Current Trends in MASS Spectrometry

SUPPLEMENT TO
October 2014 LCGC North America | LCGC Europe | Spectroscopy

GC–MS Analysis of an Herbal Medicine

Screening Clandestine Drug Operations with Portable Ambient Sampling MS

Targeted Protein Quantification Using MS with Data-Independent Acquisition

Determination of Methylxanthines and Cotinine in Human Plasma by SPE and LC–MS-MS

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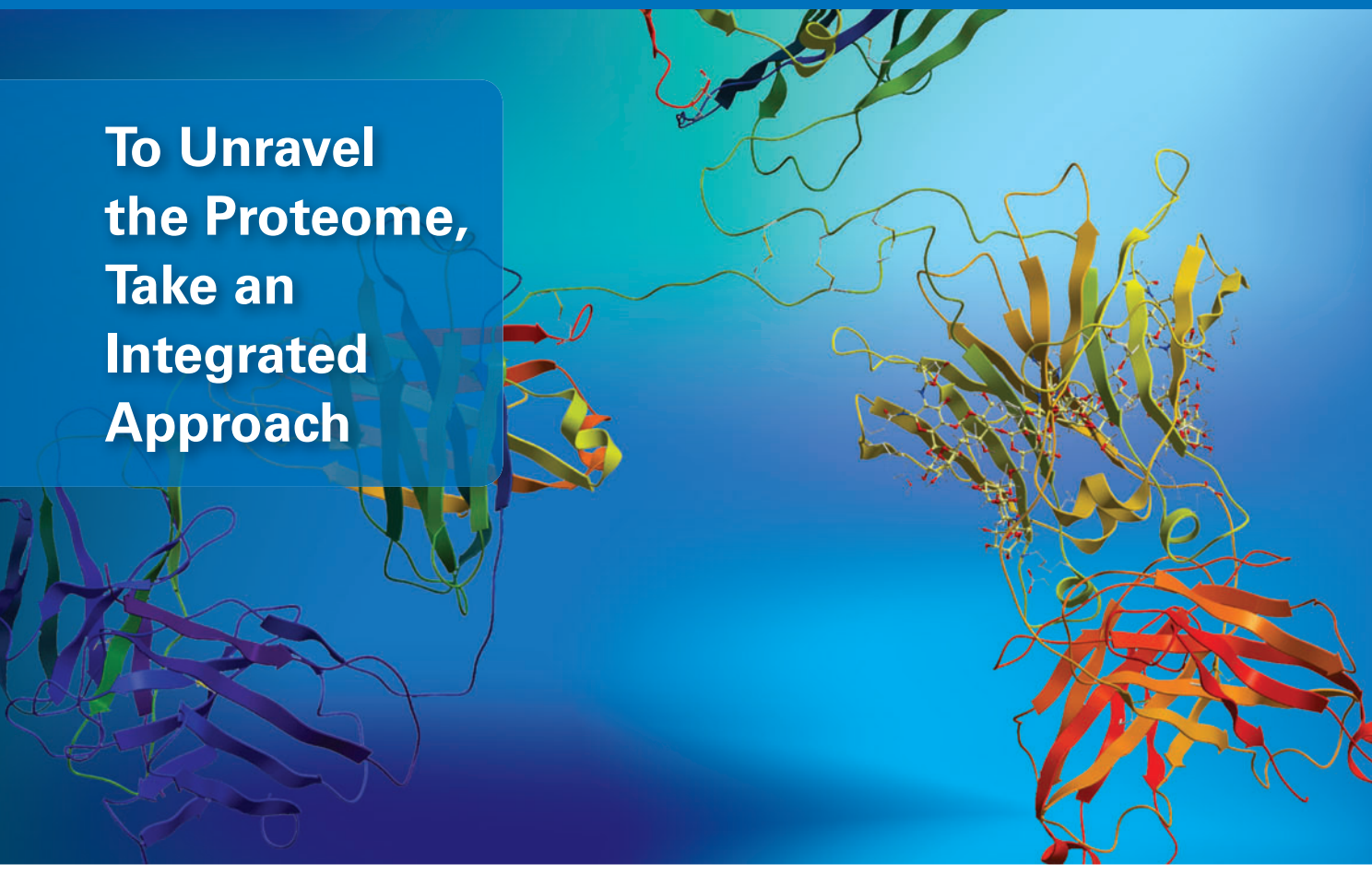

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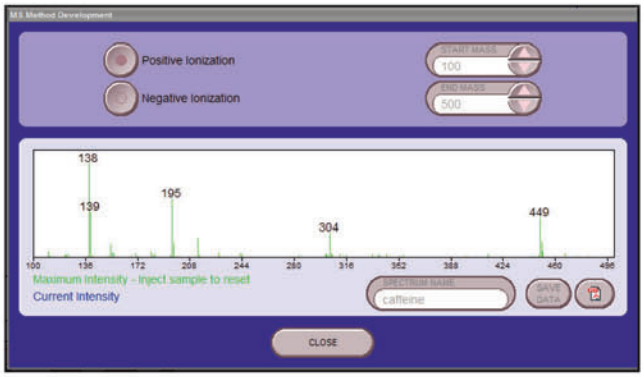

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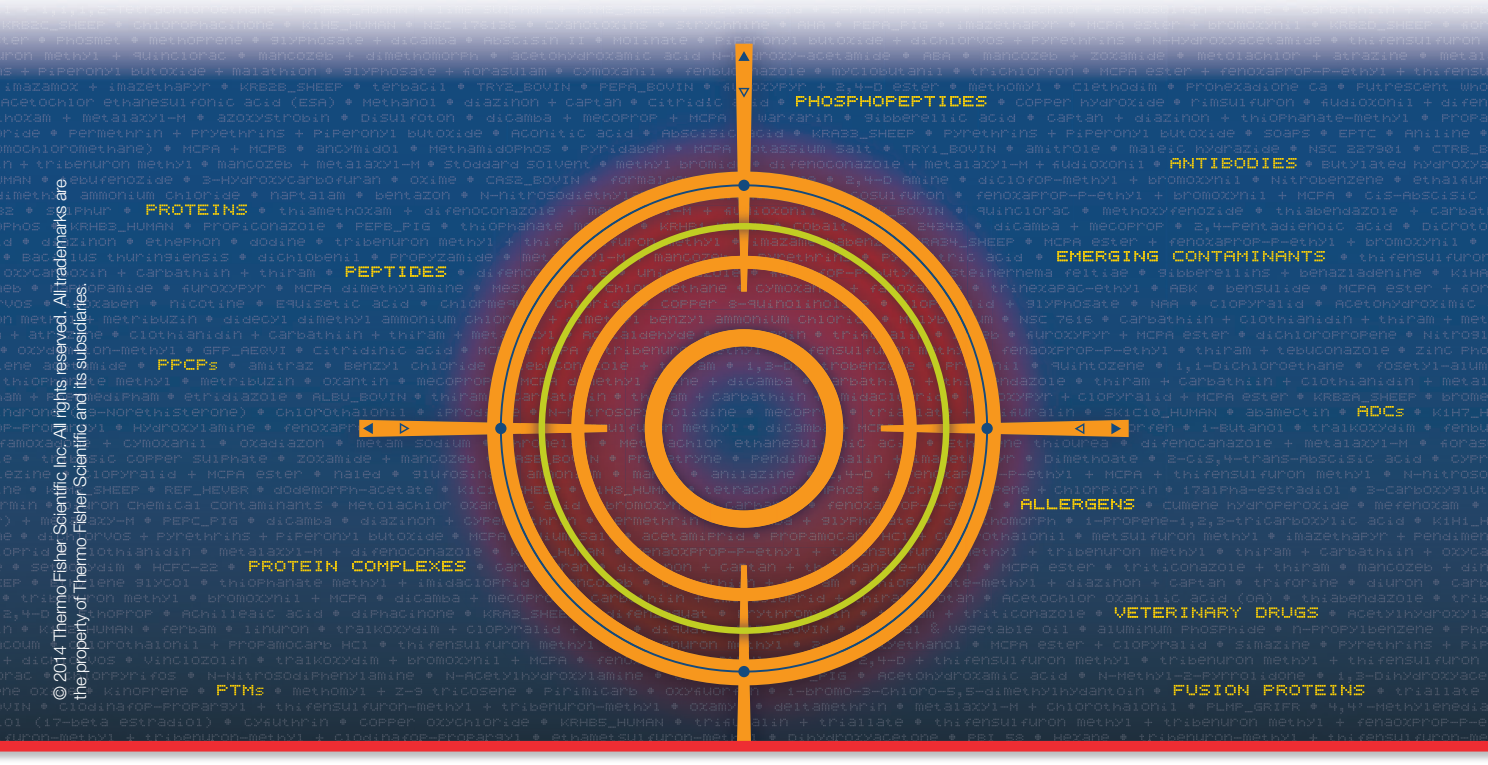

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Application of Ambient Sampling Portable Mass Spectrometry Toward On-Site Screening of Clandestine Drug Operations

Worldwide trends in illicit drug use and production have shifted toward an increase in synthetic analogues and the emergence of new variations in their manufacture. Regulation of common precursors and existing psychoactive substances have led to modifications in clandestine synthetic pathways to use alternate starting materials and produce new structural variants that mimic previous illicit chemicals in physical and physiological properties, while not being officially scheduled through the U.S. Drug Enforcement Agency (DEA). The application of ambient sampling portable mass spectrometry (MS) systems to assist in combating clandestine drug operations represents a potential improvement toward on-site evidence screening over traditional colorimetric or solubility-based field kits, providing superior chemical discrimination with high accuracy, while simultaneously yielding information regarding cutting agents that may also be present. Coupling ambient ionization methods to these portable systems allows direct screening of potential evidence present in any state, from unknown white powders to the volatile solvents used during drug extraction and cleanup.

Seth E. Hall and Christopher C. Mulligan

Recent trends in illicit drug usage have shown an increasing prevalence of synthetic analogs throughout the world. For example, seizures from clandestine methamphetamine synthesis operations in the United States quadrupled over a one year period from 2010 to 2011, and multiple countries throughout Europe have recently reported the seizure and dismantling of clandestine laboratories within their borders for the first time (1). In addition to traditional amphetamine-type stimulants, various new drugs of abuse and modifications of traditional illicit chemicals have gained prominence throughout the world, notably the class of synthetic cathinones marketed as bath salts or plant food (2). Many of the new psychoactives follow the same pattern of being labeled as "not for ingestion" to delay scheduling as controlled substances and to maximize the time they can be sold as "legal highs." After the drug has been scheduled, its usage can gradually decline as it is replaced by a new synthetic analog with a structure just dissimilar enough to circumvent current scheduling, yet possessing moieties that are mimetic in regards to physiological effects (1). Alternately, scheduling of commonly used precursors for target clandestine synthetic drugs is undertaken only to prompt the creation of new synthetic

pathways that utilize legal or easily obtainable starting materials. For example, methamphetamine synthesis through reductive amination of phenyl-2-propanone has become more common with increasing controls on pseudoephedrine-containing pharmaceuticals because of legislation like the 2005 "Combat Methamphetamine Epidemic Act." Furthermore, the analysis of seized pills sold as ecstasy are frequently found to contain other illicit psychoactive substances with more readily available precursors (such as methamphetamine) in addition to 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) (1).

The ongoing variability in illicit drug production represents a challenge to forensic analysis and investigation. Evidentiary analysis of controlled substances accounted for the second highest percentage (33%) of requests submitted to publicly funded forensic laboratories as of the 2009 Census of Publicly Funded Crime Labs, behind only forensic biology requests (34%). Additionally, requests for controlled substance analysis was the second highest evidence category in terms of the ever-growing backlog of casework, forming an estimated 12% of all backlogged samples across the nation (3). While many factors contribute to these backlogs, one major consideration is the large number of samples gathered during evidence collection and the lengthy sample preparation and analysis times required with current analytical methods. Accepted protocols for forensic laboratory analysis typically involve hyphenated mass spectrometry techniques, particularly gas chromatography–mass spectrometry (GC–MS) and liquid chromatography (LC)–MS, often requiring up to 30 min per chromatographic run in addition to the necessary sample preparation constraints (4).

On-site presumptive testing is commonly used not only to aid law enforcement officers in obtaining warrants, but also to prioritize samples that most likely contain chemicals of interest. This, in turn, allows increased efficiency by sending samples of higher relevance to off-site laboratories for confirmation. Because of their easily observable changes and simplistic procedures, colorimetric tests form the majority of presumptive tests (5), yet these field tests possess significant disadvantages. For example, the cobalt thiocyanate test for cocaine (commonly referred to as the Scott test) can produce target-specific color changes from other legal and innocuous substances, including lidocaine and diphenhydramine (found in over-the-counter allergy medicine) (6). Even with the inclusion of additional steps to the test procedure to increase specificity, it can still yield ambiguous results depending on the amount of powdered sample used, placing the burden of proper technique on end users. The Marquis test yields multiple color changes unique to different drugs of abuse, but results can be highly subjective, requiring the use of Munsell color diagrams to distinguish similar colors (7). Because of the higher likelihood of false positive

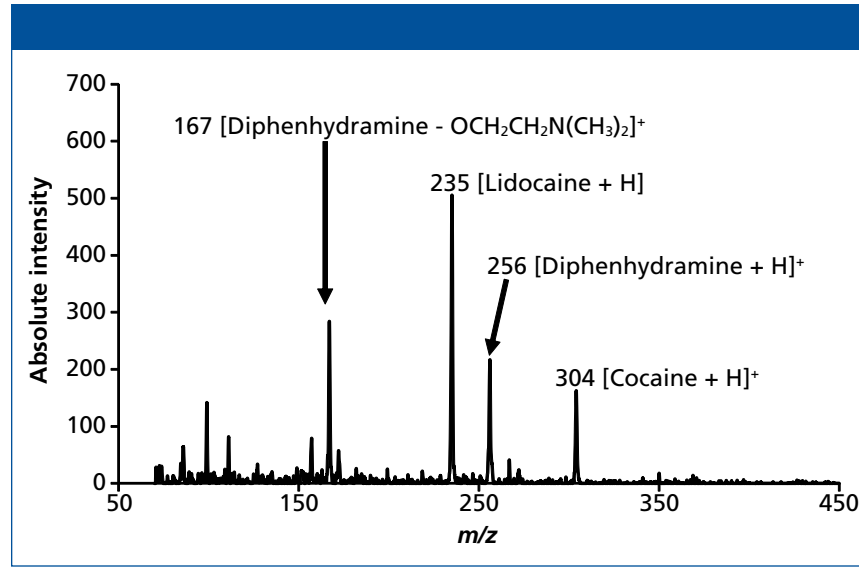

Figure 1: DESI-MS spectrum of a physical transfer swab used to sample a residue of 750 ng each of cocaine, diphenhydramine, and lidocaine swabbed off of a glass substrate.

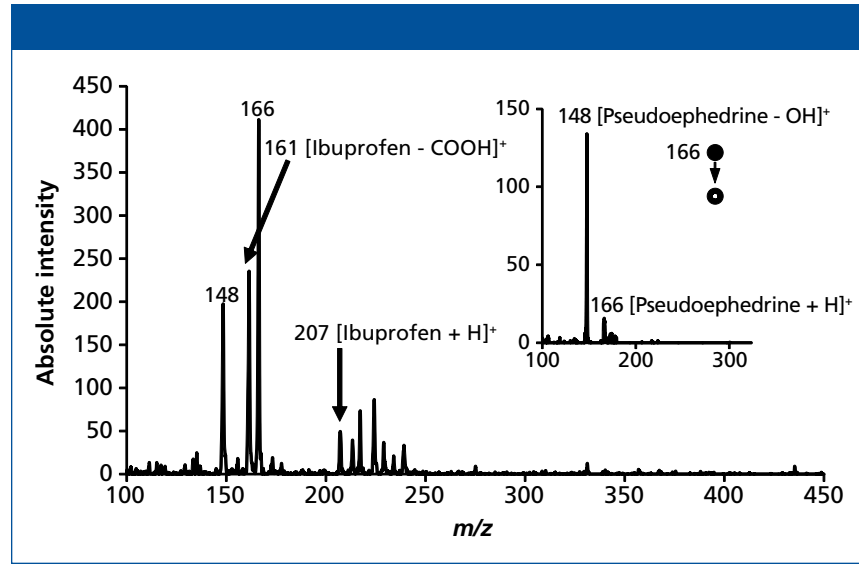

Figure 2: DESI-MS spectrum of an allergy and sinus pill containing ibuprofen and pseudoephedrine. MS-MS spectrum of pseudoephedrine (inset).

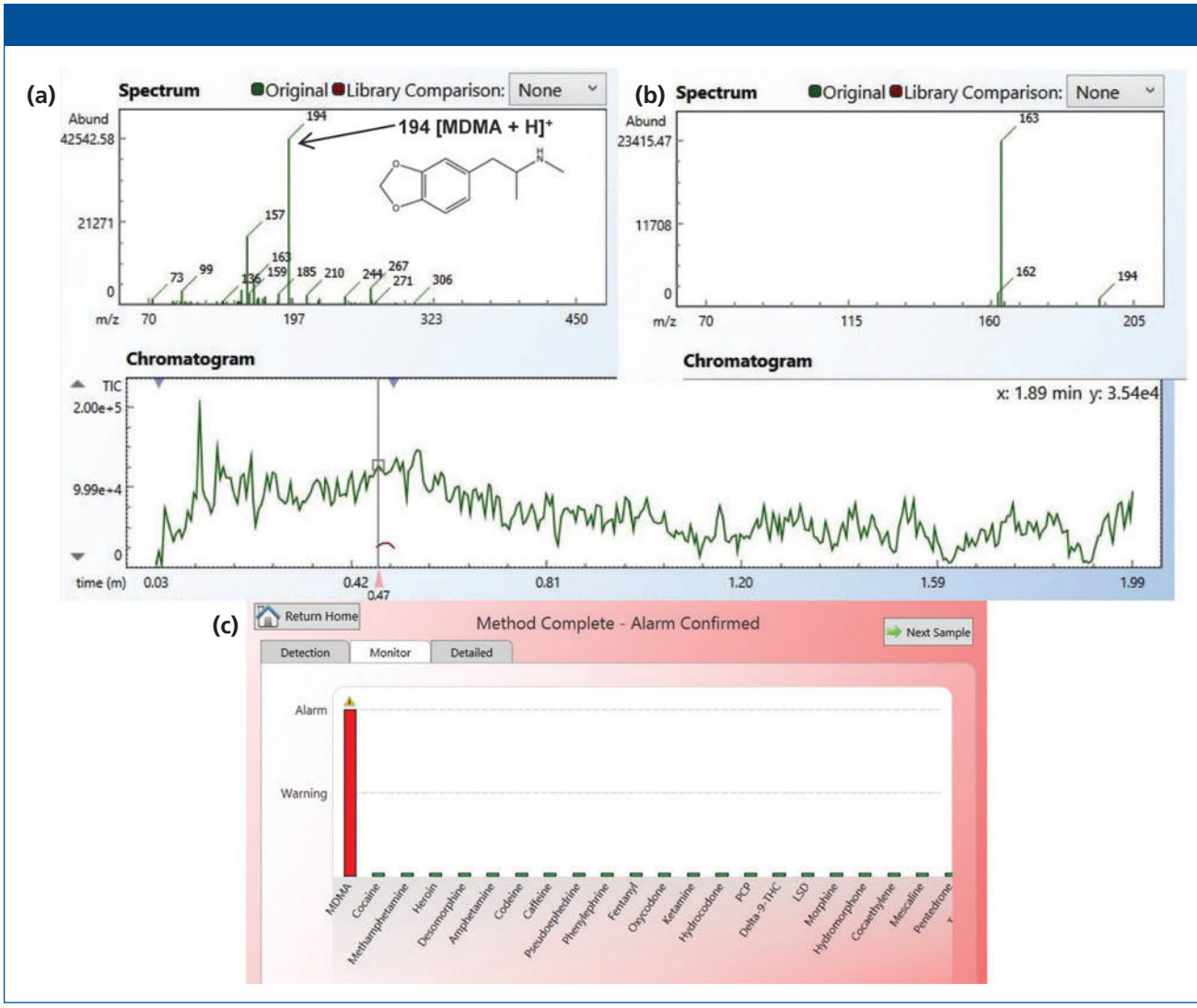

Figure 3: Software screenshots from the analysis of MDMA using a simplified automated end user method: (a) MS spectrum and (b) MS^2 spectrum from the DESI-MS analysis, showing the stored spectral data from the experiment; (c) visual identification screen presented to user after analysis, using "red light/green light" indications for simplification.

or negative responses, a selection of multiple tests must be performed on each sample. Furthermore, tests may require hazardous reagents, such as concentrated acids that must be disposed of after testing, or unique reagents and special preparations for specific controlled substances (7,8).

Major gains in accuracy and precision toward unknown evidentiary analysis could be realized by incorporating portable analytical instrumentation, particularly MS systems (2,9–11) that are adaptable to newly developed ambient ionization methods (12–14) like desorption electrospray ionization (DESI-MS). This work describes the application of a portable ambient sampling MS system coupled with ambient ionization methods for use in the rapid screening of controlled substances and chemicals related to their manufacture. The sensitivity and specificity of MS coupled with the direct sample analysis afforded to DESI has high potential for usage in on-site forensic evidentiary analysis, providing significant advantages over traditional field testing kits while plausibly reducing the burden and backlog of today's forensic laboratory system.

Sample Preparation

Standard solutions of drugs of abuse and related compounds, including cocaine, MDMA, methamphetamine, and lidocaine, were prepared through serial dilution in methanol of chemical standards purchased from Cerilliant Corporation. Additionally, bulk diphenhydramine powder was purchased from Sigma Aldrich and dissolved in methanol to prepare standard solutions. Known masses of analyte were then deposited by spotting 1–10 µL aliquots of standard solutions upon surfaces of interest. Following complete evaporation of the solvent, residues were sampled for DESI-MS analysis using polyurethane foam transfer swabs purchased from Berkshire Corporation. An over-the-counter pharmaceutical containing pseudoephedrine was also purchased and ground into a pow-

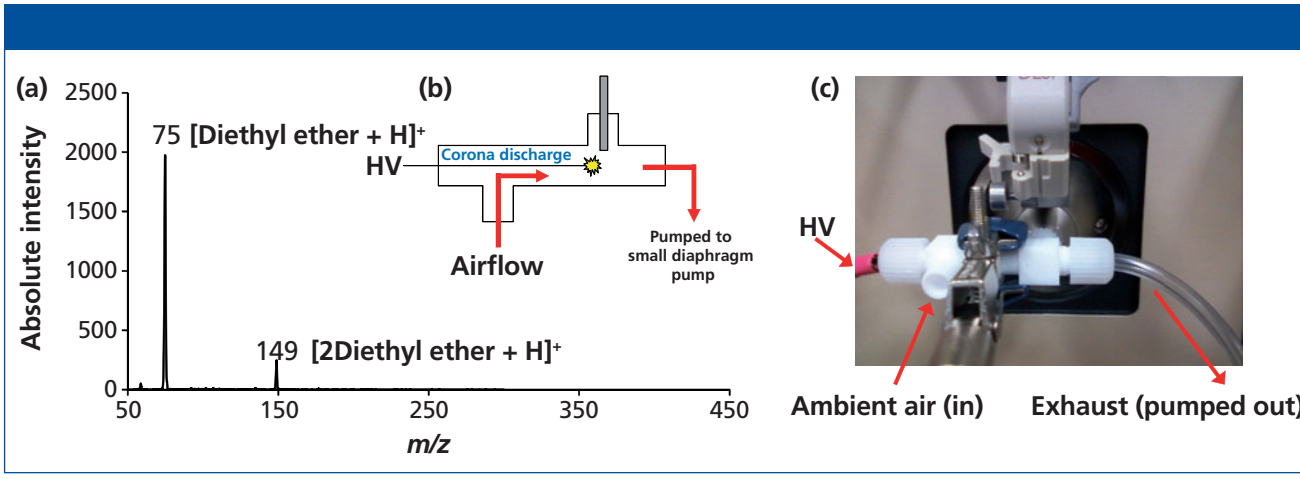

Figure 4: (a) Direct APCI-MS analysis of diethyl ether vapor, resulting in very simple spectra. (b) Diagram of the constructed APCI source, utilizing a corona discharge to produce analyte ions. (c) Photograph of the APCI source coupled to the MS system.

der that was subsequently swabbed and analyzed using DESI-MS without further preparation.

For transfer swab analysis, the swab was first lightly moistened with ~5 µL of methanol. Surfaces of interest were first swabbed along a horizontal axis in a zigzag motion while slowly rotating the swab back and forth to expose all of one face of the swab. The opposite face of the swab was then used to sample the surface along its vertical axis. Swabs were then immediately introduced to the DESI source using a positioning guide and analyzed with slow rotation of the swab to expose the entirety of the swab surface area to the DESI spray. For atmospheric pressure chemical ionization (APCI) studies, gaseous samples from solvents of interest (Sigma Aldrich) were collected from the headspace of storage containers containing the bulk liquids.

Instrumentation

All presented data was collected on a Griffin AI-MS 1.2 cylindrical ion trap mass spectrometer (FLIR Systems). The size (24 in. × 20 in. × 15 in., L × W × H), weight (98 lb), and ruggedness of this instrument makes it an amenable platform for field-based, crime scene investigation applications, and technical specifications have been reported elsewhere (2). DESI analyses were performed using the incorporated ESI-DESI ionization source without modification, which fixes the spray angle at 55° in respect to the sample or surface under investigation. Parameters used for DESI-MS included a spray solvent of 1:1 water–methanol with 0.1% (v/v) formic acid set to a 3-µL/min solvent flow rate, a spray voltage of 4 kV, and nebulizing gas (nitrogen) pressure of 100 psi. Spectra were recorded over an *m/z* 70–450 range, and tandem MS (MS-MS) data were collected on all analytes for identification purposes.

Table I: Detection limits for methamphetamine residue samples from surfaces of interest to clandestine laboratory operations using physical transfer swabbing

Substrate	Detection Limit
Glass	800 ng
Aluminum foil	500 ng
Carbon steel	500 ng
Polypropylene bottle	1000 ng
Nonstick pan	750 ng

Additionally, a custom APCI source was used to enable the analysis of volatile solvents commonly employed in the extraction and drying steps of clandestine syntheses. The home-built source used a small diaphragm pump (KNF Neuberger) to pull ambient air in proximity to a sample of interest into the ionization source through a chemically inert perfluoroalkoxy alkanes [PFA] polymer sampling tube. The sampled air passes over a tungsten needle to which a 4-kV voltage is applied to generate a corona discharge toward the inlet capillary of the MS system. Gaseous analytes present in the sampled air are ionized via charge-exchange with naturally occurring reagent ions formed in the discharge region and transported into the MS inlet (15,16), while exhausted sample is expelled through the pump to allow continuous, stand-alone analysis.

End-User Methods

To provide an analytical technique amenable to field use by potentially nontechnical users, automated instrumental methods were created to detect and confirm chemicals of relevance to drugs of abuse and clandestine synthesis via MS and MS-MS spectral matching. Methods initially search for the presence of protonated ions corresponding to known analytes in the base MS scan. If criteria including a minimum number of scans observed and surpassed intensity threshold are met, the control software indicates a "warning" of a possible target substance and automatically performs the collection of MS-MS spectra for identification purposes; these criteria are optimized and stored in the software for each potential analyte. The collected MS-MS spectra are compared in real time to an onboard spectral library, "alarming"

the verified presence of a target analyte based on known fragmentation patterns collected on analytical standards. After the prompted MS-MS spectral search, the instrument then reverts back to base MS scanning to detect any additional controlled substances or cutting agents that may be present within the sample, and this mode of operation continues for a preset time or until disrupted by the user. After sample screening is complete, users are presented with visual indications of any recorded warnings (yellow light) and alarms (red light) for target compounds. Of importance, at no time are end users required to perform spectral interpretation; all collected data are saved for chain of custody purposes and are instantly accessible.

Results and Discussion

The proposed supplantation of current presumptive tests with MS-based portable instrumentation has the potential to solve the false positive or negative responses that currently hinder field determinations. As mentioned earlier, the Scott test for cocaine will signify the presence of cocaine even when compounds such as diphenhydramine and lidocaine are present. To demonstrate the capability of DESI-capable portable systems toward routine drug evidence identification, a mock sample consisting of 750 ng each of cocaine, diphenhydramine, and lidocaine was deposited as a residue on a glass substrate (19.4 cm^2 total area). After physical contact with the slide, the transfer swab was placed into the DESI ionization source without further preparation, resulting in the spectra seen in Figure 1. Protonated molecules for cocaine, diphenhydramine, and lidocaine are clearly seen at *m/z* 304, 256, and 235, respectively, and all analytes were confirmed via MS-MS (not shown). Also seen is an in-source fragment of diphenhydramine, yielding a signature at *m/z* 167 stemming from the breakage of the ether linkage from the protonated precursor. In addition to being potential sources of false positives for the Scott test for cocaine, diphenhydramine and lidocaine may both be present in cocaine samples as cutting agents; for example, lidocaine is commonly used because of its ability to mimic the numbing properties of cocaine. This result also shows the power of the technique toward detecting and identifying adulterants besides the drug of abuse, information that can be helpful in determining the geographical origin of some seizures (17).

The sensitivity afforded to the technique makes it well suited toward trace residue analysis, which could be advantageous in the assessment of past clandestine laboratory operations. Using the physical transfer swab method, residues from precursor chemicals and illicit products can be rapidly probed and detected. As seen in Table I, methamphetamine residues, seen in DESI-MS spectra as the protonated molecule at *m/z* 150, can be detected from a variety of surfaces including aluminum foil and PTFE-coated cookware at detection limits as low at 500 ng. Limits of detection (LODs) from surfaces are dramatically affected by the properties of the substrate. Relatively smooth surfaces like glass or nonstick cookware allow relatively low LODs to be obtained for drugs of abuse, while porous or geometrically complex surfaces can hinder physical transfer.

While trace analysis has benefits in forensic investigations, most routine evidence will consist of bulk samples (for example, an unknown "white powder"). To show applicability of the system to these types of evidence, powdered pharmaceutical tablets commonly used in clandestine syntheses were successfully investigated. Figure 2 shows the resultant DESI-MS spectrum from a swab used to sample roughly 0.250 g of a powdered Aleve Allergy and Sinus (Bayer Healthcare LLC) tablet, which contains 30 mg and 200 mg of the active ingredients pseudoephedrine and ibuprofen, respectively. After swab transfer, a gentle stream of canned air was then used to remove any loose powder from the swab to prevent clogging of the MS inlet capillary. The spectrum features peaks corresponding to the protonated molecules of both ibuprofen (*m/z* 207) and pseudoephedrine (*m/z* 166), as well as in-source fragment peaks at *m/z* 161 from loss of the carboxylic acid group of ibuprofen and at *m/z* 148 from the loss of a hydroxyl from pseudoephedrine. The inset in Figure 2 shows representative MS-MS spectra obtained on the MS system for protonated pseudoephedrine, yielding the majority fragment ion at *m/z* 148. Although care must be taken to avoid overloading the swab with powder and potentially blocking the inlet capillary, DESI analysis and the swabbing procedure was applicable to bulk powder analysis without preparatory work involving extraction and filtration. This technique can be extended to powdered drugs of abuse, as well, such as our recent work with seized synthetic cathinones (2).

For a truly field-amenable technique, simplification must go beyond just the sampling step, particularly in regards to the user software interface. Figure 3 depicts screenshots of the developed "red light/green light" interface used to analyze a 200-ng residue of MDMA swabbed off of a glass substrate. During the ~2 min a user is prompted to present the swab sample, both MS (Figure 3, MDMA seen at *m/z* 194) and MS-MS (Figure 3b) data have been collected by the instrument and compared to the onboard spectral database. When both MS and MS-MS data match a compound of interest in the database, a color-coded prompt will "alarm" red for its confirmed presence, as seen in Figure 3c. During typical operation of the user interface, only the color-coded prompt is seen, but collected spectra are readily viewable if desired by an advanced operator.

Although DESI-MS has the potential to screen solid and liquid forensic analytes with this portable system, it does not provide application

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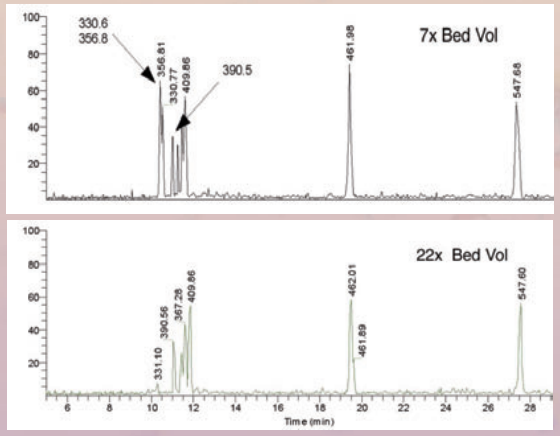

Column: TARGA® C18, 300µm x 30mm Trap
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Gradient: 98% water, 0.1% FA, 2% ACN to ACN, 0.1% FA

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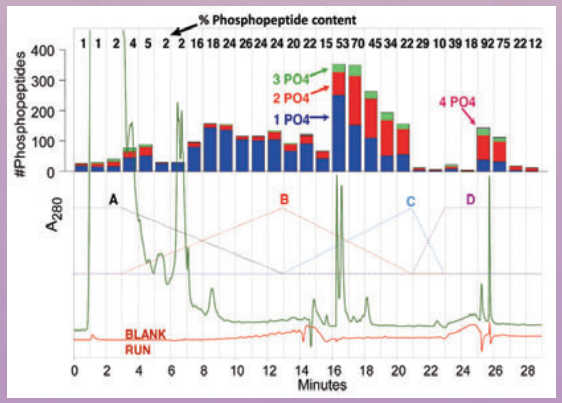

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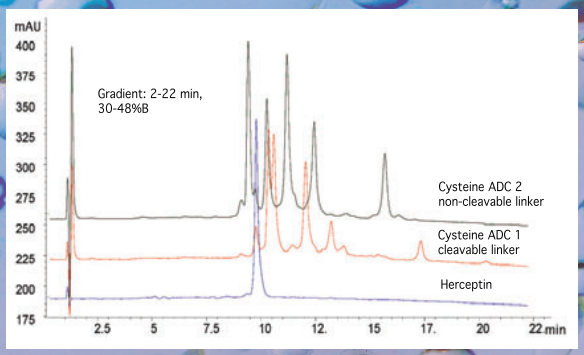

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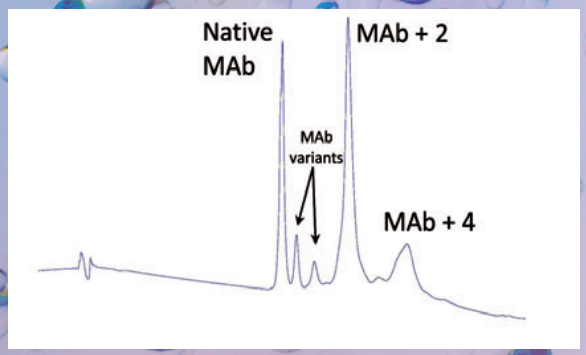

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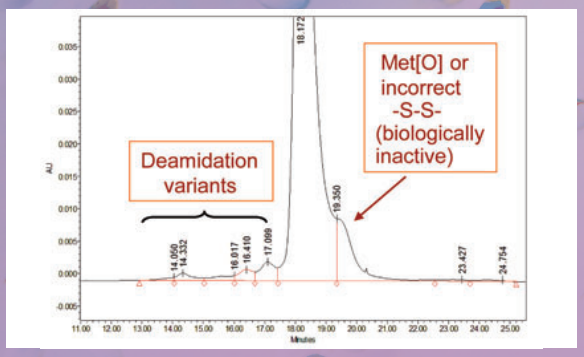

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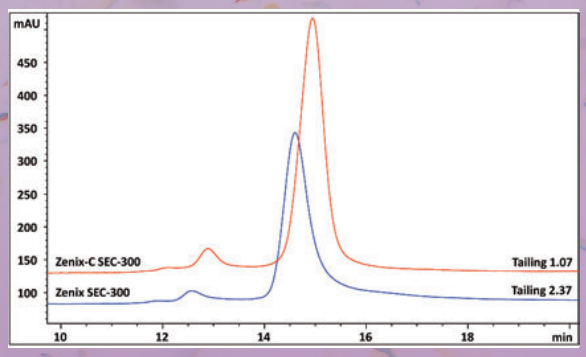
p/n: P104CT0315
Gradient:
30-145mM NH_4OAc, pH 4, 40% ACN; 30°C

MAb Aggregates by SEC



Zenix™ SEC 300 & Zenix™-C SEC-300
(7.8 x 300 mm); 0.5mL/min
Sample: Peggylated Exenatide (PEG 23 KDa)
Isocratic: 50mM NH_4OAc : ACN 90:10 (v/v)

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to gaseous or highly volatile species. As several commercially available solvents are used for liquid–liquid extraction and drying steps in clandestine syntheses, the ability to identify these materials on-site could be of interest. To accomplish screening of volatile solvents with the portable system, a home-built APCI source was constructed and demonstrated. Figure 4a shows the resultant APCI-MS spectrum from the analysis of diethyl ether headspace, which was introduced into the source from the glass storage bottle by use of a sampling tube and external pumping. The simple spectrum collected shows protonated ethyl ether at *m/z* 75, as well as a protonated dimer at *m/z* 149 because of the relatively high gas concentration of the analyte. Figure 4b shows a representation of the constructed source, including the direction of gas flow and placement of corona discharge relative to the MS inlet. Spectra were obtained within seconds of collecting undiluted sample vapor, and signal response rapidly goes back to noise level after the sample tube is removed from the diethyl ether source, showing little to no carryover. The APCI source can be attached over the inlet capillary after the DESI source is lifted to its maintenance position, allowing quick switching of ionization sources in a "plug and play" manner to obtain information about solvents used during a clandestine synthesis; a photograph of the APCI coupling is shown in Figure 4c.

Conclusions

A portable, ambient sampling MS system coupled to DESI-MS was implemented for the screening of both residues and bulk samples of controlled substances and their synthetic precursors. Analysis was rapid and highly accurate when using MS-MS confirmation, surpassing that obtained with the current presumptive test. Additionally, DESI was capable of analyzing multiple types of drugs of abuse using a general-purpose spray solvent. Potential cutting agents and impurities from clandestine precursors were detected, suggesting the possibility of providing supplemental information on a controlled substance's origin or manufacturer. Pharmaceutical tablets and potential retail sources of a synthetic precursor for clandestine drug manufacturing were also successfully identified with no complication from the inactive ingredients and binders present. Automated methods were developed to enable simplified operation by nontechnical end users, using color-coded "red light/green light" indication of any target chemical present. Furthermore, a custom-built APCI source was constructed and demonstrated for the rapid identification of common solvents used in clandestine synthetic methods.

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GC–MS Analysis of an Herbal Medicinal Remedy to Identify Potential Toxic Compounds

In most countries, herbal medicinal products (HMPs) are introduced into the market without proper scientific evaluation or enforced safety and toxicological studies. Consumers can purchase them without a prescription totally oblivious of the potential hazards that HMPs could pose. In this study, the constituents of an unlabeled HMP obtained from a Somalian patient were evaluated. The HMP was attributed to the *Commiphora molmol* species from Somalia. The HMP is used as a topical antimicrobial rub for wound treatment. Various analytical techniques were utilized to isolate and identify its constituents. Volatile components were isolated using both steam distillation and Soxhlet extraction. The extracts were analyzed using a gas chromatography–mass spectrometry (GC–MS) system with a 5% phenyl-methylpolysiloxane capillary column and electron ionization. Compounds were identified by comparison of their mass spectra with a built-in National Institute of Standards and Technology (NIST) mass spectra library. Many sesquiterpenes related to *C. molmol* were identified. Only a few compounds not characteristic of *C. molmol* were detected.

Irene W. Kimaru and Hang P. Nguyen

Herbal medicinal products (HMPs) consist of complex mixtures of one or more plants that contain a range of therapeutically active ingredients arising from plant parts or crude plant extracts. The utilization of HMPs in the treatment of certain human diseases has become common place, particularly in developing countries (1). This is a result of the high costs and side effects of most modern drugs and the fact that HMPs are perceived as effective and safer alternatives. Although HMPs are generally assumed to be safe, they could contain toxic and potentially dangerous constituents (2–4). The majority of HMPs are self-prescribed and are used to treat and manage both minor and long-term illnesses. There is reason for concern over their quality because it can affect their effectiveness and safety.

The greater Rochester, New York, area is home to a large refugee population, including Somali and Congolese people from Africa and Burmese people from Myanmar, which is located in Southeast Asia. These groups of people use many different herbal home remedies from their native countries and, in turn, are significant consumers of HMPs. In these countries, HMPs are introduced into the market without proper scientific evalua-

tions and without any enforced safety and toxicological studies. Consumers can purchase HMPs without prescriptions. Some of these HMPs may be contaminated by other plant species, microorganisms, or contaminants such as pesticides and heavy metals, which could be potentially risky to the patient.

The aim of the present study was to isolate and identify the constituents of an HMP to evaluate the chemical ingredients and identify any potentially toxic compounds. The HMP was obtained from a Somalian patient by a health-care provider from a clinic in Rochester, New York. The most significant issue that the health-care provider faced was that the HMP used by the patient was unlabeled and the supplier was unknown. To the health-care provider, this issue raised many safety concerns for the patient. The patient identified the sample as "malmal" for treatment of skin infection, wounds, and colic pain. The HMP was a hard pale-yellow resin attributed to the oleo-gum resin of *Commiphora myrrha* (Nees) Engl. var. *molmol* species, a native to northeast Africa (chiefly Somalia). The *Commiphora molmol* (Nees) Engl. species is composed of small trees and native to northeastern Africa and Southwestern Asia (5). The bark of these trees and shrubs has fragrant resin that is known for its essential oils (6). These oils are mostly known for their medicinal value (5,6).

Globally, *C. molmol* is one of the most effective herbal medicines for the treatment of sore throats, canker sores, and gingivitis (7,8). It confers many health benefits including treatment of acne, boils, arthritis, wounds, toothaches, and numerous other illnesses (7,8). Many researchers have identified the phytochemicals of resins found in *Commiphora* species (5,6,9–12) and similarly in *C. molmol* (5–8,11–13). The phytochemicals found in the resin of *C. molmol* species are made of a mixture of volatile compounds. Researchers have documented these phytochemicals to consist of monoterpenoids, including α-pinene, camphene, β-pinene, myrcene, and limonene, and sesquiterpenoids, which are generally categorized into these groups: germacrane, eudesmane, guaiane, cadinane, elemane, bisabolane, and oplopane (7,8,11,13). Some of these volatile compounds have been found to exhibit antibacterial and antifungal properties against Gram-positive and Gram-negative bacteria as well as anesthetic and antihyperglycemic properties (5,6).

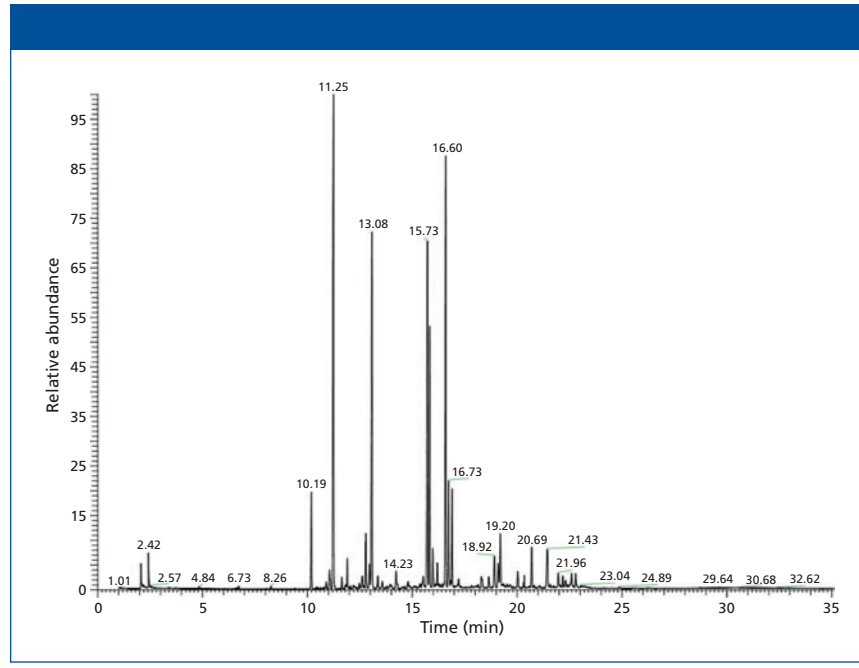

Figure 1: Total ion chromatogram for the steam distillation extract of *C. molmol* resin.

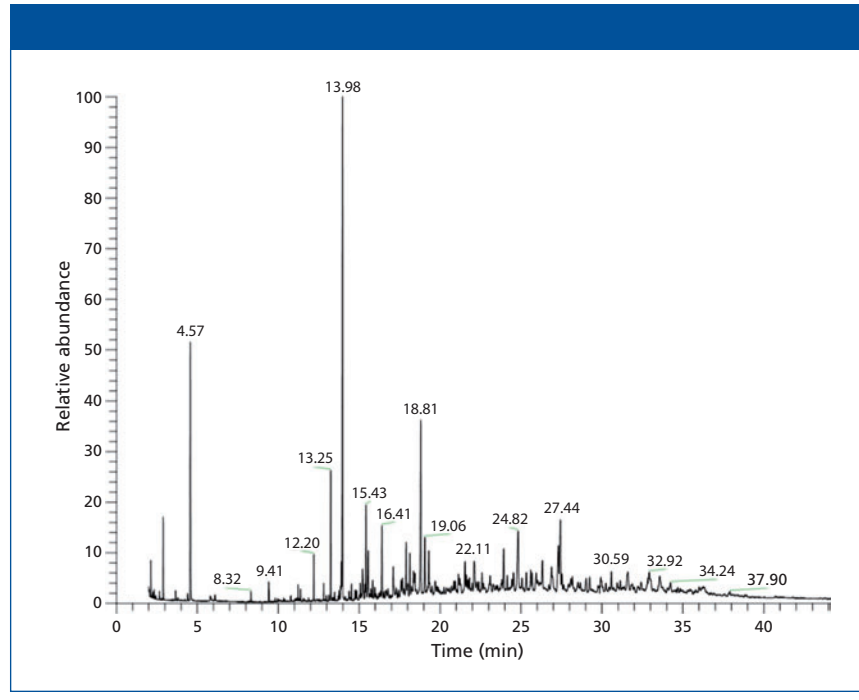

Figure 2: Total ion chromatogram for the ethyl acetate Soxhlet extract of *C. molmol* resin.

Gas chromatography coupled with mass spectrometry (GC–MS) is a commonly used technique for separating and identifying the components of complex volatile mixtures. GC–MS can be a valuable tool in natural product research assisting in the separation and identification of isolated components. Plant essential oils are volatile and well suited to GC–MS analyses. GC is known for its high-resolution separation of structurally similar sesquiterpenes, which are the main constituents of plant essential oils. The use of electron ionization (EI) in MS produces distinctive mass spectral fragmentation patterns enabling mass spec-

Table I: Compounds isolated from *C. molmol* resin using steam distillation

Retention Time (min)	Compound	Compound Nature*	Pharmacological Activity†
2.42	Acetonyldimethylcarbinol		
10.19	δ-Elemene	Sesquiterpene	
10.90	α-Copaene	Sesquiterpene	Anti-plasmodial
11.05	β-Bourbonene	Sesquiterpene	
11.25	β-Elemene	Sesquiterpene	Antitumor
11.63	α-Ylangene		
11.90	γ-Elemene	Sesquiterpene	
12.61	γ-Muurolene	Sesquiterpene	Antifungal
12.76	α-Selinene	Sesquiterpene	
12.94	γ-Gurjunene	Sesquiterpene	
13.06	Isogermafurene or curzerene	Sesquiterpene	Analgesic effects
13.34	γ-Cadinene	Sesquiterpene	
13.57	δ-Cadinene	Sesquiterpene	
14.23	Elemol	Sesquiterpene	
15.51	Cubenol		
15.73	4,4′-Dimethyl-2,2′-dimethylenebicyclohexyl-3-3′-diene		
15.96	T-Cardinol	Sesquiterpene	Antiplasmodial, antifungal, smooth muscle relaxing effect
16.19	Epiglobulol		
16.60	Elemol	Sesquiterpene	
16.73	α-Bulnesene		
17.23	Ledene oxide – (II)	Sesquiterpene	Antibacterial, antioxidant, antifungal
18.30	γ-Eudesmol	Sesquiterpene	
18.64	Eicosapentanoic acid methyl ester	Omega-3 fatty acid	
18.92	Cycloisolongifolene, 8,9-dehydro-9-fomyl	Sesquiterpene	
19.20	Cycloisolongifolene, 8,9-dehydro-9-fomyl	Sesquiterpene	
20.02	Germacrene	Sesquiterpene	
20.33	Strophanthidol		
20.69	Germacrene	Sesquiterpene	
21.43	Aromadendrene oxide		
21.96	Docosahexaenoic acid methyl ester	Omega-3 fatty acid	
22.57	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl		

Compounds identified through mass spectral library matching for the peaks in Figure 1.
*Classification of compounds obtained from references 21 and 22.
†Pharmacological activity information obtained from references 23 and 24.

tra for unknowns to be searched against libraries of EI spectra to attain identification. It is important that the constituents of the essential oils of *C. molmol* species be accurately identified. The chemical constituents of these oils can be separated and precisely identified using GC–MS to determine the quality as well as detect the presence of any harmful chemicals. In the present work, the volatile phytochemicals isolated from an unlabeled HMP obtained from a patient and attributed to *C. molmol* species (Somalian myrrh) from Africa were examined. The phytochemicals were isolated using both steam distillation and Soxhlet extraction. GC–MS was used to separate and identify these phytochemicals and detect any potentially harmful compounds.

Experimental

A resin-based HMP identified as *C. molmol* obtained from a Somalian patient was provided by a health-care provider from a clinic in Rochester, New York, along with the patient's description of how it should be prepared and administered. Steam distillation was used to extract highly volatile and loosely bound volatile organic components of the resin. Steam distillation was chosen because it is analogous to the boiling method that the patient stated to have used to isolate the essential oil from the resin. The patient informed the healthcare provider that to collect the oil, they have to boil the "malmal" and let it cool down before applying the oil to the skin.

The resin (~10 g) was coarsely powdered and extracted using steam distillation and the oil layer was collected from the distillate for GC–MS analysis. Soxhlet extraction was used to extract volatile as well as semivolatile and highly bound volatile organic compounds that could not be isolated effectively using steam distillation. The coarsely powdered resin (~10 g) was extracted using ethyl acetate for 48 h. The ethyl acetate extract was collected and concentrated using rotary evaporation to get a brown mass. The concentrated extract was dissolved in the minimum amount of ethyl acetate for GC–MS analysis.

GC–MS analysis was carried out using a Thermo Fisher Scientific Trace 1300 ISQ single-quadrupole GC–MS system with a built in National Institute of Standards and Technology (NIST) library. The GC–MS system was equipped with a 30 m × 0.25 mm, 0.25-µm d_f HP-5MS UI (5% phenyl)-methylpolysiloxane capillary column (Agilent Technologies, Inc.). To separate the extracts, the capillary column temperature was programmed as follows: 40 °C for 2 min; ramped at 10 °C/min to 140 °C at a step size of 20 °C with a 3-min hold at each consecutive step; ramped to 200 °C at a step size of 10 °C; and maintained at the same rate and hold time with

a final temperature ramp to 260 °C with a 2-min hold time at 220 °C and 250 °C. The injector temperature was set at 220 °C. The carrier gas used was helium at a flow rate of 1.5 mL/min. The concentrated extracts were injected with a 1-µL volume using a split (47:1) injection approach with a split flow of 70.1 mL/min. The instrument was operated using an electron ionization source with the mass transfer line and ion source temperatures set at 250 °C and 220 °C, respectively. The MS data were collected from *m/z* 30 to 600 with a solvent cutoff of 2 min. Data acquisition and processing was performed using XCalibur software (Thermo Fisher Scientific). The separated components were identified through mass spectral comparison with a built in NIST 11 MS library for XCalibur (Thermo Fisher Scientific).

Results and Discussion

In this study, steam distillation of the HMP attributed to *C. molmol* resin produced colorless viscous oil that gave a characteristic myrrh odor. This oil and the ethyl acetate extract obtained through Soxhlet extraction of the resin were both individually analyzed by GC–MS. The GC–MS total ion chromatograms (TICs) of the colorless oil and ethyl acetate extract are presented in Figures 1 and 2, respectively. GC–MS analyses of the extracts led to the identification of 60 compounds. These compounds are listed in Tables I and II according to their elution order from the capillary column. The extracts contained a complex mixture consisting of a high proportion of sesquiterpenes, whereas the fraction of monoterpenes was small. This is in line with the fractions of phytochemicals documented for *C. molmol* by El-Ashry and colleagues (7), Hanuš and colleagues (11), Shen and colleagues (8), and Morteza-Semnani and colleagues (13).

Research has shown that sesquiterpenes are responsible for antimicrobial activities, smooth muscle relaxing, and analgesic effects (8). The resin evaluated in this study contained one sesquiterpene, isogermafurene or curzerene, which is known to have analgesic effects (14). Hossain and colleagues (15) have documented that the sesquiterpenes α-cadinol, caryophyllene oxide, spathulenol, β-guaiene, and ledene oxide-(II) exhibit important antifungal activities. These sesquiterpenes were also identified in the resin evaluated in the present study. Limonene was the only monoterpene identified in the present investigation. Other constituents identified in this work included two lactones — deoxysericealactone and benzofuranacetic acid — as well as two fatty acid methyl esters — eicosapentanoic acid methyl ester and docosahexaenoic acid methyl ester. Other free fatty acids were

Table II: Compounds isolated from *C. molmol* resin using Soxhlet extraction

Retention Time (min)	Compound	Compound Nature*	Pharmacological Activity†
2.13	Isobutyl formate		
2.89	*sec*-Butyl nitrite		
4.57	Acetic acid butyl ester		
6.10	Dimethylfulvene		
8.32	Isobutyl methacrylate		
9.41	Ethyl-3-propylacrolein		
11.23	Decamethylcyclopentasiloxane		
12.20	Limonene	Monoterpene	
13.25	γ-Elemene	Sesquiterpene	
13.89	Germacrene-D-4-ol	Sesquiterpene	
13.92	β-Bourbonene	Sesquiterpene	
13.98	α-Bulnesene		
14.39	β-Ylangene		
14.52	γ-Gurjunene	Sesquiterpene	
14.76	β-Copaene	Sesquiterpene	
15.23	γ-Muurolene	Sesquiterpene	Antifungal
15.43	β-Eudesmene	Sesquiterpene	
15.83	γ-Cadinene	Sesquiterpene	
16.41	Elemol	Sesquiterpene	
17.11	Caryophyllene oxide	Sesquiterpene	Antitumor, anesthetic, antibacterial, anti-inflammatory, analgesic, antioxidant
17.71	Parthenin	Sesquiterpene lactone	
17.91	Spathulenol	Sesquiterpene	Antimicrobial, immunomodulatory and antitumor
18.14	T-Cadinol	Sesquiterpene	Smooth muscle relaxing effect, antifungal, anti-plasmodial
18.35	β-Eudesmol	Sesquiterpene	
18.81	Elemol	Sesquiterpene	
19.06	γ-Elemene	Sesquiterpene	
19.31	Rotundene		
21.14	β-Guaiene	Sesquiterpene	Antifungal
21.53	Norethynodrel	Steroidal progestin	
22.11	Cycloisolongifolene, 8,9-dehydro-9-formyl	Sesquiterpene	
22.57	Benzofuranacetic acid	Lactone	
23.94	Isoaromadendrene epoxide	Sesquiterpene	
24.82	Aromadendrene oxide	Sesquiterpene	
25.69	Octahydroanthracene	PAH	
26.33	Eicosapentaenoate	Fatty acid	
26.90	Octahydro-naphthalenol	PAH	
27.30	Methyl 6,9-octadecadienoate	Fatty acid	
27.44	Phenanthrenone	PAH	
27.53	Eicosatriynoic acid	Fatty acid	
30.59	Phenyloctahydro-naphthalenone	Aromatic ketone	
31.60	Deoxysericealactone	Lactone	

Compounds identified through mass spectral library matching for the peaks in Figure 2. PAH = polycyclic aromatic hydrocarbon. *Classification of compounds obtained from references 21 and 22. †Pharmacological activity information obtained from references 23 and 24.

also identified, including methyl 6,9-octadecadienoate and eicosatriynoic acid. In a study by Ammar and colleagues (16), such fatty acids were also detected in the phytochemical study of *Commiphora myrrha* also known as *C. molmol* and attributed to adulteration or bad storage conditions that resulted in the degradation of high molecular weight compounds.

Parthenin, a sesquiterpene lactone that is known for its cytotoxicity (17), was also identified in the present study. Parthenin is an active constituent of *Parthenium hysterophorus*, a weed that is known to cause numerous health hazards (18). The presence of parthenin in the resin could be attributed to the contamination of the resin by the weed, which is very prolific and now is found in Africa, Australia, Asia, and the Pacific islands. A few other compounds that are not commonly found in the resin of *C. molmol* were also identified in the present study. These include acetonyldimethylcarbinol, isobutyl formate, *sec*-butyl nitrite, acetic acid butyl ester, dimethylfulvene, isobutyl methacrylate, ethyl-3-propylacrolein, strophanthidol, decamethylcyclopentasiloxane, phenanthrenone, octahydro-naphthalenone, and 1,2,3,4,5,6,7,8-octahydroanthracene, which probably were found because of the contamination of the resin by other plant species and degradation resulting from poor storage conditions or adulteration. A report by C. R. Ganellin and D. J. Triggle (19) shows that acetonyldimethylcarbinol has been isolated from the sleep grass species *Stipa vaseyi*. Similarly, octahydroanthracene, octahydro-naphthalenone, and phenanthrenone are analogs of polycyclic aromatic hydrocarbons (PAHs) and could potentially be harmful because of the reported toxicity of PAHs (20).

Conclusion

The aim of this investigation was to qualitatively determine the chemical constituents of an unlabeled herbal medicinal remedy obtained from a Somalian patient and attributed to the *C. molmol* species. Volatile constituents were isolated from the resin-based herbal medicinal remedy using both steam distillation and Soxhlet extraction and were analyzed using GC–MS. Many compounds were detected with the major constituents being sesquiterpenes. These sesquiterpenes were similar to those that have previously been documented for *C. molmol* species (7,8,11,13). Several of these sesquiterpenes have been reported to show analgesic, anti-inflammatory, and antifungal activities (14,15). A small number of compounds that are not related to those typically found in *C. molmol* were identified. The presence of these compounds is indicative of the potential for contamination that can affect the quality, efficacy, and safety of such an unlabeled and unregulated herbal medicinal remedy. This investigation confirms that if extracts of the herbal medicinal remedy obtained from the patient and credited to *C. molmol* are to be used for medical purposes, safety and toxicity concerns must be taken into account.

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Large-Scale Targeted Protein Quantification Using Wide Selected-Ion Monitoring Data-Independent Acquisition

This article describes the development of a new data-independent acquisition (DIA) workflow for protein quantification that uses a mass spectrometer that combines three types of mass analyzers to achieve lower limits of detection (LOD), higher sensitivity, more accurate quantitative results, wider dynamic range, and better reproducibility than existing high-resolution accurate-mass (HRAM) tandem mass spectrometry (MS-MS) DIA workflows. The combined use of a mass-resolving quadrupole, orbital trap mass analyzer, collision cell, and linear ion trap with appropriate software was shown to minimize interferences caused by product ions from coeluted background compounds and significantly improve the analysis of highly complex samples.

Reiko Kiyonami, Michael Senko, Vlad Zabrouskov, Andreas F.R. Hühmer, Jarrett Egertson, Sonia Ting, and Michael MacCoss

Proteomics studies are rapidly turning from qualitative to largely quantitative experiments to better understand the functions and interactions of proteins in biological systems and verify long lists of putative biomarkers. The extreme complexity and large dynamic range of proteins in standard biological samples challenge traditional data-dependent workflows. They necessitate very fast tandem mass spectrometry (MS-MS) with simultaneous high sensitivity and reproducibility. Without these, it is difficult to achieve the depth of sample interrogation necessary to identify and quantify the same targets in replicate runs to accurately determine differences among samples.

Recently, several data-independent acquisition (DIA) approaches have been explored to increase reproducibility and comprehensiveness for better quantification (1,2). One such approach acquires only high-resolution, accurate-mass (HRAM) MS-MS data (no full scans) generated with very wide isolation windows (~25 *m/z*). These data are then interrogated for quantification using extracted ion chromatograms of targeted fragment ions only. The quantitative performance from using only HRAM MS-MS data generated with wide isolation windows is potentially compromised by interfering product ions from coeluted background compounds. For highly complex biological

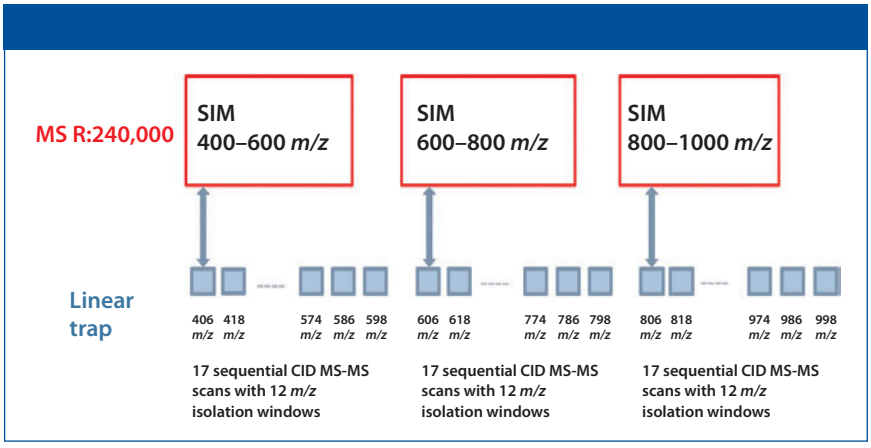

Figure 1: WiSIM-DIA workflow schematic for the collection of HRAM SIM scans at 240k resolution while simultaneously acquiring 17 sequential CID.

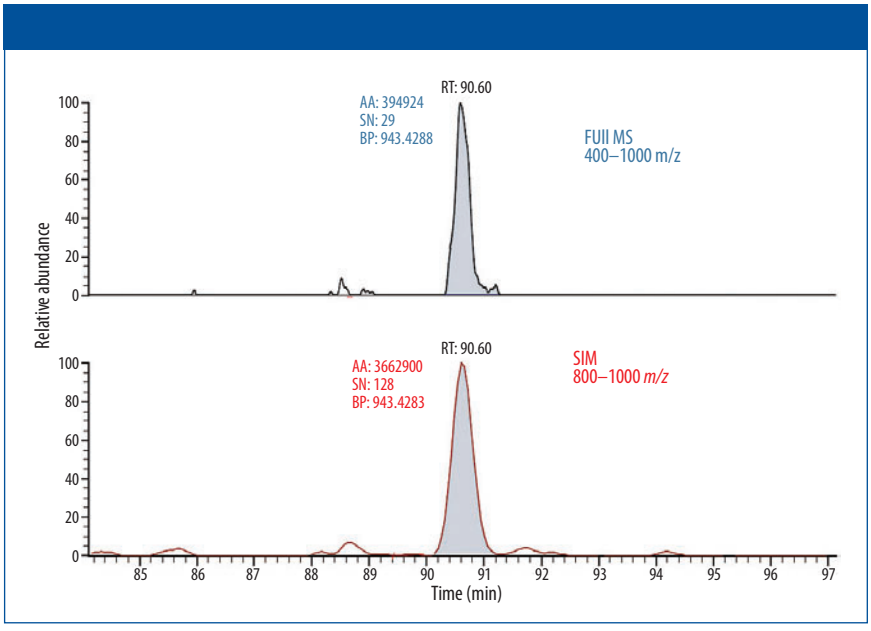

Figure 2: Significant increase in sensitivity using wide-window SIM scans compared to full MS scans, MS-MS scans of 12 *m/z* isolation.

samples, a portion of the targeted fragment ions are potentially contaminated by fragment ions from non-targeted peptides that coelute in the same retention time window and are coisolated within a 25 *m/z* wide window. These interferences can result in higher limits of detection (LOD) and limits of quantitation (LOQ), less accurate quantitative results, and narrower dynamic range (2,3).

Recent MS architecture, which combines a mass resolving quadrupole, an orbital trap mass analyzer, a collision cell, and a linear ion trap mass analyzer, was applied to achieve lower LOD and high selectivity. The orbital trap detector is designed to provide a resolving power of 240,000 at a scan rate of 1.5 Hz. The linear ion trap can collect more than 20 high-quality collision-induced dissociation (CID) MS-MS spectra in 1 s. This architecture enables parallel orbital trap selected-ion monitoring (SIM) scanning with rapid and sensitive targeted ion trap MS-MS detection to generate data with the resolution and sensitivity required for accurate data-independent analyses.

A DIA workflow was developed that takes advantage of this architecture. It simultaneously collects HRAM SIM spectra with wide isolation windows in the orbital trap detector and CID MS-MS spectra in the linear ion trap detector. This method is referred to as wide selected-ion monitoring, data-independent acquisition, or WiSIM-DIA. The WiSIM-DIA method consists of three SIM scans acquired with extremely high resolution and mass accuracy, and with wide isolation windows (240,000 resolution full width at half maximum [FWHM] at *m/z* 200, and <3 ppm, respectively). It covers all precursor ions between *m/z* 400 and

Table I: Experimental conditions

Nano-LC Conditions	
System	Thermo Scientific EASY-nLC 1000
Column	Thermo Scientific EASY-Spray PepMap C18 column (50 cm × 75 µm, 2-µm particle size)
Flow rate	300 nL/min
Buffer A	0.1% formic acid–water
Buffer B	0.1% formic acid–acetonitrile
Gradient	2% B in 5 min, 2% B to 20% B in 100 min, 20% B to 32% B in 20 min
Sample loading	Directly loaded on column
Injection amt.	1 µL
MS Conditions	
System	Thermo Scientific Orbitrap Fusion MS system equipped with a Thermo Scientific EASY-Spray source
Capillary temperature	275 °C
Spray voltage	1800 V
S-Lens RF level	60
FT	SIM
Resolution	240,000
AGC target	3×10^4
Isolation width	200 *m/z*
Maximum ion injection time	50 ms
CID	MS-MS
Rapid CID	MS-MS, AGC target 5×10^4
Isolation width	12 *m/z*
Maximum ion injection time	47 ms
Collision energy (%)	30
Software	Thermo Scientific Pinpoint software version 1.3 Thermo Scientific Proteome Discoverer

1000 while increasing the sensitivity relative to standard full-scan analysis. The use of a wide SIM window for the collection of ions in the first step of this method leads to the selective enrichment of analytes in the selected SIM mass range and enables the detection and quantification of low abundance peptides in the final targeted extraction step. In parallel with each SIM spectrum, 17 sequential ion trap MS-MS spectra with 12 *m/z* isolation windows are acquired to detect fragment ions across each associated 200 *m/z* SIM mass range (Figure 1). Quantification of targeted proteins after data acquisition is carried out using extracted ion chromatograms of HRAM SIM data with a ±5 ppm window. Simultaneous peptide sequence confirmations are carried out using CID MS-MS relying on a spectral library.

The main advantages of the WiSIM-DIA workflow, relative to the wide-window MS-MS-only DIA workflows, are that there is enough time to acquire HRAM precursor data for accurate and sensitive detection and quantification, while the fast and sensitive MS-MS can be used for reliable confirmation. As a result, the quantitative performance of this method does not suffer from the drawbacks of wide-window MS-MS-only DIA workflows. All quantitative analyses are carried out using HRAM SIM data. The CID MS-MS data are used only for peptide confirmation. Therefore, the fragment ion contamination caused from nontargeted, coeluted peptides does not impact the quantitative results. This WiSIM-DIA approach increases selectivity, sensitivity, and reproducibility, ultimately improving the accuracy and throughput of the quantification experiments by using precursor ions collected with very high (240,000) resolving power. The fragment ions detected in the WiSIM-DIA approach, which are used only for confirmation of identity, are from narrower precursor populations (12 *m/z*), and are detected by a more sensitive mass analyzer. Therefore, this WiSIM-DIA approach can quantify all detected peptide precursor peaks with high sensitivity and selectivity, yielding a complete and reproducible quantitative data set in a single sample injection. In addition, all MS-MS product ion information is recorded for sequence confirmation of any peptide of interest within the mass range of *m/z* 400–1000 by subsequent confirmatory matching of specific product ions of an easy-to-generate, sample-specific spectral library. The details of this unique WiSIM-DIA workflow and its quantitative performances are reported here.

Experimental

Sample Preparation

Sample 1 (a dilution series for evaluating detection limits and linear dynamic range): A mixture of 14 isotopically labeled synthetic peptides was spiked into a complex *E. coli* digest (500 ng/µL) at five different concen-

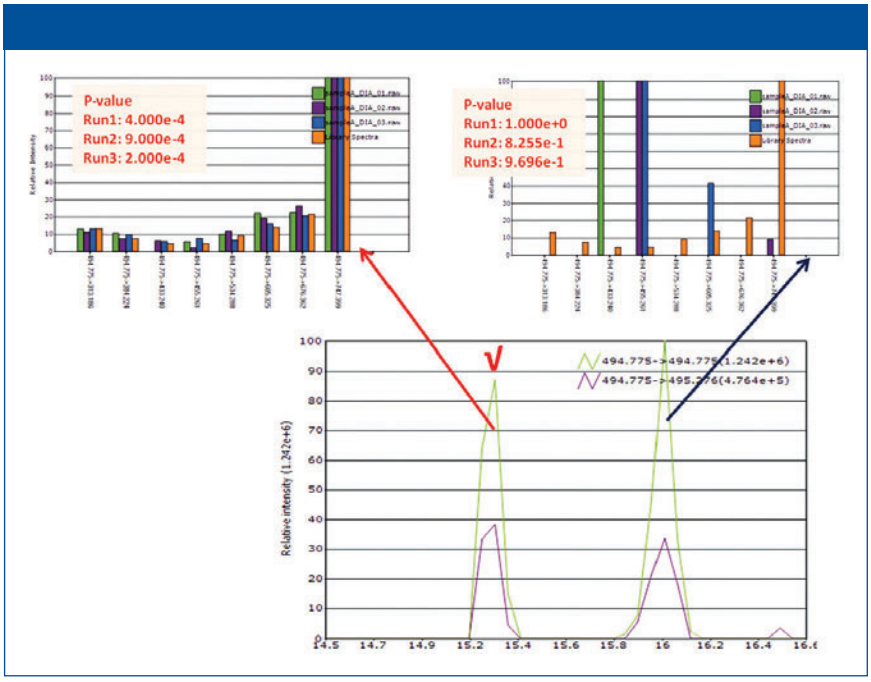

Figure 3: WiSIM-DIA verifies peptide identification using spectral matching. Confident peptide sequence confirmation by extracting the eight most intense fragment ions from the DIA-CID MS-MS data and matching the fragment ion distribution to the CID MS-MS spectrum stored in the spectral library. The peptide eluted at 15.3 min is confirmed to be the correct target with *p*-values less than 1×10^4. The peak with the precursor ion mass at 16 min elution time is a contaminant.

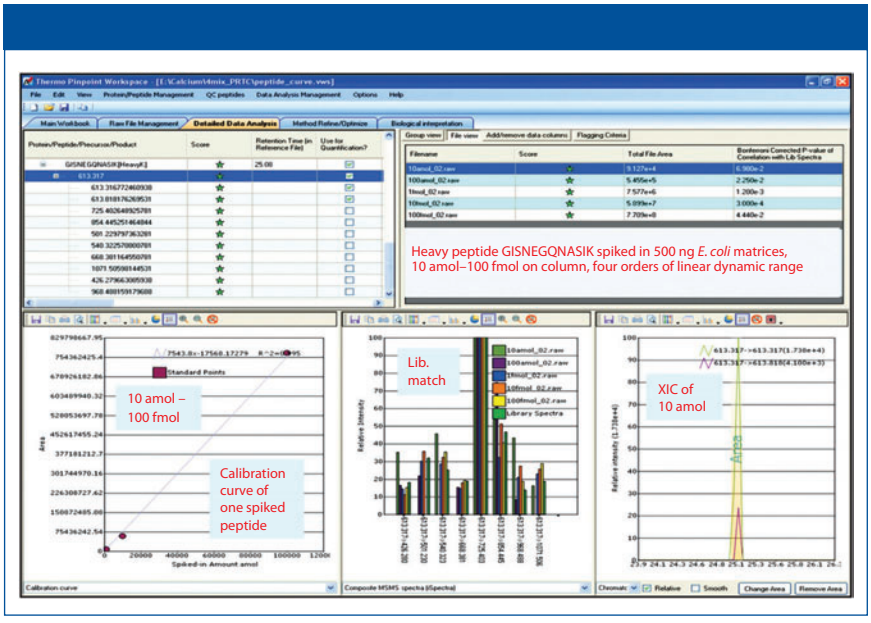

Figure 4: WiSIM-DIA workflow provides 10 amol LOD with four orders of magnitude linear dynamic range width in parallel.

trations (0.01, 0.1, 1, 10, and 100 fmol/µL) to generate a dilution series.

Sample 2 (for evaluating detection and quantification of low- to high-abundance proteins in a complex mixture): A digest of six proteins with concentrations covering five orders of magnitude (0.01, 0.1, 1, 10, 100, and 1000 fmol/µL) was spiked into an *E. coli* digest (500 ng/µL).

Sample 3 (a sample set for large-scale relative quantification comparison of separate complex mixtures): An *E. coli* digest was analyzed at 250 ng and 500 ng on column.

Table I shows the experimental conditions for the nano-LC, MS, and software systems used.

Six experiments were conducted: Three Orbitrap analyzer (Thermo Fisher Scientific) SIM experiments (experiments: 1, 3, and 5) covered the precursor mass range of *m/z* 400–1000. Each SIM experiment was followed by a targeted MS^n experiment, which carried out 17 consecutive CID MS-MS acquisitions using a predefined precursor ion inclusion list (Figure 2). This covered the associated Orbitrap analyzer SIM experiment mass range (experiments: 2, 4, and 6). The instrument method setup can be downloaded at http://planetorbitrap.com.

Data Processing

Dedicated software was used for targeted qualitative and quantitative data extraction after data acquisition. A spectral library containing precursor ion and CID MS-MS information was established using previous discovery data collected on a similar MS system. The extracted-ion chromatograms of isotope ^{12}C and ^{13}C precursor ions per targeted peptide were used for quantification with a ±5 ppm window. The eight most intense fragment ions (b and y types) detected from discovery data for each peptide were used for confirmation through spectral library matching.

Results and Discussion

Decreased Detection Limits with SIM Acquisitions Relative to Full MS Acquisitions

The newly developed WiSIM-DIA workflow provided quantitative results with a label-free approach. Standard, label-free experiments rely on full-scan data for quantification of the precursors; this often compromises the detection of low-intensity ions during the elution of more intense species. The WiSIM-DIA approach was developed to alleviate this issue through the use of sequential SIM acquisitions for quantification. Unlike a full mass-range acquisition, the SIM acquisition with a 200 *m/z* isolation window effectively "en-

riches" all ions in that window while excluding all other ions outside the mass range of interest. As a result, the SIM acquisition, even with a 200 *m/z* isolation window, can provide much higher sensitivity for detecting low-abundance peptides compared to full-mass-range acquisitions. An example of this is shown in Figure 2 where simply using a SIM acquisition allows for a nearly fivefold increase in signal-to-noise ratio. When applicable, the expected retention time per targeted peptide was also considered when extracting the quantitative information.

Peptide Sequence Verification Using Ion Trap CID MS-MS

For peptide sequence confirmation, the data-independent CID MS-MS acquisitions with a 12 *m/z* window over each *m/z* 200 SIM window were used. Unlike data-dependent analysis that requires triggering of a specific precursor for fragmentation and detection, leading to a lack of reproducibility between replicates, data-independent MS-MS acquisitions are scheduled and cover the complete mass range in every analysis. Eight of the most intense fragment ions (b and y types) identified in discovery (data-dependent) experiments were targeted in the ion trap CID MS-MS data using a ±500 ppm extraction window. They were then used for sequence verification through spectral library matching. A *p*-value (probability of random spectral matching) was calculated using standard statistical methods to compare the multiple product ion distribution extracted from the DIA MS-MS data with MS-MS data of a peptide stored in a spectra library (4). A smaller *p*-value represented a better match between the observed data and the spectral library. A peptide with a *p*-value of less than 0.1 was considered to be identified with high confidence by the library match (3). The ion trap CID MS-MS system provided high sensitivity and good spectral quality without prior knowledge of precursor ion charge state, unlike collision cell fragmentation. It was able to confirm the targeted peptide sequences with sufficient selectivity through the spectral library matches (Figure 3).

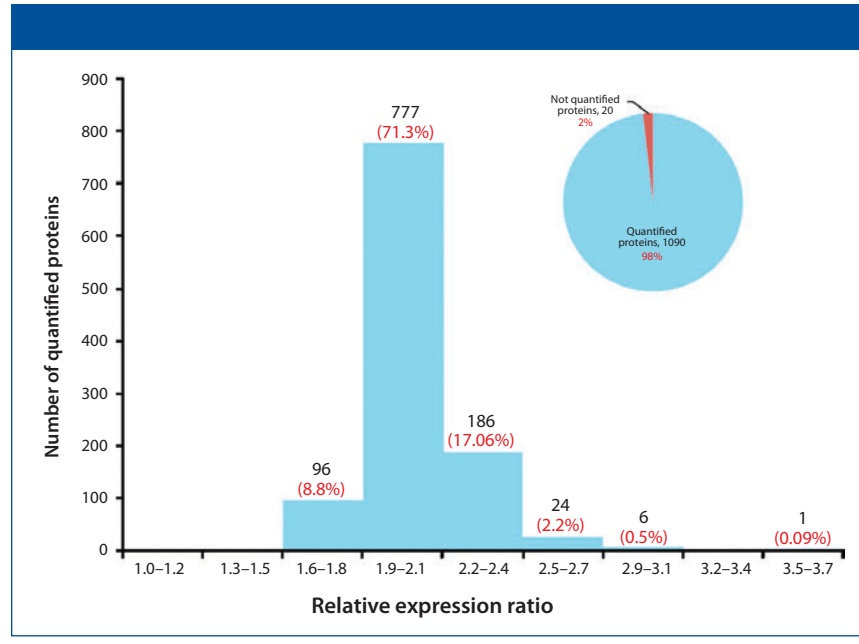

Figure 5: Quantification summary for quantifying 1100 *E. coli* proteins.

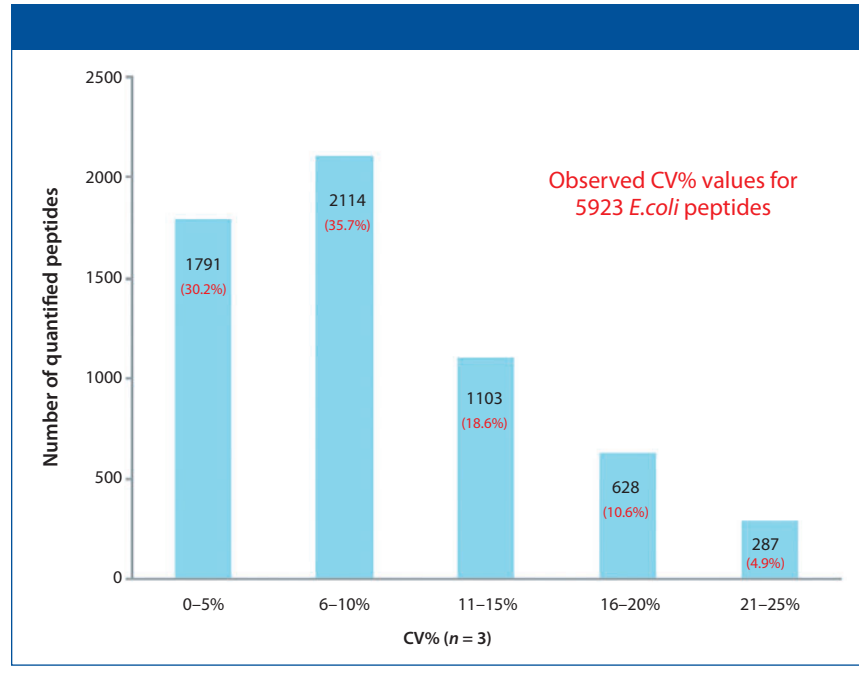

Figure 6: Coefficient of variation of 5923 quantified *E. coli* peptides.

Evaluation of Detection Limits and Linear Dynamic Range of the WiSIM-DIA Workflow in a Complex Mixture

The detection limits and the quantitative dynamic range of the unique WiSIM-DIA workflow were evaluated using a dilution series of a mixture of 14 isotopically labeled synthesized peptides spiked into *E. coli* digests (500 ng/µL) at five different concentrations (0.01, 0.1, 1, 10, and 100 fmol/µL). In addition to the decreased detection limit as a result of using SIM acquisitions, the very high resolving power of 240,000 enabled unambiguous detection of targeted peptide peaks from matrix interferences, even at the lowest 10 amol concentration level. As a result, LODs down to 10 amol were achieved. Table II shows

Table II: Coefficients of variation for the 14 isotopically labeled peptides spiked into a complex matrix over five orders of magnitude

Peptide	Precursor Ion (*m/z*)	CV% (*n* = 3)				
		0.01 fmol	0.1 fmol	1 fmol	10 fmol	100 fmol
SSAAPPPPPR[HeavyR]	493.768	31	7	1	10	2
HVLTSIGEK[HeavyK]	496.287	36	5	5	5	6
IGDYAGIK[HeavyK]	422.736	24	12	2	6	7
TASEFDSAIAQDK[HeavyK]	695.832	9	2	3	4	1
SAAGAFGPELSR[HeavyR]	586.800	46	14	6	4	2
ELGQSGVDTYLQTK[HeavyK]	1773.896	44	3	2	7	1
GLILVGGYGTR[HeavyR]	558.326	40	6	5	9	5
GILFVGSGVSGGEEGAR[HeavyR]	801.411	17	9	4	8	3
SFANQPLEVVYSK[HeavyK]	745.392	21	7	0	9	4
LTILEELR[HeavyR]	498.802	12	5	6	8	4
GISNEGQNASIK[HeavyK]	613.317	7	12	3	5	5
NGFILDGFPR[HeavyR]	573.302	49	5	2	12	1
ELASGLSFPVGFK[HeavyK]	680.373	36	7	4	13	4
LSSEAPALFQFDLK[HeavyK]	787.421	63	22	13	10	1

the observed coefficient of variation (CV) of each spiked peptide from triplicate runs at each concentration level. All spiked peptides were detected at the lowest concentration level tested (10 amol). The CVs of all spiked peptides at 100 amol were less than 15% with the exception of one peptide (22%) (Table II). With the low LOD and high selectivity of the DIA workflow, each spiked peptide was able to be detected over four orders of linear dynamic range (Figure 4).

Detection and Quantification of Low- to High-Abundance Proteins in a Complex Mixture in a Single Run with the WiSIM-DIA Workflow

To further evaluate the ability of the new WiSIM-DIA workflow to quantify targeted peptides over a wide dynamic range in a single run, six bovine protein digests were spiked into a 500-ng *E. coli* matrix at an abundance level that spanned over five orders of dynamic range (low-abundance to high-abundance proteins). Among the six spiked proteins, bovine serum albumin (BSA) was spiked at the lowest abundance level (10 amol on column) and beta-lactoglobulin was spiked at the highest abundance level (1 pmol on column) (Table III). The sample was run in triplicate using the WiSIM-DIA workflow. The HRAM SIM acquisitions reproducibly provided low limits of detection, high selectivity, and wide dynamic range for all six spiked proteins over five orders of magnitude of dynamic range. Table III summarizes the detected peptides per spiked protein and observed %CVs. Approximately 90% of quantified peptides gave %CVs less than 10%.

Large-Scale Relative Quantification Using the WiSIM-DIA Workflow

To further evaluate the analytical precision and quantitative accuracy of the WiSIM-DIA workflow when applied to large-scale quantitative experiments, a 500-ng on column *E. coli* digest and a 250-ng on column *E. coli* digest were compared. Each sample was run in triplicate using the WiSIM-DIA instrument method for quantification.

For targeted *E. coli* peptide selection and spectral library generation, a 1-µg *E. coli* sample was run in triplicate with a standard shotgun data-dependent acquisition (DDA) experiment using the orbital trap analyzer full scan and ion trap CID MS-MS analyses. A database search of the DDA raw files was performed against an *E. coli* database. The SEQUEST HT search engine was used. A target decoy peptide spectral match (PSM) validator (0.01 FDR strict–0.05 FDR relaxed) was used for PSM validation. The search results of the triplicate runs were combined and 1100 unique *E. coli* proteins were identified, which included at least two identified peptides with high confidence, number 1 peptide rank, and minimal cross-correlation scores (2.0 for charge 2, 2.25 for charge 3, and 2.5 for charge 4). These peptides were selected as the quantitative targets for targeted extraction in the large-scale quantitative comparison of *E. coli* digests using the WiSIM DIA workflow.

Search results were used to generate a sample-specific spectral library. Only the MS-MS spectra that passed the minimal cross-correlation scores were imported into the spectral library. All peptides in the spectral library were then used for the targeted data extraction of the WiSIM-DIA data. The extracted ion chromatograms of the ^{12}C and ^{13}C isotopes for the precursor ions of each targeted peptide were used for quantification with a ±5 ppm window. The eight most-intense fragment ions (b and y types larger than 200 *m/z*) detected from the discovery data were used for peptide sequence confirmation through spectral library match within the 12 *m/z* CID MS-MS spectra.

The relative expression ratios of targeted proteins between the 250 ng *E. coli* digest and 500 ng *E. coli* digest and CVs of each sample were calculated automatically. The *p*-value for each targeted peptide was also determined based on the spectrum match result. In this example, 5923 targeted *E. coli* peptides were identified with *p*-values less than 0.1 and %CVs ≤25% and were used to determine the relative expression ratios between samples. These 5923 identified peptides represent 1090 *E. coli* proteins yielding a 98% success rate for quantifying a total of 1100 targeted proteins (Figure 6). More than 97% of quantified proteins gave exceptional quantitative accuracy for the detection of the twofold expression change expected between the two samples (Figure 5). In addition, 85% of the 5923 quantified peptides gave %CVs less than 15% (Figure 6).

Conclusion

A unique data-independent acquisition workflow that collects HRAM wide-window SIM data and rapid CID MS-MS data in parallel on the recent architecture MS, which combines a mass resolving quadrupole, an orbital trap mass analyzer, and a linear ion trap mass analyzer was developed. The method setup is simple, generic, and applicable to different types of samples. Any precursor ions detected by HRAM SIM acquisition can be quantified using an extracted ion chromatogram with a ±5 ppm window and simultaneously confirmed using DIA-CID MS-MS by applying a targeted data extraction approach post acquisition.

By quantifying using high-resolution (240,000) data that provides higher mass accuracy for separating precursor ions from background interferences, low limits of detection and high selectivity were achieved. We observed 10 amol on-column LOD and LOQ and four orders of linear dynamic range with the developed DIA workflow. The reproducible qualitative and quantitative record for each sample allowed large-scale quantification of more than 1000 proteins of interest with excellent analytical precision and great quantitative accuracy.

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Table III: Detection and quantification of six low- to high-abundance bovine proteins in a complex mixture over five orders of dynamic concentrations in a single experiment

	Retention Time	CV% (n = 3)	Run 1	Run 2	Run 3
>spIP02769IALBU_BOVIN serum albumin (10 amol on column)					
HLVDEPQNLIK	51.06	22	8.40E+05	1.32E+06	1.41E+06
>spIP02662ICASA1_BOVIN alpha-S1-casein (100 amol on column)					
FFVAPFPEVFGK	110.66	8	2.33E+06	2.39E+06	2.00E+06
>spIP00366IDHE3_vovin glutamate dehydrogenase (1 fmol on column)					
LQHGTILGFPK	56.78	21	5.47E+06	5.55E+06	3.37E+06
GASIVEDK	21.11	6	1.02E+07	8.98E+06	9.11E+06
MVEGFFDR	68.04	7	3.46E+06	3.07E+06	2.94E+06
RDDGSWEVIEGYR	67.64	28	1.91E+06	3.46E+06	2.03E+06
HGGTIPIVPTAEFQDR	68.8	1	3.38E+08	3.43E+08	3.43E+08
C[Carboxymethyl]AVVDVPFGG	73.73	14	1.63E+07	1.60E+07	1.17E+07
YSTDVSVDEVK	46.35	10	2.11E+07	1.70E+07	1.77E+07
>spIP00921ICAH2_BOVIN carbonic anhydrase (10 fmol on column)					
VLDALDSIK	65.52	4	2.39E+08	2.34E+08	2.15E+08
VGDANPALQK	23.66	7	1.89E+08	1.66E+08	1.64E+08
EPISVSSQQMLK	55.15	9	1.07E+07	8.92E+06	8.69E+06
AVVQDPALKPLALVYGEATSR	91.67	1	3.46E+07	3.54E+07	3.06E+10
YGDFGTAAQQPDGLAVVGVFI	113.84	10	3.60E+06	4.54E+06	4.26E+06
>SPIP80025IPERL_BOVIN lactoperozidase (100 fmol on column)					
SPALGAANR	20.17	3	1.05E+09	9.81E+08	9.94E+08
LFQPTHK	19.72	11	5.30E+08	4.64E+08	4.09E+08
DGGIDPLVR	58.98	5	1.18E+08	1.09E+09	1.05E+09
IHGFDLAAINLQR	79.44	3	1.14E+09	1.13E+09	1.06E+09
GFC[Carboxymethyl]GLSQPK	49.46	9.00E+00	1.57E+09	1.32E+09	1.29E+09
RSPALGAANR	14.27	13	5.65E+08	5.66E+08	4.24E+08
IVGYLDEEGVLDQNR	76.67	1	3.49E+08	3.49E+08	3.39E+08
VPC[Carboxymethyl]FLAGDFR	88.27	6	1.73E+09	1.88E+09	1.63+09
LIC[Carboxymethyl]DNTHITK	33.78	3	3.56E+08	3.78E+08	3.56E+08
DYLPIVLGSEMQK	94.67	7	1.11E+08	1.24E+08	1.32E+08
FWWENPGVFTEK	102.01	5	4.59E+08	4.84E+08	4.24E+08
AGFVC[Carboxymethyl]PIPPY	77.27	3	2.22E+09	2.24E+09	2.08E+09
spIP02754ILACB_beta_lactoglobulin (1 pmol on column)					
IDALNENK	25.32	11	9.15E+09	8.75E+09	6.98E+09
TPEVDDEALEKFDK	59.56	5	2.17E+10	2.13E+10	1.95E+10
VLVLDTDYKK	46.5	4	1.29E+09	1.20+09	1.16E+09
TPEVDDEALEK	41.33	6	4.28E+10	3.72E+10	3.76E+10
WENGEC[Carboxymethyl]AQK	22.78	5	3.64E+08	3.30E+08	3.30E+08
LSFNPTQLEEQC[Carboxymethyl]	89.46	2	4.13E+10	4.15E+10	4.00E+10

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Simultaneous Determination of Methylxanthines and Cotinine in Human Plasma by Solid-Phase Extraction Followed by LC–MS-MS

A multiresidue method has been developed and validated for the analysis of methylxanthines (caffeine and its metabolites) and cotinine in human plasma. The method involves off-line solid-phase extraction (SPE) and analysis by liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) with electrospray ionization (ESI) in positive mode. The developed and validated method was fast, selective, and convenient for the simultaneous determination of cotinine, caffeine, and its metabolites in human plasma. The method has been successfully applied to the analysis of 500 samples from pregnant women in a clinical study.

Rossana Bossi and Bodil Hammer Bech

The determination of methylxanthines in human plasma or urine is based on liquid chromatography (LC) coupled to spectrophotometric detection (UV) or mass spectrometry (MS). UV detection provides low selectivity since endogenous compounds present in biological fluids may interfere with the target analytes. Liquid chromatography coupled to single mass spectrometry (LC–MS) (1,2) or tandem mass spectrometry (LC–MS-MS) (3,4) has been recently applied for the analysis of methylxanthines in human biofluids.

LC–MS-MS provides better sensitivity and selectivity compared to LC–MS. Published LC–MS and LC–MS-MS methods are based on direct injection of the sample before LC column-switching (2) or centrifugation–filtration followed by dilution of the sample to reduce matrix impact on the chromatographic determination (3,4).

The most frequently used technique for the determination of cotinine in human fluids is gas chromatography coupled to mass spectrometry (GC–MS). Recently, LC–MS and LC–MS-MS have been successfully applied for the analysis of cotinine in biological matrices (5).

The analytical method described in this paper is based on solid-phase extraction (SPE) followed by LC–MS-MS analysis with electrospray ionization (ESI) and allows a simultaneous determination of cotinine and four methylxanthines in human plasma, which is useful to assess human exposure to tobacco smoke and coffee consumption. The validated method was successfully applied to the analysis of plasma from pregnant women in a clinic study.

Experimental

The samples used for these analyses were taken from 500 pregnant women (recruited in the period 1997 to 2002).

An ethylenediaminetetraacetic acid (EDTA) blood sample was taken from the women at their first antenatal visit at their general practitioners. The blood sample was forwarded on to the biological bank for the Danish National Birth Cohort (6). After recruitment at the biological bank, the sample was centrifuged and separated in plasma and buffy coat, which were cooled with liquid nitrogen and stored at -30 °C. The Scientific Ethic Committee of Central Denmark approved the study (M-20080113).

Chemicals

Caffeine, theobromine, 1,7-dimethylxanthine, theophylline, and cotinine were purchased from Sigma Aldrich as neat compounds or solutions in methanol. Theobromine D_6, 1,7-dimethylxanthine D_6, and cotinine D_3 were from Sigma Aldrich. Caffeine $^{13}C_3$ and theophylline $^{13}C_2$ $^{15}N_2$ were from Cambridge Isotope Laboratories. High performance liquid chromatography (HPLC)-grade methanol and acetic acid were from Merck. Pure water used in the LC mobile phase and in extraction was purified with Super Q apparatus (Millipore). Primary stock solutions of the analytes were prepared in methanol or 50:50 (v/v) methanol–water for theobromine. The internal standard stock solution was prepared in methanol and further diluted with water to obtain a working solution at a concentration of 100 ng/mL. Calibration standards were prepared by spiking 100 µL of rabbit serum (Sigma Aldrich) at the following concentrations: 40 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, and 800 ng/mL. The calibration standards followed the same extraction procedure as the samples and were extracted every second batch of samples.

Instrumentation

The LC–MS-MS system consisted of an Agilent 1200 series LC system (Agilent Technologies) and a QTrap 5500 triple-quadrupole mass spectrometer (AB Sciex) equipped with an ESI source. The analytes were separated on a 150 mm × 2.1 mm, 2.7-µm d_p Ascentis Express RP Amide column (Supelco). Analyst software 1.5.1 (AB Sciex) was used for system control and data processing.

The composition of the mobile phase was 83% A (1% acetic acid in water) and 17% B (methanol) and the flow rate was 0.2 mL/min run isocratically. The injection volume was 10 µL.

The ESI source was operated in positive mode at a temperature of 600 °C and an ion spray voltage of +4500 V. Nitrogen was used as the collision gas. The analyses were performed with a multiple reaction monitoring (MRM) method that monitored two mass transitions (parent ion and product ion) for each analyte, with the exception of theobromine for which only one transition ion was obtained. The values of the voltages applied to the sampling cone, focusing lenses, collision cell, and quadrupoles were optimized in MRM mode by direct infusion of a solution containing the analytes. The precursors and product ions for each analyte, together with the applied collision energy, are summarized in Table I. Detection

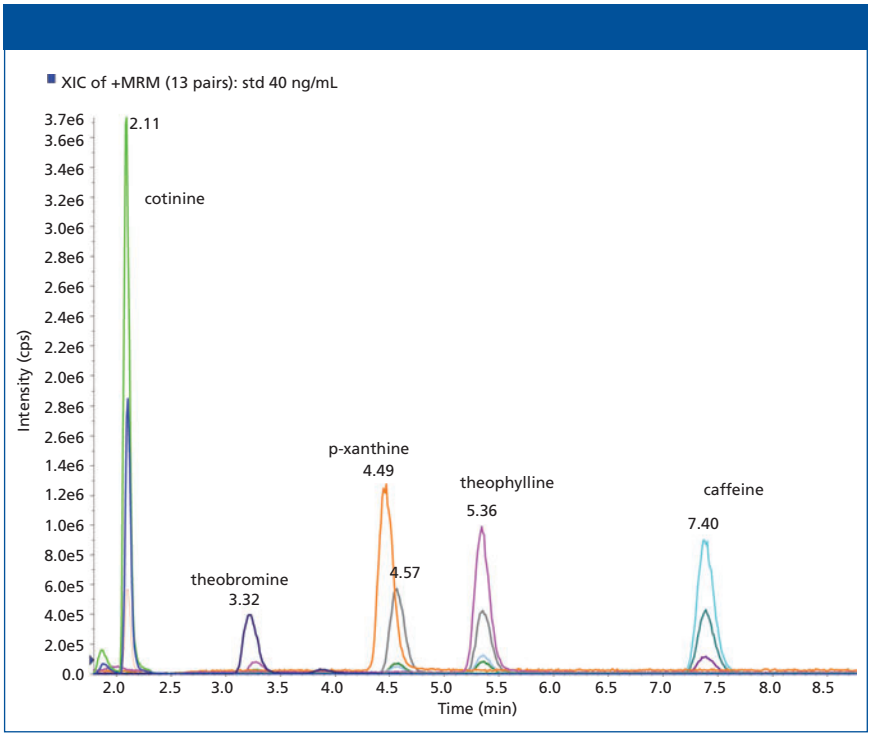

Figure 1: Total ion chromatogram of a 40-ng/mL standard. The different colors correspond to the different traces for the single transition ions (*m/z*). The traces for the six isotope-labeled compounds are included.

was based on retention time and the most abundant mass transition corresponding to an authentic standard. Confirmation of analyte identity was based on the relative response of the secondary mass transition to the primary mass transition.

Sample Preparation

Plasma samples (100 µL) were spiked with the labeled standards to a final concentration of 100 ng/mL, and 1 mL of water was added to the sample. Extraction was performed with Discovery DSC 18 SPE columns, 500 mg/3 cc (Supelco), previously conditioned with 2 mL of methanol followed by 2 mL of water. The analytes were eluted with 2 mL of methanol. The solvent was evaporated to dryness using nitrogen at 25 °C and reconstituted in 200 µL of HPLC mobile phase.

Quantification and Quality Control

Quantification of the analytes was done using response factors calculated from a five-point calibration curve consisting of blank samples (rabbit serum) spiked with the analytes in the concentration range 20–800 ng/mL and extracted following the same procedure as for the samples. Peak area ratios of target analytes and the respective internal standards were calculated at each concentration. Blank samples consisting of rabbit serum spiked with the isotope-labeled standards were analyzed with each batch of samples. Random samples were extracted and analyzed in duplicate for each batch of samples.

Results and Discussion

Optimization of the MS-MS parameters by direct infusion showed that all the investigated compounds produced stable $[M + H]^+$ ions using electrospray ionization in positive mode. For all compounds, with the exception of theobromine, which only produced one product ion, the two most abundant product ions were chosen and optimized for MRM analysis. For the isotope-labeled compounds only one product ion was selected (Table I).

The compounds paraxanthine and theophylline had one common transition ion (*m/z* 181>124), so it was necessary to optimize the analysis to obtain a complete separation of these two compounds. A C18 analytical column was tested first, but the separation of paraxanthine and theophylline was incomplete. With an amide column using isocratic elution (Figure 1) a complete baseline resolution of all compounds was obtained. However, one consequence of using isocratic elution was the build up of high pressure in the analytical column. It was therefore necessary to routinely rinse the column with 100% methanol for a couple of hours after approximately 100 injections.

The linearity of the method was tested in the concentration range 40–800 ng/mL. The calibration curves were linear ($R^2 > 0.99$) for all transition ions, with the exception of the two transition ions of cotinine, which were linear only up to 400 ng/mL. However, the concentrations of some analytes (caffeine, paraxanthine, and theobromine) exceeded the linear range in nearly half of the samples. In these cases the samples were diluted and analyzed again.

The presence of matrix components in plasma may cause problems with the LC separation and the MS detection if the sample is directly injected into the LC–MS-MS system without cleanup or dilution. Analytical methods, including time-consuming sample pretreatment, are not suitable for conducting clinical studies where large amounts of samples have to be analyzed.

Some published studies have chosen the approach of direct injection of the previous dilution of the samples (3) or automated cleanup with back-flush column switching (2). On-line methods with automated cleanup are particularly useful when high sample throughput is required in the laboratory. In the case of clinical studies involving a variety of analyses of plasma from one individual, the amount of sample available for each specific analysis is often limited (often around 50–100 µL). Therefore, in case of failure or a problem in the on-line analysis, the injection cannot be repeated. We selected off-line SPE for sample cleanup before the LC–MS-MS analysis. We started with a sample volume of 100 µL with only a factor 1:2 dilution, which still allowed for low detection limits and removed matrix interferences that could significantly decrease the response of

the analytes when using electrospray ionization. The final volume of 100 µL allowed sample injections to be repeated in the case of instrument failure. The detection limit of the method was calculated as 3× the standard deviation of blank samples (n = 55) analyzed together with the samples (Table II). The detection limits achieved with this method were in the low nanogram-per-milliliter range, which is lower than previously published studies have presented (2,3). The recoveries of the analytes were between 39% and 96%, with cotinine presenting the lowest recovery (Table II). This low recovery is probably a result of the relatively high polarity of this compound with consequent breakthrough during the SPE step. Since the corresponding isotope-labeled compound was added to samples and matrix-matched calibration standards for each analyte, no correction of sample recovery was needed, given the unchanged ratio between nonlabeled and labeled compounds during the overall extraction procedure. An isotope-labeled compound has the same physiochemical properties of the correspondent unlabeled compound. Therefore, the behavior (recovery in this case) of labeled and unlabeled compounds is expected to be the same during sample extraction.

The precision of the method (coefficient of variance, RSD%) was calculated using the results of the analysis of samples randomly selected and analyzed in duplicate following the guidelines from Eurachem/WELAC (7). The calculated values for the precision of the method ranged from 6% to 18%.

Table I: MS-MS parameters and LC retention time for the target compounds. The most abundant parent ion is the first listed.

Compound	Parent Ion (*m/z*)	Product Ions (*m/z*)	Collision Voltage (eV)	Retention Time (min)
Cotinine	177	80, 98	33, 29	2.11
Caffeine	195	138, 110	27, 31	7.40
Theobromine	181	110	29	3.32
Theophylline	181	124, 96	27, 31	5.36
1,7-Paraxanthine	181	69, 124	41, 27	4.57
Cotinine D_3	180	80	33	2.11
Caffeine $^{13}C_3$	198	140	27	7.40
Theobromine D_6	187	144	27	3.32
Theophylline $^{13}C_2$ $^{15}N_2$	184	125	27	5.36
1,7-Paraxanthine D_6	187	127	27	4.49

Table II: Recovery % (± RSD%), precision (defined as RSD% at low and high level, respectively), and method detection limit (MDL) of the method

Compound	Recovery (%)	RSD (%)	MDL (ng/mL)
Cotinine	39 (± 5.1)	6	2.0
Caffeine	95 (± 4.2)	13	6.3
Theobromine	96 (± 1.8)	9	3.7
Theophylline	91 (± 1.8)	18	1.7
1,7-Paraxanthine	78 (± 2.2)	9	2.5

Conclusions

The developed and validated method proved to be rapid, selective, and convenient for the simultaneous determination of cotinine, caffeine, and its metabolites in human serum samples. The addition of the corresponding isotope-labeled compound for each analyte, together with the quantification of matrix-matched standards, reduces the uncertainty that results from matrix effects and variable recoveries. The method has been successfully applied to the analysis of 500 samples from pregnant women in a clinical study.

Acknowledgments

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Keeping Water Safe: Detecting Pharmaceutical and Personal Care Products in Water Using Liquid Chromatography–Mass Spectrometry

Joe Anacleto, Zicheng Yang, Helen (Qingyu) Sun, and Kefei Wang, Bruker Daltonics

The Problem with PPCPs

Pharmaceutical and personal care products (PPCPs) are products used for personal health or cosmetic reasons. This category includes a broad group of chemical substances such as human and veterinary medicines and cosmetics. The presence of PPCPs in environmental and potable water is a widespread concern due to the potentially harmful environmental effects. Evidence suggests PPCPs are linked to some ecological damage such as the delayed development in fish (1).

To ensure the safety of water, PPCP concentrations are stringently monitored by environmental regulatory bodies, including the United States Environmental Protection Agency (US EPA) (2). Detection of PPCPs is traditionally a complicated process due to the range of substances potentially present. Here we explore a simple, more convenient method than traditional solid phase extraction (SPE) based methods for highly sensitive PPCP detection, using triple quadrupole liquid chromatography–mass spectrometry (LC–MS-MS).

Tables Ia & Ib: Instrumentation set up for analysis of PPCPs in clean water

Mass Spectrometer Parameters (EVOQ Elite)	
HV	4000 V
Cone gas flow	15 units
Cone gas temperature	300 °C
Heated probe gas flow	40 units
Heated probe temperature	450 °C
Nebulizer gas flow	50 units
Exhaust gas	On
Q2 pressure	1.5 mTorr (argon)
Chromatography Parameters (Advance UHPLC)	
Trap column	YMC-Pack ODS-AQ, 3 µm, 35 mm × 2.0 mm I.D.
Column temperature	40 °C
Injection volume	400 µL
Flow rate	400 µL/min
Solvent A	2 mM ammonium formate, 0.1% FA in water
Solvent B	2 mM ammonium formate, 0.1% FA in MeOH
Solvent C	2 mM ammonium formate, 0.1% FA in water
Gradient conditions	0.0 min, 10% B 0.2 min, 10% B 0.8 min, 25% B 8.0 min, 95% B 9.0 min, 95% B 9.1 min, 10% B 12.0 min, 10% B

Figure 1: PPCPs in environmental water and nearby soil is a widespread concern.

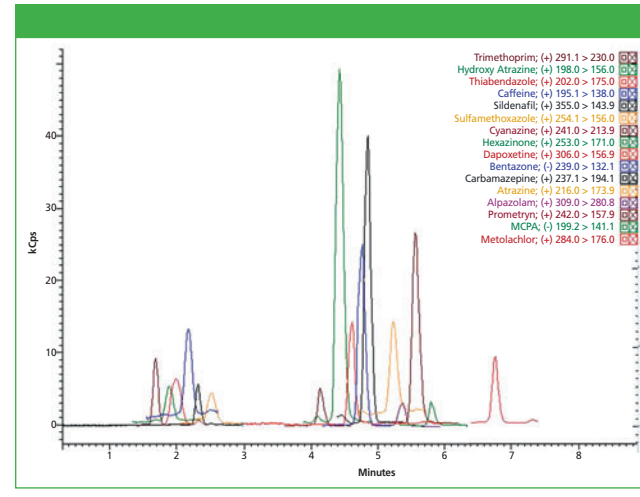

Figure 2: Selected MRM chromatograms for PPCPs at 2 ppt.

Detecting PPCPs

Conventional methods of PPCP detection in clean water have followed the defined EPA 1694 "template" for analysis which requires the pre-concentration of large volume water samples and tedious solid phase extraction cleanup, followed by liquid chromatography–mass spectrometry analysis in order to achieve the low ng/L (ppt)

Table II: Test results for selected PPCPs in real water samples

Compound Name	Tap Water 1	Tap Water 2	Creek Water	Bottle Water
Trimethoprim	<2	<2	5	<2
Hydroxyatrazine	4	<2	7	<2
Thiabendazole	ND	<2	<2	<2
Ciproxacin	ND	ND	ND	ND
Caffeine	ND	<2	<2	10
Sildenafil	ND	ND	ND	<2
Sulfamethoxazole	<2	<2	ND	<2
Cyanazine	ND	ND	ND	<2
Simazine	3	<2	5	ND
Metribuzin	ND	ND	ND	ND
Hexazinone	17	3	3	ND
Dapoxetine	ND	ND	ND	ND
Bentazone	ND	ND	ND	ND
Ametryn	ND	ND	<2	ND
Carboxine	ND	ND	ND	ND
Carbamazepine	<2	<2	<2	ND
Atrazine	<2	ND	ND	ND
Alpazolam	ND	ND	ND	ND
Diuron	9	<2	6.2	ND
Prometryn	ND	ND	ND	<2
2,4-D	9	<2	13	<2
MCPA	<2	<2	<2	ND
Mecoprop	<2	<2	11	2
Metolachlor	22	<2	<2	<2
Pyriproxifen	ND	<2	ND	<2

level detection necessary to comply with regulations (3).

Bruker has explored how LC–MS-MS can be employed specifically for the analysis of PPCPs in clean water. PPCPs were detected at 1–2 ppt with a linear response up to 200 or 500 ppt. Excellent system robustness was obtained throughout the extended method development and sample analysis period.

Case Study: Using LC–MS-MS to Analyze PPCPs in Clean Water

The study was carried out using ultrahigh-performance liquid chromatography (UHPLC) with an integrated on-line extraction (OLE) option coupled to a triple quadrupole mass spectrometer. The OLE module enables convenient method-driven on-line sample cleanup or sample pre-concentration.

Several water samples were analyzed for a range of PPCP species, including tap water samples along with bottles and creek water. Samples were analyzed targeting a wide range of PPCP species representing compounds displaying varied properties and concentrations. Tables Ia and Ib illustrate the Advance UHPLC and EVOQ instrumentation set up respectively.

All of the PPCPs were detected at 2 ppt or better with the injection of 0.4 mL samples with a linear response range up to 200 or 500 ppt.

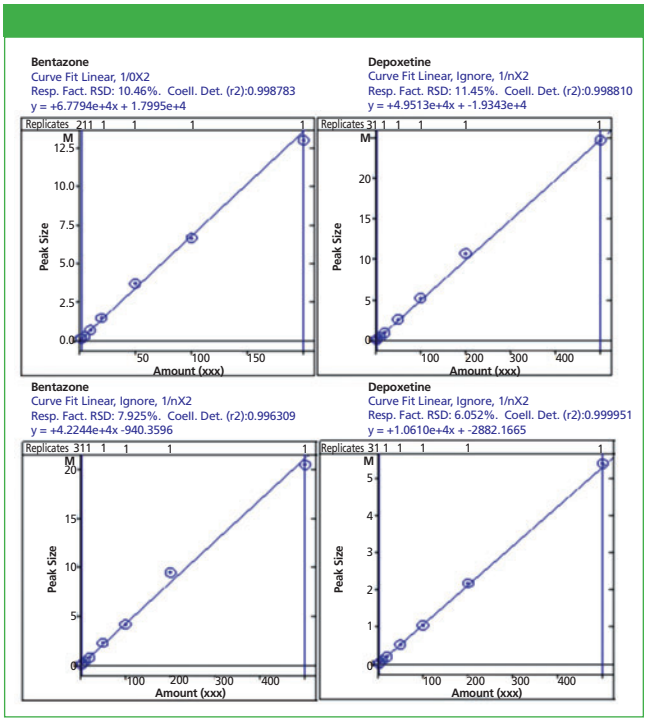

Figure 3: Selected calibration curves.

The fast polarity switch can analyze positive and negative PPCPs in the same analytical segment with excellent linear response for both polarities (Figures 2 and 3). The results for the analysis of tap, creek, and bottled waters are shown in Table II.

Conclusion

The Bruker Advance UHPLC with OLE coupled to EVOQ LC–MS-MS detected PPCP samples at 2 ppt or better within 0.4 mL samples. Excellent linearity, sensitivity, and robustness were achieved throughout. The technique presents a more convenient and simpler approach to PPCP analysis than traditional SPE-based methods.

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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

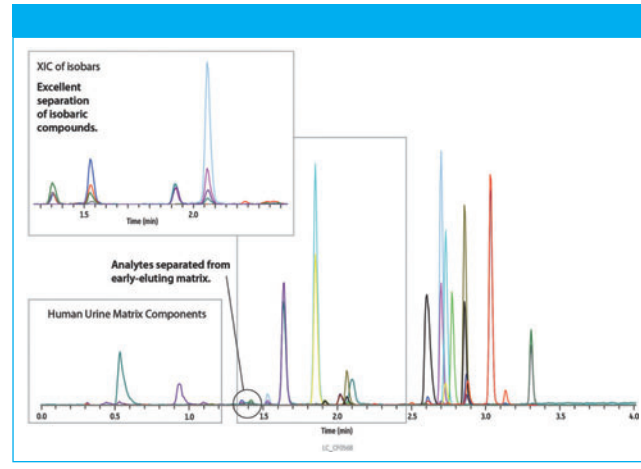

Figure 1: Baseline resolution of isobaric pain management drugs in sub-5-min runs on the Raptor™ Biphenyl column.

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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Information Rich Flash Chromatography I: Mass Directed Fractionation

Jack Silver, Teledyne ISCO

Mass spectrometers have long been used for detection and collection when coupled with liquid chromatography. Nearly universal detection of compounds is achieved when MS is combined with UV detection. The introduction of lower-cost mass spectrometers allows automated flash chromatography to enjoy the advantages of mass directed purification. These include collection of only the desired product utilizing a targeted molecular weight as a trigger. For natural products, known compounds can be identified and ignored during purification allowing the chemist to isolate only those materials with molecular weights that would suggest they are novel. Mass-directed purification of synthesized compounds is also demonstrated.

Background

There is a need for a flash chromatography detector that allows researchers to identify compounds as they are purified so time is not wasted concentrating the product of a side reaction. Mass spectrometers are useful as detectors because the molecular weight of a synthesized compound is known. Used in conjunction with UV detection, specific compounds can be collected without additional confirmation of the compound identity after elution. Likewise, in natural products, certain species are known to produce certain compounds. Knowledge of these molecular weights allows the user to screen potentially interesting compounds during elution.

Experimental and Results

All experiments were run on a Combi*Flash* Rf+ PurIon system controlled by Peak*Trak*®.

Single Ion Current (SIC)

SIC is an experimental run where the mass spectrometer is programmed to generate a data trace from a narrow range of mass-to-charge ratio values. This generates a trace specific for a molecular weight and allows purification of a single compound. As a demonstration, caffeine and theophylline were dissolved in methanol and adsorbed onto Celite 545 to make a 10% sample. To determine the appropriate molecular weight to collect caffeine, the Mass Spectrometer Method Development function in Peak*Trak* was used.

The mass spectrum from the method development screen verified that the $[M+H]^+$ ion for caffeine was seen. Since the method development program allows a user to directly inject a sample into the mass spectrometer, it is also useful for monitoring the progress of a reaction.

Extracted Ion Current (XIC)

The XIC was run in the same fashion as the single ion experiment except 4.5 g alkaloids on Celite was run. The mass spectrometer was programmed to collect compounds within the range of 180–200 daltons (Da). The mass spectrometer was able to detect both compounds.

Figure 1: Combi*Flash* Rf+ PurIon.

Synthesis of Benzidine and Purification by XIC

Nitrobenzene was dissolved in methanol containing an iodine crystal and placed in a round bottom flask fitted with a reflux condenser. Magnesium powder was added. After the reaction, additional methanol was added.

After an additional minute of reaction time, the mixture was heated, additional magnesium powder was added, and the reaction ran for more time. The mixture was poured into a beaker and the flask was washed, which was added to the reaction mix. Glacial acetic acid was added and the mixture was rotary evaporated to remove the methanol. Concentrated hydrochloric acid was added and the mixture was heated. Concentrated ammonium hydroxide was added until the solution was basic and then extracted with ethyl acetate.

The combined extracts were dried over magnesium sulfate and evaporated to yield 4.87 g of product. The mixture was adsorbed onto silica. The mass spectrometer detection range was set to 175–300 Da; the carrier solvent was methanol containing 0.1% formic acid. The recovery from the FCMS system was 0.76 g.

Conclusion

FCMS is a useful tool for validating the results of a reaction as well as ensuring the desired reaction product is collected. All purifications were performed with ACS grade solvents with standard flash columns. Nothing was found from these columns or solvents that interfered with the purification by mass spectroscopy.

The Combi*Flash* Rf+ PurIon system acquires a full range mass spectrum from 50–1200 Da even if SIC is run allowing evaluation of interesting peaks during the purification.

To download the full application note, visit: http://info.teledyneisco.com/PurIonAN93

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MASS SPECTROMETRY SPOTLIGHT

Unraveling the Links Between Diet and Human Health Using LC–MS-MS

We recently spoke to Gary Duncan and Wendy Russell of the Rowett Institute of Nutrition & Health in Aberdeen, Scotland, about the significance of phytochemical bioavailability to human health and the important role of liquid chromatography linked to tandem mass spectrometry (LC–MS-MS) in their research.

Why are you using a quantitative metabolomics approach to study diet and human health?

Russell: We only need to look around us to see that nutritional-related disorders such as obesity, diabetes, cardiovascular disease, and some cancers are emerging as a public health crisis, particularly in Scotland. Understanding the balance between diet and human health is vital, if we are to establish evidence for the provision of healthy food. As chemists, it is clear to us that we must understand the complexity of the human diet, the availability of compounds from the food matrix, and how these are absorbed and transformed in the body. Without this information it will be impossible to relate dietary composition with the overall impact of diet on human health.

Duncan: For the first time, advances in analytical methodology can provide this information and we have adopted a quantitative metabolomics approach to achieve this. Genomic analysis provides an understanding of predisposition to disease and proteomics informs on disease occurrence. Metabolomics has always been an excellent predictor of disease and is equally likely to deliver information on the status of human health.

How important is phytochemical bioavailability to human health and in food and beverage analysis?

Russell: Our analysis of the diet considers the major macronutrients (carbohydrate, protein, and fat) and micronutrient minerals and vitamins. However, the non-nutrient phytochemicals are considered to contribute to the prevention of many diet-related diseases (1). These are thought to be preventative based on their bioactive properties (that is, anti-oxidants and anti-inflammatories). One important mechanism appears to be through their anti-inflammatory activity, as low-grade chronic inflammation appears to be a critical component of disease development and progression (2). We provide detailed information on the phytochemical profiles of both the diet and dietary-derived metabolites in the body (3). This can inform the food and beverage industry to provide healthier products to the market in the form of improved commonly consumed ready meals, as well as regularly consumed snacks and drinks.

What challenges have you faced in this work?

Duncan: It is important for us that we use a targeted approach that includes a comprehensive range of metabolites that are considered both beneficial and detrimental to human health. This requires the analysis of a complex and increasingly growing number of metabolites. The analytical methodology is challenging and constantly evolving as we introduce new groups of compounds to our data set. In addition, we want to report how these compounds are transformed (both metabolically and by the gut bacteria) and distributed in the body (4,5). This not only requires the identification of the derivatives of the parent phytochemicals, but also the analysis of highly variable biological samples, where sensitivity and stability are important issues.

With bowel disorders on the rise, have you seen a trend toward a more healthy diet and knowledge of what is essentially "good for you" in your research?

Russell: Lifestyle choices and, in particular, lack of physical activity and poor diet are contributing factors to the high incidence of bowel disease worldwide. There is increasing evidence that the gut microbiota play a major role in these disorders and that the dietary metabolites they produce might be a contributing factor. We have shown from human studies that high protein diets that do not contain enough dietary fiber are likely to be detrimental to gut health in the long term (5). In these studies, we demonstrated using both targeted and liquid chromatography–tandem mass spectrometry (LC–MS-MS) that there were increased hazardous metabolites and less anti-inflammatory and cancer-protective compounds present in the gut when people ate these high-protein, low-fiber diets. We are currently run-

TOM FULLUM/GETTY IMAGES

ning similar studies that are funded by the Scottish Government; The Food, Land, and People Programme is investigating components of the Scottish diet, predominantly fruits, vegetables, and cereals (6). By understanding the composition of these foods, the impact of plant breeding, and food production processes we aim to provide healthier and more sustainable food.

Why is liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) your analytical method of choice?

Duncan: Since its inception and, more significantly, its commercialization, LC–MS has played an integral part in the analysis, identification, and quantification of natural products. The power of the LC–MS technique, or more accurately liquid chromatography combined with tandem mass spectrometry (LC–MS–MS), lies in its ability to separate complex, biological mixtures, and for pure compounds to be ionized directly from the liquid phase into the gaseous phase, using the mass spectrometer to identify or quantify the compound.

In the late 1980s, several groups had developed LC–MS methods to identify metabolites in various subclasses of phytochemicals but it wasn't until the late 1990s that the true value of LC–MS, in the analysis of phytochemicals, started to become more apparent. Papers were published utilizing the capability of the technique to perform large-scale screening of phytochemicals in complex mixtures.

With the development of ever more powerful and sensitive mass spectrometers, the analysis of phytochemicals using mass spectrometry diverged into two distinct categories: The metabolomic–qualitative approach using high-resolution mass spectrometry to identify large numbers of metabolites from a single analysis, and the targeted–quantitative approach looking at known metabolites using triple-quadrupole mass spectrometry. As the hardware on mass spectrometers has improved (detectors now have the capability of measuring as little as 1 pg on column), so has the power of the computers and software that operate them. Faster scan speeds with shorter dwell times (the time the system spends looking at an individual mass) lead to more analyses being performed during a single scan and now a large number of metabolites from many different types of groups can be measured by LC–MS with unparalleled sensitivity. Our work has focused on the quantitative analysis of phytochemicals utilizing the selectivity of the multiple reaction monitoring (MRM) method with triple-quadrupole mass spectrometry to look at a large and diverse range of dietary constituents found in both the food we eat and in the human body. LC–MS-MS provides the ideal platform to measure these metabolites, which are sometimes only present in small concentrations, with the high-throughput analysis required for large-scale human studies (8).

Where will your research into human health and food analysis take you in the future?

Russell: Understanding the human metabolic phenotype (the metabotype) will allow us to assess the specific contribution of diet on the metabolites bioavailable in the body and their translated effect on general health. We anticipate that by correlating the individual compounds found in foods with their observed bioactivity, we will be able to provide the knowledge required for the food and beverage industry to provide healthier and more sustainable food for a growing and increasingly unhealthy population. Up until now, we have focused on characterizing the metabotype, in terms of exogenous metabolites, predominantly of dietary origin. Our more recent work has identified the impact of diet on certain endogenous molecules produced by the human body (prostanoids, lysophosphatidylinositols, and bile acids) (9,10). From an analytical perspective, this work is likely to be more challenging, as the molecules are generally present in very low concentrations, are less stable, and are subject to inter-individual variation. From a dietary standpoint, we aim to provide food which will benefit health across the population. However, an understanding of endogenous metabolism may highlight a future role for more personalized nutritional strategies.

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This interview has been edited for length and clarity. ■

PRODUCTS & RESOURCES

LC mass detector

The Acquity QDa mass detector from Waters is designed for liquid chromatography applications. According to the company, the detector is small, affordable, and easy to use. **Waters Corporation,** Milford, MA; www.waters.com/qda

X-ray generator module

The uX ultracompact X-ray generator module from Spellman High Voltage is designed to provide a 0 to 50 kV/65 kV high voltage output at 2 mA limited to 50, 65, or 75 W. According to the company, the module uses closed loop filament control circuitry to provide a highly regulated beam current. **Spellman High Voltage Electronics Corporation,** Hauppauge, NY; www.spellmanhv.com/uX

Cartridge-based trap system

The cartridge-based EXP nano trap system from Optimize Technologies is designed to remove detergents or salts that can affect the ionization process in an ultra-high-pressure liquid chromatography–mass spectrometry analysis. According to the company, the hardware is rated to greater than 20,000 psi (1400 bar). Users reportedly can exchange trap columns by hand without breaking any fluid connections. **Optimize Technologies,** Oregon City, OR; www.optimizetech.com

MALDI TOF-TOF mass spectrometer

The MALDI-7090 MALDI TOF-TOF mass spectrometer from Shimadzu is designed for proteomics and tissue imaging research. According to the company, the system features a solid-state laser, 2-kHz acquisition speed in both MS and MS-MS modes, an integrated 10-plate loader, and the company's MALDI Solutions software. **Shimadzu Scientific Instruments,** Columbia, MD; www.ssi.shimadzu.com

Flash chromatography system

Teledyne Isco's CombiFlash Rf+ PurIon flash chromatography system is designed to identify a peak of interest at the time of elution with targeted fraction collection. According to the company, the system is supplied with its PeakTrak software, which allows access to commands from a single touchscreen. **Teledyne Isco,** Lincoln, NE; www.isco.com

UHPLC system

Thermo Fisher Scientific's Vanquish UHPLC system is designed to be used as a standalone system or with the latest mass spectrometers. According to the company, the system uses the company's Accucore UHPLC columns, which have 1.5-µm solid-core particles, to take advantage of its 1500 bar (22,000 psi) maximum pump pressure and flow rate up to 5 mL/min. **Thermo Fisher Scientific,** San Jose, CA; www.thermofisher.com/vanquish

GC–APCI MS interface

The second-generation GC–APCI II MS interface from Bruker is designed with a flexible heated GC transfer line for ease of instrument positioning. According to the company, the interface offers coupling of GC systems to any Bruker ESI system, including accurate-mass QTOF systems, without tools. **Bruker Corporation,** Fremont, CA; www.bruker.com

Sample introduction system

The PerkinElmer AxION direct sample analysis system is designed to eliminate all of the steps before performing MS sample analysis. According to the company, the design of the system is based on a field-free atmospheric pressure chemical ionization (APCI) source, which provides high sensitivity and reduced background noise. The system can be combined with the company's AxIon 2 TOF-MS system for applications in environmental, pharmaceutical, food, industrial, and forensics analyses. **PerkinElmer,** Waltham, MA; www.perkinelmer.com

Calendar of Events

10–14 October 2014
Asilomar Conference on Mass Spectrometry: Advances in Glycomics and Glycoproteomics: Methods and Applications
Pacific Grove, CA
www.asms.org/conferences/asilomar-conference/asilomar-conference-homepage

13–16 October 2014
First Biotechnology World Symposium and 9° Encuentro Nacional de Biotecnología
Tlaxcala, Mexico
www.9encuentro.cibatlaxcala.ipn.mx/english/meeting.aspx

14–15 October 2014
Gulf Coast Conference
Galveston, TX
www.gulfcoastconference.com

29–30 October 2014
Clinical Applications of Mass Spectrometry
Barcelona, Spain
selectbiosciences.com/conferences/index.aspx?conf=CAMS2014

2–6 November 2014
2014 AAPS Annual Meeting and Exposition
San Diego, CA
www.aaps.org/annualmeeting/

6–7 November 2014
ASMS Fall Workshop Ion Mobility Mass Spectrometry
Seattle, WA
www.asms.org/conferences/fall-workshop/fall-workshop-homepage

11–13 November 2014
The 5th Filtration & Separation Asia Combined with the 8th China International Filtration Exhibition & Conference
Shanghai, China
www.chinaexhibition.com/Official_Site/11-4219-Filtration_and_Separation_2014_-_The_5th_Filtration_and_Separation_Asia_and_The_8th_China_International_Filtration_Exhibition_and_Conference.html

17–19 November 2014
Eastern Analytical Symposium and Exposition (EAS)
Somerset, NJ
www.eas.org

22–25 January 2015
ASMS Sanibel Conference: Security and Forensic Applications of Mass Spectrometry
Clearwater Beach, FL
www.asms.org/conferences/sanibel-conference/sanibel-conference-homepage

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