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Adding a new separation dimension to MS and LC-MS: What is the utility of ion mobility spectrometry?

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28 **LIST OF ABBREVIATIONS**

- 29 2D-LC: two-dimensional liquid chromatography
- 30 AD: alzheimer disease
- 31 ADC: antibody drug conjugate
- 32 AmtB: ammonia channel
- 33 APCI: atmospheric pressure chemical ionization
- 34 APols: amphipatic polymers
- 35 AqpZ: aquaporin Z
- 36 A·T: adenine thymine
- 37 ATD: arrival time distribution
- 38 bsAb: bispecific antibodies
- 39 BSM: bovine sumaxillary mucin
- 40 CCS: collisional cross section
- 41 CDL: cardiolipin
- 42 C·G: cytosine-guanine
- 43 CID: collision induced dissociation
- 44 CIU: collision induced unfolding
- 45 CRISP : clustered regularly interspaced short palindromic
- 46 CRISPR : clustered regularly interspaced short palindromic repeats
- 47 CZE: capillary zone electrophoresis
- 48 DAR: drug-to-antibody-ratio
- 49 DBD: DNA-binding domain
- 50 DC: differential current
- 51 DDA: data-dependent acquisition
- 52 DESI: desorption electrospray ionisation
- 53 DFT: density functional theory
- 54 DHSS: diffuse hard spheres scattering
- 55 DIA: data-independent acquisition

- 56 DIMS: differential ion mobility spectrometry
- 57 DMA: differential mobility analyser
- 58 DMS: differential mobility spectrometry
- 59 DNA: deoxyribonucleic acid
- 60 $^{DT}CCS_{He}$: collisional cross section calculated with drift tube ion mobility spectrometry and helium as buffer gas
- 61
- 62 $^{DT}CCS_{N_2}$: collisional cross section calculated with drift tube ion mobility spectrometry and nitrogen as buffer gas
- 63
- 64 DTIMS: drift tube ion mobility spectrometry
- 65 DTM: diffuse trajectory method
- 66 EED: electron excitation dissociation
- 67 EHSS: exact hard sphere scattering
- 68 ESI: electrospray ionization
- 69 ETD: electron-transfer dissociation
- 70 FAIMS : field asymmetric waveform ion mobility spectrometry
- 71 FDA: food and drug administration
- 72 FID: flame ionization detector
- 73 FRET: fluorescence resonance energy transfer
- 74 GC: gas chromatography
- 75 HCP: host cell proteins
- 76 HD-DDA: high definition data direct analysis
- 77 HDX-MS: hydrogen/deuterium exchange mass spectrometry
- 78 HIC: hydrophobic interaction chromatography
- 79 HMGA: high-mobility group A
- 80 HRMS: high resolution mass spectrometry
- 81 IAPP: islet amyloid polypeptide
- 82 IDP: intrinsically disordered proteins
- 83 IgG: immunoglobulin G

- 84 IM: ion mobility
- 85 IM-MS: ion mobility - mass spectrometry
- 86 IMS: ion mobility spectrometry
- 87 IMS-QTOF: ion mobility spectrometry quadrupole time-of-flight
- 88 IR: infrared radiation
- 89 IRMPD: infrared multiphoton dissociation
- 90 LC: liquid chromatography
- 91 LC-IMS-MS: liquid chromatography - ion mobility - mass spectrometry
- 92 LC-MS: liquid chromatography-mass spectrometry
- 93 LCPA: local collision probability approximation
- 94 LOD: limit of detection
- 95 mAb: monoclonal antibody
- 96 MALDI: matrix-assisted laser desorption ionisation
- 97 MC: Monte Carlo
- 98 MD: molecular dynamics
- 99 MS: mass spectrometry
- 100 MS/MS: tandem mass spectrometry
- 101 MscL: mechanosensitive channel of large conductance
- 102 NMR: nuclear magnetic resonance
- 103 PA: projection approximation
- 104 PASEF: parallel accumulation–serial fragmentation
- 105 PDB: protein data bank
- 106 PG: phosphatidylglycerol
- 107 PSA: projection superimposition approximation
- 108 PTM: posttranslational modifications
- 109 QM: quantum mechanical
- 110 QqTOF: quadrupole-time-of-flight
- 111 QTRAP: triple-quadrupole linear ion trap mass spectrometer

- 112 RF: radio frequency
- 113 rhGH: recombinant human growth hormone
- 114 RNA: ribonucleic acid
- 115 SASA: solvent-accessible surface area
- 116 SEC: size exclusion chromatography
- 117 SFC: supercritical fluid chromatography
- 118 SID: surface induced dissociation
- 119 SLIM: structures for lossless ion manipulations
- 120 sno: small nucleolar
- 121 SRIG: stacked-ring ion guide
- 122 sRNPs: small nucleolar ribonucleoprotein particles
- 123 T₁₀: thymine 10
- 124 T2DM: type 2 diabetes mellitus
- 125 Td: Townsends unit
- 126 TIMS: trapped ion mobility spectrometry
- 127 TIMS-MS: trapped ion mobility spectrometry - mass spectrometry
- 128 ^{TIM}CCS_{N₂}: collisional cross section calculated with trapped ion mobility spectrometry and
- 129 nitrogen as buffer gas
- 130 TM: trajectory method
- 131 TOF: time-of-flight
- 132 TRAP: trp RNA binding attenuation protein
- 133 TTR: tetrameric transthyretin
- 134 ^{TW}CCS_{N₂}: collisional cross section calculated with travelling wave ion mobility spectrometry
- 135 and nitrogen as buffer gas
- 136 TWIMS: travelling wave ion mobility spectrometry
- 137 VUVPD: vacuum ultraviolet photo dissociation
- 138 VT-IM-MS: variable temperature ion mobility-mass spectrometry
- 139 wt: wild type

140 XUV: extreme ultraviolet light

141 **Abstract**

142 Ion mobility spectrometry is an analytical technique known for more than 100 years, which
143 entails separating ions in the gas phase based on their size, shape and charge. While IMS
144 alone can be useful for some applications (mostly security analysis for detecting certain
145 classes of narcotics and explosives), it becomes even more powerful in combination with MS
146 and HPLC. Indeed, the limited resolving power of ion mobility spectrometry alone can be
147 tackled when combining this analytical strategy with MS or LC-MS.

148 Over the last few years, the hyphenation of ion mobility spectrometry to MS or LC-MS attracted
149 more and more interest, with significant progresses in both technical advances and pioneering
150 applications. This review describes the theoretical background, available technologies and
151 future capabilities of these techniques. It also highlights a wide range of applications, from
152 small molecules (natural products, metabolites, glycans, lipids) to large biomolecules (proteins,
153 protein complexes, biopharmaceuticals, oligonucleotides).

154

155 1. Introduction

156 During the last decade, ion mobility spectrometry (IMS) has been applied in several fields,
157 starting from the analysis of chemical warfare agents, explosives and illicit drugs to the analysis
158 of biological samples [1–4]. Nowadays, the increased complexity of the samples (e.g. natural
159 products analysis, biopharmaceutical characterization, -omic samples such as proteins mixture
160 in proteomics or biofluids in metabolomics or lipidomics, *etc*) demands for analytical methods
161 able to offer very high resolving power to achieve analytical goals and acquire sufficient
162 information from the sample.

163 The addition of IMS dimension to the existing analytical setup can represent an attractive
164 strategy to achieve this goal. [4] Indeed, IMS is a gas-phase separation technique that allows
165 the separation of ions generated by a source (frequently by electrospray ionization, ESI) driven
166 through an ion mobility cell filled with an inert gas (generally nitrogen or helium) under the
167 influence of an electric field. Once in the IM drift cell, ions are separated based on their mobility
168 that accounts for their size, shape and charge. Then, based on the type of IMS separator, the
169 mobility can also allow the determination of the collisional cross section (CCS), a physical
170 property related to the shape of the ion for a given compound in a specific gas environment.
171 The CCS is an important property that could provide an improved characterization of different
172 compounds, such as small molecules, proteins, lipids or glycans, as well as a better
173 understanding of the intramolecular forces [5,6]. A detailed discussion about CCS
174 determination will be addressed in section 2.

175 As illustrated in Figure 1, IMS combined with mass spectrometry (MS), which is also known as
176 IMSxMS or IM-MS, has seen a spectacular growth in the last 20 years. Indeed, the information
177 obtained from IMS and MS can be considered as complementary and IMS can potentially allow
178 the separation of isobaric ions (unresolved by MS) based on their shape differences [7].
179 Besides IMS and MS, a third separation dimension, such as liquid chromatography (LC) or
180 another front-end separation technique such as supercritical fluid chromatography (SFC), gas
181 chromatography (GC), or capillary zone electrophoresis (CZE) [8] can also be included in the
182 analytical setup. As shown in Figure 1, this three-dimensional separation (LC-IM-MS) is still

183 less widespread than IMS alone or IM-MS, but is beginning to attract increasing interest for the
184 analyses of complex samples. As highlighted in Figure 2, LC-IM-MS allows the separation of
185 the different constituents of a mixture based on their lipophilicity (LC), shape (IMS), mass (MS)
186 and charge (IMS and MS). It also offers the possibility to visualize the analytical results in
187 several dimensions, using a plot of MS spectra information vs. IM drift times, for each
188 chromatographic peak.

189 Another important aspect is the time scale of IMS (in the milliseconds range), which fits very
190 well between the chromatographic step (in the minutes range) and MS experiment (in the
191 microseconds time scale). Because of the fast duty cycle of time-of-flight based mass
192 spectrometer (TOF and QqTOF), these instruments appear to be the most suitable analysers
193 for IM-MS [6].

194 This review paper describes the theoretical background of IM-MS technology, available
195 instrumentation and future capabilities of IM-MS. It also highlights a wide range of recent
196 applications of IM-MS and LC-IM-MS, ranging from small molecules to large biomolecules.

197

198 **2. Description of the available ion mobility technologies**

199 Several ion mobility technologies have been developed in the last decades, each having its
200 own principle of operation and design. According to May and McLean [9], there are three main
201 separation concepts employed in IMS, namely i) time-dispersive methods, which generate an
202 arrival time spectrum, with all ions drifting along the same pathway (i.e. drift tube ion mobility
203 spectrometry (DTIMS), travelling wave ion mobility spectrometry (TWIMS)), ii) space-
204 dispersive methods, which separate ions along different drift paths, based on their mobility
205 differences (i.e. field asymmetric waveform ion mobility spectrometry (FAIMS), differential
206 mobility analyser (DMA)) and iii) ion trapping followed by selective release, which traps ions
207 within a pressurized region and selectively ejects the ions based on their mobility differences
208 (i.e. trapped ion mobility spectrometry (TIMS)). All the IMS techniques described in this section
209 can be coupled with MS [7], but performance and possibilities may vary, particularly in terms
210 of achievable limits of detection (LOD), resolving power (peak capacity) and possibility to

211 accurately calculate CCS values. The main advantages, limitations and a list of available
212 commercial instruments for the different IMS technologies are described in this section and
213 summarized in Table 1. Last but not least, it is important to keep in mind that DTIMS and
214 TWIMS are the main choice for proteins, but have also been successfully used for small
215 molecules. On the other hand, FAIMS, DMA are only dedicated to the analysis of small
216 molecules. TIMS is a relatively new strategy, which also has the potential to be used for both
217 proteins and small molecules. However, it has only been scarcely employed for proteins and
218 more investigations are needed to prove its applicability for large biomolecules.

219

220 2.1. Drift tube ion mobility spectrometry

221 Drift tube ion mobility (DTIMS) is the historical and simplest form of IMS that was assembled
222 for the first time in the 1970s, originally in combination with a single quadrupole mass analyser
223 [10]. The schematic design of DTIMS is shown in Figure 3a. This technology is composed of
224 a tube (the drift cell) filled with an inert gas, namely the buffer gas. [2] Nitrogen or helium are
225 the most common buffer gas in DTIMS [11]. The DTIMS cell is composed of a series of piled
226 electrodes, and each electrode generates a weak uniform electric field (5-100 V) [2] able to
227 induce the movement of the ions through the tube. For example, on the Agilent DTIMS-MS
228 instrument, 1000-1400 V is typically used in a 78 cm drift cell, corresponding to a field strength
229 of around 13-18 V/cm. The mobility of ions along the cell is determined by the number of
230 collisions with the buffer gas, which slows down the ions: For a given charge, compact ions
231 drift faster than elongated ones, because they experience fewer collisions with the buffer gas.
232 [5] The time required to cross the cell is defined as the drift time and the separation of the ions
233 occurs as a function of their shape, which is physically related to the mobility (K) or rotationally
234 averaged CCS value. However, the electric field has to be maintained sufficiently low to
235 observe a good correlation between mobility and shape/size.[7] As ions exit the tube, they are
236 analysed and detected as a function of time, yielding an arrival time distribution (ATD). The
237 first home-made DTIMS generally suffered from an inherent lack of sensitivity, mainly due to
238 diffusion issues and loss in duty cycle that was related to the pulsed analysis. However, in

239 2014 a commercial DTIMS, using nitrogen drift gas and offering a good compromise between
240 sensitivity, length of drift tube, and optics, was developed by Agilent Technologies. In
241 particular, the instrument incorporates a tandem ion funnel interface before the drift tube that
242 acts as a focusing and accumulation region, and a second ion funnel after the drift tube that
243 refocuses the ions through an hexapole ion guide prior to reaching the QqTOFMS. The Agilent
244 DTIMS-MS instrument includes a QqTOF mass analyser, offers a high precision of CCS values
245 (below 0.5% [12]), a high resolving power (thanks to the length of the tube extended to 78 cm
246 combined with the precision electronics) and sensitivity compatible for protein/peptide
247 analyses [11,13]. Another DTIMS instrument commercially available is manufactured by
248 TOFWerk (Switzerland). This instrument contains a 20 cm drift cell operated at high pressure
249 (i.e. 1-1.4 atm) and elevated temperature (e.g. 60°C), which provides greater resolution, while
250 maintaining low-field conditions, but is limited to low molecular weight molecules.

251

252 2.2. Travelling wave ion mobility spectrometry

253 Traveling wave ion mobility (TWIMS) is a more recent IMS technology that was first described
254 in 2004 [14] and made commercially available in 2006 (Waters Synapt HDMS systems) [15].
255 This high performance instrument has significantly advanced the field of IM-MS thanks to its
256 versatility and widespread availability [16]. Similarly to DTIMS, TWIMS is a time-dispersive
257 method, even though it uses a dynamic application of the electric field to separate the ions in
258 gaseous phase (often nitrogen and occasionally helium). It consists of a stacked-ring ion guide
259 (SRIG) where positive and negative radio frequency (RF) voltages are periodically applied to
260 adjacent ring electrodes to provide radial ion confinement while a pulsed differential current
261 (DC) voltage is applied to axially propel the ions [2]. Therefore, rather than applying a linear
262 electric field through the full length of the mobility cell, like in DTIMS, the electric field in TWIMS
263 forms a wave whose magnitude and speed determine the ions separation, as illustrated in
264 Figure 3b. By surfing on the waves, larger ions experience larger friction with the buffer gas
265 and slip more often behind the waves, thus taking more time to exit the mobility cell as
266 compared to smaller ions. In TWIMS, CCS can also be determined from the drift time, but only

267 after calibration by using a set of reference compounds of known CCS values. In addition, the
268 TWIMS technology takes less space than DTIMS (DTIMS needs around 1 meter to be installed
269 in front of the mass spectrometer) and is more versatile, since it allows to perform CID before
270 and after Ion mobility (CID could only be performed after ion mobility with DTIMS) on the
271 commercial instruments (this is a choice of the constructors rather than a theoretical limitation).
272 As described in [9], the original Waters TWIMS technology underwent a major design
273 modification in 2009 (Synapt G2), allowing 4-fold improvement of the accessible resolution,
274 thanks to the use of higher electric fields and higher gas pressure [17]. In 2011 and 2013, two
275 new instruments were released by Waters, namely Synapt G2-S and G2-Si, allowing an
276 improvement of the ion transmission through the IM-MS and a wide range of experimental
277 options [9]. Finally, Waters has released a benchtop instrument in 2016 (Vion IMS QToF),
278 which includes in part the technology of the Synapt, but with a different design (the mobility
279 cell is located before the first quadrupole of the QqTOF/MS). Indeed, this instrument is
280 dedicated to non-experts who want to add an IMS dimension (determination of CCS values,
281 removal of spectral interferences) to routine analyses often performed in MS or LC-MS.

282

283 2.3. Field asymmetric waveform ion mobility spectrometry

284 Field asymmetric waveform ion mobility spectrometry (FAIMS) is also known as differential ion
285 mobility spectrometry (DMS or DIMS). The principle of operation of FAIMS is illustrated in
286 Figure 3c [18]. FAIMS utilizes two electrodes between which a high asymmetric electric field
287 (up to 10000 V/cm, also called dispersion field) is applied at reduced pressure (but can also
288 operate at atmospheric pressure) [19,20] and room temperature. The carrier gas (often
289 nitrogen, but there is a lot of flexibility to use some alternative gas or even dopants) is injected
290 with the ions in the same direction. A compensation voltage is then superimposed to the
291 dispersion field and it can be tuned for ions with a particular mobility [21]. By applying a suitable
292 compensation voltage, the trajectory of the desired ions can be corrected along the axis of the
293 cell to permit these ions to leave the cell. All other ions will migrate away from the straight line

294 due to their different mobility. A full spectrum can finally be collected by scanning the
295 compensation voltage.

296 Unlike DTIMS and TWIMS, FAIMS does not separate ions based directly on their mobility, but
297 rather according to the ratio of mobilities in a low-field and high-field. Therefore, the FAIMS
298 separation is much more orthogonal to m/z than other IMS modes (see section 8.2 for more
299 explanations). However, FAIMS is actually considered to be a filtering technique (or selective
300 scanning technique) rather than structure characterization tool, since calculation of CCS is not
301 possible [2]. Very high resolutions of up to several hundreds were reported in FAIMS, but this
302 was only possible when a mixture of gases was employed (He/N₂) [22]. In addition, FAIMS
303 resolution cannot be directly compared to that of any other IMS technique described in this
304 review, since the separation phenomenon is different as is the scaling of the axes [16].

305 Several companies have commercialized FAIMS devices, including Thermo Fisher Scientific,
306 AB SCIEX and Owlstone. However, the most active provider is certainly SCIEX which utilises
307 FAIMS technology (SelexION) in several MS platforms, including QTRAP (Sciex 5500, 6500
308 and 6500+ series) and QqTOFs (Sciex TripleTOF 5600+ and 6600) analysers. On Thermo
309 Fisher Scientific instruments, the FAIMS interface can be added to existing triple quadrupole,
310 ion trap MS analysers and high-resolution orbital ion trap mass analysers [23].

311

312 2.4. Differential mobility analyser

313 Differential mobility analyser (DMA) is another form of IMS where the ions with different
314 mobilities are separated in space (rather than in time) at atmospheric pressure and relatively
315 low electric field. Two cylindrical and concentric metal electrodes constitute the usual DMA
316 design. A planar scheme of the DMA design is shown in the Figure 3d [24]. As illustrated in
317 this figure, the ions are injected along an electrical field (directed from top to bottom of the
318 scheme) between the two electrodes. An orthogonal flow of sheath gas from the left to the right
319 of the scheme pushes ions down from an inlet slit to the exit slit. Then, the ions whose mobility
320 matches the length from inlet slit to exit slit, at the used buffer gas flow and the electric field,
321 will reach the exit slit and will be directed towards the MS analyser [25].

322 The other ions, which do not match this mobility, will be eliminated on the lens that acts as a
323 wall. Only the ions with the targeted mobility will leave the DMA and the variation of the electric
324 field permits to select ions with different mobilities. Similarly to FAIMS, DMA is also considered
325 as a selective filter, but operates in the low-field regime. An important limitation of DMA is that
326 it does not allow the transmission of ions having diverse mobilities. The commercial availability
327 of DMA technology is relatively limited, and only a company called SEADM sells DMA systems
328 that can be directly coupled to MS.

329

330 2.5. Trapped ion mobility spectrometry

331 Trapped ion mobility spectrometry (TIMS) is a technology that appeared very recently on the
332 market, which is based on the use of a non-uniform electric field [16]. In contrast to DTIMS or
333 TWIMS, TIMS uses a high gas flow (exclusively nitrogen) to carry the ions of interest into the
334 drift cell. A low electric field is applied in the meantime in the opposite direction with the aim of
335 trapping and separating the ions as a function of their size-to-charge ratio. Then the electric
336 field is gradually decreased allowing the release of the ions from high (large CCS) to small
337 (small CCS) size-to-charge ratios [5]. A schematic of the separation process is shown in Figure
338 3e.

339 The TIMS-MS approach is still in early development and commercial devices have only been
340 released recently by Bruker (timsTOF) [26].

341

342 The advantages and limitations of these different IM-MS techniques above described are
343 summarized in Table 1.

344

345 3. The importance of collision cross section

346 3.1. Basic collision cross section equation

347 Ion mobility is a separation technique that allows analytes to be identified according to their
348 mass, charge and shape. The principle of IMS is often compared to that of electrophoresis,
349 even though the separation is conducted in the gas phase within a millisecond timescale.

350 Considering a pool of gas-phase ions moving in a chamber filled with a buffer gas and under
351 the influence of an electric field (E), the mobility (K) is defined as the proportionality coefficient
352 between the strength of the electric field E and the velocity v_d attained by the ions:

$$v_d = K \cdot E \quad (1)$$

354
355 K is usually reported as the reduced mobility (K_0), upon normalization to standard pressure
356 and temperature ($p_0 = 760$ Torr and $T_0 = 273.15$ K):

$$K_0 = K \cdot \frac{p}{p_0} \cdot \frac{T_0}{T} \quad (2)$$

357
358 In addition, K_0 can be related to the collision system parameters, accounting for the analyte
359 characteristics under the experimental conditions, by the Mason-Schamp modification of the
360 fundamental zero-field equation [27]:

$$K_0 = \frac{3q}{16N_0} \cdot \left(\frac{2\pi}{k_B T}\right)^{\frac{1}{2}} \cdot \left(\frac{1}{m} + \frac{1}{M}\right)^{\frac{1}{2}} \cdot \frac{1}{\Omega} \quad (3)$$

362
363 where $q = ze$ is the ionic charge (z is the nominal ion charge and e is the elementary charge),
364 N_0 is the gas density number at p_0 and T_0 , k_B is the Boltzmann's constant, T is the gas
365 temperature, m is the mass of the buffer gas, while M is the one of the ion, and Ω is the collision
366 cross section (CCS).

367 While q and M take into account the charge and the mass of the ion, respectively, CCS (Ω) is
368 the physical quantity taking into account its "size and shape". CCS represents indeed the
369 effective area of the ion colliding with the molecules of the buffer gas. The higher the CCS, the
370 higher will be the number of collisions and thus more pronounced the effects on the ions that
371 will be slowed down by friction upon collisions. The CCS is thus a distinctive characteristic of
372 each ion (this is true for small molecules) and it is considered as an important structural

373 descriptor obtainable by IMS. However, it is important to keep in mind that the case of proteins
374 is more complex, since the activation conditions can influence the CCS and different CCS of
375 the same ion can co-exist. CCS can also be compared with other structural data obtained with
376 different analytical techniques, such as nuclear magnetic resonance (NMR), small-angle X-ray
377 scattering (SAXS), electron microscopy and X-ray crystallography. This point will be further
378 discussed in section 3.3. It is worth noticing that, according to Equations 1 – 3, the mobility (K)
379 depends on the density number of the drift gas (N), and the proportional linearity between v_d
380 and E is only valid in the low electric field limit (E/N ratio ≤ 2 Townsends (Td)). As the electric
381 field is increasing, K is no longer constant and becomes field-dependent. Thus, the simple
382 relationship between K_0 and $1/\Omega$ breaks down. This point is particularly important to understand
383 the limits of the experimental CCS determination and how to properly deduce it from measured
384 drift times on different types of IMS.

385

386 3.2. Experimental collision cross section determination

387 As described in section 1, several types of ion mobility separators can be coupled to MS. All
388 of these configurations allow the separation of the analytes based on their conformations, but
389 only a few of them can be used to determine CCS values. In this section, our goal was to
390 critically consider whether the IMS hardware allows the determination of experimental CCS. In
391 general, if the analyser operates in the low field limit (E/N ratio < 2 Td) at all times and if the
392 field applied is constant, CCS might be directly determined from measured drift times (like in
393 DTIMS). If the IMS analyser operates in the low field limit, but with non-constant field due to
394 the application of additional radio frequencies (RF) or potentials, then a calibration with ions of
395 known CCS is required (like in TWIMS, DMA and TIMS). Finally, CCS cannot be derived at all
396 from the mobility data if the analyser operates at high field (like in FAIMS). The first two
397 scenarios allowing experimental CCS determination are discussed in further detail.

398

399 (i) *Low field limit at all times and application of constant field.* The best example of this IMS
400 configuration is the DTIMS. Knowing the drift time (t_d , time spent by the ions in the drift tube)
401 and that $v_d = L/t_d$ (where L is the drift tube length), Equation 2 can be expressed as follow:
402

$$K_0 = \frac{L}{t_d E} \cdot \frac{p}{p_0} \cdot \frac{T_0}{T} \quad (4)$$

403
404 with consequent CCS determination obtainable by K_0 following Equation 3. Theoretically,
405 DTIMS analysers offer the most accurate CCS measurement using a stepped-field approach
406 without the need for calibration, assuming that all the parameters involved are accurately
407 known and carefully controlled. If not, a calibration with ions of known CCS could be necessary
408 also in this case. For online analysis (e.g. LC-IM-MS), a single-field calibration with ions of
409 known CCS is used to provide a regression model for converting drift time and mass into CCS.
410 In addition, the drift tube can operate with a wide range of neutral gases (e.g. nitrogen, helium,
411 carbon dioxide), thus CCS values are a conditional property. In this regard, users are beginning
412 to ensure appropriate reporting of CCS values with specific reference to the gas and type of
413 IM used (e.g. $^{DT}CCS_{He}$ or $^{DT}CCS_{N_2}$).

414 An important inter-laboratory study has been recently published [12] to highlight the trueness
415 and reproducibility of CCS measurement with DTIMS. In this work, 120 unique ion species (i.e.
416 metabolites, peptides, lipids and proteins, representing a wide range of masses, chemical
417 compositions and structures) were monitored in both positive and negative ionization modes,
418 and the reproducibility of $^{DT}CCS_{N_2}$ values were evaluated across three laboratories equipped
419 with commercial DTIMS-MS instruments. It appears that the average RSD on CCS values was
420 equal to only 0.29% for all ion species across the three laboratories equipped with the same
421 type of instrument. The trueness of the values, obtained with the commercial DTIMS-MS
422 instruments, was comprised between 0.34% and 0.54%, depending whether a calibration was
423 applied or not. These results clearly illustrate the potential of DTIMS-MS for providing a

424 molecular identifier for a broad range of applications, including untargeted screening
425 workflows.

426

427 (ii) *Low field limit with non-constant field.* The best examples for this IMS configuration are
428 TWIMS, DMA and TIMS. Due to the non-linearity of the electric field that complicates the
429 equations describing the ion trajectories in TWIMS, CCS cannot be directly measured from the
430 drift times, but they can be evaluated after calibration with ions of known CCS and under
431 defined conditions of the drift cell (gas pressure and temperature, drift cell length, wave height
432 and speed). Ruotolo *et al.* reported a detailed TWIMS calibration protocol to which readers
433 may refer to [28]. Generally, proper calibrants should cover the IM drift time range of the ions
434 of interest, but also being physically and chemically related to the analytes and best bracket
435 the m/z of the compound to be analysed, especially when small differences in CCSs are
436 expected [29]. The most reliable calibration is obtained by using reference CCS values
437 obtained by DTIMS operating with nitrogen as buffer gas ($^{DT}CCS_{N_2}$), since TWIMS cannot
438 operate with helium and is more often commercialised with nitrogen, thus giving access to
439 $^{TW}CCS_{N_2}$ measures. The accuracy of CCS values is an important topic now that these values
440 are becoming an additional reference parameter in automated peak assignment of database
441 for -omics sciences.

442 Similarly, a calibration is needed for obtaining CCS values in DMA. As described in [30], a
443 single calibrant ion (such as tetraheptylammonium⁺) could be used to link the applied voltage
444 to the mobility of the ions transmitted, assuming that pressure and temperature inside the DMA
445 classification zone are known and controlled.

446

447 TIMS is another example of ion mobility analyser operating in the low field limit, but with no-
448 constant field. In TIMS, an axial electric field is applied to accumulate, trap and elute the ions
449 according to their Ω/z ratio (see section 1.5 for additional hardware details). Unlike the
450 configurations introduced previously, larger ions (high CCS) migrate first with respect to the
451 smaller ions (low CCS). Moreover, similar to TWIMS, CCS determination can be obtained only

452 upon proper calibration with ions of known CCS as TIMS also operates with nitrogen as buffer
453 gas. Since the commercialisation of this analyser is still relatively recent, there are still very
454 few sources reporting $^{TIM}CCS_{N_2}$ values [5].

455

456 3.3. Prediction of collision cross section values

457 Even though the experimental CCS values represent an interesting structural parameter
458 obtainable by IMS, it is not sufficient to allow any structural interpretation on its own. To
459 disclose the conformation and shape adopted by the ions in the gas-phase, the experimentally
460 derived CCS needs to be compared with computationally derived CCS values obtained from
461 atomistic models. Nowadays, this approach is the gold-standard if using IM-MS as analytical
462 technique for “conformation analysis” [2,4,5,31–41].

463 The first challenging task is the achievement of reliable atomistic models. A plethora of
464 computational approaches might be applied for this purpose, including quantum mechanical
465 (QM) methods such as (but not limited to) density functional theory (DFT), *ab-initio* or *semi-*
466 *empirical* methods and classical methods such as Monte Carlo (MC) and molecular dynamics
467 (MD). The method of choice is at the discretion of the user, but it is mainly driven by the size
468 of the system under investigation. Generally, QM methods offer the highest level of accuracy,
469 but they are extremely time-consuming and usually viable only for small systems, whereas
470 classical methods are instead more suitable for large systems such as biomolecules [42]. A
471 detailed evaluation of the computational approaches is beyond the scope of this review, but it
472 is worth noting that extensive proficiency in the field of computational chemistry is required to
473 obtain reliable atomistic models, thus several research groups (using IMS as structural tool)
474 have already taken into account the possibility to have a modeller in their group or to work in
475 close collaboration with a modelling team.

476 The second challenging task is the reliable determination of computationally derived CCS.
477 Indeed, once a pool of atomistic models, presumably representing the experimental gas-phase
478 conformations, have been selected through a proper computational method, a CCS calculation
479 algorithm needs to be applied to finally obtain theoretical CCS values (CCS_{THE} or CCS_{CAL})

480 which can be compared to the experimentally derived CCS (CCS_{EXP} or CCS_{IMS}). Several CCS
481 calculation models might be applied for this purpose. The “historical” models (still widely known
482 and used) are the trajectory method (TM) [43,44], the exact hard sphere scattering (EHSS)
483 [44], and the projection approximation (PA) [43,45,46], all initially parametrised for helium as
484 buffer gas and available in the MOBCAL suite of programs [47]. In general, the three algorithms
485 allow simulation of the collisions between the buffer gas and the randomly oriented structure
486 of the ion under investigation. However, during the collisions, multiple scattering events and
487 long-range interactions also occur, and the three models differ in the assumptions and the
488 grade of approximations of all these events, as depicted in Figure 4. TM [43,44] is an explicit
489 model which can be considered as the most rigorous and physically appealing method, since
490 it accounts for both long-range interactions and multiple scattering events. It is the method of
491 choice for small molecules calculations, but it becomes prohibitively slow if applied to large
492 molecules (> 1000 atoms), except if parallelization of computation is available. EHSS [48] is a
493 hard sphere collisions based method, meaning that both buffer gas and ion under investigation
494 are modelled as hard spheres. It computes the multiple scattering events, but it does not take
495 into account the long-range interactions, offering a reasonable balance between CCS accuracy
496 and time requested for the calculation, also for large molecules (even if an over-estimation of
497 CCS could arise in this latter case). Finally, PA [43,45,46] is a non-explicit method that does
498 not consider the multiple scattering events, or long-range interactions, but works by projecting
499 the “shadow” of the ion onto a plane to find the average area of all the possible orientations of
500 the molecule under investigation. It is the fastest method, but can lead to a systematic
501 underestimation of the CCS, especially for molecules presenting cavities. PA is generally
502 recommended for CCS determination of small molecules (10-200 atoms) with convex surfaces,
503 even though a good correlation has been reported between TM and PA when dealing with
504 CCS calculation of macromolecules [49].

505 During the last few years, optimizations of these three algorithms and also their
506 parametrizations have been reported, especially to answer to the need of including nitrogen
507 as collision gas [50–54]. In the meantime, new algorithms have also been proposed, such as

508 the projection superimposition approximation (PSA) [55–57], and the local collision probability
509 approximation (LCPA) [58], both non-explicit methods; the diffuse hard spheres scattering
510 (DHSS) [59] that is a hard sphere collisions based method; and the diffuse trajectory method
511 (DTM) [59] that is a physically explicit method. Furthermore, new CCS calculators are still
512 being developed with the goal to provide the most reliable CCS calculation possible, especially
513 for large biomolecules and with nitrogen as collision gas. This is the case of the IMPACT
514 software [49] that has been specifically developed for the CCS calculation of large
515 biomolecules. Indeed, IMPACT calculates CCS using an octree-augmented projection
516 approximation with an exceptional boost to speed for large targets. “Collidoscope” [60] is an
517 alternative TM based CCS calculator using parallel processing and optimized trajectory
518 sampling, implementing both helium and nitrogen as collision gas option.

519 It is hard to compare the performance of all these approaches, but accuracy and computing
520 time are clearly strongly dependent on the molecular size. What is clear is that this *gold rush*
521 indicates the increasing and intriguing involvement of IM-MS in the field of structural
522 characterization.

523

524 **4. Resolution and separation performance of ion mobility spectrometry**

525 4.1. Basic resolution equations in ion mobility

526 The resolving power of IM is defined in the same fundamental way as for mass spectrometry,
527 but using mobility related parameters (e.g. $t_d/\Delta t_d$ instead of $m/\Delta m$). IM resolution has also been
528 historically described using a variety of approaches beginning with chromatographically-
529 equivalent terms including plate height theory [61,62]. However, due to the fundamental
530 differences in ion mobility analyser principles, different models are necessary to describe the
531 achievable resolving power. As a number of recent reviews have comprehensively described
532 fundamentals behind IMS separation and resolution capabilities [8,9,16], only key theoretical
533 equations for describing IMS resolution relevant to commercially available IM-MS
534 instrumentation and their underpinning studies are highlighted in this section.

535 The nature of low-field drift tube ion mobility lends itself particularly well to understanding IM
536 resolution as the diffusion-based band broadening can be described by similar equations to
537 that used for isocratic linear chromatographic separations. Following historical development of
538 DTIMS instrumentation and seminal theory [27,63], the conditional resolution model [64]
539 provides a fundamentally-sound basis for describing the evolution of zone width in an ideal
540 case and the maximum obtainable sample peak capacity can thus be readily calculated
541 according to critical experimental parameters (e.g. drift gas, tube length, temperature,
542 pressure, sample type). However, limitations for this model are well-known, including the
543 influence of ion trapping and release processes, as well as additional broadening of the ion
544 packages between the IM and mass analysers. A semi-empirical model designed to account
545 for these effects was developed by the group of Hill [65] and offers a means to better
546 understand instrumental contributions to non-ideal performance, as shown in the recent study
547 of May *et al.* [66]. Resolution on current commercially available DTIMS instrumentation is in
548 the order of 40-60 for low pressure cells for singly charged compounds (Agilent) and up to 250
549 for high pressure cells (TOFWerk). In Table 2 [21,27,64–71], various aspects of these
550 experimental differences can be rationalised according to the relevant parameters influencing
551 DTIMS resolution. Nevertheless, attempts to improve resolution should also be considered
552 alongside other practical considerations. For example, use of above ambient pressure results
553 in improved resolution, but will also result in substantial losses in transmission [72].

554 In the case of TWIMS, the fundamentals and achievable resolution are described in detail in
555 the publication of Shvartsburg and Smith [67]. Unlike in DTIMS, the measured drift velocity is
556 proportional to the square of the ion mobility. This yields a resolution term that is dependent
557 upon the ion mobility (scaling as $K^{1/2}$) and allows the IM resolution to be practically optimized
558 according to the spread of mobilities to be measured. Further technical changes in the design
559 of commercially available TWIMS devices has allowed improved resolution (40-60) to be
560 achieved with consideration to CCS measurement stability and the optimization of the ratio of
561 wave height and wave velocity given thorough investigation [17,68]. Furthermore, exciting
562 developments in building serpentine paths of several meters in length seem to be the way

563 forward to achieve resolution of several 100s for next generation TWIMS devices as effective
564 IM length can be increased enormously without increasing the instrument footprint [73].

565 A resolution equation for TIMS was derived by Hernandez *et al.*, whereby the resolution for a
566 given ion is proportional to the gas velocity (v_g) and to the fourth root of the length of the electric
567 field gradient [69]. As ions of different mobilities will be trapped using different gas velocities,
568 the achievable resolution will differ accordingly. Finally, the achievable velocity is primarily
569 controlled by v_g , the electric field ramp speed, and the RF confinement. Experimental studies
570 have demonstrated good agreement with this model and resolution of up to 300 under
571 optimized conditions [74]. However, it must be noted that this very high resolution is only
572 achievable for a narrow m/z (respectively mobility) range. When used for generic, full mass
573 scan IM-MS experiments then the resolution will be in the range of 50-70.

574 Finally, as described in the review of May and McLean, IMS resolution has limitations as a
575 standalone metric for comparing different types of IMS analysers [9]. In particular, the
576 achievable resolution for DMA and FAIMS are more difficult to compare. An excellent overview
577 of DMA resolution theory is found in the aerosol science literature [75], while some recent
578 examples have demonstrated resolving powers in the order of 65 using DMA-MS with
579 optimized plate designs [76]. The fundamentals of compensation field-based resolving power
580 for FAIMS were outlined in a computational study from Shvartsburg and Smith [77] with some
581 recent contributions also highlighting some of the limitations of using classical resolving power
582 to describe the performance of FAIMS devices [78]. Although not implemented in routine
583 FAIMS analysis, one recent study demonstrated an improvement in the resolving power of
584 FAIMS from 20 up to 400-500 by the use of optimized gas mixtures and extended separation
585 times [22], while another recent work utilized ion focusing strategies to yield up to 37%
586 improvement in resolution [79].

587 A summary of the most relevant IMS resolution equations is provided in Table 2.

588

589 4.2. Separation performance in ion mobility

590 Based on established differences in CCS values of molecules and IM resolution models, the
591 theoretical limitations of various ion mobility analysers can be considered according to the
592 instrument design parameters. Many early studies have assessed peak capacity in some form
593 for IM-MS analysis, with some of them focusing specifically on LC-IM-MS [80–82]. Akin to the
594 chromatographic definition [83], peak capacity within IM is simply a theoretical estimate of the
595 maximum number of peaks from the sample class of interest that can be fully resolved side-
596 by-side within a single scan. In combination with resolution and/or peak capacity models for
597 different types of ion mobility devices, improvements in separation potential can be modelled
598 and practically verified. An excellent example of the improvements from increasing path length
599 can be seen in the development of serpentine paths for TWIMS separation of ions [73]. With
600 effective lengths of several meters, quite elevated resolution separations have been realised,
601 while still maintaining sub-second IMS separation times.

602 One of the great promises of combining IM with MS is the possibility to resolve and individually
603 interrogate isobaric species present in a single sample. For such considerations, the IM
604 resolving power and measurement precision are the most critical factors. The majority of
605 publications on IM-MS continue to use experimentally determined IM resolving power as a
606 means to broadly describe instrument performance, but it is critical to point out that the nature
607 of the ion (charge state), ion gating/trapping, and the IM principle used can all influence the
608 measured resolving power for a given measurement. In many cases, more relevant information
609 for users can be gained by focusing on critical pair (peak-to-peak) resolution within the
610 molecular class of interest, as originally postulated by Karasek and Kim [84]. Practically, a
611 number of studies have demonstrated that isomers exhibiting minor differences in CCS can be
612 differentiated with excellent repeatability precision when analysed separately. Conversely,
613 measurements of mixtures of isomers often yield only broadened IMS peaks as the differences
614 in CCS are in the order of <2% and cannot be resolved by most IM-MS instrumentation (Figure
615 5) [82]. In a recent contribution, the group of McLean provided theoretical estimations for the
616 required resolution to achieve sufficient IM separation such as small molecules and explored
617 the necessary advances in IM resolution required in some details [85]. In particular, the

618 separation challenges associated with different isobaric separation problems are discussed
619 with reference to the degree of separation required. The differences in CCS values for isomer
620 pairs of particular classes (e.g. diastereoisomers) were estimated to vary from 0.3 up to 6.9%,
621 and further suggest that an instrument with a resolving power of 250 should be capable of
622 resolving approximately three quarters of the considered isomer pairs. Thus, as modelling
623 developments and improvements in measurement accuracy established by inter-laboratory
624 comparisons continue to inform our understanding of IMS behaviour, we expect that instrument
625 design can mature in order to address this challenge. Nevertheless, it is clear that chemical
626 separation (e.g. LC) remains critical in many cases for the analysis of complex sample analysis
627 in order to correctly assign mass spectral features to individual compounds, and also to reduce
628 ion suppression which is obviously not mitigated by ion mobility separation.

629

630 **5. Formation of protomers – a potential issue in ion mobility spectrometry?**

631 When considering a singly charged species in positive electrospray ionization mode (ESI+),
632 the molecules have to be first protonated, and the added proton, which gives the charge, is
633 located at the molecule site having generally the largest gas-phase basicity. However, a
634 significant number of substances possess several protonation sites, which can lead to different
635 isomers in the gas phase, also known as protomers. As reported in [4], the average number of
636 protomers for more than 100,000 structures of biologically relevant compounds contained in
637 the drug data report database was equal to 6.3 per compound, which means that the
638 prevalence of protomers in the small molecule chemical space is important. In addition, it has
639 been reported that the protonation sites (location of charges) can have a significant effect on
640 the conformation of an ion [4]. In MS and HRMS, the protomers cannot be distinguished since
641 they possess different conformation, but the m/z ratios remain identical. However, when adding
642 an IMS dimension to the separation, the protomers can be differentiated based on their shapes,
643 leading to several peaks observed on the mobilogram. This behaviour is well illustrated in
644 Figure 6, showing the mobilogram of ciprofloxacin (fluoroquinolone antibiotic), which reveals
645 the presence of two well separated gas-phase species (protomers), with their respective sites

646 of protonation in the positive ionization mode (on the cyclic ketone and the piperazine moieties)
647 and their corresponding drift times [86]. In this example, these two different gas phase
648 components, although they only differ by their site of protonation, were well separated in IMS
649 (1.14 ms difference in drift time). The experimental IMS data of ciprofloxacin were compared
650 with theoretical calculations using molecular modelling (density functional theory) and
651 subsequent CCS calculations using projection approximation. Besides ciprofloxacin, there are
652 several other examples reported in the literature.

653 In differential mobility spectrometry, two protomers were observed for the 4-aminobenzoic acid
654 [87]. In this study, the authors have demonstrated that the relative abundance of each protomer
655 depends on the nature of the solvent employed in ESI. Various proportions of acetonitrile/water
656 and methanol/water were tested and the following conclusions were drawn: in presence of a
657 protic solvent (MeOH), mostly O-protonation of 4-aminobenzoic acid occurs, while the
658 presence of aprotic solvent (acetonitrile) favours the formation of N-protonated 4-
659 aminobenzoic acid. This behaviour can be attributed to the stabilization of one of the two
660 protonated forms in a given solvent [88]. Similar observations on the influence of ESI solvent
661 on the relative abundance of protomers were reported for benzocaine, a local anaesthetic [89].
662 The authors proved that the differences in drift times observed for the two species were
663 attributed to a different charge distribution within the molecules. They also highlighted that the
664 permittivity of the solvent was a key point since a highly protic solvent (such as MeOH/water)
665 facilitates the ionization of the most basic group (amide nitrogen) into the gas phase, while the
666 carbonyl oxygen becomes energetically favourable for protonation in the presence of a less
667 protic solvent (ACN/water), when the relative permittivity decreases.

668 Apart from differences observed between protomer abundance when altering the nature of the
669 ESI solvent, different fragmentation behaviours were also reported for the protomers. This has
670 been highlighted in [87], where some differences in the amount of residual precursor ion and
671 in some fragment ions, as well as the presence of unique ions were observed for one of the
672 two protomers of 4-aminobenzoic acid. These differences in MS/MS spectra of protomers were
673 also highlighted in [90], with ciprofloxacin as a case study. The formation, separation and

674 characterization of the protomers of aniline and three different porphyrins in presence of
675 different organic solvents and using polarizable CO₂ as the drift gas was shown in [91]. The
676 observed protomers of aniline were characterized *via* structurally diagnostic collision-induced
677 dissociation (CID), thanks to post-TWIMS CID, while the pre-TWIMS CID allows the
678 determination of protomers ratio from equilibrium in solution or formed in the gas phase. For
679 aniline, the two protomers, namely N-protonated and unusual ring-protonated species, exist
680 as a ratio of 5:1 in solution equilibrium. This ratio was found to be substantially different in the
681 gas phase at non-equilibrium conditions. The same conclusions were drawn for porphyrins,
682 showing that pre- and post-TWIMS CID may be considered as a useful structural tool for
683 investigating protomers in more details.

684 In conclusion, IMS can be considered as a valuable analytical tool for the separation and
685 characterization of protomers. This feature could be particularly attractive for those who are
686 interested in determining the most favourable site of protonation or deprotonation of molecules,
687 which exhibit several acidic and basic sites. Therefore, the characterization of protomers is of
688 great fundamental relevance, to better understand compound stability and reactivity. However,
689 in analytical chemistry, the presence of various protomers for one single molecule is not
690 welcome, since it will surely add complexity to the mobilogram and MS spectra, making the
691 data interpretation difficult. Indeed, several peaks could potentially be observed for one single
692 molecule on the mobilogram. If these different species cannot be totally resolved in IMS, the
693 corresponding mobility peak will appear broad and distorted (shouldering) on the mobilogram.
694 Currently, the IMS resolution is relatively low (often below 50), and therefore, the different
695 protomers are often not separated at all and are analyzed as a single peak in IMS. However,
696 if (as expected) the IMS resolution will be significantly enhanced with the next generation of
697 instruments (see section 8.1.), numerous protomers will be observed for one given substance,
698 which will create some additional issues in terms of data interpretation. Finally, the number of
699 studies dealing with the understanding of protomers formation, separation and characterization
700 in IMS and its prevalence is still too limited and deserves some additional work from specialists.

701 In addition, it is important to consider that the same effect (protomer formation) can also takes
702 place with proteins and protein complexes. This is of great interest, because of the mechanism
703 whereby charges migrate upon gas-phase unfolding of the protein (collision induced unfolding,
704 CIU). More details will be provided on this particular mechanism in section 7, dedicated to the
705 characterization of large biomolecules.

706

707 **6. Application of ion mobility spectrometry to the analysis of small molecules**

708 6.1. Metabolomics and related applications

709 Global metabolomic studies are a challenging task that aims to analyze as much of the
710 metabolome of interest as possible often within a single analysis [92]. For this reason, high
711 resolution mass spectrometry in combination with chromatographic separation has become
712 the essential tool for successful metabolite annotation and relative quantification tasks [93,94].
713 IM-MS in combination with LC attempts to directly address the challenge of limited metabolome
714 coverage within a single analysis, by increasing overall method selectivity, which was outlined
715 in recent contributions on this topic [9,95]. The high chemical diversity of small metabolites
716 (e.g. encompassing amino acids, small organic acids, sugars, nucleosides, nucleotides,
717 carbohydrates, sugar phosphates, vitamins, but also lipids) and their widely differing molecular
718 properties (e.g. polarity, acid/base character) provides particular challenges for the
719 development of IM-MS and LC-IM-MS workflows as in-source fragments, dimers and adducts
720 can be separated by IM, which would not occur when using LC-MS alone. Thus, the correct
721 re-grouping of these signal features and interpretation of data from such workflows remains a
722 challenging task that has not yet been solved. The earliest literature examples using this
723 combination for metabolomics demonstrated increased confidence in metabolite annotation
724 via drift time or CCS alignment, but also limitations in transmission and assignment of
725 molecular formulae to adducts [72,96,97].

726 Building on a landmark interlaboratory study [98], Paglia and Astarita recently published a
727 protocol using LC-TWIMS-MS for both metabolomic and lipidomic studies using this platform,
728 which presents details on all procedures from calibration, tuning, analysis up to data

729 processing including the derivation of TMCCS_{N₂} values [3]. Other recent works focusing on
730 method development for global metabolomics have investigated the use of commercially
731 available TWIMS-MS instrumentation for urine profiling [99] and DTIMS-MS for human plasma
732 and HaCaT cells [100], whereby improvements in signal-to-noise and some confirmed
733 examples of isobar separations have been demonstrated. Other notable recent studies have
734 focused on non-targeted metabolomic assessment of placental samples [101], and serum
735 profiling [102]. The potential of LC-FAIMS-MS was also explored in a very recent contribution
736 with a broad outlook toward different “omics” approaches [103].

737 In addition to high resolution mass data, as commercially-available IM-MS instrumentation also
738 includes a quadrupole and collision cell (e.g. IMS-QTOF), the potential of using both IM-MS
739 with fragment ions associated to the precursor ion (i.e. aligned by drift time) can become part
740 of standardized workflows in order to utilize retention time, CCS, MS and MS/MS information
741 for metabolite annotation. However, data processing to make use of all of this information for
742 annotating metabolites in fully non-targeted workflows is time consuming and remains a
743 current roadblock for method development.

744 Aside from global metabolomic profiling, several groups have also focused on characterization
745 of secondary metabolites including food and plant studies [104–107]. Xiao *et al.* studied the
746 thermal degradation of beta-carotene using TWIMS-MS and putatively identified several
747 breakdown products [107]. Causon *et al.* demonstrated preliminary developments for wine
748 metabolomic studies using DTIMS, where excellent day-to-day repeatability precision (<0.3%)
749 and agreement between CCS determined using standards and online measurement of real
750 samples (<0.5%) was observed [104]. Covington *et al.* recently reviewed strategies utilized for
751 secondary metabolism and concluded that IM-MS is likely to play a role in natural products
752 dereplication [108]. Structural isomers and epimers of steroids, oxysterols and related
753 hormones have also been in focus within some recent IM-MS studies demonstrating the
754 suitability of available instrumentation for a diverse range of applications [109–111]. In all of
755 these examples, cleaner mass spectra and use of CCS as an identification point are
756 highlighted to have potential for increasing confidence in confirming metabolite identity. Thus,

757 it is of critical importance for establishment of user-friendly LC-IM-MS metabolomic workflows
758 that the introduction of CCS as a conditional identification point for metabolite annotation
759 involves careful establishment of consensus CCS values to populate libraries for global use. It
760 is therefore expected that combined efforts in building reference CCS libraries for different
761 types of IM-MS instrumentation will emerge in order to provide validated methods supporting
762 metabolomic-related studies.

763 The area of drug metabolism has also been studied using IM-MS in recent contributions with
764 particular focus on isomeric variants. Reading *et al.* studied the gas phase conformation of
765 drug metabolites using TWIMS-MS, with a focus on correlating experimental data with
766 computational models for pharmaceutical structural characterization (Figure 7) [112]. Lee *et*
767 *al.* investigated metabolites of the anticancer drug Paclitaxel to study hydroxylation at different
768 sites using TWIMS-MS and supported their results with theoretical calculations [113]. Beucher
769 *et al.* studied non-steroidal selective androgen receptor modulators, which are prohibited by
770 the World Anti-Doping Agency, but remain readily available and potentially used in livestock
771 production for growth promotion [114]. Interestingly, the authors were able to successfully
772 couple supercritical fluid chromatography (SFC) separation with TWIMS-MS for the analysis
773 of bovine urine and suggested that CCS has potential within this workflow to be used as an
774 identification parameter within a screening method.

775

776 6.2. Glycan analysis

777 Glycans constitute a large class of biomolecules with a broad chemical diversity. They are
778 involved in many biological processes, such as embryonic development, cell-cell recognition,
779 inflammation and fertilization, among others [115,116]. Glycans can be covalently bound with
780 proteins to form glycoconjugates and hence affect protein folding mechanism and protein
781 stabilization. An abnormal conjugation between proteins and glycans can lead to a modification
782 of the tertiary structure having an impact on their biological response.

783 Structural characterization of glycans is the first step towards the understanding of their
784 biological activity. Due to their structural heterogeneity, the elucidation of glycan structures

785 remains a challenging task. Liquid chromatography combined with tandem mass spectrometry
786 has been widely used for glycan analysis [117–119]. Nevertheless, different chromatographic
787 columns may be required to provide sufficient separation of isomers, which cannot be obtained
788 with tandem MS, leading to a low analysis throughput. Recent advances in IM-MS have
789 allowed the improvement of the structural characterization and separation of carbohydrates
790 have been reported since then [5,120]. Clemmer and coworkers first published the reduced
791 mobilities of negatively charged trisaccharides and their fragments by using a home-built drift
792 tube instrument [121]. In 2003, Hill and coworkers separated 21 different carbohydrate
793 standards using a non-commercial ion mobility instrument as well [122]. Overall, these results
794 showed the capabilities and the potential of IM-MS for the analysis of carbohydrates.

795 The interest on IM-MS especially increased after the advent of commercial instruments. A large
796 number of studies dealing with carbohydrate analysis in IM-MS have been reported [120] since
797 then [5]. Resolving power is the major issue of ion mobility in the analysis of carbohydrates.
798 Several strategies have been developed to circumvent this problem. Formation of non-covalent
799 complexes with metal ions has been largely used to induce alternative carbohydrate
800 conformations and thus, enhance isomer separation [123–125]. This approach has been used
801 for comparing the effect of different cations on the CCS of several milk sugar isomers [123], to
802 study the influence of the metal cation on carbohydrate structure. The sodium adducts
803 presented larger CCS compared to lithium adducts, but Na⁺ was not found to be a suitable
804 cation to provide the best isomeric carbohydrate separation. Altogether, it was concluded that
805 separation of isomers does not only depend on cation radius, but also on the stereochemistry
806 of the compounds. Hoffman *et al.* studied a set of six trisaccharide isomers with different
807 composition in the non-reducing-end moiety [126]. IM-MS experiments were performed in
808 positive and negative ion modes to evaluate the influence of the ion polarity. In addition, two
809 kinds of trisaccharide adducts were formed with Na⁺ and Cl⁻ to improve the separation of the
810 precursor ions. Overall, IM provided a better separation of configurational and linkage isomers
811 in negative ion mode, compared to sodium adducts or protonated trisaccharides. Interestingly,
812 similar ATDs were observed for [M-H]⁺ and [M-Na]⁺ sodium adducts, whilst an impact on the

813 general structure of trisaccharides was observed upon chloride adduct formation. Another
814 additional interest about IM highlighted in this work was its ability to quantify small amounts of
815 carbohydrate impurities from a given mixture. Product ions in a relative concentration of 0.1%
816 could indeed be efficiently detected and quantified by analyzing the corresponding ATDs.

817 The versatility of certain IM-MS instruments allows the fragmentation of ions before the IM cell
818 (pre-mobility CID) [127,128] and/or after (post-mobility CID) [129,130] to provide in-depth
819 insights about the structure of precursor carbohydrates and also to generate specific tandem
820 mass spectra. For instance, oligosaccharide-alditols from bovine sumaxillary mucin (BSM)
821 were separated by HPLC and analyzed by IM-MS in the negative ion mode [128]. Some
822 specific precursor ions were post-mobility fragmented to generate specific tandem mass
823 spectra from isomeric precursor ions. CID was also performed upon IM separation giving rise
824 to isomeric mobility peaks. The mobility resolved product ions were also fragmented in the
825 transfer cell, generating further structural information. A more recent study from Harvey *et al.*
826 performed a post-mobility fragmentation of hybrid and complex glycans obtained from five
827 different glycoproteins [130].

828 The previous works were mainly focused on improving the separation and identification of
829 isomeric species and the characterization of the global structure from IM measurements.
830 However, IM can also be used as a filter, to reduce the complexity associated with the
831 carbohydrate mass spectra [11,127,130,131]. Interactions between neutral buffer gases and
832 ions strongly depend on the chemical nature of the analytes, leading to different mobility
833 behaviors. May *et al.* calculated almost 600 CCS values that comprise different molecules
834 such as lipids, peptides, carbohydrates and quaternary ammonium salts, using a drift tube
835 coupled to a quadrupole time-of-flight mass spectrometer [11]. Above 1200 Da, these four
836 classes exhibit different gas-phase packing trends that were clearly distinguishable by IM. In
837 addition, multiply charged ions will exhibit higher mobilities than singly charged ones,
838 describing specific drift time/mass-to-charge trends that can be individually extracted from the
839 global mass spectrum. This example is clearly observed in the analysis of N-glycosides from
840 bovine fetuin studied by Harvey *et al.* in the negative ion mode [130]. Several well-defined

841 regions are displayed in the driftscope, corresponding to different N-glycan charge states. It is
842 worth pointing out that filter-role of ion mobility not only facilitates the interpretation of mass
843 spectra but also allows the detection of minor species that could not be observed in classical
844 MS conditions by improving the signal-to-noise ratio.

845 Alternative fragmentation techniques to CID are required to produce more diagnostic cross-
846 ring fragments such as electron excitation dissociation (EED) [132], vacuum ultraviolet
847 photodissociation (VUVPD) [133], and extreme ultraviolet light (XUV) [134] or spectroscopic
848 techniques such as infrared multiple photon dissociation (IRMPD) [78], to produce
849 characteristic IR spectra to elucidate the structure of the carbohydrate isomers. Further
850 improvements in separation and identification of glycan isomers can be achieved by coupling
851 two IMS dimensions, as recently demonstrated [135,136]. IMS-CID-IMS-MS experiments can
852 be performed with these experimental set-ups to study the structural heterogeneity of complex
853 glycan mixtures, where the separation of the precursor ions is not fully achieved in classical
854 IM-MS experiments.

855

856 6.3. Lipid analysis

857 Lipids occupy a distinct conformational space in IM-MS analysis, due to their relatively high
858 flexibility and low density (i.e. large CCS for their m/z). Moreover, aside from the broad class
859 separation requirements for lipid analysis (triacylglycerides, phospholipids, free fatty acids,
860 ceramides, sphingomyelins,...), there is great structural diversity within individual classes
861 influencing their conformation including different alkyl chain lengths, degree of saturation, and
862 double bond position. In this regard, IM-MS offers great potential to investigate this diversity
863 that may not be revealed by conventional analysis. Application of IM-MS to lipidomics has been
864 recently reviewed by Paglia et al [137,138] and the reader is referred to these two contributions
865 for a comprehensive overview of this topic. Astarita and Paglia also recently published an
866 advanced lipidomic protocol using TWIMS-MS in combination with liquid chromatography [3],
867 while excellent insight into CCS calibration strategies for lipidomics using this type of
868 instrument was recently published [139]. In a particularly novel approach, the group of Murphy

869 has demonstrated the use of product ion mobility for lipidomic workflows, whereby collisional
870 activation is used to generate product ions for which a CCS value can be determined to aid
871 with lipid identification using a TWIMS-MS platform [140,141].

872 Some very recent results from DTIMS studies have indicated the challenges associated with
873 separating minor differences in CCS (e.g. double bond position isomers) due to the sub-1%
874 differences in CCS of many isomers. Kyle *et al.* studied a wide range of lipid classes using the
875 Agilent DTIMS instrument demonstrating the possibilities for a wide range of lipid isomer types
876 and the separation potential offered with current resolution limitations [142]. Dried blood spots
877 were the focus of a second study from this group involving longitudinal samples from diabetic
878 patients also assessed by DTIMS [143]. Stable isotope-labelling was used for profiling of fatty
879 acids using DTIMS, where the similarity of drift times was used to help associate labeled with
880 unlabeled acids in normal and cancerous human thyroid tissue samples [144]. Impressive
881 resolution of acyl chain- and double bond positional isomers of lipids has been demonstrated
882 on the TOFWerk DTIMS platform [145]. A resolution of >250 was calculated for isomeric lipids
883 measured under low-field conditions with the instrument operated with Hadamard multiplexing,
884 which requires sophisticated signal deconvolution approaches.

885 As is the case for metabolomic studies, standardization of CCS values must become a key
886 focus for building of global lipid libraries for identity confirmation and is already a targeted focus
887 of some computational modelling studies [146]. Nevertheless, some of the subtleties of gas-
888 phase lipid conformation have not yet been resolved between theoretical and experimental
889 considerations and ongoing effort is needed to reconcile these aspects. The commercial
890 availability of high-precision instrumentation utilizing different types of IMS analysers is already
891 playing a key role in supporting such work.

892 FAIMS offers a complementary approach in comparison to TWIMS and DTIMS and has also
893 been used for targeted studies of lipids by several authors for interrogating isobaric lipid
894 species as essentially a “mobility filter” prior to MS experiments. In one such study, Steiner *et al.*
895 used differential-mobility in combination with a state-of-the-art ozonolysis strategy to probe
896 the structure of deoxysphingosine [147]. This type of study underlines the structural diversity

897 within a single lipid class that can influence biological behavior that can benefit from IM-MS as
898 a powerful analytical approach (Figure 8). A very recent study has also demonstrated some
899 promising results using FAIMS for resolution of a wider range of isomeric lipid classes [148].
900 Finally, due the complementarity of different available IM-MS analysers, the potential for
901 revealing previously unknown biological information using IM-MS has started to emerge in
902 recent literature and further insightful publications in this area are expected.

903

904 6.4. Environmental analysis

905 IMS has a long history for usage in field applications entailing rapid environmental
906 assessments. The recent review of Marquez-Sillero *et al.* presents IMS applications focusing
907 on environmental analysis [149]. While IMS alone has great potential for applications requiring
908 portable analysis, IM-MS and LC-IM-MS have excellent potential to be used for lab-based
909 screening workflows, particularly for trace analysis in complex samples. The group of Schmitz
910 has recently demonstrated a powerful approach by combining two online separation methods
911 (LC+LC or GC+GC) with DTIMS-MS [150,151]. Complex wastewater samples were
912 successfully screened using an in-house CCS database with excellent repeatability of CCS
913 and mass spectra, while their more recent study used GC+GC with atmospheric pressure
914 chemical ionization (APCI) for the screening of various compounds, including pesticides.
915 Further discussion of the potential of multidimensional chromatography in combination with
916 IM-MS is presented in section 8.3. Grimm *et al.* used TWIMS-MS for the study of of unknown
917 or variable composition biological materials (UVCBs), complex reaction products, and
918 biological materials in petroleum samples [152]. This workflow allowed mass-to-charge, drift
919 time, and Hydrogen/Carbon ratio to be used to support compound classification and inter-
920 samples comparisons. While such studies offer exciting results, for such approaches to
921 become broadly accepted in standardized methods, CCS will require standardization as an
922 identification point in order to be used alongside retention time and MS identification
923 parameters within regulated methods.

924 The substantial challenge of exposomic studies, where 1000s of compounds including
925 xenobiotics, secondary metabolites, and transformation products which remain currently
926 unidentified, is a focus of high-throughput IM-MS studies whereby computational prediction of
927 CCS values is suggested to be a way forward to allow rapid screening of samples [153]. Such
928 a workflow requires substantial computational effort to predict CCS values, but some authors
929 have presented promising results using different approaches in addressing this major
930 challenge [154–156]. The workflow demonstrated by Zhou et al [156] was particularly in-depth
931 as DTIMS experimental data from a total of ~400 compounds were used to generate molecular
932 descriptors to train a machine-learning algorithm, which matched CCS values measured on
933 their instrument with a median relative error of 3%. Alternative approaches to modelling of CCS
934 are discussed in Section 3.3.

935

936 6.5. Clinical analysis

937 A number of studies have investigated the use of IMS and IM-MS for the analysis of samples
938 of clinical origin and the potential of these technologies for such applications was recently
939 reviewed by Chouinard *et al.* [110]. As is the case for environmental applications, standalone
940 IMS instrumentation is considered to be attractive for clinical applications due to its portability,
941 ease of use and low cost. Rapid sampling of exhaled breath is one example that has already
942 explored the use of this technology since the 1980s [157]. In the case of IM-MS, some of the
943 more promising investigations for clinical applications appearing within the last few years are
944 aiming toward the use of IM-MS for imaging using desorption electrospray ionisation (DESI) or
945 matrix-assisted laser desorption ionisation (MALDI) in combination with IM-MS [158–160].
946 Apart from these developments, many of the goals and challenges within clinical applications
947 using IM-MS also overlap with those of the related “omic” studies, which are presented in the
948 elsewhere relevant sections of this review.

949

950 **7. Application of ion mobility spectrometry to the characterization of large** 951 **biomolecules**

952 7.1. Separation of isobaric peptides

953 One of the most important challenge of structural biology is the elucidation of the protein
954 structure/function relationship. Posttranslational modifications (PTM) like phosphorylation,
955 acetylation, or methylation can be involved in modification of the protein structure and thereby
956 have a straight impact on their function. Identification and precise location are essential
957 information to provide a thorough PTM characterization. For this purpose, LC has been used
958 prior to MS/MS to separate precursor peptides, however this approach often fails to separate
959 and identify positional variants, especially when isobaric peptides exhibit no specific
960 reporter/diagnostic fragments [154]. A newer approach based on the implementation of IMS
961 has been used to provide an additional separation dimension to the characterization of large
962 biomolecules. Ruotolo *et al.* studied [154] a large set of phosphorylated and non-
963 phosphorylated peptides using a MALDI-DT-MS mass spectrometer. The contour plots show
964 that phosphorylated peptides exhibit lower drift times compared to non-phosphorylated
965 peptides with the same m/z . Unfortunately, the resolving power of the technique [161–163]
966 only allows the efficient separation of some phosphorylated variants. The separation capability
967 of IM was further improved by using FAIMS coupled to MS [23,164]. Three different
968 phosphorylated peptides from τ protein (VAVVRTpPPKSPSSAK, VAVVRTPPKSpPSSAK,
969 VAVVRTPPKSPSpSAK) were efficiently separated under the influence of the asymmetric
970 electric field and by modifying the He/N₂ ratio of the buffer gas [165]. The best separation was
971 achieved with a mixture of gas containing 40/60 v/v of He/N₂. The identification of each
972 phosphorylated variant was performed after ETD fragmentation. It is worth noting that ETD
973 spectra of each individual phosphorylated peptide do not exhibit unique fragments that allow
974 unambiguously identification of the precursor ions, thereby, an efficient separation is required
975 prior to ETD fragmentation. More challenging PTM characterization was envisaged with the
976 same experimental set-up, like methylated [166] and acetylated peptides [167]. Both
977 modifications were studied on histone tails, more precisely in the 1-21 and 21-44 segments of
978 H3 tails, and 1-25 segment of H4 respectively. Positional variants of methylated and acetylated
979 peptides were separated using different He/N₂ or H₂/N₂ gas ratios, to improve ion separation.

980 Overall, very narrow peaks and a baseline separation could be achieved after addition of He
981 to the buffer gas (from 0% to 40% v/v). It is worth mentioning that methylated or acetylated
982 peptides are +14 and +42 Da modification, respectively over “middle down” sequences (up to
983 3 KDa) which means that methyl variation accounts for 0.5% of the mass, requiring high
984 resolving power to provide an efficient separation. These results show the utility of IM-MS to
985 improve detection of positional isomers of modified peptides and identification from a complex
986 peptide mixture.

987

988 7.2. Adding an ion mobility dimension for improved peak capacity in high throughput 989 large scale proteomics

990 Modern proteomic analysis consists of identification and simultaneous quantification of
991 thousands of proteins present in complex protein mixtures, with many applications in molecular
992 and cellular biology [168–170]. Up to now, most proteomic studies rely on bottom-up analysis:
993 complex protein mixtures are digested into peptides that are further analyzed by reversed
994 phase liquid chromatography coupled to nanoelectrospray ionization tandem mass
995 spectrometry (nanoLC-MS/MS) platforms. Hybrid tandem mass spectrometers such as ion
996 trap-orbitrap, quadrupole-orbitrap and quadrupole-time-of-flight (QqTOF) instruments have
997 dominated the field of large scale high throughput proteomics. These configurations offer high
998 data acquisition speed and high mass accuracy for peptide precursor and fragment ions.
999 However, this classical bottom-up proteomic workflow presents a series of caveats : i) several
1000 peptides can co-elute from the chromatography column and ii) MS/MS acquisitions are mostly
1001 performed in a data-dependent (DDA) way, meaning that most intense ions are fragmented
1002 one at a time, the others being entirely discarded. Limited scan speeds and sensitivities of the
1003 mass spectrometers mostly account for this under-sampling, leading to subsequent lower
1004 sensitivities. Thus, improving the performance of new instrumentation relies on increasing the
1005 rate of MS/MS acquisition without information loss (transmission) on tandem mass spectra.
1006 Although ion trap instruments may still offer higher absolute sensitivity owing to their ability to
1007 accumulate precursor ions to a desired level prior to fragmentation (a feature that is not

1008 available on QqTOF instruments), TOF instruments have the potential for very fast data
1009 acquisition (up to 100 Hz for QqTOFs) [171] and high in-spectral dynamic range, both of which
1010 are highly desirable for the analysis of the very complex samples [172]. IMS can then be
1011 exploited to increase speed, selectivity, and sensitivity of MS/MS experiments [2,173,174].
1012 The integration of TWIMS into DDA acquisition schemes [14], termed high definition or HD-
1013 DDA [175] has removed the long standing limitation of low duty cycles of QqTOF instruments
1014 and leads to considerably improved sensitivity and productivity of proteomic analysis on such
1015 instruments [14]. The actual speed of HD-DDA is 30 Hz, mainly owing to the time required for
1016 on-the-fly data processing within the instrument control software. In the TWIMS configuration,
1017 charge separation capabilities of TWIMS cells are used to separate singly charged from
1018 multiply charged ions of tryptic peptides.

1019 Data-independent acquisition (DIA) modes, in which fragment ion spectra of all detectable
1020 precursor ions within an LC-MS run regardless of any specific characteristics (intensity, charge
1021 state and so on) are generated, [176] have also been reported as alternatives to circumvent
1022 under-sampling of MS/MS experiments. MS^E is one of the first reported DIA approach and
1023 consists of two alternating MS scans recorded at low and high collision energy across the full
1024 mass range, providing precursor and product ion information, respectively [177]. The
1025 integration of IMS to DIA workflows has been referred to as high-definition MS^E (HDMS^E). IMS
1026 provides here an additional dimension of separation that increases the overall system peak
1027 capacity in LC-MS workflows [174], offering the possibility to fragment precursor ions after IMS.
1028 IMS not only allows the resolution of co-eluting isobaric species, but also enables the use of
1029 drift-time information [178] for the alignment of precursor and product ions (in addition to
1030 retention times in chromatography). This drastically increases the number of identified peptides
1031 and proteins in complex samples as compared to MS^E workflows without IMS [179]. Optimized
1032 HDMS^E approaches achieved similar proteome coverage as compared with state-of-the-art
1033 DDA methods and displayed excellent reproducibility and overlap between technical replicates
1034 [180,181]. Figure 9 presents an example of HDMS^E analysis of a 400 ng Hela digest, leading
1035 to the identification of more than 38440 peptides corresponding to 3490 unique proteins.

1036 Thorough overview of the use of IMS-DIA-based label-free quantification and its applications
1037 in biomedical research and clinical proteomics have been reported elsewhere [182,183].
1038 Similarly, IMS-DIA has also been reported for enhanced impurity detection in protein
1039 biotherapeutics (Host Cell Proteins, HCP), as they present a high dynamic range between the
1040 very abundant therapeutic protein and its low abundance impurities. HCPs present at a
1041 concentration as low as 1 ppm (1 ng HCP/mg mAb) were successfully identified and quantified
1042 in the reference NIST monoclonal antibody and Infliximab [13]. Recently, Haynes *et al.*
1043 described the use of a new mode of IMS-DIA acquisition: instead of performing IMS-DIA at a
1044 fixed velocity TWIMS settings, ramping the traveling-wave velocity increased drift space
1045 occupancy, amplifying resolution by 16%, peak capacity by nearly 50%, and peptide/protein
1046 identifications by 40%, opening new perspectives for variable-velocity TWIMS in proteomics
1047 analysis [184]. To summarize, IMS adds an orthogonal analytical dimension, reduces ion
1048 interference to improve reproducibility, peak capacity, and peptide identifications to rival
1049 modern hybrid quadrupole orbitrap systems.

1050 A variant of the HDMS^E approach has been proposed using a TIMS device [185], introducing
1051 the concept of parallel accumulation–serial fragmentation (PASEF) consisting of sub-
1052 millisecond quadrupole serial selection and fragmentation of multiple precursors in single 50
1053 ms TIMS scans [185]. PASEF enables hundreds of MS/MS events per second at full sensitivity.
1054 The PASEF workflow presents a series of advantages among which: i) no loss of sensitivity,
1055 ii) the resulting spectra are fully precursor mass resolved and iii) good compatibility with
1056 reporter ion based chemical multiplexing strategies (such as iTRAQ or TMT). A 10-fold gain
1057 should be achievable by PASEF in shotgun proteomics experiments, as a consequence of
1058 increased sequencing speed, without a decrease in sensitivity. However, modeling suggests
1059 that a combination of targeting more precursors and targeting weak precursors repeatedly will
1060 be most effective. The PASEF principle could be applied to any IM-MS configuration with the
1061 required sub-millisecond scan speed in the MS read out.

1062

1063 7.3. Ion mobility spectrometry for the characterization of gas phase protein structures

1064 ESI-IM-MS has been mostly used to study the 3D architecture and conformational properties
1065 of many proteins and biomolecular complexes, through the measurement of their averaged
1066 CCS [28]. However, over years, the question “is protein conformation altered from the solution
1067 to the gas phase of the mass spectrometer?” became central to avoid misinterpretation of IM
1068 data. Several studies that compared IM measured CCS to either calculated CCS from PDB
1069 structures or in vacuum molecular dynamics (MD) generated CCSs brought partial answers to
1070 this question. One common observation is that when the charge increases, the molecules
1071 undergo Coulomb-induced unfolding to adopt extended helical structures [186,187] and
1072 ultimately string-like extended structures at very high charge states [188,189]. At low charge
1073 states, however, the proteins usually remain compact, with sizes that are expected for
1074 condensed phase native structures. Thus, depending on the type of proteins/complexes of
1075 interest, the gas phase collapse and/or unfolding of proteins is more or less pronounced. It is
1076 now generally accepted that 3D structures of globular proteins (like TRAP, TTR, SAP, integral
1077 membrane proteins) [190] survive the transfer into the gas phase without major compactions
1078 or unfolding at low energies [191], as attested by the number of success stories based on
1079 agreement between IMS-measured CCSs and calculated ones. More recently, Devine *et al.*
1080 [192] showed that non-globular proteins (like monoclonal antibodies or proteins with flexible
1081 hinge regions in between more structured domains) and RNA molecules undergo strong
1082 compaction in the gas phase (CCS values measured underestimate the physical size of the
1083 solution structure and modeled data of the biomolecule), highlighting a potential caveat in
1084 studying these specific biomolecules with this technique. However, IMS is not directly sensitive
1085 to protein secondary structure, and observing a compact structure does not guarantee that the
1086 secondary structure accurately reflects that of the native form. To go ahead with keeping
1087 secondary structures from the solution to the gas phase, optical/IR spectroscopy, a direct
1088 method that allows distinguishing helices from β -sheets, has been coupled to IMS [193]. In this
1089 configuration, IMS is used to select the global shape of proteins, followed by IR spectroscopy
1090 to probe their secondary structure. On two reference proteins, myoglobin (85% of α -helix) and
1091 β -lactoglobulin (60% β -sheets), IMS combined to IR showed that secondary along with tertiary

1092 structures are retained in the condensed gas-phase. When IMS is combined to gas-phase
1093 infrared multiple photon dissociation (IRMPD) spectroscopy, gas-phase Coulomb-driven
1094 unzipping to a defined secondary structure is a general feature of all highly charged protein
1095 ions [194].

1096

1097 7.4. ion mobility spectrometry for proteins and protein-ligand complexes

1098 7.4.1. Ion mobility spectrometry for intrinsically disordered proteins

1099 Intrinsically disordered proteins (IDPs) have been the scope of many IM-MS studies (for review
1100 see [195,196]). Indeed IDPs, which are associated with a wide range of major disorders
1101 including cancer and amyloid-related disorders, are flexible proteins that can adopt many
1102 different conformations in order to bind to different physiological partners. This conformational
1103 change along with absolute conformation(s) characterization and detection of populations of
1104 conformations can be uniquely monitored by IM-MS. Bernstein *et al.* [197] performed IM-MS
1105 experiments on α -synuclein that indicate how the size of its conformations differed with charge
1106 state at initial solutions of neutral and acidic pH. IM-MS has also been used to study the
1107 structure of the DBD (DNA-binding domain) of the tumour-suppressor protein p53 with and
1108 without the functional zinc [198]. IM-MS showed smaller CCSs (12.3% in CCS) for the p53
1109 without zinc than for the zinc-bound form. Other research using IM-MS to study IDPs has
1110 examined changes in conformational equilibria of the intrinsically disordered HMGA (high-
1111 mobility group A) chromatin factors [199]. IM-MS measurements of wild type and of a C-
1112 terminal truncated (shorter) HGMA2 were recorded. Despite the increase in size of WT with
1113 respect to the C-terminal truncated HGMA2, IM-MS revealed a more compact structure for the
1114 C-terminal shorter form, indicating that the presence of the C-terminal tail is here responsible
1115 for further compacting HGMA2, which could be correlated to a reduction in oncogenic activity.
1116 D'urzi *et al.* reported the use of IM-MS to characterize the complex between the intrinsically
1117 disordered N_{TAIL} domain and the phosphoprotein X domain (P_{XD}) from measles virus (MeV).
1118 IM-MS revealed distinct conformers, yielding the estimation of the solvent-accessible surface
1119 area (SASA) in solution and the average CCS in the gas phase. Computational modeling of

1120 the complex in solution, based on experimental IM-MS constraints, were generated and
1121 provided the first attempt to modeling the entire N_{TAIL} domain bound to P_{XD} at atomic resolution.
1122 IM-MS is also well-positioned to assess IDP/ligand interactions and conformations induced
1123 upon ligand binding. Dickinson *et al.* have demonstrated the use of IM-MS to characterize the
1124 effect of two inhibitors RITA and Nutlin-3 on their respective binding partners: p53 and MDM2
1125 [200]. While no Np53:RITA complex was observed in native MS experiments, an alteration in
1126 the conformational distributions (through variations in ^{DT}CCS_{He} values) adopted by Np53 in the
1127 gas phase was observed. This finding supports the hypothesis that RITAs mode of action
1128 proceeds via a conformational change in p53, which was further confirmed by a combination
1129 of complementary biophysical techniques. Similarly, DTIMS analysis revealed that, in the
1130 absence of Nutlin-3, every charge state of N-MDM2 shows at least two conformations. Again,
1131 upon binding to Nutlin-3, the protein undergoes a compaction event similar to that observed
1132 with RITA and Np53. This multi-technique approach highlights the inherent disorder in these
1133 systems; and in particular exemplifies the power of IM-MS as a technique to study transient
1134 interactions between small molecule inhibitors and IDPs. For instance, Knapman *et al.*
1135 reported the use of IM-MS to probe the conformational characteristics of two IDPs, apo-
1136 cytochrome *c* and apo-osteocalcin, which are both structured when bound to their respective
1137 substrates, but disordered in their unbound-states [201]. In both cases, the intrinsically
1138 unstructured apo-states exist as multiple conformations with larger cross-sectional areas than
1139 their holo-analogues, suggesting that IDPs do not preclude the formation of preferred
1140 conformations. Together, these examples illustrate the scope and utility of IM-MS for studying
1141 the characteristics and properties of IDPs, whose analysis by other techniques is limited.
1142 Another feature that can be monitored by IM-MS is the tendency of IDP to form amyloid fibrils,
1143 the protein aggregates involved in the onset of neurodegenerative diseases such as
1144 Parkinson's and Alzheimer's (for review see [202]). Due to their dynamic, polydisperse, and
1145 polymorphic nature, these oligomers are very challenging to characterize using traditional
1146 condensed-phase methods. IM-MS based approaches can be used to study amyloid
1147 formation, providing information about the assembly pathway and the size and shape of

1148 presumably toxic oligomers. IM-MS was first used to separate, analyze and measure CCSs of
1149 individual oligomer species present in equilibrium [202]. A remarkable IM-MS study is reported
1150 by Leney *et al.* [203] and compares the oligomers formed from wild-type $\beta 2m$ (beta-2-
1151 microglobulin) with those formed from a variant of the protein (H51A), during in vitro fibril
1152 assembly. While H51A was shown to exhibit a two-fold increase in the lag-time of fibril
1153 formation, no differences were observed by IM-MS for the oligomers of the two proteins (similar
1154 CCSs). Real-time native MS was the only technique able to highlight the differences in the
1155 kinetics of the lag phase between the two protein variants. Similarly, IM-MS was used to
1156 investigate the formation of heterogeneous pre-fibrillar, oligomeric species involving human
1157 amylin (also known as islet amyloid polypeptide, hIAPP) and the amyloid-beta peptide ($A\beta$ -
1158 40), two pathogenic factors in Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD),
1159 respectively [204]. Oligomerization of human islet amyloid polypeptide (IAPP), being
1160 considered as a pathogenic process in type II diabetes, has also been extensively investigated
1161 by IM-MS [205,206]. Time-course MS and IM-MS were reported to probe the early aggregation
1162 states of an amyloidogenic endecapeptide derived from amino acid residues 105-115 of the
1163 human plasma protein transthyretin [207]. Early $A\beta$ oligomers, which are thought to be
1164 responsible for the neurodegeneration of AD patients, have also been analyzed using IM-MS
1165 [31]. IM-MS revealed for instance that full-length $A\beta 42$ aggregates faster and is more cytotoxic
1166 than the shorter $A\beta 40$ variant, which could be related to a compact stable tetramer formation
1167 for the $A\beta 40$ and a rather "open tetramer" that can evolve to an hexamer for the $A\beta 42$. More
1168 recently, Illes-Toth *et al.* have characterized by IM-MS a specific population of α -syn oligomers,
1169 which was shown to have the ability to induce intracellular aggregation [208]. Structurally, the
1170 MS compatible oligomers populated a range of species from dimers to hexamers. Lower-order
1171 oligomers were structurally diverse and consistent with unstructured assemblies. Higher-order
1172 oligomers were shown to be compact with ring-like structures. The observation of this compact
1173 state may explain how this natively disordered protein is able to transfer pathology from cell to
1174 cell and avoid degradation by cellular proteases.

1175 IM-MS provides valuable data on the ion's overall size and shape, but it does not yield direct
1176 information about the underlying internal structure. To obtain such data, IM-MS was recently
1177 used as a preselection tool for a further analysis using orthogonal, structure-sensitive gas-
1178 phase techniques, like action fluorescence resonance energy transfer (FRET) spectroscopy
1179 [209] or gas phase infrared spectroscopy [210]. IM-MS can also be successfully applied for
1180 high-throughput inhibitor screening. IM-MS is capable of high-throughput binding screening
1181 with a rate of up to 5000 molecules per day [211,212], providing not only conformational
1182 characteristics but also binding stoichiometries and specificities.

1183 To summarize, IM-MS provides a useful insight into events that occur during the early stages
1184 of aggregation including delineating the structure of the monomer, identifying oligomer
1185 distributions, and revealing mechanistic details of the aggregation process.

1186

1187 7.4.2. Ion mobility spectrometry for protein-ligand interactions

1188 The study of protein–ligand interactions is of particular interest for the pharmaceutical industry,
1189 especially in the context of drug development. Native MS has there also been demonstrated
1190 to be a pivotal technique providing relevant information about stoichiometry of interaction, site-
1191 specificity of ligand-binding and, in appropriate cases, affinities (through dissociation constant,
1192 K_D , measurements) between the target protein and the ligand [212,213]. As the binding of
1193 ligands often induces a conformational change of the protein, the hyphenation of IMS to native
1194 MS could bring an additional level of conformational characterization, in addition to the
1195 classical binding screening (for review see [213,214]). A first solution to track the
1196 conformational change of a protein upon ligand binding is thus to perform IM-MS and measure
1197 CCS values for the free protein and the ligand-bound form. However, this simple and basic
1198 idea is not straightforward as ligand binding not always induce major global conformational
1199 changes, but rather very localized conformational changes with exceedingly low impact on the
1200 global CCS measurements (below 5% CCS differences). In addition, the resolution of the IMS
1201 cell is here of paramount importance. As a consequence, careful optimization of the IM cell to
1202 distinguish between protein (P) and protein:ligand (P:L) are required to resolve CCS changes

1203 lower than 2% for P:P and P:L complexes [29,68,215]. Despite these limitations, the use of IM-
1204 MS to discern conformational tightening on the c-MYC:MAX leucine zipper formation due to
1205 ligand binding has been previously reported by Harvey *et al.* [216] and along with these
1206 findings, provides an exciting prospect as a method for screening inhibitors to conformational
1207 dynamic systems. Collision induced unfolding (CIU) experiments have proven to be an
1208 interesting IM-based alternative to tackle systems involving very subtle conformational
1209 changes (<2%). CIU is an IMS-based gas phase method that aims at collisional heating of
1210 ions, typically through the application of an accelerating electric field that results in higher-
1211 energy collisions with background gas. Ions undergo unfolding and the resulting conformers
1212 are separated in the IMS cell. Two ions that share similar initial CCS values can be readily
1213 differentiated through the CCSs and collision energies associated with the unfolded protein
1214 forms populated during CIU. The comparison of CIU fingerprints (IM drift times as a function
1215 of accelerating voltages) obtained for P and different P:L systems can help to differentiate
1216 conformational populations of very close CCS (<1%, [217]). Ruotolo first showed that CIU
1217 experiments allow differentiation of the effects of ligand binding not normally observed by MS
1218 alone on wild-type (wt) and disease-associated variants of tetrameric transthyretin (TTR) [218].
1219 CIU experiments were able to show that the natural thyroxine ligand of TTR stabilizes wt TTR
1220 but also disease-associated TTR variants. Similar experiments were reported for ligand
1221 binding on model proteins like FK-binding protein, lysozyme or myoglobin [219]. More recently,
1222 CIU experiments have been reported for the identification of kinase inhibitors that specifically
1223 bind to the inactive conformation of the protein. CIU patterns allowed distinguishing between
1224 inhibitors with similar K_D values, suggesting the use of IMS-based CIU fingerprints as a new
1225 assay capable of both rapidly detecting inhibitor binding and classifying the resultant kinase
1226 binding modes. Since then CIU experiments have been reported in advanced drug discovery
1227 workflow [220], highlighting the stabilizing role of lipids in the context of membrane protein
1228 complexes [221] or the cooperative stabilization effects upon sugar binding in the concanavalin
1229 A tetramer [222]. CIU appears as a method allowing to “resolve the unresolved through gas-
1230 phase measurements” [213].

1231

1232 7.4.3. Ion mobility spectrometry for membrane proteins

1233 IM-MS is also a well-established strategy to study the global conformation of membrane
1234 proteins and the characterization of their interactions with ligand/lipids [223–225]. Membrane
1235 proteins are involved in many vital cellular processes, such as trafficking and signal
1236 transduction [226,227]. Due to the high hydrophobicity associated to their structure, membrane
1237 proteins are transferred into the mass spectrometer encapsulated within detergent micelles to
1238 preserve their native-like structure [167]. Detergent aggregates strongly suppress ionization in
1239 ESI and need to be removed to perform MS analysis. Therefore, ionization source conditions
1240 such as pressure, temperature, and acceleration voltages are critical for membrane protein
1241 analysis. The protective role of detergent aggregates was clearly showed in the case of the
1242 transmembrane complex BtuC2D2 [223]. After increasing the number of collisions to produce
1243 the release of the complex from the micelle, the intact tetramer can be observed. The same
1244 ion channel (BtuC2D2) and a transporter (KirBac3.1) were characterized by Robinson and
1245 coworkers [224] to compare the average CCS of both tetramers after release from the
1246 detergent micelles. Different activation energies were applied and the overall size of both
1247 tetramer remained constant. The same experiment was performed upon activation of their
1248 respective trimers. In this case, KirBac3.1 trimer exhibited one single distribution that remained
1249 constant through ion activation, however, a bimodal distribution was observed for the BtuC2D2
1250 trimer when the acceleration voltage was set to 240 V being consistent with two conformer
1251 populations. Experimental CCS of BtuC2D2 and KirBac3.1 trimers were smaller compared to
1252 the theoretical ones, suggesting that trimer structures collapsed after the removal of one
1253 subunit.

1254 CCS measurements played a critical role in the modeling strategy of full length OmpA (FL-
1255 OmpA) dimer structure [167]. Native MS data showed monomeric and dimeric populations
1256 while further IM-MS experiments revealed compact dimers and extended and compact
1257 monomers. The same experiment was carried out for the N-terminal domain (TM-OmpA),
1258 where only the monomer structure could be observed. These results combined with native MS

1259 confirmed that dimer interface was located in the C-terminal 188-276 amino acid sequence. A
1260 low-resolution structure for the FL-OmpA dimer assembly was proposed based on IM-MS data
1261 as well as cross-linking constraints. The theoretical model of the FL-OmpA dimer is sustained
1262 by the good agreement between experimental and theoretical CCS.

1263 Amphipatic polymers (APols) have been shown to be a suitable alternative to detergent
1264 aggregates for preserving membrane protein water solubility [228]. Watkinson *et al.* studied
1265 the influence of several APols on the structure and activity of three outer membrane proteins
1266 (Omp), namely PagP, OmpT, and tOmpA [229]. The catalytic activity of these three Omp
1267 strongly varies as a consequence of amphipols interactions, despite the fact that circular
1268 dichroism data indicate that PagP, OmpT, and tOmpA native structures is preserved in the
1269 amphipols. The structures of the released Omp were assessed by ESI-IMS-MS. The
1270 experimental CCS of the lower charge states were in good agreement with theoretical CCS
1271 from protein data bank (PDB)-coordinates indicating that APols tested in this study do not
1272 induce any significant structural changes.

1273 Membrane proteins have shown strong interactions with lipids [167,230]. They play an
1274 essential role in membrane protein structure, folding, and activity [230,231]. Laganowsky and
1275 coworkers studied the selectivity of lipid binding to membrane proteins by using native MS and
1276 IMS. The stability of the mechanosensitive channel of large conductance (MscL), aquaporin Z
1277 (AqpZ) and the ammonia channel (AmtB) was assessed upon lipid binding by monitoring the
1278 unfolding process of the membrane proteins as a function of collision voltage (CIU
1279 experiments). CIU fingerprints revealed that upon the addition of CDL and PG, the stabilisation
1280 energy of AmtB dramatically increased. This result is based on the increase of the collision
1281 voltage required to induce unfolding process of the lipid complexes compared to the apo
1282 structures. In the case of AqpZ and MscL, the same stabilization effect was observed after the
1283 addition of the different lipids. These results emphasize the interest on CIU experiments to
1284 study the selectivity of membrane proteins toward lipids, and how this interaction can modulate
1285 their function. In this case, the trap cell was used for releasing membrane proteins from
1286 detergent micelles as well as to induce protein unfolding. To avoid the presence of detergent

1287 aggregates in the trap cell, “in-source” fragmentation was performed by increasing the source
1288 temperature up to 120 V [225] (Figure 10), thus detergent aggregates are removed before the
1289 entrance of the ions in the trap cell. Ions are subsequently activated (CIU experiments) with or
1290 without previous ion selection (IMS-MS or MS-IMS-MS respectively) to monitor the unfolding
1291 process of membrane proteins as a function of the activation energy. Stabilization of AmtB
1292 upon lipid binding could be evidenced by comparing the transitions of the CIU profiles. The “in-
1293 source” fragmentation allowed performing MS-IMS-MS CIU profiles to evaluate the
1294 stabilization energy provided upon lipid binding.

1295 Overall, for all these works, experimental CCS for membrane proteins were measured through
1296 the use of well-defined IM calibration protocols [232,233]. However, membrane protein ions
1297 exhibit lower mobility compared to soluble proteins with similar molecular weights [223] as a
1298 result of their low charge state. In a recent paper from Allison *et al.*, it was reported that the
1299 average mobility of membrane proteins was about 30% lower than soluble proteins used for
1300 TWIMS calibration [190]. Similar native-like membrane protein standards should be used to
1301 provide an accurate CCS measurement. Due to the lack of available membrane protein
1302 standards, they suggest to use larger calibrants for reducing the error associated with the long-
1303 range extrapolation of the calibration function.

1304

1305 7.5. Ion mobility spectrometry for large protein assemblies and its integration to 1306 structural biology programs

1307 For protein analysis, IM-MS is mostly used to characterize conformational states or to monitor
1308 conformational changes induced upon any modification of the protein environment
1309 (experimental conditions, oligomerization, ligand binding, RNA/DNA binding, protein partner
1310 binding, etc. [41]). As the number of publications reporting the use of IM-MS for protein
1311 characterization has tremendously increased in the past 10 years, it is no more possible to
1312 draw an exhaustive picture of the possibilities of IM-MS for protein conformational
1313 characterization. IM-MS can be used to determine changes in mobility and thus conformation
1314 and CCS, properties such as conformational dynamics, folding and unfolding intermediates,

1315 ligand-induced conformational changes, aggregation intermediates and quaternary structures
1316 (topology) [2,28,41]. We thus tried to focus on some relevant works performed over the past
1317 10 years that could serve as case studies.

1318 Native MS has been particularly useful to study the dynamics of assembly or disassembly of
1319 several macromolecular machineries, giving direct access to the stoichiometry of intermediates
1320 of assembly/disassembly through accurate mass measurement. When combined with IMS,
1321 native IM-MS enables monitoring of global conformational changes occurring during the
1322 assembly/disassembly process through accurate CCS measurements. Plots representing the
1323 CCS as a function of mass are often used to highlight assembly/disassembly events. In such
1324 representation, CCS is expected to follow a linear increase as a function of the compound
1325 mass, thus events that happen at constant CCS further suggest a major conformational
1326 change. An example of such IM-MS application is given by Van Duijn et al [234], who reported
1327 the use of native MS in combination to IMS for the study of the *E. coli* Cascade complex (405
1328 kDa) and the *P. aeruginosa* Csy CRISP-complex (350 kDa) immune systems. They monitored
1329 Cascade complex disassembly either under gas-phase or in-solution dissociation experiments
1330 using native IM-MS. The results allowed to create subunit connectivity models of both the
1331 Cascade and Csy CRISPR immune systems. They compared experimentally measured
1332 CCS_{EXP} of Cascade sub-modules with values calculated using molecular modeling on the basis
1333 of the cryo-EM structure of Cascade. Finally, the interaction between Cascade and its target
1334 ssDNA was next monitored by IM-MS, highlighting a small but significant structural change for
1335 Cascade upon its association to ssDNA as evidenced by the measured CCS_{EXP} (3 nm²
1336 experimental compared to 9 nm² expected from calculations), which can only be explained by
1337 an altered conformation of the complex.

1338 Several case studies based on structural information derived from IM-MS to build three-
1339 dimensional models have been reported, including the analysis of the full clamp loader
1340 complex ($\gamma\delta\epsilon\psi\chi$, 254 kDa) in interaction with a single-stranded DNA (ssDNA) binding protein
1341 (SSB4) [235] (Figure 11), the analysis of the trp RNA binding attenuation protein (TRAP) [41],

1342 the study of ribonucleoprotein particles (sno/sRNPs) [236] or to decipher the conformation and
1343 assembly of viruses with molecular weights ranging from 1.8 MDa up to 18 MDa [237–239].
1344 Most of these studies were performed on TWIMS instruments. However, the group of
1345 Fernandez-Lima recently reported for the first time the use of TIMS instruments for accurate
1346 CCS measurements on protein complexes [240]. As CCS values alone give no detailed
1347 information on subunit structure within the complex, IM-MS analyses can be coupled to gas-
1348 phase activation techniques including collision induced dissociation (CID) and more recently
1349 surface induced dissociation (SID) to generate sub-complexes, SID being more adapted to
1350 retain native-like structures in the gas phase [241,242]. In addition, crosslinking strategies have
1351 been proven to be valuable to capture transient complex, protein complexes modified with
1352 either charged crosslinkers or tags, but not those treated with neutral crosslinkers, exhibiting
1353 enhanced stability in the gas phase relative to CIU [243].

1354 Overall, these studies of large heteromeric complexes analyzed by IM-MS, coupled with
1355 integrative modelling, highlight the potential of such an approach to reveal structural features
1356 of previously unknown complexes of high biological importance.

1357 IM-MS can be considered today as a complementary low-resolution technique for structural
1358 biology. Indeed, hybrid approaches in structural biology have gained considerable interest for
1359 uncovering the molecular architectures of large and transient biological systems. MS-based
1360 methods, and in particular IM-MS, and structural electron microscopy can complement
1361 conventional tools, such as X-ray crystallography and NMR spectroscopy [244,245]. Today,
1362 IM-MS is often applied to the structural elucidation of protein assemblies that have failed high-
1363 throughput crystallization or NMR spectroscopy screens. In the interpretation of the IM data
1364 for multiprotein complexes, there are two main steps to interpret IM for multiprotein complexes:
1365 the conversion of drift times into CCS values, and their comparison with model protein
1366 architectures.

1367 Such IM-MS and MS/MS measurements are often integrated with other sources of structural
1368 information to build comprehensive models of intact multiprotein complexes that remain

1369 refractory to more classical structural biology techniques. For instance, CCS restraints from
1370 IM-MS have been successfully combined to distance constraints from chemical crosslinking
1371 approaches to determine the best model among hundreds or thousands of computationally
1372 generated models for the study of the proteasomal base [246] or for the outer membrane
1373 protein A from *Escherichia coli* [247]. Combining IM-MS to solution hydrogen/deuterium
1374 exchange MS (HDX-MS) seems also appealing and has been reported for the characterization
1375 of flexible serpins [248] or on the Kai circadian complex [249]. For this latter system, native
1376 MS revealed that the complex formed between two of the components (KaiB and KaiC) is a
1377 dodecamer; HDX-MS provided their binding interface and finally the structural restraints
1378 derived from HDX were then combined with IM-derived CCSs and used as input for the
1379 HADDOCK web server to generate models of the KaiC6B6 complex. IM-MS is an emergent
1380 technology for structural proteomics, capable of assessing multiprotein topologies from
1381 complex mixtures using minute amounts of sample. Computational analysis is a key aspect of
1382 IM-MS data interpretation, and developments in this area are likely to be rapid over the next
1383 5 years.

1384

1385 7.6. Biopharmaceuticals

1386 MS performed under non-denaturing conditions (native MS) has emerged as a promising
1387 technique for mAb-related compound characterization [250,251]. As a logical evolution,
1388 hyphenation of IMS to native MS was also rapidly implemented for biopharmaceutical analysis.
1389 Since IMS provides a global view of conformational differences, it is a good choice for
1390 preliminary investigations of mAb conformations, where the shape and size of the molecule
1391 are of interest. IMS can either be used qualitatively mostly to address heterogeneity issues,
1392 but also in a quantitative way, to determine CCS values or to directly evaluate the relative
1393 amounts of the different species present in solution from IMS data. IMS can also serve to
1394 address more fundamental issues through collision induced unfolding (CIU) experiments, in
1395 which collisional heating is applied in the gas-phase to unfold the protein, which is
1396 subsequently separated by IM and then analyzed by MS. A typical CIU plot presents IM drift

1397 times as a function of increasing collisional energies applied in the region in front of the IM cell
1398 [252]. CIU fingerprints represent the amount of protein unfolding as a function of collisional
1399 energy applied. Those unfolding patterns have proven to be highly sensitive to small variations
1400 in protein structure that cannot be easily resolved by IMS alone. Figure 12 summarizes the
1401 IMS-based information that can be obtained for biopharmaceutical characterization.

1402

1403 *IMS for disulfide bridge heterogeneity* - The first application of IMS for mAb structural
1404 characterization was reported in 2010 for the rapid characterization of disulfide variants in
1405 intact IgG2 mAbs [253]. IMS revealed 2 to 3 gas-phase conformer populations for IgG2s,
1406 whereas a single gas-phase conformer was revealed for either an IgG1 or a mutated IgG2,
1407 providing strong evidence that the observed IgG2 gas-phase conformers are related to
1408 disulfide bond heterogeneity. Similarly, Jones *et al.* have used IMS to rapidly provide
1409 information on the higher order structure of a series of wild type and mutant IgG2 mAbs [254].

1410

1411 *IMS for global conformational characterization* - Given that IM is fast and gives a global view
1412 of mAb-related compound conformation, several groups have addressed the global
1413 conformation of mAb, ADC or bispecific antibodies compounds using IMS, attempting to
1414 attribute CCSs to given mAb isotype conformations and even to particular conformational
1415 heterogeneities. Pritchard *et al.* used this approach to detect three monomer conformations
1416 and two dimers for a commercial rhGH-specific antibody [255]. A quantitative analysis of the
1417 ATDs revealed that ~80% of the species present in the sample was in the second monomer
1418 conformation. Pacholarz *et al.* reported structural differences between IgG1 and IgG4 based
1419 on IMS measurements [38]. Interestingly, the $^{DT}CCS_{He}$ values obtained on a linear drift tube
1420 IM instrument in helium (61.1–83.2 nm²) were in agreement with those measured on TWIMS
1421 instruments by other groups for different IgGs, which demonstrates the robustness of the CCSs
1422 calculated from properly calibrated IM-MS data. However, the experimentally IMS-estimated
1423 CCS values were shown to be significantly lower than the CCS determined either from crystal
1424 structures or from MD simulation [38,192]. This has recently been explained by the flexibility

1425 of the hinge region which collapses in the gas phase, such that the measured CCS of the mAb
1426 is substantially less than both the predicted CCS from its crystal structure and the in vacuum
1427 MD simulation [192].

1428
1429 *Real-time IMS to monitor dynamic processes* – IMS has also been employed to monitor
1430 dynamic processes such as the IgG4 Fab-arm exchange that produces bispecific mAbs,
1431 constituted of two different half-antibody arms, each of which recognizes a distinct antigen, a
1432 particularly valuable property for therapeutic applications. In combination with time-resolved
1433 native MS, IMS was used to monitor the formation of the bispecific mAbs in real time [256], the
1434 CCSs of the bsAb (66.9 nm²) being intermediate to those of the original mAbs (65.6 and 69.6
1435 nm²).

1436
1437 *IMS for ADC drug conjugation heterogeneity assessment* - IMS can also be of great interest
1438 for the characterization of drug conjugation heterogeneity/homogeneity for antibody-drug
1439 conjugate characterization. IMS can provide several information, among which the global
1440 conformation of all detected species including conjugated and unconjugated forms or the
1441 global conjugation profile and average drug-to-antibody-ratio (DAR) through a semi-
1442 quantitative interpretation of IMS data (integration of ATDs areas). Debaene *et al.* reported the
1443 IMS characterization of brentuximab vedotin, the reference cysteine ADC [257]. The different
1444 ^{TW}CCS of the multiple drug loads were calculated, evidencing slight conformational changes
1445 that occur upon payload binding. The average DAR determined from IMS was 3.7 ± 0.1 (vs.
1446 4.0 expected), consistent with values obtained from other MS methods. In addition,
1447 hydrophobic interaction chromatography (HIC) fractions were collected and analyzed
1448 individually by IMS, revealing conformational homogeneity of each drug load species. Marcoux
1449 *et al.* next performed a similar study on trastuzumab emtansine (T-DM1), the reference lysine
1450 ADC already on the market [258]. The detailed structural picture provided by IM-MS was
1451 complemented by molecular modeling. As for the cysteine compound, native IMS highlights
1452 the increase in heterogeneity that occurs when trastuzumab is conjugated with DM1. The

1453 resulting drug distribution profile and average DAR (3.4 ± 0.2) are also consistent with values
1454 obtained from other MS methods (average DAR of 3.5). Recently, extensive conformational
1455 characterization by IMS of a new generation site-specific ADC enabled detection of different
1456 IM drift times for unconjugated mAb and the DAR4 site-specific ADC [259]. Again, average
1457 DAR values along with drug load distributions could be estimated. Altogether, these studies
1458 demonstrate the capabilities of native IMS to directly access site-specific ADC conformational
1459 information through the measurement of IM drift times and CCS calculations.

1460

1461 *Qualitative IMS for comparability studies* - IMS is a forefront methodology for qualitative batch
1462 to batch comparison. In case of comparability studies between originators and biosimilars
1463 (copy versions of mAb), IMS can address global conformational identity. IMS has been
1464 employed to highlight conformational heterogeneity in a biosimilar version of trastuzumab.
1465 Different IM drift times were observed for trastuzumab and its biosimilar version, which could
1466 be attributed to the presence of additional species bearing 0 or 1 glycan per Fc on the biosimilar
1467 version [260]. Similarly, a combination of MS-based approaches, including native MS and IMS
1468 were used to compare the originator compound Remicade (infliximab, Janssen) to its biosimilar
1469 product Remsima (Celltrion) [261]. Native IMS revealed similar IM drift times for mAb
1470 fragments, monomers, and dimers in both sample. Extensive comparative studies of
1471 monoclonal antibody drug products representing different IgG subclasses, manufacturers, and
1472 lots were also reported by Fergusson *et al.* [262] using a set of IMS-based metrics to assess
1473 reproducibility and robustness issues of IMS. IMS enabled detection of differences between
1474 monoclonal antibodies with the same target protein but different production techniques, as well
1475 as products with different targets. All of these studies conclude that native IMS analysis
1476 presents the advantage to require only a minimal (when manual buffer exchange is used) or
1477 no (when size exclusion is coupled to IMS using volatile salts) sample preparation, along with
1478 a global conformational characterization of the studied mAb-related compounds.

1479

1480 *CIU experiments and variable-temperature IMS to study mAb unfolding patterns* - IMS
1481 methodologies based on CIU experiments have been reported to address rapid conformational
1482 characterization of mAbs [263]. CIU experiments provide specific unfolding fingerprints that
1483 can be used to distinguish similar conformations, for instance mAb isotypes (IgG1 vs IgG2 vs
1484 IgG3 vs IgG4) with different numbers and/or patterns of disulfide bonding and levels of
1485 glycosylation. CIU experiments have also been reported for site-specific ADC characterization
1486 [259]. Even if different IM drift times were obtained for the ADC and its unconjugated mAb
1487 counterpart, they both exhibited similar intact CCS, which did not allow distinction between a
1488 mass effect or a real conformational change induced upon drug conjugation. CIU experiments
1489 were thus performed on both ADC and its unconjugated mAb version. Different CIU unfolding
1490 patterns were obtained for the unconjugated mAb compared to the site-specific DAR4 ADC,
1491 revealing increased ADC stability towards unfolding as compared with the unconjugated mAb.
1492 Here, CIU experiments allowed circumventing the lack of IM resolution through the
1493 establishment of unfolding patterns. CIU experiments were also used for comparability studies
1494 between biosimilar and originator [261]. Pisupati *et al.* compared the CIU patterns of the
1495 originator Remicade and its biosimilar version Remsina. Both compounds revealed remarkably
1496 similar patterns. While CIU patterns were globally similar (two unfolded states at nearly
1497 identical voltages for each CIU transition), a marked difference in the variance associated with
1498 the CIU fingerprint for Remsima at low collision voltages was observed. CIU experiments can
1499 thus be used for batch-to-batch comparison or more generally for comparability studies. Due
1500 to its higher reproducibility compared to traditional collision cross section IMS studies, CIU
1501 experiments remain a promising technique for advanced analytical characterization of protein
1502 therapeutics [262].

1503 Variable Temperature IMS (VT-IM-MS) were also reported to address mAb conformation and
1504 conformational transitions induced upon IM cell temperature variations [264]. In this work, the
1505 thermal stability of three types of intact IgG molecules (with the same antigen binding
1506 specificity) has been probed by variable-temperature IM-MS. In these experiments, mass
1507 spectra and IM plots were acquired, while the temperature of the helium gas in the drift tube

1508 was increased (from 300 to 550 K). While the native mass spectra showed no significant
1509 change as the temperature was increased (other than improved desolvation), the $^{DT}CCS_{He}$ of
1510 the IgG1 and IgG4 isotypes decreased because of compaction. The maximum collapse
1511 occurred at a higher temperature for IgG1 (400 K) than for IgG4 (360 K), in agreement with
1512 solution-phase studies in which IgG1 was found to be more thermally stable than IgG4.
1513 Differences in the extent of unfolding (an increase and widening of $^{DT}CCS_{He}$) among the IgG
1514 subclasses were also observed at high buffer gas temperatures, IgG4 unfolding at a lower
1515 temperature and to a greater extent than IgG1. VT-IM-MS provides insights into mAbs
1516 structural thermodynamics and can be considered as a promising tool for thermal-stability
1517 studies for proteins of therapeutic interest.

1518
1519 In conclusion, IMS is an interesting methodology for the characterization of intact antibodies
1520 and related formats (bispecific, ADCs) and may be useful to routinely fingerprint higher order
1521 structures.

1522 Reproducibility of IMS measurements and mobilograms, as well as the suitability of IMS to
1523 differentiate between and/or characterize different monoclonal antibody drug products has
1524 been evaluated by the FDA [262], evidencing that the use of summed charge state datasets
1525 and the analysis of metrics beyond drift time should be preferred for a more comprehensive
1526 comparative study between different monoclonal antibody drug products. Also the added value
1527 of CIU experiments, that have proved to be more reproducible than classical IMS
1528 measurements (IM drift times and CCS), offers new possibilities for mAb-related compound
1529 conformational characterization. IMS measurements can finally be extended to investigate
1530 conformational changes induced upon antigen binding to individual mAb isoforms in order to
1531 gain more structural insights.

1532
1533 7.7. Oligonucleotides
1534 IM-MS applied to oligonucleotides has been mainly used to gain conformational insights of
1535 such highly polymorphic macromolecule. Indeed, besides the classical and widely known

1536 double-stranded DNA sequence (duplex), a plethora of other helical structures can be found,
1537 such as hairpin, pseudoknot, triplex, cruciform (Holliday junction) and G-quadruplex structures.
1538 Most of these DNA conformations have been studied by IM-MS and molecular dynamics,
1539 highlighting a more dynamic and complex behaviour in the gas-phase as compared to proteins
1540 [265].

1541 One of the first research article was published by Clemmer *et al.* [266]. They reported a
1542 conformational study of an oligonucleotide comprising ten thymine nucleotides (T_{10}). The
1543 authors highlighted that the gas-phase conformations observed by IM-MS were strongly
1544 dependent of the charge states: lower charge states corresponded to globular conformers
1545 (lower CCS) and higher charge states to elongated forms (higher CCS). Moreover, by
1546 comparing the experimental CCS (Ω_{EXP}) with calculated CCS (Ω_{THE}) of conformers generated
1547 by modelling technique (by using one of the first parametrisation of EHSS model), [48] a strong
1548 dependency was found between the gas-phase conformations and the number and especially
1549 the locations of charged sites. It should be noted that charge states observed by IM-MS reflect
1550 the extension of the neutralization of the ionizable groups. Whereas in proteins, the ionizable
1551 groups are represented by the N- and C-terminal residues plus the basic and acidic side
1552 chains, in nucleic acids they are represented by the phosphodiester linkages that are all
1553 potentially and equally ionizable. In the case of T_{10} , neutralization could occur along the nine
1554 phosphate groups with a number of combination equal to $9!/n!(9-n)!$, thus several protomers
1555 could arise for each unique charge state. Authors calculated theoretical CCS (Ω_{THE}) for several
1556 different charge site assignments and they found that conformers having the same charge
1557 state (-5) showed a compact conformation ($\Omega_{\text{THE}} = 501 \pm 19 \text{ \AA}$) when having close located
1558 charges, and a most open conformation ($\Omega_{\text{THE}} = 608 \pm 21 \text{ \AA}$) when charges were more evenly
1559 distributed throughout the oligomer. Open conformation was found to match better with the
1560 experimental values ($\Omega_{\text{EXP}} = 627 \pm 6 \text{ \AA}$) and thus it was accordingly assigned [266]. Although
1561 the authors themselves questioned the absolute accuracy of the calculations, the concepts
1562 highlighted on number and locations of charged sites affecting the conformers shape were

1563 fundamental to understand how different from solution nucleic acids might behave in the gas-
1564 phase.

1565 Duplex stability and its behaviour in gas-phase has also been investigated by IM-MS and
1566 molecular modelling; initial contributions in this direction came from Bowers group [267–269].
1567 First studies involving d(CG)_n-d(CG)_n duplexes [267] demonstrated that a globular form is the
1568 dominant conformer for up to 8-mer length oligomer, whereas for longer duplexes only the
1569 helical structure was observed, keeping quite good memory of the solution structure. Then, the
1570 effect of the oligomer sequence composition on the overall conformation of the duplex was
1571 investigated by studying a series of 14-mer duplexes containing both C·G and A·T pairs in
1572 different sequences [268,269]. It turns out that the A·T pairs were preferentially broken over
1573 C·G pairs and they appear more easily disrupted when placed at the ends of the duplex, while
1574 forming bubble if placed at the middle of the sequence. Therefore, overall results showed that
1575 duplex helicity was usually maintained in the gas-phase on the ms timescale but the structures
1576 stability was dependent of the length of the oligomer, its bases composition and its sequence
1577 order. However, as highlighted in [270], the structural comparison between the duplex native
1578 forms in solution and the conformations assumed in the gas-phase remained questionable,
1579 since the experiments were performed in non-native solution conditions. Indeed, a more recent
1580 contribution on duplex nucleic acids behaviour in the gas-phase upon native electrospray mass
1581 spectrometry came from the Orozco and Gabelica groups [270]. A compelling study on short
1582 (12-bp) and long (36-bp) DNA and RNA duplexes was performed by ESI-DTIM-MS, and a
1583 significant compaction of the duplex nucleic acids was observed for ions sprayed from native
1584 solution conditions. Ω_{THE} of the structures adopted in solution were obtained upon MD
1585 simulation carried out in water and then compared to Ω_{EXP} ($^{\text{DT}}\text{CCS}_{\text{He}}$) that were found
1586 significantly lower than the predicted values. To investigate the behaviour of the duplexes in
1587 the gas-phase, an extensive molecular modelling study was performed, including DFT and
1588 semi-empirical calculations for small helices and short duplexes plus MD simulations for long
1589 duplexes. Finally, authors were able to demonstrate that the compaction experimentally
1590 observed by ESI-IM-MS was related to the self-solvation of the phosphates groups occurring

1591 through new phosphate-phosphate interactions that undermine the Coulomb-driven expansion
1592 of the duplexes. These metastable conformations arose in part during the desolvation process
1593 and rearranged by self-solvation while in the gas-phase, thus resulting significantly compacted
1594 compared to the starting structures [270]. Similarly, Devine *et al.* described the gas-phase
1595 compaction of two 35-mer RNAs having similar mass, but different sequences and secondary
1596 structures [192]. Reference Ω_{THE} values of the two 35-mer RNAs were obtained from an NMR
1597 and a crystal structure, respectively. Nevertheless, ESI-TWIMS-MS of the RNAs yielded to
1598 identical Ω_{EXP} ($^{\text{TW}}\text{CCS}_{\text{N}_2}$) that were significantly lower than the predicted values, hence
1599 accounting for a severe compaction of the RNAs in the gas-phase. However, beyond
1600 mentioning the collapse of the RNAs, authors did not investigate further on the RNA behaviour
1601 in the gas-phase by molecular modelling studies.

1602 A quite different gas-phase behaviour was observed for higher-ordered oligonucleotide
1603 structures, namely triplex and G-quadruplex structures. In [271] Ω_{THE} obtained upon an
1604 extensive (90 μs) MD simulation were compared with Ω_{EXP} ($^{\text{DT}}\text{CCS}_{\text{He}}$) obtained by ESI-DTIM-
1605 MS experiments, to validate the theoretical data and assess the stability and the structure of a
1606 triplex in the gas-phase. A very good agreement was found between theoretical and
1607 experimentally derived CCS. A 12-mer and 18-mer triplexes were used for the study and the
1608 overall results suggested that the gas-phase structures maintained an excellent memory of the
1609 solution structures, together with a well-preserved helicity, even though a significant number
1610 of structural alterations were observed. Indeed, a slight compaction of the triplex (in respect to
1611 the solution structure) was observed for the most abundant charge states, but no triplex
1612 disruption was observed in any case.

1613 Even a higher DNA structure stability in the gas-phase was observed for the G-quadruplex
1614 structures that were monitored by both ESI-IM-MS and molecular modelling [272–275]. G-
1615 quadruplexes are characterised by the formation of G-tetrads (four planar guanines held
1616 together by Hoogsteen hydrogen bonds) that stack on top of each other to form rigid and well
1617 organised structures, thanks also to the presence of cations placed in-between the G-tetrads
1618 that further stabilise the structure. These particular DNA conformations can be formed, starting

1619 from G-rich oligonucleotides, they can be intra-, bi-, or tetra-molecular, and they are related to
1620 cellular ageing and transformations. Evidences of G-quadruplex structure preservation in the
1621 gas-phase were reported by several groups [276,277] and mainly related to the preservation
1622 of the inner cations in-between the G-tetrads [278]. Moreover, MD simulation (0.5 – 1 μ s)
1623 studies validated this preservation and proved the ability of the G-quadruplexes to keep an
1624 excellent memory of their solution structure [279], with a general excellent agreement between
1625 theoretical and experimentally derived CCS [39,268,272]. Then, the stability of G-
1626 quadruplexes in the gas-phase was furthermore validated after testing a series of potential
1627 ligands that were found to form stable complexes maintained also upon transfer in the gas-
1628 phase [280].

1629

1630 **8. The future of ion mobility spectrometry**

1631 8.1. Some potential solutions to improve ion mobility resolution

1632 One of the major constraints of IMS is the limited resolution of commercial instruments. This
1633 low separation power obviously limits the applicability of IMS, as it remains difficult to
1634 differentiate structurally related compounds (species having mobilities differences of less than
1635 1-2% are hardly distinguishable today [215]). Besides resolving isobaric compounds, a higher
1636 resolving power could also contribute to increasing the dynamic range of the measurements
1637 [8].

1638 To circumvent this limitation, there are many IMS prototypes that have been proposed by
1639 academic laboratories and providers of MS instrumentation, which are well described in the
1640 recent review of McLean and May [9]. However, it is important to keep in mind that achieving
1641 higher resolution comes at the expense of mobility range and/or sensitivity reduction. In this
1642 section, only the technical solutions that have the potential to be transformed into commercial
1643 products, at least from our point of view, will be described. Indeed, we have to keep in mind
1644 that the speed of translating IM-MS prototypes into commercial instruments in the last 5 years
1645 has increased dramatically.

1646 As described in the first sections of this review, the DTIMS resolution is directly proportional to
1647 the square root of drift tube length and applied electric field. Therefore, there have been a lot
1648 of attempts to increase these parameters in DTIMS. However, when increasing drift tube
1649 length, the transmission may be drastically reduced (ion losses), or only a very narrow mobility
1650 range could be investigated, thus limiting the molecular coverage. In addition, a drift tube length
1651 of several meters, as described by Kemper [281] may be impractical for common laboratory
1652 space and requires very high voltages. To avoid the use of high voltages, the TWIMS
1653 technology was found to be more adapted in combination with long IMS cell. Recently, the
1654 group of Smith *et al.* have constructed a 13 m long serpentine TWIMS drift path, including 44
1655 "U" turns to limit the required laboratory space [73]. In addition, the 13 m tube was included in
1656 a structure for lossless ion transmission (SLIM), to assist in ion transmission and manipulation,
1657 thus enabling a very limited loss in sensitivity. They demonstrated a baseline resolution for two
1658 isomeric sugars and also employed this new device for resolving peptides of an albumin tryptic
1659 digest. According to the authors, the resolution was increased by a factor of 9, in comparison
1660 with commercial DTIMS instrument having a tube length of 80 cm.

1661 Another promising strategy to improve the current resolving power of IMS may be the use of a
1662 cyclic TWIMS design to limit laboratory space. Using this approach, very high resolution can
1663 be achieved after an important number of cycles (up to 12) on a circular TWIMS drift tube of
1664 around 1 meter [9]. One of the major limitations of the circular drift tube geometry may be
1665 related to the fact that the ions of high mobility of the next cycle can lap over the ions of low
1666 mobility from the previous cycle. Since the TWIMS operates in a time dispersive manner, this
1667 point is less critical, but one of the main limitations of this design remains that the measurable
1668 mobility range decreases with every cycle needed to improve the resolution.

1669 Another strategy to improve IMS resolution is to modify the gas nature since the latter could
1670 strongly impact the separation power in FAIMS. In particular, Shvartsburg and co-workers have
1671 improved FAIMS measurements by using non-traditional buffer gases (mixtures of He/ N₂ or
1672 H₂/N₂). They showed that when helium rich-gases and elevated electric fields were used, the
1673 resolution was significantly increased, allowing the separation of previously unresolved

1674 conformers of a proteolytic digest [282,283]. To avoid the electrical breakdown which is
1675 inevitable when a high proportion of He is used, mixtures of N₂ and up to 90% H₂ were also
1676 successfully employed [284]. Some other authors have also demonstrated that drift gas with
1677 high polarizability (carbon dioxide or argon) allow achieving better resolution and peak capacity
1678 in DTIMS and TWIMS, due to the extended interactions that take place [81,285]. As example,
1679 Eberlin *et al.* show a separation of disaccharide isomers in TWIMS with carbon dioxide, while
1680 it was not possible with nitrogen [286].

1681

1682 8.2. Improving orthogonality between ion mobility and mass spectrometry

1683 Besides its limited resolving power, another important constraint of IMS is the lack of
1684 orthogonality with MS, since the shape of molecules in a gas phase (IMS separation criteria)
1685 is strongly correlated to its mass (MS separation criteria). In this sense, FAIMS offers a better
1686 orthogonality to MS, due to a different operation principle, in comparison with DTIMS, TWIMS
1687 and TIMS. Some authors have clearly demonstrated that the gas nature in IMS could also
1688 impact selectivity, in addition to the improvement of resolving power discussed in section 5.1.
1689 The most remarkable example to date has been reported by Hill *et al.* who demonstrated the
1690 separation of enantiomers in DTIMS, by doping the drift gas with a chiral selector (2-butanol)
1691 [287]. Some other research groups have also tested different drift gases (i.e. nitrogen, helium,
1692 argon, methane) in DTIMS for separating peptides from a tryptic digest, but they showed that
1693 selectivity was less affected by the gas nature, when the ions belong to the same biomolecular
1694 class [288].

1695 In FAIMS, the selectivity could be drastically modified when adding different polar modifiers
1696 (alcohols, acetonitrile, acetone...) to the drift gas (nitrogen) [289,290]. This was well illustrated
1697 by Varesio *et al.* who considered a mixture of eight pharmaceutical compounds having all a
1698 mass of 316 Da, as a case study. As illustrated in Figure 13, with pure nitrogen as transport
1699 gas, the resolving power for the eight compounds with the same nominal mass was close to
1700 zero and the eight analytes experienced a relatively similar differential mobility in the 10 V
1701 compensation voltage window [291]. When adding only 1.5% organic modifier, the separation

1702 was drastically improved, particularly with isopropanol. The separation enhancement was
1703 probably due to the formation of transient clusters between the analytes of interest and the
1704 polar modifier molecules. In this example, the overall IMS separation was quite good, despite
1705 the fact that the drugs possess the same masses, because their chemical properties were
1706 different as well as their interactions with the modifiers. In another study [292], 30 drugs of
1707 abuse were investigated in FAIMS using various modifiers (i.e. acetone, acetonitrile and
1708 methanol) in the drift gas. The best IMS separation was achieved with 1.5% methanol, but this
1709 example also demonstrates the limited resolving power of FAIMS (the maximum peak capacity
1710 was less than 20). Despite the poor peak capacity, some isomeric metabolites such as
1711 hydroxylated or desmethylated ones were partially resolved, and glucuronide metabolites were
1712 differentiated from the parent drug, preventing the overestimation of the latter in case of in-
1713 source fragmentation. Finally, Chen *et al.* have also used FAIMS for the detection and
1714 quantification of nine structurally related amphetamine-type stimulants in urine in presence of
1715 dopants (i.e. MeOH, propanol, butanol, hexanol, heptanol) and various transport gas (i.e.
1716 nitrogen, helium, argon, carbon dioxide) [293]. The authors concluded that the combination of
1717 He and MeOH allows achieving the best sensitivity and selectivity for the investigated mixture
1718 of compounds.

1719 All the above mentioned studies illustrate the potential of using alternative drift gas natures or
1720 adding modifiers to the drift gas in IMS and DMS to tune selectivity. It is also clear that there
1721 are further combinations of gas and additives that could be explored to improve orthogonality
1722 between IMS and MS and some additional studies in this particular field will be helpful to make
1723 progress. However, it is also important to keep in mind that some of the current instruments
1724 on the market are not compatible with a variety of gases and modifiers and can only be used
1725 with pure nitrogen as drift gas (i.e. Waters VION, Bruker TIMS...).

1726

1727 8.3. Combining two-dimensional LC and ion mobility - mass spectrometry (4
1728 dimensional analytical approach)

1729 Because the samples to be investigated in the -omic field (i.e. lipidomics, proteomics or
1730 metabolomics), in natural products analysis or in biopharmaceuticals characterization are
1731 extremely complex, there is a need to develop analytical strategies able to separate the highest
1732 number of compounds. On the chromatographic side, comprehensive 2D-LC strategy has
1733 emerged over the last few years, as a promising (but complex) alternative to very high-
1734 resolution 1D separation [294–299]. Beyond separation techniques, IMS and HRMS become
1735 ever popular and powerful, particularly for untargeted analysis of complex samples.

1736 To simultaneously take advantage of these chromatographic and mass spectrometric
1737 advances, Stephan *et al.* [105,151] have recently combined in series 2D-LC and IMS-HRMS,
1738 enabling the characterization of the most complex samples in four dimensions. According to
1739 the authors, there are many constraints when implementing such a complex analytical setup.

1740 In particular, there is a need to improve the software for managing the LC+LC-IMS/MS setup
1741 and above all the data treatment. Nevertheless, they have successfully applied the strategy to
1742 characterize plant extract (*Ginkgo Biloba*) and achieved impressive performance, with a peak
1743 capacity of 8,700 for an analysis time of only two hours [105]. In addition, for each peak
1744 observed on the 2D-LC chromatogram, the CCS and m/z values were also available, as
1745 illustrated in Figure 14. Stephan *et al.* have also applied the same strategy for the analysis of
1746 wastewater [151]. They performed the analysis of wastewater sample in “regular” LC-IMS/MS
1747 and discovered 22 different pollutants using a database containing about 500 drug-like
1748 compounds and pesticides. When using the newly developed LC+LC-IMS/MS strategy, 53
1749 different compounds were identified using the exact mass and CCS values in the database.

1750 In the future, it is expected that this type of approach will be investigated for the
1751 characterization of -omic samples, as the number of detected features could certainly be
1752 strongly enhanced when using these multiple separation dimensions. Another application
1753 could be the analytical characterization of biopharmaceuticals, including monoclonal
1754 antibodies, antibody-drug conjugates, and bispecific antibodies. The addition of IMS to the
1755 more commonly used 2D-LC-MS setup would be of particular interest because it could help to
1756 differentiate isobaric compounds that are not chromatographically separated [298]. Recently,

1757 an innovative multidimensional analytical approach combining comprehensive on-line two
1758 dimensional chromatography that consists of hydrophobic interaction chromatography (HIC)
1759 and size exclusion chromatography (SEC) to ion mobility and mass spectrometry (IM-MS) was
1760 applied for performing analytical characterization of ADCs under non-denaturing conditions.
1761 This promising approach allows obtaining in-depth and detailed information on ADC samples
1762 that cannot be reached from HIC-UV or non-denaturing mass spectrometry alone. The all-in-
1763 one analytical strategy described here permits a deep, straightforward and rapid
1764 characterization of complex ADC samples [300].

1765

1766 8.4. Improvement of data interpretation workflows and software

1767 As underlined in the previous sections, there are currently a number of new IM-MS techniques
1768 and technologies under development with the aim of further improving the performance in
1769 terms of resolution, sensitivity and orthogonality between IMS and MS. However, one of the
1770 remaining limitations of IM-MS instrument is often represented by the software, which requires
1771 intensive development for improving speed and user-friendliness, especially when technical
1772 possibilities continue to grow.

1773 Indeed, when performing LC-IM-MS experiments, the data are extremely complex as they are
1774 acquired on many different dimensions (i.e. retention times in LC; drift times in IMS; exact
1775 mass, MS/MS spectra and signal intensity in MS). Not all softwares are well developed for 4-
1776 dimensional (retention time, drift time, m/z, abundance) alignment of molecular features
1777 (entities) across different samples. This means that doing high-quality statistical interpretation
1778 during non-targeted (discovery) analysis can be extremely challenging or even impossible
1779 without innovation using open-source tools.

1780 In addition, files processing of large data sets may be critical. In particular, peak picking
1781 (feature finding) can be very slow and algorithms optimization is still a topic of prime interest.
1782 In this regard, Vivo-Truyols and co-workers reported the Bayesian approach for features
1783 finding in GC-HRMS [301] or GCxGC-FID [302], that could be envisaged for application in LC-
1784 IM-MS data treatment in the future in order to improve the peak picking process throughput.

1785 For processing MS/MS data alongside IMS information (e.g. LC-IMS-QqTOF/MS), some
1786 solutions are proposed by the IM-MS instruments providers. For example, Waters proposes
1787 the MS^E approach, which records data without discrimination or preselection, while Agilent
1788 uses an approach called "alternating frames", where two MS analyses (high energy and low
1789 energy) can be obtained within a single acquisition in order to associate MS/MS and MS
1790 spectra via drift time alignment. Such approaches can be already implemented into analytical
1791 methods, but software-based extraction of all the information together is awaiting for further
1792 developments and it will also require careful validation for non-targeted workflows.
1793 Another important area of software development regards the data deconvolution strategies,
1794 which allows the extraction of additional information from partially resolved ion mobility data,
1795 taking into account the orthogonal information obtained from ion fragmentation taking place
1796 after the ion mobility cell. This promising approach can allow the reconstruction of ion mobility
1797 spectra for individual species, especially for the challenging topic of isomer differentiation, as
1798 reported in [66].

1799

1800 **9. Conclusion**

1801 This review paper summarizes the current status of IM-MS and main application fields where
1802 the technology has been employed to date. As discussed, the main benefits of IM-MS are the
1803 following: i) Improving the confidence in peak annotation thanks to the use of CCS values in
1804 addition to retention time, exact mass and fragment spectra, ii) Reducing the complexity of
1805 MS/MS spectra, and rapid association of fragments with their monoisotopic precursors by
1806 taking into account the mobility alignment, iii) Increasing the overall resolution of the analysis,
1807 since MS and HRMS are obviously unable to separate isobaric compounds, iv) In-depth
1808 studies of the gas phase conformation of large biomolecules such as proteins and
1809 oligonucleotides and comparison of calculated and experimental CCS values.

1810 Among the applications reported in this contribution, adding an IMS dimension looks
1811 particularly attractive in metabolomics for improving peak annotation confidence. It can also
1812 offer a higher resolving power for a few families of compounds having relatively high flexibility

1813 and containing a substantial number of isomers. Among them, lipids and glycans are
1814 particularly well adapted to IMS and have been studied extensively during the last few years.
1815 Finally, due to the inherent complexity of protein biopharmaceuticals and oligonucleotides,
1816 there will certainly be even more interest for IMS in the coming years in order to increase the
1817 amount and quality of information that can be obtained from their analysis.

1818 As illustrated throughout this paper, the field of IMS is very active and instrumental advances
1819 are being regularly published. In this regard, one of the remaining bottlenecks of IMS is its
1820 limited resolution. Therefore, there is a need for longer IMS drift cells, or alternative IMS
1821 designs, as an order of magnitude improvement in resolving power would clearly revolutionize
1822 the field [16]. Another important limitation of the technology is the poor orthogonality between
1823 IMS and MS. To improve this aspect, the addition of dopants (small amount of organic modifier)
1824 to the IMS drift gas appears as a promising strategy, but this solution is unfortunately not
1825 compatible with all IM-MS instruments. Finally, even though the sensitivity achieved in IM-MS
1826 has been drastically improved over the last few years, there could still be some sensitivity loss
1827 when adding an IMS dimension prior to MS. To avoid, or at least limit this issue, Smith *et al.*
1828 have recently developed some IMS structures with lossless ion manipulations (SLIM), which
1829 seems certain to be implemented in the next IMS generation [303].

1830

1831

1832

1833 **The authors have declared no conflict of interest.**

1834

1835 **10. References**

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- 2765

2766 **11. Figure captions**

2767

2768 Figure 1: Number of papers published since 1995 in the field of IMS, IM-MS and LC-IM-MS. In
2769 this figure, the 2 years gap, mean all papers published within 2 years. Source: Scifinder scholar
2770 2017. Date of information gathering: June 2017.

2771

2772 Figure 2: Schematic representation of a three-dimensional analysis, including a liquid
2773 chromatography separation, followed by ion mobility discrimination and time of flight mass
2774 spectrometry detection. A 3D representation of the data was also included in this
2775 representation.

2776

2777 Figure 3: Schematic representation of the commercially available IMS technology, classified
2778 as time-dispersive methods (DTIMS, TWIMS), space-dispersive methods (FAIMS, DMA) and
2779 ion trapping methods (TIMS). This figure was adapted from [2,5,18,24], with permissions from
2780 the Publishers.

2781

2782 Figure 4: Schematic representation of the three atomistic models employed for estimating gas-
2783 phase conformations, allowing calculation of CCS values. A: projection approximation (PA), a
2784 non-explicit method working by projecting the “shadow” of the ion onto a plane to find the
2785 average area of all the possible orientations of the molecule under investigation. B: exact hard
2786 sphere scattering (EHSS), a hard sphere collisions based method that computes the multiple
2787 scattering events without taking into account the long-range interactions. C: trajectory method
2788 (TM), an explicit model which accounts for both long-range interactions and multiple scattering
2789 events. The arrows represents the buffer gas atom trajectories.

2790

2791 Figure 5: (a) Calculated absolute CCS differences required to satisfy various peak-to-peak
2792 resolution conditions based on experimental CCS data for positively-charge ions based on
2793 conditional resolution model for current Agilent DTIMS instrument. Symbol key: metabolites

2794 (filled squares), peptides (open stars), and lipids (open circles). A peak-to-peak R_s value of
2795 0.6 reflects conditions allowing both CCS values to be reliably determined for co-eluting
2796 isobaric species, while 1.5 represents baseline resolution. (b) Corresponding calculated
2797 percentage CCS differences for the same data. Reprinted from J. Chromatogr. A, Vol. 1416,
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2799 chromatography combined with drift tube ion mobility-mass spectrometry., pp 47-56 (ref [82]).
2800 Copyright 2015, with permission from Elsevier.

2801
2802 Figure 6: Intensity vs. drift time for the protomers of ciprofloxacin, with the respective sites of
2803 protonation on the cyclic ketone and the piperazine moiety in the positive ionization mode.
2804 CCS values of the two protomers were also reported in this figure. This figure was adapted
2805 from [86], with permission from Waters corporation. However, a modification was applied by
2806 the authors of this application note on the original figure (Figure 5) found in [86], since one of
2807 the hypothesized protonation site was wrongly assigned in their original work.

2808
2809 Figure 7: CCSs of drug glucuronide metabolite structural isomers with different adducts and
2810 polarity. (A) Structures of drug glucuronide metabolite structural isomers. Overlays of
2811 representative arrival time distributions at 50 V drift voltage for (B) naringenin-4'-glucuronide
2812 (4') and naringenin-7-glucuronide (7), MW (Da) = 448.5 and for (C) β -3-estradiol-glucuronide
2813 (3) and β -17-estradiol-glucuronide (17), MW (Da) = 448.5. ATD full width half maxima and
2814 DTCCSHe averages are inset. Errors quoted are the standard deviations from at least two
2815 interday repeated measurements. Reproduced from Reading, E., Munoz-Muriedas, J.,
2816 Roberts, A. D., Dear, G. J., Robinson, C. V., Beaumont, C., Anal. Chem. 2016, 88, 2273–2280
2817 (ref [112]). Copyright 2016 American Chemical Society.

2818
2819 Figure 8: ESI(+) results from a mixture of D3-labeled hydrolyzed HEK cell lipid extracts spiked
2820 with unlabeled synthetic standards, SPH m18:1(4E)(3OH) and SPH m18:1(14E)(3OH). (A)
2821 Ionograms showing separation in voltage space of the $[M+H]^+$ ions formed from the two lipid

2822 isomers. Each of the synthetic lipids spiked into the extract produced a peak in the black trace
2823 (m/z 284) and similar features were observed for the labeled HEK293 extract shown in the red
2824 trace (m/z 287). (B) Ozonolysis (OzID) spectra obtained for synthetic standard (black
2825 spectrum) and the D3-labeled extract (red spectrum) corresponding to the first feature in the
2826 ionogram (i.e., CV = 17 V ozone reaction time of 100 ms). (C) OzID spectra obtained for
2827 synthetic standard (black spectrum) and the D3-labeled extract (red spectrum) corresponding
2828 to the second feature in the ionogram (i.e., CV = 20 V ozone reaction time of 15 s). OzID
2829 product ions characteristic of the locations of the carbon-carbon double bonds are indicated
2830 by closed circles and enable assignment of the position of unsaturation to (Δ 14)-position (B)
2831 and (Δ 4)-position (C). Adapted from [147], with permission from the American Society for
2832 Biochemistry and Molecular Biology.

2833

2834 Figure 9: HDMS^E analysis of 400 ng Hela digest. IMS additional dimension provides an
2835 increasing number of identified peptides and proteins (38440 and 3490 respectively) with a
2836 FDR of 1% at a protein level using PLGS algorithm.

2837

2838 Figure 10: Workflow utilized for IMS-MS analysis of membrane protein-lipid interaction by
2839 Laganowsky *et al.* Proteins are released from micelles by rising the source temperature and
2840 subsequently activated in the trap cell. The in-source fragmentation allows performing of MS-
2841 IM-MS analysis on specific ions.

2842

2843 Figure 11: A general picture of the interaction of the full clamp loader and SSB4 before and
2844 after the addition of ssDNA. Mass spectra and the corresponding countour plots are depicted
2845 for a) $y_{3\delta\delta'}\psi\chi$ -SSB4 and b) $y_{3\delta\delta'}\psi\chi$ -SSB4-SSDNA₂. The three dimension structure proposed
2846 for c) $y_{3\delta\delta'}\psi\chi$ -SSB4 and e) $y_{3\delta\delta'}\psi\chi$ -SSB4-SSDNA₂ complexes from molecular modeling.
2847 Measured drift time (d) for the 36+ charge state of $y_{3\delta\delta'}\psi\chi$ -SSB4(blue) and $y_{3\delta\delta'}\psi\chi$ -SSB4-
2848 SSDNA₂ (red). A significant increase in measured collision cross section of the clamp loader-
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2851 Mass Spectrometry Reveals Structural Features of the Clamp Loader in Complex with Single-
2852 Stranded DNA Binding Protein, pp 4790-4801 (ref [235]). Copyright 2013, with permission from
2853 Elsevier.

2854
2855 Figure 12: Insights about biopharmaceutical mAbs and ADC characterization based on IM-MS
2856 data. a) Analysis of global conformation differences induced by disulfide bridge heterogeneity
2857 (Reproduced from Bagal, D., Valliere-Douglass, J. F., Balland, A., Schnier, P. D., Anal. Chem.
2858 2010, 82, 6751–6755. (ref [253]). Copyright 2010 American Chemical Society.) b)
2859 Benchmarking of different conjugation strategies by IM-MS, c) CIU fingerprint to evaluate gas-
2860 phase unfolding process, d) analysis of protein aggregation (oligomerization), e)
2861 characterization of the global conformation of gas-phase ions.

2862
2863 Figure 13: Separation of a mixture of 8 pharmaceutical compounds having a nominal mass of
2864 316 by DMS coupled to a QqQLIT MS. DMS separation: (a) without polar modifier (nitrogen),
2865 (b) with 1.5% of MeOH as modifier, (c) with 1.5% of EtOH as modifier, and (d) with 1.5% of i-
2866 PrOH as modifier. With kind permission from Springer science + business media: Anal.
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2868 spectrometry, Vol 402, 2012, 2555-2564, Varesio, E., Le Blanc, J. C. Y., Hopfgartner, G.
2869 Figure 3 (ref [291]).

2870
2871 Figure 14: Heat map of an LC + LC-IM-qTOF measurement of a *Ginkgo biloba* extract (a). The
2872 separation on the second dimension column of one fraction collected between 4 and 8 min
2873 (eluting from the second dimension between 8 and 12 min) from the sample is zoomed in (b).
2874 2D plot of m/z vs. drift time extracted from 9.63 to 9.76 min (c). The zoomed in image shows
2875 the separation of two peaks with the same m/z in the IM dimension (d). With kind permission
2876 from Springer science + business media: Anal. Bioanal. Chem., A novel four-dimensional

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2878 Jakob, C., Hippler, J., Schmitz, O. J. Figure 4 (ref [105]).

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2880 **12. Short biography of authors**

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2882 **Dr Valentina D'Atri** obtained her PhD in industrial and molecular biotechnologies at the
2883 University of Naples Federico II, Italy. She is now Postdoc at the University of Geneva,
2884 Switzerland. Her expertise includes mass spectrometry, ion mobility-mass spectrometry,
2885 molecular modelling and separation science, including HPLC, UHPLC, and HILIC. Her current
2886 research activity is in the field of biopharmaceutical proteins; in particular, her interest focuses
2887 on the analytical characterization of mAbs, ADCs, and fusion proteins by LC-MS and LC-IM-
2888 MS.

2889

2890 **Dr Tim Causon** obtained his PhD at the University of Tasmania, Australia. He is now Senior
2891 Scientist in the group of Instrumental Analytical Chemistry and Metabolomics at the
2892 Department of Chemistry of the University of Natural Resources and Life Sciences in Austria.
2893 His research interests centre on mass spectrometry, ion mobility-mass spectrometry and
2894 separation science, including analytical method development for metabolomics, as well as
2895 fundamental analytical studies.

2896

2897 **Dr. Oscar Hernandez Alba** has joined the BioOrganic Mass Spectrometry Laboratory
2898 (LSMBO) at the Hubert Curien Pluridisciplinary Institute (IPHC, University of Strasbourg,
2899 CNRS UMR7178) in Strasbourg as a postdoctoral researcher. He obtained his PhD degree at
2900 the University of Paris-Sud. His thesis was focused on developing a multimodal approach
2901 based on a single mass spectrometer, which incorporates ion mobility spectrometry and
2902 infrared spectroscopy in order to characterize the structure of mobility- and mass-selected
2903 molecular ions. He is currently working on the characterization of biopharmaceuticals and
2904 noncovalent protein/protein complex using native mass spectrometry and ion mobility
2905 spectrometry.

2906

2907 **Aline Mutabazi** graduated from the School of Pharmaceutical Sciences of the University of
2908 Geneva as a pharmacist in 2016. After a master thesis about the development and validation
2909 of a LC-MS method for the analysis of neonicotinoids in honey, she joined Jean-Luc Veuthey
2910 and Davy Guillarme's team in January 2017 to begin a Ph.D. thesis focused on ion mobility
2911 applied to the analysis of pharmaceutical compounds.

2912
2913 **Prof. Jean-Luc Veuthey** is professor at the School of Pharmaceutical Sciences, University of
2914 Geneva, Switzerland. He also acted as President of the School of Pharmaceutical Sciences,
2915 Vice-Dean of the Faculty of Sciences and finally Vice-Rector of the University of Geneva. His
2916 research domains are: development of separation techniques in pharmaceutical sciences, and
2917 more precisely: study of the impact of sample preparation procedures in the analytical
2918 process; fundamental studies in liquid and supercritical chromatography; separation
2919 techniques coupled with mass spectrometry; analysis of drugs and drugs of abuse in different
2920 matrices. He published more than 300 articles in peer-review journals.

2921
2922 **Dr. Sarah Cianféroni** holds a PhD degree in analytical chemistry from the University of
2923 Strasbourg, France. She is now a CNRS research director and heads the BioOrganic Mass
2924 Spectrometry Laboratory (LSMBO) at the Hubert Curien Pluridisciplinary Institute (IPHC,
2925 University of Strasbourg, CNRS UMR7178) in Strasbourg. Her expertise focused on
2926 developments in structural mass spectrometry (native MS, ion mobility, H/D exchange and
2927 crosslinking) and applications of advanced native mass spectrometry and native ion-mobility
2928 mass spectrometry methodologies for biological noncovalent complex characterization, and
2929 especially biopharmaceuticals (mAb-related compounds). She is a co-author of more than 110
2930 scientific papers related to mass spectrometry analysis of proteins.

2931
2932 **Dr Davy Guillarme** holds a PhD degree in analytical chemistry from the University of Lyon,
2933 France. He is now senior lecturer at the University of Geneva in Switzerland. He authored 190
2934 journal articles related to pharmaceutical analysis. His expertise includes HPLC, UHPLC,

2935 HILIC, LC-MS, SFC, analysis of proteins and mAbs. He is an editorial advisory board member
2936 of several journals including Journal of Chromatography A, Journal of Separation Science, LC-
2937 GC North America and others...
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