Current Trends in MASS Spectrometry

SUPPLEMENT TO
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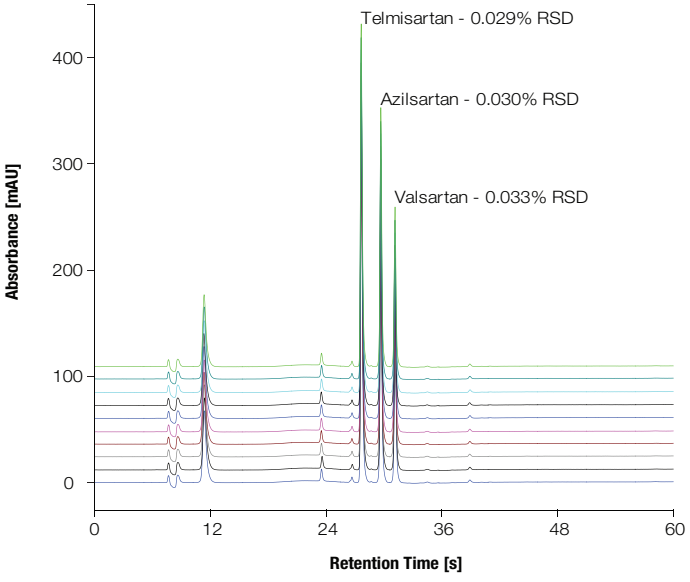

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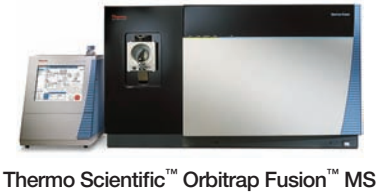

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Rapid Determination of 24 Synthetic and Natural Cannabinoids for LC–MS-MS Screening in Natural Products and Drug Inspection Applications

Marijuana, the common or slang term for cannabis in its herbal form, is one of the most widely used illicit drugs in the world. Synthetic cannabinoids have similar psychotropic effects to the natural ones and are rapidly being integrated by the illicit market. To deal with their expanding number and diversity, a targeted and untargeted liquid chromatography–tandem mass spectrometry (LC–MS-MS) screening method was developed, allowing for the simultaneous analysis of 24 synthetic and natural cannabinoids in 8 min for a wide variety of samples such as herbal smoking mixtures, incense sticks, serums, and cannabis plant material. The particular advantage of this LC–MS-MS method is that the full scan event in the MS acquisition provides accurate masses for all detected species and thus allows postanalysis identification of initially untargeted compounds.

Philippe Lebel, Karen C. Waldron, and Alexandra Furtos

The primary psychoactive compound in cannabis is Δ^9-tetrahydrocannabinol (THC), which exerts its activity by interacting with the CB1 and CB2 cannabinoid receptors in the brain. Synthetic analogs of THC, when smoked or orally ingested, can mimic the psychotropic effects of cannabis by binding the same receptors (1). Cannabimimetic compounds can be found in herbal mixtures (incense stick, cigarette, Spice, K2), serums, and other matrices, often with no indication of their presence. The European monitoring center for drugs and drug addiction reported in 2009 (2) that spice products (3) were generally used by teenagers and young adults because it enabled them to pass drug-screening tests. Many governments have taken legal action to control specific synthetic cannabinoids. In the United States, they have been listed as Schedule I drugs on the Controlled Substances Act, and in Canada as Schedule II drugs. However, small structural modifications from the controlled substance result in new analogs being legal. Therefore, in May 2012 the United States amended the Act and proposed a bill to place all cannabimimetic agents as Schedule I drugs (4,5).

Given the structural similarities between THC and its synthetic analogs, identification of the compounds in seized samples is a continuous challenge for government agencies. As quickly as analogs are added to controlled substance lists, new ones are synthesized, making their monitoring a moving target (6–9). These new generation analogs are obviously not present in mass spectrometry (MS) or UV spectra libraries; therefore, routine methods for screening cannabimimetics in both products and biofluids rapidly become less effective.

The most common methods of cannabinoid analysis use gas chromatography (GC)–MS (10,11) and liquid chromatography (LC)–MS (9,12–14). GC–MS approaches use MS spectra of known cannabinoids for identification, and most LC–MS approaches are used in multiple reaction monitoring (MRM) mode. Both are targeted methods that are limited to screening known species and are therefore always one step behind in the monitoring of this dynamic designer drug

Table I: Spectra with associated structures searched with NIST search software

Standard Name (Molecular Formula)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)
AKB48 *N*-(5-fluoropentyl) analog ($C_{23}H_{30}FN_3O$)	384.245	134.97	Not applicable
AM-694 ($C_{20}H_{19}FINO$)	436.057	**230.93**, 309.25, 234.11	202.99, 93.96
AM-694 3-iodo isomer ($C_{20}H_{19}FINO$)	436.057	**230.95**, 202.96	202.96, 220.89, 94.06
AM-1220 ($C_{26}H_{26}N_{2O}$)	383.212	**286.20**, 154.99, 112.00	154.96, 157.97, 127.00
AM-2201 ($C_{24}H_{22}FNO$)	360.176	155.00, **232.12**, 126.92	144.05, 212.11, 176.16
AM-2201 2′-naphthyl isomer ($C_{24}H_{22}FNO$)	360.176	154.98, **232.1**8, 126.87	143.93, 176.28
AM-2201 *N*-(4-fluoropentyl) isomer ($C_{24}H_{22}FNO$)	360.176	155.01, **232.15**, 340.27	212.12, 144.05, 176.01
CP 47,497 – H_2O ($C_{21}H_{32}O$)	301.253	175.07, **261.23**	233.21
(±)-CP 47,497-C8-homolog – H_2O ($C_{22}H_{34}O$)	315.268	175.11, 241.23	Not applicable
(±)-epi CP 47,497-C8-homolog – H_2O ($C_{21}H_{32}O$)	301.253	175.06, 261.22	Not applicable
(±) -3-epi CP 47,497-C8-homolog – H_2O ($C_{22}H_{34}O$)	315.268	175.11, 241.23	Not applicable
JWH-018 ($C_{24}H_{23}NO$)	342.185	154.89, **214.08**, 126.92	143.89, 157.98
JWH-019 ($C_{25}H_{25}NO$)	356.201	154.91, **228.10**, 126.94	143.92, 157.93,129.96
JWH-073 ($C_{23}H_{21}NO$)	328.17	154.92, **200.05**, 126.91	143.92, 158.04
JWH-081 ($C_{25}H_{25}NO_2$)	372.196	185.06, **214.12**, 157.06	144.04, 158.06, 200.07
JWH-122 ($C_{25}H_{25}NO$)	356.201	168.98, **214.0**7, 140.95	143.88, 158.00
JWH-200 ($C_{25}H_{24}N_2O_2$)	385.191	155.05, **298.12**, 127.05	268.11, 283.10, 200.08
JWH-210 ($C_{26}H_{27}NO$)	370.217	182.99, **214.09**, 155.00	143.93, 158.01, 116.01
JWH-250 ($C_{22}H_{25}NO_2$)	336.196	120.91, **188.06**, 131.87	131.99, 117.91
STS-135 ($C_{24}H_{31}FN_2O$)	383.249	**232.12**, 135.02, 206.12	143.94, 212.13, 158.07
Cannabidiol (CBD) ($C_{21}H_{30}O_2$)	315.232	**259.21**, 193.06, 233.15	231.12, 217.06, 189.06
Cannabigerol (CBG) ($C_{21}H_{32}O_2$)	317.248	**193.10**, 207.09, 261.24	122.90, 136.89
Cannabinol (CBN) ($C_{21}H_{26}O_2$)	311.201	293.19, **223.10**, 241.12	195.03, 208.02, 205.04
Δ^9-Tetrahydrocannabinol (Δ-9-THC) ($C_{21}H_{30}O_2$)	315.232	**259.18**, 193.07, 233.13	231.08, 217.16, 189.00

market. To address this shortcoming, we report a targeted–untargeted high-resolution MS approach to screen a variety of samples for cannabinoid-like compounds. The current method includes a high-resolution, nontarget scan that allows identification of all species if present in the sample and targets a selection of 20 synthetic and four natural cannabinoids (Figure 1).

Experimental

Reagents and Standards

LC–MS-grade methanol and acetonitrile were from J.T. Baker (TekniScience) and LC–MS-grade formic acid (98%) was from

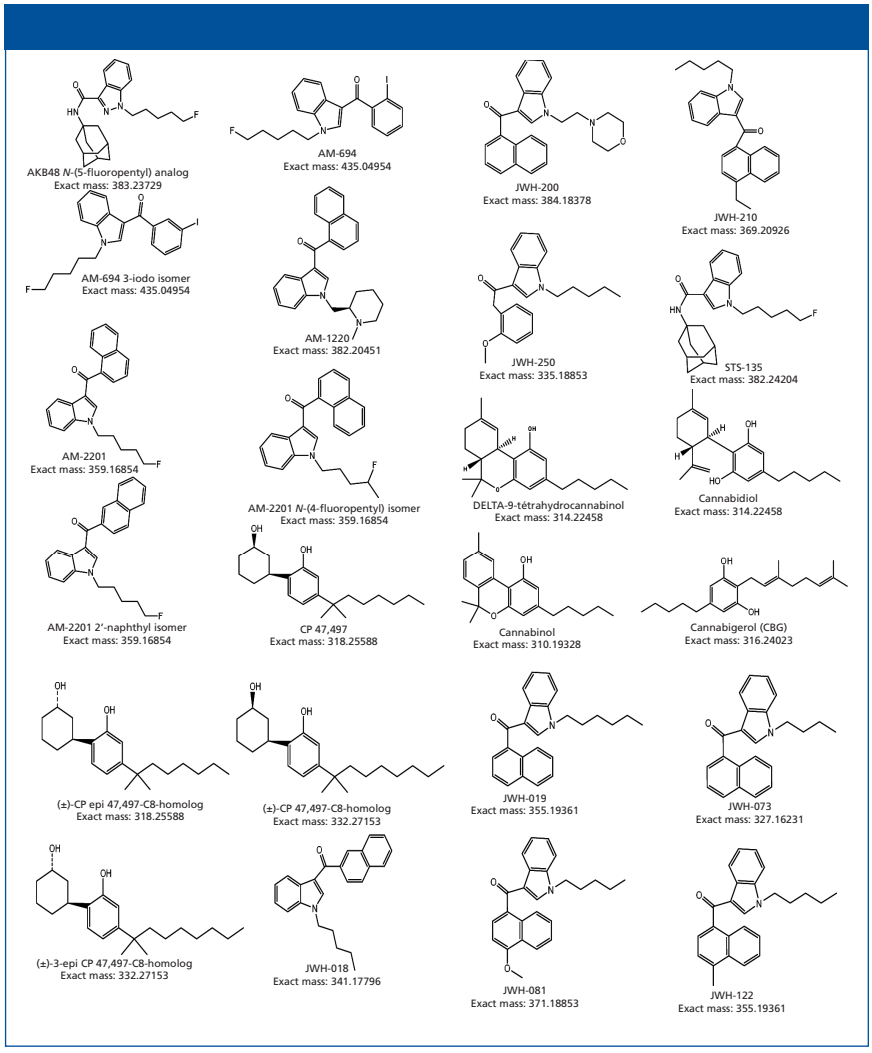

Figure 1: Chemical structures and accurate masses of the 24 synthetic and natural cannabinoids investigated.

Fluka (Sigma-Aldrich). High performance liquid chromatography (HPLC)-grade water from a Milli-Q Reference A+ system (Fisher Scientific) was used to prepare all aqueous solutions and mobile phases.

A total of 24 standards were investigated. JWH-018 and JWH-210 were obtained from Toronto Research Chemicals Inc. AKB48 *N*-(5-fluoropentyl) analog, AM-694, AM-694 3-iodo isomer, AM-1220, AM-2201, AM-2201 2′-naphthyl isomer, AM-2201 *N*-(4-fluoropentyl) isomer, CP 47,497, (±)-CP 47,497-C8-homolog, (±)-epi CP 47,497-C8-homolog, (±) 3-epi CP 47,497-C8-homolog, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-250, and STS-135 were obtained from Cayman Chemical. Δ^9-Tetrahydrocannabinol and cannabinol were purchased from Alltech/Grace. Cannabidiol was purchased from Lipomed and cannabigerol was purchased from THC PHARM.

Stock solutions of individual standards were prepared separately in 10-mL volumetric flasks at an approximate concentration of 100 µg/mL in methanol. Diluted stock solutions (100 ng/mL to 1 µg/mL) were directly infused into the mass spectrometer for adjustment of the experimental parameters for each analyte. A standard mixture of the 24 components was also prepared and injected to adjust the chromatographic separation of all analogs.

Sample Preparation

To determine the effectiveness and robustness of the LC–MS-MS method, 10 seized samples and a cannabis sample were analyzed. The samples were present in two forms: tablets and herbal products (incense stick, cigarette, and a cannabis plant). They were finely ground, then aliquots of 5–10 mg of the resulting powder were transferred to 10-mL volumetric flasks and dissolved in 70:20:10 methanol–water–acetonitrile containing 1% formic acid. Following this, the solutions were vortexed for 2 min, sonicated for 10 min, and vortexed again for 3 min. The supernatant was filtered through a 0.45-µm pore polytetrafluoroethylene (PTFE) syringe filter (Phenomenex). Herbal samples required an additional centrifugation step at 3500 rpm for 10 min to avoid mass overloading of the syringe filter. Filtrates were diluted 10–100-fold in 80:20 water–acetonitrile, the initial mobile phase, before injection.

LC–MS-MS Operating Conditions

Data were acquired on an LTQ Orbitrap XL mass spectrometer coupled to an Acella HPLC system (Thermo Scientific). Xcalibur 2.1 and Thermo LTQ Tune Plus 2.5.5 software (Thermo Scientific) were used to control the system and process the data. External mass calibration was used throughout the project. Four analytical columns were initially tested for their chromatographic performance: 100 mm × 2.1 mm, 3.5-µm d_p XTerra C18 and 100 mm × 2.1 mm, 1.7-µm d_p Acquity BEH C18 columns, both from Waters; a 75 mm × 2.1 mm, 2.6-µm d_p Kinetex C18 column from Phenomenex; and a 100 mm × 2.1 mm, 2.6-µm d_p Accucore aQ C18 column from Thermo Scientific. Two eluent systems were tested during method development, water–methanol and water–acetonitrile, both containing 0.1% formic acid, under generic gradient conditions (5–95% organic).

Optimized separations were carried out using the Accucore aQ column coupled to a 4 mm × 2.0 mm Phenomenex C18 guard column, both maintained at 40 °C, and the water–acetonitrile gradient. The autosampler temperature was set at 10 °C to avoid sample degradation. Eluents consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B), and the initial mobile phase contained 20% B. The following gradient elution was applied at a flow rate of 350 µL/min: 20–58% B over 1 min, held at 58% B for 1 min, increased to 85% B over 1 min, then held at 85% B for 2 min. Eluent B was then returned to 20% B over 0.2 min. The system was allowed to reequilibrate for 2.8 min, giving a total cycle time of 8.0 min. The injection volume was 3–5 µL. A needle wash

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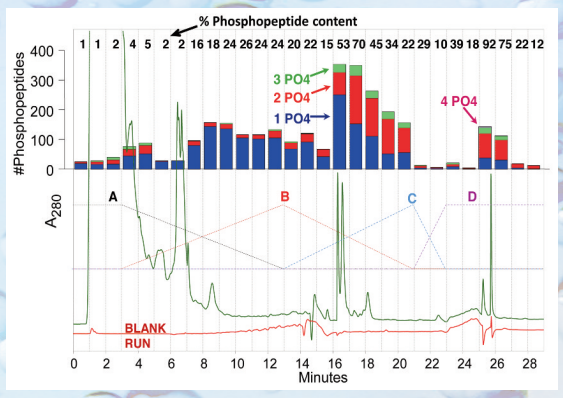

Sample: Tryptic Digest
Column: ERLIC-WAX microSPE p/n: SEM HIL-DE
Load in 85% ACN, 0.1% FA. Elute 10% ACN pH 2, 100mM Na-methylphosphonate
Desalt on MACROspin TARGA® C18 p/n: SMM SS18R
Analytical column: PolyWAX LP™ 4.6 x 100mm, 5µm, 300Å, p/n: P104WX0503

DISPLACEMENT CHROMATOGRAPHY VS. SELECTIVE ELUTION

MASS RELATIVE TO COLUMN SIZE
Excess capacity; everything retained. Separation through selective elution.

VS. +3

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EXAMPLE: ERLIC-CAT chromatography

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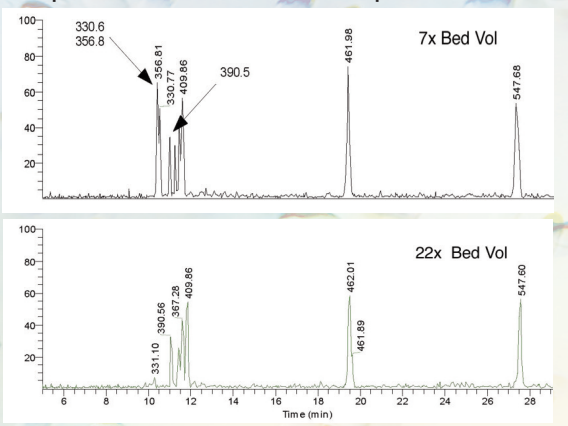

Column: TARGA® C18, 300µm x 30mm Trap
Sample Solvent: 0.1% FA water (volumes as shown). Trap Column in 0.1% FA water
Gradient: 98% water, 0.1% FA, 2% ACN to ACN, 0.1% FA

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Product Formats	Sample Capacity	Packed Volume	Void Volume	Elution Volume
UltraMicroSpin™ Column	3-30µg	25µL	12µL	2-25µL
MicroSpin™/TIP Column	5-60µg	50µL	25µL	5-50µL
MacroSpin™ Column	30-300µg	180µL	90µL	25-180µL
96-Well Spin Plate	10-100µg	75µL	35µL	7-75µL
96-Well MACROspin Plate	25-250µg	200µL	100µL	25-200µL
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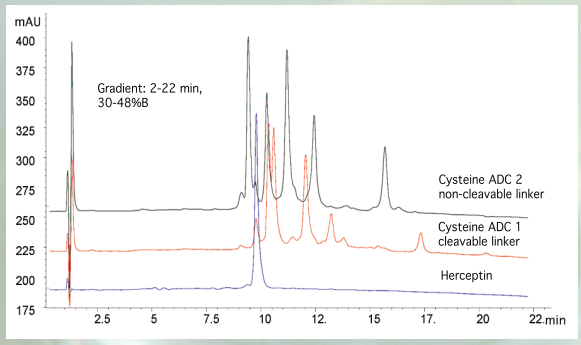
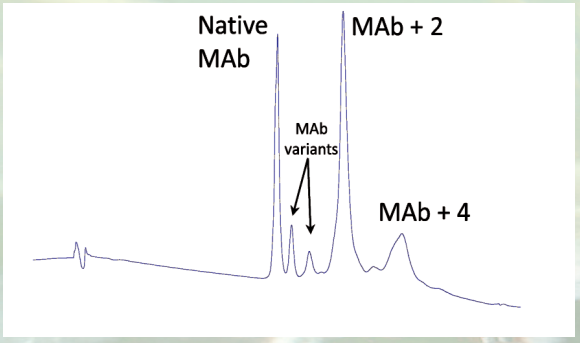


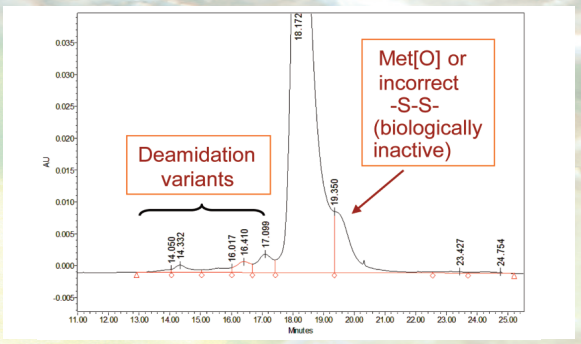
PolyRP™ 1000, 5µm, 1000Å, 4.6 x 100mm
p/n: T 260950-4610
Gradient:
A: 0.1% TFA; B: 0.1% TFA, ACN; 80°C

Native Conjugates by HIC



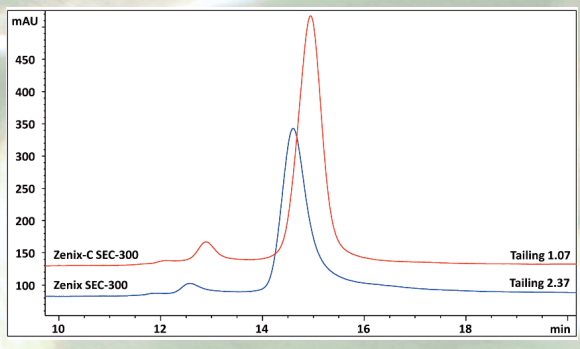
PolyPROPYL A™, 3µm, 1500Å, 4.6 x 100mm
p/n: P104PR0315
Segmented Gradient:
A: 0.9M $NaSO_4$; B: 50mM $NaPO_4$, pH 6

Deamidation by IEX



PolyCAT A®, 3µm, 1500Å, 4.6 x 100mm
p/n: P104CT0315
Gradient:
30-145mM NH_4OAc, pH 4, 40% ACN; 30°C

Aggregates by SEC



Zenix™ SEC 300 & Zenix™-C SEC-300
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Isocratic: 50mM NH_4OAc : ACN 90:10 (v/v)

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step using 70:20:10 methanol–water–acetonitrile was included in the method. A 5-µL blank, consisting of the initial mobile phase, was injected after each sample to monitor and reduce any potential carryover.

The electrospray interface was operated in positive ion mode. Nitrogen was used as both sheath gas and auxiliary gas while helium was used as collision gas. Using direct infusion, instrumental parameters were adjusted semiautomatically for every analyte using the tune tool in the LTQ Tune Plus software. The parent ions of all analytes showed similar behavior due to their similar structures. After screening every compound individually, the experimental parameters of the full scan event that were found to be suitable for all analytes were set to the following values: sheath and auxiliary gas at flow rates of 44 and 17 (instrument units), respectively; spray voltage, +3500 V; capillary temperature, 310 °C; capillary voltage, 28 V; tube lens, 101. The MS^2 and MS^3 transitions for every compound were also determined using the tune tool by varying the normalized collision energy. They ranged between 25% and 33% after they were optimized. Therefore, a three-step collision energy function set at 25%, 30%, and 35% was used to perform average fragmentation on every compound. The ion transitions MS^2 and MS^3 for each standard are shown in Table I, where the MS^3 transitions arise from the MS^2 value shown in boldface type. Mass spectra were acquired from *m/z* 50 to 1000 using two scan events: the first was a Fourier transform (FT)-MS full scan for accurate mass detection and the second was a data dependent step with MS-MS acquired only for precursors from the parent mass list with a dynamic exclusion of 10 s. Every standard was then injected onto the column individually to determine its retention time and confirm the parent ion accurate mass, MS^2 and MS^3. To determine if there were any interactions between the compounds, a mixture of the 24 standards was injected.

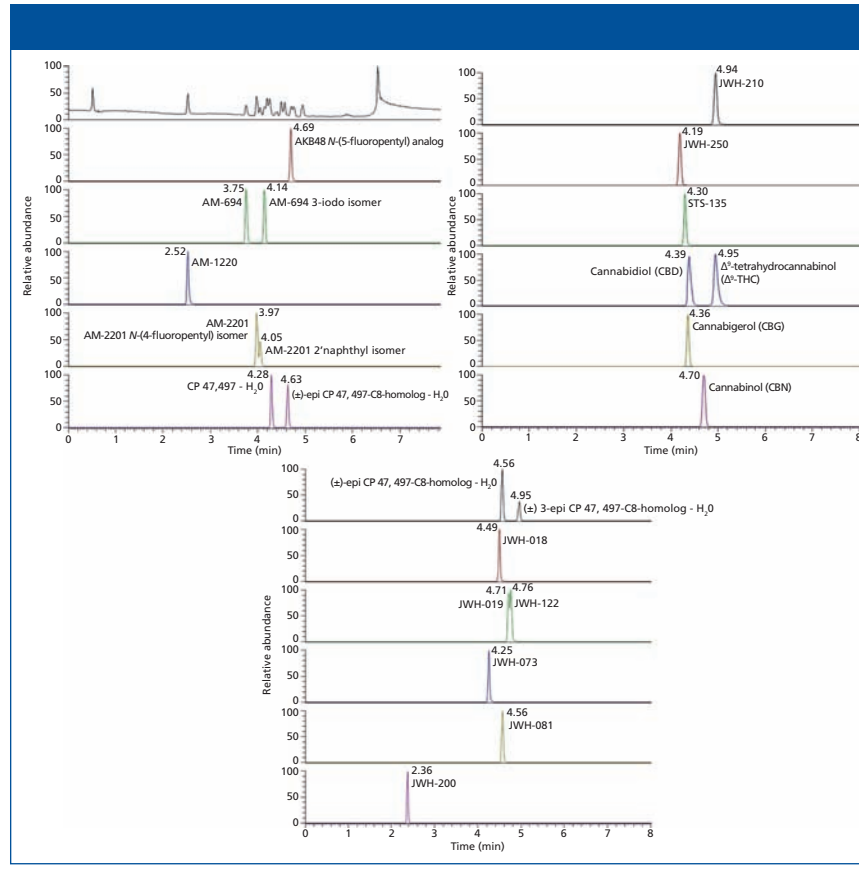

Figure 2: Typical chromatograms obtained by LC–MS-MS in positive electrospray mode for a mixture containing all 24 analytes. The upper trace of the first panel represents the total ion chromatogram (TIC), followed by the extracted ion chromatograms of all the compounds spread over three panels for ease of presentation (parent ions shown).

Results and Discussion

Method Development and Validation

As seen in Figure 1, many of the 24 compounds have similar structures, which makes the chromatographic separation challenging. Between the mobile phases tested, the acetonitrile gradient gave better selectivity. Between the columns tested, the Accucore aQ gave the best selectivity and retention of early eluted analytes.

Selectivity was further enhanced by coupling the optimized chromatographic method to the high-mass-resolution orbital ion trap mass spectrometer. By individual direct infusion of the standards, the final optimized parameters for full-scan detection (values presented in the LC–MS-MS operating conditions section) were selected to provide appropriate sensitivity for all 24 components in a single analysis. The mass accuracy across the whole study was within 3 ppm.

When operating the orbital ion trap MS system at a resolution of 30,000, approximately 1.5 scans (points) per second were obtained for the mixture, containing 24 standards analyzed simultaneously. This acquisition scan speed was well suited for the narrow peaks (5–8 s wide) produced by the core–shell particle chromatography and allowed a very reliable quantification and improved method sensitivity. The well selected parent-fragment transitions helped to enhance the method selectivity. As seen in Figure 2 (spread across 3 panels for ease of peak identification), it was possible to positively identify 24 cannabinoids by differentiating them with a combination of accurate mass, retention time, and fragmentation pattern (MS^2). For example, AM-2201 and AM-2201 *N*-(4-fluoropentyl) have the same accurate mass and retention time and very similar fragmentation patterns, as shown in Figure 3. However, a signal at *m/z* 340, generated by the loss of fluorine from a secondary carbon, is only present in the MS^2 of AM-2201 *N*-(4-fluoropentyl) and allows distinction between the two. Similarly, JWH-019 and JWH-122 are eluted within 0.05 min of each other and have the same accurate mass. Without reinjecting, they could be identified by their completely different MS^2 fragmentation patterns (Figure 4). The MS^3 data were acquired but not actually needed for the screening method. In the eventuality of samples with a new analog of identical parent ion mass, retention time, and MS^2, the MS^3 data are available

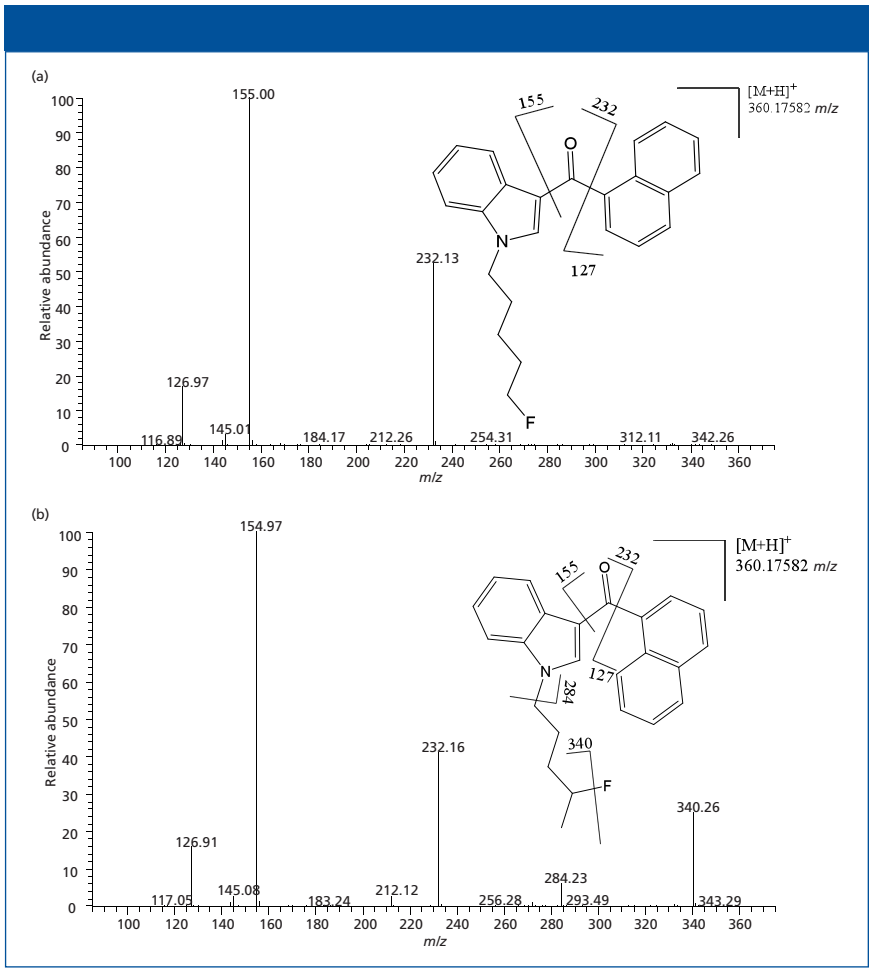

Figure 3: Fragmentation pattern of two isomers: (a) AM-2201 and (b) AM-2201 *N*-(4-fluoropentyl) isomer.

for identification.

Figure 2 shows all compounds eluted between 2.36 and 4.94 min. Adulterated natural remedies can contain new synthetic drug substances of unknown polarity. It is therefore necessary to use caution when developing fast chromatographic separations; if additional yet-to-be characterized synthetic cannabinoids are present, excessive coelution could compromise their postacquisition identification using the untargeted full scan event in the acquisition method.

Matrix effects were evaluated in four samples (tablet and herbal based) spiked with the 24 analytes before extraction. A comparison of the chromatograms to that of the standard mixture revealed no difference in peak areas for the 24 analytes. To evaluate carryover, blanks injected after samples were used. No carryover was detected at analyte concentrations below 50 ng/mL whereas most compounds presented 0.1–0.3% carryover at 250 ng/mL and 0.4–1.0% at 1000 ng/mL. Therefore, we suggest that two blank injections be used between samples at high concentrations. The linearity of 15 compounds, selected to be representative of the 24, spiked into an herbal matrix, was evaluated across a concentration range of 0.01–1000 ng/mL ($n = 21$). Linear regression was applied by plotting the peak area responses of each compound as a function of its respective concentration and applying a $1/x$ weighting factor. Calibration curve correlation coefficients (r^2) ranged between 0.996 and 0.999. Detection limits (calculated at signal-to-noise ratio of 3) were between 0.03 ng/mL and 0.61 ng/mL for all but one compound regardless of the presence of matrix; the limit of detection (LOD) for (±) 3-epi-CP 47,497-C8-homolog was 1.52 ng/mL.

It was important to develop a simple and economical extraction method that was also fast and effective with good recovery to complement the rapid LC–MS-MS separation. For 12 cannabinoids, selected to be representative of the 24, recoveries ranged between 92–110% across three concentrations (50, 250, and 1000 ng/mL).

The precision and accuracy of the method were evaluated for 10 representative compounds at 50, 250, and 1000 ng/mL. Each concentration level was prepared in five replicates by spiking the appropriate amount of analyte standard into a matrix (tablet or herbal based) and each sample was injected five times. Intra-assay precision ranged from 1.2–4.0% relative standard deviation (RSD) across the three concentration levels. The procedure was repeated the following week to determine the interassay precision, which ranged from 2.9% to 9.7% RSD. Even though only 10 compounds were spiked for method validation, all 24 were injected numerous times individually, and together, during the method development with no significant changes (that is, within repeatability error) in peak areas and retention times. For example, after 25 injections of standard AM-694 (five replicates for each of the five different samples), essentially no change in retention time (0.09% RSD intra-assay and interassay) and only a very small change in peak area (1.7% RSD) was observed. The same results were obtained for the other compounds (data not shown). The method accuracy, determined by comparing the calculated concentration with the true value spiked into the matrix was within 10% for all 10 compounds at all concentrations tested.

Application to the Analysis of Seized Samples

We used the rapid LC–MS-MS screening method to analyze 11 seized samples (four incense sticks, two cigarettes, three herbal samples, one cannabis sample, and one tablet), all from nonregistered sources. Seven were found to be positive for cannabinoids. In one of the incense sticks, two synthetic cannabinoids were detected: JWH-018 at 4.48 min and JWH-073 at 4.24 min, as shown in Figure 5. These two synthetic analogs are controlled substances and, as highlighted in the introduction, their presence in these products is illegal. The incense sticks were therefore designated for withdrawal from distribution.

In the chromatographic separation of the cannabis plant sample (Figure 6), a number of endogenous cannabinoids were detected. THC, the main psychoac-

tive compound, was eluted at 4.93 min. Cannabigerol was detected at 4.36 min, and unlike THC, it does not mimic psychosis symptoms even though it interacts with the CB2 receptor in the brain (1). Cannabidiol, identified at 4.48 min, is another natural cannabinoid that is highly abundant in cannabis, up to 40% in the plant extract; however, it is not psychoactive. Cannabinol, which also interacts with the CB2 receptor, is usually present at low concentrations in fresh cannabis samples, but its abundance increases as it is formed by THC oxidative degradation by exposure to light or air (13). It was identified at 4.69 min with approximately a fivefold higher signal compared to THC (Figure 6), which suggests that the cannabis sample had undergone extensive oxidation.

The cannabis plant sample was previously analyzed by validated thin-layer chromatography (TLC) and GC–MS methods in a local government laboratory. The other samples in this study were previously analyzed by two validated HPLC–photodiode array (PDA) detection and GC–MS routine methods from the same government laboratory. The results obtained with the current rapid LC–MS-MS approach were in perfect agreement with the routine methods in that there were no false positive or false negative results; however, the new approach was four times faster than the validated HPLC–PDA method, six times more rapid than the validated GC–MS method, and more sensitive than both methods. Most cannabinoid analysis methods screen for metabolites in biological samples (such as urine, blood, and hair) because the parent compounds are rapidly metabolized (11,15). For screening suspected adulterated herbal mixtures, identification of the parent drug is required. Several rapid LC–MS-MS methods for cannabinoid screening have been reported recently in which 15–20 parent compounds and, in some cases, over 20 metabolites, have been separated in times ranging from 8 to 20 min (12,14,16). The large majority of these are MRM-based approaches, which means they are targeted and thus monitor only analogs predefined in the method; they are entirely inefficient for detecting new-generation cannabinoid analogs.

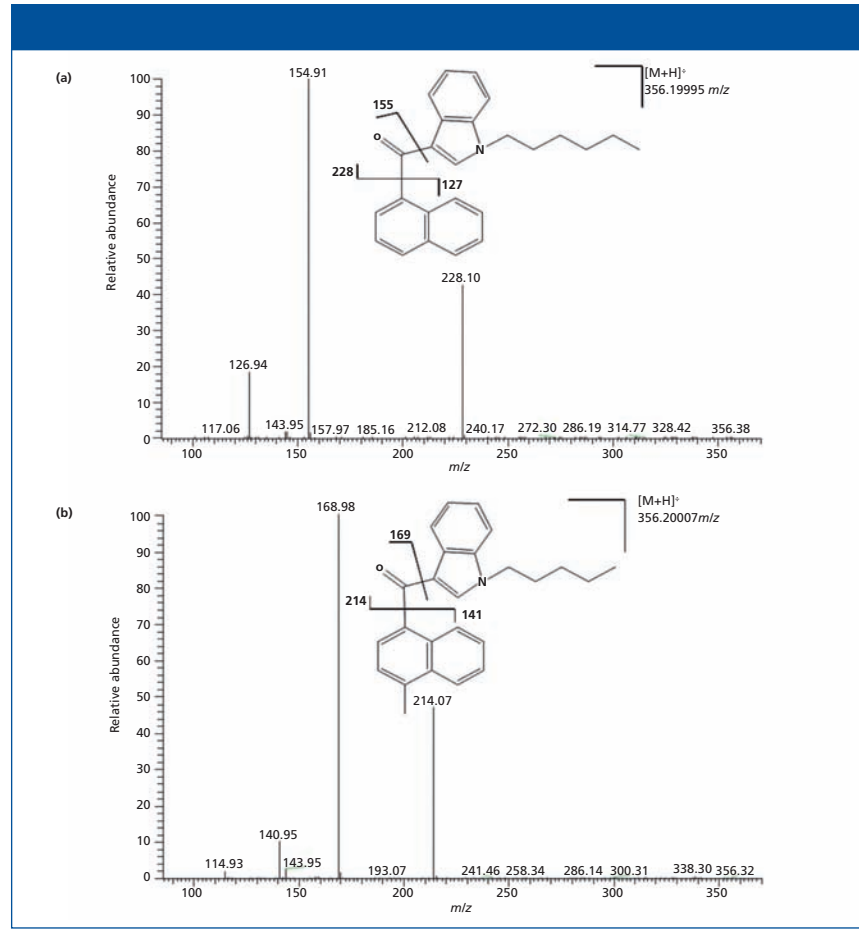

Figure 4: Fragmentation pattern of two isomers: (a) JWH-019 and (b) JWH-122.

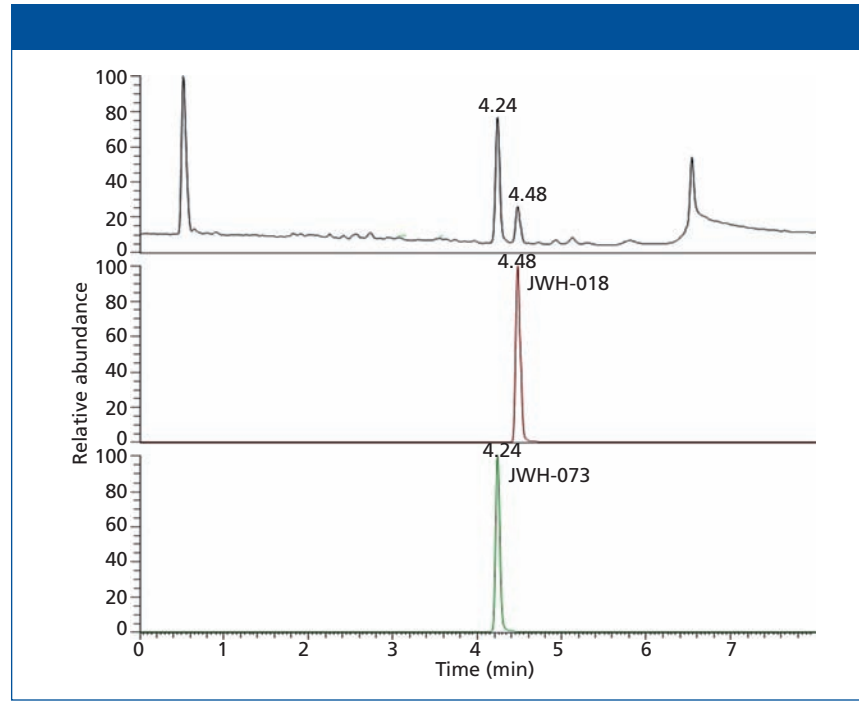

Figure 5: Illegal product (incense stick) showing the presence of controlled synthetic cannabinoids: JWH-018 and JWH-073. The uppermost panel represents the TIC and the lower two extracted chromatograms showing detection of JWH-018 (middle panel) and JWH-073 (lower panel).

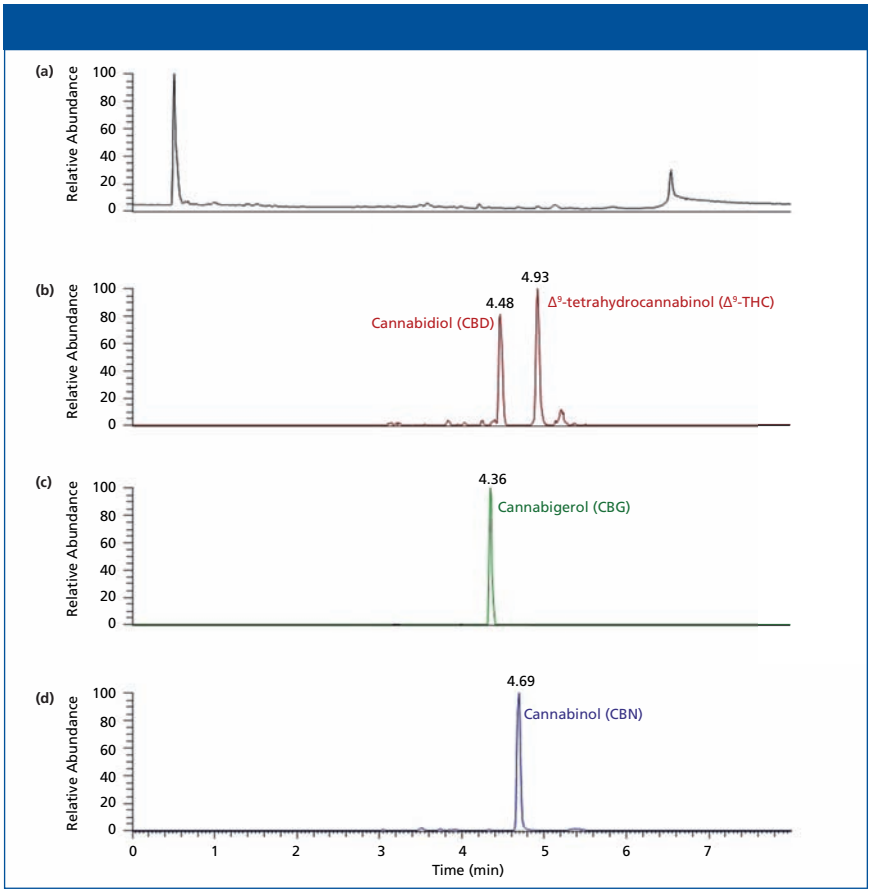

Figure 6: Cannabis sample showing the presence of THC, cannabidiol, cannabigerol, and cannabinol: (a) TIC, (b–d) extracted ion chromatograms showing the detection of the four cannabinoid compounds.

Conclusion

The development of a rapid, selective, and sensitive LC–MS-MS screening method for simultaneous identification of 24 synthetic and natural cannabinoids present in herbal smoking mixtures, incense sticks, and tablet matrices has been described. The high mass-resolution power of an orbital ion trap mass spectrometer and its compatibility with the high peak efficiency provided by core–shell particle chromatography contributed to the excellent selectivity of the method. Additional confidence in analyte identification for the 24 compounds targeted was provided by the fragmentation patterns. The novelty of this work lies in the high-resolution full-scan event in the acquisition method that allows postanalysis identification, in an untargeted approach, of additional species in the complex matrices. This is highly relevant to the current application because of the emergence of new generations of cannabinoid analogs that try to be one step ahead of the controlled substances act. The need for such untargeted screening approaches is starting to be recognized by the analytical community as shown in recent reports by Scheidweiler and colleagues (15) and Shanks and colleagues (8). Our rapid LC–MS-MS screening method has the potential to be an indispensable forensic analysis tool for identifying illegal drug substances and adulterated products.

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A Sensitive and Cost-Effective LC–MS-MS Method for Determination of 1α,25-Dihydroxyvitamin D_3 in Human Plasma

The biologically active form of vitamin D is an important analytical target in both research and clinical practice. However, quantification of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has been challenging because of the extremely low levels in circulation — in the low picograms-per-milliliter range. Here, we report a sensitive, time-efficient, and cost-effective liquid chromatography–tandem mass spectrometry (LC–MS-MS) method that combines the use of immunoaffinity extraction, 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) derivatization, and methylamine adduction.

Jenny P. Dai, Allan Xu, Eric J. Battaglioli, Bruce A. Stanley, and Robin T. Wilson

Vitamin D_3 plays an important role in the maintenance of bone health by promoting the absorption and metabolism of calcium and phosphate (1,2). Vitamin D_3 is either produced in skin (under the influence of UV light) or absorbed from dietary or supplement intake (3,4). The primary circulating metabolite, 25-hydroxyvitamin D_3 ($25(OH)D_3$), is formed following a first hydroxylation step in liver microsomes. The active form, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is formed in a second hydroxylation step, which occurs primarily in the kidney (Figure 1). Through upregulation of a wide variety of genes, $1,25(OH)_2D_3$ is responsible for most of the biological action of vitamin D_3. Areas of influence of the active metabolite include immune function, cell proliferation, differentiation, and apoptosis (5).

Circulating concentrations of $1,25(OH)_2D_3$ are very low (low picograms per milliliter) (6) in normal human plasma. These extremely low concentrations and the potential interference from other vitamin D_3 metabolites have made the accurate quantification of $1,25(OH)_2D_3$ a challenge. Previous progress to increase the sensitivity of liquid chromatography–tandem mass spectrometry (LC–MS-MS) assays has been made by either using a more sensitive derivatization reagent (7) or by using the microflow LC–MS-MS (8) concept to improve the instrument sensitivity. But there are drawbacks associated with those methods, including cost, availability, the stability of the agents used, and the complexity of the system involved. Herein, we report a sensitive and selective LC–MS-MS method using immunoaffinity extraction (IAE), 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) derivatization, and methylamine (CH_3NH_2) adduction for the LC–MS-MS analysis. Our method is easier to use than current methods and offers significant advantages in cost effectiveness. The recovery and selectivity of the IAE are high for $1,25(OH)_2D_3$ and PTAD is a stable and commercially available reagent.

Experimental

The finalized experimental conditions for IAE, PTAD derivatization, and methylamine adduction in the LC–MS-MS analysis of $1,25(OH)_2D_3$ are described below.

Immunoaffinity Extraction

Samples were extracted using ImmunoTube extraction kits (Immundiagnostik AG, distributed by ALPCO). Internal standards (IS) consisted of 20 µL of a 10-ng/mL solution of 26,26,26,27,27,27-hexadeuterium-1α,25-dihydroxyvitamin D_3 (d_6-$1,25(OH)_2D_3$) (Chemaphor) dissolved in acetonitrile and

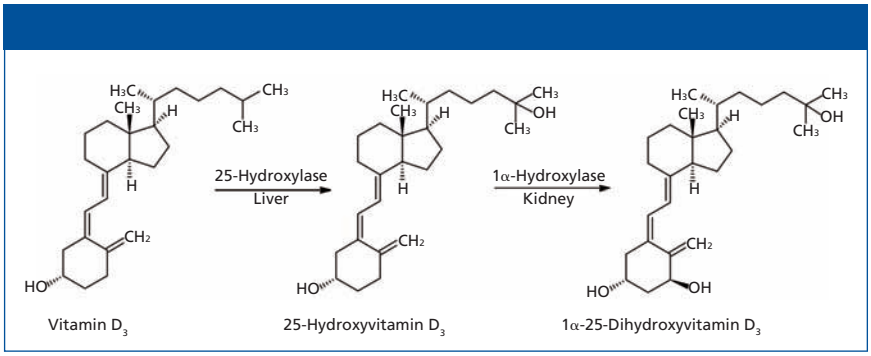

Figure 1: Metabolic pathway of 1α,25-dihydroxyvitamin D_3.

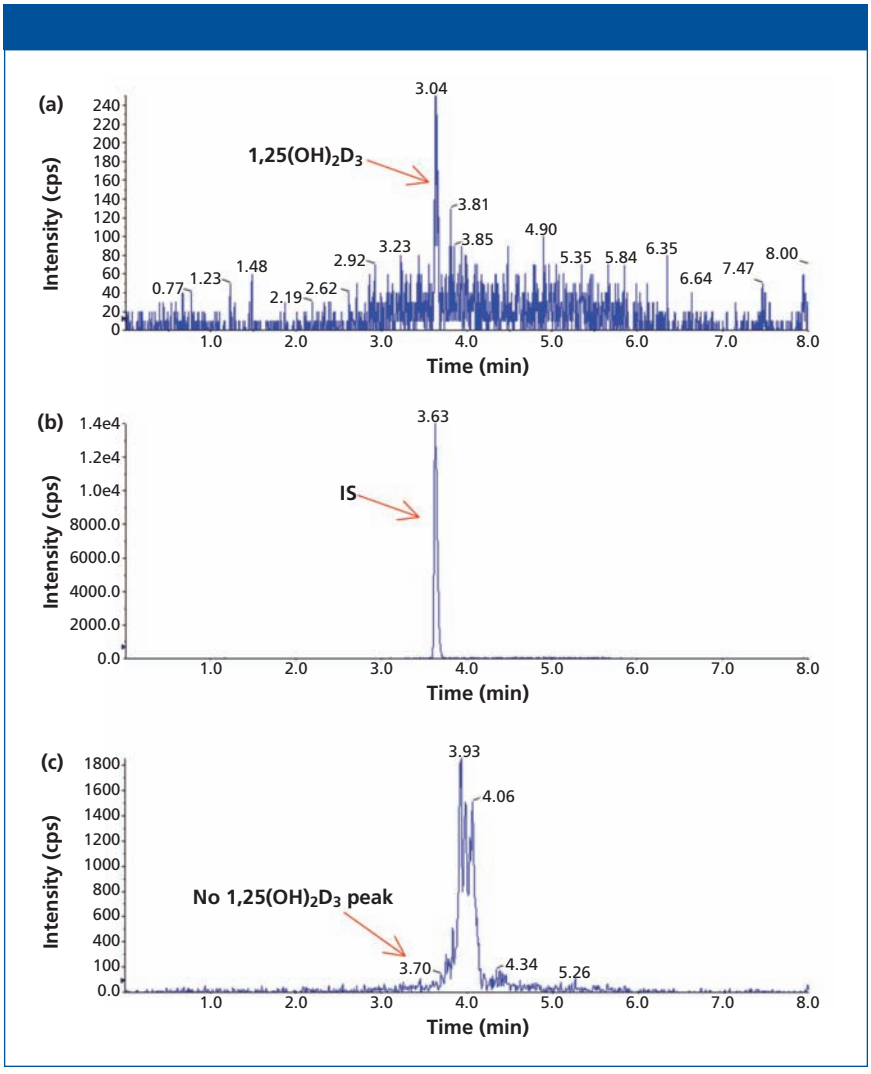

Figure 2: A comparison of the chromatograms of a sample containing 5 pg/mL of $1,25(OH)_2D_3$ extracted by different methods: (a) IAE, (b) IAE, and (c) SPE.

were spiked to 0.5 mL of human plasma sample. The sample was vortexed and centrifuged before being transferred into the ImmunoTubes. The ImmunoTubes were incubated on a rotator at room temperature for 1 h, then washed with 3 × 500 µL of WASHSOL (the wash solution provided by the manufacturer in the extraction kit), and finally eluted with 250 µL of ELUREAG (the elution reagent provided by the manufacturer in the extraction kit). The eluent was dried completely on a Speed Vac concentrator (Savant SPD121P-115, Thermo Electron Corporation), and then 50 µL of PTAD (0.2 mg/mL in acetonitrile) was added. The reaction was then held at room temperature for 2 h and finally terminated with 50 µL of water. Then the samples were transferred to high performance liquid chromatography (HPLC) vials containing inserts, and 10 µL was injected into the LC–MS-MS system. Next, $1,25(OH)_2D_3$ calibration standards were prepared by spiking 50 µL of $1,25(OH)_2D_3$ standard (Cerilliant) at different concentrations in acetonitrile into a 500-µL aliquot of vitamin D–depleted blank human serum (Golden West Biologicals Inc., VD-DDC Mass Spect Gold MSG 1000) at concentrations of 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, and 1000.0 pg/mL. The remaining procedures were carried out identically to those performed for the plasma samples.

LC–MS-MS Conditions

The LC–MS-MS system consisted of an Agilent 1100 system coupled to a tandem quadrupole 4000 Qtrap MS system (AB Sciex). Chromatographic separations were performed on a 50 mm × 2.1 mm, 2.7-µm Waters Cortecs C18+ column (90 Å), with the column temperature held at 60 °C. Mobile-phase A was 0.1% formic acid and 1 mM methylamine in water, and mobile-phase B was 0.1% formic acid in methanol.

The gradient was as follows: 0–0.1 min, 50% B; 2 min, 90% B: 2.5–4.0 min, 100% B; 4.1–8.0 min, 50% B. The MS system was operated in positive electrospray ionization mode with an ion spray voltage of 5500 V. Nitrogen was used as the collision gas. The optimized source conditions were as follows (arbitrary units if not specified): gas 1: 50; gas 2: 40; curtain gas (CUR): 30; collision gas (CAD): 10; and temperature (TEM): 400. The optimized compound conditions were declustering potential (DP): 50; collision energy (CE): 32; entrance potential (EP): 10; collision cell exit potential (CXP): 8; and dwell time: 100 ms for all analytes. The multiple reaction monitoring (MRM) transitions used were *m/z* 623.4→314.1 for $1,25(OH)_2D_3$ and *m/z* 629.4→314.1 for d_6-$1,25(OH)_2D_3$. Data were processed with Analyst software version 1.5.1 (AB Sciex).

Results and Discussion

Selection of Sample Cleanup Method

Before using IAE to separate $1,25(OH)_2D_3$ from its matrix interferences, several different solid-phase extraction (SPE) cartridges were evaluated. IAE was clearly superior in sample cleanup and sensitivity (Figure 2). The $1,25(OH)_2D_3$ peak was detected in the sample (containing 5 pg/mL of $1,25(OH)_2D_3$) treated with IAE (Figure 2a), but not in the sample treated with SPE (Figure 2c). Figure 2b is the peak of IS for the retention time identification purpose for the main peak of $1,25(OH)_2D_3$. No other interference peaks were observed near the main peak from a plasma sample treated with IAE (Figure 3c). Unlike SPE, which separates analytes based on their polarity, the immunoaffinity assay targets dihydroxyvitamin D metabolites only, resulting in higher selectivity. A cleaner sample made the subsequent LC–MS-MS analysis easier since an extensive separation on an LC column is not necessary. The run time was shortened by 50%, resulting in greater throughput efficiency and less cost to the investigator. The IAE procedures provided by the manufacturer were straightforward and easy to use with minimum preparation. IAE is a key step to improve the assay sensitivity, making it possible to measure $1,25(OH)_2D_3$ in human plasma on the mass spectrometer. These results were not achieved with any of the tested SPE cartridges used previously as sample cleanup methods.

Selection of the Derivatization Reagent and Use of Mobile-Phase Additive

Besides the improvement in the sample cleanup method, using a sensitive derivatizing reagent is another key to improve the sensitivity. At the beginning, the Amplifex Diene (AD, AB Sciex) derivatizing reagent was used for the assay since it was able to provide a 10-fold higher signal-to-noise ratio compared to PTAD (7) (Figure 4). However, because of the cost, the current availability of the AD derivatizing reagent, and its low stability following exposure to ambient air, the choice of derivatizing reagent went back to PTAD since our intention was to develop a method with great practicality and relative low cost while still meeting the assay detection limit requirement. To achieve this goal,

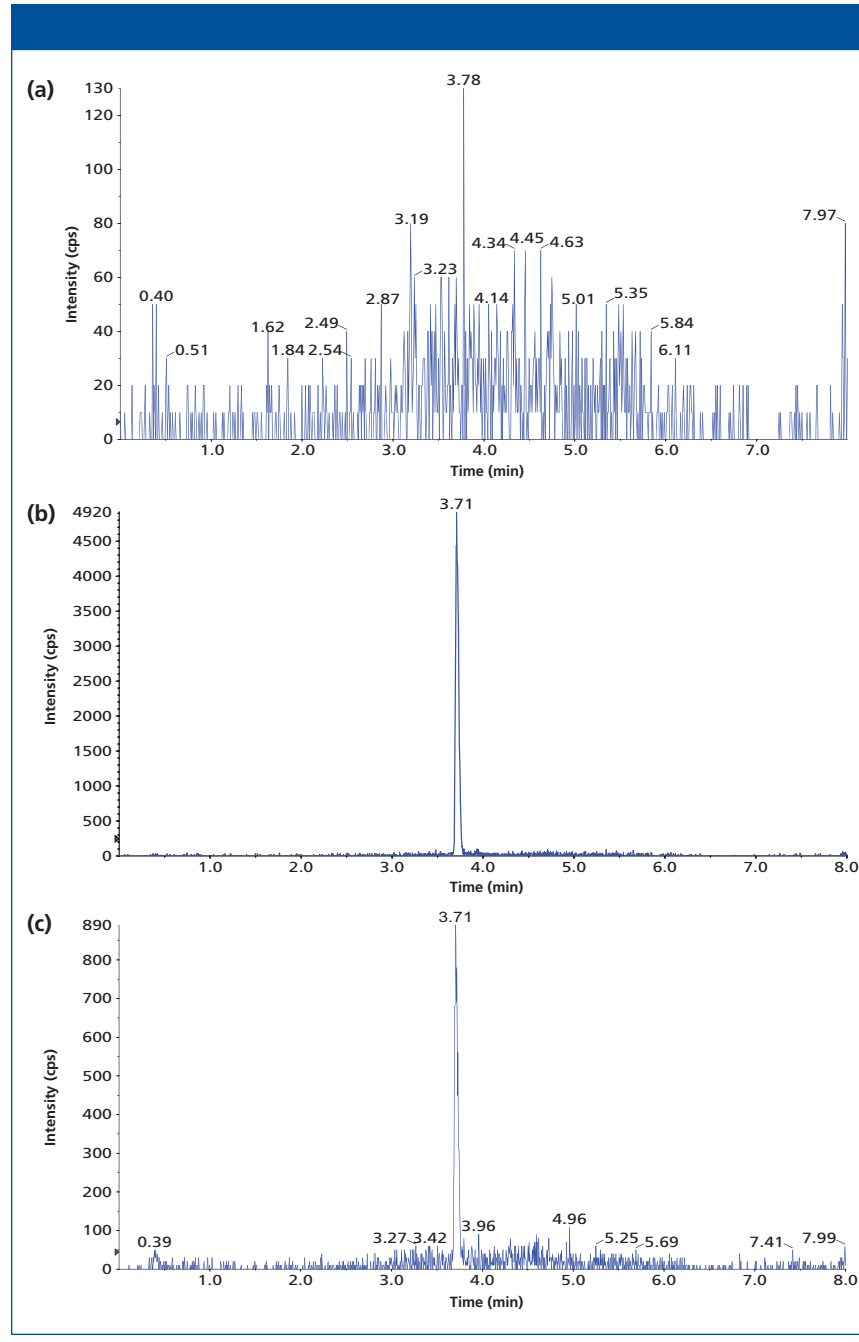

Figure 3: Example chromatograms of $1,25(OH)_2D_3$ in human plasma treated with IAE: (a) blank human plasma, (b) a standard, and (c) a patient sample.

methylamine was used as a mobile-phase additive to enhance assay sensitivity after the PTAD derivatization. When methylamine was added to the mobile phase, the derivative provided methylammonium adduct ions ($[M+ CH_3NH_2]^+$, *m/z* 623.4) as the base peak ion (9). The intensities of the protonated molecules, other adduct ions, and the dehydrated ion significantly decreased. In summary, the combination of PTAD derivatization and the addition of methylamine to the mobile phase provided sensitive and reliable measurement of $1,25(OH)_2D_3$ on the mass spectrometer with a lower limit of quantification (LLOQ) of 5 pg/mL in human plasma.

Optimization of the Conditions for PTAD Derivatization and LC–MS-MS Analysis Using Methylamine as an Additive

To obtain the maximum sensitivity, the amount of PTAD added for the derivatization, the concentration of methylamine in

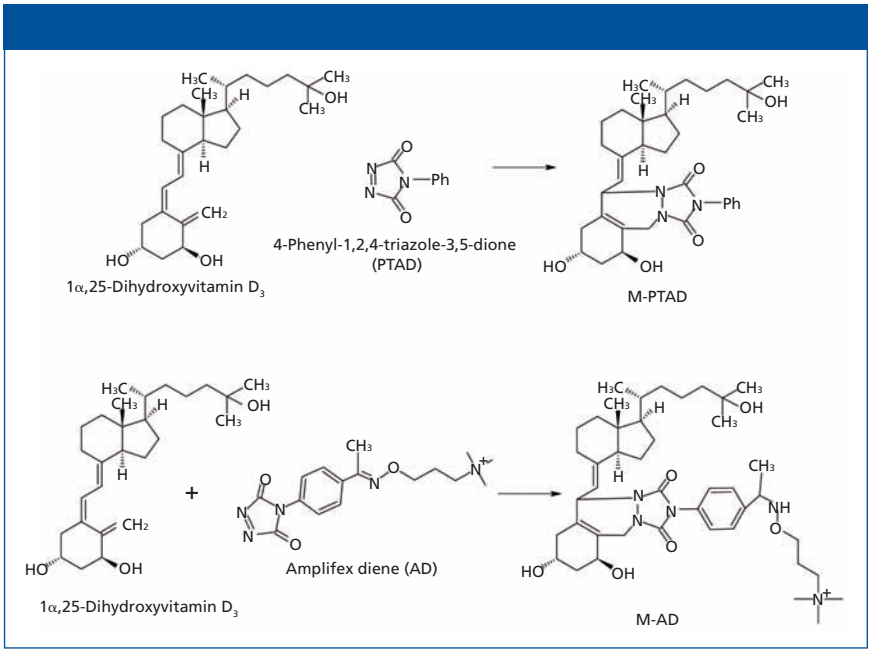

Figure 4: Derivatization of 1α,25-dihydroxyvitamin D_3 with AD and PTAD.

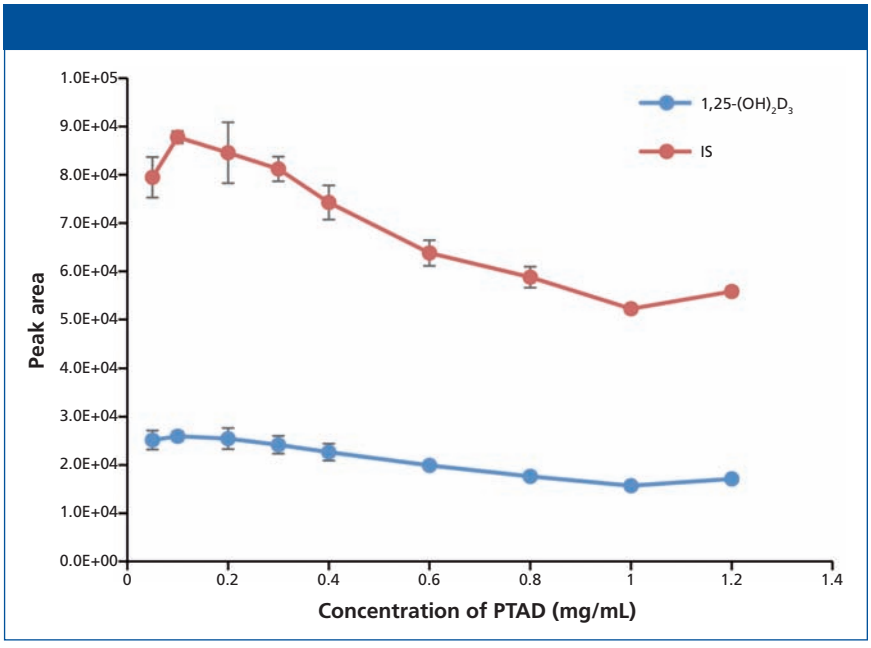

Figure 5: Optimization of PTAD concentration in derivatization. Error bars represent standard deviations of three replicates.

the mobile phase, the type of organic solvent in mobile-phase B, and the gradient were optimized to yield the highest signal-to-noise ratios and the lowest detection limit with repeatable results in a relatively short run time. For PTAD optimization, 50 µL of PTAD at different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/mL in acetonitrile) was added to identical samples spiked with 500 pg/mL of $1,25(OH)_2D_3$ and the IS. The samples were derivatized and analyzed according to the conditions described in the experimental section. The results are shown in Figure 5. Our data show that 0.1–0.2 mg/mL PTAD gave the highest peak area for both analyte and IS, and 50 µL of a 0.2-mg/mL solution of PTAD was chosen for the IAE sample to ensure that a sufficient derivatizing reagent was available in all reactions.

To optimize the methylamine concentration in the mobile phase, aliquots of the same sample were injected and analyzed on the LC–MS-MS system with different concentrations of methylamine in mobile-phase A. The optimal value is 1 mM methylamine in mobile-phase A because it gave the highest peak area for both analyte and IS (Figure 6).

Several different columns, organic components in mobile-phase B, and gradients were also evaluated to maximize the sensitivity. It was found that the column mentioned in the experimental section gave the best performance overall in terms of peak shape, separation, and column pressure. The detection sensitivity was shown to be higher when methanol instead of acetonitrile was used in mobile-phase B. Since IAE yielded a cleaner sample than other sample cleanup methods, and since PTAD is less hydrophilic than AD, 50% methanol was used as the initial gradient condition, resulting in a faster gradient that reduced the run time from 16 min (using AD as the derivatizing reagent) to 8 min (using PTAD as the derivatizing reagent) (see Figure 7). The 8-min run time is also significantly shorter than other methods reported for LC–MS-MS analysis of $1,25(OH)_2D_3$ (7,8,10).

A Comparison of the $1,25(OH)_2D_3$ Results Obtained Using Different Derivatization Reagents

To make sure that the new method (using PTAD derivatization and 1 mM methylamine in mobile-phase A) with a shorter run time (8 min) was able to replace the initial method (using AD derivatization) developed for $1,25(OH)_2D_3$ analysis, six patient samples at low, middle, and high levels of $1,25(OH)_2D_3$ were analyzed in both ways to compare the results side by side. By doing so, after the IAE sample cleanup, one set of the six patient samples went through the derivatization with PTAD and was analyzed by LC–MS-MS using the conditions described in the experimental section above. Another set of the samples went through the derivatization with AD and were analyzed on the same LC–MS-MS system using the optimized conditions, which was different from the one used for the PTAD method. Since the "best" chromatographic conditions (mainly the column and the mobile phases) developed for the PTAD method

are not the best for the AD-derivatized samples, the optimized chromatographic conditions for the AD method are listed below. The column used was a 100 mm × 2.1 mm, 2.6-µm Phenomenex Kinetex C18 column (100 Å), mobile-phase A was 0.1% formic acid in water, and mobile-phase B was 0.1% formic acid in acetonitrile. The gradient was as follows: 0 min, 5% B; 0.85 min, 35% B; 6.0 min, 45% B; 6.5–10 min, 95% B, 10.1–16.0 min, 5% B. The optimized source conditions were as follows: gas 1: 40; gas 2: 60; CUR: 30; CAD: 11; and TEM: 450. The optimized compound conditions were as follows: DP: 70; CE: 40; EP: 13; and CXP: 11 for MRM transition 748.6→689.5 ($1,25(OH)_2D_3$). For MRM transition 754.6→689.5 (d_6-$1,25(OH)_2D_3$), the conditions were DP: 65; CE: 50; EP: 11; and CXP: 9. Each patient sample was analyzed in triplicate, with the average reported in Table I. The results in Table I indicate that the percent difference of the results from the two methods is <9%, which is below the acceptance limit (±15%) generally set for LC–MS-MS analysis. The accuracy of the new method (using PTAD and methylamine) is very comparable to that of the initial method developed for $1,25(OH)_2D_3$ in which the more sensitive derivatizing reagent AD was used. Both methods have the same LLOQ, which is 5 pg/mL for $1,25(OH)_2D_3$. The calibration curve was linear over the range of 5.0–1000 pg/mL (Table II). Therefore, it is possible to use the existing low-cost reagent PTAD to replace the more expensive AD for the measurement of $1,25(OH)_2D_3$ in human plasma as long as methylamine is added in the mobile phase.

Method Validation

The last step in the method development was method validation to test for robustness and reproducibility. The method was validated for linearity, sensitivity, accuracy, precision, selectivity, recovery, and matrix effect. Excellent linearity was obtained for the eight-point calibration curve constructed by plotting the peak area ratio of $1,25(OH)_2D_3$ to its internal standard (d_6-$1,25(OH)_2D_3$) versus the corresponding concentration ratio and fitting the data using linear least-squares regression with a $1/x$ weighting factor.

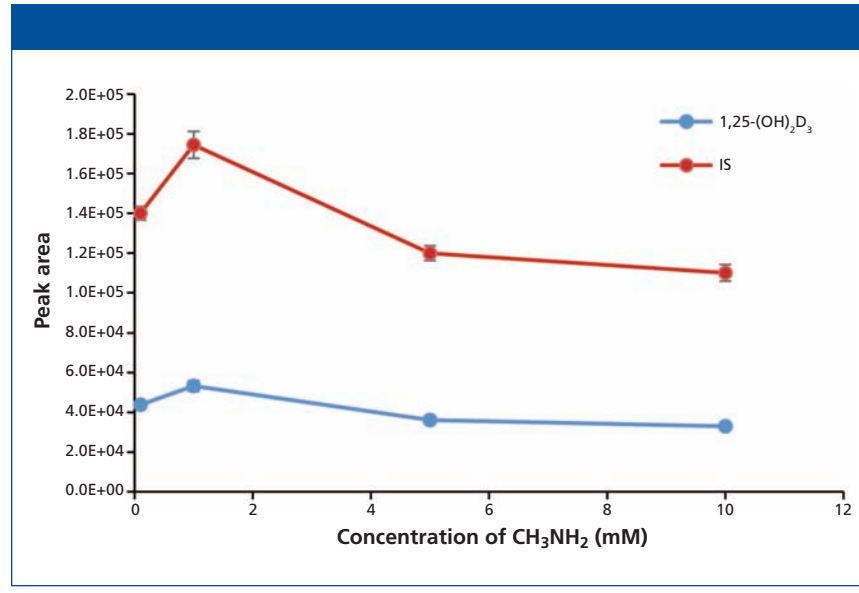

Figure 6: Optimization of methylamine concentration in the mobile phase. Error bars represent standard deviations of three replicates.

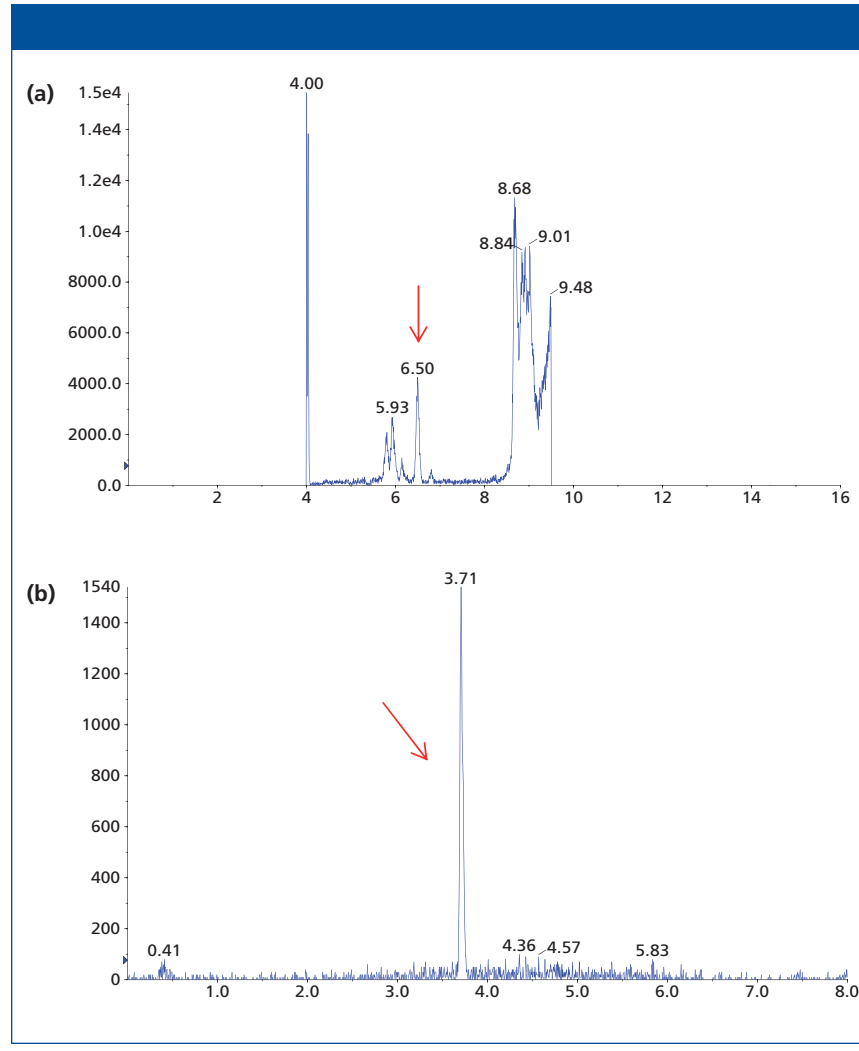

Figure 7: Mass chromatograms of the LC–MS-MS analysis of a patient sample derivatized with (a) AD and (b) PTAD. The arrows indicate the $1,25(OH)_2D_3$ peak.

Table I: A comparison of the $1,25(OH)_2D_3$ results (pg/mL) obtained from different derivatizing reagents

Patient Sample Number	Derivatizing Reagent		% Difference of the Results Between PTAD and AD
	AD	PTAD	
1	27.2	28.1	3.3
2	26.7	26.4	-1.1
3	41.5	38.8	-6.5
4	41.8	44.9	7.3
5	57.2	61.8	8.1
6	70.4	70.4	0.0

Table II: Precision and accuracy for calibration standards of $1,25(OH)_2D_3$ in human plasma from three validation batches

Analysis Group	Theoretical Concentration (pg/mL)							
	5	10	25	50	100	250	500	1000
	Measured Concentration (pg/mL)							
1	5.42	10.6	22.9	48.4	97.8	246	503	1010
2	5.62	9.43	23.9	49.0	98.1	253	502	999
3	4.88	10.1	23.8	52.3	99.0	252	514	984
n	3	3	3	3	3	3	3	3
Mean	5.31	10.0	23.5	49.9	98.3	250	506	998
RSD (%)	7.2	5.8	2.3	4.2	0.6	1.5	1.3	1.3
Accuracy	106.1	100.4	94.1	99.8	98.3	100.1	101.3	99.8

Table III: Precision and accuracy for the QC samples of $1,25(OH)_2D_3$ in human plasma

Analysis Group	Statistics	Theoretical Concentration (pg/mL)			
		5.0	15.0	200.0	350.0
001	*n*	6	6	6	6
	Intra-assay mean	5.2	13.4	191.3	340.7
	RSD (%)	6.3	2.8	3.1	1.5
	Accuracy (%)	104.2	89.2	95.7	97.3
002	*n*	6	6	6	6
	Intra-assay mean	5.3	14.7	198.5	345.2
	RSD (%)	7.0	7.6	3.2	1.7
	Accuracy (%)	105.2	98.1	99.3	98.6
003	*n*	6	6	6	6
	Intra-assay mean	5.1	14.4	199.2	345.7
	RSD (%)	6.5	9.2	3.2	1.6
	Accuracy (%)	102.7	95.7	99.6	98.8
Overall	*n*	18	18	18	18
	Interassay mean	5.2	14.2	196.3	343.8
	RSD (%)	6.3	7.7	3.5	1.6
	Accuracy (%)	104.2	94.3	98.2	98.2

For three consecutive batches, the calibration curves showed an overall accuracy of 94.1–106.1% with RSD ≤7.2% over the concentration range of 5.0–1000.0 pg/mL (Table II). The correlation coefficient (r^2) of the linear regression was ≥ 0.9998. The LLOQ was taken as the lowest calibration concentration that passed acceptance criteria with a signal-to-noise ratio of at least 5:1. The LLOQ was found to be 5.0 pg/mL. The LLOQ obtained on the mass spectrometer is low enough to accomplish the $1,25(OH)_2D_3$ measurement in human plasma since the level of $1,25(OH)_2D_3$ in most human plasma samples is 15–60 pg/mL (10). In addition, our technique differs from the prior method (7) because it is faster and more cost effective.

Method accuracy and precision were evaluated using quality control (QC) samples prepared by spiking $1,25(OH)_2D_3$ into the blank plasma at four concentration levels (5.0, 15.0, 200.0, and 350.0 pg/mL) to serve as QC-LLOQ, low QC, mid QC, and high QC. Three consecutive batches were prepared and each batch contained a freshly prepared calibration curve and six replicates of QC samples at the four levels. Intra-assay precision was calculated by obtaining the relative standard deviation (RSD) of the six replicates of each QC level, and intra-assay accuracy was calculated by averaging the accuracies of six replicates of each QC level against the fresh curve. Interassay precision was calculated by obtaining the RSD of all 18 replicates at each QC level from all three batches, with overall accuracy calculated by averaging the accuracies of all 18 replicates at each QC level from all three batches. The method was found to be highly accurate and precise. The accuracy of 89.2–105.2% (of the theoretical value) and precision of 1.5–9.2% RSD for intra-assay, and accuracy of 94.3–104.2% and precision of 1.6–7.7% RSD for interassay were obtained for all QC levels including LLOQ (Table III).

The method selectivity was evaluated by assaying blank plasma sample without an internal standard. No interfering peaks were detected at the retention time of interest (Figure 3). To evaluate the recovery of the immunoaffinity step plus matrix effects, three samples were each prepared in triplicate. Sample 1 was a blank plasma

sample spiked with $1,25(OH)_2D_3$ (500.0 pg/mL) and an internal standard. Sample 2 was a blank plasma sample without IS. Sample 3 was water without IS. All three samples were processed according to the procedures described in the experimental section above; however, for samples 2 and 3, instead of adding derivatization solutions, a solution containing $1,25(OH)_2D_3$ and the IS at concentrations that mimic the concentrations of sample 1 in the final extract were added right before being dried down for derivatization with PTAD. These samples are referred to as the postextraction spike sample (for sample 2) and the pure solution sample (for sample 3), respectively. The mean peak area counts of $1,25(OH)_2D_3$ and the IS from three replicates of sample 1 were compared with the corresponding mean peak area counts from three replicates of sample 2 to calculate the extraction recovery. As shown in Table IV, a recovery of 83.2% for $1,25(OH)_2D_3$ and 81.6% for the IS were achieved. Although the recovery of $1,25(OH)_2D_3$ was lower than 100%, the percent recovery was very consistent for the three replicates performed. At the LLOQ detection limit, only the sample extracted by the immunoaffinity tubes showed a peak, which was not shown with other sample treatment methods like SPE (Figure 3). Similarly, matrix effects were calculated as follows: mean peak area from the postextraction spike samples minus the mean peak area from the pure solution samples, then divided by mean peak area from the pure solution samples and multiplied by 100. Positive values indicate percent enhancement and negative values indicate percent suppression. A matrix effect of 7.22% for $1,25(OH)_2D_3$ and 13.42% for the IS was observed, and all three matrix lots showed very similar matrix effects. Although the matrix effect is a negative number, which indicates ion suppression for the samples treated by IAE, there was no other major interfering peak observed near the main peak (Figure 3) in the real patient sample. These data demonstrate the selectivity, robustness, and ruggedness of the method.

Table IV: Immunoaffinity extraction recovery and matrix effect for $1,25(OH)_2D_3$ and d_6-$1,25(OH)_2D_3$ in human plasma

Statistics	$1,25(OH)_2D_3$	d_6-$1,25(OH)_2D_3$
RSD (%)*	3.7/4.0	5.0/4.7
Recovery (%)	83.2	81.6
RSD (%)†	3.3/3.1	4.2/1.6
Matrix effect (%)	-7.2	-13.4

*RSD: RSD of three replicates of sample 1/ RSD of three replicates of sample 2.
†RSD: RSD of three replicates of sample 2/ RSD of 3 replicates of sample 3.

Conclusion

This work represents the first report to combine the advantage of IAE, PTAD derivatization, and methylamine adduction in the LC–MS-MS analysis of $1,25(OH)_2D_3$ in human plasma. First, IAE is a key step for improving the assay sensitivity and eliminating interfering metabolites that cannot be eliminated by SPE. Second, methylamine addition in the mobile phase resulted in the same LLOQ when comparing AD alone to PTAD with methylamine. The results from PTAD are the same as that from AD. In contrast, the cost of the PTAD plus methylamine method was significantly lower than the original AD method, needing only half the run time (8 min versus 16 min). In addition, this method can be broadly adopted in a majority of LC–MS-MS laboratories. In conclusion, the PTAD plus methylamine method has significantly lower cost and greater practicality.

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Development of a Fast LC–MS-MS Screen for Common Drugs of Abuse as an Alternative to Immunoassay Screening

Enzyme immunoassay (EIA) is a conventional drug screening technique, but it can be limited by cross-reactivity that can lead to high false positive rates. To improve the selectivity and efficiency of a urine screen for drugs of abuse, mass spectrometry (MS) was investigated as an alternative. An existing multianalyte MS method for amphetamine, benzoylecgonine, 3,4-methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, and phencyclidine (PCP) was modified to optimize for speed and to be a competitive screen.

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Recently, many new direct-analysis mass spectrometry (MS) techniques have been developed primarily for screening purposes, such as high-throughput systems (1–10), laser diode thermal desorption (LDTD) (11–19), direct analysis in real time (DART, JEOL) (20–32), and desorption electrospray ionization (DESI) (20,33–44). These techniques have proven to be time efficient and useful in drug sample analysis (2,6,10,13,15,16,20–23,26,28,32,34,35,37,41,42,44). However, all of these techniques require special and expensive equipment in addition to a typical MS system that most often is purchased in tandem with a liquid chromatography (LC) system. Another downside is that these techniques can require a specific sample "holder." For example, one high-throughput approach currently only uses 96- or 384-well plates, and the LDTD system has its own special plate as well, which may not be ideal for small-volume assays because the need for automated liquid handling becomes a necessary additional expense. This additional equipment cost for smaller laboratories may not always be feasible. Therefore, a less-expensive and time-efficient LC–MS-MS alternative screening technique was investigated.

Using conventional LC system components and short columns or commercially available guard columns, a rapid on-line sample preparation and injection (ROSPI) method was developed. ROSPI was developed using the following guidelines:

- The method cycle time had to be less than 2 min and ideally ~1 min.
- Short columns were used to help reach the method cycle time and maintain system pressures.
- Complete separation of the peaks or interferences was not necessary.
- Only one transition was used per analyte or internal standard (IS) to ensure a sufficient number of points across the peak.
- The number of IS compounds was minimized.
- Two-point calibration near the cutoff was used for semiquantitation to determine the positivity of samples.

A current LC–MS-MS confirmation method that included commonly abused drugs was used as a proof of concept for the ROSPI method. This method included seven analytes, shown in Figure 1: amphetamine, benzoylecgonine (a cocaine metabolite), 3,4-methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, phencyclidine (PCP). The method also included five IS compounds: amphetamine-D5, benzoylecgonine-D3, MDMA-D5, methamphetamine-D5, and PCP-D5. Typically, the compounds in this method would be screened by four different enzyme immunoassay (EIA) tests: amphetamines

(amphetamine and methamphetamine), MDMA (MDA, MDEA, and MDMA), benzoylecgonine, and PCP. However, with MS all of these compounds can be screened with a single injection in less than 2 min. Furthermore, implementing with existing equipment and less expensive guard columns affords rapid screening and improved cost efficiency. Using the aforementioned goals, a ROSPI method was developed for these drugs and compared to EIA for selectivity and sensitivity using authentic patient samples.

Experimental

Materials

Amphetamine, amphetamine-D5, benzoylecgonine, benzoylecgonine-D3, MDA, MDEA, MDMA, MDMA-D5, methamphetamine, methamphetamine-D5, PCP, and PCP-D5 controls were all purchased from Cerilliant. LC solvents, acetonitrile, and formic acid were purchased from Fisher Scientific and methanol was purchased from EMD Millipore. Drug-free, normal human urine was purchased from UTAK Laboratories. All water was acquired from an in-house Barnstead Nanopure purification unit from Thermo Scientific. Polypropylene 700-µL vials and snap caps with septa were purchased from VWR International.

Samples were run on an Agilent 6460 QQQ mass spectrometer with an Agilent 1200 Series Binary Pump, a column compartment with a CTCPal autosampler, and an Agilent Jet Stream electrospray ionization (ESI) source. Columns were purchased from Phenomenex and included a 30 mm × 3.00 mm, 2.6-µm d_p, 100-Å Kinetex C18 column for the ROSPI method and a 50 mm × 3.00 mm, 2.6-µm d_p, 100-Å Kinetex C18 column for the confirmation method. Both columns were used in tandem with a Phenomenex SecurityGuard Ultra C18 guard column and holder. Data were analyzed on Agilent MassHunter Quantitative Analysis software.

Methods

Aliquots of drug-free urine were fortified with amphetamine, benzoylecgonine, MDA, MDEA, MDMA, methamphetamine, and PCP at various concentrations for the calibration curves and a quality control (QC). For the confirmation method, all compounds except PCP were prepared at concentrations of 25, 50, 100, 150, 500, 1000, and 2500 ng/mL. PCP was prepared at additional concentrations of 4 and 10 ng/mL and excluded from the 2500-ng/mL point. For the screen method, all compounds except PCP were prepared at concentrations of 100, 150, and 300 ng/mL; PCP was prepared at concentrations of 25, 50, and 150 ng/mL. All samples were diluted 20× with 500-ng/mL amphetamine-D5, benzoylecgonine-D3, MDMA-D5, methamphetamine-D5, and PCP-D5 in water and centrifuged before a 20-µL injection onto the column. The gradient for the confirmation method was 1.87 min with a 3.12 min cycle time. The gradient was improved to 0.8 min with a ~1.7 min cycle time for the screening method. The flow rate was the same for both methods at 0.6 mL/min and the solvents used were 0.1% formic acid in water for solvent A and 50:50 acetonitrile–methanol + 0.1% formic acid for solvent B. Additionally, a wash 1 solvent of 50:50 methanol–water and a wash 2 solvent of 100% methanol were used on the CTCPal system. The additional cycle time for the methods is related to the washing and injection cycle of the CTCPal system.

In the confirmation method, two multiple reaction monitoring (MRM) transitions were used for every analyte, including the IS compounds. In the screening method, the quantifier ion transition, or the most abundant transition, was used for every analyte, including the IS compound. For the simplicity of the screening method, only benzoylecgonine-D3 was used as the IS compound in the processing method, even though all IS compounds were present in the sample. This was done to demonstrate the ability to decrease cost for the screen by using a single IS compound instead of five. Data were acquired in positive ESI mode and all MS conditions remained the same for both methods.

Because the confirmation method was previously validated, validation experiments were performed only for this screening method and included limits, precision and accuracy, and patient comparison. For the limits experiment, five replicates of each point "lower" limit of quantitation (LOQ), screen cutoff, QC, and upper limit of linearity (ULOL/carryover) were averaged and the %CV was calculated for each analyte. Precision and accuracy were assessed across three days using three replicates of only the screen cutoff and the QC for each analyte. Finally, a comparison of 344 authentic patient positive samples was correlated to the EIA results and confirmation results to determine the false positive and false negative rates as applicable.

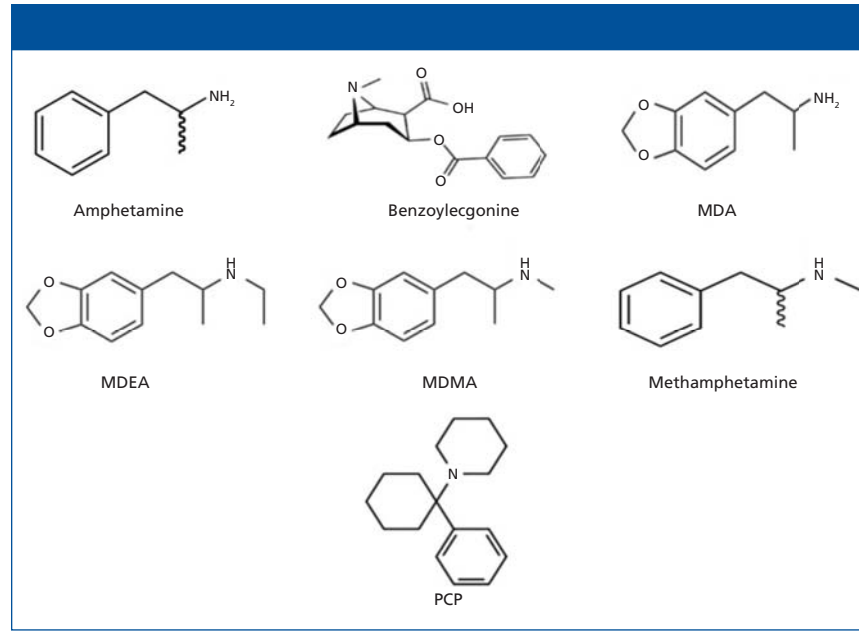

Figure 1: Structures of drugs analyzed.

Table I: Limits data from ROSPI single-point calibrator MS screen

Analyte	LOQ/LOD (24/4 ng/mL)		Screen Cutoff (100/25 ng/mL)		QC (150/50 ng/mL)		ULOL/ULOC (10,000/2500/1000 ng/mL)	
	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
Amphetamine	27.0	6.9%	106.7	3.5%	177.1	2.2%	11627.3	8.0%
Benzoylecgonine	24.4	1.7%	94.2	4.1%	170.5	2.6%	10504.1	7.5%
MDA	24.1	9.3%	103.8	3.2%	168.3	2.9%	2581.0	2.7%
MDEA	25.8	3.0%	96.1	3.2%	168.2	3.6%	2356.7	3.0%
MDMA	23.6	7.6%	105.7	4.5%	173.4	1.6%	9753.9	9.5%
Methamphetamine	23.2	6.7%	98.0	2.3%	168.1	4.0%	9173.6	8.1%
PCP	3.5	5.1%	23.3	4.2%	57.7	3.2%	1016.4	1.7%

Five replicates of each data point were averaged.
Lower concentrations apply to PCP only in the LOQ (4 ng/mL), screen cutoff (25 ng/mL), and QC (50 ng/mL).
ULOL/ULOC: 10,000 ng/mL applies to amphetamine, benzoylecgonine, MDMA, and methamphetamine; 2500 ng/mL applies to MDA and MDEA; 1000 ng/mL applies to PCP.

Table II: Precision and accuracy data from ROSPI single-point calibrator MS screen

Analyte	Day 1				Day 2			
	Screen Cutoff (100/25 ng/mL)		QC (150/50 ng/mL)		Screen Cutoff (100/25 ng/mL)		QC (150/50 ng/mL)	
	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
Amphetamine	101.8	3.8%	179.0	6.1%	92.7	13.6%	169.9	6.3%
Benzoylecgonine	99.4	2.4%	167.6	2.2%	98.2	6.5%	169.0	4.0%
MDA	99.2	4.2%	176.9	4.2%	90.7	13.5%	163.5	5.0%
MDEA	105.5	3.0%	189.6	2.1%	99.8	6.2%	182.5	5.0%
MDMA	106.2	7.0%	176.9	1.9%	95.0	11.8%	164.7	6.0%
Methamphetamine	97.3	3.5%	171.1	1.8%	96.8	10.2%	178.2	4.0%
PCP	22.8	4.2%	52.4	11.0%	21.7	12.1%	51.9	13.4%

Analyte	Day 3				Intraday			
	Screen Cutoff (100/25 ng/mL)		QC (150/50 ng/mL)		Screen Cutoff (100/25 ng/mL)		QC (150/50 ng/mL)	
	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
Amphetamine	98.1	8.2%	172.2	5.6%	97.5	3.8%	173.7	2.2%
Benzoylecgonine	93.1	1.7%	157.5	2.1%	96.9	2.8%	164.7	3.1%
MDA	92.9	9.4%	164.3	8.1%	94.3	3.8%	168.2	3.6%
MDEA	94.6	1.9%	163.5	5.5%	100.0	4.5%	178.5	6.2%
MDMA	94.0	3.5%	160.6	5.1%	98.4	5.6%	167.4	4.1%
Methamphetamine	94.0	3.1%	160.9	10.0%	96.0	1.5%	170.1	4.2%
PCP	21.0	4.6%	48.1	7.1%	21.8	3.4%	50.8	3.8%

Three replicates of each data point were averaged.
Lower concentrations apply to PCP only in the LOQ (4 ng/mL), screen cutoff (25 ng/mL), and QC (50 ng/mL).

Results and Discussion

All analytes seemed to work well with a linear fit through the origin using the single-point calibrator to provide a two-point calibration and benzoylecgonine-D3 as the sole internal standard. Data for the limits of validation can be seen in Table I. The LOQ was 25 ng/mL for every analyte except PCP, which was 4 ng/mL. The MS screen cutoff was set at 100 ng/mL for all analytes except PCP, which was set at 25 ng/mL. All analytes had a concentration of 150 ng/mL in the QC, except PCP, which was at 50 ng/mL. Carryover/ULOL was established at 10,000 ng/mL for amphetamine, benzoylecgonine, MDMA, and methamphetamine; 2500 ng/mL for MDA and MDEA; and 1000 ng/mL for PCP. Because the screen method was concerned more with accuracy in quantitation at the screen cutoff and used a lower calibration point, the carryover/ULOL was just assessed for precision (coefficient of variation [%CV]) and carryover criteria. If the accuracy failed outside of the tolerance, it was deemed acceptable as long as it would not change the positivity of the sample. However, it appeared that even though the single-point calibrator was at a significantly lower concentration than the ULOL, 2–3× the cutoff, the linear range held up to be the same as the origi-

Table III: Patient comparison of EIA screen versus ROSPI MS-screen

Analyte	EIA Screen				MS Screen				Overlap of False Postives
	Cutoff	Number of Positives	% False Positives	% False Negatives	Cutoff	Number of Positives	% False Positives	% False Negatives	
Amphetamine	500 ng/mL	205	41.9%	N/A	100 ng/mL	125	4.8%	0.8%*	50.0%
Methamphetamine	500 ng/mL				100 ng/mL	47	48.9%	N/A	65.2%
MDA	500 ng/mL	12	100.0%	N/A	100 ng/mL	0	N/A	N/A	0.0%
MDEA	500 ng/mL				100 ng/mL	1	100.0%	N/A	0.0%
MDMA	500 ng/mL				100 ng/mL	1	100.0%	N/A	0.0%
Benzoylecgonine	150 ng/mL	60	6.6%	N/A	100 ng/mL	63	11.1%	N/A	57.1%
PCP	25 ng/mL	3	100.0%	N/A	25 ng/mL	1	100.0%	N/A	100.0%

*False negative sample quantitative value was above LOD and within 20% of the cut-off
%FP = (#FP/TP)*100; %FN = (#FN/TP)*100; Overlap = (FP in both EIA and MS Screen/#MS Screen FP)*100
FP: False positive, TP: Total positives, FN: False negative; N/A: Not available

nal method, >1000 ng/mL depending on the analyte. Requirements for passing included that accuracy of the quantitation be within ±25% and %CV be within ±15%. The passing of the limits of validation indicated that using a two-point calibration with a single IS compound could be successfully used for semiquantitation at least for the analytes in this method.

The same acceptance criteria of quantitation within ±25% and %CV within ±15% was still used when assessing the precision and accuracy validation data that can be seen in Table II. However, it was noted that since the QC was a production control that was prepared at a different date and with different working stocks compared to the curve and other calibrators that were also used for quantitation, there was a slightly higher bias with the QC sample. Occasionally, the quantitation accuracy criteria were widened to ±30%, but the %CV criteria were not changed for the precision and accuracy experiment. While this would not have passed the validation to allow use of the method for production, it at least provided a proof-of-concept understanding of the ability to use the two-point calibration with a single IS compound over the course of several days, which was successful based on the precision. Had a new QC solution been prepared from the same stocks, it is reasonable to assume that the accuracy would have passed within the 25% acceptance criteria for all analytes. Furthermore, additional data points (that is, 10 versus 3) would be used to fully validate the method in precision and accuracy before its use in a production environment.

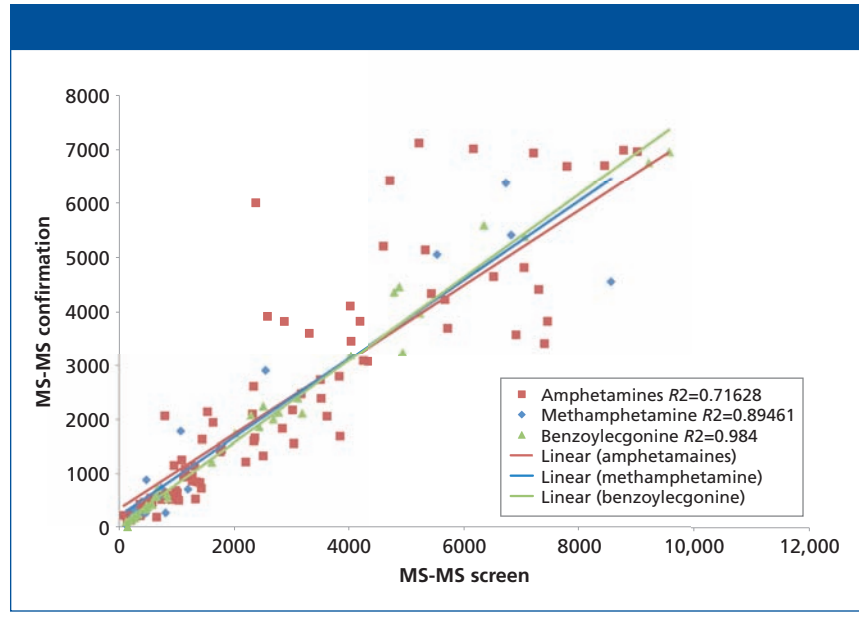

Figure 2: Patient quantitation correlation of MS confirmation and ROSPI MS-screen of amphetamine, benzoylecgonine, and methamphetamine.

The last proof-of-concept data that were considered were patient comparison data. There were two different assessments of this data. First, a correlation of quantitation between the MS confirmation method and the MS screen method were looked at, which is displayed in Figure 2. Only amphetamine, benzoylecgonine, and methamphetamine had enough patient positives to make the correlation meaningful. Overall, correlation was better than 70% for all three analytes. Benzoylecgonine even had ~98% correlation agreement, which could be because of the fact that benzoylecgonine-D3 was used as the internal standard. Because of the lower correlation with amphetamine and methamphetamine (~71% and ~89%, respectively), it also could be considered if using either the amphetamine-D5 or the methamphetamine-D5 as the IS compound or, in addition to the benzoylecgonine-D3, could improve those correlations, if desired. However, since there tends to be little to no correlation between values from EIA and MS confirmation methods, it could be argued that even ~71% correlation is acceptable.

Secondly, the patient data from the MS screen were compared to the EIA data to determine the false positive (FPR) and false negative (FNR) rates. It should be noted that all samples

were positive biased samples, because they screened positive for at least one of the class compounds by EIA and were then sent for MS confirmation. The samples prepared for the MS confirmation method were then reinjected on the MS screen method to determine whether they screened positive by the MS screen method as well. It should also be noted when assessing the data that the cutoff values used for the EIA and MS screen methods were different in that the MS screen cutoff values are lower than the EIA cutoff values as can be seen in Table III. In general, the MS screen values were lowered to be similar to the MS confirmation reporting cutoff values, with the exception of the PCP cutoff of 25 ng/mL which remained the same between EIA, the MS screen, and MS confirmation. A summary of the data can be seen in Table IV. In general, the MS screen has a lower FPR (%FPR = [number of false positives/total number of positives] × 100). There are a few exceptions. Methamphetamine increases a little from the EIA screen, which could be because the MS screen cutoff was 100 ng/mL and the confirmation cutoff was 125 ng/mL for these samples. Because of the high correlation in quantitation between the two methods, it could be that there is a systematic bias for a higher FPR. Adjusting the cutoff to be 125 ng/mL or higher could help reduce this bias. Interferences, such as phentermine, could also be investigated to determine if that is affecting the FPR for methamphetamine. As for the MDEA and MDMA, while the %FPR was not different, it should be noted that the total number of FPs did decrease significantly from 12 to 1 and the same observation was made for PCP. Benzoylecgonine is the only analyte that actually had a higher incidence of the number of FPs and FPR. It is unclear why this was observed. Unlike methamphetamine, the MS screen and MS confirmation cutoffs are the same, so perhaps there is an interfering compound that could be causing a slight increase in FPs, which would need to be investigated further. Additionally, the FPs for both the EIA and MS screen were compared to determine if the same samples were proving to be problematic by both methods; however, it seemed only about 50% of the FPs were the same between methods, indicating that the reason for a sample being a FP is not the same between methods and that each method has its own flaws and weaknesses.

One false negative was observed for amphetamine; this was a case where one sample screened positive by EIA and confirmed positive with the validated MS method, but screened negative by the ROSPI MS method developed here. It should be noted that the quantitative value determined by the single-point calibration was within 20% of the cutoff value, so perhaps using a different IS compound could improve the accuracy and correlation between patient samples to reduce the change of FNs. However, the FNR (%FN = [false negative/total positives] × 100) is still low enough to be considered acceptable. The FNR could not be calculated for EIA in this validation because any sample that screens negative by EIA is not processed by the MS confirmation method.

Conclusion

Overall, the data show that ROSPI is a viable MS screening alternative to EIA and could provide cost savings and improvements in FPR. Further validation experiments should be considered before using this method in a production environment. In particular, to be more time efficient, using a guard cartridge that is shorter (for example, 4 mm) and an autosampler alternative could be investigated to improve the cycle time further. Many autosamplers now have a much shorter cycle time, ~10–20 s, compared to the one used in our study, which can only be shortened to ~0.9 min. However, what was established here still met the study's goals and reduced the time from the confirmation method by ~50%. Increasing the flow rate or investigating duplexing and multiplexing could also provide more time savings. As for additional validation experiments, a more extensive precision and accuracy experiment with more concentrations and replicates along with an interference study appears to be the most crucial. However, matrix effect and signal suppression experiments should be considered as well, especially if a shorter cartridge is used that increases the coelution of interferences and signal suppressing compounds, which was observed in high-throughput MS (6), the logical endpoint of shortening the column.

The time required for completion of a single screen (that is, one sample) was dictated by the length of the guard column inasmuch as the flow rate was not changed. While the cycle time observed herein met the criteria for an effective screen, faster times have been observed in work using guard "filters" as short as 4 mm. This experimental work has also shown that the optimal combination of "column" length and flow rate remain empirical with issues such as tailing and peak shape requiring specific combinations of these parameters. The smaller "columns" used herein are less expensive than conventional analytical columns and seem to hold up well to the dilute and shoot paradigm used in this work. Sample preparation (that is, solid-phase extraction or supported liquid extraction) can be used, but it would increase the cost per sample markedly. Lastly, the sample can be prepared in vials or 96-well plates of conventional format to further simplify the process and minimize the expense.

This ROSPI proof of concept also showed that using a two-point calibration curve with a singular IS compound can afford sufficient semiquantitation used in a screening setting to determine the positivity of samples. While these compounds were successful in maintaining the original MS confirmation method ULOL and carryover limit, there may be some compounds that do not have the same behavior, but within a reasonable range around the cutoff value quantitation can be achieved with the same accuracy as an MS confirmation method. It was also demonstrated that while EIA values are known to have little to no correlation to MS quantitative methods, it can be seen here that MS screen methods have high correlation between the MS screen value and the MS quantitative value. This could have an impact on the cutoff values used for screening relative to the MS confirmation cutoff. Typically, EIA screen cutoffs are higher than MS confirmation meth-

ods; however, the correlation data indicates that MS screen cutoff should be lower than EIA screen cutoffs and more similar to the MS confirmation cutoff. For the compounds that have lower positivity rates, MDA, MDEA, MDMA, and PCP that were not correlated, a benefit for these compounds is the overall reduction in the number of positives and that now the cost of these screens is included with the higher volume-positive screens of amphetamine, methamphetamine, and benzoylecgonine. This is beneficial in cost-savings since there is a limited shelf-live for the screening solutions that could go bad with these lower demanding screens. With an MS-based screen there is not as much concern for the expiration of standards, especially since they can be used for the other compounds as well. Overall, with proper method development and validation ROSPI-MS screening is a reasonable time- and cost-efficient alternative to EIA screening.

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An Investigation into the Use of Alternate Carrier Gases for the Determination of Volatile Organic Compounds in Water by GC–MS

This study focuses on United States Environmental Protection Agency (US EPA) Method 524.3 for volatile organic compounds (VOCs) in water using gas chromatography–mass spectrometry (GC–MS). The GC sample introduction and separation technique specified by the EPA is purge and trap and the detector is MS. This article discusses optimizing MS and chromatographic separation parameters when using alternative carrier gases. In particular, it discusses the results of using nitrogen and hydrogen as alternate carrier gases to helium for EPA Method 524.3 for VOC analysis and specifically the impact of MS and chromatography parameters on the optimization process. Data are presented showing that nitrogen could be a more suitable carrier gas than hydrogen when compared to helium.

Lee Marotta, Tom Hartlein, Jacob Rebholz, Roger Bardsley, and Robert Thomas

The United States Environmental Protection Agency (US EPA) has established several methods to ensure safe and contaminant-free drinking water. One of these methods is described in EPA Method 524.3 (1), the determination of 75 volatile organic compounds (VOCs) using purge-and-trap sample concentration and gas chromatography (GC) coupled with mass spectrometry (MS). In this method, the volatile components are concentrated onto a trap, which is then heated to elute the compounds onto a GC column, typically using helium as a carrier gas, where they are separated and finally detected by MS. However, the concerns of a helium shortage and the significant increase in market prices have resulted in chromatographers investigating the use of other carrier gases to see if acceptable performance can be achieved. This article describes a suite of MS and chromatographic separation optimization experiments that were performed to better understand whether hydrogen and nitrogen can be used as alternative carrier gases to helium and, in particular, how they compare from a performance perspective when monitoring VOCs according to EPA Method 524.3.

What Are Some of the Challenges to Using Hydrogen and Nitrogen?

Even though hydrogen has excellent chromatographic separation efficiency with the widest range of useful linear velocities when compared to other carrier gases, it is extremely reactive and poses a safety risk, and as a result may not be the most suitable choice. In addition, the pumping efficiency of hydrogen is less than that of helium. Pumping efficiency is an important consideration because a good vacuum is critical to minimize collisions between target analytes and the carrier gas during electron ionization (EI) spectral acquisition. Additional collisions may result in atypical EI spectrum for targets and degraded sensitivity, mass resolution, and linear dynamic range. Furthermore, collisions in the source between the analytes and hydrogen atoms present may result in protonation. For these reasons, there are concerns about meeting the method tuning criterion using hydrogen as the carrier gas.

Nitrogen also has its challenges. The heavier nitrogen molecule may scatter the analyte ions to a greater degree in the ionization source and in the analyzer, and may degrade the detection limits for the same analyte masses compared to he-

lium. Finally, nitrogen's narrower range of useful linear velocities than helium or hydrogen can be easily overcome by careful flow rate selection.

Another concern with using nitrogen is that it is more easily ionized than helium and therefore changes the space charge conditions in the ion source. Ionization of nitrogen within the source can also reduce the number of electrons available to ionize target compounds. In addition, nitrogen can cause scattering of molecular target ions in the analyzer. Tuning MS conditions can help to alleviate some of these problems, but unfortunately it may also defocus the electron beam, which can lead to less efficient analyte ionization. All of these limitations can cause reduced sensitivity when switching from helium to nitrogen as a carrier gas. Table I shows a comparison of the three gases, with regard to a number of performance metrics.

Table I: Brief comparison of performance capability of helium, hydrogen, and nitrogen

Parameter	Helium	Hydrogen	Nitrogen
Safety	Safe	Caution	Safe
Source	Cylinder	Cylinder or generator	Cylinder or generator
Cost	Expensive	Cost effective	Cost effective
Supply	Concern	Not applicable	Not applicable
Column choices	Wide to narrow bore	Narrow bore	Narrow bore
Inertness	Inert	Highly reactive	Inert
BFB/DFTPP	Passes	Passes	Passes

Table II: Purge-and-trap instrumental conditions used in this study.

Purge		Desorb	
Sparge vessel heater	On	Desorb time	0.5 min
Sparge vessel temp.	40 °C	Bake	
Pre-purge time	1.00 min	Bake time	3.00 min
Dry purge time	1.00 min	Bake flow	400 mL/min
		Bake temp.	270 °C

Investigation Objective

The goal of the study was to meet all EPA Method 524.3 validation protocols using hydrogen and nitrogen as carrier gases by overcoming the traditional limitations described previously. The drinking water method was chosen because it has the most aggressive detection limits of any of the EPA methods for VOCs. It was believed that low detection capability was the most critical challenge to overcome, and if Method 524.3 requirements could

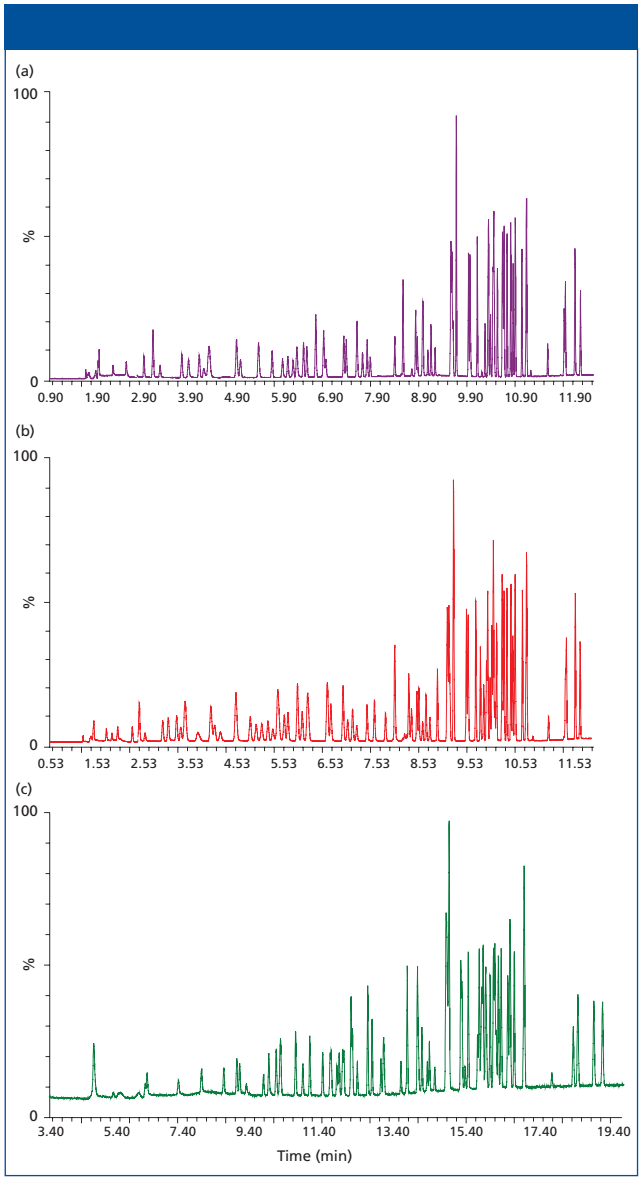

Figure 1: TICs of the Method 524.3 20 ppb test mix for (a) helium, (b) hydrogen, and (c) nitrogen.

be met, performance metrics for other EPA methods could also be achieved.

Experimental Conditions

Using a 20 ppb Method 524.3 standard, GC–MS conditions were established that provided adequate target component resolution and comparable peak efficiency for nitrogen and hydrogen compared to the reference helium data. The purge-and-trap multimatrix sample preparation system (Atomx, Teledyne-Tekmar) conditions are listed in Table II; the GC–MS (Clarus SQ 8 GC–MS, PerkinElmer) conditions are shown in Table III.

A narrow-bore column with a low flow rate was used, which reduced the amount of nitrogen and hydrogen molecules in the source. This approach resulted in improved vacuum efficiency, increased sensitivity, and reduced protonation, thus overcoming the mass spectral challenges of these carrier gases.

To prove this concept an example is provided using nitrogen.

Table III: GC and MS instrument conditions

Carrier Gas	Helium	Hydrogen	Nitrogen
GC Conditions			
Oven parameters	35 °C for 4.0 min, ramp at 16 °C/min to 85 °C, ramp at 30 °C/min to 210 °C, hold 3.71 min	35 °C for 4.0 min, ramp at 16 °C/min to 85 °C, ramp at 30 °C/min to 210 °C, hold 3.71 min	35 °C for 1.0 min, ramp at 10 °C/min to 60 °C, ramp at 20 °C/min to 230 °C, hold 5 min
Inlet temperature	220 °C	220 °C	220 °C
Carrier flow	0.4 mL/min	0.2 mL/min	0.3 mL/min
MS Conditions			
Ionization	EI+	EI+	EI+
Mass range	35 to 270 *m/z*	35 to 270 *m/z*	35 to 270 *m/z*
Scans across peak	12 to 15 scans	12 to 15 scans	12 to 15 scans
Source temperature	260 °C	260 °C	280 °C
Transfer line temp.	240 °C	240 °C	240 °C

Two columns were investigated. The first was a 20 m × 0.18 mm, 1-µm d_f Restek VMS column, coupled to a narrow-bore piece of deactivated fused silica. The VMS column offered excellent separation of the compounds of interest, and the deactivated fused silica offered the increased backpressure necessary to operate the inlet at the low flow rates being investigated. Since connectors can often bring ease of use issues, the method was also developed on a 40 m × 0.18 mm, 1-µm d_f Restek VMS column. The 40-m length provided enough back pressure that the added fused silica was unnecessary. Figure 1 shows the total ion chromatograms (TIC) of the EPA Method 524.3 20 ppb test mix for each of the three carrier gases.

To investigate the effect of reducing the amount of nitrogen in the source, the target compound intensities were compared using nitrogen column flow rates of 0.3, 0.4, 0.6, and 0.8 mL/min. Ultimately, 0.3 mL/min was selected as the nitrogen flow rate because it provided efficient peak widths and target compound separation at a vacuum of 1.8×10^{-5} Torr.

Calibration

The EPA tuning criterion for bromofluorobenzene was met for each carrier gas before calibration and was also continuously verified during the experiments. The results are displayed in Table IV.

EPA Method 524.3 allows the use of a first order, 1/*x* weighted calibration, which was used in our analysis. Calibrations ranged from 0.2 µg/L to 40 µg/L for all three carrier gas studies. Seven concentration levels were used for the helium and hydrogen studies, and five concentration levels were used for the nitrogen study.

The following additional experiments were carried out on all three carrier gases to ensure Method 524.3 initial demonstration of capability (IDC) was met:

- Recovery and precision of the midpoint standard was performed using the 20 ppb standard.
- The minimum reporting limits (MRL) of 0.2 ppb and 0.5 ppb were investigated by injecting seven replicates of each concentration to calculate the upper and lower limits for prediction interval of results (UPIR and LPIR, respectively).
- All concentration levels were processed as quality control (QC) checks using the respective calibration.
- A blank was analyzed after the high-level standard and several times during the experiments to ensure system cleanliness and determine if any carryover was occurring.

Discussion and Results

Using the optimum linear velocity for nitrogen, the chromatographic performance is very similar to helium on both columns tested; therefore, chromatographically, nitrogen works for this method, as demonstrated by the TICs in Figure 1. Additionally, the tuning criteria were met using all three carrier gases as demonstrated in Table IV. Therefore, under these GC–MS conditions, hydrogen protonation is not a concern for bromofluorobenzene tuning.

An increase in nitrogen flow rate resulted in a decrease in target response and an increase in the background of N_3^+ (*m/z* 42). This result suggests that the increase of nitrogen molecules in the source decreased the response of the target analyte compounds, which could be attributed to a combination of increased electron consumption by nitrogen ionization, resulting in reduced target analyte ionization, and increased collisions of nitrogen molecules and ions with the analytes.

Further study is required to determine the cause of this decrease in response and will be the focus of future work.

Calibrations were qualified as required under EPA Method 524.3 (section 10.1.10). Table V contains the correlation coefficients and the reporting limit results. The range of correlation coefficients was 0.9990–1.0000 across the components. In addition, Table V contains the signal-to-noise ratio (S/N) achieved at the reporting limit. Please note a shorter list of targets is tabulated for space requirements; however, the full list of 75 compounds is available in the on-line version of this article.

The method precision and accuracy criteria in Method 524.3 for the midpoint standard are ±20% and 80–120%, respectively. The performance criteria for all three carriers at the midpoint were exceeded. The precision results on selected targets are presented in Table VI. The results for both UPIR and LPIR criteria, calculated using the MDL, which are a part of the EPA Method 524.3 IDC requirements (section 9.2), are also displayed in Table VI. The method limits for the UPIR and LPIR are from 50–150%.

The MRL criteria for the 0.2 ppb concentration were met for all 75 target analytes using helium as a carrier gas. For nitrogen, 72 of the target analytes met these criteria for the 0.2 ppb concentration, whereas the three remaining targets met the criteria for a concentration of 0.5 ppb. For hydrogen, 67 of the target analytes met these criteria for the 0.2 ppb concentration, whereas the remaining eight targets met the criteria for the concentration of 0.5 ppb. Even though these exceptions met the ±50% criteria at the 0.2 ppb standard concentration, they did not meet the LPIR and UPIR criteria, so for those three targets in nitrogen and eight targets in hydrogen, an MRL of 0.5 ppb was chosen. Since the 0.2 ppb calibration point was linear, this point was maintained on the calibration.

Table IV: The tuning criterion for bromofluorobenzene demonstrates that the tuning criteria of Method 524.3 passed for all three carrier gases

Mass	Reference Mass	Range	Relative Abundance (%)		
			Helium	Hydrogen	Nitrogen
50	BPI	Report only	12.1	15.9	14.5
75	BPI	Report only	45.7	51.7	46.9
95	BPI	100%	100.0	100.0	100.0
96	95	≥5%–≤9%	5.9	6.5	7.6
173	174	<2%	0.6	0.6	0.9
174	95	>50%–<100%	86.0	75.8	87.7
175	174	≥5%–≤9%	7.4	6.2	7.3
176	174	>95%–<105%	98.3	101.4	100.8
177	176	≥5%–≤10%	6.2	5.4	5.7

Conclusion

The results of this study show that by using optimized GC–MS conditions, hydrogen and nitrogen are appropriate to use as alternate carrier gases to helium for EPA Method 524.3 analysis. With

Table V: Correlation coefficients, reporting limit results, and signal-to-noise ratios (S/N)

Compound Name	Helium Carrier Gas			Hydrogen Carrier Gas			Nitrogen Carrier Gas		
	Linearity 0.2–40 ppb	0.2 ppb std.	S/N at 0.2 ppb	Linearity 0.2–40 ppb	0.2 ppb std.	S/N at 0.2 ppb	Linearity 0.2–40 ppb	0.2ppb std.	S/N at 0.2 ppb
Vinyl chloride	0.9999	0.21	470:1	0.9998	0.21	175:1	0.9998	0.19	55:1
1,3-Butadiene	0.9998	0.23	360:1	0.9996	0.20	73:1	1.0000	0.20	36:1
Carbon disulfide	1.0000	0.21	250:1	0.9999	0.23	222:1	1.0000	0.20	230:1
Methylene chloride	0.9999	0.20	440:1	0.9999	0.20	93:1	1.0000	0.20	480:1
Methyl *tert*-butyl ether	0.9999	0.20	110:1	0.9994	0.21	350:1	0.9999	0.19	68:1
Bromochloromethane	1.0000	0.19	230:1	0.9999	0.23	429:1	0.9998	0.19	105:1
Chloroform	0.9999	0.19	260:1	1.0000	0.22	85:1	1.0000	0.20	308:1
Benzene	0.9998	0.20	120:1	1.0000	0.21	460:1	1.0000	0.21	134:1
Toluene	1.0000	0.20	550:1	1.0000	0.20	457:1	0.9999	0.18	208:1
1,3-Dichloropropane	0.9999	0.21	705:1	0.9996	0.22	212:1	0.9999	0.20	189:1
Ethylbenzene	0.9999	0.22	405:1	0.9999	0.19	576:1	1.0000	0.21	215:1
4-Chlorotoluene	0.9997	0.19	500:1	0.9999	0.20	293:1	0.9999	0.23	710:1
1,4-Dichlorobenzene	1.0000	0.20	860:1	0.9998	0.23	367:1	0.9999	0.21	265:1
Naphthalene	1.0000	0.21	820:1	0.9996	0.24	399:1	0.9997	0.24	246:1

Table VI: Precision performance criteria for the midpoint standard and LPIR and UPIR requirements

Compound Name	Helium Carrier Gas			Hydrogen Carrier Gas			Nitrogen Carrier Gas		
	%RSD at midpoint	LPIR	UPIR	%RSD at midpoint	LPIR	UPIR	%RSD at midpoint	LPIR	UPIR
Vinyl chloride	3.86	73	126	7.57	78	105	5.88	56	128
1,3-Butadiene	3.72	84	133	2.26	75	100	2.52	67	126
Carbon disulfide	5.05	54	92	3.24	96	115	2.20	88	130
Methylene chloride	3.01	70	112	4.71	87	100	3.13	74	123
Methyl *tert*-butyl ether	1.52	86	116	3.75	97	105	2.65	72	130
Bromochloromethane	1.88	57	115	2.07	68	115	2.38	66	137
Chloroform	1.68	62	111	1.89	93	110	2.27	85	133
Benzene	2.08	77	96	2.31	78	105	2.45	73	111
Toluene	1.67	89	127	4.65	82	100	2.12	67	98
1,3-Dichloropropane	1.44	90	148	1.81	97	110	1.95	58	122
Ethylbenzene	2.00	107	138	1.06	66	95	2.34	60	105
4-Chlorotoluene	0.87	57	101	6.02	87	100	2.22	104	123
1,4-Dichlorobenzene	0.72	91	118	2.26	82	115	1.98	79	117
Naphthalene	0.63	79	128	1.46	103	120	1.84	107	134

these optimized conditions the sensitivity limitations of these two gases have been overcome. Since nitrogen is more inert, less expensive, and doesn't have the safety concerns of hydrogen, it is recommended as the superior option for helium carrier gas replacement.

Acknowledgments

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Quantitative Analysis of Natural Cannabinoids Using LC–MS-MS

Jonathan Edwardsen, Shimadzu Scientific Instruments

Quantitative analysis of natural cannabinoids was conducted using the LCMS-8050 triple quadrupole mass spectrometer. A lower limit of quantitation (LLOQ) of 1–4 ng/mL was achieved depending on the specific cannabinoid. This method showed certain medicinal oils or tinctures available over the internet contained naturally occurring cannabinoids.

The analysis of natural cannabinoids is necessary not only because of potential medical uses for these compounds, but also in the regulation and quality control testing of products containing these compounds. To ensure the authenticity, quality, and amount of each cannabinoid contained in the product, an LC–MS-MS method was developed using the Shimadzu LCMS-8050 triple quadrupole mass spectrometer.

Experimental Conditions

After diluting in methanol neat standards of the naturally occurring cannabinoids, flow injection analysis was used to optimize source, CID conditions, and product ion selection. Optimized LC conditions were developed empirically and a 3-min gradient method was developed using a Restek column. Using solvent standards, calibration curves were created and various medical tinctures were then analyzed.

Results and Discussion

Cannabinoid optimization identified one quantifier and two qualifier ions for each naturally occurring cannabinoid. The two ions were selected based on ion intensity and repeatability across multiple collision energies. The precursor ions selected were the $[M+H]^+$ for all of the cannabinoid compounds. Following MRM optimization and development of chromatographic conditions, a standard curve was generated for each cannabinoid with $n = 6$.

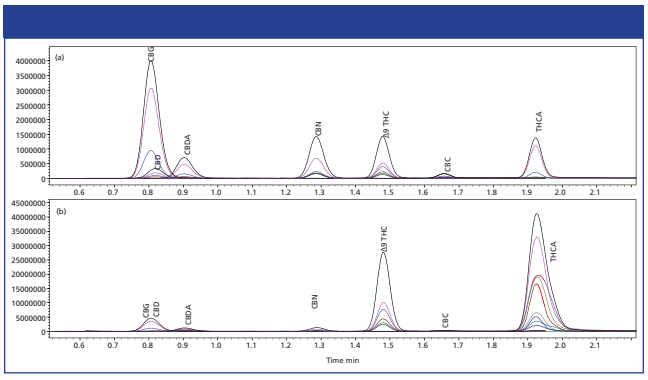

Figure 1: (a) Chromatogram of seven cannabinoids at 100 ng/mL in solvent. (b) Chromatogram of a commercially available tincture containing seven cannabinoids.

The lower limits of quantitation (LLOQ) were established for each cannabinoid at 1 ng/mL except for CBC, which was 4 ng/mL. The minimum signal-to-noise ratio for all of the cannabinoids was determined to be greater than or equal to 20:1. Calibration curve weighting of either 1/Concentration (1/C) or 1/Concentration^2 (1/C^2) was applied.

The chromatographic method that was developed yielded baseline separation of six of the seven cannabinoids with CBG (*m/z* 317.25) and CBD (*m/z* 314.95) co-eluting. Even though these peaks co-eluted, the LCMS-8050 was able to identify and quantify them without complete baseline separation. All seven of the cannabinoids were detected in the commercially available tincture purchased online (Figure 1). The concentrations for each cannabinoid are presented in Table I. There was no measurable carryover in the blank injected immediately after the highest level standard.

Conclusion

This work demonstrates a rapid method for the detection of naturally occurring cannabinoids by using the Shimadzu LCMS-8050. All seven cannabinoids were detected at levels as low as 1 ng/mL (1 pg on column) with a S/N of at least 20:1. This method is useful for quantitating cannabinoids in raw or commercial products.

Table I: Quantitative results for each cannabinoid at the limit of quantitation and the concentration of the commercial tincture.

Quantitative Results at LLOQ ($n = 6$)						
Compound	**LOD (ng/mL)**	**%RSD**	**%Accuracy**	**S/N**	**Weighting**	**Commercial Tincture**
CBN	1	4.516099	99.998± 4.2%	58.96	1/C²	0.016% ± 0.001%
THCA	1	7.023558	99.998 ± 9.1%	21.14	1/C	0.452% ± 0.018%
CBDA	1	6.671582	100.001 ± 5.7%	70.42	1/C²	0.019% ± 0.001%
Δ9 THC	1	6.414479	99.997 ± 6.3%	85.89	1/C²	0.370% ± 0.021%
CBG	1	3.666911	100.000 ± 3.7%	2397.6	1/C²	0.018% ± 0.0004%
CBD	1	7.770838	100.123 ± 6.8%	107.4	1/C²	0.006% ± 0.001%
CBC	2.5	8.193242	100.006± 5.7%	70.64	1/C	0.029% ± 0.006%

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Accurate Pain Management Analysis in Under 5 Min on Raptor™ Biphenyl Superficially Porous Particle LC Columns

Sharon Lupo, Ty Kahler, and Paul Connolly, Restek Corporation

Pain management LC analyses can be difficult to optimize due to the limited selectivity of C18 and phenyl-hexyl phases. In contrast, the selectivity of Raptor™ Biphenyl superficially porous particle (SPP) LC columns provides complete resolution of isobaric pain medications with a total cycle time of 5 min.

Accurate, reliable analysis of pain medications is a key component in monitoring appropriate medical use and preventing drug diversion and abuse. As the demand for fast, multicomponent methods grows, LC–MS-MS methods are increasingly desired for pain management and therapeutic drug monitoring due to the low detection limits that can be achieved with this highly sensitive and selective technique. However, despite the selectivity offered by mass spectrometry, hydrophilic matrix components can still interfere with early-eluting drug compounds resulting in ion suppression. In addition, isobaric pairs must be chromatographically separated for positive identification. The need for highly selective and accurate methods makes LC column selection critical.

While C18 and phenyl-hexyl phases are frequently used for bioanalytical LC–MS-MS applications, Restek's Biphenyl phase offers better aromatic retention and selectivity for pharmaceutical and drug-like compounds, giving it a significant advantage over other phases for the analysis of pain management medications or other drugs of abuse. The Biphenyl phase, originally developed a decade ago by Restek, has recently been combined with Raptor™ SPP ("core-shell") silica particles to allow for faster separations without the need for expensive UHPLC instrumentation.

Table I: Mobile phase gradient

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	90	10
1.50	0.6	55	45
2.50	0.6	0	100
3.70	0.6	0	100
3.71	0.6	90	10
5.00	0.6	90	10

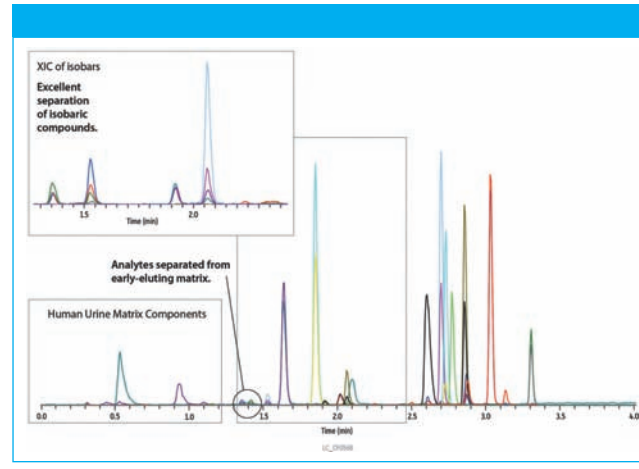

Figure 1: Baseline resolution of isobaric pain management drugs in sub-5-min runs on the Raptor™ Biphenyl column.

Here, we demonstrate the fast, selective separation of commonly tested pain drugs that can be achieved using the new Raptor™ SPP Biphenyl LC column.

Experimental Conditions

A standard containing multiple pain management drugs was prepared in blank human urine and diluted with mobile phase as follows, urine:mobile phase A:mobile phase B (17:76:7). The final concentration for all analytes was 10 ng/mL except for lorazepam, which was 100 ng/mL. Samples were then analyzed by LC–MS-MS using an AB SCIEX API 4000™ MS-MS in ESI+ mode. Chromatographic conditions, retention times, and mass transitions are presented here and in Tables I and II:

Column: Raptor™ Biphenyl, 50 mm × 3.0 mm i.d. × 2.7 µm
Sample: Fortified urine
Inj. vol.: 10 µL
Inj. temp.: 30 °C
Mobile phase A: Water + 0.1% formic acid
Mobile phase B: Methanol + 0.1% formic acid

Results

As shown in Figure 1, 18 commonly tested pain management drugs were analyzed with the last compound eluting in less than 3.5 min, giving a total cycle time of 5 min

Table II: Analyte retention times and transitions

Peaks	t_R (min)	Precursor Ion	Product Ion 1	Product Ion 2
Morphine*	1.34	286.2	152.3	165.3
Oxymorphone	1.40	302.1	227.3	198.2
Hydromorphone*	1.52	286.1	185.3	128.2
Amphetamine	1.62	136.0	91.3	119.2
Methamphetamine	1.84	150.0	91.2	119.3
Codeine*	1.91	300.2	165.4	153.2
Oxycodone	2.02	316.1	241.3	256.4
Hydrocodone*	2.06	300.1	199.3	128.3
Norbuprenorphine	2.59	414.1	83.4	101.0
Meprobamate	2.61	219.0	158.4	97.2
Fentanyl	2.70	337.2	188.4	105.2
Buprenorphine	2.70	468.3	396.4	414.5
Flurazepam	2.73	388.2	315.2	288.3
Sufentanil	2.77	387.2	238.5	111.3
Methadone	2.86	310.2	265.3	105.3
Carisoprodol	2.87	261.2	176.3	158.1
Lorazepam	3.03	321.0	275.4	303.1
Diazepam	3.31	285.1	193.2	153.9

*An extracted ion chromatogram (XIC) of these isobars is presented in the inset of Figure 1.

on Restek's Raptor™ SPP Biphenyl LC column. Analyte retention times are presented in Table II. Important isobaric pairs (morphine/hydromorphone and codeine/hydrocodone) were completely resolved and eluted as symmetrical peaks, allowing accurate identification and integration. In addition, early-eluting compounds such as morphine, oxymorphone, and hydromorphone are separated from hydrophilic matrix interferences, resulting in decreased ion-suppression and increased sensitivity. Similar analyses on C18 and phenyl-hexyl columns often exhibit poor peak shape and resolution (for example, peak tailing between closely eluting isobars), which makes identification and accurate quantification more difficult.

Conclusions

Complete separation of critical pain management drug analytes from hydrophilic matrix components and isobaric interferences was achieved using the new Raptor™ SPP Biphenyl LC column in less than 5 min. The fast, complete separations produced in this method allow accurate quantification of pain management drugs and support increased sample throughput and improved lab productivity.

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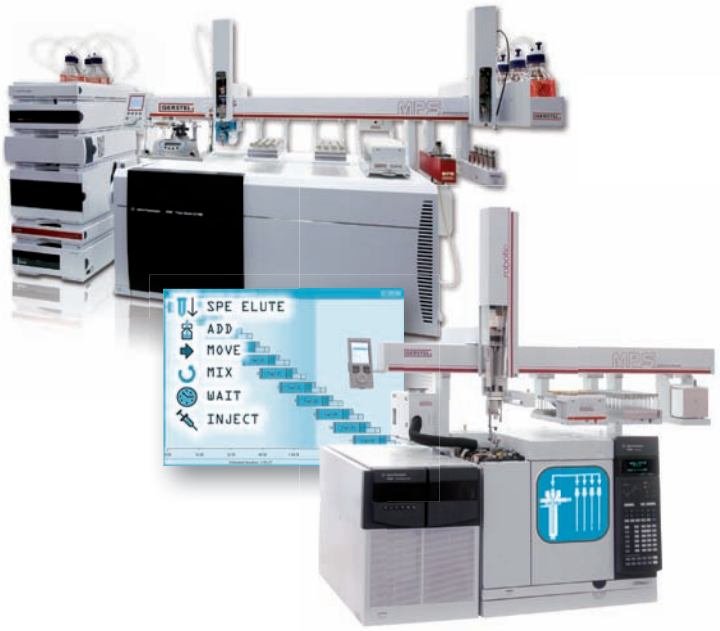

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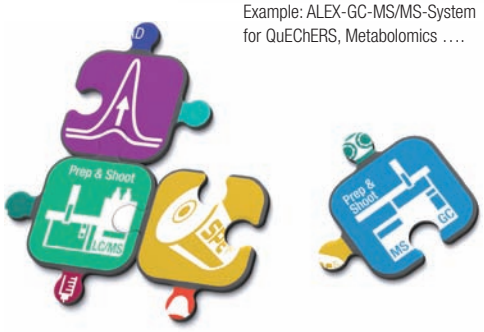

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