
Amoxapine	014028-44-5
Amoxapine AC	999128-02-8
Amphetamine	000060-15-1
Amphetamine AC	999107-02-7
Ampyrone	000083-07-8
Ampyrone AC	000083-15-8
Ampyrone-2AC	999240-02-7
Anhydroecgonine methyl ester	043021-26-7
Anileridine	000144-14-9
Anisindione	000117-37-3
Antazoline	000091-75-8
Antazoline AC	999408-02-5
Antipyrine	000060-80-0
Apomorphine 2TMS	074841-68-2
Aprobarbital	000077-02-1
Aprobarbital 2TMS	999180-02-8
Atenolol formyl artifact	999459-02-8
Atomoxetine	083015-26-3
Atomoxetine AC	999257-02-2
Atovaquone	953233-18-4
Atovaquone TMS	999409-02-8
Atropine	000051-55-8
Atropine TMS	055334-03-7
Azacyclonol	000115-46-8
Azatadine	003964-81-6
Barbital	000057-44-3
BDMPEA	066142-81-2
BDMPEA AC	999357-02-7
BDMPEA formyl artifact	999378-02-8
Bemegride	000064-65-3
Benzocaine	000094-09-7
Benzoylecgonine	000519-09-5
Benzoylecgonine TMS	999462-02-1
Benzphetamine	000156-08-1
Benzquinamide	000063-12-7
Benztropine	000086-13-5
Benzydamine	000642-72-8
Benzylpiperazine	002759-28-6
Benzylpiperazine AC	999129-02-1
Betahistine	005579-84-0
Betahistine AC	999439-02-0
Betaxolol	063659-18-7
Betaxolol formyl artifact	999436-02-1
Biperiden	000514-65-8
Bisacodyl	000603-50-9
Bisoprolol	066722-44-9
Bromazepam	001812-30-2

* Compounds for which a real CAS number could not be found were given a contrived one beginning with 999. These are not real CAS numbers.

Compound name	CAS number
Bromazepam TMS	999158-02-0
Bromdiphenhydramine	000118-23-0
Bromocriptine breakdown	025614-03-3
Bromperidol	010457-90-6
Brompheniramine	000086-22-6
Brucine	000357-57-3
Buclizine	000082-95-1
Bupivacaine	002180-92-9
Buprenorphine	052485-79-7
Buprenorphine TMS	999159-02-3
Bupropion	034911-55-2
Buspirone	036505-84-7
Butabarbital	000125-40-6
Butabarbital 2TMS	052988-92-8
Butacaine	000149-16-6
Butalbital	000077-26-9
Butalbital 2TMS	052937-70-9
Butethal	000077-28-1
Butorphanol	042408-82-2
Butorphanol TMS	100013-72-3
Caffeine	000058-08-2
Canrenone	000976-71-6
Canrenone TMS	999413-02-4
Cantharidin	000056-25-7
Carbamazepine	000298-46-4
Carbamazepine-M (formyl-acridine)	999243-02-6
Carbinoxamine	000486-16-8
Carbromal-M/artifact	999196-02-0
Carisoprodol	000078-44-4
Carisoprodol artifact	999401-02-4
Cathinone AC	999485-02-8
Celecoxib	169590-42-5
Cetirizine methanol adduct	083881-46-3
Cetirizine TMS	999183-02-7
Chlophedianol	000791-35-5
Chlophedianol TMS	999464-02-7
Chloramphenicol 2TMS	021196-84-9
Chlorcyclizine	000082-93-9
Chlordiazepoxide	000058-25-3
Chlordiazepoxide artifact (desoxo)	999197-02-3
Chlormezanone	000080-77-3
Chlormezanone artifact	999245-02-2
Chloroamphetamine	000064-12-0
Chloroamphetamine AC	999414-02-7
Chlorophenylpiperazine	038212-33-8
Chlorophenylpiperazine AC	999486-02-1
Chloroprocaine, 2-	000133-16-4
Chloroquine	000054-05-7
Chlorpheniramine	000132-22-9
Chlorphenisin	000104-29-0
Chlorphentermine	000461-78-9
Chlorphentermine AC	999130-02-8
Chlorpropamide artifact-2	999246-02-5
Chlorprothixene	000113-59-7
Chlorzoxazone	000095-25-0
Cholesterol	000057-88-5
Cholesterol TMS	001856-05-9
Cinnarizine	000298-57-7
Cisapride	081098-60-4
Citalopram	059729-33-8
Clemastine	015686-51-8
Clemizole	000442-52-4
Clenbuterol	037148-27-9
Clenbuterol AC	999360-02-0
Clobazam	022316-47-8
Clofibrate	000637-07-0
Clomipramine	000303-49-1
Clonazepam	001622-61-3
Clonazepam TMS	999184-02-0
Clonazepam-M (amino-)	004959-17-5
Clonazepam-M (amino) - TMS	999175-02-9
Clonidine	004205-90-7
Clonidine 2AC	999131-02-1
Clonidine AC	999132-02-4
Clopidogrel	113665-84-2
Clozapine	005786-21-0
Clozapine AC	999133-02-7
Cocaethylene	000529-38-4
Cocaine	000050-36-2
Codeine	000076-57-3
Codeine TMS	074367-14-9
Colchicine	000064-86-8

Compound name	CAS number
Colchicine breakdown	999532-02-4
Coniine	000458-88-8
Coniine AC	999361-02-3
Cotinine	000486-56-6
Cyclandelate	000456-59-7
Cyclandelate TMS	999442-02-3
Cyclizine	000082-92-8
Cyclobenzaprine	000303-53-7
Cyclophosphamide	000050-18-0
Cyclophosphamide -HCL	999379-02-1
Cyheptamide	007199-29-3
Cyproheptadine	000129-03-3
Dapsone	000080-08-0
Debrisoquine AC	999415-02-0
Desalkylflurazepam AC	999298-02-1
Desethyllidocaine (MegX)	999044-02-9
Desethyllidocaine AC (MegX)	999263-02-4
Desipramine	000050-47-5
Desipramine AC	999108-02-0
Desmethylclomipramine	000303-48-0
Desmethylclomipramine AC	999134-02-0
Desmethylclozapine	006104-71-8
Desmethyldoxepin (cis)	999516-02-8
Desmethyldoxepin (cis) AC	999517-02-1
Desmethyldoxepin (trans)	001225-56-5
Desmethyldoxepin (trans) AC	999443-02-6
Desmethylselegiline	999072-02-5
Desmethylselegiline AC	999147-02-3
Desmethylsertraline	091797-58-9
Desmethyltramadol, O-	999018-02-9
Desmethyltramadol, O- 2TMS	999444-02-9
Desmethyltrimipramine	999019-02-2
Desmethyltrimipramine AC	999445-02-2
Dextromethorphan	000125-71-3
Diacetylmorphine	000561-27-3
Diazepam	000439-14-5
Dichlorophene	000097-23-4
Dichlorophene TMS	999237-02-4
Diclofenac -H2O	999200-02-1
Diclofenac TMS	999222-02-5
Dicyclomine	000077-19-0
Diethyltryptamine	000061-51-8
Dihydrocodeine	000125-28-0
Dihydroxy-4-methylcoumarin, 7, 8 - TMS	999236-02-1
Diiodohydroxyquin	000083-73-8
Diltiazem	042399-41-7
Dimethadione	000695-53-4
Diphenadione	000082-66-6
Diphenhydramine	000058-73-1
Diphenidol	000972-02-1
Diphenidol TMS	999417-02-6
Diphenoxylate	000915-30-0
Diphenylpyraline	000147-20-6
Disopyramide	003737-09-5
Donepezil	120014-06-4
Dothiepin	000113-53-1
Doxapram	000309-29-5
Doxepin (cis)	999515-02-5
Doxepin (trans)	001668-19-5
Doxylamine	000469-21-6
Dyphylline	000479-18-5
Dyphylline TMS	999446-02-5
Ecgonine methyl ester	106293-60-1
Ecgonine methyl ester TMS	999162-02-6
Efavirenz	154598-52-4
Efavirenz AC	999489-02-0
Efavirenz TMS	999505-02-1
Emetine	000483-18-1
Encainide	999034-02-5
Ephedrine	000299-42-3
Ephedrine 2AC	055133-90-9
Epinephrine AC	999111-02-3
Ergonovine AC	999447-02-8
Estazolam	029975-16-4
Ethacrynic Acid TMS	999227-02-0
Ethambutol AC	999261-02-8
Ethamivan	000304-84-7
Ethinamate	000126-52-3
Ethopropazine	000522-00-9
Ethosuximide	000077-67-8
Ethotoin	000086-35-1
Ethyl-2-malonamide, 2-	068692-83-1

Compound name	CAS number
Ethyl-2-malonamide, 2- TMS	999418-02-9
Ethylamphetamine	000457-87-4
Ethylamphetamine AC	999148-02-6
Ethylecgonine	999037-02-4
Ethylecgonine TMS	999448-02-1
Ethylmorphine	000076-58-4
Ethylmorphine TMS	999221-02-2
Etodolac TMS	999212-02-1
Etofylline	000519-37-9
Etofylline TMS	077630-35-4
Etomidate	033125-97-2
Eucatropine Isomer 1	999038-02-7
Eucatropine Isomer 1 TMS	999278-02-3
Eucatropine Isomer 2	999277-02-0
Eucatropine Isomer 2 TMS	999518-02-4
Felbamate artifact 1	999250-02-1
Felbamate artifact 2	999251-02-4
Felbamate artifact 3	999252-02-7
Felodipine	072509-76-3
Felodipine-M/artifact (dehydro-)	999296-02-5
Fenfluramine	000458-24-2
Fenfluramine AC	999139-02-5
Fenoprofen	031879-05-7
Fenoprofen TMS	999310-02-0
Fentanyl	000437-38-7
Finasteride	098319-26-7
Flavoxate	015301-69-6
Flavoxate-M/artifact (HOOC-) ME	999279-02-6
Flecainide	054143-55-4
Flecainide AC	999140-02-2
Flumazenil	078755-81-4
Flunarizine	052468-60-7
Flunitrazepam	001622-62-4
Fluoxetine	054910-89-3
Fluoxetine AC	999141-02-5
Flupenthixol	002709-56-0
Flupentixol TMS	999387-02-9
Fluphenazine	000069-23-8
Fluphenazine TMS	999280-02-3
Fluphenazine-M (ring)	000092-30-8
Flurazepam	017617-23-1
Flurazepam-M (desalkyl-)	002886-65-9
Flurazepam-M (HO-ethyl-)	020971-53-3
Flurbiprofen	005104-49-4
Flutamide	013311-84-7
Flutamide TMS	999467-02-6
Fluvoxamine	054739-18-3
Fluvoxamine AC	999262-02-1
Furazolidone	000067-45-8
Furosemide 2TMS	999214-02-7
Gemfibrozil	025812-30-0
Gemfibrozil AC	999389-02-5
Glutethimide	000077-21-4
Griseofulvin	000126-07-8
Guaifenesin	000093-14-1
Guaifenesin 2TMS	107966-19-8
Guanethidine	000055-65-2
Haloperidol	000052-86-8
Harmaline	000304-21-2
Harmaline AC	999301-02-9
Harmine	000442-51-3
Hexobarbital	000056-29-1
Hexobarbital TMS	999469-02-2
Hexylresorcinol	000136-77-6
Hexylresorcinol 3TMS	999422-02-5
Homatropine	000087-00-3
Homatropine TMS	999282-02-9
Hydrastine	000118-08-1
Hydrocodone	000125-29-1
Hydromorphone	000466-99-9
Hydromorphone enol 2TMS	999513-02-9
Hydromorphone TMS	221209-08-1
Hydroxychloroquine AC	999512-02-6
Hydroxyethylflurazepam TMS	999204-02-3
Hydroxyloxapine, 8-	999053-02-0
Hydroxyzine	000068-88-2
Hydroxyzine AC	999113-02-9
Ibuprofen	015687-27-1
Ibuprofen TMS	999165-02-5
Iminostilbene	000256-96-2
Imipramine	000050-49-7
Indomethacin TMS	999318-02-4

Compound name	CAS number
Isocarboxazid	000059-63-2
Isometheptene AC	999265-02-0
Isoniazid	000054-85-3
Isoniazid 2AC	999266-02-3
Isoniazid AC	999254-02-3
Isoproterenol 2TMS	999424-02-1
Isoxsuprine	000395-28-8
Isoxsuprine TMS	999319-02-7
Ketamine	006740-88-1
Ketamine AC	999114-02-2
Ketoprofen TMS	999320-02-4
Ketorolac TMS	999215-02-0
Ketotifen	034580-13-7
Lamotrigine	084057-84-1
Lamotrigine 2AC	999255-02-6
Laudanosine	020412-65-1
Levallorphan	000152-02-3
Levallorphan TMS	999321-02-7
Levetiracetam	102767-28-2
Levorphanol	000077-07-6
Levorphanol TMS	999223-02-8
Lidocaine	000137-58-6
Loratadine	079794-75-5
Lorazepam	000846-49-1
Lorazepam 2TMS	999202-02-7
Lorcainide	059729-31-6
Lormetazepam	000848-75-9
Loxapine	001977-10-2
Ly170222	999123-02-3
Lysergide (LSD)	000050-37-3
Maprotiline	010262-69-8
Maprotiline AC	999366-02-8
Mazindol	022232-71-9
MBDB	100031-29-2
MBDB AC	999142-02-8
Mecamylamine	000060-40-2
Meclizine	000569-65-3
Meclofenamic acid TMS	999322-02-0
Medazepam	002898-12-6
Mefenamic acid TMS	999324-02-6
Mefloquine	053230-10-7

Compound name	CAS number
Memantine	019982-08-2
Memantine AC	999115-02-5
Meperidine	000057-42-1
Mephenesin	000059-47-2
Mephenesin 2TMS	999325-02-9
Mephentermine	000100-92-5
Mephentermine AC	999143-02-1
Mephenytoin	000050-12-4
Mephobarbital	000115-38-8
Mepivacaine	000096-88-8
Meprobamate	000057-53-4
Mescaline	000054-04-6
Mescaline AC	999511-02-3
Mescaline formyl artifact	999284-02-5
Mesuximide-M (nor)	001497-17-2
Metaproterenol AC	999391-02-5
Metaxalone	001665-48-1
Metaxalone AC	999116-02-8
Methadone	000076-99-3
Methadone-M (EDDP)	999058-02-5
Methamphetamine	000537-46-2
Methamphetamine AC	999117-02-1
Methapyrilene	000091-80-5
Methaqualone	000072-44-6
Metharbital	000050-11-3
Metharbital TMS	999186-02-6
Methazolamide	000554-57-4
Methcathinone AC	999300-02-6
Methcathinone-M (HO-) 2AC	005650-44-2
Methdilazine	001982-37-2
Methimazole	000060-56-0
Methimazole AC	999368-02-4
Methocarbamol 2TMS	999285-02-8
Methohexital	000151-83-7
Methohexital TMS	999425-02-4
Methotrimpeprazine	000060-99-1
Methoxyverapamil	016662-47-8
Methsuximide	000077-41-8
Methylaminorex, 4-	029493-77-4
Methylaminorex, 4- 2AC	999508-02-0
Methylaminorex, 4- AC	999510-02-0

Compound name	CAS number
Methylenedioxyamphetamine AC	999479-02-6
Methylenedioxyamphetamine (MDA)	004764-17-4
Methylenedioxyethylamphetamine	014089-52-2
Methylenedioxyethylamphetamine AC	999481-02-6
Methylenedioxymethamphetamine AC	999480-02-3
Methylenedioxymethamphetamine (MDMA)	042542-10-9
Methylephedrine	000552-79-4
Methylephedrine AC	999370-02-4
Methyl-nicotine	999065-02-0
Methylphenidate	000113-45-1
Methylphenidate AC	999144-02-4
Methylphenobarbtial	999509-02-3
Methylprimidone	059026-32-3
Methylprimidone 2TMS	999286-02-1
Methyprylon	000125-64-4
Metoclopramide	000364-62-5
Metoclopramide AC	999145-02-7
Metoprolol 2AC	999306-02-4
Metronidazole	000443-48-1
Metronidazole TMS	999450-02-1
Mexiletine	031828-71-4
Mexiletine AC	999146-02-0
Mianserin	024219-97-4
Mianserin-M (nor-)	999015-02-0
Mianserin-M (nor-) AC	999364-02-2
Midazolam	059467-70-8
Mirtazapine	061337-67-5
Moclobemide	071320-77-9
Molindone	007416-34-4
Morphine	000057-27-2
Morphine 2TMS	055449-66-6
Muconic acid TMS	999166-02-8
N,N-Dimethyl-5-methoxy-tryptamine	001019-45-0
N,N-Dimethyltryptamine	000061-50-7
Nabumetone	042924-53-8
N-Acetylprocainamide	999070-02-9
Nadolol 3TMS	999287-02-4
Nalbuphine	020594-83-6
Nalbuphine 2TMS	999167-02-1
Nalidixic acid	000389-08-2
Nalidixic acid TMS	999238-02-7

Compound name	CAS number
Nalorphine	000062-67-9
Nalorphine 2TMS	999473-02-8
Naloxone	000465-65-6
Naloxone TMS	999427-02-0
Naltrexol, beta-	999406-20-9
Naltrexol, beta- 2TMS	999405-02-6
Naltrexol, beta- 3TMS	999520-02-4
Naltrexone	016590-41-3
Naltrexone 2TMS	999328-02-8
Naltrexone 3TMS	999523-02-3
Naltrexone TMS	999522-02-0
Naproxen ME	999295-02-2
Naproxen TMS	074793-83-2
Nevirapine	129618-40-2
Nevirapine TMS	999451-02-4
Niclosamide	000050-65-7
Nicotinamide	000098-92-0
Nicotine	000054-11-5
Nifedipine	021829-25-4
Nikethamide	000059-26-7
Nimodipine	066085-59-4
Nimodipine-M/artifact	999340-02-2
Nitrazepam	000146-22-5
Nitrazepam TMS	999288-02-7
Nomifensine	024526-64-5
Nomifensine AC	999371-02-7
Noralfentanil	061086-18-8
Noralfentanil AC	999150-02-6
Norchlordiazepoxide	016300-25-7
Norchlordiazepoxide AC	999525-02-9
Norchlordiazepoxide breakdown	999524-02-6
Norchlordiazepoxide breakdown AC	999372-02-0
Norclozapine 2AC	999135-02-3
Norclozapine AC	999136-02-6
Norcodeine	000467-15-2
Norcodeine 2AC	999118-02-4
Nordiazepam	001088-11-5
Nordiazepam TMS	999207-02-2
Norepinephrine 2AC	999119-02-7
Norepinephrine 3AC	999528-02-8
Norfenfluramine	001886-26-6

Compound name	CAS number
Norfenfluramine AC	999120-02-4
Norfentanyl	999076-02-7
Norfentanyl AC	999272-02-5
Norfluoxetine	999077-02-0
Norfluoxetine AC	999121-02-7
Norketamine	999078-02-3
Norketamine AC	999494-02-9
Normeperidine	000077-17-8
Normeperidine AC	999122-02-0
Normetanephrine AC	999373-02-3
Normethsuximide TMS	999429-02-6
Noroxycodone	057664-96-7
Noroxycodone AC	999495-02-2
Norpropoxyphene	999079-02-6
Norpropoxyphene breakdown 1	999530-02-8
Norpropoxyphene breakdown 2	999531-02-1
Norpropoxypheneamide	999080-02-3
Norpseudoephedrine	000492-41-1
Norpseudoephedrine AC	999081-02-6
Norpseudoephedrine artifact	999478-02-3
Nortriptyline	000072-69-5
Nortriptyline AC	999151-02-9
Norvenlafaxine	130198-38-8
Norverapamil	067018-85-3
Norverapamil AC	999488-02-7
Olanzapine	132539-06-1
Opipramol TMS	999226-02-7
Orphenadrine	000083-98-7
Ortho-cotinine	999083-02-2
Oxazepam	000604-75-1
Oxazepam 2TMS	999168-02-4
Oxcarbamazepine	028721-07-5
Oxprenolol 2AC	999374-02-6
Oxybutynin	005633-20-5
Oxycodone	000076-42-6
Oxycodone enol 2TMS	999514-02-2
Oxycodone TMS	221209-10-5
Oxymorphone	000076-41-5
Oxymorphone 2TMS	999521-02-7
Oxymorphone TMS	999208-02-5
Papaverine	000058-74-2

Compound name	CAS number
Paramethadione	000115-67-3
Pargyline	000555-57-7
Paroxetine	061869-08-7
Paroxetine AC	999124-02-6
Pemoline	002152-34-3
Pentachlorophenol	000087-86-5
Pentazocine	000359-83-1
Pentazocine TMS	100013-72-2
Pentobarbital	000076-74-4
Pentobarbital 2TMS	052937-68-5
Pentoxifylline	006493-05-6
Pentylenetetrazole	000054-95-5
Pergolide	066104-22-1
Perphenazine TMS	999291-02-0
Phenacemide	000063-98-9
Phenacetin	000062-44-2
Phenacetin AC	999496-02-5
Phenacetin TMS	999504-02-8
Phenazopyridine	000094-78-0
Phenazopyridine AC	999303-02-5
Phencyclidine	000077-10-1
Phencyclidine artifact	000771-98-2
Phendimetrazine	000634-03-7
Phenelzine AC	999304-02-8
Phenindione	000083-12-5
Pheniramine	000086-21-5
Phenmetrazine	000134-49-6
Phenmetrazine AC	999090-02-7
Phenobarbital	000050-06-6
Phenobarbital 2TMS	052937-73-2
Phenolphthalein	000077-09-8
Phenolphthalein 2TMS	999292-02-3
Phenoxybenzamine	000059-96-1
Phensuximide	000086-34-0
Phentermine	000122-09-8
Phentermine AC	999152-02-2
Phenylacetamide	000103-81-1
Phenylbutazone	000050-33-9
Phenylbutazone artifact	999338-02-2
Phenylbutazone artifact TMS	999198-02-6
Phenylbutazone TMS	074810-87-0

Compound name	CAS number
Phenylephrine 3AC	999091-02-0
Phenylethylamine, beta-	000064-04-0
Phenylethylamine, beta AC	999343-02-1
Phenylpropanolamine	999498-02-1
Phenylpropanolamine AC	999092-02-3
Phenyltoloxamine	000092-12-6
Phenytoin	000057-41-0
Phenytoin 2TMS	063435-72-3
Pilocarpine	000092-13-7
Pindolol	013523-86-9
Pindolol formyl artifact	999458-02-5
PMA TMS	999172-02-0
p-Methoxyamphetamine	000064-13-1
Prazepam	002955-38-6
Prilocaine	000721-50-6
Primidone	000125-33-7
Probenecid TMS	999294-02-9
Procainamide	000051-06-9
Procaine	000059-46-1
Prochlorperazine	000058-38-8
Procyclidine	000077-37-2
Procyclidine artifact (dehydro-)	999460-02-5
Procyclidine TMS	999454-02-3
Promazine	000058-40-2
Promethazine	000060-87-7
Propantheline bromide	000050-34-0
Propiomazine	000362-29-8
Propofol	002078-54-8
Propoxur	000114-26-1
Propoxur-M/artifact	999393-02-1
Propoxyphene	000469-62-5
Propylamphetamine	051799-32-7
Propylamphetamine AC	999302-02-2
Protriptyline	000438-60-8
Protriptyline AC	999273-02-8
Pseudoephedrine	000090-82-4
Pseudoephedrine 2AC	999500-02-6
Pseudoephedrine formyl artifact	999483-02-2
Psilocin 2TMS	999192-02-8
Psilocybin 3TMS	999193-02-1
Pyrazinamide	000098-96-4
Pyrilamine	000091-84-9
Pyrimethamine	000058-14-0
Quetiapine	999097-02-8
Quetiapine TMS	999527-02-5
Quinacrine	000083-89-6
Quinidine	000056-54-2
Quinine	000130-95-0
Ramelteon	999274-02-1
Reboxetine	098769-81-4
Ritodrine 3TMS	999218-02-9
Rofecoxib	162011-90-7
Ropivacaine	132112-35-7
Salbutamol 3TMS	999394-02-4
Salicylamide	000065-45-2
Salicylamide 2TMS	055887-58-6
Salicylic acid 2TMS	003789-85-3
Salicylic acid ethylester	000118-61-6
Salicylic acid methylester	000119-36-8
Scopolamine	000051-34-3
Scopolamine TMS	999194-02-4
Secobarbital	000076-73-3
Secobarbital 2TMS	052937-71-0
Selegiline	014611-51-9
Selegiline-M (HO-) AC	999482-02-9
Sertraline	079617-96-2
Sertraline AC	999125-02-9
Sertraline-M (nor-) AC	999109-02-3
Sildenafil TMS	999213-02-4
SKF-525a	000302-33-0
Strychnine	000057-24-9
Sufentanil	056030-54-7
Sulfadiazine	000068-35-9
Sulfadimethoxine	000122-11-2
Sulfamethazine	000057-68-1
Sulfamethazine AC	999501-02-9
Sulfamethoxazole	000723-46-6
Sulfanilamide	000063-74-1
Sulfapyridine	000144-83-2
Sulfathiazole	000072-14-0
Sulfinpyrazone	000057-96-5
Tacrine	000321-64-2

Compound name	CAS number
Talbutal	000115-44-6
Tamoxifen	010540-29-1
Temazepam	000846-50-4
Temazepam artifact-2	020927-53-1
Temazepam TMS	035147-95-6
Terbinafine	091161-71-6
Terfenadine TMS	999220-02-9
Teriflunomide AC	999502-02-2
Tetracaine	000094-24-6
Tetrahydrocannabinol	001972-08-3
Tetrahydrocannabinol TMS	999529-02-1
Tetrahydrozoline	000084-22-0
Tetrahydrozoline AC	999398-02-6
Thebaine	000115-37-7
Theobromine	000083-67-0
Theophyline	000058-55-9
Thiamylal	000077-27-0
Thiethylperazine	001420-55-9
Thiopental	000076-75-5
Thioridazine	000050-52-2
Thonzylamine	000091-85-0
Ticlopidine	055142-85-3
Tiletamine	014176-49-9
Timolol TMS	999399-02-9
Tocainide	041708-72-9
Tocainide AC	999375-02-9
Tolazoline	000059-98-3
Topiramate artifact (-SO_2NH)	020880-92-6
Topiramate breakdown	097240-79-4
Tramadol	027203-92-5
Tramadol TMS	999336-02-6
Tranylcypromine	000155-09-9
Tranylcypromine AC	999305-02-1
Trazodone	019794-93-5
Triamterene	000396-01-0
Triazolam	028911-01-5
Trifluoperazine	000117-89-5
Triflupromazine	000146-54-3
Trihexyphenidyl	000144-11-6
Trimeprazine	000084-96-8
Trimethobenzamide	000138-56-7
Trimethoprim	000738-70-5
Trimipramine	000739-71-9
Tripelenamine	000091-81-6
Triprolidine	000486-12-4
Tropacocaine	000537-26-8
Tryptamine	000061-54-1
Tryptamine 2AC	999352-02-2
Tryptamine AC	999353-02-5
Tryptophan, D- AC	999519-02-7
Valproic acid	000099-66-1
Venlafaxine	093413-69-5
Venlafaxine TMS	999173-02-3
Verapamil	000052-53-9
Vigabatrin AC	999376-02-2
Warfarin	000081-81-2
Warfarin artifact	000122-57-6
Warfarin TMS	036307-79-6
Xanthinol TMS	999239-02-0
Xylazine	007361-61-7
Yohimbine	000146-48-5
Yohimibine TMS	999457-02-2
Zaleplon	151319-34-5
Zolazepam	031352-82-6
Zolpidem	082626-48-0
Zomepirac -CO_2	999355-02-1
Zonisamide	068291-97-4
Zonisamide AC	999354-02-8
Zopiclone	043200-80-2
Zotepine	026615-21-4

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- Determination of Buprenorphine, Norbuprenorphine, and Their Glucuronides in Urine Using LC/MS/MS
- Determination of Cocaine and Metabolites in Urine Using Electrospray LC/MS

Applications by Technique

LC/MS

Analysis of Anabolic Agents in Urine by LC/MS/MS

Application

Forensics/Doping Control

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Abstract

The use of the Agilent 1200 Series liquid chromatography (LC) system coupled to the 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of the G1948B electrospray ionization (ESI) source is demonstrated in the analysis of anabolic substances in urine. The high degree of sensitivity of the QQQ instrument allows for excellent quantitation and linearity for meeting Minimum Required Performance Levels (MRPLs) for each compound as specified by the World Anti-Doping Agency (WADA). For increased chromatographic resolution and speed, a 2.1 mm id C18 column with a 3.5-µm particle size is employed. The seven compounds, including a designated internal standard, all elute within 10 minutes at a flow rate of 0.4 mL/min.

Introduction

The use of anabolic substances for performance enhancement in sports seems to be a ubiquitous topic of discussion. While their use is a growing problem in high school and collegiate athletics, their use at the professional level is an ongoing controversy. It is therefore the mandate of agencies like WADA to ensure that fair competition is being maintained by monitoring the possible use of banned substances like anabolic compounds.

Traditionally, doping control analysis for anabolic substances, including steroids, in urine includes screening by derivatization and GC/MS [1], followed by confirmation of the presumptive positive using high-resolution magnetic sector GC/MS in EI mode [2]. The high purchase and operational costs of high-resolution magnetic sector instruments make alternative techniques like LC/MS attractive for confirming the presence of the banned compounds.

More than 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily analyzed using GC/MS but are amenable to LC/MS. The analysis of some of these compounds is very challenging, as they must be detected and confirmed at MRPLs of 2 ng/mL or lower in urine.

This work describes the results of using the Agilent LC/QQQ instrument for detection and confirmation of a number of anabolic substances at the

WADA MRPL or, more specifically, covering the 1/2 × – 10 × MRPL range. The anabolic compounds analyzed in this work are listed in Table 1 along with their MRPLs.

Some previous work [3] used the TOF to analyze these compounds and found that accurate mass could be used for both screening and confirmation. However, the QQQ is more specific with MS/MS, increasing the confidence in confirmation and quantitating compounds of interest.

Table 1. Minimum Required Performance Levels (ng/mL of urine)

Compound	MRPL
Clenbuterol	2
19-norandrosterone	1
4β-OH-stanozolol	10
Tetrahydrogestrinone (THG)	10
Methyl testosterone metabolite (MeTest metabolite) or 17α-methyl-5β-androstane-3α,17β-diol	2
Epimetendiol	2
Methyl testosterone – Internal Standard	NA

In this study all compounds are steroids except for clenbuterol.

The structures of the compounds analyzed in this work are shown in Figure 1. Based on the results of work presented elsewhere [4], a derivatizing agent is used on these samples, but only reacts with 19-norandrosterone to improve sensitivity. The derivatizing agent, known as Girard's Reagent P (Sigma Aldrich, St. Louis, MO), reacts with ketone groups to form a quaternary amine, which is more easily ionized by ESI.

Experimental

Sample Preparation

The anabolic agents and their metabolites are purchased from Sigma Aldrich (St. Louis, MO), Steraloids (Newport, RI), and the National Measurement Institute (Sydney, Australia). Girard's Reagant P (GRP) is purchased from Sigma Aldrich and β-glucuronidase is purchased from Roche (Indianapolis, IN).

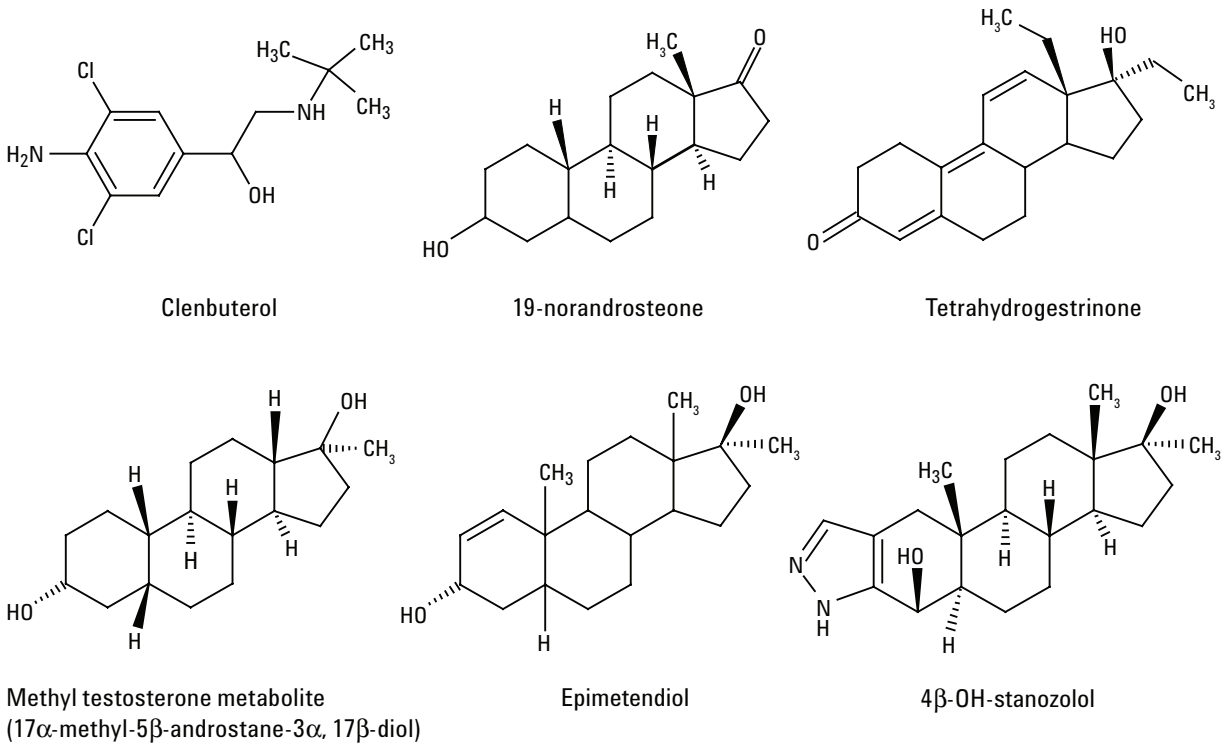

Figure 1. Structures of anabolic substances analyzed in this work.

To 3 mL urine negative control sample, 1 mL 0.8 M potassium phosphate buffer, pH 7.0, is added. A further 25 µL β-glucuronidase is added and then the mixture is incubated at 50 °C for one hour. A 750-µL mixture of 20% (w/v) $K_2CO_3/KHCO_3$ (1:1) mixture is then added. Extract with methyl-t-butyl ether and then remove and dry the organic extract.

The same extraction procedure used for GC/MS screening is employed except that the compounds are not derivatized as usual for GC/MS analysis. Rather, the samples are dried and then reconstituted in 100 µL of LC mobile phase.

As part of the reconstitution step for LC/MS/MS analysis, 20 µL methanol, followed by 8 µL of 1M GRP in 50 mM ammonium acetate buffer, pH 4.2, is added. Incubation at room temperature for one hour is then followed by LC/MS/MS analysis.

Of the compounds analyzed, only the 19-norandrosterone is reactive with the GRP derivative. This compound has been problematic in LC/MS/MS analysis and the GRP improves sensitivity.

The MeTest internal standard has a fixed concentration of 10 ng/mL.

LC/MS Method Details

LC Conditions

Agilent 1200 Series binary pump SL, wellplate sampler, thermostatted column compartment, inline filter 0.5 µm between needle seat and injector valve.

Column:	Agilent ZORBAX XDB-CN 2.1 × 100 mm, 3.5 µm (p/n 961764-905)
Column temp:	50 °C
Mobile phase:	A = 0.1% formic acid in water B = 0.1% formic acid in methanol
Flow rate:	0.4 mL/min; injection vol: 2 µL

Gradient:

Time (min)	%B
0–1	5
3	15
3.01	40
12	50
15	95

Stop time = 15 min; Post-run time = 3 min.

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	40 psig
Drying gas flow:	9 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 resolution:	0.7 amu
Q2 resolution:	0.7 amu

MRM transitions shown in Table 2. Chromatographic retention times (RTs), fragmentor (Frag), collision energy (CE), and dwell times are included. Time segments in which the MRM transitions are implemented are also noted.

Results and Discussion

The chromatographic elution profile of all compounds at their equivalent 10 × MRPL is shown in Figure 2. The responses vary quite significantly among the compounds and the background interference from the matrix is evident.

Concentration levels ranging from 1/2 × to 10 × MRPL are run in triplicate injections. The results for clenbuterol are shown in Figures 3a to 3c. Linearity over this range has a correlation coefficient of $R^2 > 0.999$ using the most conservative

Table 2. Data Acquisition Parameters for MRM Transitions

Compound	RT (min)	MRM	Frag (V)	CE (V)	Dwell (msec)
Segment 1 (0–4.0 min)					
Clenbuterol	2.74	277.0 > 203.1	100	15	200
Segment 2 (4.0–6.3 min)					
19-norandrosterone	5.82	410.3 > 331.3	130	30	75
Segment 3 (6.3– 6.93 min)					
4β-OH-stanozolol	6.64	345.2 > 327.2	140	15	200
Segment 4 (6.93–7.55 min)					
MeTest (IStd)	7.19	303.2 > 97.1	140	25	75
Segment 5 (7.55–8.8 min)					
THG	7.88	313.2 > 295.1	150	15	100
MeTest metabolite	8.08	271.2 > 161.2	110	20	100
Segment 6 (8.8–12.0 min)					
Epimetendiol	9.47	269.2 > 105.1	90	20	200

curve fit settings of linear, ignored origin, and no weighting. A closer look at the reproducibility of the lowest three level replicates is included in Figure 3a. The limit of detection (LOD), which is defined here as being a peak-to-peak signal-to-noise (S/N) ratio of 3:1, the S/N of the lowest level (1/2 × MRPL) is measured first. Then the same factor that is applied to this S/N, in order to obtain a S/N of 3:1, is also applied to the lowest level.

For example, in Figure 3b the S/N is nearly 60:1 for all three injections at the 1/2 × MRPL. A factor of 20 is applied to achieve 3:1 so that the LOD is 1/20th the concentration of this level, or 1/40 × MRPL.

To determine the on-column injection amount it should be noted that the original sample corresponds to 3 mL of urine. Since the MRPL of clenbuterol is 2 ng/mL, according to Table 1, then the 1/2 × MRPL contains 3 ng clenbuterol in the 3 mL urine sample. Following extraction and evaporating to dryness, this 3 ng of clenbuterol is reconstituted in 100 µL of LC mobile phase. Of this volume, 2 µL is injected. Therefore, the on-column injection amount of clenbuterol at the 1/2 × MRPL corresponds to 2/100 × 3 ng = 60 pg. The LOD is therefore 1/20 × 60 pg, or about 3 pg on-column.

The LOD for clenbuterol is given in Figure 3b. Note that the negative quality control (NQC) is also shown as evidence that the calculated S/N is justifiable.

Figure 3c shows the replicate injections at the lowest three levels.

The results for THG, MeTest metabolite, epimetendiol, and 4β-OH-stanozolol are shown in Figures 4, 5, 6, and 7, respectively.

As can be seen from Figure 5a, the 1/2 x MRPL does not appear to be a limit of detection because an S/N of 3:1 does not seem possible. However, in comparison to the matrix blank (NegQC) this level is certainly detectable. For this reason, including the fact that the 1/2 × MRPL replicate injections are at the lowest end of the range investigated and linear with the curve fit, the 1/2 × MRPL of the MeTest metabolite is considered the LOD.

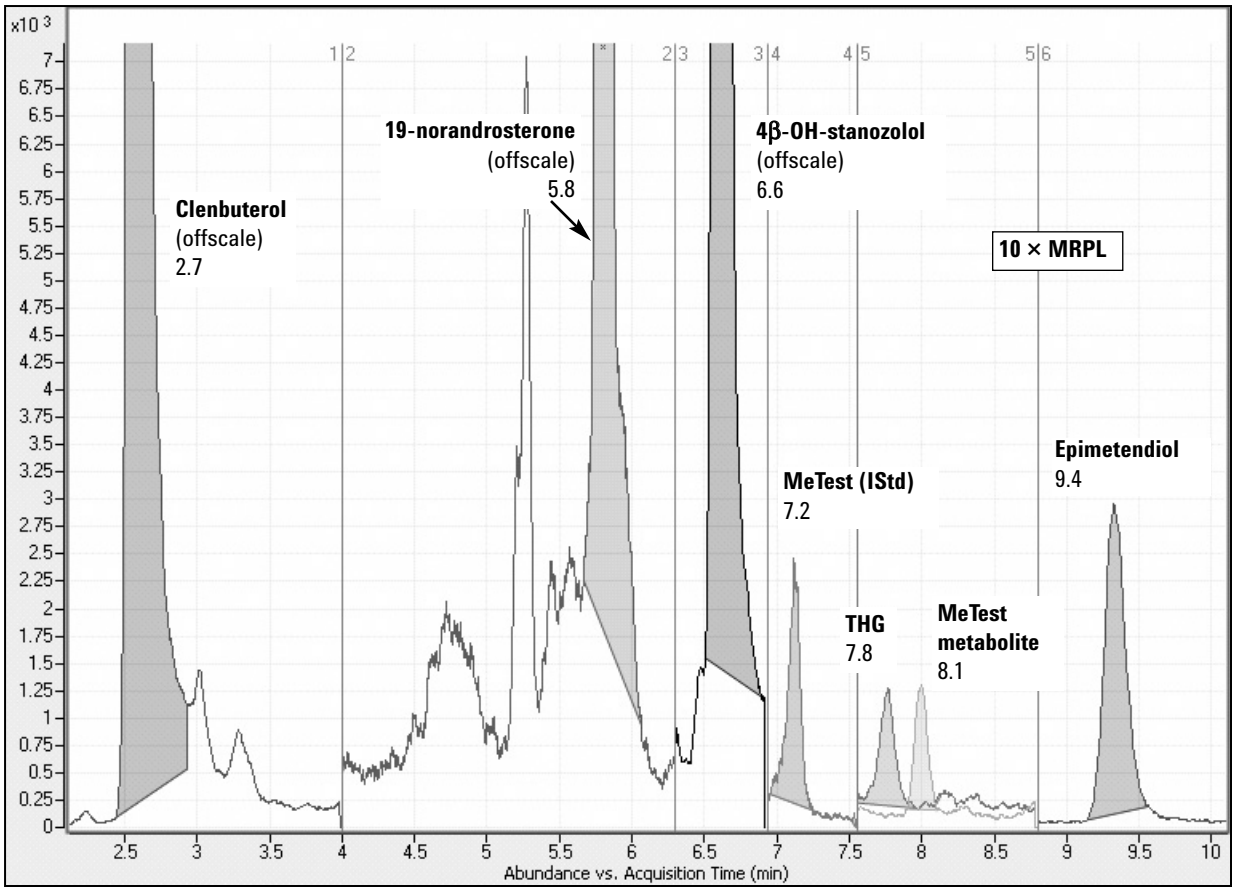

Figure 2. Chromatographic profile of 10 × MRPL extract in urine.

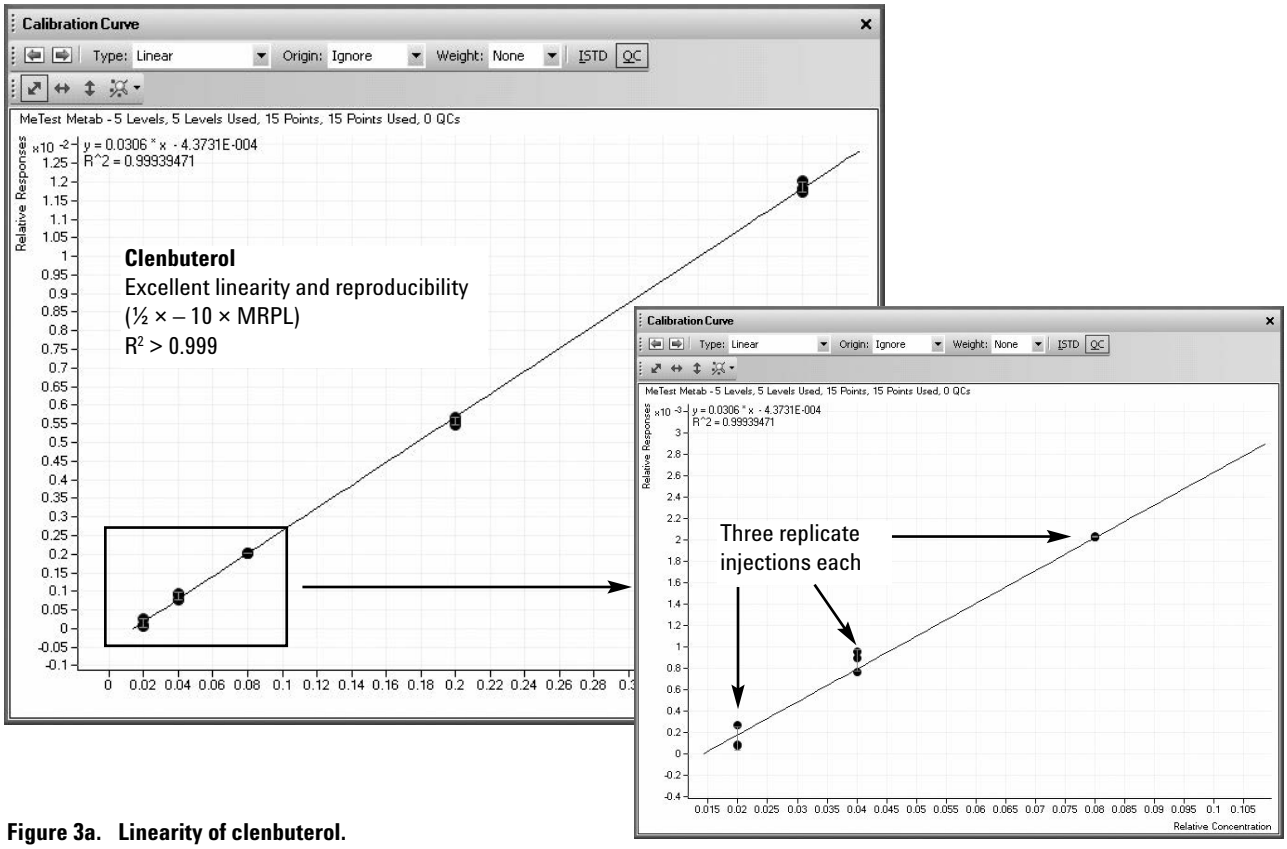

Figure 3a. Linearity of clenbuterol.

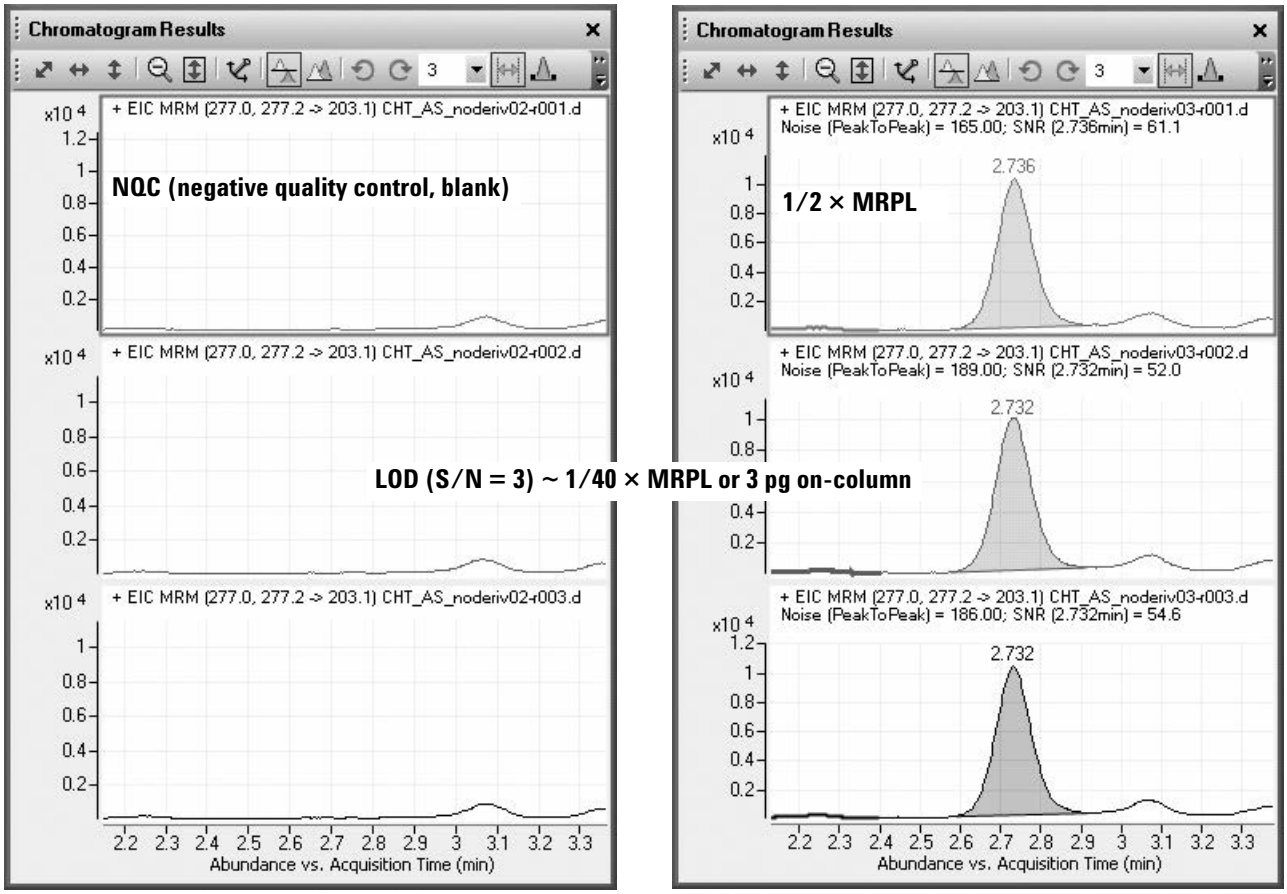

Figure 3b. Estimate of LOD for clenbuterol.

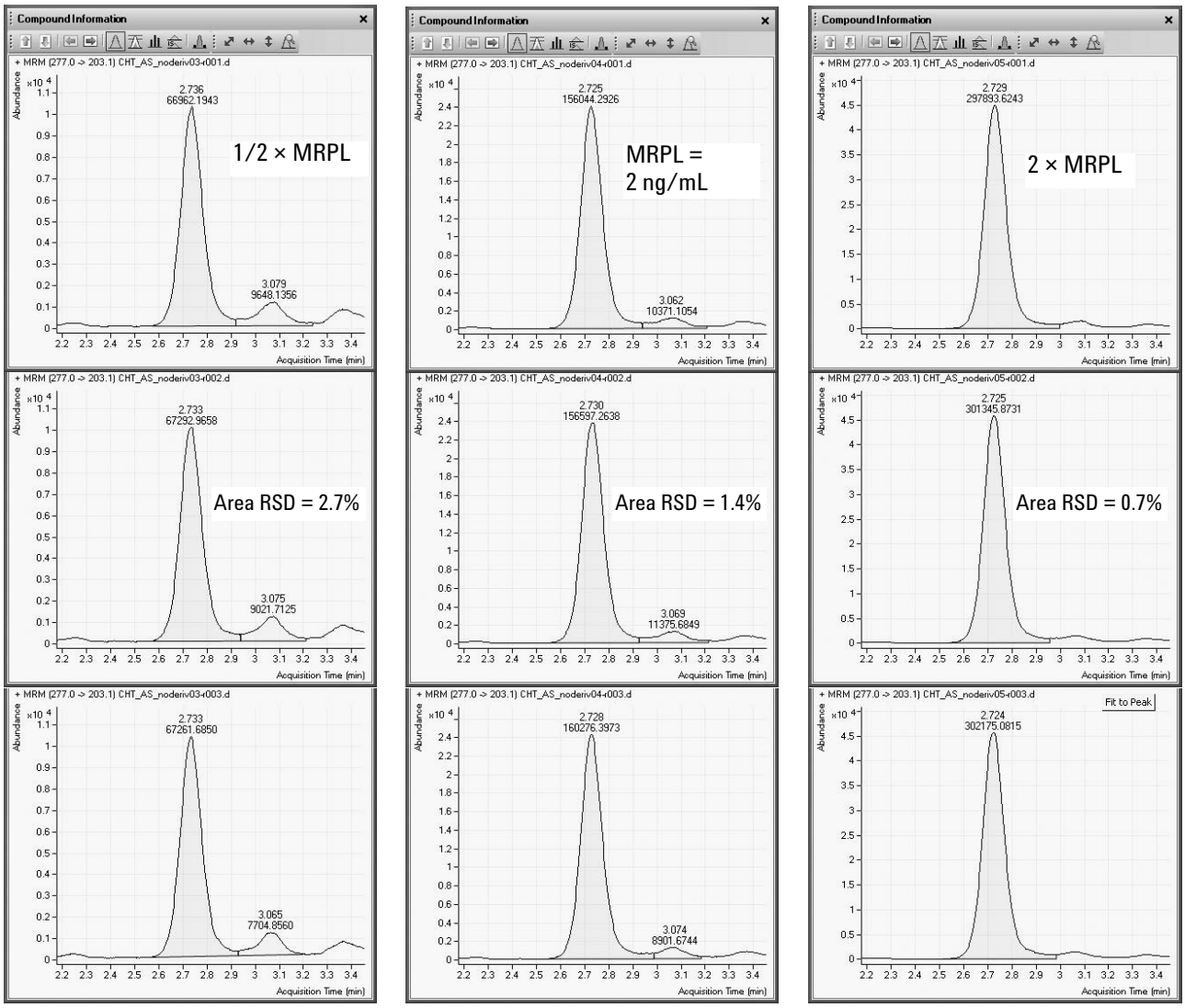

Figure 3c. Triplicate injections of the lowest three levels of clenbuterol.

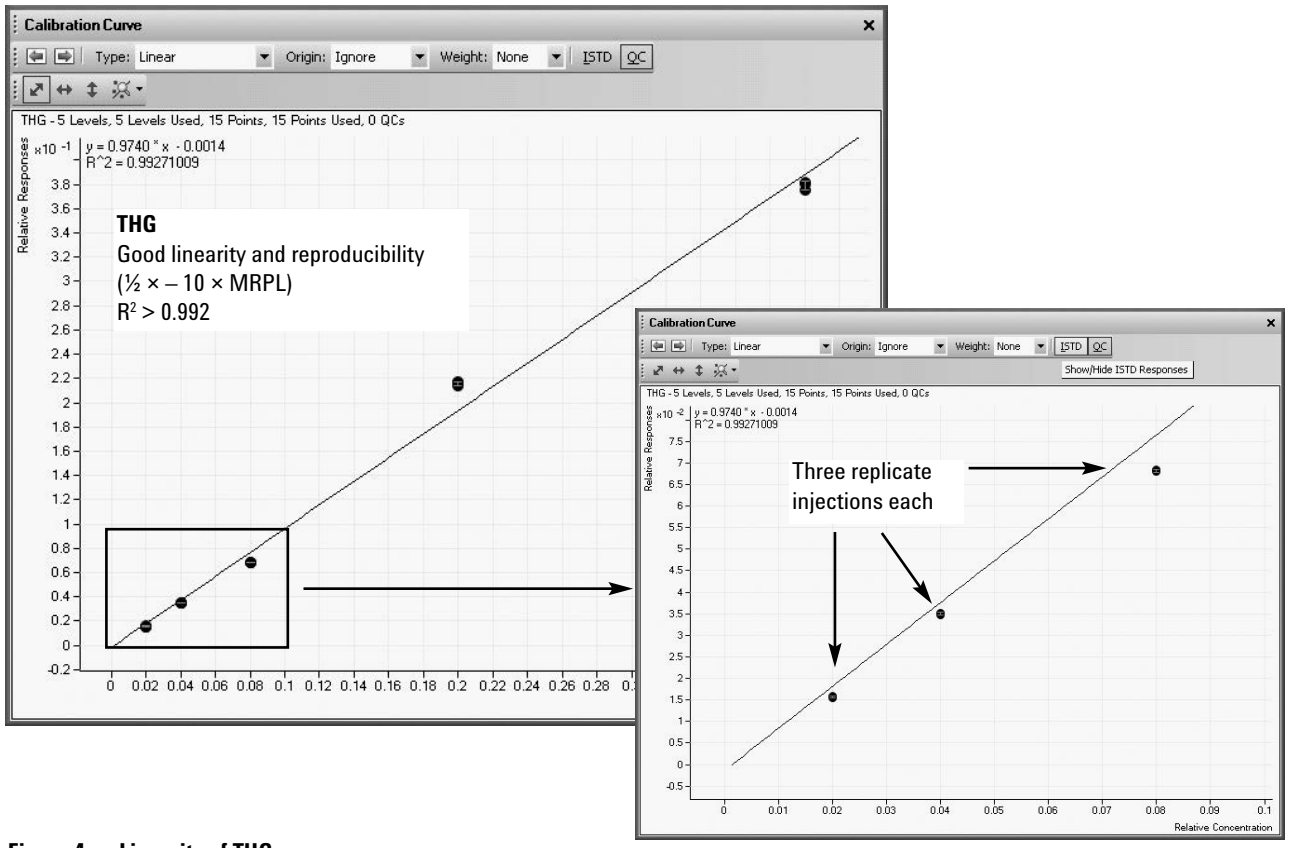

Figure 4a. Linearity of THG.

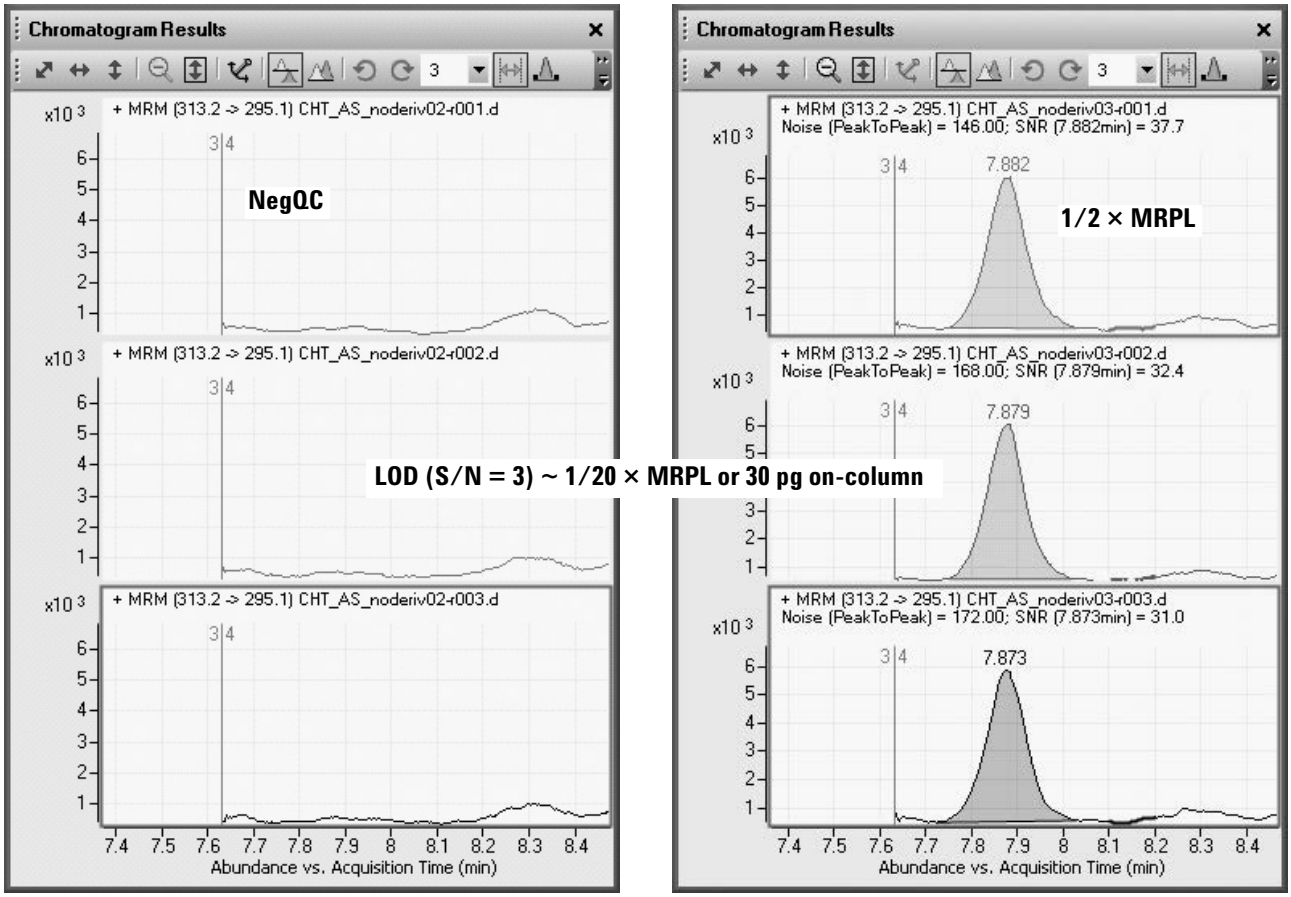

Figure 4b. Estimate of LOD for THG.

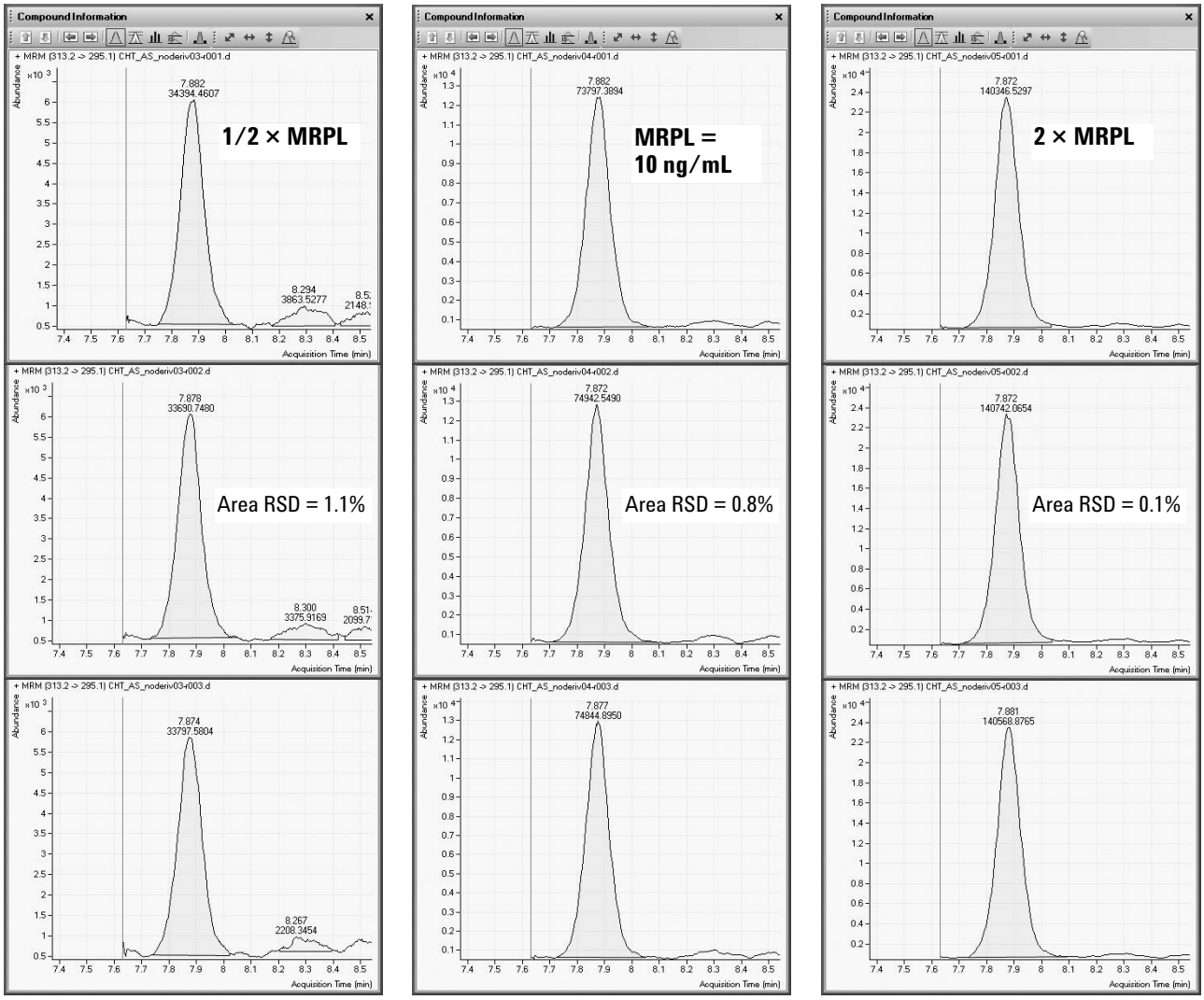

Figure 4c. Triplicate injections of the lowest three levels of THG.

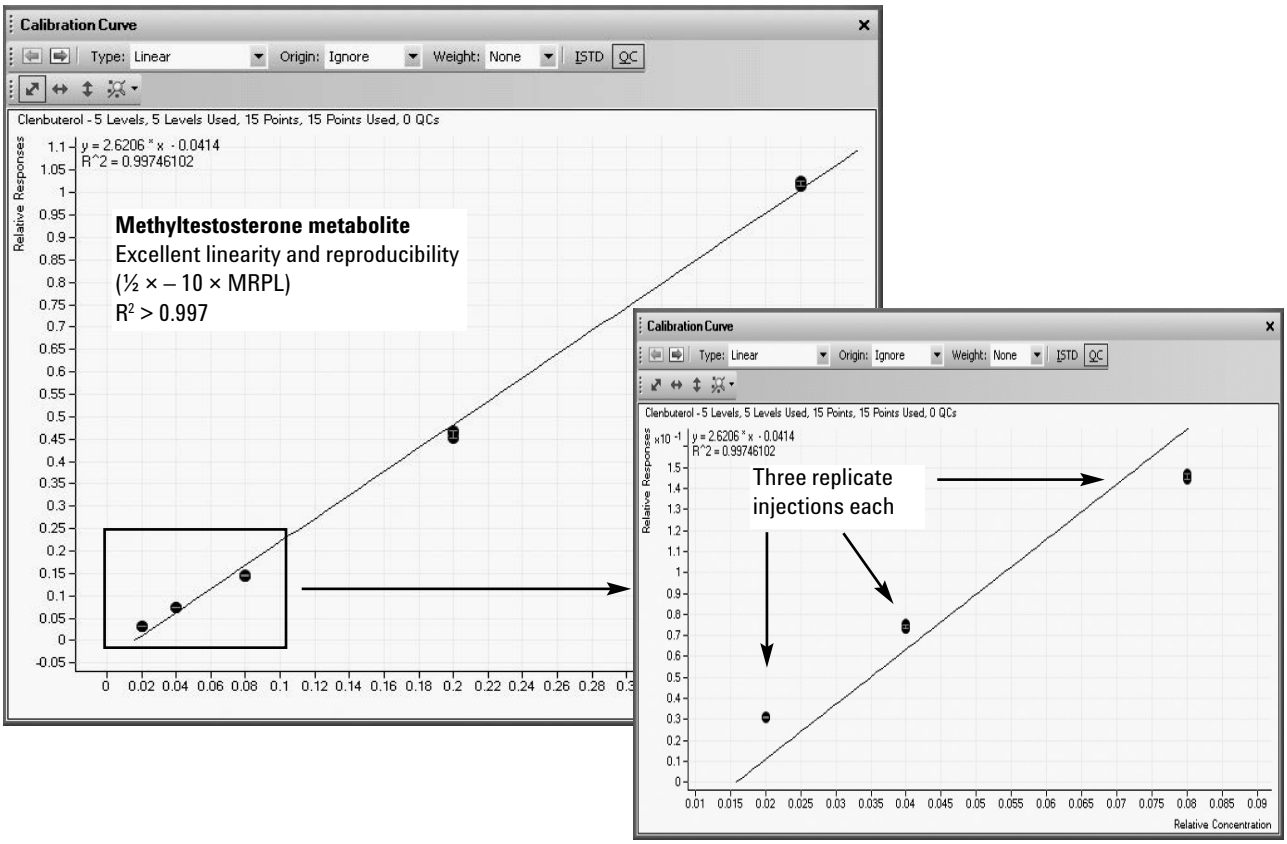

Figure 5a. Linearity of methyltestosterone metabolite.

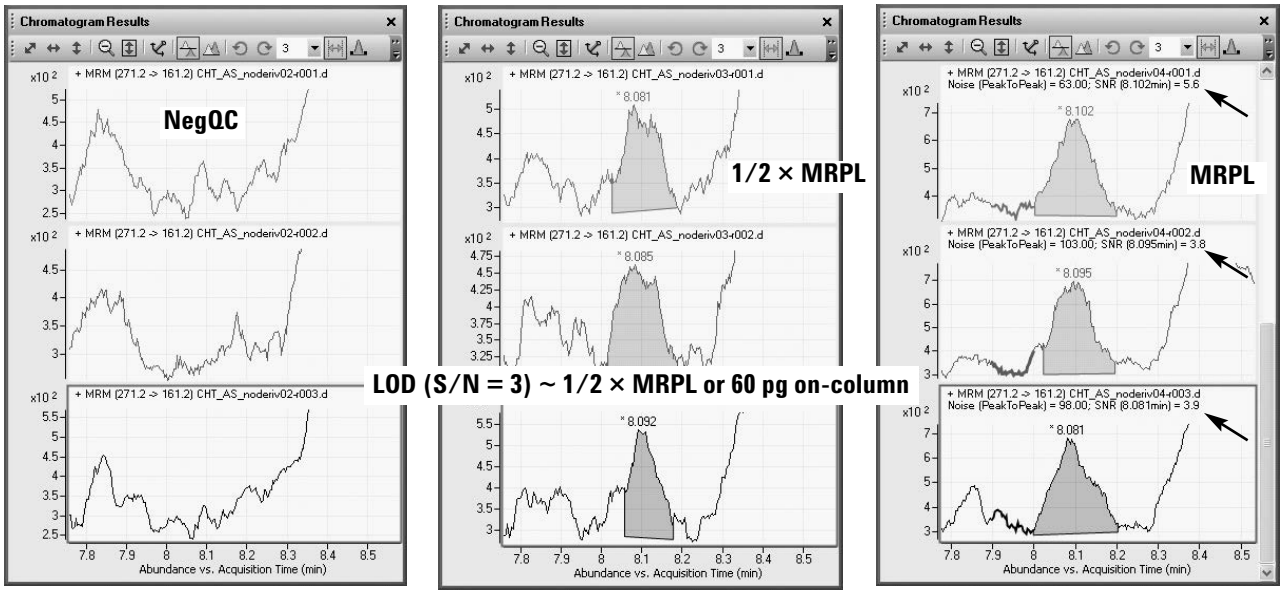

Figure 5b. Estimate of LOD for methyltestosterone metabolite.

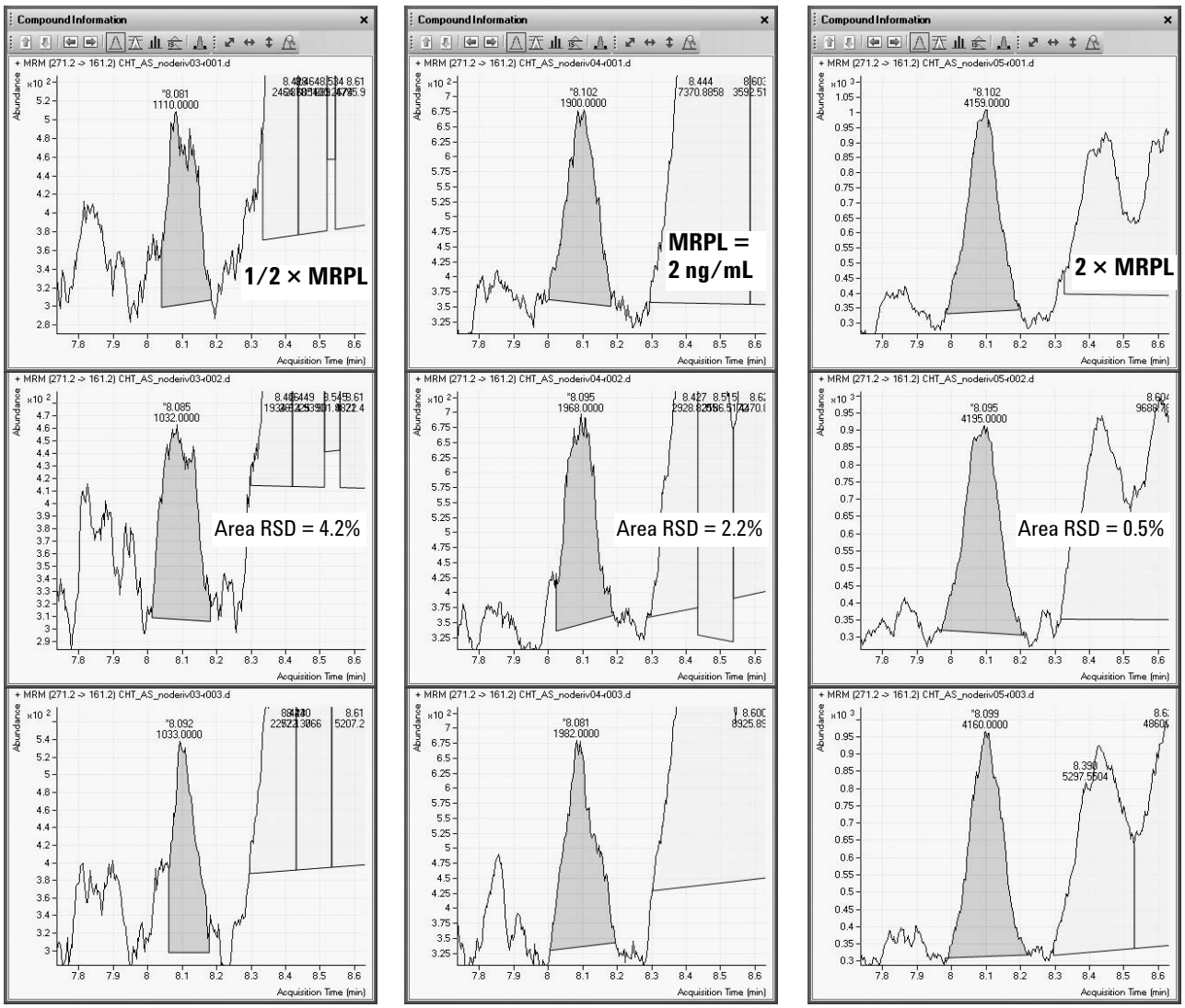

Figure 5c. Triplicate injections of the lowest three levels of methyltestosterone metabolite.

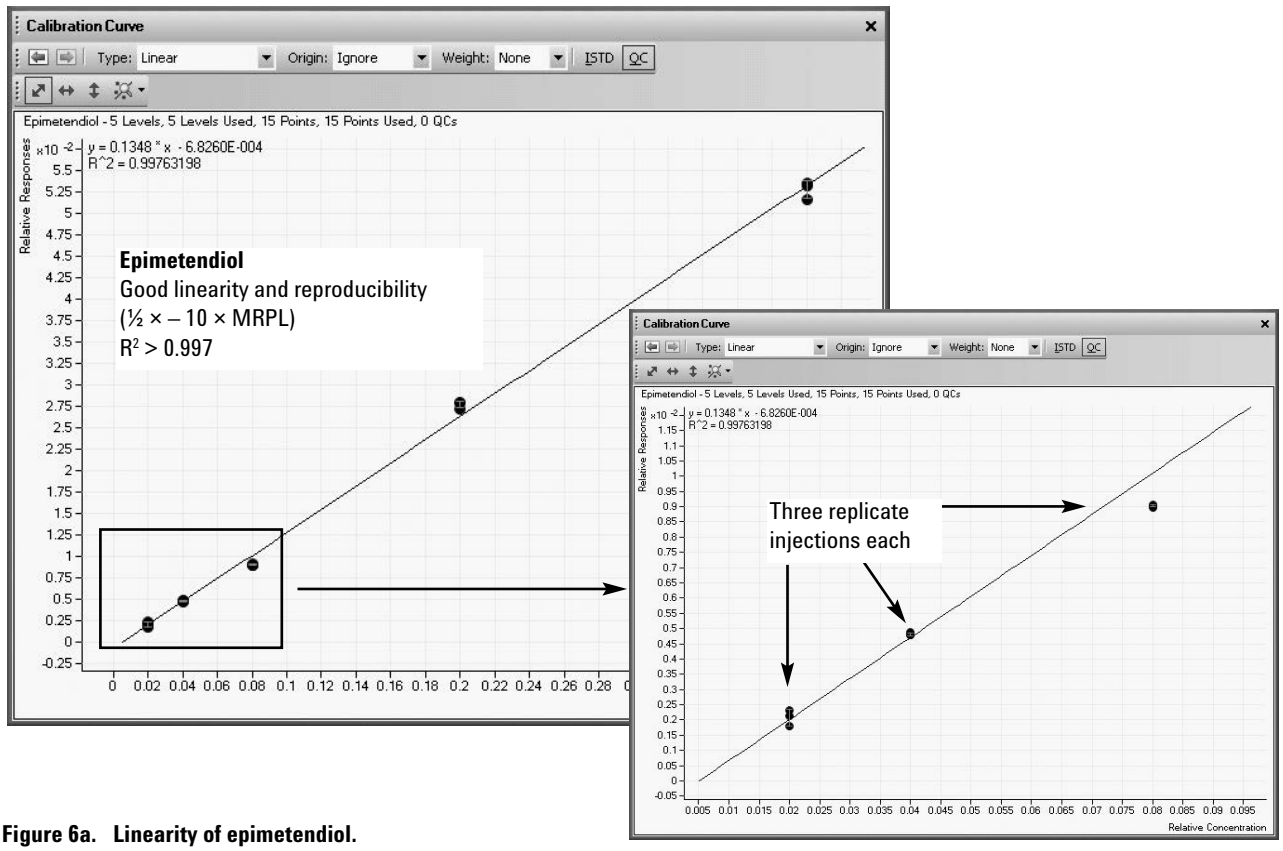

Figure 6a. Linearity of epimetendiol.

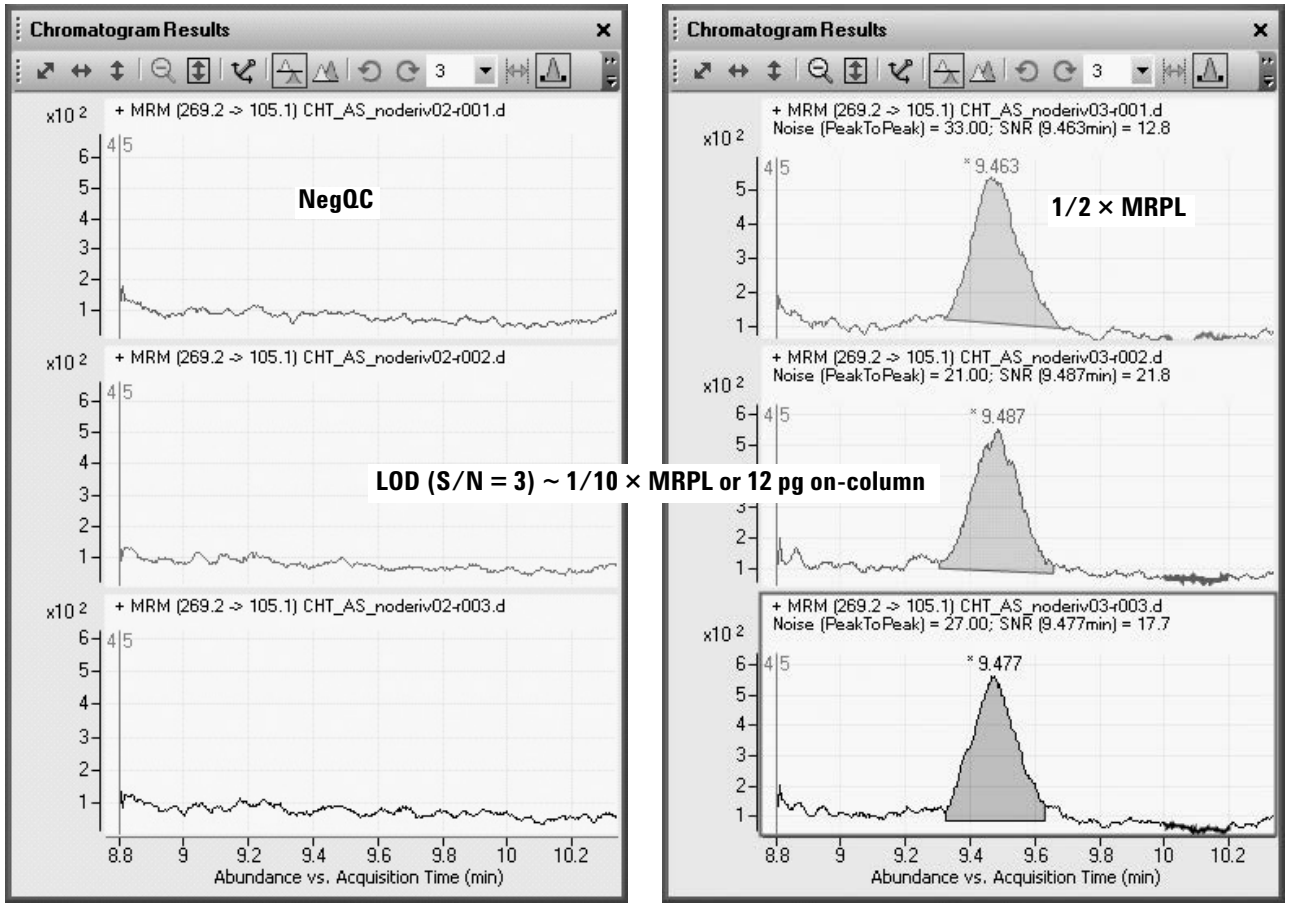

Figure 6b. Estimate of LOD for epimetendiol.

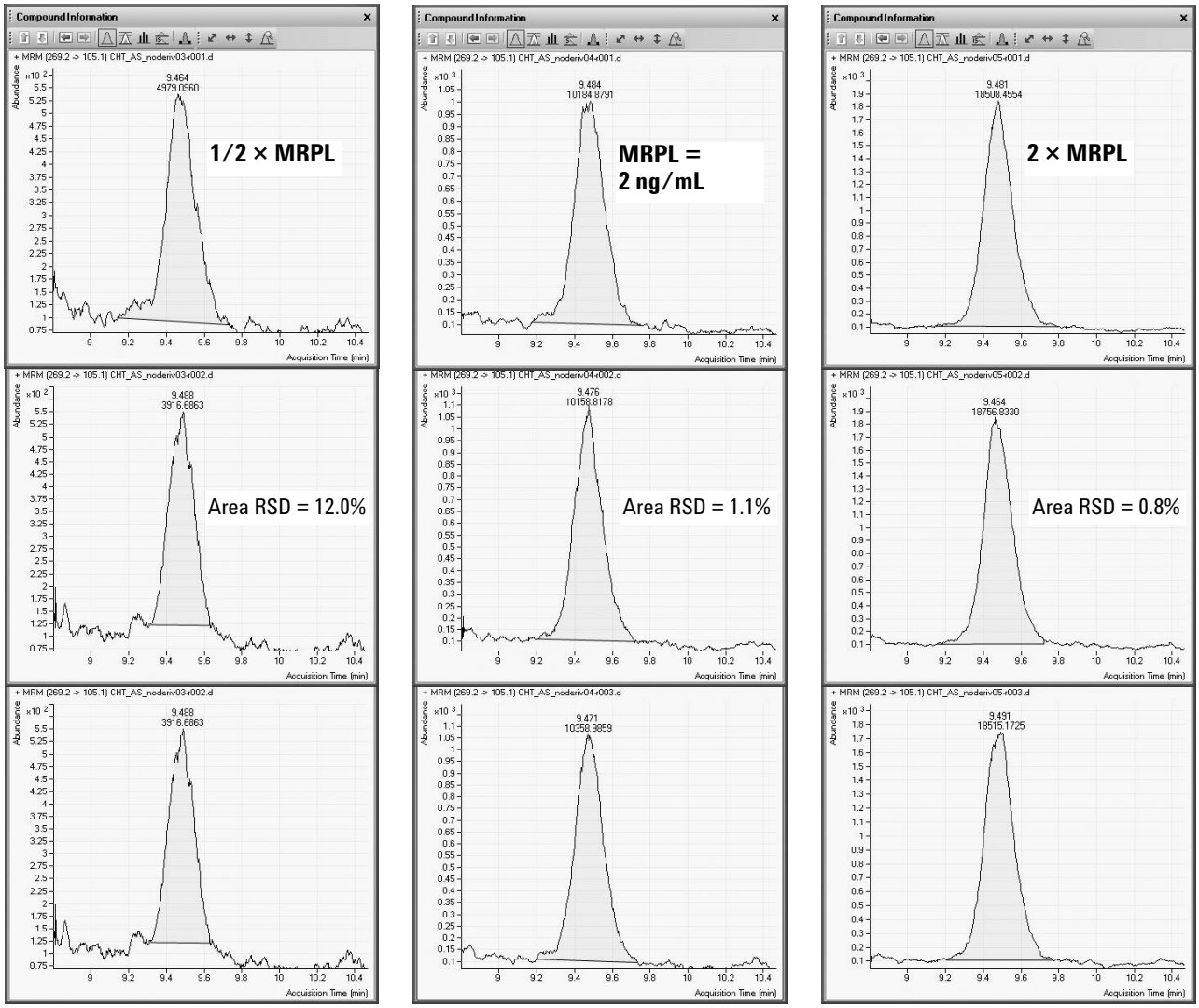

Figure 6c. Triplicate injections of the lowest three levels of epimetendiol.

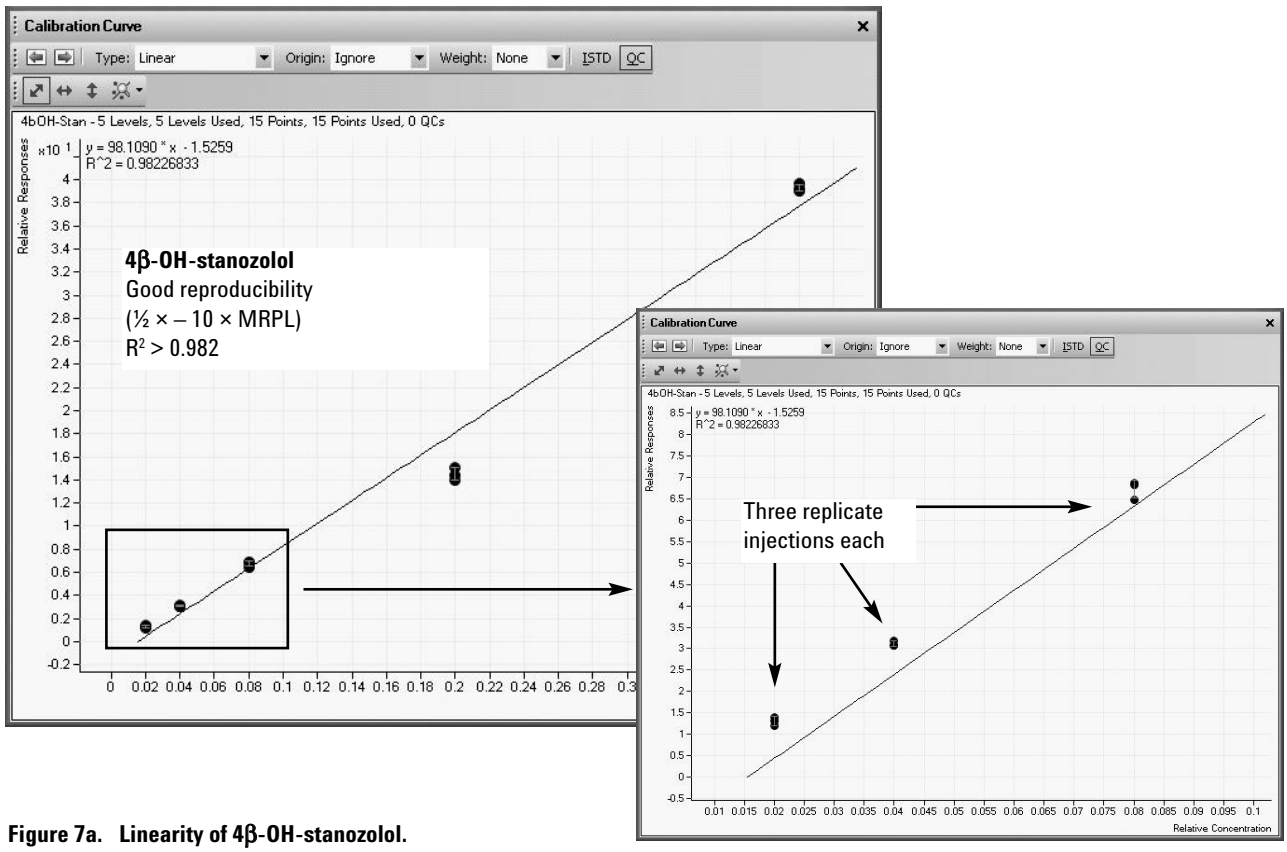

Figure 7a. Linearity of 4β-OH-stanozolol.

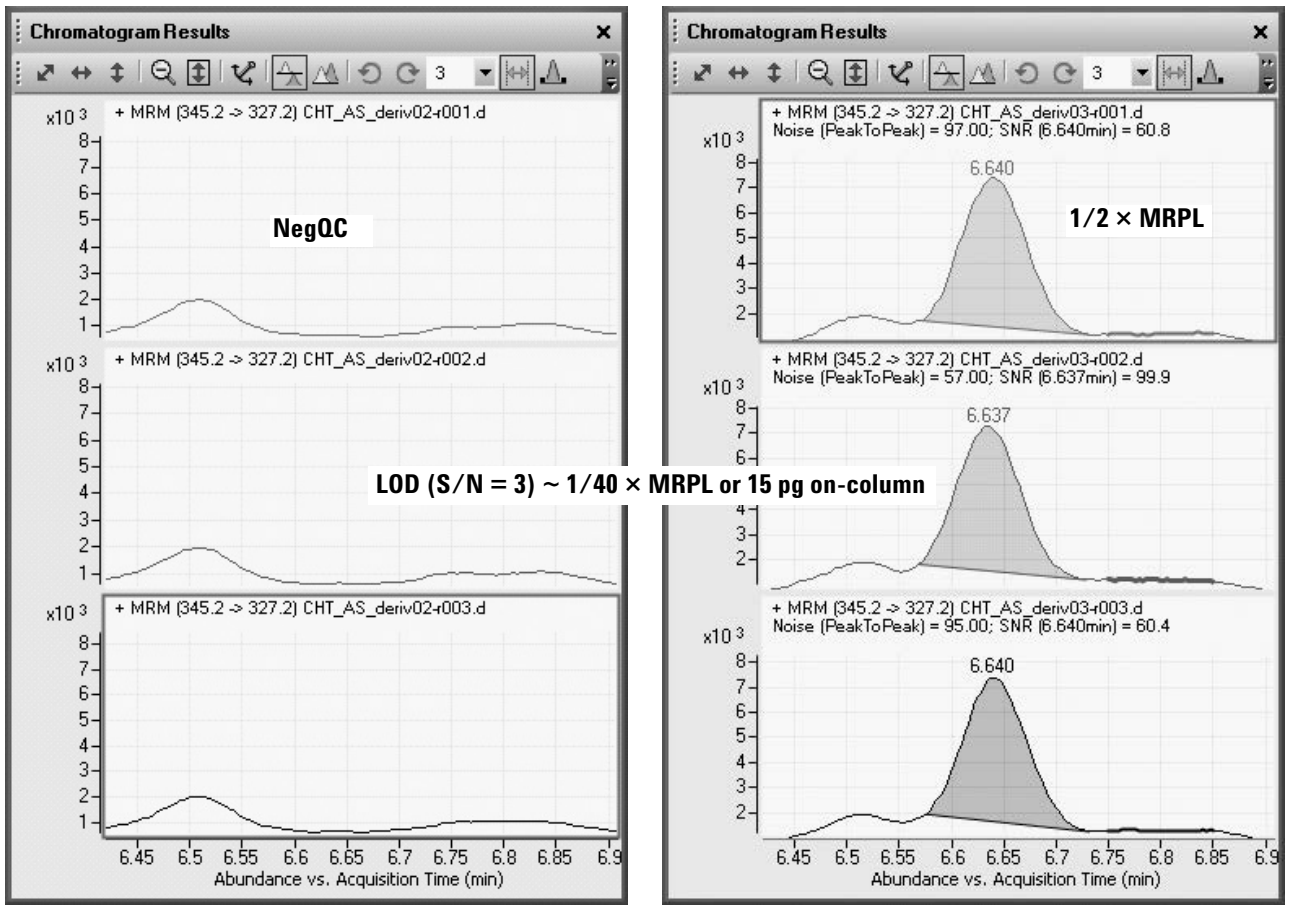

Figure 7b. Estimate of LOD for 4β-OH-stanozolol.

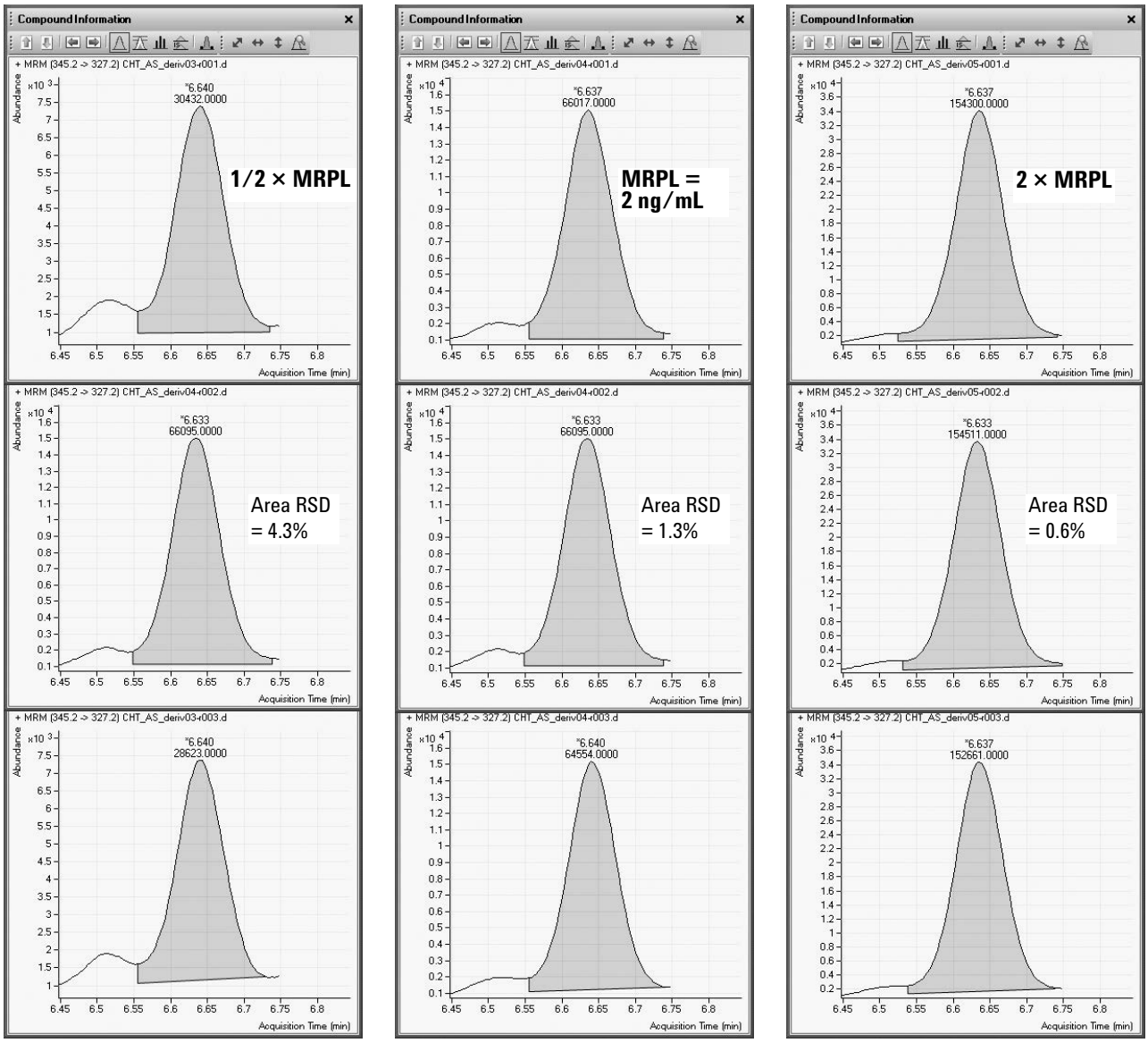

Figure 7c. Triplicate injections of the lowest three levels of 4β-OH-stanozolol.

In Figure 8 the reason for using the GRP derivative is shown by comparing the sensitivity of analyzing the 19-norandrosterone with and without the derivative.

Figures 9a to 9c show the linearity, LOD, and the lowest three level replicate injections for 19-norandrosterone. In Figure 9b we see noticeable signal in the negative quality control. However, this signal definitely comes from the matrix itself as it is not seen in the solvent blank.

The results for all compounds are summarized in Table 3.

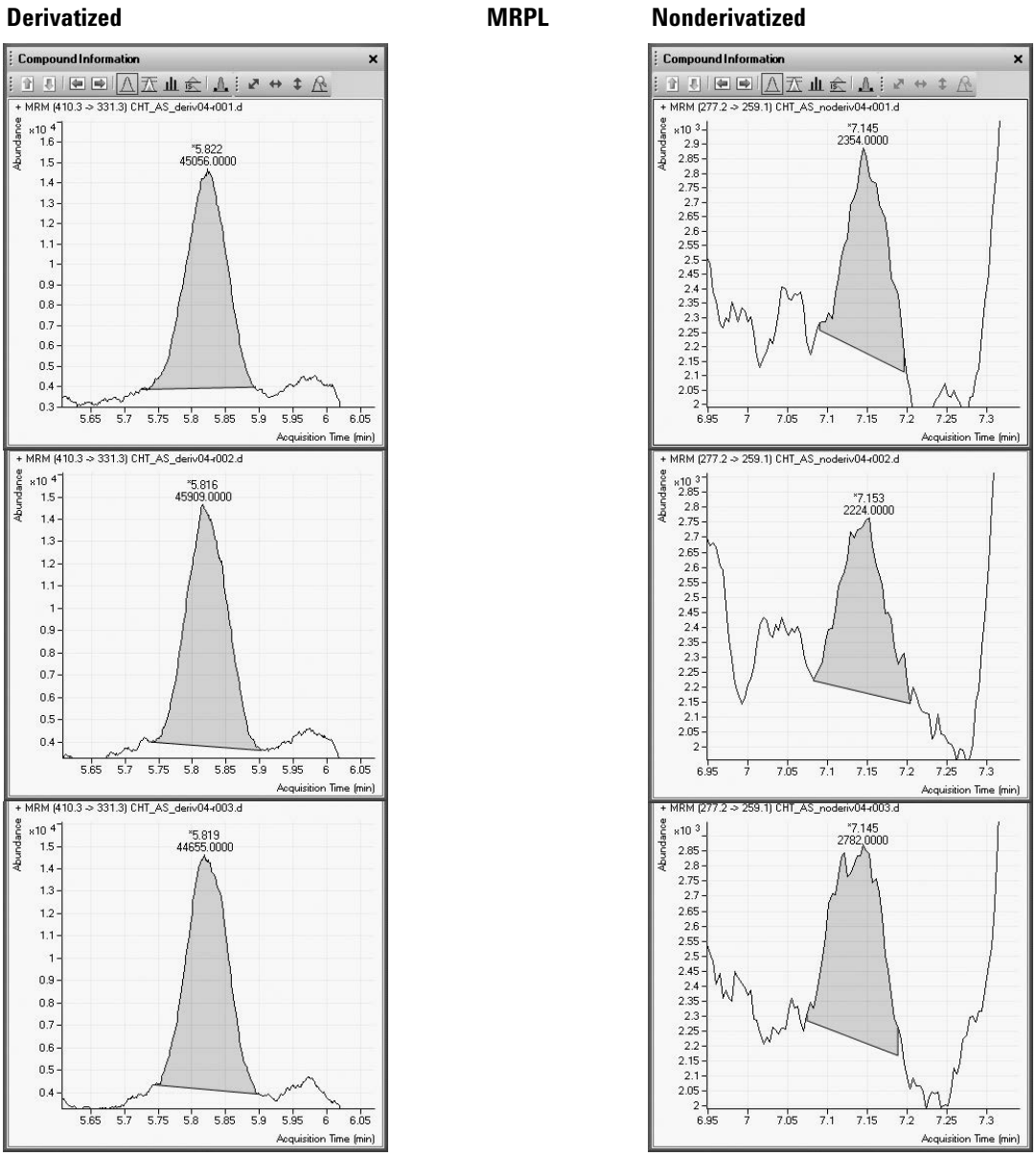

Figure 8. Comparison of signal response for the derivatized (left) versus nonderivatized forms of 19-norandrosterone.

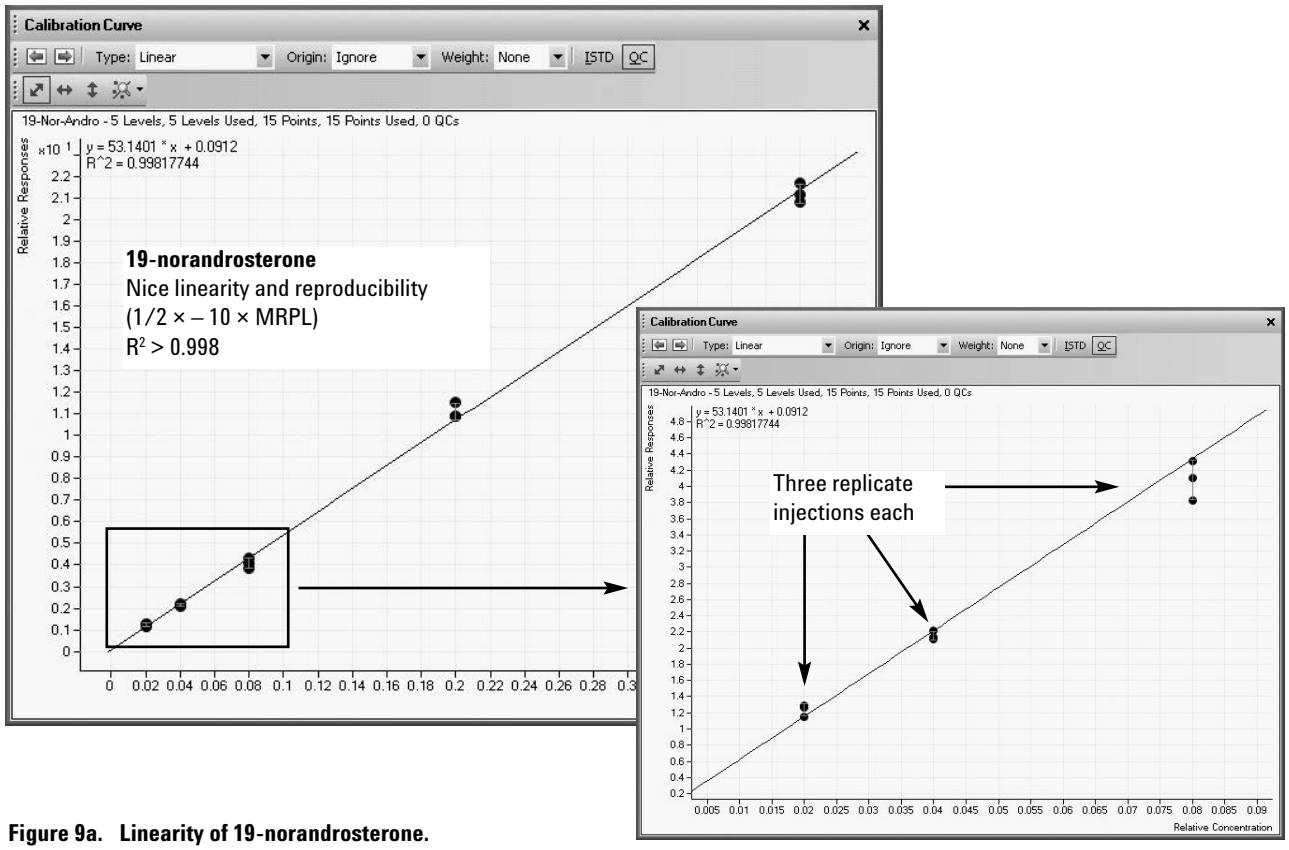

Figure 9a. Linearity of 19-norandrosterone.

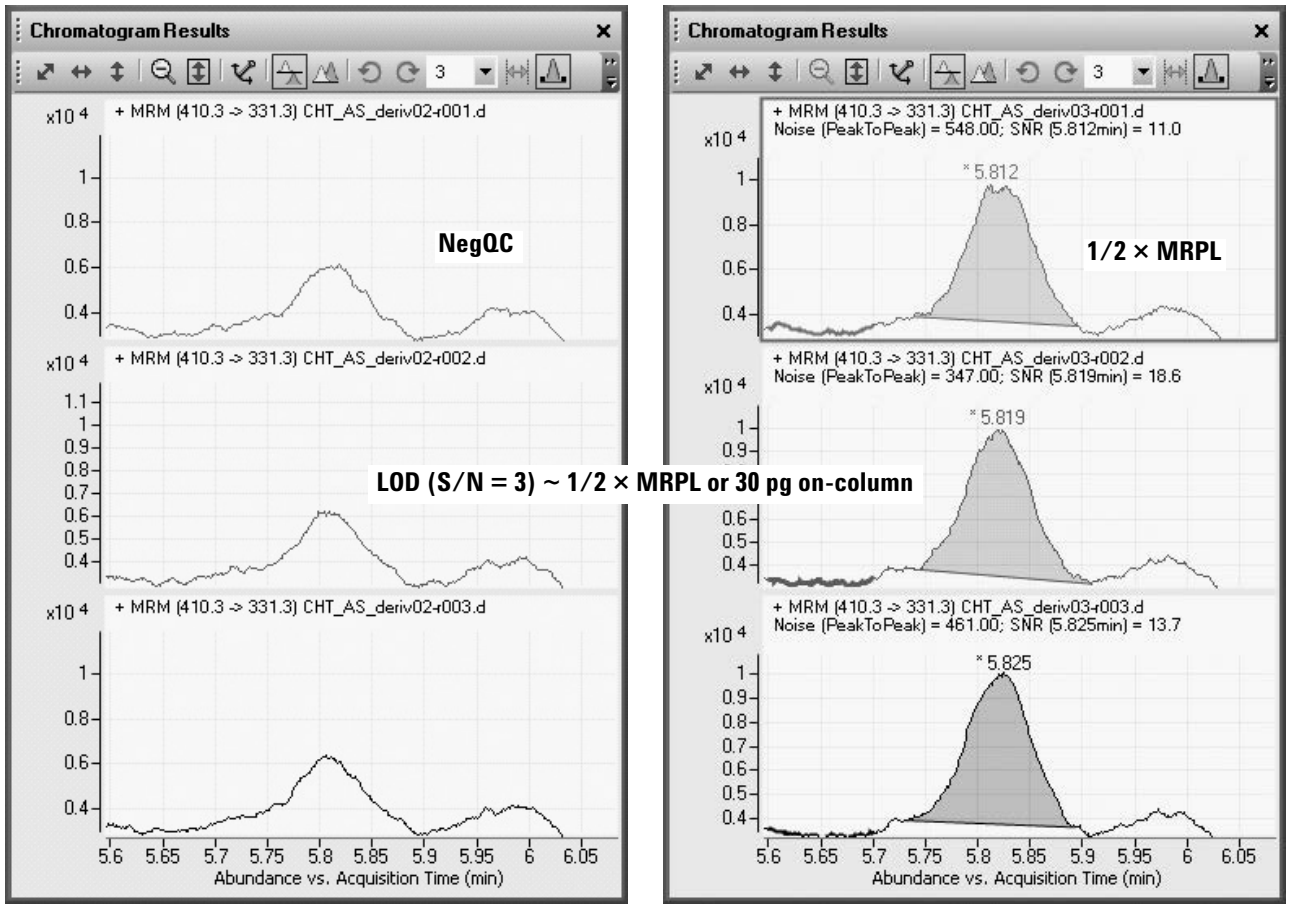

Figure 9b. Estimate of LOD for 19-norandrosterone.

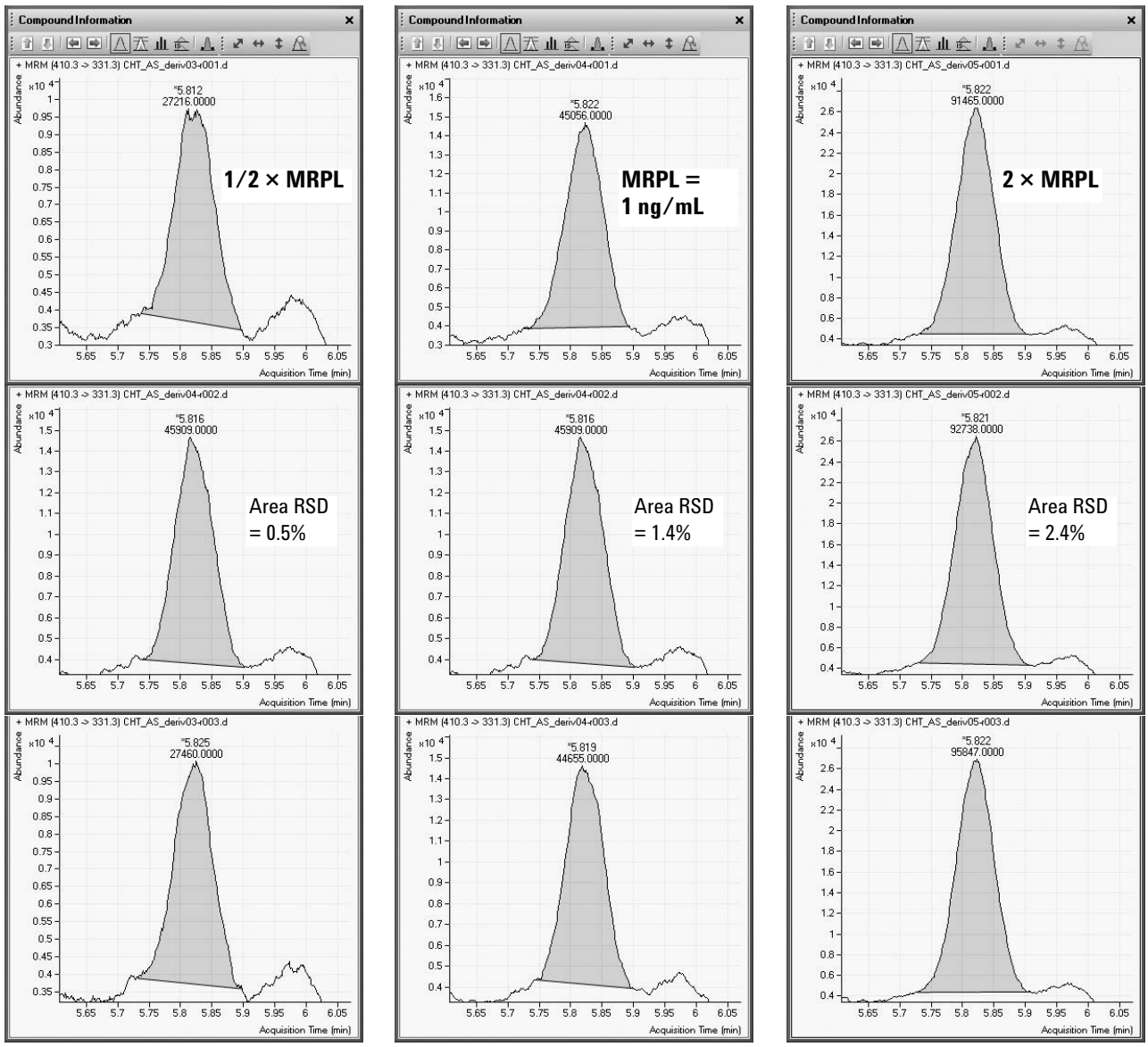

Figure 9c. Triplicate injections of the lowest three levels of 19-norandrosterone.

Table 3. Linearity, Reproducibility, and Calculated Sensitivity for All Compounds Analyzed

Compound	Linearity R^2	% RSD at 1/2 × MRPL	LOD on-column (pg)	LOD MRPL (×)
Clenbuterol	> 0.999	1.1	3	1/40
THG	> 0.992	1.1	30	1/20
MeTest metabolite	> 0.997	4.2	60	1/2
Epimetendiol	> 0.997	12.0	12	1/10
4β-OH-stanozolol	> 0.982	4.3	15	1/40
19-norandrosterone	> 0.998	0.5	30	1/2

Conclusions

The analysis of anabolic substances in urine can be difficult and may require the sensitivity of a triple quadrupole mass spectrometer as seen in this work. Linearity over the range of 1/2 × to 10 × MRPL for each compound is demonstrated and shown to be very good, especially for clenbuterol, which has a correlation coefficient of more than 0.999. The liquid chromatography in this work only uses solvents of water and methanol, with the addition of formic acid for a simple gradient. Limits of detection at levels lower than the minimum required performance levels are demonstrated with percent relative standard deviations of peak areas ranging from 12.0% to as low as 0.5%. The addition of Girard's Reagent P solution shows a marked improvement in sensitivity for the 19-norandrosterone compound.

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Determination of Buprenorphine, Norbuprenorphine, and Their Glucuronides in Urine Using LC/MS/MS

Application

Forensics

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Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of buprenorphine, its metabolite norbuprenorphine, and their glucuronides in urine using the Agilent 6410 Triple Quadrupole Mass Spectrometer in electrospray mode is described. Sample preparation included dilution of the urine samples in deionized water for direct injection into the LC/MS/MS system. Since the glucuronides are monitored in the same assay as the free drugs, no hydrolysis or extraction was necessary. To our knowledge, the procedure is the first to include the simultaneous monitoring of a qualifying ion for the parent drug, which is required to be present within a specific ratio to the primary ion for acceptable identification (± 20%). The Agilent MassHunter software allows the transitions to be monitored and automatically calculated into ratios, which must fall within the range of the calibration standards in order to be considered positive. While monitoring a qualifying ion naturally inhibits the sensitivity of the assay, the additional confidence in the result is a critical factor in forensic analysis.

Introduction

Buprenorphine is a member of the opioid family of drugs for the treatment of chronic pain, and in heroin addiction, as an alternative to methadone. It is metabolized to norbuprenorphine, and both species undergo extensive conjugation with glucuronide before urine excretion. The simultaneous determination of buprenorphine, norbuprenorphine, and methadone has recently been published [1]. Liquid chromatographic methods for the detection of buprenorphine in urine have predominantly been directed towards the free drug following hydrolysis, centrifugation, and/or extraction [2–4]. However, in 2003, Kronstrand et al. were the first to report on the detection of both free and conjugated compounds in urine using LC/MS/MS, noting that a low concentration of 20 ng/mL of free compounds seemed appropriate for the testing of patients. They improved the detection limit by hydrolyzing the specimens and subjecting them to solid phase extraction [5].

In this work, we present a rapid method, sensitive to 1 ng/mL of urine, for the detection of buprenorphine, norbuprenorphine, and their glucuronides in urine involving simple dilution of authentic urine samples with deionized water. Two transitions per compound are monitored for the free drugs and one transition for the glucuronides. The monitoring of the qualifying ion and calculation of its ratio to the intensity of the primary transition are integral parts of the software package and necessary for forensic identification.

The method is simple, sensitive, and rapid, with all analytes being determined in less than 8 minutes.

Experimental

Materials and Methods

Standards and Reagents

D4-Buprenorphine (D4-BUP); D3-Norbuprenorphine (D3-NBUP); BUP; NBUP; BUP glucuronide; and NBUP glucuronide were purchased from Cerilliant (Round Rock, TX). All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

Internal standard mix: D4-BUP; D3-NBUP (1,000 ng/mL)

Unlabelled drugs: BUP, NBUP, BUP glucuronide, NBUP glucuronide

Extraction Procedure–Urine

To urine (0.1 mL), add deionized water (0.35 mL) and 0.1 mL internal standard (1 µg/mL)

Calibration Curve:

a)	Negative:	0.1 mL D4-BUP; D3- NBUP
b)	1 ng/mL:	0.1 mL D4-BUP and D3-NBUP 10 µL of BUP, NBUP, and their glucuronides (100 ng/mL)
c)	5 ng/mL:	0.1 mL D4-BUP and D3-NBUP 5 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
d)	10 ng/mL:	0.1 mL D4-BUP and D3-NBUP 10 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
e)	20 ng/mL:	0.1 mL D4-BUP and D3-NBUP 20 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
f)	40 ng/mL:	0.1 mL D4-BUP and D3-NBUP 40 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)
g)	100 ng/mL:	0.1 mL D4-BUP and D3-NBUP 100 µL of BUP, NBUP, and their glucuronides (1,000 ng/mL)

Analytical Procedure

Instrument:	Agilent 1200 Series RRLC; 6410 Triple Quadrupole Mass Spectrometer

LC Conditions:

Column:	ZORBAX Eclipse XDB C18 4.6 mm × 50 mm × 1.8 µm (PN: 922795-902)
Dimensions:	4.6 mm × 50 mm × 1.8 µm
Column temp:	40 °C
Injection volume:	5 µL
Solvent flow rate:	0.8 mL/min

Pump Program:

Time (minutes)	% 20 mM ammonium formate (A)	% Methanol (B)
0	40	60
2.5	40	60
5	0	100
8.5	0	100
10	40	60
Post time:	3 min	

Mass Spectrometer Conditions:

Operation:	Electrospray (ESI) positive mode
Gas temperature:	300 °C
Gas flow (N_2):	6 L/min
Nebulizer pressure:	50 psi
Capillary voltage:	4500 V
Dwell Time:	50 ms

The MRM transition settings are shown in Table 1. The NBUP and BUP have both quant and qual (in parenthesis) product ions.

Table 1. Buprenorphine Acquisition Parameters

Compound	Precursor ion	Fragment ion	RT (min)	Fragment voltage (V)	CE (V)
Group 1					
D3-NBUP	417.4	399.3	1.16	240	40
NBUP 3 gluc	590.5	414.4	0.73	240	40
NBUP	414.4	340.4	1.17	240	35
		(187.2)	1.17	240	40
Group 2					
D4-BUP	472.5	400.4	6.62	240	45
BUP 3 gluc	644.5	468.4	5.21	240	40
BUP	468.4	414.4	6.68	240	35
		(396.1)	6.68	240	55

( ) Qualifier ratios must be within 20% of calibration point

LC/MS/MS Method Validation

The analytical method was validated according to standard protocols, whereby the limit of quantitation, linearity range, correlation, and intra- and inter-day precision were determined via multiple replicates over a period of 4 days. The slope of the calibration curve was not forced through the origin. The equation of the calibration curves and correlation coefficients (R^2) are shown in Table 2; the precision and accuracy of the assay are shown in Table 3. The assay was robust, precise, and accurate at the selected point of 10 ng/mL and was linear over the range of 5 to 100 ng/mL. The precision for all drugs was less than 20% both within day and between days, with most showing a variation of less than 10%. The limit of quantitation was 5 ng/mL; the limit of detection was 1 ng/mL.

Figure 1 shows a typical calibration curve for buprenorphine, with a correlation coefficient of 0.9984.

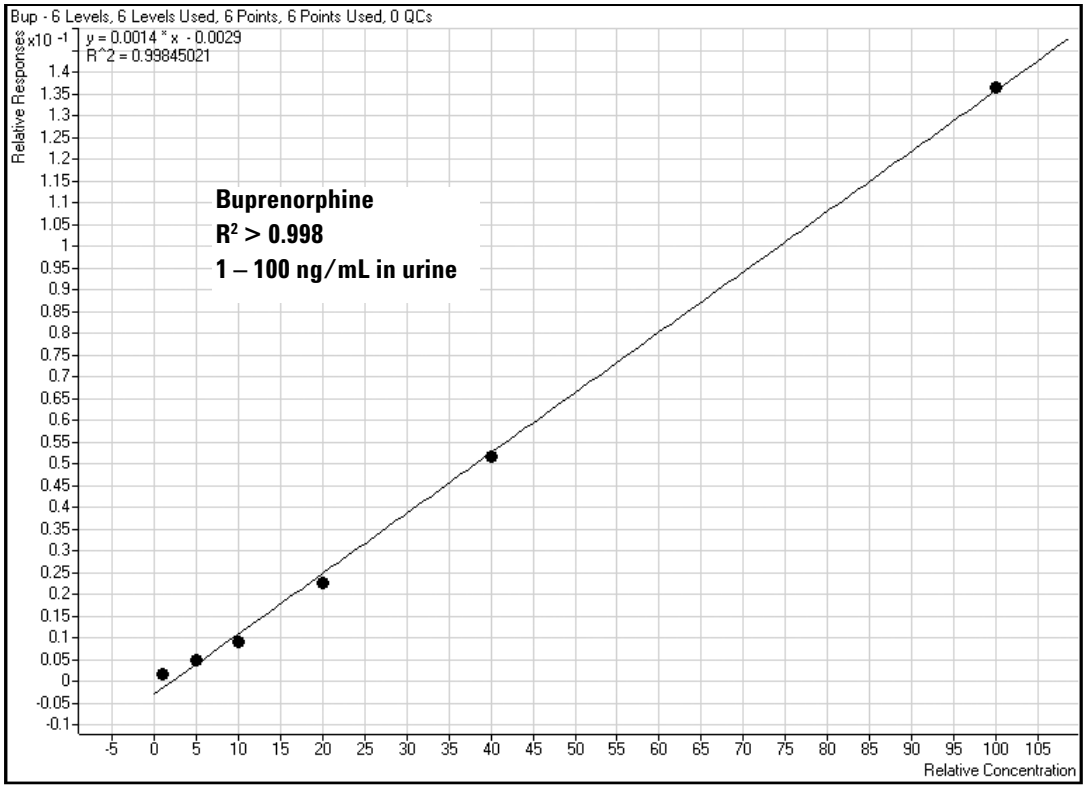

Figure 1. Calibration curve for free buprenorphine in urine.

Table 2. Linearity, Correlation Coefficient, and Acceptable Qualifier Ratio for Buprenorphine and Related Compounds in Urine

Drug	Calibration equation	Correlation coefficient (R^2)	Acceptable qualifier ratio (20%)
Buprenorphine	Y = 0.0065x – 0.005	0.9984	35.4 (28.3–42.5)
Norbuprenorphine	Y = 0.0068x – 0.0036	0.9995	44.9 (35.9–53.9)
Buprenorphine 3 glucuronide	Y = 0.0226x – 0.0064	0.9927	
Norbuprenorphine 3 glucuronide	Y = 0.0013x – 0.0039	0.9948	

Table 3. Inter-Day Precision (10 ng/mL Control Specimens; n = 10)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Buprenorphine	10.74	1.38	12.85
Norbuprenorphine	10.08	1.36	13.51
Buprenorphine glucuronide	12.68	2.41	19.02
Norbuprenorphine glucuronide	11.1	1.84	16.55

Intra-Day Precision (n = 5)

Drug	Mean recovery (ng/mL)	SD	Precision (%)
Buprenorphine	10.22	0.58	5.64
Norbuprenorphine	8.76	0.57	6.54
Buprenorphine glucuronide	10	0.8	7.04
Norbuprenorphine glucuronide	8.98	0.61	6.75

Discussion

The instrumentation allowed the rapid determination of buprenorphine, norbuprenorphine, and their glucuronides at low concentration, as is required for these drugs. The chromatographic separation produced by the small particle analytical column allowed separation of the peaks in each group segment (Figure 2). The software provided with the instrument is able to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, the identification is rejected. This is an additional feature of the triple quadrupole mass spectrometer, which is extremely important in forensic analysis where court challenges to laboratory data are frequent. Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in blood. The software plots the ratio in the chromatographic window, so the operator is able to assess positivity visually using the “uncertainty” band imposed by the software (Figure 3).

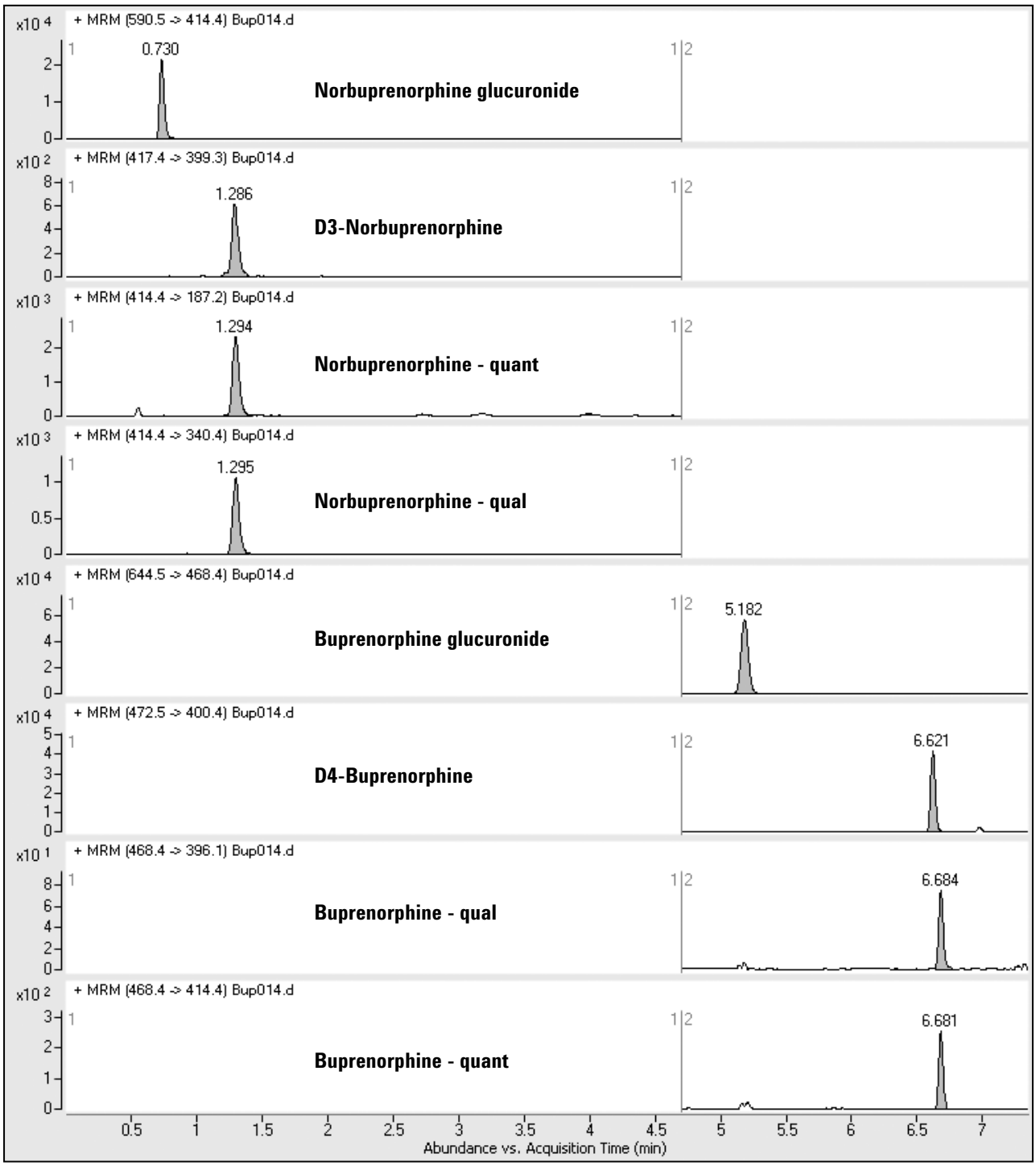

Figure 2. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide extracted from authentic urine specimen.

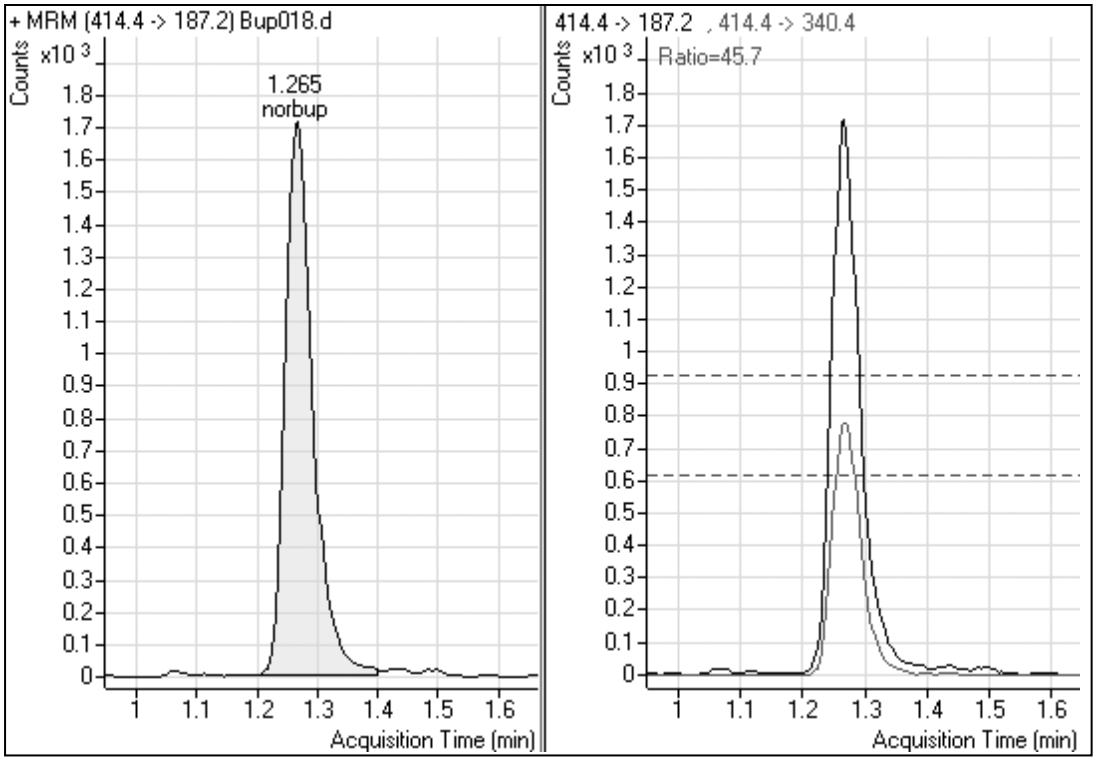

Figure 3. Free norbuprenorphine (98 ng/mL) in urine: quantitation ion at left and overlay of quantitation ion with qualifier ion at right.

Conclusions

The procedure described is suitable for the detection of buprenorphine and norbuprenorphine glucuronides in urine, without need for hydrolysis or extraction using an Agilent Technologies 6410 Triple Quadrupole LC/MS/MS system. This is the first method, which includes qualifying ions required to be present within a specific ratio, for the identification of buprenorphine and norbuprenorphine at low concentration in urine. The method is in routine use in our laboratory.

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Determination of Cocaine and Metabolites in Urine Using Electrospray LC/MS

Application Note

Drug Testing

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John M. Hughes
Agilent Technologies

Abstract

A rapid, simple, and sensitive electrospray LC/MS method has been developed for the quantitative analysis of cocaine and benzoylecgonine in urine using electrospray with the Agilent 1100 LC/MSD system. Urine samples were extracted using solid phase extraction cartridges, and the drug and metabolite were analyzed without derivatization using an isocratic separation and selected ion monitoring (SIM).

Introduction

Cocaine is a widely abused drug for which two metabolites, benzoylecgonine (BE) and norcocaine, are frequently analyzed as markers of cocaine use. The well-established GC/MS analysis of cocaine and BE requires derivatization of the metabolite. Derivatization adds additional variables from the derivatization process and can also introduce aggressive derivatizing reagents into the analytical system. These basic molecules show excellent sensitivity in electrospray mass spectrometry, and the analysis of cocaine and both metabolites can be carried out without a derivatization step. The same solid-phase extraction (SPE) developed for the GC/MS analysis can be used for the LC/MS analysis.

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Materials and Methods

The Agilent 1100 Series system included a binary pump, vacuum degasser, autosampler, thermostatted column compartment, diode-array detector, and an LC/MSD. The LC/MSD was used with the electrospray ionization (ESI) source. The diode-array detector was used during method development only. Complete system control and data evaluation was carried out using the Agilent ChemStation for LC/MS.

Sample Preparation and Extraction

Drug-free urine was fortified with known concentrations of the analytes for preparation of standard curves. Control samples were fortified with known concentrations of the analytes prepared from separate lots of stock solutions. Clean-Screen SPE columns (ZSDAU020, United Chemical Technologies) were conditioned with 3 mL of methanol and 3 mL of Milli-Q water, followed by 1 mL of 100 mM phosphate buffer, pH 6. Urine (1 mL) was mixed with 1 mL of the phosphate buffer, spiked with deuterated internal standards (cocaine-d3 and benzoylecgonine-d3) and loaded on the conditioned column. The column was sequentially washed with 2 mL of Milli-Q water, 2 mL of 100 mM HCl, and 3 mL of methanol. The column bed was dried at full vacuum for five minutes, and the analytes were eluted with 3 mL of dichloromethane/isopropanol/ammonium hydroxide (78/20/2). The eluate was evaporated to dryness with a stream of air at 40°C. The final sample residue was reconstituted in 50 µL of LC mobile phase, and 20 µL was injected for analysis by LC/MS.

Results and Discussion

In the analysis of cocaine metabolites, it is important to be able to distinguish the isobaric BE and norcocaine to allow accurate interpretation of results. The chromatography for this method was therefore optimized to separate BE from norcocaine, and isocratic conditions were found which allow for rapid analysis without column re-equilibration. Figure 1 shows the separation of cocaine, norcocaine and BE using these conditions.

MS parameters which were optimized for this analysis included fragmentor voltage (to give the most intense protonated molecule for each analyte), capillary voltage (for maximum signal), and spray chamber parameters (for maximum signal with minimum noise).

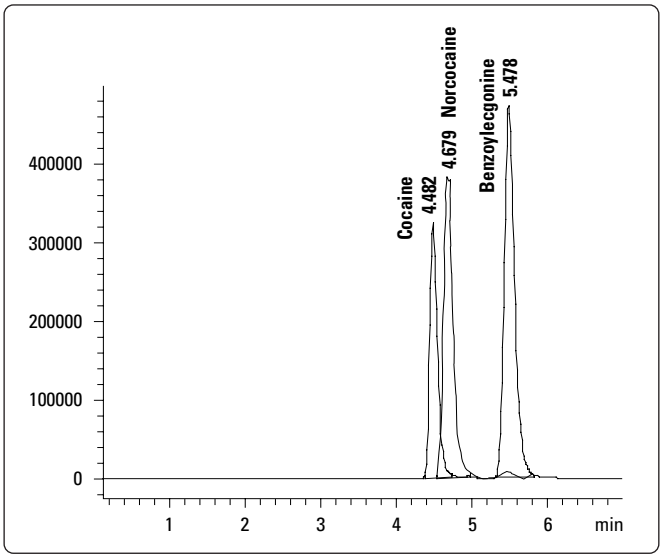

Figure 1. Isocratic separation of cocaine, norcocaine and BE.

Chromatographic Conditions	
Column:	Metasil Basic 3 µm, 3 × 150 mm (Metachem)
Mobile phase:	A = 0.1% formic acid in water
	B = methanol
Isocratic:	51% B
Flow rate:	0.2 mL/min
Column temp:	40°C
Injection vol:	20 µl
Diode-array detector:	signal: 234, 8 nm; reference: 360, 100 nm
MS Conditions	
Source:	ESI
Ionization mode:	positive
Vcap:	1500 V
Nebulizer:	20 psig
Drying gas flow:	10 L/min
Drying gas temp:	300°C
SIM ions:	*m/z* 290.1 (BE and norcocaine)
	m/z 293.1 (BE-d3)
	m/z 304.1 (cocaine)
	m/z 307.1 (cocaine –d3)
Peak width:	0.10 min
Time filter:	On
Fragmentor:	70 V

Figure 2 shows the extracted ion chromatograms (EICs) for blank urine fortified with the internal standards. Figure 3 shows the EICs for a urine standard fortified at 25 ng/mL.

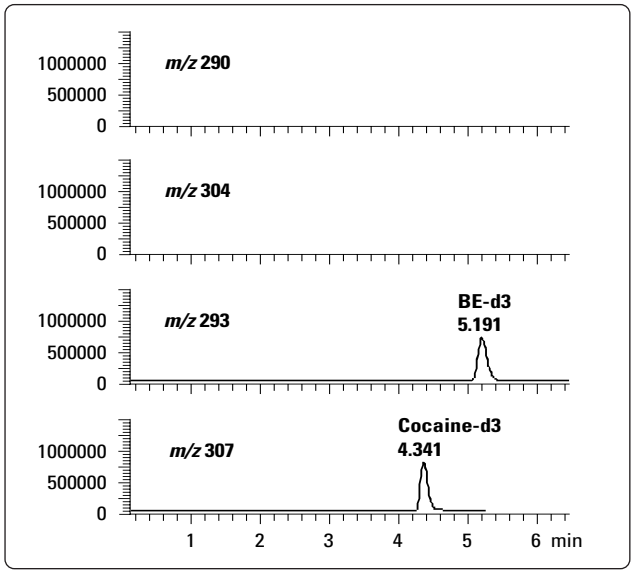

Figure 2. Extracted ion chromatograms of blank urine extract.

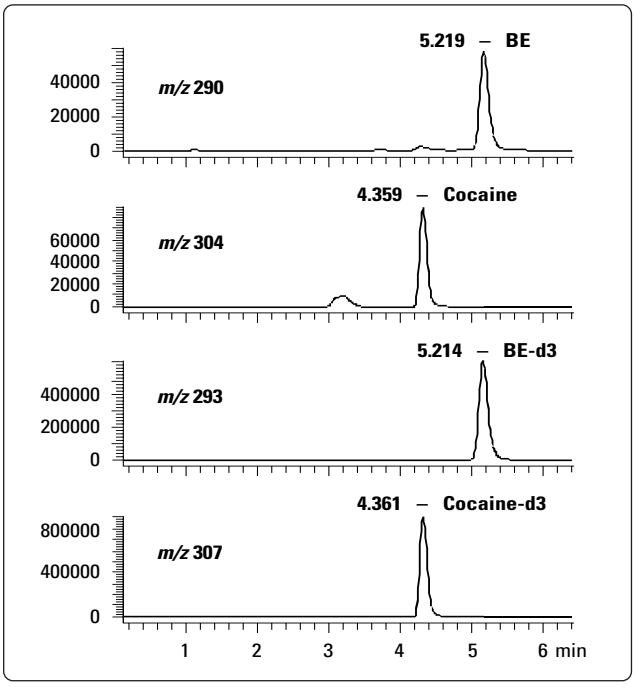

Figure 3. Extracted ion chromatograms of fortified urine extract (25 ng/mL).

The calibration range used for this analysis was 25–1000 ng/mL for both cocaine and BE. The calibration curves were linear across the calibration range without special weighting or curve treatment. Figure 4 shows typical calibration curves for cocaine and BE, with correlation coefficients (r^2) greater than 0.99 (0.99925 for cocaine and 0.99491 for BE).

Figure 5 shows the EICs of a positive urine sample found to contain 640 ng/mL cocaine and approximately 2700 ng/mL BE. The BE quantitation is an estimate, as the concentration is above the calibrated range of the method. Note that norcocaine can be clearly identified because it is chromatographically separated from benzoylecgonine which has the same mass.

Quality control samples fortified with 50 ng/mL and 150 ng/mL of each analyte gave quantitation results within 12% of the target concentration for cocaine and 3% for BE (see Table 1). Coefficients of variation were 7.1% and 5.1% for cocaine and BE respectively as shown in Table 1.

Table 1. Method accuracy and precision. Target concentrations were 50 ng/mL for cocaine and 150 ng/mL for BE.

	Cocaine	BE
	48.25	146.47
	47.06	155.69
	47.41	158.97
	46.21	148.50
	38.80	147.29
	40.89	146.57
	41.38	167.06
	42.68	159.81
Mean	44.085	153.795
Std Dev	3.570	7.734
C.V.*	7.1%	5.1%

*coefficient of variation = (mean/target)*100

These results compare well with an established GC/MS assay in which intra-assay coefficients of variation were less than 7% for both analytes when tested at 10, 25, 100, and 200 ng/mL.[1] The GC/MS assay gave quantitation results within 4% of the target concentration for cocaine and 5% for BE.

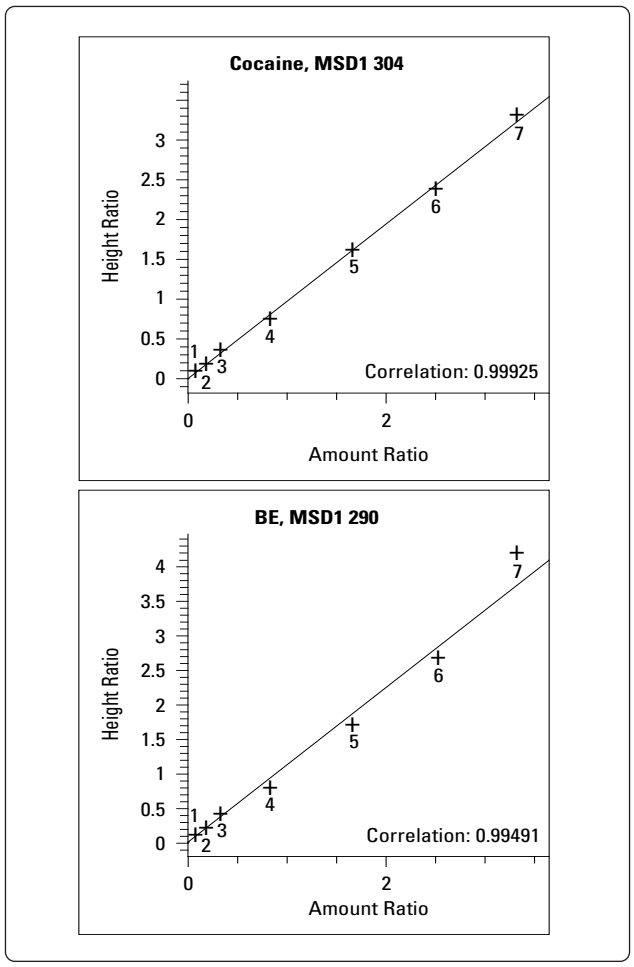

Figure 4. Calibration curves for cocaine and BE.

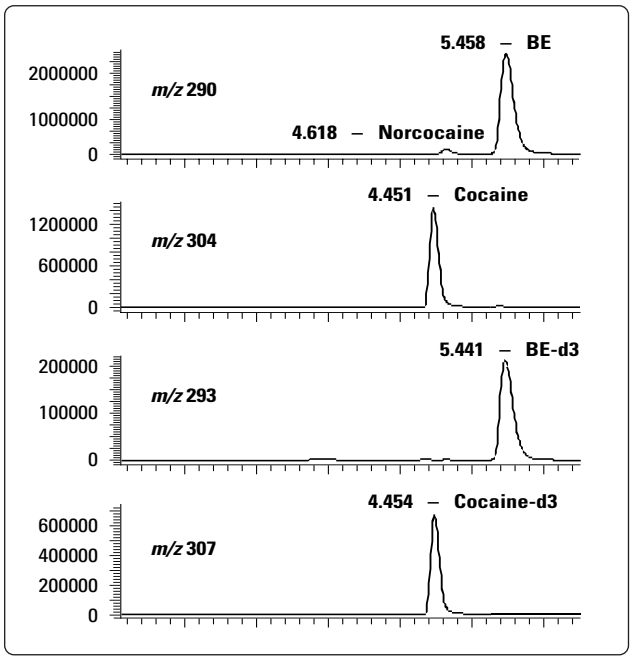

Figure 5. Extracted ion chromatograms from the extract of a positive urine sample.

Conclusions

This note describes an electrospray LC/MS method suitable for routine measurements of cocaine, BE and norcocaine in urine. The assay has a linear range of 25–1000 ng/mL and the precision and accuracy of this method compare favorably to those of the well-established GC/MS method for cocaine and BE. The sample preparation uses previously-described solid phase extraction technology widely used in forensic laboratories and requires no special modifications. In comparison to an existing GC/MS method for these analytes, the LC/MS method is simpler because it does not require derivatization, which involves aggressive reagents, derivatization time, and additional variability. In addition, the overall cycle time for one analysis is shorter for the LC/MS method than for the GC/MS method. This LC/MS method offers several advantages over traditional GC/MS assays with comparable quality of data.

References

1. Crouch, D.J.; Alburges, M.E.; Spanbauer, A.C.; Rollins, D.E.; Moody, D.E.; Chasin, A.A. *Journal of Analytical Toxicology* 1995, 19, 352–358.

Acknowledgments

The authors would like to thank Christine Miller of Agilent Technologies for review and helpful comments.

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- The First Accurate Mass MS/MS Library for Forensics and Toxicology Using the Agilent 6500 Series Accurate Mass Q-TOF LC/MS
- Accurate Mass Measurement for Analyzing Drugs of Abuse by LC/Time-of-Flight Mass Spectrometry
- Screening and Confirmation of Anabolic Steroids Using Accurate Mass LC/MS
- An Application Kit for the Screening of Samples for Analytes of Forensic and Toxicological Interest using TOF or Q-TOF LC/MS with a Personal Forensics/Toxicology Database
- Quantitative Analysis of Opiates in Urine Using Accurate Mass LC/MSD TOF
- Development of a Screening Analysis by LC Time-Of-Flight MS for Drugs of Abuse
- High Resolution Quantitative Analysis of B2-Agonist in Human Urine by LC/MS Using the Agilent LC/MSD TOF

Applications by Technique

LC/TOF & LC/QTOF

Database and Library Searching for Screening Toxins and Drugs-of-Abuse

The First Accurate Mass MS/MS Library for Forensics and Toxicology Using the Agilent 6500 Series Accurate Mass Q-TOF LC/MS

The Broecker, Herre, & Pragst Personal Compound Database and Library virtually eliminates false positives and provides confident identification without standards.

Screening and identifying the large number of compounds that are of concern to forensic scientists and toxicologists is a formidable undertaking. The Agilent 6500 Accurate Mass Q-TOF LC/MS with the Forensic and Toxicology Personal Compound Database and Library (PCDL) can screen and identify both the parent compound and resulting metabolites. There are over 7500 compounds in the database and over 2600 of them contain MS/MS spectra. Any of the Agilent Q-TOF LC/MS instruments can collect high resolution MS and MS/MS spectra with mass accuracies better than 3 ppm even for MS/MS fragments. Samples can be run and the database and library searched using Auto MS/MS and MassHunter Qualitative Analysis, which are powerful data mining tools that positively identify compounds with accurate mass of both precursor and fragment ion information.

Auto MS/MS precursor ions trigger MS/MS spectra to be collected under user defined conditions. All single MS ions detected are mined to determine if they represent compounds and if they do are searched against the database of compounds using exact molecular weight and the possible adducts. The MS/MS spectra are then searched for library matches and identified with both a forward and reverse score. Direct graphic and tabular inspection of the matches can be made. The power of the high quality data collected, data mining approaches, and the library allow a difficult task to be completed in hours versus days, with the confidence of a direct match from Agilent instrument to instrument.

Key Benefits

- Agilent 6500 Series Accurate Mass Q-TOF LC/MS provides the sensitivity needed with full spectra to determine toxins or drugs present in bodily fluids
- The Broecker, Herre & Pragst PCDL provides the greatest number of relevant compounds for screening and identification
- The database contains over 7500 compounds and metabolites with accurate mass MS/MS spectra for more than 2600 of them
- The library can identify a large number of compounds quickly
- False positives are virtually eliminated with confident identification of accurate MS/MS library search results
- Comprehensive workflows meet the needs of the specific analysis: Auto MS/MS for rapid screening Targeted MS/MS for focused analysis

Our measure is your success.

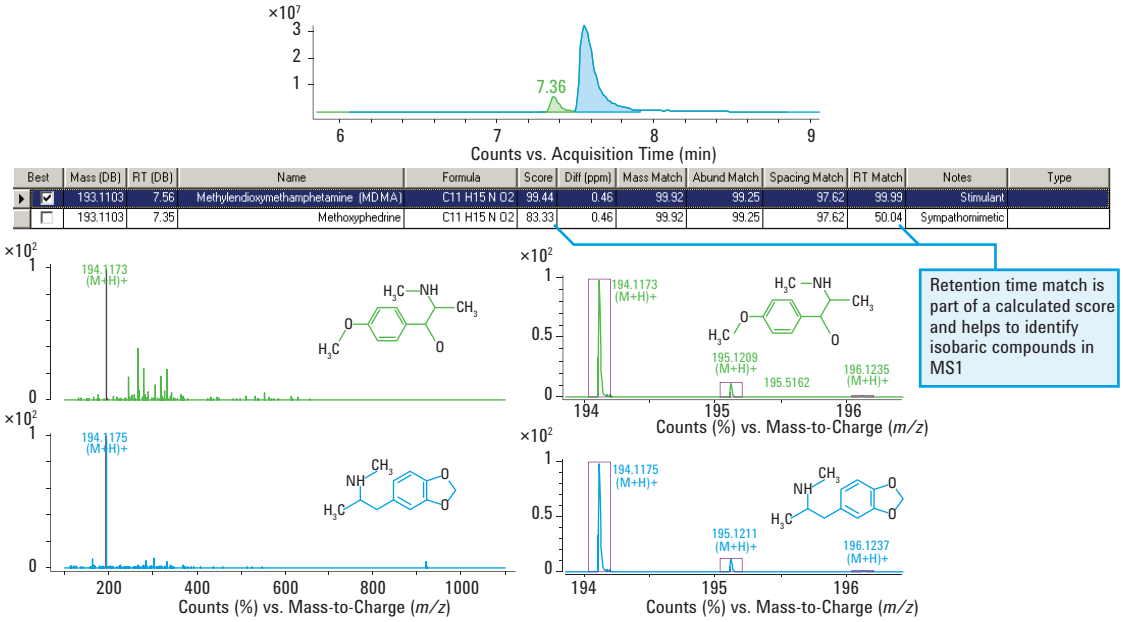

Figure 1: Single MS accurate mass data provides molecular formula but cannot determine isomers.

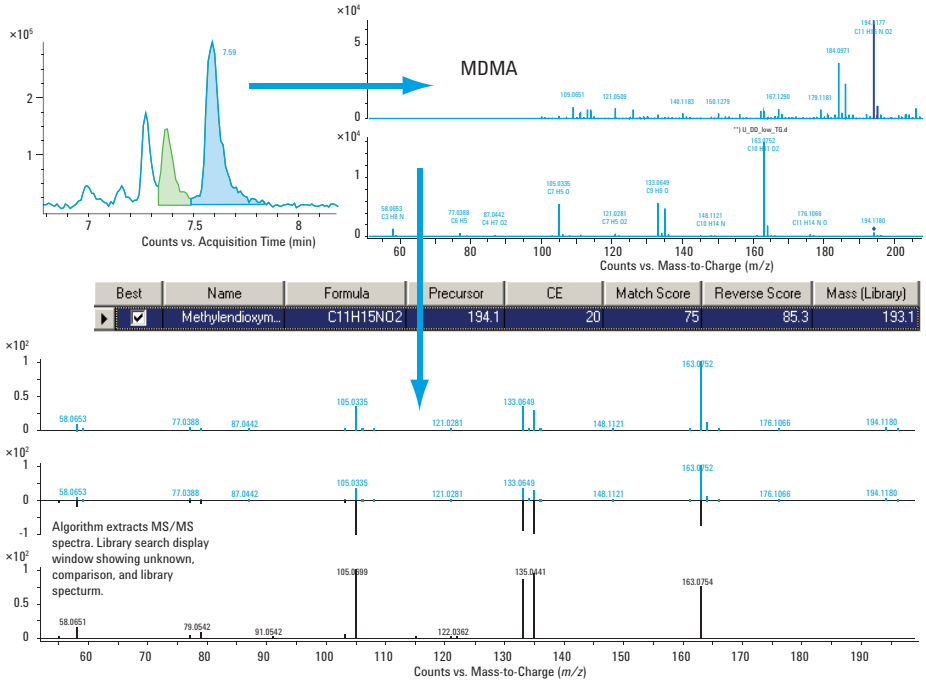

Figure 2: Detection of methoxyphedrine and MDMA isomers not distinguishable with a database search only without standards and retention time. With library, MDMA is readily identified.

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Accurate Mass Measurement for Analyzing Drugs of Abuse by LC/Time-of-Flight Mass Spectrometry

Technical Overview

Forensic

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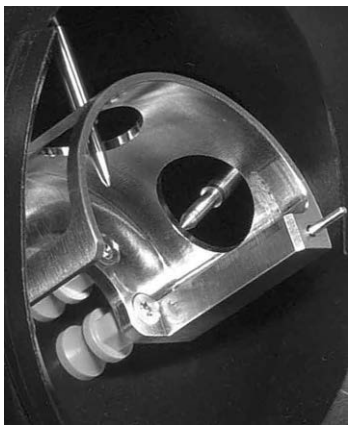
2850 Centerville Road
Wilmington, DE 19808-1610
USA

The new Agilent 1100LC/MSD TOF system



Dual-electrospray source

In the world of forensic analysis, the unambiguous identification of chemical substances is crucial. For substance abuse, this is simply a matter of providing scientific evidence indicating innocence or guilt in the possession and/or sale of controlled substances. For toxicology, this can include treatment of poison victims, identification of adulterants in tampered products, and crimes involving substance abuse. In addition, toxicological screens of postmortem samples can provide scientific evidence supporting the commission of a crime. To protect the innocent and to prosecute the guilty, both the identification of unknown compounds and the confirmation of suspect compounds are imperative. The new Agilent 1100 liquid chromatography/ mass selective detector Time of Flight (LC/MSD TOF) system can provide important information for the forensic analyst towards identification and confirmation.

The 1100 LC/MSD TOF provides mass measurements with the accuracy and resolution to distinguish empirical formulas and separate interfering compounds with the same nominal mass. Figure 1

shows 14 basic drugs of abuse (DA) separated using the LC/MSD TOF; Table 1 gives the identity of these compounds along with their retention times (RTs). Chromatographic separation is not complete in some cases, as indicated in the total ion chromatogram (TIC) between 3.3 and 4.0 minutes. However, examination of extracted ion profiles for each of the $[M+H]^+$ ions shows that each compound can be identified without interference. In the case of the isomers, hydrocodone and codeine, the extracted ion chromatogram shown in Figure 2 clearly demonstrates that what the mass spectrometer cannot do, chromatography can. These data also demonstrate the high degree of mass measurement reproducibility achieved within a chromatographic run. The averaged mass spectrum across the chromatographic peak of these two isomers is identical, as shown in Figure 3.

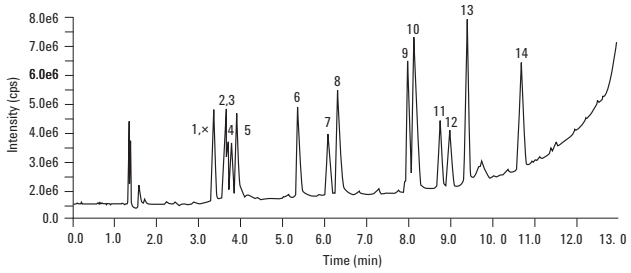


Figure 1. TIC of basic DA. LC/ES-MS TOF separation of 14 basic DA used as targeting compounds in toxicological sample screening.

Conditions

Column	ZORBAX Eclipse C18 XDB, 150 × 4.6 mm id, 5 µm
Part number	993967-902
Agilent 1100 binary pump flow	0.9 mL/min
Solvents	A = 0.1% Formic acid in water B = 0.1% Formic acid in acetonitrile
Gradient	10% B to 20% B in 1 min, then to 70% B in 11 min, then to 100% in 12 min; run time = 13 min
Agilent 1100 wellplate autosampler injection volumes	From 0.1 µL to 10 µL

Table 1. Compounds* and RT as Found in Figure 1.

Peak Number	RT (min)	Compound
1	3.37	Hydrocodone
x	3.40	Unknown with M + H = 166, 1221
2	3.64	Oxycodone
3	3.70	Amphetamine
4	3.81	Codeine
5	3.94	Methamphetamine
6	5.41	Cocaine
7	6.12	CE
8	6.36	PCP
9	8.04	Propoxyphene
10	8.18	Methadone
11	8.81	Alprazolam
12	9.00	Nordiazepam
13	9.46	Methaqualone
14	10.86	Diazepam

*Special thanks to Lucas Zarwell of the Washington D.C. Medical Examiners Office for providing the reference material.

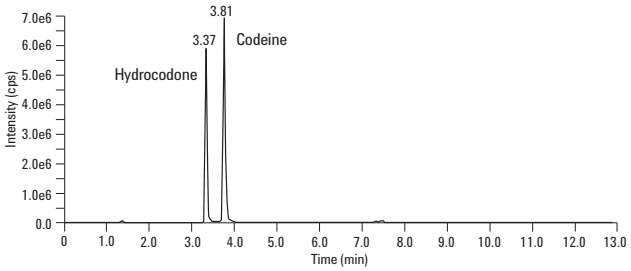


Figure 2. Extracted ion chromatogram. Ion chromatogram of *m/z* 300.0–300.2 extracted from the data shown in Figure 1. Isomers are chromatographically separated, facilitating their identification.

Because the exact mass of isomers is the same (meaning they have the same empirical formula), the mass spectrometer cannot distinguish between them. On the other hand, compounds with the same nominal mass that have different atoms will be distinguished by an accurate mass measurement. When this happens, mass resolution (M/ΔM) from >4000 (at *m/z* 200) to >10000 (at *m/z* 2722), is sufficient to resolve most nominal mass coeluting compounds. Resolution of ~7000 for *m/z* 300 in Figure 3 shows the high level of separation between the C12 and C13 isotopes.

The Agilent 1100 LC/MSD TOF system provides accurate mass measurement through highly stable electronics, a flight tube constructed with a very low coefficient of thermal expansion, and a design that maximizes consistent measurement of flight

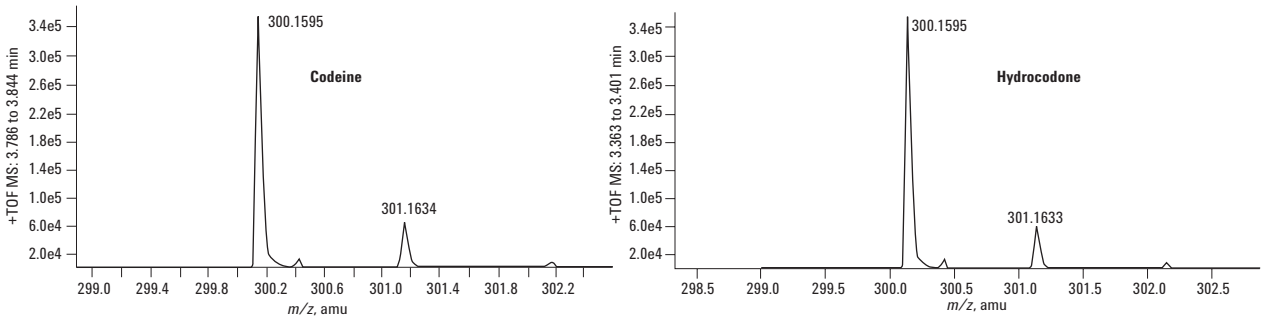


Figure 3. Mass spectra of narcotic isomers. Spectra of codeine and hydrocodone isomers representing an average spectra taken across the chromatographic peak.

time. This includes algorithms designed to minimize the error involved in calculating the center of the distribution of the ions' flight times. The Agilent calibrant delivery system (CDS) and a continuously infused reference material, a lockmass, ensure that every spectrum that is collected is automatically calibrated and stored. The reference material, compounds used in our patented calibration solution, though any reference material is acceptable, is infused at both a low concentration and low flow through the second of a dual-electrospray source. This assures that effects like ion suppression will not be caused by the lockmass. In addition, the use of analog-to-digital conversion provides a dynamic range of almost three orders of magnitude. Table 2 shows the mass accuracy achieved from 50 pg of these compounds to 50-ng injected on-column. Accuracy of better than 5 ppm is achieved for amphetamine and better than 2 ppm for oxycodone. Note that for low mass measurements, the number of possible empirical formulas is far less and a 5-ppm range is more than sufficient. At a higher mass, the possibilities increase and a lower range for error is needed to provide confirmation or suggest a reasonable empirical formula to aid the identification of an unknown.

Table 2. Accurate Mass Measurements vs. Concentration of Some DA in Reference Material Using Targeted Automatic Search of Empirical Formula

Peak Number	**3**	**5**	**1**	**4**	**2**
Compound	Amphetamine	Methanphetamine	Hydrocodone	Codeine	Oxycodone
Nominal *(m/z)*	136.10	150.10	300.15	300.15	316.15
Conc. (ng-injected)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)
50.00	–4.97	–2.41	0.94	0.94	1.32
25.00	3.53	–2.49	1.17	1.60	0.27
5.00	–4.71	–3.01	0.37	0.37	0.04
5.00	–4.53	–3.03	0.47	0.37	–0.16
5.00	4.53	–3.05	0.30	0.30	–0.75
2.50	5.00	–2.78	1.60	1.60	–0.42
0.50	–4.23	–2.20	1.34	1.90	0.04
0.50	–5.01	–2.48	1.11	1.27	–0.33
0.25	–5.70	–2.69	0.95	1.27	–1.16
0.05	5.00	–5.42	3.60	2.26	0.53

Figure 4 shows a response vs. concentration plot for codeine. The highly-linear response indicates this instrument can also be used for quantitative analysis. Note that the 50-ng injection was excluded, with this compound and others, because of detector saturation. For these compounds at saturated concentrations, accurate mass measurement was made at the edges of the chromatographic peak with an automated script. Also, a detection limit was not set and the 50-pg injection was made as an arbitrary low standard. The LC/MSD TOF specification for reserpine is 10 pg at a signal to noise ratio of 10:1. With the instrument's high mass resolution and seamless auto-calibration of every spectrum collected, selectivity of the extracted ion is increased.

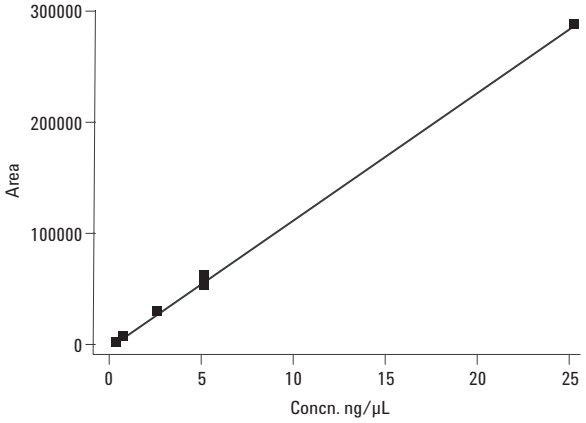


Figure 4. Codeine linearity. Plot of codeine extracted ion (ms/300.0–300.2) chromatographic peaks measured from 50-pg injected on-column to 25-ng on-column. TOF detector saturated at 50 ng.

Conclusion

The new Agilent LC/MSD TOF provides routine and seamless accurate mass measurement for confirmation of these drugs of abuse. Very high sensitivity is achieved and, with the TOF detection, all data are “full scan,” allowing compounds that are not targeted to be detected. The system offers a wide dynamic range capable of providing accurate mass measurements across that range without having to match lock-mass signal intensity with analyte intensity. Finally, a linear response is achieved within a concentration range below detector and electrospray saturation.

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Screening and Confirmation of Anabolic Steroids Using Accurate Mass LC/MS

Application

Forensics

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Abstract

The use of the Agilent LC/MSD TOF, an orthogonal-axis time-of-flight (oa-TOF) mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) source, is described for the analysis of four anabolic steroids and one internal standard, in urine extracts at a 1–2 ng/mL concentration. The high degree of mass accuracy (<3 ppm) of the TOF instrument allows for both excellent confirmation by empirical formula determination and quantitation using extracted ion chromatograms (EICs). The accurate mass capability of the LC/MSD TOF produces EICs with sufficiently narrow mass widths to exclude most chemical noise contributions. For increased chromatographic resolution and speed, a C18 column with a 1.8-micron particle size was employed.

Introduction

The use of anabolic substances for performance enhancement in sports is receiving increasing attention [1, 3]. Their use is an avowed problem in some professional sports, a growing problem in high school and collegiate athletics, and an area of doping in which new compounds are continually being introduced (for example, the BALCO/THG controversy in the USA and issues with nutritional supplements). The typical doping control analysis for anabolic steroids and related substances in urine includes screening by gas chromatography/mass spectrometry (GC/MS) [2], followed by confirmation of the screening result using high-resolution magnetic sector GC/MS in EI mode [4]. Because of the high initial and operational costs of high-resolution magnetic sector instruments, alternative techniques for the confirmation of screening results are being explored [5].

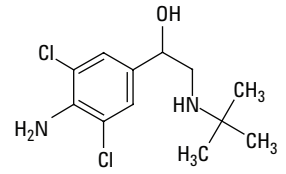
Tandem GC/MS is an alternative to high resolution GC/MS, because tandem GC/MS is an established technique for drug confirmation, and because selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) acquisitions with ion ratio calculations provide high confidence in the identification. Liquid chromatography/mass spectrometry (LC/MS) has also been used as an alternative confirmation technology with the advantages that it uses a different mode of chromatographic separation and different ionization techniques [6].

Accurate-mass API LC/MS has significant advantages because it provides not only the same spectral information, such as isotope ratios and presence of heteroatoms as previous methods, but also allows for the highly specific detection of the intact molecule and confirmation of the empirical formula. Approximately 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily detected or confirmed using GC/MS, but are amenable to LC/MS [7]. The analysis of many of these compounds is further complicated because they must be detected and confirmed at a 2-ng/mL concentration or lower in urine [the Minimum Required Performance Level (MRPL) for a laboratory, in the World Anti-Doping Agency (WADA) program]. This application note describes the results of using an Agilent LC/MSD TOF instrument for detection and confirmation of a number of analytically challenging anabolic substances at the WADA MRPL.

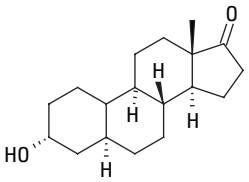
The anabolic substances analyzed in this work and their structures are shown in Figure 1.

Most of these analytes, including the internal standard (ISTD), showed better sensitivity using APCI than electrospray ionization (ESI). This might be expected for these relatively non-polar analytes that contain no basic groups. APCI is also less susceptible to ion suppression from co-eluting endogenous materials, and tends to give simpler spectra than ESI (free of complicating adducts such as Na^+ and K^+).

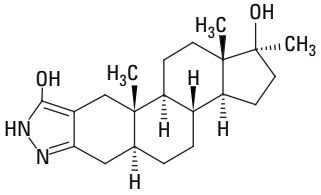
One complication of APCI can be the loss of water from the initially-formed protonated molecule due to either the thermal effect of the heated vaporizer, or to the ionization driven by the corona discharge of APCI. For some compounds, water loss is even observed in the milder ESI mode which involves ionization in the liquid phase and less contact of the analyte with heat. However, the mass



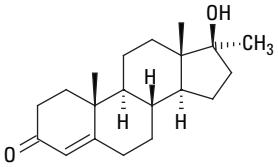
Clenbuterol



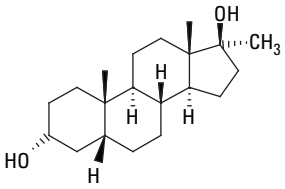
19-norandrosterone



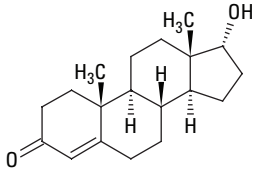
3'-OH stanozolol



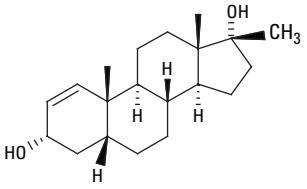
Methyltestosterone
(internal standard)



Methyltestosterone
metabolite
(17α-methyl-5β-androstane-3α,17β-diol)



Epitestosterone



Epimetendiol
(methandrostenolone
metabolite)

Figure 1. Compounds analyzed.

measurement of water-loss ions still retains its mass accuracy and the ion ratios for the $[M+H]^+$ and the subsequent water-loss ions are reproducible with this system. It is interesting to note that more water loss occurred with the later-eluting steroids rather than with the more polar early-eluting compounds.

Experimental

Sample Preparation

The sample preparation method used in this study was developed at the Center for Human Toxicology, Sports Medicine Research and Testing Laboratory, for screening of steroids in urine by GC/MS. The same sample preparation was used for the LC/MS analysis, except derivatization was unnecessary. The internal standard (20 µL of 10 ng/µL methyltestosterone) was added to 3 mL of urine, followed by 1 mL of 0.15M sodium acetate, pH 5. This solution was vortexed-mixed and then transferred to an Extrelut-3 column (Merck, VWR catalog number 48219-494, pkg of 100) connected in-line to an amino SPE column (J. T. Baker, VWR catalog number JH7088-3, pkg of 50) also containing 1 g of sodium sulfate. After an 8-minute delay, the columns were eluted with 9 mL of diethyl ether into a 13 × 100 mm silanized conical glass tube. For LC/MS, the final extracts were simply evaporated to dryness with a stream of nitrogen at 40°C. The tubes were capped and sent by overnight courier to the Agilent laboratory (Pleasanton, CA) where they were stored at –10°C while awaiting analysis. The residues were reconstituted in 100 µL of the initial mobile phase just prior to analysis.

LC/MS Method Details

The API-TOF system consisted of an Agilent 1100 LC system (vacuum degasser, binary pump, wellplate autosampler, thermostatted column compartment, and diode array UV-VIS detector), interfaced to a G1969A LC/MSD TOF mass spectrometer. The mass spectrometer was operated with either the orthogonal ESI or APCI sources. The instrument was autotuned weekly using the automatic built-in calibrant delivery system and Agilent-developed calibrant compounds. The mass axis was calibrated daily using the same mix and an automatic calibration routine. Spectra were internally mass-corrected in real time using an automatically-introduced reference mass solution containing two known compounds (purine and calibrant HP-921) bracketing the mass range of interest at *m/z* 121.050873 and 922.009798, respectively. The optimized LC, MS, and APCI conditions are shown in Table 1.

Table 1. LC/MS Conditions for the Analysis of Anabolic Substances in Urine

LC Conditions	
Column:	Agilent ZORBAX RRHT SB-C18 2.1 × 50 mm, 1.8 µm (Agilent part number 822700-902)
Mobile phase:	A = 0.1% Formic acid/water B = Methanol
Flow rate:	0.4 mL/min
Col temp:	55°C
Gradient:	55% B, hold 5 min 55% to 75% B from 5 to 9 minutes
Analysis time:	14 min
Post-time:	5 min
Injection volume:	4 µL
MS Conditions	
Ionization mode:	Positive APCI (final method)
Capillary voltage:	3500 V
Vaporizer temp:	450°C
Corona current:	4 µA
Nebulizer:	60 psig
Drying gas flow:	5 L/min
Drying gas temp:	350°C
Scan:	*m/z* 100–1000, 10,000 transients/scan (0.89 sec/scan)
Reference masses:	121 and 922 (added post-column at 5 µL/min, 10 µM solution)
Fragmentor:	150 V [no collision-induced-ionization (CID)]
Skimmer:	60 V (default)
Octopole RF:	250 V (default)

Results and Discussion

Accurate-mass API-TOF LC/MS is commonly used for empirical formula determination and confirmation of naturally-occurring and synthetic molecules. The instrument used here was specifically designed to be as easy to use as a quadrupole GC/MS or LC/MS, by virtue of features such as automatic tuning and calibration, automatic reference mass correction, and improved instrument stability resulting from its mechanical and electronic design. The instrument has a mass resolution of approximately 7000 in the *m/z* range of the anabolic steroids, a routine mass accuracy of 3 ppm or less, and operates in full scan mode. Full scan has the advantage over MRM-MS/MS of not being a target analysis acquisition. Therefore, additional compounds can be detected without modifying the acquisition method or developing and optimizing specific MS/MS parameters.

Initial studies using unextracted standards of these and other steroids with ESI demonstrated that the instrument could measure the *m/z* of the $[M+H]^+$ ion to 3 ppm accuracy or less under routine unattended operation. However, the analytes included in this work showed better sensitivity using APCI. Theoretically, APCI might have been predicted to be the ionization mode of choice because the analytes tested are relatively nonpolar, and many contain no readily ionizable functional groups. Also, APCI is less susceptible to ion suppression from coeluting endogenous materials and tends to give simpler spectra than ESI (free of complicating adducts such as Na^+ and K^+).

Figure 2 shows the base peak chromatogram of an unextracted standard equivalent to 16 ng/mL, if extracted from a 3-mL urine sample. An objective of this method development was to obtain separation of the target analytes in less than 15 minutes. Although this was achieved, it was unexpectedly challenging due to the range of polarities from clenbuterol to epimetendiol. In future work we plan to evaluate additional LC columns to improve the separation between epitestosterone and the internal standard while maintaining a reasonable analysis time.

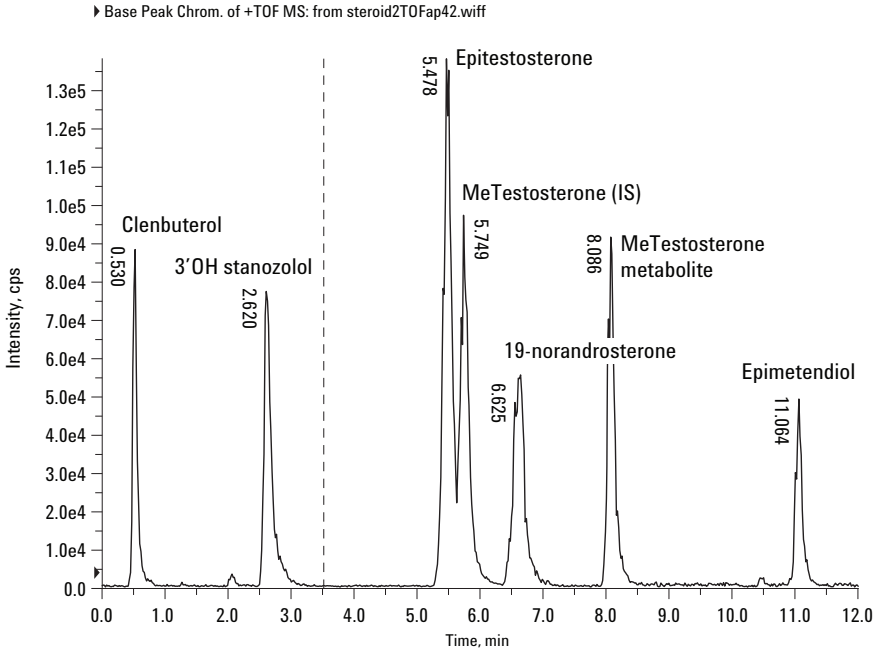


Figure 2. Base peak chromatogram of anabolics mix (unextracted standard equivalent to 16-ng/mL urine).

The vaporizer temperature and corona current were optimized for the best sensitivity across the range of analytes. In these studies, no in-source collision induced dissociation (CID) was used but in-source CID will be evaluated in future studies as a mechanism for generating ions for potential ion ratio calculations. The detection limits for these compounds were also improved by optimizing the number of transients/scan while maintaining a sufficient number of scans to accurately describe each chromatographic peak. In addition, increasing the photomultiplier voltage by only 50 V improved the detection limits without a significant increase in background noise.

An example of the typical performance of the LC/MSD TOF is shown in Figure 3 for the compound epitestosterone. The APCI spectrum shows both the $[M+H]^+$ protonated molecule and the less abundant $[M+H-18]^+$ water-loss ion. Applying the formula for mass accuracy shown in the Figure demonstrates a mass error of –1.04 ppm for the measurement of the $[M+H]^+$ ion.

The inset in Figure 3 also shows a mass resolution of 6890 measured for the $[M+H]^+$, and the large separation between the $[M+H]^+$ ion and the ^{13}C isotope peak at *m/z* 290. The resolution (R) at *m/z* 289.2159 can be calculated by dividing the *m/z* value by the full width at half maximum (FWHM or $w_{1/2}$) in Da. That is, $R = M/w_{1/2}$ where M = 289.2159 and ΔM is the width of the mass peak at half maximum, or half of its intensity. In this case, $w_{1/2}$ = 0.042 Da, so that R = 289.2159/0.042 = 6890. The accurate mass and ion ratio for the ^{13}C peak also have to match for the empirical formula calculated from the $[M+H]^+$ to be a correct assignment.

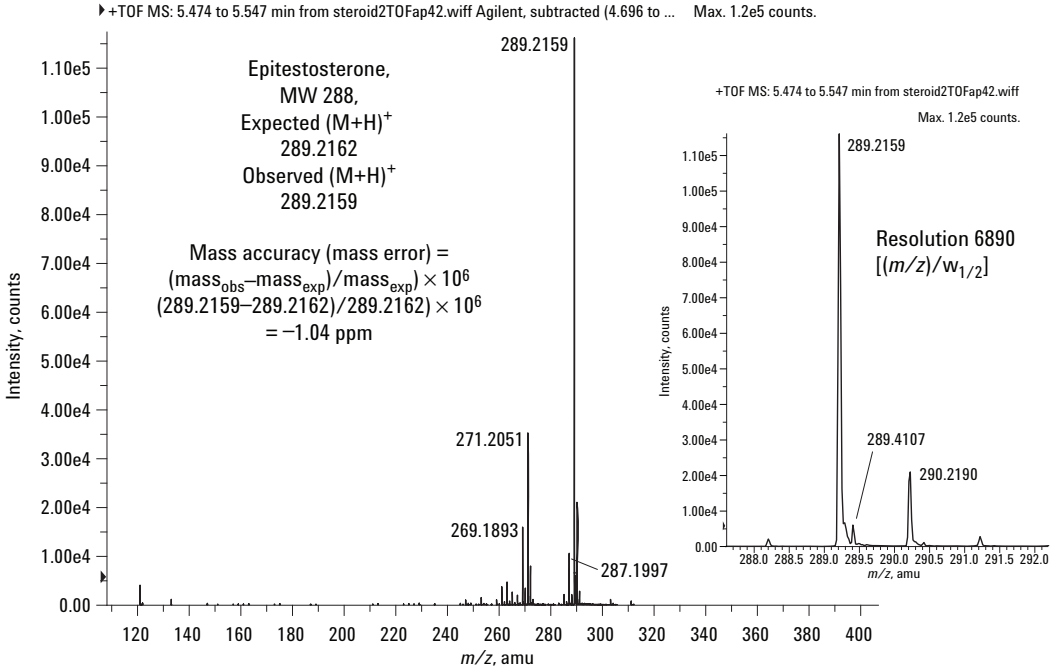


Figure 3. Typical APCI spectrum and TOF performance for epitestosterone, $[M+H]^+$ = 289.2162. Also shown is a water-loss fragment at *m/z* 271.2051.

Although there was some water loss observed in the spectrum of epitestosterone (the *m/z* 271 ion), for 19-norandrosterone [Figure 4], the most prominent ions in the spectrum are those at *m/z* 259.2049 and 241.1949. These represent the loss of one and two water molecules, respectively, from the protonated molecule. The loss of water was steroid-dependent and could not be entirely eliminated even by using ESI or by adjusting source conditions such as voltages and solvent-evaporation parameters.

Also note in Figure 4 the presence of the reference mass ions at 121 and 922, used to calibrate the mass axis for every scan in order to maintain mass accuracy throughout the acquisition. These ions are two compounds added automatically post-column as a dilute solution using a separate LC pump and a zero-dead-volume mixing tee at the MS system's inlet filter. In this case, the compounds are purine and HP-921 (a member of the Agilent API calibrant series), which are supplied with the LC/MSD TOF in a reference mass kit. Only a few thousand counts of reference mass are necessary to automatically calibrate each spectrum in real time. The instrument automatically reports any failure to find the reference masses on the screen and in a log file.

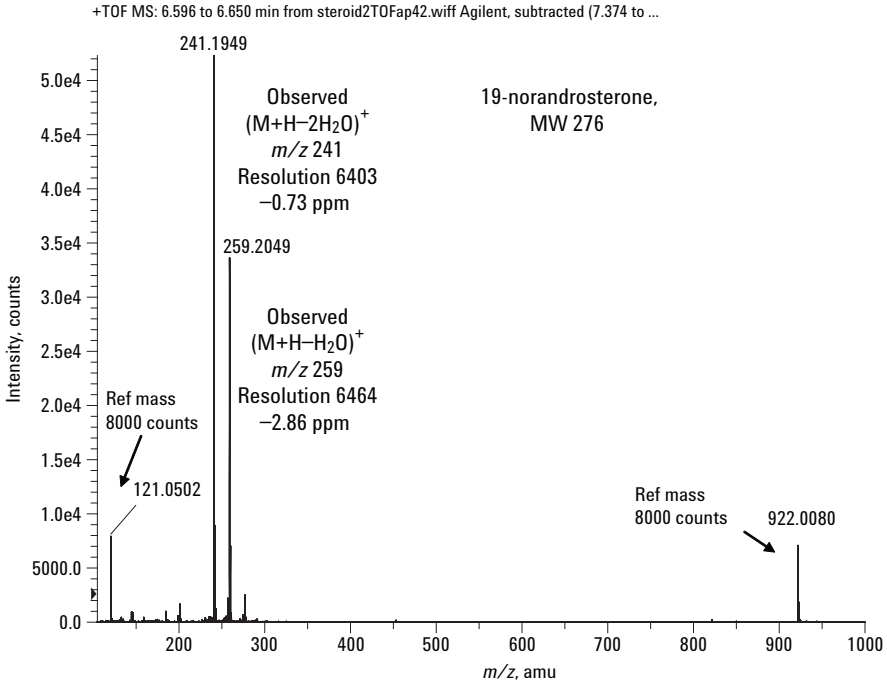


Figure 4. Typical APCI spectrum for 19-norandrosterone, MW 276, showing two water-loss ions. Reference masses at *m/z* 121 and 922 also shown.

A base peak chromatogram of an extracted steroid-free control urine fortified with epitestosterone, 19-norandrosterone, methyltestosterone metabolite and epimetendiol at 50 ng/mL, and 66 ng/mL of the methyltestosterone ISTD, is shown in Figure 5. Clenbuterol and 3'-hydroxystanozolol are not shown in the Figure because they were poorly recovered with the extraction procedure used at this stage of the method development. Because of the reduced recovery, these analytes are not shown in the subsequent Figures. Although epitestosterone and the internal standard appear unresolved chromatographically in the base peak chromatogram, they were resolved by their extracted ion chromatograms (EICs).

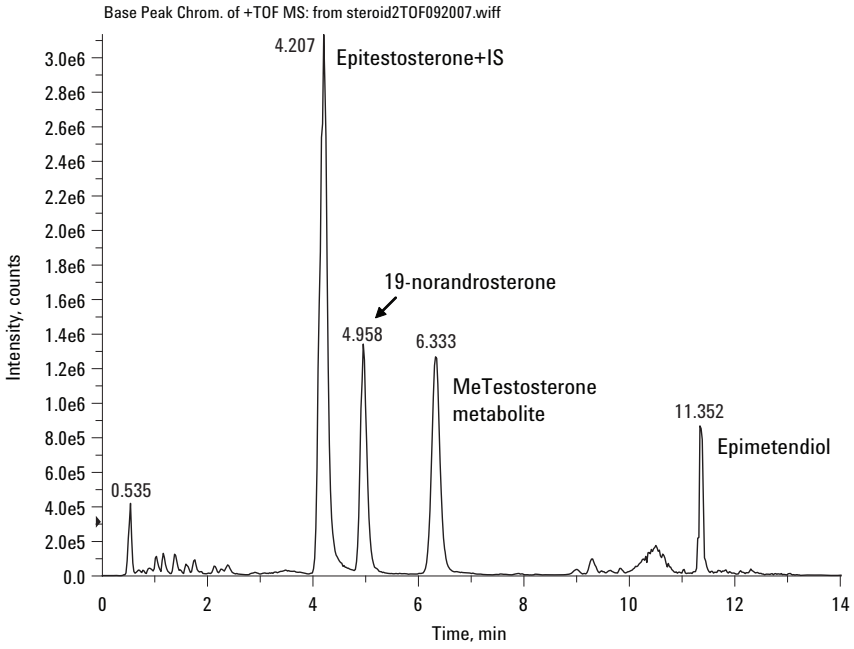


Figure 5. Extract of 50 ng/mL urine. Analytes are visible in base peak chromatogram.

Figure 6 shows the EICs from an extract of urine that was fortified with 2 ng/mL of epitestosterone, methyltestosterone metabolite and epimetendiol, and 1 ng/mL (the MRPL) of 19-norandrosterone. As shown, the EICs had 50,000–100,000 count intensities and excellent signal-to-noise, which permitted easy peak detection and location of spectra. For quantitation, one can take advantage of the high degree of mass accuracy with the TOF by designating narrow mass widths for the EICs. In this study, we used mass widths of 1 mDa (~3 ppm) for the EICs. For example, for 19-norandrosterone detection, the $[M+H - 2H_2O]^+$ ion at 241.1949 was selected, and the EIC mass window was (241.1944 to 241.1954). By using the TOF's resolving power for selectivity, nearly all of the chemical noise was eliminated and the S/N vastly improved.

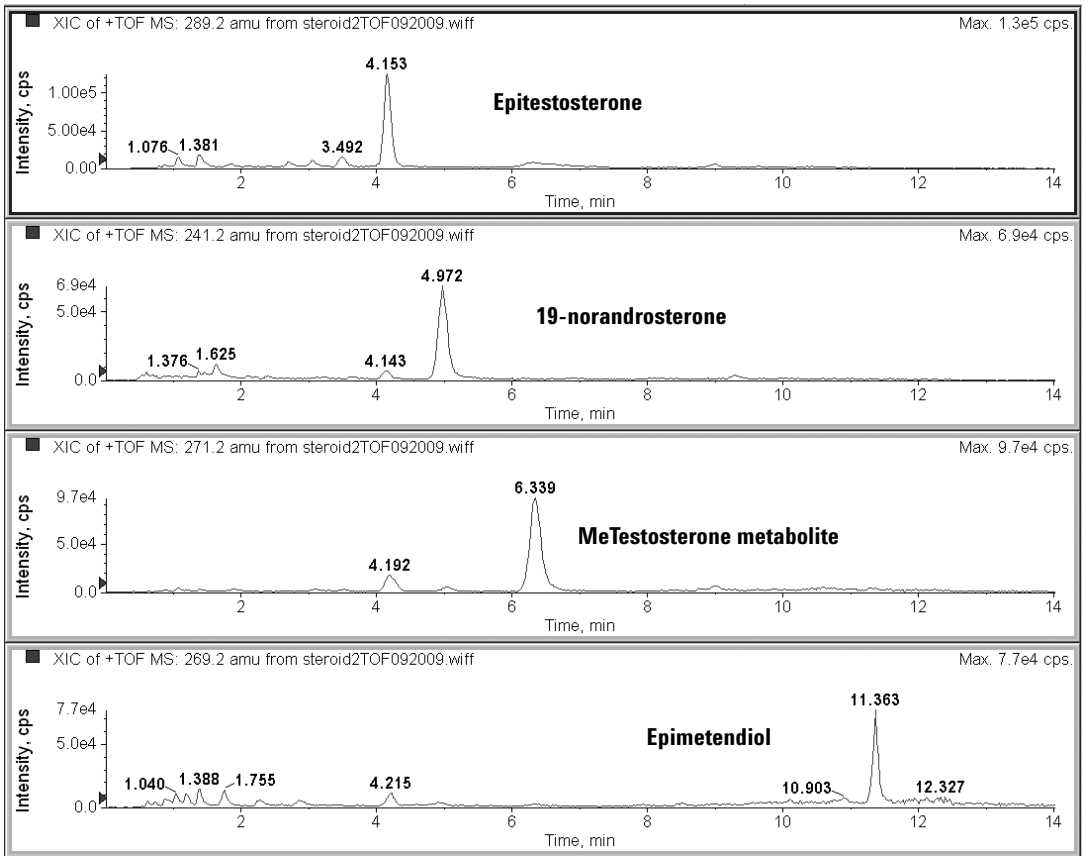


Figure 6. Extract of 2 ng/mL urine (1 ng/mL 19-norandrosterone). Analytes detected using accurate mass EICs (1 mDa width).

Figure 7 shows the spectra corresponding to the extracted analytes shown in Figure 6. Note the mass accuracy results that were calculated automatically by the TOF's data analysis method. These spectra were obtained from an estimated 240 pg of each analyte on-column, assuming 100% recovery (120 pg for 19-norandrosterone). Even at such a low concentration and in a urine extract, the mass accuracy was better than 2 ppm for each analyte. This demonstrates that there were no matrix interferences with the mass measurements even with simple and fast chromatography.

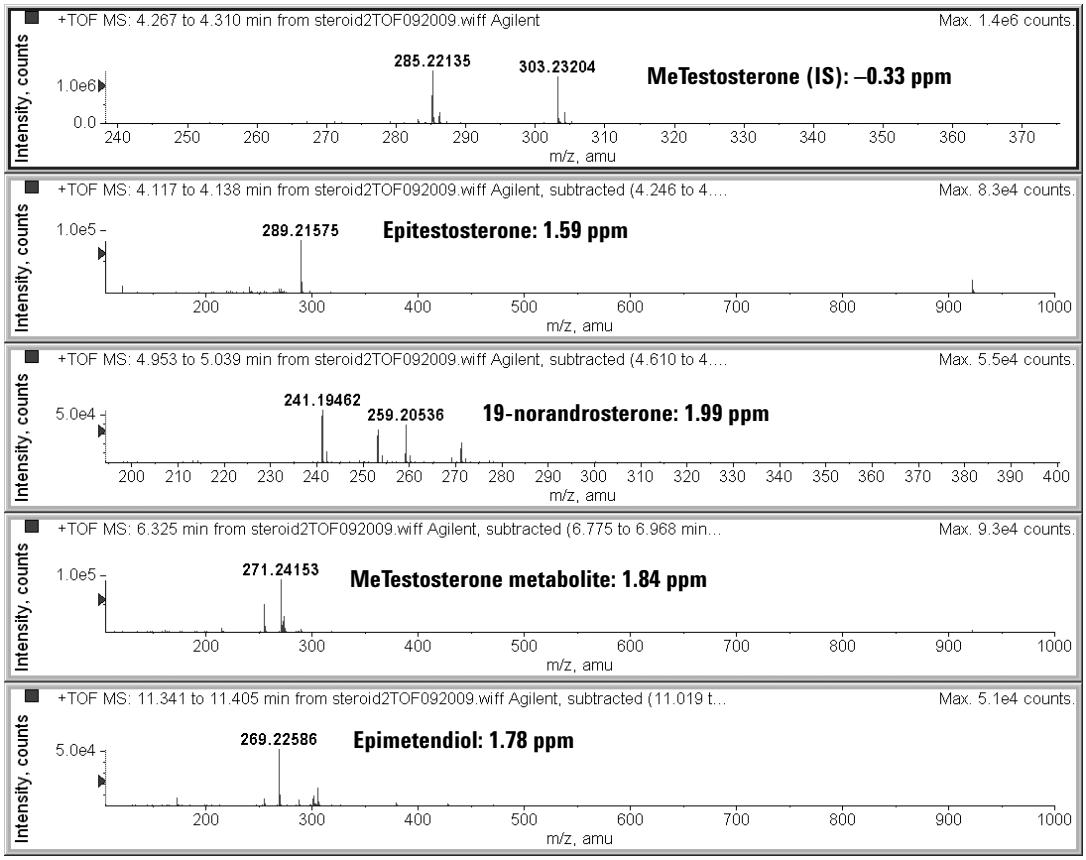


Figure 7. Spectra from 2 ng/mL urine extract (1 ng/mL 19-norandrosterone) (240/120 pg on-column) [see Figure 6].

One final experiment was performed to estimate the detection limit for the internal standard, epitestosterone, 19-norandrosterone, methyltestosterone metabolite, and epimetendiol using the developed LC/MSD TOF method. Serial dilutions of an unextracted standard were prepared and analyzed down to the concentration corresponding to 0.16 ng/mL extracted from 3 mL of urine. A 4-µL injection corresponding to 20 pg on-column was analyzed. The accurate-mass (1 mDa width) EICs are shown in Figure 8 and still exhibit excellent signal-to-noise.

It is possible that the method could be further improved by using 3.5-µm columns, rather than the 1.8-µm particle column used in this study, which would allow for larger injection volumes. The choice of columns would depend on the need for more sensitivity with larger injections, but might sacrifice the speed and resolution achieved with the 1.8-µm column.

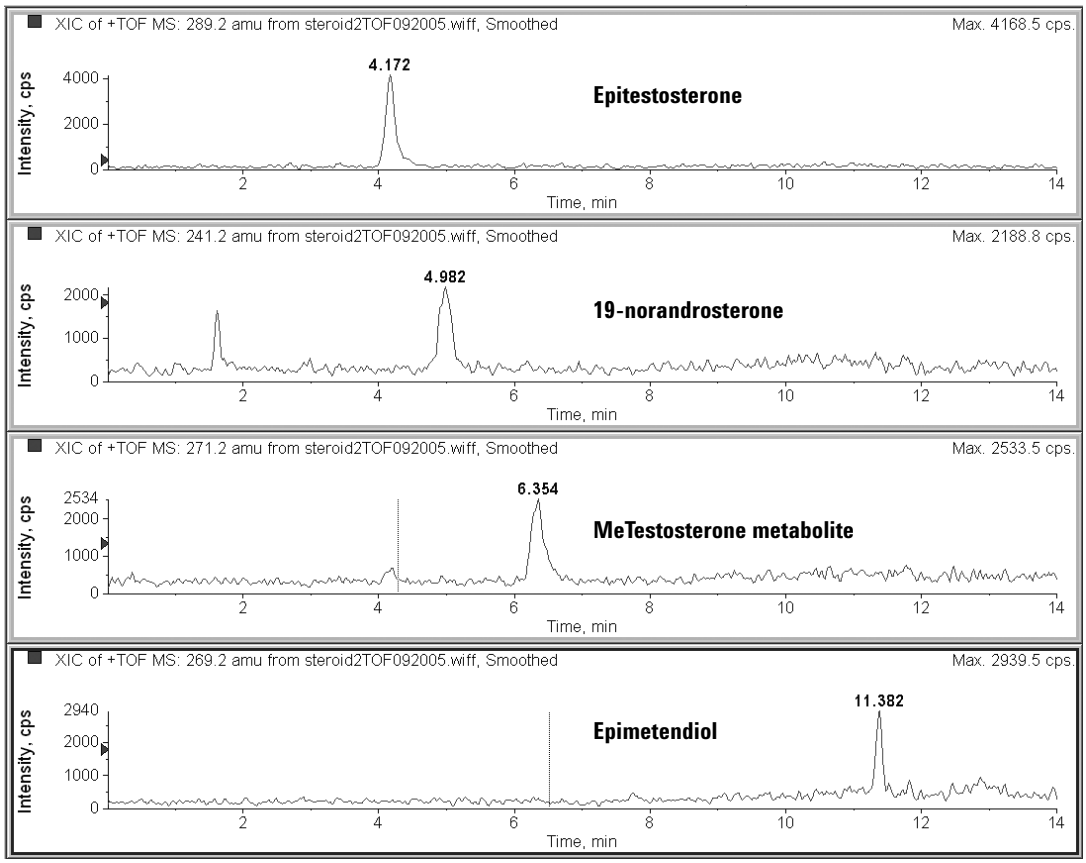


Figure 8. Accurate-mass EICs (1 mDA width) from 0.16-ng/mL unextracted standard (20 pg on-column).

Conclusions

Using a standard sample preparation method developed for GC/MS screening, but without derivatization, an LC/MS method for an easy-to-use, bench top API-TOF instrument readily detected a representative group of anabolic substances at 1–2 ng/mL concentrations in urine. This sensitivity was achieved by capitalizing on the resolution of the LC/MSD TOF that allowed for accurate mass analysis, EICs with extremely narrow mass windows, and enhanced S/N. The analysis consumed only 4 µL of the 100 µL of reconstituted extract, which allowed for re-analysis, analysis of replicates, or different analyses from a single extraction. The use of "accurate-mass EICs" (that is, EICs with an *m/z* width of 1 mDa) allowed for specific detection of the target steroids in a complex sample. The spectra obtained at these low ng/mL concentrations all showed mass errors of less than 2 ppm.

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Agilent Technologies

An Application Kit for the Screening of Samples for Analytes of Forensic and Toxicological Interest using TOF or Q-TOF LC/MS with a Personal Forensics/Toxicology Database

Application Note

Forensics and Toxicology

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Abstract

A Forensic and Toxicological screening application kit has been developed for use with the Agilent TOF and Q-TOF Mass Spectrometers which contains an accurate mass database with a content of around 6700 analytes. The aim of the MassHunter Personal Forensics and Toxicology Database Kit is to provide a user with a sufficient starting point for the analysis of samples for which the ability to detect and identify from a large array of forensic and toxicological analytes is necessary. The combined system allows the user to create custom databases containing retention times of compounds of interest for smaller and more specific suites of analytes according to specific requirements. A test mix containing analytes of forensic interest, to demonstrate the functionality of the MassHunter Personal Forensics and Toxicology Database Kit, together with an example of a general screening method for common drugs of abuse is provided.

Agilent Technologies

Introduction

The application of high definition accurate mass spectrometers, such as time-of-flight (TOF) and quadrupole time-of-flight (Q-TOF), to screening, discovery and confirmation in the areas of forensics and toxicology has become more desirable given the indiscriminant and non-targeted nature of their full spectral data capture. Indeed, given the highly accurate and sensitive mass measurement of modern TOF and Q-TOF instruments (sub 2-ppm mass accuracy, pg on-column sensitivity and high resolution) in combination with powerful software data mining tools, post acquisition screening techniques are easier to perform reliably with a higher number of analytes in one analytical method. The lists of potential toxins are large and typically depend on the area of analytical focus such as work-place drug testing, doping control, post-mortem toxicology, or explosives.

Accurate single-stage mass spectrometry (MS) mass measurements identify monoisotopic adducts to a high confirmatory degree, and databases can be built to accommodate various suites of forensic and toxicological analytes of interest. They are obtained from both TOF and Q-TOF LC/MS instruments. In contrast LC/MS/MS with a triple quadrupole MS in its most sensitive mode, multi-reaction monitoring (MRM), provides targeted screening and confirmation only.[1]

This application note describes the Agilent MassHunter Personal Forensics and Toxicology Database Kit for Forensic and Toxicological Screening and Identification which contains the accurate mass (AM) details for around 6700 analytes of forensic and toxicological interest. The content was gathered upon advice from many leading institutions and knowledge bases world-wide and contains information such as common names, monoisotopic mass, compound formulas, CAS & Chemspider IDs, chemical structure and in most cases the IUPAC nomenclature. In addition to accurate mass, the ability to add retention time for a chromatographic method to every analyte for extra search confirmation is a built-in functionality of the MassHunter Personal Compound and Library (PCDL) program interfaces. This allows accurate mass retention time (AMRT) data mining routines. Furthermore, an analyst can use the database content 'as is' for non-targeted screening or create smaller custom and more targeted databases from the read-only supplied database. Custom databases can be edited by changing entries, adding, and deleting entries and semi-automatically updating retention times for particular analytes and methods. [2] The analyst can create as many custom databases with LC-dependent retention times as needed.

This application note describes the typical use of the MassHunter Personal Forensics and Toxicology Database Kit through a few analytical screening work flow examples.

Experimental

The analysis results outlined in this application note were obtained using an Agilent 6230 Time-of-Flight LC/MS coupled to an Agilent 1200 SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter Workstation equipped with the [G6855AA] MassHunter Personal Forensics and Toxicology Database Kit.

Sample preparation

An ampoule from the LC/MS Toxicology Test Mix [p/n 5190-0470] which is included in the MassHunter Personal Forensics and Toxicology Database Kit [G6855AA] was opened and 10 µL of the 1 µg/mL (1 ppm) solution was diluted to a concentration of 100 ng/mL (100 ppb) using 990 µl of pure LC/MS grade methanol to create a clean solvent standard for method checkout purposes.

Table 1 outlines the composition of the LC/MS Toxicology Test Mix [p/n 5190-0470] which is intended to cover a wide and representative range of forensic analyte classes.

Table 1. LC/MS Toxicology Test Mix components (1 µg/ml)

Compound Name	Formula	Mass
3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107
Cocaine	$C_{17}H_{21}NO_4$	303.14706
Codeine	$C_{18}H_{21}NO_3$	299.15214
delta9-Tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458
Diazepam	$C_{16}H_{13}ClN_2O$	284.07164
Heroin	$C_{21}H_{23}NO_5$	369.15762
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723
Methadone	$C_{21}H_{27}NO$	309.20926
Methamphetamine	$C_{10}H_{15}N$	149.12045
Methylendioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091
Oxycodone	$C_{18}H_{21}NO_4$	315.14706
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.1987
Phentermine	$C_{10}H_{15}N$	149.12045
Proadifen	$C_{23}H_{31}NO_2$	353.23548
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316

Reagents and chemicals

Burdick & Jackson LC/MS grade acetonitrile together with de-ionized water (locally produced 18.1 MΩ) were used for mobile phases. Buffers were freshly prepared using a high purity source of formic acid and ammonium formate.

Instrument settings and MS acquisition method parameters

LC conditions

Column:	Zorbax Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm [p/n - 959764-902]
Column Temperature:	60 °C
Mobile Phase	A: 5 mM NH_4 formate/0.01% Formic acid in water B: 0.01% formic acid in acetonitrile
Flow Rate:	0.5 ml/min

Gradient program:

Time	A	B	Flow rate
Initial	90%	10%	0.5 ml/min
0.5 min	85%	15%	0.5 ml/min
3.0 min	50%	50%	0.5 ml/min
4.0min	5%	95%	0.5 ml/min
6.0min	5%	95%	0.5 ml/min

Injection volume:	1 µL (with 5 second needle wash in flushport)
Analysis time:	6.0 min
Post Time:	2.0 min
Overall Cycle time:	8.0 min

6230 TOF MS conditions

Source conditions:

Electrospray AP-ESI (using Agilent Jet Stream Technology):

Positive ionization polarity	
Sheath gas temperature and flow:	380°C, 12 L/min
Nozzle voltage:	500 V
Drying gas temperature and flow:	320°C, 8 L/min
Nebulizer gas pressure:	27 psi
Capillary voltage:	3750 V
Fragmentor voltage:	150 V

Electrospray AP-ESI:

Positive ionization polarity	
Drying gas temperature and flow:	350°C, 12 L/min
Nebulizer gas pressure:	30 psi
Capillary voltage:	2000 V
Fragmentor voltage:	150 V

MS acquisition method parameters:

Reference ion mass enabled:	121.050873, 922.009798
Acquisition mode:	MS1
Minimum mass value:	50 *m/z*
Maximum mass value:	1050 *m/z*
Scan rate:	3 Hz

All other instrument operating parameters were taken care of by Agilent's autotune functionality and subsequent mass calibration using standard settings.

Results and discussion

Fast and easy start up with Agilent LC/MS Toxicology Test Mix

The LC/MS Toxicology Test Mix [p/n 5190-0470] is included in the MassHunter Personal Forensics and Toxicology Database Kit [G6855AA] to rapidly implement the method and verify that acquisition and data analysis methodology is correctly set up. The LC/MS Toxicology Test Mix contains a representative range of components from 25 forensic analyte classes. (See Table 1). MS screening depends on accurate mass results from the TOF or Q-TOF. Therefore, the use of appropriate reference ions as outlined in the 'Experimental conditions' section obtains the most accurate results.

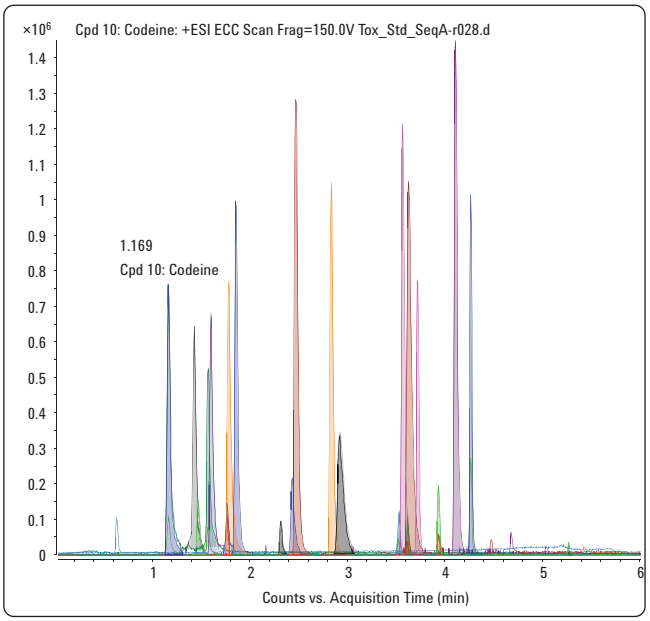


Figure 1. Extracted compound chromatogram of LC/MS Toxicology Test Mix.

In compliance with the methodology outlined in the experimental section, a 1-ul injection of the 100 ng/ml LC/MS Toxicology Test Mix equates to a 100 pg on-column injection amount. Figure 1 shows an overlay of the expected extracted compound chromatograms for the LC/MS Toxicology Test Mix. A standard method is included for TOF and Q-TOF as part of the MassHunter Personal Forensics and Toxicology Database Kit. These can be loaded so that all conditions are correct and the user can reproduce the analysis.

These methods are acquisition only methods and correspond to the instrument configuration as outlined in the experimental section of this application note. Appropriate settings must be manually input if a different instrument configuration is used. Similar results will demonstrate that the system is working properly.

Personal Compound Database and Library (PCDL) Software interface

Outline

An 'open database' dialog box appears after invoking the PCDL interface from the desktop icon. It is best to choose the pre-installed Forensic.cdb from the MassHunter\database directory. Figure 2 illustrates the single search view of the software interface. The screen shows a list of search results for 'amphetamine'. There are seven views available to the user, however, for the scope of this application note, only the first four (tabs to the left) that are directly applicable to AMRT functionality will be described. These views are switched on this flat user interface by clicking on the appropriate tab: Single Search, Batch Search, Batch Summary, or Edit Compounds.

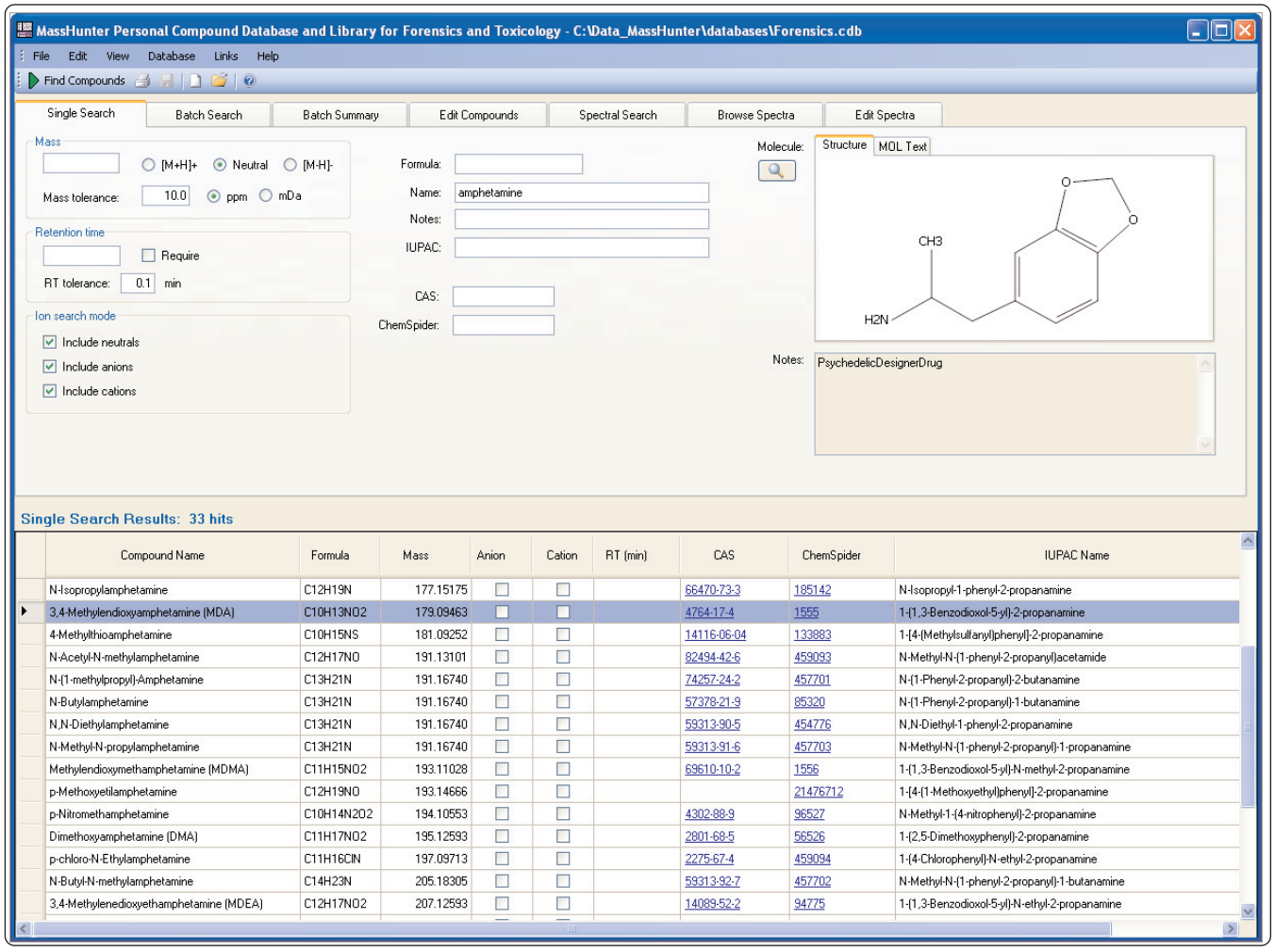


Figure 2 Single Manual Search view of the PCDL software interface.

Any field or combination of fields in the upper portion of the Single Search tab (Figure 2.) can be used to manually search the loaded database. Table 2 lists all available search fields from the PCDL single search view. The powerful search algorithm also handles partial names (eg. 'amph' will return all database entries containing this letter string.)

Note: To view the entire contents of the loaded database, a single search invoked with all empty search fields will allow the user to display the entire database content.

Table 2. All available search fields for PCDL single search.

Search Fields Available (Single Search View)	Value
Mass	Measured mass (*m/z*)
Retention time	(minutes)
Formula	Empirical Formula
Name	Common name of compound (or part thereof)
Notes	Compound class or description
IUPAC	IUPAC or commonly recognized compound name
CAS	Unique CAS number
ChemSpider	Unique ChemSpider ID

Workflow A. Manual (Single Mass Search)

Using PCDL Program

Single search would normally be used manually by obtaining a measured mass from a measured or observed spectrum in MassHunter Qualitative Analysis program and typing it in to the mass search field. Figure 3 illustrates this manual application of the MassHunter Qualitative Analysis program and PCDL single search capability for observed masses.

In this example, a compound peak was identified in MassHunter Qualitative Analysis program from positive polarity TOF data, the spectrum was extracted, and the observed mass of 244.205770 *m/z* was searched against the PCDL database (including cations) for [M+H]+ adducts using a mass tolerance of 10 ppm.

The search returns an accurate mass match with phencyclidine (PCP) and with a mass deviation (or delta mass) of 0.85 ppm between the measured and theoretical database values.

More detailed information of single search capability can be found in Agilent G6855AA MassHunter Personal Forensics and Toxicology Database and Kit Quick Start Guides [3,4] .

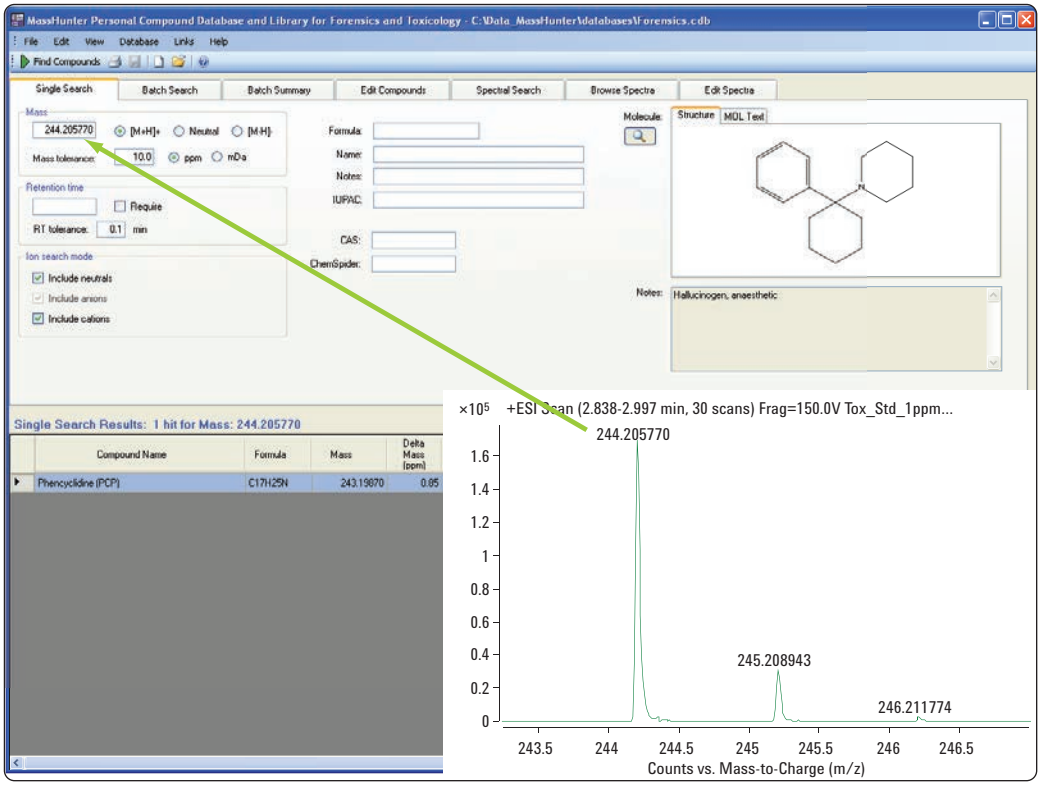


Figure 3. Manual search of observed mass.

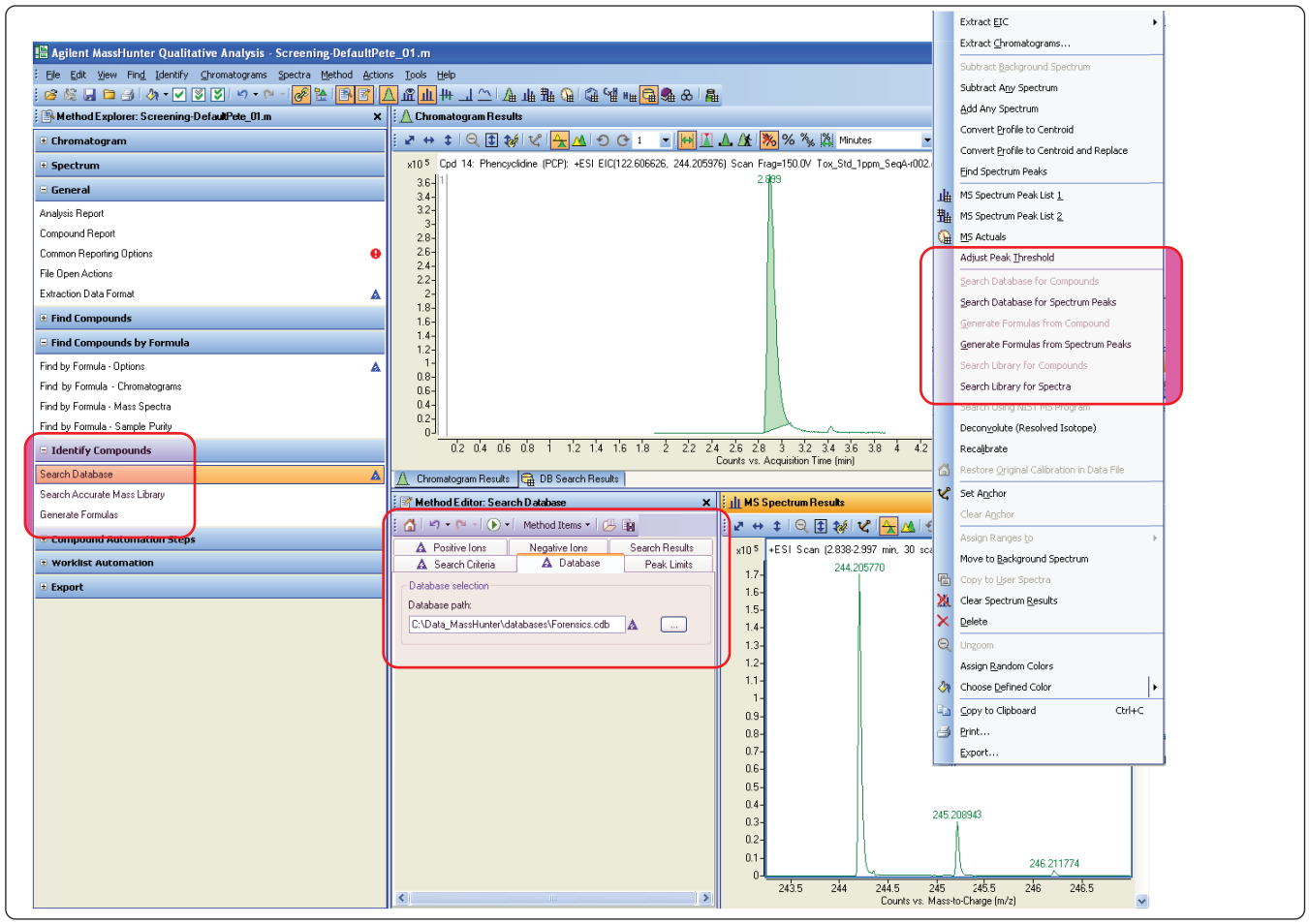


Figure 4a. Manual Search of observed mass using MassHunter Qualitative Analysis program.

Single manual search of database using MassHunter Qualitative Analysis program.

To obtain a seamless single spectral peak database search via MassHunter Qualitative Analysis program, the database must be specified in the qualitative analysis method editor. Compatible software versions are B.03.01 or higher. Figures 4a through 4d illustrate the settings used for this example.

Figure 4a shows the typical MassHunter Qualitative Analysis program view containing the chromatographic peak in question together with its manually extracted spectrum. On the left side of the screen shot, the 'Identify Compounds' method explorer options have been expanded and the 'Search Database' method editor was selected. In the method editor, the required AMRT database was specified as 'forensic.cdb'.

Figure 4b shows the mass tolerance window and the search criteria that can be selected, such as 'mass only' or 'mass with retention time'.

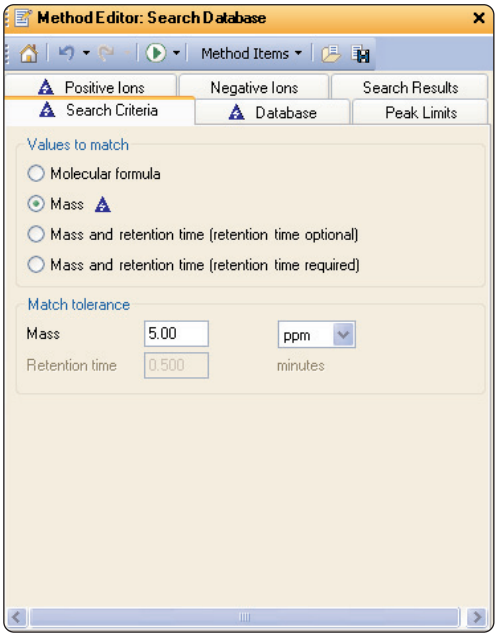


Figure 4b. Manual Search Criteria Settings.

Figure 4c illustrates more adduct and charge state options required for the database search.

Right-click in the spectrum window and a shortcut menu appears against the specified AMRT database (Figure 4a.) This menu has various options including 'Search database for spectrum peaks'. Selection of this option automatically invokes the database search. In Figure 4d the spectrum peak has been identified as PCP, with 0.87 ppm mass deviation and a spectral combined score of 99.36 out of 100 indicating extra confirmation of identity.

To calculate this score, three distinct score components were considered: Mass Match, Abundance Match, and Spacing Match with values of 99.61, 98.61, and 99.79, respectively. These are individually displayed in Figure 4d.

For trustworthy results, the software scores the database matches based on the similarity of each of the isotopic masses (Mass Match), isotope ratios (Abund Match), isotope spacing (Spacing Match), and optionally the retention time (RT Match).

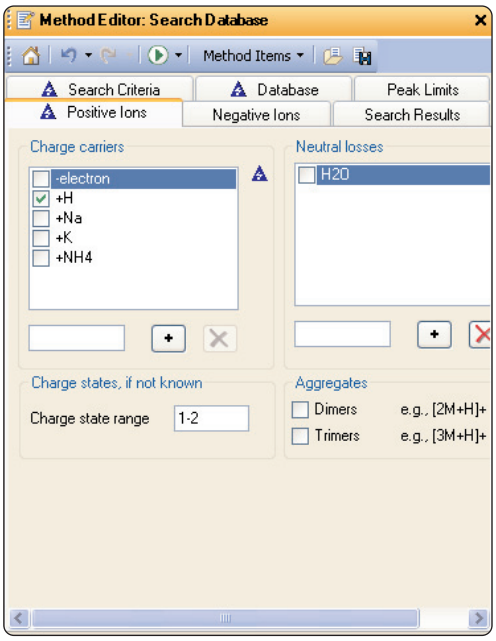


Figure 4c. Manual Search Adduct Selection.

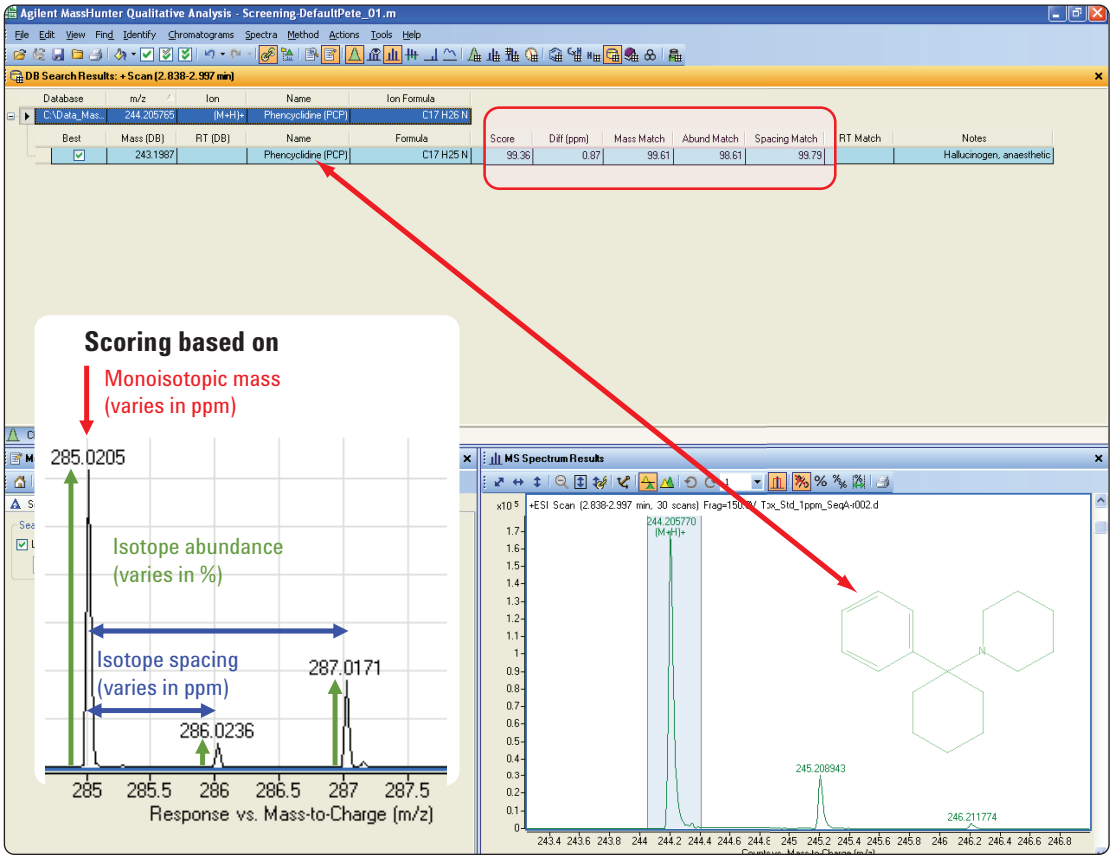


Figure 4d. Manual Database Search Results using MassHunter Qualitative Analysis program.

Isotope spacing is another important component of the scoring algorithm. The mass spacing from the M to the M+1 and M+2 isotopes can be measured with low-ppm accuracy. Any small mass shifts affect all isotopes equally, so this measurement is independent of overall mass axis shifts. This is outlined graphically in Figure 4d.

In this example, a single AMRT database result of phencyclidine (PCP) was returned, together with its structure which is optionally overlaid on the peak spectrum as shown in Figure 4d and can be displayed if selected in the reporting options.

More detailed information about MassHunter Qualitative analysis program database searching can be found in the MassHunter Qualitative Analysis Program Help Files or user guides [5].

Workflow B. Data mining using 'Molecular Feature Extractor' (MFE)

Batch PCDL searches (tabs 2 & 3) are designed for database searching and identification using an accurate mass list created from an automated data mining algorithm such as the Agilent Molecular feature extractor (MFE.) Such algorithms are extremely powerful, especially with complex data derived from difficult sample matrices, such as blood extracts. For the remainder of this application note, only batch searches invoked from inside the MassHunter Qualitative Analysis program interface will be outlined and described. For information on how to perform batch searches within the PCDL interface, please refer to the PCD application note [2].

Data mining algorithms such as MFE automatically search and 'mine' complex sets of single-stage MS data to determine and distinguish most likely and 'real' compound peaks from continuous background interferences. Combinations of adducts can be selected as part of the compound identification protocol to provide added assurance of compound validity.

Other data mining algorithms such as 'find by MS/MS' and 'find by Targeted MS/MS' are integral options included as part of the MassHunter Qualitative Analysis program software. The algorithms are dependent on the mode of operation and nature of the instrument being used. 'Find by Formula' compound search routines are described in the 'Workflow C' section of this application note.

For illustrative purposes, the LC/MS Toxicology Test Mix was analyzed under the conditions outlined in the experimental section. The data file was loaded into MassHunter Qualitative Analysis program. The 'Find by Molecular Feature' method editor was opened under the method explorer in the 'Find Compounds' section (see Figures 5a & 5b).

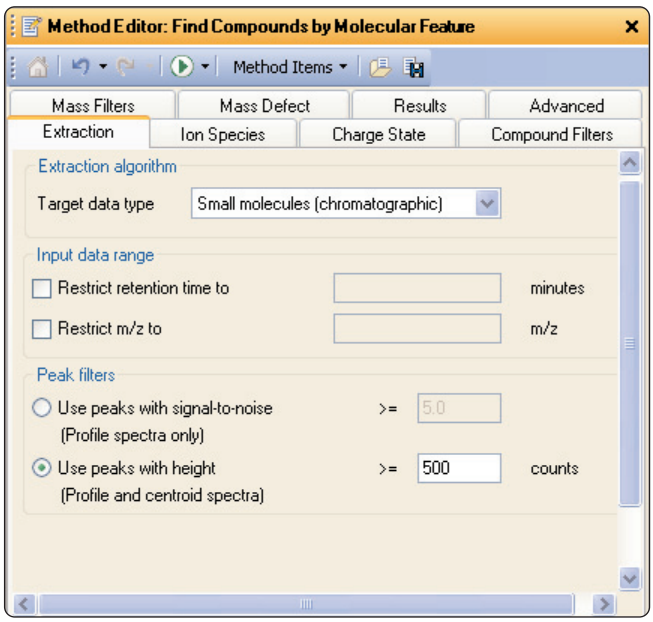


Figure 5a. MFE extraction parameters.

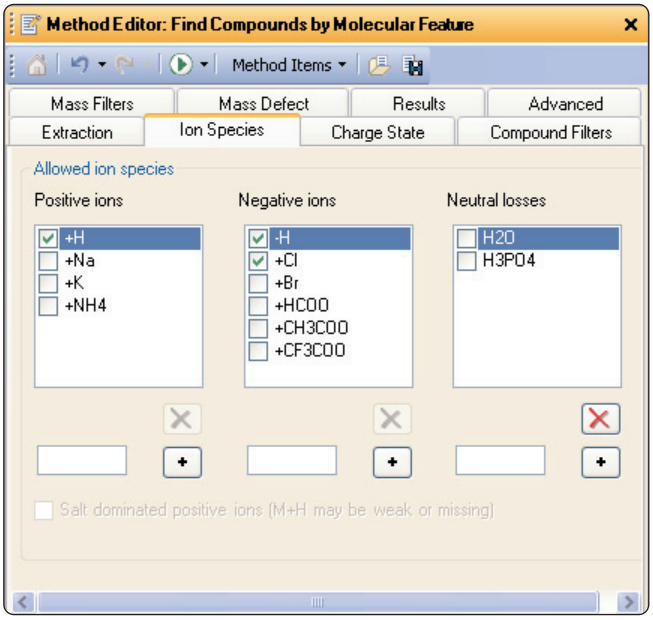


Figure 5b. MFE ion species setup.

A very aggressive setting of absolute peak height threshold (>500 counts) was used in this example (see Figure 5a), together with the small molecules algorithm (chromatographic) which yielded over 3000 possible compound hits. By raising this threshold amount, less abundant analytes may remain undetected. Conversely with a higher threshold the number of potential false positives are greatly reduced. Only [M+H]+ adducts were searched in this instance, however,

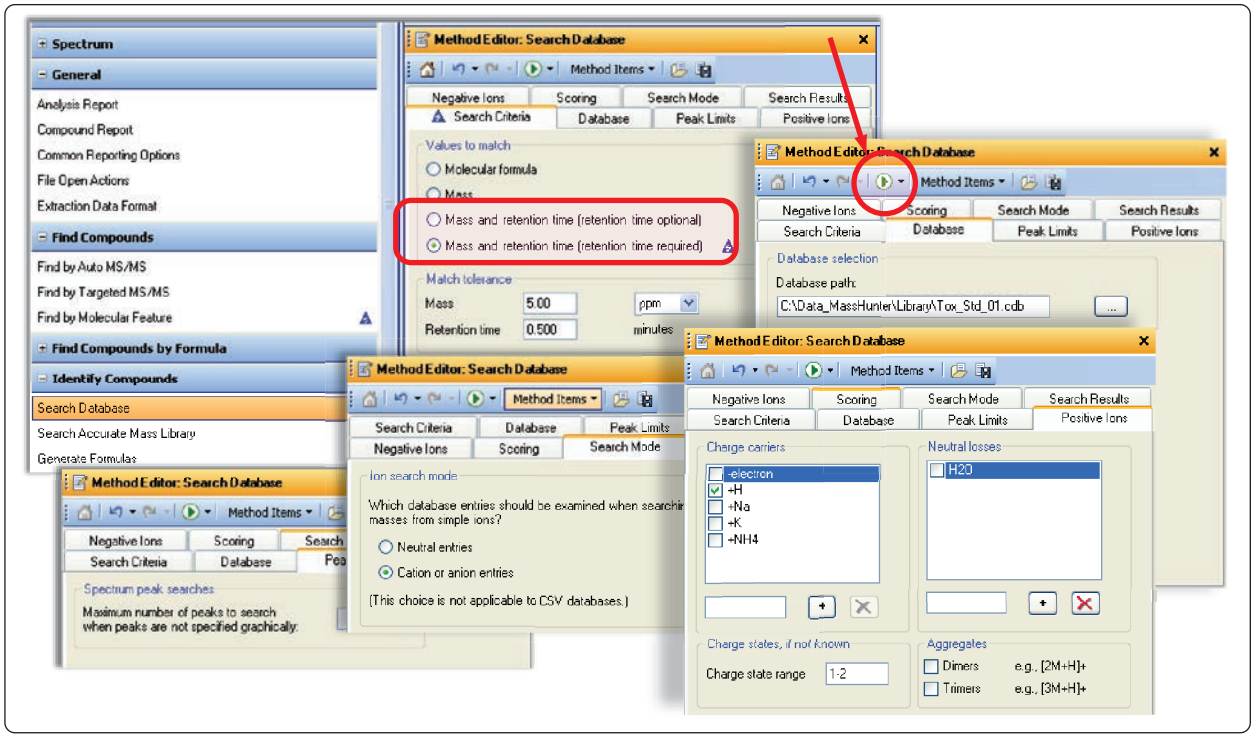


Figure 6. MFE compound database search settings.

further confidence could have been sought (see Figure 5b) by choosing additional adducts such as Na+ and NH4+.

No compound, mass filters or mass defect filters were specified for this search and a maximum charge state of 1 was specified in the MFE method setup. The next step after MFE search was to specify the forensic AMRT database (see Figure 6) in the identify compound/search database method editor, highlight all of the MFE-found compounds and search each compound against its content. A mass and retention time (RT) match was specified, since RT database values had already been pre-determined by analyzing individual standards and inserted into a customized compound database.

Figure 7 illustrates the results obtained from the MFE operation invoked by pressing the green 'process' button highlighted in the title bar of the MFE method editor (Figure 6).

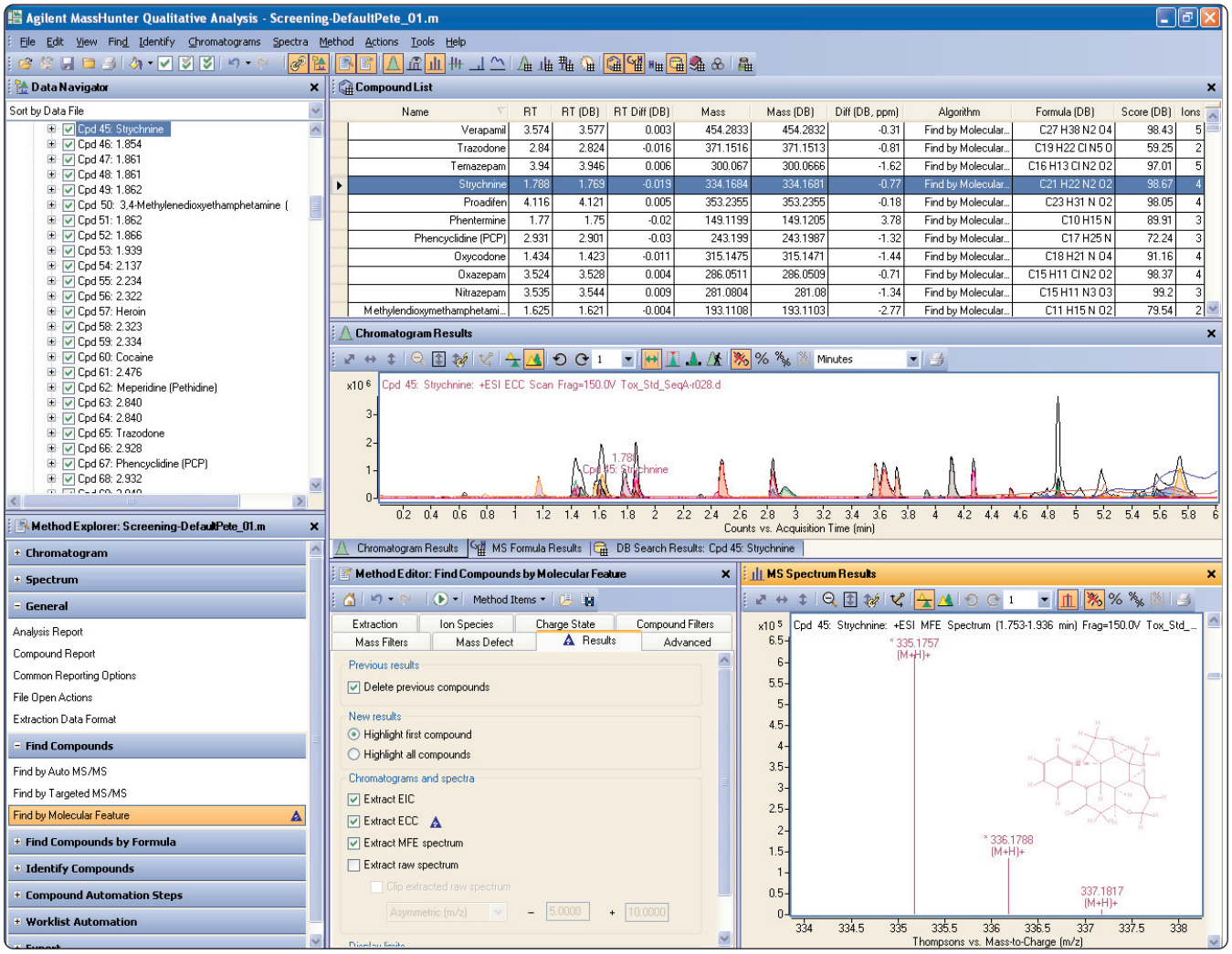


Figure 7. MFE compound database search results using MassHunter Qualitative Analysis program.

These results are detailed in Table 3 and show that all 25 compounds of the LC/MS Toxicology Test Mix were identified for this sample injection. This confirms that the data analysis settings for the find and identify steps are appropriate for the identification process. Many of the 3000+ compounds identified by MFE did not find any PCDL matches as expected and the data analysis option of excluding non-positives was used to report only the database hits.

Isobaric compounds such as codeine/hydrocodone and methamphetamine/phentermine were also correctly identified and distinguished automatically, by using the retention capability of the PCDL database and by inputting the predetermined retention time of each analyte for this chromatographic methodology as outlined in the Agilent G6855AA MassHunter Personal Forensics and Toxicology Database Quick Start Guide [3].

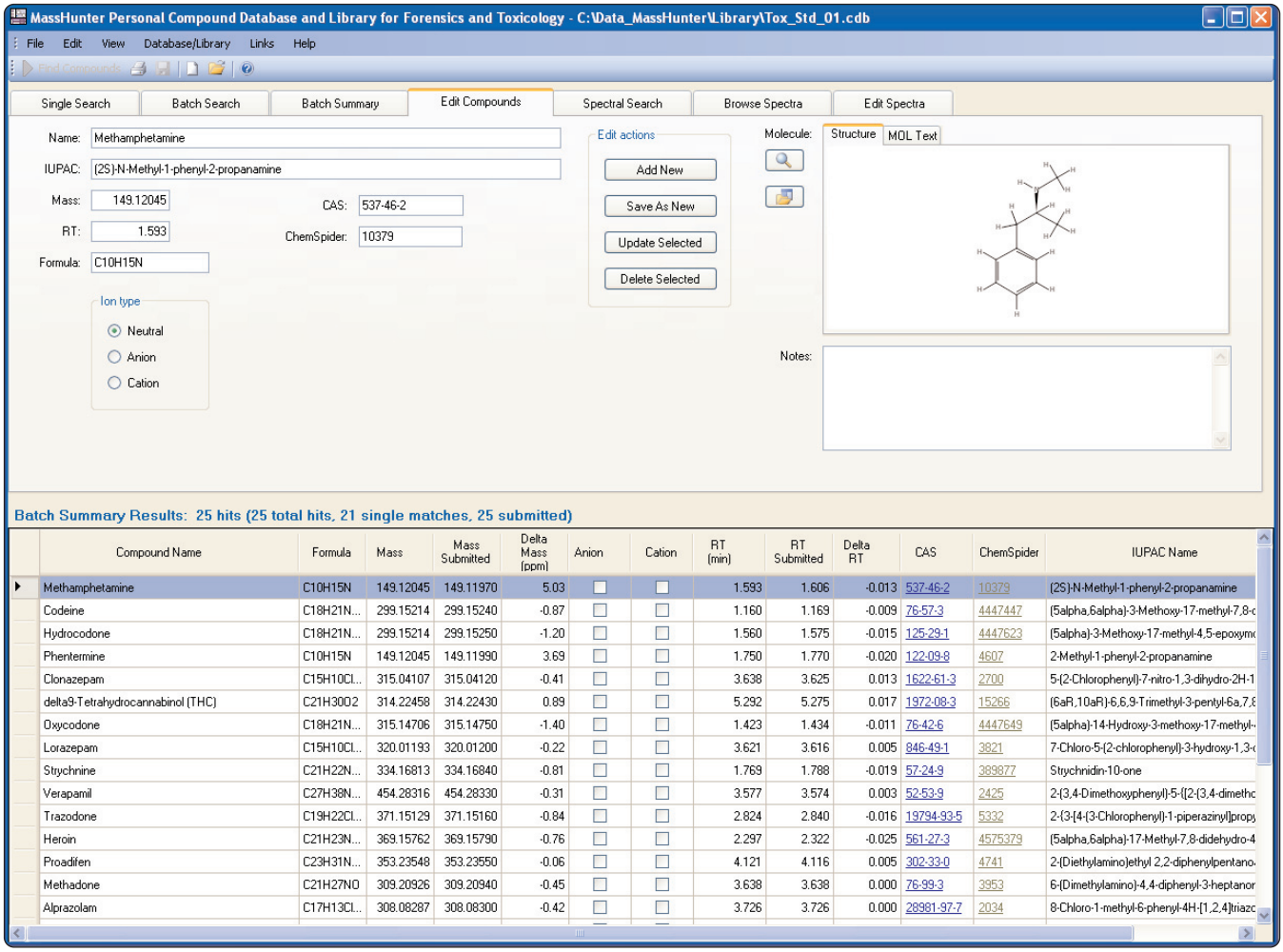
Table 3. MFE compound and database search results.

Name	RT	RT (DB)	RT Diff (DB)	Mass	Mass (DB)	Diff (DB, ppm)	Formula (DB)	Score (DB)
Verapamil	3.574	3.577	0.003	454.2833	454.2832	-0.31	$C_{27}H_{38}N_2O_4$	98.43
Trazodone	2.84	2.824	-0.016	371.1516	371.1513	-0.81	$C_{19}H_{22}ClN_5O$	59.25
Temazepam	3.94	3.946	0.006	300.067	300.0666	-1.62	$C_{16}H_{13}ClN_2O_2$	97.01
Strychnine	1.788	1.769	-0.019	334.1684	334.1681	-0.77	$C_{21}H_{22}N_2O_2$	98.67
Proadifen	4.116	4.121	0.005	353.2355	353.2355	-0.18	$C_{23}H_{31}NO_2$	98.05
Phentermine	1.77	1.75	-0.02	149.1199	149.1205	3.78	$C_{10}H_{15}N$	89.91
Phencyclidine (PCP)	2.931	2.901	-0.03	243.199	243.1987	-1.32	$C_{17}H_{25}N$	72.24
Oxycodone	1.434	1.423	-0.011	315.1475	315.1471	-1.44	$C_{18}H_{21}NO_4$	91.16
Oxazepam	3.524	3.528	0.004	286.0511	286.0509	-0.71	$C_{15}H_{11}ClN_2O_2$	98.37
Nitrazepam	3.535	3.544	0.009	281.0804	281.08	-1.34	$C_{15}H_{11}N_3O_3$	99.2
Methylendioxymethamphetamine (MDMA)	1.625	1.621	-0.004	193.1108	193.1103	-2.77	$C_{11}H_{15}NO_2$	79.54
Methamphetamine	1.606	1.593	-0.013	149.1197	149.1205	4.82	$C_{10}H_{15}N$	81.88
Methadone	3.638	3.638	0	309.2094	309.2093	-0.61	$C_{21}H_{27}NO$	99.67
Meperidine (Pethidine)	2.477	2.456	-0.021	247.1577	247.1572	-1.7	$C_{15}H_{21}NO_2$	97.91
Lorazepam	3.616	3.621	0.005	320.012	320.0119	-0.19	$C_{15}H_{10}Cl_2N_2O_2$	98.27
Hydrocodone	1.575	1.56	-0.015	299.1525	299.1521	-1.2	$C_{18}H_{21}NO_3$	85.2
Heroin	2.322	2.297	-0.025	369.1579	369.1576	-0.63	$C_{21}H_{23}NO_5$	98.97
Diazepam	4.272	4.275	0.003	284.072	284.0716	-1.36	$C_{16}H_{13}ClN_2O$	58.97
delta9-Tetrahydrocannabinol (THC)	5.275	5.292	0.017	314.2243	314.2246	0.94	$C_{21}H_30O_2$	94.83
Codeine	1.169	1.16	-0.009	299.1524	299.1521	-0.72	$C_{18}H_{21}NO_3$	72.49
Cocaine	2.44	2.418	-0.022	303.1475	303.1471	-1.29	$C_{17}H_{21}NO_4$	98.03
Clonazepam	3.625	3.638	0.013	315.0412	315.0411	-0.42	$C_{15}H_{10}ClN_3O_3$	98.72
Alprazolam	3.726	3.726	0	308.083	308.0829	-0.33	$C_{17}H_{13}ClN_4$	96.77
3,4-Methylenedioxyethamphetamine (MDEA)	1.862	1.846	-0.016	207.1263	207.1259	-1.8	$C_{12}H_{17}NO_2$	97.4
3,4-Methylendioxyamphetamine (MDA)	1.474	1.473	-0.001	179.095	179.0946	-2.23	$C_{10}H_{13}NO_2$	86.15

Customized databases with user-added retention times

One of the benefits of the Agilent Personal Forensics and Toxicology Database is that it can be saved to a user customized form. To create a read-write customizable database the user selects New Database from the PCDL File menu. The PCDL program then allows selection of an existing database and the naming of a new database. A description can also be given. When 'Create' is selected, the database with the new name contains all the entries of the selected database. In this way multiple custom or smaller, more targeted databases can be created depending on the analytes of interest. A technical note on the Pesticide PCD [2] shows how users can run standards with unique chromatographic conditions and easily update or insert retention times in their custom database.

Customizing and updating PCDL AMRT compound data is accomplished by using tab 4 (from left) of the PCDL program interface. This is shown in Figure 8, where the options of 'Add New', 'Save as New', 'Update Selected' and 'Delete Selected' are clearly present. When 'Allow Editing' is activated from the 'Database/Library' pull-down menu, any of the displayed information fields in the users' custom database can be changed, added to or deleted. Furthermore, the ability to insert '*.mol' molecular diagrams to any new database entry is possible from the 'Edit Compounds' tab.



Compound Name	Formula	Mass	Mass Submitted	Delta Mass (ppm)	Anion	Cation	RT (min)	RT Submitted	Delta RT	CAS	ChemSpider	IUPAC Name
Methamphetamine	C10H15N	149.12045	149.11970	5.03			1.593	1.606	-0.013	537-46-2	10379	(2S)-N-Methyl-1-phenyl-2-propanamine
Codeine	C18H21N...	299.15214	299.15240	-0.87			1.160	1.169	-0.009	76-57-3	4447447	(5alpha,6alpha)-3-Methoxy-17-methyl-7,8-d
Hydrocodone	C18H21N...	299.15214	299.15250	-1.20			1.560	1.575	-0.015	125-29-1	4447623	(5alpha)-3-Methoxy-17-methyl-4,5-epoxym
Phentermine	C10H15N	149.12045	149.11990	3.69			1.750	1.770	-0.020	122-09-8	4607	2-Methyl-1-phenyl-2-propanamine
Clonazepam	C15H10Cl...	315.04107	315.04120	-0.41			3.638	3.625	0.013	1622-61-3	2700	5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1
delta9-Tetrahydrocannabinol (THC)	C21H30O2	314.22458	314.22430	0.89			5.292	5.275	0.017	1972-08-3	15266	(6aR,10aR)-6,6,9-Trimethyl-3-pentyl-6a,7,8
Oxycodone	C18H21N...	315.14706	315.14750	-1.40			1.423	1.434	-0.011	76-42-6	4447649	(5alpha)-14-Hydroxy-3-methoxy-17-methyl-
Lorazepam	C15H10Cl...	320.01193	320.01200	-0.22			3.621	3.616	0.005	846-49-1	3821	7-Chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-d
Strychnine	C21H22N...	334.16813	334.16840	-0.81			1.769	1.788	-0.019	57-24-9	389877	Strychnidin-10-one
Verapamil	C27H38N...	454.28316	454.28330	-0.31			3.577	3.574	0.003	52-53-9	2425	2-(3,4-Dimethoxyphenyl)-5-[[2-(3,4-dimetho
Trazodone	C19H22Cl...	371.15129	371.15160	-0.84			2.824	2.840	-0.016	19794-93-5	5332	2-(3-[4-(3-Chlorophenyl)-1-piperazinyl]propy
Heroin	C21H23N...	369.15762	369.15790	-0.76			2.297	2.322	-0.025	561-27-3	4575379	(5alpha,6alpha)-17-Methyl-7,8-didehydro-4
Proadifen	C23H31N...	353.23548	353.23550	-0.06			4.121	4.116	0.005	302-33-0	4741	2-(Diethylamino)ethyl 2,2-diphenylpentano
Methadone	C21H27NO	309.20926	309.20940	-0.45			3.638	3.638	0.000	76-99-3	3953	6-(Dimethylamino)-4,4-diphenyl-3-heptanor
Alprazolam	C17H13Cl...	308.08287	308.08300	-0.42			3.726	3.726	0.000	28981-97-7	2034	8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazo

Figure 8. Edit Compounds PCDL interface tab.

Workflow C. Data mining using 'Find by Formula' (FBF)

The 'Find by Formula' data-mining algorithm of the MassHunter Qualitative Analysis program uses a pre-defined empirical formula (or list of formulae) to search TOF and Q-TOF (MS) data files for evidence that peaks may be present. The PCDL-format databases can also be specified as the list of empirical formulae. Depending on the size and content of the database, FBF can take slightly longer than the MFE approach. However, FBF is highly accurate and sensitive especially at very low analyte concentration levels.

Figure 9 illustrates the results screen displayed after a 'Find by Formula' search has been undertaken using the LC/MS Toxicology Test Mix data file. All 25 compounds were matched with accurate mass, abundance and isotopic spacing in a combined score (shown) together with retention time. The DA method editor settings used for this FBF analysis are shown in Figure 10, where 'Tox_std_01.cdb' was a custom PCDL-format database.

When reporting the results, FBF assesses the chromatographic peak shape and isotopic match scores and returns the best match, even if there are several peaks displayed in the extracted compound chromatogram of similar mass.

Additional adducts [M+Na]+, [M+NH4]+ and [2M+H]+ were used during this FBF data screen. The extra information is displayed in the spectrum view and results table to provide added confirmatory evidence. Figure 9 shows the Temazepam spectrum which displays both [M+H]+ and [M+Na]+ adducts.

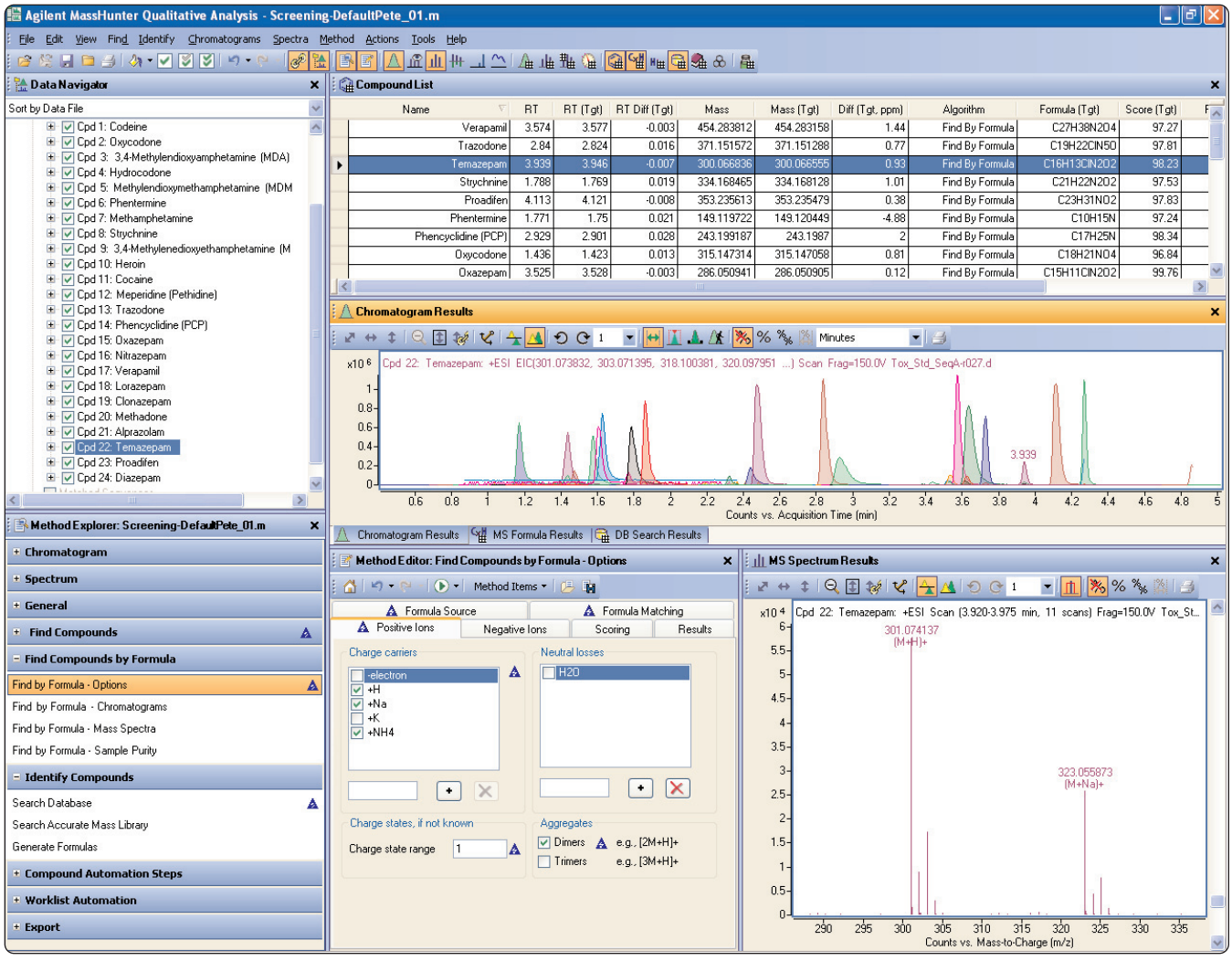


Figure 9. Find By Formula Database search results, MassHunter Qualitative Analysis program.

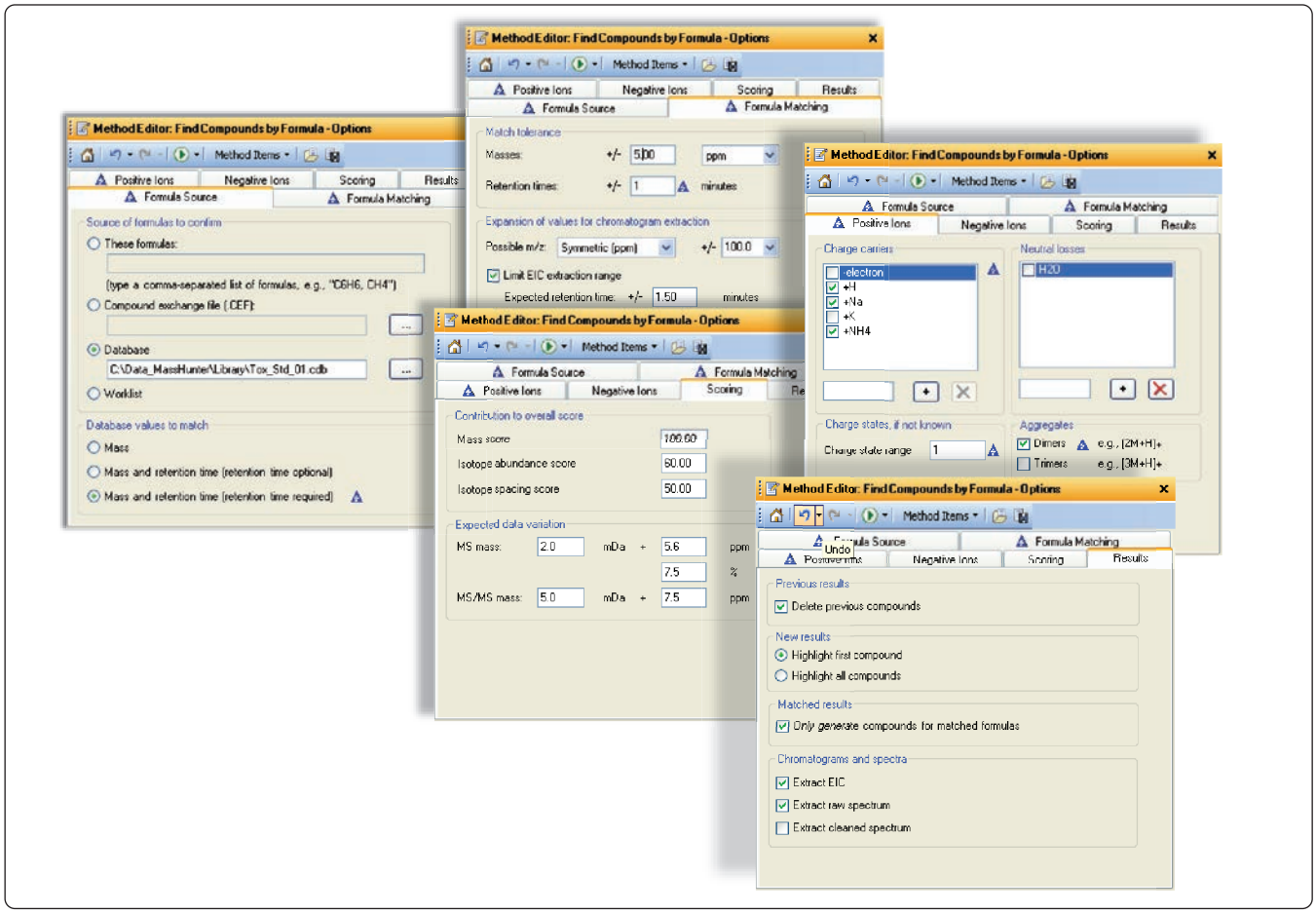


Figure 10. Find By Formula Database search - Method editor settings.

More in-depth information can be obtained from MassHunter Qualitative Analysis program Help files or Agilent MassHunter Workstation Software Qualitative Analysis Familiarization Guide [5].

Reporting

Manual, MFE and FBF database searching all use the identical method of compound reporting options in the MassHunter Qualitative Analysis program software interface. Figure 11 details the reporting options which are based upon the standard compound report template 'CompoundReportWithIdentificationHits.xlsx'. Under the General section of the method explorer, the 'Common reporting options' link opens the corresponding method editor pane, shown on the left side of Figure 11. MassHunter Qualitative Analysis program treats search algorithm data and database searches as compound-centric data. Therefore, to report the results the appropriate compound report template must be chosen. In this example, the correct report template is displayed.

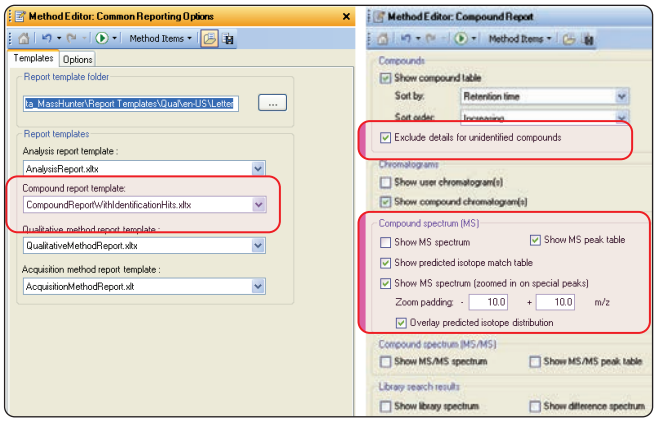


Figure 11. Common compound reporting options for Manual/MFE/FBF PCDL Searches.

More specific content can then be specified by choosing the information required for the Toxicology screen report using the 'Compound Report' options of the method editor (shown on the right in Figure 11).

Decisions about the report content are decided here. For example, if the check box for 'Exclude Details for Unidentified Compounds' is activated, then only positive PCDL identifications will be reported. The option to report compound extracted chromatograms, individual MS spectra, or summary results and individual compound tables is also determined from the compound report method editor.

Once all the correct settings have been achieved for the reporting of results, the green button (circled in Figure 12) activates the 'printing dialogue' window which gives various options for directing the output of the data file results. The user can choose to send results directly to a specified printer or save the results in excel format or public distribution format (pdf). Alternatively, the results report can be processed by choosing the 'Print Compound Report' option from the drop-down 'File' menu.

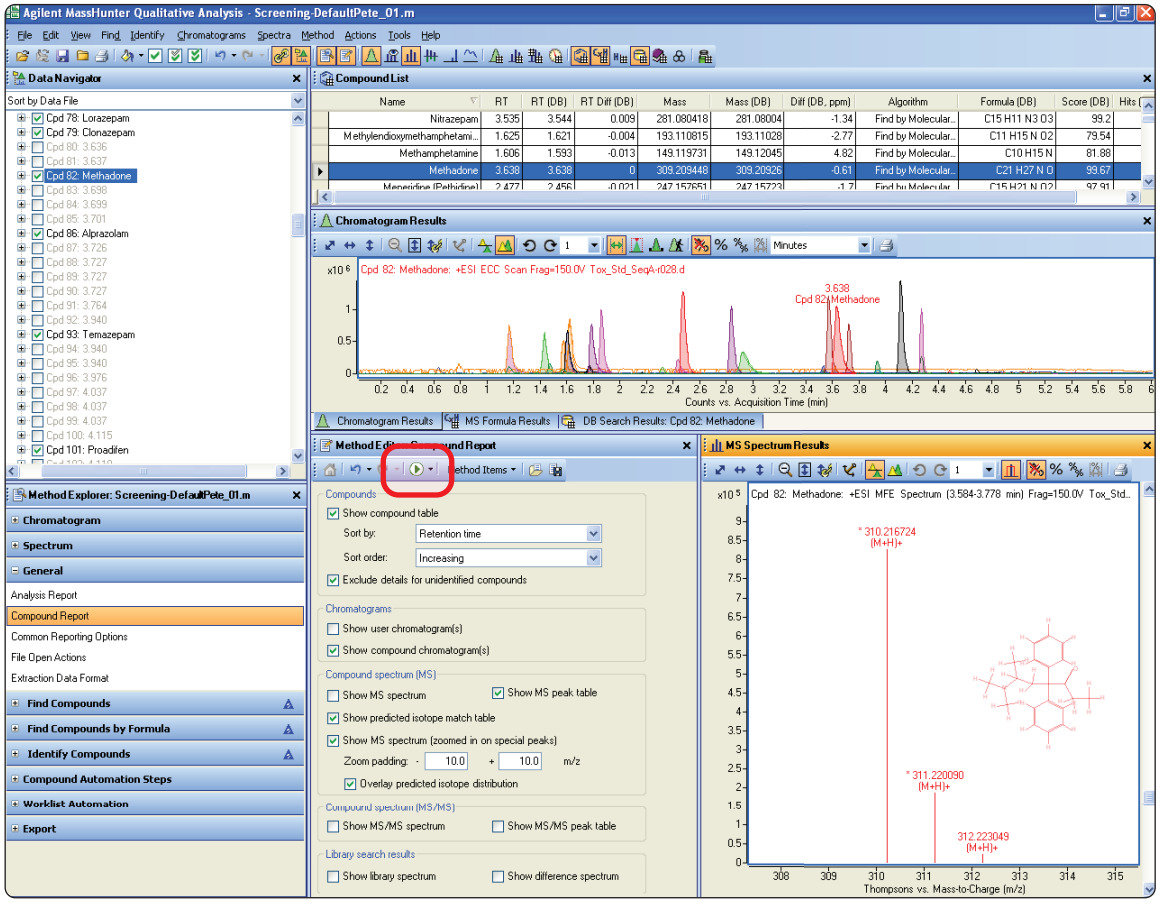


Figure 12. Compound Reporting for Manual/MFE/FBF PCDL Searches.

Figure 13 illustrates a typical report summary front page for the LC/MS Toxicology Test Mix.

Qualitative Compound Report

Data File	Tox_Std_SeqA-r028.d	**Sample Name**	Tox_Std_100pg
Sample Type	Sample	**Position**	Vial 2
Instrument Name	SEIKO-90500107	**User Name**	Pete Stone
Acq Method	PStone_Tox_Std_01.m	**Acquired Time**	7/17/2009 9:41:03 PM
IRM Calibration Status	Success	**DA Method**	Screening-DefaultPete_01.m
Comment			

Compound Table

Compound Label	RT	Mass	Name	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 10: Codeine	1.169	299.1524	Codeine	C18 H21 N O3	-0.72	2
Cpd 17: Oxycodone	1.434	315.1475	Oxycodone	C18 H21 N O4	-1.44	1
Cpd 23: 3,4-Methylendioxyamphetamine (MDA)	1.474	179.095	3,4-Methylendioxyamphetamine (MDA)	C10 H13 N O2	-2.23	1
Cpd 29: Hydrocodone	1.575	299.1525	Hydrocodone	C18 H21 N O3	-1.2	2
Cpd 34: Methamphetamine	1.606	149.1197	Methamphetamine	C10 H15 N	4.82	2
Cpd 39: Methylendioxymethampheta mine (MDMA)	1.625	193.1108	Methylendioxymethampheta mine (MDMA)	C11 H15 N O2	-2.77	1
Cpd 41: Phentermine	1.77	149.1199	Phentermine	C10 H15 N	3.78	2
Cpd 45: Strychnine	1.788	334.1684	Strychnine	C21 H22 N2 O2	-0.77	1
Cpd 50: 3,4-Methylenedioxyethamphetami ne (MDEA)	1.862	207.1263	3,4-Methylenedioxyethamphetami ne (MDEA)	C12 H17 N O2	-1.8	1
Cpd 57: Heroin	2.322	369.1579	Heroin	C21 H23 N O5	-0.63	1
Cpd 60: Cocaine	2.44	303.1475	Cocaine	C17 H21 N O4	-1.29	1
Cpd 62: Meperidine (Pethidine)	2.477	247.1577	Meperidine (Pethidine)	C15 H21 N O2	-1.7	1
Cpd 65: Trazodone	2.84	371.1516	Trazodone	C19 H22 Cl N5 O	-0.81	1
Cpd 67: Phencyclidine (PCP)	2.931	243.199	Phencyclidine (PCP)	C17 H25 N	-1.32	1
Cpd 73: Oxazepam	3.524	286.0511	Oxazepam	C15 H11 Cl N2 O2	-0.71	1
Cpd 74: Nitrazepam	3.535	281.0804	Nitrazepam	C15 H11 N3 O3	-1.34	1
Cpd 76: Verapamil	3.574	454.2833	Verapamil	C27 H38 N2 O4	-0.31	1
Cpd 78: Lorazepam	3.616	320.012	Lorazepam	C15 H10 Cl2 N2 O2	-0.19	1
Cpd 79: Clonazepam	3.625	315.0412	Clonazepam	C15 H10 Cl N3 O3	-0.42	1
Cpd 82: Methadone	3.638	309.2094	Methadone	C21 H27 N O	-0.61	1
Cpd 86: Alprazolam	3.726	308.083	Alprazolam	C17 H13 Cl N4	-0.33	1
Cpd 93: Temazepam	3.94	300.067	Temazepam	C16 H13 Cl N2 O2	-1.62	1
Cpd 101: Proadifen	4.116	353.2355	Proadifen	C23 H31 N O2	-0.18	1
Cpd 110: Diazepam	4.272	284.072	Diazepam	C16 H13 Cl N2 O	-1.36	1
Cpd 243: delta9-Tetrahydrocannabinol (THC)	5.275	314.2243	delta9-Tetrahydrocannabinol (THC)	C21 H30 O2	0.94	1

Compound Label	Name	RT	Algorithm	Mass
Cpd 10: Codeine	Codeine	1.169	Find by Molecular Feature	299.1524

Figure 13. Output Report from MFE/Database search.

Worklist Automation:

Once the analyst or operator has decided on the correct settings for all aspects of the data mining routines, the PCDL search options and reporting options (outlined in this application note) can be saved to one convenient data analysis method. This method can be used for repetitive and consistent data manipulation from week to week. This is achieved by choosing the 'Save As' option from the drop-down 'Method' menu in the MassHunter Qualitative Analysis program interface. This method will then open as the default DA method when the MassHunter Qualitative Analysis program is started until another DA method is saved or loaded.

An added advantage to saving reprocessing options is the 'Worklist Automation' functionality built into the MassHunter Qualitative Analysis program. Figure 14 outlines the setup of Worklist automation and specifically addresses a routine that would automatically interrogate a data file using MFE and PCDL database search followed by reporting of results to the specified printer or data file location.

In this example, a list of automatic data analysis steps are defined in order of operation, as they would be undertaken manually.

First, the sample data file is loaded, and all previous results (if any) are cleared. Next, the 'Find by MFE' routine according to the saved DA method setup is performed with the compound results searched against the PCDL database specified in the DA method. Finally, any results are automatically sent to a final report, the format of which has been determined and also saved to the DA method.

Two further steps must be performed to run such a worklist automation routine automatically during sample data acquisition.

First, the DA analysis method and the Worklist Automation routine must be saved into the acquisition method by using the 'Save As' option from the 'Method' menu and selecting the MassHunter acquisition method name. Once 'OK' is

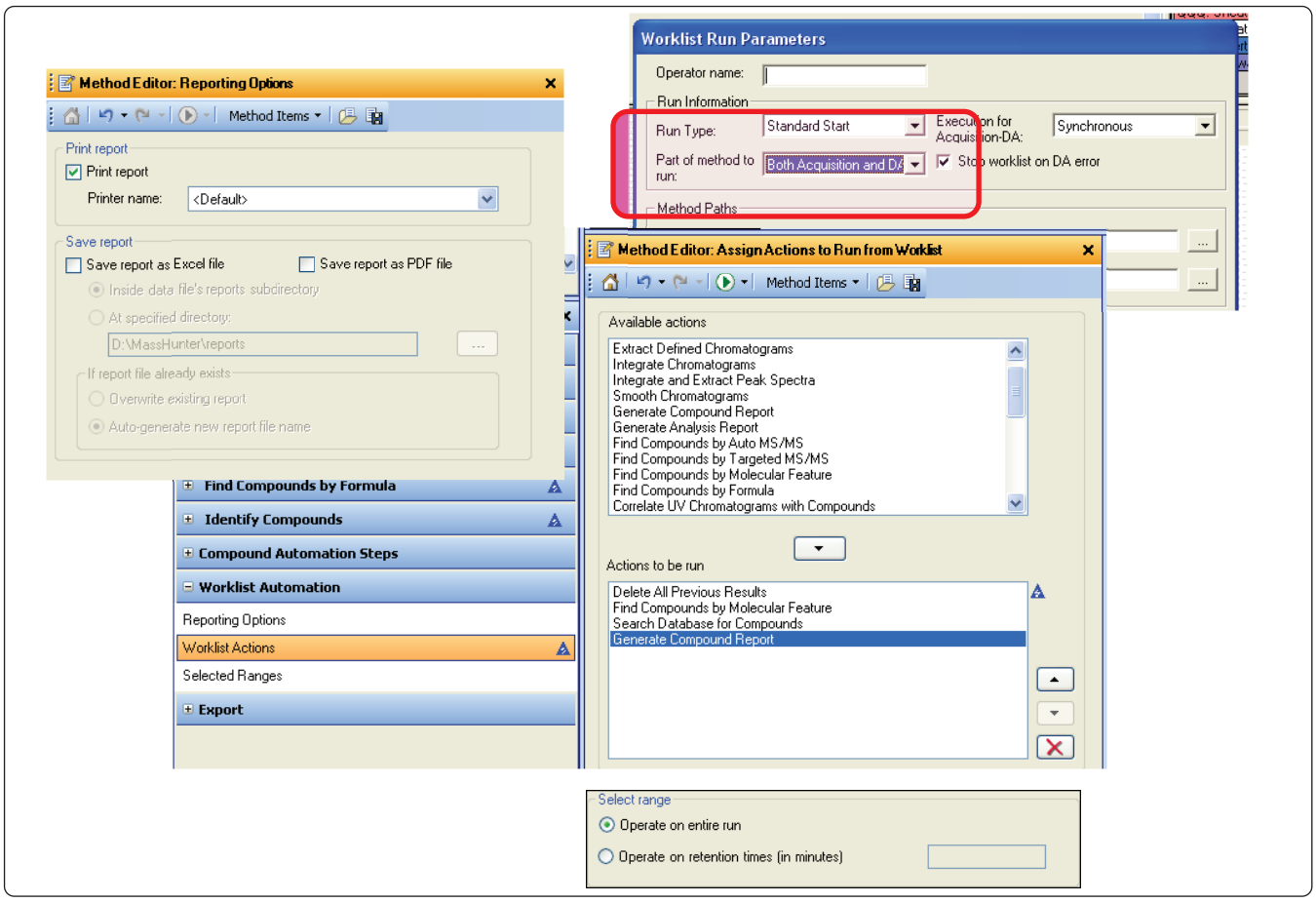


Figure 14. Worklist automation method setup.

selected, the data analysis method becomes an integral part of the Acquisition method.

Finally, to automatically perform Worklist Data Analysis during data acquisition, the 'Worklist Run Parameters' window must be opened from the 'Worklist' Menu of MassHunter Acquisition software. Figure 14 shows a screen capture of this window with the settings highlighted so that the DA routine will operate 'Parts of method to Run - Both Acquisition and DA'. The data analysis has the option to be run 'Synchronously' or 'Asyncronously'.

Conclusions

The Agilent MassHunter Personal Forensics and Toxicology Database Kit has been developed to provide comprehensive screening of samples for both targeted and non-targeted approaches. The database includes accurate mass data for around 6700 compounds of potential interest and gives the user flexibility in its use.

The MassHunter Personal Forensics and Toxicology Database Kit offers:

- Fast and easy startup of complex analyses
- A comprehensive database of around 6700 compounds including
  - Chemical structures, formulas and exact masses
  - Direct Chemical Internet links to PUBCHEM and ChemSpider
  - IUPAC names
  - The ability to create MS/MS spectral libraries
  - Complete customization with additions/deletions of retention time for chromatographic conditions developed by the user
- Results can be searched from within the PCDL software interface or directly from the MassHunter Qualitative Analysis program.
- Results can be data-mined with powerful searching tools, such as the Molecular Feature Extractor and Find by Formula
- Searches of the database can be partially or completely automated using MassHunter Qualitative Analysis program and the MassHunter Acquisition Worklist

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Agilent Technologies

Quantitative Analysis of Opiates in Urine Using Accurate Mass LC/MSD TOF

Application

Forensics

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Abstract

Urine samples were quantitatively analyzed at the 6 ng/mL level using liquid chromatography/mass selective detector time-of-flight. The advantage of accurate mass measurement to enhance selectivity is presented. The instrumental detection limit is 2 pg on-column with a signal/noise ratio of 5:1.

Introduction

Until now quantitative time-of-flight (TOF) has not been broadly applicable. This application demonstrates that the Agilent liquid chromatography/mass selective detector time-of-flight (LC/MSD TOF) can routinely quantify compounds at low levels in matrices important to the forensic scientist. Both direct injection of urine and solid phase extraction (SPE) are performed to demonstrate the robustness, sensitivity, and selectivity of the LC/MSD TOF.

Experimental

Sample preparation

Direct injection samples were spiked at the specified concentrations with no further handling. Accubond II Evidex SPE Cartridges (part number 188-2946) were used as per extraction protocol for opiates (see step-by-step instructions that comes with cartridges). Five milliliters of either blank or spiked urine was treated with 0.5 mL concentrated HCl, 0.75 mL 10 N NaOH, and then adjusted to pH 6.5-7.5 with 2.5 mL 0.5 M phosphoric acid. The heating step was not included because acid hydrolysis of glucuronides were not expected. After conditioning, this solution was loaded onto the cartridge, rinsed, and then eluted with the prescribed solution of methylene chloride/isopropanol/ammonium hydroxide. The eluant was taken to dryness with nitrogen (no heat) and then reconstituted in 0.5 mL 40:60 water:acetonitrile.

Instrument

Agilent 1100 Series LC/MSD TOF with Agilent 1100 binary pump and well plate autosampler

Table 1 Experimental Conditions

LC Conditions	
Column	ZORBAX XDB-C18, 2.1 mm × 50 mm, 3.5 µm P/N 971700-902
Mobile Phases	A: Acetonitrile with 0.1 % formic acid B: Water with 0.1 % formic acid
Gradient	35% to 95% A in 5 min, then to 100% in 6 min
Flow rate:	0.35 mL/min

MS Conditions

Standard autotune conditions with calibrant delivery system providing constant low flow of ~2 µM purine and HP-921 calibrant to dual ESI for continuous auto-calibration

Results

Shown in Figure 1 (upper panel) is the total ion chromatogram (TIC) and in the lower panel, overlaid extracted ion chromatograms (EICs) for morphine, codeine, and acetylmorphine, in a direct injection of urine at 300 ng/mL. The EIC has a mass window of 20 ppm (~ ±0.002 u). Accurate mass spectra for these opiates is given in Figure 2. Table 1 shows the quantitative results obtained with direct injection. Table 2 shows the results obtained with the solid phase extraction (SPE).

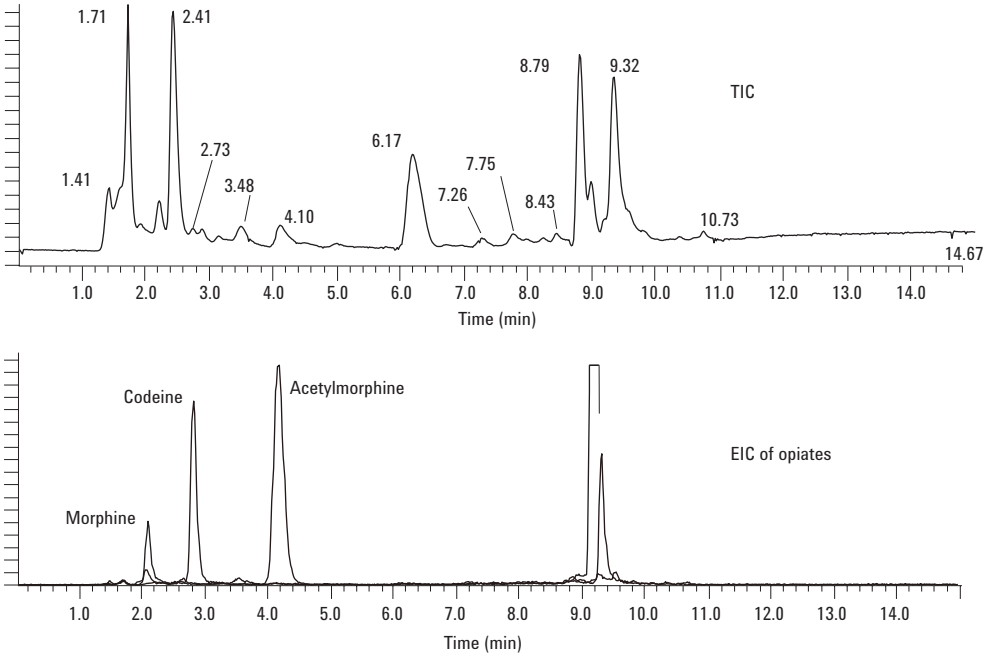

Figure 1. The upper panel shows the TIC of a direct urine injection spiked with 300 ng/mL of each opiate. The lower panel shows the EIC of each compound.

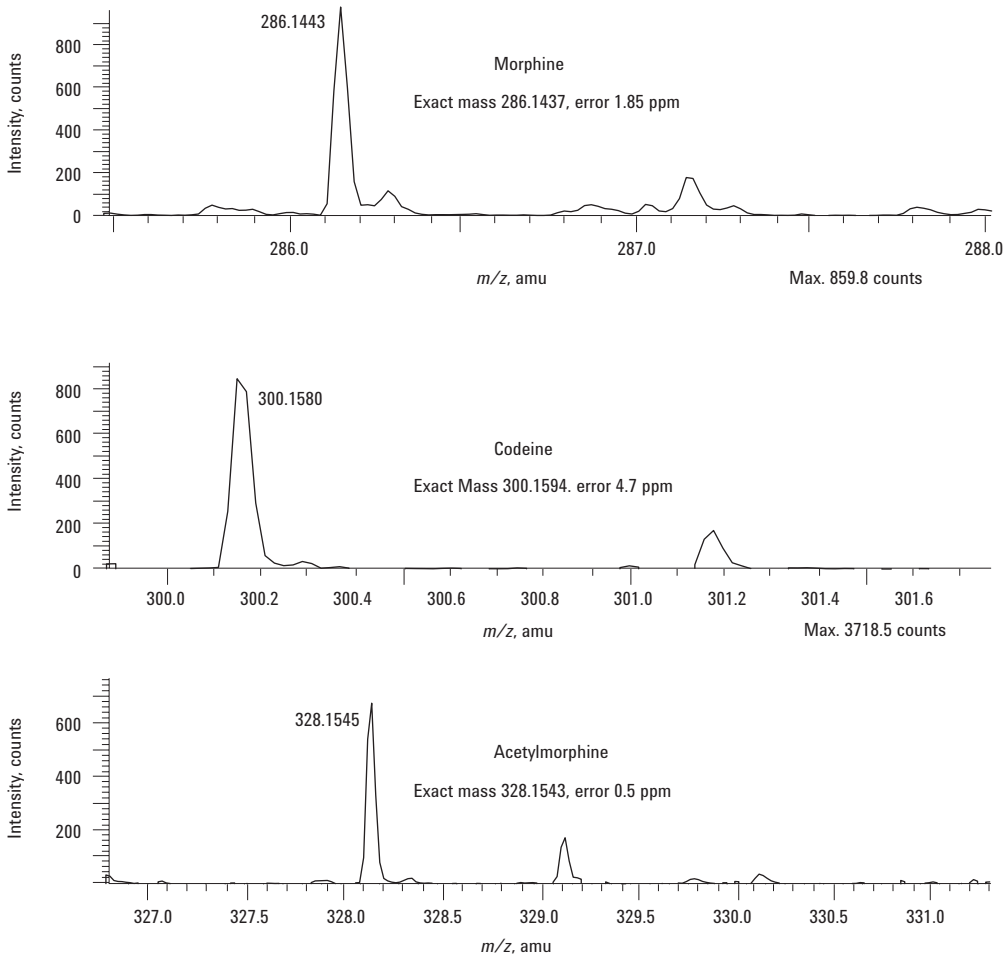

Figure 2. Mass spectra of M+H ions for opiates showing both mass resolution and mass accuracy at 2 pg on-column.

Table 2. Quantitative results (in ng/mL) of spikes at 1000 ng/mL and 300 ng/mL obtained by LC/MSD TOF direct injection of urine.

Urine direct injection (Spike 1000 ng/mL)				Urine direct injection (Spike 300 ng/mL)			
	Morphine	**Codeine**	**Acetylmorphine**		**Morphine**	**Codeine**	**Acetylmorphine**
	241	446	715		66.7	93.8	176
	222	402	653		78.5	94.8	203
	238	426	683		73.7	93	199
	195	338	687		73.7	96.5	201
	200	351	588		76.6	94.3	185
Mean	219.2	392.6	665.2	**Mean**	73.8	94.5	192.8
SD	21.2	46.8	48.4	**SD**	4.5	1.3	11.8
RSD (%)	**9.7**	**11.9**	**7.3**	**RSD (%)**	**6.1**	**1.4**	**6.1**

These are typical concentration and cut-off range of immunoassay. Note that difference between spiked value and measured concentration represents degree of ion suppression at source.

Table 3. Quantitative results of spikes at 6 ng/mL and 60 ng/mL obtained by LC/MSD TOF with Accubond Evidex SPE sample preparation.

Accubond Evidex 5 mL Urine (Spike 6 ng/mL) Expected Conc. 60 pg/µL				Accubond Evidex 5 mL Urine (Spike 60 ng/mL) Expected Conc. 600 pg/µL			
	Morphine	**Codeine**	**Acetylmorphine**		**Morphine**	**Codeine**	**Acetylmorphine**
	6.97	8.62	3.74		508	499	182
	8.56	9.57	4.21		567	543	193
	10	8.41	4.03		525	504	183
	9.24	8.5	3.81		521	502	191
	7.07	8.15	3.48		595	532	193
	9.46	8.99	3.5		591	532	192
	7.66	8.91	3.79		582	540	196
Mean	8.4	8.7	3.8	**Mean**	555.6	521.7	190.0
SD	1.2	0.5	0.3	**SD**	36.6	19.2	5.4
RSD (%)	**14.4**	**5.3**	**6.9**	**RSD (%)**	**6.6**	**3.7**	**2.8**

Difference in spiked value and measured concentration represents both recovery of SPE method and ion suppression (if any).

Conclusions

The data shown demonstrates the ability of LC/MSD TOF to confirm - with accurate mass measurement, and quantify- with selective narrow mass window.

- Direct injection of urine shows the robustness of the LC/MSD TOF.
- Typical clean-up (SPE) shows excellent sensitivity.
- High-mass resolution and accuracy (of every spectrum) provides the selectivity for reduction of chemical noise for quantitation and confirmation.

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Agilent Technologies

Development of a Screening Analysis by LC Time-Of-Flight MS for Drugs of Abuse

Application

Forensics

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Abstract

The screening for drugs of abuse in human samples has relevance in much of today's society; employers, police and prison officials, and forensic pathologists all rely on the accuracy of drug screening. Currently, the most common method of this analysis is a straight-forward immunoassay technique, which although allowing for a rapid turnaround of screening samples, involves a slower confirmatory test of derivatization and detection by gas chromatography/mass spectrometry (GC/MS).

This application note presents the potential for the Agilent Time-of-Flight Mass Spectrometer (LC/MSD TOF) for use as both a screening and a confirmation tool in one analytical run of 30 minutes.

Introduction

History has shown that the human race has a long-standing fascination with the consumption and experimentation of mind and body altering substances. Traditionally, all of these substances occurred naturally. Even today, many drugs of choice for this use are derived directly from natural substances. One of the most common of these, *cannabis*, is reported to have been tried on at least one occasion by up to 32% of the American population, and amongst the younger generation, 56% of high school senior students. These large numbers and the associated law enforcement issues have resulted in the decriminalization of its private use in many states and territories, but this does not diminish the potential impact on workplace performance or motor skills impairment, which may result in serious accidents. An extensive review of the illicit drug market in 25 major U.S. cities is provided in the Office of National Drug Control Policy Document "Pulse Check" [1].

Over the last 100 years, the physiological effects of many of the current illicit drugs were evaluated and reviewed, resulting in their subsequent banishment from society. During this time, a plethora of new drugs were developed, many finding wide acceptance within the medical community for the treatment of specific ailments. Unfortunately, the undesirable side effects of addiction or long-term abuse were often associated with the use of these drugs. The opiate class of drugs, which provided substantial improvement for the comfort of many patients, is an excellent example of one such class, as they are highly addictive and subject to abuse.

Interest in the analysis of drugs of abuse covers many areas, all with different concerns in the results obtained. Some of the areas of significance include:

- Workplace screening
- Therapeutic monitoring
- Forensic pathology
- Accident investigation
- Crime scene investigation

Today, screening of drugs of abuse is performed through a variety of methods, with the most common lab-based technique being an Enzyme Multiple Immunoassay Test (EMIT), with a confirmatory analysis by GC/MS, if required. This immunoassay technique allows for screening to be performed and reported in as little as 2 hours, yet more commonly a 36–48 hour turnaround time is required. A further disadvantage of the EMIT technique is that it lacks the specificity to identify anything more than the class of drug detected.

The current analytical confirmatory technique of GC/MS was developed in order to achieve the sensitivity and specificity required to accurately determine the exact type and level of the drug compound, within the class indicated by the immunoassay technique. In order to achieve this detection, many of the drugs require derivatization to ensure adequate volatility and/or thermal stability required for GC analysis. See Table 1.

Table 1. National Institute of Drug Analysis Compound Class and Detection Limit Summary

Compound class	Detection limits (ng/mL)	Confirmation
Amphetamines	1000	EMIT/GC/MS
Barbiturates	300–3000	EMIT/GC/MS
Cocaine	300	EMIT/GC/MS
Methadone	300	EMIT/GC/MS
Opiates	300	EMIT/GC/MS
Phencyclidine	25	EMIT/GC/MS
Propoxyphene	300	EMIT/GC/MS
Benzodiazepines	300	EMIT/GC/MS
Methaqualone	300	EMIT/GC/MS
Cannabinoids	50	EMIT/GC/MS

Recently published Agilent application notes have shown the potential of LC/MS for the screening analysis and therapeutic monitoring of drugs of abuse using a single quadrupole instrument [2, 3]. Numerous other publications discuss selected drugs of abuse, or drug classes, illustrating the potential for the technique to one day replace GC/MS as either the confirmatory tool or as both the screening and confirmatory tool in one analysis.

Accurate mass measurement, such as that provided by the Agilent LC/MSD TOF, greatly increases the confidence of identification because it inherently limits the possible number of candidate compounds. The better the precision and accuracy of the mass measurement, the fewer the number of compounds theoretically possible for a given accurate mass. This is particularly useful for the analysis of samples from a variety of sources, each with their own potential interferences, such as those encountered with explosives residue analysis.

This application note provides an overview of the power of the Agilent TOF mass spectrometer for the screening and confirmation analysis of drugs of abuse. The TOF mass spectrometer provides accurate mass determinations (<3 ppm) with good linearity, proving its use as an excellent tool for the detection, confirmation, and quantitation of different drug classes. The method used here is not intended to represent one that will determine the lowest possible level of any one particular analyte or class of analytes, but rather is a procedure that could be expanded to cover a wider range of components used in screening analyses.

The compounds studied and their molecular formulas are shown in Table 2.

Table 2. Compounds Included in Study

Compound	Molecular formula	Drug class
α-hydroxyalprazolam	$C_{17}H_{13}N_4OCl$	Benzodiazepine
7-Aminoclonazepam	$C_{15}H_{12}N_3OCl$	Benzodiazepine
Diazapam	$C_{16}H_{13}N_2OCl$	Benzodiazepine
Oxazepam	$C_{15}H_{11}N_2O_2Cl$	Benzodiazepine
Temazepam	$C_{16}H_{13}N_2O_2Cl$	Benzodiazepine
7-Aminoflunitrazepam	$C_{16}H_{14}N_3OF$	Benzodiazepine
7-Aminonitrazepam	$C_{15}H_{13}N_3O$	Benzodiazepine
dl-11-nor-9-carboxy-δ-9-THC	$C_{21}H_{28}O_4$	Cannabinoid
Codeine	$C_{18}H_{21}NO_3$	Opiate
Morphine 3β-d-glucuronide	$C_{23}H_{27}NO_9$	Opiate
6-acetylmorphine	$C_{19}H_{21}NO_4$	Opiate
EDDP perchlorate	$C_{20}H_{24}NO_4Cl$	Opiate
(+)-ephedrine	$C_{10}H_{15}NO$	Stimulant
Fenfluramine	$C_{12}H_{16}NF_3$	Stimulant
dl-MBDB:HCL	$C_{12}H_{18}NO_2Cl$	Stimulant
(±) BDB Hydrochloride	$C_{11}H_{16}NO_2Cl$	Stimulant
dl-MDEA	$C_{12}H_{17}NO_2$	Stimulant
dl-MDA	$C_{10}H_{13}NO_2$	Stimulant
dl-MDMA	$C_{11}H_{15}NO_2$	Stimulant
dl-Methamphetamine	$C_{10}H_{15}N$	Stimulant
dl-Amphetamine	$C_9H_{13}N$	Stimulant
Phentermine	$C_{10}H_{15}N$	Stimulant
(+)-Psuedoephedrine	$C_{10}H_{15}NO$	Stimulant
(–)-Cotinine	$C_{10}H_{12}N_2O$	Other
4'-Hydroxynordiazepam	$C_{15}H_{11}ClN_2O_2$	Benzodiazepine
Nordiazepam	$C_{15}H_{11}N_2OCl$	Benzodiazepine
Flunitrazepam	$C_{16}H_{12}N_3O_3F$	Benzodiazepine
Flurazepam	$C_{21}H_{23}N_3OClF$	Benzodiazepine
Desalkylflurazepam	$C_{15}H_{10}N_2OClF$	Benzodiazepine
(–)-δ-9-THC	$C_{21}H_{30}O_2$	Cannabinoid
(±)-11-hydroxy-δ-9-THC	$C_{21}H_{30}O_3$	Cannabinoid
Cocaine	$C_{17}H_{21}NO_4$	Cocaine
Benzoylecgonine	$C_{16}H_{19}NO_4$	Cocaine
Buprenorphine	$C_{29}H_{41}NO_4$	Opiate
Morphine	$C_{17}H_{19}NO_3$	Opiate
Normorphine	$C_{16}H_{17}NO_3$	Opiate
Meperidine	$C_{15}H_{21}NO_2$	Opiate
Normeperidine	$C_{14}H_{19}NO_2$	Opiate
dl-Methadone	$C_{21}H_{27}NO$	Opiate
EMPD	$C_{19}H_{21}N$	Opiate
Naloxone	$C_{19}H_{21}NO_4$	Opiate
Oxycodone	$C_{18}H_{21}NO_4$	Opiate
LSD	$C_{20}H_{25}N_3O$	Hallucinogen
Iso-LSD	$C_{20}H_{25}N_3O$	Hallucinogen
(±)-phenylpropanolamine:HCL	$C_9H_{13}NO$:HCl	Stimulant
Fluoxetine:HCL	$C_{17}H_{18}F_3NO$:HCl	Prosac
GHB	$C_4H_7O_3Na$	Other
(–)-Nicotine	$C_{10}H_{14}N_2$	Other

Methodology

The work undertaken in this study was performed on an Agilent 1100 system consisting of:

Binary pump
Standard auto-sampler
Thermostated column compartment
Diode Array Detector (DAD)
G1969 LC/MSD TOF.

Instrument Conditions

Pump

Time (min)	% Water (0.1% formic acid)	% Methanol (0.1% formic acid)	Flow rate (mL/min)
0	90	10	0.4
4	90	10	0.4
22	0	100	0.4
29	0	100	0.4
29.6	90	10	0.4
30	90	10	0.4

Post time:	5 minutes
Total run time:	35 minutes
Injection volume:	10 µL, with needle wash
Column temperature:	30 °C
Column:	ZORBAX SB-AQ, 150 mm × 2.1 mm × 3.5 µm

MS Detection

Ionization	**ESI Positive**
Gas temp	350 °C
Drying gas	10 L/min
Nebulizer pressure	40 psig
Capillary V (+ve)	3500 V

MS Conditions

Scan *m/z* range	100–1000
Fragmentor	125 V
Storage mode	Profile
Skimmer	60 V
Oct RF	200 V

Reference Mass Introduction with LC-TOF

The Agilent TOF MS uses a reference mass in the generation of reliable high level accurate mass. The electro-spray source for the TOF is a unique dual spray assembly that allows the simultaneous constant introduction of a reference mass component.

The reference mix 1 used in these experiments consists of 2 mL of purine and 0.8 mL of HP-0921. This mixture was prepared in 1 L of 90:10 methanol:water to better represent the mobile phase.

The control software enables the use of the following reference masses:

Positive Ion Detection
121.050873
922.009798

Analysis of Drugs of Abuse by LC-TOF MS

An overwhelming advantage of using TOF MS for the trace level detection of any component is the confirmatory information that is provided through accurate mass. An example of this mass accuracy is shown in Table 3, where the observed masses for each component are detected, and their deviation from the theoretical masses for the adduct are shown.

The ability to closely match the expected mass and the observed mass provides the analyst with a higher level of confidence in the assignment given to a chromatographic peak. In the screening for components such as drugs, which may have a significant impact on the life of a person, this additional confidence is of great importance. This capability also allows the possibility of using this technique as a screening tool for a wide range of components.

Table 3. Theoretical Accurate Mass, Observed Mass and Mass Error

Compound	Monoisotopic mass	Retention time	Adduct	Observed mass	Adduct accurate mass	Mass error (ppm)
α-Hydroxyalprazolam	324.0778	17.76	$[M+H]^+$	325.0852	325.0850	0.41
7-Aminoclonazepam	285.0669	13.32	$[M+H]^+$	286.0739	286.0741	–0.93
Diazapam	284.0716	19.15	$[M+H]^+$	285.0796	285.0789	2.39
Oxazepam	286.0509	17.4	$[M+H]^+$	287.0579	287.0581	–0.98
Temazepam	300.0666	18.2	$[M+H]^+$	301.0741	301.0738	0.89
7-Aminoflunitrazepam	283.1121	15.3	$[M+H]^+$	284.1191	284.1093	–0.94
7-Aminonitrazepam	251.1059	8.82	$[M+H]^+$	252.1134	251.1131	1.04
dl-11-nor-9-carboxy-δ-9-THC	344.1988	21.38	$[M+H]^+$	345.2061	345.2060	0.18
Codeine	299.1521	5.5	$[M+H]^+$	300.1592	300.1594	–0.73
Morphine 3 β-d-glucuronide	461.1686	1.7	$[M+H]^+$	462.1764	462.1758	1.17
6-Acetylmorphine	327.1471	8.9	$[M+H]^+$	328.1542	328.1543	–0.41
EDDP perchlorate	377.1394	15.42	$[M-O_4Cl]^+$	278.1909	278.1903	2.06
(+)-Ephedrine	165.1154	2.46	$[M+H]^+$	166.1225	166.1226	–0.85
Fenfluramine	231.1235	12.9	$[M+H]^+$	232.1303	232.1307	–1.98
dl-MBDB:HCL	243.1026	10.63	$[M-Cl]^+$	208.1337	208.1332	2.37
(±) BDB hydrochloride	229.087	9.5	$[M-Cl]^+$	194.1181	194.1175	2.81
dl-MDEA	207.1259	9.6	$[M+H]^+$	208.1332	208.1332	–0.03
dl-MDA	179.0946	4.9	$[M+H]^+$	180.1019	180.1191	–0.03
dl-MDMA	193.1103	6.4	$[M+H]^+$	194.1174	194.1175	–0.08
dl-Methamphetamine	149.1204	3.85	$[M+H]^+$	150.1281	150.1277	2.49
dl-Amphetamine	135.1048	3.05	$[M+H]^+$	136.1125	136.112	3.11
Phentermine	149.1204	5.34	$[M+H]^+$	150.1278	150.1277	0.49
(+)-Psuedoephedrine	165.1154	2.76	$[M+H]^+$	166.1231	166.1226	2.76
(–)-Cotinine	176.095	2.56	$[M+H]^+$	177.1023	177.1022	0.34
4'-Hydroxynordiazepam	286.0509	14.23	$[M+H]^+$	287.0582	287.0581	0.06
Nordiazepam	270.056	18.1	$[M+H]^+$	271.0634	271.0632	0.49
Flunitrazepam	313.0863	18.1	$[M+H]^+$	314.0924	314.0935	–3.65
Flurazepam	387.1514	15.23	$[M+H]^+$	388.1591	388.1586	1.17
Desalkylflurazepam	288.0466	18.2	$[M+H]^+$	289.0535	289.0538	–1.19
(–)-δ-9-THC	314.2246	22.31	$[M+H]^+$	315.2328	315.2318	2.99
(±)-11-hydroxy-δ-9-THC	330.2195	21.07	$[M+H]^+$	331.2267	331.2267	–0.22
Cocaine	303.1471	12.6	$[M+H]^+$	304.1545	304.1543	0.54
Benzoylecgonine	289.1314	12.1	$[M+H]^+$	290.1386	290.1386	–0.29
Buprenorphine	467.3036	16.11	$[M+H]^+$	468.3107	468.3108	–0.29
Morphine	285.1365	2.2	$[M+H]^+$	286.1438	286.1437	–0.1
Normorphine	271.1208	2	$[M+H]^+$	272.1286	272.1281	1.7
Meperidine	247.1572	12.4	$[M+H]^+$	248.1652	248.1645	2.8
Normeperidine	233.1416	12.5	$[M+H]^+$	234.1493	234.1488	1.9
dl-Methadone	309.2093	16.72	$[M+H]^+$	310.2166	310.2165	0.19
EMPD	263.1674	16.4	$[M+H]^+$	264.1754	264.1746	2.7
Naloxone	327.1471	5.74	$[M+H]^+$	328.1541	328.1543	–0.72
Oxycodone	315.1471	7.2	$[M+H]^+$	316.1547	316.1543	1.15
LSD	323.1998	14.7	$[M+H]^+$	324.2073	324.207	0.8
Iso-LSD	323.1998	14.55	$[M+H]^+$	324.2078	324.207	2.34
(±)-Phenylpropanolamine:HCL	187.0764	1.95	$[M-Cl]^+$	152.1069	152.1069	–0.59
Fluoxetine:HCL	345.1107	16.3	$[M-Cl]^+$	310.1412	310.1413	1.85
GHB	126.0293	1.72	$[M+H]^+$	127.0369	127.0365	2.63
(–)-Nicotine	162.1157	1.6	$[M+H]^+$	163.1233	163.1229	1.99
2-oxo-3-hydroxy-LSD	355.1896	n.d.			Not detected in +veESI	

A greater than two-fold increase in sensitivity for many components is seen with the narrowing of the mass-extraction window. Figure 1 shows the reduction in noise that is observed with the extraction of a smaller mass range for flunitrazepam, commonly known as Rohypnol, a date-rape drug. The ability of TOF-MS to accurately determine the presence of components such as Rohypnol at low levels may assist with investigations into reported abuse of the illicit substance, and prove to be a critical factor in confirmation when dealing with complex matrices.

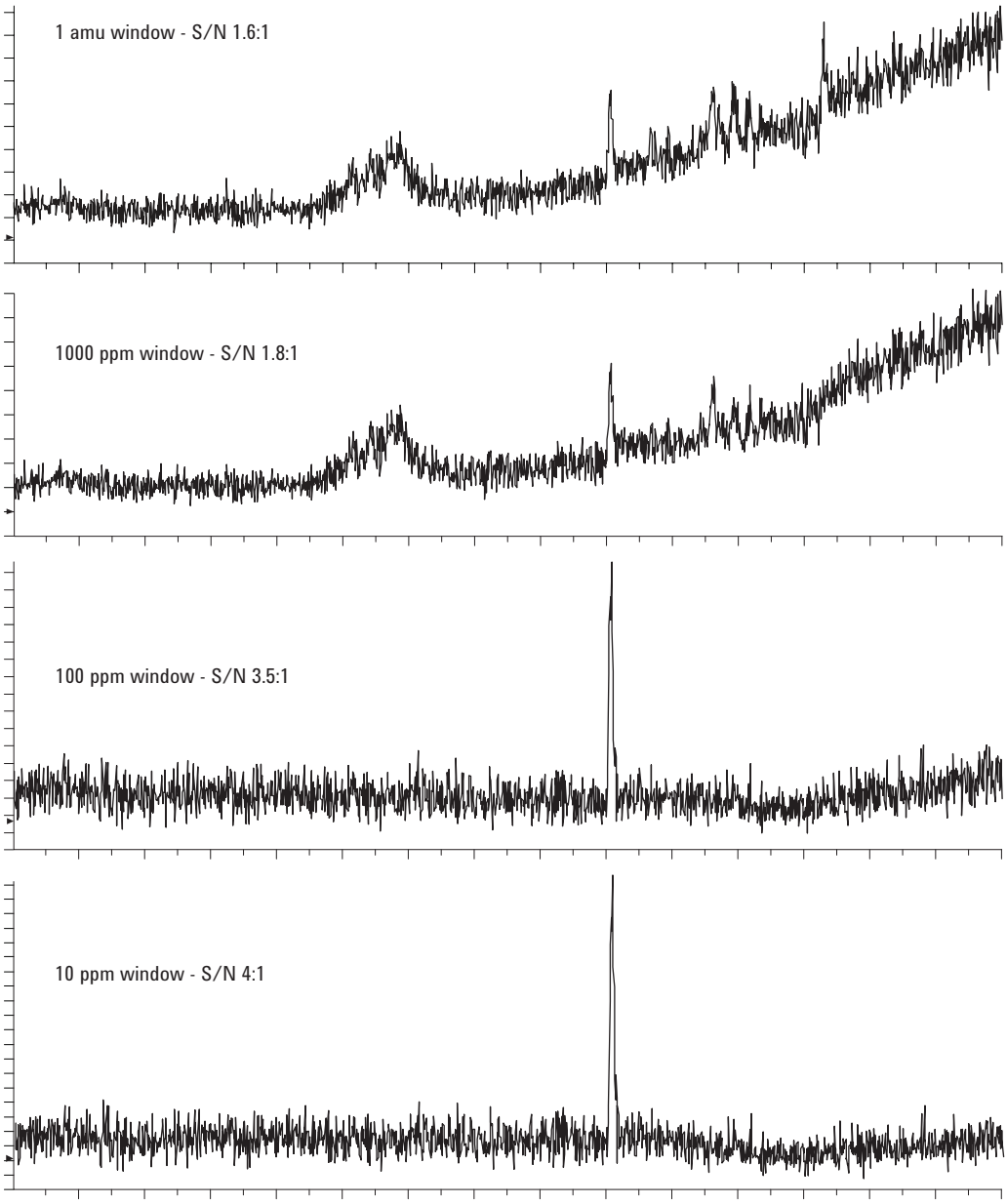


Figure 1. Effect of extracted ion range on noise – 1 ng/mL flunitrazepam.

TOF Linearity

TOF-MS has traditionally been considered as unsuitable for quantitation due to the use of time-to-digital conversion of data. The Agilent TOF MS uses analog-to-digital conversion, allowing for far better quantitative data than the alternative technology of time-to-digital conversion. Several of the components analyzed by TOF were tested for linearity as part of this study. Figures 2–5 show the linearity of four selected components, most displaying linearity over three orders of magnitude from 1 ng/mL to 1000 ng/mL. However, some components, such as δ-9-THC and fluoxetine, only exhibit a narrower linear range, a result of their ionization behavior (Figures 6 and 7). Nevertheless, regression values of over 0.999 were seen for each of these components.

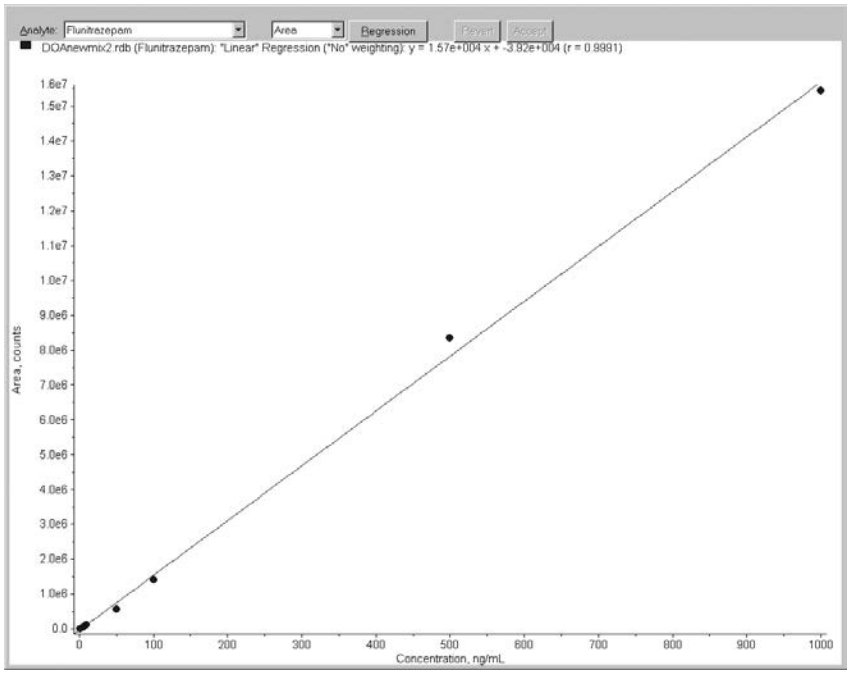


Figure 2. Calibration curve for flunitrazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

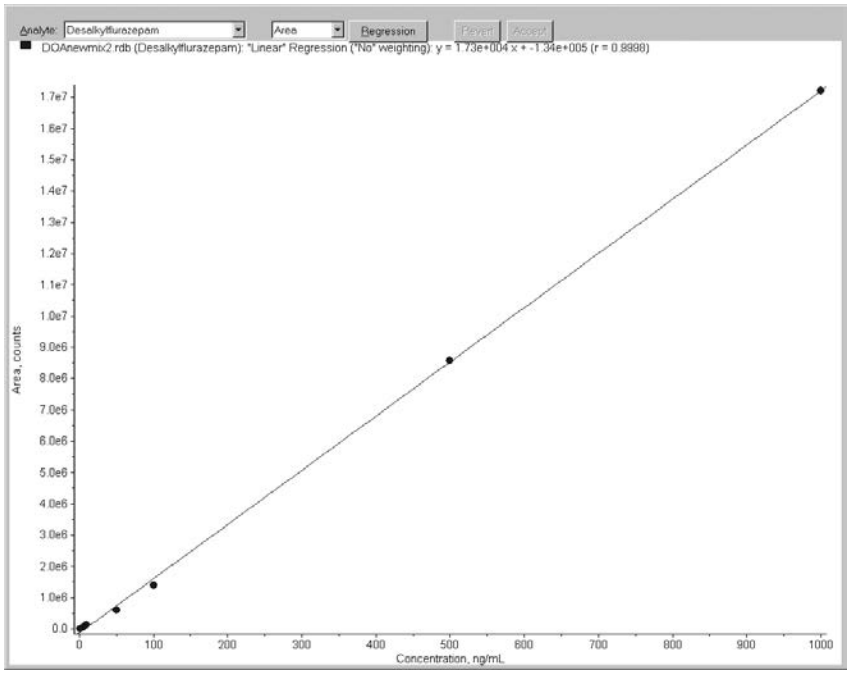


Figure 3. Calibration curve for desalkylflurazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

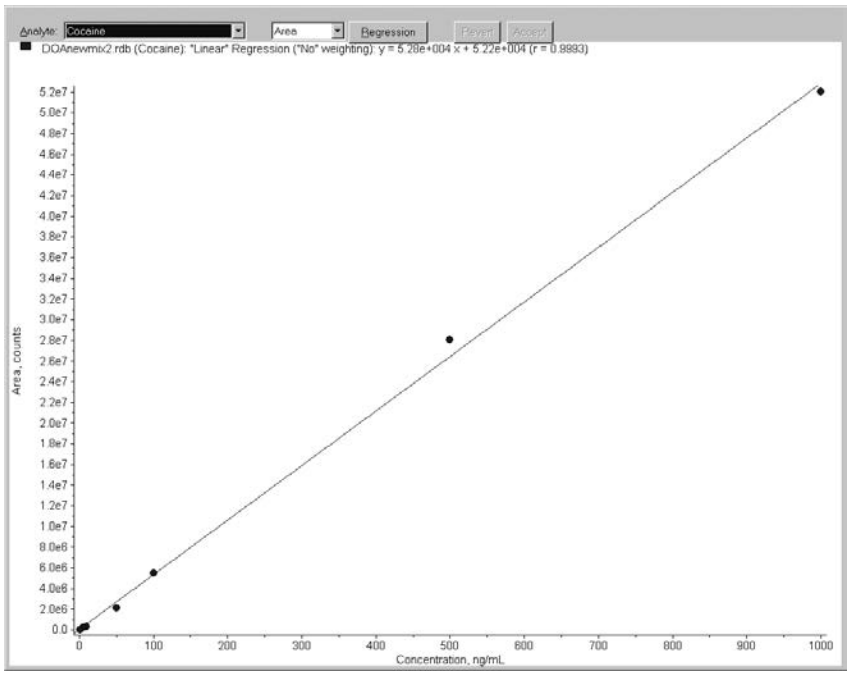


Figure 4. Calibration curve for cocaine from 1 ng/mL to 1000 ng/mL with TOF-MS.

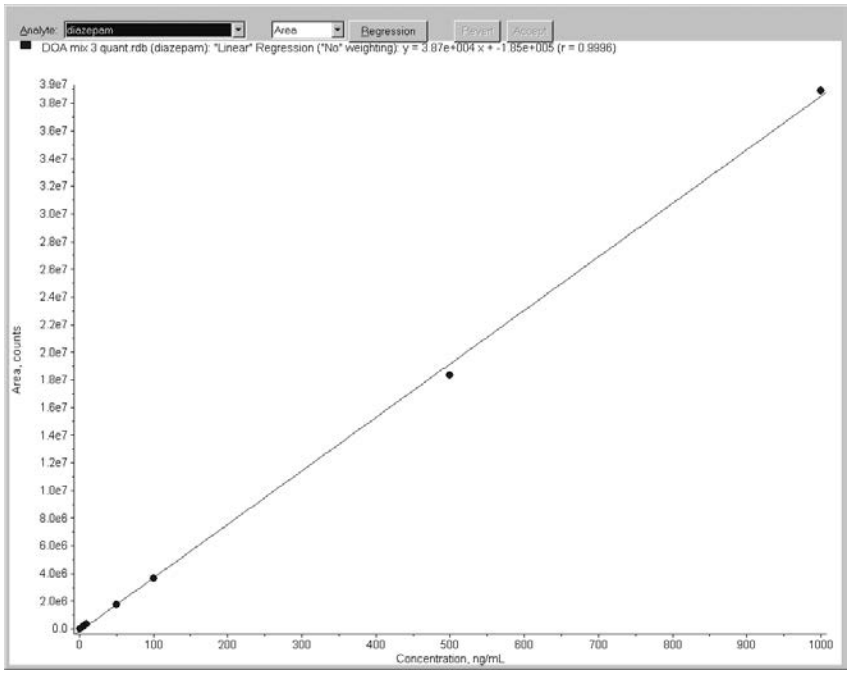


Figure 5. Calibration curve for diazepam from 1 ng/mL to 1000 ng/mL with TOF-MS.

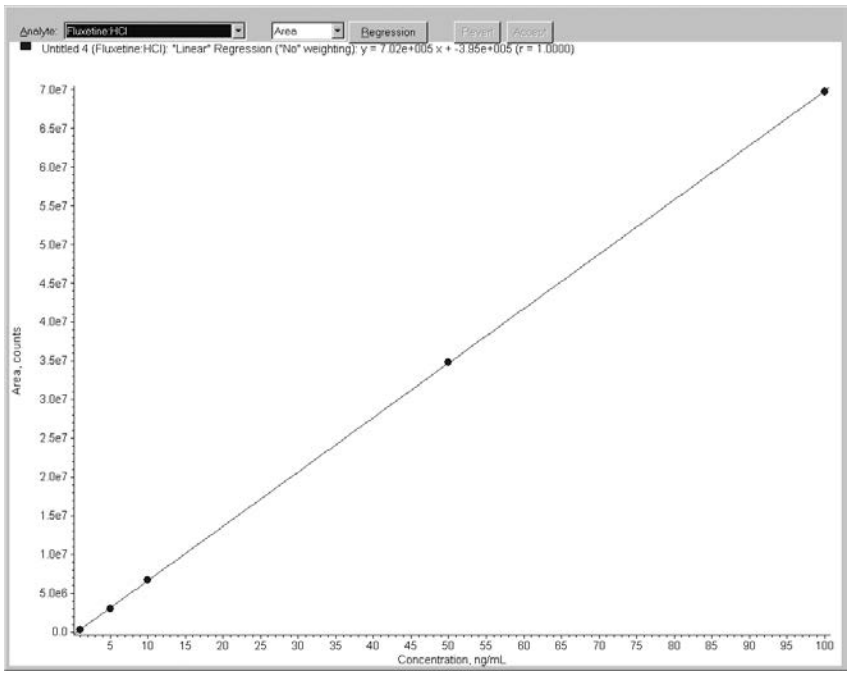


Figure 6. Calibration curve for (–)-δ-9-THC from 1 ng/mL to 100 ng/mL with TOF-MS.

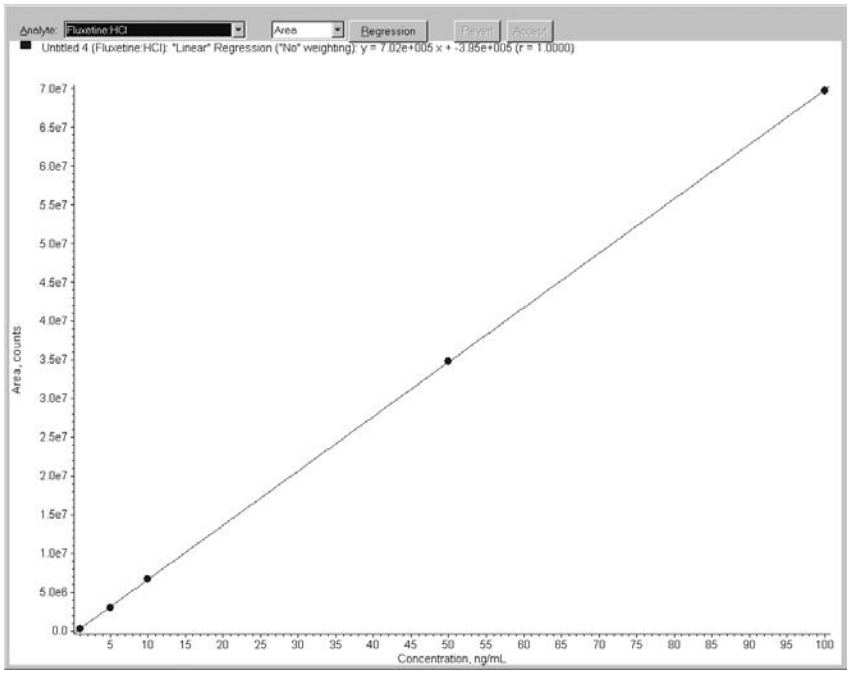
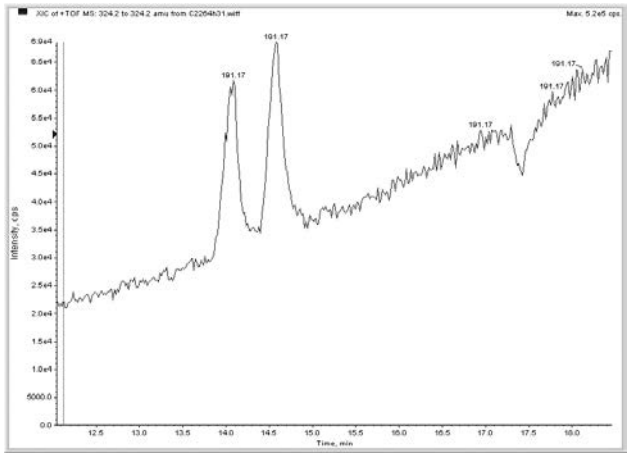
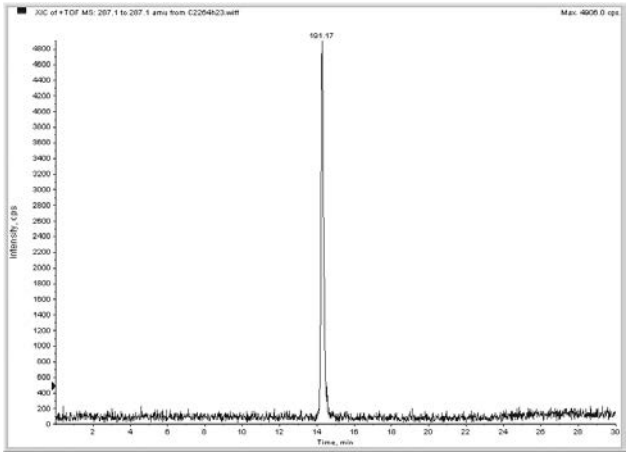


Figure 7. Calibration curve for fluoxetine from 1 ng/mL to 100 ng/mL with TOF-MS.

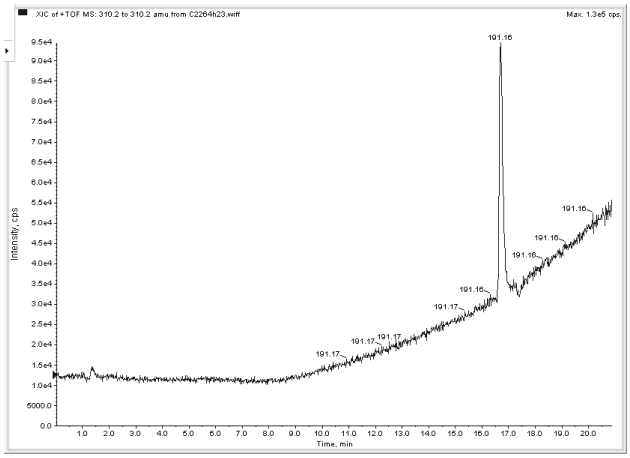
Chromatogram examples for four components at 1 ng/mL are shown below in Figure 8 with 10 ppm extraction windows.



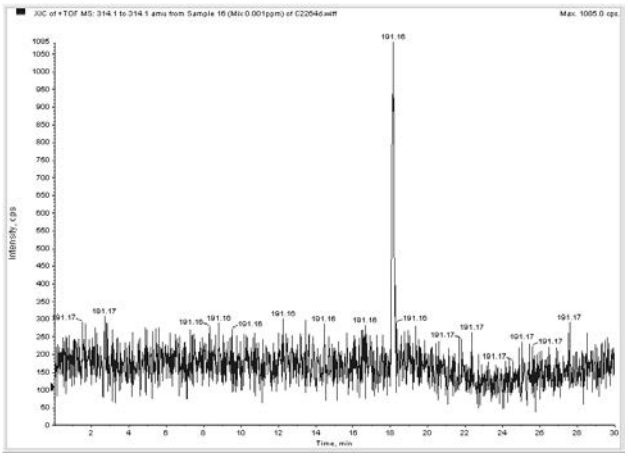
1 ng/mL iso-LSD and LSD (respectively)



1 ng/mL 4′-hydroxynordiazepam



1 ng/mL dl methadone



1 ng/mL flunitrazepam (Rohypnol)

Figure 8. Ten ppm extraction window of 1 ng/mL solutions of four drugs of abuse.

Table 4 summarizes the limits of detection (LOD) for each of the components analyzed under this method. Note that while the method has not been optimized for any one component, it is designed to provide a broad screening tool for the analysis of drugs of abuse.

Table 4. LOD for Components by LC/MSD TOF

Component	Accurate mass	LOD (ng/mL)
α-Hydroxyalprazolam	325.085	5
7-Aminoclonazepam	286.0741	1
Diazapam	285.0789	1
Oxazepam	287.0581	5
Temazepam	301.0738	5
7-Aminoflunitrazepam	284.1093	5
7-Aminonitrazepam	251.1131	1
dl-11-nor-9-carboxy-δ-9-THC	345.206	5
Codeine	300.1594	50
Morphine 3β-d-glucuronide	462.1758	5
6-Acetylmorphine	328.1543	10
EDDP perchlorate	278.1903	1
(+)-Ephedrine	166.1226	20
Fenfluramine	232.1307	1
dl-MBDB:HCL	208.1332	5
(±) BDB Hydrochloride	194.1175	10
dl-MDEA	208.1332	10
dl-MDA	180.1191	50
dl-MDMA	194.1175	50
dl-Methamphetamine	150.1277	20
dl-Amphetamine	136.112	50
Phentermine	150.1277	20
(+)-Psuedoephedrine	166.1226	20
(–)-Cotinine	177.1022	20
4'-Hydroxynordiazepam	287.0581	0.5
Nordiazepam	271.0632	0.5
Flunitrazepam	314.0935	1
Flurazepam	388.1586	1
Desalkylflurazepam	289.0538	1
(–)-δ-9-THC	315.2318	1
(±)-11-Hydroxy-δ-9-THC	331.2267	1
Cocaine	304.1543	1
Benzoylecgonine	290.1386	5
Buprenorphine	468.3108	1
Morphine	286.1437	10
Normorphine	272.1281	10
Meperidine	248.1645	1
Normeperidine	234.1488	1
dl-Methadone	310.2165	1
EMPD	264.1746	1
Naloxone	328.1543	5
Oxycodone	316.1543	20
LSD	324.207	1
Iso-LSD	324.207	1
(±)-Phenylpropanolamine:HCL	152.1069	1
Fluoxetine:HCL	310.1413	1
GHB	127.0365	20
(–)-Nicotine	163.1229	5
2-oxo-3-hydroxy-LSD	Not detected in +veESI	ND

Detection of Drugs of Abuse in Bodily Fluids

Urine is the matrix of choice for the detection of drugs of abuse in areas such as workplace screening, and was therefore chosen to evaluate the LC/TOF MS method developed. To present a "worst case scenario", neat urine was spiked for this analysis and run directly with no sample clean-up. This would not normally be done; however, it was used as an illustration of the method's ability to provide a quick screening result without cleanup.

A further discussion of a solid phase extraction (SPE) sample preparation method that may be considered can be found in Agilent Technologies application note 5989-2260EN [4].

In the first instance, neat and spiked urine was scanned for both cocaine and benzoylegonine, a metabolite of cocaine (Figures 9 and 10). Recoveries of the spiked samples (100 ng/mL – representing a level lower than the traditional EMIT screen) were both approximately 100%.

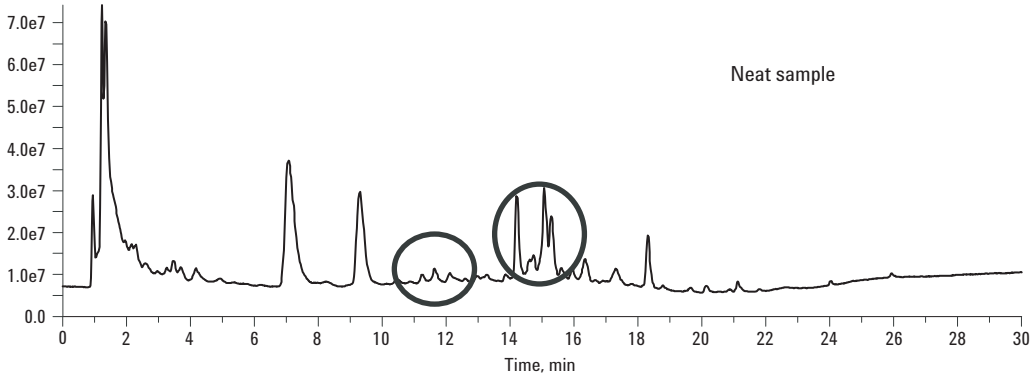


Figure 9. TIC of Blank Urine – Injected neat – circle shows expected retention time of analytes of interest.

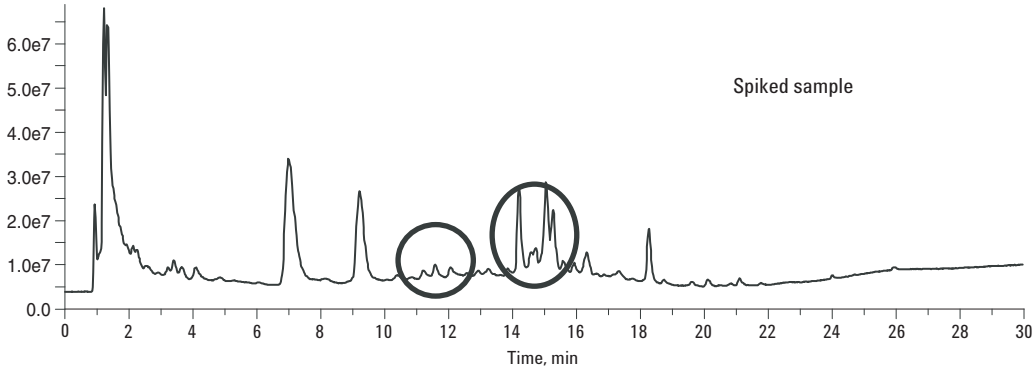


Figure 10. TIC of Neat Urine with 100 ng/mL spike of cocaine, benzoylecognine, flunitrazepam, and 7-aminoflunitrazepam.

It can be seen from Figures 11 and 12 that the ability to narrow the mass extraction window greatly reduces the noise for a given mass, and with retention time information can provide a high level of confidence in the assignment of a component.

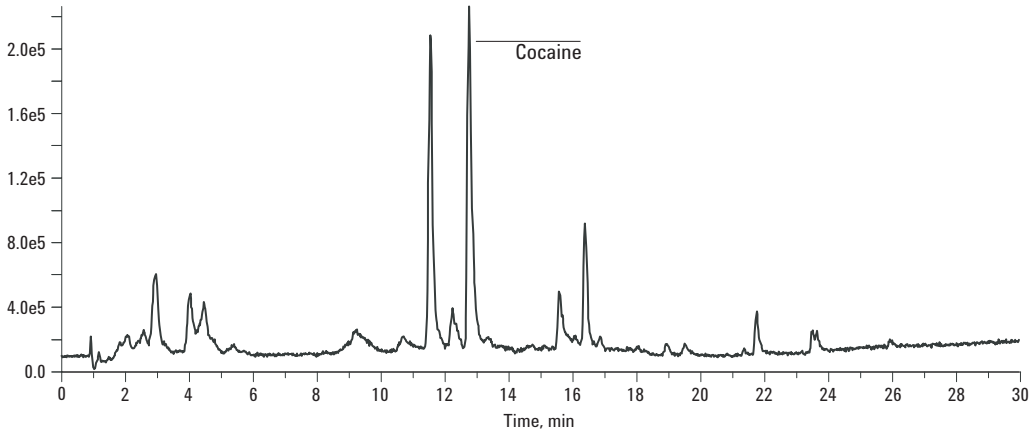


Figure 11. One amu extraction window of scanned target mass 304.1543 – cocaine.

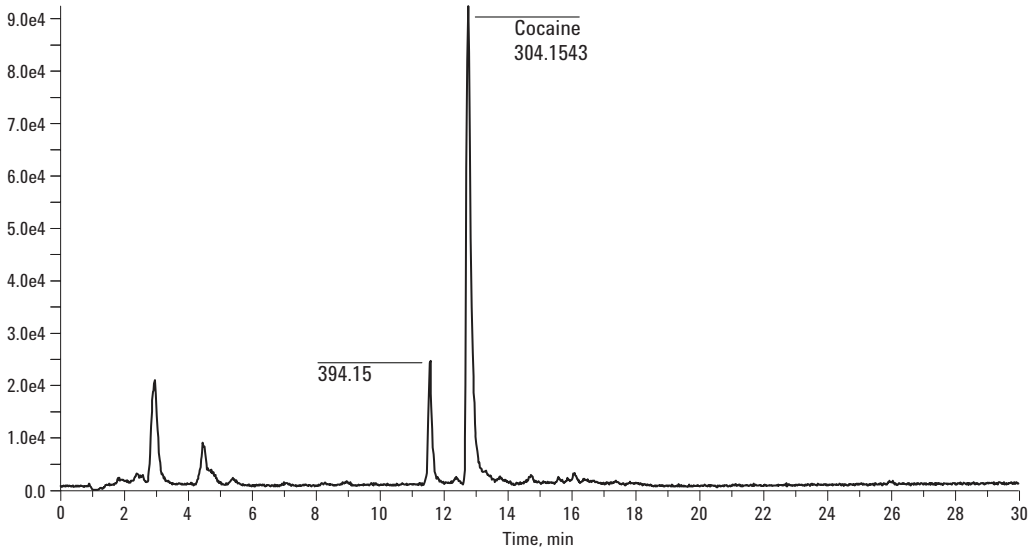


Figure 12. Ten ppm extraction window of scanned target mass 304.1543 – cocaine.

In the instance with Figure 12, the larger peak at approximately 12.9 minutes shows an excellent match with cocaine (mass error of ~0.2 ppm), while the earlier peak at 11.5 minutes has a mass of 394.15 and a fragmentation product in the extraction window (Figures 13 and 14).

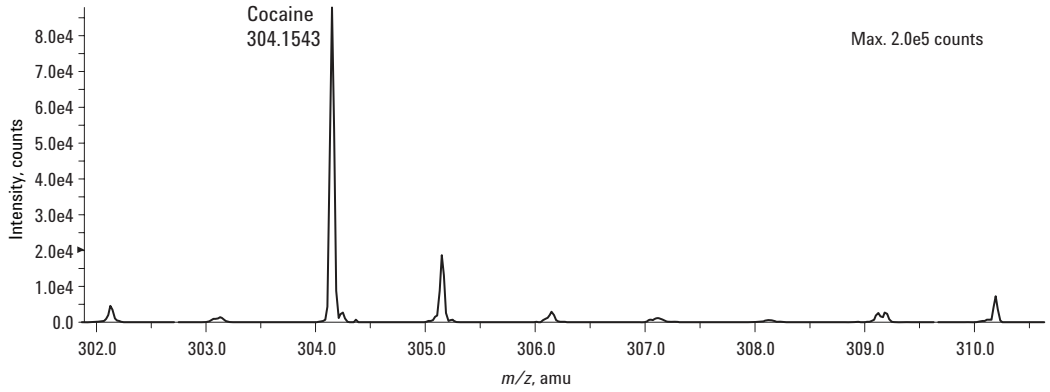


Figure 13. Confirmation of mass of cocaine.

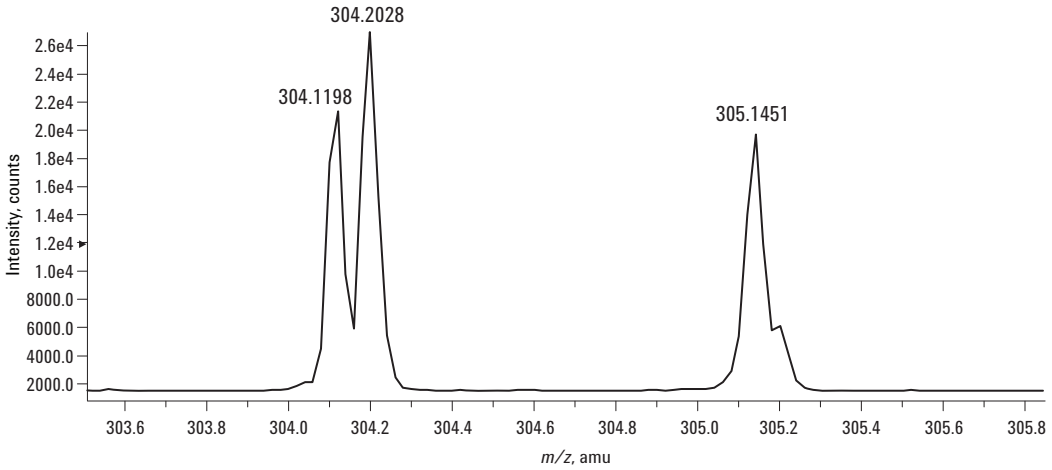


Figure 14. Mass spectrum 304 – 305 amu for peak at 11.9 minutes – no match of mass for cocaine.

A second drug that was of particular interest was flunitrazepam, another substance used as a date rape drug (Rohypnol), and one of its metabolites, 7-aminoflunitrazepam. Again, these components were spiked at 100 ng/mL into neat urine and injected directly. The recoveries achieved for these two components were approximately 40%, which is likely a result of ion suppression in the source. However, due to the excellent detection limits possible, even with up to 60% suppression of the signal, a clear peak can be seen for both compounds at 100 ng/mL (a level well below the EMIT screening reporting limits) when extracting a narrow mass window (Figures 15 and 16).

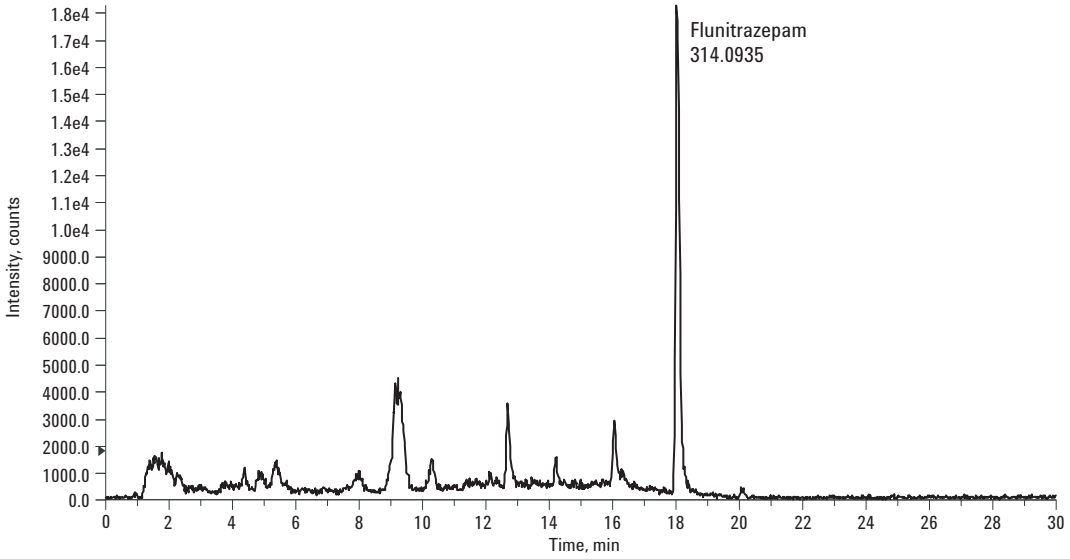


Figure 15. Ten ppm extraction window of scanned target mass 314.0935, flunitrazepam in neat urine. Retention time helps confirm presence at 18.1 minutes.

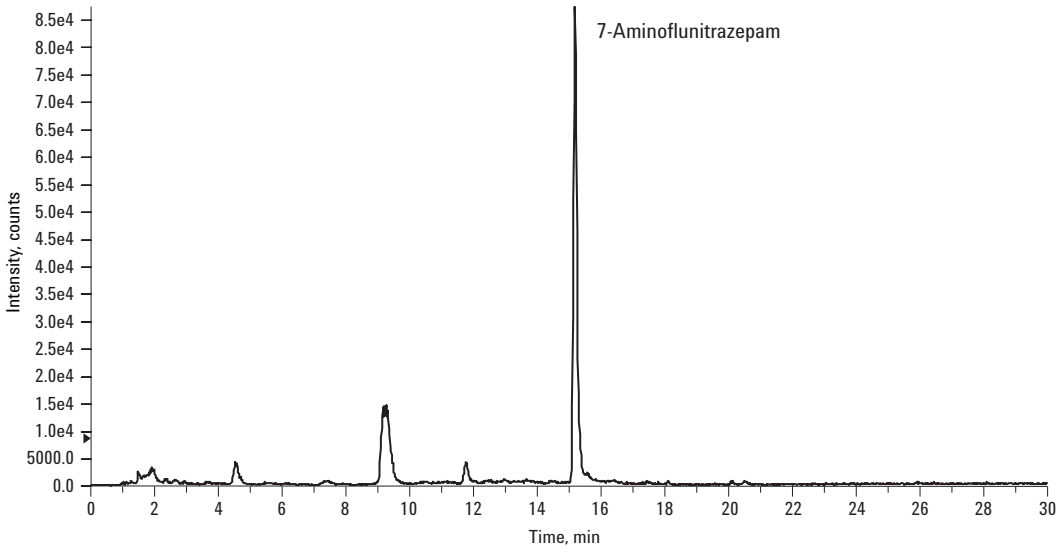


Figure 16. Ten ppm extraction window of scanned target mass 284.1191, 7-aminoflunitrazepam in neat urine. Retention time helps confirm presence at 15.3 minutes.

The second common matrix encountered in the screening of drugs of abuse is blood and plasma. To test the method when analyzing plasma, a sample was spiked with desalkylflurazepam. Sample preparation was again kept to a minimum, with a simple acetonitrile precipitation performed on the sample prior to injection. The effect of the mass extraction window on the detection of peaks, seen in Figures 17 to 20, shows the removal of interferences from the spiked plasma sample.

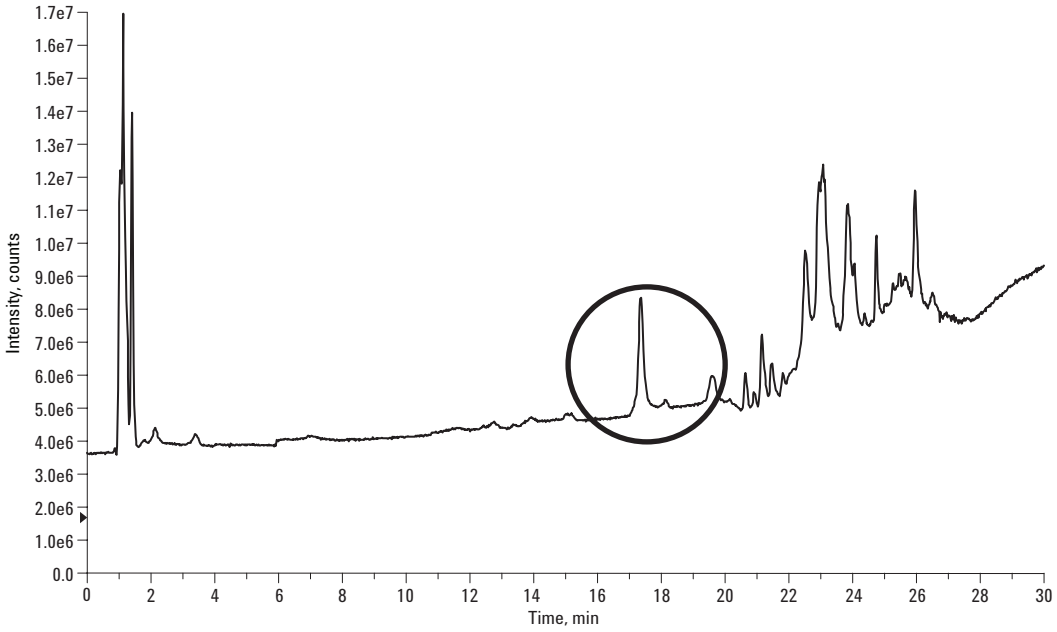


Figure 17. TIC of unspiked plasma.

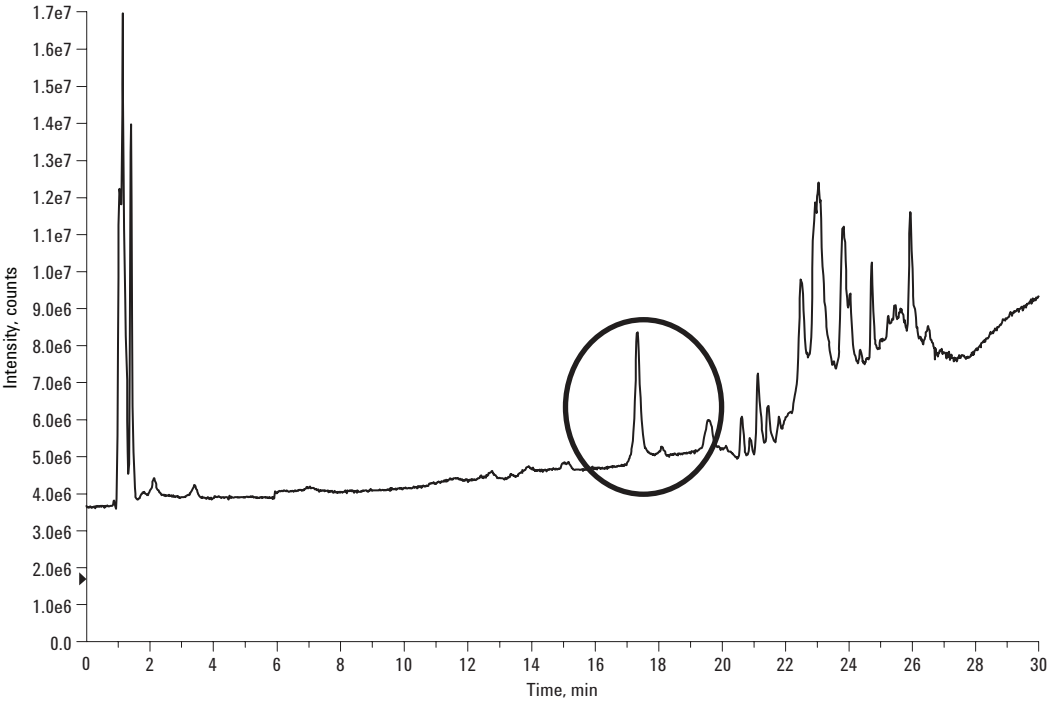


Figure 18. TIC of plasma spiked with 200 ng/mL of desalkylflurazepam.

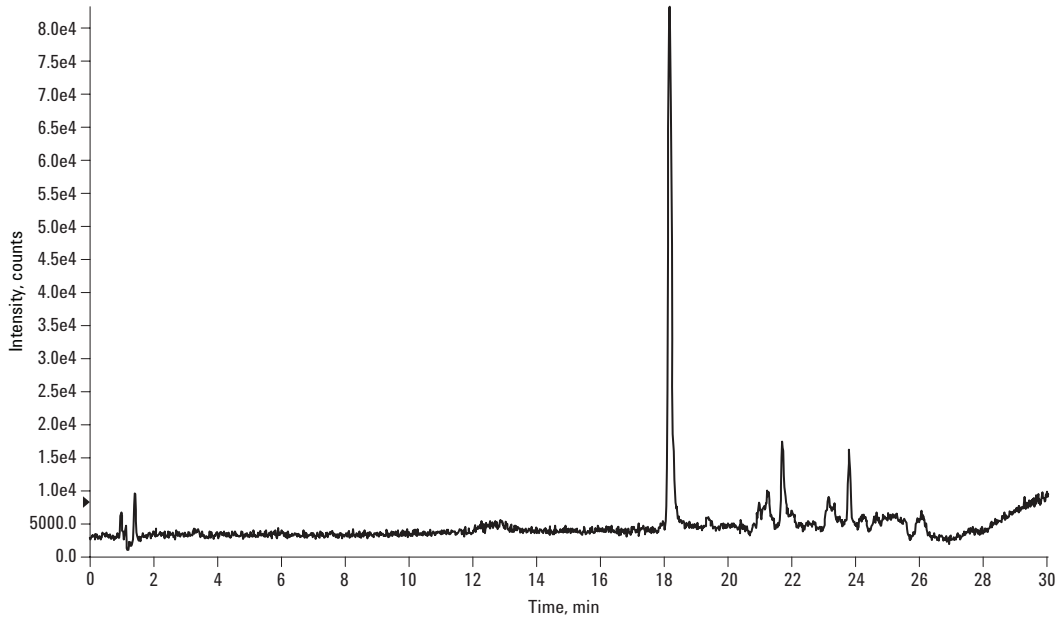


Figure 19. Mass extraction window (0.1 amu) of spiked plasma sample (346 ppm mass window).

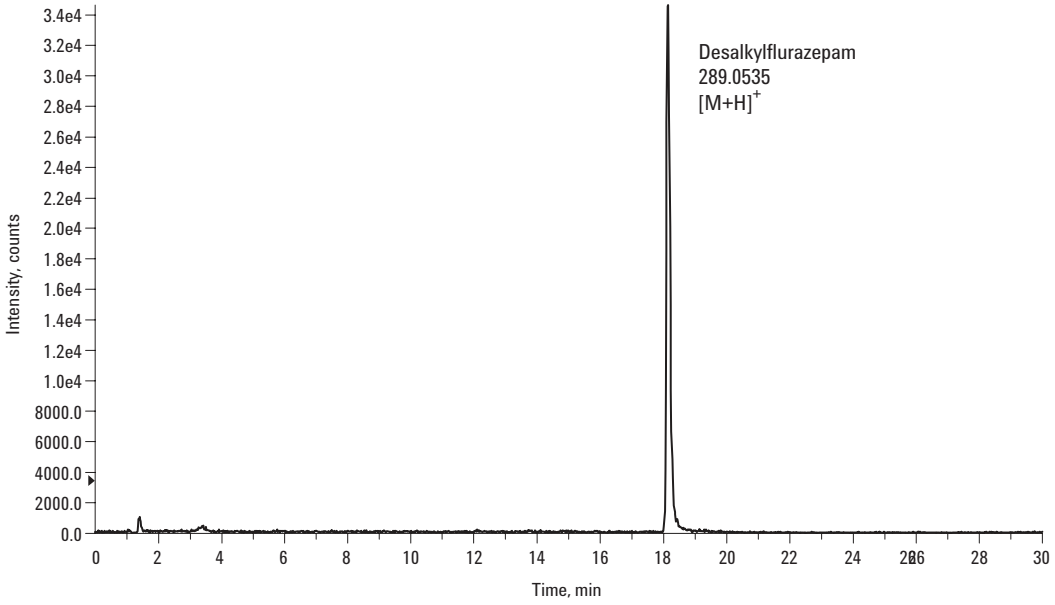


Figure 20. Ten ppm (0.003 amu) mass extraction window of spike plasma – note minimal noise.

Analysis of Coronial Samples – Using a Screener Database

The instrumentation and software provides the user the ability to create a screener database for all components they wish to automatically screen for. The minimum requirement for this database is the empirical formula and name for the component of interest, although the inclusion of a retention time will assist with confidence in the confirmation and reduce analysis time.

Several samples were acquired from the local coronial office to test the procedure that was developed. These samples were provided as butyl chloride extracts of blood samples obtained from deceased persons, for screening using the developed method.

The coronial samples supplied were screened using a database created from the 48 components analyzed under this method.

Sample Preparation

Samples were obtained from 1-mL blood volumes, liquid-liquid extracted with 6–8 mL of butyl chloride following centrifugation. Organic layer evaporated to dryness and then reconstituted in 100-µL mobile phase for a final 10-fold concentration.

Sample 1

Sample 1 was known to contain amphetamine, codeine, diazepam, and nordiazepam from the previous analysis performed at the coronial office. In addition to the four previously reported components, the screen also indicated the presence of

- Nicotine
- Cotinine
- Acetylmorphine
- Ephedrine
- Methamphetamine
- Pseudoephedrine

The total ion chromatogram (TIC) for this sample is shown in Figure 21.

An excerpt of the screen report is shown in Figure 22 with the details for cotinine. For each component included in the compound database, the screening report displays the extracted ion chromatogram, spectra of detected peak, and enlarged spectra of the target mass. This is accompanied by a summary table with the mass and retention time error. In this instance, an excellent match is seen for both retention time and mass.

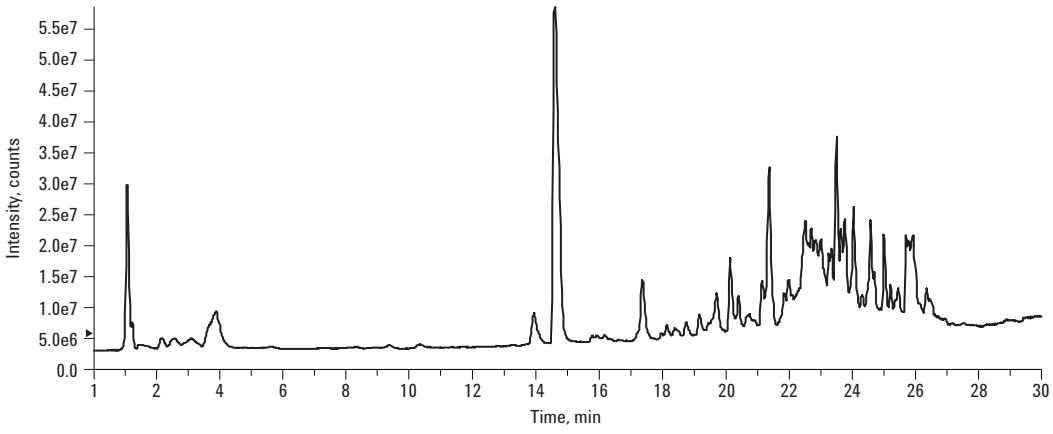
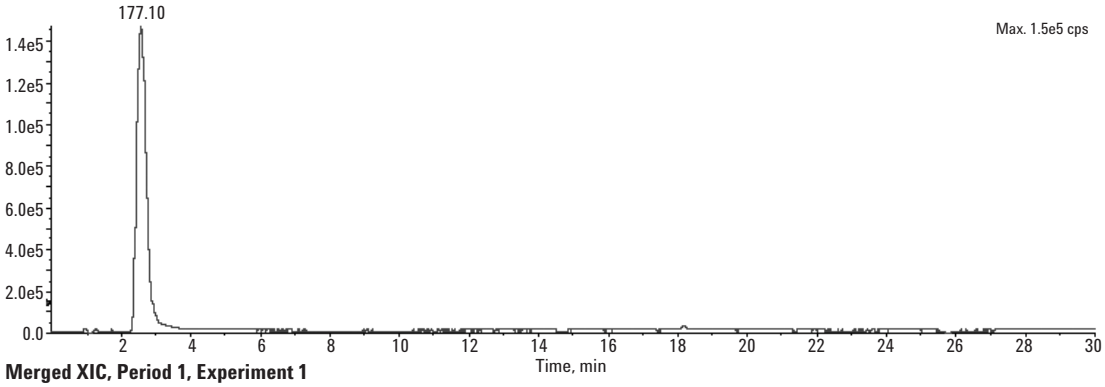
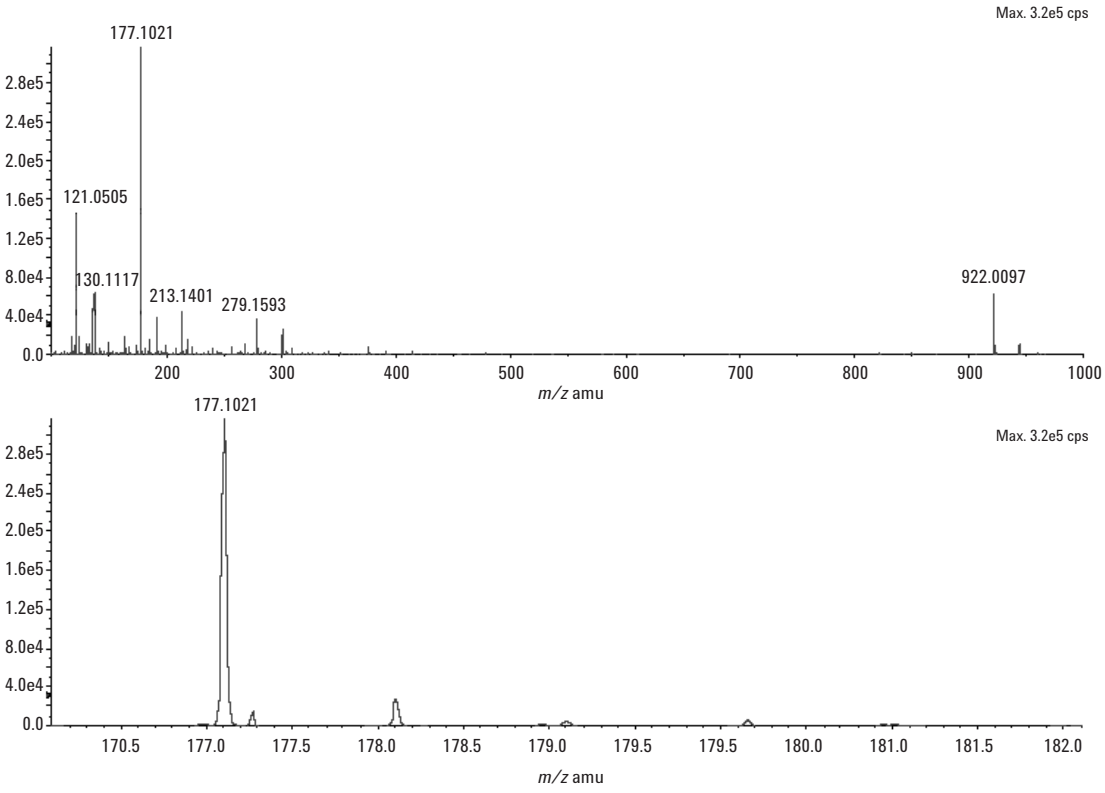


Figure 21. TIC of Coronial Sample 1.



Merged XIC, Period 1, Experiment 1



Formula	Compound name	Mass	Peak RT (min)	Peak area	Description	
$C_{10}H_{12}N_2O$	(-)-colinine	176.09496	2.57	2.78259 E7	_	
Species	**Abundance (counts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**	**Ret. time error (min)**
$[M + H]^+$	316885.14	177.10224	177.10215	-0.00009	-0.51	0.01

Figure 22. Excerpt from screener report of coronial Sample 1 showing confirmation of the presence of cotinine.

The results obtained from the analysis of Sample 1 suggest the deceased was a smoker, with the presence of both the nicotine and cotinine in the sample.

Sample 2

Sample 2 was known to contain citralopram, codeine, doxylamine, and tramadol from the previous analysis at the coronial office. In addition to the four previously reported compounds, screening with the database further showed the presence of

- Diazepam
- 6-acetylmorphine
- MDMA
- Methampethamine
- Cotinine
- Meperidine
- nicotine

A TIC of the sample is shown in Figure 23. It could again be inferred that this patient was a smoker.

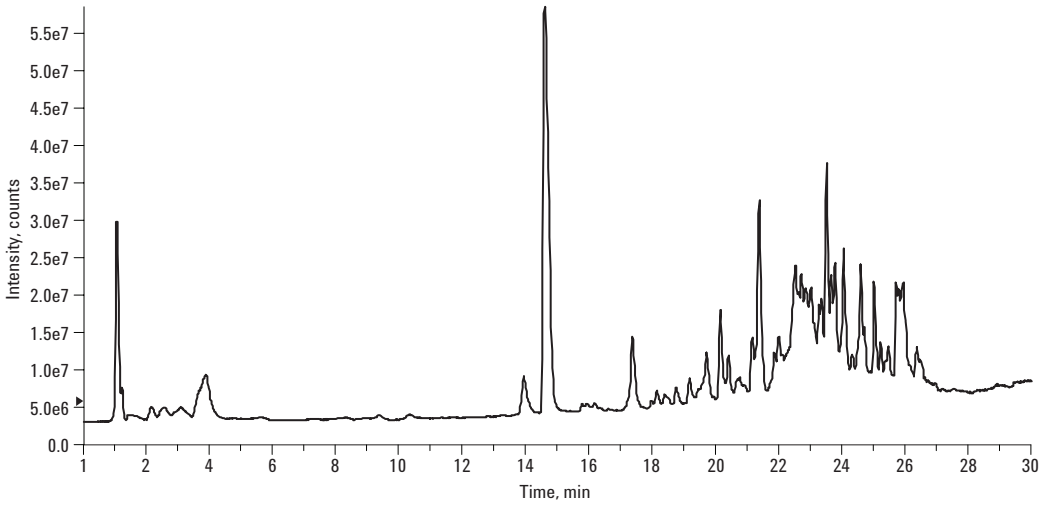
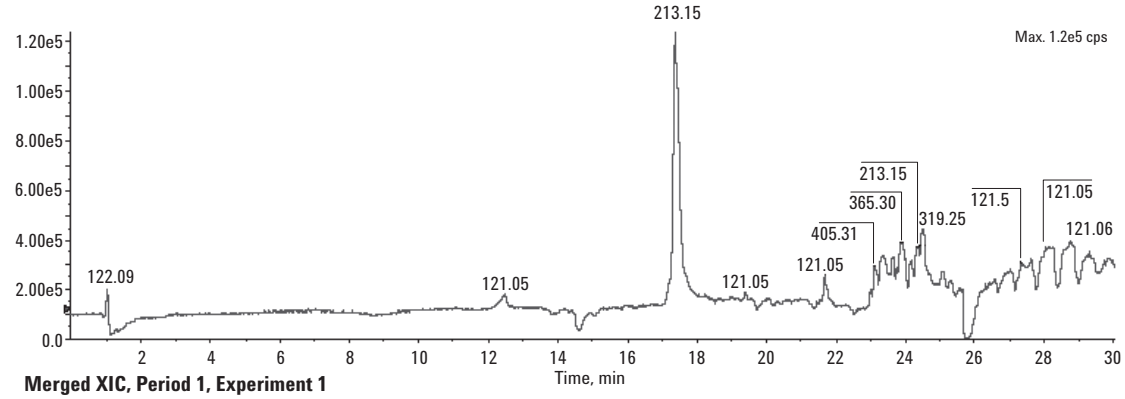
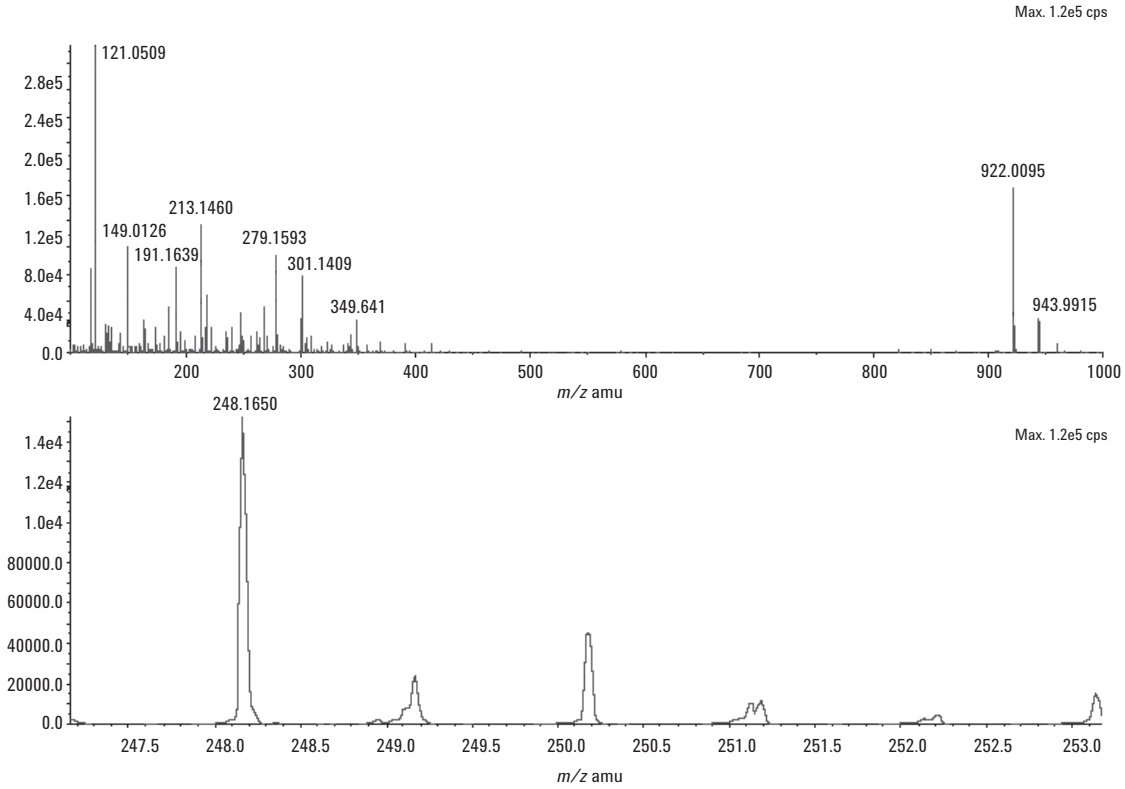


Figure 23. TIC of coronial Sample 2.

An excerpt of the screener report, in this instance for the confirmation of meperidine, is shown in Figure 24. Again, an excellent match to both retention time and mass can be seen.





Formula	Compound name	Mass	Peak RT (min)	Peak area	Description	
$C_{15}H_{21}NO_2$	Meperidine	247.15723	12.47	4.20401 E5	_	
Species	**Abundance (counts)**	**Ion mass**	**Measured mass**	**Error (mDa)**	**Error (ppm)**	**Ret. time error (min)**
$[M + H]^+$	15313.85	248.16451	248.16498	0.00047	1.90	0.07

Figure 24. Excerpt from screener report indicating presence of meperidine.

Conclusions

The analysis of drugs of abuse is important in many different areas of our society, from law enforcement to medical monitoring. Current analytical techniques use a two-step screening and confirmation procedure to achieve the required specificity and sensitivity required. This application note has investigated 48 of the more common drugs of abuse and their applicability for determination through LC-TOF MS. It is not intended to be a comprehensive study of all possible components, but provides an excellent launching pad for the inclusion of the full gamut of possibilities

This application note shows the potential of the Agilent LC-TOF-MS as a single tool for both screening and confirmatory analysis, with quantitative information, often at levels below those currently analyzed for today. As a final example of the power of this technique, real-life coronial samples were evaluated under a screening protocol, with an additional seven components other than those previously reported by the coronial office detected.

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Agilent Technologies

High Resolution Quantitative Analysis of ß$_2$-Agonist in Human Urine by LC/MS Using the Agilent LC/MSD TOF

Application

Forensics

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Abstract

A liquid chromatography/mass spectrometry (LC/MS) method was developed for the sensitive qualitative and quantitative analysis of clenbuterol, mabuterol, bambuterol, and ractopamin to demonstrate the utility of an orthogonal-axis electrospray time-of-flight mass spectrometer (oa-ESI-TOF) for doping analysis. The limit of quantitation for these compounds was 2 ng/mL in human urine, well below control levels. In addition, the ability to measure exact mass, to accuracies better than 3 ppm at all levels and in sample matrix was demonstrated.

Introduction

Beta$_2$-agonists such as clenbuterol, mabuterol, bambuterol, and other sympathomimetic agents are commonly used for the treatment of pulmonary diseases such as bronchial asthma. In 1992, sympathomimetics were added to the list of prohibited substances by the World Anti-Doping Agency (IOC). They are classified as stimulants and anabolic agents with specific regulations in terms of competition and out-of-competition testing.

A LC/MS method was developed for the sensitive, qualitative and quantitative analysis of clenbuterol, mabuterol, bambuterol, and ractopamine to demonstrate the utility of an oa-ESI-TOF mass spectrometer for doping analysis.

Experimental

All LC/MS experiments were performed using an Agilent LC/MSD TOF mass spectrometer coupled to an Agilent 1100 Series LC system. The TOF was operated with an orthogonal electrospray source in positive ion mode. A gradient method was used for chromatography. Clenpenterol was used as an internal standard (ISTD) for all analytes. Method conditions are given in Table 1.

Table 1. Experimental Conditions

HPLC	**Agilent 1100**
Column	ZORBAX XDB-C18, 50 mm × 2.1 mm × 3.5 µm
Solvents	A: Water + 5 mM of NH_4OAc + 0.1% of acetic acid B: Acetonitrile + 0.1%-acetic acid
Gradient	5% A, 0–7 min; 80% A, 7–9 min
Flow rate:	0.3 mL/min
Injection:	20 µL out of 1000 µL
Agilent LC/MSD TOF system	
Ionization mode:	Positive ESI
Nebulizer pressure:	45 psi
Drying gas flow:	11 L/min
Drying gas temperature:	350 °C
Full scan mode	100 amu–1000 amu
Automatic reference	Ion 122 and Ion 922
Quantitation	
Bambuterol	EIC of 368.20750 - 368.22959, Ret-Time: 6.3 min
Clenbuterol	EIC of 277.07858 - 277.09520, Ret-Time: 5.8 min
Mabuterol	EIC of 311.10392 - 311.12258, Ret-Time: 6.45 min
Ractopamine	EIC of 302.16600 - 302.18413, Ret-Time: 5.35 min
Clenpenterol (ISTD)	EIC of 291.09381 - 291.11128 + EIC of 293.09080 - 293.10839, Ret-Time: 6.4 min
Calibration curves:	All weighted 1/x

Sample Preparation

Samples were prepared according to the protocol established by the Institute of Biochemistry, German Sport University [1]. Using this protocol, to a volume of 5 mL of urine, 300 µL of 10 M hydrochloric acid and 250 ng of clenpenterol were added; and then the sample was incubated at 80 °C for 45 min. After cooling to ambient temperature, 5 mL of t-butyl methyl ether was added, the mixture was shaken for 15 min, centrifuged at 620*g* for 5 min, and the organic layer was discarded. To the remaining aqueous phase, 0.65 mL of 5M aqueous KOH, 1 mL of t-butanol, 500 mg of a mixture of K_2CO_3 and $NaHCO_3$ (2:1, w/w), 2 g of NaCl, and 5 mL of t-butyl methyl ether were added. The mixture was shaken for 15 min and centrifuged at 620*g* for 10 min. The organic layer was transferred to a fresh tube and evaporated to dryness at 50 °C, under vacuum. The dry residue was dissolved in 1 mL of 0.06 M HCl, transferred to HPLC vials, and 20 µL injected into the LC/MS system.

Chemicals

Hydrochloric acid 32% (for example, Merck, 100319)

t-Butanol

Potassium hydroxide, 85%

Potassium carbonate

Sodium hydrogencarbonate, anhydrous

Sodium chloride, (Merck)

t-Butyl methyl ether, distilled before use, (KMF, St. Augustin, Germany)

Sodium acetate, anhydrous, (Sigma)

Results and Discussion

Several ß$_2$-agonists need to be identified in urine samples for doping control in sports. The structures of the compounds used to demonstrate this methodology are given in Figure 1. A liquid-liquid extraction was used to extract the ß$_2$-agonists from the urine matrix. The TOF mass spectrometer was used for identification with accurate mass measurement and to quantify the analytes under control. This instrument operates only in full-scan mode, allowing detection of any ionizable compound in the mass range. No special method setup was necessary for the mass spectrometer. This allows for the addition of more analytes without changing the acquisition method.

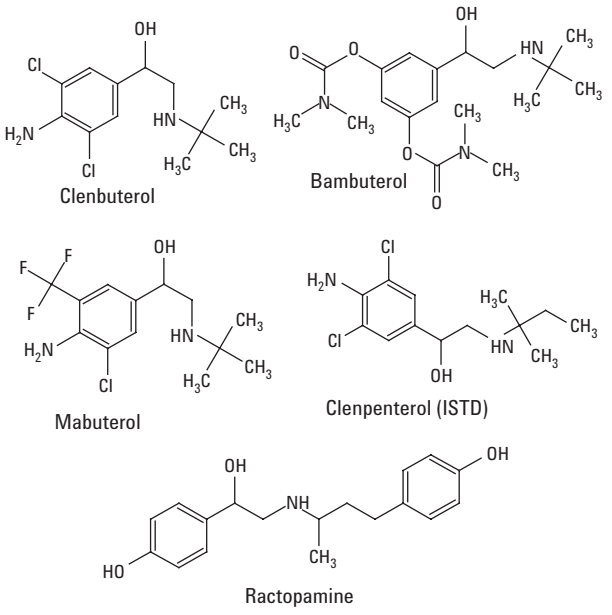

Figure 1. Chemical structures of the investigated ß^2-agonists.

Quantitation was performed by selecting the quasi-molecular ion to create extracted ion chromatograms (EICs) for each analyte and ISTD. Figure 2 displays example EICs from a 2-ng/mL spiked urine sample. Figure 3 shows calibration curves based on spiked urine samples as well as the statistics in Table 2. The IOC list of prohibited substances in sports gives the limit of detection (LOD) for some of the ß$_2$-agonists. Clenbuterol and salbutamol are referred to as anabolic agents. For the latter, a threshold of 1 µg/mL is established. The capability shown here is well below those levels.

Identification was performed by using an automated empirical formula search routine. Added to the runtime work list, this allows for an automated empirical formula calculation, post-acquisition, reported in html, and a comma-separated value spreadsheet for further manipulation. Figure 4 displays a plot showing the calculated mass accuracy for standards as well as spiked urine samples. It shows that the system is able to routinely measure mass accuracy to better than 3 ppm in both standards and sample matrix.

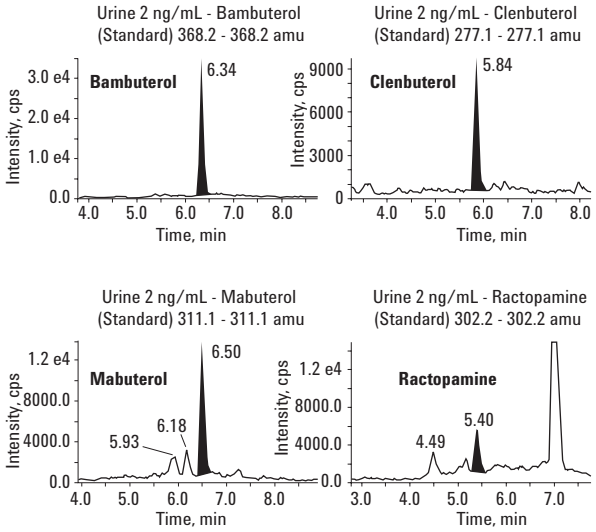

Figure 2. Representative EICs of a spiked urine sample at the 2 ng/mL level.

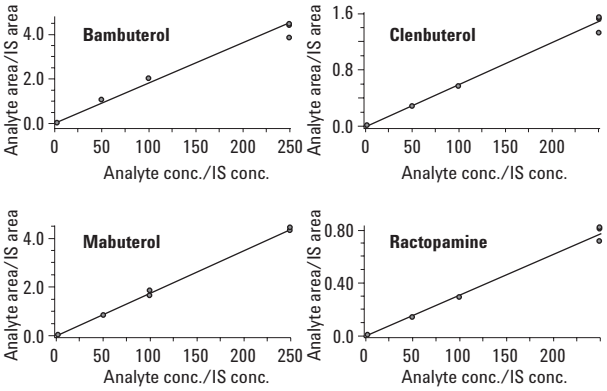

Figure 3. Calibration curves based on spiked urine samples (2 ng/mL-250 ng/mL).

Table 2. Calibration Curve Statistics

Compound	Expected conc. ng/mL	No. of values	Low ng/mL	High ng/mL	Mean ng/mL	Standard Deviation	%CV	%Accuracy
Bambuterol	2	5	1.59	1.67	1.65	0.035	2.104	82.40
	50	5	58.11	58.56	58.36	0.185	0.318	116.72
	100	5	110.50	111.93	111.27	0.640	0.575	111.27
	250	5	211.46	247.25	232.40	17.934	7.717	92.96
Mabuterol	2	5	2.02	2.09	2.06	0.026	1.243	102.90
	50	5	48.72	49.11	48.92	0.188	0.385	97.85
	100	5	95.62	108.17	98.39	4.807	4.886	98.39
	250	5	250.48	256.32	253.69	2.674	1.054	101.47
Clenbuterol	2	5	2.05	2.17	2.09	0.051	2.455	104.50
	50	5	47.65	48.77	48.24	0.422	0.876	96.49
	100	5	96.27	98.05	97.25	0.715	0.736	97.25
	250	5	226.79	263.17	254.41	15.545	6.110	101.77
Ractopamine	2	5	2.14	2.24	2.21	0.040	1.832	110.51
	50	5	44.27	45.78	45.09	0.705	1.563	90.18
	100	5	95.13	96.59	95.71	0.632	0.661	95.71
	250	5	232.60	267.43	258.99	14.825	5.724	103.60

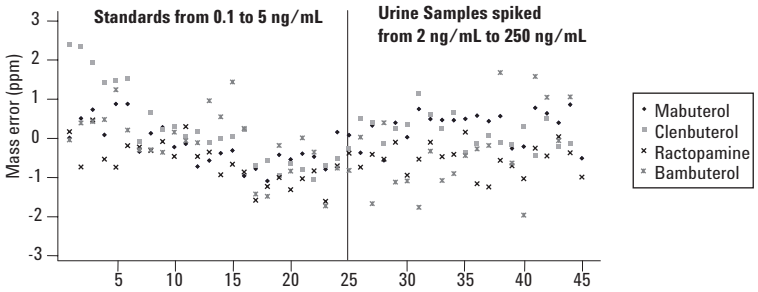

Figure 4. Mass Accuracy on an 18-hour sequence for the investigated ß$_2$-agonists.

Conclusion

A LC/MS method was developed for the qualitative and quantitative measurement of ß$_2$-agonists in human urine using the Agilent LC/MSD TOF system. The LOQ for bambuterol, clenbuterol, mabuterol and ractopamine was 2 ng/mL in human urine, well below control levels. In addition, the ability to measure exact mass, to accuracies better than 3 ppm at all levels and in sample matrix was demonstrated

Reference

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DOPING CONTROL

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- Screening of Corticosteroids in Urine by Positive Atmospheric Pressure Chemical Ionization LC/MS/MS
- Quantitative Analysis of Opiates in Urine Using RRHT LC/MS/MS
- 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
- Analysis of (+)-11-Nor-9-Carboxy-Delta-9-THC in Urine by Negative Ion Electrospray LC/MS/MS
- Rapid Screening of Amphetamine Drugs in Urine by Positive Ion Electrospray LC/MS/MS

Applications by Technique

LC/QQQ

Screening of Corticosteroids in Urine by Positive Atmospheric Pressure Chemical Ionization LC/MS/MS

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Introduction

Corticosteroids are a class of components often abused and misused in sport. They are very potent drugs in the treatment of inflammations and asthma [1]. Corticosteroids can have an effect on the nervous system and can improve an athlete's ability to concentrate and perform in endurance and power events [2,3]. Moreover, corticosteroids can alleviate pain in general. To prevent their misuse related to euphoria and pain suppression, the anti-doping governing bodies are restricting the use of corticosteroids. Systemic use of corticosteroids is forbidden in all circumstances. However, when medically necessary, local and intra-articular injections or dermatological preparations are allowed under the approval of a therapeutic use exemption [4].

The samples collected for doping control are mainly urine samples because large sample volumes can be collected in a non-invasive way. Therefore, the abuse of corticosteroids is analyzed using urine samples.

A simple and sensitive LC/MS/MS method for the screening of 17 corticosteroids is described below. The method is able to detect corticosteroids from the doping control urine samples at 20 ng/mL – below the WADA minimum required performance level (MRPL), which is 30 ng/mL.

Instrumentation

- Varian ProStar™ 430 AutoSampler
- Varian ProStar™ 210 Solvent Delivery Modules
- Varian 1200L LC/MS equipped with Atmospheric Pressure Chemical Ionization (APCI) source
- Harvard Syringe Pump model 11

Materials and Reagents

- Standards of corticosteroids, from Sigma-Aldrich, USA
- Methanol, gradient grade for liquid chromatography, from Merck, Germany
- Water supplied by a Simplicity 185 ultrapure water system, from Millipore, Great Britain
- α-glucuronidase from E. Coli K12, from Roche Diagnostics, Germany
- All other chemicals are pro analysis or HPLC grade

Sample Preparation

The samples are prepared by a standard procedure for steroids. A 2 mL urine sample is transferred in a tube. A 40 µL aliquot of a 10 ppm desoximetasone (internal standard) solution, 1 mL phosphate buffer 0.8M pH 7.0 and 25 µL beta-glucuronidase are added, the mixture is vigorously vortexed and kept for 1 hour at 50 °C for enzymatic hydrolysis. (The enzymatic hydrolysis step is needed since the corticosteroids are mainly excreted in a conjugated form with the glucuronic acid). Adding 750 µL of 20% buffer K_2CO_3/ $KHCO_3$ (1:1), which brings the pH around 9, stops the hydrolysis. Next, 5 mL of tertbutylmethylether is added, and the mixture shaken for 15 min. After centrifugation, the organic layer is transferred to another tube and evaporated to dryness. The remaining residue is dissolved in 100 µL mobile phase (20:80, solvent A / solvent B). Then, 10 µL is injected in LC/MS/MS [5].

HPLC Conditions

Column	ChromSep SS 100x2.0 mm with guard column ChromSep OmniSpher 3 C18 (Varian Part No. CP27839)
Solvent A	0.1% acetic acid : 5 mM ammonium acetate in water (v/v)
Solvent B	Methanol

LC Program	Time (min:sec)	%A	%B
	0:00	70	30
	0:30	70	30
	1:00	50	50
	16:00	30	70
	17:00	30	70
	17:06	70	30
	22:00	70	30

Flow	0.25 mL/min
Mixer	250 µL
Injection Volume	10 µL
Injection Solvent	20% solvent A / 80% solvent B

MS Parameters

Ionization Mode	APCI negative
Collision Gas	1.5 mTorr Argon
Housing	50 °C
API Drying Gas	12 psi at 150 °C
API Nebulizing Gas	58 psi at 400 °C
Auxiliary Gas	17 psi
Scan Time	1 - 1.7s
SIM Width	0.7 amu
Corona current	5 µA
Shield	600 V
Capillary	Tuned Values
Detector	1500 V

Scan parameters

No.	RT (min)	Corticosteroid	Capillary (V)	Precursor Ion	Product Ion	CE (V)
1	5.5	Triamcinolone	-30	453.2	345 363	23 12
2	7.3	Prednisone	-30	417.2	327 357	18 8
3	7.6	Cortisone	-25	419.2	329	16
4	8.7	Prednisolone	-20	419.2	329 295	16 36.5
5	8.7	Hydrocortisone	-25	421.2	331	19
6	10.4	Flumethasone	-40	469.2	379 305	19 41
7	10.8	Betamethasone + Dexamethasone	-40	451.2	361 307	19 33
8	11.1	Triamcinolone acetonide	-25	493.2	375 413	14 22
9	11.1	Fludrocortisone acetate	-35	481.2	349 341	25.5 21
10	11.2	Metilprednisolone	-25	433.2	343 309	17.5 37
11	11.3	Bechlomethasone	-55	467.2	377 341	14 21.5
12	11.8	Flunisolide	-55	493.2	375 357	14 21
13	12.0	Fluorometolone	-40	435.2	59 355	12 16
14	12.1	Flurandrenolide	-35	495.2	377 359	14 20
ISTD	12.6	Desoximetasone	-10	435.2	355	16
15	15.5	Fluocinolone acetonide acetate	-55	553.2	375 355	18 24
16	15.6	Budesonide	-50	489.2	357 339	13.5 19
17	17.9	Fluticasone propionate	-50	559.2	413 433	22.5 15.5

The scan method is divided in 3 segments of acquisition:

- 0 - 9.5 min 7 transitions Scan time 1s
- 9.5 - 15 min 17 transitions Scan time 1.7s
- 15 - 20 min 6 transitions Scan time 1s

Results and Discussion

In order to develop the MS parameters, 10 ppm solutions of each corticosteroid were prepared in a 20% buffer A / 80% methanol mixture. The mixture was meant to mimic the mobile phase that would elute with the compound of interest in an actual LC/MS analysis. The 10 ppm solutions were directly injected in the APCI with a syringe pump at a 50 µL/min rate. First, the most appropriate precursor ion was selected from the parent scan, and the capillary voltage was optimized for its highest abundance. Second, the product ions were selected and the collision energies optimized by the MS/MS breakdown automatic procedure.

Two MS/MS product ions, instead of one, are used to monitor each corticosteroid in order to better eliminate the false positives. Only cortisone and hydrocortisone, which are endogenous corticosteroids, are monitored with one ion. Three ions were not used in order not to increase the scan time. The confirmation of the positive sample can be done by a confirmation method specific for the suspected corticosteroid monitoring at least three of its MS/MS transitions.

Particular care was taken to separate the prednisolone from cortisone. The two corticosteroids have the same molecular masses, and cortisone gives an abundant peak on the transition (-) 419.2>329 of prednisolone. The triamcinolone acetonide-flunisolide and fluorometholone-desoximethasone pairs also share their transitions, but are separated by their retention times. The epimeres betamethasone and dexamethasone have similar retention times and mass spectra, and they could not be separated in the LC/MS conditions described.

Figure 1 (page 3) shows the LC/MS analysis of a blank urine sample spiked with 20 ng/mL of each corticosteroid (except cortisone and hydrocortisone, which are endogenous) and 200 ng/mL internal standard. There are no matrix interferences, and the abundances and signal/noise ratios are satisfactory for all compounds of interest even with a standard gain of the detector. In order to increase the reliability of the result, the confirmation of a positive sample can be done with the detector set on high gain.

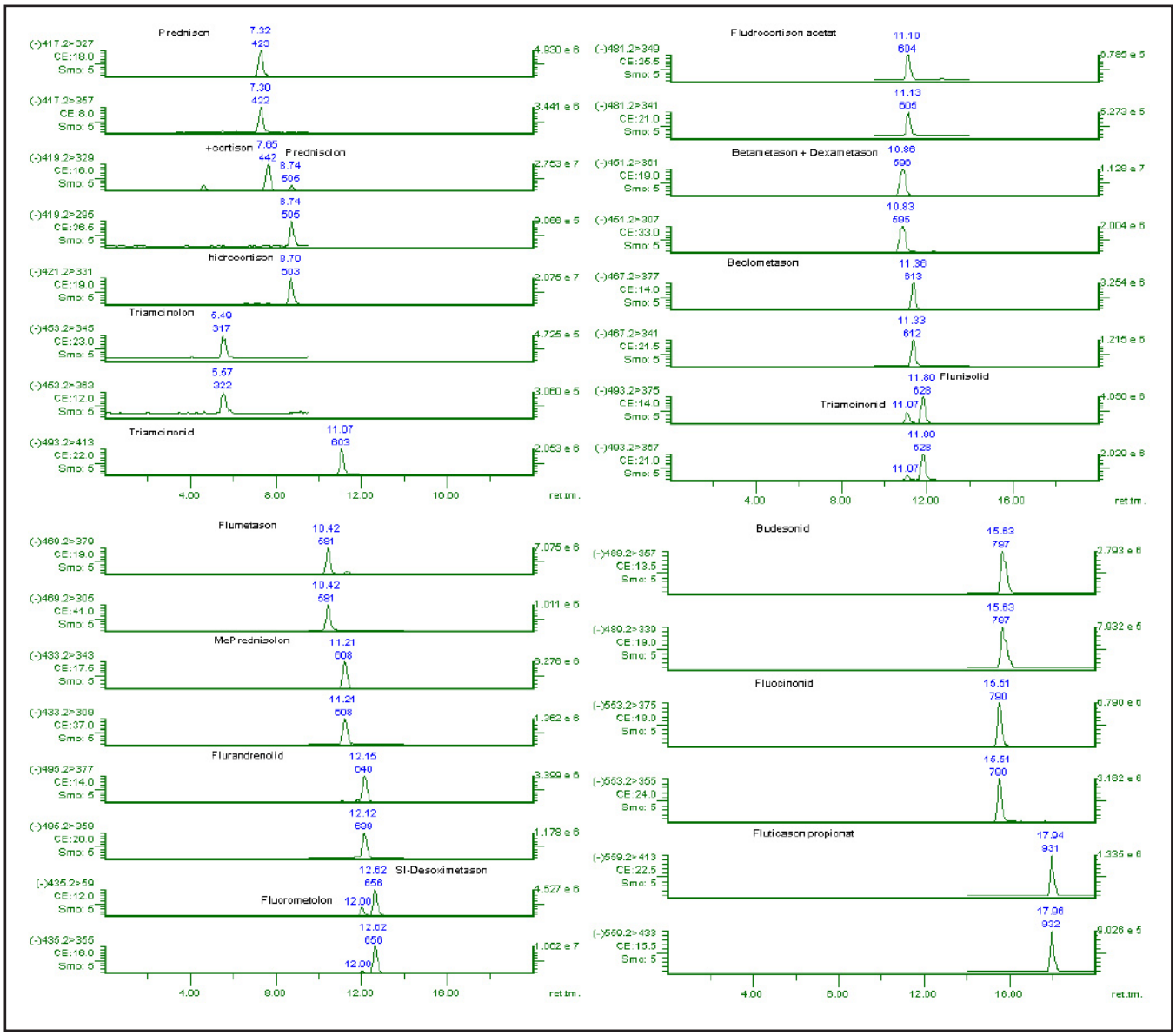

Figure 1. Analysis of a blank urine sample spiked with 20 ng/mL corticosteroids

Conclusion

The LC/MS/MS method described in this application note is simple and sensitive. In one run this method can screen for 17 corticosteroids and easily detect them below the WADA's MRPL, 30 ng/mL. The Varian 1200L system proved to be an essential tool for a doping control laboratory.

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Quantitative Analysis of Opiates in Urine Using RRHT LC / MS / MS

Application

Forensics

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Abstract

An Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) is used to analyze several opiates in urine. A simple isocratic liquid chromatography elution is carried out to detect all seven analytes and their respective internal standards in less than 3.5 minutes using Rapid Resolution High-Throughput liquid chromatography with a ZORBAX C18, 2.1 × 50 mm, 1.8-µm particle size column. Both quantifier and qualifier ions are monitored for each analyte, with the requirement that the qualifier / quantifier ion ratio be within ± 20% for confirming their presence in samples. Except for 6-acetylmorphine (6-MAM), all calibration standards are extracted in matrix and range from 1 to 150 pg / µL in urine. The range for 6-MAM is 0.067 to 10 pg / µL. Following extraction, which corresponds to a factor of 6.78 decrease in concentration, the injected concentrations range from 0.147 to 22.12 pg / µL, or 147 ppt to 22.12 ppb. For 6-MAM, this corresponds to 9.8 ppt to 1.5 ppb. All compounds show very good linearity (R^2 > 0.99).

Introduction

Opiates are drug compounds commonly used for sedation and pain relief and may be obtained both legally as prescription medication or illegally. Their abuse can often lead to addiction. For several reasons, including therapeutic drug monitoring, driving under the influence of drugs, and workplace drug testing, these compounds are commonly analyzed, particularly in urine due to ease of sample availability and volume. For testing in the area of forensics it is often necessary to provide additional confirmation of the presence of these compounds beyond their quantitative values exceeding defined cutoff values.

The triple quadrupole mass spectrometer (QQQ) provides the most sensitive form of quantitation by acquiring the signal corresponding to the highest response product ion (quantifier) from the fragmentation of the analyte precursor ion. This transition is known as multiple reaction monitoring (MRM). However, by acquiring additional signal corresponding to the next highest product ion (qualifier), enough information may be considered available for confirmation, particularly if the ratio of signal between the two product ions is consistent between the calibration standards and the unknown samples. Using the QQQ to acquire MRM signals for both the quantifier and qualifier ions can result in both quantitation and confirmation simultaneously.

The Agilent MassHunter software includes user-definable ion ratio confirmation in the quantitative analysis program as shown in Figure 1. The default tolerance for confirmation is ± 20% of the derived ion ratio, but this may be customized for the particular user. Additionally, up to four different product ions may be used as qualifiers. In this work, the default value of ± 20% is used, along with only one qualifier ion.

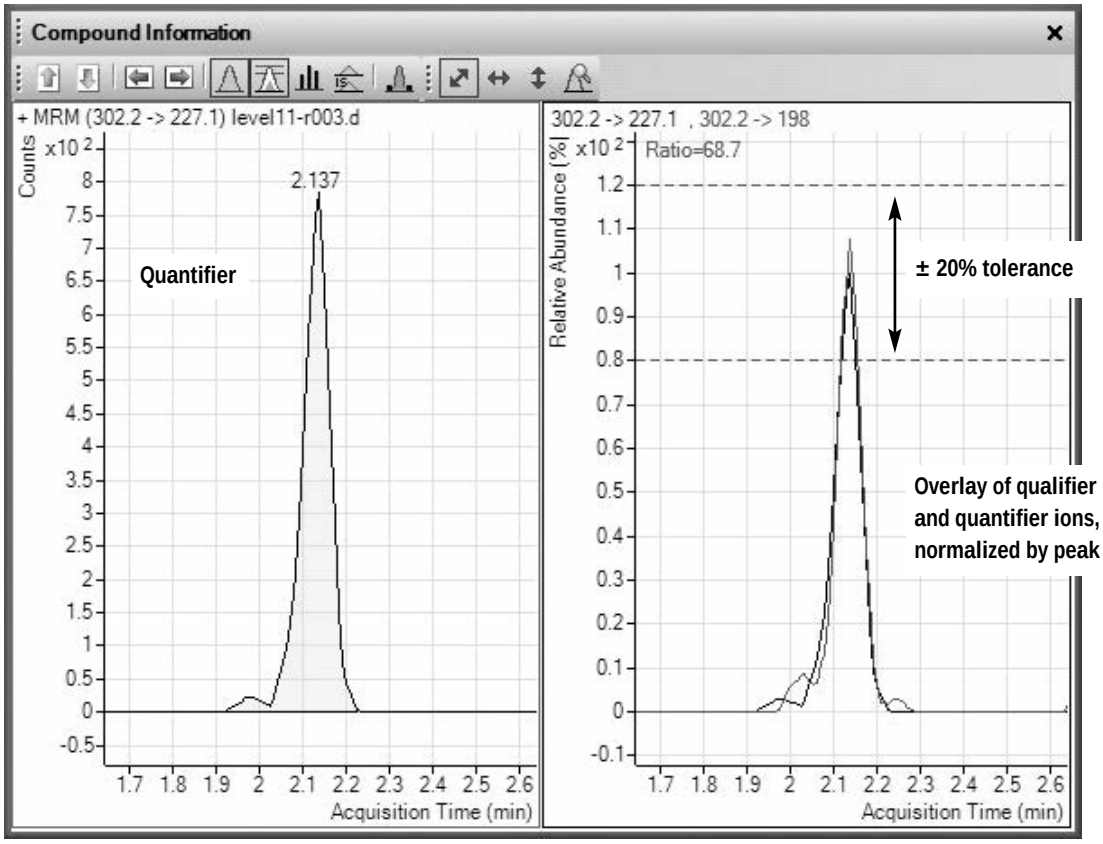


Figure 1. Qualifier / quantifier ion ratios for confirmation of oxymorphone.

Several opiates in urine, including morphine, oxymorphone, hydromorphone, codeine, oxycodone, hydrocodone, and 6-acetylmorphine (6-MAM), a metabolite of heroin, are analyzed in this work. The corresponding structures are shown in Figure 2. A deuterated chemical analog for each compound is included to account for extraction efficiency and matrix interference. A qualifier ion for each internal standard is not necessary and is therefore not analyzed.

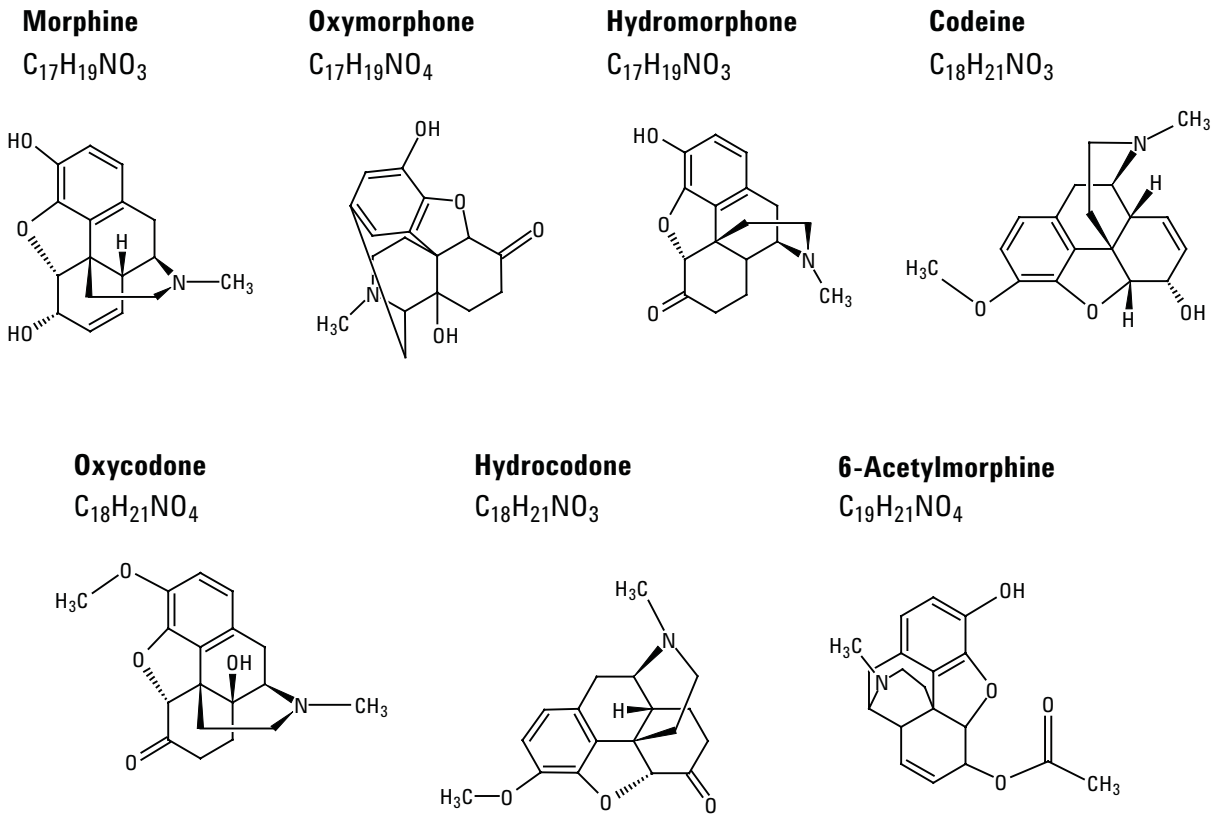


Figure 2. Structures of the opiates analyzed in this work.

This work uses a gradient LC analysis consisting of only water and acetonitrile (no modifiers) to elute all analytes and corresponding internal standards in less than 3.5 min on a Rapid Resolution High-Throughput (RRHT) LC column with a 1.8-µm particle size. The complete cycle time from one injection to the next is about 8 minutes. The compounds are analyzed using an electrospray ionization source in positive ion mode. Parameters associated with this ion source, like drying gas, are standard for the LC flow rate of 0.4 mL/min, in which the samples are introduced into the mass spectrometer.

Voltage settings for maximum ion transfer between the ion source and the mass analyzer components of the QQQ instrument are set using the autotune capability of the instrument to optimize signal intensity, resolution, and mass assignment across a wide mass range. One parameter requiring optimization for each analyte is the fragmentor voltage, which is located in the ion transfer optics between the ion source and the mass analyzer. This optimization results in the maximum response of the precursor ion of interest incident upon the first quadrupole of the QQQ mass analyzer. The fragmentor voltage of 110 V worked best for all analytes.

Once this is done, the optimal collision energy for fragmenting the precursor to form the highest possible response of a product ion is obtained. The mass spectrometer method development is now complete for the quantifier ion. Repeat optimizaton of the collision energy for the second most-abundant product ion and both MRM transitions are thus derived for one compound. Both steps in optimization may be carried out by flow injection analysis.

Experimental

Sample Preparation

Urine samples spiked with the opiate compounds were provided at the following labeled concentrations: 1, 5, 10, 50, 100, and 150 pg/µL, and a factor of 15 times lower for 6-MAM. These samples were then processed using the following procedure:

1. Start with 250-µL sample size
2. Add 500 µL sodium acetate buffer
3. Add 20 µL glucuronidase
4. Add 75 µL of internal standard mixture at 500 ng/mL concentration (de-ionized water)
5. Vortex
6. Incubate at 60 °C for 20 minutes
7. Add 850 µL de-ionized water
8. Vortex and spin down
9. Place 200 µL of supernatant in sample vial

All prepared samples provided by customer.

This procedure dilutes the samples by a factor of 6.78 so that a 1 pg/µL concentration in urine has an actual concentration of 147 fg/µL for injection. Upon addition of internal standards and extraction, the starting concentrations in urine now correspond to the following concentrations for injection: 0.147, 0.737, 1.47, 7.37, 14.7, and 22.12 pg/µL. With a 5-µL injection volume (see LC Conditions), this range then corresponds to 0.737, 3.685, 7.35, 36.85, 73.5, and 110.6 pg on-column. For 6-MAM, all of these values are a factor of 15 lower.

LC / MS Method Details

LC Conditions

Agilent 1200 Series binary pump, degasser, wellplate sampler, and thermostatted column compartment

Column:	Agilent ZORBAX SB-C18, 2.1 × 50 mm, 1.8-µm particle size (PN: 822700-902)
Column temp:	50 °C
Mobile phase:	A = water B = acetonitrile
Flow rate:	0.4 mL/min
Injection volume:	5 µL

Gradient:

Time (min)	%B	
0	2	
4	40	Stop time: 6.1 min
4.1	90	Post time: 2.0 min
6	90	
6.1	2	

Needle wash (25:75 water/methanol)–flush port 10 seconds

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	60 psig
Drying gas flow:	11 L/min
Drying gas temp:	350 °C
V_{cap}:	2000 V

Resolution (FWHM): Q1 = 0.7; Q2 = 0.7
Dwell time for all MRM transitions = 50 msec
Fragmentor voltage for all transitions = 110 V

The MRM transitions for each compound are listed in Table 1 by retention time. Those product ions in parentheses are used as qualifiers. The retention times are included. Note that 6-MAM, or 6-monoacetylmorphine, is abbreviated as 6-MAM.

Table 1. MRM Mode Parameters for Opiates

Segment	Compound	Transition	Collision energy (V)	Retention time (min)
1 (0 min)	D3-morphine	289.2 > 152.1	75	1.851
	Morphine	286.2 > 152.1 (128.0)	75 (73)	1.862
	D3-oxymorphone	305.2 > 230.1	33	2.138
	Oxymorphone	302.2 > 227.1 (198.0)	33 (55)	2.146
	D3-hydromorphone	289.2 > 157.1	50	2.379
	Hydromorphone	286.2 > 185.0 (157.0)	33 (50)	2.385
2 (2.65 min)	D3-codeine	303.2 > 152.0	75	2.908
	Codeine	300.2 > 152.0 (115.0)	75 (85)	2.912
	D3-oxycodone	319.2 > 244.1	30	3.109
	Oxycodone	316.2 > 241.0 (256.0)	30 (27)	3.120
	D6-6-MAM	334.2 > 165.1	40	3.161
	6-MAM	328.2 > 165.0 (211.0)	40 (27)	3.168
	D3-hydrocodone	303.2 > 199.1	28	3.245
	Hydrocodone	300.2 > 199.0 (128.0)	28 (73)	3.249

Results and Discussion

The calibration curves for all seven compounds are shown in Figures 3A through 3G, including expanded views of the lowest three levels. All calibration curves are generated using a linear fit, no inclusion of the origin, and a 1/x weighting. All curves have linearity coefficients of at least 0.99 and show good reproducibility and accuracy at the lowest levels. One exception is 6-MAM, which only showed signal for two of the three injections at the lowest level (49 fg on-column). However, the corresponding concentration in urine is 0.067 pg/µL (0.067 ng/mL), which is much lower than the 10 ng/mL confirmatory cutoff level for workplace testing proposed by the U.S. Substance Abuse Mental Health Services Administration (SAMHSA).

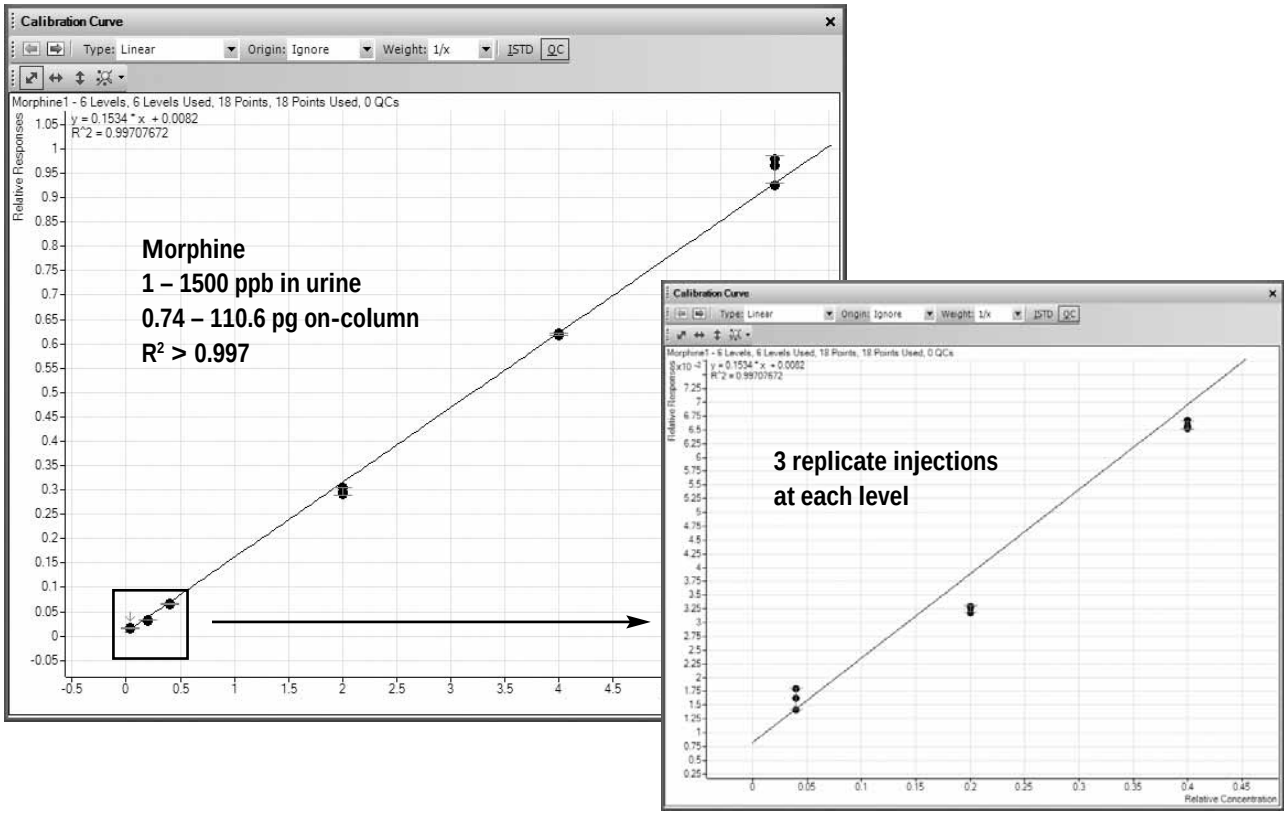


Figure 3A. Linearity of morphine in urine. Injection concentration range = 147 ppt – 22 ppb.

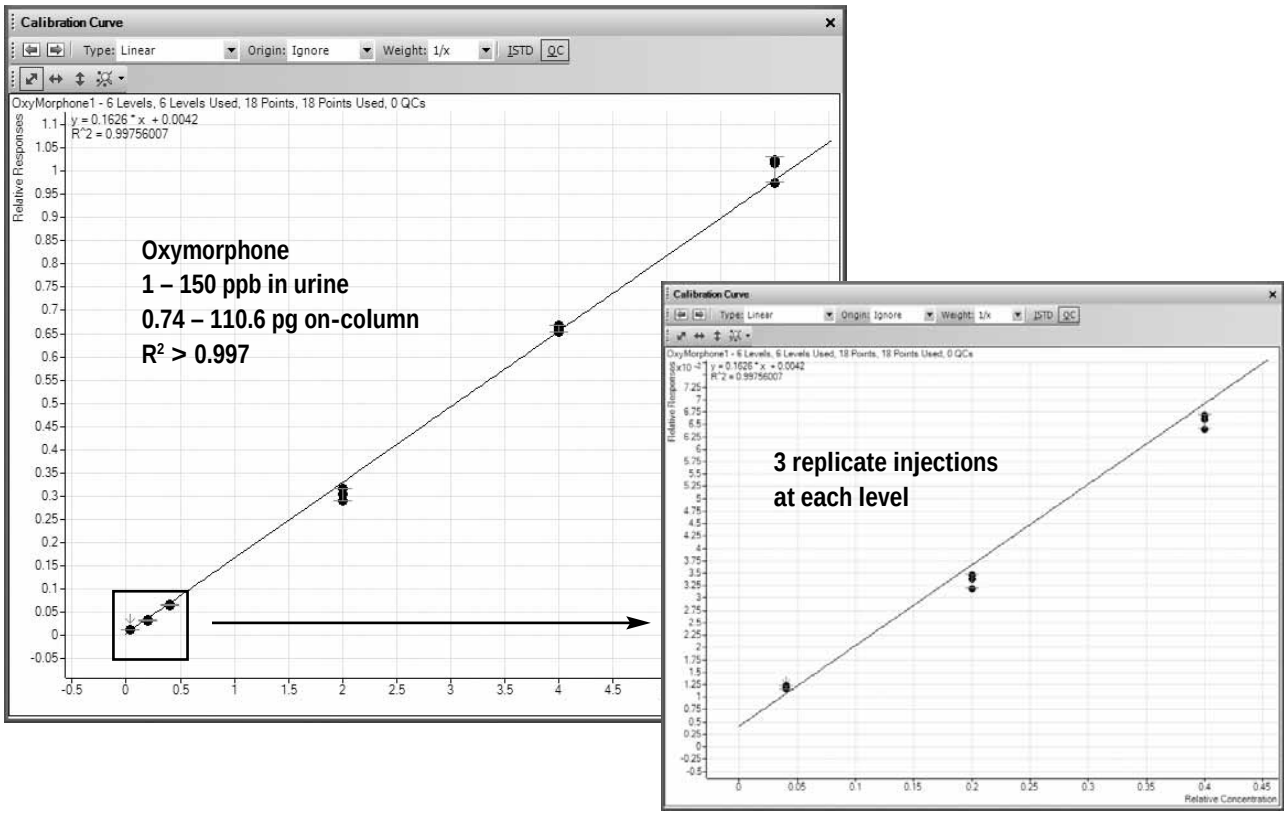


Figure 3B. Linearity of oxymorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

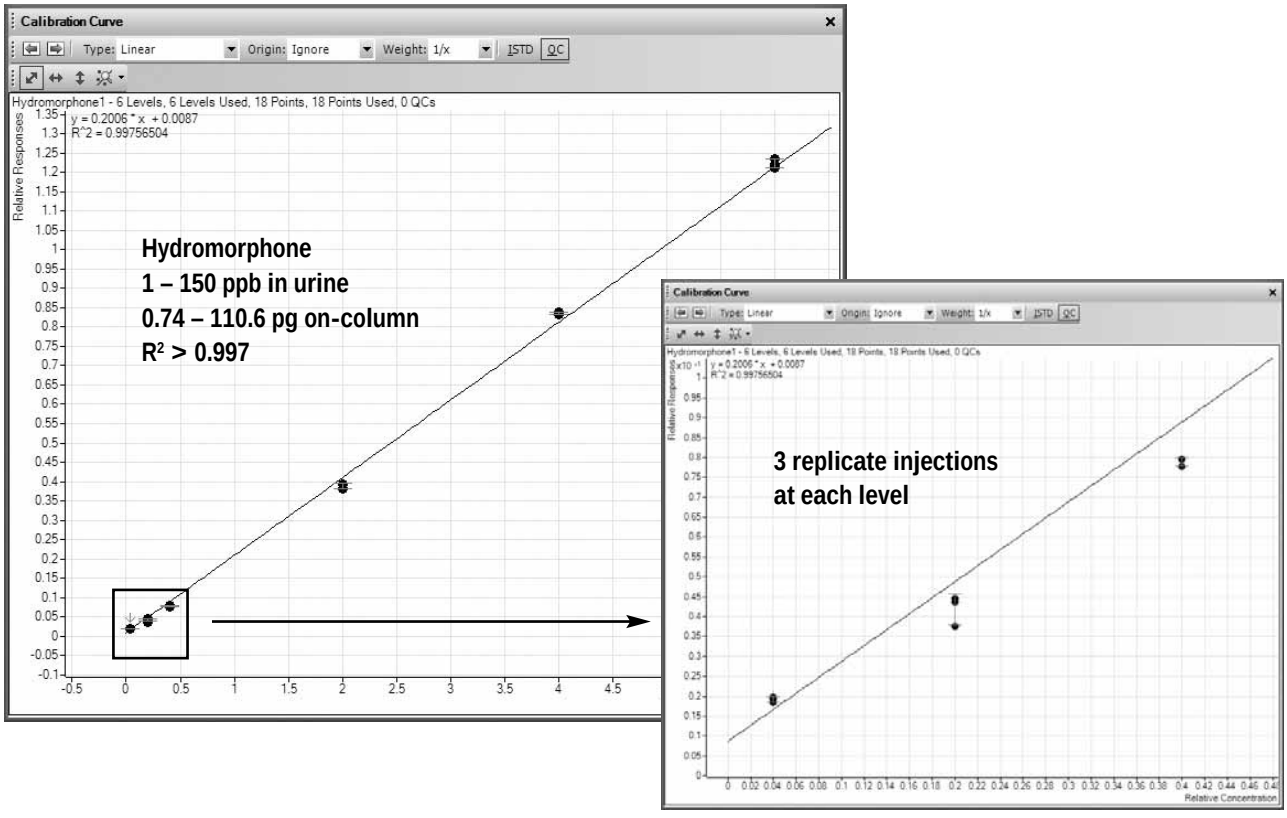


Figure 3C. Linearity of hydromorphone in urine. Injection concentration range = 147 ppt – 22 ppb.

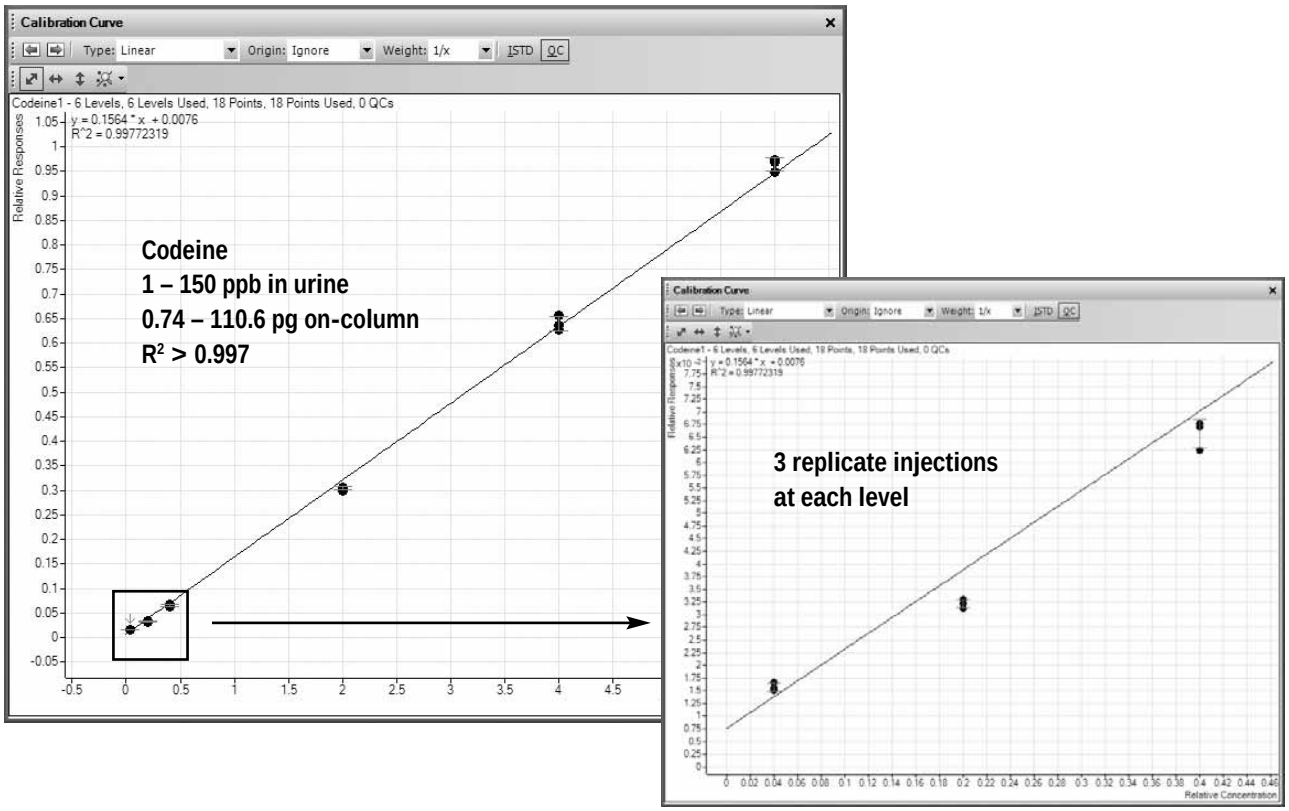


Figure 3D. Linearity of codeine in urine. Injection concentration range = 147 ppt – 22 ppb.

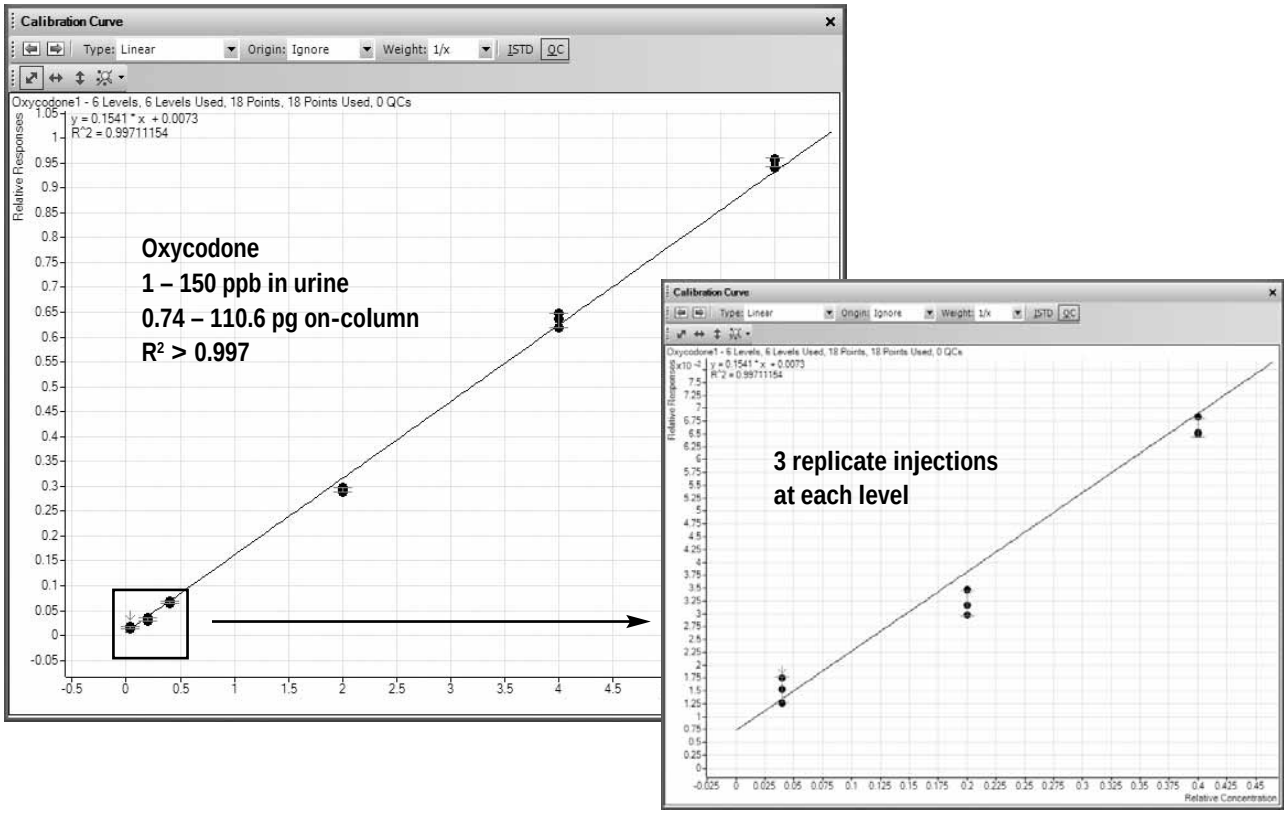


Figure 3E. Linearity of oxycodone in urine. Injection concentration range = 147 ppt – 22 ppb.

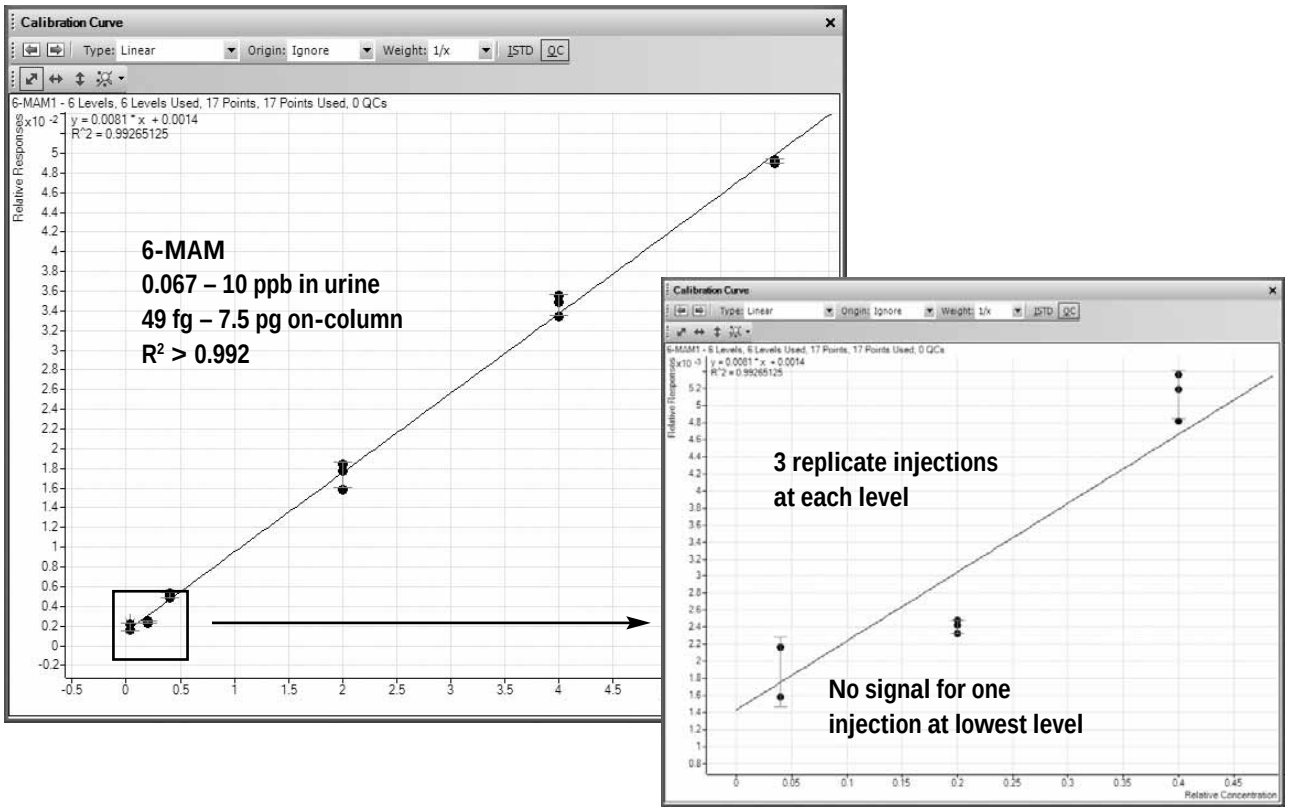


Figure 3F. Linearity of 6-MAM in urine. Injection concentration range = 9.8 ppt – 1.5 ppb.

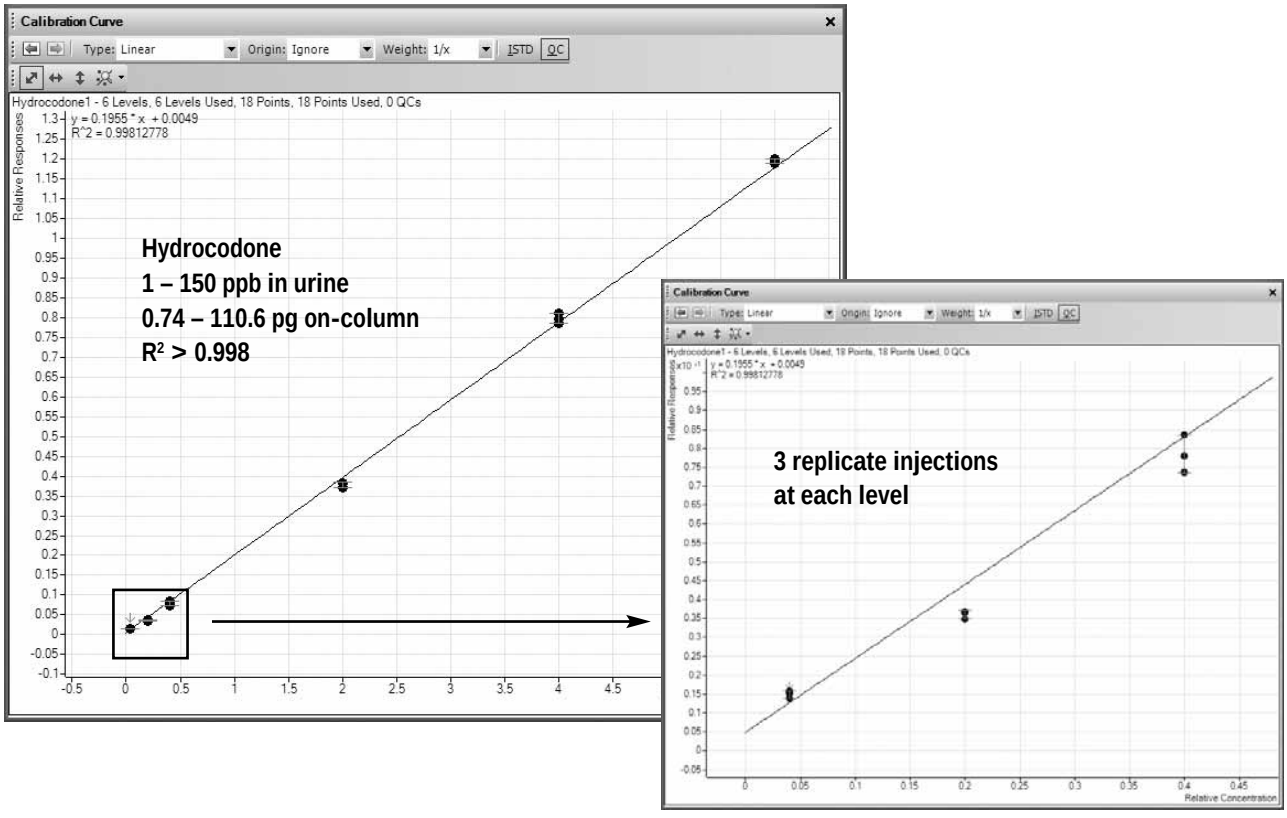
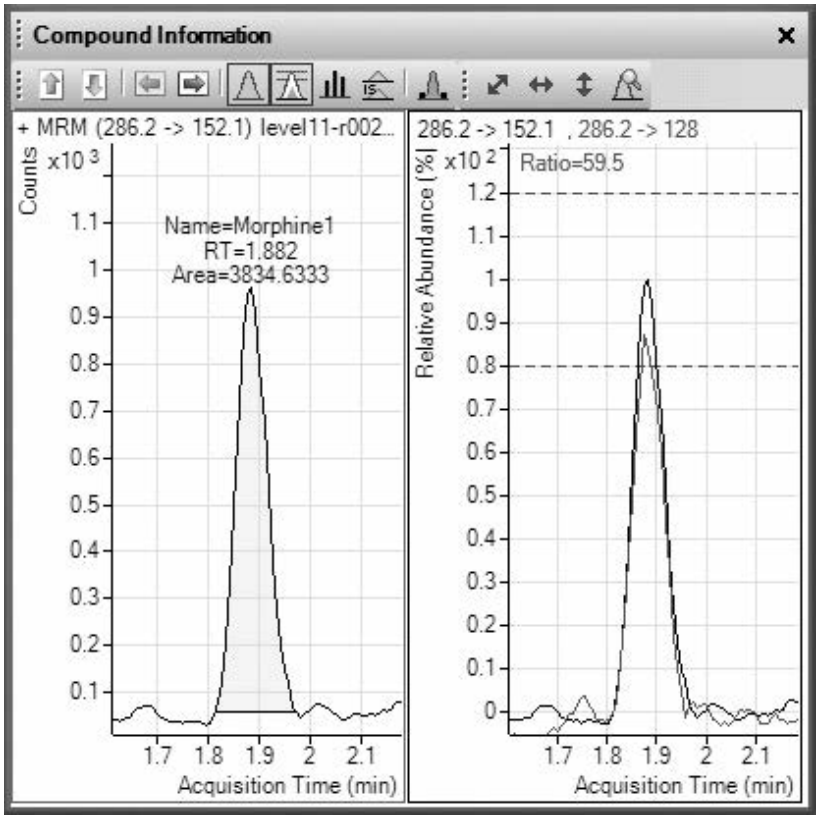


Figure 3G. Linearity of hydrocodone in urine. Injection concentration range = 147 ppt – 22 ppb.

Confirmation is carried out by examining the qualifier/quantifier ion ratio and making sure it stays within ± 20% of the determined value for each analyte. For example, after optimizing the MRM transitions for both product ions of morphine, it is automatically determined by the MassHunter Quantitative Analysis that the ratio of the qualifier peak to that of the quantifier should be 0.7%, or 70%. Applying a ± 20% tolerance to this ratio means that all calibration standards and samples analyzed in this batch should have a ratio of 0.56 to 0.84 in order to confirm the presence of morphine. The lowest calibration levels that consistently satisfy the confirmation requirement for each analyte are shown in Figures 4A through 4G.

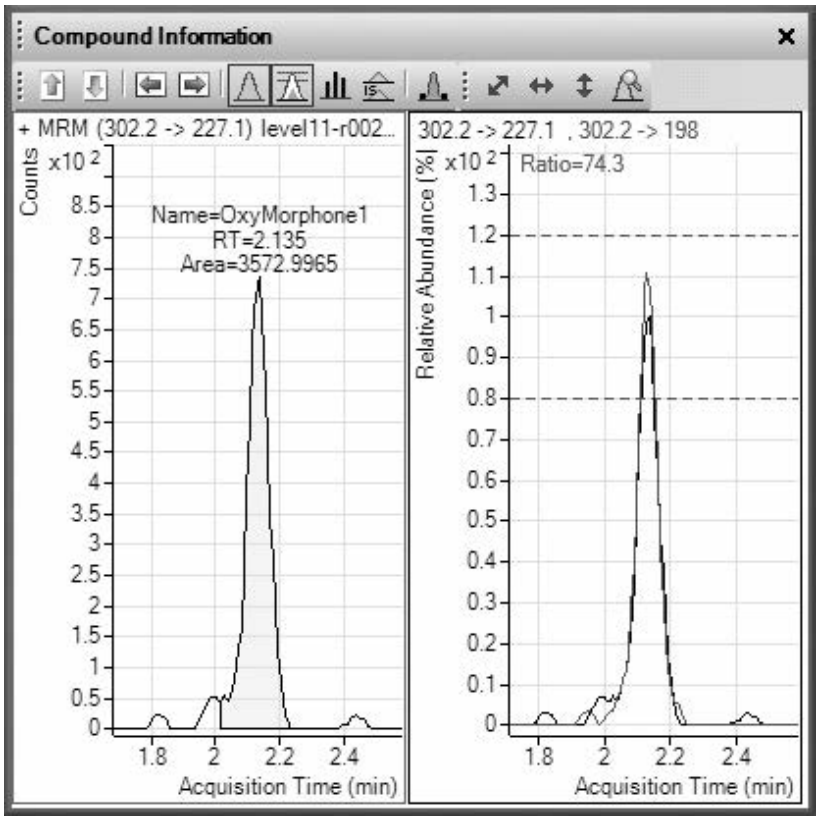
Note that with the exception of oxycodone and 6-MAM, the confirmation ion ratio for all analytes is satisfied at the corresponding lowest calibration levels of 1 pg/µL in urine. For oxycodone and 6-MAM, the lowest levels are 5 and 0.3 pg/µL, respectively.

Limits of detection (shown in Figures 5A through 5G) are also determined for this work using the quantifier ion of each analyte and based on a visual determination of peak-to-peak signal-to-noise ratio of at least 3:1 and a peak area %RSD (percent relative standard deviation) of 30 or less. The results for all analytes except oxycodone and 6-MAM are based on eight 1-µL injections at 147 fg on-column each. These correspond to original concentrations in urine of 1 pg/µL. For oxycodone, the LOD is determined from the triplicate 5-µL injections of the calibration level corresponding to 1 pg/µL (see Figure 5E). Like oxycodone, the LOD of 6-MAM is seen at a 5-µL injection, but of the 0.067 pg/µL level. However, only two of the three injections had signal so an area %RSD was not calculated. These values are further tabulated in Table 2.



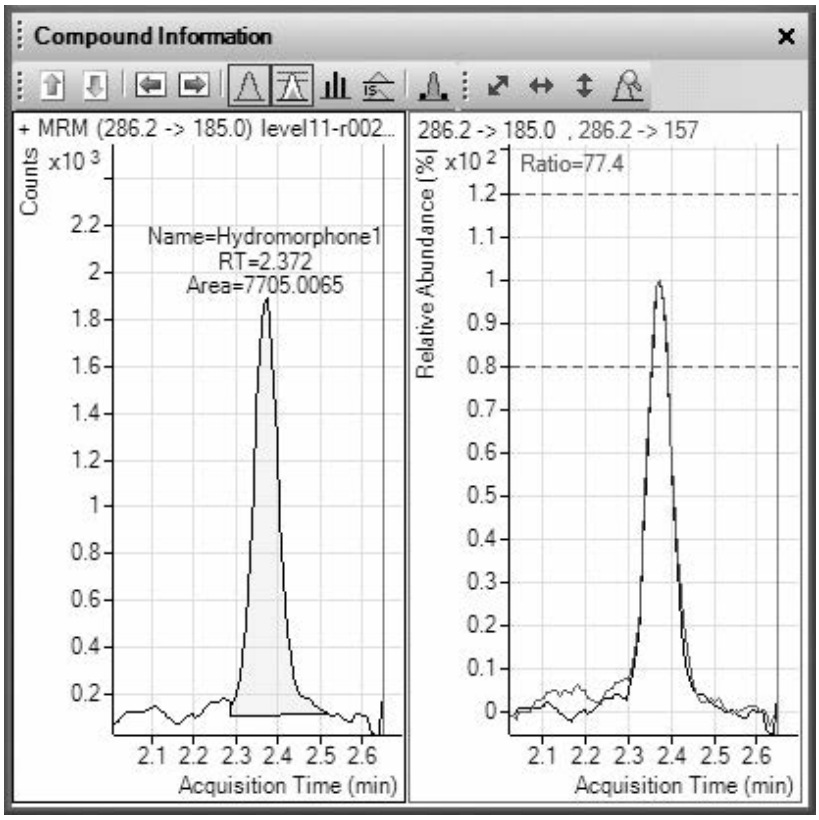
0.74 fg on-column

Figure 4A. Confirmation of morphine at 1 pg / µL (147 fg / µL).



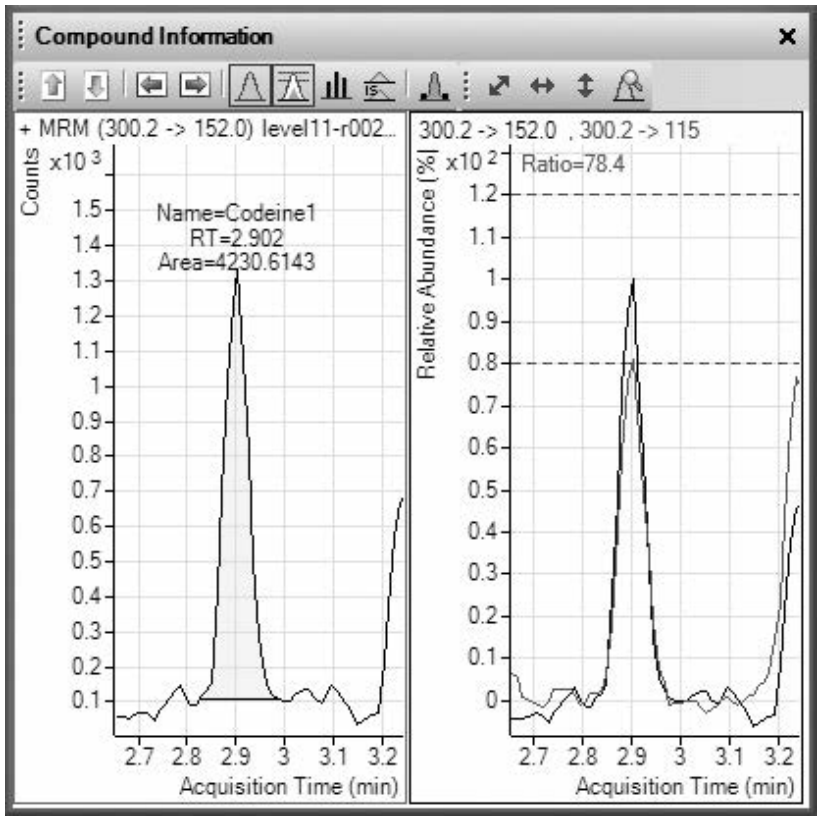
0.74 fg on-column

Figure 4B. Confirmation of oxymorphone at 1 pg / µL (147 fg / µL).



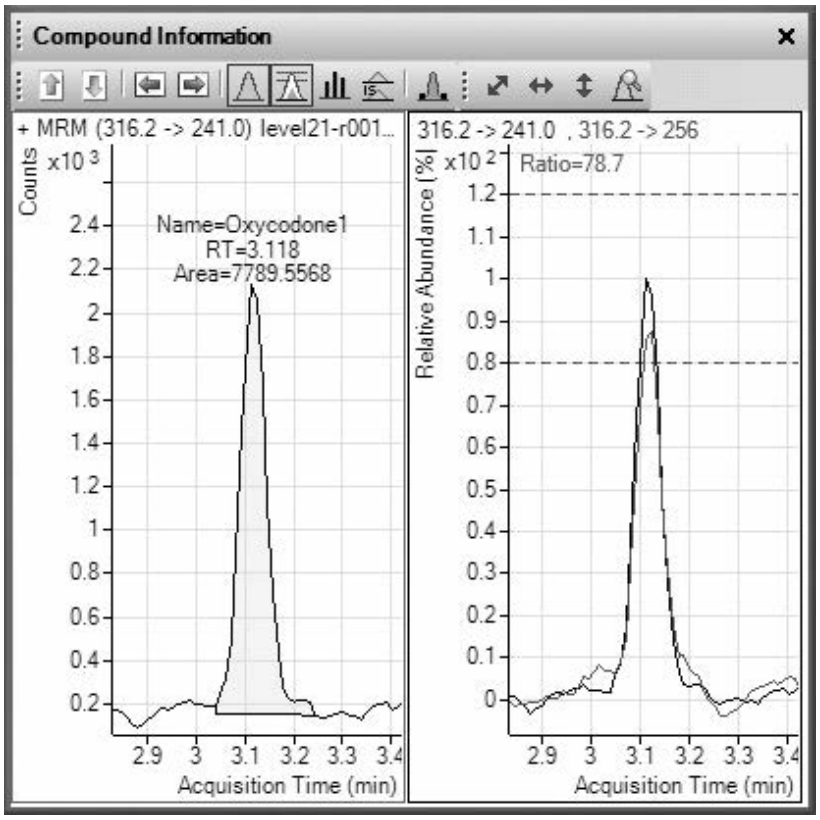
0.74 fg on-column

Figure 4C. Confirmation of hydromorphone at 1 pg / µL (147 fg / µL).



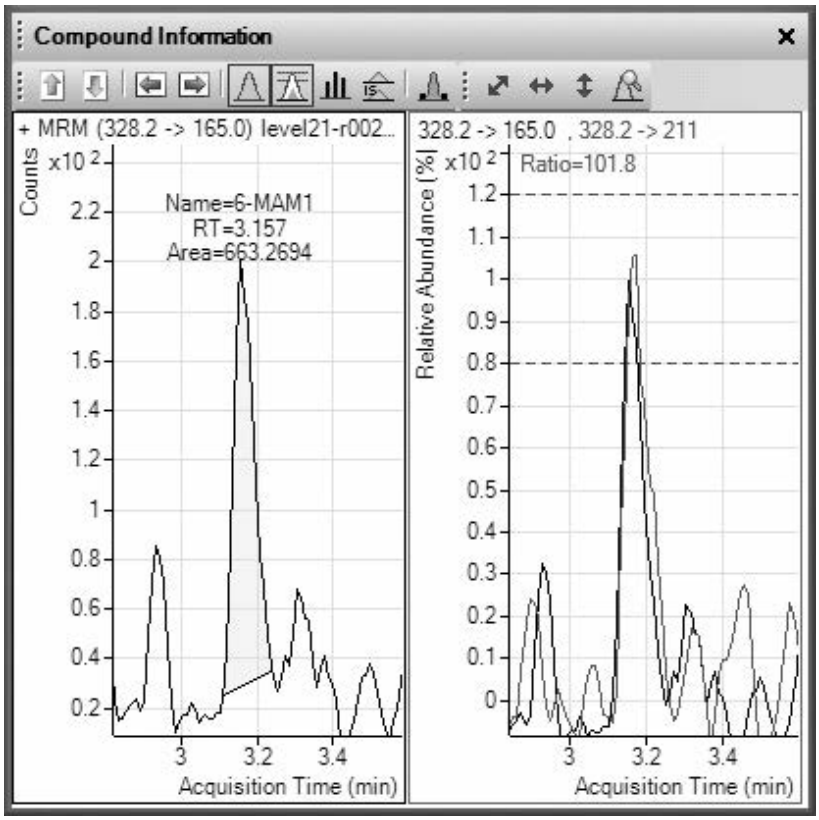
0.74 fg on-column

Figure 4D. Confirmation of codeine at 1 pg / µL (147 fg / µL).



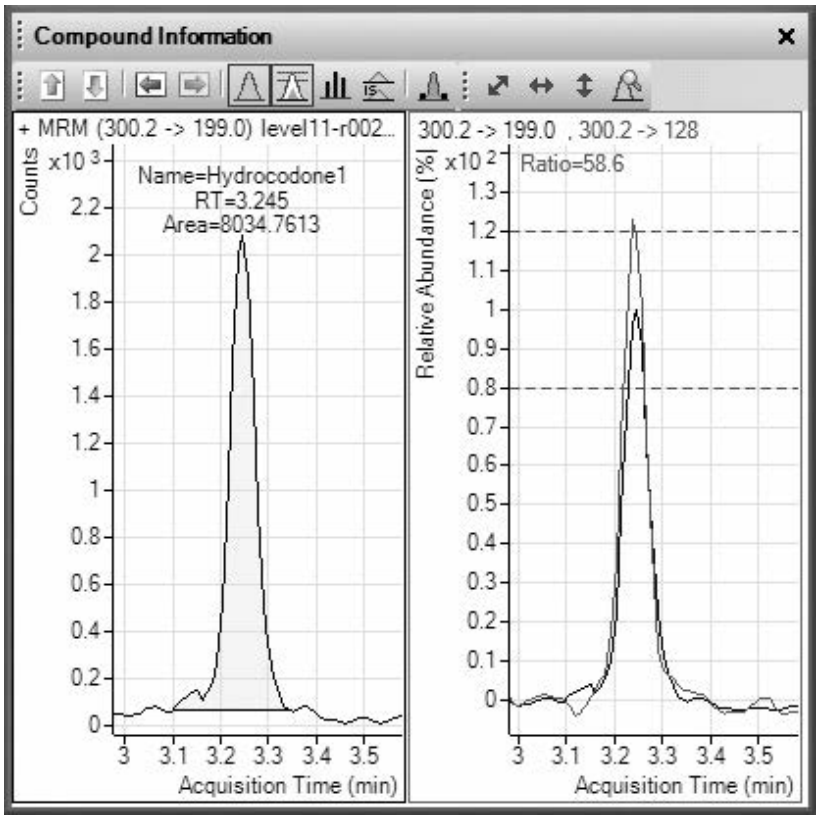
3.7 pg on-column

Figure 4E. Confirmation of oxycodone at 5 pg / µL (737 fg / µL).



245 fg on-column

Figure 4F. Confirmation of 6-MAM at 0.3 pg / µL (49 fg / µL).



0.74 pg on-column

Figure 4G. Confirmation of hydrocodone at 1 pg / µL (147 fg / µL).

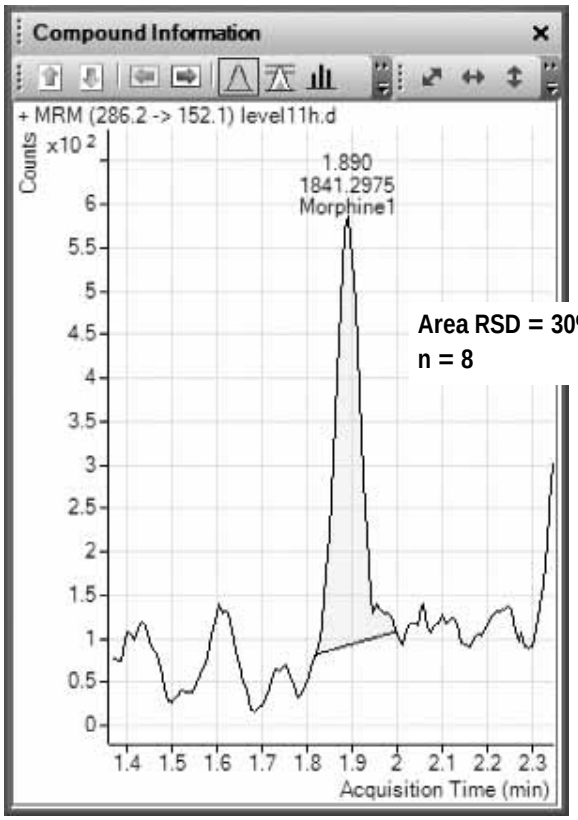


Figure 5A. LOD of morphine at 147 fg on-column.

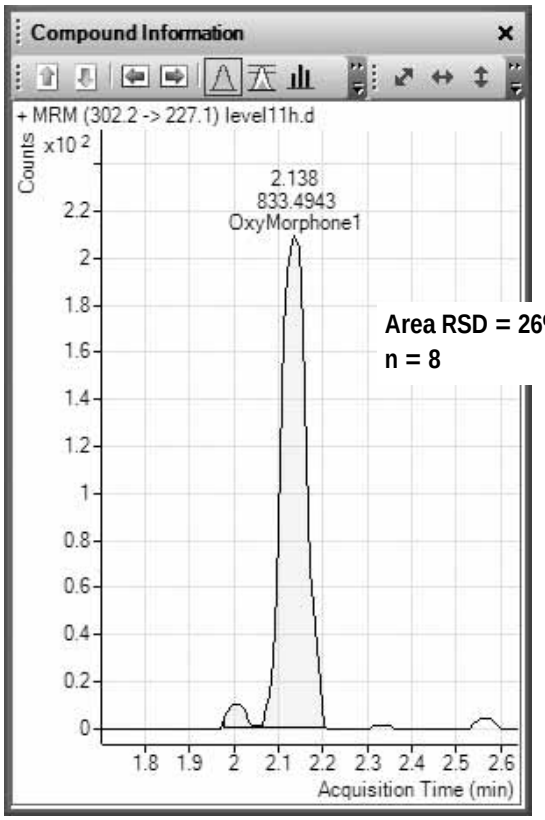


Figure 5B. LOD of oxymorphone at 147 fg on-column.

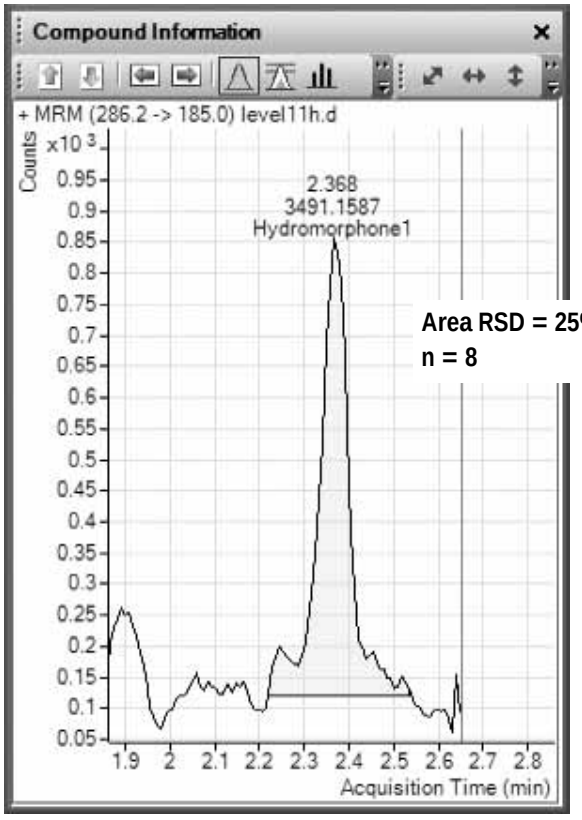


Figure 5C. LOD of hydromorphone at 147 fg on-column.

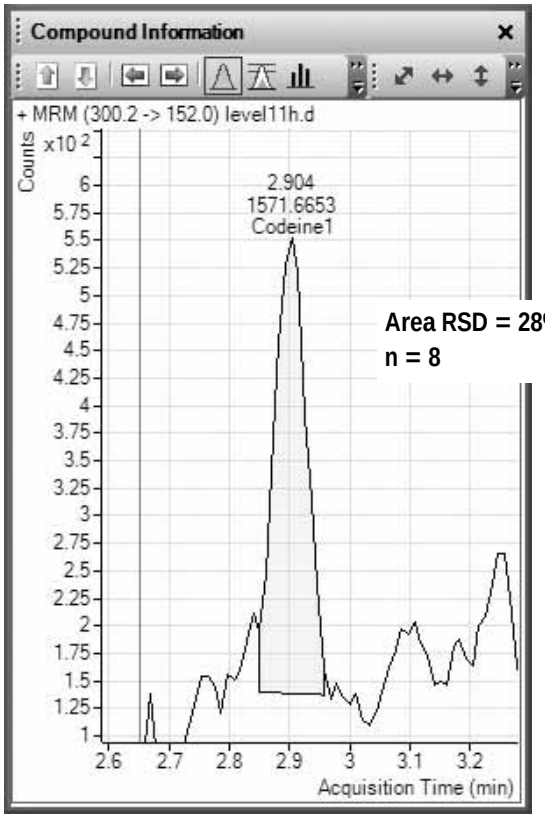


Figure 5D. LOD of codeine at 147 fg on-column.

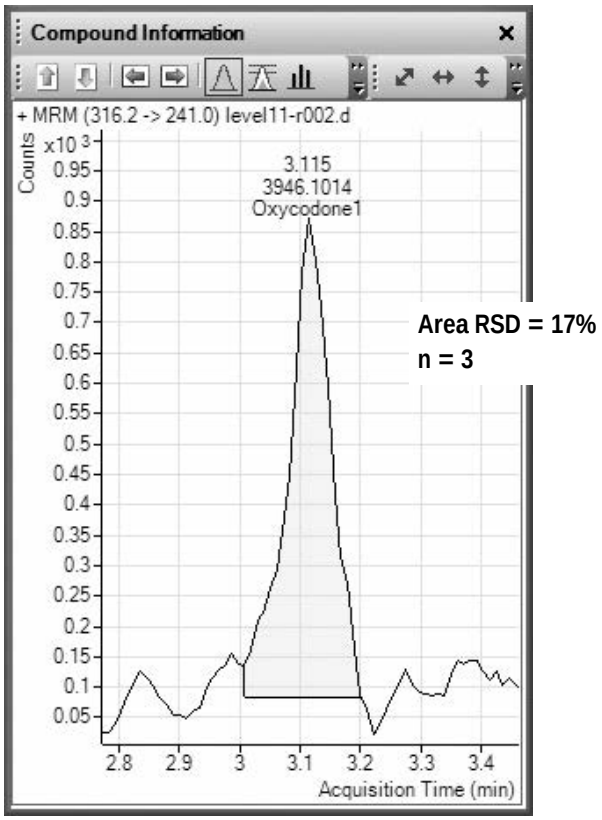


Figure 5E. LOD of oxycodone at 737 fg on-column.

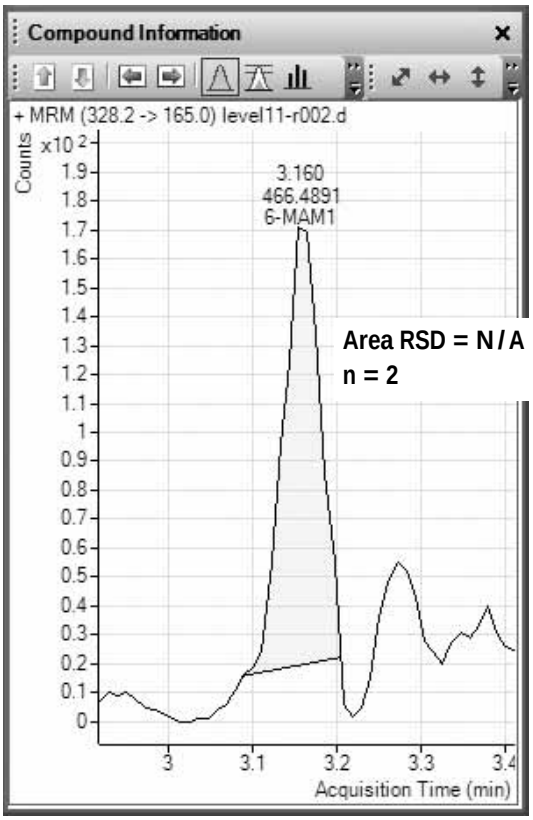


Figure 5F. LOD of 6-MAM at 49 fg on-column. Peak area %RSD not applicable because only two of three injections contained signal.

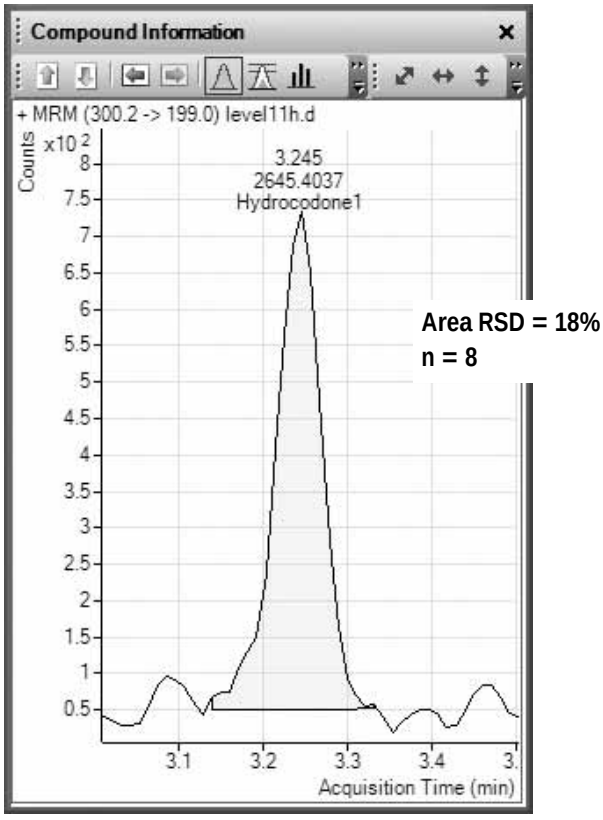


Figure 5G. LOD of hydrocodone at 147 fg on-column.

Table 2. Determined Limits of Detection (LODs) in Urine for Each Analyte

Analyte	LOD (fg on-column)
Morphine	147
Oxymorphone	147
Hydromorphone	147
Codeine	147
Oxycodone	737
6-MAM	49
Hydrocodone	147

Conclusions

Opiates are successfully analyzed in the presence of urine. Good linearity ($R^2 > 0.99$) is obtained for all compounds over two orders magnitude in concentration range, which is 1 to 150 ppb for all analytes except 6-MAM; for 6-MAM this range is 0.067 to 10 ppb. After processing the samples and considering the 5-µL injection volume, this range corresponds to 0.74 to 110.6 pg on-column (49 fg to 7.5 pg for 6-MAM). The calibration curve fitting is carried out with no inclusion of the origin, a linear fit, and a 1/x weighting. At the lowest levels very good reproducibility and accuracy is demonstrated. Limits of detection are less than 1 pg on-column for all analytes. The Agilent 6410 QQQ is an excellent instrument for sensitive quantitation in a relatively dirty matrix.

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For more details concerning this application, please contact Michael Zumwalt at Agilent Technologies, Inc.

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Agilent Technologies

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

Application Note

Forensic and Toxicology

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Abstract

A Forensic and Toxicological screening application kit has been developed for use with the Agilent 6400 Series triple quadrupole (QQQ) LC/MS systems which contains a database of optimized MRM transitions for approximately 200 analytes of forensic and toxicological interest. The database content is mainly focused on controlled substances and drugs of abuse. The aim of this application kit is to provide a user with a solid starting point for building analysis methods where the ability to screen for a large array of forensic and toxicological analytes is necessary. Typical results obtained from such a method created by using the database are described using serial dilutions of a test mix containing analytes of forensic interest.

Agilent Technologies

Introduction

Lists of potential toxins and analytes of forensic interest can be extremely large and typically depend on the area of analytical screening focus (for example, workplace drug testing, doping control, postmortem toxicology, explosive residues, and so forth). Often, the concentration levels of such target analytes are challenging and low, which can be further impacted by a complex sample matrix or the quantity of sample obtained.

The most sensitive liquid chromatography/mass spectrometry (LC/MS) screening or quantitation techniques are those based around triple quadrupole (QQQ) LC/MS/MS instruments, where a second stage of MS (post fragmentation from a collision cell) acts as an effective method of eliminating background chemical noise that is not associated with the target precursor and fragment ions. This technique is commonly referred to as Multiple Reaction Monitoring (MRM.) Instruments using each quadrupole as targeted mass filters in this manner are an effective and widely accepted technique for forensic and toxicological studies of challenging sample matrices and concentration levels.

QQQ MS instruments, however, operate by focusing a finite amount of time on only one MRM transition before the next MRM transition is selected in turn. Once the complete list of target MRM transitions has been monitored, then the MRM list is repeated or cycled until the end of the chromatographic analysis or until a new retention time segment begins that contains different MRM transitions. The amount of finite time given to any specific MRM transition is referred to as dwell time and can be uniquely specified for every MRM transition.

The chromatographic consideration with regard to dwell time and overall MRM cycle time is one of peak width or resolution, normally referred to as full width at half maximum (FWHM). Statistically, higher numbers of data points measured across a chromatographic peak will provide more accurate and reproducible results. This means that the overall cycle time of the MRM target list must be sufficiently low to achieve this, relative to the particular chromatography used. Furthermore, each MRM transition dwell time must be high enough to output ion statistics of high quality and precision.

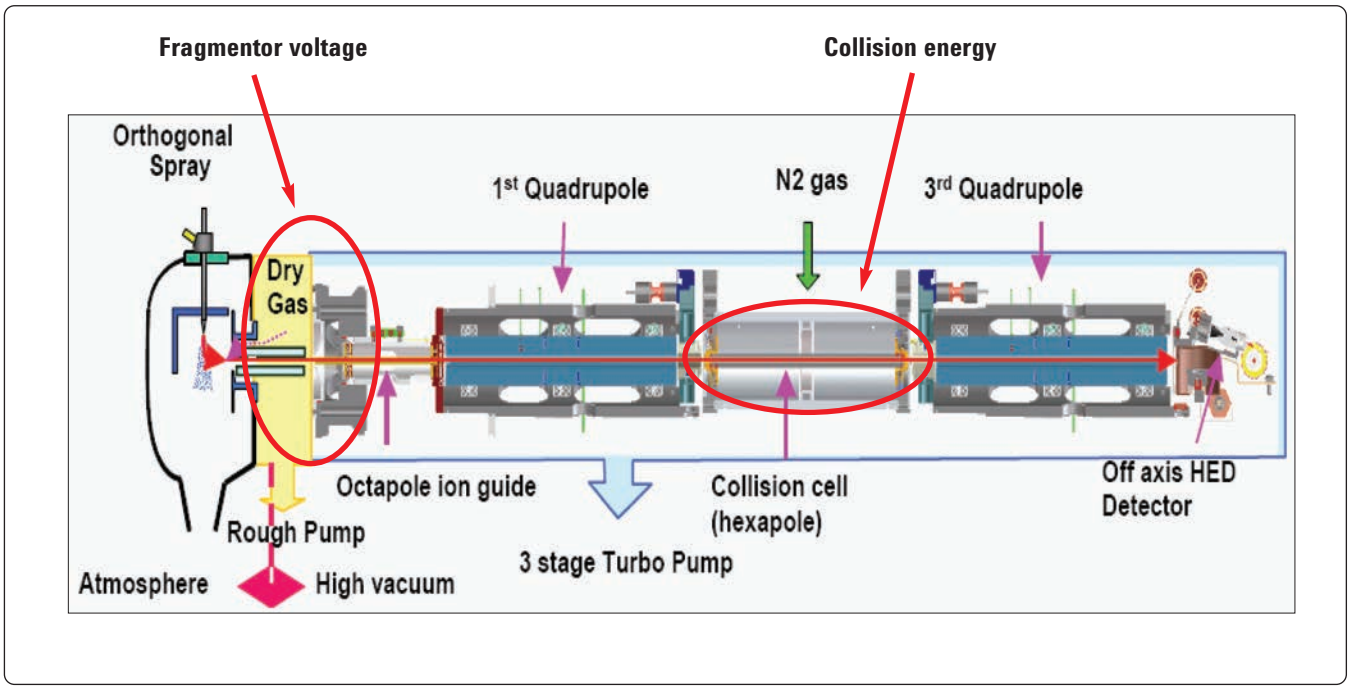

Figure 1. Two key optimized MRM transition settings.

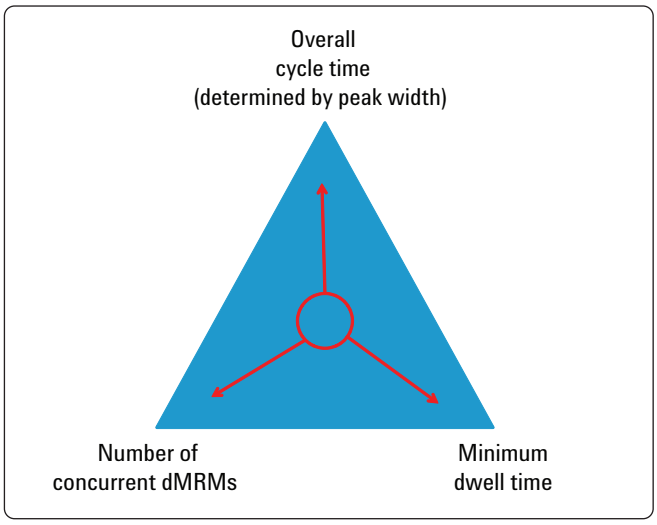

Figure 2. Compromise between cycle time, peak width, dwell time and number of MRM transitions.

Therefore, compromise between cycle time, dwell time and ultimately the total number of MRM transitions is often required especially with larger suites of analytes in a target screen assay (Figure 2). For this reason, Agilent Technologies introduced Dynamic MRM (dMRM) [1] functionality on the Agilent 6400 Series QQQ LC/MS system. Dynamic MRM is a technique where each ion transition has an associated retention time window (delta RT) where it is dynamically switched on and off without impacting a constant data cycle time. Since the complete list of ion transitions is unlikely to be cycled through at any given chromatographic retention time, then the result is normally higher dwell time for every transition and higher data quality when compared to normal MRM methods. Figure 3 graphically illustrates the Dynamic MRM principle.

Herein are described the results obtained from an analysis method using the Agilent MassHunter Forensic and Toxicological Dynamic MRM Database Kit (G1734AA) with optimized MRM transitions from the database inserted directly into the acquisition method. More detailed instruction on the creation of such methods are outlined in the G1734AA

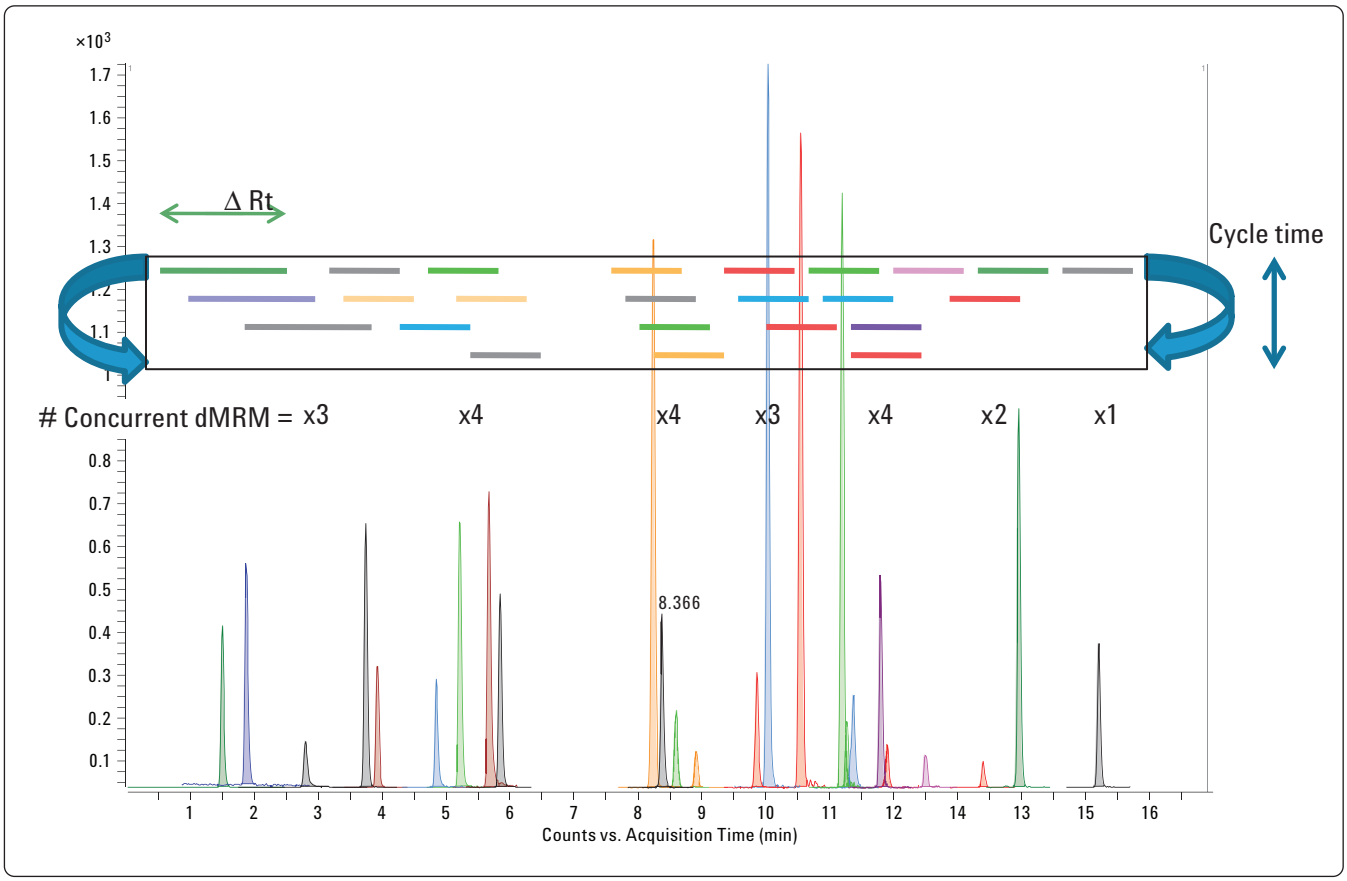

Figure 3. Illustration of Dynamic MRM principle.

MassHunter Forensic & Toxicology Dynamic MRM Database Kit Quick Start Guide [2]. Confirmatory evidence was obtained by using the two most abundant MRM transitions for use as quantifier and qualifier ions, the ratio of which are indicative of the analyte of interest. This application note aims to describe typical results using an LC/MS Forensic & Toxicology Test Mix.

Experimental

The analysis results outlined in this application note were obtained using an Agilent 6460 QQQ LC/MS coupled to an Agilent 1200SL Series LC system. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system equipped with the MassHunter Optimizer program (Rev. B.02.01) and the [G1734AA] forensic & toxicology Dynamic MRM application kit.

Sample Preparation

An ampoule from the LC/MS Forensics & Toxicology Test Mix [p/n 5190-0470] which is included in the Forensic and Toxicology application kit [G1734AA] was opened and 100 µL of the 1 µg/mL (1ppm) solution was diluted to a concentration of 10 ng/mL (10 ppb) using 9.9 mL of pure LC/MS grade methanol to create a clean solvent standard for method checkout purposes.

Appropriate serial dilutions from the original LC/MS Forensic & Toxicology Test Mix were created for the purposes of quantitation. These are listed in Table 1.

Table 1. Dilution Series of LC/MS Forensic & Toxicology Test Mix

Data File	Type	Level	Vol. (uL)	Conc.	Units
LCMS_Forensic and Toxicology Test Mix 10fg.d	Cal	1	1	10	fg on-column
LCMS_Forensic and Toxicology Test Mix 25fg.d	Cal	2	1	25	fg on-column
LCMS_Forensic and Toxicology Test Mix 50fg.d	Cal	3	1	50	fg on-column
LCMS_Forensic and Toxicology Test Mix 100fg.d	Cal	4	1	100	fg on-column
LCMS_Forensic and Toxicology Test Mix 250fg.d	Cal	5	1	250	fg on-column
LCMS_Forensic and Toxicology Test Mix 500fg.d	Cal	6	1	500	fg on-column
LCMS_Forensic and Toxicology Test Mix 1pg.d	Cal	7	1	1000	fg on-column
LCMS_Forensic and Toxicology Test Mix 5pg.d	Cal	8	1	5000	fg on-column
LCMS_Forensic and Toxicology Test Mix 10pg.d	Cal	9	1	10000	fg on-column
LCMS_Forensic and Toxicology Test Mix 25pg.d	Cal	10	1	25000	fg on-column
LCMS_Forensic and Toxicology Test Mix 50pg.d	Cal	11	1	50000	fg on-column

Table 2 outlines the composition of the LC/MS Toxicology Test Mix [p/n 5190-0470] which is intended to cover a wide and representative range of forensic analyte classes.

Table 2. LC/MS Forensics & Toxicology Test Mix Components (1µg/mL)

Compound Name	Formula	Mass
3,4-Methylendioxyamphetamine (MDA)	$C_{10}H_{13}NO_2$	179.09463
3,4-Methylenedioxyethamphetamine (MDEA)	$C_{12}H_{17}NO_2$	207.12593
Alprazolam	$C_{17}H_{13}ClN_4$	308.08287
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.04107
Cocaine	$C_{17}H_{21}NO_4$	303.14706
Codeine	$C_{18}H_{21}NO_3$	299.15214
delta9-Tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	314.22458
Diazepam	$C_{16}H_{13}ClN_{2O}$	284.07164
Heroin	$C_{21}H_{23}NO_5$	369.15762
Hydrocodone	$C_{18}H_{21}NO_3$	299.15214
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	320.01193
Meperidine (Pethidine)	$C_{15}H_{21}NO_2$	247.15723
Methadone	$C_{21}H_{27}NO$	309.20926
Methamphetamine	$C_{10}H_{15}N$	149.12045
Methylendioxymethamphetamine (MDMA)	$C_{11}H_{15}NO_2$	193.11028
Nitrazepam	$C_{15}H_{11}N_3O_3$	281.08004
Oxazepam	$C_{15}H_{11}ClN_2O_2$	286.05091
Oxycodone	$C_{18}H_{21}NO_4$	315.14706
Phencyclidine (PCP)	$C_{17}H_{25}N$	243.1987
Phentermine	$C_{10}H_{15}N$	149.12045
Proadifen	$C_{23}H_{31}NO_2$	353.23548
Strychnine	$C_{21}H_{22}N_2O_2$	334.16813
Temazepam	$C_{16}H_{13}ClN_2O_2$	300.06656
Trazodone	$C_{19}H_{22}ClN_5O$	371.15129
Verapamil	$C_{27}H_{38}N_2O_4$	454.28316

Reagents and Chemicals

Burdick & Jackson LC/MS grade acetonitrile together with deionized water (locally produced 18.1 MΩ) were used for mobile phases. Buffers were freshly prepared using a high purity source of formic acid and ammonium formate.

Instrumentation

LC Conditions

Column:	Agilent Zorbax Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm [p/n - 959764-902]
Column temperature:	60 °C
Mobile phase	A: 5 mM NH_4 formate/0.01% Formic acid in water B: 0.01% formic acid in acetonitrile
Flow rate:	0.5 mL/min

Gradient program:

Time (min)	A (%)	B (%)	Flow rate mL/min
Initial	90	10	0.5
0.5	85	15	0.5
3.0	50	50	0.5
4.0	5	95	0.5
6.0	5	95	0.5

Injection volume:	1 µL (with 5 second needle wash in flushport)
Analysis time:	6.0 min
Post time:	2.0 min
Overall cycle time:	8.0 min

6460 QQQ LC/MS Conditions

Source Conditions:
Electrospray AP-ESI (using Agilent Jet Stream Technology):

Positive ionization polarity

Sheath gas temperature and flow:	380 °C, 12 L/min
Nozzle voltage:	500 V
Drying gas temperature and flow:	320 °C, 8 L/min
Nebulizer gas pressure:	27 psi
Capillary voltage:	3750 V
Fragmentor voltage:	150 V

6410 QQQ LC/MS Conditions
(Results not included in this application note.)

Source Conditions:
Electrospray AP-ESI:

Positive ionization polarity

Drying gas temperature and flow:	350 °C, 12 L/min
Nebulizer gas pressure:	30 psi
Capillary voltage:	2000 V
Fragmentor voltage:	150 V

All other instrument operating parameters were taken care of by Agilent's autotune functionality and subsequent mass calibration using standard settings.

Dynamic MRM Acquisition Method Parameters

Table 3. Dynamic MRM Method Conditions

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Codeine	–	300.2	Unit	165.1	Unit	158	45	1.11	0.4	Positive
Codeine	–	300.2	Unit	58.1	Unit	158	29	1.11	0.4	Positive
Oxycodone	–	316.2	Unit	298.1	Unit	143	17	1.285	0.4	Positive
Oxycodone	–	316.2	Unit	256.1	Unit	143	25	1.285	0.4	Positive
δ-Amphetamine	–	136.1	Unit	119.1	Unit	66	5	1.296	0.4	Positive
δ-Amphetamine	–	136.1	Unit	91	Unit	66	17	1.296	0.4	Positive
MDA	–	180.1	Unit	163	Unit	61	5	1.332	0.4	Positive
MDA	–	180.1	Unit	105	Unit	61	21	1.332	0.4	Positive
Hydrocodone	–	300.2	Unit	199	Unit	159	29	1.4	0.4	Positive
Hydrocodone	–	300.2	Unit	128	Unit	159	65	1.4	0.4	Positive
Methamphetamine	–	150.1	Unit	119	Unit	92	5	1.45	0.4	Positive
Methamphetamine	–	150.1	Unit	91	Unit	92	17	1.45	0.4	Positive
MDMA	–	194.1	Unit	163	Unit	97	9	1.468	0.4	Positive
MDMA	–	194.1	Unit	105	Unit	97	25	1.468	0.4	Positive
Strychnine	–	335.2	Unit	184	Unit	195	41	1.629	0.4	Positive
Strychnine	–	335.2	Unit	156	Unit	195	53	1.629	0.4	Positive
MDEA	–	208.1	Unit	163	Unit	107	9	1.735	0.4	Positive
MDEA		208.1	Unit	105	Unit	107	25	1.735	0.4	Positive
Heroine		370.2	Unit	268.1	Unit	149	37	2.256	0.4	Positive
Heroin		370.2	Unit	165	Unit	149	61	2.256	0.4	Positive
Cocaine		304.2	Unit	182.1	Unit	138	17	2.376	0.4	Positive
Cocaine		304.2	Unit	77	Unit	138	61	2.376	0.4	Positive
Meperidine		248.2	Unit	220.1	Unit	128	21	2.419	0.4	Positive
Meperidine		248.2	Unit	174.1	Unit	128	17	2.419	0.4	Positive
Trazodone		372.2	Unit	176	Unit	159	25	2.797	0.4	Positive
Trazodone		372.2	Unit	148	Unit	159	37	2.797	0.4	Positive
PCP		244.2	Unit	91	Unit	86	41	2.876	0.4	Positive
PCP	–	244.2	Unit	86.1	Unit	86	9	2.876	0.4	Positive
Oxazepam	–	287	Unit	269	Unit	150	12	3.53	0.4	Positive
Oxazepam	–	287	Unit	241	Unit	150	20	3.53	0.4	Positive
Nitrazepam	–	282.1	Unit	236.1	Unit	148	25	3.542	0.4	Positive
Nitrazepam	–	282.1	Unit	180	Unit	148	41	3.542	0.4	Positive
Verapamil	–	455.3	Unit	165	Unit	158	37	3.554	0.4	Positive
Verapamil	–	455.3	Unit	150	Unit	158	45	3.554	0.4	Positive
Methadone	–	310.2	Unit	265.1	Unit	112	9	3.61	0.4	Positive
Methadone	–	310.2	Unit	105	Unit	112	29	3.61	0.4	Positive
Lorazepam	–	321	Unit	275	Unit	102	21	3.626	0.4	Positive
Lorazepam	–	321	Unit	194	Unit	102	49	3.626	0.4	Positive
Alprazolam	–	309.1	Unit	281	Unit	179	25	3.727	0.4	Positive
Alprazolam	–	309.1	Unit	205	Unit	179	49	3.727	0.4	Positive
Temazepam	–	301.1	Unit	255.1	Unit	117	29	3.941	0.4	Positive

Table 3. Dynamic MRM Method Conditions (continued)

Compound name	ISTD?	Prec ion	MS1 res	Prod ion	MS2 res	Frag (V)	CE (V)	Rett ime	Ret window	Polarity
Temazepam	–	301.1	Unit	177	Unit	117	45	3.941	0.4	Positive
Proadifen	–	354.2	Unit	167	Unit	153	29	4.088	0.4	Positive
Proadifen	–	354.2	Unit	91.1	Unit	153	45	4.088	0.4	Positive
Diazepam	–	285.1	Unit	193	Unit	169	45	4.268	0.4	Positive
Diazepam	–	285.1	Unit	154	Unit	169	25	4.268	0.4	Positive
THC	–	315.2	Unit	193.2	Unit	150	20	5.277	0.4	Positive
THC	–	315.2	Unit	123.3	Unit	150	30	5.277	0.4	Positive

Results and discussion

Fast and easy startup with Agilent Test Mix

In order to rapidly implement and verify that acquisition and data analysis methodology is correctly set up, the LC/MS Forensics & Toxicology Test Mix [p/n 5190-0470] is included in the Forensic and Toxicology Dynamic MRM application kit [G1734AA] which contains a representative range of forensic analyte classes of 25 components (Table 2).

To create a method from first principles, the required transitions are selected from the database browser window (Figure 4). Once each selection has been made, the transitions are transferred to the acquisition method by clicking the 'Import' button to the bottom right of the browser window. An example of an acquisition method is illustrated in Figure 5.

Detailed information on this operation is contained in the MassHunter Forensic and Toxicology Dynamic MRM Database Kit Quick Start Guide [2].

Using the methodology outlined in the experimental section, a 1-uL injection of the 10 ng/mL LC/MS Forensics & Toxicology Test Mix equates to a 10 pg on-column injection amount. Figure 6 illustrates a typical overlay of extracted compound chromatograms for the test mix. A prepared method for QQQ is included in the application kit. When this method is loaded all conditions are correct and the user is able to reproduce the analysis.*

*These methods are acquisition-only and correspond to the instrument configuration as outlined in the experimental section of this application note. Appropriate settings must be manually input if a different instrument configuration is used. Similar results will demonstrate that the system is working properly.

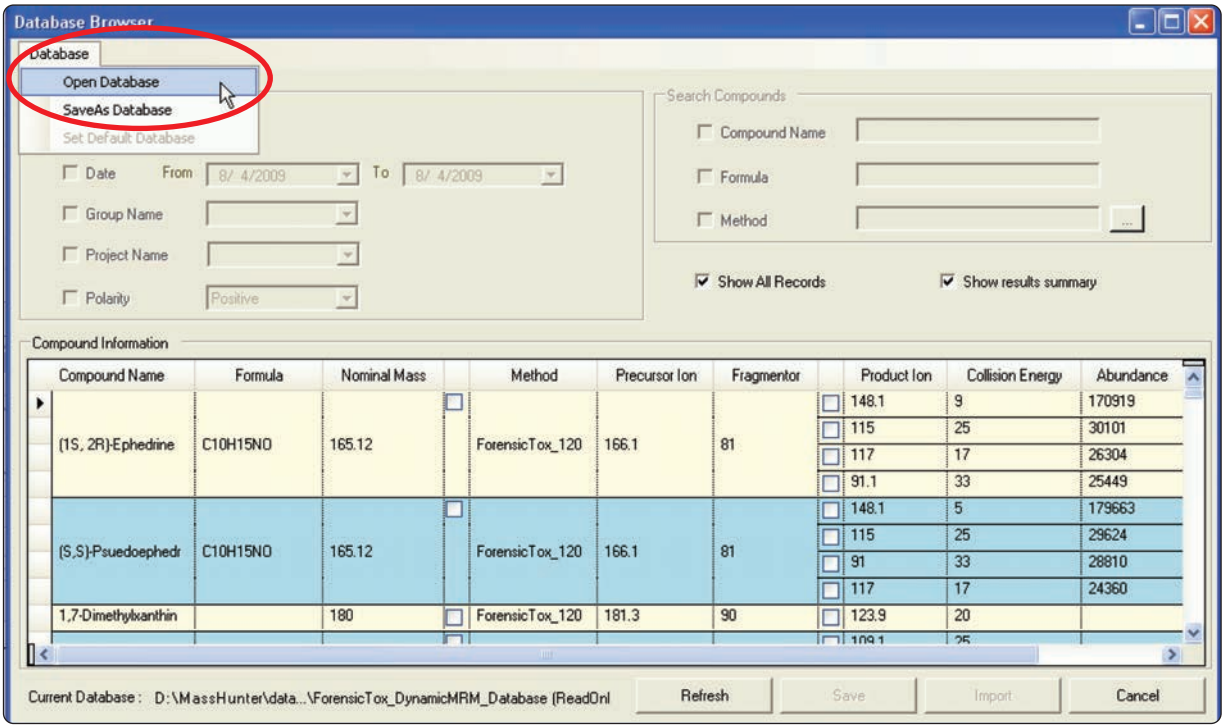

Figure 4. Compound MRM database browser containing 200 forensic analytes.

Acquisition | Source | Chromatogram | Instrument | Diagnostics

Scan segments

Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragmentor	Collision Energy	Ret Time (min)	Delta Ret Time	Polarity
Alprazolam	☐	309.1	Unit	281	Unit	179	25	3.715	1	Positive
Cocaine	☐	304.2	Unit	182.1	Unit	138	17	2.358	1	Positive
d-Amphetamine	☐	136.1	Unit	91	Unit	66	17	1.278	1	Positive
Diazepam	☐	285.1	Unit	154	Unit	169	25	4.269	1	Positive
Heroin	☐	370.2	Unit	165	Unit	149	61	2.236	1	Positive
Hydrocodone	☐	300.2	Unit	199	Unit	159	29	1.38	1	Positive
Lorazepam	☐	321	Unit	275	Unit	102	21	3.61	1	Positive
MDA	☐	180.1	Unit	163	Unit	61	5	1.311	1	Positive
MDEA	☐	208.1	Unit	163	Unit	107	9	1.72	1	Positive

Dynamic MRM Parameters

Cycle Time 500 ms

Figure 5. Scan segments table with Dynamic MRM transitions imported database browser.

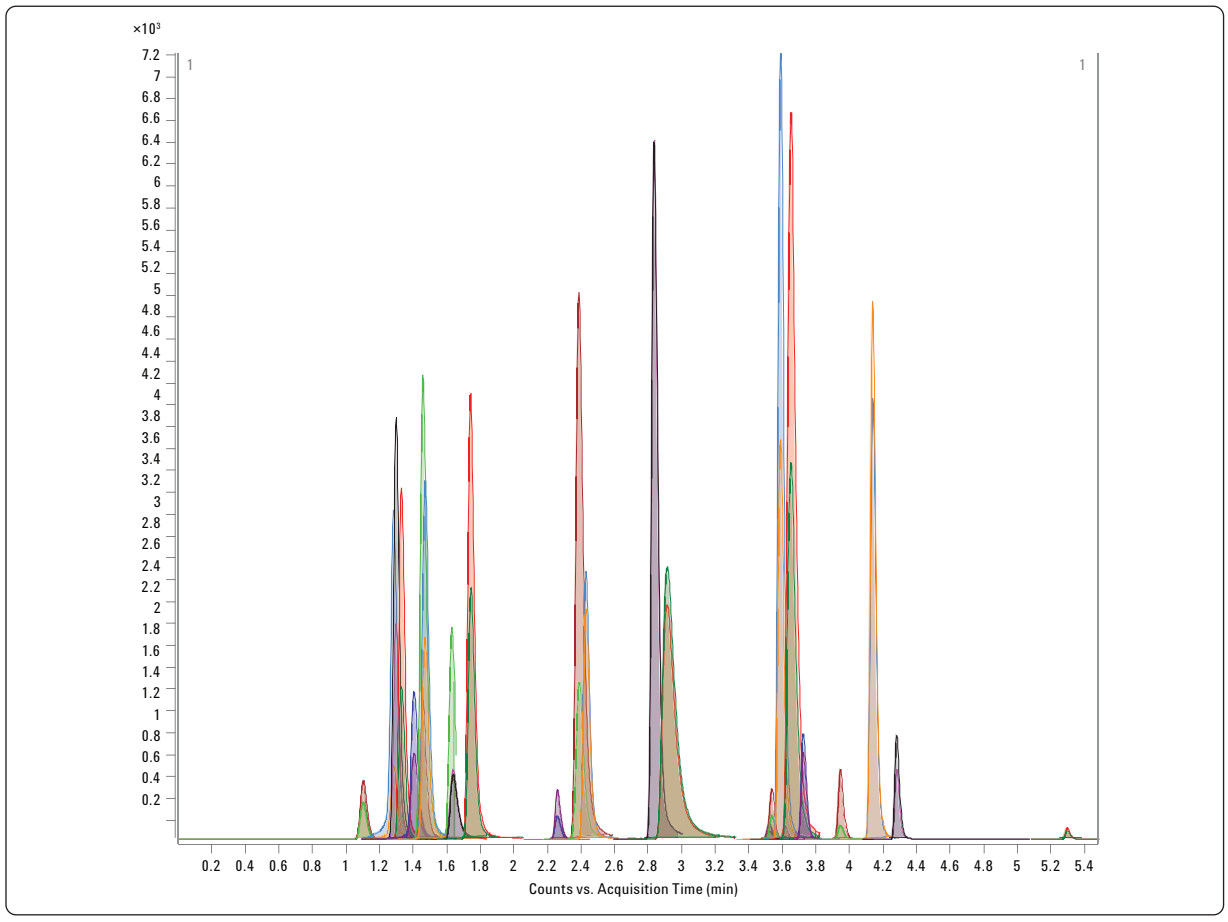

Figure 6. Example LC/MS Forensics and Toxicology test mix 10 pg on-column extracted ion chromatogram (overlay).

Quantitative analysis and standard curves

By using a Dynamic MRM acquisition method, the series of LC/MS Forensic and Toxicology Test Mix dilutions (Table 1) were analyzed according to the procedure outlined in the experimental section. All 50 Dynamic MRM transitions were used and Table 4 summarizes the results for the limits of detection and linearity of each component in the 25-component test mix.

Table 4. Limits of Detection and Calibration Linearity Results

Compound Name	Limit of Detection (fg on-column)	Linearity Correlation
3,4-Methylendioxyamphetamine (MDA)	50	0.99817
3,4-Methylenedioxyethamphetamine (MDEA)	10	0.99743
Alprazolam	50	0.99755
Clonazepam	100	0.99501
Cocaine	10	0.99755
Codeine	50	0.99841
δ9-Tetrahydrocannabinol (THC)	50	0.99869
Diazepam	10	0.99896
Heroin	25	0.99863
Hydrocodone	25	0.99493
Lorazepam	100	0.99601
Meperidine (Pethidine)	10	0.99687
Methadone	10	0.99666
Methamphetamine	10	0.98750
Methylendioxymethamphetamine (MDMA)	25	0.99217
Nitrazepam	25	0.99712
Oxazepam	250	0.99544
Oxycodone	50	0.99804
Phencyclidine (PCP)	25	0.99659
Phentermine	50	0.99898
Proadifen	<5	0.99772
Strychnine	50	0.99496
Temazepam	25	0.99751
Trazodone	<5	0.99777
Verapamil	<5	0.99787

Figures 7 through 10 illustrate the calibration curves through the range of 10-50000 fg on-column for six of the analytes from the LC/MS Forensic and Toxicology Test Mix.

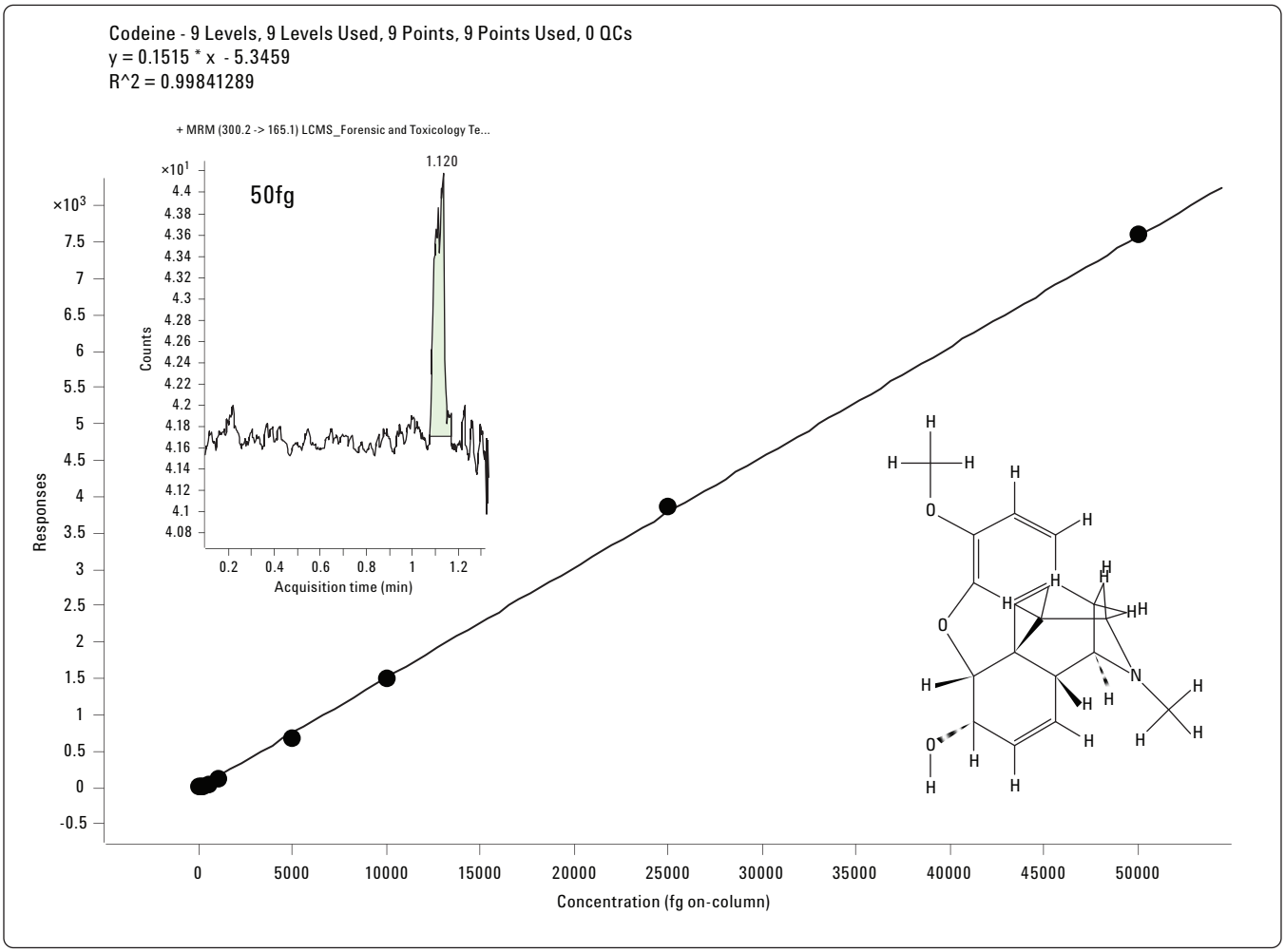

Figure 7. Calibration curve and LOD chromatogram, codeine.

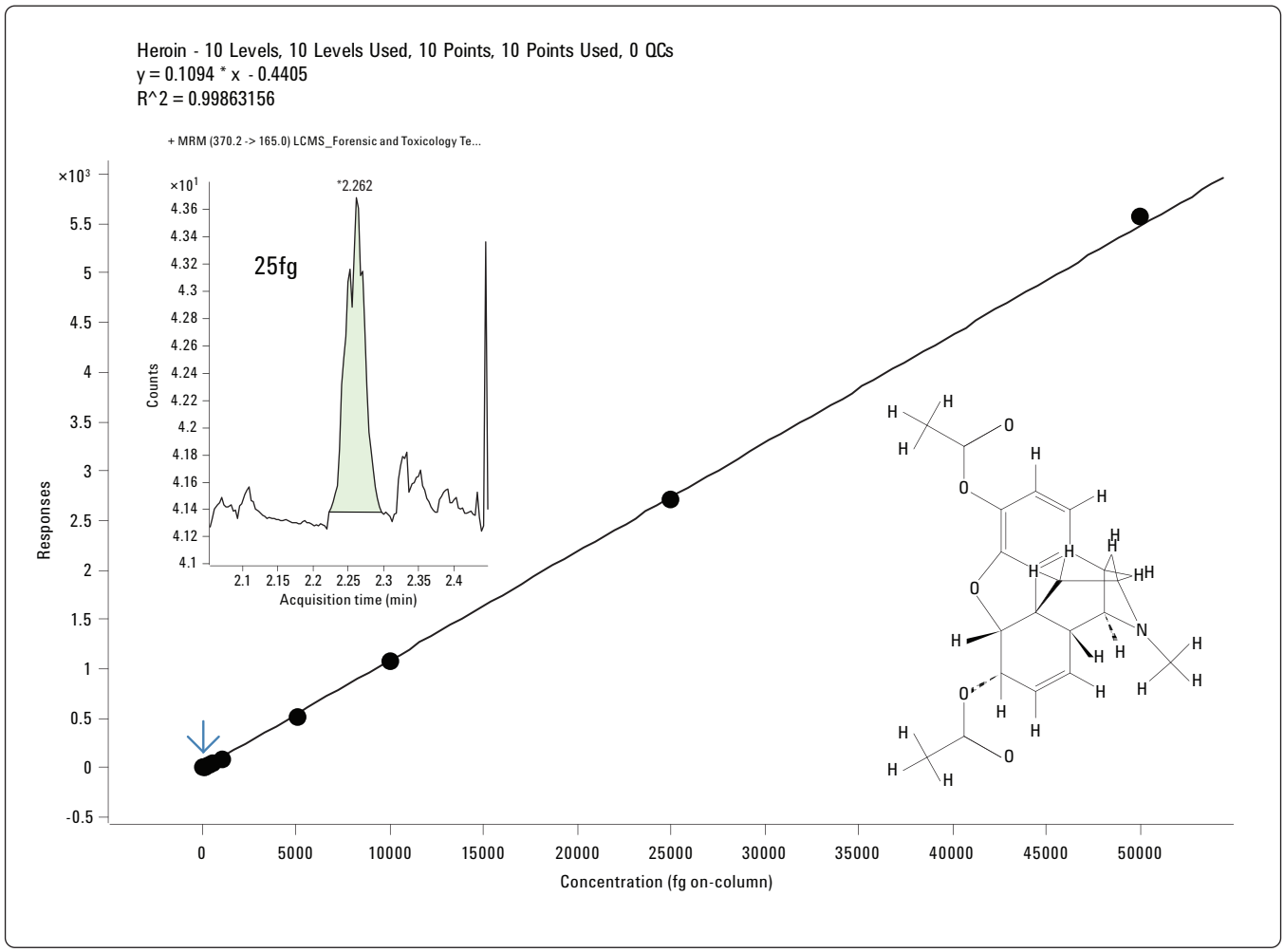

Figure 8. Calibration curve and LOD chromatogram, heroin.

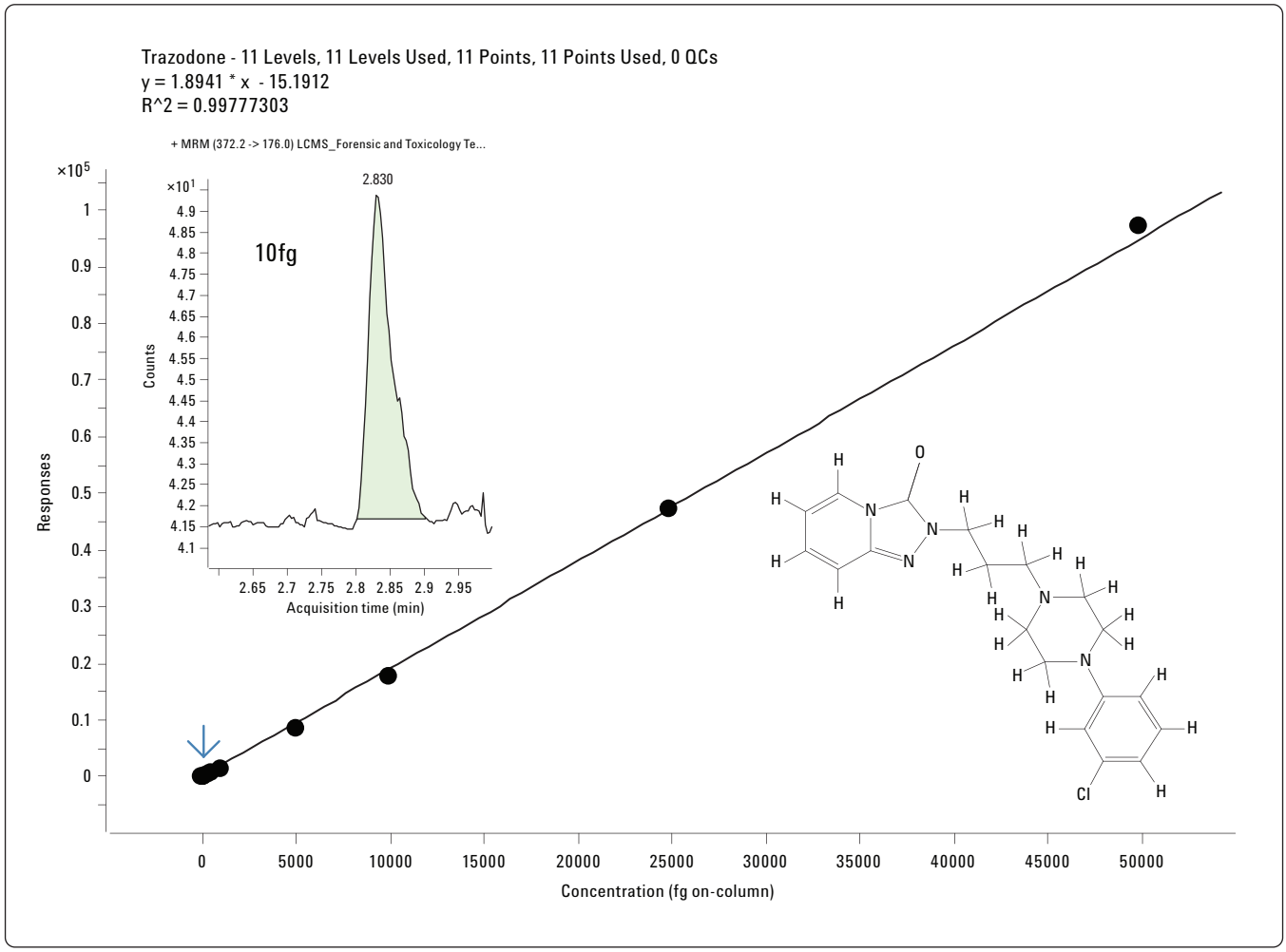

Figure 9. Calibration curve and LOD chromatogram, trazodone.

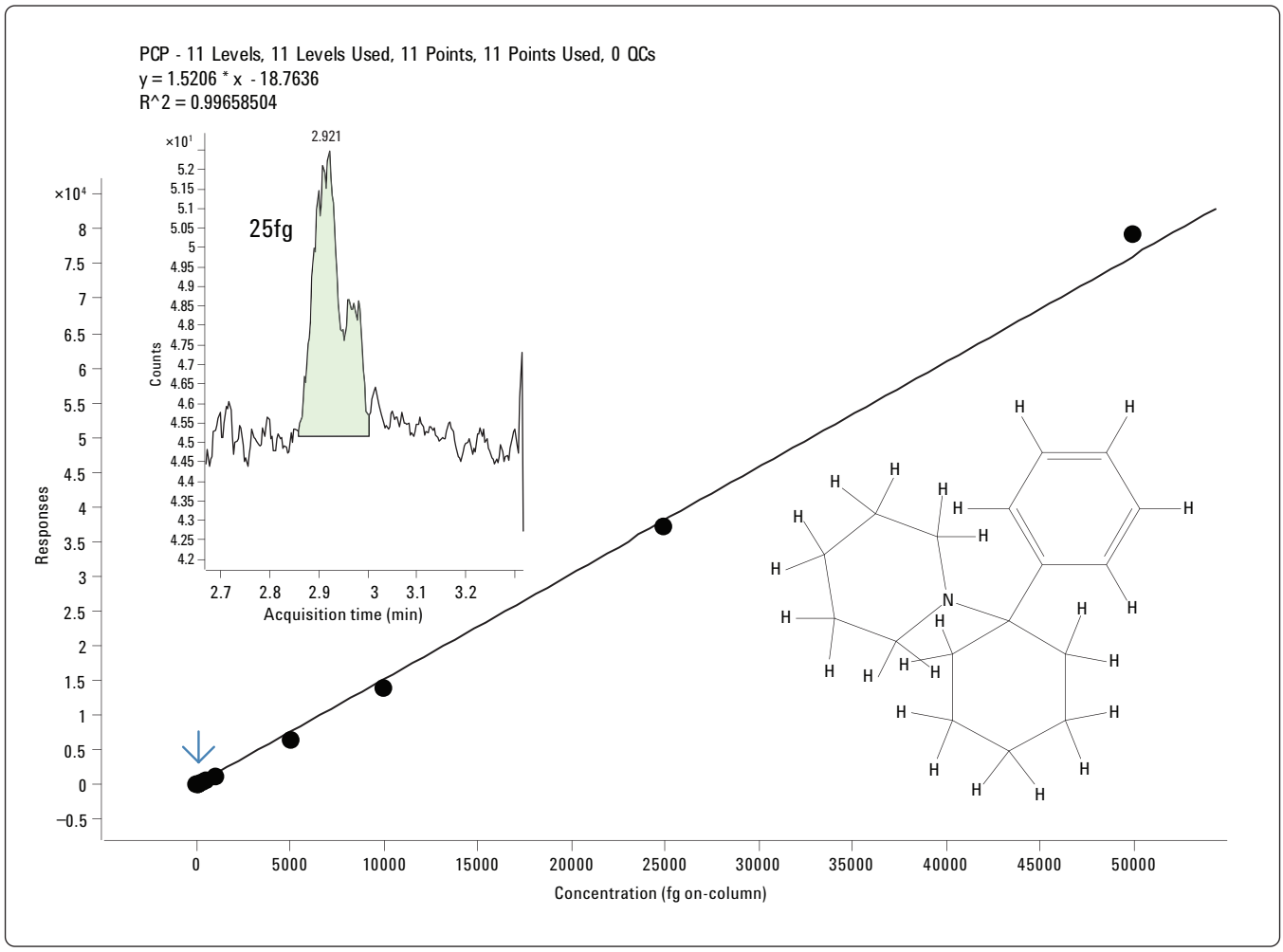

Figure 10. Calibration curve and LOD chromatogram, phencyclidine (PCP).

Conclusions

The Agilent MassHunter Forensic & Toxicology Dynamic MRM Database Kit provides a user with faster method development capability for 200 forensic analytes with up to 4 MRM transitions for each. These methods can be used equally for screening or for more focused and dedicated analyte quantitation dependant on specific needs.

This application note briefly outlines the type of results that could be obtained by using database optimized MRM parameters with the appropriate chromatography conditions and MS ion source settings.

The kit offers:

- Fast and easy startup of complex analyses.
- An optimized MRM transition database of approximately 200 forensic compounds.
- Completely customizable with additional optimized transitions to the database.
- Example chromatography with ready to use methods inclusive of test sample and chromatography column.
- Automatic re-optimization of transition parameters using the MassHunter Optimizer program for particular instrument conditions and method revalidation.

References

1. "New Dynamic MRM Mode Improves Data Quality and Triple Quad Quantification in Complex Analyses," Agilent application note publication 5990-3595EN.
2. "Agilent G1734AA MassHunter Forensics and Toxicology Dynamic MRM Database Kit Quick Start Guide." Agilent Technologies publication 5990-4265EN

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LC/MS Application Note

Number 13
February 2004

Analysis of (±)-11-Nor-9-Carboxy-Delta-9-THC in Urine by Negative Ion Electrospray LC/MS/MS

J. Beck and C. Schmidt, Varian, Inc.

Introduction

Marijuana is one of the most commonly used illegal recreational drugs in the United States. The compound that gives the "high" from smoking marijuana is tetrahydrocannabinol or THC. Toxicology, forensic, and clinical labs are often asked to analyze urine samples for the presence of one of the metabolites of THC, most frequently the carboxy form.

Traditionally, samples are screened for THC metabolites by immunoassay and confirmed using GC/MS. GC/MS, while the current standard for THC metabolite testing, requires time consuming sample derivatization prior to analysis. LC/MS provides the same specificity and sensitivity without the need for a derivatization process.

A simple, high throughput LC/MS/MS method is described here for the detection and quantitation of (±)-11-nor-9-carboxy-delta-9-THC in urine.

Instrumentation

- Varian ProStar 410 AutoSampler
- Varian ProStar 210 Solvent Delivery Modules
- Varian 1200L LC/MS/MS equipped with ESI source

Materials and Reagents

- Standard solution: 0.1 mg/mL (±)-11-Nor-9-Carboxy-Delta-9-THC (Catalog No. T-006), from Cerilliant Corp., Texas, USA.
- Internal standard (IS) solution: 500 ng/mL (±)-11-Nor-9-Carboxy-Delta-9-THC-d9 in methanol, a gift from Norchem Drug Testing, Flagstaff, AZ.
- Test samples: samples containing various amounts of (±)-11-Nor-9-Carboxy-Delta-9-THC, also gifts from Norchem Drug Testing, Flagstaff, AZ.
- All other chemicals are reagent grade or HPLC grade.

Sample Preparation

Serial dilutions of the standard solution of carboxy-THC (THCC) were prepared in deionized water. The concentrations of the samples ranged from 1 ng/mL (1 ppb) to 1000 ng/mL. A 50 µL aliquot of the internal standard solution was added to 1 mL of each sample. A 20 µL aliquot was injected directly onto the column for analysis.

Test samples from Norchem Drug Testing were prepared in 1:4 dilutions of pooled urine with deionized water.

HPLC Conditions

Column	Varian Pursuit Diphenyl 3 µm, 50 x 2 mm (Varian Part No. A3041-050X020)			
Solvent A	deionized water			
Solvent B	methanol			
LC Program	Time (min:sec)	%A	%B	Flow (mL/min)
	0:00	60	40	0.2
	0:30	60	40	0.2
	1:00	5	95	0.2
	3:00	5	95	0.2
	3:01	60	40	0.2
	6:30	60	40	0.2
Injection Volume	20 µL			

MS Parameters

Ionization Mode	ESI negative
Collision Gas	2.0 mTorr Argon
API Drying Gas	25 psi at 325 °C
API Nebulizing Gas	51 psi
Scan Time	0.5 sec
SIM Width	0.7 amu
Needle	-4200V
Capillary	-30V
Detector	1620V

Scan Parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
(±)-Carboxy-THC	343.5	299	17.5
(±)-Carboxy-THC-d9	352.5	308	20.5

Results and Discussion

The LC gradient for this analysis results in a retention time of 4.6 minutes for the THCC analyte and the IS peak. Chromatograms for the 1 ng/mL sample and 25 ng/mL IS (20 pg and 500 pg on column, respectively) are shown in Figure 1. This level is 50 times below the proposed drug cutoff level for the initial immunoassay screen published by the Substance Abuse and Mental Health Services Administration (SAMHSA)[1] and 15 times below the proposed cutoff level for the GC/MS confirmatory test.

The LC/MS/MS method described here is linear from 1 ppb to 1000 ppb as shown in Figure 2. Each calibration standard was run in triplicate and the three data points were averaged.

A series of test samples ranging from 1 ng/mL to 800 ng/mL were run on the 1200L LC/MS/MS. The calculated and actual values are shown in Table 1.

A representative chromatogram for the Norchem Drug Testing samples is shown in Figure 3. At the 1 ng/mL LOQ level, no interference is observed, demonstrating the specificity of the LC/MS/MS method.

Conclusion

The LC/MS/MS method presented in this application note is very simple and sensitive. The method eliminates the need for a time-consuming derivatization step which can take an hour or more. The Varian 1200L LC/MS/MS can be a powerful tool in forensic, clinical, and toxicology laboratories offering significant cost and time savings.

MRM Chromatograms of Standards

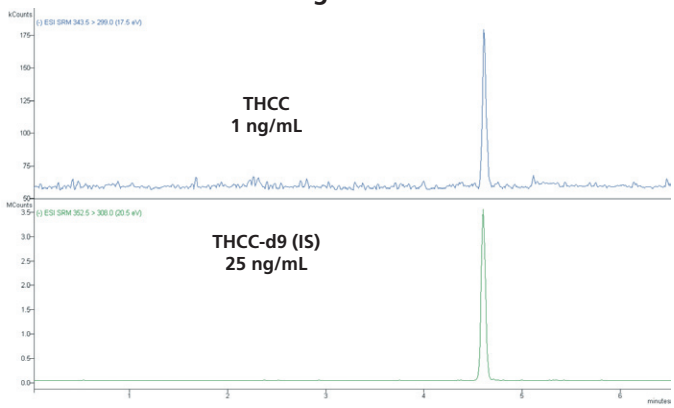

Figure 1. Signal-to-noise (300:1 RMS) is excellent at the lowest calibration level of 1 ng/mL for THCC.

Standard Calibration Curve for THCC

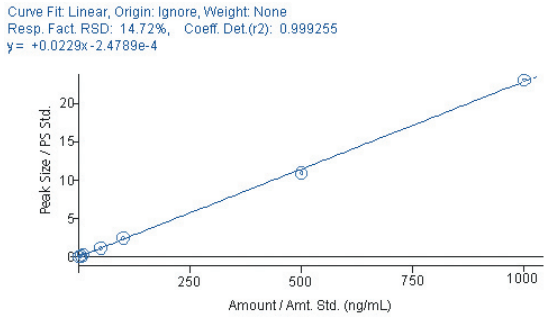

Figure 2. Seven calibration levels for THCC (1, 5, 10, 50, 100, 500, and 1000 ng/mL) with 25 ng/mL internal standard.

MRM Chromatogram of Test Sample

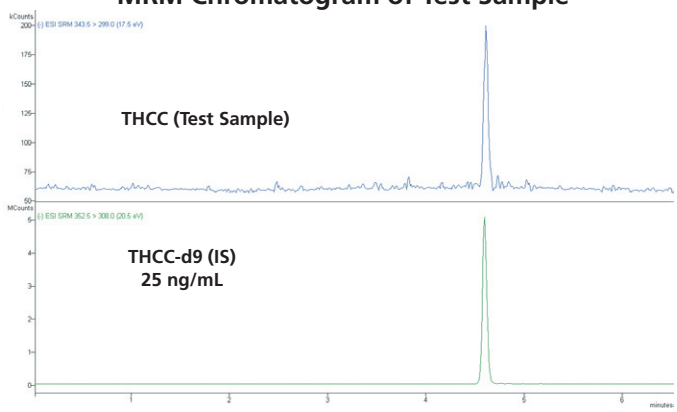

Figure 3. For the THCC test sample in diluted urine at 1 ng/mL, the calculated value based on the calibration curve is 1.1 ng/mL.

Results of LC/MS/MS Study of THCC

Sample ID	Calculated Amount (ng/mL)	Actual Amount (ng/mL)
F	1.1	1.0
E	5.4	5.5
G	10.9	11.0
D	15.3	15.0
C	106.0	100.0
B	411.0	400.0
A	802.0	800.0

Table 1. The calculated results correspond very well to the actual concentration of the spiked samples provided by Norchem Drug Testing.

Acknowledgement

The authors would like thank Dr. A. Fischinger, Norchem Drug Testing, Flagstaff, AZ for kindly supplying technical advice and the THCC test samples for this study.

Reference

1. http://workplace.samhsa.gov/ResourceCenter/DT/FA/GuidelinesDraft4.htm

These data represent typical results.
For further information, contact your local Varian Sales Office.

LC/MS Application Note

Number 10.r1
December 2003

Rapid Screening of Amphetamine Drugs in Urine by Positive Ion Electrospray LC/MS/MS

Z. Yang and S. Sadjadi, Varian, Inc.

Introduction

Amphetamine drugs are often abused and misused in sports, in the work place, and by recreational users. Forensic, clinical, and doping laboratories are frequently asked to analyze for the presence of amphetamines in urine. Urine samples are most common because large sample volumes can be collected non-invasively. These drugs generally remain detectable in urine for two to three days longer than in blood. For most clinical and forensic applications, initial screening is done by immunoassay with presumptive positive samples confirmed by a second, more specific method such as gas chromatography/mass spectrometry (GC/MS).

A simple and sensitive LC/MS/MS method is described below for high throughput identification and quantitation amphetamine drugs in urine. A rapid and effective solid-phase extraction (SPE) procedure using Focus™ was used to extract amphetamines from urine samples.

Instrumentation

- Varian ProStar 410 AutoSampler
- Varian ProStar 210 Isocratic Solvent Delivery Modules
- Varian 1200L LC/MS equipped with ESI source

Materials and Reagents

- Standard solutions: 1.0 mg/mL ((±)-Amphetamine, (±)-Methamphetamine, 1S,2R(+)-Ephedrine, (±)-MDMA, (±)-MDA and (±)-MDEA), from Cerilliant Corp., Texas, USA.
- Internal standard (IS) solutions: 0.1 mg/mL ((±)-Amphetamine-D5, (±)-Methamphetamine-D5, 1S,2R(+)-Ephedrine-D3 HCl, (±)-MDA-D5, (±)-MDMA-D5 and (±)-MDEA-D5), from Cerilliant Corp., Texas, USA.
- All other chemicals are reagent grade or HPLC grade.
- Focus™ Solid Phase Extraction Cartridges (Varian Part No. A5306021).
- In-house vacuum or vacuum pump (Varian Part No. WL2012B01).
- Vac Elut 20 Manifold with the standard Glass Basin (Varian Part No. 12234505) and Collection Rack for 13 x 75 mm test tubes (Varian Part No. 12234507).

Sample Preparation

A 100 µL aliquot of a 500 ng/mL deuterated internal standards solution was transferred into individually labeled tubes (double blank tube was urine only). To each tube, a 1 mL of urine sample followed by 0.1 mL of 0.1 N KOH solution was added and mixed by vortex.

The mixture was loaded onto the sorbent bed of an activated 3 mL Focus cartridge pretreated with 1 mL of methanol followed by a 1 mL deionized water wash under gentle vacuum of1 to 2 in. Hg. Next, the sorbent bed was washed with 2 x 1 mL acetonitrile/water (10:90, v/v) under gentle vacuum.

The analyte was collected in a 2 mL autosampler vial by eluting with 2 x 100 µL elution solvent (acetonitrile/methanol/water/formic acid (22:68:9:1, v/v) under gentle vacuum. The sorbent bed was then flushed with 600 µL of water under vacuum to wash off the elution solvent and dilute the sample for injection. A 10 µL aliquot was injected directly for analysis.

HPLC Conditions

Column	MonoChrom MS 5 µm, 50 x 2 mm (Varian Part No. A2080050X020)			
Mixer	250 µL static mixer			
Solvent A	0.2% formic acid:10mM NH_4 OAc in water (v/v)			
Solvent B	acetonitrile/methanol (1:1, v/v)			
LC Program	Time (min:sec)	%A	%B	Flow (mL/min)
	0:00	75	25	0.25
	6:00	75	25	0.25
Injection Volume	10 µL			
Injection Solvent	acetonitrile/methanol/water/formic acid (5.5:17:77.25:0.25, v/v)			

MS Parameters

Ionization Mode	ESI positive
Collision Gas	2.0 mTorr Argon
API Drying Gas	30 psi at 380 °C
API Nebulizing Gas	59 psi
Scan Time	1.8 sec
SIM Width	0.7 amu
Needle	5000V
Shield	600V
Capillary	30V
Detector	1800V

Scan Parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
(±)-Amphetamine	136	91	14.0
	136	119	6.5
(±)-Amphetamine-D5	141	96	12.5
(±)-Methamphetamine	150	91	17.0
	150	119	9.0
(±)-Methamphetamine-D5	155	92	16.5
1S,2R(+)-Ephedrine	166	117	17.0
	166	148	10.0
1S,2R(+)-Ephedrine-D3	169	151	9.5
(±)-MDA	180	105	20.5
	180	163	9.0
(±)-MDA-D5	185	168	9.0
(±)-MDMA	194	135	19.0
	194	163	10.0
(±)-MDMA-D5	199	165	10.5
(±)-MDEA	208	135	18.0
	208	163	11.5
(±)-MDEA-D5	213	163	12.0

MRM Chromatograms of Amphetamines

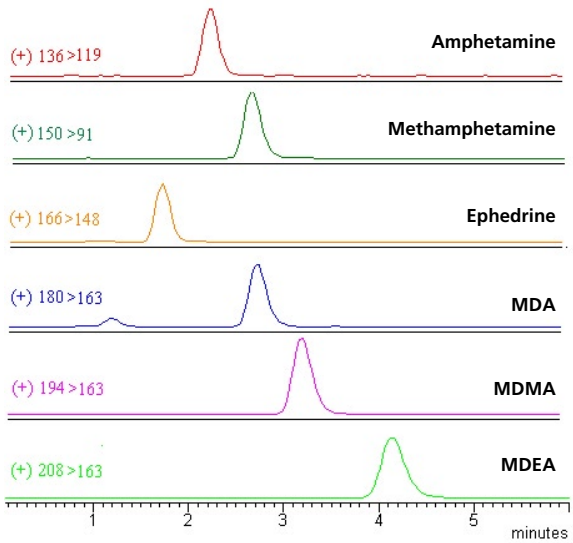

Figure 1. Good separations with short run time and no matrix interferences. Sample: spiked 50 ng/mL in urine.

Example of a Tox Report for Methamphetamine

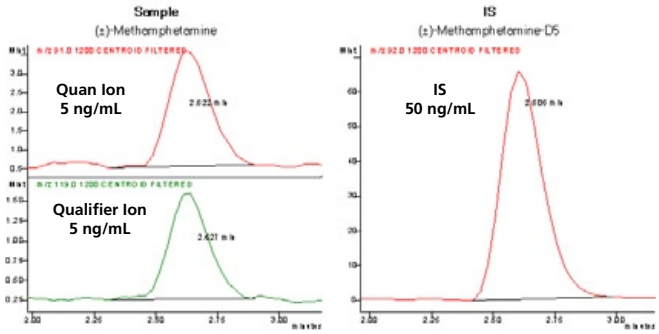

Figure 2. The positive identification was confirmed by retention time matching of the Quan ion with the confirmatory qualifier ion. The IS was used to measure and calculate recovery. Also, the IS was used to provide additional confirmation by retention time as a reference marker.

Example of a Standard Calibration Curve for Methamphetamine

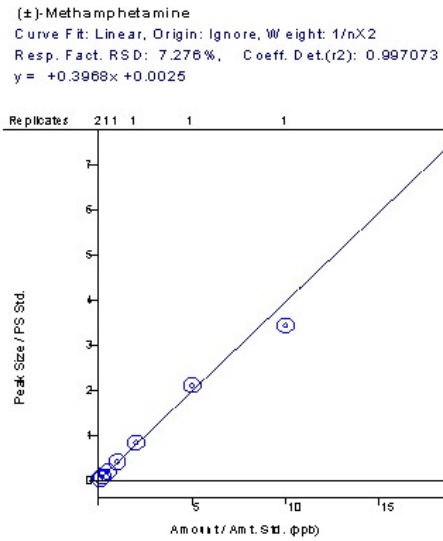

Figure 3. Eight calibration levels (5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL) standard with 50 ng/mL internal standard.

Example of Breakdown Curve for Methamphetamine

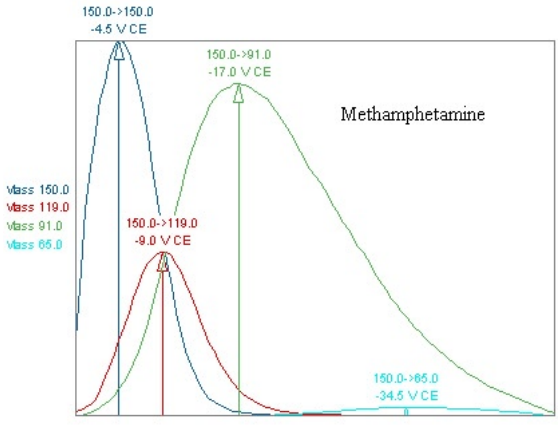

Figure 4. In this typical MS breakdown curve, methamphetamine gives two intense product ions, 150>91 and 150>119.

Results and Discussion

The LC method used a six-minute run cycle time with the first peak at 1.65 minutes and the last peak at 4.12 minutes (Figure 1). The two product ions for each analyte can be quantitatively analyzed at the level of 5 ng/mL in urine (Figure 2, about 50 pg on-column). This level is 50 times below the proposed drug cutoff levels published by the Substance Abuse and Mental Health Services Administration (SAMHSA).[1] Eight concentration levels were used to generate the calibration curves for the standard. The linearity of the detector response and the response factor-Relative Standard Deviation (rf-RSD) are excellent (Table 1, Figure 3).

The recovery of the drugs from urine was > 85%. The eluent from the Focus cartridge can be injected directly into LC/MS system without derivatization, evaporation, and reconstitute steps. The 96-well format Focus can be used for automation and high-throughput screening.

Only two product ions were used for this analysis because amphetamine and methamphetamine only give two intense product ions (Figure 4, Table 2) while ephedrine, MDA, MDMA, and MDEA produce multiple intense product ions (Table 2). Run-to-run retention time is very reproducible with a <1.4% RSD. Two product ions with a retention time match can be strong evidence for positive identification of amphetamine drugs (Figure 2). Both the urine double blank and the blank with IS show no interference of the analysis at low quantitation level (LQL). For the standard calibration curve, the LQL is 5 ng/mL and upper quantitation level (UQL) is 1000 ng/mL. This LC/MS/MS method is very sensitive and can be possibly adapted to other body fluid analysis for amphetamines, such as sweat and oral fluid which have confirmatory drug cutoff levels of 25 ng/mL and 50 ng/mL, respectively.

Conclusion

The LC/MS/MS method described in this application note is simple and sensitive. This method can quantitatively analyze amphetamine drugs at 50 times below the drug cutoff levels in urine. The Varian SPE and 1200L LC/MS/MS system demonstrated excellent performance for the urinalysis of amphetamines. The system can be a useful tool for forensics, clinical, and doping laboratories.

Reference

1. http://workplace.samhsa.gov/ResourceCenter/DT/FA/GuidelinesDraft4.htm

*ng/mL=ppb

Combined Results of LC/MS Study of Amphetamines

	Retention Time		Curve Parameters				Drug Cutoff Levels in Urine	
Drug Name	min	RSD (%)	R^2	rf-RSD (%)	LQL (ng/mL)	S/N (5 ng/mL)	Initial (ng/mL)	Confirmatory (ng/mL)
Amphetamine	2.16	0.91	0.995	7.96	5	163	500	250
Methamphetamine	2.57	0.94	0.997	7.29	5	1242	500	250
Ephedrine	1.65	0.94	0.999	3.79	5	1031	500	250
MDA	2.67	1.22	0.999	9.39	5	521	500	250
MDMA	3.14	1.24	0.999	1.86	5	729	500	250
MDEA	4.12	1.40	0.999	4.84	5	440	500	250

Table 1. Run-to-run retention time over 13 injections was very reproducible. The linearity of the detector response and the response factor-RSD are excellent. The LQL of this method is 50 times below the proposed drug cutoff levels as published by the SAMHSA.

Summary of Breakdown Data by Ion Transition, Ion Intensity, and Collision Energy (V)

Amphetamine	Methamphetamine	Ephedrine	MDA	MDMA	MDEA
136>91, 100%, -14.5	150>150, 100%, -4.5	166>148, 100%, -10.0	180>163, 100%, -9.0	194>163, 100%, -10.0	208>163, 100%, -11.5
136>136, 64.26%, -4.5	150>91, 88.54%, -17.0	166>166, 75.85%, -4.0	188>188, 50.46%, -4.0	194>194, 95.26%, -4.5	208>208, 89.31%, -4.0
136>119, 63.06%, -7.5	150>119, 43.65%, -9.0	166>117, 15.39%, 17.0	180>105, 32.34% -20.5	194>105, 33.3%, -22.0	208>105, 31.77%, -23.0
136>65, 3.99%, -32.5	150>65, 2.26%, -34.5	166>115, 14.5%, -24.0	180>133, 29.25%, -16.0	194>135, 29.93%, -19.0	208>135, 29.97%, -18.0
		166>133, 13.68%, -18.5	180>135, 29.5%, -16.0	194>133, 29.74%, -18.5	208>133, 29.11%, -18.0
		166>91, 8.82%, -29.0	180>77, 6.83%, -32.0	194>77, 6.34%, -35.5	208>103, 8.03%, -33.0

Table 2. Amphetamine and methamphetamine only give two intense product ions while ephedrine, MDA, MDMA, and MDEA produce multiple intense product ions.

These data represent typical results.
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- Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Applications by Analyte

Benzodiazepines

Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Authors

William Hudson
Agilent Technologies, Inc.

Introduction

Benzodiazepines are typically used as minor tranquilizers, hypnotics, muscle relaxants and anticonvulsants. The usefulness of benzodiazepines in the management of anxiety can often lead to abuse and dependence. Benzodiazepines are a large class of drugs and include compounds such as diazepam (Valium), chlordiazepoxide (Librium), oxazepam (Serax), lorazepam (Ativan), alprazolam (Xanax), clonazepam (Clonopin) and others. 1,4-benzodiazepines, like diazepam, nordiazepam and temazepam, are metabolized and excreted as oxazepam and oxazepam glucuronide. The nitrobenzodiazepines, like clonazepam and flunitrazepam, are metabolized to a 7-amino metabolite in urine. Flurazepam is rapidly desalkylated.

Quantitative analysis of benzodiazepines in urine by LC/MS can be difficult due to the high level of matrix components. Organic salts as well as pigments and proteins cause ion suppression and the loss of signal intensity. Bond Elut Plexa PCX SPE products are a new addition to the Plexa family based on a polymeric cation exchanger. Plexa PCX products use a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantification of basic compounds.

In addition, Plexa PCX products offer faster and highly reproducible flow rates, resulting in excellent tube-to-tube and well-to-well performance. Plexa PCX products exhibit significantly reduced ion suppression because their highly polar, hydroxylated surfaces are entirely amide-free. Therefore, the particle exterior minimizes strong binding of proteins and phospholipids. An LC/MS/MS method is presented for the quantitative determination of benzodiazepines and their target metabolites in human urine specimens with Plexa PCX tubes. Hydrolysis may also be necessary by adding 5000 units of b-glucuronidase to a 1 M acetic acid (pH=3.8) buffered urine sample. The sample is vortexed and incubated for 2 hours at 60 °C prior to extraction.

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Materials and Methods

Table 1. SPE Reagents and Solutions

2% Formic Acid	Add 2 mL of concentrated formic acid to 100 mL of DI water
Methanol	Reagent grade or better
50% Methanol	Add 50 mL of methanol to 50 mL of DI water
5% Ammonia in Methanol	Add 5 mL of concentrated ammonia to 100 mL of methanol
Bond Elut Plexa PCX 30 mg 3 mL tube (part number 12108303)	

Table 2. SPE Method

Sample Pre-treatment	1 mL human urine. Dilute 1:2 with 2% formic acid.
Condition	1. 1 mL CH_30H 2. 1 mL H_2O
Wash 1	2 mL 2% formic acid
Wash 2	2 mL 50% CH_3OH in water
Elution	1 mL 5% NH_3 in methanol

All samples are evaporated to dryness and reconstituted in 200 µL of 50:50 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 0.1% Formic acid
B: Methanol

Gradient:
t = 0-1 min 40% A : 60% B
t = 2.0-4.30 min 20% A : 80% B
t = 4.31-5.30 min 40% A : 60% B

Flow Rate: 0.2 mL/min

Column: Pursuit XRs$^{Ultra\ 2.8}$ C18, 100 x 2.0 mm (part number A7511100X020)

Table 3: MS Conditions

Transition ions and collision energy were:

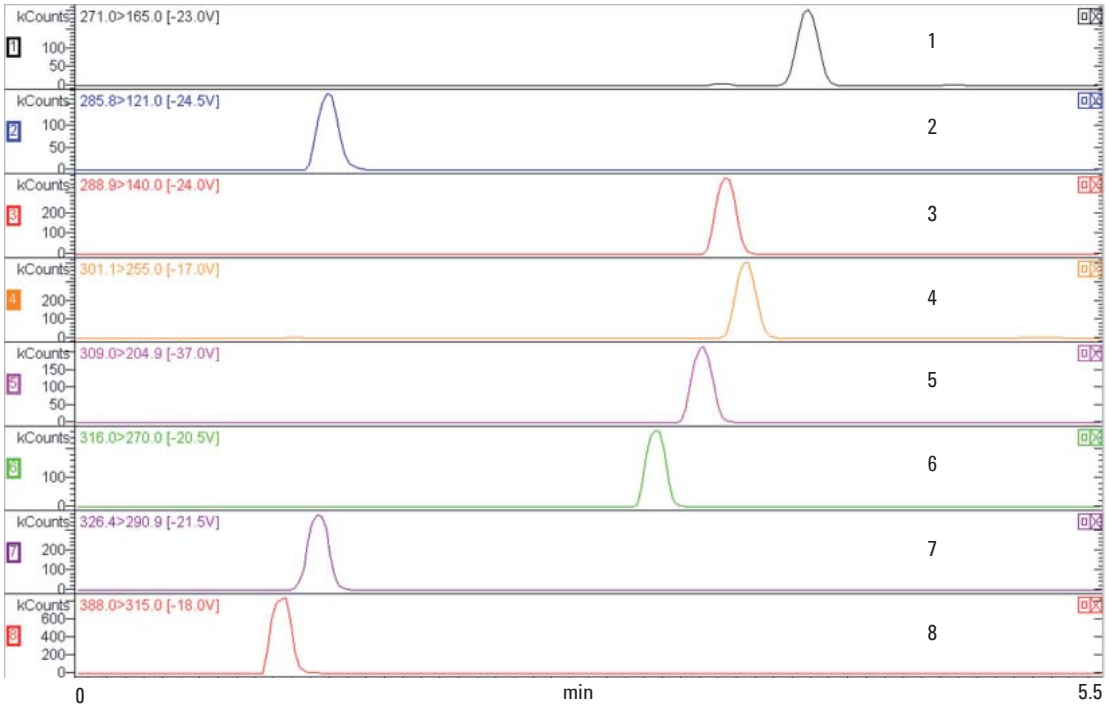
Compound	Q1	Q3	CE
Clonazepam	316.0	270.0	16.5 V
7-Aminoclonazepam	285.8	121.0	24.5 V
Flurazepam	388.0	315.0	18.0 V
Desalkylflurazepam	288.9	140.0	24.0 V
Midazolam	326.4	290.9	21.5 V
Alprazolam	309.0	204.9	37.0 V
Flunitrazepam	314.0	268.0	21.0 V
7-Aminoflunitrazepam	284.1	135.0	22.0 V
Chlordiazepoxide	300.3	227.0	19.5 V
Diazepam	285.0	222.0	20.5 V
Lorazepam	321.0	274.9	18.0 V
Oxazepam	286.8	241.0	16.5 V
Nordiazepam	271.0	165.0	23.0 V
Temazepam	301.0	255.0	17.0 V

Capillary: 70 V
Dry Gas Temperature: 350 °C, 30 psi
CID: Argon
Polarity: Negative

The procedure describes a method for extracting and determining fourteen different benzodiazepines in human urine. The Limit of Detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a 1st order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to 3 orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 4, the extractions produced very reproducibly high recoveries.

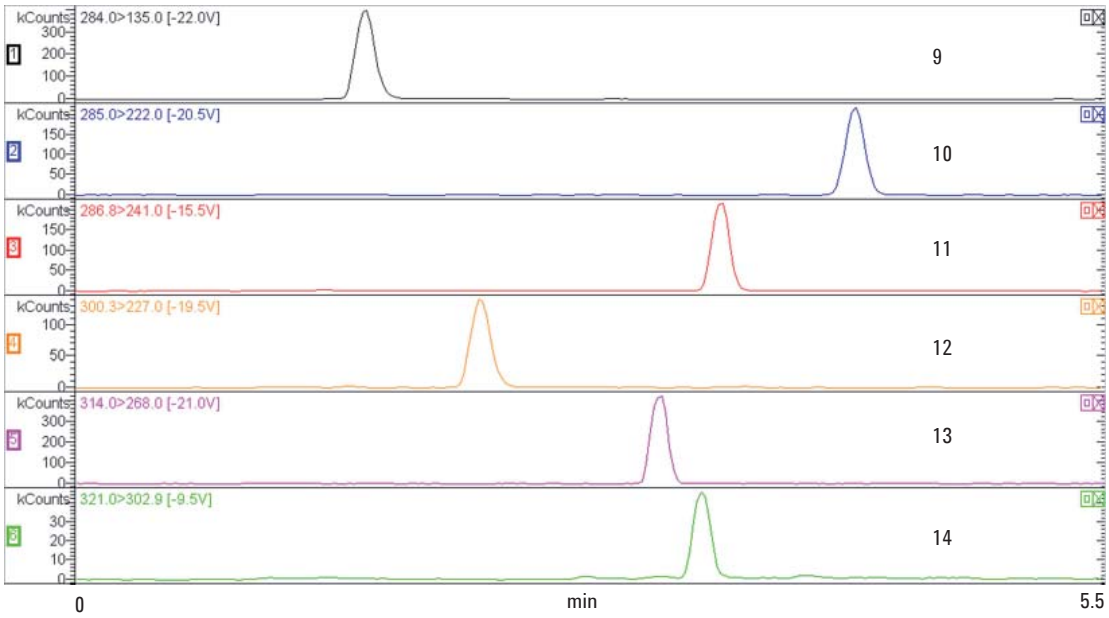
Table 4. Analyte relative recoveries

Analyte	% Rec (1 ng/mL)	% RSD	% Rec (100 ng/mL)	% RSD
Clonazepam	116	13	103	7
7-Aminoclonazepam	102	10	99	2
Flurazepam	117	14	106	8
Desalkylflurazepam	115	13	99	6
Midazolam	108	13	110	4
Nordiazepam	113	15	107	7
Alprazolam	113	17	110	8
Flunitrazepam	107	16	101	3
7-Aminoflunitrazepam	112	18	95	9
Chordiazepoxide	119	15	92	10
Diazepam	111	12	99	8
Temazepam	118	4	97	8
Lorazepam	102	14	94	10
Oxazepam	113	10	97	5



Peak Identification
1. Nordiazepam
2. 7-Aminoclonazepam
3. Desalkylflurazepam
4. Temazepam
5. Alprazolam
6. Clonazepam
7. Midazolam
8. Flurazepam

Figure 1a. Chromatograms of a 100 ng/mL urine extract (peaks 1-8)



Peak Identification
9. 7-Aminoflunitrazepam
10. Diazepam
11. Oxazepam
12. Chlordiazepoxide
13. Flunitrazepam
14. Lorazepam

Figure 1b. Chromatograms of a 100 ng/mL urine extract (peaks 9-14)

Conclusions

Bond Elut Plexa PCX products are a useful tool for high throughput SPE applications, which require analysis at low analyte levels, need validated reproducibility, and that must be quickly implemented with minimal method development. Bond Elut Plexa products meet these requirements and are therefore highly recommended for clinical and toxicological work.

With Bond Elut Plexa PCX, a generic drug extraction protocol can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent. Polar interferences and proteins are washed away with an acidic, aqueous solution. A wash with 50% aqueous methanol is possible without significant loss of analytes. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, ammoniated methanol is used to disrupt the cation exchange interaction, resulting in the elution of the benzodiazepines.

Flow rate is fast because Bond Elut Plexa PCX particles have much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent tube-to-tube reproducibility. Bond Elut Plexa tubes are therefore a useful tool for high throughput SPE applications, which require analysis at low analyte levels, validated reproducibility and quick implementation, with minimal method development.

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- Rapid Screening of Amphetamine Drugs in Urine by Positive Ion Electrospray LC/MS/MS

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- The First Accurate Mass MS/MS Library for Forensics and Toxicology Using the Agilent 6500 Series Accurate Mass Q-TOF LC/MS
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Productivity Tools

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Productivity Tools

Improving Productivity and Extending Column Life with Backflush

Application Brief

Chin-Kai Meng

All Industries

A previous application note [1] has shown that multiple GC signals and MS signals can be acquired from a single sample injection. When a 3-way splitter is connected to the end of a column, column effluent can be directed proportionally to two GC detectors as well as the MSD. This multi-signal configuration provides full-scan data for library searching, SIM data for quantitation, and element selective detector data for excellent selectivity and sensitivity from complex matrices.

The system used in this study consists of a 7683ALS, a 7890A GC with split/splitless inlet, 3-way splitter, μECD, dual flame photometric detector (DFPD), and a 5975C MSD. Figure 1 shows four chromatograms from a single injection of a milk extract. The synchronous SIM/scan feature of the 5975C MSD provides data useful for both screening (full scan data) and quantitation (SIM data). DFPD provides both P and S signals without the need to switch light filters.

Noticeably in the full scan TIC in Figure 1, a significant number of matrix peaks were observed after 32 minutes. It is not uncommon to add a "bake-out" oven ramp to clean the column after analyzing complex samples. The bake-out period is used to quickly push the late eluters out of the column to be ready for the next injection. Therefore, it is common to use a higher oven temperature than required for the analysis and an extended bake-out period at the end of a normal

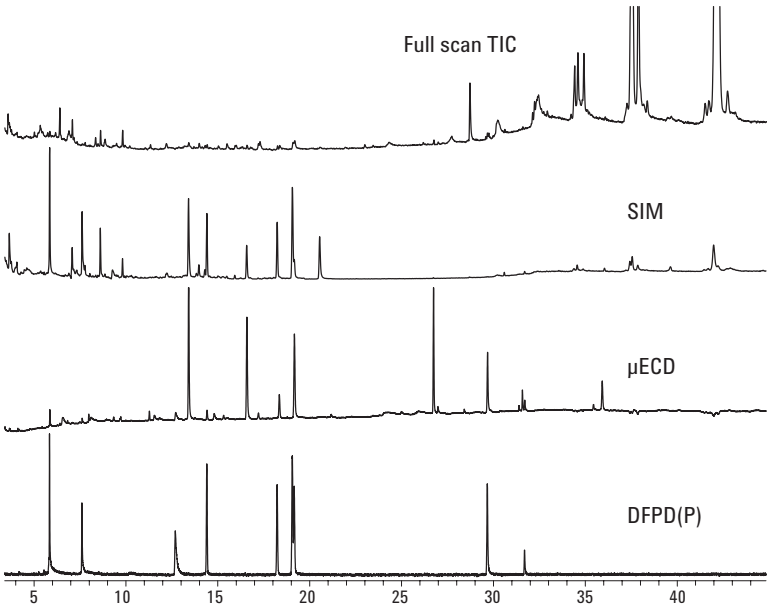

Figure 1. Four chromatograms collected simultaneously from a single injection of a milk extract.

Highlights

- Backflush – a simple technique to remove high boilers from the column faster and at a lower column temperature to cut down analysis time and increase column lifetime.
- The milk extract example shows that a 7-minute 280 °C backflush cleaned the column as well as a 33-minute 320 °C bake-out. The cycle time was reduced by more than 30%.
- Using backflush, excess column bleed and heavy residues will not be introduced into the MSD, thus reducing ion source contamination.

over program to clean out the column, which adds to the cycle time and shortens the column lifetime. Adding the bake-out period to the milk extract analysis, additional matrix peaks were observed even up to 72 minutes, while target compounds already eluted before 42 minutes. This means that 30 minutes were lost in productivity for each injection.

Backflush [2] is a simple technique to drastically decrease the cycle time by reversing the column flow to push the late eluters out of the inlet end of the column. Late eluters stay near the front of the column until the oven temperature is high enough to move them through the column. When the column flow is reversed before the late eluters start to move down the column, these late eluters will take less time and at a lower oven temperature to exit the inlet end of the column.

There are many benefits in using backflush:

- Cycle time is reduced (no bake-out period, cooling down from a lower oven temperature)
- Column bleed is reduced (no high-temperature bake-out needed), resulting longer column life
- Ghost peaks are eliminated (no high boilers carryover into subsequent runs)
- Contamination that goes into the detector is minimized, which is especially valuable for the MSD (less ion source cleaning)

Figure 2 shows three total ion chromatograms from the Agilent 7890A GC/5975C MSD. The top chromatogram is a milk extract analysis with all the target compounds eluted before 42 minutes (over program goes to 280 °C). However, an additional 33-minute bake-out period at 320 °C was needed to move the high boilers out of the column. This bake-out period was almost as long as the required time to elute all target compounds. The middle chromatogram is the same milk extract analysis stopped at 42 minutes with a 7-minute backflush post-run at 280 °C added to the analysis. The bottom chromatogram is a blank run after the backflushing was completed. The blank run shows that the column was very clean after backflushing. The example shows that a 7-minute backflush cleaned the column as well as a 33-minute bake-out.

The milk extract example in Figure 2 illustrates the backflush technique in reducing cycle time and column bleed. The cycle time was reduced by more than 30% and the column was kept at 280 °C, without going to the bake-out temperature of 320 °C. A column effluent splitter or QuickSwap is required to do the backflush.

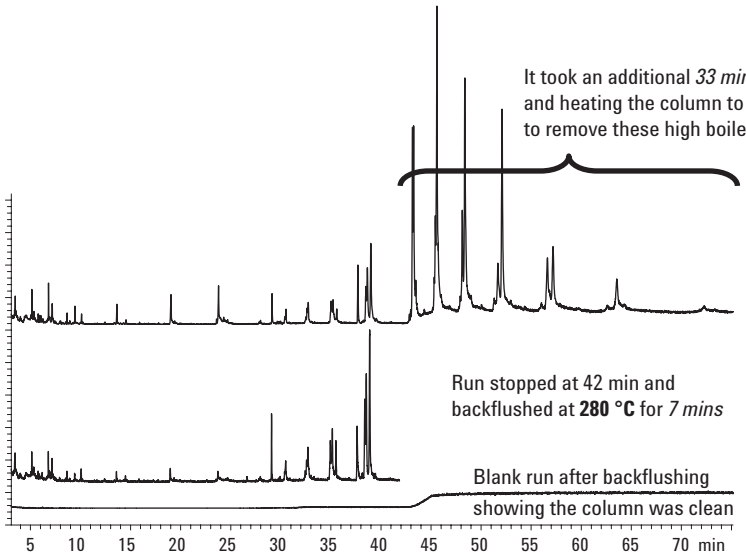

Figure 2. Three total ion chromatograms comparing the results with and without backflush.

References

1. Chin-Kai Meng and Bruce Quimby, "Identifying Pesticides with Full Scan, SIM, μECD, and FPD from a Single Injection," Agilent Application Note, 5989-3299EN, July 2005.
2. Matthew Klee, "Simplified Backflush Using Agilent 6890 GC Post Run Command," Agilent Application Note, 5989-5111EN, June 2006.

Acknowledgement

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Improved Data Quality Through Automated Sample Preparation

Application Note

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Abstract

Sample preparation tasks can be extremely time-consuming and are often prone to errors, leading to poor reproducibility and accuracy. Many of these tasks, such as calibration curve generation, sample dilution, internal standard addition, or sample derivatization are performed daily, requiring significant resources as well. The Agilent 7696 Sample Prep WorkBench can perform many common sample prep tasks with better accuracy and precision than most manual methods, while using significantly fewer reagents and requiring less time from the operator. To demonstrate this, three sample preparation tasks were adapted for use on the Agilent 7696 Sample Prep WorkBench and yielded the same, if not better, results than the manual methods for accuracy and precision.

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Introduction

The Agilent 7696 Sample Prep WorkBench can perform many sample preparation tasks for either gas chromatographic (GC) or liquid chromatographic (LC) analyses. The Agilent 7696 Sample Prep WorkBench consists of two liquid dispensing modules, a single vial heater capable of reaching 80 °C, a single vial mixer, and barcode reader (Figure 1). This enables dilutions/aliquoting, liquid addition, heating for derivatization or digestion, liquid/liquid extractions, and sample mixing. Individual racks can also be heated and/or cooled. This sample preparation instrument can perform tasks with the same accuracy and precision as the Agilent 7693A Automatic Liquid Sampler only in an offline setting instead of on top of a GC [1]. Many sample preparation tasks such as sample dilution, calibration curve standard generation, and sample derivatization within both fields can be time consuming and resource intensive. Automating these procedures with the Agilent 7696 Sample Prep WorkBench therefore is beneficial in many ways.

Figure 1. The Agilent 7696 Sample Prep WorkBench.

A side-by-side comparison of manual and automated methods was performed for three common sample prep applications to demonstrate the improved data quality achieved through automated sample preparation. Sample dilution, calibration curve standard generation, and derivatizations were performed with success on the Agilent 7696 Sample Prep WorkBench.

Experimental

Three common sample preparation tasks were performed with the Agilent 7696 Sample Prep WorkBench. First, sample dilutions and internal standard additions were performed for analysis by both GC and LC. For the GC samples, 50 µL each of isooctane and a standard solution containing four analytes were added to an empty 2-mL autosampler vial. Additionally 0.5 µL of an internal standard solution (ISTD) containing three analytes was added to the vial. The solution was mixed using the onboard mixer before transferring the vials to a GC for analysis. The samples for LC followed a similar procedure. To an empty 2-mL autosampler vial, 187.5 µL of acetonitrile, 62.5 µL of a pesticide standard, and 125 µL of an ISTD were added. The sample was mixed before being transferred to an LC for analysis. For both of these sample dilutions, n=10.

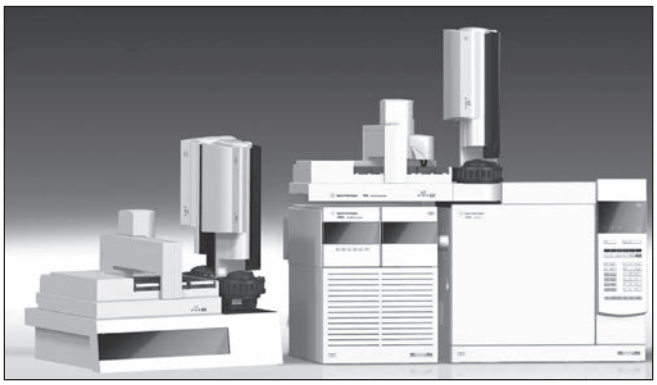

Figure 2. The Agilent 7696 Sample Prep WorkBench with a gas chromatograph and mass spectrometer.

Second, generic calibration curves for the GC were made in triplicate via linear dilution both manually in 10-mL volumetric flasks and with the Agilent 7696 Sample Prep WorkBench. To make the standards manually, small amounts of hexane was added to six clean, dry 10-mL volumetric flasks. Varying amounts of a stock solution containing five analytes at 5 mg/mL, ranging from 0.1 to 1 mL, were added using serological pipets. The flasks were diluted to the mark with hexane to yield concentrations of 50, 100, 200, 300, 400, and 500 ppm. For the automated method, 100 µL of hexane was added to six empty 2-mL autosampler vials. Again, varying amounts of the stock solution, ranging from 1 to 10 µL, was added to the vials yielding approximately the same concentrations.

Figure 3. The Agilent 7696 Sample Prep WorkBench with a liquid chromatograph.

Third, derivatization of fatty acids via silylation reaction was performed. For the manual prep, 100 µL of a silylating reagent was added to approximately 0.5 mL of a free fatty acid solution using an automatic pipettor. The solutions were heated to 70 °C using a heated block. The same derivatization was performed with the Agilent 7696 Sample Prep WorkBench using the single vial heater.

Results and Discussion

GC and LC Sample Dilution

For the 10 samples diluted for GC and LC analysis, the dispensed solvent, standard solution, and ISTD, was measured gravimetrically to determine the reproducibility of the dispensing action. Dispensing 50 µL with a 250 µL syringe results in a 0.5% relative standard deviation (RSD) for the 10 samples measured by weight. The samples were diluted within 1% accuracy, determined from the peak areas. The ISTD exhibited a slightly higher RSD. Dispensing 0.5 µL with a 25 µL syringe resulted in an RSD of 2% for the 10 samples. If a smaller syringe had been used to dispense the ISTD, a lower RSD, closer to that obtained when dispensing the solvent and standard, would have resulted. The added ISTD did not affect the accuracy of the diluted sample (Figure 4).

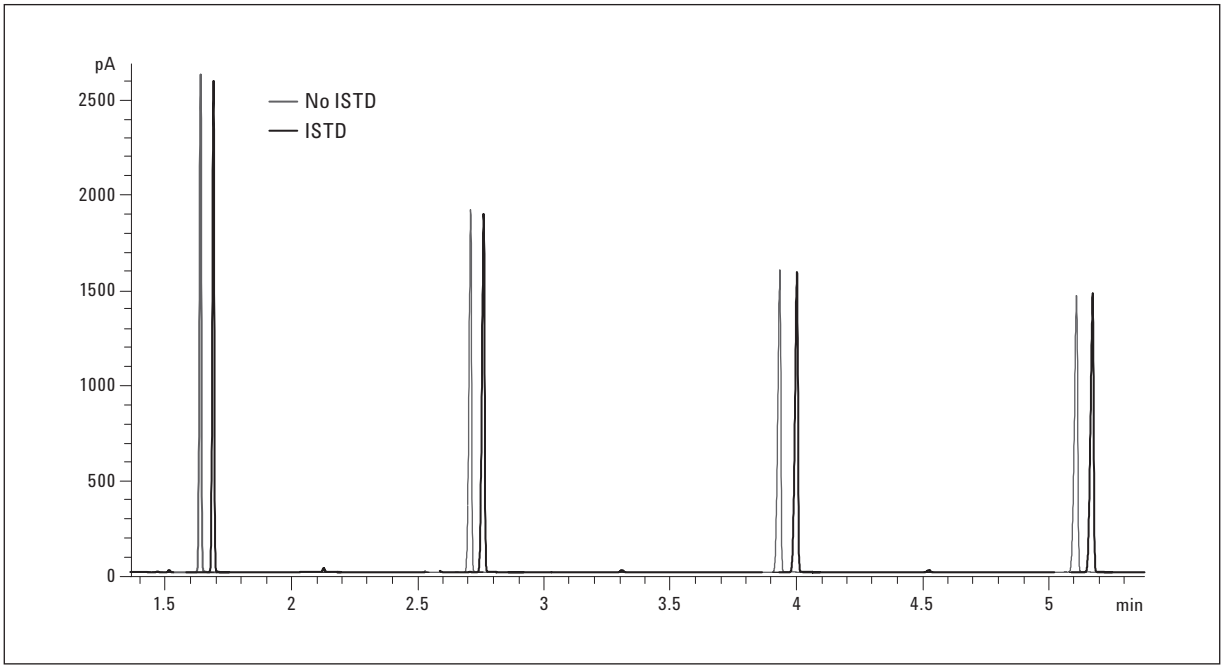

Figure 4. GC chromatograms (slightly offset) are shown for a standard solution dispensed and diluted with and without an ISTD added. No difference in peak areas are observed.

For the 10 samples diluted for LC analysis, similar results were obtained. Dispensing all three volumes with a 250 µL syringe resulted in a RSD of <0.5%, determined gravimetrically. By examining the peak areas after analysis, the dilutions were found to be accurate within 2% (Figure 5).

Calibration Curve Standard Preparation

Three sets of standards were made both manually and with the Agilent 7696 Sample Prep WorkBench. Comparing the three standard sets on the same plot highlighted the increased reproducibility of the Agilent 7696 Sample Prep WorkBench (Figure 6). While each individual curve yielded R^2 values of 0.999, when plotted together the R^2 value was reduced to 0.934 for the manually prepared standards. In contrast, the three curves prepared by the Agilent 7696 Sample prep WorkBench also yielded R^2 values of 0.999 for the individual curves, but when plotted together, the R^2 value was only reduced to 0.997.

Additionally, the relative response factor (RRF) was calculated for each set of standards. Calculating the RSD of the RRFs provides a measure of linearity and reproducibility. The individual calibration curves yielded good RSDs (<5%), demonstrating linear relationships. However, when comparing the three calibration curves together the superiority of the 7696 Sample Prep WorkBench made standards is evident. The average RSD of the RRFs for the three curves made manually was 16%; the three calibration curves made with the 7696 Sample Prep WorkBench gave an average RRF RSD of 4%.

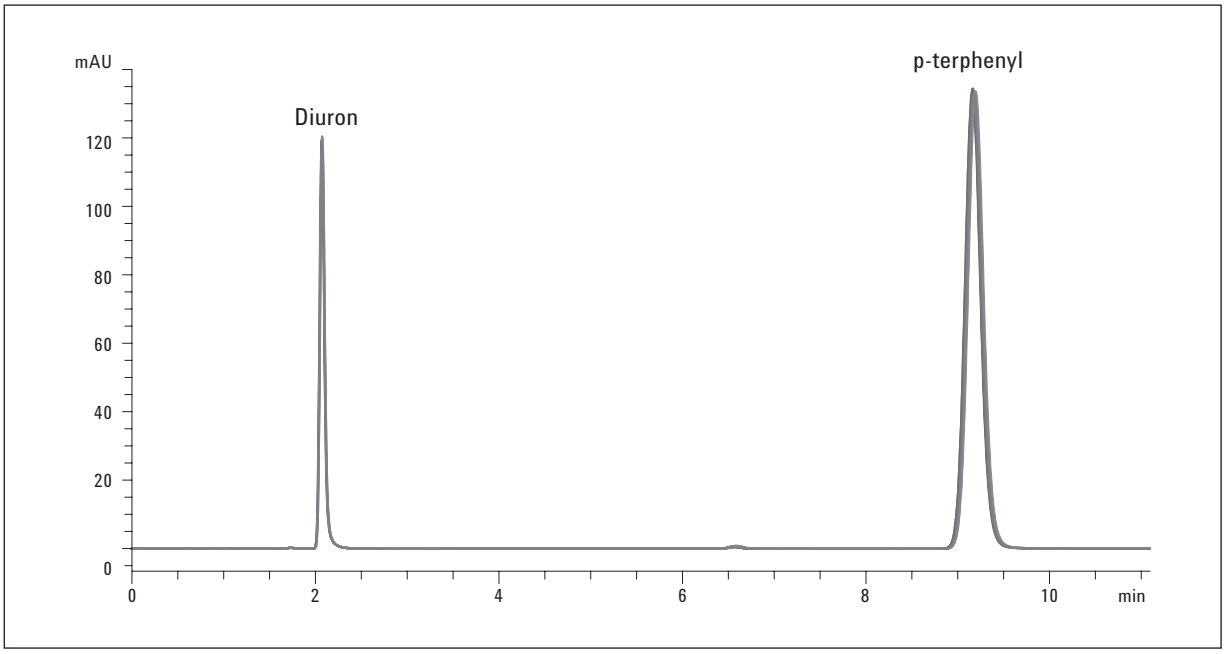

Figure 5. LC Chromatograms are shown for a diluted pesticide standard with an ISTD added. Excellent reproducibility was observed for the five samples shown.

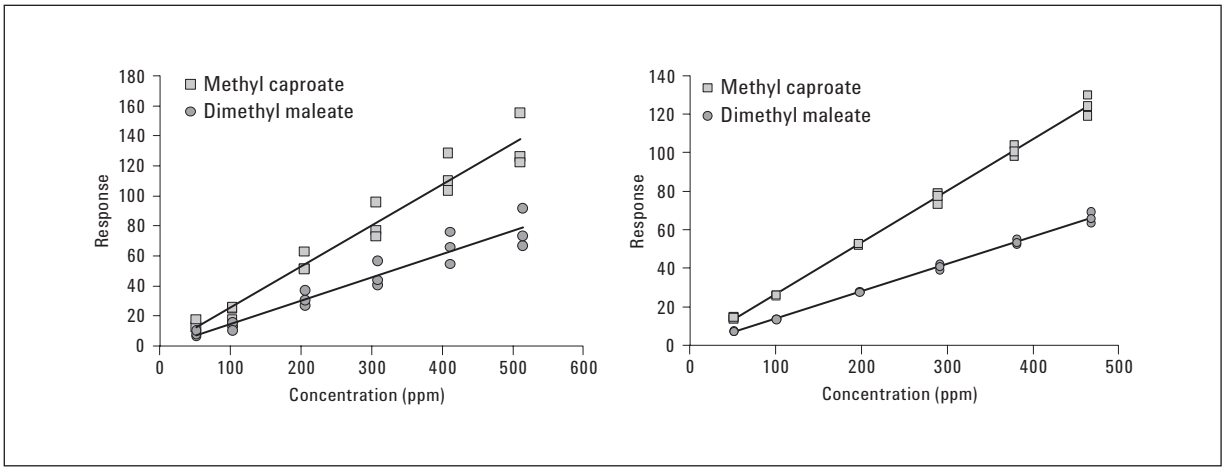

Figure 6. Two calibration curves are shown for two representative analytes. The curves on the right, prepared with the Agilent 7696 Sample Prep WorkBench, are visibly more reproducible than the curves made manually on the left.

Fatty Acid Derivatization

For sample derivatization, identical results were obtained whether the sample was derivatized manually or with the Agilent 7696 Sample Prep WorkBench. For a set of four fatty acids, no discrimination was observed in either method when derivatizing with a silylating reagent (Table 1). However, as seen with other sample preparation tasks, the Agilent 7696 Sample Prep WorkBench is more reproducible in its liquid delivery. The RSD from the peak areas for the three samples prepared manually 0.9%. The RSD for the three samples prepared with the Agilent 7696 Sample Prep WorkBench was 0.7%.

Table 1. After normalizing the fatty acid peak areas to myristic acid, no discrimination was observed from automating the derivatization

Analyte	Ratio-manual	Ratio-automated
Capric acid	0.92	0.92
Capric acid	1.2	1.2
Myristic acid	1.0	1.0
Palmitic acid	1.1	1.1

Conclusions

The three sample preparation tasks presented in this application note highlight the increased reproducibility achieved by automation with the Agilent 7696 Sample Prep WorkBench. Sample dilutions are accurate and reproducible, calibration curve standards are more linear with fewer errors, and sample derivatizations can be performed without analyte discrimination. However, additional benefits can be reaped through sample prep automation with the Agilent 7696 Sample Prep WorkBench.

By automating calibration curve standard preparation, solvent and reagent usage is significantly reduced. Instead of using >60 mL of solvent to make up standards in 10-mL flasks, only 600 µL of solvent was used, excluding the wash vials. This can result in substantial cost savings for laboratories. Additionally, calibrations curve standards required approximately half the time to complete with the Agilent 7696 Sample Prep WorkBench, compared to making up the standards manually. While the other automated sample prep tasks require the same amount of time to complete as the manual methods, the Agilent 7696 Sample Prep WorkBench frees the operator to perform other tasks, such as experiment design or data analysis.

Overall there are many benefits to sample prep automation with the Agilent 7696 Sample Prep WorkBench. While freeing personnel to perform other tasks and reduced solvent usage are important, the largest benefit comes from the reproducibility and accuracy achieved with this system. The automated methods showed better reproducibility and accuracy with fewer errors, thereby improving the quality of the data.

Reference

1. Susanne Moyer, Dale Synder, Rebecca Veeneman, and Bill Wilson, "Typical Injection Performance for the Agilent 7693A Autoinjector," Agilent Technologies Publication 5990-4606EN.

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Retention Time Locking: Concepts and Applications

Application

Gas Chromatography

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Abstract

The concepts and applications of retention time locking (RTL) are described. RTL simplifies the process of transferring methods from chromatographic instrument to chromatographic instrument, column to column, and detector to detector. The analysis of impurities in styrene according to ASTM D 5135 is used to demonstrate the efficacy of the approach. Using RTL, the retention times matched within an average of 0.16% (0.02–0.03 minute) in constant pressure modes.

Key Words

Retention time locking, method validation, styrene analysis, ASTM D 5135, capillary gas chromatography, laboratory productivity

Introduction

Retention time is the fundamental qualitative measurement of chromatography. Most peak identification is performed by comparing the retention time of the unknown peak with that of a standard. It is much easier to identify peaks and validate methods if there is no variation in the retention time of each analyte.

However, shifts in retention time occur frequently. Routine maintenance procedures such as column trimming alter retention times. In a multi-instrument laboratory running duplicate methods, the retention times for each instrument will differ from each other, even when run under nominally identical conditions. These differences in retention times mean that each instrument must have a separate calibration and integration event table, making it time-consuming to transfer methods from one instrument to another. Differences in retention time also complicate comparison of data between instruments and over time.

Retention time locking (RTL) is the ability to very closely match chromatographic retention times in any Agilent 6890 gas chromatograph (GC) system to those in another 6890 GC system with the same nominal column.

There are several subtle effects that combine to cause retention time differences between similarly configured GC systems. Columns of the same part number can vary slightly in length, diameter, and film thickness.

GC pneumatics can have small variations in the actual inlet pressure applied at a given setpoint. The actual temperature of the GC oven also has minute but real deviations from the indicated value. The sum of these and other effects result in the observed retention time differences between similarly configured GC systems.

The pneumatics and oven temperature control of the 6890 GC have advanced the state of the art in GC hardware accuracy and precision. Agilent's advances in fused silica capillary column technology have resulted in highly reproducible column-to-column retention characteristics. With these advances, retention time precision for a given peak in a single GC setup is usually better than 0.01 minute. However, even with these advances in columns and instrument hardware, the sum of the effects mentioned above can cause retention time differences between identically configured GC systems of as much as 0.4 minute.

It would be impractical to control all of the instrument and column variables to a degree where retention time differences between similarly configured GC systems are removed. There is, however, a means of greatly reducing these differences. By making an adjustment in the inlet pressure, the retention times on a given GC setup can be closely matched to those of a similarly configured GC system. RTL is based on this principle. The process of RTL is to determine what adjustment in inlet pressure is necessary to achieve the desired match in retention times. Agilent RTL software (G2080AA), which integrates into the Agilent GC ChemStation (version A.05.02 or later), provides the tool required to determine the correct inlet pressure quickly and simply.

There are several advantages gained by using RTL in the laboratory. Peak identification becomes easier and more reliable. It is easier to compare data both between instruments and over time. Comparison of data when using different detectors for analyte identification is simplified. Transferring methods from instrument to instrument or laboratory to laboratory is easier because calibration time windows normally will not require readjustment. Validation of system performance is easier. With "locked" GC methods, the development and use of retention time data bases for unknown identification is much more straightforward.

To maintain a locked method, RTL should be performed whenever:

- The column is changed or trimmed
- The method is installed on a new instrument
- A detector of different outlet pressure is used
- System performance is validated
- Troubleshooting chromatographic problems

To lock a given method for the first-time or for the reasons below, one must first develop a retention time versus pressure (RT vs. P) calibration.

Even when using columns with the same part number (same id, stationary phase type, phase ratio, and same nominal length), separate/different locking calibration curves are needed when using:

- Systems with different column outlet pressures (FID/atmospheric, MSD/vacuum, AED/elevated)
- Columns differing from the "nominal" length by more than 15% (e.g., due to trimming)
- Systems where the predicted locking pressure falls outside the range of the current calibration

A specific solute (usually one found in the normal method calibration standard) must be chosen and then used for both developing the locking calibration and locking all future systems. The solute, or target peak, should be easily identifiable, symmetrical, and should elute in the most critical part of the chromatogram. Solutes that are very polar or subject to degradation should be avoided.

Once the target solute has been chosen and all other chromatographic parameters of the method have been determined, five calibration runs are performed. The runs are made at conditions identical to the nominal method except that four of the runs are made at different pressures. The pressures used are typically:

- Target pressure – 20%
- Target pressure – 10%
- Target pressure (nominal method pressure)
- Target pressure + 10%
- Target pressure + 20%

The retention time of the target compound is determined for each run. The resulting five pairs of inlet pressures and corresponding retention times are entered into the ChemStation software to generate an RTL calibration file.

Figure 1 shows the dialog box used to enter the calibration data. After the data is entered, a plot is displayed, as shown in figure 2. The maximum departure of the fitted curve from the data is given for both time and pressure. If the fit is acceptable, the retention time versus pressure calibration is stored and becomes part of the GC

method. This calibration need only be generated once. Subsequent users of the method can use this calibration when running the method on a similar instrument setup, regardless of location.

To relock a system or lock a new one:

1. Set up the method conditions and run a standard containing the target compound.
2. Enter the actual retention time of the target compound into the "(Re)Lock current method" dialog box (see figure 3).
3. Update the 6890 method with the new calculated pressure, and save the method.
4. Validate the retention time lock by injecting the standard at the new pressure, and compare the retention time obtained to the desired retention time.
5. Repeat steps 2 to 4, if necessary.

A Note on Constant Flow versus Constant Pressure Modes of EPC Operation

Many GC chromatographers prefer to use the "constant flow mode" of EPC operation. In this mode, inlet pressure increases automatically to maintain constant outlet flow rate as the oven temperature increases during the run. Constant flow mode reduces run time and ensures that flow-sensitive detectors see a constant column effluent flow.

The "constant pressure" mode of EPC operation is also popular. In this mode, the pressure remains constant during the run (outlet flow will decrease as temperature increases). For those wishing to reduce run time in constant pressure mode, a higher pressure can be chosen. For

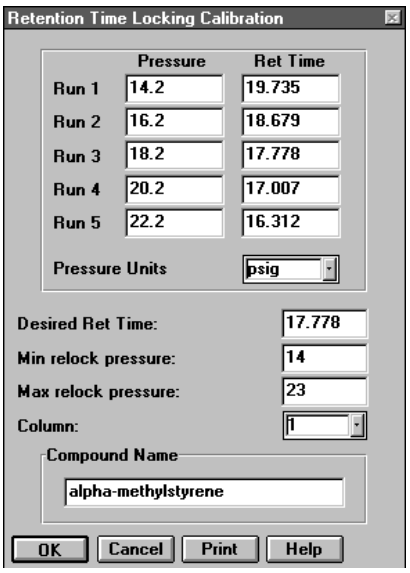

Figure 1. Dialog box used for entering retention time locking calibration data

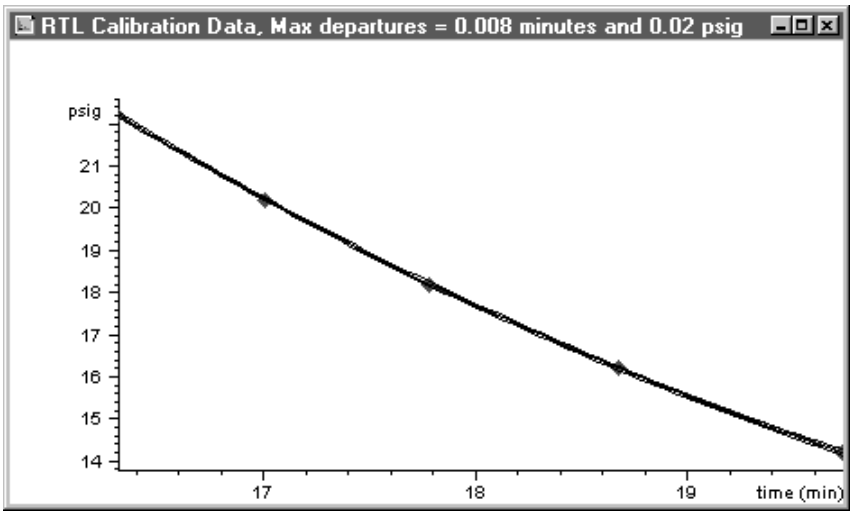

Figure 2. Plot of calibration data as displayed by RTL software

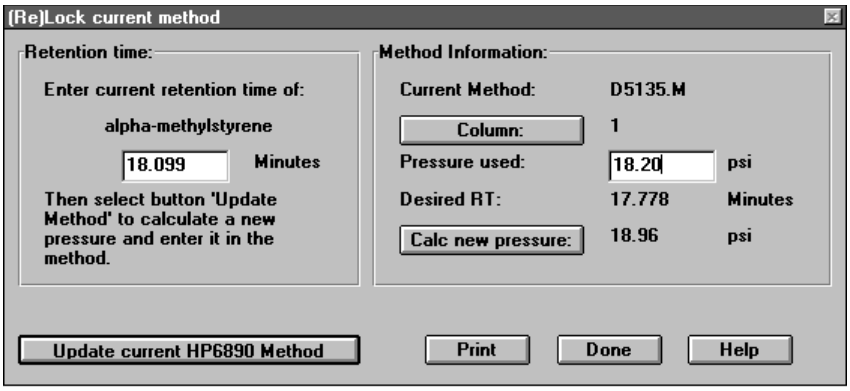

Figure 3. Dialog box used to calculate locking pressure and update the 6890 method

flow-sensitive detectors, one can set "constant column flow + makeup" via the 6890 keyboard or ChemStation. In this mode, the makeup flow is increased as the column flow decreases to keep the sum of the two constant.

The underlying theory of RTL predicts that constant pressure mode of EPC provides the closest matching of retention times. If one desires to compare data from systems with very different configurations, such as GC/FID to GC/MSD, it is best to use constant pressure mode. As can be seen from the styrene analysis data herein, retention time matching between systems of the same configuration (GC/FID, in this case) is still quite good in the constant flow mode.

This application note shows the use of RTL to lock retention times between multiple chromatographic instruments, columns, and detector types and demonstrates RTL in both constant flow and constant pressure modes.

Experimental

Two 6890 Series GC systems were used. Each system was equipped with:

- Electronic pneumatics control (EPC)
- Split/splitless inlet (250 °C, He carrier gas, split 80:1)
- Automatic liquid sampler
- GC ChemStation (version A.05.02)
- Flame ionization detector (FID)
- 60 m × 0.32 mm, 0.5 mm HP-INNOWax column (part no. 19091N-216)
- Temperature program: 80 °C (9 min), 5 °C/min to 150 °C

The inlet pressures/flows used are indicated with each chromatogram.

A third 6890 Series GC was also used. This system was equipped with an Agilent 5973 mass selective detector (MSD) and was used for peak identification. The GC-MSD chromatographic parameters used were the same as the GC systems noted above except for the inlet pressures as indicated.

Results and Discussion

GC-FID to GC-FID Locking

Figure 4 shows the original chromatogram (GC system 1) obtained from running a styrene sample under the conditions specified in ASTM D 5135.[1] Many of the typical impurities found in styrene are found here. The phenylacetylene peak represents about 60 ppm. The peaks are identified in table 1.

The sample was then run at four other pressures to collect the five data pairs for RTL calibration. Because this method was run in constant flow mode, the pressures entered into the RTL software were the initial pressures. The α-methylstyrene peak (peak 10) was chosen as the target compound. The calibration data are shown in figure 1.

The method conditions and RTL calibration were then moved to GC system 2, a different GC and column. The sample was run at the original method inlet pressure of 18.2 psi. The chromatogram obtained using this scouting run is overlaid on the original chromatogram in figure 5. The retention times shifted about 0.3 minute on the second GC. This is a typical result obtained when trying to replicate an analysis on a second instrument or with a second column.

The retention time of α-methylstyrene was entered into the RTL software

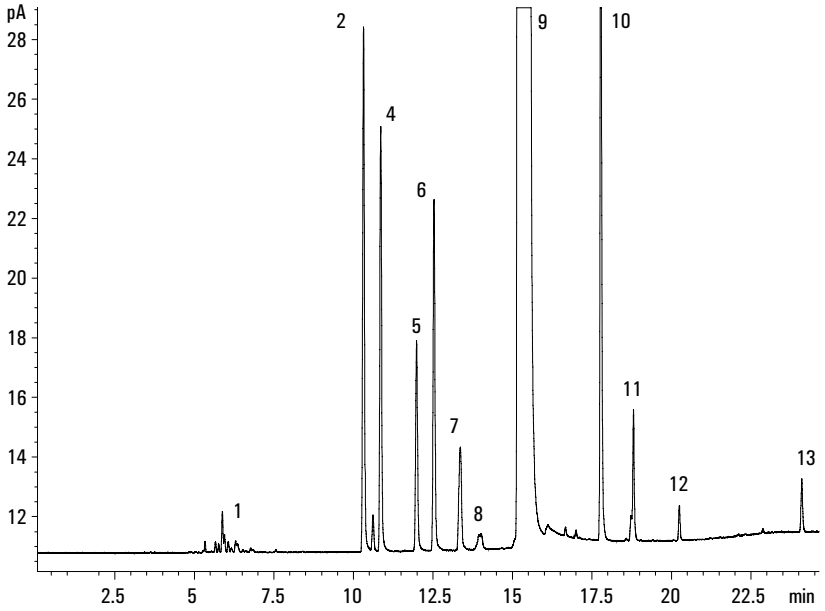

Figure 4. Styrene sample run on GC system 1 at 18.2 psi initial pressure, constant flow mode

dialog box on GC system 2, as shown in figure 3. The RTL software indicated the initial pressure should be modified from 18.2 psi to 18.96 psi. The new initial pressure was entered into the method and saved.

Figure 6 compares the chromatograms obtained from the original run and after locking retention times using the α-methylstyrene. Table 2 compares the retention times before and after using this approach. The retention times are now closely matched.

GC-FID to GC-MSD Locking

A second experiment was conducted to lock the original method from GC system 1 to the GC-MSD. This is useful for identification of unknown impurities that show up in the FID chromatogram. For example, there is a shoulder evident on the front side of the phenylacetylene peak in figure 4. It would simplify locating the impurity in the GC-MSD data if the retention times closely matched that of the GC-FID.

Because constant pressure mode is preferred when comparing data from FID and MSD systems, constant pressure mode was chosen, and the styrene sample was re-run on GC system 1 at 18.2 psi for reference.

The next step was to determine the chromatographic conditions to be used on the GC-MSD. The Agilent method translation software tool was used to calculate the conditions necessary to have the peaks elute in the identical order on the two systems.[2,3] Because the retention times need to match, the dead time and temperature program used for running the GC-MSD must be the same as the GC

Table 1. Peak Identities for Figure 4

Peak #	Name	Peak #	Name
1	Nonaromatics	8	p/m-Ethyltoluene
2	Ethylbenzene	9	Styrene
3	p-Xylene	10	α-Methylstyrene
4	m-Xylene	11	Phenylacetylene
5	i-Propylbenzene	12	β-Methylstyrene
6	o-Xylene	13	Benzaldehyde
7	n-Propylbenzene		

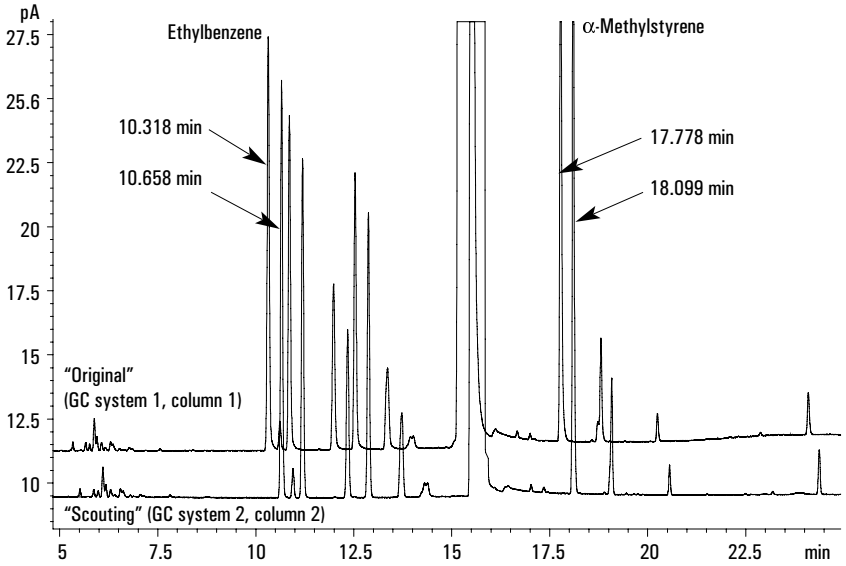

Figure 5. Comparison of original chromatogram on GC system 1 with GC system 2 before retention time locking

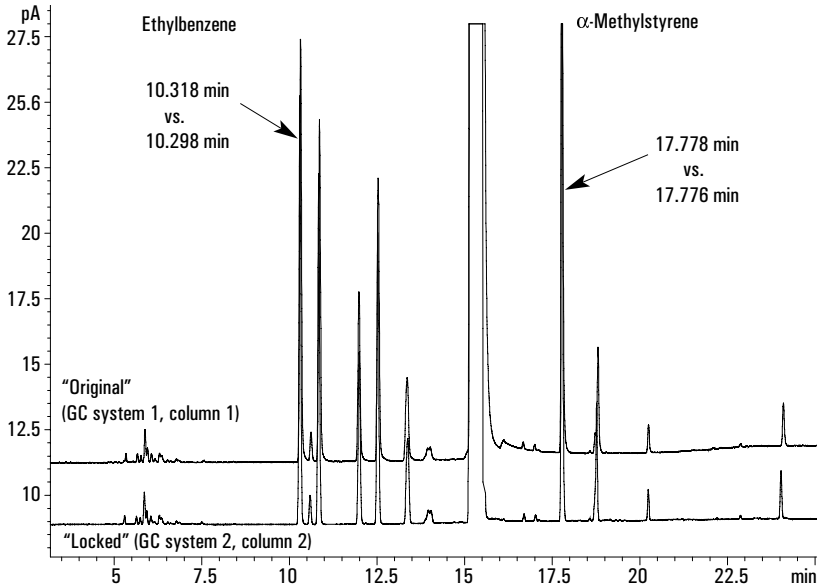

Figure 6. Comparison of original chromatogram on GC system 1 with GC System 2 after retention time locking

method. The pressure used, however, will be different due to the difference in column outlet pressure. The GC-MSD inlet pressure is calculated using the "none" mode of the method translation software (figure 7). In this mode, the holdup time between the two columns was forced to be identical to the GC-FID. This gives a speed gain of 1. The pressure calculated for use on the GC-MSD was 8.44 psi. Note that this calculated pressure is only the nominal pressure required to get similar retention times, not the exact locking pressure.

A different RTL calibration is required for GC-MSD because the outlet pressure is vacuum, and that of the FID is atmospheric pressure. Five runs were made on the GC-MSD system bracketing the 8.44 psi nominal method pressure. Because the GC-MSD used in this study was not equipped with RTL software, a dummy method was created in GC system 1 and the GC-MSD RTL calibration data was entered into it. A scouting run of the Styrene sample was made on the GC-MSD, and the α-methylstyrene retention time was used for locking. The locking inlet pressure calculated with the dummy method was 7.9 psi and was entered into the GC-MSD.

Figure 8 shows the resulting matched chromatograms from the GC-FID and GC-MSD. As seen in table 3, the retention times are now closely matched within 0.02 minute.

Figure 9 shows the MSD first choice of library search result of the impurity that created the shoulder on the front side of the Phenylacetylene peak. RTL ensured that this shoulder remained separated on the MSD system and eluted at the same time for easy comparison to the FID results.

Table 2. GC-FID Retention Times Before and After Locking for Styrene Impurities (Constant Flow Conditions). Chromatograms Shown in Figures 4, 5, and 6.

Component	Original Run GC 1/Column 1 18.2 psi	GC2–GC1 Before RTL	Scouting Run GC 2/Column 2 18.2 psi	GC2–GC1 After RTL	Locking Run GC 2/Column 2 19.0 psi
Ethylbenzene	10.318	0.340	10.658	–0.020	10.298
p-Xylene	10.616	0.333	10.949	–0.026	10.590
m-Xylene	10.858	0.337	11.195	–0.022	10.836
i-Propylbenzene	11.985	0.359	12.344	+0.005	11.990
o-Xylene	12.533	0.345	12.878	–0.012	12.521
n-Propylbenzene	13..360	0.364	13.724	–0.016	13.376
α-Methylstyrene*	17.778	0.321	18.099	–0.002	17.776
Phenylacetylene	18.806	0.275	19.081	–0.040	18.766
β-Methylstyrene	20.248	0.310	20.558	–0.006	20.242
Benzaldehyde	24.097	0.279	24.376	–0.069	24.028
Average Δ		0.326		0.028	

* Used in locking calculation

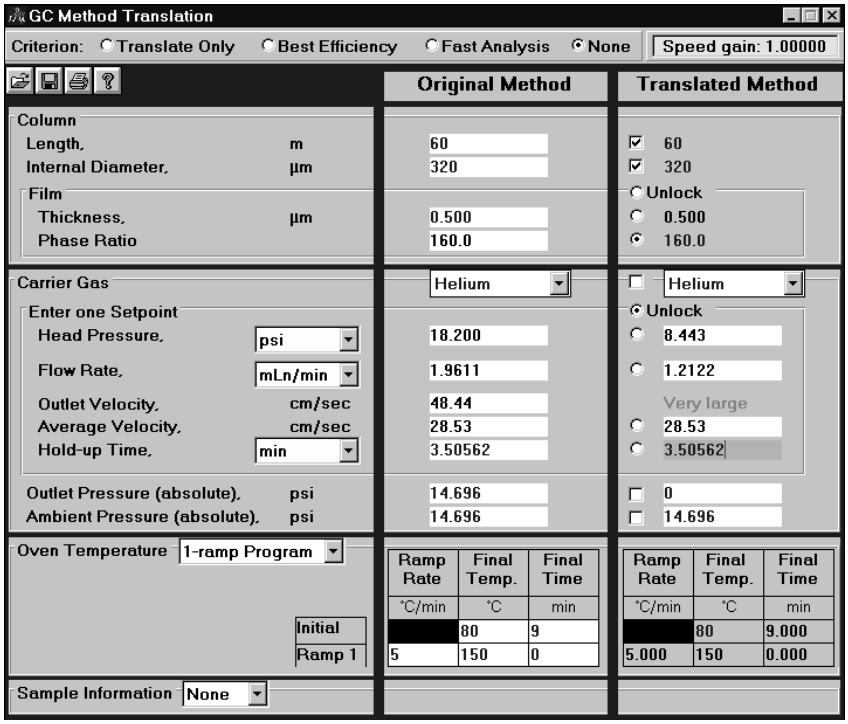

Figure 7. Method translation software provides scaled conditions for GC systems with different configurations

Conclusions

Retention time locking facilitates replicating results from instrument to instrument, from column to column, and from detector to detector by locking retention times. The retention times of a styrene sample analyzed according to ASTM D 5135 matched to within 0.06 minute after locking.

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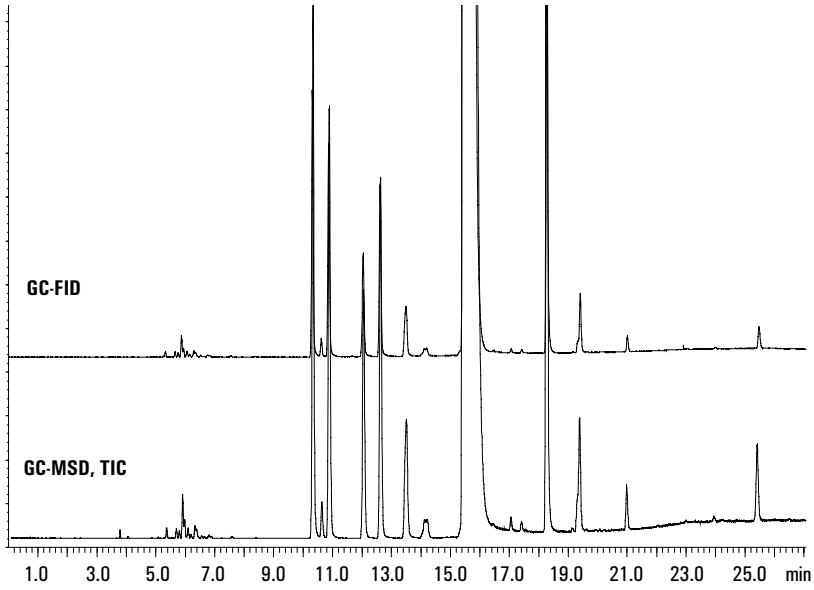

Figure 8. Comparison of chromatogram on GC system 1 with GC-MSD system after retention time locking, Constant Pressure Mode

Table 3. GC-FID vs. GC-MSD, Method Translated then Locked—Retention Times (Constant Pressure Conditions)

Component	GC-FID Original 18.2 psi	GC-MSD 7.9 psi	RT Difference min
Ethylbenzene	10.315	10.338	0.023
p-Xylene	10.620	10.642	0.022
m-Xylene	10.869	10.890	0.021
i-Propylbenzene	12.038	12.053	0.015
o-Xylene	12.613	12.630	0.017
n-Propylbenzene	13.492	13.508	0.016
a-Methylstyrene*	18.276	18.267	–0.009
Phenylacetylene	19.406	19.389	–0.017
b-Methylstyrene	21.008	20.987	–0.011
Benzaldehyde	25.475	25.415	–0.060
		Average	0.021

* Used in locking calculation

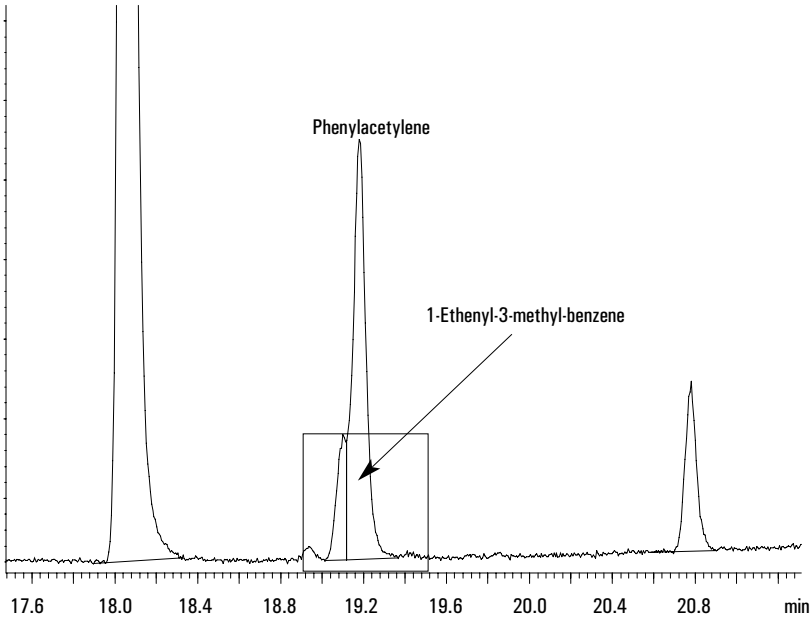

Figure 9. GC-MSD identification of impurity in shoulder of phenylacetylene peak

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Improving the Effectiveness of Method Translation for Fast and High Resolution Separations

Application

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Abstract

The increased availability of sub-2-micron (STM) columns and increased demand for methods friendly to mass spectrometers has led to strong trend toward conversion of existing HPLC methods to smaller diameter and smaller particle size columns. While the conversion is a simple mathematical exercise requiring the scaling flow rates, gradient times and injection volumes, many users observe less than perfect results. Here we look closely at the problem and propose calculations that improve the speed and/or resolution in a more predictable and beneficial way.

Introduction

Methods developed on older columns packed with large 5- or 10-µm particles are often good candidates for modernization by replacing these columns with smaller dimension columns packed with smaller particle sizes. The potential benefits include reduced analysis time and solvent consumption, improved sensitivity and greater compatibility with mass spectrometer ionization sources.

Simplistically, a column of 250-mm length and containing 5-µm particles can be replaced by a 150-mm length column packed with 3-µm particles. If the ratio of length to particle size is equal, the two columns are considered to have equal resolving power. Solvent consumption is reduced by L1/L2, here about 1.6-fold reduction in solvent usage per analysis. If an equal mass of analyte can then be successfully injected, the sensitivity should also increase by 1.6-fold due to reduced dilution of the peak as it travels through a smaller column of equal efficiency.

LC/MS (Liquid Chromatography/Mass Spectrometry) ionization sources, especially the electrospray ionization mode, have demonstrated greater sensitivity at lower flow rates than typically used in normal LC/UV (UltraViolet UV/VIS optical detection) methods, so it may also be advantageous to reduce the internal diameter of a column to allow timely analysis at lower flow rates. The relationship of flow rate between different column diameters is shown in Equation 1.

$$\text{Flow}_{\text{col. 1}} \times \left[\frac{\text{Diam.}_{\text{column2}}}{\text{Diam.}_{\text{column1}}}\right]^2 = \text{Flow}_{\text{col. 2}} \quad \text{(eq. 1)}$$

The combined effect of reduced length and diameter contributes to a reduction in solvent consumption and, again assuming the same analyte mass can be injected on the smaller column, a proportional increase in peak response. We normally scale the injection mass to the size of the column,

though, and a proportional injection volume would be calculated from the ratio of the void volumes of the two columns, multiplied by the injection volume on the original column.

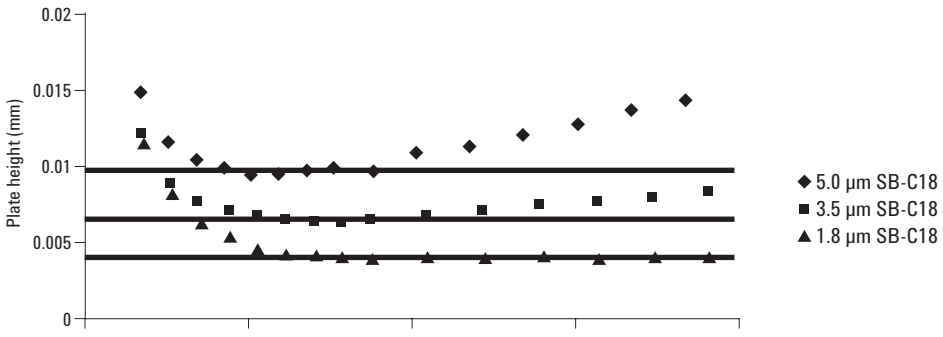
$$\text{Inj. vol.}_{\text{col. 1}} \times \left[\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}}\right] = \text{Inj. vol.}_{\text{col. 2}} \quad \text{(eq. 2)}$$

For isocratic separations, the above conditions will normally result in a successful conversion of the method with little or no change in overall resolution. If one wishes to improve the outcome of the method conversion, though, there are several other parameters that should be considered. The first of these parameters is the column efficiency relative to flow rate, or more correctly efficiency to linear velocity, as commonly defined by van Deemter [1] and others, and the second is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column.

Van Deemter observed and mathematically expressed the relationship of column efficiency to a variety of parameters, but we are most interested here in his observations that there is an optimum linear velocity for any given particle size, in a well-packed HPLC column, and that the optimum linear velocity increases as the particle size decreases. Graphically, this is often represented in van Deemter plots as shown in Figure 1, a modified version of the original plot [2].

In Figure 1 we observe that the linear velocity at which 5-µm materials are most efficient, under the conditions used by the authors, is about 1 mm/sec. For 3.5-µm materials the optimum linear velocity is about 1.7 mm/sec and has a less distinct optimum value, suggesting that 3.5-µm materials would give a more consistent column efficiency over a wider flow range. For the 1.8-µm materials, the minimum plate height, or maximum efficiency, is a broad range beginning at about 2 mm/sec and continuing past the range of the presented data. The practical application of this information is that a reduction in particle size, as discussed earlier, can often be further optimized by increasing the linear velocity which results in a further reduction in analysis time. This increase in elution speed will decrease absolute peak width and may require the user to increase data acquisition rates and reduce signal filtering parameters to ensure that the chromatographic separation is accurately recorded in the acquisition data file.

The second important consideration is the often overlooked effect of extracolumn dispersion on the observed or empirical efficiency of the column. As column volume is reduced, peak elution volumes are proportionately reduced. If smaller particle sizes are also employed there is a further reduction in the expected peak volume. The liquid chromatograph, and particularly the areas where the analytes will traverse, is a collection of various connecting capillaries and fittings which will cause a measurable amount of bandspreading. From the injector to the detector flow cell, the cumulative dispersion that occurs degrades the column performance and results in observed efficiencies that can be far below the values that would be estimated by purely theoretical means. It is fairly typical to see a measured dispersion of 20 to 100 µL in an HPLC system. This has a disproportionate effect on the smallest columns and smallest particle sizes, both of which are expected to yield the smallest



Lin. vel.	mm/sec	1	2	3	4	5
4.6 mm	mL/min	0.7	1.4	2.1	2.8	3.5
3 mm	mL/min	0.3	0.6	0.9	1.2	1.5
2.1 mm	mL/min	0.14	0.29	0.44	0.58	0.73
1 mm	mL/min	0.033	0.066	0.1	0.133	0.166

Figure 1. van Deemter plot with various flow rates and particle sizes.

possible peak volumes. Care must be taken by the user to minimize the extracolumn volume and to reduce, where practical, the number of connecting fittings and the volume of injection valves and detector flow cells.

For gradient elution separations, where the mobile phase composition increases through the initial part of the analysis until the analytes of interest have been eluted from the column, successful method conversion to smaller columns requires that the gradient slope be preserved. While many publications have referred to gradient slope in terms of % change per minute, it is more useful to express it as % change per column volume. In this way, the change in column volume during method conversion can be used to accurately render the new gradient condition. If we think of each line of a gradient table as a segment, we can express the gradient by the following equation:

$$\%\ \text{Gradient slope} = \left[\frac{(\text{End}\% - \text{Start}\%)}{\#\text{Column volumes}} \right] \quad \text{(eq. 3)}$$

Note that the use of % change per column volume rather than % change per minute frees the user to control gradient slope by altering gradient time and/or gradient flow rate. A large value for gradient slope yields very fast gradients with minimal resolution, while lower gradient slopes produce higher resolution at the expense of increased solvent consumption and somewhat reduced sensitivity. Longer analysis time may also result unless the gradient slope is reduced by increasing the flow rate, within acceptable operating pressure ranges, rather than by increasing the gradient time.

Resolution increases with shallow gradients because the effective capacity factor, k*, is increased. Much like in isocratic separations, where the capacity term is called k', a higher value directly increases resolution. The effect is quite dramatic up to a k value of about 5 to 10, after which little improvement is observed. In the subsequent examples, we will see the results associated with the calculations discussed above.

Experimental Conditions

System

Agilent 1200 Series Rapid Resolution LC consisting of:
G1379B micro degasser
G1312B binary pump SL
G1367C autosampler SL, with thermostatic temperature control
G1316B Thermostatted column compartment SL
G1315C UV/VIS diode array detector SL, flow cell as indicated in individual chromatograms
ChemStation 32-bit version B.02.01

Columns

Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm
Agilent ZORBAX SB-C18, 3.0 mm × 150 mm, 3.5 µm

Mobile phase conditions

Organic solvent:	Acetonitrile
Aqueous solvent:	25 mm phosphoric acid in Milli-Q water

Gradient Conditions

Gradient slope:	7.8% or 2.3% per column volume, as indicated. See individual chromatograms for flow rate and time

Sample

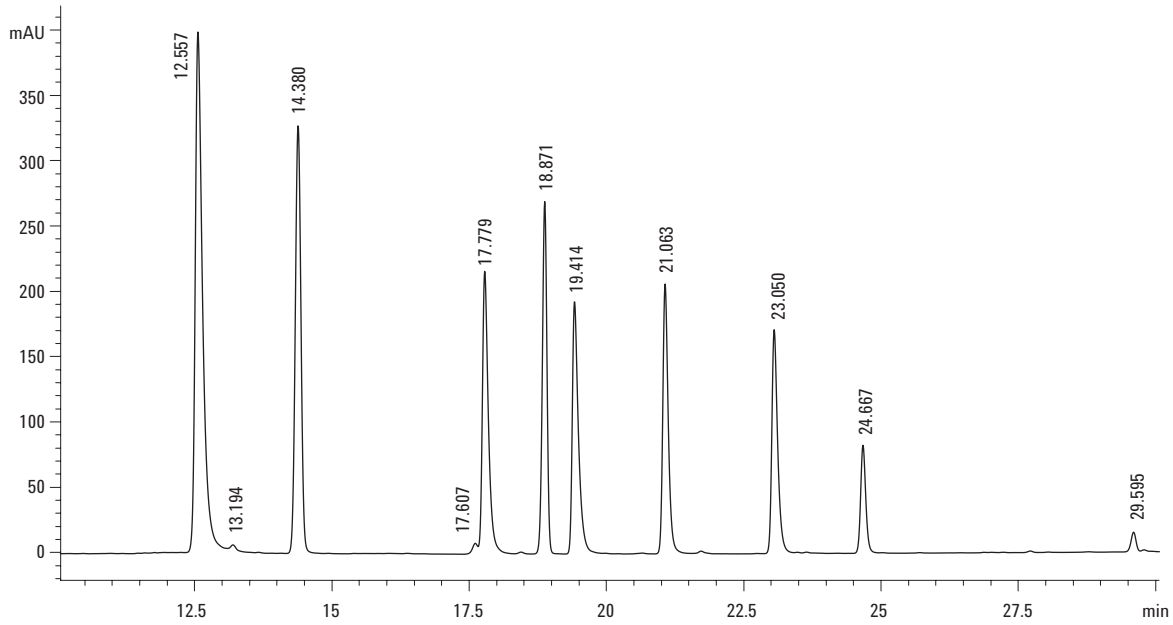
Standard mixture of chlorinated phenoxy acid herbicides, 100 µg/mL in methanol

Results

The separation was initially performed on a standard 4.6 × 250 mm, 5-µm ZORBAX SB-C18 column thermostatted to 25 °C (Figure 2) using conditions referenced in US EPA Method 555. The method was then scaled in flow and time for exact translation to a 3.0 × 150 mm, 3.5-µm column (Figure 3). Solvent consumption is reduced from 60 mL to 15.5 mL per analysis.

The separation was then re-optimized for faster separation with the identical slope, 7.8%, by increasing the flow rate from 0.43 to 1.42 mL/min, and proportionately reducing the gradient time (Figure 4). Finally, increased resolution is demonstrated by keeping the original times used in Figure 3 with the increased flow rate (Figure 5). This yields a gradient with identical time but a reduced slope of 2.3%. The increased resolution of peaks 4 and 5 is readily apparent.

The conditions in Figure 4, 7.8% slope at increased linear velocity on 3.0 × 150 mm, 3.5-µm material, yield a separation with comparable resolution to the original 4.6 × 250 mm method, but with only a 12-minute total analysis time. This is excellent for



Conditions

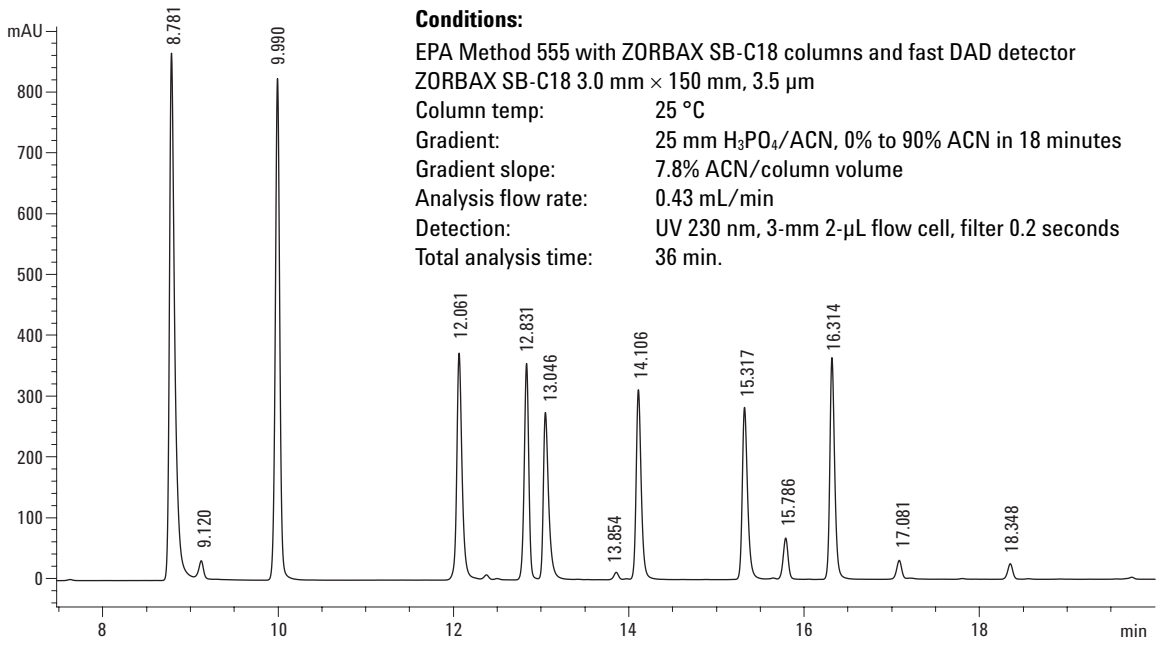
EPA Method 555 with ZORBAX SB-C18 columns and fast DAD detector
ZORBAX SB-C18 4.6 mm × 250 mm, 5 µm

Column temp:	25 °C
Gradient:	10% to 90% ACN vs. 25 mM H_3PO_4
Gradient slope:	7.8% ACN/column volume
Analysis flow rate:	1 mL/min

Group A Compounds

Total analysis time:	60 min
Detection:	UV 230 nm, 10-mm 13-µL flow cell, filter 2 seconds (default)

Figure 2. Gradient separation of herbicides on 4.6 × 250 mm 5-µm ZORBAX SB-C18.



Conditions:

EPA Method 555 with ZORBAX SB-C18 columns and fast DAD detector
ZORBAX SB-C18 3.0 mm × 150 mm, 3.5 µm

Column temp:	25 °C
Gradient:	25 mm H_3PO_4/ACN, 0% to 90% ACN in 18 minutes
Gradient slope:	7.8% ACN/column volume
Analysis flow rate:	0.43 mL/min
Detection:	UV 230 nm, 3-mm 2-µL flow cell, filter 0.2 seconds
Total analysis time:	36 min.

Figure 3. Gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

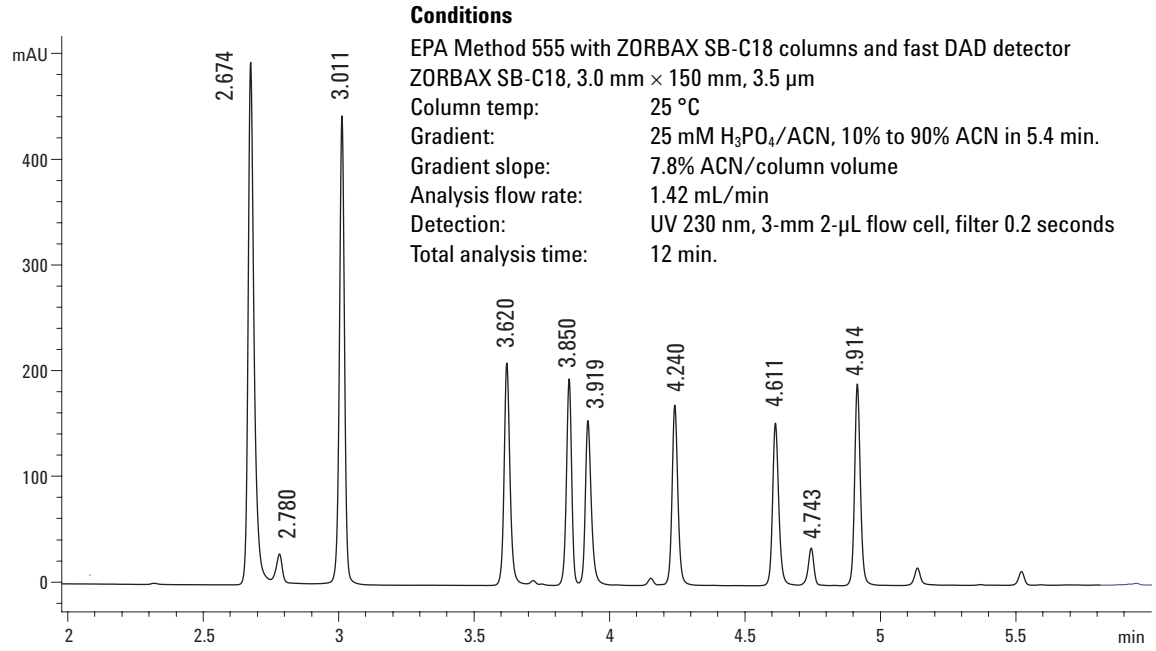


Figure 4. High speed gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

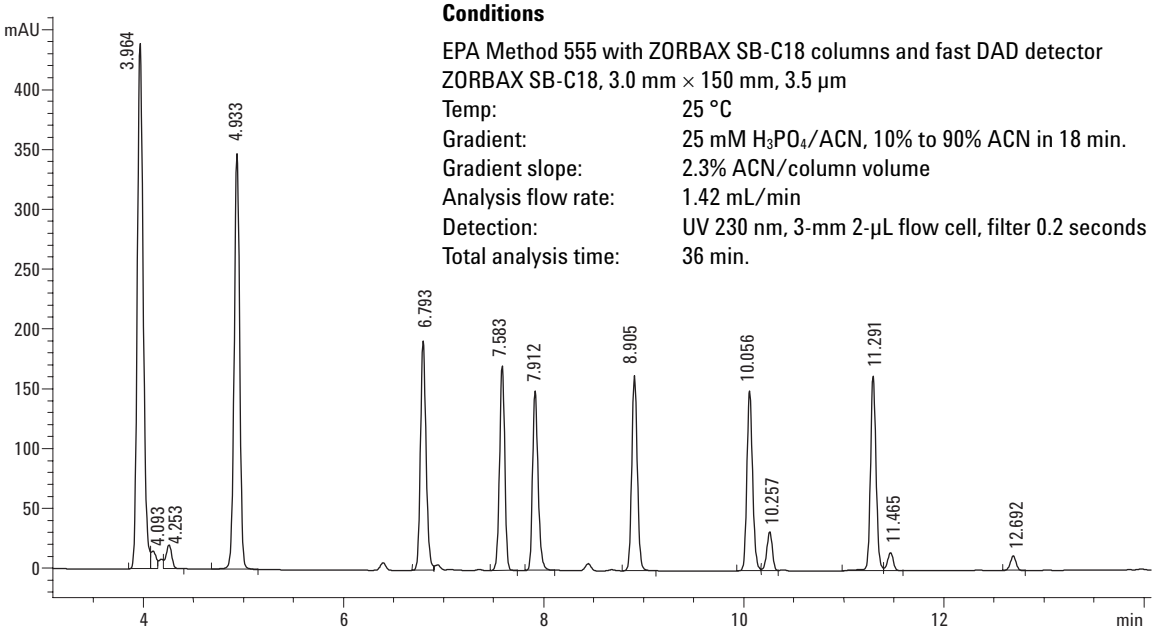


Figure 5. Reduced slope gradient separation of herbicides on 3.0 × 150 mm, 3.5-µm ZORBAX SB-C18.

high throughput screening and quantitation of a large number of samples. Figure 5, with the gradient slope reduced to 2.3%, results in a high-resolution separation with a calculated R value of 3.3 vs. the standard 3.0 × 150 mm separation value of 1.9, for the critical pair seen in Figure 5 at 7.5 to 8 minutes.

In Table 1 the column has been replaced with a low dead volume connecting union in a system fitted with 0.12-mm id capillary tubing at all points of sample contact. A 1-µL injection of dilute actone is made to determine the bandspreading contribution of the system, with various flow cells. Multiple flow cells were tested, and the average result reported, where possible. The elution volume summarizes the total volume of all tubing in the system. While the absolute volume from the 2-µL to the 13-µL flow cells is 11 µL, we observe an increase of 15 to 16 µL because of the larger diameter inlet tubing integral to the larger volume flow cells.

Table 1. Volumetric Measurements of Various Flow Cells

Flow cell	Elution volume (µL)	Half height width (µL)	5 Sigma width (µL)
New SL 2 µL 3 mm	11	5	12
Micro 6 mm 1.7 µL (n = 2)	14	6	18
Semi-micro 6 mm 5 µL (n = 2)	13	6.5	18.5
Standard 10 mm 13 µL	26	11	26
New SL 10 mm 13 µL	27	11	25

Conclusion

Careful analysis of the existing gradient conditions, coupled with an awareness of the need to accurately calculate new flow and gradient conditions can lead to an easy and reliable conversion of existing methods to new faster or higher resolution conditions. In addition, awareness of extracolumn dispersion, especially with small and high resolution columns, will ensure good column efficiency which is critical to a successful translation of the method.

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1. J. J. van Deemter, F. J. Zuiderweg, A. Klinkenberg, Chemical Engineering Science 1956, 5, 271–289
2. The Influence of Sub-Two Micron Particles on HPLC Performance, Agilent Technologies, application note 5989-9251EN, May 2003

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Improving GC-MS Method Robustness and Cycle Times Using Capillary Flow Technology and Backflushing

Application Note

Environmental

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Abstract

This application note demonstrates the customer benefits from using Capillary Flow Technology to provide backflushing of high-boiling materials in GC and GC/MS analyses. Benefits include reduction in chromatographic cycle times, a reduction in system column maintenance, and extended GC column life. If a GC/MS system is utilized, the author has experienced an increase in the number of samples analyzed before ion source maintenance is required.

Agilent Technologies

Introduction

A critical component of the GC/MS analysis of any sample that contains large amounts of matrix material is the sample preparation. Environmental samples such as soils and sediments require not only extraction, but may also require multiple cleanup steps in order to present as clean an extract as possible for injection in to the GC/MS system.

Any remaining matrix in the sample extract can have deleterious effects on the GC sample inlet, column, and the ion source of the mass spectrometer. Traditionally, these high-boiling matrix materials are removed from the capillary column by a long bake-out period after the analytes of interest have eluted. This long bake-out process causes thermal stress to the column and also drives the matrix material towards the ion source, where it will eventually affect system performance. Moreover, should any material remain in the column after the bake-out process, it can cause loss of chromatographic peak shape and retention time shifting of target analytes. This shifting of retention time is particularly troublesome if the mass spectrometer is being used in the selected ion monitoring (SIM) mode (as with a single quadrupole GC/MS) or in the multiple reaction monitoring (MRM) mode (as with a triple quadrupole GC/MS).

This paper demonstrates how high-boiling matrix materials can be removed from the column quickly and effectively – between sample injections – by using capillary flow technology and capillary column backflushing.

Figure 1 shows a schematic diagram of the GC/MS system used. The 15-m analytical column was connected to the EPC split/splitless inlet and a capillary flow technology two-way splitter (p/n G3180B or G1540 option number 889).

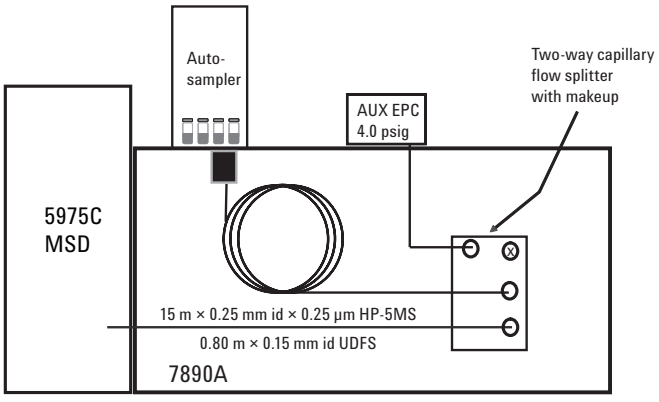

Figure 1. Schematic diagram of GC-MS system.

A short length of uncoated, deactivated fused silica (UDFS) capillary column is used as a restrictor between the splitter and the MS. Note carefully how the connections are made at the splitter. The X represents a port on the splitter plate that is closed off with a SilTite metal ferrule and stainless steel wire plug.

Backflushing in this example was accomplished during a post-run period by a combination of increasing oven temperature, reducing the inlet pressure of the analytical column, and increasing the pressure applied to the splitter plate.

Experimental

The full analytical conditions, both with and without post-run backflush set-points, are shown in Table 1.

Table 1. GC/MS Analysis Conditions

Gas chromatograph	Agilent 7890A
Columns	(1) 15.0 m × 0.25 µm id × 0.25 µm HP-5MS Ultra Inert (19091S-431SI) Inlet Front split/splitless, outlet 2-way Capillary Flow Device
	(2) 0.80 m × 0.15 mm id uncoated deactivated fused silica inlet two-way capillary flow device at 4.0 psig outlet vacuum
Carrier gas	Helium
Carrier gas mode	Constant pressure
Flow rate	17.18 psi
Injection port	EPC split/splitless
Autosampler	Agilent 7683A
Injection mode	Splitless, purge delay 0.5 min Purge flow 50.0 mL/min at 0.5 min
Injection volume	2.0 µL
Injection port liner	4 mm single-taper splitless liner (5181-3316)
Oven program °C (min)	70 (1) – 50 °C /min – 150 (0) 6 – 200 (0) – 16 – 280 (0) °C
Mass spectrometer	Agilent 5975C MSD
MS interface	280 °C
MS source	230 °C
MS quad 1	150 °C
Backflush conditions (1)	Post-run, 10 min, AUX 60 psig, oven 320 °C
Backflush conditions (2)	Post-run, 6 min, AUX 80 psig, oven 320 °C
Detection mode	EI full scan; mass range 40:550 amu
EI tune	Gain factor = 1

Results and Discussions

Experiment 1: No Backflushing Employed

In the first experiment, an extracted sediment sample was analyzed in full-scan mode to show the extent of the matrix problem. No backflushing was employed.

Before any sediment was injected, a system blank (no injection) followed by a 2-µL solvent blank was made. In the absence of the actual hexane solvent used to prepare the sediment extract, hexane that was not particularly clean was used. The TICs are shown overlaid in Figure 2, system blank in black, and solvent blank in gray. These chromatograms show that the system is free from high-boiling matrix material.

Following the blanks, a single injection of the sediment extract was made without backflushing; the TIC is shown in Figure 3. Note the very high abundance of the matrix and that when the analysis finishes, there is still a significant amount of matrix material to elute from the column.

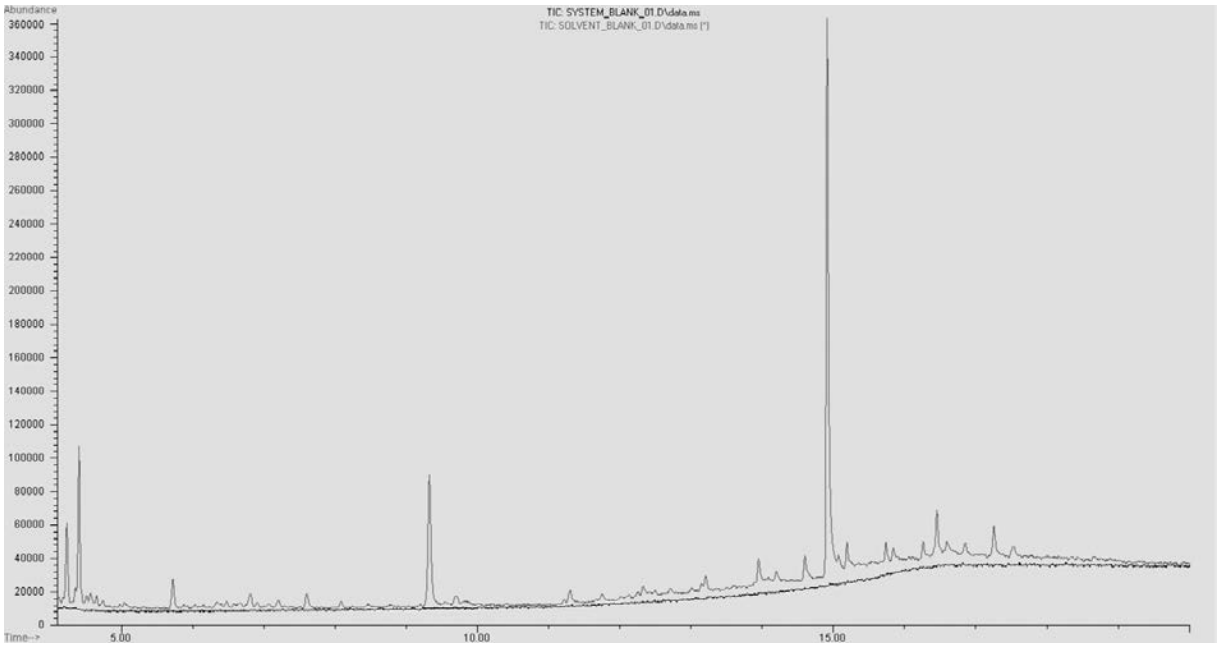

Figure 2. System blank and solvent blank TICs.

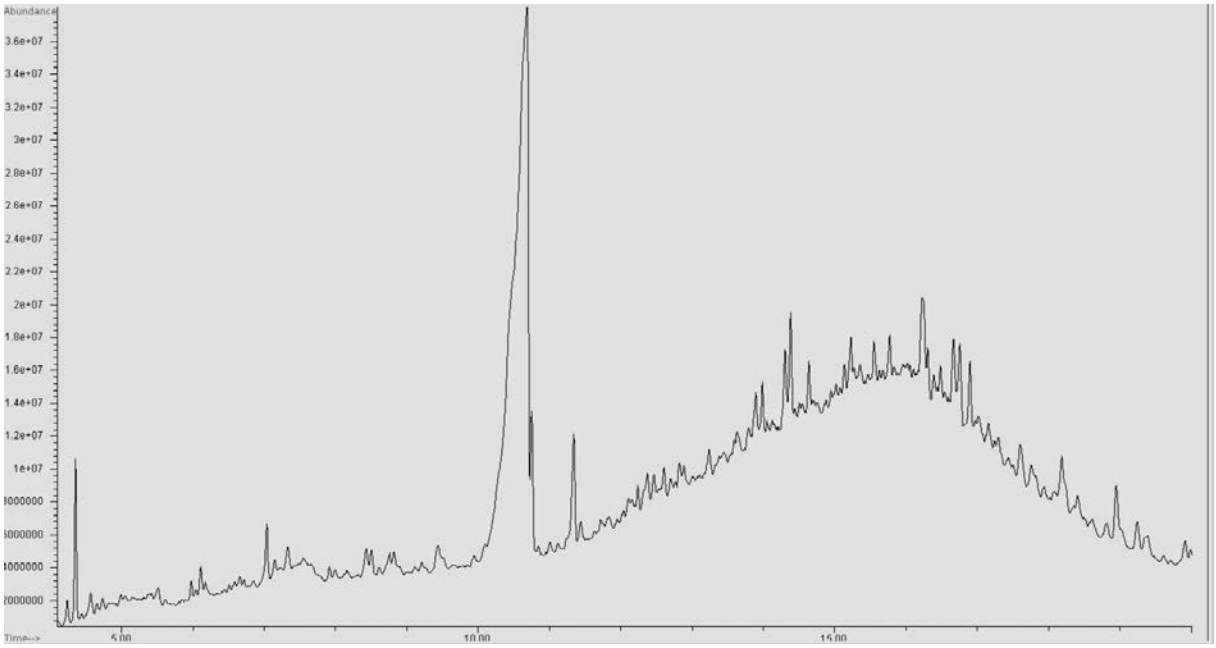

Figure 3. Sediment extract TIC.

The sediment extract injection was followed by a series of hexane blank injections. The first seven hexane blank TICs are shown overlaid in Figure 4 with the solvent blank before the sediment was injected into the GC/MS system.

Figure 5 shows that after the eighth solvent blank injection, the system has almost recovered to the level of background before the sediment sample was injected.

The original solvent blank TIC is shown in black, the eighth solvent blank TIC after the sediment injection is shown in gray.

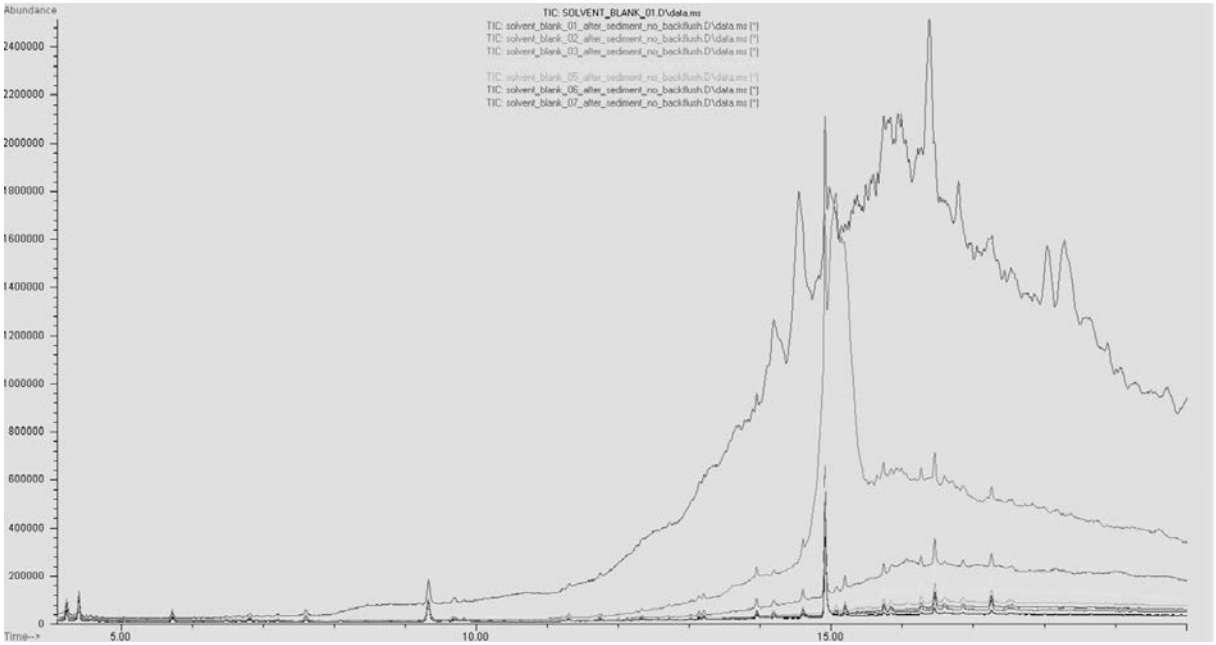

Figure 4. Successive solvent blank injections.

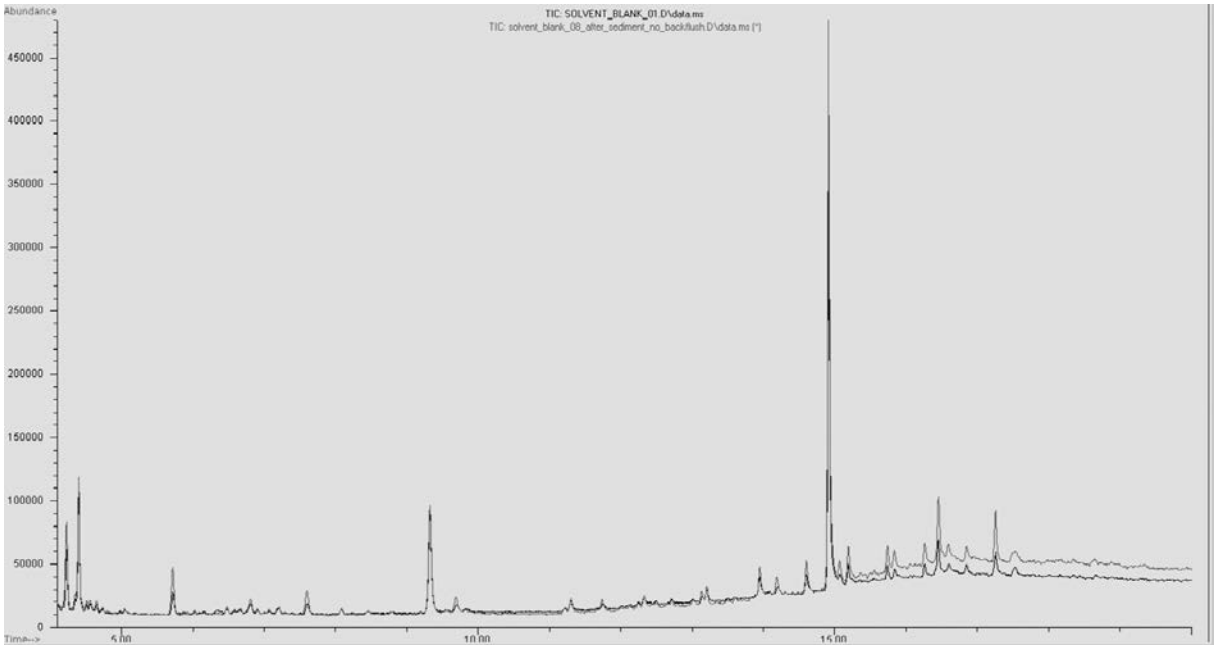

Figure 5. Eighth solvent blank and original solvent blank TICs

Experiment 2: Backflushing Employed

Backflushing was enabled during a post-run period by increasing column oven temperature, reducing the inlet pressure of the analytical column, and increasing the gas pressure applied to the splitter plate.

The 7890A instrument control software includes simple and easy-to-use screens to help set up post-run backflushing conditions. Figure 6 shows the configuration of columns and connections with the GC oven.

Figure 7 shows the actual backflushing conditions, namely the post-run oven temperature (320 °C), post-run inlet pressure for the analytical column (1 psig), post-run pressure applied to the splitter device (60 psig), and post-run time (10 minutes). The figure also shows the number of column-volumes of carrier gas that will backflush the analytical column.

Note that using the backflushing conditions shown in Figure 7 (320 °C, column pressure 1 psig, and splitter pressure 60 psig for 10 minutes), that 59.4 column volumes of carrier gas was used to backflush the column during the post-run period. This backflush time may have been more than necessary. Alternate conditions were also investigated and are presented later.

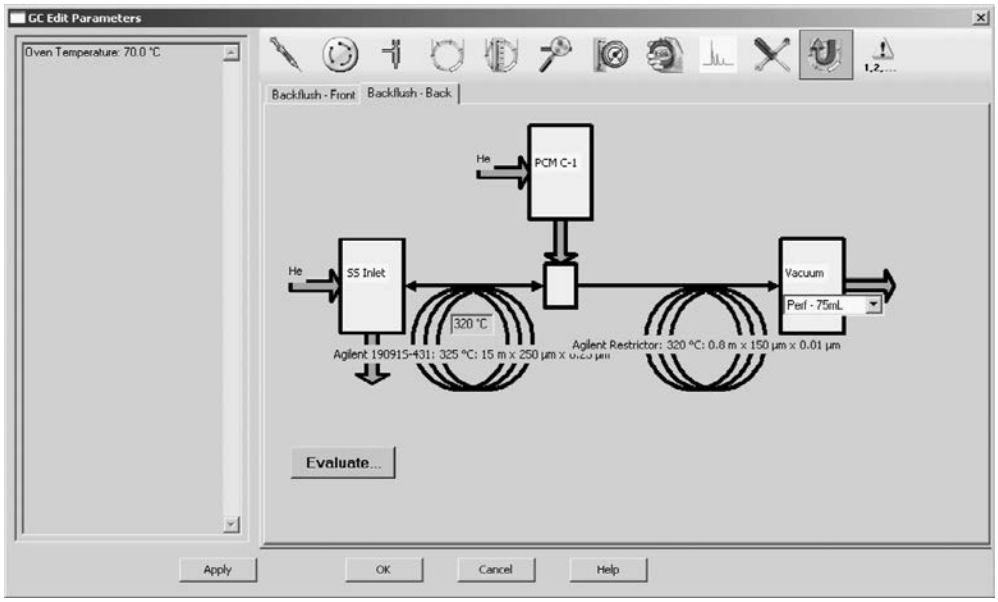

Figure 6. Post-run backflushing screen number 1.

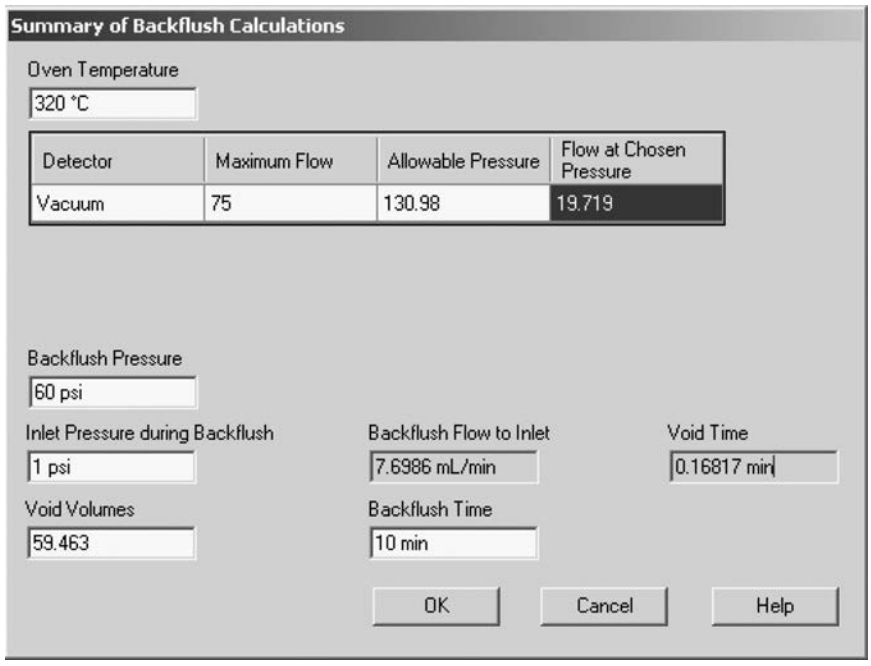

Figure 7. Post-run backflushing screen number 2.

Before applying the backflush conditions to the method the user is presented with a convenient summary of the back-flush conditions. See Figure 8.

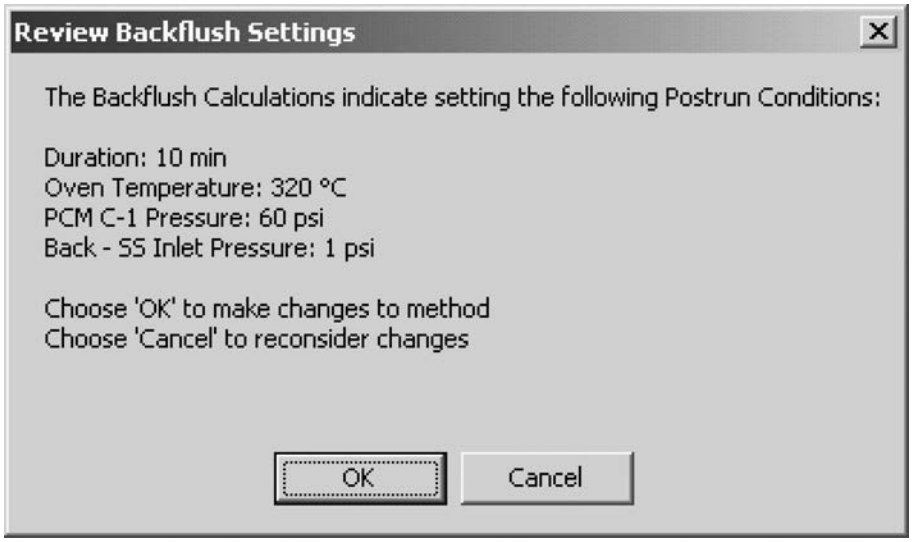

Figure 8. Post-run backflushing screen number 3.

Another injection of the sediment including backflush was made followed by a blank injection of solvent. Figure 9 shows the overlaid TIC of the original solvent blank (black) overlaid on the solvent blank after the sediment injection (gray).

No evidence of any matrix material is indicated, demonstrating that all the high-boiling matrix material had been effectively removed by backflushing.

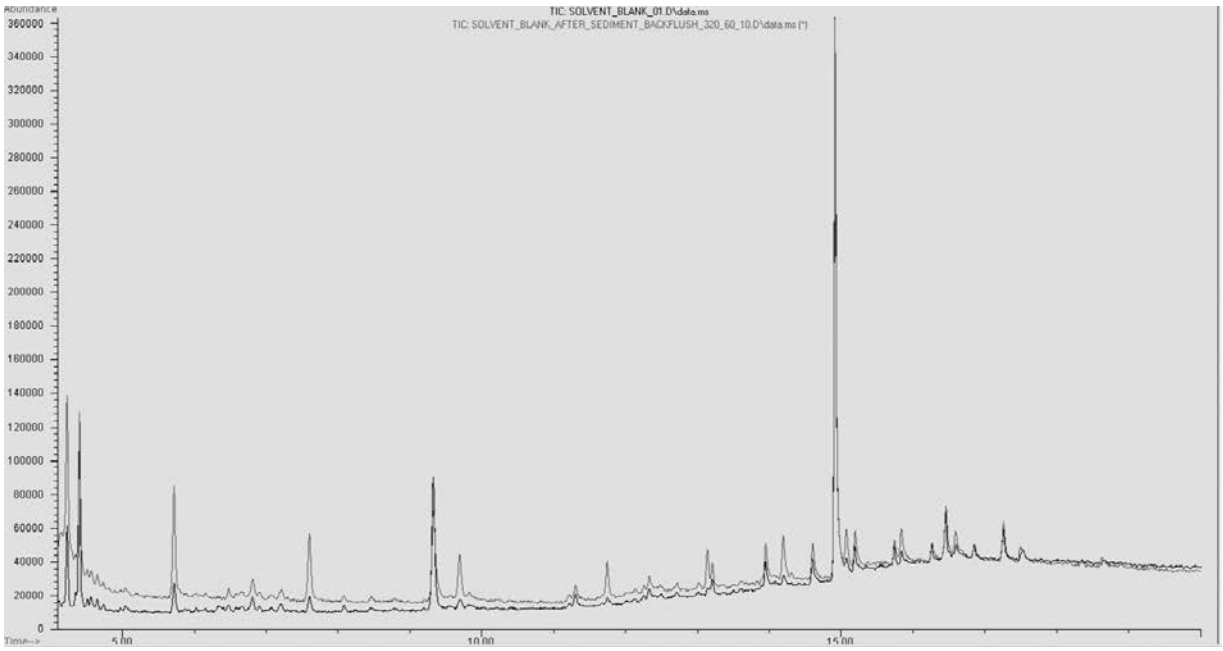

Figure 9. Original solvent blank TIC and solvent blank after sediment injection with post-run backflush (1).

Experiment 3: Backflushing Employed

In order to reduce cycle time for the method, the backflush conditions were modified by increasing the backflush pressure to 80 psig and holding for 6 minutes.

Note that using the backflushing conditions shown in Figure 10 (320 °C, column pressure 1 psig, and splitter pressure 80 psig for 6 minutes), that 46.6 column volumes of carrier gas was used to backflush the column during the post-run period.

Another injection of the sediment was made, followed by a blank injection of solvent. Figure 11 shows the overlaid TIC of the original solvent blank (black) overlaid on the solvent blank after the sediment injection (gray).

No evidence of any matrix material is indicated, demonstrating that all the high-boiling matrix material has been removed by backflushing with the more aggressive conditions as well. These conditions reduced the cycle time for this method 4 minutes compared to the backflushing conditions used in Experiment 1.

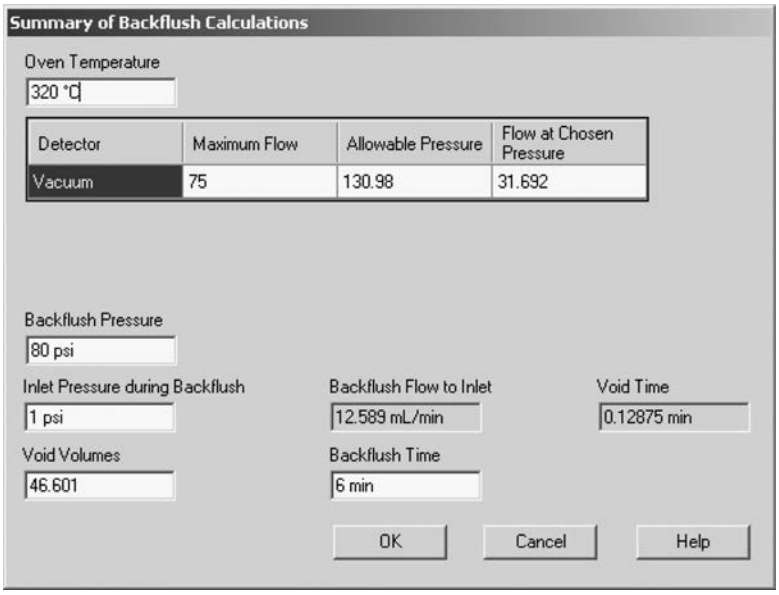

Figure 10. Post-run backflushing screen conditions number 2.

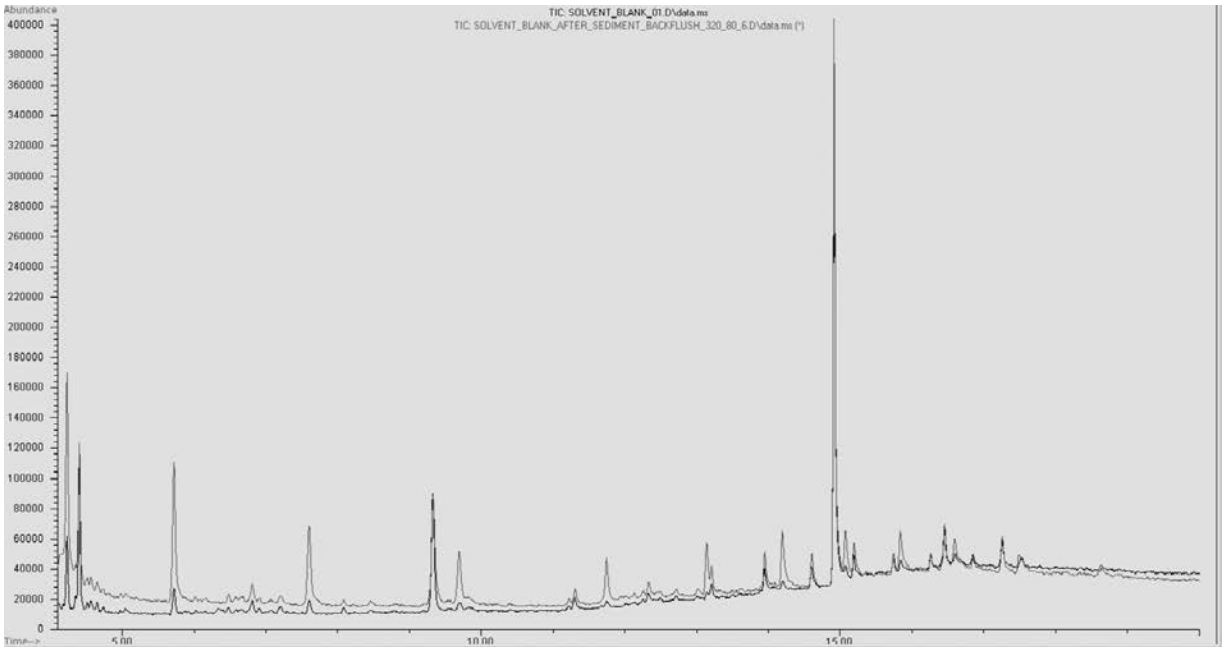

Figure 11. Original solvent blank TIC and solvent blank after sediment injection with post-run backflush (2).

Conclusions

Post-run backflushing was shown to effectively eliminate high-boiling sample matrix in a short amount of time. The major benefits of GC capillary column post-run backflushing include:

- Agilent's capillary flow technology and GC software enable easy and robust setup of GC backflushing.
- Compared to long bake-out periods with flow in the forward direction, a short period of backflushing can remove high-boiling matrix materials more effectively without contaminating the MS ion source.
- Chromatographic cycle time is reduced, columns stay clean, and the integrity of target analyte peak shapes and retention times are maintained.
- For this particular sediment extract the GC column was free of sample matrix after a backflush period of 6 minutes.
- Less system maintenance (ion source cleaning) is required.

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The 5973N inert MSD: Using Higher Ion Source Temperatures

Application

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Abstract

The new 5973N inert MSD and ChemStation software (G1701DA) offers the capability of operating the ion source at higher temperatures. This feature, combined with the improved inertness of the source, can provide the user with improvements in analysis, if exploited coherently. This application note provides advice and examples of how to explore the utility of ion source temperature.

Introduction

The default ion source temperature of 230 °C is commonly applied in electron impact (EI) ionization on the 5973 MSD platforms. The new Inert Source when used with the new revision of the ChemStation software (rev. DA) allows ion source temperature to be set to a maximum of 300 °C. As with all advances, there are advantages and disadvantages in operating at higher source temperatures. This note will address several general aspects in EI operation.

Tuning

Figures 1 and 2 show the results for autotuning the Inert Source at the standard 230 °C ion source temperature and the 300 °C temperature limit of the new source (quadrupole temperature 200 °C). The higher temperature for the source produces a perfluorotributylamine (PFTBA) spectrum that shows lower abundances of the higher mass fragments, which is not entirely unexpected. The *m/z* 219 fragment has dropped to an abundance comparable to the *m/z* 69 ion and the ion at *m/z* 502 has dropped about 50%. This is to be expected as the internal energy of the calibrating gas has increased. Note, however, that the isotopic ratios are maintained.

The user should also expect to see a higher background in the higher temperature tunes. A portion of the background will be due to ions associated with column bleed. Bleed, which usually condenses in the source, now is volatized and will appear as an increase in background and baseline.

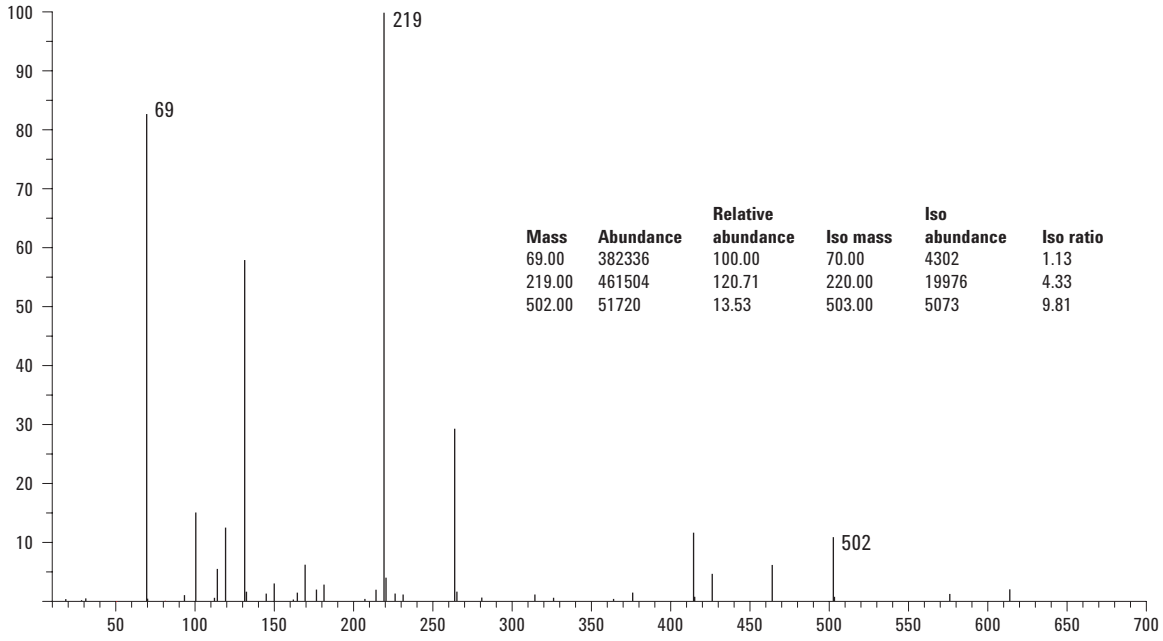

Figure 1. Autotune results for an ion source temperature of 230 °C.

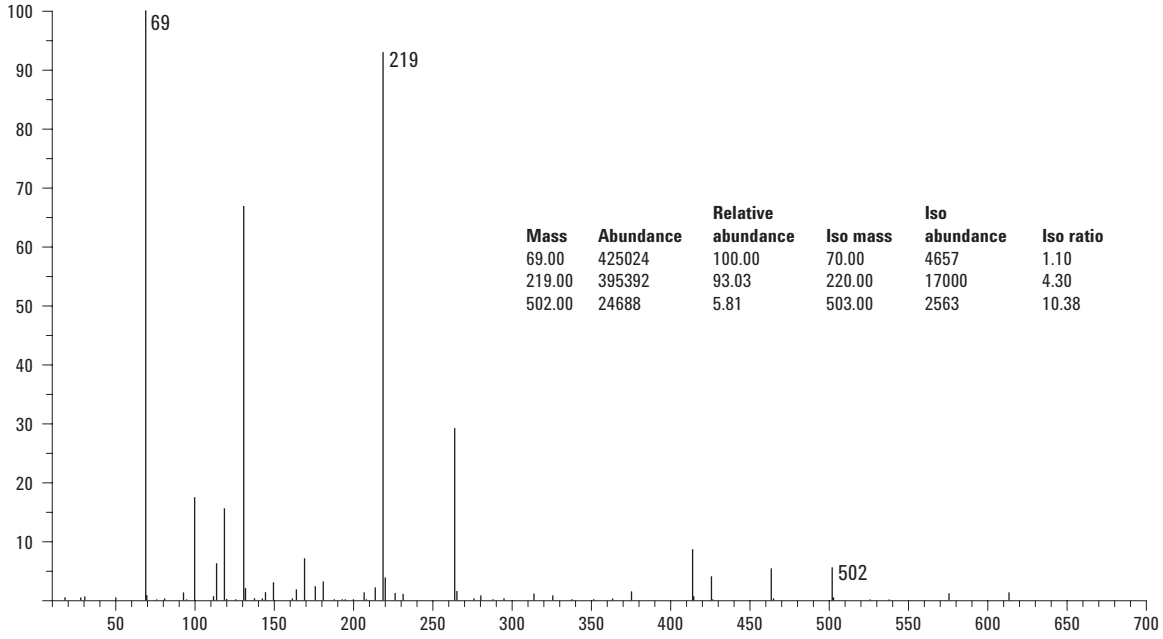

Figure 2. Autotune results for an ion source temperature of 300 °C.

Implications for Analytical Applications

Although the tuning compound showed a spectral change that favored more fragmentation, and all compounds could be expected to be influenced similarly, there are some advantages that can occur for less fragile compounds, especially those that have higher boiling points and are late eluting in GC. Analysis of the class of compounds known as "persistent organic pollutants" (POPs) is likely to benefit from higher source temperatures.

To illustrate the aspects that need to be examined, consider the six polychlorinated biphenyls (PCBs) acquired in full-scan and presented in Figure 3. The overlaid reconstructed total-ion-current chromatograms (RTICCs) suggest that the higher source temperature increases the total response for the later eluting PCBs but produces little enhancement for the early eluters. This could be due to more fragmentation and may not necessarily be useful if the increase in the RTIC is due to lower mass fragments since these lower mass ions are usually compromised by interferences. A calculation of the signal/noise (S/N) for the RTICCs shows that while there is an increase in signal at the source higher temperature, there is also an increase in the background noise and the result is a lower S/N ratio for the higher source temperature.

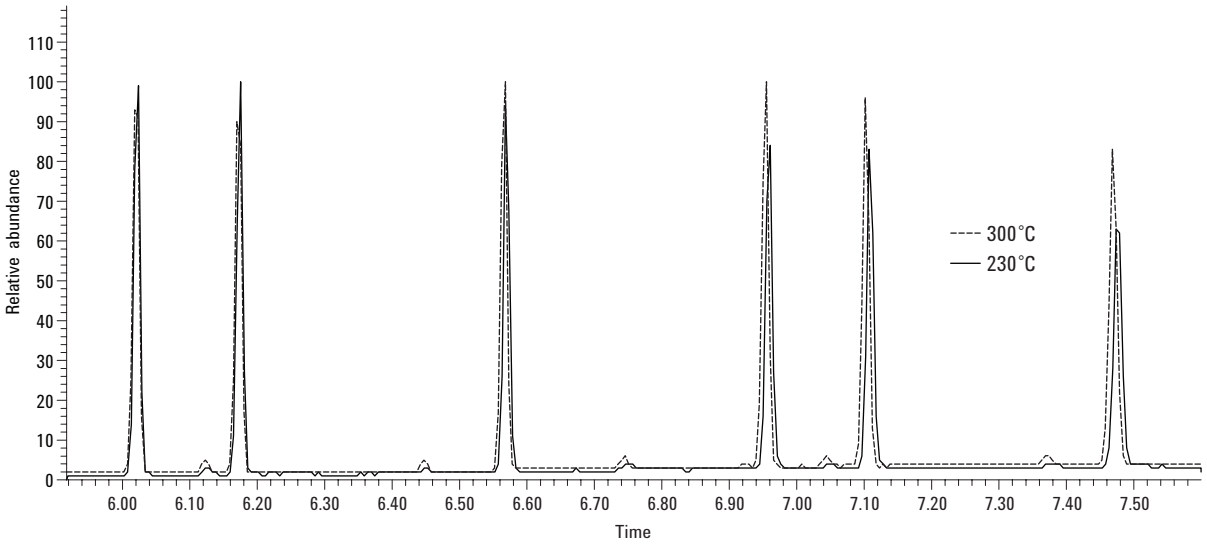

Figure 3. Overlaid RTICC of six PCBs acquired in full-scan (50–505 amu) at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram, the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

Figure 4 shows the same analytes acquired in selected-ion-monitoring mode (SIM) using three ions for each component (M, M+2 or M–2, and M–70). The same trend appears with an enhancement apparent in signal for the later eluting PCBs but little increase for the earlier PCBs. Now, however, the RTIC for the SIM acquisition does show a higher S/N ratio for these later PCBs. As opposed to the full-scan acquisition, the SIM mode acquisition at higher source temperature does increase signal for the ions of interest and, because there was no increase in background, a useful S/N increase was obtained. As always, the guiding principle that an increase in signal is only useful if it exceeds the concomitant increase in background holds. This is clearly illustrated by the third PCB, the pentachlorobiphenyl (Cl_5–B). Figure 5 shows the behavior of the signal and background for the two source temperatures for one of the pentachlorobiphenyl confirming ions. The higher source temperature raises the signal and the background for this ion of interest over the lower temperature but fortunately signal increases faster than background. In this case, the background is due to column bleed components and is unavoidable but fortunately not very intense. This may or may not be the case in sample analysis.

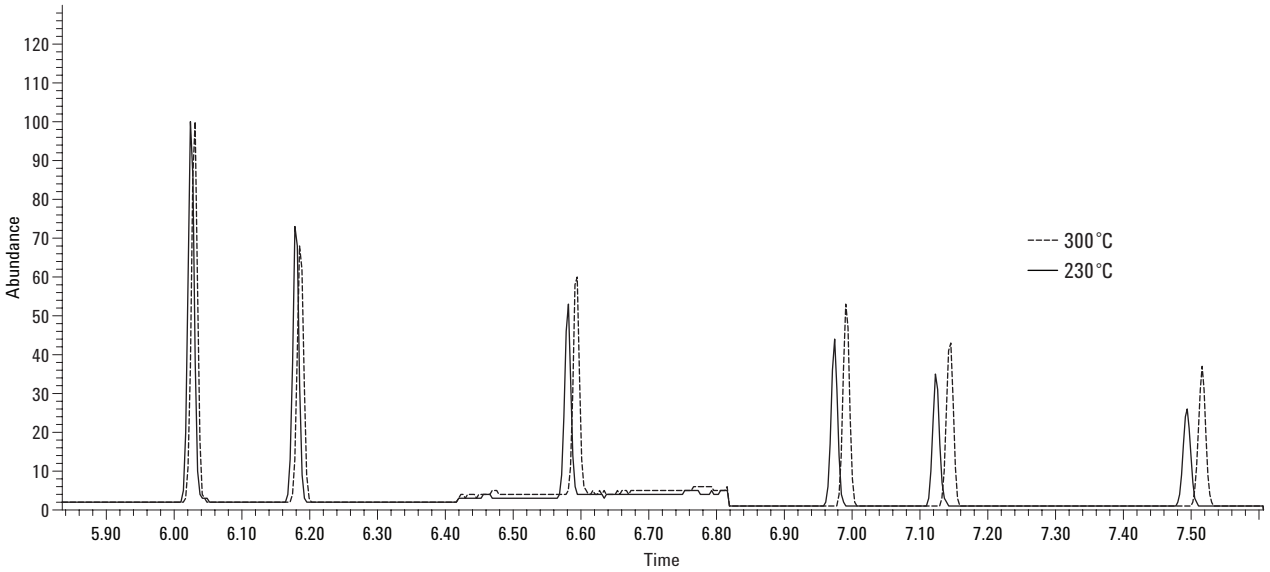

Figure 4. Overlaid RTICC of six PCBs acquired in SIM at source temperatures of 230 °C and 300 °C. From left to right, or earlier to later, in the chromatogram the PCBs consist of a Cl_3-Biphenyl, Cl_4-B, Cl_5-B, Cl_6-B, another Cl_6-B and a Cl_7-B.

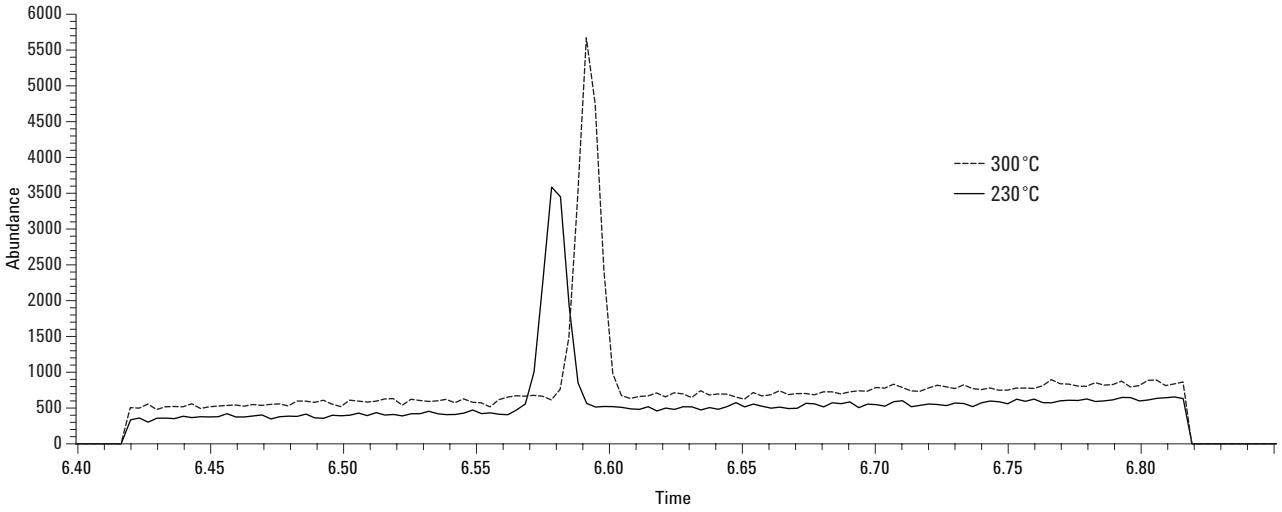

Figure 5. Overlaid extracted ion-current chromatograms of one ion (M-70) for the pentachlorobiphenyl acquired in SIM at source temperatures of 230 °C and 300 °C.

The detection limits for many late eluting, "high-boiling" compounds that will improve by implementing higher source temperatures (for example, PAHs, terphenyls, etc.). As an illustration of the enhancement for very "high-boiling" compounds, consider the 6-ring benzenoid hydrocarbon (PAH), coronene (CAS 191-07-1). This compound is difficult to determine due to low response and poor chromatography, although it is present in many sediment samples. Figure 6 shows overlaid RICCs for acquisitions of coronene at 230 °C and 300 °C. Although the peak area is the same, the enhanced Gaussian peak shape achieved at 300 °C improves detection.

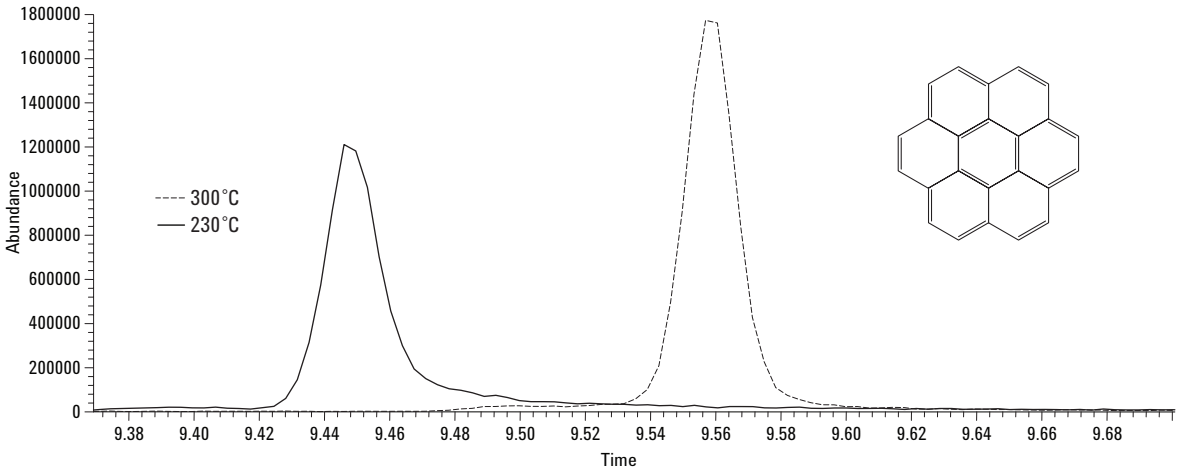

Figure 6. Overlaid extracted ion-current chromatograms of one ion (*m/z* 300) for coronene acquired in full scan at source temperatures of 230 °C, and 300 °C.

Source "Bakeout"

There may be considerable temptation to use the higher source temperature for source “cleaning” by “baking”. In other words, when the user notices a higher background in the source or a reduction in response, the ill-conceived approach of baking the source clean may come to mind. The result will be that “garbage” coating the source will be volatized further into the analyzer; the other lenses will get dirtier, as will the multiplier, etc. “Baking” is not a substitute for mechanical cleaning of the source. However, baking a source after a cleaning is a good approach and a macro that provides this option is given in Table 1. After a source has been cleaned, and the MS system pumped down and checked to be leak free, this macro can be implemented either manually or in a sequence. (Note that the temperature limits in the tune file need to be altered to 300 and 200 for source and quadrupole, respectively). Manually the bakeout is called from the command line in TOP by –

```
macro "bake.mac"  <enter>
bake 2  <enter>
```

The “2” calls for a 2 hour bakeout, and which can be set to anytime the user requires.

Copy the lines in Table 1 into Notepad and save the file as BAKE.MAC in the MSDCHEM\MSEXE directory. The “!” indicates a comment (line) which is not executed. Note that the temperature limits, which reside in the tune file, must be edited to allow the higher settings.

Table 1. ChemStation Macro for Baking the Source and Quadrupole After Source Maintenance

```
name Bake
! this macro sets the source and quad temps to their maximum and holds for a set period
parameter hours def 6                  ! default setting is 6 hours -this is customizable
msinsctl "mstemp QUAD, , , 200"        ! sets the quad temperature to bake at 200C
synchronize
msinsctl "mstemp SOURCE, , , 300"      ! sets the source temperature to bake at 300C
synchronize
SLEEP hours*60*60                      ! bakes for set period
msinsctl "mstemp QUAD, , , 150"        ! sets the quad temperature to operating temp at 150C
synchronize
msinsctl "mstemp SOURCE, , , 230"      ! sets the source temperature to operating temp at 230C
synchronize
return
```

Usually a source cleaning is executed at the end of the working day, and the system pumped down overnight for operation the next day. In this case, a "pumpdown sequence" is useful. After the system is confirmed to be leak-tight, this sequence is loaded and executed which bakes the source and quad overnight, then executes an Autotune, and then makes a few injections of a checkout standard to confirm system performance. In this way, the analyst returns the next day to review data about the system prior to beginning new analyses. An example of this is given in Figure 7.

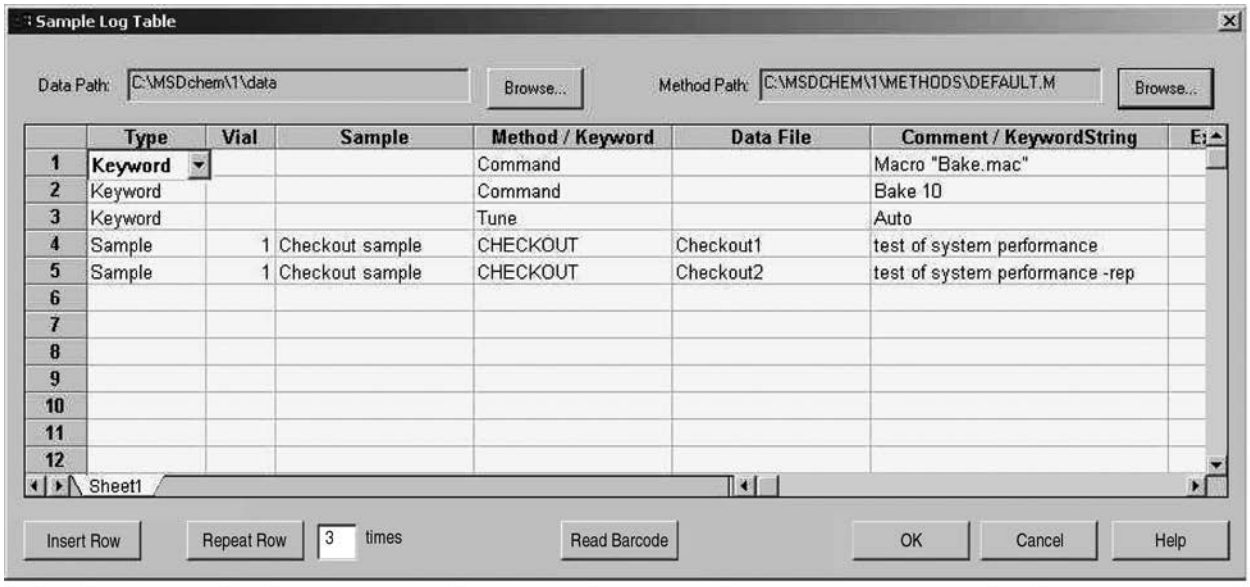

Figure 7. Pumpdown sequence table using source bakeout.

Line 1 Loads the Bake macro. Line 2 sets the bake time to 10 hours. After the bake, (Line 3) an autotune is executed. Lines 4 and 5 run the system performance method, CHECKOUT.M, on the system checkout standard. Note: after the system has been cleaned and leak-checked, the CHECKOUT.M method should be loaded, THEN this sequence should be run!

Conclusions

The increased source temperature limit available on the 5973N inert MSD can provide improved detection limits for common, late-eluting, recalcitrant compounds such as the POPs when properly applied. A requirement, that must be explored, is that the higher source temperatures do not increase compound fragmentation or reduce the intensity of the (useful) higher mass ions. These improvements are most likely to be realized in SIM acquisitions where the increased background that must result from higher source temperatures is not as likely to affect the signal.

This application note also describes a programmed bake-out of the source and quadrupole that can be automatically implemented after source cleaning. This bake-out provides a rapid lowering of the air-water background and can be used within the sequence table as part of the instrument performance checkout.

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Agilent Technologies

Enhanced Reliability of Forensic Drug Testing Using Retention Time Locking

Application

Gas Chromatography

January 1998

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Abstract

Retention time locking (RTL) is used to lock retention times when transferring methods from chromatographic instrument to chromatographic instrument, column to column, and detector to detector. The retention times for a derivatized cocaine standard were locked when the method was translated from an Agilent 6890/Agilent 5972 GC-MSD (gas chromatograph-mass selective detector) system to other Agilent GC-MSD systems and when performing column trimming as a routine maintenance procedure on the same system. The retention times, under both circumstances, matched the original retention times within 0.01 minute after locking.

Key Words

Retention time locking, RTL, method validation, forensic drug testing, capillary gas chromatography, MSD, laboratory productivity, cocaine.

Introduction

In large drug testing laboratories, several instruments are used simultaneously to analyze or screen for cocaine and/or its derivatives. To increase laboratory productivity and improve ease of sample identification and quantitation, laboratories could benefit in many ways by matching retention times of analytes and internal standards from instrument to instrument, day to day, and location to location. This is easily achieved using the G2080AA retention time locking (RTL) software tool for GC systems with electronic pneumatics control (EPC) using the Agilent GC ChemStation (version A.05.02).

RTL provides the ability to match chromatographic retention times exactly in any 6890 GC system to those in another chromatographic system with the same nominal column. The RTL software allows rapid, accurate locking of all retention times using columns of the same stationary phase and dimensions (same part number). The concepts, uses, and requirements for RTL have been discussed elsewhere.[1] This application note examines the use of RTL to prepare the retention time vs. pressure (RT vs. P) calculation for a derivatized cocaine standard sample from a method that was optimized for a 6890/ 5972 GC-MSD system. Retention times for the derivatized cocaine sample were easily locked when the same method was transferred to a 6890/Agilent 5973 GC-MSD system or an 5890/5972 GC-MSD system. In addition, retention times of target cocaine derivatives were again locked every time when column trimming was performed in the same GC system.

At the time of this study, the software used to accomplish locking was not available for the GC/ MSD system. A duplicate method was created on a separate GC ChemStation to develop the RTL relationships for the data collected from the GC/MSD systems. The RTL software residing on the GC ChemStation was also used to calculate the recommended locking pressure for the GC/MSD system based on the RT vs. P relationship and the results from the GC/MSD scouting run.

Experimental

Samples of benzoylecgonine (BE) containing cocaine and cocaine-d3 (internal standard) were prepared and analyzed in accordance with methods reported previously.[2,3]

For the analysis of trimethylsilyl derivatives of BE samples, GC systems equipped with split/splitless inlets (splitless mode) and mass selective detectors (5972 MSD or 5973 MSD) were used. A GC/MSD ChemStation was used for instrument control and data acquisition. The experimental conditions for the GC methods are given in table 1.

Table 1. Experimental Conditions

Automatic sampler	Agilent 7673B, 10-µL syringe, 1-µL injection, viscosity delay: 1 sec
Inlet	Split/splitless inlet, 250 °C, splitless mode
Carrier	Helium, constant pressure (1.2 mL/min at 150 °C), vacuum compensation: on
Column	Agilent HP-5MS, 30 m x 0.25 mm x 0.25 µm (part no. 19091S-433)
Oven	150 °C (0.5 min), 20 °C/min to 290 °C (4 min)
Detector	Direct column interface to MSD at 290 °C, autotune with 600 V above autotune voltage, SIM mode with 25-second dwell time
Software	G2070AA GC ChemStation A.05.02, G2080AA RTL software for GC ChemStation, and G1701AA MSD ChemStation A.03.00

Results and Discussion

In this study, a testing laboratory wanted to lock the retention times of the trimethylsilyl derivative of cocaine and its internal standard at 6.70 minutes on any Agilent GC-MSD system. The chemist developing the locking method first ran five analyses using different column head pressures to establish an RT vs. P relationship. The RT vs. P data were manually entered into the RTL software resident on a separate GC ChemStation. The RTL software RT vs. P calibration screen is shown in figure 1. The RT vs. P information is saved in the method and is valid for any Agilent GC system using the same method and nominal column. The result of this RT vs. P relationship is shown in figure 2. Once this relationship is developed, it can be used to lock any other system of the same configuration. To do that, the method is loaded and a scouting run is done at the method's nominal pressure.

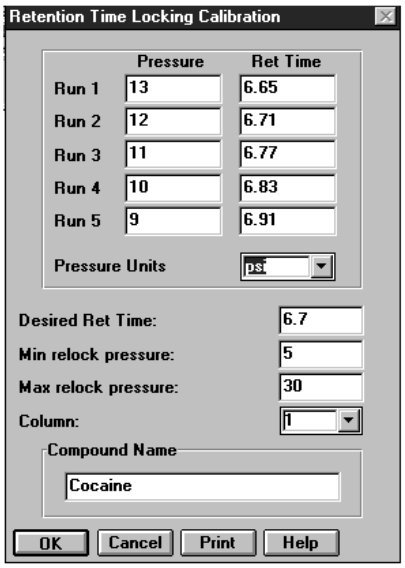

Figure 1. Preparation of retention time locking calibration using the Agilent GC ChemStation.

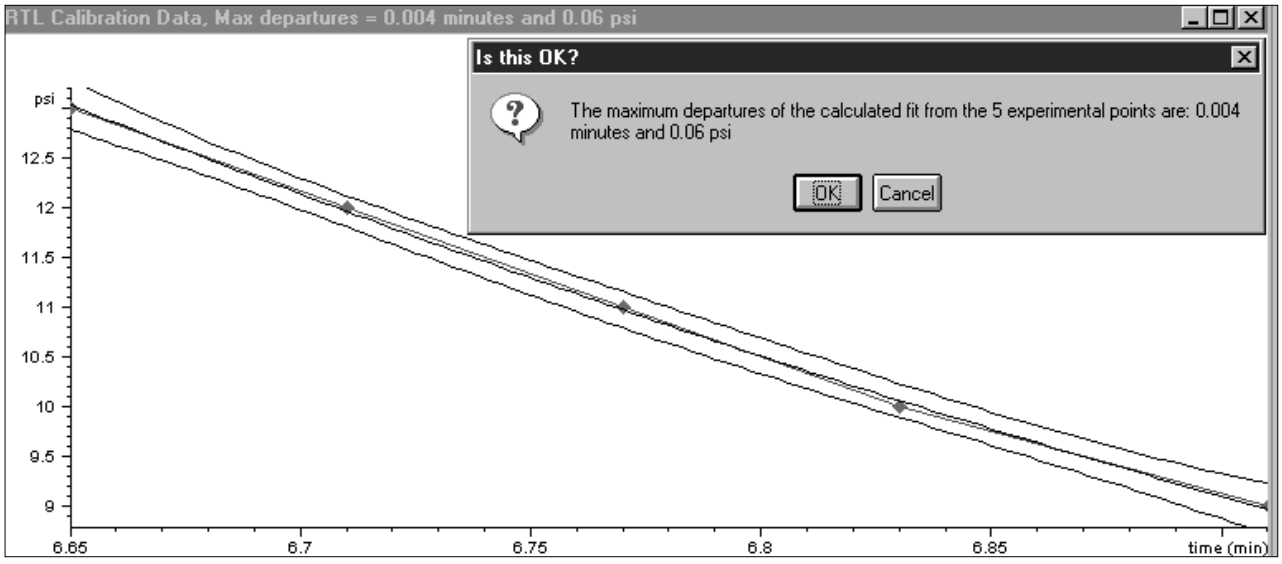

Figure 2. RT vs. P relationship for retention time locking.

On system I (6890/5972 GC-MSD), a scouting run using a head pressure of 12.00 psi yielded a retention time of cocaine at 6.71 min (see table 2). Based on this data set, the RTL software (on the separate GC ChemStation) recommended a column pressure of 12.17 psi for the method (see figure 3).

A column pressure of 12.2 psi was manually entered into the GC/MSD and the extracted ion chromatograms shown in figure 4 were produced. The RT for cocaine was found at 6.69 minutes. In the next five runs at 12.20 psi, the retention time for cocaine was reproducibly obtained at exactly 6.69 min. Extracted ion chromatograms of these runs (m/z 240/256 and 243/259) confirmed that the peak eluting at 6.69 min was indeed cocaine and its internal standard, cocaine-d3 (see figure 4).

Similarly, a good RT match was obtained on system II (6890/ 5973 GC-MSD system) shown in table 2. The same technique was used to

Table 2. Retention Time Locking When Transferring a Method to Different Systems

	Scouting Run		(Re)Lock Run	
	Pressure (psi)	Retention Time(min)	Pressure (psi)	Retention Time(min)
System I	12.00	6.71	12.20	6.69
System II	12.20	7.57	9.10	6.70
System III	12.20	7.33	18.77	6.80
			20.39*	6.69*

* Second (Re)Lock run to fine-tune the method because the experimental retention time falls outside the RT vs. P curve

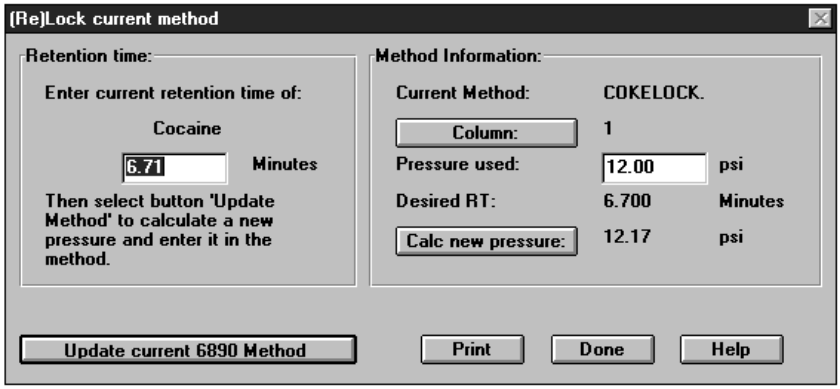

Figure 3. Column pressure recommendation for retention time locking and method update (as found on the RTL software for the GC ChemStation).

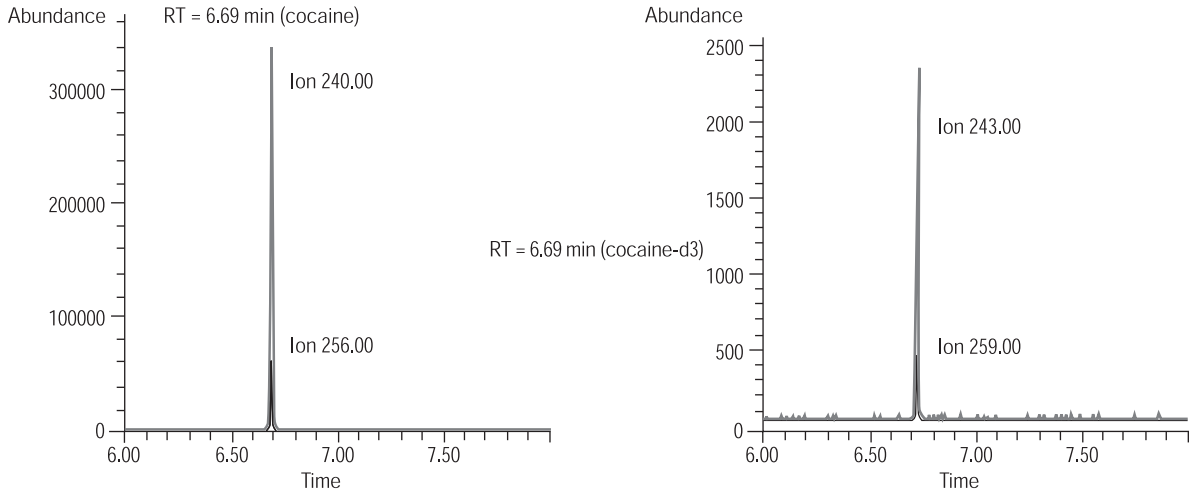

Figure 4. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System I (6890/5972 GC-MSD)

match RT on system III (5890 GC/5972 MSD system)-also shown in table 2. The results shown suggest that system III may have experienced some hardware irregularity. However, the RTL software was able to correct for minor system trouble and to provide recommendations for matching RTs after a third trial to finetune RT matching (see table 2). The recommended column head pressure of 20.39 psi was used to obtain a match for cocaine at 6.69 min for system III. The extracted ion chromatograms confirmed that the analytes that eluted at 6.69 minutes at this column head pressure of 20.39 psi on system III were cocaine and its internal standard, cocaine-d3 (see figure 5).

A routine system maintenance task such as cutting a short length from the head of the analytical column is performed regularly in testing laboratories. The RTs of cocaine were closely matched after each column trimming was performed (see table 3). Each time, a scouting run followed by a (Re)Lock run were performed and an RT of 6.70 min was achieved for cocaine.

First, cocaine was locked at 6.69 min with a pressure of 12.20 psi on system I using an Agilent HP-5MS column with a nominal length of 30 meters. When 14 inches of the column were trimmed off, a scouting run at 12.20 psi yielded an RT of 6.66 min for cocaine. To lock the system, the RTL software suggested a new column pressure of 11.48 psi. The (Re)Lock run yielded an RT of 6.70 min for cocaine (see table 3), and the system was locked again.

The column was trimmed a second time by an additional 14 inches, and a scouting run followed by a (Re)Lock run were performed. The resulting RT for cocaine was locked at 6.70 min with the recommended pressure of 11.00 psi. Finally, the column was trimmed by an additional 28 inches (for a total of 56 inches cut from the 30-meter column). Again, the RT for cocaine was easily locked at 6.70 min with a column pressure of 9.10 psi, which was recommended by the RTL software and manually entered into the GC/MSD (see table 3).

Table 3. Pressures and Retention Times after Performing System Maintenance on System I

Tasks	Scouting Run		(Re)Lock Run	
	Pressure (psi)	Retention Time (min)	Pressure (psi)	Retention Time (min)
Column length, 30 meters			12.20	6.69
Cut 14 inches (30 m - 14 in.)	12.20	6.66	11.48	6.70
Cut additional 14 inches (30 m - 28 in.)	11.50	6.66	11.00	6.70
Cut additional 28 inches (30 m - 56 in.)	11.00	6.60	9.10	6.70

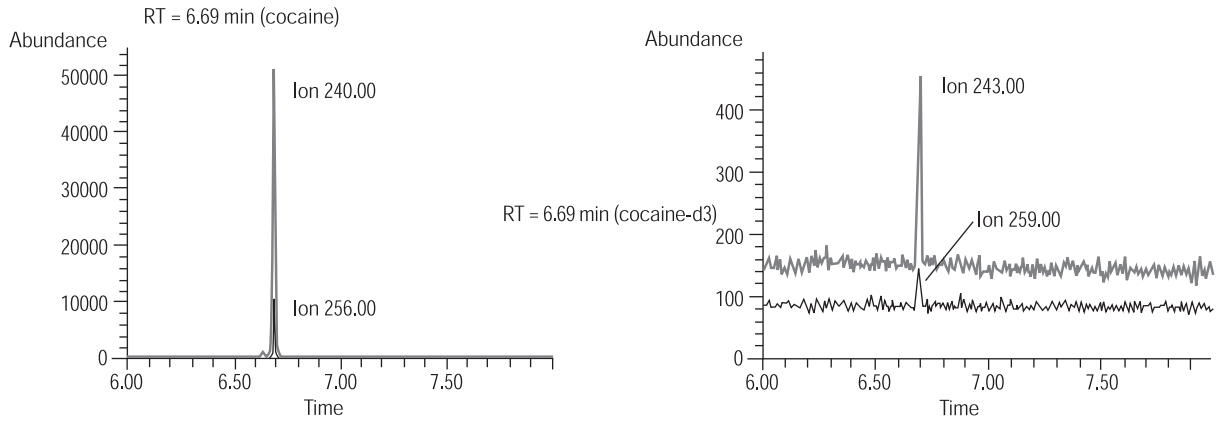

Figure 5. Extracted ion chromatograms of derivatized cocaine and internal standard (cocaine-d3) on System III (5890/5972 GC-MSD).

Conclusions

The G2080AA retention time locking software tool was used to lock retention times for a derivatized cocaine sample analyzed on three different GC/MSD systems and after column trimming. The retention times under these circumstances matched the original retention time within 0.01 minute. The ability to lock retention times means that one calibration can be used for multiple systems, and data from the past can be easily compared to new data.

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2. Goldberger, B., and Miles. W. S., "Benzoylecgonine in Urine: Validating a PrepStation/GC/ MSD System that Integrates Automated Sample Preparation with Analysis," Agilent Technologies, Application Note 228-338, Publication (23) 5964-3705E, October 1995.
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Innovating the HP Way

Fast and Ultra-fast Analysis with the Agilent 1200 Series Rapid Resolution LC System Compared to a Conventional Agilent 1100 Series LC System Using Sub 2-µm Particle Columns

Application Note

A. G. Huesgen

Abstract

Due to an increasing workload in many analytical laboratories, a need to develop analytical methods faster has arisen. Furthermore, developing faster methods for standard columns is critical. Faster method development for faster LC methods is a requirement that can be met with state-of-the-art LC equipment. Even though conventional LC equipment can also provide fast methods, better performance and time savings can be obtained on specially designed LC systems with wider pressure and temperature ranges and lower delay volume - predominantly with 2.1-mm ID columns, where typically lower flow rates are used than on 4.6-mm ID columns. This Application Note shows that shorter run times, shorter equilibration times, and consequently shorter cycle times and more sample throughput are obtained using the Agilent 1200 Series Rapid Resolution LC (RRLC) system.

Agilent Technologies

Introduction

Due to an increasing workload in many analytical laboratories, a need to develop analytical methods faster has arisen. Furthermore, developing faster methods for standard columns is critical. Increasingly more applications are carried out using LC/MS systems, therefore there is also a demand to use narrow-bore columns for full compatibility with most MS engines. Narrow-bore columns with an internal diameter of 2.1 mm and lower have high demands in respect to low delay volumes and dispersion volumes before and after the column. In the following experiment an example is given, showing how fast methods can be developed on an LC system taking advantage of higher pressure and temperature limits of state-of-the-art equipment. In addition, speed and performance comparisons are made between a conventional Agilent 1100 Series LC system and an Agilent 1200 Series Rapid Resolution LC system, using 4.6-mm ID columns and 2.-mm ID columns packed with 1.8-µm particles.

Experimental

An Agilent 1200 Series RRLC system was used with the following modules:

- Agilent 1200 Series binary pump SL with vacuum degasser for applications using 1.8-µm particle columns up to 150-mm length and with internal diameters from 2.1 to 4.6 mm
- Agilent 1200 Series high-performance autosampler SL for highest area precision
- Agilent 1200 Series thermostatted column compartment SL with wide temperature range from 10 degrees below ambient up to 100 °C
- Agilent 1200 Series diode-array detector SL for 80-Hz operation, including new data protection tool
- ZORBAX SB C-18 columns with different internal diameters and 50-mm length, packed with 1.8-µm particles
- Low dispersion kit for optimized conditions for 2.1-mm ID columns (Agilent part number G1316-68744)

An Agilent 1100 Series LC system was used with the following modules:

- Agilent 1100 Series binary pump with vacuum degasser
- Agilent 1100 Series well-plate autosampler
- Agilent 1100 Series thermostatted column compartment
- Agilent 1100 Series diode-array detector B
- Low dispersion kit for optimized conditions for 2.1-mm ID columns (Agilent part number 5065-9947)

Results and discussion

In the past the Agilent 1100 Series LC system was frequently used for fast and ultra-fast analysis[1]. The instrument is very well suited specifically for the analysis of compounds using short 4.6-mm ID column packed with 1.8-µm particles, and run times below one minute. Cycle times below two minutes were achieved. The Agilent 1200 Series RRLC system is a newly developed LC system with a wider pressure and temperature range, lower system delay volumes and improved noise for the DAD system. Due to these advancements, speed and performance have improved compared to an Agilent 1100 Series LC system, especially for columns with an internal diameter of 2.1 mm.

Experiments using a 4.6-mm ID column

Both instruments were set up in a standard configuration with mixers and 0.17-mm ID flow capillaries installed. Typically the same parameters can be used to optimize an LC method for speed and resolution. These parameters are flow rate, column temperature, gradient profile and other instrument-specific parameters such as switching the autosampler delay volume out of the flow path after the sample has reached the top of the column (ADVR=automatic delay volume reduction). Gradient changes can therefore reach the column much faster. A typical example of how a fast method can be developed is given in figure 1. The objective is to achieve fast cycle times and a minimum resolution of 2 for all peaks.

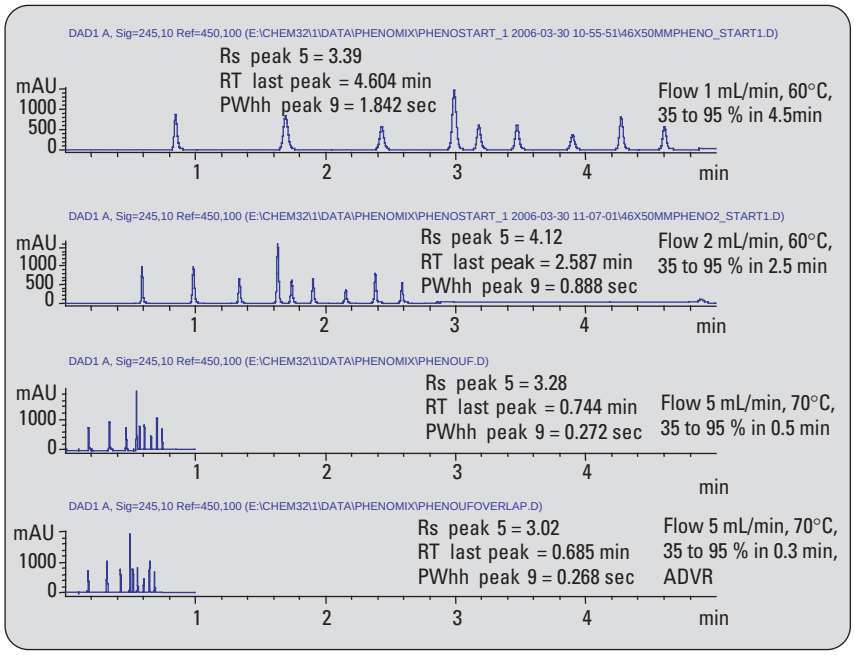

Figure 1
Method development of an ultra fast LC method.

Chromatographic conditions:

Test sample:	Set of 9 compounds; 100 ng/µL each; dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3: Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6.Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 4.6 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O + Solvent B: ACN Gradient: 35 to 95 % B using different profiles
Autosampler:	Injection volume: 1 µL Wash 5 sec for needle exterior flush out factor 20
Thermostatted column compartment:	Temperature: different temperatures
Diode array detector	B and diode-array detector SL: Signal: 245/10 nm Ref 450/100 nm

Optimization of all of the above-mentioned parameters on both systems resulted in the chromatograms shown in figure 2. The pressure limit of 400 bar on the Agilent 1100 Series LC system restricts the maximum possible flow. 5 mL/min flow was not possible, even though the column temperature was set to 80 °C, which is the upper limit for the 1100 Series column compartment. The Agilent 1200 Series RRLC system can be operated with up to 600 bar and up to 100 °C. Applying a flow rate of 5 mL/min can be done without reaching the 600 bar pressure limit at elevated temperatures. In addition, due to design changes, the noise level of the Agilent 1200 Series DAD SL has significantly improved compared to the Agilent 1100 Series DAD B.
The performance for both systems is shown in table 1.

Resolution and noise have improved with the Agilent 1200 Series RRLC system, whereas run and cycle times are comparable. The noise level of the 1200 Series RRLC system can be further reduced using the post column cooling device[2]. The device adapts the temperature of the column effluent to the temperature of the optical unit. This further reduces the noise level, especially if high flow rates and high temperatures are used. Another possibility to reduce cycle time is to enable the overlapped injection features, which is possible with both systems.

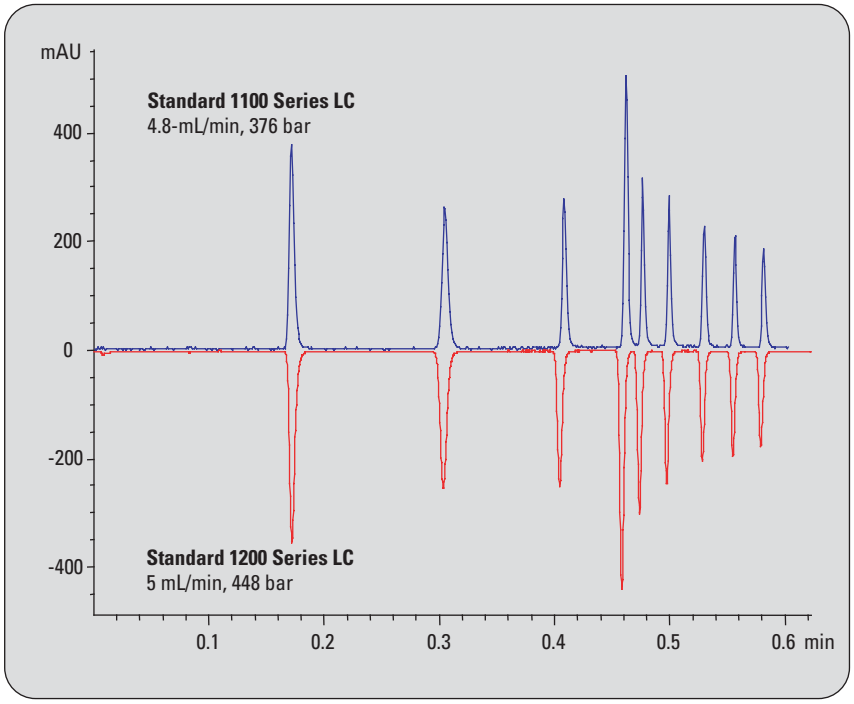

Figure 2
Standard Agilent 1200 Series RRLC system vs. Agilent 1100 Series LC system: analysis of phenone mix on 4.6-mm ID column packed with 1.8-µm particles.

Chromatographic conditions:

Test sample:	Set of 9 compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3: Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 4.6 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O, Solvent B: ACN Gradient: 35 to 95 % B in 0.3 min
Autosampler:	Injection volume: 1 µL Wash 5 sec for needle exterior, flush-out factor 20
Thermostatted column Compartment:	Temperature: 80 °C
Detector DAD B and DAD SL:	Signal: 245/10 nm Ref 450/100 nm

_Parameter	Standard 1100 Series 80 °C 4.8 mL/min	Standard 1200 Series 80 °C 5 mL/min
Flow rate	4.8 mL/min	5 mL/min
Run time	0.60 min	0.60 min
Cycle time	1 min 37 sec	1 min 37 sec
Rs Peak 5	2.22	2.30
PW1/2 peak 9	0.00378 min	0.00375 min
PW1/2 peak 1	0.00458 min	0.00486 min
Noise PtoP	6.2021mAU	0.7930 mAU
Backpressure	376 bar	448 bar
Injection volume	1 µL	1 µL
DAD data rate	20 Hz, path 10 mm	80 Hz, path 10 mm

Table 1
Performance comparison for 4.6-mm ID column.

Furthermore, column switching valves can be installed in the ovens, which provides even higher sample throughput using 2 columns for analysis. A sample is analyzed on the first column, while the second column is regenerated using a second pump. If the analysis on the first column is completed, the next injection can be immediately performed on the previously equilibrated second column.

Experiments using 2.1-mm ID column

Columns with an internal diameter of 2.1 mm and lower have high demands regarding low delay volumes and dispersion volumes before and after the column. Using columns with an internal diameter of 2.1 mm, the Agilent 1100 Series binary LC system must be optimized without using a mixer or only a mixer with a significantly smaller volume and capillaries with smaller IDs for all flow connections. Nevertheless, cycle times below 2 minutes could barely be achieved using columns packed with 1.8 µm particles and 50 mm length. This was mainly due to the pressure limitation of 400 bar for the Agilent 1100 Series LC system. In addition, the delay volume of the 1100 Series LC system is a drawback for fast run and equilibration times. With the introduction of the Agilent 1200 Series RRLC system this gap was closed. Now using narrow bore columns packed with 1.8-µm particles, run times below 0.5 min are possible, with higher flow rates and elevated temperatures. Both systems are compared using the same column and optimized instrument configurations. To allow for optimized conditions for both systems, the following set-ups were used:

Configuration of the Agilent 1100 Series LC system:

- The mixer was replaced by a short capillary with an internal diameter of 0.12 mm (Agilent part number G1312-67301)
- Seat and seat capillary were replaced by 0.12-mm ID parts (well-plate seat, Agilent part number G1367-87104, and seat capillary, Agilent part number G1313-87103)
- The capillary from the injector to the column compartment was replaced with a 0.12-mm ID capillary (Agilent part number 01090-87610)
- The 0.17-mm ID capillary from the column compartment to the column was exchanged with a capillary with an internal diameter of 0.12 mm (Agilent part number G1316-87303)
- The column was connected to the detector using the detector inlet capillary.
- A 1.7-µL cell with a path length of 6 mm was used as the detector cell.

Configuration of the Agilent 1200 Series RRLC system:

- The low delay volume configuration for the pump was set up with a 120-µL delay volume (mixer and damper were moved out of the flow path).
- Two flow capillaries were replaced with 0.12-mm ID capillaries, all included in the Agilent 1200 Series low dispersion kit (Agilent part number G1316-68744).
- The seat capillary was also replaced with a 0.12-mm ID capillary (included in kit Agilent part number G1316-68744)
- The DAD SL 2 µL flow cell with a 3-mm path length was used. The inlet capillary was directly connected to the column outlet.

The same 2.1 x 50 mm column was used for both systems. The flow rate was set so that the backpressure was close to the limit of each system. Automated delay volume reduction (ADVR) was selected in the injector setup screen for both systems. The injection volume was set to 1 µL for the Agilent 1100 Series LC system, and to 2 µL for the Agilent 1200 Series RRLC system to compensate for the lower path length of the 1200 Series 2-µL flow cell.

In figure 3 an overlay of the chromatograms obtained from both systems is shown. In table 2 the performance for both system is recorded.

The chromatograms in figure 3 clearly demonstrate the advantages of the Agilent 1200 Series RRLC system, using 2.1-mm ID columns, packed with 1.8-µm particles. Faster run times and cycle times are possible, due to the fact that higher flow rates can be obtained with the Agilent 1200 Series RRLC system. Table 2 indicates that the cycle time for the Agilent 1200 Series RRLC system is only half that of the Agilent 1100 Series LC system. In addition, the resolution of the 5th peak and also peak width at half height is significantly improved at higher flow rates.

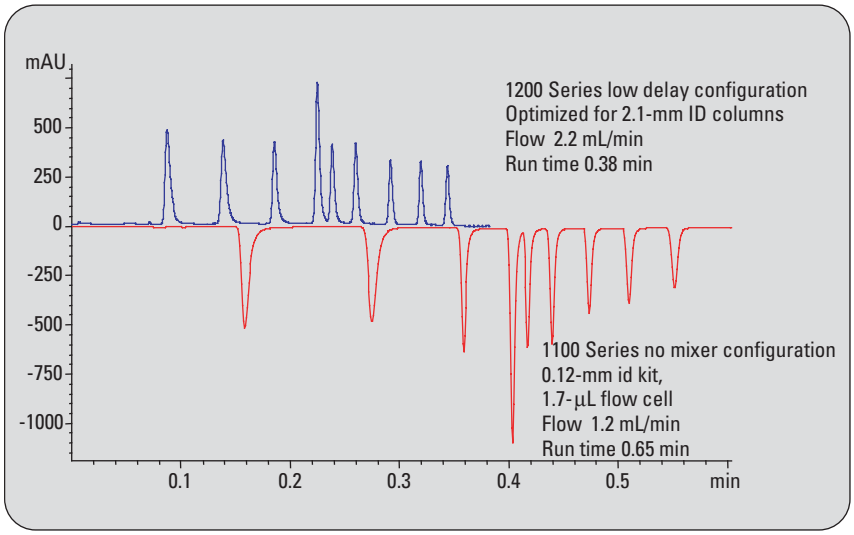

Figure 3
Analysis performed with a 2.1-mm ID column with the optimized Agilent 1200 Series RRLC system and the optimized Agilent 1100 Series LC system using automated delay volume reduction for both systems.

Chromatographic conditions:

Test sample:	Set of 9 compounds, 100 ng/µL each, dissolved in water/ACN (65/35) 1. Acetanilide, 2. Acetophenone, 3. Propiophenone, 4. Butyrophenone, 5. Benzophenone, 6. Valerophenone, 7. Hexanophenone, 8. Heptanophenone, 9. Octanophenone
Column:	50 x 2.1 mm ZORBAX SB C-18, 1.8 µm for 600 bar operation
Pump:	Solvent A: H_2O , Solvent B: ACN Gradient: 35 to 95 % B in 0.3 min
Autosampler:	Injection volume: 1 and 2 µL Wash 5 sec for needle exterior, flush out factor 20
Thermostatted column compartment:	Temperature: 80 and 95 °C
Detector DAD B and DAD SL:	Signal: 245/10 nm Ref 450/100 nm

Parameter	1100 Series, optimized, no mixer, ADVR, 80 °C	1200 Series, optimized, low delay volume configuration, ADVR, 95 °C
Flow rate	1.2 mL/min	2.2 mL/min
Run time	0.65 min	0.38 min
Cycle time	2 min 33 sec	1 min 16 sec
Rs Peak 5	1.86	2.15
PW1/2 peak 9	0.00556 min	0.00328 min
PW1/2 peak 1	0.00729 min	0.0049 min
Noise PtoP	0.1 mAU	0.2 mAU
Backpressure	370 bar	570 bar
Injection volume	1 µL	2 µL
DAD data rate	20 Hz, path 6 mm	80 Hz, path 3 mm

Table 2
Performance comparison using a 2.-mm ID column.

Conclusions

Faster method development for faster LC methods is a requirement that can be met with state-of-the-art LC equipment. Even though conventional LC equipment can also provide fast methods, better performance and time savings can be obtained on specially designed LC systems with wider pressure and temperature ranges. Predominantly with 2.1-mm ID columns, where typically lower flow rates are used than on 4.6-mm ID columns, an LC system like the Agilent 1200 Series RRLC system provides significantly lower delay volumes. Shorter run times and shorter equilibration times, and consequently shorter cycle times and more sample throughput are obtained.

References

1.
Anabel Fandino, "Ultra-fast liquid chromatography using the Agilent 1100 Series HPLC system and 1.8-um ZORBAX SB C18 Rapid Resolution HT columns", *Agilent Application Note, publication number 5989-1603EN*, **2004.**

2.
A.G.Hüsgen, "Agilent 1200 Series column compartment SL with temperature control up to 100 °C and post-column cooling for lowest baseline noise", *Agilent Application Note, publication number 5989-5034EN*, **2006.**

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Agilent Technologies

Achieving fastest analyses with the Agilent 1200 Series Rapid Resolution LC system and 2.1-mm id columns

Application Note

Michael Frank

Abstract

The need to increase the daily throughputs of LC systems is a constant desire. Now, with the Agilent 1200 Series Rapid Resolution LC system highest throughputs are possible, and in combination with the Agilent ZORBAX RRHT columns and the increased pressure and temperature range of the LC system, excellent chromatographic resolution can be achieved even at run times below one minute.

This Application Note describes the correct set-up of the instrument which is the key for optimal results with narrow bore columns, such as a 2.1 mm x 50 mm column packed with sub two micron particles. Peak capacities in the range of fifty in analysis times as short as 24 seconds and peak widths as narrow as 200 milliseconds are shown. The well-balanced use of all possible module options to achieve shortest cycle times with throughputs far beyond 1500 samples per day is described.

Agilent Technologies

Introduction

Particularly analytical service laboratories in the pharmaceutical industry, responsible for analyzing chemical libraries[1] or performing MS based quantifications of certain ADME-properties and drug metabolism studies of drug candidates[2] are faced with the challenge to increase their throughput, but also to maintain a high chromatographic resolution. In 2003 Agilent Technologies introduced sub two micron particles in their RRHT column series. Because of the small particle size, the chromatographic resolution obtainable with these columns is superior to standard particle sizes such as 3.5 µm or even 5 µm. Due to a unique silica manufacturing process, Agilent ZORBAX RRHT columns show a significantly reduced backpressure, if compared to similar column dimensions of other manufacturers. Excellent chromatographic results are achieved in a very short analysis time with the Agilent 1200 Series Rapid Resolution LC system, which facilitates an increased pressure range and flow rates from 0.05 up to 5 mL/min using column diameters ranging from 2.1-mm id up to 4.6-mm id. This Application Note will focus on 2.1-mm id columns only.
Not only are the run times of the analyses important for high throughput, but also the overhead time. The Agilent 1200 Series Rapid Resolution LC system can be optimized to achieve highest throughputs with exceptionally good overall system performance.

Experimental

An important issue when dealing with narrow bore columns, especially in gradient mode where smallest peak widths can be achieved, is to have small extra column volumes. This also includes any volumes in front of the sampling device, because any volume after the solvent mixing point will increase the time for the gradient composition to reach the column. This results in an increased run time. The Agilent 1200 Series Rapid Resolution LC system can be reconfigured within a few minutes to provide appropriate system volumes for different column ids. Here, the pumps are set-up in the low delay volume configuration with an internal volume of approximately 120 µL. All other modules are optimized for lowest delay volumes by using the low delay volume capillary kit (G1316-68744). Consequently, only capillaries of 0.12 mm id are used beyond the injection valve. In the Agilent 1200 Series thermostatted column compartment SL the newly introduced low dispersion heat exchangers with 1.6 µL internal volume were used. In some experiments, the Agilent 1200 Series Rapid Resolution LC is set up for alternating column regeneration to achieve highest throughput using the ACR-capillary kit (G1316-68721) and 2.1-mm id columns[3]. The high pressure rated 2-position/10-port valve in the thermostatted column compartment was only placed into the flow path if alternating column regeneration was used indeed.

The instrument set-up is as follows (figure 1):

- Agilent 1200 Series binary pump SL with the new Agilent 1200 Series micro vacuum degasser
- Agilent 1200 Series high performance autosampler SL
- Agilent 1200 Series thermostatted column compartment SL, equipped with a high pressure, 2-position/10-port valve, facilitating alternating column regeneration
- Agilent 1200 Series diode-array detector SL with a 2-µL/3-mm cell
- ZORBAX SB C18, 2.1 mm id x 50 mm, 1.8 µm

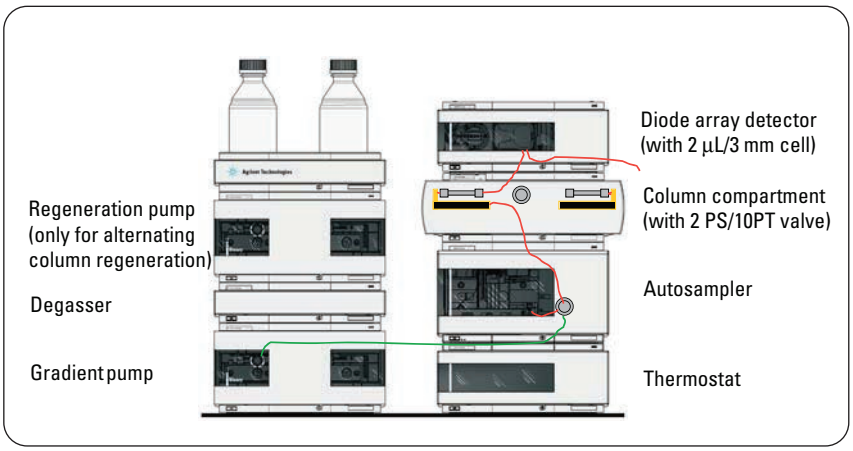

Figure 1
System setup with low delay volume for high speed applications using 2.1-mm id columns with lengths from 20 to 50 mm.

The Agilent 1200 Series binary pump SL is designed to fulfill the demands for high throughput, highest performance, optimum resolution and lowest pump ripple. The pump hardware is significantly different from the standard binary pump. In the Agilent 1200 Series binary pump SL the pressure transducer is separate from the damper which has been modified to have a lower delay volume (pressure dependent ranging from 80-280 µL). In this study the pumps were used in the low delay volume configuration without the mixer and damper in the flow path. In contrast to the standard binary pump the pump heads of the binary pump SL have an additional damping coil (500 µL volume each) to allow damping in the low delay volume configuration. This does not add to the gradient delay volume because it is before the mixing point. Anyhow, pressure ripples are also strongly suppressed by the Electronic Damping Control (EDC). The pressure range of the pump and all other modules is increased to 600 bar.

Only one sample, the so-called "phenone-mix", was used in the course of this study to keep variations low. The sample consists of nine compounds: acetanilid, acetophenone, propiophenone, butyrophenone, benzophenone, valerophenone, hexanophenone, heptanophenone and octanophenone. Unless otherwise stated, the concentration was 0.1 µg/µL for each compound except butyrophenone which was 0.2 µg/µL. The solvent was water-acetonitril 2:1.

Results and discussion

The most frequently sold particle size in chromatographic columns today is 5 µm. Of course, fast and ultra fast LC is also possible with columns packed with particles of these larger diameters – the reduced back pressure is even beneficial to allow higher flow rates. However, resolution will be sacrificed because conditions are usually far on the right side of the van-Deemter-optimum. Here, the big advantage of the RRHT columns with particles of less than 2 µm diameter is proven. The van Deemter optimum is shifted further to the right and the curve is much flatter at the onset because the "resistance of mass transfer" term is diminished (figure 2). In figure 3 the analysis on a 2.1-mm id column with 1.8-µm particles is compared to the linear scaled analysis on the same stationary phase but on 5 µm particles packed in a 4.6-mm id-column. The gain in resolution is obvious – from Rs = 2.1 up to Rs = 3.5 for the critical pair which matches the theoretically expected value of a 1.66 fold increase in resolution. Also note that there is a saving in solvent consumption of 8.6 mL in the "standard" HPLC analysis and only 1.8 mL in the ultra fast HPLC analysis.

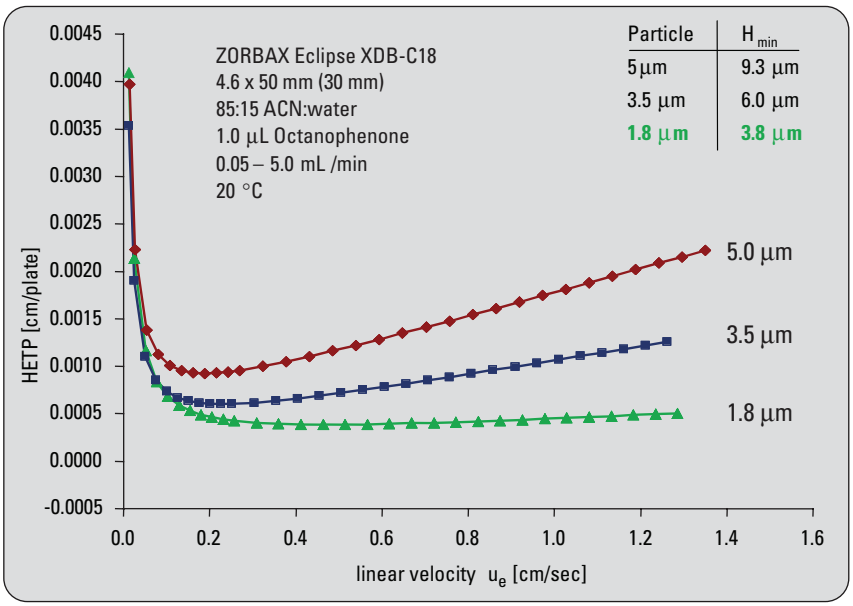

Figure 2
Van Deemter curves of columns packed with 1.8 µm, 3.5 µm and 5.0 µm particles.

For gradient separation the dependencies of the capacity factor can be expressed as:

$$k^* = 0.87 \cdot tg \cdot \frac{F}{Vm \cdot \Delta\%B \cdot S}$$

(tg = gradient time, F = flow rate, Vm = column void volume, △%B = gradient steepness, S = solvent and solute dependent factor)

If the product of the gradient time and flow rate, the so-called gradient volume, is kept constant together with all other parameters, the gradient time might be decreased while the flow rate is increased. Thus, the capacity factors of two compounds will stay constant and if no large alteration of the plate height occurs, the resolution will not change significantly, either. The final point is the big advantage of the sub two micron particles – the van-Deemter curve is nearly flat on the right side of the minimum (figure 2) and flow rates can be increased with only little increase in plate heights. However, the equation is an empirical one and deviations may occur especially under extreme conditions.

With a two-step approach, highest gradient speeds with virtually no loss or only little loss in resolution can be achieved. In the first step, start from a medium temperature and begin to increase the flow rate up to the pressure maximum. Subsequently the temperature should be increased to lower the viscosity of the solvent and then the flow rate is increased again. It may be worthwhile to check the resolution with two identical gradients but with different temperatures to see the influence of the temperature change on the resolution which may be very compound dependent. In figure 4 the result of this approach is shown. A nearly 7-fold increase in separation speed could be achieved with still baseline separation of the critical pair before meeting the pressure and temperature limit (the maximum temperature is a function of flow, temperature, number of controlled Peltier elements and of the heat capacity of the solvent used).

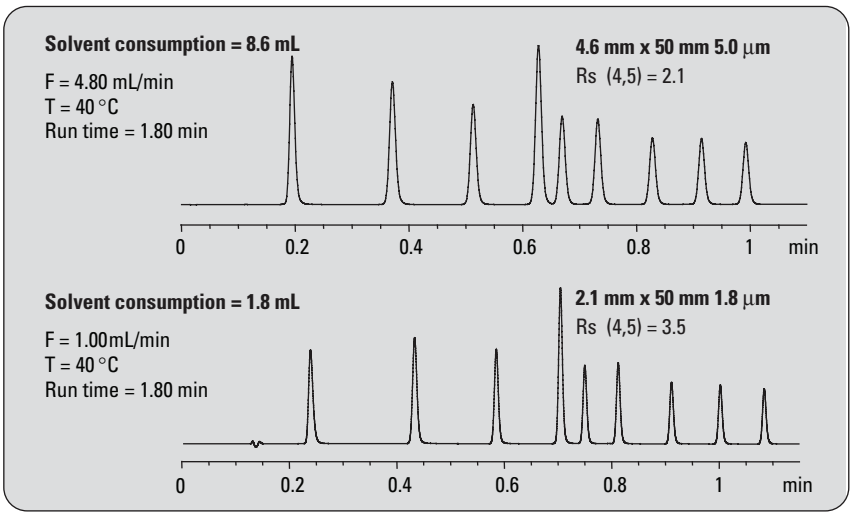

Figure 3
Analysis with 1.8-µm particle column vs. 5.0 µm particle column.

Conditions:	4.6-mm id column used on standard Agilent 1200 system	
Solvent:	A = Water, B = ACN	
Temperature:	40 °C	
Column:	**2.1 mm x 50 mm, 1.8 µm**	**4.6 mm x 50 mm, 5.0 µm**
Flow:	1.0 mL/min	4.8 mL/min (scaled from 2.1 mm col.)
Gradient:	0.00 min 35 %B	0.00 min 35 %B
	0.90 min 95 %B	0.90 min 95 %B
	1.10 min 95 %B	1.10 min 95 %B
	1.11 min 35 % B	1.11 min 35 % B
Stoptime:	1.15 min	1.15 min
Posttime:	0.70 min	0.70 min
Wavelength:	245 nm (8), ref. 450 nm (100)	245 nm (8), ref. 450 nm (80)
Peakwidth:	>0.0025 min (0.05 s res.time), 80 Hz	>0.01 min (>0.2 s), 20 Hz
Injection volume:	1 µL	5 µL (not scaled)

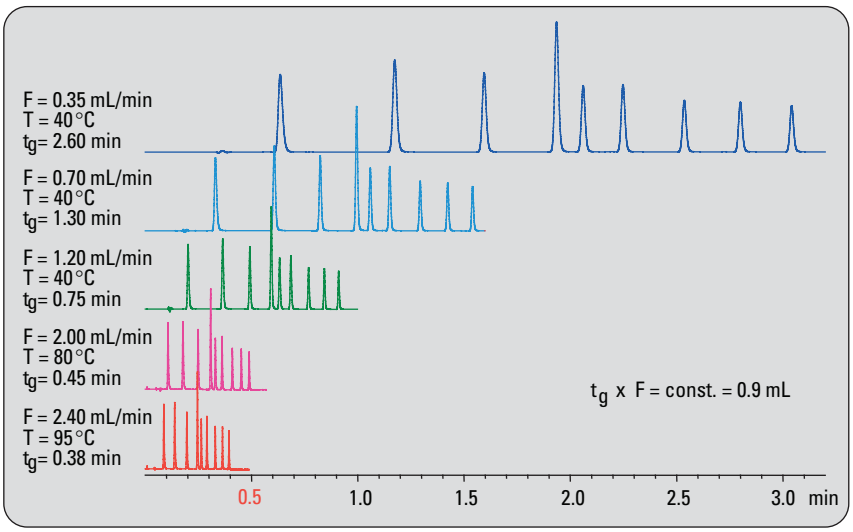

Figure 4
Increasing separation speed by increasing temperature and flow rate while decreasing gradient time.

Conditions:
Solvent: A = water, B = ACN
Temp.: 40 °C, 80 °C, 95 °C
Flow: 0.35, 0.70, 1.20, 2.00, 2.40 mL/min
Gradient: 0.00 min 35 %B
2.60 min 95 %B
3.20 min 95 %B
3.21 min 35 %B
Time values for F = 0.35 mL/min. For all other flow rates times are scaled so that (tg x F) = 0.90 mL
Stop time: 3.20 min
Post time: 2.00 min
Wavelength: 245 nm (8), Ref. 450 nm (100)
Peak width: >0.0025 min (0.05 s response time), 80 Hz

The last chromatogram is enlarged in figure 5 and reveals the details of this separation. The first peak is eluted after only five seconds and peaks with a width at half height of less than 200 ms are achievable. Within twenty-four seconds nine compounds are separated with a peak capacity in the range of fifty.

Retention time precision at highest analysis speed

High analysis speed is meaningless without precision. One basic performance criteria for HPLC pumps is the precision of gradient formation measured by the precision of retention times of repeated gradients. However, the stability of the column temperature must also be taken into consideration, because temperature fluctuations will also influence the retention times of a given sample. In table 1 and figure 6 the results from the 10-fold repeated analysis of a standard sample are listed and since the deviation between individual runs is so small, the octanophenone peak is enlarged in a separate window. This sample contains compounds that are both not retained and refer to isocratically eluted compounds found at the starting conditions of the gradient, as well as highly unpolar and strongly retained compounds. The analyses

Conditions:

Solvent:	A = Water, B = ACN
Temp.:	40 °C, 80 °C
Flow:	0.35 mL/min, 1.20 mL/min, 2.0 mL/min
Gradient:	0.00 min 35%B
	2.60 min 95%B
	3.20 min 95%B
	3.21 min 35%B
	Time values for F = 0.35 mL/min.
	For all other flow rates times are
	scaled so that (time x flow) = 0.90 mL
Stop time:	3.20 min
Post time:	2.00 min
Injection vol.:	1.0 µL

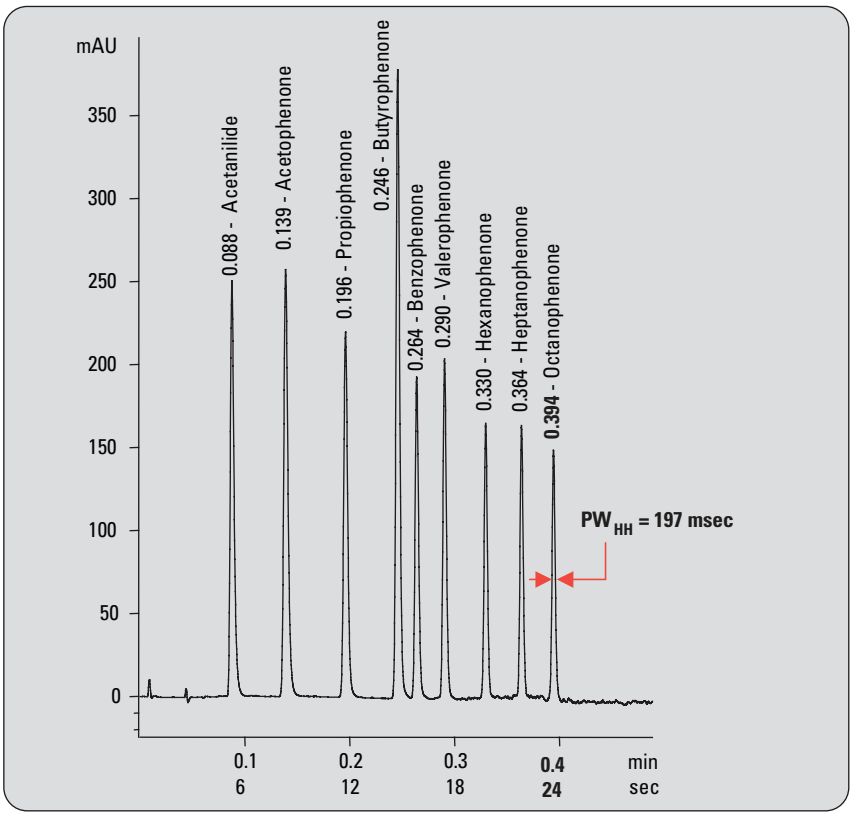

Figure 5
Separation of a nine compound mixture under ultra fast conditions.

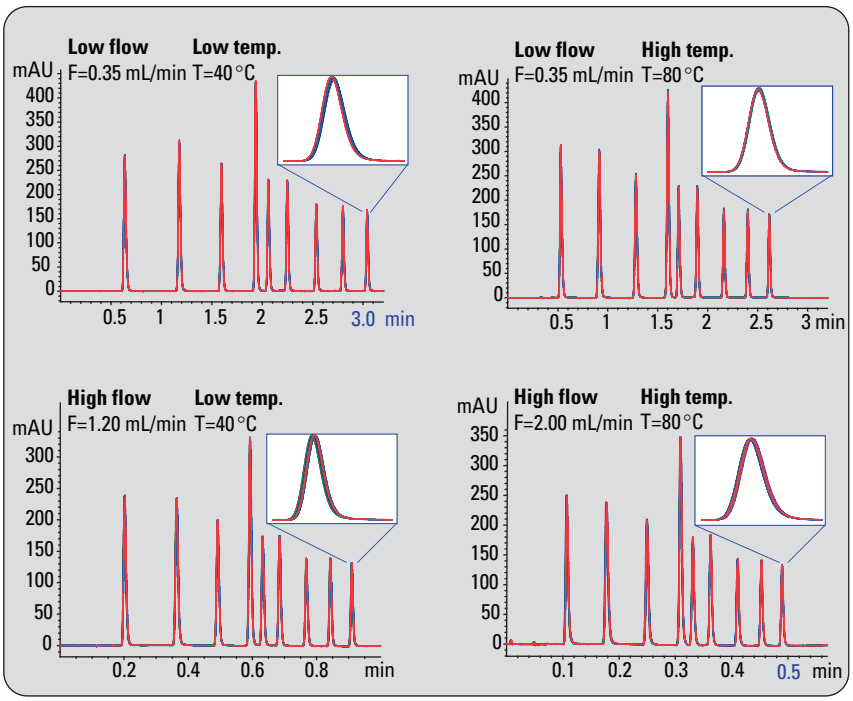

Figure 6
Overlaid chromatograms of the repeated analysis of a 9 compound mixture under various conditions.

were done at high and low flow rates as well as with high and low temperatures as in the examples shown earlier. In all cases the mean retention time precision is below 0.3 % RSD, which was the specification of the Agilent 1100 Series LC system. Of course, the results are also in line with the specifications for the new Agilent 1200 Series Rapid Resolution LC system which is < 0.07 % RSD or < 0.02 min SD, whichever is met first. At these high gradient speeds, the SD criteria are always met. The RSD criteria are also met for both fast-LC gradients of 2.6 min duration (0.35 mL/min flow rate). Even at ultra-fast gradient speeds, the retention time precisions are still below or only slightly higher than 0.1% RSD (table 1).

Improving the cycle-time

Not only is the gradient speed important when dealing with high-throughput analysis but furthermore the over all cycle time of the entire system, which is the time between two consecutive analyses. A good method to measure the cycle time is by using the time stamp the data file is assigned by the operating system of the computer. Clearly, optimizing the cycle time has some drawbacks. For example, extensive needle cleaning procedures are in contradiction with a high sampling speed. Table 2 gives an overview of important parameters influencing the cycle time. Using 1.8-µm particle size columns together with an optimized HPLC system very short run times can be achieved without sacrificing chromatographic resolution. Combining short run times together with low overhead times will result in a high daily throughput. In figure 7 the cycle time and daily throughput is shown for two

	0.35 mL/min, 40°C		0.35 mL/min, 80°C		1.20 mL/min, 40°C		2.00 mL/min, 80°C	
	SD	% RSD	SD	% RSD	SD	% RSD	SD	% RSD
Average	0.00107	0.067	0.00084	0.070	0.00048	0.098	0.00031	0.134

Table 1
Standard deviations (mAU) and %RSD (n=10) of the retention times under different chromatographic conditions in temperature and flow.

Module	Parameter	Effect on cycle time	Other effects
Pump	Low delay volume setting	Reduced retention times, run time can be shortened, reduced cycle time	Increased pressure ripple, slightly increased mixing noise if modifiers such as TFA are used.
Autosampler	Automatic Delay Volume Reduction (ADVR) – activated	Reduced delay volume, reduced retention times, run time can be shortened, reduced cycle time	Increased carry-over
	ADVR activated and Overlapped Injection (OI)	Enables parallel sampling, thus reduces the cycle time independently of the below listed settings (as long as the overall sampling speed does not exceed the gradient and post time)	Increased carry-over
	no OI – Needle Wash	Increased sampling time with increasing wash time	Reduced carry-over with longer needle wash time
	no OI – Equilibration time	Increased sampling time with increased equilibration time	Better injection precision with longer equilibration time
	no OI – Draw/Eject speed	Low speed causes increased sampling time	Low speed results in better injection precision
Column compartment	Alternating column regeneration	Saves column wash-out and equilibration time, reduces cycle time enormously	Additional hardware required, slightly increased extra column volume, slightly different retention times between columns possible
Detector	Pre-run and/or post-run balance	Increased cycle time	Baseline drifts possible if not applied
	Spectral data acquisition with high data rate, small band width and broad wavelength range large data files	Depending on computer power and additional processes running might increase cycle time because of writing speed	Reduced information content if no spectral data acquired or with lower resolution
Software	Data analysis with acquisition	Increased cycle time, depending on computer power and number of peaks	Data analysis has to be done offline is no set
	Save method with data	Slightly increased cycle time	Information is missing if method is not saved
	Execution of pre-run or post-run macros	Increased cycle time, depending on macro	Depending on macro
System	LC controlled over local network between computer and LC (and MS) only	Faster data and method transfer between computer and LC because of reduced net work traffic reduced cycle time	Additional hardware might be necessary (use independent acquisition computer)
	Number of detectors	More detectors produce a higher data amount and lower the data transfer speed, resulting in higher cycle times	More detectors higher information content

Table 2
Influence of various parameters on the overall cycle time.

different methods – both giving virtually the same resolution. The first method (0.45 min gradient) utilizes alternating column regeneration and high temperatures to allow high flow rates and speed optimized settings. A cycle time of 49 s could be achieved, resulting in a theoretical daily throughput of more than 1700 samples per day. The second method (0.90 min gradient) does not use high temperatures or alternating column regeneration and the time saving of some simple and often forgotten method options are shown. By optimizing these parameters the real cycle time gets as close to 8 s to the run time (stop time plus post time) and allows a daily throughput of more than 700 samples per day. By sub-optimal method set up this can easily drop to below 500 samples per day if options like automatic delay volume reduction, overlapped injection or offline data-analysis are not used.

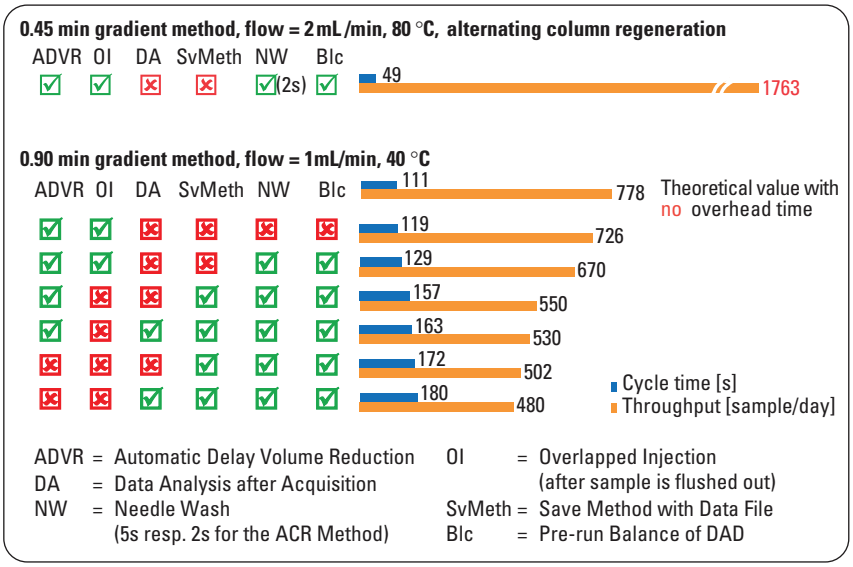

Figure 7
Cycle time and daily throughput optimization.

Chromatographic conditions:

Alternating Column Regeneration Method

Solvent:	A = Water, B = ACN	
Temp.:	80 °C	
Flow:	2.0 mL/min	
ADVR:	Yes	
Gradient:	**Gradient-Pump**	**Regeneration-Pump**
	0.00 min 35 %B	0.00 min 35 %B
	0.45 min 95 %B	0.01 min 95 %B
	0.46 min 35 %B	0.11 min 95 %B
	0.57 min 35 %B	0.12 min 35 %B
Stoptime:	0.57 min	no limit
Posttime:	off	off
Wavelength:	245 nm (8), ref. 450 nm (100)	
Peak width:	> 0.0025 min (0.05 s response time), 80 Hz	
Spectra:	none	
Injection volume:	1.0 µL	
Injector:	Overlapped injection, 2 s needle wash, sample flush-out factor = 10, draw/eject speed = 100 µL/min	
Valve:	next position	

No Alternating Column Regeneration Method

Solvent:	A = Water, B = ACN	
Temp.:	40 °C	
Flow:	1.0 mL/min	
ADVR:	Yes	No
Gradient:	0.00 min 35 %B	0.00 min 35 %B
	0.90 min 95 %B	0.90 min 95 %B
	1.10 min 95 %B	1.10 min 95 %B
	1.11 min 35 %B	1.11 min 35 %B
Stoptime:	1.15 min	*1.40 min (add. 300 µL extra column volume, increased retention times)*
Posttime:	0.70 min	0.70 min
Wavelength:	245 nm (8), ref. 450 nm (100)	
Peak width:	> 0.0025 min (0.05 s response time), 80 Hz	
Spectra:	all, 190-500 nm, BW = 1 nm	
Injection volume:	1.0 µL	
Injector:	See figure 7, 2 s equilibration time	

Conclusion

The Agilent 1200 Series Rapid Resolution LC system is a powerful tool to achieve highest chromatographic resolutions and also highest throughputs. The extended pressure range allows the usage of columns packed with stationary phases with particles sizes below 2 µm, for example, Agilent RRHT columns with particle sizes of 1.8 µm. These columns not only allow an increase in linear flow rates with virtually no loss in resolution but also have an inherently higher resolution compared to 3.5 µm or even 5.0 µm particle sizes. The possibility to switch the pump into its low delay volume configuration allows the use of the entire bandwidth of today's widely used column ids – from 4.6 mm down to 2.1 mm and even 1.0 mm. As illustrated above, the system has uncompromised performance characteristics even at highest gradient speeds.

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www.agilent.com/chem/1200rr

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Agilent Technologies

Combined EI and CI Using a Single Source

Technical Overview

Chris Sandy

Agilent Technologies

Introduction

The Agilent 5973x gas chromatograph/mass selective detectors (GC/MSDs) come with sources optimized for electron ionization (EI) and chemical ionization (CI). However, there are occasions where another ionization mode is desired without changing sources. This note demonstrates the capability of acquiring high-quality EI spectra with the CI source.

Data Acquisition

An Agilent 5973 inert MSD with a CI source was set up for the experiments. The following process was used to tune the MS:

1. Perform the CI autotune at the normal methane reagent gas flow rate (typically at a mass flow controller (MFC) setting of 20%).
2. Reduce the CI flow to 2%.
3. Set the emission current to 250 µa.
4. In Manual Tune, ramp the repeller from 0–5 volts for the mass 69 ion.
5. Set the repeller voltage to the maximum value.
6. Turn off the CI gas.
7. Save tune file.
8. Associate tune file with method.

Data was acquired in positive CI (PCI) and EI modes. Figure 1 shows the CI and EI total ion chromatograms using the CI source. The major and minor peaks are easily comparable in the two chromatograms.

Figure 2 shows the CI spectrum for Hexadecanolide (MW = 254) with the expected adduct ions for methane. Note the relatively large response for the 255 ion. As expected, there is little fragmentation due to the soft ionization.

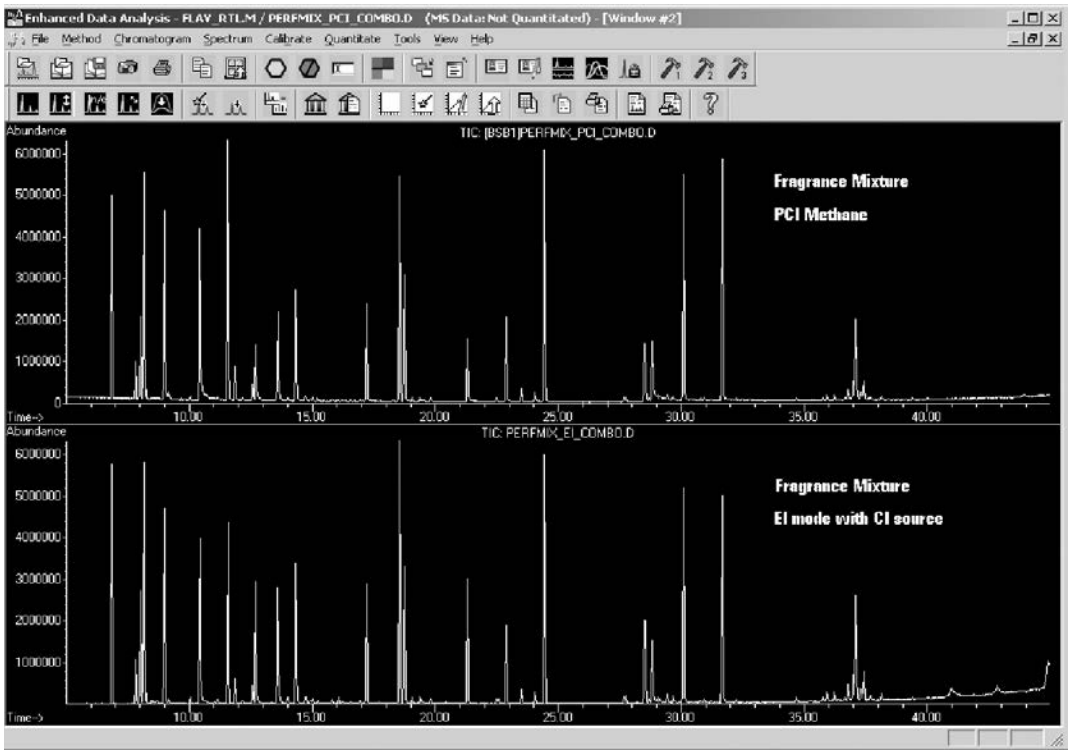

Figure 1. PCI and EI total ion chromatograms using the CI source.

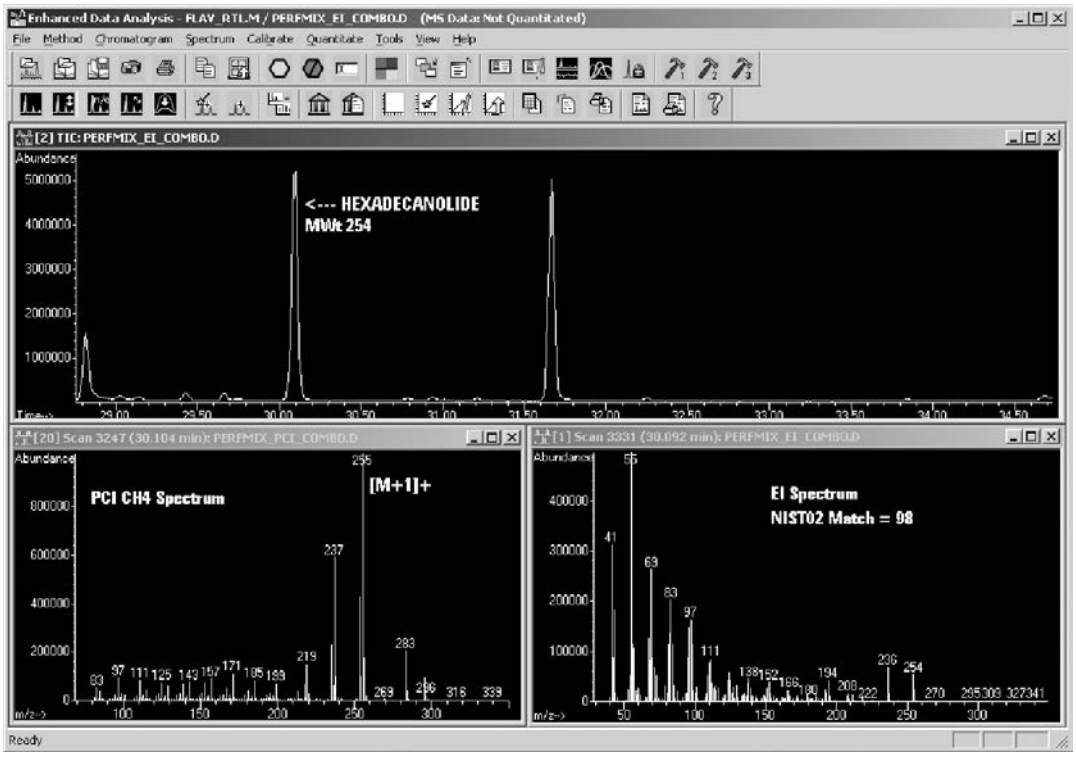

Figure 2. PCI and EI spectra for Hexadeconolide.

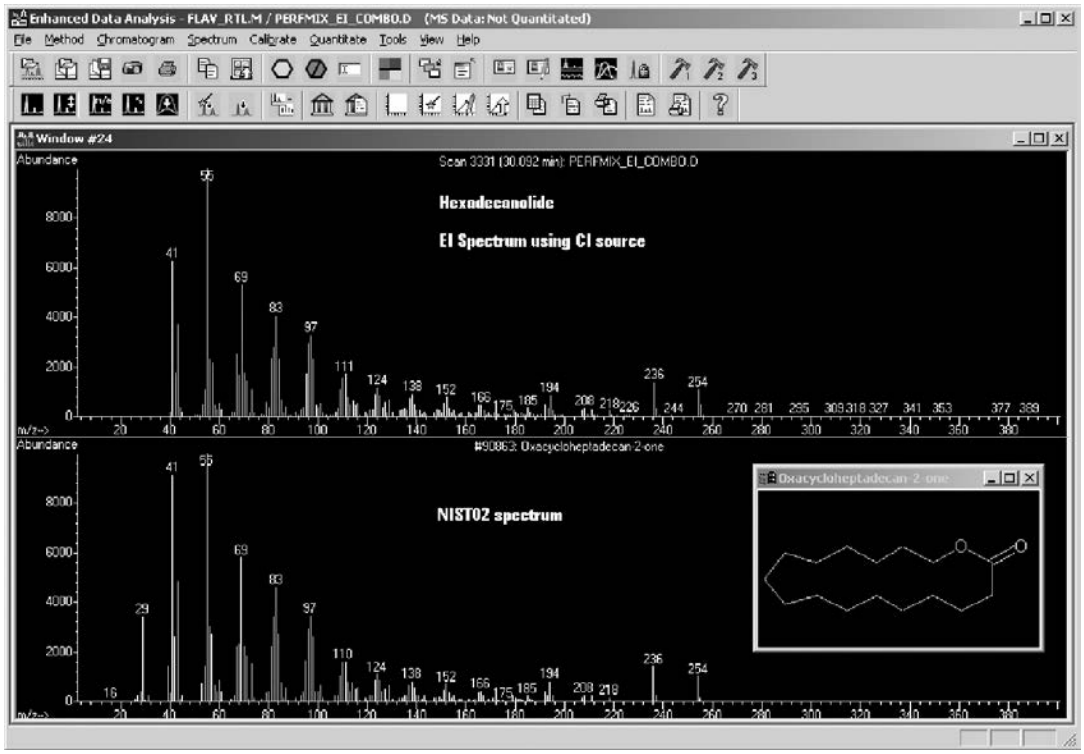

Figure 3. Acquired EI spectrum compared to the NIST02 library reference spectrum.

The EI data in Figure 3 shows much more fragmentation useful for compound identification. The response for 255 is relatively small. Using the NIST02 library, the EI reference spectra for Hexadecanolide (Oxacyclohelptadecan-2-one) was retrieved with a 98% quality match.

Summary

This data demonstrates the Agilent 5973 inert GC/MSD's ability to acquire high quality EI spectra using the CI source. The EI spectra can be searched against standard libraries for identification while the CI spectra provide molecular weight information. The ability to acquire both types of data without changing sources results in increased productivity.

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www.agilent.com/chem

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Agilent Technologies

The Benefits of Achieving High Mass Accuracy at High Speed Using Agilent's TOF-MS Technology

Application Note

Edgar Naegele

Abstract

Measuring accurate molecular mass by mass spectrometry and calculating the corresponding empirical formula is an important step in the identification process of small molecules in a variety of application fields. Depending on the accuracy of mass measurement, significant empirical formulas can be calculated in low numbers. This Application Note will discuss the benefits of using the Agilent 6210 TOF mass spectrometer in combination with the Agilent 1200 Series Rapid Resolution LC system for compound identification in various applications.

Agilent Technologies

Introduction

Reliable empirical formula confirmation necessitates setting a mass accuracy limit, which takes the acceptable uncertainty of the accurate molecular mass measurement into consideration[1]. This results in more accurate mass measurement with decreasing relative mass error and requires fewer possibilities to consider for an empirical formula (table 1).

Mass accuracy [ppm]	Empirical formulae
100	138
50	67
25	32
10	15
5	7
2	2

Table 1
Mass accuracy vs. number of calculated empirical formulae for reserpine ($C_{33}H_{40}N_2O_9$ M=608.2734; within $C_{1\text{-}100}H_{2\text{-}200}N_{0\text{-}10}O_{0\text{-}10}$).

The current generation of comparably easy-to-use and inexpensive ESI orthogonal acceleration TOF (oaTOF) instruments are capable of handling this task. This was clearly demonstrated by a comparison study of different types of MS instruments, which are used for the determination of accurate mass of small molecules[2]. Innovations in TOF technology introduced during the past several years, like the orthogonal acceleration TOF technology with an analog-to-digital (ADC) converter, made this progress possible[3]. This Application Note will demonstrate the benefits of using the Agilent 6210 time-of-flight mass spectrometer in combination with the Agilent 1200 Series Rapid Resolution LC (RRLC) system and their impact on compound identification in various applications.

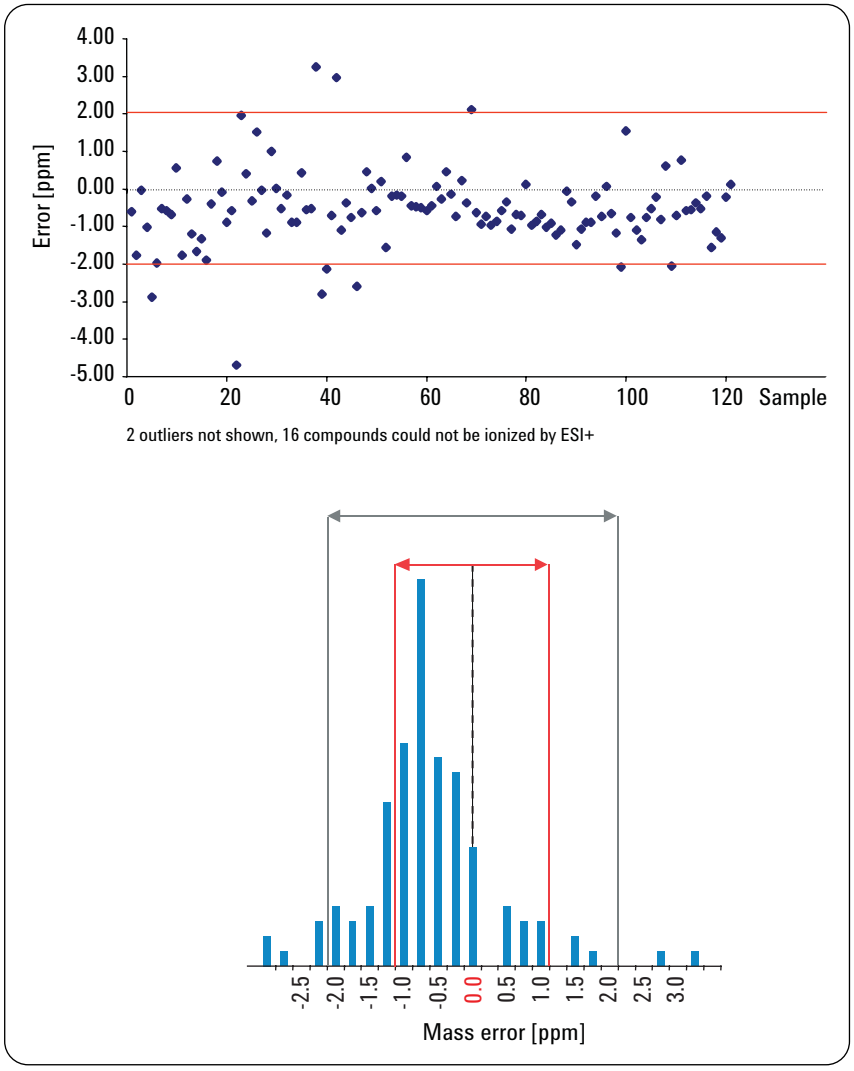

Figure 1
A) Mass accuracy errors as returned by an automatically generated report.
B) Histogram of the mass accuracy errors of the analysis of 140 real chemical library samples of a pharmaceutical company.

Results and discussion

When using a TOF mass spectrometer, attention is certainly focussed on the accurate mass. Figure 1A shows the achieved mass accuracy errors of the analysis of 140 members of a chemical library used in a screening campaign. More compelling is the histogram of these samples as shown in figure 1B. More than 71 % of the analyzed compounds have a mass accuracy error in the range of ± 1.0 ppm. This efficiency enables the chemist to narrow down the number of possible calculated empirical formulas for confirming the identity of a compound[4]. Analysis times below one

minute could be achieved, with high peak capacities above forty in just 39 seconds, both in the UV and in the MS chromatogram (figure 2) by using a method which includes alternating column regeneration, MS TOF data acquisition at 40 Hz, and DAD data acquisition at 80 Hz.

Application examples

- Analysis of complex samples with the MassHunter software, which allows extraction of molecular mass data and their detailed analysis[5] (figure 3).
- Detection and identification of minor impurities in pharmaceutical compounds generated during stability testing, production, formulation or storage of the final drug compound (Agilent publication numbers 5989-2348EN and 5989-5617EN).
- Statistical evaluation of achieved TOF mass accuracies with a real sample of less than 2 ppm (Agilent publication number 5989-3561EN).
- Simultaneous determination of metabolic stability and metabolite identification by high speed and high resolution (Agilent publication number 5989-5110EN).
- Automated screening of clinical body fluid samples for administered drugs (Agilent publica tion number 5989-5835EN).
- Identification of natural products from complex plant extracts (Agilent publication number 5989-4506EN).
- A complete overview of TOF applications is published in a compendium (Agilent publication number 5989-2549EN).

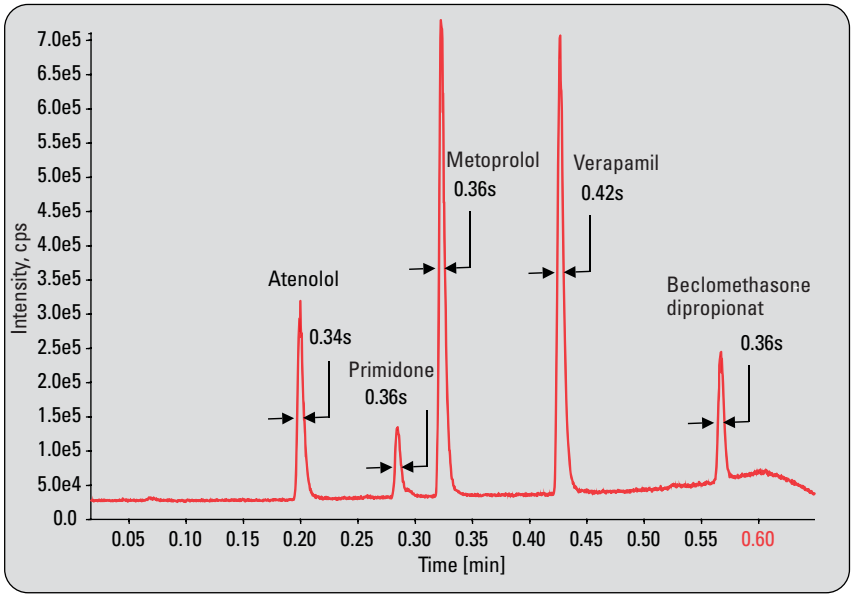

Figure 2
TIC chromatogram (40-Hz data rate of the 6210 TOF mass spectrometer, 80-Hz data rate of the DAD) with PWHH values for the TIC.

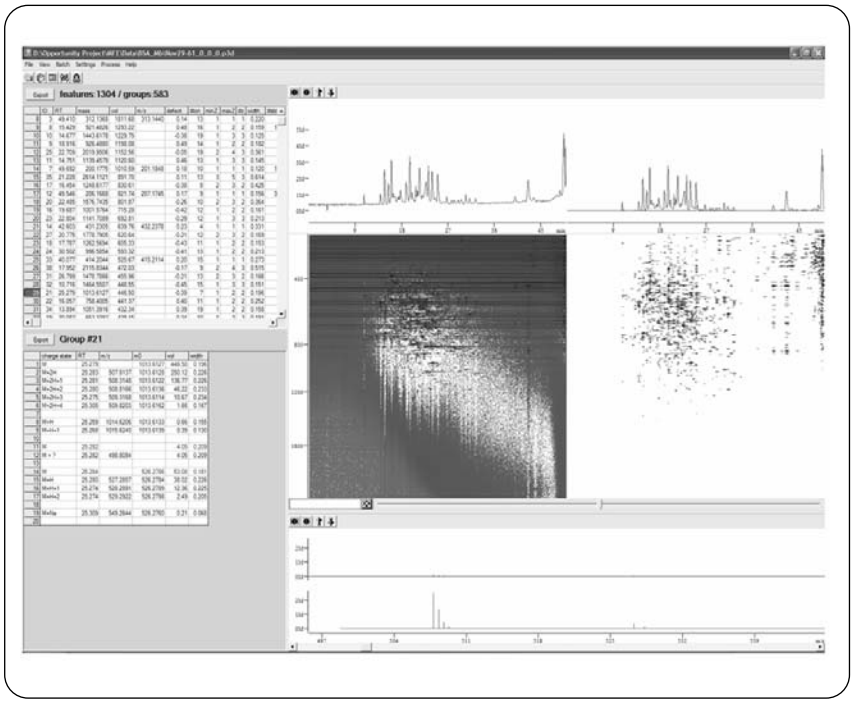

Figure 3
MassHunter software for analysis of complex samples.

Conclusion

- It is possible to rapidly acquire molecular mass data with highest mass accuracy in the single digit ppm error range with the Agilent 6210 TOF. This allows the unambiguous calculation of empirical formulas for compound confirmation.
- It is possible to measure mass differences with highest resolution with the Agilent 6210 TOF instrument. This allows the separation of compounds, which have a similar mass and distinguish between their empirical formulas.
- It is possible to acquire date with up to 40 Hz acquisition rate with the Agilent 6210 TOF. This permits the instrument to be used in ultra-fast LC separation applications.
- The principal benefits are accurate time-of-flight mass measurement, high resolution and high speed data acquisi tion, which can be used over a broad range of applications, such as library screening, screening of clinical samples, metabolite stability and metabolite identification, identification of minor impurities in drugs and natural product analysis.

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www.agilent.com/chem/tof

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Agilent Technologies

Can "Deconvolution" Improve GC/MS Detectability?

Application Note

All Industries

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Abstract

This study uses 35 pesticides spiked in spinach extracts at the 50 ppb level to find the optimal AMDIS deconvolution settings. Additional advantages of using deconvolution versus MSD ChemStation, to find more compounds in an extract are also discussed.

The detectability of compounds in a complex matrix is significantly improved with deconvolution. This can also be viewed as better or increased sensitivity through improved selectivity versus the background.

Agilent's MSD ChemStation add-on - Deconvolution Reporting Software (DRS) runs AMDIS automatically to generate an easy-to-read quantitation report.

Agilent Technologies

Introduction

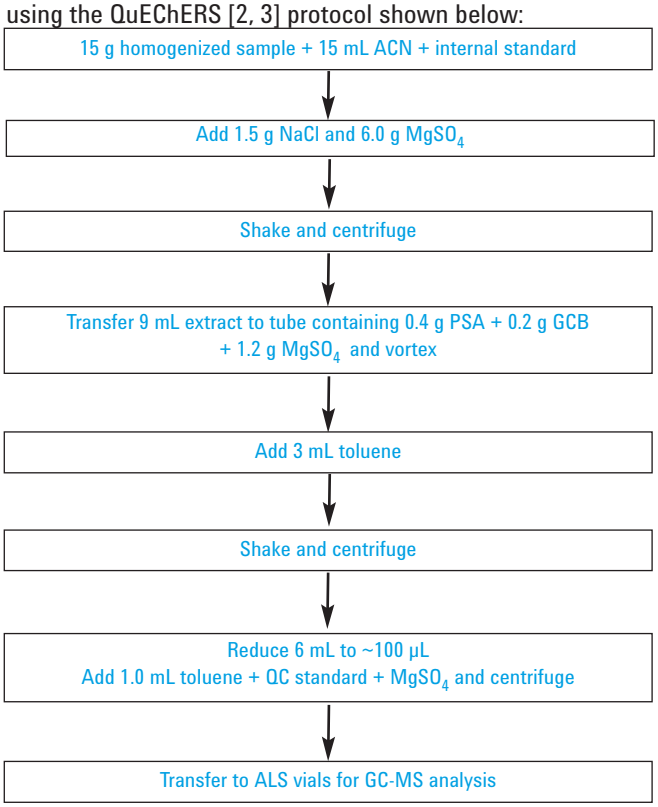
Instrument detectability is usually determined by the amount of sample injected, the responses from the detector and matrix interferences. The signal-to-noise ratio (S/N) can be used to gauge the sensitivity of an instrument in a clean sample. The presence of matrix alters this sensitivity due to a lack of selectivity between compounds of interest and background.

In a multiresidue analysis, the data reviewing process is also very important in confirming the hits found by the software and reviewing the integration and quantitation for accuracy.

Agilent Deconvolution Reporting Software (DRS) has been proven as a powerful data processing tool for finding trace compounds in complex matrices [1]. In this study, results from the Automated Mass spectral Deconvolution and Identification System (AMDIS), part of DRS is closely studied and compared to the results from ChemStation. The goal is to determine if deconvolution (DRS) can provide better results (detectability) than routine ChemStation data processing.

Experimental

Spinach extracts (see Acknowledgement) were prepared using the QuEChERS [2, 3] protocol shown below:



Thirty-five pesticides were spiked into spinach extract at 50 ppb (pg/µL).

Instrument parameters

GC: **7890A**

Autoinjector: 7693A
Retention gap: 2 m × 0.25 mm id Siltek capillary tubing
Column: HP-5MS UI (ultra inert), 15 m × 0.25 mm, 0.25 µm (from inlet to Purged Union) Agilent p/n 19091S-431 UI

Oven ramp:	Rate (°C/min)	Temp (°C)	Time (min)
Initial		100	1.6
Ramp 1	50	150	0
Ramp 2	6	200	0
Ramp 1	16	280	5

Run time: 20.933 min
Inlet: Multimode Inlet (MMI) at 17.73 psi (Retention Time Locked), constant pressure mode
RT locking: Chlorpyrifos-methyl locked to 8.297 min
Liner: Helix double taper, deactivated (Agilent p/n 5188-5398)
Injection mode: 2-µL cold splitless (fast injection)

Inlet temp. ramp:	Rate °C/min	Temp °C	Time min
Initial		50	0.01
Ramp 1	720	300	hold

Septum purge: 3 mL/min
Purged Union: 4 psi (PCM)
Split vent: 50 mL/min at 0.75 min
Gas saver: 20 mL/min after 4 min
Cryo on: Cryo use temperature 150 °C; time out at 15 min

Backflush

Postrun: 5 min
Oven: 280 °C
Purged Union: 70 psi
MMI: 2 psi
Restrictor: 0.7 m × 0.15 mm deactivated fused silica tubing (from Purged Union to MSD)

MSD: **5975C**

Solvent delay: 2.5 min
EMV mode: Gain Factor = 2
Mass Range: Full scan, 45-550
Threshold: 0
Sample number: 2 A/D Samples 4
Transfer Line: 280 °C
Source: 300 °C
Quad: 200 °C

Deconvolution

Deconvolution is a process for extracting ions from a complex total ion chromatogram (TIC), even with the target compound signal at trace levels. The software used for this technique is AMDIS developed by NIST (National Institute of Standards and Technology) [4].

As a review, let's look at the deconvolution process. AMDIS considers the peak shapes of all extracted ions and their apex retention times (RT). In this example, only some of the extracted ion chromatograms (EICs) are overlaid for clarity with the apex spectrum (Figure 1A).

Ion 160 EIC has the same RT as ions 50, 170 and 280, but has a different peak shape. Ion 185 has a different peak shape and an earlier RT. Ions 75 and 310 have similar peak shapes but they have different RTs.

Figure 1A

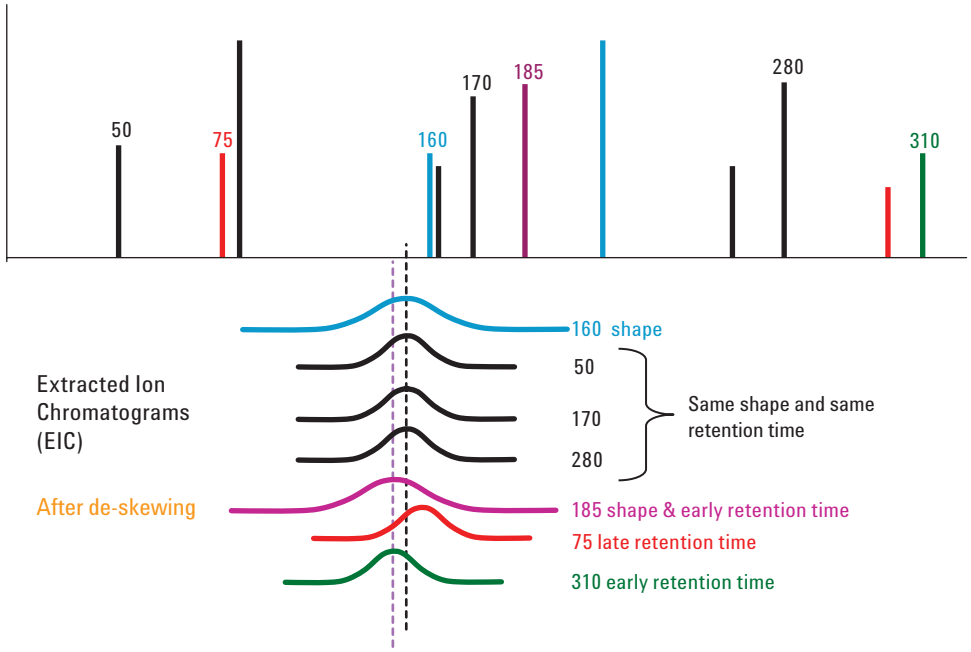


Figure 1B shows the EICs after the different peak shapes or RTs are eliminated from Figure 1A. Ions 50, 170, 280 and a few others remain.

Figure 1B

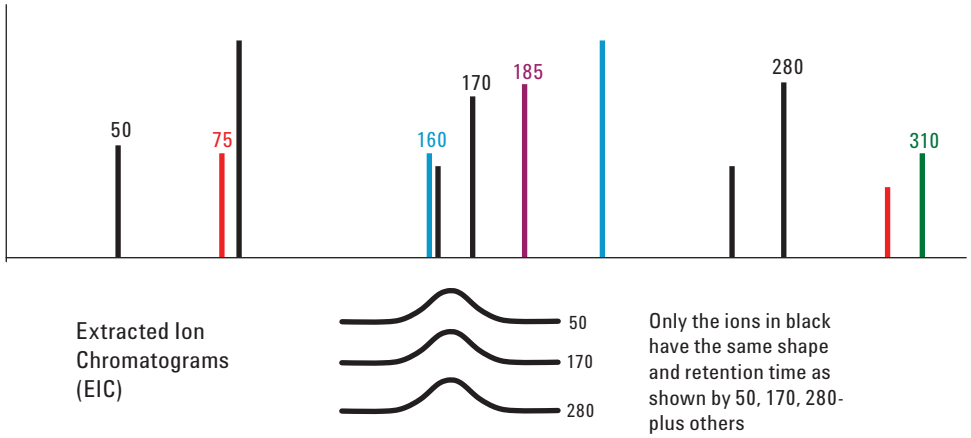


Figure 1A-1C. Simplified deconvolution process (continued).

Figure 1C shows all of the ions in black that have similar peak shapes and RTs, within the criteria set earlier by the analyst. These are grouped together and referred to as a component by AMDIS.

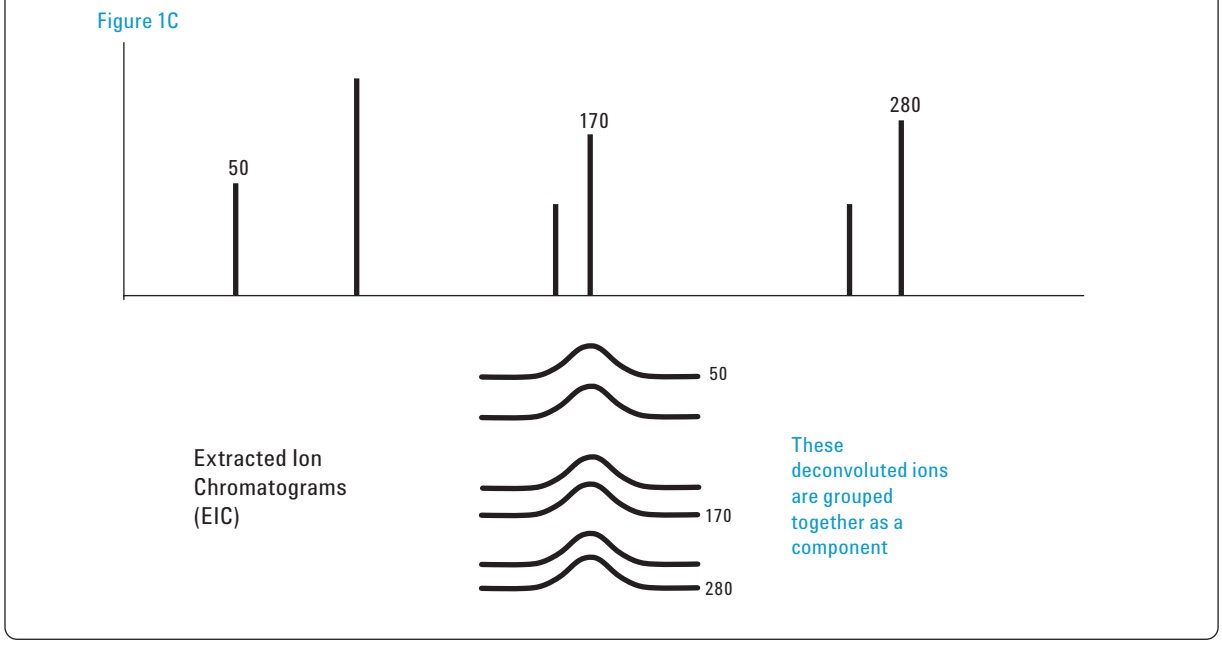


Figure 1A-1C. *Simplified deconvolution process (continued).*

Deconvolution finds the components from a complex TIC. Each component is searched against a retention time locking (RTL) library in AMDIS format. In addition to spectral matching, the locked RT can also be used as a criterion for hits. Depending on the match factor from the search, target compounds can be identified or flagged in a complex TIC. The power of deconvolution is appreciated while comparing the top two spectra in Figure 2. The raw scan or original nondeconvoluted scan is shown on top. The clean scan, that is the deconvoluted component, is shown in the middle. The bottom scan is the identified compound in the AMDIS library. Without deconvolution, the analyst would visually compare the background subtracted raw scan and library scans for confirmation. It would be very difficult, if not impossible, to say that Fenbuconazole, the target compound in this example, is present using that type of comparison.

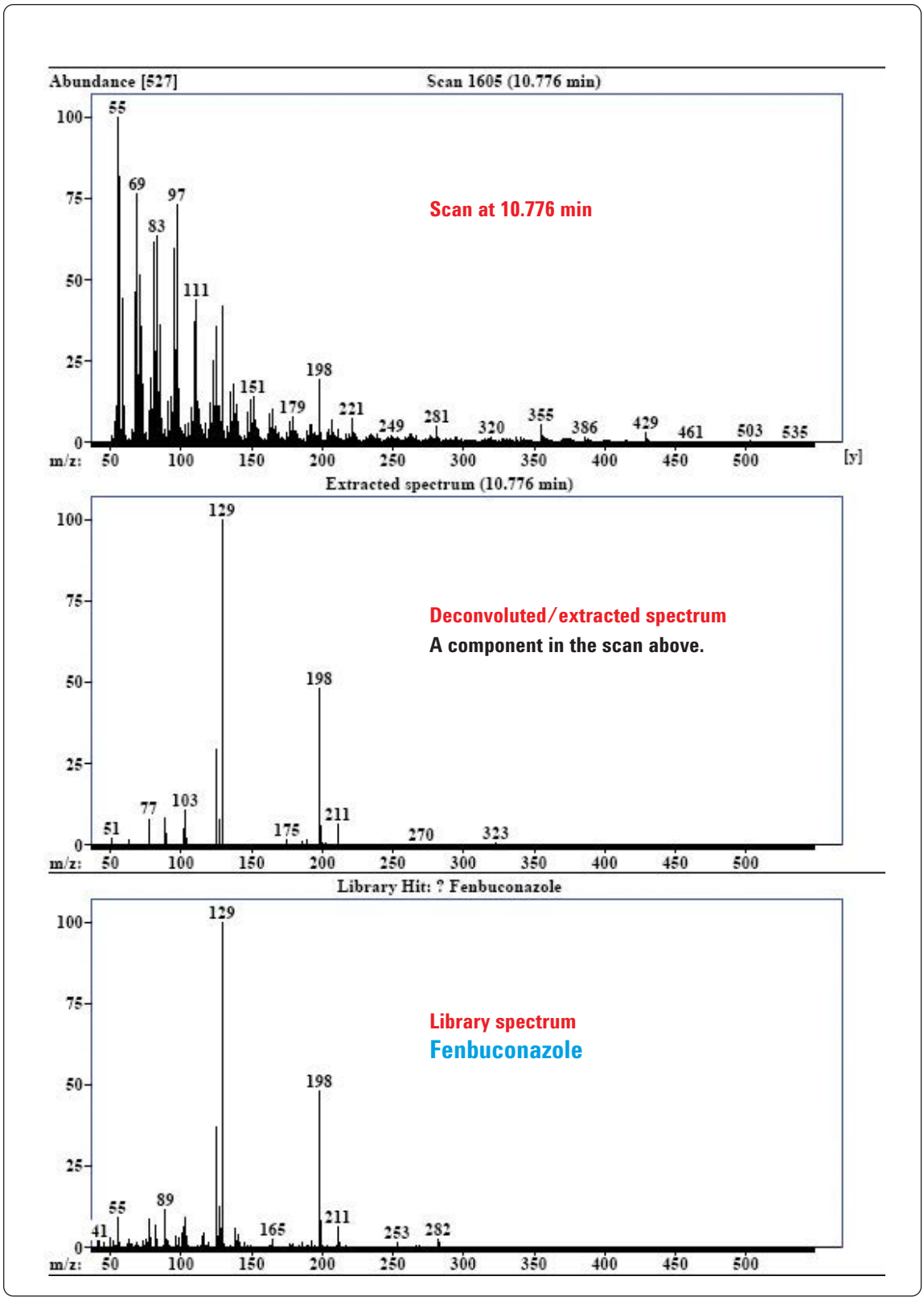


Figure 2. Comparison of raw, deconvoluted, and library spectra.

AMDIS Settings

Previous publications that discussed the power of using deconvolution to screen complex matrices, did not discuss specific AMDIS settings to define components [1, 5, 6]. In this study, several settings (that is, resolution, sensitivity, and shape requirements) are compared to find the maximum number of spiked compounds. The minimum match factor is set to 30 and the retention time window is limited to ± 30 seconds (RI window is set to 30) to qualify the hits from the retention time library search (Figure 3). The expected retention times of the compounds in the library database are obtained in acetone solvent without a retention gap. The samples in this study are in toluene solvent with a retention gap. Therefore, the retention time window is set wider than the normal 10 or 15 seconds, at ± 30 seconds.

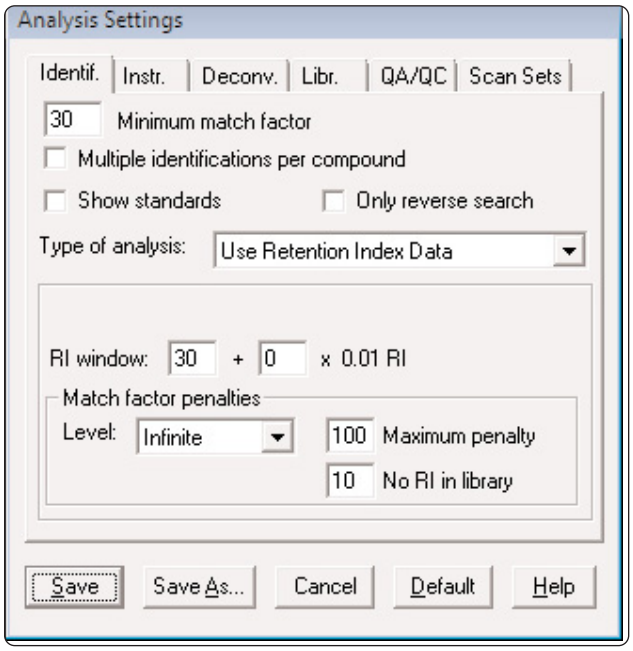


Figure 3. AMDIS identification settings.

Figures 4 and 5 describe some of the parameters in the AMDIS deconvolution tab. In this article, "1 M H M" means: adjacent peak subtraction = 1, resolution = medium, sensitivity = high, shape requirements = medium.

Settings can be optimized for chromatographic resolution, peak shape, retention time windows, acceptance criteria, and so forth. Settings can be saved to "ini" files. The chemist has control over the deconvolution and identification process by varying numerous AMDIS settings. Most of these parameter settings are not independent; so changing one parameter can affect another.

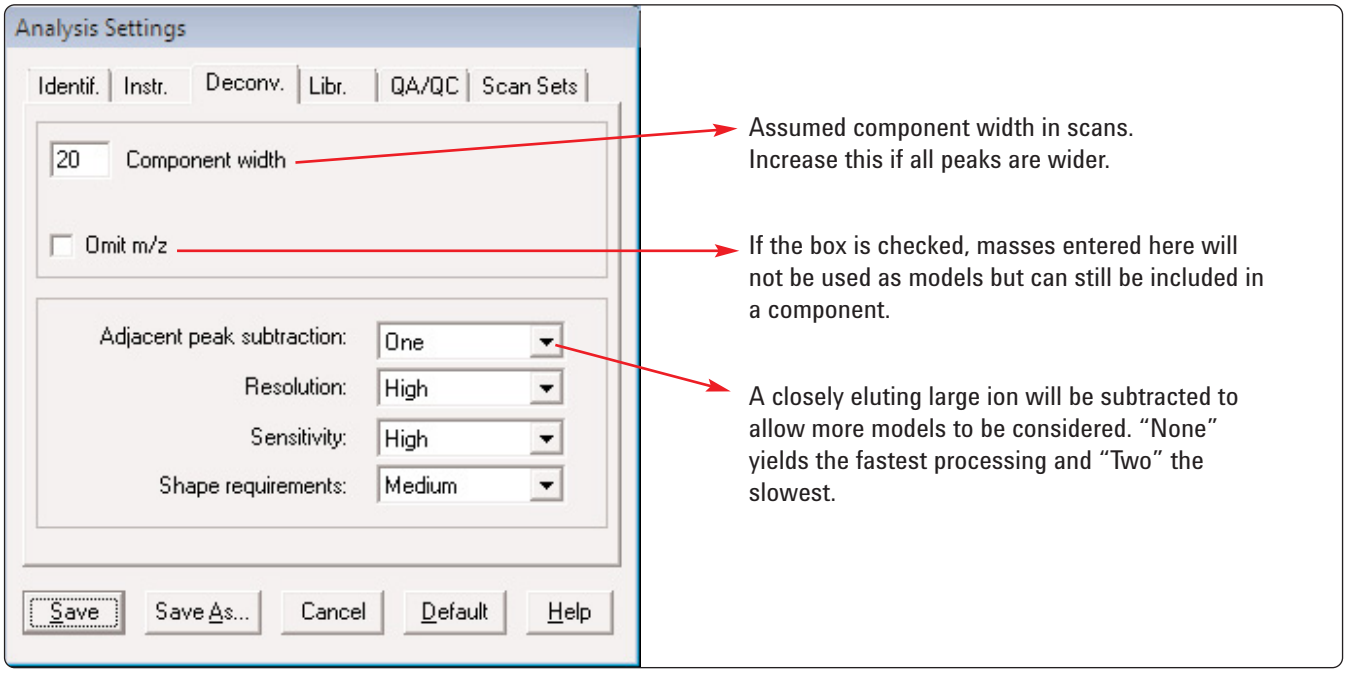


Figure 4. AMDIS deconvolution settings.

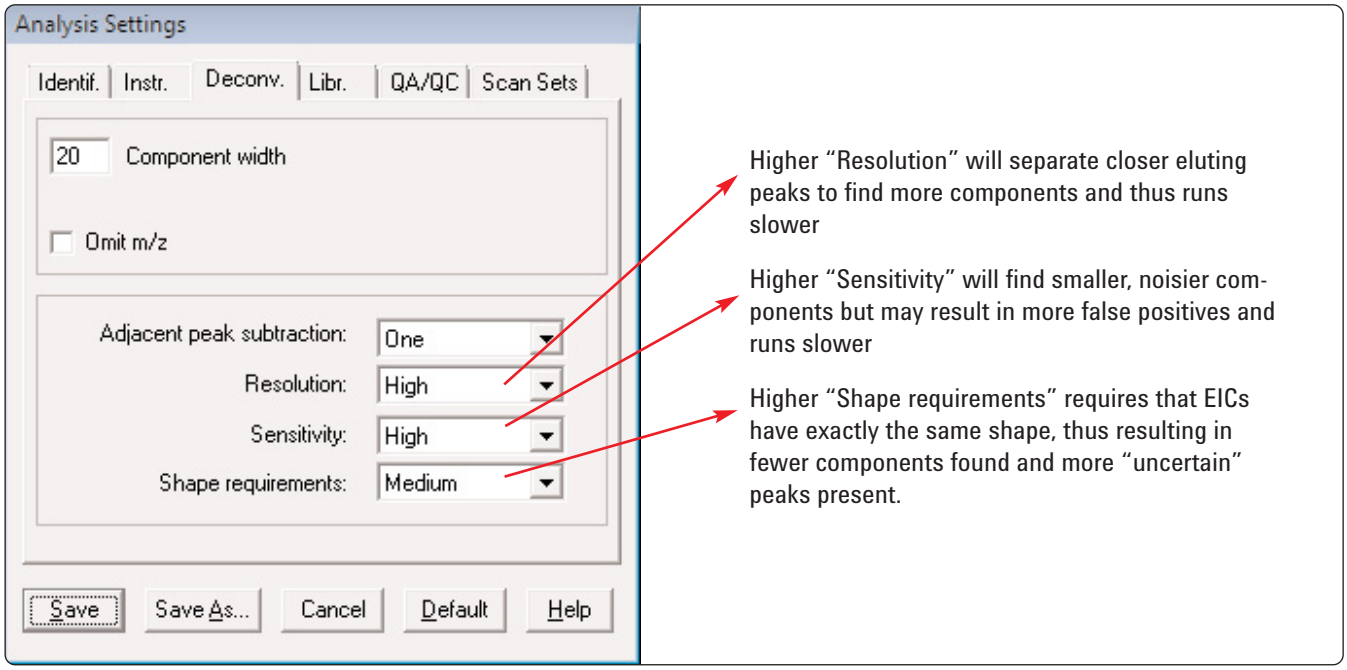


Figure 5. AMDIS deconvolution settings.

Results and Discussion

Deconvolution Settings

Figure 6 shows effects on match factors (y-axis) due to variation of adjacent peak subtraction and sensitivity across 35 pesticides (x-axis). This figure shows two things:

– The adjacent peak subtraction (1 or 2) makes little difference in match factor

– The sensitivity setting (very high and high) makes little difference in match factor

In the next few figures, the AMDIS setting is varied one at a time to observe the number of pesticides found. The reference point is the optimal setting (HHM) where the maximum number of hits were obtained.

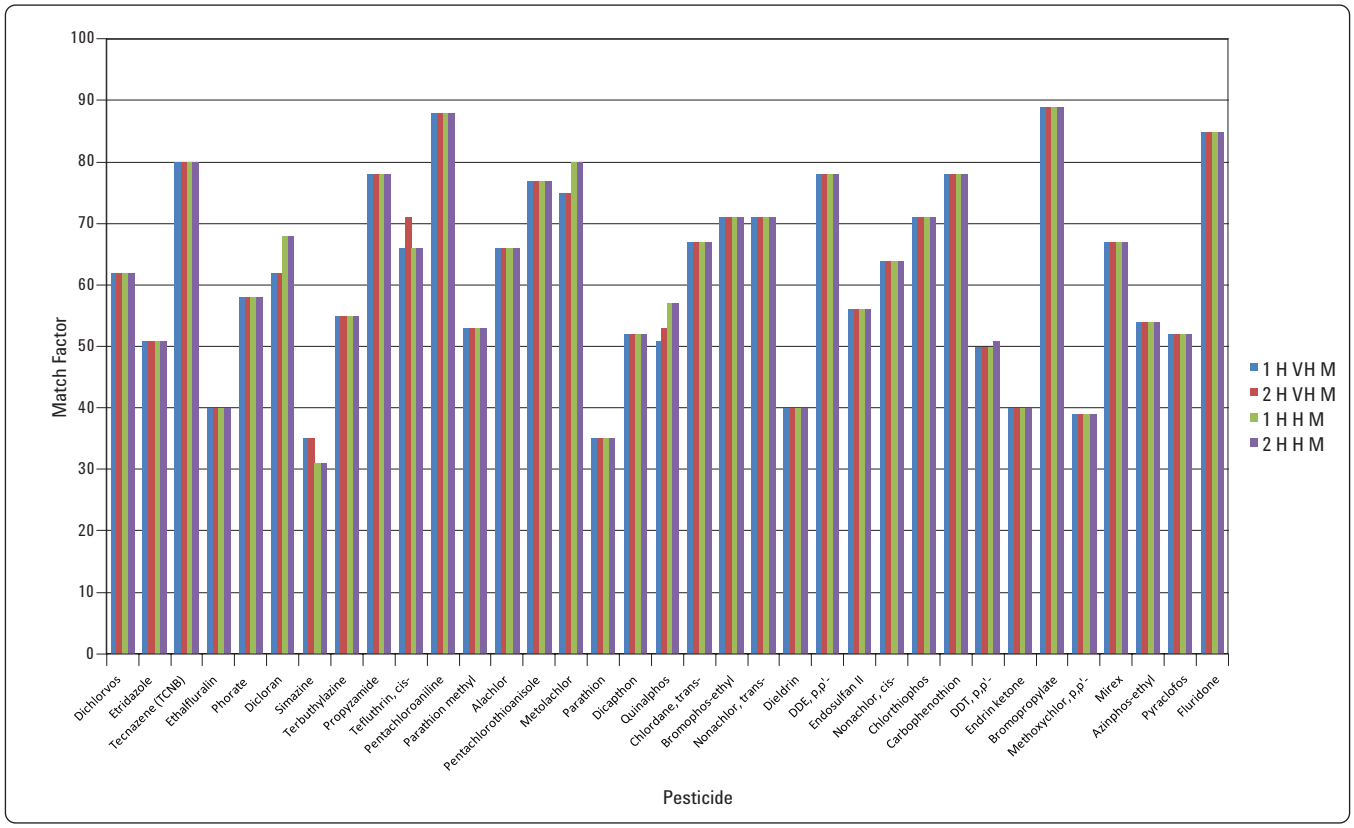


Figure 6. Comparison of match factors with four AMDIS settings.

Figure 7 shows that keeping the sensitivity and peak requirements the same, and lowering the resolution from H to M will find fewer targets. The number of targets found is in the yellow circle. A resolution setting of "low" yields even fewer targets.

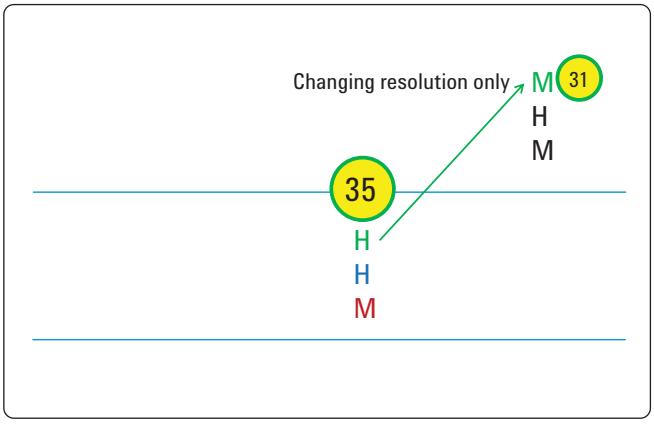


Figure 7. Number of compounds found by varying resolution.

Figure 8 shows that while keeping the resolution and peak requirement constant, lowering the sensitivity from H to M will find fewer targets. However, increasing the sensitivity from H to VH does not affect the number of targets found, similar to that in Figure 6.

Figure 9 shows that while keeping the resolution and sensitivity the same, lowering or increasing the peak shape requirement from M to L or H will find less targets.

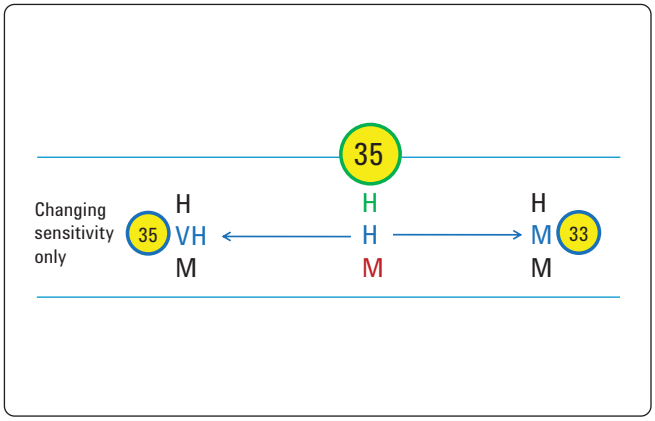


Figure 8. Number of compounds found by varying sensitivity.

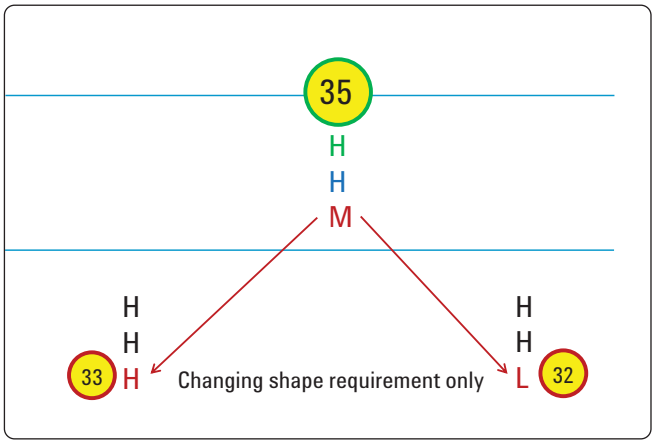


Figure 9. Number of compounds found by varying peak shape.

In addition to the number of targets found, we should look at the Average Match Factor (AMF) of all the targets found. The AMF is the number in the green triangle. Figure 10 shows that there is no significant variation in AMFs except in HHH mode (58.5) which is much lower than others (>61.6). This supports that HHM is still the optimal setting, considering processing speed and number of false positives.

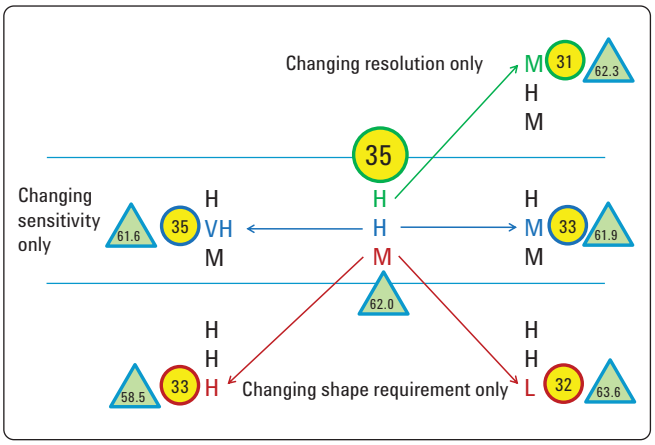


Figure 10. Comparison of average match factors with AMDIS settings.

ChemStation Quant settings

Figure 11 shows part of the "Edit Compound" screen in the MSD ChemStation. This shows the quant database for locating and confirming compounds using three ion ratios of each target analyte. The RT window is specified in the upper box and the ions and ion ratios are specified in the lower box.

As shown in Figure 11, the Extraction RT window is set to ± 0.5 min and the Qualifier Ion (Q1, Q2, and Q3), % Uncertainty is set to Absolute 50%. In ChemStation, the

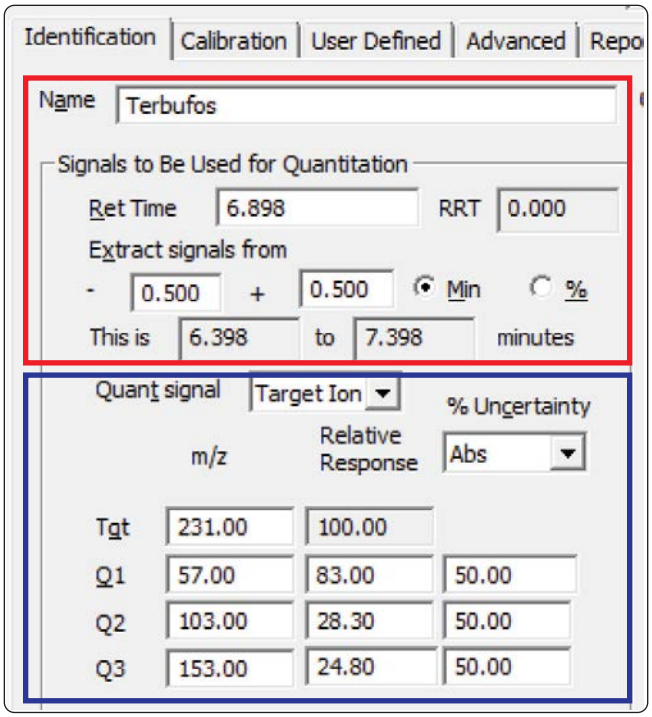


Figure 11. Target compound RT and ion setup.

target compound identification is based on four ions and three qualifier ion ratios. However, the target compound identification in AMDIS (Figure 2) was based on the full spectral library match which is more dependable.

Another key parameter in quantitation is the "Quantitation subtraction method" which is set to "Avg first and last" and not shown here.

Figure 12 is an overlay of four ions (Quant and Qualifiers) from ChemStation and the quant ion from AMDIS (in magenta).

Due to the chemical background, the four ions from ChemStation have offset and noisy baselines, which will affect the peak integration and proper quantitation results.

In comparison, the magenta trace is the deconvoluted quant ion from AMDIS. The chemical noise had been removed in the deconvolution process. It shows a flat baseline and accurate integration. There are other advantages of using deconvolution in GC/MS analysis as discussed below.

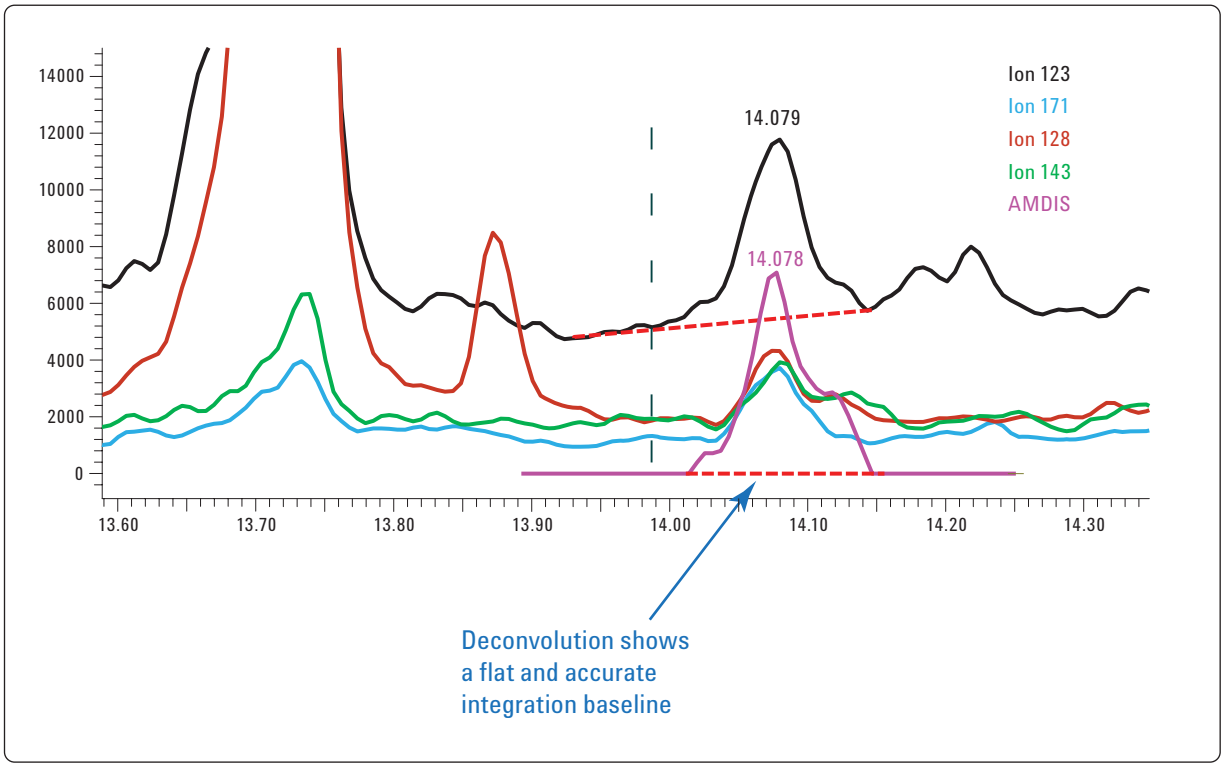


Figure 12. Target, qualifier and AMDIS deconvoluted EIC overlay.

Additional Advantages of Using Deconvolution

Finds more compounds than ChemStation does

In Figure 13, ChemStation did not integrate ion 109 (ChemStation target ion) at the expected RT, therefore, the compound was not found. AMDIS found Fonofos correctly, at 6.898 min. The qualifier ion ratios at this RT also match that required by ChemStation for identification.

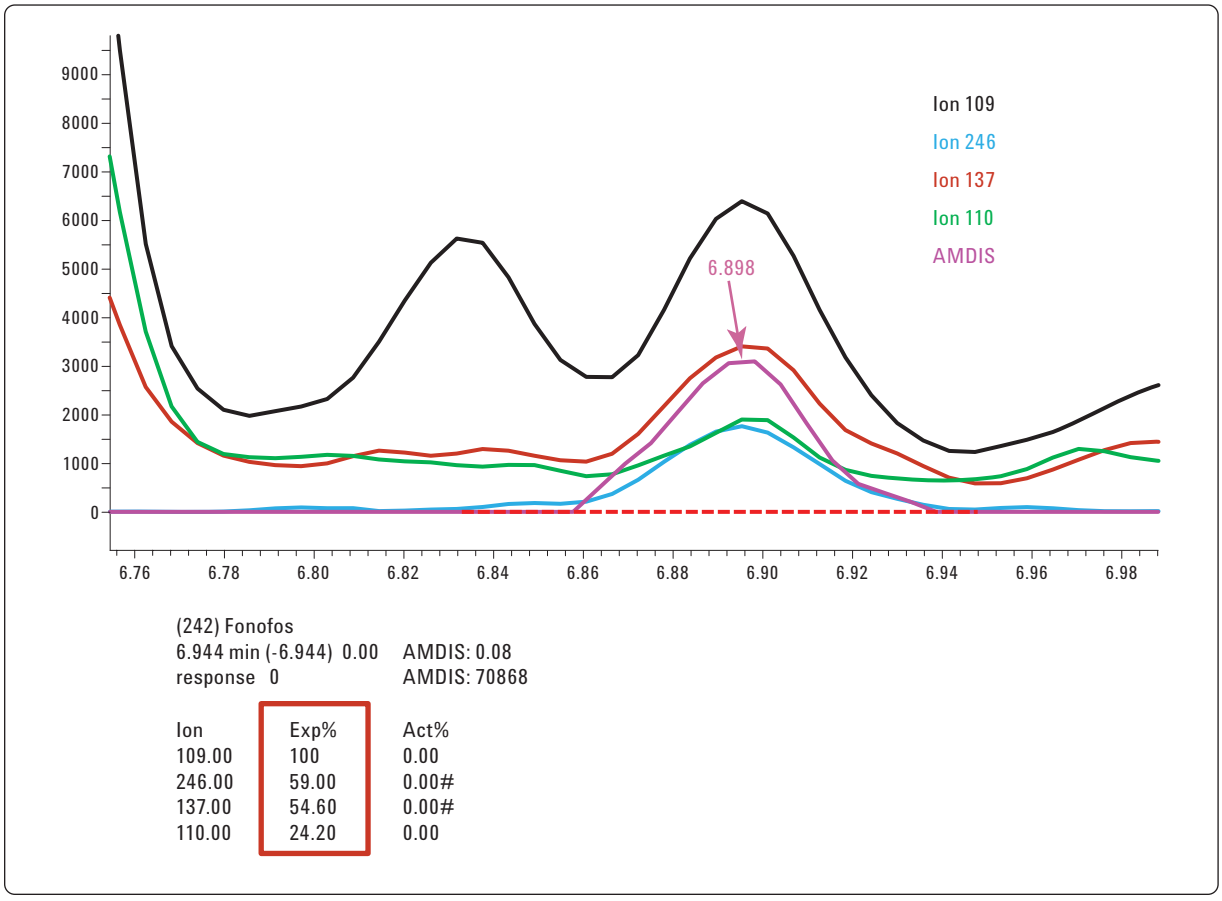


Figure 13. Target, qualifier and AMDIS deconvoluted EIC overlay.

Finds the correct peak

In Figure 14, from the size and location of the three qualifier ions, it is obvious that ChemStation picked the wrong peak (at RT = 4.067) to quantitate. However, AMDIS found a peak (at RT = 3.873) whose ion ratios are in agreement with the ChemStation qualifier ions. Again, this demonstrates that the AMDIS full-spectrum matching process is a more robust approach for identifing a compound in a complex matrix.

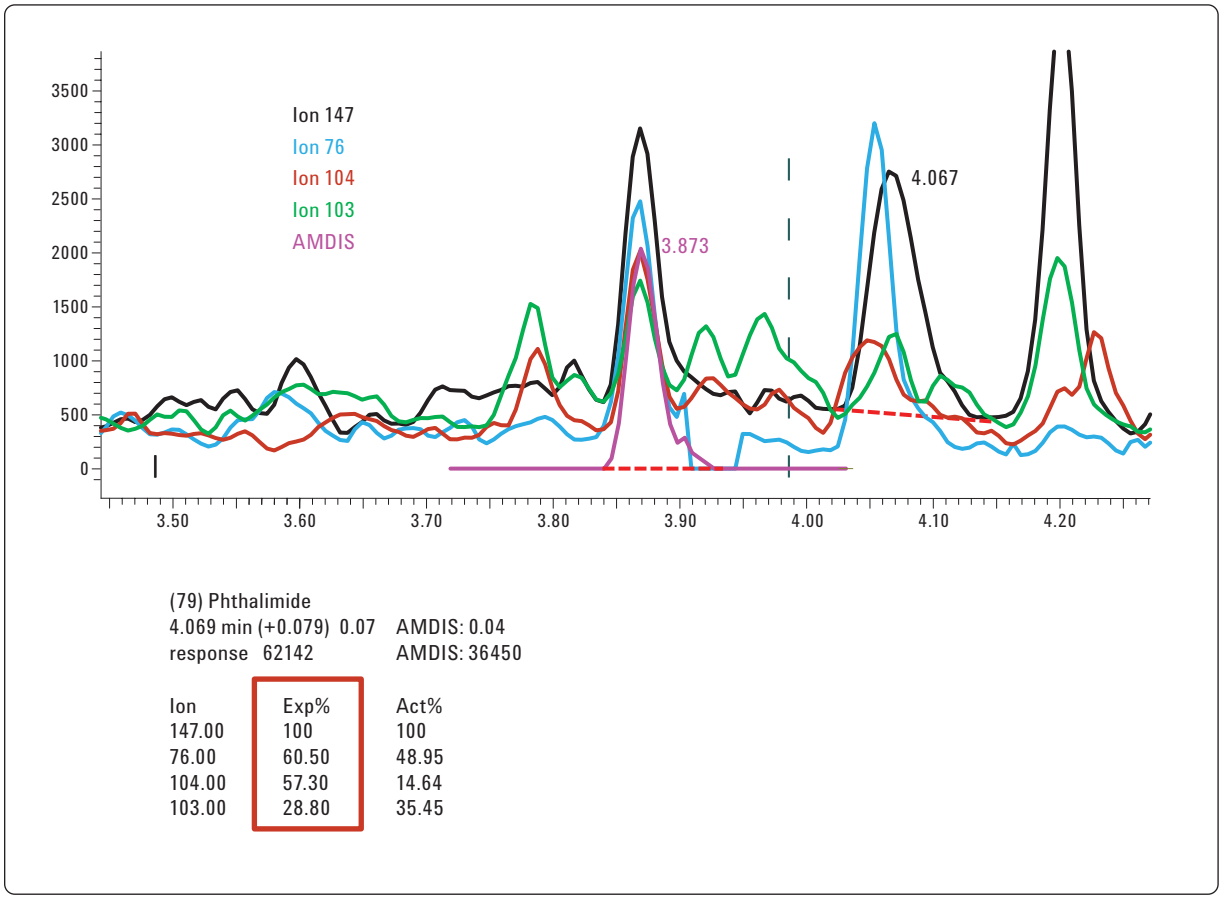


Figure 14. Target, qualifier and AMDIS deconvoluted EIC overlay.

Higher discrimination power than ChemStation

In Figure 15, the target ion (ion 235) is overwhelmed by the matrix background (shown as a large fronting peak). ChemStation was not able to differentiate the ion 235 contribution from the background or the compound; therefore it integrated the distorted peak. Due to the rising baseline, ChemStation integrated a large area of chemical background as the "target compound signal". On the other hand, AMDIS was able to deconvolute the compound signal away from the background ion and remove noise properly before the integration. This provides a more reliable quant result.

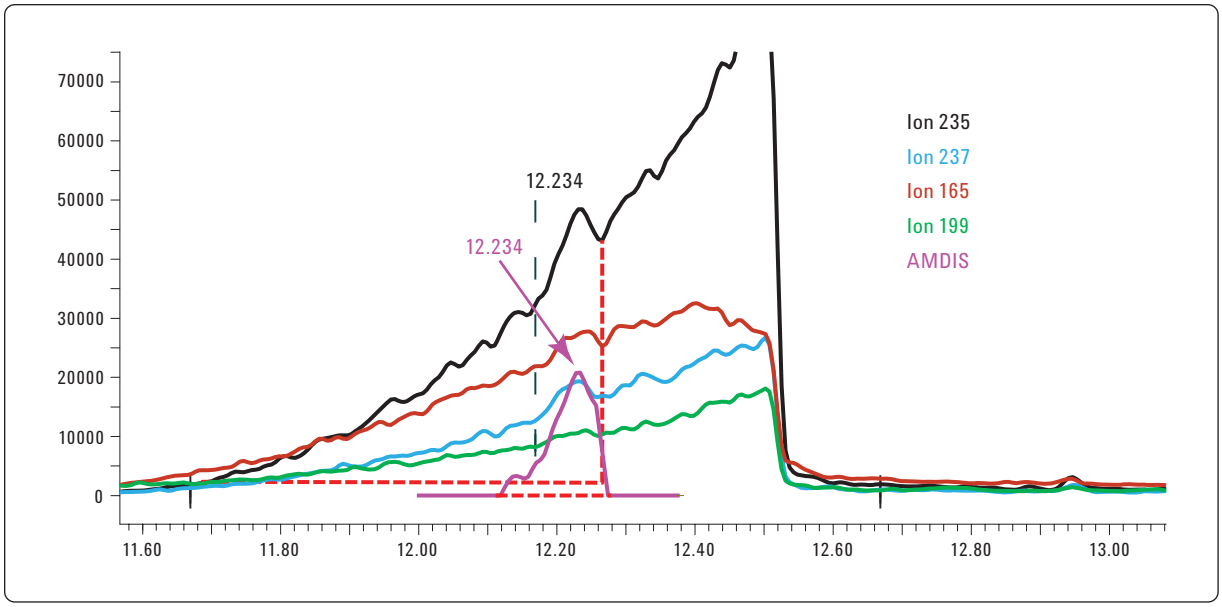


Figure 15. Target, qualifier and AMDIS deconvoluted EIC overlay.

Deconvoluted ion is noise-free, thus easier to integrate for more reliable quantitation results

In Figure 16, ChemStation and AMDIS found the same peak. Due to the noisy baseline, ChemStation drew the integration baseline (red dash line) incorrectly. Again, deconvolution removes chemical noise first, and can therefore, integrate the peak easily and reliably.

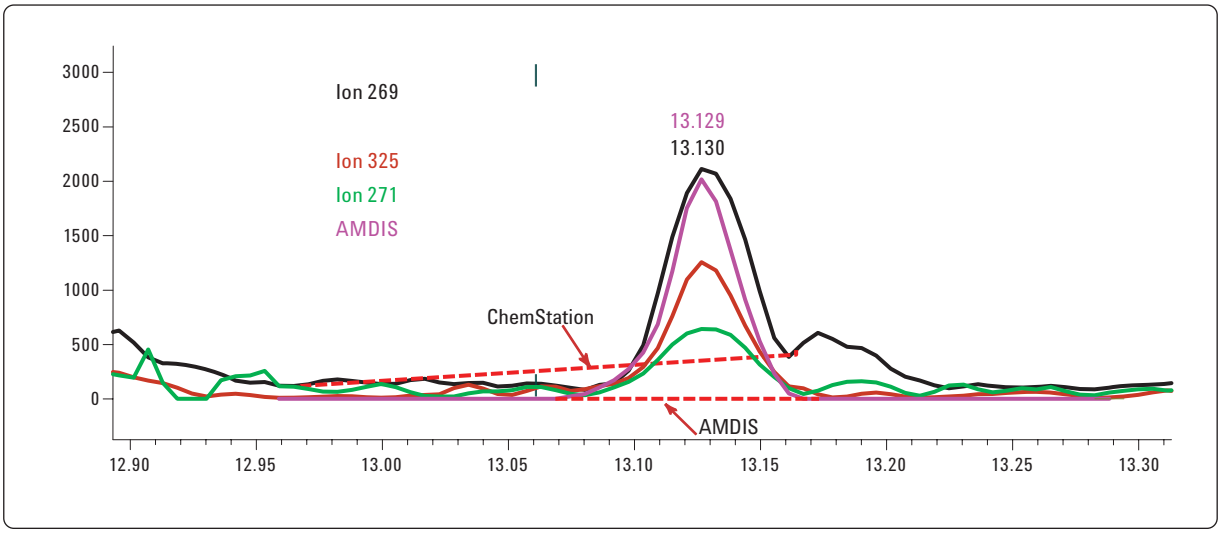


Figure 16. Target, qualifier and AMDIS deconvoluted EIC overlay.

Agilent's ChemStation add-on - Deconvolution Reporting Software (DRS) incorporates AMDIS deconvolution. Therefore, the above AMDIS advantages are automatically captured in DRS data processing which combines results from ChemStation, AMDIS, and NIST MS Search into one report.

Comparing number of compounds found between ChemStation and AMDIS

Figure 17 is a summary of the hits from ChemStation and AMDIS under four different settings, respectively. The blue bars represent the number of false positives and the red bars represent the number of actual target compounds found. On the left side of the graph, the settings of ChemStation are Ion Ratio Uncertainty. Although the absolute 30% and 50% increase the total number of compounds found, only about half of the 35 targets are found. The analyst is forced to review more hits and does not gain any additional information. The entire target list of 900+ compounds must be reviewed for false negatives. The right side of the graph shows that the four AMDIS settings gave similar results. In each case, all 35 targets were found with a reasonable number of false positives. There were no false negatives. The analyst must only review the positives, which is a significant time savings. This shows that AMDIS (DRS) is much more capable than ChemStation in finding target compounds in a complex matrix. AMDIS (DRS) provides better detectability and faster data processing.

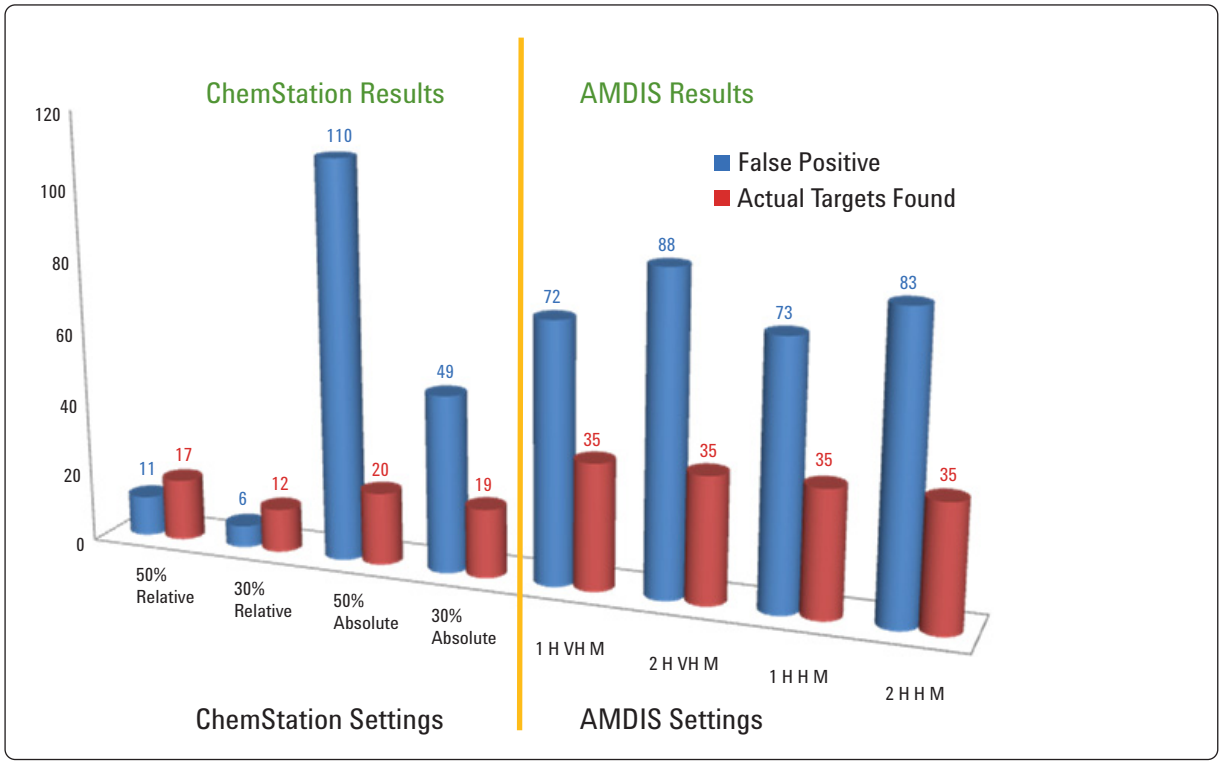


Figure 17. Overall comparison of AMDIS and MSD ChemStation compounds found.

Conclusions

- AMDIS finds more target compounds than ChemStation in a complex matrix. Deconvolution (DRS) provides a cleaned peak to integrate properly giving more reliable results.
- AMDIS did not miss any target compounds at the 50 ppb level using scan data. This minimizes the time an analyst must spend reviewing results.
- Confirmation of compounds is done in significantly less time with deconvoluted component spectra available.
- The detectability of compounds in a complex matrix is significantly improved with deconvolution. This can also be viewed as better or increased sensitivity through improved selectivity versus the background.
- Deconvolution Reporting Software (DRS) automates the deconvolution (AMDIS) process to produce an easy-to-read quantitation report.

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