

Automated Solid-Phase Extraction-Liquid Chromatography–Tandem Mass Spectrometry Analysis of 11-nor- Δ^9 -Tetrahydrocannabinol-9-Carboxylic Acid in Human Urine Specimens: Application to a High-Throughput Urine Analysis Laboratory*

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Abstract

An automated solid-phase extraction coupled with liquid chromatography and tandem mass spectrometry (SPE-LC–MS–MS) method for the analysis of 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in human urine specimens was developed. The method was linear ($R^2 = 0.9986$) to 1000 ng/mL with no carryover evidenced at 2000 ng/mL. Limits of quantification and detection were found to be 2 ng/mL. Interrun precision was evaluated at the 15 ng/mL level over nine batches spanning 15 days ($n = 45$). The coefficient of variation (%CV) was found to be 5.5% over the course of the validation. Intrarun precision of a 15 ng/mL control ($n = 5$) ranged from 0.58% CV to 7.4% CV for the same set of analytical batches. Interference was tested using (\pm)-11-hydroxy- Δ^9 -tetrahydrocannabinol, cannabidiol, ($-$)- Δ^8 -tetrahydrocannabinol, and cannabinal. One hundred and nineteen specimens previously found to contain THC-COOH by a previously validated gas chromatographic mass spectrometry (GC–MS) procedure were compared to the SPE-LC–MS–MS method. Excellent agreement was found ($R^2 = 0.9925$) for the parallel comparison study. The automated SPE procedure eliminates the human factors of specimen handling, extraction, and derivatization, thereby reducing labor costs and rework resulting from human error or technique issues. Additionally, method runtime is greatly reduced (e.g., during parallel studies the SPE-LC–MS–MS instrument was often finished with analysis by the time the technician finished the offline SPE and derivatization procedure prior to the GC–MS analysis).

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Introduction

Marijuana is the most commonly abused illicit substance in the United States with 14.8 million United States residents admitting use (1). According to the Substance Abuse and Mental Health Services Administration, marijuana use is most prevalent among young people, rising during the teen years and peaking at age 20 with 1 in 5 users reporting usage within the last month (1–4). Because the military services draw significant numbers of recruits from this age group and the risks associated with marijuana use are considered to be a detriment to safety, performance, and morale, the Department of Defense monitors the illicit use of marijuana in service members by analyzing for 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in a random urine analysis program (5). Positive specimens reported in this program are predominantly THC-COOH (46.5%), so the need for accurate and rapid testing is required to return results to submitting commands as efficiently as possible (6). Efforts to improve the efficient analysis of THC-COOH have progressed from liquid–liquid extraction (7) to solid-phase extraction (SPE), which is commonly used in most forensic laboratories today (8), and the use of fast gas chromatography (GC) (9).

Automation of solid-phase extraction for GC–mass spectrometry (MS) analysis has been implemented with some advantages, but derivatization for GC–MS complicates the procedures as it must be carried out under a chemical fume hood with noxious chemicals and requires additional time to perform (10).

Liquid chromatography (LC)–tandem MS techniques require no derivatization step and are readily adaptable to automation for drugs of abuse in urine (11,12). Automation reduces processing time by allowing one specimen to be prepared while another specimen is being analyzed. Automa-

tion of the extraction step not only reduces time and labor costs but also increases forensic integrity in that technicians only intervene in matters of specimen handling for performing hydrolysis and the loading of samples onto the instrument. The method presented here reduces the amount of time for the complete analysis of THC-COOH specimens by 50%, virtually eliminating extraction time constraints and technician manipulation of specimens.

Materials and Methods

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile and water and reagent-grade formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Reagent-grade hydrochloric acid, potassium hydroxide, acetic acid, isooctane, ammonium hydroxide, and 23% tetramethylammonium hydroxide in methanol were purchased from J.T. Baker (Phillipsburg, NJ). Anhydrous isopropanol was purchased from Gallade Chemical (Escondido, CA). Omni-Solv methanol, ethyl acetate, and acetonitrile for GC-MS procedures were purchased from EMD (Gibbstown, NJ). Dimethyl sulfoxide and iodomethane were purchased from Sigma Aldrich (St. Louis, MO).

Standards and controls

Stock methanolic solutions of THC-COOH, THC-COOH-d₃, and 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid glucuronide (1 mg/mL) were purchased from Cerilliant (Round Rock, TX). All runs contained a THC-COOH calibrator at 15 ng/mL, a low control at 7 ng/mL, a negative control at 0 ng/mL, and a high control at 600 ng/mL. Additionally, a hydrolysis control containing the glucuronide was included in each run at 18.8 ng/mL. The internal standard stock solution was formulated at 600 ng/mL. Compounds tested for interference [(±)-11-hydroxy- Δ^9 -tetrahydrocannabinol, (-)- Δ^8 -tetrahydrocannabinol, cannabidiol, and cannabinol] were purchased from Cerilliant and formulated at 100 ng/mL. All standards, controls, and the calibrator were spiked into certified drug-free urine obtained from Roche Diagnostics (Indianapolis, IN). Analyte concentrations for specimens and controls were made using the relative response of a single-point calibration of THC-COOH at 15 ng/mL and THC-COOH-d₃ at 40 ng/mL in certified drug-free urine.

Sample and internal standard preparation

For analysis, 200 μ L internal standard and 100 μ L of 45% KOH were added to 3 mL of each specimen and control. Specimens and controls were vortex mixed, then incubated in a 50–60°C water bath for 15 min to effect hydrolysis of any glucuronidated analyte. After hydrolysis, 140 μ L concentrated HCl was added to the specimens and controls for LC analysis only.

Automated SPE system

The automated SPE system has been described in previous publications (11,12). The Spark Holland (Emmen, The Nether-

lands) Symbiosis Pharma system contains a refrigerated specimen compartment, a flow-through autosampler, binary HPLC pumps, and an automated SPE cartridge exchange device (ACE). The ACE system uses two SPE cartridge clamps to ensure a freshly extracted cartridge is always available for analysis. In one clamp, a fresh cartridge was activated and equilibrated, and a specimen loaded onto the cartridge. Cartridge washing also occurred in this clamp. The cartridge was then transferred to a second clamp where it was eluted with the analytical solvent flow.

Spark Holland Hysphere C8 EC-SE 10 cartridges were used on the automated SPE system. The cartridge contains 10 μ m end-capped silica-based C8 bonded phase material. Each cartridge was conditioned with 1 mL of acetonitrile at a flow rate of 5 mL/min. The cartridge was then equilibrated with 1 mL of 5:95 (v/v) acetonitrile/0.2% formic acid in water at a flow rate of 5 mL/min. Five-hundred microliters of specimen was loaded onto the cartridge with 1 mL of 20:80 (v/v) acetonitrile/0.2% formic acid in water at a rate of 1 mL/min. The cartridge was washed with 1 mL 40:60 (v/v) acetonitrile/0.2% formic acid in water at a flow rate of 5 mL/min using the solvent selection module. The cartridge contents were eluted using a process called gradient elution, which couples the SPE cartridge with the analytical column solvent flow. The composition of the elution solvent gradient is described in the LC chromatographic parameters section. Gradient elution was performed for 2.5 min. After elution of the SPE cartridge, the clamp was flushed with 500 μ L 5:95 (v/v) acetonitrile/0.2% formic acid in water.

Carryover was prevented by washing the autosampler loop and valve alternately with 700 μ L of 40% acetonitrile/0.2% formic acid in water, 1400 μ L of 40:40:10:10 (v/v/v/v) acetonitrile/methanol/isopropanol/water, and then repeating the first solvent mixture. During the last two solvent applications, the valve mechanism was activated to effectively wet all surfaces.

LC chromatographic parameters

Analytical chromatography was performed on a Waters (Milford, MA) Xbridge C8 column (2.1 mm in diameter, 50 mm in length, 3.5- μ m particle size). The analytical column was preceded by a Waters 2.1-mm \times 10-mm guard column with matching sorbent. The initial solvent system composition was 50:50 (v/v) acetonitrile/0.2% formic acid in water. The solvent composition was changed linearly to 85:15 (v/v) acetonitrile/0.2% formic acid in water over a 3-min span and held for 30 s. At 3.5 min, the gradient was returned linearly to the initial condition at 4.0 min and was then held for 1 min. All solvent flow was directed to the detector source and held constant at 0.4 mL/min. System suitability parameters required the retention times to be \pm 2% of the calibrator retention time and peak symmetry between 0.5 and 2.0 by the peak asymmetry factor calculation method (13). The drug qualifying ion was required to be within \pm 20% of those obtained from the calibrator. Chromatographic interference was limited to less than 10% of analyte peak height.

Tandem MS conditions

Tandem MS detection was carried out using a Waters Quattro Micro triple-quadrupole MS system in positive electrospray

ionization mode. The THC-COOH response was monitored by multiple reaction monitoring (MRM) transitions of m/z 345.20 to m/z 326.90 and m/z 299.00. The first transition was used as the quantifying ion and the second transition as the qualifying ion. Internal standard response was measured by the transition from m/z 348.20 to m/z 302.00. An MRM chromatogram of a standard containing 15 ng/mL of THC-COOH and 40 ng/mL of THC-COOH- d_3 is shown in Figure 1. Capillary voltage was 3.50 kV, cone voltage was 25 V, extractor voltage was 3.0 V, and dwell time was 0.2 s for all analytes. Collision voltage was 20 V for all transitions except for the THC-COOH quantifying ion, for which the collision voltage was held at 15 V.

Extraction and analysis by GC-MS

All SPE extractions for GC-MS analysis were performed using the Speedisk positive-pressure manifold (SPEware, San Pedro, CA). Specimens and controls were pipetted and hydrolyzed identically to the SPE-LC-MS-MS procedure, except that the addition of hydrochloric acid was omitted. The tubes were centrifuged at 1500–2000 rpm in a Silencer 2110 centrifuge (GFMD, Troy, MI) for 3–5 min to separate any solid material in suspension. The contents were applied directly to SPEware Cerex-THC cartridge, a proprietary anion-exchange resin. The specimens were loaded at 2–5 psi. The cartridges were washed sequentially with 1 mL of 84:15:1 (v/v/v) water/acetonitrile/ammonium hydroxide, 1 mL of methanol, and 1 mL of ethyl acetate, and then dried for 3 min at 20–25 psi. The cartridges were then eluted with 80:18:2 (v/v/v) isooctane/ethyl acetate/acetic acid. The eluate was evaporated to dryness using a stream of 99.99% medical-grade nitrogen gas (Westair, San Diego, CA) at 50–60°C, then dissolved into 100 μ L of 1:20 (v/v) tetramethylammonium hydroxide/dimethylsulfoxide. Twenty microliters of iodomethane was added as a derivatizing agent for GC. Two-hundred microliters of 0.1 N HCl and 2 mL isooctane were added to each tube. The tubes were capped and vortex mixed for 2 min before centrifuging at 1500–2000 rpm for 5 min. The tubes were placed in a dry ice/isopropanol bath until frozen, and the upper organic phase was decanted to a clean disposable test tube. The eluate was evaporated to dryness at 50–60°C under a stream of nitrogen gas. Specimens were then reconstituted with 50 μ L

of ethyl acetate and transferred to automated liquid sampler vials.

The samples were analyzed with an Agilent (Little Falls, DE) 6890 GC equipped with a 7683 autosampler and an Agilent J&W DB-XLB capillary column (15 m \times 0.25 mm \times 0.25- μ m film thickness) coupled to an Agilent 5975 mass selective detector in the selected ion monitoring (SIM) mode. The injection port was held at 290°C with a split ratio of 20:1, using helium as the carrier gas at a constant pressure of 3.0 psi. The oven was operated isothermally at a temperature of 280°C. The transfer line temperature to the mass selective detector was maintained at 300°C. Quantification of THC-COOH in specimens and controls was accomplished using the ratio of m/z 375 to m/z 372 from the 15 ng/mL calibrator multiplied by the cutoff level and the inverse ratio from the specimen. The qualifier ions for THC-COOH were m/z 313 and m/z 360, which were required to be within $\pm 20\%$ of those obtained from the calibrator. System suitability parameters required the retention times to be $\pm 2\%$ of the calibrator retention time and peak symmetry between 0.5 and 2.0 by the peak asymmetry factor calculation method (13).

Results and Discussion

The automated SPE-LC-MS-MS assay was evaluated for intrarun precision at 5, 7, 15, 18.8, 25, and 50 ng/mL ($n = 5$) for each data point. The mean value and standard deviation at each control value were used to calculate the percent %CV. Data are summarized in Table I. Intrarun precision of a 15 ng/mL control ($n = 5$) was also evaluated over nine batches spanning 15 days. The % CV ranged from 0.58% to 7.4%. Interrun precision was evaluated at the 15 ng/mL level over the same nine analytical batches ($n = 45$). The %CV was found to be 5.5%. The limits of quantification (LOQ) and detection (LOD) for the automated SPE-LC-MS-MS method were determined by analyzing, in quintuplicate, six concentration levels (0, 1, 2, 5, 10, and 15 ng/mL) of drug-free urine spiked with THC-COOH. The LOQ was defined as the lowest level of analyte correctly quantifiable within $\pm 20\%$ of the nominal value, with the qualifying ion ratio within $\pm 20\%$ of the calibrator

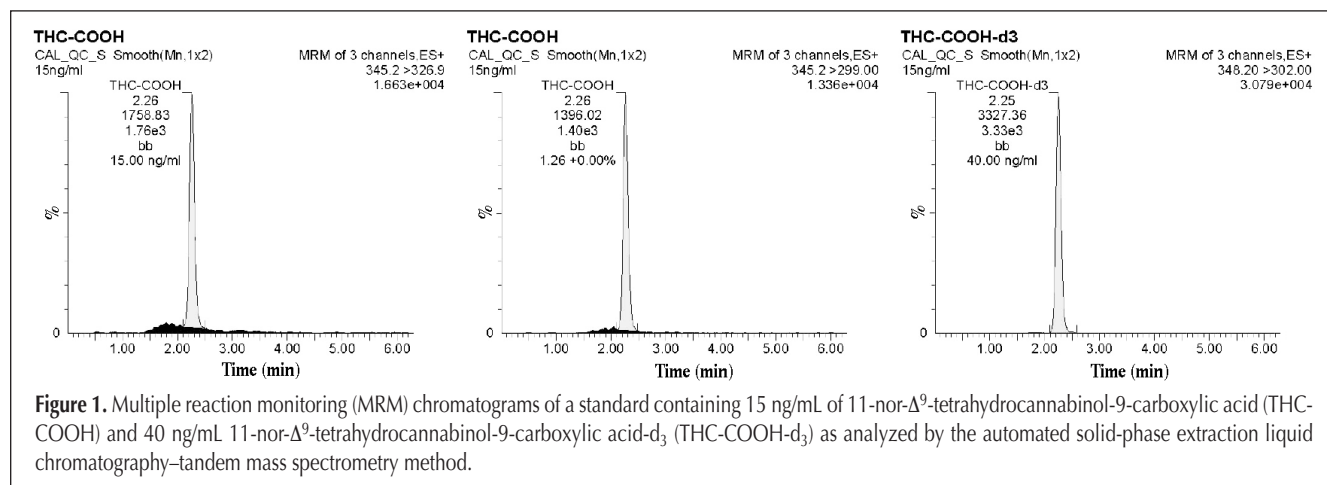


Figure 1. Multiple reaction monitoring (MRM) chromatograms of a standard containing 15 ng/mL of 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and 40 ng/mL 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid- d_3 (THC-COOH- d_3) as analyzed by the automated solid-phase extraction liquid chromatography-tandem mass spectrometry method.

qualifying ion ratio. The LOD was the lowest level of analyte detectable that had only the qualifying ion ratio restriction applied. The LOQ and LOD were 2 ng/mL. The linearity of detector response was evaluated by analyzing increasing concentrations of THC-COOH prepared at 7, 15, 18.8, 50, 100, 600, 1000, and 2000 ng/mL ($n = 5$ for each data point). The data were fit with a linear least-squares regression. The detector response was linear to 1000 ng/mL with a slope of 0.95 and a correlation coefficient of 0.9986.

Method specificity was investigated using compounds structural similar to THC-COOH. The potential interferences from (\pm)-11-hydroxy- Δ^9 -tetrahydrocannabinol, cannabidiol, (-)- Δ^8 -tetrahydrocannabinol, and cannabinol were analyzed at a concentration 100 ng/mL alone and in the presence of 7.5 ng/mL of THC-COOH and evaluated for their potential of interference with quantification or analytical acceptability. None of the four compounds produced interference with the correct identification or quantification of THC-COOH.

The potential for carryover was evaluated by the extraction

Analyte (ng/mL)	Mean	%CV
5	5.2	6.4
7	6.0	1.8
15	14.8	1.6
18.8	17.4	2.2
25	22.8	2.9
50	42.9	2.0

* $n = 5$ replicates.

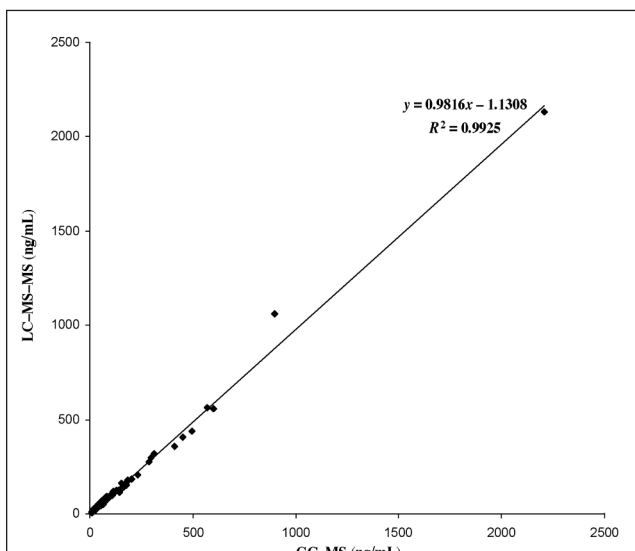


Figure 2. Correlation of 119 authentic human urine specimens previously confirmed positive for 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) using the standard GC-MS confirmatory method and the automated SPE-LC-MS-MS method described in this paper. Linear regression analysis ($R^2 > 0.9925$) demonstrates data concordance between both analytical methods.

and injection of a 2000 ng/mL THC-COOH control followed by three drug-free urine specimens containing the internal standard. No carry over was detected in any of the three samples following the control.

The potential for ion suppression or enhancement was evaluated using postcolumn infusion as described by Bonfiglio et al. (14). Drug-free urine was extracted and eluted onto the analytical column, after which THC-COOH (100 ng/mL) at a flow rate of 50 μ L/min was infused prior to the detector inlet. There was no ion suppression of THC-COOH noted during the analytical run time.

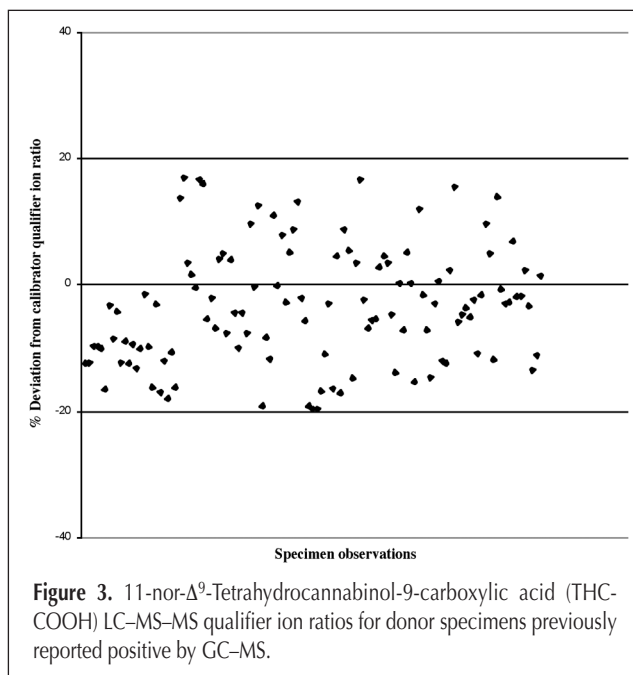


Figure 3. 11-nor- Δ^9 -Tetrahydrocannabinol-9-carboxylic acid (THC-COOH) LC-MS-MS qualifier ion ratios for donor specimens previously reported positive by GC-MS.

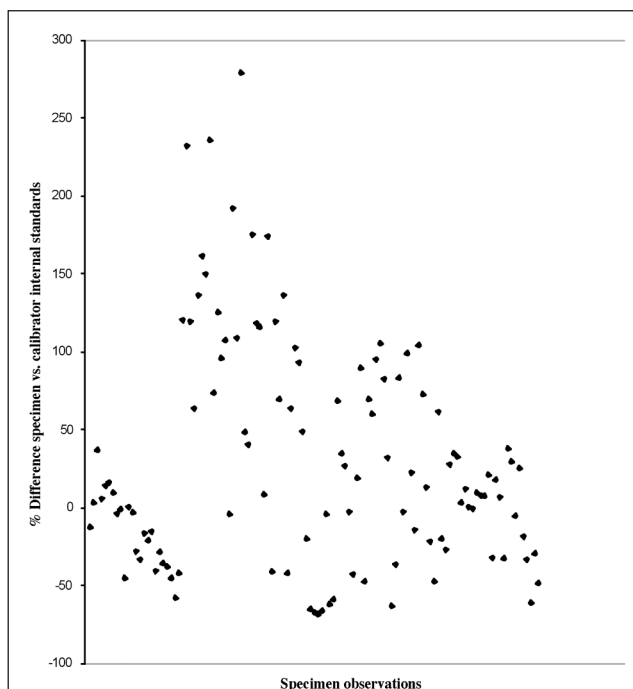


Figure 4. Percent deviation of internal standard response for specimens versus internal standard response of calibrator.

A comparative study of 119 specimens previously found to contain THC-COOH ranging from 6.7 to 2019 ng/mL was performed in parallel with the previously validated GC-MS and the SPE-LC-MS-MS method. Samples that exceeded the assay's limit of linearity (LOL) (1000 ng/mL) were diluted below the LOL with drug-free certified human urine and re-analyzed. The concordance of the data generated by both methods was excellent with a correlation coefficient of 0.9925 and a slope of 0.98 (Figure 2). Importantly, both methods produce the same number of positive samples and negative samples both above and below the cutoff concentration (15 ng/mL). Notably, three of the specimens analyzed by the SPE-LC-MS-MS method exhibited interference on the m/z 345.2 to m/z 299 qualifier transition that was not resolved to 10% of peak height from the chromatographic baseline. However, the qualifier ion ratio was within acceptable limits for these specimens. One of the specimens was below the administrative cutoff and was not investigated further. The interference in one of the two remaining specimens was adequately resolved using a longer analytical column, but the additional runtime incurred offset the advantages of the method developed in this communication. There were no chromatographic issues with these samples when analyzed by GC-MS.

The percent deviation of the qualifying ion ratio (ratio of the areas obtained from the transition of m/z 345.20 to m/z 326.90 to that of m/z 345.20 to m/z 299) for the authentic human urine specimens were plotted in Figure 3. As required by Department of Defense Standard Operating Procedure, all ratios were within $\pm 20\%$ of that obtained for the calibrator.

The percent increase or decrease of the internal standard response of each authentic human urine specimen relative to that obtained from the calibrator is shown in Figure 4. In general, the data are consistent with our laboratory's current GC-MS acceptance criterion that requires an internal standard response within one-quarter and four times of the calibrator internal standard response.

Conclusions

Although the automated SPE-LC-MS-MS method provides comparable results to the GC-MS method in regards to instrument run time, linearity, precision, accuracy, LOD, and LOQ, the SPE-LC-MS-MS provides a 50% reduction in the amount of time required to take the specimens from extraction through analysis and reporting. During the comparative studies the SPE-LC-MS-MS instrument was often finished with analysis by the time the technician finished the offline GC-MS SPE and derivatization procedure. The only non-automated steps in this procedure are the addition of internal standard and the hydrolysis procedure. However, as communicated previously (12), these steps may also be automated. Because human interaction is minimized, the possibility of manual error in the laboratory is greatly reduced and the repro-

ducibility of sample analysis is improved. Additionally, the use of noxious derivatizing reagents is eliminated, increasing workplace safety, reducing laboratory footprint, and eliminating the procurement and storage of hazardous materials.

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References

- Office of the National Drug Control Policy. *2008 Marijuana Sourcebook*, Rockville, MD, 2008.
- A.J. Budney, R. Roffman, R.S. Stephens, and D. Walker. Marijuana dependence and its treatment *Addict. Sci. Clin. Pract.* **4(1)**: 4–16 (2007).
- Office of Applied Studies, Substance Abuse and Mental Health Services Administration, *National Survey on Drug Use and Health*, January 2004.
- Office of Applied Studies, Substance Abuse and Mental Health Services Administration, *National Survey on Drug Use and Health*, January 2008.
- Department of Defense, *Instruction 1010.16*, December 1994.
- Office of the Assistant Secretary of Defense for Health Affairs, *Status of Drug Use in the Department of Defense Personnel: Fiscal Year 2007 Drug Testing Statistical Report*, 2008.
- S.B. Needleman, K. Goodin, and W. Severino. Liquid-liquid extraction systems for THC-COOH and benzoylecgonine. *J. Anal. Toxicol.* **15**: 179–181 (1991).
- P.R. Stout, C.K. Horn, and K.L. Klette. Solid-phase extraction and GC-MS analysis of THC-COOH method optimized for a high-throughput forensic drug-testing laboratory. *J. Anal. Toxicol.* **25**: 550–554 (2001).
- M.H. Jamerson, R.M. Welton, C.L. Morris-Kukoski, and K.L. Klette. Rapid quantification of urinary 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid using fast gas chromatography-mass spectrometry. *J. Anal. Toxicol.* **29**: 664–668 (2005).
- Handbook of Derivatives for Chromatography*, 2nd ed., K. Blau and J. Halket, Eds. Wiley, Hoboken, NJ, 1993.
- E. Jagerdeo, M.A. Montgomery, M. Sibum, T.A. Sasaki, and M.A. LeBeau. Rapid analysis of cocaine and metabolites in urine using a completely automated solid-phase extraction-high-performance liquid chromatography-tandem mass spectrometry method. *J. Anal. Toxicol.* **32**: 570–576 (2008).
- P.V. Robandt, L.J. Reda, and K.L. Klette. Complete automation of solid-phase extraction with subsequent liquid chromatography-tandem mass spectrometry for the quantification of benzoylecgonine, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine, and norbenzoylecgonine in urine—application to a high-throughput urine analysis laboratory. *J. Anal. Toxicol.* **32**: 577–585 (2008).
- R.J. Flanagan, R. Whelpton, I.D. Watson, and A.A. Taylor. *Fundamentals of Analytical Toxicology*. Wiley, Hoboken, NJ, 2008.
- R. Bonfiglio, R.C. King, T.V. Olah, and K. Merkle. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun.*