QTRAP® Technology

A COMPENDIUM OF RELATED RESEARCH APPLICATIONS

SCIEX

Overview

QTRAP® Technology

Discover QTRAP, the only triple quadrupole technology in the world powered by a linear ion trap (LIT), combining the MRM sensitivity of a triple quadrupole system but enhanced with unique and powerful workflows for better data, complete confidence, unrivalled efficiency, and ultimate performance.

Contents

QTRAP® Applications & Methods

Challenges Faced by Analytical Testing Labs

Have you ever had second thoughts about whether the data you acquired are true?
Are you sure that your peak is truly your analyte of interest?
Do you ever wish you could confirm your result apart from MRM, retention times, and ion ratios?
Are you experiencing matrix interferences that effect your ability to detect and quantify?

Addressing the Challenges

The solution to these common challenges: SCIEX QTRAP® technology delivers the gold standard in quantitation and offers confirmational experiments to enhance your workflows. Used in laboratories all over the world, the QTRAP is the instrument of choice for many companies and institutions that rely on its power, accuracy, reproducibility, and robustness to acquire the best possible data across key applications in pharmaceutical drug discovery and development, food testing, environmental monitoring, protein quantitation, and more.

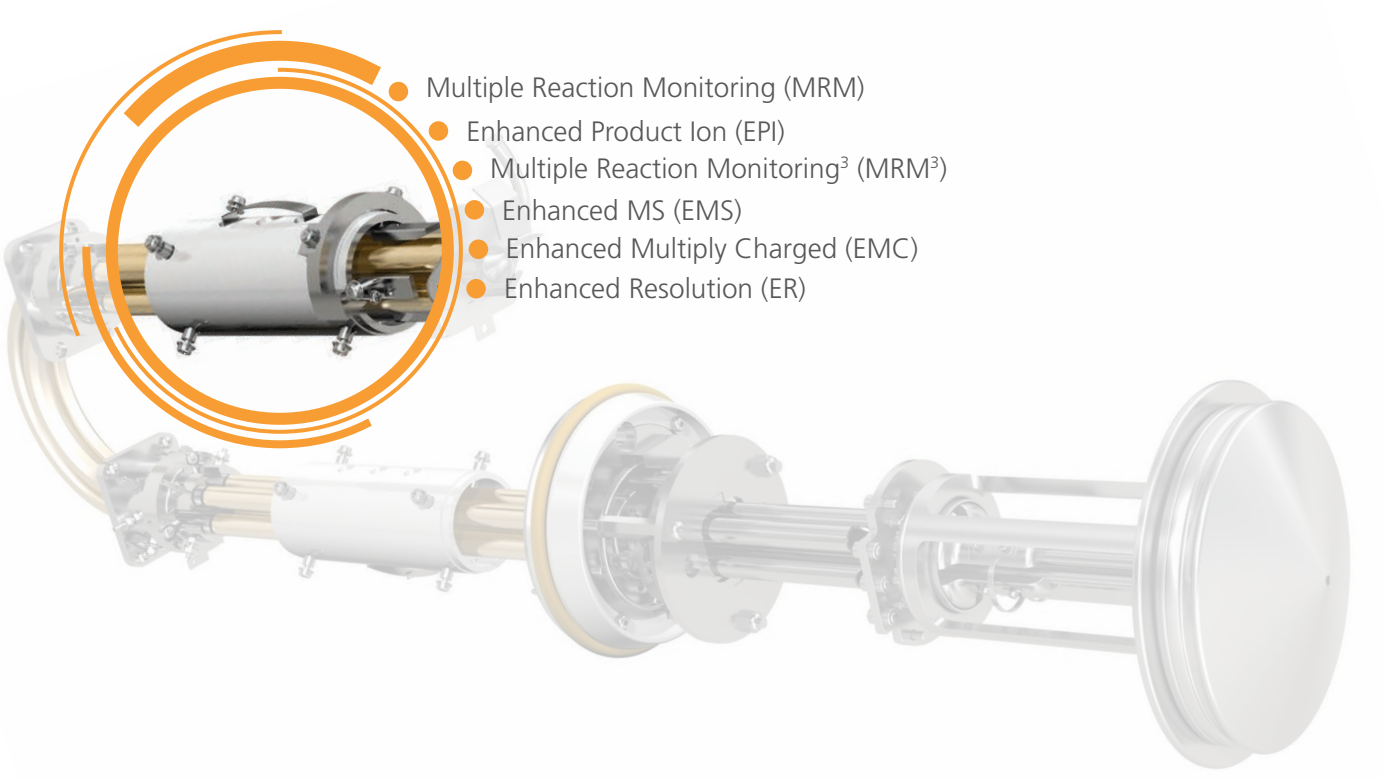
In this compendium you will discover the functions of the QTRAP supported by methods and application notes which will show why QTRAP is more than just a triple quad.

QTRAP® Technology Explained

What makes a QTRAP system so unique? While having the capability to function like a standard triple quad LC-MS/MS, it also doubles as a linear ion trap (LIT), proprietary technology that can perform a multitude of additional workflows beyond basic multiple reaction monitoring (MRM) for better specificity and quantitative performance.

QTRAP technology packs more than twice the functionality of a standard triple quad system.

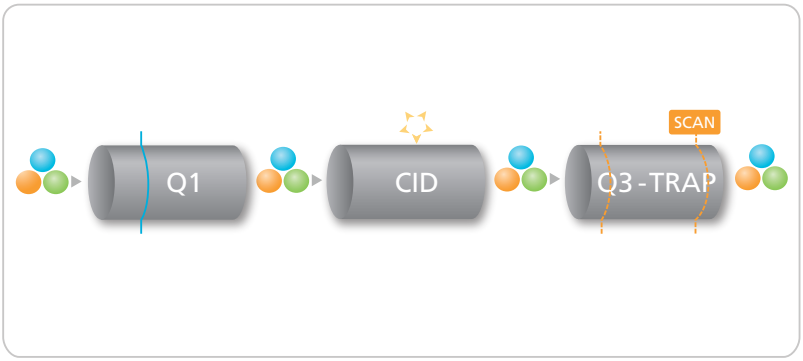
Scan Type	Triple Quad	QTRAP
Precursor	•	•
MRM	•	•
Neutral Loss	•	•
Product Ion	•	•
Enhanced MS (EMS)		•
Enhanced Multiply Charged (EMC)		•
Enhanced Resolution		•
Enhanced Product Ion		•
MS^3 (MS/MS/MS) and MRM^3		•

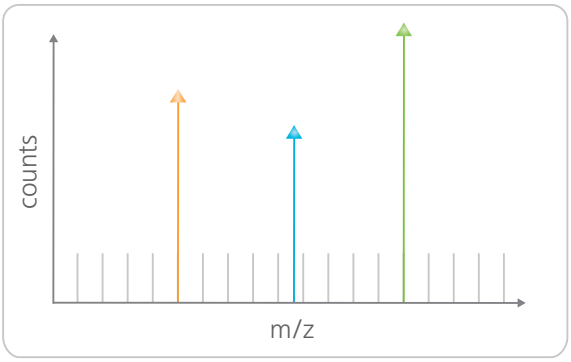


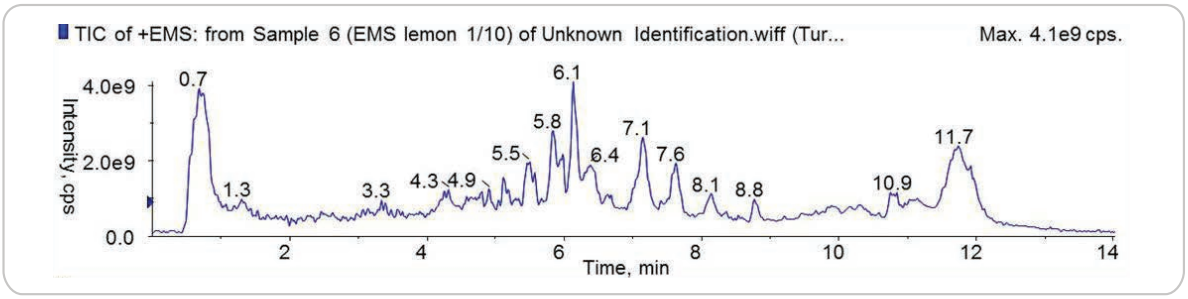
Unique Scan Functions to Accelerate Your Lab's Performance

Enhanced MS Scan (EMS)

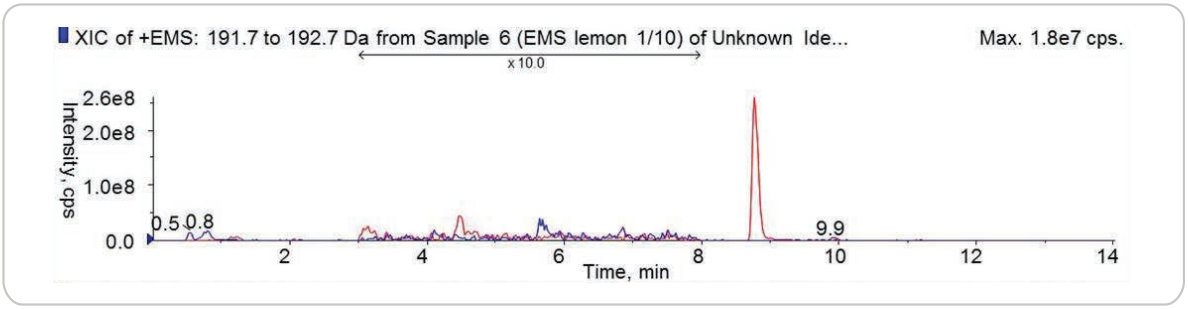
The enhanced MS scan is the standard QTRAP® MS scan where ions are transmitted from the source (Turbo V® or IoDrive®) through the RF mode quadrupoles into the ion trap. The ion trap is filled, and the ions are scanned out axially to the detector. The scan type delivers a highly sensitive full scan for the detection of unknown analytes when your conventional MS/MS system struggles to see a response.



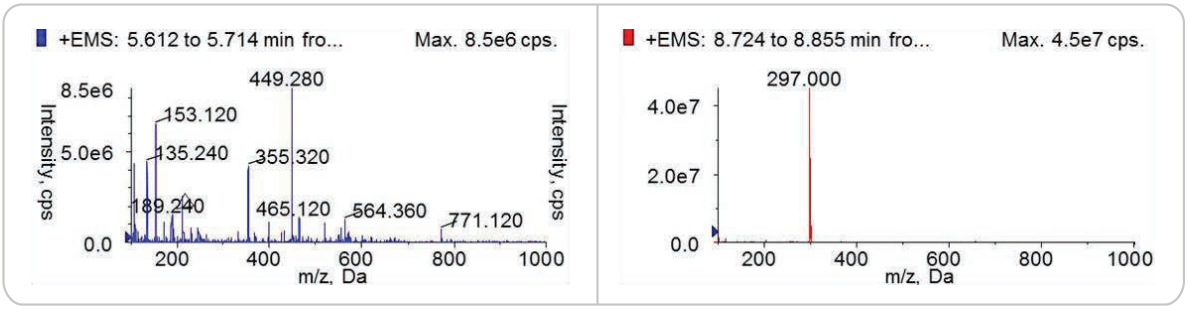




The total ion chromatogram (TIC) for the sample



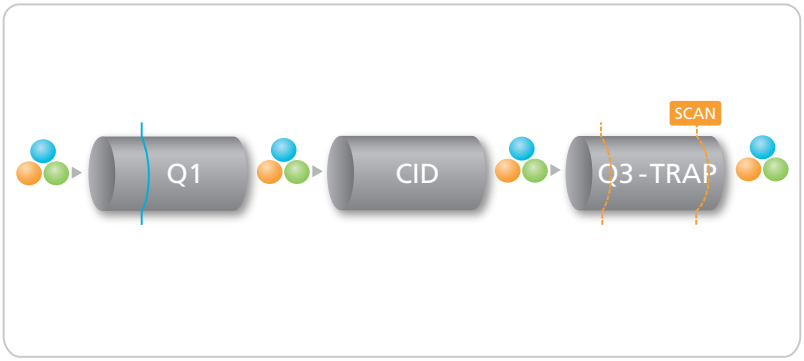
The extracted ion chromatogram (XIC) from the EMS using a range of 191.7 to 192.7 Da

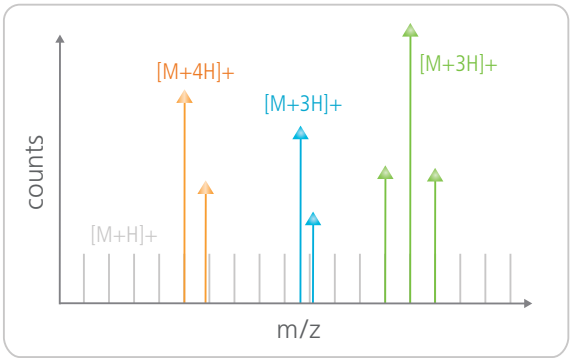


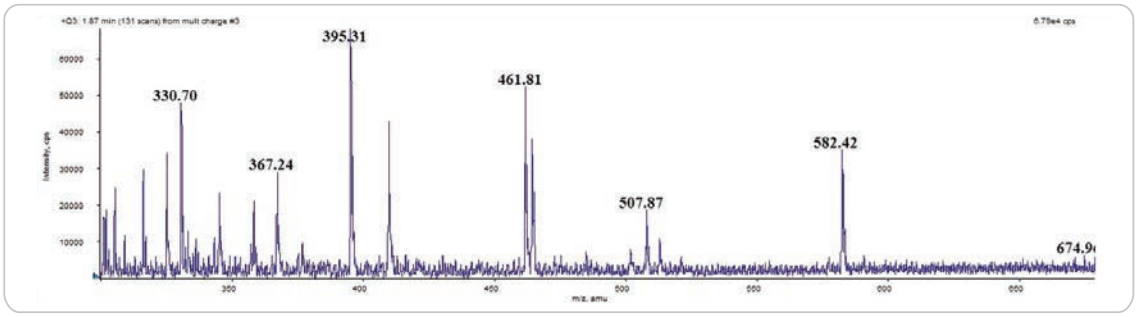
The spectra acquired from the EMS which can be used to understand the potential structure of the detected compound

Enhanced Multiply Charged Scan (EMC)

An EMC scan is a unique QTRAP® function that can be used to improve the signal/noise ratio on ions which are multiply charged. As in the EMS, the Ions are transmitted from the source to the LIT. Once the ion trap has been filled, the singly charged ions are removed, leaving the multiply charged ions behind. The ions are then scanned out axially to the detector.



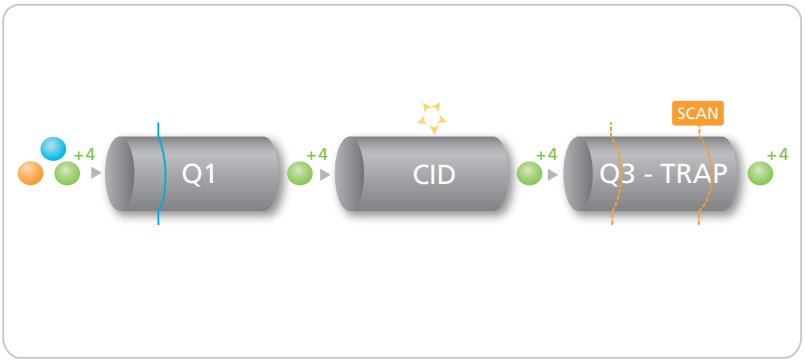




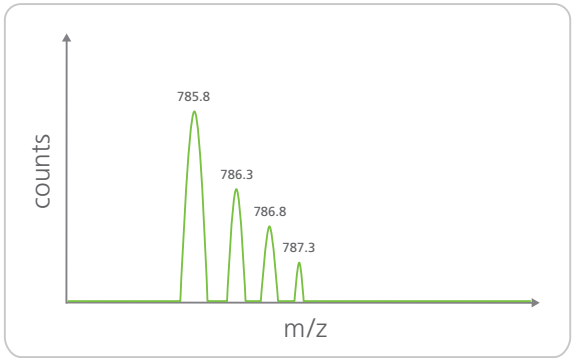
Only see what you need to see, the EMC scan filters out all of the single charged species so only the multiply charged ions remain.

Enhanced Resolution Scan (ER)

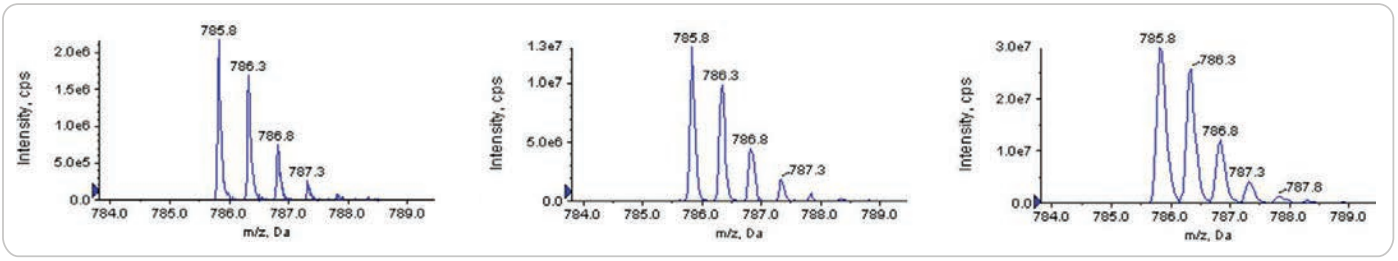
Using the enhanced resolution scan mode allows for high resolution MS to be obtained for an ion of interest. This scan is important in the acquisition of an accurate molecular weight and to obtain both the value of the charge state and the accurate mass to charge ratio (m/z), This scan type is most useful when trying to determine structural information, perform database searches, perform peptide sequencing, among many other applications.



Schematic of the path of the ions through the QTRAP



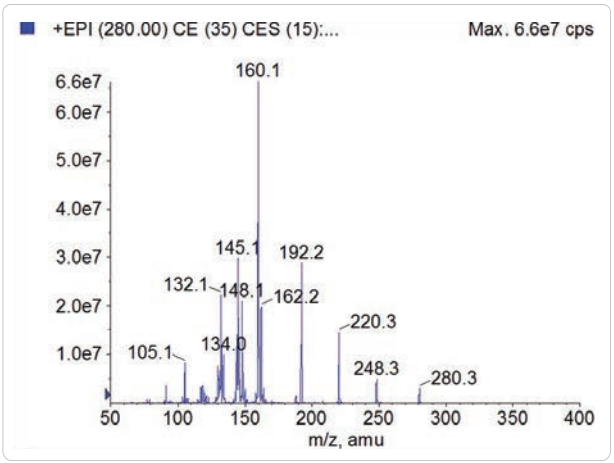
Example of the QTRAP will resolve the analyte



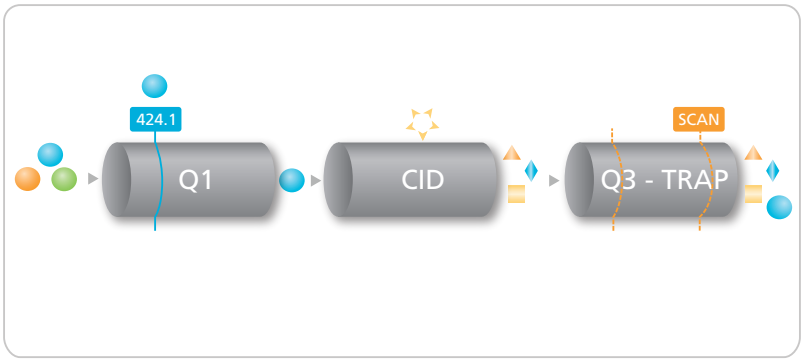
Acquired data using various parameters for an ER scan at 50, 250 and 1000 Da/sec

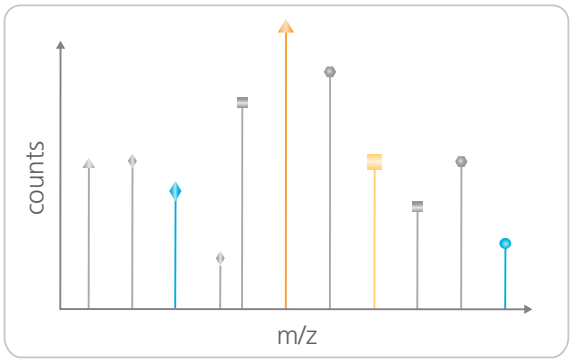
Enhanced Product Ion (EPI)

The enhanced product ion scan is a trap scan used to obtain high quality MS/MS spectrum on a specific ion. The fragmentation is done in the collision cell and provides information-rich MS/MS spectral data. The fragmented ions generated are captured in the LIT and then scanned out. This unique scan type is key to identification experiments and perfectly complements a quantitative workflow. The EPI scan delivers high sensitivity, high mass accuracy, and fast scanning when compared to a non-enhanced basic product ion scan.



Acquire high quality MS/MS spectra so you have a unique thumbprint of your analyte and build a vast library of compound structural information.

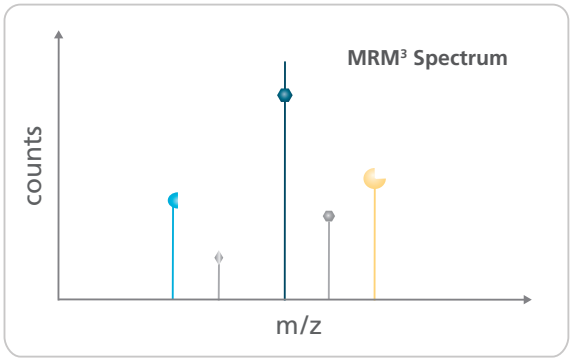


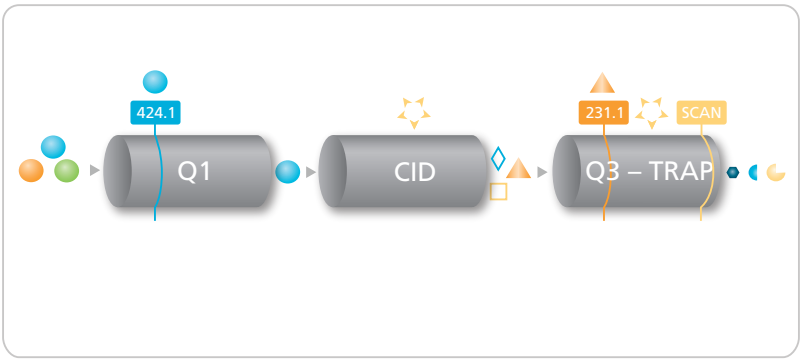


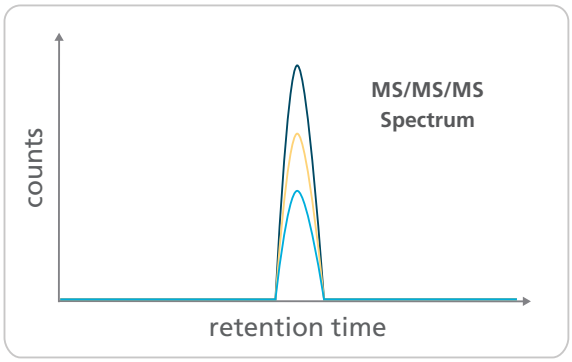
MS³ (MS/MS/MS) and MRM³

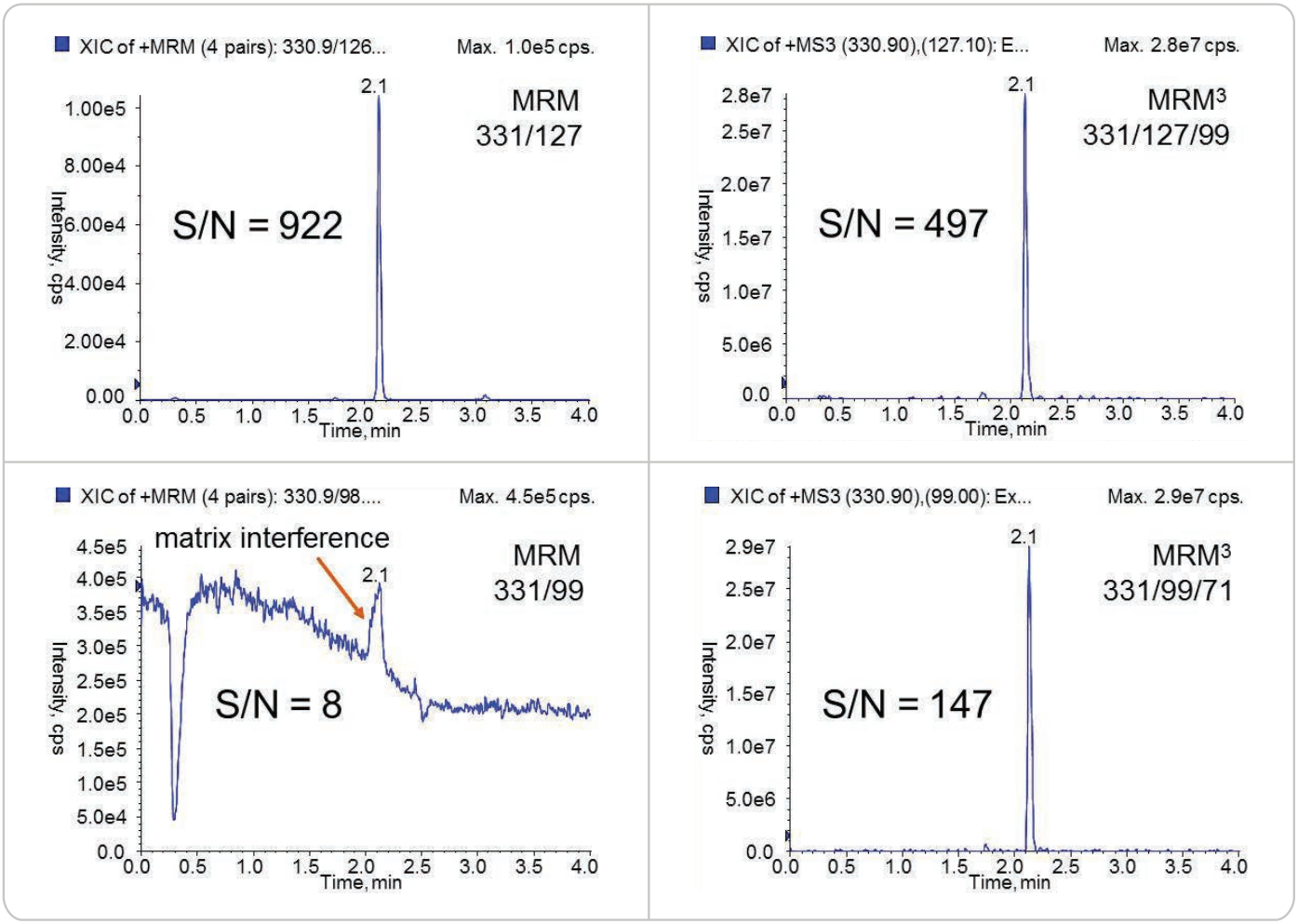
MRM³ is an effective scan solution for the quantitation of analytes when high background and interferences make standard MRM quantitation difficult. MRM³ can remove the interference andenable a much lower detection of the particular analyte.

This very powerful tool allows you to obtain additional structural information from your sample. In particular this scan mode is important in the characterization of modified peptides such as glycopeptides.



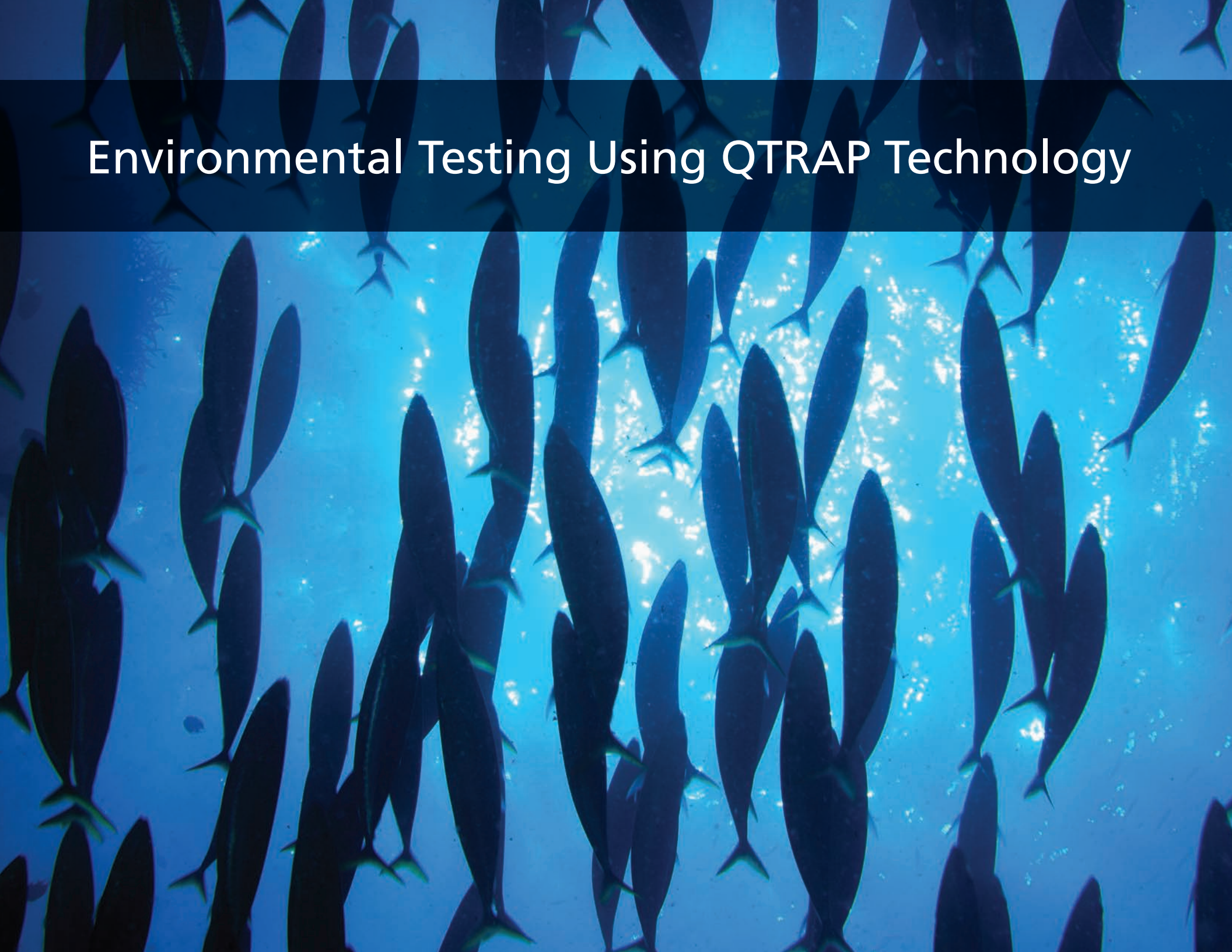






Left panel shows 2 MRM transitions for a compound, where the confirmation MRM transition (bottom panel) shows high background from matrix interferences. By monitoring the 2nd generation MRM, or MRM3, the matrix interferences are virtually eliminated, and the analyte is detected with high S/N for accurate quantitation.

Environmental Testing Using QTRAP Technology



Food and Environmental

Quantitation and Identification of Legal and Illicit Drugs in Wastewater in the low Nanogram per Liter Range using Large Volume Direct Injection and QTRAP® Technology

Jean-Daniel Berset[1], Michael Scherer[2], and André Schreiber[3]
[1] Water and Soil Protection Laboratory (WSPL), Office of Water and Waste Management, Bern (Switzerland); [2] AB SCIEX Brugg (Switzerland); [3] AB SCIEX Concord, Ontario (Canada)

Overview

The present application note describes the optimization of the front-end HPLC methodology by improving the separation of legal and illicit drugs such as cocaine, MDMA and methamphetamine and its important metabolites such as benzoylecgonine and monoacetylmorphine using an unusual 5 µm particle core-shell column with 4.6 mm ID. The SCIEX QTRAP® 5500 system was used to detect target compounds in Multiple Reaction Monitoring (MRM) mode. To overcome saturation effects observed for high sensitive MRM transitions the collision energies (CE) were detuned for some compounds. Different acquisition modes such as *Scheduled* MRM™ and *Scheduled* MRM™ Pro with Information Dependent Acquisition (IDA) of MS/MS full scan spectra were explored. Thus, the analytes could be quantified in a traditional way using two MRM transitions in MultiQuant™ 3.0 software, and additionally, QTRAP® MS/MS spectra could be used for identification at trace levels in MasterView™ 1.1 software. The optimized method was successfully applied to the measurement of drugs in influent wastewater samples collected during a party event (Street Parade Zürich).

Introduction

Drug abuse is a global problem with major negative impacts on human health and social welfare. Illicit drugs are substances for which nonmedical use is prohibited by national or international laws. Important groups of illicit drugs are opioids, cocaine, cannabis, amphetamines and ecstasy (MDMA). Among those, amphetamines and MDMA currently demand the most attention by law enforcement agencies.[1] In Europe, the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) is the reference point on drugs and drug addiction.[2]

For drug consumption, questionnaire-based surveys have traditionally been performed to estimate drug use. However, it is recognized that this method is not sufficient to monitor trends in drug use quickly and adequately and therefore complementary data from other sources are needed.[3]

Since several years, the chemical analysis of influent-wastewater for the combined excretions products of illicit drugs has become a potent approach for monitoring patterns and trends of drugs consumed in a community.[3, 4] Meanwhile, the study of spatial differences and temporal changes in illicit drug use through the method of wastewater analysis, also called sewage epidemiology, is becoming an important tool to estimate drug consumption in Europe.

Nowadays, LC-MS/MS has become the method of choice for the quantitative determination of illicit drugs in aqueous matrices.[6] The *Scheduled* MRM™ algorithm using unique fragment ions and specific retention times of the molecules has evolved as a promising method for the reliable quantitation of compounds in water matrices.[7] Large volume direct injection (LVDI) techniques together with the exceptional sensitivity of the SCIEX QTRAP® 5500 system allow limits of quantitation (LOQ) in the low ng/L range.[8, 9] Finally, the acquisition of MS/MS spectra using Enhanced Product Ion scanning (EPI) in the Linear Ion Trap of the QTRAP® mass spectrometer provides additional confidence of the presence of the analytes under investigation.

The Street Parade Zürich is an interesting event to study such trends because the wastewater influent is strongly affected due to the relationship between number of inhabitants (approx. 300000) and visitors (approx. 950000).

Experimental

Standards and Internal Standards (IS)

Target analytes (morphine, monoacetylmorphine, amphetamine, methamphetamine, codeine, monoacetylcodeine, MDMA, cocaine, benzoylecgonine, methadone, EDDP, and mephedrone) as well as their corresponding deuterated IS were obtained as solutions in methanol or acetonitrile from Lipomed, Arlesheim, Switzerland. Working standard and calibration solutions were freshly prepared by appropriate dilution with methanol and water (purified using a water purification system from ELGA, Villmergen, Switzerland).

Sampling and sample preparation

Wastewater samples were obtained from the Zürich-Werdhölzli sewage treatment plant (STP) and immediately acidified to pH 2 using HCl, filtered and stored in the dark at 4°C until analysis. For the analysis 1 mL of wastewater sample was transferred to an HPLC vial, diluted 1:1 and 1:10 respectively, with ELGA water, and 10 µL of deuterated IS added. The final concentration of the IS was 500 ng/L. For ion suppression studies samples were also diluted 1:1 or 1:10.

Samples were collected as 24 h composite influent wastewater samples over 7 days between Wednesday July 30th (SP1) and Tuesday August 5th, 2014 (SP7). Sample SP 4 corresponds to the day of the Street Parade event and SP 5 to the day after.

LC Separation

A Dionex Ultimate 3000 HPLC system with a binary gradient pump, autosampler and column oven (30°C) was used for the chromatographic separation.

The LC method was completely redesigned. In the previous method a Phenomenex Synergi Hydro-RP column 100 x 2.1 mm 2.5 µm was used.[6] In this study, a core-shell LC column, Phenomenex Kinetex C18, 100 x 4.6 mm, 5 µm, was applied. Mobile phase A was water + 0.1% formic acid + 2 mM ammonium formate and mobile phase B acetonitrile. A flow rate 900 µL/min was used. The gradient with a total run time of 12 minutes is listed in Table 1. The injection volume was set to 100 µL.

Table 1. LC gradient

Step	Time (min)	A (%)	B (%)
0	0.0	98	2
1	1.0	98	2
2	7.0	35	65
3	7.1	0	100
4	9.0	0	100
5	9.1	98	2
6	12.0	98	2

MS/MS Detection

A SCIEX QTRAP® 5500 system with Turbo V™ source with ESI probe was used. The target compounds were detected in positive polarity. The ion source parameters were optimized for the new LC conditions using the Compound Optimization (FIA) function in Analyst® software.

Table 2. Ion source parameters

Parameter	Value
Curtain Gas (CUR)	30 psi
IonSpray voltage (IS)	3000 V
Temperature (TEM)	650°C
Nebulizer Gas (GS1)	70 psi
Heater Gas (GS2)	70 psi

Two characteristic MRM transitions were monitored for each analyte, and 1 MRM transition for each internal standard (Table 3). The MRM transitions were taken over from the existing method[6] and MRM transitions of mephedrone and the corresponding IS were added.

The *Scheduled* MRM™ algorithm was activated to monitor compounds only around the expected retention time to maximize dwell times and optimize the cycle time of the methods.

Table 3. MRM transitions and retention time (RT)

Compound	RT (min)	Q1	Q3
Morphine (MOR)	3.4	286	152
	3.4	286	165
Codeine (COD)	4.2	300	215
	4.2	300	165
Amphetamine (AMP)	4.3	136	91
	4.3	136	119
Monoacetylmorphine (MAM)	4.5	328	165
	4.5	328	211
Methamphetamine (MAMP)	4.5	150	91
	4.5	150	119
MDMA	4.6	194	163
	4.6	194	105
Mephedrone (MEP)	4.7	178	160
	4.7	178	145
Benzoylecgonine (BEC)	4.9	290	168
	4.9	290	105
Monoacetylcodeine (MAC)	5.2	342	225
	5.2	342	165
Cocaine (COC)	5.4	304	182
	5.4	304	105
EDDP	6.4	278	234
	6.4	278	249
Methadone (MET)	6.7	310	265
	6.7	310	105
IS Morphine	3.4	289	152
IS Codeine	4.2	303	215
IS Amphetamine	4.3	139	122
IS Monoacetylmorphine	4.5	331	165
IS Methamphetamine	4.5	155	92
IS MDMA	4.6	199	165
IS Mephedrone	4.7	181	163
IS Benzoylecgonine	4.9	293	171
IS Monoacetylcodeine	5.2	345	225
IS Cocaine	5.4	307	185
IS EDDP	6.4	281	234
IS Methadone	6.7	313	268

The *Scheduled* MRM™ pro algorithm in Analyst® software (version 1.6.2) was used as an alternative survey scan for information dependent acquisition of MS/MS full scan spectra for identification. Two MRM transitions were monitored and at the same time EPI spectra were recorded when a signal exceeds a compound specific threshold. The setup of this methods is described in a separate note.[10] The thresholds of the internal standards were set very high to exclude them from MS/MS acquisition.

Results and Discussion

Optimization of LC Conditions

The column dimension 100 x 4.6 mm allowed large volume direct injection without the breakthrough of polar compounds like morphine. The void time of the column was approximately 1 minute, and the elution window of the analytes was between 3.4 and 6.8 minutes.

The 5 µm core-shell material resulted in very sharp chromatographic peaks of approximately 4 seconds width (Figure 1) while the column pressure was very low, ~100 bar, at 900 µL/min and 30°C.

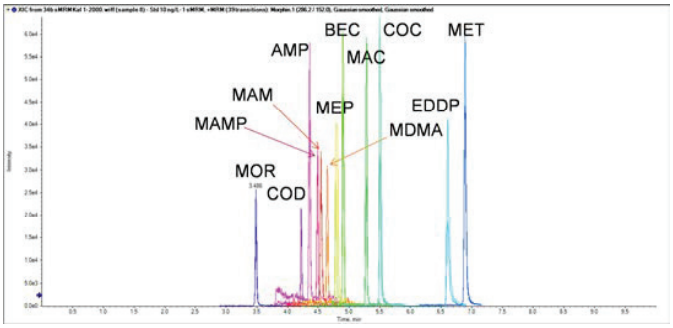


Figure 1. Example chromatogram of a 10 ng/L standard

The optimization of the LC conditions lead to intensity gains up to a factor of 10 for early eluting compounds like morphine and a factor of 2 for medium and late eluting compounds like MDMA (Figure 2). Signal-to-Noise (S/N) values were increased by a factor of 2 to 10.

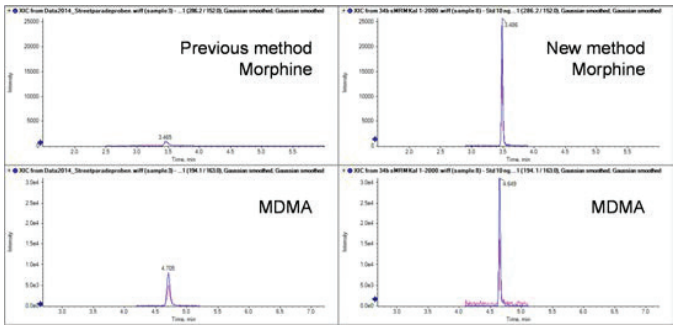


Figure 2. Sensitivity gains for morphine and MDMA by optimizing LC conditions, previous method (left) and newly optimized method (right)

Detuning of Collision Energies (CE)

With sharper and higher LC signals some compounds (e.g. MDMA, benzoylecgonine, cocaine) could cause detector saturation when present at higher concentration in water samples. Some high sensitivity transitions were detuned to minimize this effect and maintain linear dynamic range for quantitation.

The example shown in Figure 3 shows the detuning of CE for benzoylecgonine.

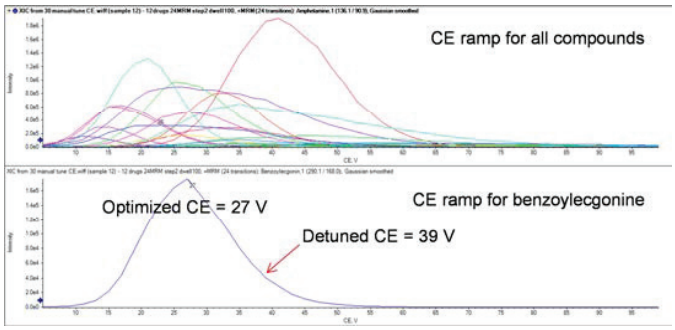


Figure 3. Detuning of CE to minimize detector saturation for benzoylecgonine

Method Data

Method data are listed in Table 4 and summarized below.

Table 4. Sensitivity (S/N calculated using 3x standard deviation in PeakView® software), repeatability (coefficient of variation, %CV) and linearity from 1 to 1000 ng/L (linear fit with 1/x weighting, coefficient of regression, r, using the *Scheduled* MRM™ and the *Scheduled* MRM™-IDA-MS/MS method)

Compound	S/N at 1 ng/L	%CV at 10 ng/L	r (MRM)	r (IDA)
Morphine 1	35	2.5	0.999	0.994
Morphine 2	22	1.4	0.999	0.991
Codeine 1	33	4.4	0.999	0.998
Codeine 2	14	7.1	0.999	0.993
Amphetamine 1	4	4.2	0.997	0.996
Amphetamine 2	8	5.5	0.999	0.997
Monoacetylmorphine 1	14	11.4	0.996	0.992
Monoacetylmorphine 2	15	9.3	0.999	0.995
Methamphetamine 1	3	6.4	0.995	0.994
Methamphetamine 2	17	4.8	0.995	0.993
MDMA 1	24	4.3	0.999	0.993
MDMA 2	2.5	7.7	0.997	0.998
Mephedrone 1	28	6.6	1.000	0.997
Mephedrone 2	14	4.4	0.999	0.994
Benzoylecgonine 1	37	5.9	0.999	0.996
Benzoylecgonine 2	18	5.9	0.999	0.997
Monoacetylcodeine 1	62	4.9	0.998	0.997
Monoacetylcodeine 2	7	4.2	0.998	0.996
Cocaine 1	72	1.9	0.999	0.996
Cocaine 2	15	3.1	1.000	0.998
EDDP 1	47	2.7	0.998	0.998
EDDP 2	43	2.7	0.999	0.998
Methadone 1	43	3.5	0.996	0.985
Methadone 2	22	2.7	0.996	0.989

- Linearity and working range: 1 ng/L to 1000 ng/L for all compounds (except amphetamine and methamphetamine). This corresponds to 2-2000 ng/L for 1:1 diluted samples, and 10-10000 ng/L for 1:10 diluted samples.

- Limits of quantitation (LOQ): 1 ng/L; S/N > 10 for all compounds (except amphetamine.1 S/N=4, methamphetamine.1 S/N=3)
- Linearity: r > 0.994
- Accuracy of the standards from 1 to 1000 ng/L: between 80 and 120%
- Precision: RSD% typically between 2.5 and 6% for 6 consecutive injections of a standard at 10 ng/L (except acetylmorphine)

Ion suppression

Matrix effects have been investigated by T-infusion experiments. Matrix load of wastewater samples can strongly differ from sample to sample. Generally dilution is necessary to minimize ion suppression when large volume direct injection is used. Figure 4 shows that some suppression effects can still be observed in the elution window of the analytes with a 1:1 dilution. But with dilution 1:10 nearly no ion suppression was observed.

It can also be seen that strong matrix effects are present in the range of the void time up to a retention time of 2.0 min. However, the earliest eluting compound morphine has a retention time of 3.4 min and is not affected by ion suppression.

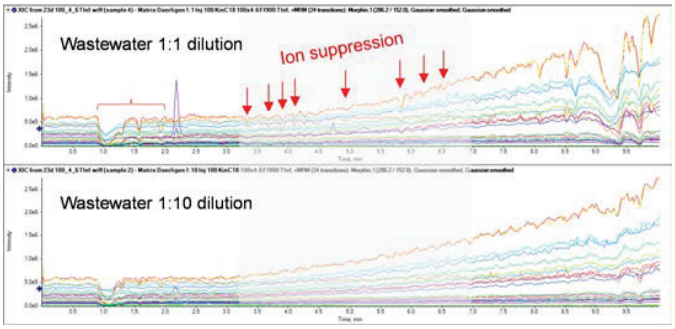


Figure 4. Investigation of matrix effects

Street Parade Results

The gain in sensitivity and the lower detection limits help to detect low levels of illegal drugs. Figure 5 shows the day 5 sample of the Street Parade 2014, diluted 1:10.

Figure 6 shows the profile of MDMA (ecstasy), benzoylecgonine (metabolite of cocaine) and monoacetylmorphine (metabolite of heroin) over the time period of the Street Parade. These profiles indicate different consumption amounts during the event resulting in different wastewater profiles.

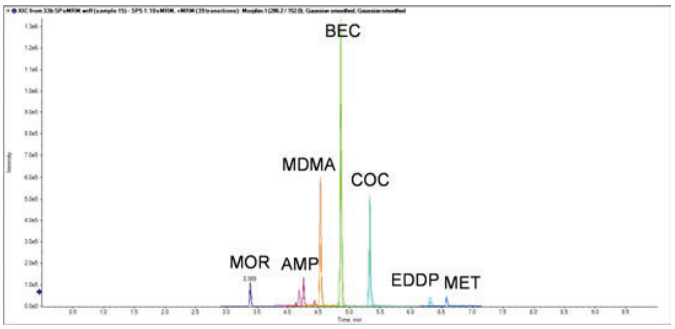


Figure 5. Day 5 sample of the Street Parade Zürich, injected directly after 1:10 dilution

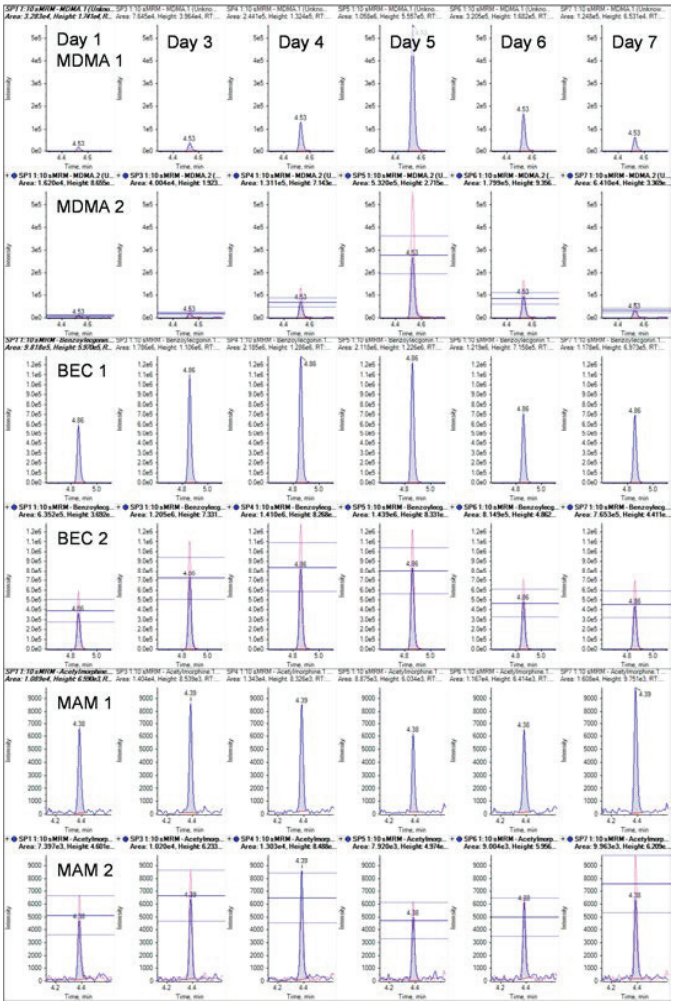


Figure 6. Profile of MDMA (ecstasy), benzoylecgonine (metabolite of cocaine) and monoacetylmorphine (metabolite of heroin) over the time period of the Street Parade. Data indicate high consumption of MDMA and increased consumption of cocaine during the event. The concentration of MAM in wastewater was relatively constant. The peak review in MultiQuant™ software allows reviewing MRM ratios and tolerance levels for compound identification.

Compound Identification

Commonly at least two MRM transitions are monitored per compound and the ratio of quantifier and qualifier ion is used for compound identification. Guidelines define identification criteria and tolerance levels for ion ratios.[11] However, MRM ratios are not always unambiguous. Matrix interferences might disturb one of the two transitions, and thus, ion ratio identification fails. In addition, ion ratios are often falsified at the upper end of the linear dynamic range because of detector saturation. On the other hand, the qualifier MRM can be too weak to be used for identification at the lower end of the dynamic range.

With the *Scheduled* MRM™ pro-IDA-MS/MS workflow it is possible to monitor two transitions for each compound and use the ratio for identification. In addition, QTRAP® MS/MS full scan spectra are collected automatically. These spectra can be searched against mass spectral libraries in MasterView™ software for increased confidence in identification.

The IDA triggering works effectively due to individual thresholds for each compound. Figure 7 shows that chromatograms acquired in IDA mode have slightly less data points across the LC peak at the time where the MS/MS spectrum was acquired, but still enough data points for accurate and reproducible quantitation. Results presented in Table 4 also show that linearity was not compromised when using the IDA method.

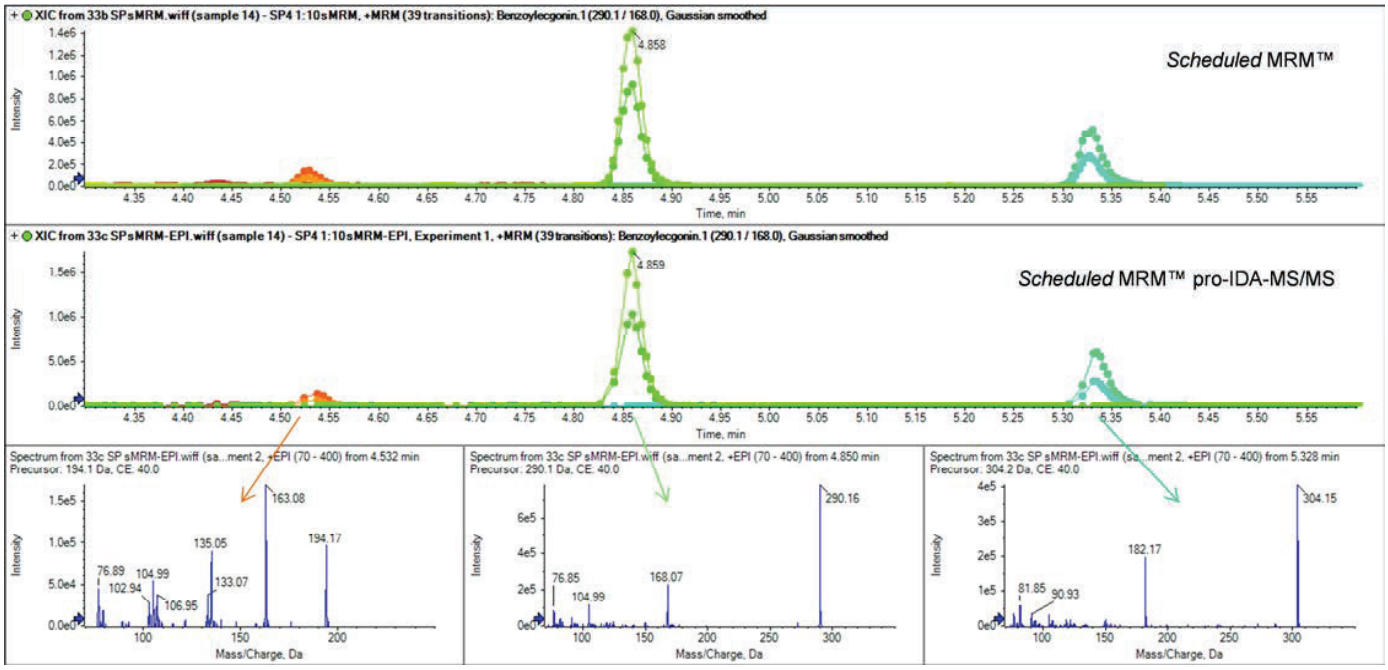


Figure 7. Day 4 sample of the Street Parade Zürich, quantitative data quality is not compromised when using *Scheduled* MRM™ or *Scheduled* MRM™ pro-IDA-MS/MS since sufficient number of data points is acquired using both workflows (top and middle), the IDA methods provides additional information for compound identification (bottom left to right: MS/MS of MDMA, benzoylecgonine, and cocaine)

Standard samples were injected to find out at what concentration compounds can be identified using retention time matching and MS/MS library searching. At 1 ng/L, 9 of the 12 drugs could be clearly identified (no MS/MS spectra were acquired for codeine, amphetamine and monoacetylmorphine) at 5 ng/L, all of the 12 compounds were identified with high confidence (Figure 8).

Figure 9 shows the day 7 sample, diluted 1:10 prior LC-MS/MS analysis. MDMA was identified with high confidence, although the concentration in the injected sample was only 7 ng/L, which corresponds to 70 ng/L in the undiluted sample.

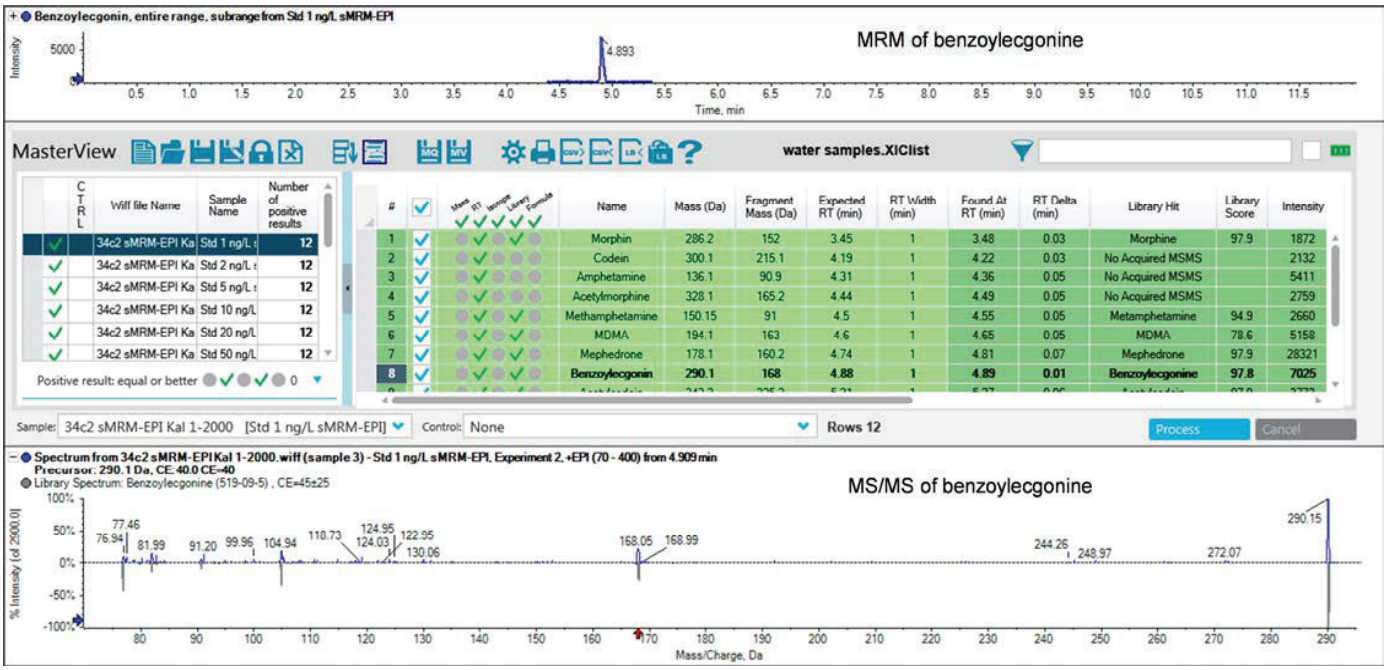


Figure 8. Identification based on retention time matching and MS/MS library searching in MasterView™ software, 9 out of 12 compounds were identified at 1 ng/L, all 12 compounds were identified at 5 ng/L, the example shows the MRM (retention time error = 0.01 min) and MS/MS spectrum (FIT = 97.8%) of benzoylecgonine at a concentration of 1 ng/L

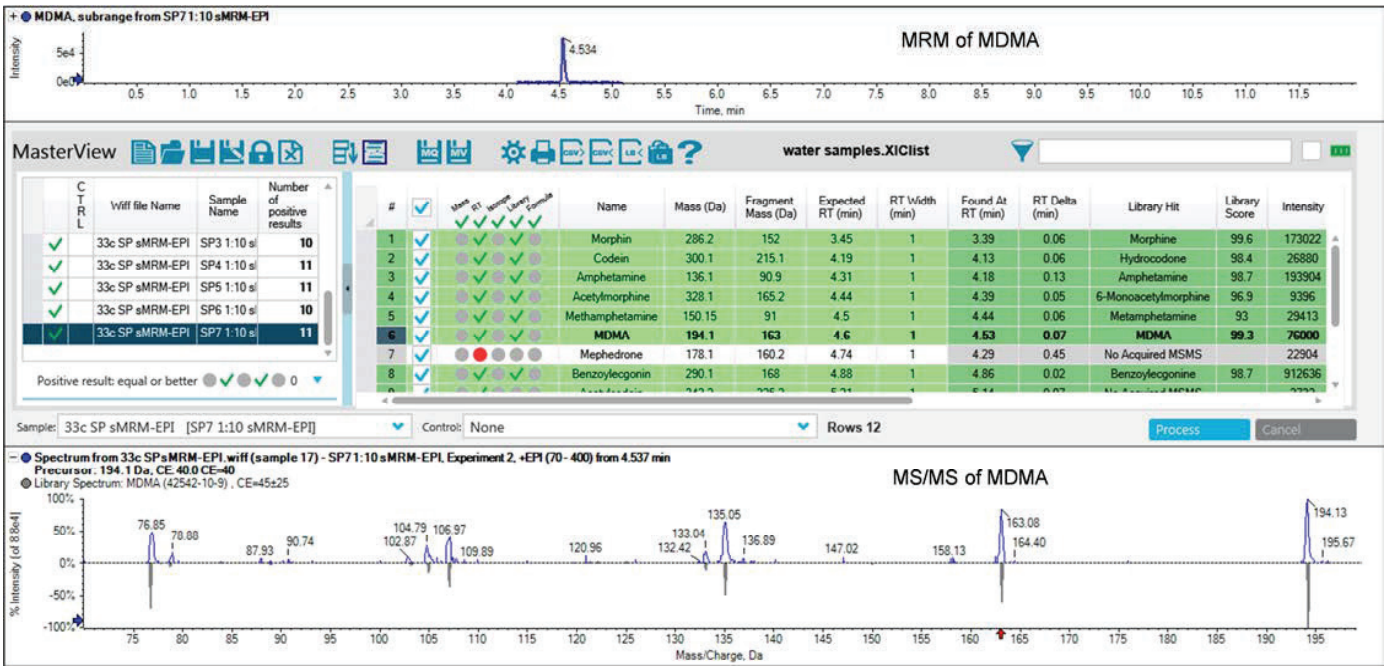


Figure 9. Identification based on retention time matching and MS/MS library searching in MasterView™ software, MDMA was identified with a retention time error of 0.07 min and a library FIT of 99.3%

Summary

The existing method for the determination of drugs of abuse in complex matrices like wastewater by large volume direct injection was significantly improved. Sensitivity gains and S/N gains by a factor of up to 10 were obtained by optimizing the LC and ion source conditions. This was achieved by using a Phenomenex Kinetex core-shell column with 4.6 mm ID and a high-flow method design. The column backpressure was only ~100 bar due to the use of 5 µm particles and therefore, the method can be run on a traditional LC systems.

The collision energies of high abundant MRM transitions were detuned to avoid detector saturation. Two method workflows were developed using *Scheduled* MRM™ and *Scheduled* MRM™ pro-IDA-MS/MS. Both method allow accurate and reproducible quantitation down to low ng/L range and identification based on ion ratios. The IDA method offers the additional benefit of identifying target analytes based on MS/MS library searching resulting in increased confidence in results.

The method was successfully applied to the determination of drugs of abuse wastewater during a party event (Street Parade Zürich 2014). Different profiles were observed for different drugs indicating different consumption during the time period of the event.

Acknowledgement

The authors wish to thank the people of the Sewage Treatment Plant Werdhölzli, Zurich for the daily wastewater sampling during the Street Parade.

References

1 R.Pal, M.Megharaj, K.P.Kirkbride and R.Naidu: 'Illicit drugs and the environment - a review' Science of the Total Environment 463 (2013) 1079-1092

2 European Monitoring Centre for Drugs and Drug Addiction, Lisbon http://www.emcdda.europa.eu/

3 Chr. Ort, A.L.N. van Nuijs, J.D. Berset et al.: 'Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis' Addiction 109 (2014) 1338-1352

4 S. Castiglioni, K.V. Thomas, B. Kasprzyk-Hordern, L. Vandam and P. Griffiths: 'Testing wastewater to detect illicit drugs: State of the art, potential and research needs' Science of the Total Environment 487 (2014) 613-620

5 D.R. Baker, L. Barron and B. Kasprzyk-Hordern: 'Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A : chemical analysis and drug use estimates' Science of The Total Environment 487 (2014) 629-641

6 J.D. Berset and A. Schreiber: 'Determination of Selected Illegal Drugs and its Important Metabolites in Waste Water by Large Volume Direct Injection HPLC-MS/MS' Application Note AB Sciex (2010) # 0990310-01

7 J.D. Berset, R.Brenneisen and Chr. Mathieu: 'Analysis of Ilicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-MS/MS)' Chemosphere 81 (2010) 859-866

8 S. Dulaurent, J.M. Gaulier, L. Imbert, A. Morla and G. Lachâtre: 'Simultaneous determination of Δ9-tetrahydrocannabinol, cannabidiol, cannabinol and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in hair using liquid chromatography–tandem mass spectrometry' Forensic Science International 236 (2014) 151-156

9 A. Schreiber and R. Kern: 'Quantitation and Identification of Pharmaceuticals and Personal Care Products (PPCP) in Water Samples' Application Note AB Sciex (2010) # 1790210-01

10 'Using Your QTRAP® LC/MS/MS System at Full Potential - A Quick-Start Guide to Upgrade an MRM Acquisition Method to a Scheduled MRM Pro-IDA-MS/MS Acquisition Method in 6 Easy Steps using Analyst® Software' Application Note AB Sciex (2014) # 10160214-01

11 European Guideline 2002/657/EC

Food & beverage testing using QTRAP technology

Food and Environmental

Advanced Data Acquisition and Data Processing Workflows to Identify, Quantify and Confirm Pesticide Residues

André Schreiber[1] and Lauryn Bailey[2]
[1]AB SCIEX Concord, Ontario (Canada); [2]AB SCIEX Framingham, Massachusetts (USA)

Overview

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. Pesticide residues may pose a potential threat to human health. Modern analytical techniques, such as QuEChERS extraction followed by LC-MS/MS, allow screening for pesticides in a variety of food matrices.[1-3]

Here we present a new and powerful workflow to identify, quantify and confirm the presence of 400 pesticides utilizing generic QuEChERS extraction and LC-MS/MS analysis with the AB SCIEX QTRAP® 6500 system using the *Scheduled* MRM™ Pro algorithm and Information Dependent Acquisition (IDA) of full scan MS/MS spectra. High confidence in identification and confirmation was achieved by automatically calculating the ratio of quantifier and qualifier ions and searching MS/MS spectral libraries in MultiQuant™ and MasterView™ software. Qualitative method performance was verified using guideline SANCO/12571/2013 guideline.[4]

Introduction

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. After application pesticides may remain on agricultural products or accumulate in the environment, posing a potential threat to human health. Consequently, government agencies, food producers and food retailers have the duty to ensure that pesticide residues occurring in food are below established maximum residue limits set by Codex Alimentarius, the European Union, the US EPA, or by the Japanese Ministry of Health, Labour and Welfare.

There is a demand for powerful and rapid analytical methods that can identify pesticides with high confidence in a broad range of food matrices and quantify them at low concentrations with good accuracy and reproducibility.

A new analytical workflow was developed to screen for 400 pesticides in fruit, vegetable, tea and spices utilizing generic QuEChERS extraction, UHPLC separation using a core-shell particle column, and MS/MS detection with the AB SCIEX QTRAP® 6500 system. The *Scheduled* MRM™ Pro algorithm was used to acquire ~800 MRM transitions to accurately quantify target pesticides and identify them based on the characteristic ratio of quantifier and qualifier ions. The *Scheduled* MRM™ data were also used to automatically acquire full scan MS/MS spectra to allow data to be searched against spectral libraries. The data processing in MultiQuant™ and MasterView™ software was used as a confirmatory tool to enhance confidence in quantitative and qualitative results.

Experimental

Sample Preparation

A pesticide standard containing ~400 compounds was used for method development and sample analysis.

Store-bought food samples were extracted using a QuEChERS procedure based on the European standard method 15662.[5]

- 10 g of frozen homogenized sample
- Addition of water to increase the water content of the sample to approximately 10 g
- Addition of 10 mL acetonitrile and internal standard
- Extraction by vigorous shaking for 1 min
- Addition of Phenomenex roQ™ QuEChERS kit buffer-salt mix (KS0-8909) and immediate vigorous shaking for 1 min
- Centrifugation for 10 min at 9000 rpm

- Transfer of a 1 mL aliquot of the sample extract into a tube containing Phenomenex roQ™ dSPE kit (KS0-8916, 8913, 8914 or 8915 depending on sample type)
- Cleanup by vigorous shaking for 30 sec
- Transfer of 100 µL of the cleaned sample extract into an autosampler vial
- 10x dilution with water prior LC-MS/MS analysis

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into food samples and used to verify method performance for identification and confirmation.

LC Separation

- Separation using a Phenomenex Kinetex Biphenyl (100 x 2.1 mm, 2.6u) column
- Gradient water/methanol with 5 mM ammonium formate with a total run time of 15 min (Table 1)
- Injection volume of 10 µL

Table 1. LC gradient conditions at a flow rate of 500 µL/min

Step	Time	A (%)	B (%)
0	0.0	90	10
1	10	10.0	90
2	13	10	90
3	13.1	90	10
5	15	90	10

MS/MS Detection

Samples were analyzed with two separate methods utilizing the AB SCIEX QTRAP® 6500 system with IonDrive™ Turbo V ion source using the electrospray ionization probe. The following gas settings were used: CUR 30 psi, Gas1 50 psi, Gas2 65 psi, CAD high.

The ion source temperature was set to 300°C to avoid degradation of thermally fragile pesticides such as Avermectin.

Method 1: *Scheduled* MRM™ Pro algorithm monitoring 2 transitions for each target pesticide (Figure 1)

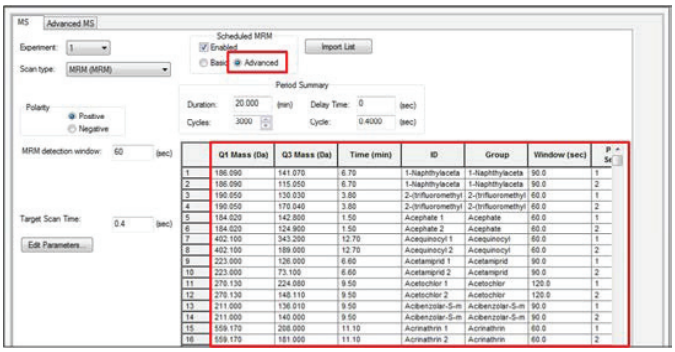


Figure 1. Acquisition method editor to build a method using the *Scheduled* MRM™ Pro algorithm

- Compound dependent detection window to match LC peak width and shape
- Compound dependent threshold for dynamic window extension and MRM-triggered MRM
- Target scan time of 0.4 sec to monitor ~800 transitions

Method 2: *Scheduled* MRM™-IDA-MS/MS to collect additional MS/MS information for identification (Figure 2)

- Information dependent acquisition of the most intense precursor ion detected in the MRM survey
- Dynamic background subtraction with a threshold of 1000 cps in methods without using an inclusion list (screening methods)
- Dynamic background subtraction with a threshold of >1000000 cps in methods when using an inclusion list, threshold of 100 cps for every compound in the inclusion list (confirmatory methods)

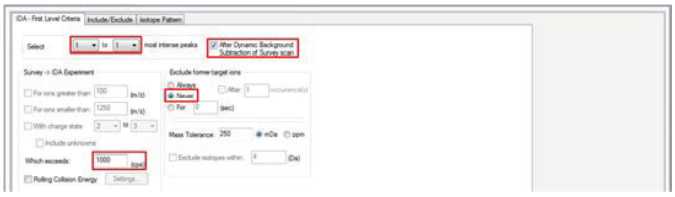


Figure 2. Acquisition method editor to build a method using (IDA)

MS/MS spectra were acquired in Enhanced Product Ion (EPI) scanning mode using a scan speed of 10000 Da/s. Dynamic fill time was used to achieve good quality spectra of compounds present at low and high concentrations. Highly characteristic MS/MS spectra were achieved using a collision energy (CE) of 35 V with collision energy spread (CES) of 15 V.

Data Processing

MultiQuant™ software version 3.0 was used for quantitative analysis and automatic MRM ratio calculation. MasterView™ software version 1.1 was used for MS/MS library searching.

MS/MS spectra were searched against the MS/MS spectra were search against the iMethod™ Pesticide Library version 2.1.

Results and Discussion

Compound Coverage

An example chromatogram of a solvent standard at 1 ng/mL is shown in Figure 3.

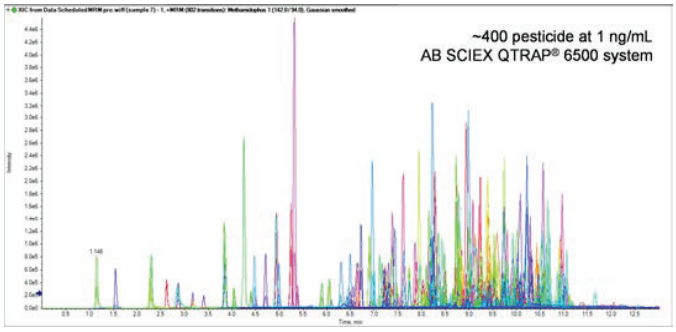


Figure 3. Approximately 400 pesticides detected using 800 MRM transition with the *Scheduled* MRM™ Pro algorithm

Approximately 800 MRM transitions were monitored using the *Scheduled* MRM™ Pro algorithm. This allows quantitation and identification of 400 pesticides in a single LC-MS/MS run while using the ratio of quantifier and qualifier transitions. Further optimization of the gradient profile is planned to spread late eluting compounds more evenly through the chromatogram to extend the method to a total of 500 compounds (1000 MRM transitions).

The example chromatograms shown in Figure 4 highlight the advantage of setting compound dependent detection windows to match LC peak width and shape. Pesticides with wider peaks or partly separated isomers were detected using a longer window, while narrow peaks were detected using a shorter window to enhance scheduling of transitions for best data quality.

Quantitative Results

Solvent standards were injected at a concentration ranging from 0.1 to 100 ng/mL. Example calibration lines are shown in Figure 5. Linear regression with 1/x weighting was used and points with accuracy values outside 80 to 120% were excluded. The coefficient of regression was typically higher than 0.99.

All target compounds had limits of quantitation (LOQ) of at least 1 ng/mL, for most compounds the estimated LOQ was much lower than 0.1 ng/mL (Signal-to-Noise, S/N >10). Example chromatograms and S/N at 1 ng/mL are shown in Figure 4 and Table 1.

Table 1. Signal-to-Noise (S/N) and Coefficient of Variation (%CV) for selected pesticides at a concentration of 1 ng/mL

Pesticide	S/N at 1 ng/mL	%CV at 1 ng/mL
Acephate	276	1.18
Avermectin	16.2	6.16
Bitertanol	44.9	6.12
Carbendazim	8090	1.70
Carbofuran	2670	1.52
Clethodim E	249	4.18
Clethodim Z	295	2.02
Difenoconazole	314	8.65
Dimethoate	19100	0.98
Dimethomorph	844	1.71
Imidacloprid	1430	0.49
Lufenuron	17.6	4.79
Omethoate	19800	1.22
Oxadixyl	1290	2.39
Permethrin	128	5.91
Propamocarb	1540	0.44
Propazine	2190	1.92
Pymetrozine	2600	1.66
Spinosyn A	661	3.10
Spinosyn D	253	4.47
Spiroxamine	2740	2.62
Thiabendazole	831	2.32

- Transfer of a 1 mL aliquot of the sample extract into a tube containing Phenomenex roQ™ dSPE kit (KS0-8916, 8913, 8914 or 8915 depending on sample type)
- Cleanup by vigorous shaking for 30 sec
- Transfer of 100 µL of the cleaned sample extract into an autosampler vial
- 10x dilution with water prior LC-MS/MS analysis

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into food samples and used to verify method performance for identification and confirmation.

LC Separation

- Separation using a Phenomenex Kinetex Biphenyl (100 x 2.1 mm, 2.6u) column
- Gradient water/methanol with 5 mM ammonium formate with a total run time of 15 min (Table 1)
- Injection volume of 10 µL

Table 1. LC gradient conditions at a flow rate of 500 µL/min

Step	Time	A (%)	B (%)
0	0.0	90	10
1	10	10.0	90
2	13	10	90
3	13.1	90	10
5	15	90	10

MS/MS Detection

Samples were analyzed with two separate methods utilizing the AB SCIEX QTRAP® 6500 system with IonDrive™ Turbo V ion source using the electrospray ionization probe. The following gas settings were used: CUR 30 psi, Gas1 50 psi, Gas2 65 psi, CAD high.

The ion source temperature was set to 300°C to avoid degradation of thermally fragile pesticides such as Avermectin.

Method 1: *Scheduled* MRM™ Pro algorithm monitoring 2 transitions for each target pesticide (Figure 1)

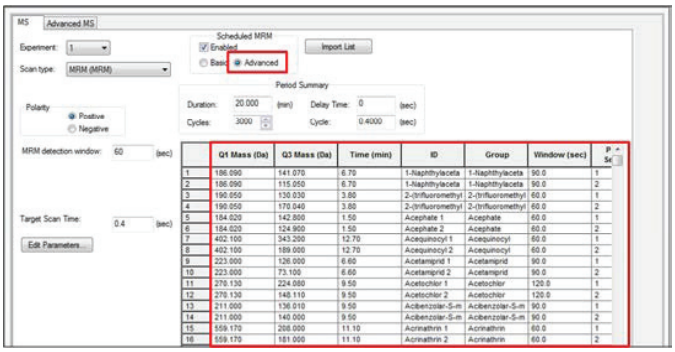


Figure 1. Acquisition method editor to build a method using the *Scheduled* MRM™ Pro algorithm

- Compound dependent detection window to match LC peak width and shape
- Compound dependent threshold for dynamic window extension and MRM-triggered MRM
- Target scan time of 0.4 sec to monitor ~800 transitions

Method 2: *Scheduled* MRM™-IDA-MS/MS to collect additional MS/MS information for identification (Figure 2)

- Information dependent acquisition of the most intense precursor ion detected in the MRM survey
- Dynamic background subtraction with a threshold of 1000 cps in methods without using an inclusion list (screening methods)
- Dynamic background subtraction with a threshold of >1000000 cps in methods when using an inclusion list, threshold of 100 cps for every compound in the inclusion list (confirmatory methods)

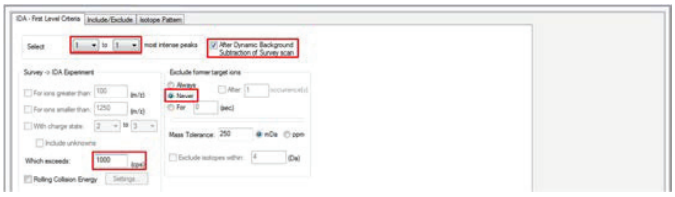


Figure 2. Acquisition method editor to build a method using (IDA)

MS/MS spectra were acquired in Enhanced Product Ion (EPI) scanning mode using a scan speed of 10000 Da/s. Dynamic fill time was used to achieve good quality spectra of compounds present at low and high concentrations. Highly characteristic MS/MS spectra were achieved using a collision energy (CE) of 35 V with collision energy spread (CES) of 15 V.

Despite the high selectivity of MRM detection, there is always a risk of false positive or negative findings due to interfering matrix signals. To increase confidence in identification or to confirm MRM ratio results, highly sensitive MS/MS spectra can be acquired on QTRAP® systems and searched against mass spectral libraries. Full scan MS/MS spectra contain more structural information of a detected compound resulting in a more confident identification.

Full scan spectra were acquired using and *Scheduled* MRM™-IDA-MS/MS method (Figure 6). This way quantitative (MRM peak area) and qualitative information (MRM ratio and MS/MS full scan spectrum) can be collected at the same time. Data processing was performed in MasterView™ software. A library PUR value of 70% or higher was used for positive identification.

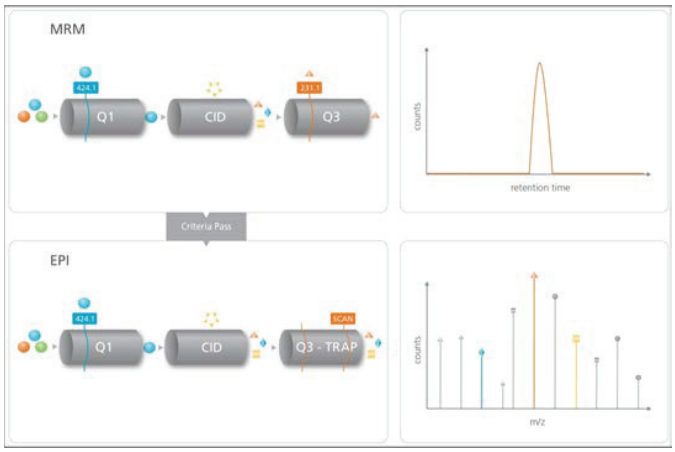


Figure 6. Information Dependent Acquisition (IDA) of MS/MS spectra using an MRM survey scan on a QTRAP® system

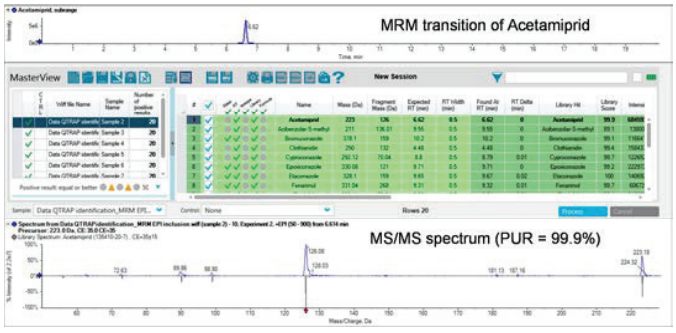


Figure 7. Processing of *Scheduled* MRM™ and MS/MS data in MasterView™ software, compound identification is achieved through automatic retention time matching and MS/MS library searching

Verification of Qualitative Method Performance

Mix D of the SCIEX iD*Quant*™ kit for pesticide analysis, containing 20 compounds, was spiked into carrot, grapes, grapefruit, red pepper, and spinach extract at 10 µg/kg.[7]

The results of identification based on retention time matching, MRM ratio comparison, and MS/MS library searching are summarized in Table 2. All 20 pesticides were confidentially identified in all 5 spiked samples. The average retention time error ranged from 0.008 to 0.024%, the average MRM ratio error from 5.09 to 6.30%, and the average MS/MS PUR from to 95.9 to 98.5%.

Table 2. Pesticides identified in different spiked food samples based on retention time (RT) matching with a tolerance of 0.2 min, MRM ratio comparison, and MS/MS library searching for qualitative method validation

Pesticides in Carrot	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	1.7	97.7
Acibenzolar-S-methyl	9.56	0.01	0.35	6.5	62.7
Bromuconazole	10.20	0.00	0.16	9.2	99.5
Clothianidin	4.48	0.00	0.35	5.6	98.1
Cyproconazole	8.84	0.04	0.58	8.4	100.0
Epoxiconazole	9.73	0.02	0.35	5.2	95.6
Etaconazole	9.68	0.03	0.17	3.2	99.6
Fenarimol	**9.30**	**0.01**	**0.26**	**36.7**	**99.7**
Flutriafol	8.04	0.01	0.59	6.0	99.8
Imazalil	9.98	0.01	0.57	1.8	97.9
Imidacloprid	6.04	0.00	0.81	0.9	98.7
Metribuzin	6.97	0.01	0.43	2.6	100.0
Myclobutanil	9.04	0.00	0.76	7.5	99.5
Nitenpyram	4.38	0.00	0.86	3.2	94.3
Paclobutrazol	8.41	0.01	0.19	6.5	100.0
Pyrimethanil	8.57	0.00	0.53	3.2	99.5
Thiacloprid	7.43	0.01	0.11	3.7	99.8
Thiamethoxam	4.97	0.00	0.35	1.1	98.8
Triadimenol	8.46	0.00	0.38	0.4	100.0
Triticonazole	9.14	0.02	0.07	3.3	98.6
Average		0.009		5.84	96.99

Pesticide in Grapes	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.64	0.02	0.20	1.3	98.0
Acibenzolar-S-methyl	9.59	0.04	0.39	4.9	96.1
Bromuconazole	10.23	0.03	0.13	7.5	98.6
Clothianidin	4.49	0.01	0.36	2.4	97.4
Cyproconazole	8.81	0.01	0.61	14.2	99.0
Epoxiconazole	9.75	0.04	0.33	0.2	74.6
Etaconazole	9.69	0.04	0.16	1.3	97.7
Fenarimol	**9.33**	**0.02**	**0.25**	**33.3**	**99.3**
Flutriafol	8.06	0.03	0.56	1.7	100.0
Imazalil	10.01	0.02	0.58	3.6	98.8
Imidacloprid	6.05	0.01	0.81	0.7	98.7
Metribuzin	6.98	0.02	0.43	3.2	100.0
Myclobutanil	9.05	0.01	0.78	11.0	100.0
Nitenpyram	4.39	0.01	0.85	1.3	95.2
Paclobutrazol	8.44	0.04	0.17	4.9	100.0
Pyrimethanil	8.60	0.03	0.51	7.8	99.5
Thiacloprid	7.44	0.02	0.12	10.7	99.8
Thiamethoxam	4.98	0.01	0.34	2.1	99.3
Triadimenol	8.50	0.04	0.39	2.3	99.2
Triticonazole	9.15	0.03	0.09	11.7	99.7
Average		0.024		6.30	97.55

Pesticide in Grapefruit	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	0.0	99.4
Acibenzolar-S-methyl	9.53	0.02	0.40	5.1	80.0
Bromuconazole	10.22	0.02	0.14	5.2	99.8
Clothianidin	4.48	0.00	0.36	2.2	98.1
Cyproconazole	**8.77**	**0.03**	**0.57**	**6.9**	**50.3**
Epoxiconazole	9.70	0.01	0.34	2.3	99.5
Etaconazole	9.66	0.01	0.17	1.8	99.3
Fenarimol	**9.30**	**0.01**	**0.24**	**27.8**	**99.7**
Flutriafol	8.04	0.01	0.62	11.3	100.0
Imazalil	9.99	0.00	0.60	7.5	98.8
Imidacloprid	6.04	0.00	0.79	1.5	99.5
Metribuzin	6.96	0.00	0.46	10.4	100.0
Myclobutanil	9.05	0.01	0.72	1.5	99.6
Nitenpyram	4.38	0.00	0.84	0.5	95.6
Paclobutrazol	8.40	0.00	0.16	8.3	100.0
Pyrimethanil	8.56	0.01	0.55	0.5	99.5
Thiacloprid	7.42	0.00	0.11	1.7	100.0
Thiamethoxam	4.97	0.00	0.34	3.4	98.5
Triadimenol	8.45	0.01	0.36	6.3	99.7
Triticonazole	9.12	0.00	0.08	4.2	100
Average		0.008		5.42	95.87

Pesticide in Red Pepper	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.63	0.01	0.20	0.2	99.5
Acibenzolar-S-methyl	9.55	0.00	0.41	8.3	71.4
Bromuconazole	10.20	0.00	0.14	5.0	99.1
Clothianidin	4.49	0.01	0.35	3.5	98.0
Cyproconazole	8.88	0.08	0.61	14.8	98.9
Epoxiconazole	9.72	0.01	0.35	6.7	96.5
Etaconazole	9.66	0.01	0.18	7.0	99.2
Fenarimol	**9.30**	**0.01**	**0.25**	**33.4**	**94.4**
Flutriafol	8.04	0.01	0.57	3.7	99.9
Imazalil	9.98	0.01	0.59	6.7	98.0
Imidacloprid	6.05	0.01	0.80	0.0	99.1
Metribuzin	6.97	0.01	0.42	1.7	100.0
Myclobutanil	9.04	0.00	0.70	1.6	99.8
Nitenpyram	4.39	0.01	0.84	1.0	95.9
Paclobutrazol	8.40	0.00	0.17	4.6	100.0
Pyrimethanil	8.57	0.00	0.54	1.9	99.5
Thiacloprid	7.43	0.01	0.12	4.3	100.0
Thiamethoxam	4.98	0.01	0.34	3.6	99.2
Triadimenol	8.45	0.01	0.36	6.8	100.0
Triticonazole	9.14	0.02	0.08	4.7	99.0
Average		0.012		5.98	97.37

Pesticide in Spinach	RT (min)	RT Error	MRM Ratio	% Ratio Error	MS/MS PUR (%)
Acetamiprid	6.61	0.01	0.20	0.0	99.6
Acibenzolar-S-methyl	9.57	0.02	0.34	8.9	95.5

Bromuconazole	10.21	0.01	0.13	10.1	98.4
Clothianidin	4.47	0.01	0.36	1.3	98.7
Cyproconazole	8.75	0.05	0.54	1.3	99.7
Epoxiconazole	9.70	0.01	0.33	0.1	99.8
Etaconazole	9.67	0.02	0.17	0.7	89.6
Fenarimol	**9.31**	**0.00**	**0.25**	**32.0**	**96.9**
Flutriafol	8.03	0.00	0.56	1.4	99.4
Imazalil	9.99	0.00	0.63	13.1	98.8
Imidacloprid	6.03	0.01	0.82	2.0	97.9
Metribuzin	6.96	0.00	0.44	4.7	100.0
Myclobutanil	9.04	0.00	0.72	1.7	99.9
Nitenpyram	4.38	0.00	0.85	1.5	97.0
Paclobutrazol	8.42	0.02	0.18	1.4	100.0
Pyrimethanil	8.58	0.01	0.55	0.5	99.5
Thiacloprid	7.42	0.00	0.12	5.5	99.8
Thiamethoxam	4.96	0.01	0.34	2.6	99.3
Triadimenol	8.49	0.03	0.34	12.2	100.0
Triticonazole	9.12	0.00	0.08	1.0	100.0
Average		0.011		5.09	98.5

Bold and green = positive identification (RT error < 0.2 min, ratio error <30%, MS/MS PUR >70%

Bold and yellow = questionable identification (MS/MS PUR <70%),

Bold and red = no identification (ratio error >30%)

However, very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels.

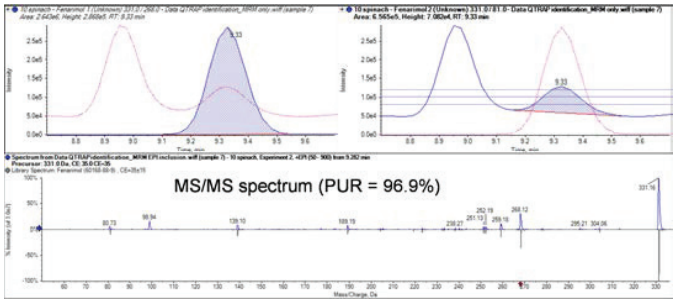


Figure 8. Detection of Fenarimol in spiked spinach: the MRM ratio was slightly out of the 30% tolerance due to high background and a closely eluting interfering matrix peak, but MS/MS library searching confirmed the presence of the detected pesticide.

For example Fenarimol was detected in all samples with matching retention time but the MRM ratio was outside or very close to the 30% tolerance due to high background and a closely eluting interfering matrix peak (Figure 8). But the analysis of a second sample extract to acquire MS/MS spectra confirmed the presence of Fenarimol with excellent library PUR well above 90% (94.4 to 99.7%).

Cyproconazole was identified in the grapefruit sample with matching retention time but the MS/MS PUR value was below the tolerance level (50.3%). Figure 9 shows the MS/MS review in MasterView™ software which helped to identify an isobaric matrix interference causing the low library search PUR. The analysis of a second sample extract confirmed the presence of Cyproconazole by MRM ratio matching (0.569 vs. theoretical 0.532).

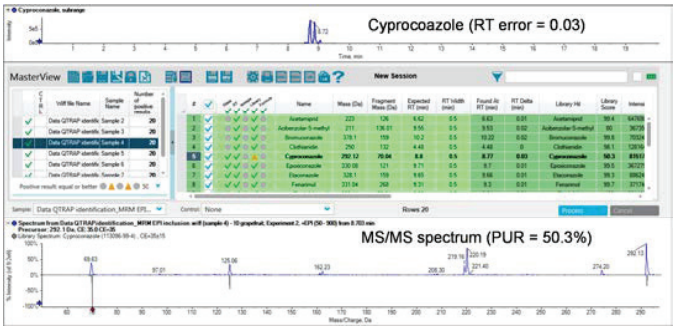


Figure 9. Detection of Cyproconazole in grapefruit: the MS/MS library search resulted in a PUR value of 50.3% only, however, review of spectra revealed in isobaric matrix interference, the MRM ratio error of 6.9% further confirmed the presence of the pesticide.

These two data examples highlight the complementary nature of identification using MRM ratios and MS/MS library searching. Both methods, utilizing the *Scheduled* MRM™ Pro algorithm and *Scheduled* MRM™-IDA-MS/MS, are suitable to quantify and identify pesticides in food samples. However, matrix interferences and high background can result in questionable identification. The analysis of a second sample extract using the alternative approach greatly enhances identification making it a viable tool for confirmation. Such a confirmation method is especially important if the target pesticide is not amenable to an orthogonal method, such as GC-MS.

Application to Incurred Food Samples

Store-bought food samples were extracted using a QuEChERS procedure. Extracts were diluted 10x to minimize possible matrix effects and analyzed by LC-MS/MS using the two described methods utilizing the *Scheduled* MRM™ Pro algorithm and the *Scheduled* MRM™-IDA-MS/MS approach.

Results are summarized in Table 3.

Table 3. Pesticides identified in different incurred food samples based on retention time matching, MRM ratio comparison, and MS/MS library searching

Sample	Pesticide	Conc. (µg/kg)	RT Error	% Ratio Error	MS/MS PUR (%)
Avocado	Azoxystrobin	55.0	0.07	3.9	99.2
	Imidacloprid	6.2	0.01	0.6	95.2
Banana	Bifenthrin	26.8	0.12	9.4	73.0
	Fenpropimorph	12.2	0.08	4.6	99.7
	Imazalil	120	0.08	4.2	97.0
	Thiabendazole	37.3	0.00	0.7	100
Carrot	Linuron	14.3	0.07	1.9	95.1
Grapefruit	Fenbuconazole	5.1	0.05	9.8	75.4
	Imazalil	900	0.08	7.3	97.7
	Thiabendazole	269	0.01	2.3	100
Grapes 1	Fenhexamid	711	0.04	10.4	100
	Pyrimethanil	**226**	**0.06**	**32.8**	**99.4**
	Quinoxyfen	5.9	0.02	7.8	99.4
	Trifloxystrobin	16.2	0.03	4.0	99.2
Grapes 2	Boscalid	15.9	0.07	8.9	78.7
	Fenhexamid	363	0.05	11.4	100
	Myclobutanil	14.2	0.05	0.86	70.7
	Pyrimethanil	687	0.07	28.2	99.5
	Spirotetramat metabolite	6.0	0.04	7.1	not in library
	Tebuconazole	7.1	0.33	11.6	75.4
Lemon	Imazalil	981	1.00	0.8	98.8
	Thiabendazole	7.6	0.20	0.59	99.5
Onion	no pesticides detected				
Orange	Imazalil	1830		4.4	
	Thiabendazole	3110		13.2	
Pepper 1	Acetamiprid	8.9	0.04	3.4	98.6
	Boscalid	9.8	0.06	7.2	82.8
	Clothianidin	6.0	0.00	7.6	87.2
	Imidacloprid	9.1	0.05	0.7	80.8
	Myclobutanil	17.3	0.03	9.0	86.4
	Pyriproxyfen	11.7	0.00	2.4	87.6
	Thiamethoxam	10.6	0.02	0.9	83.5
Pepper 2	Boscalid	47.6	0.06	4.2	87.2
	Pyraclostrobin	21.5	0.03	0.6	80.2
Spinach	**Boscalid**	**14.9**	**0.07**	**21.3**	**14.9**
	Dimethomorph	53.7	0.17	6.2	79.0
	Fenamidone	755	0.02	5.9	99.2
	Imidacloprid	217	0.04	0.8	98.0
	Permethrin	**1060**	**0.10**	**1.4**	**17.0**
Tomato	no pesticides detected				

Four pesticides were identified in the avocado samples based on retention time matching and MS/MS library searching. Confirmatory analysis and quantitation was performed using the *Scheduled* MRM™ Pro method and MRM ratio calculation (Figure 9).

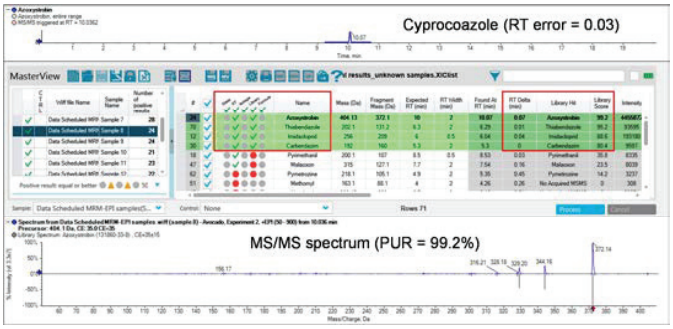


Figure 9. Identification of Azoxystrobin, Imidacloprid, Thiabendazole, and Carbendazim in an avocado sample based on retention time matching and MS/MS library searching, results were confirmed using MRM ratio calculation (note: Thiabendazole and Carbendazim were present below 5 µg/kg)

Four pesticides were identified and quantified in the grapes samples using the *Scheduled* MRM™ Pro method. The example presented in Figure 10 shows the results for Pyrimethanil. It can be seen in the Peak Review window that the MRM ratio is outside the 30% tolerance.

We performed confirmatory analysis of a second sample extract using the *Scheduled* MRM™-IDA-MS/MS approach. Figure 10 shows the excellent MS/MS library match with a PUR 99.4% confirming the presence of Pyrimethanil.

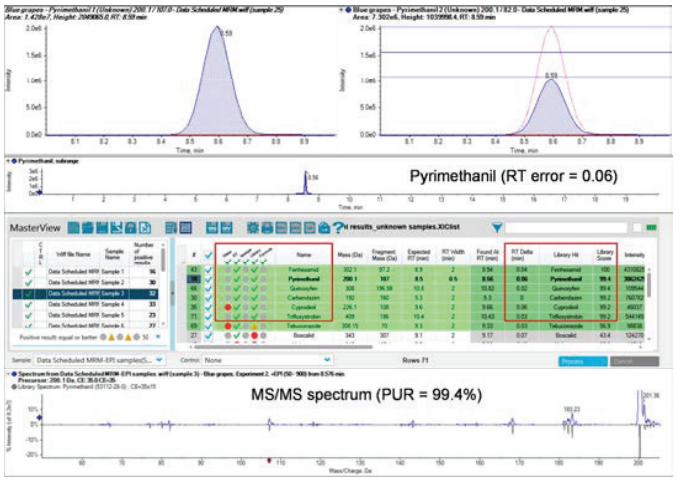


Figure 10. Fenhexamid, Pyrimethanil, Quinoxyfen, and Trifloxystrobin were identified based on MRM ratios and quantified in a grapes sample, the MRM ratio of Pyrimethanil were slightly outside the 30% tolerance (top), however, second analysis using MS/MS library searching confirmed the presence of Pyrimethanil (bottom)

Figure 11 and 12 highlight the complementary nature of MRM ratio and MS/MS library searching for identification.

Figure 11. Boscalid was detected in a spinach samples with a concentration of 14.9 µg/kg, the ion ratio of 21.3 is inside the 30% tolerance (top), however, the MS/MS library searching with a PUR of 14.9% indicated strong matrix interference and suggest that Boscalid is not present in the sample (bottom)

Boscalid was detected in spinach. The ion ratio was inside the 30% tolerance, however, the MS/MS library searching with a PUR of 14.9% indicated strong matrix interference and suggested that Boscalid was not present in the sample.

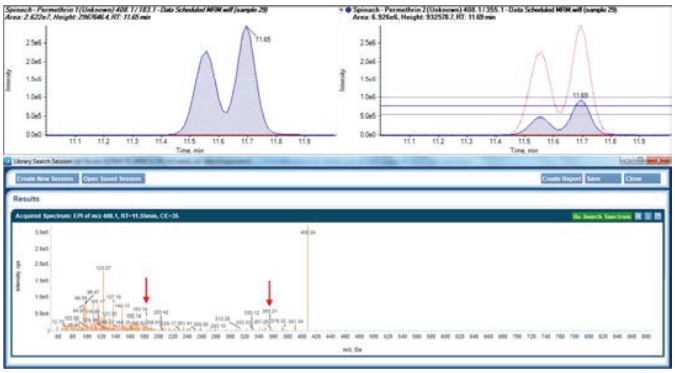


Figure 12. Permethrin was detected in the spinach sample at a high concentration of 1060 µg/kg, the identification using MRM ratio was positive but the MS/MS library searching indicates strong matrix interferences, manual searching in LibraryView™ software confirms the presence the presence of both characteristic ions in the MS/MS spectrum, further confidence is gained through the presence of characteristic isomers in the LC profile

Permethrin was detected in the spinach sample at a high concentration of 1060 µg/kg (above the MRL of 50 µg/kg set by the EU[8]). MRM ratio and library searching are in disagreement for compound identification. Manual evaluation of the MS/MS spectrum in LibraryView™ software confirms the presence of both characteristic fragment ions in the MS/MS spectrum suggesting that Permethrin is present in the sample. The characteristic LC profile of Permethrin isomers further helps compound identification (Figure 12). Since the high level detected is a violation of the maximum residue level additional confirmation is recommend, which can be achieved by using an alternative LC separation setup and the acquisition of additional confirmatory MRM transitions using the *Scheduled* MRM™ Pro algorithm.

Summary

A QuEChERS and LC-MS/MS based method for the analysis of approximately 400 pesticides in food samples was developed.

The method used the AB SCIEX QTRAP® 6500 system utilizing the *Scheduled* MRM™ Pro algorithm and information dependent acquisition of full scan MS/MS spectra allowing quantitation and confident identification.

The method provide sufficient speed and sensitivity to quantify all ~400 pesticides at a concentration of 1 µg/kg in 10x diluted QuEChERS extract of food samples. Good linearity was observed for most compounds from 0.1 to 100 ng/mL with coefficient of variation typically well below 10%.

Qualitative method performance was verified by 20 compounds, into 5 different matrices at a concentration of 10 µg/kg. All compounds were confidentially identified in all samples using the dual method approach. Retention time errors observed were well below the 0.2 min tolerance. Very few pesticides required confirmatory analysis since the identification criteria were slightly outside of tolerance levels (MRM ratio tolerance of 30% or library PUR value of less than 70%). However, these results highlight the complementary nature of MRM ratios and MS/MS full scan offering a possibility for confirmatory analysis.

Last but not least store-bought food samples were analyzed. Automatic identification, quantitation, and confirmation were performed in MultiQuant™ and MasterView™ software.

References

1 M. Anastassiades, et al.: 'Fast and easy multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce' J. AOAC Int. 86 (2003) 412-431

2 St. Lehotay: 'Determination of Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate: Collaborative Study' J. AOAC Int. 90 (2007) 485-520

3 J. Wong et al.: 'Development and Interlaboratory Validation of a QuEChERS-Based Liquid Chromatography−Tandem Mass Spectrometry Method for Multiresidue Pesticide Analysis' J. Agric. Food Chem. 58 (2010) 5897-5903

4 SANCO/12571/2013: 'Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed.'

5 CSN EN 15662: 'Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method' (2008)

6 2002/657/EC: European Commission Decision 'concerning the performance of analytical methods and the interpretation of results'

7. A. Schreiber et al.: 'Using the iD*Quant*™ Standards Kit for Pesticide Analysis to Analyze Residues in Fruits and Vegetable Samples' Application Note AB SCIEX (2011) #3370211-01

8 http://ec.europa.eu/sanco_pesticides

Forensics testing using QTRAP technology

Detecting a New Wave of K2/Spice in Human Urine

An Analytical Method for the Identification of JWH-018, JWH-073, JWH-081 and JWH-250 using the QTRAP® LC-MS/MS System

Alexandre Wang, Brent Dawson, Hua-Fen Liu
SCIEX, Redwood City, CA

Purpose

This application note describes an updated version of the screening method for the active ingredients in K2/Spice blends. Previously we have developed a method focused on the detection of JWH-018 and JWH-073 in human urine. This has now been expanded to include JWH-081 and JWH-250, as well as their metabolites. This screening method takes advantage of the QTRAP® system to perform an information dependent acquisition (IDA) using multiple reaction monitoring (MRM) as a survey scan, and automatically triggering an enhanced product ion (EPI) scan. EPI spectra are searched against an MS/MS spectral library for confirmation.

Introduction

In 2010, the Drug Enforcement Administration announced that they would be temporarily controlling five synthetic cannabinoids: JWH-018, JWH-073, JWH-200, CP-47 and CP47-C8 homologue. Meanwhile, other as-yet unregulated chemicals emerged to replace the controlled substances, including JWH-081 and JWH-250. Similar to JWH-018 and JWH-073, these new chemicals also act as cannabinoid agonists at both the CB1 and CB2 receptors in the brain, causing feelings of euphoria and

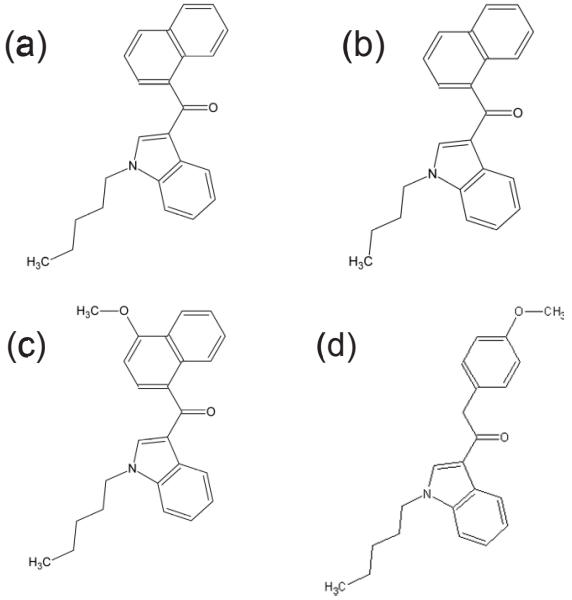


Figure 1. Chemical structures of (a) JWH-018, (b) JWH-073, (c) JWH-081, and (d), JWH-250.

Figure 2. QTRAP® system technology enables identification, characterization, confirmation and quantitation of low abundance analytes.

clarity. With the emergence and widespread abuse of these new chemicals, it became necessary to expand our original screening method in order to detect all four active ingredients, as well as their metabolites. The major challenge was that little or no parent compounds were observed in human urine after a few hours of dose, making it essential to include metabolites of every active ingredient in the screening method.

Key Features of Hybrid Linear Ion Trap Technology

- Exceptional triple quadrupole and ion trap sensitivity allows identification, characterization, confirmation, and quantitation of low abundance analytes with a high degree of confidence.
- Powerful workflows enable fast, efficient identification, characterization, confirmation, and quantitation—all in a single experiment.
- LINAC® collision cell permits greatly reduced dwell times without a loss in sensitivity allowing multi-target analyses.
- Broad linear dynamic range provides true triple quadrupole quantitation performance and enhances identification of ions in complex matrices.
- Powerful advanced scan modes, including neutral loss and precursor ion scans, can be used in flexible combinations to achieve unprecedented selectivity.

Experimental Conditions

The identification of metabolites of the active ingredients JWH-081 and JWH-250 was accomplished using the enhanced sensitivity and resolution provided by the QTRAP® 5500 system and the TripleTOF® 5600 system, however the final screening method was developed for the 4000 QTRAP® system. JWH-081 and JWH-250 were incubated in human liver microsomes and hepatocytes to produce the phase 1 and phase 2 metabolites. These samples were then analyzed using a predictive MRM list of probable metabolite transitions as the survey scan. MRM transitions producing a signal above a pre-determined threshold triggered an enhanced product ion (EPI) scan taking advantage of the linear ion trap. The MS/MS spectrum for each identified metabolite was also added to a searchable library.

Based on the results, the major metabolites for JWH-081 and JWH-250 were added to the existing 4000 QTRAP® method containing metabolites for JWH-018 and JWH-073. Chromatographic separation was achieved on a Restek Ultra Biphenyl column, 5µ 50mm x 2.1mm, with a linear gradient and a flow rate of 0.5mL/min. Mobile phase A consisted of water, 0.1% formic acid and mobile phase B consisted of acetonitrile, 0.1% formic acid.

Table 1. HPLC Gradient for K2 Screening Method

Time	%A	%B
0	90	10
0.5	90	10
6	10	90
7.5	10	90
7.6	90	10
9	90	10

Results and Discussion

Identification and screening of K2 metabolites were challenging for many reasons including the presence of multiple active ingredients, short half-life for the parent compound, lack of standards as well as lack of control samples for the positive urine specimens. To resolve these challenges, JWH-081 and JWH-250 were incubated in human liver microsomes and hepatocytes and the *in vitro* metabolite pathway was identified for each individual compound.

Table 2. List of Major Metabolites for JWH-081 Included in the Screening Method.

Peak ID	Biotransformation	Mass Shift	Expected *m/z*
M1	Demethylation	-14	358.2
M2	Oxidation + Demethylation	2	374.2
M3	Oxidation 1	16	388.2
M4	Oxidation 2	16	388.2
M5	Oxidation + Hydrogenation	18	390.2
M6	Carboxylation 1	30	402.2
M7	Carboxylation 2	30	402.2
M8	Di-oxidation 1	32	404.2
M9	Di-oxidation 2	32	404.2
M10	Demethylation + Glucuronidation	162	534.2
M11	Oxidation + Demethylation + Glucuronidation	178	550.2
M12	Oxidation + Glucuronidation	194	564.2
M13	Carboxylation + Glucuronidation	206	578.2
M14	Di-oxidation + Glucuronidation	208	580.2
	JWH-081	0	372.2

Table 3. List of Major Metabolites for JWH-250 Included in the Screening Method.

Peak ID	Biotransformation	Mass Shift	Expected *m/z*
M1	Demethylation	-14	322.2
M2	Oxidation + Demethylation	2	338.2
M3	Oxidation 1	16	352.2
M4	Oxidation 2	16	352.2
M5	Carboxylation	30	366.2
M6	Di-oxidation	32	368.2
M7	Sulfonation + Oxidation	96	432.2
M8	Demethylation + Glucuronidation	162	498.2
M9	Oxidation + Glucuronidation	194	528.2
M10	Carboxylation + Glucuronidation	206	542.2
M11	Di-oxidation + Glucuronidation	208	544.2
	JWH-250	0	336.2

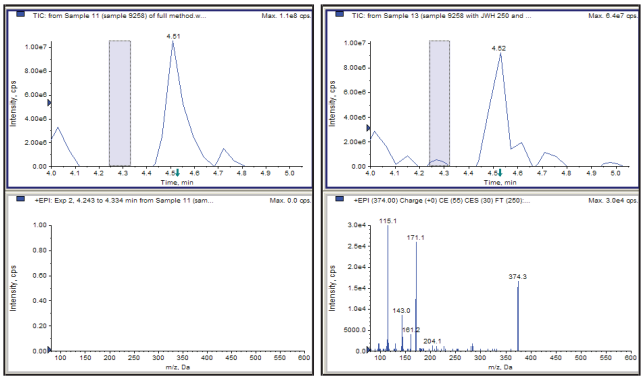
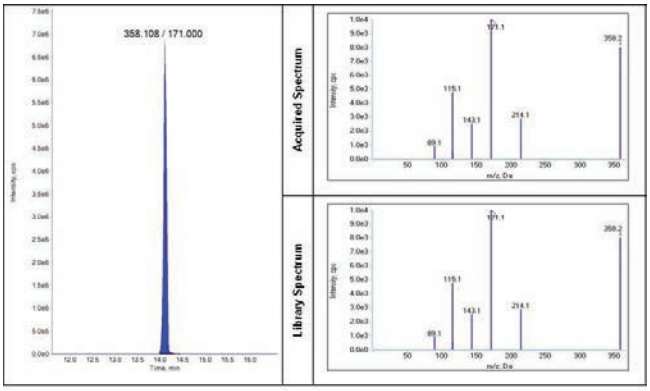


Figure 3. Chromatograms (top) and extracted EPI spectra (bottom) for a blank urine sample (left) and a urine sample spiked with the microsomal incubation of JWH-081 (right). The chromatogram and spectrum on the right show the presence of the demethylation+oxidation metabolite of JWH-081.



	Compound Name	Peak Area	Purity(%)	Visual Check?
1	JWH-081 - Demethylation	3.00e+007	90.7	

Figure 4. MS2 library search result with a positive match for a metabolite of JWH-081.

The *in vitro* incubation samples were analyzed by using a targeted MRM-IDA-EPI approach where LightSight® software was used to generate a list of possible metabolites for each synthetic cannabinoid. Both JWH-081 and JWH-250 were found to be extensively metabolized by demethylation, mono-, di-hydroxylation, carboxylation, reduced di-hydroxylation and corresponding glucuronide conjugation.

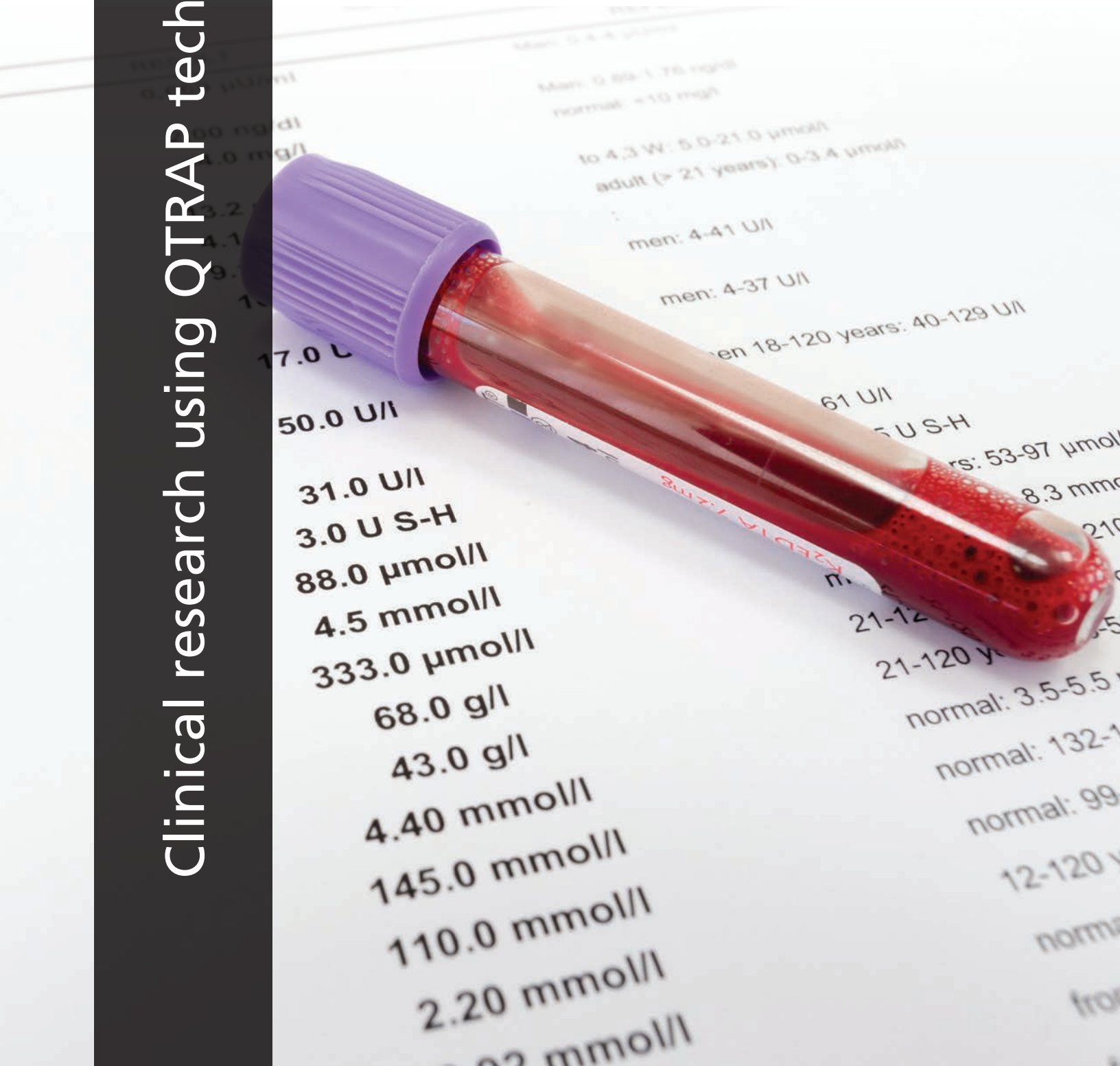
Based on the consolidated list of major metabolites for each active ingredient, the original spice/K2 method (JWH-018 and JWH-073) was updated to include the new parent compounds (JWH-081 and JWH-250) as well as the corresponding major metabolites. Screening confirmation was carried out through IDA triggering of EPI scans where the acquired MS2 spectrum was matched against a library developed from *in vitro* experiments.

The screening method was evaluated with human urine samples spiked with the microsomal incubations as well as positive urine samples diluted with mobile phase and injected directly into the 4000 QTRAP® system. Both sets of samples yielded good signal for various MRM transitions as well as positive matches for the triggered EPI spectra.

Conclusions

The updated IDA method is capable of screening for the synthetic cannabinoids JWH-018, JWH-073, JWH-081, JWH-250 and their major metabolites in a single injection using MRM transitions as survey scan, and triggering enhanced product ion scans which are automatically searched against the MS/MS library for positive confirmation. The method takes full advantage of the sensitivity of the 4000 QTRAP® system to find low level metabolites as well as the trap features of the hybrid system to generate full product ion spectra for library confirmation.

Clinical research using QTRAP technology



Analysis of Nucleoside Reverse Transcriptase Inhibitor (NRTI) Antiretroviral Drugs by LC-MS/MS

Quantitation and Confirmation Workflows using the QTRAP® 4500 LC/MS/MS System

(For Research Use Only. Not for Use in Diagnostic Procedures.)

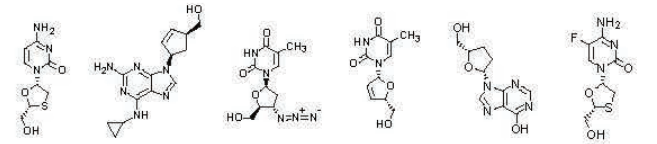
YunYun Zou, Michael Jarvis
SCIEX, Concord, Canada

Introduction

The nucleoside reverse transcriptase inhibitors (NRTI) are an important class of antiretroviral drugs, which are analogues of naturally occurring deoxynucleotides. They compete with deoxynucleotides for substrate binding and inhibit growth of the viral DNA strand by causing chain termination. These antiretroviral drugs are often administered as part of a cocktail of drugs, an approach known as Highly Active Antiretroviral Therapy (HAART), which targets multiple aspects of the viral life cycle. As such, a single method for the accurate quantitation of these drugs in human plasma is highly desirable.

In the work presented here, an LC-MS/MS method has been developed for the quantitative analysis of the NRTI antiretroviral drugs Abacavir (ABC), Didanosine (ddI), Emtricitabine (FTC), Lamivudine (3TC), Stavudine (d4T), and Zidovudine (AZT), using

Figure 1. Structures of the Nucleoside Reverse Transcriptase Inhibitor (NRTI) antiretroviral drugs.



the QTRAP 4500 hybrid triple quadrupole / linear ion trap mass spectrometer. The method employs the Multiple Reaction Monitoring (MRM) scan mode to perform quantitation using the *Scheduled* MRM™ algorithm, with limits of quantitation in plasma ranging from 0.1 to 1.0 ng/mL for the various analytes.

Employing the QTRAP 4500 linear ion trap, a reference library containing MS/MS spectra has been generated for the six NRTI antiretroviral drugs. A second analytical method has been developed, taking advantage of the speed and sensitivity of the QTRAP linear ion trap to perform the simultaneous quantitation and confirmation of these compounds. This method employed the linear ion trap in information-dependent acquisition mode, and automatically acquired MS/MS spectra using the linear ion trap for all of the detected NRTI antiretroviral drugs, which were then searched against the MS/MS library to provide confirmation of the detected compounds. This approach provides additional confidence in the identity of the compounds being quantified.

Figure 2: The LC-MS/MS analysis of NRTI antiretroviral drugs was performed using the QTRAP 4500 LC-MS/MS system (left) and SCIEX UltraLC 100 system (right).

Experimental

Sample Preparation

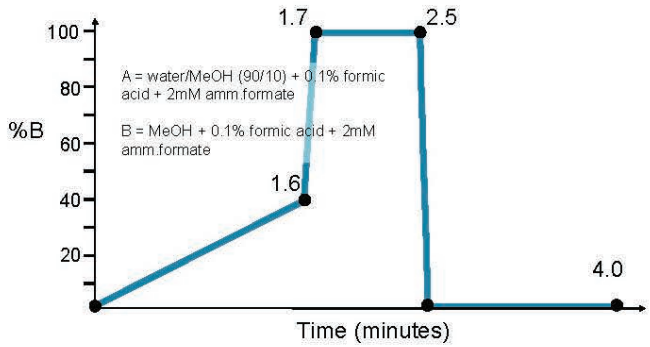
150uL of plasma was measured into a 1.5mL polypropylene Eppendorf microcentrifuge tube. 6uL of internal standard solution was added to each tube, which was then vortex mixed well. The internal standard solution consisted of 10µg/mL each of Didanosine-$^{13}C_2$ (ddI-$^{13}C_2$), Emtricitabine-^{13}C,$^{15}N_2$ (FTC-$^{13}C^{15}N_2$), Lamivudine-$^{15}N_2$,^{13}C, (3TC-$^{15}N_2$,^{13}C), Stavudine-d_3 (d4T-d_3), and Zidovudine-d_3 (AZT-d_3). 400uL of methanol was then added to each sample to precipitate the proteins, followed by vortex mixing for 3 minutes and centrifugation at 14,000 rpm for 5 minutes. The supernatant was then removed and transferred into a clean 1.5mL microcentrifuge tube and dried down under nitrogen gas at 40C. The dried sample was reconstituted using 300uL of 10:90 v/v methanol:water, vortex mixed, and centrifuged at 10,000 rpm for 5 minutes to remove any insoluble material. The reconstituted solution was injected directly onto the LC-MS/MS system.

HPLC Conditions

The SCIEX UltraLC 100 system was used, consisting of an autosampler, a binary pump, a degasser, and a column oven. The chromatic separation was accomplished using a Phenomenex Kinetex C18 column (50x3 mm, 2.6mm) with a SecurityGuard C18 cartridge (4x3 mm). The column oven was maintained at 40˚C.

The HPLC method employed a 4 minute binary flow gradient with a total flow rate of 700 mL/min. Mobile Phase A consisted of water/methanol (90/10) + 0.1% formic acid + 2mM ammonium formate. Mobile Phase B consisted of methanol + 0.1% formic acid + 2mM ammonium formate. The injection volume was set to 20uL.

Figure 3. HPLC flow gradient

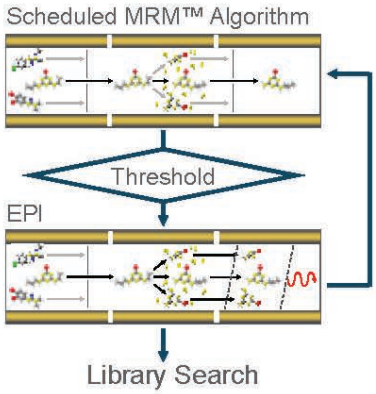


MS/MS Conditions

A SCIEX QTRAP 4500 LC-MS/MS System with Turbo V™ source was used, in positive electrospray ionization (ESI) mode. The six antiretroviral drugs were detected using 2 MRM transitions per compound. Isotopically-labeled analogues of the target compounds were used as internal standards for each of the analytes, with the exception of Abacavir. The MRM transitions are summarized in Table 1. Two LC-MS/MS methods were developed and tested:

1. LC-MS/MS method for quantitation of 6 antiretroviral drugs
   - 2 MRM transitions monitored per compound; 1 MRM transition for each internal standard
   - employs the *Scheduled* MRM™ (sMRM) algorithm.
2. LC-MS/MS method for simultaneous quantitation and confirmation of 6 antiretroviral drugs, using MS/MS library searching
   - 2 MRM transitions monitored per compound; 1 MRM transition for each internal standard
   - employs the *Scheduled* MRM™ algorithm
   - Information-Dependent Acquisition (IDA) of sensitive, high-quality MS/MS spectra for all of the detected compounds, using the QTRAP® linear ion trap;
   - MS/MS spectra are searched against a reference library for confirmation of the identity of all detected compounds

Figure 4: MRM-IDA-EPI experimental workflow for simultaneous quantitation and confirmation of analytes.



In the MRM-IDA-EPI workflow, the Multiple Reaction Monitoring (MRM) mode of operation is used as a survey scan to detect the presence of any of the target analytes. Information Dependent Acquisition (IDA) criteria are pre-configured to ensure that when the signal for a detected compound exceeds a specified threshold, an Enhanced Product Ion (EPI) MS/MS spectrum will automatically be acquired. The MS/MS spectra are searched against a reference library to confirm the identity of the detected compounds.

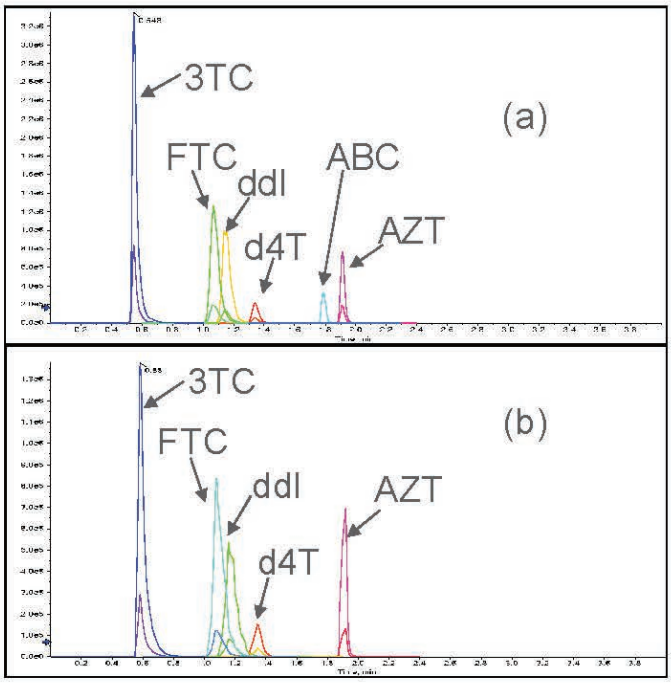
Table 1. MRM Transitions for target compounds.

Analyte	Q1	Q3	Analyte	Q1	Q3
d4T (quan)	225.1	127.0	*FTC (quan)*	248.0	130.0
d4T (qual)	225.1	110.1	*FTC (qual)*	248.0	113.0
d4T-d3	228.1	130.0	*FTC-13C,15N2*	251.0	133.0
3TC (quan)	230.0	112.0	*AZT (quan)*	268.1	127.2
3TC (qual)	230.0	95.1	*AZT (qual)*	268.1	110.1
3TC-15N2,13C	233.0	115.0	*AZT-d3*	271.0	130.0
ddI (quan)	237.1	137.0	*ABC (quan)*	287.2	191.2
ddI (qual)	237.1	110.0	*ABC (qual)*	287.2	150.2
ddI-13C2,15N	240.0	140.0			

Results

A rapid, 4-minute LC-MS/MS method was developed for the quantitation of Abacavir (ABC), Didanosine (ddI), Emtricitabine (FTC), Lamivudine (3TC), Stavudine (d4T), and Zidovudine (AZT), using the SCIEX QTRAP® 4500 LC-MS/MS System. To evaluate the method, a set of human plasma samples were spiked with a series of known concentrations of the target compounds, and these were used to generate calibration curves for each compound. The plasma samples were spiked at concentration levels ranging from 1 ng/mL to 5000 ng/mL, except for Abacavir (ABC) which was spiked at concentration levels ranging from 0.1 ng/mL to 500 ng/mL, due to its significantly stronger response compared to the other analytes.

Figure 5: Example chromatogram for a human plasma sample, spiked at 5ng/mL of each target analyte, using (a) the QTRAP 4500 system (top), and (b) the 4000 QTRAP system (bottom).



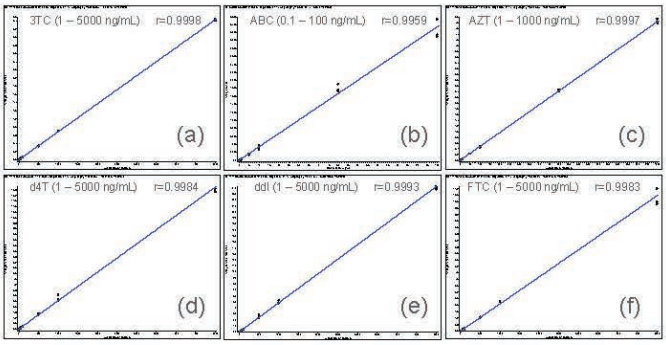
An example chromatogram for a plasma sample spiked at 5 ng/mL concentration, and analyzed with the QTRAP 4500 system, is shown in Figure 5a. The same analysis was performed on the 4000 QTRAP system, and the chromatogram for the 5ng/mL sample is shown in Figure 5b. As can be seen, the analysis on the QTRAP 4500 system provided a gain in sensitivity ranging from a factor of 1.1x (for AZT) to 2.4x (for 3TC), depending on the analyte, with an average signal gain of 1.6x. No comparison data was available for Abacavir using the 4000 QTRAP system. The calibration curves for individual analytes are shown in Figure 5.

Table 2. Signal gains on the QTRAP 4500 system in MRM mode for the analysis of antiretroviral drugs , compared to the 4000 QTRAP system.

	3TC	AZT	d4T	ddI	FTC	Average
Gain in Signal	2.4x	1.1x	*1.4x*	1.9x	1.5x	1.66x

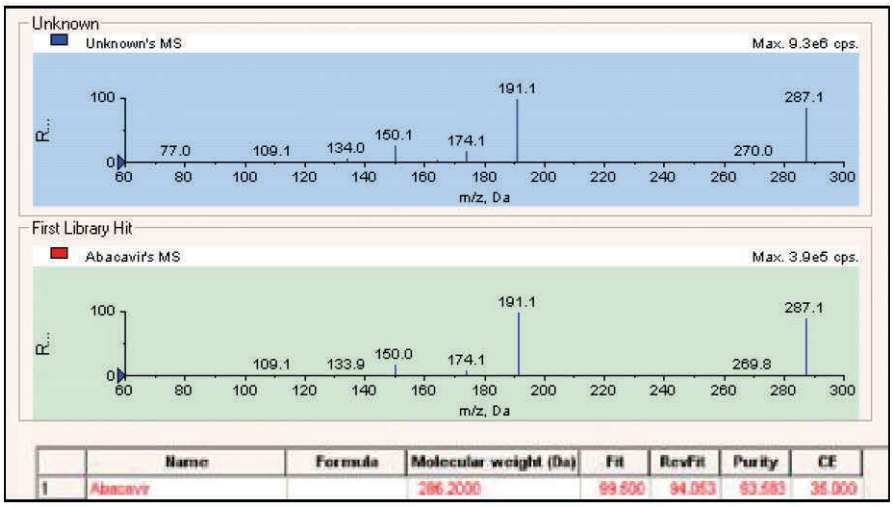
Over the entire range of concentrations covered by the method, the accuracies for all analytes ranged from 88% to 118%, and the CVs were below 10% (<12% for LLOQ). The same set of samples was stored at 4°C after the first analysis, and re-analyzed four days later. Similar results were obtained, and the accuracies and CVs remained in the same range, indicating that the method introduced here is robust.

Figure 6. Calibration curves for (a) Lamivudine (3TC), (b) Abacavir (ABC), (c) Zidovudine (AZT), (d) Stavudine (d4T), (e) Didanosine (ddI), and (f) Emtricitabine.



As mentioned previously, a second analytical method was developed, which employed the linear ion trap in information-dependent acquisition (IDA) mode, to automatically trigger the acquisition of enhanced product ion (EPI) MS/MS spectra throughout the LC-MS/MS run. Using this method, quantitation of the target compounds was performed in the usual way, using the MRM mode of operation. In addition, confirmation of the identity of each detected analyte was possible, by comparing the acquired MS/MS spectra to a reference library, thus reducing the chance of false positives.

Figure 7: MS/MS library searching results for the analysis of Abacavir (top) spiked in human plasma.

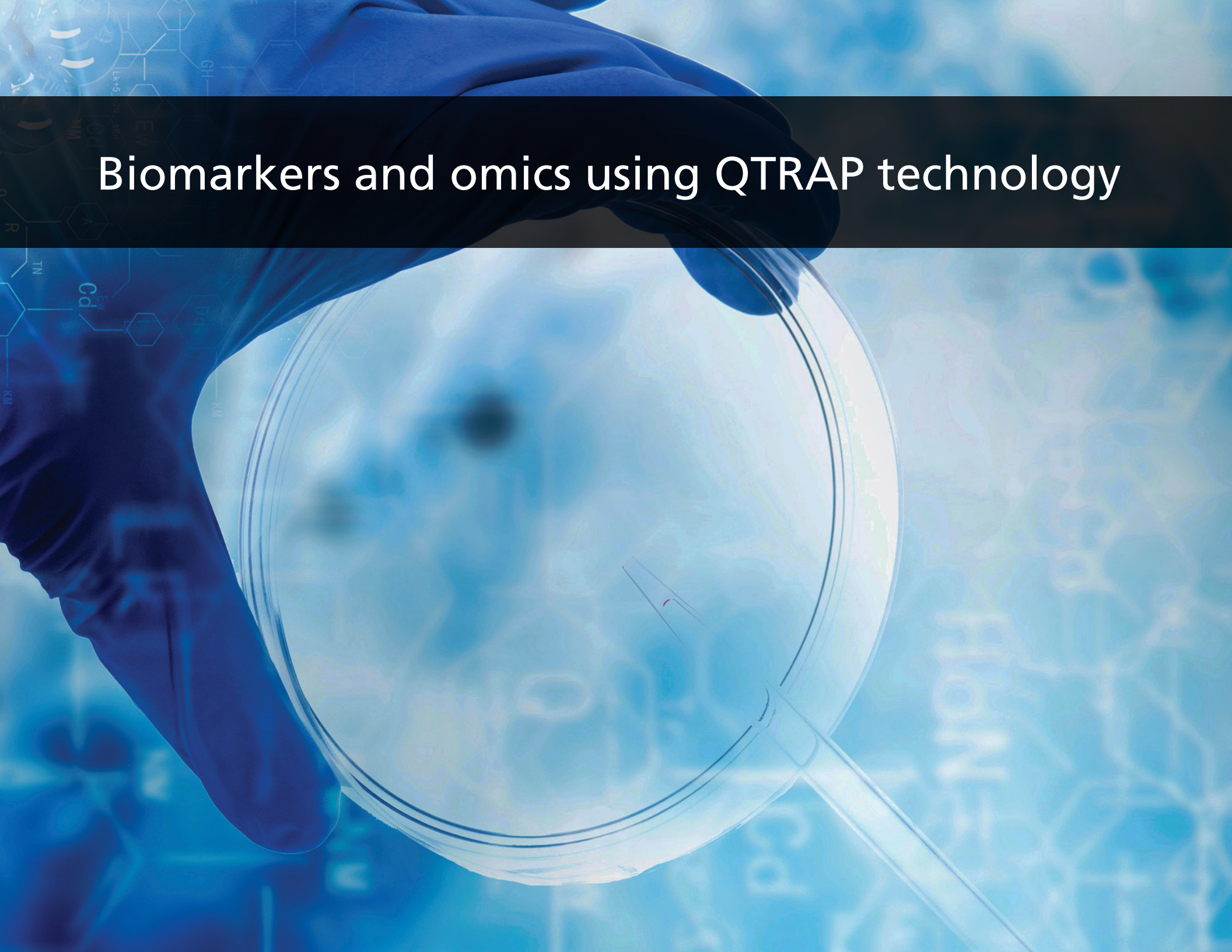


The MS/MS spectral library was generated using neat standard solutions, with individual injections for each compound to reduce any potential interferences. When EPI spectra were acquired from spiked human plasma samples and compared to the spectral library, the calculated purity scores ranged from 70-95% for all samples. Figure 7 displays a representative library search confirmation result for Abacavir, for a spiked human plasma sample.

Conclusions

A rapid and robust method for the detection and quantitation of 6 nucleoside reverse transcriptase inhibitor antiretrovirals in human plasma has been developed. A simple protein precipitation procedure followed by a reconstitution step was used to clean up the plasma matrix. Fast chromatography was combined with high sensitivity detection using an SCIEX QTRAP 4500 LC-MS/MS System, and the SCIEX UltraLC 100 UHPLC system.

The *Scheduled* MRM™ algorithm was used to automatically optimize dwell times and cycle times for best sensitivity and reproducibility. The QTRAP linear ion trap was used in information-dependent acquisition (IDA) mode, to acquire Enhanced Product Ion (MS/MS) spectra which were used for additional confirmation, to minimize the possibility of false positives. The speed and sensitivity of the QTRAP 4500 LC-MS/MS system enables the simultaneous acquisition of quantitative and confirmatory information in a single analysis, with no compromise in the quality of the data.



Biomarkers and omics using QTRAP technology

Quantification of Prostate Specific Antigen (PSA) in Non-Depleted Human Serum Using MRM^3 Analysis

High Throughput Analysis using the SCIEX QTRAP® 5500 System

Fortin T[1], Salvador A[2], Charrier JP[1], Lenz C[3], Bettsworth F[1], Lacoux X[1], Choquet-Kastylevsky G[1], Lemoine J[2].
[1]BioMerieux, France, [2]University of Lyon, France, [3]SCIEX, Germany

Over the last ten years, mass-spectrometry based proteomics has been applied to the discovery of putative protein biomarkers of disease from comparably small numbers of samples. However, there has been limited verification / validation of these putative biomarkers and therefore minimal translation of these proteins into assays of clinical utility. Immuno-based assays have been traditionally used for detection and quantification of proteins in clinical research. Developing these tests, however, is time-consuming. Analytical techniques are needed that will enable researchers to develop protein assays rapidly and produce assays with high specificity and sensitivity. This is driving a rapidly growing interest in using LC/MS-based strategies to quantify proteins.

The use of Multiple Reaction Monitoring (MRM) combined with stable isotope labeled proteins / peptides for the quantification of proteins has been actively explored over the last few years and shows great promise for clinical research. Some key requirements of protein assays for clinical research are high specificity, high robustness, very high throughput and the ability to quantify low abundance proteins in the ng/mL to pg/mL concentration range in e.g. human plasma. Also, the sample preparation must be simple and robust.

Another limitation hindering the widespread use of MRM for biomarker verification has been the widespread use of nanoflow chromatography. While it greatly increases the overall sensitivity of LC-MS/MS experiments and requires significantly reduced

amounts of sample, nanoflow chromatography does currently not offer the sample throughput, reproducibility and robustness required for implementation in clinical research laboratories.

We present here a novel approach that combines the use of analytical chromatography with a new, highly selective mass spectrometry technique called MRM^3 that is able to significantly reduce sample complexity. The approach enables robust detection of protein biomarkers from human serum at concentrations down to the low ng/ml level.

Key Features of Using MRM^3 for Quantifying of Protein Biomarkers

- Technology advancements in the QTRAP 5500 system^3 enables faster, more sensitive MRM^3
- MRM^3 analysis enables higher specificity peptide detection and therefore better quantification of peptide from highly complex samples
- Higher specificity can also reduce the fractionation required for low level detection and increase sample throughput

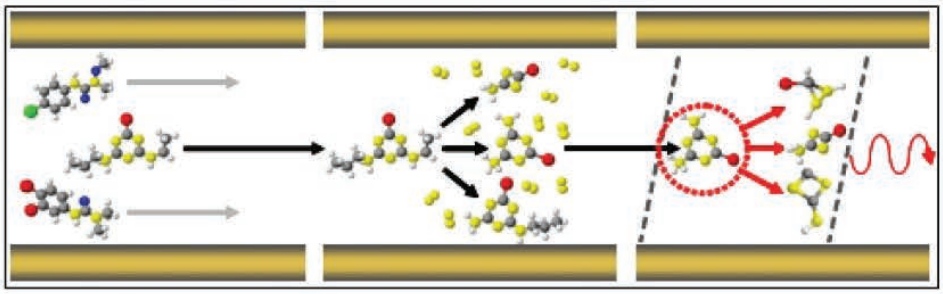


Figure 1. MRM^3 for Quantitative Analysis by LC-MS. Analyte precursor ions are selected in the Q1 quadrupole, fragmented in the Q2 collision cell, and product ions are collected in the linear ion trap (LIT). A suitable fragment ion is isolated and fragmented in a second step using resonance excitation. Second-generation product ions are collected and scanned out of the LIT to the detector.

Methods

Sample Preparation: Human serum samples were denatured with 6M urea, reduced with 30 mM dithiothreitol and alkylated with 50 mM iodoacetamide. Proteins were digested with trypsin overnight at 37 °C (1:30 w/w enzyme to substrate ratio). Samples were desalted using reversed phase cartridges (Oasis HLB 3 cm3, Waters) and fractionated using an MCX cartridge with elution at pH 5.5 using a methanol/acetate buffer mixture (Waters).[2]

Liquid Chromatography: LC-MS/MS analysis was performed using a 2.1 x 100 mm Symmetry C18 reversed phase column (3.5 µm, Waters). A 30 min linear gradient (5-40% acetonitrile in 0.1 % formic acid, 300 µL/min) was used to elute the peptides into the MS system. Total injection to injection cycle time was 45 mins. [2]

Mass Spectrometry: The eluent was analyzed using the Turbo V™ Source on the QTRAP® 5500 system in both MRM and MRM^3 modes of acquisition3. MRM analysis was performed using Unit resolution both Q1 and Q3 quadrupoles. MRM^3 analysis was performed using the MS/MS/MS scan function. The precursor ion was isolated in Q1 using unit resolution, first-generation product ions were generated in the Q2 collision cell using an optimized collision energy and trapped in the Q3 linear ion trap for 200 ms. A suitable first-generation product ion was selected and further excited by resonance excitation for 25 ms to produce second-generation fragments. These were finally scanned out of the ion trap at 10 000 Da/sec resulting in a total cycle time of 350 ms per peptide.2 Q0 Trapping was used to further increase sensitivity.

Data Processing: Data was processed using MultiQuant™ Software. MRM peak areas were integrated, either individually or summed together. MRM3 peak areas were determined by summing the integration of up to four granddaughter ions.

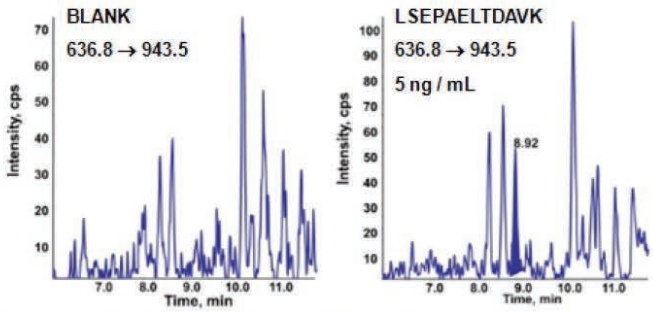


Figure 3. MRM Quantification of PSA (5 ng/mL) in Human Serum. High flow HPLC and MRM analysis can be used to detect PSA when combined with depletion, digestion, and some fractionation[1].

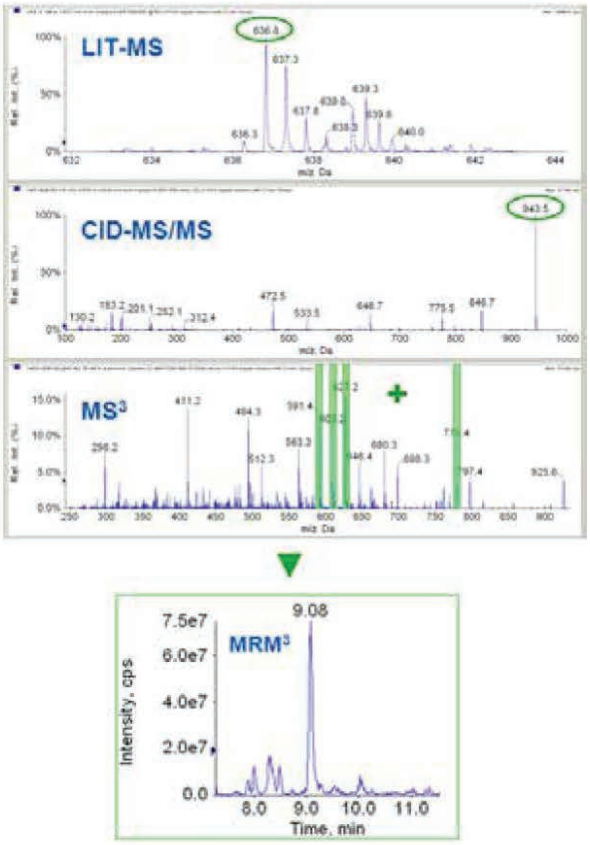


Figure 2. MRM^3 assay design. An EMS scan (top) is used to select the dominant parent ion, the most intense fragment ions are identified using the EPI mode (middle), and MS^3 fragmentation is used to select the best second-generation fragments to extract. Multiple second-generation fragments can be used to generate MRM^3 XICs.

Previous Worked Show Low Level Detection of PSA

Recently, the detection of PSA in human serum was demonstrated using LC-MRM analysis on a 4000 QTRAP system using high flow chromatography1. This method used HSA depletion followed by mixed cation exchange fractionation and a HPLC separation to enable low level detection (Figure 3). As depletion of high abundant proteins is the most difficult step to automate, exploring additional strategies to remove this step are key workflow improvements. Here, the use of MRM^3 analysis3 (Figure 2) has been explored for the detection of PSA and other low abundance proteins in human serum.

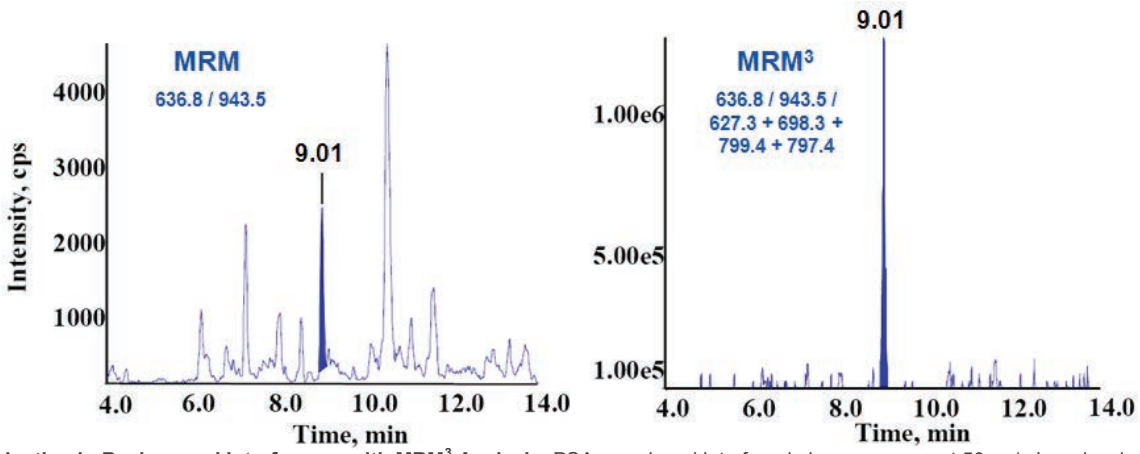
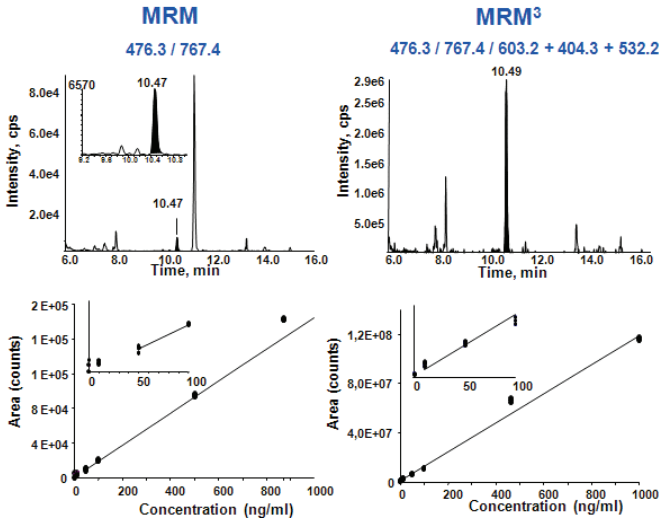


Figure 4. Reduction in Background Interference with MRM^3 Analysis. PSA was dosed into female human serum at 50 ng/mL and a significant reduction in background was observed.

MRM^3 for PSA Quantification

To assess first the intrinsic gain in specificity of detection of the MRM^3 method compared to the conventional MRM operating mode, a trypsin digest of a human female serum was spiked with trypsin hydrolyzed bacterial protein models TP171, TP574, TP435, core NS4, and human prostate specific antigen (PSA) over three decades of concentration, ranging from 0 to 1000 ng/mL. On average, moving from MRM to MRM^3 resulted in a 3- to 5-fold improvement of the limits of detection and quantification for the five model proteins[2]. Removal of background interferences / noise is illustrated for PSA in Figure 4. The standard concentration curve data from CoreNS4 protein illustrates the typical improvement (Figure 5). The reduction in background in the LC chromatograms of the MRM and MRM3 data can be observed in the top panes (Figure 5). The middle panes show the standard concentration curves using both acquisition techniques. The table in the lower pane shows the precision and accuracy for both concentration curves, highlighting the improvements in LLOQ achieved by the more selective MRM^3 experiment over the standard MRM.



	concentration (ng/mL)	10	50	100	500	1000
MRM^3	mean	11.3	47.0	92.4	516.7	992.7
	precision[a]	16.0	6.7	6.4	3.2	0.8
	accuracy[b]	13.2	−5.9	−7.6	3.3	−0.7
MRM	mean		54.7	101.1	511.3	994
	precision[a]		8.17	19.3	1.3	0.4
	accuracy[b]		9.4	1.1	2.3	−0.6

Figure 5. Improved Detection of ALESFWAK peptide from CoreNS4 in Human Plasma. MRM analysis yielded a quantitation limit (LLOQ) of 50 ng/mL and MRM^3 analysis results in a LLOQ of 10 ng/mL.

In order to reach the desired limit of quantitation of 4 ng/mL, we applied a previously optimized proteotypic peptide enrichment based on mixed-cation exchange solid phase extraction on a cartridge format just after trypsin hydrolysis of the crude sera2. This extraction phase could also be easily implemented online using an additional chromatographic module. This extraction step combined with MRM^3 analysis enabled the removal of the immune-depletion step from the sample preparation strategy.

Strong Correlation with ELISA Assays

The MRM^3 quantitation assay was performed on blank female serum and three male samples with known prostate cancer. The measured values of PSA obtained correlated well with the values obtained from the established ELISA assay (Figure 6)2. This clearly demonstrates the potential of the MRM^3 approach for the detection and accurate quantification of low-level protein biomarkers in body fluids

Conclusions

- Quantification of proteins in human serum at low ng/mL concentration levels has been demonstrated using high flow chromatography and LC-MRM^3 analysis.
- Critical detection levels of 4-10 ng/mL of circulating PSA were accurately determined in human serum using a robust two step sample preparation protocol combined with LC-MRM^3 analysis
- Improvements in speed and sensitivity to the QTRAP® 5500 system make MRM^3 analysis a robust quantitative strategy for peptides in complex matrices when significant background / interferences are present.

References

1. Fortin T et al. (2009) Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. *Mol. Cell. Prot.* **8**, 1006.
2. Fortin T et al. (2009) Multiple reaction monitoring cubed for protein quantitation at the low nanogram/milliliter level in nondepleted human serum. *Anal. Chem.* **15**, 9343.
3. MRM^3 Quantitation for Highest Specificity in Complex Matrices. SCIEX Technical Note, Publication 0920210-01

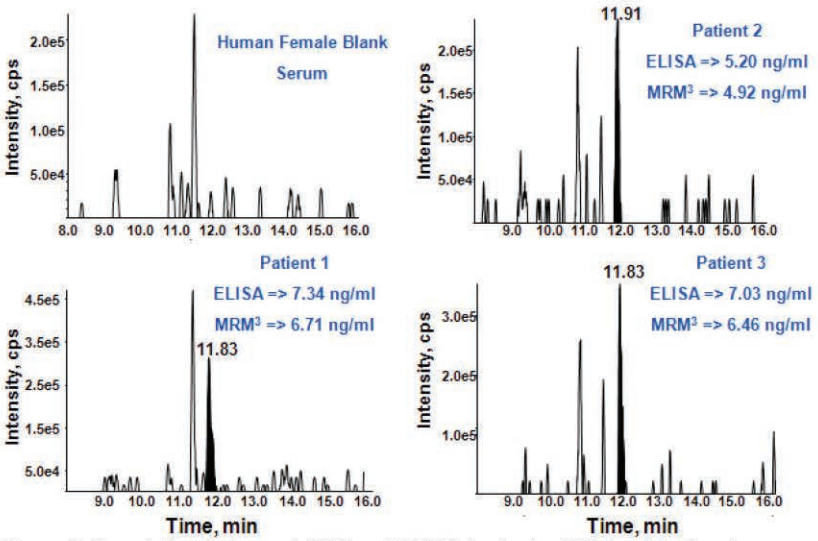


Figure 6. Correlation between LCMS and ELISA Analysis. PSA levels in female serum and three patients diagnosed with prostate cancer were analyzed with both LC-MRM3 and a clinically accepted ELISA assays. Both techniques produced very similar measurements for circulating PSA levels.

Rapid LC-MS/MS Analysis of Free Amino Acids in Extracellular Matrix

Quantitative, Fast, Sensitive and Robust Analysis of Free Amino Acids on the QTRAP® 6500+ System

Catherine S. Lane
SCIEX, Warrington, UK

Introduction

A rapid, robust and simple method is described for the quantitative measurement of 17 amino acids without prior derivatization. The method was applied to the analysis of cell supernatant from purified peripheral blood mononuclear cells (PBMCs). Unique identification of analytes, including isobaric species, was obtained using the QTRAP MIDAS™ functionality.

Experimental

Samples: Amino Acid standard solution (AAS18, Sigma), containing 2.5 µmol/ml each of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine, and 1.25 µmol/ml L-cystine. Sample was diluted for analysis in 0.1% formic acid in water. Cell supernatant from purified PBMCs (10,000,000 cells) was subjected to methanol precipitation, and the supernatant removed. Supernatant was diluted 1 in 50 in 0.1% formic acid in water for analysis.

Chromatography: MicroLC 200 system (Eksigent), with ACE 3 AQ column (HiChrom), 0.5 x 150 mm; flow rate 20 µl/min. Mobile phases: 0.1% formic acid in water (A), 0.1% formic acid in acetonitrile (B). Gradient: 2 to 20% B over 5 minutes, total analysis time 10 minutes. Injection volume 2 µl.

Mass Spectrometry: QTRAP® 6500+ LC-MS/MS system (SCIEX), with IonDrive™ Turbo V source equipped with Eksigent 50 µm i.d. electrode. Data acquired in MRM (Table 1) or MIDAS mode. Peak integration using MultiQuant™ software v3.0.2.

Table 1. Optimized MRM transitions for 17 free amino acids.

Amino Acid	Parent ion *m/z*	Fragment ion *m/z*	DP (V)	EP (V)	CE (V)	CXP (V)
Glycine	76.1	30	6	7.6	19	14
L-Alanine	90.1	44	6	4.5	17	6
L-Serine	106.1	60	6	10.5	15.5	7
L-Proline	116.1	70	20	13.5	21	10
L-Valine	118.1	55	11	13.5	27	8
L-Threonine	120.1	103.2	105	14.5	25	7
L-Leucine/Isoleucine	132.1	86	8	14.5	13	10
Isoleucine 2	132.1	69	8	14.5	23	8
L-Aspartic Acid	134.1	74	7	14.5	19	10
L-Lysine	147.1	84	16	13.5	23	10
L-Glutamic Acid	148.1	84	21	14.5	21	10
L-Methionine	150.2	104	6	12	15	12
L-Histidine	156.1	110	16	13	19	12
L-Phenylalanine	166.1	103	11	14	37	12
L-Arginine	175.2	70	40	11	27	8
L-Tyrosine	182.2	165.2	20	11	13	8
L-Cystine	241.2	152.1	25	14	19	10

Results

MRM extracted ion chromatograms for 17 amino acids are shown in Figure 1. The method was applied to the analysis of amino acids in PBMC cell supernatant (Figure 2). Using the MIDAS Workflow, full scan linear ion trap MS/MS data can be used to confirm the identity of target analytes, for example isobaric amino acids L-leucine and L-isoleucine (Figure 2).

MRM extracted ion chromatograms for four amino acids at concentrations close to their limits of detection (LODs), and corresponding linearity data, are shown in Figures 3 and 4, respectively. LODs were calculated for 17 amino acids, and their concentrations measured in diluted PBMC cell supernatant (Table 2).

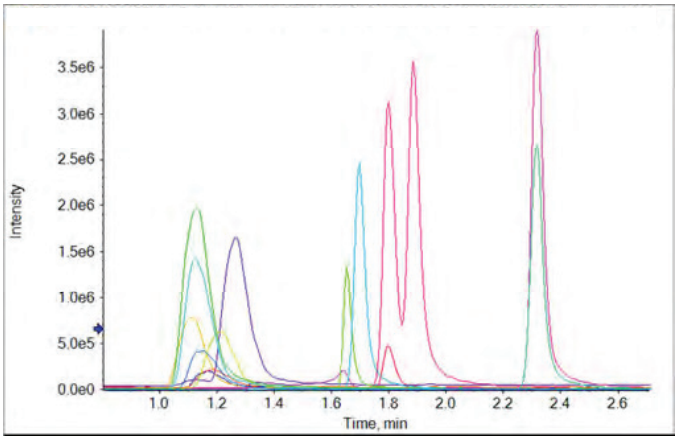


Figure 1. MRM Extracted ion chromatogram for 17 amino acids, each at 0.5 pmol on-column (except for cystine, 0.25 pmol on-column).

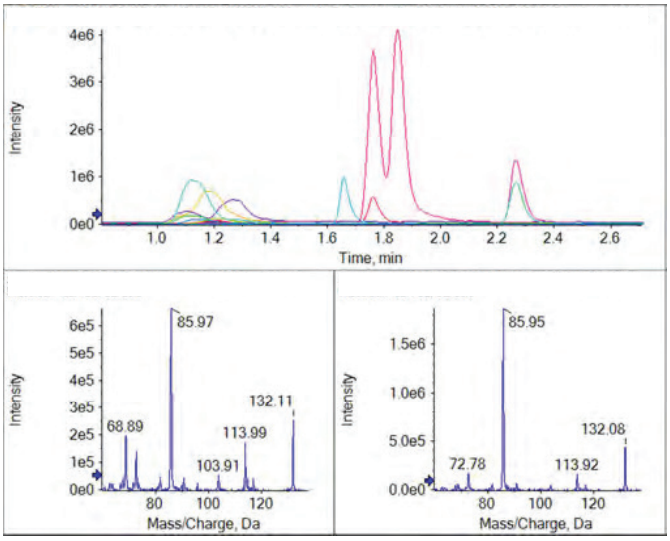
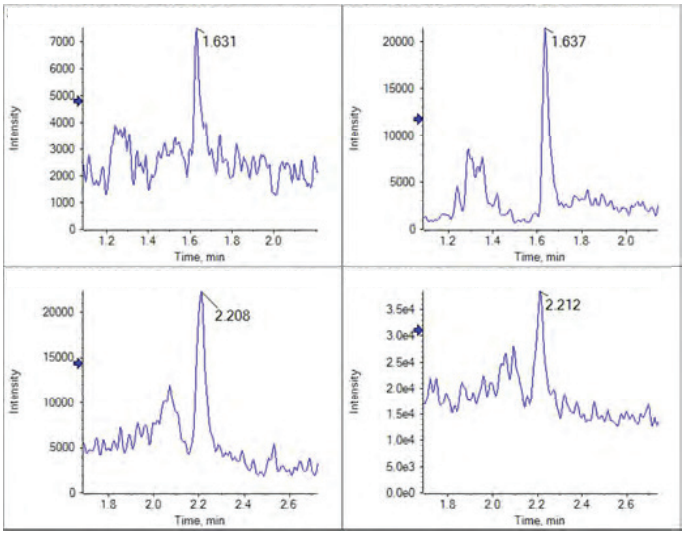


Figure 2. The MIDAS Workflow. Using the MIDAS Workflow, a set of MRM transitions are used as a survey scan to trigger the acquisition of high sensitivity linear ion trap MS/MS data. These data allow conclusive identification of analytes, including isobaric structures. Top panel, MRM extracted ion chromatogram for 17 amino acids in PBMC cell supernatant (diluted 1 in 50). Bottom panel, left to right, full scan linear ion trap MS/MS data generated at 1.75 min and 1.88 min, corresponding to isobaric amino acids L-isoleucine and L-leucine. The L-isoleucine diagnostic fragment ion at *m/z* 69 can be observed in the MS/MS spectrum on the left.



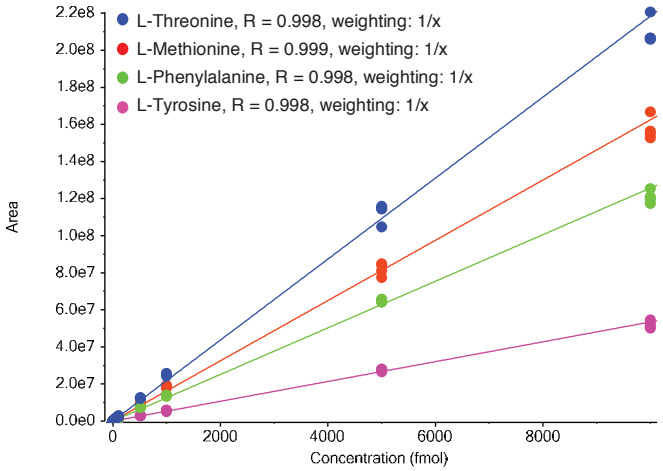


Figure 4. Linearity for L-threonine, L-methionine, L-phenylalanine and L-tyrosine over 4 orders of magnitude linear dynamic range (1 fmol on-column to 10 pmol on-column).

Table 2. Limits of detection for 17 free amino acids and their concentrations measured in diluted PBMC cell supernatant (1 in 50).

Amino Acid	LOD (fmol)	Mean conc. (n=3) in diluted PBMC cell supernatant (fmol/µl)	Peak Area %CV	Calculated conc. in undiluted PBMC cell supernatant (pmol/µl)
Glycine	<1000	<LOD	-	-
L-Alanine	<1000	<LOD	-	-
L-Serine	50	21	7.7	1.0
L-Proline	2.5	96	4.7	4.8
L-Valine	25	105	6.1	5.2
L-Threonine	1	97	7.3	4.9
L-Leucine	2.5	338	3.2	17
Isoleucine	2.5	329	1.5	16
L-Aspartic Acid	10	26	4.9	1.3
L-Lysine	5	305	2.2	15
L-Glutamic Acid	5	55	4.8	2.8
L-Methionine	1	3	6.2	0.17
L-Histidine	5	23	4.0	1.1
L-Phenylalanine	1	100	5.1	5.0
L-Arginine	2.5	220	5.4	11
L-Tyrosine	1	97	1.4	4.8
L-Cystine	1.25	36	6.8	1.8

Conclusions

The method described here allows rapid, robust and sensitive quantitation of 17 amino acids without derivatization. Use of the MIDAS functionality, unique to QTRAP systems, allows unambiguous analyte identification. The method has been successfully applied to the quantitation of amino acids in a sample of PBMC cell supernatant.

Drug discovery using QTRAP technology

Quantification of the Therapeutic Peptide Exenatide in Human Plasma

MRM³ Quantitation for Highest Selectivity in Complex Mixtures on the SCIEX QTRAP® 5500 System

Yan Xu, John Paul Gutierrez, Tian-Sheng Lu, Haiqing Ding, Katie Piening, Erin Goodin, Xiuying Chen, Kristin Miller, Yong-Xi Li
Medpace Bioanalytical Laboratories, Cincinnati, OH

With increasing focus on biotherapeutics, there is greater interest in using LCMS for the quantitative analysis of proteins and peptides in pharmaceutical research. Exenatide is a large therapeutic peptide that has been approved for the treatment of Diabetes mellitus type 1 and 2. This peptide enhances glucose-dependent insulin secretion by the pancreatic beta-cell, acting as a regulator of glucose metabolism and insulin secretion.

In recent years, the plasma concentrations of exenatide were measured by ligand-binding assays, such as immunoenzymetric assays used for pharmacokinetic studies. However, there are specificity and selectivity risks with these types of analysis since certain compounds may have similar physiochemical properties. For this reason, an MRM³ LCMS strategy[1] that will ensure higher selectivity in peptide detection in plasma was evaluated using the SCIEX QTRAP 5500 System.

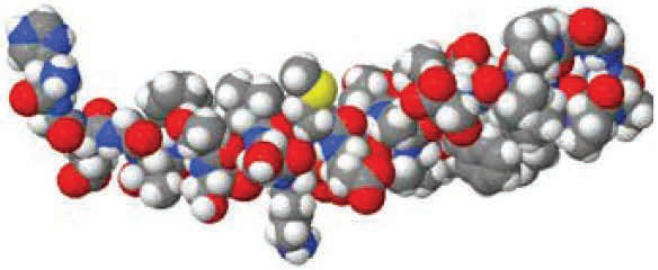


Figure 1. Structure of Exenatide. Exenatide is a large 39 amino acid peptide (MW = 4186.6 Da) that acts as a regulator of glucose metabolism and insulin secretion.

Key Features of MRM³ for Quantifying Large Therapeutic Peptides

- Because of the multiple fragmentation steps in MRM3, higher selectivity is achieved.
- Improvements to the QTRAP 5500 systems has enabled faster and more sensitivity MRM³ analysis
- Detection limits in very complex matrices can often be improved using MRM³ analysis by removing interferences at the low end of the concentration curve.
- Unlike MS³ on traditional ion traps, the unique hybrid triple quadrupole – linear ion trap design allows Q1 to be used for precursor ion selection (unit resolution), and Q2 for the first fragmentation step in a transmission mode. This allows higher speed, greater selectivity, and more flexibility in the choice of the first product ion since there is no low mass cut-off associated with the first fragmentation step in Q2, and higher collision energies can be used.

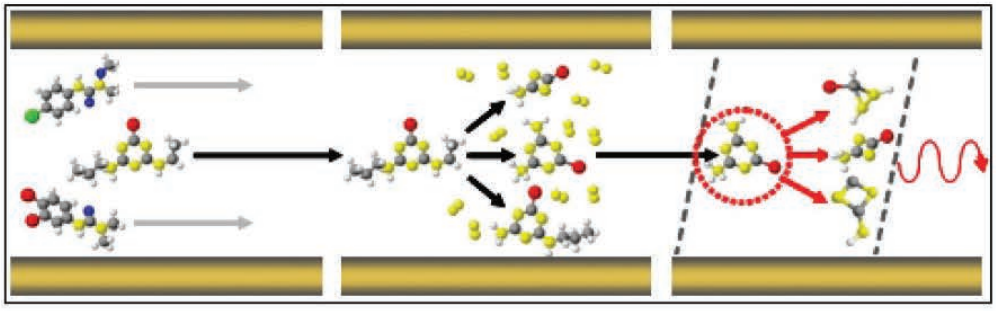


Figure 2. MRM³ for Quantitative Analysis by LCMS. Parent ion is first selected in the Q1 quadrupole, then fragmented in Q2 collision cell. Product ions are trapped then isolated in the linear ion trap, followed by excitation to perform the second fragmentation step. 2nd generation product ions are scanned out to the detector.

Methods

Sample Preparation: Exenatide was extracted from human plasma, dried in a TurboVap under nitrogen and reconstituted. In all steps, pH values and organic phase were carefully controlled.

Liquid Chromatography:

- Shimadzu UFLC LC-20ACXR
- Reverse phase C-18 2.0 x 30 mm, 5 µ
- Flow rate: 0.6 mL / min.
- Injection volume: 5 µL
- Mobile Phase A
  - 0.1% Formic acid in water
- Mobile Phase B
  - 0.1% Formic acid in Methanol
- Gradient 2 – 95% B in 5 minutes

Mass Spectrometry: LC-MS analysis was done on the SCIEX QTRAP® 5500 system using the MRM^3 acquisition strategy[1]. Using the MS scan type, the trap was filled using Dynamic Fill Time (DFT) and the instrument was scanned at 10,000 Da/sec. The trap excitation time was 25 ms, giving a total cycle time of 0.17 sec. The transition ions used for the MRM^3 analysis were 838 → 396 → 202.

Assay Development Results

In Enhanced MS (EMS) mode, the multiple charged parent ion $[M+5H]^{5+}$ at m/z 838.3 was selected as the first precursor (Figure 2, top). When this charge state is fragmented, the predominant product ion m/z 396.4 was chosen as the second precursor (Enhanced Product ion (EPI) scan, Figure 3, middle). The m/z 396.4 was fragmented in LIT to generate MS3 spectrum (Figure 3, bottom). The major fragment ion m/z 202.2 was selected as the second generation product ion for the MS^3 quantification. The principle of MRM^3 analysis as performed on the QTRAP 5500 system is demonstrated in Figure 1.

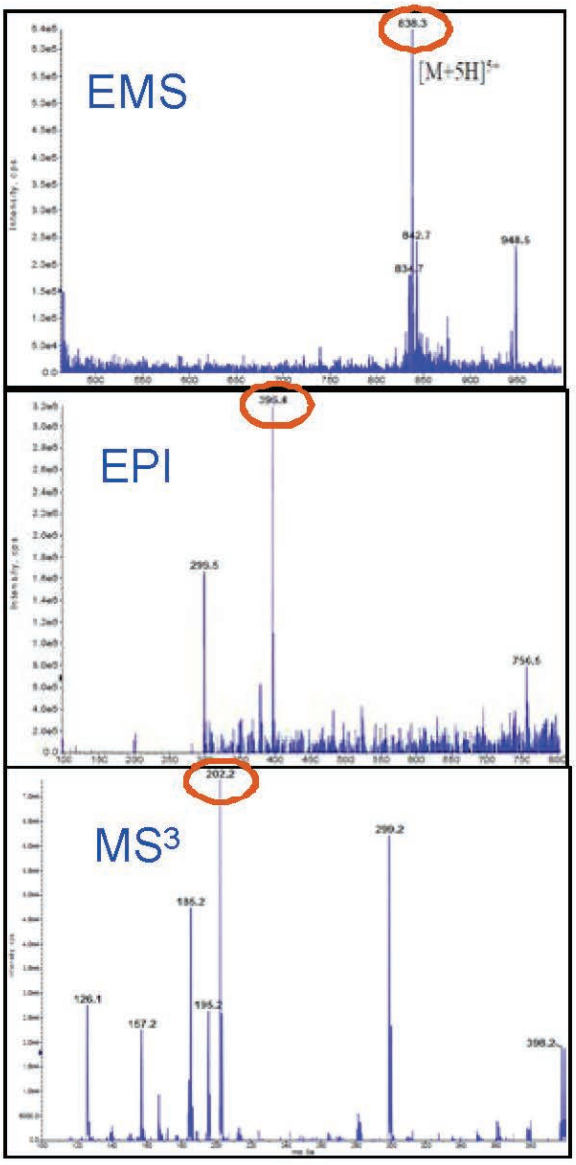


Figure 3. MRM^3 assay design. EMS scan (top) is used to select the dominant parent ion, the most intense fragment ions are identified using the EPI mode (middle), and MS^3 fragmentation is used to select the best secondary fragments to extract.

Assay Performance for Exenatide

Use of MRM^3 analysis resulted in significantly improved selectivity of detection for exenatide in human plasma extracts. Figure 4 shows a comparison of MRM^3 vs. traditional MRM quantitation. Baseline was lower and chromatographic interference from the plasma matrix was completely eliminated in MRM^3. The fast scanning speed of the QTRAP® 5500 system (10 000 Da/sec) provided a sufficient number of data points across the analyte chromatographic peak for good reproducibility of quantitation.

The improved detection performance resulted in excellent assay performance at the LLOQ and four QC levels as shown in Table 1. Accuracy and %CV for six replicates demonstrate that the MRM^3 approach is capable of quantitative performance suitable for development-grade bioanalytical assays.

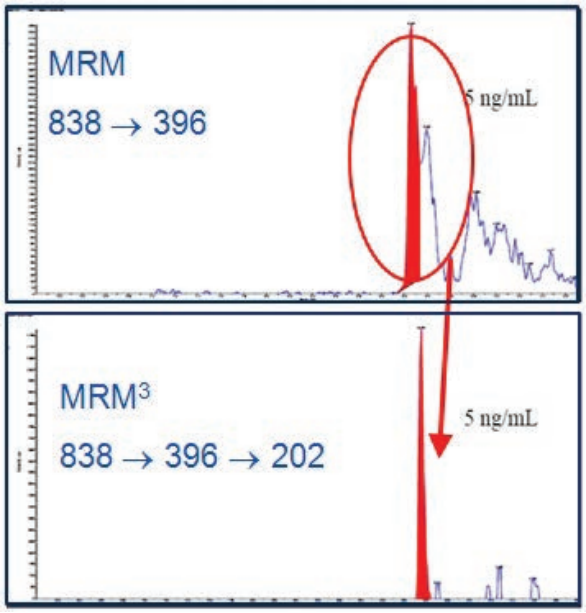


Figure 4. MRM^3 Significantly Improved Selectivity of Detection in Plasma. Elimination of chromatographic interferences and background noise improves the LOQ for exenatide in plasma.

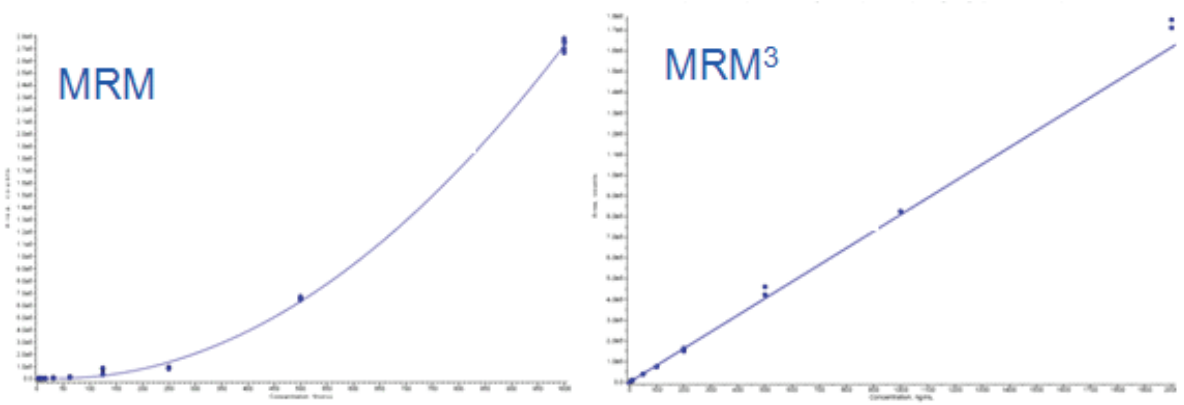


Figure 5. Calibration Curves for Exenatide in Human Plasma using MRM and MRM^3 Analysis. (Left) MRM from 250-1000 ng/mL and (Right) MRM^3 from 5 – 1000 ng/mL showing significantly better linearity (R2 = 0.996).

Conclusions

- A bioanalytical assay for Exenatide in human plasma was successfully developed using MRM^3 analysis.
- The increased selectivity of MRM^3 allowed for the elimination of baseline noise and chromatographic interference, resulting in superior analytical performance compared to traditional MRM.
- MRM^3 demonstrated the potential for excellent linearity achieving a calibration range of 5-2000 ng/mL, compared with a range of less than 250-1000 ng/mL for traditional MRM with this analyte.
- Accuracy and reproducibility of the MRM^3 assay was compatible with the requirements for a development stage bioanalytical assay.

Table 1. Accuracy and Reproducibility of the MRM^3 assay. The reproducibility achieved on the quality control samples demonstrates that MRM^3 analysis is compatible with validated bioanalysis.

	LLOQ	LQC	MQC	MHQC	HQC
Conc (ng/mL)	5.00	15.0	50.0	800	1800
	5.14	15.9	46.4	779	1650
	4.32	16.9	47.2	767	1549
	5.65	12.0	41.7	821	1521
	4.54	13.5	43.7	729	1641
	3.69	17.1	50.0	658	1745
	4.22	17.4	45.3	751	1672
Mean	4.59	15.5	45.7	751	1630
SD	0.701	2.22	2.85	54.8	82.4
CV	15.3%	14.3%	6.2%	7.3%	5.1%
RE	-8.2%	3.2%	-8.6%	-6.1%	-9.5%

References

1. MRM^3 Quantitation for Highest Selectivity in Complex Matrices. SCIEX Technical Note, Publication 0920210-01.


push
ExionLC
SCIEX
ExionLC
SCIEX
ExionLC
SCIEX
ExionLC

Your Success is Our Success

We take it personally

As a SCIEX customer you have access to an excellent customer support organization. Wherever you are, we're there with you as a trusted partner to answer questions, provide solutions, and maximize lab productivity.

Our customer support organization has access to the latest product updates, software revisions, methods and repair procedures to make sure that you stay on top of your game.

When you have questions, we have answers.

Learn more at sciex.com/customersupport, or locate your local account representative at sciex.com/contactus

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices