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Note d'application

# Retention Time, Product Ion, and CCS Characterization of the Constituents of a FDA-Approved Small Molecule Pharmaceuticals Library

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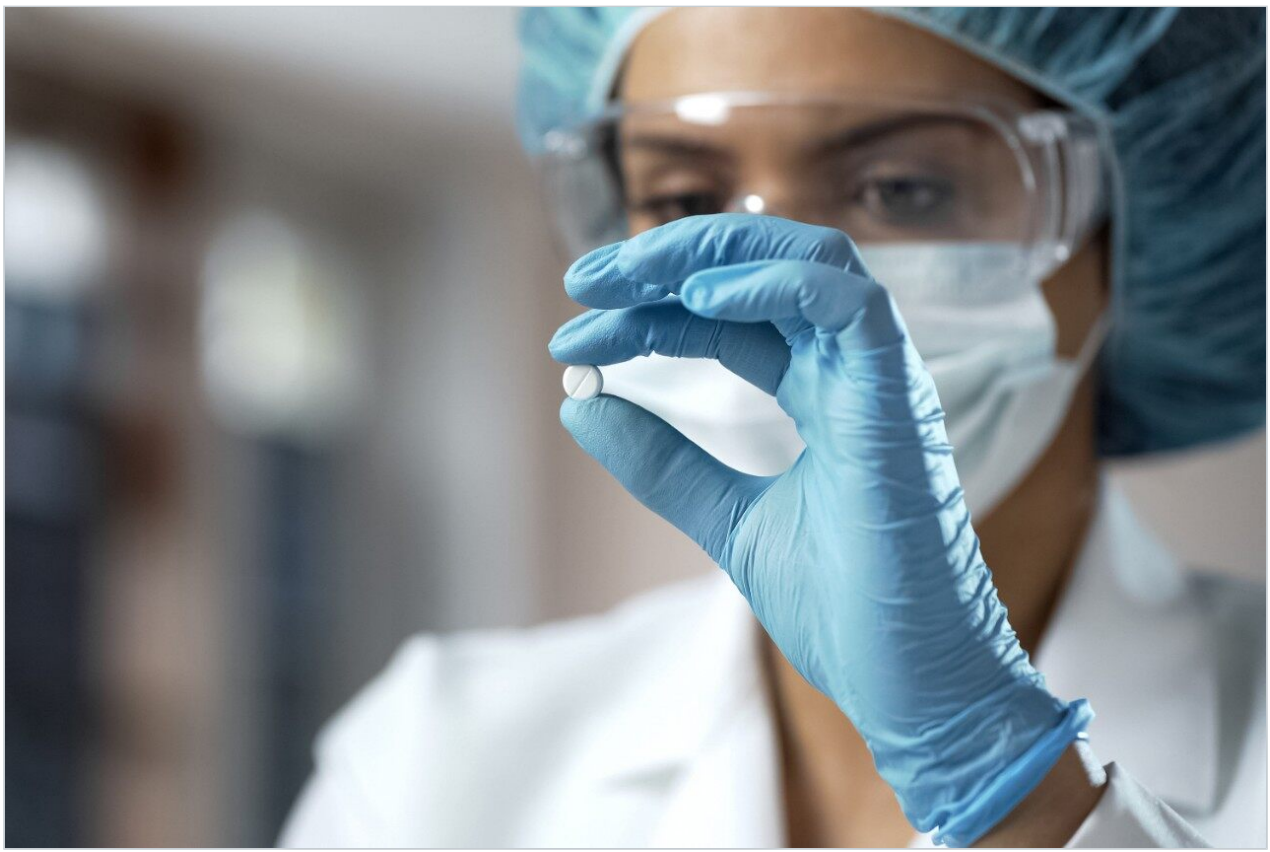

## Abstract

A set of FDA-approved small molecule pharmaceuticals was used to produce a UPLC-IM-MS library, comprising retention time $t_r$, precursor ion, product ions and CCS values for 1343 entries in ES+ mode and 950 entries in ES- mode. Ion mobility-enhanced mass spectrometry libraries incorporate additional cumulative specificity compared to conventional mass spectrometry libraries. They can be used to reduce false detection rates and increase the confidence of identification in complex matrices.

A non-targeted screening approach was performed in which CCS values were used alongside retention time, precursor, and product ions to evaluate a human urine sample. The xenobiotic exogenous components carbamazepine, carbamazepine-10, 11-epoxide, and acetaminophen were identified, as well as a variety of endogenous matrix components. When compared to the mass spectrometry library generated, ΔCCS values <1% have been obtained routinely and identification was confirmed in conjunction with a product ion count ≥1 and mass accuracy <5 ppm. The post processing workflow enabled false positive matches in the initial 60 detection results to be filtered to 5 identified compounds.

Verification of the extensive library generated has been performed using a non-targeted screen of a human urine complex biological matrix sample. The library generated can facilitate non-targeted screening to perform monitoring of therapeutic xenobiotics.


### Benefits

- Ion mobility-enabled multifactor authentication mass spectrometry libraries afford additional specificity compared to conventional mass spectrometry libraries
- Reduced false detections and increased confidence of identification in complex matrices
- The library $t_r$, precursor ion and product ion information can be applied in assays that do not incorporate ion mobility data

## Introduction

High resolution mass spectrometers (HRMS) such as quadrupole time of flight mass analyzers (Q-TOF), have become more prevalent as screening tools in clinical, forensic toxicology and metabolite identification, where the constituents of interest are present in complex biological matrices such as urine and blood.[1,2] Using non-

targeted “full scan” data acquisition thousands of detections can be made in a single analysis, and can be followed by retrospective targeted data analysis. The drive for higher sample throughput is global, requirement for improved time efficiency and cost reduction has resulted in movement towards multiclass compound analysis. This approach has been used to analyse pesticides, mycotoxins, natural plant toxins[3] and organic contaminants,[4,5] which also reside within a variety of complex sample matrices, ranging from food[6] to environmental samples such as water effluent.[7,8] The purpose of a screening method is to rapidly detect and identify target compounds in the sample under investigation, with false detection rates being kept as low as possible. Using measured properties of a compound, such as the accurate mass, isotope pattern, and product ion spectrum, appropriate filters can be applied to determine the presence of a compound in a sample. However, for compounds of interest which are present only at low concentration, within complex biological matrices, using these properties alone to achieve matrix or analyte identification may prove to be more challenging and additional method development strategies need to be employed. For such complex analyses, the extra dimension of IM separation can help to mitigate such analytical challenges, as well as generate additional identification specificity via the collision cross section (CCS).

Using a previously reported mass spectrometry library generation strategy,[9] a set of commercially available FDA approved drugs was characterized using UltraPerformance Liquid Chromatography-Ion Mobility Mass Spectrometry (UPLC-IM-MS). The strategy employed enables retention time ($t_r$), precursor ions, product ions and CCS to be determined. The commercialization of IM instrumentation (Waters Corp: SYNAPT (2006), Vion (2015), Cyclic IMS (2018)), has led to an increase in peer reviewed papers (>1250 by 2014/2015)[10-11] and analytical strategies utilizing CCS as an additional endpoint to aid identification specificity have been developed, for example pesticide screening assays.[12] The routine use of CCS for small molecule analysis has since increased across multiple areas of research including pharma (metabolism, metabolomics, lipids) and food safety (veterinary drugs, mycotoxins, steroids, natural product screening, natural toxins). CCS-searchable libraries have been generated whereby use of a CCS metric can be used to increase cumulative specificity of identification as well decrease false detections. Generation of natural product and food additives libraries has recently been presented, as well as an evaluation of the long-term robustness and reproducibility of CCS measurements.[14-17]

UPLC-IM comprises ion mobility (gas phase separation prior to MS analysis) coupled with UPLC (neutral species separation).[18,19] The timescale of UPLC (seconds), IMS (milliseconds), and time-of-flight MS (microseconds) are compatible with the requirement of high throughput analysis of complex samples. Ion mobility separation of compounds result from gas phase ions being separated within a gas-filled travelling wave ion mobility (TWIM) RF ion guide of the mass spectrometer, prior to the mass analyzer. Mobility separation is obtained by driving packets of ions through an inert buffer gas (nitrogen) or using a relatively weak electric field. The number of collisions between ions and the buffer gas cause drift time differences. The

resultant separation is based on the application of repeating DC pulses along the RF ion guide; periodically ions are overtaken by the pulses or waves, where less mobile species are overtaken more frequently than higher mobility species, hence the time to traverse the device is mobility dependent and is a function of factors such as the ion mass, charge and shape. Ion mobility provides a third dimension of separation to that of LC (hydrophobicity) and MS (*m/z*).

We have generated an extensive library of FDA approved drug small molecules and used it to perform a non-targeted urinary screen of a patient sample to identify administered pharmaceutical compounds.

## Experimental

### Sample Description

Human urine sample diluted 10:1 ($H_2O$).

Sample taken 6 hrs after medication was administered.

Carbamazepine Dosage: 2 x 200 mg tablets.

Acetaminophen Dosage: 2 x 500 mg tablets.

### LC Conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC I-Class |
| Vials: | LCMS Certified Clear Glass 12 x 32 mm Screw Neck Total Recovery Vial, with Cap and Pre-slit PTFE/Silicone Septa, 1 mL Volume, [600000671CV] |
| Column: | ACQUITY UPLC HSS T3 $C_{18}$ (100 mm x 2.1 mm, 1.8 µm) Column |
| Column temp.: | 40 °C |

| | |
|---|---|
| Sample temp.: | 4 °C |
| Injection volume: | 10 µL |
| Flow rate: | 0.5 mL/min |
| Mobile phase A: | Water (containing 0.1% formic acid v/v) |
| Mobile phase B: | Acetonitrile (containing 0.1% formic acid v/v) |

## Gradient Table

| Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| 0 | 0.5 mL/min | 99.0 | 1 | initial |
| 1 | 0.5 mL/min | 99.0 | 1 | 6 |
| 3 | 0.5 mL/min | 85 | 15 | 6 |
| 6 | 0.5 mL/min | 50 | 50 | 6 |
| 9 | 0.5 mL/min | 5 | 95 | 6 |
| 10 | 0.5 mL/min | 5 | 95 | 6 |
| 10.1 | 0.5 mL/min | 99 | 1 | 6 |
| 12 | 0.5 mL/min | 99 | 1 | 6 |

## MS Conditions

| | |
|---|---|
| MS system: | SYNAPT G2-S*i* |
| Ionization mode: | ESI+ |

| | |
|---|---|
| Acquisition range: | *m/z* 50–1200 |
| Acquisition rate: | 10 spectra per second |
| Capillary voltage: | 1.5kV |
| Desolvation temp.: | 550 °C |
| Source temp.: | 150 °C |
| Lockmass: | Leucine enkephalin (*m/z* 556.2766) |
| Acquisition mode: | HDMS$^{E}$ |
| Collision energy: | Collision energy ramp (15 to 25 eV) |
| IMS parameters: | Defaults include: T-Wave Velocity Ramp = Start: 1000 m/s End: 300m/s, T-Wave Pulse Height = 40V and a gas flow of helium 180 mL and nitrogen 90 mL (buffer gas) for the respective gas cells was used, giving an IM cell pressure of ~3.2 mBar |
| Calibration: | IMS/ToF Calibration Kit (186008113) (Waters Corp. UK) |

## Data Management

| | |
|---|---|
| Chromatography software: | MassLynx v4.2 SCN 983 |
| MS software: | MassLynx v4.2 SCN 983 |
| Informatics: | UNIFI v1.94<br>Library average CCS values were determined |

using an in-house version of UNIFI v1.94

## Results and Discussion

The small molecules library generated comprises 1453 compounds. For positive electrospray mode there are 1343 entries are included (comprised of 1277 $[M+H]^+$ and 958 $[M+Na]^+$ species). In negative ion mode the library contains 950 entries (comprised of 903 $[M-H]^-$ and 238 $[M-H+HCOO]^-$ species. (FDA Approved Drugs Profiling CCS Library <https://ims.waters.com/discover-ims-ms/ccs-libraries/> ).

The rationale for the generation of a UPLC-IM-MS library is two-fold. Primarily the library facilitates a high degree of specificity to detect the presence or absence of therapeutic xenobiotics. The library specificity also provides a route to distinguishing components of interest from the exogenous/endogenous components of complex biological matrices such as urine. The complexity of human urine matrix is illustrated in Figure 1, where the extracted base peak ion chromatogram is comprised of 1000's of major and minor intensity components (8646 candidate masses detected (> 100 counts intensity)). The corresponding ion mobility separation, illustrating the combined peak capacity of UPLC-IM, is also shown where chromatographically coeluting components are separated in the IM dimension. This facilitates generation of non-targeted single component precursor ions with corresponding product ion spectra, from the drift time and retention time aligned species. This is illustrated for the identification of carbamazepine in Figure 2.

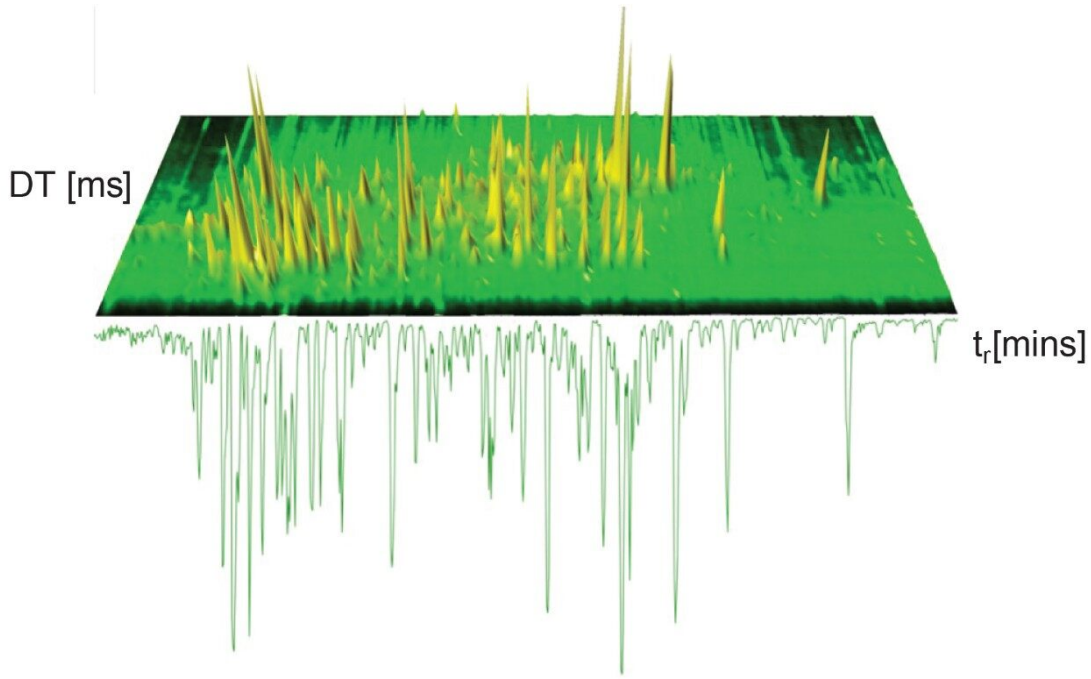


*Figure 1. UPLC-IM-MS separation obtained for non-targeted urinary screening.*

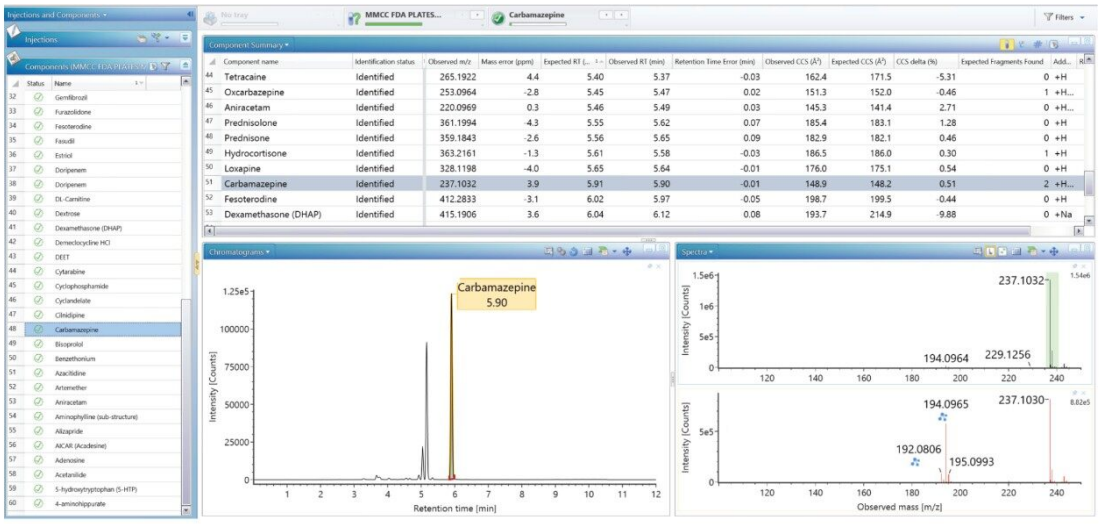


*Figure 2. Post-acquisition processing workflow filtered detection results (identified count 60) for screening using FDA approved drug small molecule library, applied tolerance $t_r$ 0.1 min, and mass accuracy +/-5pm.*

Including carbamazepine, using typical non-targeted screening tolerances of 5ppm and a retention time

tolerance 0.1 min, 60 identifications were made. Applying a 2% ΔCCS tolerance, 23 false detections were removed (see Figure 3). Application of an identification criterion of at least one expected product ion (listed in the UPLC-IM-MS library) further removes 18 false positives, resulting in 5 final detections (see Figure 4).

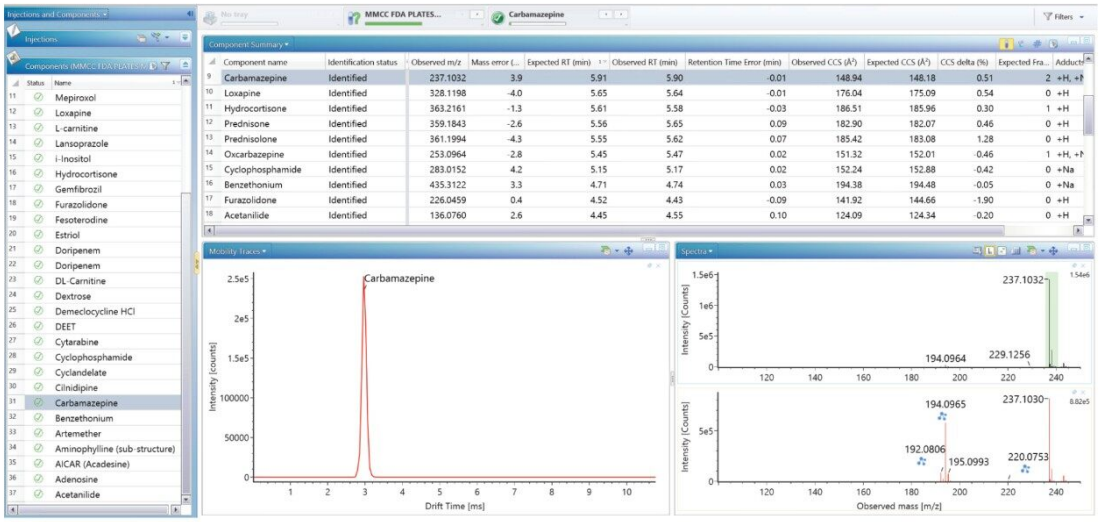


*Figure 3. Post-acquisition processing workflow filtered detection results (identified count 37) for screening against FDA approved drug small molecule library, applied tolerance $t_r$ 0.1 min, and mass accuracy +/-5pm and Δ CCS <2%.*

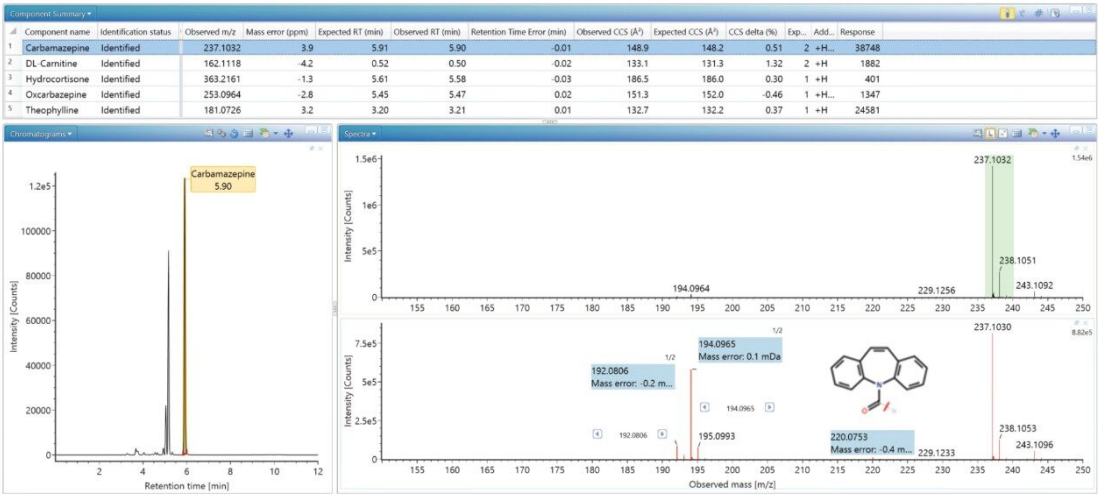


*Figure 4. Post-acquisition processing workflow filtered detection results (identified count 5) for screening against FDA approved drug small molecule library, applied tolerance $t_r$ 0.1 min, and mass accuracy +/-5pm, Δ CCS <2% and ≥1 product ion.*

The largest response was observed for carbamazepine, which was identified based on the tolerance criteria for accurate mass measurement of 5 ppm (3.9 ppm), $t_r$ tolerance of 0.1 min (0.01 min), product ion count ≥1 (2) and ΔCCS <2% (0.51%). This medication is known as an anticonvulsant or anti-epileptic drug. It is also used to relieve certain types of nerve pain. Carbamazepine is a globally prescribed medication, with 6 million prescriptions (2011) and 3.5 million (2017) in USA alone. Carbamazepine is used to prevent and control seizures. Figure 4 also shows the identification of endogenous compounds DL-carnitine (derived from an amino acid, is found in nearly all cells of the body) and hydrocortisone (a natural substance (corticosteroid hormone) made by the adrenal gland). Oxcarbazepine, was also identified, however, it is believed that the correct identification is the alternative assignment, the carbamazepine-10, 11-epoxide metabolite (CCS 151.8 Å$^2$). Exogenous theophylline was also identified with an accurate mass measurement within 3.2 ppm, retention time within 0.01 min, product ions (2) and ΔCCS (<0.37%). While Theophylline is used to prevent and treat respiratory disorders, a likely reason for its detection is that the urine was obtained from a subject that had consumed coffee. Caffeine belongs to a group of compounds known as the xanthines and is metabolised to theophylline. To further support the hypothesis of the source of theophylline in the urine sample, further interrogation of the data was performed. The precursor, product ion, and CCS values of caffeine from a natural products library were entered into the analysis method. The identification of caffeine was confirmed (see Figure 5) with accurate mass measurement (-1.0 ppm), product ions (4) and ΔCCS (0.06%). Non-targeted screen strategies typically aim for a false detection rate of <5%. The sequential application of combined filtering parameters to reduce false detections in a post processing workflow are presented in

Table 1.

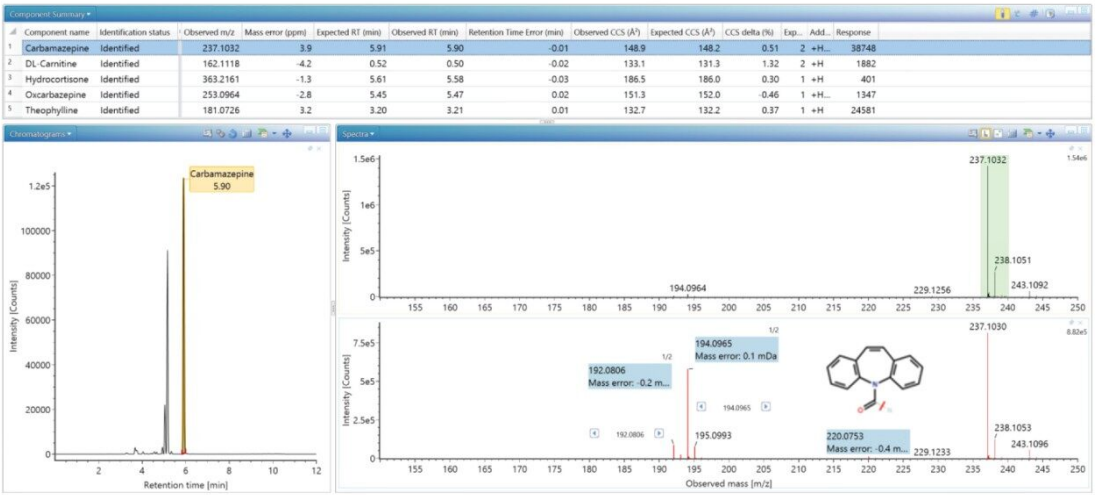


*Figure 5. Additional analytes identified utilizing natural products and forensic toxicology libraries.*

| Post processing workflow | Mass accuracy tolerance | Δ CCS | Expected | Number of detections | False detections removed |
|---|---|---|---|---|---|
| step | | | fragments | | |
| 1 | 5ppm | | | 60 | |
| 2 | 5ppm | <2% | | 37 | 23 |
| 3 | 5ppm | <2% | >1 | 5 | 55 |

*Table 1. Sequential application of combined workflow filtering parameters to reduce false detections.*

During manual data review, evidence indicating the subject was also taking acetaminophen was observed, therefore the corresponding CCS and precursor ion data (from a forensic toxicology library) was also entered into the analysis method. Retention time independent identification of acetaminophen was also confirmed using accurate mass measurement (0.3 ppm), product ion (1) and Δ CCS (0.57%). Enhanced specificity, combined with analysis flexibility is illustrated, where both acetaminophen and caffeine, were identified based on precursor ion, product ion and CCS alone, in conjunction with a wide retention time tolerance. Retention time information was not available, because the libraries from which the CCS and product ion

information were obtained for these compounds used different chromatographic conditions, to those described herein. Further confidence in detection of acetaminophen could be gained from additional identification of metabolites, acetaminophen glucuronide ($t_r$ 2.48 min, observed CCS 181 $Å^2$) and acetaminophen sulphate ($t_r$ 2.82 min, observed CCS 149.7 $Å^2$). The approach illustrates the versatility of the UPLC-IM libraries being generated and the utility of additional analytical selectivity of CCS values.

## Conclusion

A UPLC-IM-MS library of small molecules FDA-approved pharmaceuticals (1343 ES+ and 950 ES-) was generated comprising retention time $t_r$, precursor ion, product ions, and CCS values. This library can be used to facilitate non-targeted screening to perform drug monitoring and illicit drug use. Verification of the library generated has been performed using a non-targeted screen of a human urine complex biological matrix sample. The research performed has produced only one detection requiring further investigation, where oxcarbazepine was observed, which could be rationalized and reassigned. Exogenous xenobiotics and natural endogenous species have been identified; no false detections were observed. For the research performed UNIFI functionality enabled rapid reassignment of a suspected false detection. The versatility of UPLC-IM libraries has  been illustrated, using the FDA approved pharmaceutical drugs library, natural products and forensic toxicology library entries. Utilizing CCS in a UNIFI post-acquisition processing workflow to filter detection results for non-targeted screening assays provides unparalleled targeted data review flexibility, in a non-targeted analytical environment.

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