Separation and Characterization of Synthetic Cannabinoid Metabolite Isomers using SLIM High-Resolution Ion Mobility-Tandem Mass Spectrometry (HRIM-MS/MS)

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Keywords: Synthetic cannabinoids, Isomers, SLIM, Ion Mobility-Mass Spectrometry (IM-MS)

Abstract

Synthetic cannabinoids, a subclass of new psychoactive substances (NPS), are lab-made substances that are chemically similar to those found naturally in the cannabis plant. Many of these substances are illicitly manufactured and have been associated with severe health problems, prompting a need to development analytical methods capable of characterizing both known and previously undetected compounds. This work focuses on a novel Structures for Lossless Ion Manipulations (SLIM) IM-MS approach to differentiation and structural characterization of synthetic cannabinoid metabolites, specifically MDA-19/BUTINACA, JWH-018, and JWH-250 isomer groups. These different compound classes are structurally very similar, differing only in the position of one or a few functional groups; this yielded similarity in measured collision cross section (CCS) values. However, the high resolution of SLIM IM provided adequate separation of many of these isomers, such as sodiated JWH-250 metabolites N-4-OH, N-5-OH, and 5-OH which displayed CCS of 187.5, 182.5, and 202.3 $Å^2$, respectively. In challenging cases where baseline separation was precluded due to nearly identical CCS, such as for JWH-018 isomers, simple derivatization by dansyl chloride selectively reacted with the 6-OH compound to provide differentiation of all isomers using a combination of CCS and *m/z*. Finally, the opportunity to use this method for structural elucidation of unknowns was demonstrated using SLIM IM mobility-aligned MS/MS fragmentation. Different MDA-19/BUTINACA isomers were first mobility separated and then could be individually activated, yielding unique fragments for both targeted identification and structural determination. Overall, the described SLIM IM-MS/MS workflow provides significant potential as a rapid screening tool for characterization of emerging NPS such as synthetic cannabinoids and their metabolites.

Introduction

Synthetic cannabinoids are a subset of new psychoactive substances (NPS) that stimulate the same cannabinoid receptors as naturally occurring cannabis compounds like Δ^9-tetrahydrocannabinol (THC).[1–3] However, where THC is considered a partial agonist, the synthetic cannabinoids act as direct agonists which leads to potentially higher toxicity.[1] This unpredictable toxicity has resulted in higher rates of abuse and adverse effects, frequently prompting hospitalization.[2,3] Clandestine laboratories continue to develop and synthesize these compounds, often marketed as “legal highs” which can evade detection by standard drug screens.[3] However, a growing concern is the expanding molecular diversity of these substances, because it is known that the *in vivo* activity of both naturally occurring and synthetic substances differs significantly based on their chemical structure.[4,5] As such, it has become imperative to develop analytical techniques capable of identifying those cannabinoids, as well as their metabolites, in a variety of sample types.

Because of their wide-ranging potency, confident identification of specific cannabinoids in biological samples is a critical step towards linking toxicity with symptoms.[6,7] Laboratory-based testing of samples, which might include urine, blood, oral fluid, or hair, has primarily been accomplished using mass spectrometry-based methods coupled to either gas chromatography (GC-MS)[8–11] or liquid chromatography (LC-MS).[12–18] Investigation of cannabinoid metabolites is also especially important, as their relatively quick metabolism often precludes detection of parent compounds in urine. Several studies have reported on GC- and/or LC-MS analysis of metabolites from a range of synthetic cannabinoids, either through collection of urine samples following use of those substances or via simulated metabolism (i.e., with human liver microsomes or by fungal metabolism).[19–21]

In addition to MS-based methods, ion mobility spectrometry (IMS) has historically been considered the technique of choice for screening of illicit drugs at various security checkpoints, and this has been recently demonstrated for the analysis of cannabis products. Commercial standalone IMS instruments have been used to measure reduced mobility (K_0) for dozens of NPS substances including synthetic cannabinoids.[22,23] Measurement of residue on hands,[24] portable/handheld detection in plant extracts,[25]

screening of suspect materials in jails and prisons,[26,27] and metal-organic framework decorated paper-based sensors[28] constitute just a few recent examples of IMS applied to current challenges in cannabinoid analysis. But where more comprehensive separations and chemical/structural information are required, IMS has also been coupled with mass spectrometry (i.e., IM-MS); this has been especially beneficial for resolving isobaric/isomeric cannabis substances. Several different IM-MS techniques have been explored including traveling wave (TWIMS),[29,30] drift tube (DTIMS),[31,32] and differential mobility (DMS).[33,34] Structures for Lossless Ion Manipulations (SLIM) is another form of IMS recently developed at Pacific Northwest National Laboratory (PNNL) and commercialized by MOBILion Systems. The technology provides long-path (>10 m) traveling wave-based IM separations by using serpentine paths patterned on printed circuit boards.[35–39] SLIM has seen application in several areas including proteomics[40–43] and metabolomics,[44–48] but herein we report the first instance of SLIM IM for characterization of synthetic cannabinoid isomers.

2. Experimental Methods

2.1 Materials

Standards of all synthetic cannabinoid metabolites (MDA-19 N-(4-hydroxybenzoyl) metabolite, MDA-19 N-(5-hydroxyhexyl) metabolite, 4-cyano CUMYL-BUTINACA metabolite 10, APP-BUTINACA phenylpropanoic acid metabolite, JWH-018 4-hydroxyindole metabolite, JWH-018 N-(5-hydroxypentyl) metabolite, JWH-018 6-hydroxyindole metabolite, JWH-250 N-(4-hydroxypentyl) metabolite, JWH-250 N-(5-hydroxypentyl) metabolite, and JWH-250 5-hydroxyindole metabolite) were purchased from Cayman Chemical (Ann Arbor, MI) as 1 mg/mL solutions in methanol or acetonitrile. Dansyl chloride, sodium carbonate, and sodium bicarbonate were purchased from Fisher Scientific. All solvents (water with 0.1% formic acid, methanol, and acetonitrile) were Fisher Scientific Optima LC-MS grade.

2.2 Sample Preparation

All working samples were prepared at 1 µg/mL of synthetic cannabinoid metabolite, either individually or as mixtures, in 50:50 (v/v) water (0.1% formic acid)/methanol.

Derivatization reactions were performed using dansyl chloride as previously.[49] Briefly, 100 µL of a 1 mg/mL solution of dansyl chloride (in acetonitrile) was combined with 100 µL of an aqueous sodium bicarbonate buffer (100 mM) with a pH of 9, adjusted using sodium carbonate. Working samples of the synthetic cannabinoid metabolite(s) (10 µL each) were then added and vortexed for 15 seconds. The samples were incubated at 60 °C for 3 minutes, followed by gentle drying under nitrogen at room temperature. Finally, the dried product was reconstituted in 1 mL of mobile phase A (90:10 (v/v) water (0.1% formic acid)/methanol.

2.3 Instrumentation

Accurate $^{DT}CCS_{N2}$ values were collected using an Agilent 6560 drift tube (DT) IM-QTOF coupled to 1290 Infinity II UHPLC (Santa Clara, CA). Samples were introduced via direct injection (10 µL) with an LC flow rate of 500 µL/min (50:50 (v/v) water (0.1% formic acid)/methanol). Ionization was performed with an Agilent Jetstream (AJS) ESI source in positive mode, and the MS data were acquired using full scan mode. The drift tube was maintained at approx. 3.95 Torr nitrogen and approx. 27 °C, and the electric field strength was 18.5 V/cm. CCS values were measured using the established single-field method based on beta and t-fix values for the Agilent Tune Mix ions.[50] All measurements were made in triplicate to report repeatability (in terms of standard deviation). Further details of this system have been published elsewhere.[51]

High-resolution IM measurements were performed using a MOBIE HRIM SLIM (MOBILion Systems, Inc., Chadd's Ford, PA) coupled to an Agilent 6546 QTOF and Agilent 1290 UHPLC (Santa Clara, CA). Samples were introduced via direct injection (10 µL) with an LC flow rate of 500 µL/min (50:50 (v/v) water (0.1% formic acid)/methanol). Ionization was performed with an Agilent Jetstream (AJS) ESI source in positive mode. The SLIM device was maintained at 2.50 Torr nitrogen gas and 25 °C. Prior to IM separations, ions were accumulated onboard for 40 ms with a 30 kHz and 30 V_{pp} sine traveling wave (TW). SLIM IM separations (13 m) were performed using a separation TW of 20 kHz and 35 V_{pp}. MS/MS was performed by mass isolation in the quadrupole and fragmentation in the collision cell, with optimized collision energies of 5 and 25 V for protonated and sodiated ions, respectively. All measurements were made in triplicate to

report repeatability (in terms of standard deviation). Additional details of the parameters used in the ionization source region and SLIM separation device can be found in Table S1.

2.4 Data Acquisition and Processing

DT IM-QTOF data acquisition was performed using Agilent MassHunter B.09.00 (Build 9.0.9044.0) and visualized/processed in Agilent IM-MS Browser 10.0.1 (Build 10.0.1.10039). SLIM data acquisition was performed using Agilent MassHunter Acquisition Version 11.0 (Build 11.0.221.1) and MOBILion EyeOn software (0.0.2.2619-release-1.5.14.3). All SLIM data was first converted from the MOBILion .mbi file format into Agilent .d file format using the EyeOn software, and then pre-processed using the PNNL PreProcessor 4.0 (2022.02.18) (Richland, WA) with a drift bin compression of 3:1, spike removal of 2, and CCS conversion.[52] Further data visualization/processing was then performed using Agilent IM-MS Browser 10.0.1 (Build 10.0.1.10039). $^{SLIM}CCS_{N2}$ measurements were initially made by calibrating the SLIM system using the Agilent Tune Mix ions (hexakis(fluoroalkoxy)phosphazines, HFAPs), which allowed for conversion of arrival time to CCS using the PNNL PreProcessor. Briefly, this process involves plotting of experimental arrival times versus reduced $^{DT}CCS_{N2}$, which are then fit using a trinomial function. To account for observed systematic bias, a simple linear correction factor was applied, based on rescaling to the average bias within the class;[48] this provided 'corrected' $^{SLIM}CCS_{N2}$ values, which are all displayed in the main manuscript text. Uncorrected values are included in the Supporting Information Tables S2-4. Microsoft Excel was used to create all figures and to calculate the percentage CCS bias as described by Rose et al.[48]

3. Results and Discussion

3.1 SLIM IM-Based Measurement of Collision Cross Section ($^{SLIM}CCS_{N2}$)

Three groups of synthetic cannabinoid metabolite isomers were chosen for this study because they exhibit structural differences only in the arrangement of their functional groups (e.g., hydroxyl, carbonyl, and methyl groups): (a) JWH-018 isomers ($C_{24}H_{23}NO_2$, MW 357.173 Da); (b) JWH-250 isomers ($C_{22}H_{25}NO_3$, MW 351.183 Da); and (c) MDA-19 isomers ($C_{21}H_{23}N_3O_3$, MW 365.174 Da). For example, the JWH-018 metabolites

all differ in the position of a single hydroxyl group. Structures for all compounds, indicating those isomeric differences, are shown in Figure S1. SLIM-based arrival times for the protonated and sodiated species of each metabolite were used to calculate collision cross section ($^{SLIM}CCS_{N2}$) after first calibrating with the Agilent Tune Mix ions.[48,53,54] For comparison, $^{DT}CCS_{N2}$ values were also measured using a drift tube IM (DTIM) instrument, as this is widely accepted as a direct measurement technique for CCS. Moderate agreement was observed between $^{SLIM}CCS_{N2}$ and $^{DT}CCS_{N2}$ values, with all protonated and sodiated values agreeing to within 1.1% and 1.5%, respectively (Tables S2-S3). However, there was a systematic bias observed in which SLIM values were on average 0.66% and 0.77% lower than DT values for the protonated and sodiated species, respectively. This concurs with other reports from traveling wave (TW)-based systems using generic calibrants (i.e., Agilent Tune Mix ions), including with SLIM-based systems.[48] However, as recently suggested by Rose et al.,[48] in lieu of performing class-specific CCS calibration (which is often not possible without commercial availability of a wide array of standards), a calibration strategy involving a correction factor can be applied. Upon employing this strategy, we observed that the majority of $^{SLIM}CCS_{N2}$ values agreed with the corresponding $^{DT}CCS_{N2}$ values to within only ≤0.4% (Tables 1-2). Additionally, the $^{SLIM}CCS_{N2}$ values showed good repeatability across replicate measurements, with all compounds displaying relative standard deviations (RSD) of ≤0.2%.

Table 1. Experimental $^{DT}CCS_{N2}$ and corrected $^{SLIM}CCS_{N2}$ for the protonated species, $[M+H]^+$, for all metabolites. Chemical formulae and theoretical *m/z* are also included.

Metabolite	Formula	$[M+H]^+$ *m/z*	$^{DT}CCS_{N2}$ (Å^2)	$^{SLIM}CCS_{N2}$ (Å^2)	ΔCCS (%)
JWH-018 4-hydroxyindole	$C_{24}H_{23}NO_2$	358.181	189.0 ± 0.1	189.0 ± 0.2	0.02%
JWH-018 N-(5-hydroxypentyl)			187.0 ± 0.1	188.2 ± 0.2	0.66%
JWH-018 6-hydroxyindole			192.4 ± 0.1	192.3 ± 0.4	-0.07%
JWH-250 N-(4-hydroxypentyl)	$C_{22}H_{25}NO_3$	352.191	187.6 ± 0.1	187.6 ± 0.2	0.02%
JWH-250 N-(5-hydroxypentyl)			187.6 ± 0.1	187.9 ± 0.4	0.18%
JWH-250 5-hydroxyindole			191.5 ± 0.1	191.2 ± 0.3	-0.18%
MDA-19 N-(4-hydroxybenzoyl)	$C_{21}H_{23}N_3O_3$	366.182	196.1 ± 0.1	195.5 ± 0.1	-0.32%
MDA-19 N-(5-hydroxyhexyl)			189.3 ± 0.1	189.5 ± 0.1	0.13%
4-cyano CUMYL-BUTINACA			186.4 ± 0.1	ND	
APP-BUTINACA phenylpropanoic acid			187.3 ± 0.1	186.5 ± 0.4	-0.42%

Table 2. Experimental $^{DT}CCS_{N2}$ and corrected $^{SLIM}CCS_{N2}$ for the sodiated species, $[M+Na]^+$, for all metabolites. Chemical formulae and theoretical *m/z* are also included.

Metabolite	Formula	$[M+Na]^+$ *m/z*	$^{DT}CCS_{N2}$ (Å^2)	$^{SLIM}CCS_{N2}$ (Å^2)	ΔCCS (%)
JWH 018 4-hydroxyindole	$C_{24}H_{23}NO_2$	380.163	206.4 ± 0.1	206.2 ± 0.1	-0.11%
JWH 018 N-(5-hydroxypentyl)			193.9 ± 0.1	193.1 ± 0.1	-0.43%
JWH 018 6-hydroxyindole			206.3 ± 0.1	209.1 ± 0.1	1.35%
JWH 250 N-(4-hydroxypentyl)	$C_{22}H_{25}NO_3$	374.173	184.3 ± 0.1	187.5 ± 0.2	1.74%
JWH 250 N-(5-hydroxypentyl)			183.8 ± 0.1	182.5 ± 0.4	-0.71%
JWH 250 5-hydroxyindole			203.7 ± 0.1	202.3 ± 0.2	-0.67%
MDA-19 N-(4-hydroxybenzoyl)	$C_{21}H_{23}N_3O_3$	388.164	211.1 ± 0.1	210.0 ± 0.3	-0.52%
MDA-19 N-(5-hydroxyhexyl)			190.8 ± 0.1	190.0 ± 0.2	0.03%
4-cyano CUMYL-BUTINACA			195.7 ± 0.1	195.0 ± 0.2	-0.36%
APP-BUTINACA phenylpropanoic acid			194.8 ± 0.1	194.2 ± 0.1	-0.32%

3.2 SLIM IM-Based Separation of Isomers

To investigate the capabilities of the SLIM HRIM technique in differentiating the different metabolites, a comprehensive analysis of individual isomers and equimolar

mixtures was performed (Figure 1). Instrumental resolving power (CCS/ΔCCS) approached R_p ~ 200, which was expected to allow mobility separation of isomers with ΔCCS ≥ 0.5%.[55] The JWH-018 compounds were best separated as sodiated species, with the 5-OH isomer baseline resolved from the others; however, the similarity in structure between the 4-OH and 6-OH isomers precluded their baseline separation (Figure 1A). Analysis of the protonated species allowed separation of the 6-OH isomer, but significant overlap was observed for the 4-OH and 5-OH metabolites (Figure S2A), whose $^{SLIM}CCS_{N2}$ values of 189.0 and 188.2 Å^2 differed by only 0.4%; as such, neither adduct resulted in definitive resolution of all three isomers. On the other hand, the JWH-250 compounds were nearly baseline resolved as sodiated species (Figure 1B), while the N-4-OH and N-5-OH isomers overlapped as protonated species (Figure S2B) with $^{SLIM}CCS_{N2}$ values of 187.6 and 187.9 Å^2 yielding ΔCCS <0.2%. Lastly, the MDA-19 isomers were well resolved in a mixture as protonated species (Figure 1C), although 4-cyano CUMYL-BUTINACA was not observed in the protonated form; its sodiated form showed clear resolution from the N-5-OH isomer (Figure S2C).

To improve separation for those challenging isomers, simple derivatization reactions were investigated. Such reactions are commonly used to improve ionization, volatility, and/or chromatographic retention in many GC-/LC-MS applications, but more recently have also been explored by our group as IM 'shift reagents' with the goal of improving resolution.[56,57] One such derivatizing reagent, dansyl chloride, has been demonstrated to selectively react with phenolic alcohols such as those found in several of these cannabinoid metabolites.[49] This derivatization reaction was applied to the JWH-018 isomers, for which the reaction might only be expected to proceed for 4-OH and 6-OH, because the 5-OH compound lacks a phenolic alcohol group (Figure S3). The reaction product was clearly identified for the 6-OH species as its $[M_{DC}+H]^+$ ion at *m/z* 591.233 (Figure 2A), however no product was observed for the 4-OH compound presumably due to steric hindrance preventing the reaction. Both 4-OH and 5-OH were still only observed as their unreacted protonated and sodiated ions. But importantly, where the 4-OH and 6-OH species were not fully IM resolved previously (Figure 1A), this reaction selectively proceeds for the 6-OH and allows baseline resolution of all isomers (Figure 2B); the sodiated species of unreacted 4-OH and 5-OH remain separated by

mobility and the reaction product of 6-OH is differentiable by mass. In addition, the derivatization increased the ionization efficiency as evidenced by the higher abundance of the product in comparison with its unreacted equivalent.

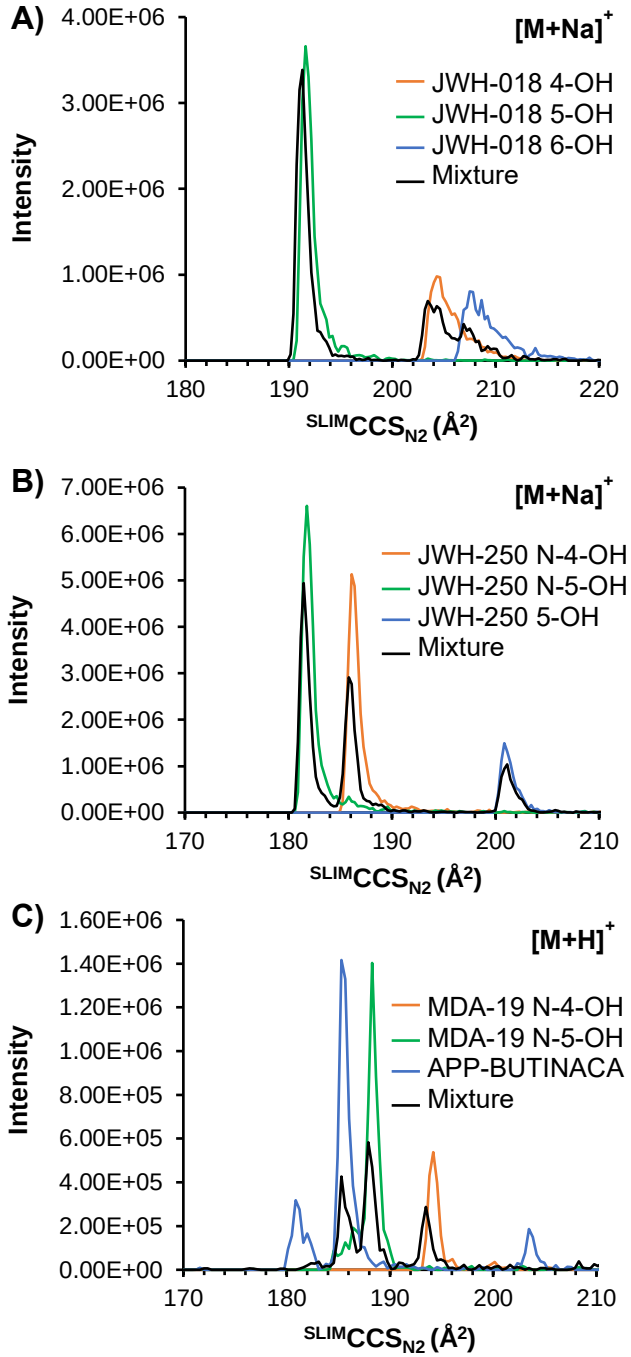

Figure 1. Optimized SLIM IM separations for individual compounds and mixtures of the (A) JWH-018 isomers as sodiated species, (B) JWH-250 isomers as sodiated species, and (C) MDA-19 isomers as protonated species.

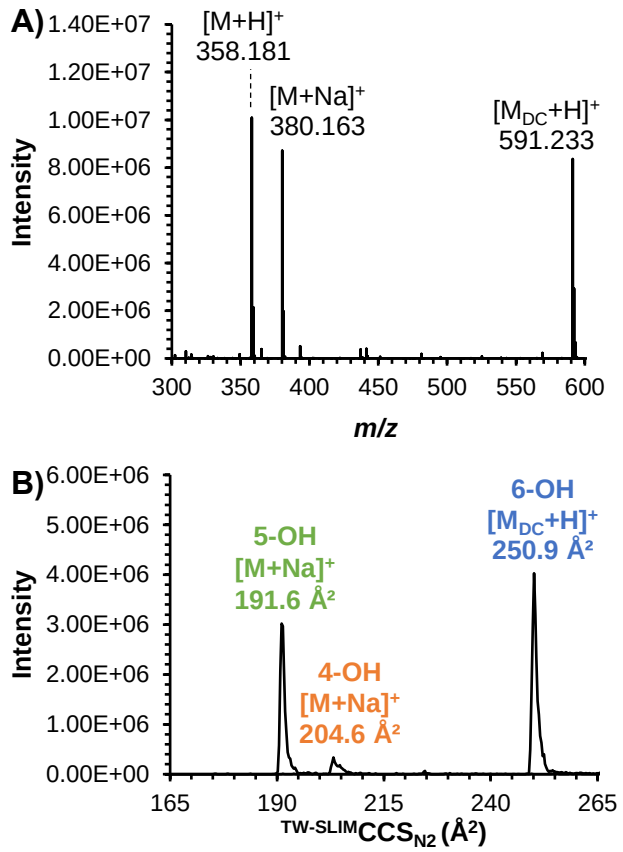

Figure 2. (A) Mass spectrum showing the successful dansyl chloride derivatization of JWH-018 6-hydroxyindole with major product ion at *m/z* 591.233; the underivatized JWH-018 N-(5-hydroxypentyl) and JWH-018 4-hydroxyindole have $[M+H]^+$ and $[M+Na]^+$ peaks at *m/z* 358.181 and 380.163, respectively. (B) The $[M+Na]^+$ species shows baseline separation between JWH 018 4-hydroxyindole and JWH 018 N-(5-hydroxypentyl), while the dansyl chloride derivatized JWH-018 6-hydroxyindole separated based on both unique *m/z* (591.233) and CCS (250.9 Å²).

3.3 Identification of Sodiated Dimers and Heterodimers Present in Mixtures

At the concentrations studied, sodiated dimers were also observed for all compounds. Their raw $^{SLIM}CCS_{N2}$ values (based on calibration with Agilent Tune Mix ions) are included in Table S4, but a similar linear correction factor was applied which yielded the values displayed in Table 3. While such dimers are unlikely to form at the low concentrations expected in biological samples, this dimerization behavior could be useful in differentiating synthetic cannabinoids in unknown drug substances, where the analysis is not sample-limited. Each individual compound displayed only a single dimer peak in its mobility spectrum, however mixing the isomers resulted in new mobility peaks, presumably due to heterodimerization. This was confirmed by systematically mixing

isomer pairs, to demonstrate the unique appearance of a single new mobility peak in each. This is evidenced for JWH-018 isomers in Figure 3A, where the dimers of 4-OH and 5-OH isomers individually show CCS of 283.7 and 262.5 $Å^2$, respectively, while their mixture also shows a unique peak at CCS 272.4 $Å^2$. Similar comparisons of 4-OH/6-OH and 5-OH/6-OH are shown in Figures 2B-C, where an intermediate mobility peak appears in the mixture, presumably due to heterodimerization not present in the individual samples. Mixing all three isomers together yielded a mobility spectrum with six resolvable features, corresponding to three homodimers and three heterodimers (Figure 2D).

Table 3. Experimental $^{DT}CCS_{N2}$ and corrected $^{SLIM}CCS_{N2}$ for the sodiated dimers, $[2M+Na]^+$, for all metabolites. Chemical formulae and theoretical *m/z* are also included.

Metabolite	Formula	$[M+Na]^+$ *m/z*	$^{DT}CCS_{N2}$ ($Å^2$)	$^{SLIM}CCS_{N2}$ ($Å^2$)	ΔCCS (%)
JWH 018 4-hydroxyindole	$C_{24}H_{23}NO_2$	737.336	281.7 ± 0.1	283.7 ± 0.4	0.71%
JWH 018 N-(5-hydroxypentyl)			261.9 ± 0.1	262.5 ± 0.1	0.25%
JWH 018 6-hydroxyindole			290.8 ± 0.1	290.6 ± 0.2	-0.08%
JWH 250 N-(4-hydroxypentyl)	$C_{22}H_{25}NO_3$	725.357	271.4 ± 0.1	272.7 ± 0.2	0.49%
JWH 250 N-(5-hydroxypentyl)			259.1 ± 0.1	261.1 ± 0.2	0.76%
JWH 250 5-hydroxyindole			267.6 ± 0.1	267.9 ± 0.2	0.12%
MDA-19 N-(4-hydroxybenzoyl)	$C_{21}H_{23}N_3O_3$	753.338	281.6 ± 0.1	280.1 ± 0.2	-0.54%
MDA-19 N-(5-hydroxyhexyl)			268.4 ± 0.1	267.3 ± 0.1	-0.39%
4-cyano CUMYL-BUTINACA			264.5 ± 0.1	262.8 ± 0.3	-0.63%
APP-BUTINACA phenylpropanoic acid			275.0 ± 0.1	273.6 ± 0.3	-0.50%

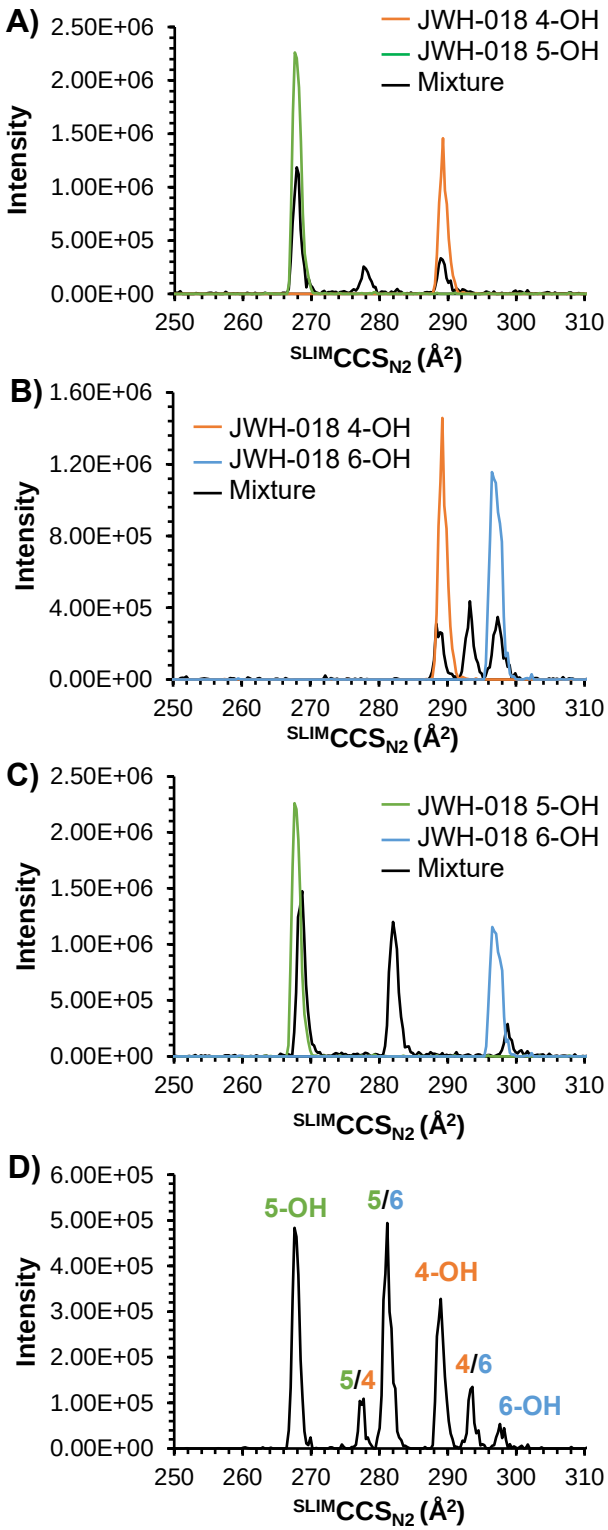

Figure 3. Sodiated dimers were observed for the JWH-018 isomers when run individually, and as paired mixtures: (A) 4-OH/5-OH, (B) 4-OH/6-OH), and (C) 5-OH/6-OH. Each individual compound displays only a single dimer mobility peak, however when run in mixtures new mobility peaks corresponding to

heterodimers are observed. (D) The mixture of all three isomers yields differentiable homodimers and heterodimers.

3.4 SLIM Ion Mobility-Aligned Tandem MS (SLIM IM-MS/MS) Studies

While the above SLIM IM-MS methods have proven useful for differentiation of several groups of known cannabinoid isomers, especially when commercial standards are available for comparison of CCS, structural characterization and identification of true unknown metabolites in complex mixtures can further benefit from including tandem mass spectrometry (MS/MS). Because the current instrument platform (MOBIE SLIM/Agilent 6546) performs mobility separations prior to mass isolation in the quadrupole and fragmentation in the collision cell, fragment ions can be easily matched to their precursor via mobility alignment. To demonstrate the selectivity of this approach for structural characterization and identification of cannabinoid metabolites, all compounds (individually and as mixtures) were subjected to SLIM IM-MS/MS analysis. Individual fragmentation spectra for protonated MDA-19 isomers are shown in Figure 4. Because the three compounds were mobility-separated, their MS/MS spectra could be individually interrogated and displayed unique product ions. As such, each could be easily resolved according to the following transitions: (1) MDA-19 N-4-OH: CCS 196, *m/z* 366 → 121; (2) MDA-19 N-5-OH: CCS 189, *m/z* 366 → 105; and (3) APP-BUTINACA : CCS 187, *m/z* 366 → 201.

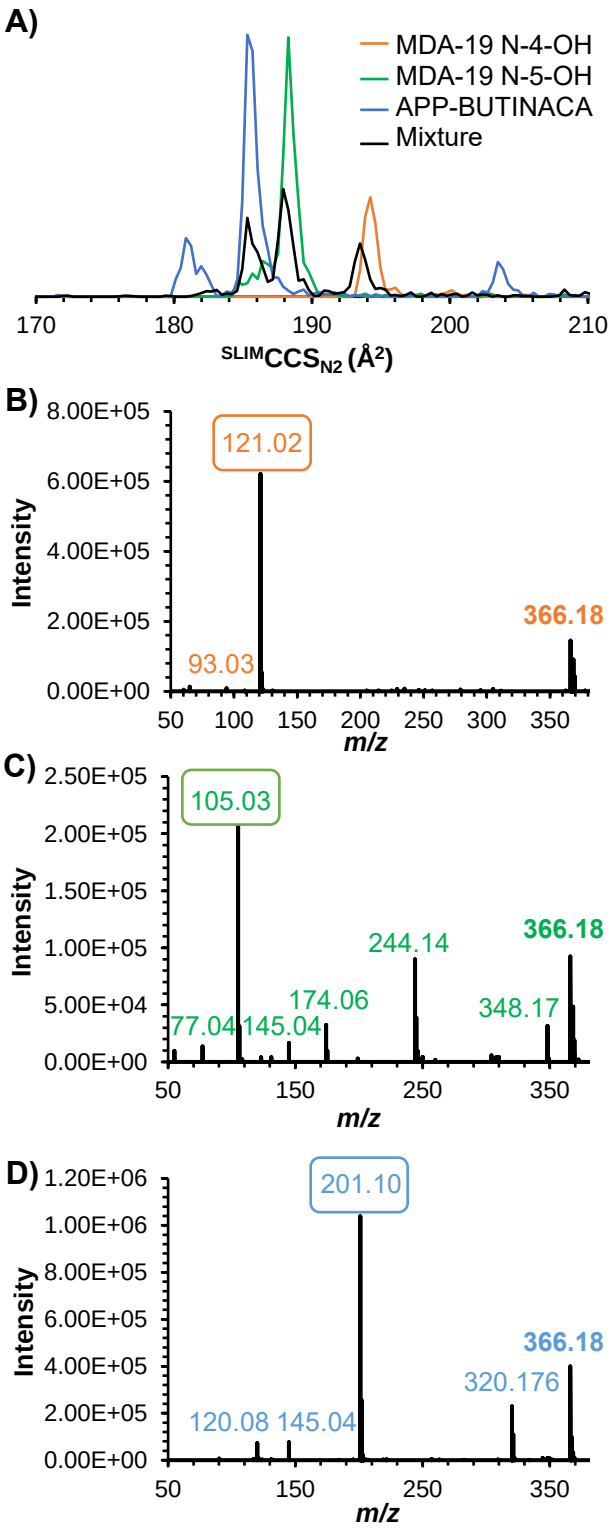

Figure 4. (A) Protonated MDA isomers were well resolved in a mixture by SLIM IM. Structural analysis could be performed by ion mobility-aligned MS/MS fragmentation for each individual IM feature. This demonstrated unique fragments for (B) MDA-19 N-4-OH at *m/z* 121, (C) MDA-19 N-5-OH at *m/z* 105, and (D) APP-BUTINACA at *m/z* 201.

CONCLUSION

Several classes of synthetic cannabinoid metabolite isomers, differing only in the position of one or a few of their functional groups, were successfully differentiated using a combination of high-resolution SLIM IM, selective derivatization reactions, dimerization, and mobility-aligned MS/MS. The unique challenges in identifying these compounds highlighted the need for such a multidimensional approach (i.e., SLIM IM-MS/MS). This approach allowed not only targeted separation, but also provided structural information (especially via MS/MS fragmentation) that could be used in future applications to identify potentially novel synthetic cannabinoids and their metabolites. Future studies will investigate the ability of this workflow to identify and quantify cannabinoid metabolites in biological samples (i.e., urine).

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge. MOBIE SLIM and Agilent 6546 QTOF parameters; Raw $^{SLIM}CCS_{N2}$ values for protonated, sodiated, and sodiated dimer species; Structures of all metabolites; Mobility separation of secondary metabolites; Reaction scheme for dansyl chloride derivatization

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Author Contributions

RA and SWL prepared and analyzed the samples. RA, SWL, and CDC processed the data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

Financial support for this work was provided by Agilent Technologies (Global Academic Research Support Program #2624930), MOBILion Systems, Inc., and Clemson University startup funds.

REFERENCES

(1) Mills, B.; Yepes, A.; Nugent, K. Synthetic Cannabinoids. *Am. J. Med. Sci*. **2015**, *350*, 59–62.

(2) Le Boisselier, R.; Alexandre, J.; Lelong-Boulouard, V.; Debruyne, D. Focus on Cannabinoids and Synthetic Cannabinoids. *Clin. Pharmacol. Ther.* **2017**, *101*, 220–229.

(3) Castaneto, M. S.; Gorelick, D. A.; Desrosiers, N. A.; Hartman, R. L.; Pirard, S.; Huestis, M. A. Synthetic Cannabinoids: Epidemiology, Pharmacodynamics, and Clinical Implications. *Drug Alc. Dep*. **2014**, *144*, 12–41.

(4) Martin, B. R.; Balster, R. L.; Razdan, R. K.; Harris, L. S.; Dewey, W. L. Behavioral Comparisons of the Stereoisomers of Tetrahydrocannabinols. *Life Sci.* **1981**, *19*, 565-574.

(5) Wiley, J. L.; Marusich, J. A.; Huffman, J. W. Moving around the Molecule: Relationship between Chemical Structure and in Vivo Activity of Synthetic Cannabinoids. *Life Sci*. **2014**, *97*, 55–63.

(6) Navarro-Tapia, E.; Codina, J.; Villanueva-Blasco, V. J.; García-Algar, Ó.; Andreu-Fernández, V. Detection of the Synthetic Cannabinoids AB-CHMINACA, ADB-CHMINACA, MDMB-CHMICA, and 5F-MDMB-PINACA in Biological Matrices: A Systematic Review. *Biology*. **2022**, *11*, 796.

(7) Zuba, D.; Byrska, B.; Maclow, M. Comparison of “Herbal Highs” Composition. *Anal. Bioanal. Chem.* **2011**, *400*, 119–126.

(8) Pellegrini, M.; Marchei, E.; Papaseit, E.; Farré, M.; Zaami, S. UHPLC-HRMS and GC-MS Screening of a Selection of Synthetic Cannabinoids and Metabolites in Urine of Consumers. *Medicina,* **2020**, *56*, 1–9.

(9) Kusano, M.; Zaitsu, K.; Yamanaka, M.; Hisatsune, K.; Asano, T.; Taki, K.; Hayashi, Y.; Tsuchihashi, H.; Ishii, A. Development of a Mass Spectrometric Hydroxyl-Position Determination Method for the Hydroxyindole Metabolites of JWH-018 by GC-MS/MS. *J. Mass Spectrom.* **2016**, *51*, 350–357.

(10) Emerson, B.; Durham, B.; Gidden, J.; Lay, J. O. Gas Chromatography-Mass Spectrometry of JWH-018 Metabolites in Urine Samples with Direct Comparison to Analytical Standards. *Forensic Sci. Int.* **2013**, *229*, 1–6.

(11) Gwak, S.; Arroyo-Mora, L. E.; Almirall, J. R. Qualitative Analysis of Seized Synthetic Cannabinoids and Synthetic Cathinones by Gas Chromatography Triple Quadrupole Tandem Mass Spectrometry. *Drug Test. Anal.* **2015**, *7*, 121–130.

(12) Atasoy, A.; Daglioglu, N.; Gören, İ. E.; Girisbay, A.; Aslan, R.; Akgur, S. A. Determination of Synthetic Cannabinoids in Randomly Urine Samples Collected from Probationers in Turkey. *Forensic Sci. Int.* **2021**, *322*, 110752.

(13) Mazzarino, M.; Torre, X. de la; Botrè, F. A Liquid Chromatography-Mass Spectrometry Method Based on Class Characteristic Fragmentation Pathways to Detect the Class of Indole-Derivative Synthetic Cannabinoids in Biological Samples. *Anal. Chim. Acta.* **2014**, *837*, 70–82.

(14) Liying, Z.; Min, S.; Baohua, S.; Hang, C.; Xin, W.; Hongxiao, D.; Ping, X.; Yan, S. Application of a UPLC-MS/MS Method for Quantitative Analysis of 29 Synthetic Cannabinoids and Their Metabolites, Such as ADB-BUTINACA and MDMB-4en-PINACA in Human Hair in Real Cases. *Forensic Sci. Int.* **2022**, *331*, 111139.

(15) Rosano, T. G.; Cooper, J. A.; Scholz, K. L.; Wood, M. Confirmation of Cannabinoids in Forensic Toxicology Casework by Isomer-Selective UPLC–MS-MS Analysis in Urine. *J. Anal. Toxicol.* **2023**, *47*, 709-718.

(16) Agostini, M.; Favretto, D.; Renzoni, C.; Vogliardi, S.; Duranti, A. Characterization of URB Series Synthetic Cannabinoids by HRMS and UHPLC–MS/MS. *Pharmaceuticals.* **2023**, *16*, 201.

(17) Liu, P.; Liu, W.; Qiao, H.; Jiang, S.; Wang, Y.; Chen, J.; Su, M.; Di, B. Simultaneous Quantification of 106 Drugs or Their Metabolites in Nail Samples by UPLC-MS/MS with High-Throughput Sample Preparation: Application to 294 Real Cases. *Anal. Chim. Acta.* **2022**, *1226*, 340170.

(18) Nzekoue, F. K.; Agostini, M.; Verboni, M.; Renzoni, C.; Alfieri, L.; Barocci, S.; Ricciutelli, M.; Caprioli, G.; Lucarini, S. A Comprehensive UHPLC–MS/MS

Screening Method for the Analysis of 98 New Psychoactive Substances and Related Compounds in Human Hair. *J. Pharm. Biomed. Anal.* **2021**, *205*, 114310.

(19) Watanabe, S.; Kuzhiumparambil, U.; Nguyen, M. A.; Cameron, J.; Fu, S. Metabolic Profile of Synthetic Cannabinoids 5F-PB-22, PB-22, XLR-11 and UR-144 by Cunninghamella Elegans. *AAPS J.* **2017**, *19*, 1148–1162.

(20) Sobolevsky, T.; Prasolov, I.; Rodchenkov, G. Detection of Urinary Metabolites of AM-2201 and UR-144, Two Novel Synthetic Cannabinoids. *Drug Test. Anal.* **2012**, *4*, 745–753.

(21) Grigoryev, A.; Savchuk, S.; Melnik, A.; Moskaleva, N.; Dzhurko, J.; Ershov, M.; Nosyrev, A.; Vedenin, A.; Izotov, B.; Zabirova, I.; Rozhanets, V. Chromatography-Mass Spectrometry Studies on the Metabolism of Synthetic Cannabinoids JWH-018 and JWH-073, Psychoactive Components of Smoking Mixtures. *J. Chrom. B,* **2011**, *879*, 1126–1136.

(22) Armenta, S.; Garrigues, S.; de la Guardia, M.; Brassier, J.; Alcalà, M.; Blanco, M.; Perez-Alfonso, C.; Galipienso, N. Detection and Characterization of Emerging Psychoactive Substances by Ion Mobility Spectrometry. *Drug Test. Anal.* **2015**, *7*, 280–289.

(23) Gwak, S.; Almirall, J. R. Rapid Screening of 35 New Psychoactive Substances by Ion Mobility Spectrometry (IMS) and Direct Analysis in Real Time (DART) Coupled to Quadrupole Time-of-Flight Mass Spectrometry (QTOF-MS). *Drug Test. Anal.* **2015**, *7*, 884–893.

(24) Sonnberg, S.; Armenta, S.; Garrigues, S.; de la Guardia, M. Detection of Tetrahydrocannabinol Residues on Hands by Ion-Mobility Spectrometry (IMS). Correlation of IMS Data with Saliva Analysis. *Anal. Bioanal. Chem.* **2015**, *407*, 5999–6008.

(25) Contreras, M. del M.; Jurado-Campos, N.; Sánchez-Carnerero Callado, C.; Arroyo-Manzanares, N.; Fernández, L.; Casano, S.; Marco, S.; Arce, L.; Ferreiro-Vera, C. Thermal Desorption-Ion Mobility Spectrometry: A Rapid Sensor for the Detection of Cannabinoids and Discrimination of Cannabis Sativa L. Chemotypes. *Sens. Actuators B. Chem.* **2018**, *273*, 1413–1424.

(26) Metternich, S.; Zörntlein, S.; Schönberger, T.; Huhn, C. Ion Mobility Spectrometry as a Fast Screening Tool for Synthetic Cannabinoids to Uncover Drug Trafficking in Jail via Herbal Mixtures, Paper, Food, and Cosmetics. *Drug Test. Anal.* **2019**, *11*, 833–846.

(27) Norman, C.; McKirdy, B.; Walker, G.; Dugard, P.; NicDaéid, N.; McKenzie, C. Large-Scale Evaluation of Ion Mobility Spectrometry for the Rapid Detection of Synthetic Cannabinoid Receptor Agonists in Infused Papers in Prisons. *Drug Test. Anal* **2021**, *13*, 644–663.

(28) Martínez-Pérez-Cejuela, H.; García-Atienza, P.; Simó-Alfonso, E. F.; Herrero-Martínez, J. M.; Armenta, S. Micro-Paper-Based Analytical Device Decorated with Metal-Organic Frameworks for the Assay of Synthetic Cannabinoids in Oral Fluids Coupled to Ion Mobility Spectrometry. *Microchimica Acta.* **2023**, *190*, 271.

(29) Tose, L. V.; Santos, N. A.; Rodrigues, R. R. T.; Murgu, M.; Gomes, A. F.; Vasconcelos, G. A.; Souza, P. C. T.; Vaz, B. G.; Romão, W. Isomeric Separation of Cannabinoids by UPLC Combined with Ionic Mobility Mass Spectrometry (TWIM-MS)—Part I. *Int. J. Mass Spectrom.* **2017**, *418*, 112–121.

(30) Kiselak, T. D.; Koerber, R.; Verbeck, G. F. Synthetic Route Sourcing of Illicit at Home Cannabidiol (CBD) Isomerization to Psychoactive Cannabinoids Using Ion Mobility-Coupled-LC–MS/MS. *Forensic Sci. Int.* **2020**, *308*, 110173.

(31) Yanini, A.; Esteve-Turrillas, F. A.; de la Guardia, M.; Armenta, S. Ion Mobility Spectrometry and High Resolution Mass-Spectrometry as Methodologies for Rapid Identification of the Last Generation of New Psychoactive Substances. *J Chrom. A.* **2018**, *1574*, 91–100.

(32) Hädener, M.; Kamrath, M. Z.; Weinmann, W.; Groessl, M. High-Resolution Ion Mobility Spectrometry for Rapid Cannabis Potency Testing. *Anal. Chem.* **2018**, *90*, 8764–8768.

(33) Mashmoushi, N.; Larry Campbell, J.; di Lorenzo, R.; Scott Hopkins, W. Rapid Separation of Cannabinoid Isomer Sets Using Differential Mobility Spectrometry and Mass Spectrometry. *Analyst.* **2022**, *147*, 2198-2206.

(34) Ieritano, C.; Thomas, P.; Hopkins, W. S. Argentination: A Silver Bullet for Cannabinoid Separation by Differential Mobility Spectrometry. *Anal. Chem.* **2023**, *95*, 8668–8678.

(35) Garimella, S. V. B.; Ibrahim, Y. M.; Webb, I. K.; Ipsen, A. B.; Chen, T. C.; Tolmachev, A. V.; Baker, E. S.; Anderson, G. A.; Smith, R. D. Ion Manipulations in Structures for Lossless Ion Manipulations (SLIM): Computational Evaluation of a 90° Turn and a Switch. *Analyst.* **2015**, *140*, 6845–6852.

(36) Deng, L.; Webb, I. K.; Garimella, S. V. B.; Hamid, A. M.; Zheng, X.; Norheim, R. V.; Prost, S. A.; Anderson, G. A.; Sandoval, J. A.; Baker, E. S.; Ibrahim, Y. M.;

Smith, R. D. Serpentine Ultralong Path with Extended Routing (SUPER) High Resolution Traveling Wave Ion Mobility-MS Using Structures for Lossless Ion Manipulations. *Anal. Chem.* **2017**, *89*, 4628–4634.

(37) Hamid, A. M.; Ibrahim, Y. M.; Garimella, S. V. B.; Webb, I. K.; Deng, L.; Chen, T. C.; Anderson, G. A.; Prost, S. A.; Norheim, R. V.; Tolmachev, A. V.; Smith, R. D. Characterization of Traveling Wave Ion Mobility Separations in Structures for Lossless Ion Manipulations. *Anal. Chem.* **2015**, *87*, 11301–11308.

(38) Chen, T. C.; Ibrahim, Y. M.; Webb, I. K.; Garimella, S. V. B.; Zhang, X.; Hamid, A. M.; Deng, L.; Karnesky, W. E.; Prost, S. A.; Sandoval, J. A.; Norheim, R. V.; Anderson, G. A.; Tolmachev, A. V.; Baker, E. S.; Smith, R. D. Mobility-Selected Ion Trapping and Enrichment Using Structures for Lossless Ion Manipulations. *Anal. Chem.* **2016**, *88*, 1728–1733.

(39) Deng, L.; Ibrahim, Y. M.; Hamid, A. M.; Garimella, S. V. B.; Webb, I. K.; Zheng, X.; Prost, S. A.; Sandoval, J. A.; Norheim, R. V.; Anderson, G. A.; Tolmachev, A. V.; Baker, E. S.; Smith, R. D. Ultra-High Resolution Ion Mobility Separations Utilizing Traveling Waves in a 13 m Serpentine Path Length Structures for Lossless Ion Manipulations Module. *Anal. Chem.* **2016**, *88*, 8957–8964.

(40) Allen, S. J.; Eaton, R. M.; Bush, M. F. Analysis of Native-Like Ions Using Structures for Lossless Ion Manipulations. *Anal. Chem.* **2016**, *88*, 9118–9126.

(41) Chouinard, C. D.; Nagy, G.; Webb, I. K.; Shi, T.; Baker, E. S.; Prost, S. A.; Liu, T.; Ibrahim, Y. M.; Smith, R. D. Improved Sensitivity and Separations for Phosphopeptides Using Online Liquid Chromotography Coupled with Structures for Lossless Ion Manipulations Ion Mobility-Mass Spectrometry. *Anal. Chem.* **2018**, *90*, 10889–10896.

(42) Nagy, G.; Kedia, K.; Attah, I. K.; Garimella, S. V. B.; Ibrahim, Y. M.; Petyuk, V. A.; Smith, R. D. Separation of β-Amyloid Tryptic Peptide Species with Isomerized and Racemized L-Aspartic Residues with Ion Mobility in Structures for Lossless Ion Manipulations. *Anal. Chem.* **2019**, *91*, 4374–4380.

(43) Nagy, G.; Chouinard, C. D.; Attah, I. K.; Webb, I. K.; Garimella, S. V. B.; Ibrahim, Y. M.; Baker, E. S.; Smith, R. D. Distinguishing Enantiomeric Amino Acids with Chiral Cyclodextrin Adducts and Structures for Lossless Ion Manipulations. *Electrophoresis.* **2018**, *39*, 3148–3155.

(44) Wojcik, R.; Webb, I. K.; Deng, L.; Garimella, S. V. B.; Prost, S. A.; Ibrahim, Y. M.; Baker, E. S.; Smith, R. D. Lipid and Glycolipid Isomer Analyses Using Ultra-High Resolution Ion Mobility Spectrometry Separations. *Int. J. Mol. Sci.* **2017**, *18*, 183.

(45) Nagy, G.; Attah, I. K.; Garimella, S. V. B.; Tang, K.; Ibrahim, Y. M.; Baker, E. S.; Smith, R. D. Unraveling the Isomeric Heterogeneity of Glycans: Ion Mobility Separations in Structures for Lossless Ion Manipulations. *Chem. Comm.* **2018**, *54*, 11701–11704.

(46) Chouinard, C. D.; Nagy, G.; Webb, I. K.; Garimella, S. V. B.; Baker, E. S.; Ibrahim, Y. M.; Smith, R. D. Rapid Ion Mobility Separations of Bile Acid Isomers Using Cyclodextrin Adducts and Structures for Lossless Ion Manipulations. *Anal. Chem.* **2018**, *90*, 11086–11091.

(47) Kedia, K.; Harris, R.; Ekroos, K.; Moser, K. W.; DeBord, D.; Tiberi, P.; Goracci, L.; Zhang, N. R.; Wang, W.; Spellman, D. S.; Bateman, K. Investigating Performance of the SLIM-Based High Resolution Ion Mobility Platform for Separation of Isomeric Phosphatidylcholine Species. *J. Am. Soc. Mass Spectrom.* **2023**, *34*, 2176-2186.

(48) Rose, B. S.; May, J. C.; Reardon, A. R.; McLean, J. A. Collision Cross-Section Calibration Strategy for Lipid Measurements in SLIM-Based High-Resolution Ion Mobility. *J. Am. Soc. Mass Spectrom.* **2022**, *33*, 1229–1237.

(49) Anari, M. R.; Bakhtiar, R.; Zhu, B.; Huskey, S.; Franklin, R. B.; Evans, D. C. Derivatization of Ethinylestradiol with Dansyl Chloride to Enhance Electrospray Ionization: Application in Trace Analysis of Ethinylestradiol in Rhesus Monkey Plasma. *Anal. Chem.* **2002**, *74*, 4136–4144.

(50) Stow, S. M.; Causon, T. J.; Zheng, X.; Kurulugama, R. T.; Mairinger, T.; May, J. C.; Rennie, E. E.; Baker, E. S.; Smith, R. D.; McLean, J. A.; Hann, S.; Fjeldsted, J. C. An Interlaboratory Evaluation of Drift Tube Ion Mobility-Mass Spectrometry Collision Cross Section Measurements. *Anal. Chem.* **2017**, *89*, 9048–9055.

(51) Aderorho, R.; Chouinard, C. D. Improved Separation of Fentanyl Isomers Using Metal Cation Adducts and High-Resolution Ion Mobility-Mass Spectrometry (IM-MS). *Drug Test. Anal.* **2023**, *In Press*. DOI: 10.1002/dta.3550

(52) Bilbao, A.; Gibbons, B. C.; Stow, S. M.; Kyle, J. E.; Bloodsworth, K. J.; Payne, S. H.; Smith, R. D.; Ibrahim, Y. M.; Baker, E. S.; Fjeldsted, J. C. A Preprocessing Tool for Enhanced Ion Mobility-Mass Spectrometry-Based Omics Workflows. *J. Prot. Res.* **2022**, *21*, 798–807.

(53) May, J. C.; Leaptrot, K. L.; Rose, B. S.; Moser, K. L. W.; Deng, L.; Maxon, L.; Debord, D.; McLean, J. A. Resolving Power and Collision Cross Section Measurement Accuracy of a Prototype High-Resolution Ion Mobility Platform Incorporating Structures for Lossless Ion Manipulation. *J. Am. Soc. Mass Spectrom.* **2021**, *32*, 1126–1137.

(54) Smith, D. P.; Knapman, T. W.; Campuzano, L.; Malham, R. W.; Berryman, J. T.; Radford, S. E.; Ashcroft, A. E. Deciphering Drift Time Measurements from Travelling Wave Ion Mobility Spectrometry- Mass Spectrometry Studies. *Eur. J. Mass Spectrom.* **2009**, *15*, 113–130.

(55) Dodds, J. N.; May, J. C.; McLean, J. A. Correlating Resolving Power, Resolution, and Collision Cross Section: Unifying Cross-Platform Assessment of Separation Efficiency in Ion Mobility Spectrometry. *Anal. Chem.* **2017**, *89*, 12176–12184.

(56) Neal, S.; Hodges, W.; Velosa, D.; Aderorho, R.; Lucas, S.; Chouinard, C. D. Improved Analysis of Derivatized Steroid Hormone Isomers Using Ion Mobility-Mass Spectrometry (IM-MS). *Anal. Bioanal. Chem.* **2023**, *415*, 6757-6769.

(57) Velosa, D. C.; Dunham, A. J.; Rivera, M. E.; Neal, S. P.; Chouinard, C. D. Improved Ion Mobility Separation and Structural Characterization of Steroids Using Derivatization Methods. *J. Am. Soc. Mass Spectrom*. **2022**, *33*, 1761-1771.