https://doi.org/10.1016/j.trac.2019.115801

Recent Advances in Differentiation of Isomers by Ion Mobility Mass Spectrometry

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Abstract

Differentiation of isomers is an important analytical task in many fields, e.g., chemistry, biology, pharmaceutical science, medicine, environmental science, food science. Ion mobility mass spectrometry (IM-MS) can resolve isobaric ions based on their shape and size and measure the collision cross section (CCS) values of ions to provide information on their intrinsic structures, and is thus a very promising tool for differentiation of isomers. In this review, the applications of IM-MS in differentiation of various isomers including glycans, lipids, peptides, proteins, as well as metabolite isomers, chiral isomers, etc. are summarized. Prospects for further development of IM-MS for differentiation of isomers are also discussed.

Keywords: Ion mobility; Mass spectrometry; Isomer; Differentiation.

1. Introduction

1.1 Importance of differentiation of isomers

Isomers are chemical species with the same formulas but different structures. Isomerism is a common phenomenon in nature and synthetic chemistry. The structural differences of isomers can lead to significant differences in their physical, chemical and thus biological, and pharmaceutical properties. For example, the different configurations of glycosidic bonds of glucose monomers in cellulose and starch make them have very dissimilar properties and functions; (S)-ibuprofen is about 160 times more potent than its (R)-enantiomer in inhibiting prostaglandin synthesis *in vitro,* and (R)-ibuprofen can undergo rapid and substantial inversion into the active (S)-enantiomer *in vivo* [1]; cisplatin and transplatin usually orientate to different binding positions of methionine upon reaction in water solution [2]. Differentiation of isomers is thus an important task in chemistry, biology, pharmaceutical science, medicine, environmental science, food science, etc.

Various techniques have been applied for differentiation of isomers, including ultraviolet and infrared spectroscopy, circular dichroism, nuclear magnetic resonance, chromatography, capillary electrophoresis, mass spectrometry (MS), etc. Compared to other techniques, MS shows significant advantages in speed, sensitivity and specificity, and has been widely used for differentiation of isomers. Isomers are detected with the same mass-to-charge (*m/z*) values in the mass spectra, and typically cannot be distinguished directly by using single-stage MS. Differentiation of isomers by conventional MS is usually achieved by comparing their reaction

tendencies with selective reagents or their fragmentation patterns in tandem MS (MS/MS) [3].

Ion mobility spectrometry (IMS) can separate gas-phase ions based on their charge, size and shape, according to their differential mobility through a buffer gas in an electric field. The coupling of ion mobility with MS (IM-MS) can thus provide an additional dimension to resolve isobaric ions that are indistinguishable by MS alone, and the collision cross section (CCS, Ω) values obtained by IM-MS offer the structural and molecular dynamics information of the ions [4, 5]. IM-MS is thus a very promising technique for differentiation of isomers. Major IM-MS techniques, including drift-time IMS (DTIMS), traveling-wave IMS (TWIMS), field asymmetric IMS (FAIMS) (also known as differentiation mass spectrometry (DMS)) and trapped ion mobility spectrometry (TIMS), have been employed for differentiation of isomers [4, 6]. Although there have been several review articles about the applications of IM-MS for analysis of a certain class of compounds such as metabolites, glycan and lipids [7-10], a comprehensive review covering the major issues in isomer analysis by IM-MS has not been available yet, which is expected to be addressed in this review.

2. Applications of IM-MS in differentiation of isomers

2.1 Glycans

Carbohydrates are a common energy source for living organisms, and glycan is the carbohydrate portion of many important biological molecules in living organisms. The inherent complexity of branch and diverse regio- and stereochemistry in glycans leads to a vast number

of possible isomers with differences in composition, connectivity or configuration, presenting a major challenge for their structural analysis. IM-MS has proved to be a powerful tool for structural characterization of glycans [10], and can play a critical role in carbohydrate chemistry and glycobiology.

Hofmann *et al.* [11] systematically investigated the separation of trisaccharides with various types of isomerization by TWIMS, and demonstrated that IM-MS in negative ion mode could be used to unambiguously identify isomerization of connectivity or configuration in trisaccharides. Deprotonated ions of trisaccharides with (1→4) connectivity and trisaccharides in α configuration were found to have shorter drift time than their (1→3) linked and β configuration counterparts, respectively (Fig.1). Quantitative results showed that the coexisting carbohydrate isomers could be detected with relative concentrations as low as 0.1%. The deprotonated or chlorine adduct ions were reported to allow better IM-MS separation of some glycan isomers than their protonated species or sodium adducts [11-13]. On IM-MS analysis of human milk oligosaccharides, Struwe *et al.* [12] employed *ab* initio molecular dynamics (MD) simulation to reveal the highly mobile position of the deprotonation site, which was believed to lead to considerably richer structural spaces of the deprotonated ions and their improved IM-MS separation.

Positive ion mode IM-MS has also been commonly used for differentiation of glycan isomers, typically with the addition of metal ions to improve the separation [14, 15]. It was found that

the metal type, the number of metal ion adducts, the ionic radii, and the ionic valence of metal ions affected the metal-induced conformational change of heparin octasaccharide, which might further alter the interactions of heparin and heparin-binding proteins [14]. Moreover, the spin state of the transition metal ions was found to have an effect on ionic radii, which was not observed on other metals investigated. A later study [15] on separation of five isomeric tetrasaccharides using a hybrid IM-MS revealed that drift time for adducts of alkaline earth metals overall consistently increased as the metal ionic radii increased, which was not observed for transition metals and alkaline metals, and that transition metal adducts yielded improved separation over glycan anion for the isomers examined.

A total of 65 CCS values for alkali metal adducts of four groups of isomeric carbohydrates were measured using a DTIM instrument [16]. The results showed that the CCS of adducts generally increased with increasing metal ion radius and appropriate selection of metal ion binders could optimize the resolution and distinction of carbohydrate isomers. A TWIMS instruments calibration protocol was proposed by Pagel et al. [17], which was achieved by spraying a set of glycans using in-source fragmentation conditions to generate a large number of glycan calibrants. Using this protocol, a series of sodiated N-glycan isomers with different conformations could be distinguished based on their CCS values when all the associated errors were taken into account [17]. For known isomeric structures, the experimental data could be compared to theoretical CCS values for predicting or matching the protonation site and structure of the observed ions. Computational methods have been applied for calculation of

CCSs, with the Trajectory Method (TM), Projection Approximation (PA) and Exact Hard Sphere Scattering (EHSS) as the prevailing algorithms [18]. For unknown structures, theoretical method based on quantum mechanical calculations, such as density-functional theory (DFT), is promising for data interpreting, understanding of IM-MS data at the atomic level and rigorous screening of the structural space [18, 19].

2.2 Lipids

Cellular lipids perform diverse biological functions, ranging from building blocks of membrane structures to critical signaling intermediates. Abundant evidence [8] has shown that the position of the unsaturated fatty acyl chain, location and orientation of double bonds are related to the specific lipid biological mechanisms and diseases. Therefore, structural elucidation of lipid isomers is essential to explore their functions. IM-MS has recently emerged as an important tool for lipid classification and isomer separation.

Lipids can be classified into specific 'trend lines' for distinct lipid classes by the drift time versus *m/z* plots using IM-MS. As shown in Fig. 2a, glycerophospholipids, glycerolipids and sphingolipids were clustered with distinct trends in atmospheric pressure DTIMS [20]. Diacyl-phospholipids (PL) include phosphoethanolamine (PE), phosphoethanolamine phosphate (PE(P)), phosphoserine (PS), phosphoglycerol (PG), phosphatidylinositol (PI) and phosphocholines (PC), among which PEs and PCs, including their *sn*-1/*sn*-2 positional isomers, *cis/trans* and R/S isomers, showed more significantly different trend lines that allowed them to

be distinguished from each other (Fig. 2b). Similar trends were also observed in a recent study involving construction of a CCS database of sphingolipid and glycerophospholipid species using high precision IM-MS [21]. Comparing with glycerophospholipids of similar masses, sphingolipids exhibited 2-6% larger CCSs (Figs. 2c and 2d), probably due to the sphingosine backbone's restriction of the *sn-1* tail length, which limited the gas-phase packing efficiency. The *cis/trans* isomer pair could also be separated using a TWIMS device created with structures for lossless ion manipulations (SLIM), with differentiation attributed to the compact serpentine ion drift path [22]. A FAIMS study showed that about 75% of triacylglycerols (TG), and PC isomers could be separated at high electric fields, including *regio*-, *sn*-, positional, and geometric pairs [23], indicating that in general the smaller fatty acyl chains in the *sn-1* position, the *cis*-oriented double bonds and the R orientations of the 2-hydroxyl group of ceramides made the lipid structures more compact. Among all the types of lipid isomers, those that differ only in the unsaturation location confronted larger difficulty and required higher resolving power for their differentiation. Ozonolysis reaction could be utilized to target sites of unsaturation in lipids to diagnose the double bound cleavage. In an online LC-ozone-induced dissociation (OzID)-IM-MS approach [24], ozone was introduced into the high-pressure trapping ion funnel region for oxidation of ionized lipids in 10 ms, allowing assignment for the positions of carbon-carbon double bonds of 11 classes of unsaturated lipids based on the predictable OzID transitions. Forming adducts could be a solution to increase the shape differences between lipid isomers as well. For instance, four pairs of TG regio-isomers could be separated using DMS with their silver adducts, and improved separation of oleic or stearic

acid could be achieved with 1-butanol or 1-propanol as the chemical modifier [25].

Explorations have been made on differentiation of lipid isomers in complex biological mixtures by IM-MS [20, 24-26]. The study via DMS showed that the regioisomeric ratios of three pairs of TG isomers containing combinations of saturated and monounsaturated fatty acyls in porcine adipose tissue samples could be measured within one minute [25]. The online OzID-IMS-MS approach also provided a possibility to reveal the presence of double bond geometrical isomers in biological samples, in which the *cis* PE isomer exhibited a lower drift time than the *trans* isomer [24]. Jeanne et al. [26] demonstrated the potential of LC-TIMS-MS for non-targeted analysis of isomeric PC and diacylglycerol (DG) lipid species in human plasma, allowing identification of lipid isomers that differed in the double bond locations/geometries as well as in the position of the acyl chain, and the performance of distinguishing PC 16:0/18:1 lipid isomers in the complex biological mixture could be comparable with that in solutions, with the small structural differences with CCS less than 1% distinguishable. The identification of lipids is still restricted to the available CCS values. To achieve a precise prediction of lipid CCSs, an LipidCCS database including 15,646 lipids and 63,434 CCS values in total was built using optimized molecular descriptors together with standard CCS values for 458 lipids [27].

2.3 Peptides and proteins

Differentiation of the same protein with PTMs in distinct localization is still a challenge in proteomics study. FAIMS was applied by Cooper et al. [28] for the analysis of a

phosphopeptide library containing numerous sequence inversions and structural isomers, and identified 35% of the peptide library, comparing with 8% by LC−MS/MS alone and 17.3% by LC-MS/MS with prefractionation using strong cation exchange chromatography. TWIMS also allowed differentiation of sialic acid linkage isomers on both N- and O-linked glycopeptides [29]. Due to the very similar drift behavior of the isomers, instrumental resolution is of higher priority than sensitivity from the view of separation. FAIMS and TIMS were combined to enable the orthogonal 2-dimension separations across field strength, charge states and CCS, allowing separation of the isotopic histone tails [30]. In addition, by adopting novel electronics to output stable waveforms platform, FAIMS using N_2 gas could be integrated with Orbitrap for the separation of histone tail isomers as well [31]. Using a novel IM-Ultraviolet photodissociation (UVPD)-MS approach to detect the human ubiquitin protein, two peaks with distinct drift times were observed for ions of the same molecule and the same charge state, representing the occurrence of isobaric fragments induced by UVPD, and MD simulation revealed that the difference was caused by the *cis/trans* isomerization of proline peptide bond [32]. In a broader perspective, these studies indicated the potential of IM-MS for structural analysis of proteins in the gas phase.

2.4 Metabolite isomers

Metabolites can be products of either endogenous physiological process or exogenous compounds. They are usually small molecules involved in numerous metabolic pathways and cellular regulatory processes in biological systems.

In 2003, FAIMS was applied for differentiation of three pairs of diastereoisomers, ephedrine and pseudoephedrine, as well as their metabolites, with reduced analysis time as it required no cleanup step and column separation [33]. With establishment of the more complete CCS database, identification and quantification of metabolite isomers become more and more practicable. The isomers could be characterized by comparing the experimental CCSs with theoretical CCSs in a standard-free method [34]. The additional separation dimension provided by IM incorporated with LC/MS could reduce the matrix effect in complex biological samples and improve the sensitivity and throughput of metabolic study. With FAIMS, the different mobility pattern of doubly sodiated adducts ($[M+2Na-H]^+$) of isobaric glucuronide and sulfate metabolites simplified their qualitative and quantitative analysis of seven anabolic-androgenic steroids in urine [35]. By adding a selective shift reagent (SSR), 4' -nitrobenzo-15-crown-5 ether, isomers of metabolite 2,3-dihydroxypropionylselenocystathionine produced by Se-rich yeast were fully resolved due to the different stability of host-guest complex formed between the isomeric analytes with the SSR. Under a 4 V collision energy in the trap cell prior to IM separation, one of the unstable crown ether complexes dissociated. The isomers could be separated and detected in different structural formation and their ratios were determined to be 11-13% and 87-89% using the method [36].

Metabolites are influenced by genes, environment, nutrition and disease state, and can be used as biomarkers for diseases. In a study about the striatal metabolomes in a Parkinson's like

disease (PD-like) rat model, dopamine, one of the biomarkers for PD-like diseases, was found to be co-existed with isomers [37]. Two peaks were observed in the selected-mass mobility spectra of *m/z* 154 for the health-control group samples, while only one peak appeared for the disease group. The missing peak was proved to be dopamine and the isomeric compound was tentatively inferred to be 2-(2,4-dihydroxyphenyl) ethylamine. The presence of this isomer suggested that the reduction of dopamine in PD might be underestimated.

Efforts have been made to build up a CCS database that can assist the annotation of metabolites. Picache et al. curated a unified CCS compendium of more than 3800 CCS values obtained from chemical standards representing a wide variety of structures spanning 14 super classes, 80 classes and 157 subclasses [38]. The graphical user interface (GUI) of the unified CCS compendium was shown in Fig. 3. This model enabled filtering and prediction of unknown biochemical species, which narrowed chemical search space by 60% while increasing the confidence in the remaining isomeric identifications from a single class.

2.5 Enantiomers

Differentiation of enantiomers typically requires the introduction of a chiral selector to form diastereomeric complexes, which can be separated by IM-MS. For example, amino acids were used as chiral selectors for the separation and quantification of terbutaline enantiomers by IM-MS [39]. The use of metal ions could normally facilitate the formation of multimeric complexes, and increased number of metal ion and chiral selector in diastereomeric complexes tended to

expand the difference of drift time and CCS values between the complexes [40]. Yu et al. [41] found that tetrameric complexes with binuclear metal could potentially improve chiral discrimination of amino acids as compared to the trimeric complexes with single metal, and that larger CCS differences and better enantioselectivity were observed for amino acids with aromatic rings or long and active side chains, suggesting that the mechanism for chiral recognition by IM-MS might be significantly different from that by MS/MS. Modified amino acids such as N-tert-butoxycarbonyl-O-benzyl-L-serine (BBS) were also used as the chiral selector using DMS to form proton-bound diastereomeric dimers for differentiation of enantiomers including cysteine and proline [42]. The separation of the two enantiomers in IM-MS allowed direct determination of enantiomeric excess or enantiomeric ratio using the two peaks [41, 42].

Introduction of chiral gas into drift tube is another approach for chiral differentiation by IM-MS. In 2006, (S)-(+)-2-butanol was used as a modifier of the drift gas to provide a chiral environment for interactions with the chiral analytes, allowing successful enantiomeric separations of atenolol, serine, methionine, threonine, methyl α-glucopyranoside, glucose, penicillamine, valinol, phenylalanine and tryptophan [43].

2.6 Others

Many pollutants, e.g., polybrominated diphenyl ether, endocrine disrupting hormones, nonylphenols, are mixtures of isomers that are detected as isobaric species in conventional MS.

However, the biologic activities of these isomers can be very different, and thus quantitation to each isomer is required for accurate assessment of their potential risks. Early in 2006, DMS was employed for analysis of halogenated aromatics and enabled differentiation of most of the studied isomeric compounds. Selected accumulation trapped ion mobility spectrometry (SA-TIMS) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) allowed reduced chemical interferences and differentiation of structural isomers of seven commonly targeted endocrine disrupting compounds, e.g., bisphenol A, (Z)- and (E)-diethylstilbestrol, hexestrol, estrone, α-estradiol, and 17-ethynylestradiol, from environmental samples [44].

For analysis of food samples, three mono-caffeoylquinic acid isomers, i.e., 5-O-caffeoylquinic acid, 3-O-caffeoylquinic acid and 4-O-caffeoylquinic acid, in fruit juice samples were separated with FAIMS using the major product ions in the negative ion MS/MS [45]. Hadamard transform ion mobility mass spectrometry (HT-IM-MS) also exhibited its high resolving power in separation of isomers with the same anthocyanidin but different hexoses, allowing profiling anthocyanins from different fruits with the mobility-mass trend lines to facilitate the discovery of unknown anthocyanin [46].

Recently, solvent-assisted electrospray ionization (SAESI) coupled with IM-MS successfully intercepted and characterized two short-lived isomers of carbinolamine and iminium species in the early stage of enamine formation [47]. The ratio of Z/E enamine intermediates was obtained

in situ, providing experimental support for the proposed reaction process, the mechanisms of organocatalytic asymmetric amination reactions and the origin of enantioselectivity.

3. Conclusions and prospects

IMS separates compounds based on their size, shape and charge in the gas phase, which complements the limitation of MS in distinguishing isomers with the same masses. The additional separation dimension provided by coupling MS with IMS has allowed IM-MS to become a powerful tool for separation of isomers. IM-MS has gained widespread applications for differentiation of isomers involved in carbohydrates, lipids, peptides, proteins, metabolites, enantiomers, and environmental and food compounds, etc. Many attempts have been made to increase the separation ability for the isomeric analytes, e.g., introduction of metals ions and selective shift reagents to enable larger drift time differences. The resolving power of IM-MS instruments is a major limitation for differentiation of isomers at this stage, and efforts have been made to enhance this by increasing path length of the drift tube, changing the drift gas, orthogonal coupling of two IMS instruments with diverse advantages, etc. Several algorithms have been developed for calculating CCS values, an important factor for characterizing structures of analytes. The improvement of the current algorithms may enhance the accuracy of matching between the theoretical and experimental CCS values, and precise elaboration of molecular structures based on their CCSs would be very helpful for IM-MS studies. CCS database is very useful for identification of unknown compounds in IM-MS, but the number of compounds in the current databases and the identification accuracy need to be further improved.

Together with the future improvements in the data processing software and the capability to efficiently extract information for structural interpretation, wider applications of IM-MS in isomer analysis can be expected, allowing discovery of more biological active isomers and better understanding of diseases and mechanisms.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 81874306), Hong Kong Research Grants Council (Nos. 153040/15P, 153348/16P, 153041/17P, C5031-14E and C4002-17G), and The University Research Facility in Chemical and Environmental Analysis and the University Research Facility in Life Sciences of The Hong Kong Polytechnic University.

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Figure captions

Figure 1. (a) Structural features of trisaccharides 1-6, with the same disaccharide core, and differences merely in the composition, connectivity, or configuration. (b) IM–MS drift-time distributions for $[M-H]^-$ ions of trisaccharides 1–6. 1→4 connectivity has shorter drift time than 1→3 connectivity (4 vs 1; 5 vs 2; 6 vs 3) and α configuration has shorter drift time than β configuration (3 vs 2; 6 vs 5) (Adapted from reference [11]).

Figure 2. (a) Plots of monoisotopic mass versus drift time for 280 lipids identified from standards and mouse uterine tissue from three lipid categories (glycerophospholipids, glycerolipids and sphingolipids); (b) further examination of 137 lipids from six subclasses within the diacyl-PL class (Adapted from reference [20]). (c) Plots of CCS versus mass for 456 lipids from 7 classes of 2 lipid categories; (d) Enlarged view of the region within the rectangle in (c) (Adapted from reference [21]).

Figure 3. (a) Compendium interface depicting measured data points classified into super classes. Trends lines according to charge state can be observed clearly. (b) Enlarged view of the area within the box and the illustration of information revealed by the data point. (c) Distribution of compounds across the 14 structural super classes (Adapted from reference [38]).

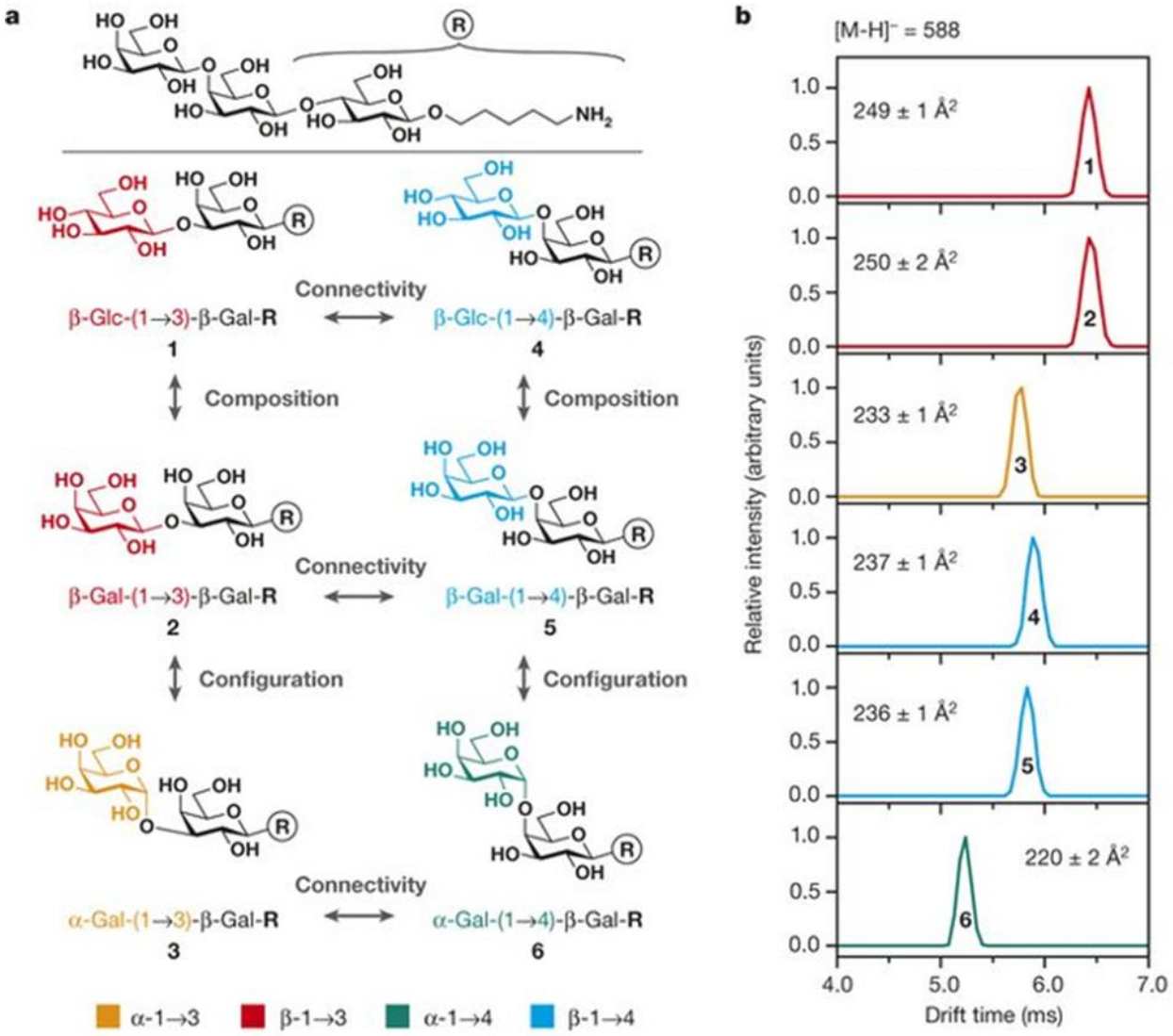
a
b
Connectivity
β-Glc-(1→3)-β-Gal-R
1
β-Glc-(1→4)-β-Gal-R
4
Composition
Composition
Connectivity
β-Gal-(1→3)-β-Gal-R
2
β-Gal-(1→4)-β-Gal-R
5
Configuration
Configuration
Connectivity
α-Gal-(1→3)-β-Gal-R
3
α-Gal-(1→4)-β-Gal-R
6
α-1→3
β-1→3
α-1→4
β-1→4
[M-H]⁻ = 588
249 ± 1 Å²
250 ± 2 Å²
233 ± 1 Å²
237 ± 1 Å²
236 ± 1 Å²
220 ± 2 Å²
Relative intensity (arbitrary units)
Drift time (ms)

Figure 1

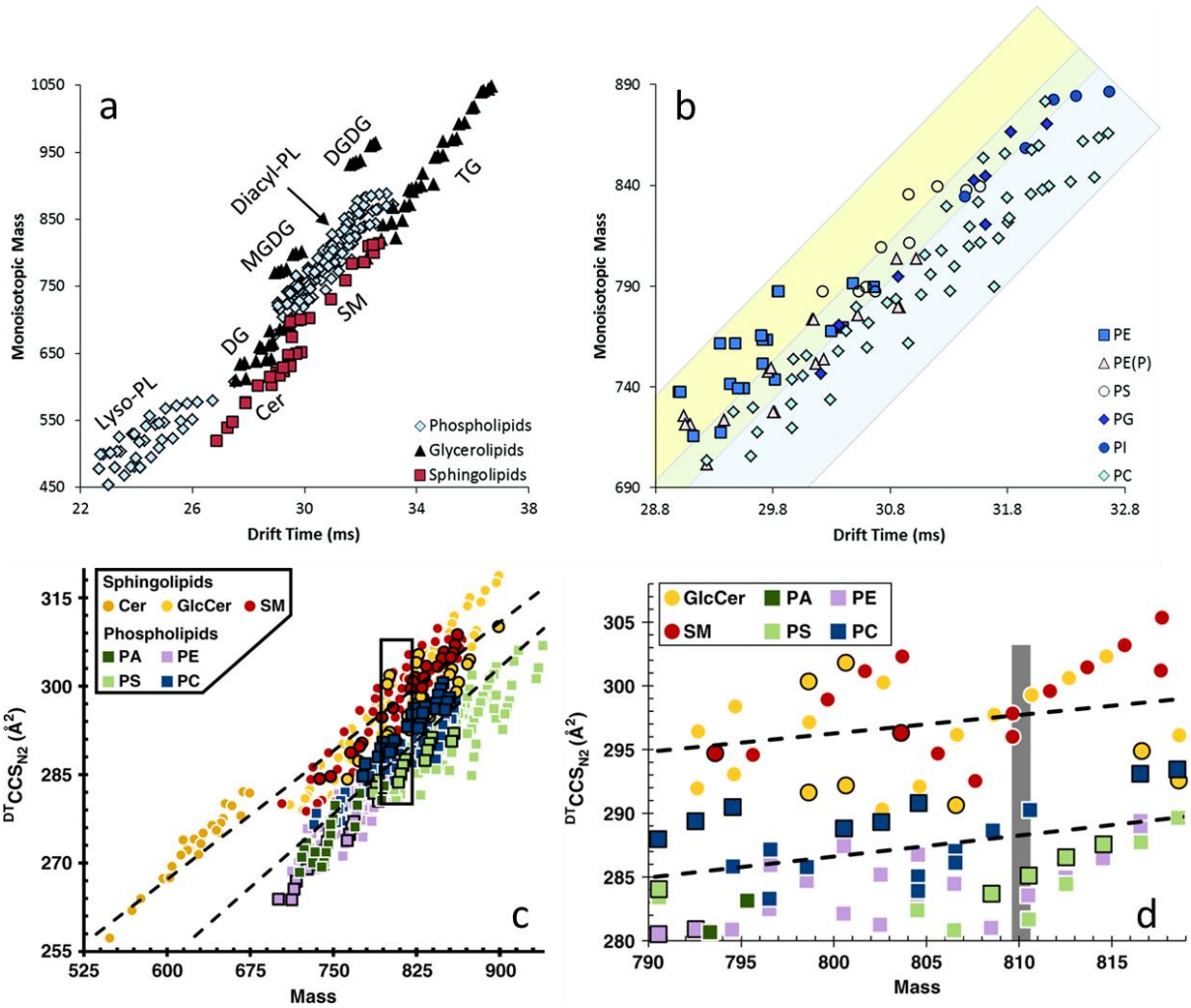
a
Monoisotopic Mass
Drift Time (ms)
Lyso-PL
DG
Cer
SM
MGDG
Diacyl-PL
DGDG
TG
Phospholipids
Glycerolipids
Sphingolipids
b
Monoisotopic Mass
Drift Time (ms)
PE
PE(P)
PS
PG
PI
PC
c
Sphingolipids
Cer
GlcCer
SM
Phospholipids
PA
PE
PS
PC
DTCCSN2 (Å2)
Mass
d
GlcCer
PA
PE
SM
PS
PC
DTCCSN2 (Å2)
Mass

Figure 2

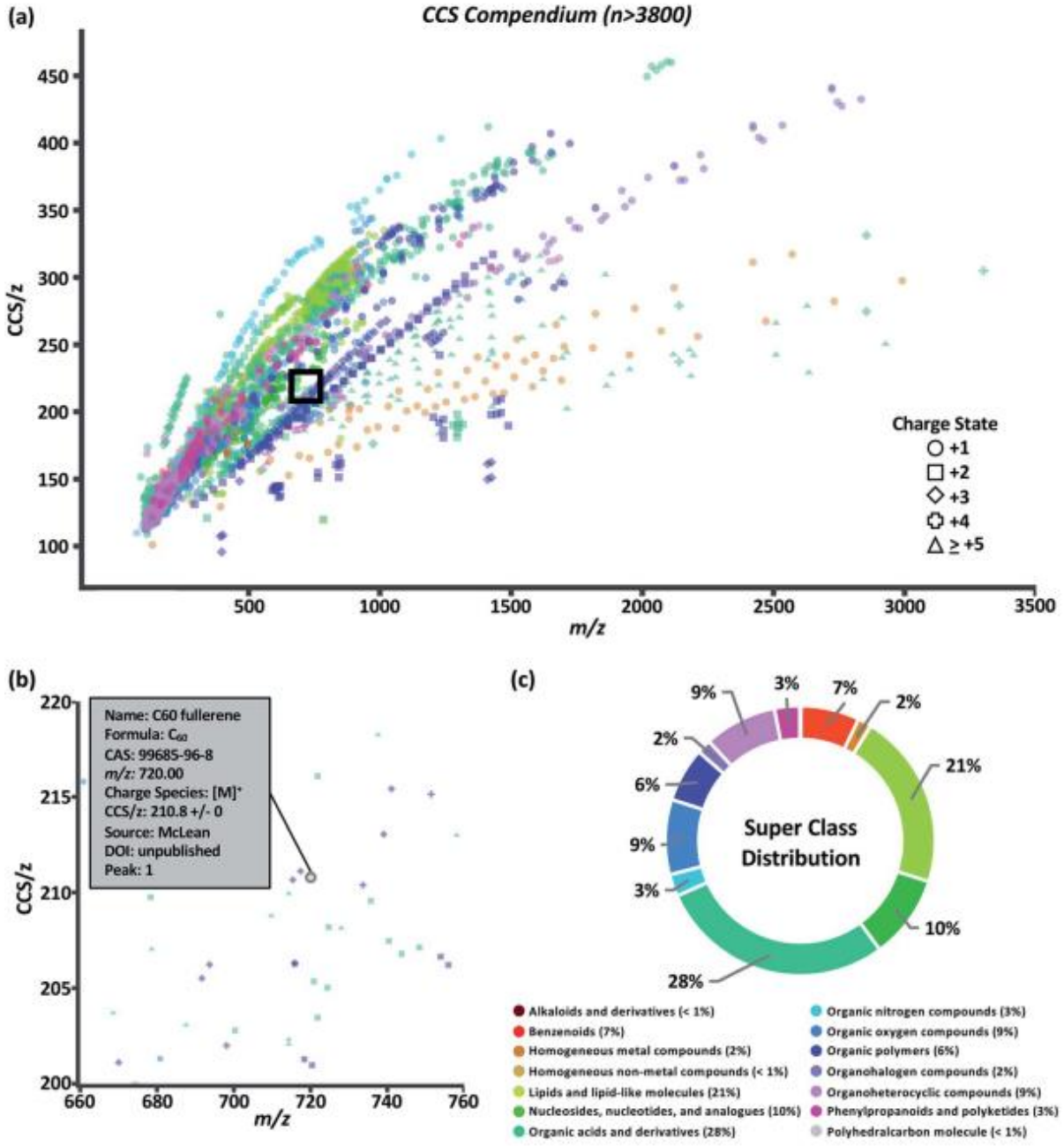
(a)
CCS Compendium (n>3800)
CCS/z
m/z
Charge State
+1
+2
+3
+4
≥ +5
(b)
Name: C60 fullerene
Formula: C60
CAS: 99685-96-8
m/z: 720.00
Charge Species: [M]+
CCS/z: 210.8 +/- 0
Source: McLean
DOI: unpublished
Peak: 1
(c)
Super Class Distribution
9%
3%
7%
2%
21%
10%
28%
3%
9%
6%
2%
Alkaloids and derivatives (< 1%)
Benzenoids (7%)
Homogeneous metal compounds (2%)
Homogeneous non-metal compounds (< 1%)
Lipids and lipid-like molecules (21%)
Nucleosides, nucleotides, and analogues (10%)
Organic acids and derivatives (28%)
Organic nitrogen compounds (3%)
Organic oxygen compounds (9%)
Organic polymers (6%)
Organohalogen compounds (2%)
Organoheterocyclic compounds (9%)
Phenylpropanoids and polyketides (3%)
Polyhedralcarbon molecule (< 1%)

Figure 3