Chapter 20

Traveling Wave Ion Mobility-Mass Spectrometry to Address Chemical Food Safety Issues

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Abstract

There is growing interest in ion mobility spectrometry in the field of food analysis as a consequence of its hyphenation with mass spectrometry and increased commercialization of ion mobility-mass spectrometry instruments. In this regard, ion mobility spectrometry brings several advantages to traditional liquid chromatography-mass spectrometry workflows, including information on the collision cross section of the detected ions. This parameter provides more confidence for the identification and confirmation of compounds, which is of great value for analytical methods applied in chemical food safety as it carries legal consequences. This protocol describes the steps to follow for the implementation of traveling wave ion mobility-mass spectrometry in food safety applications, and for which the analysis of steroid metabolites has been selected as an illustrative example.

Key words Ion mobility-mass spectrometry, LC-IM-MS methods, Collision cross section, Food safety, Steroids

1 Introduction

The successful integration of ion mobility spectrometry (IMS) into traditional liquid chromatography (LC)-mass spectrometry (MS) workflows is currently attracting the attention of researchers in many fields [1]. Furthermore, interest in this separation technique has increased exponentially in recent years due to the commercialization of hyphenated ion mobility-mass spectrometry (IM-MS) instruments, which is contributing to the development of IMS-based applications in numerous fields such as food analysis [2, 3].

IMS is an electrophoretic technique in which ionized molecules are separated based on their mobility (K) in a cell filled with a neutral gas, usually N_2, He, or CO_2, and under an electric field. The mobility of the ions depends on their charge, size, and shape

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and refers to the time required for the ions (t_d) to pass through the entire length of the mobility cell (l), as indicated in Eq. 1:

$$K = \frac{v_d}{E} = \frac{l}{t_d E} \quad (1)$$

where v_d refers to the velocity of the ions in the mobility cell and E to the applied electric field. Furthermore, the mobility of the ions is also affected by temperature and pressure as well as the physicochemical properties of the buffer gas in the mobility cell [4].

IMS can be integrated as an additional separation dimension between chromatographic separation (seconds up to minutes range) and MS analysis (microseconds range) due to its fast separation speed on the milliseconds time scale. In this context, IMS brings several advantages to traditional LC-MS methods: (1) isobaric molecules and isomers, which can co-elute in the LC dimension and cannot be distinguished in the MS dimension, can potentially separate in the IMS dimension when showing different geometric conformation in gas phase; (2) the analytes of interest are separated from the chemical background improving the detection sensitivity, as well as cleaner mass spectra are obtained; (3) IMS provides additional information to retention indexes (e.g., retention time) and mass spectra, the so-called collision cross section (CCS), increasing confidence in molecular identification or confirmation [5].

Nowadays, there are different IMS technologies hyphenated to MS that are commercially available. Each of these technologies comes with its advantages and disadvantages over the others [6], so the choice of IMS technology to use depends on the analytical question raised. In the field of chemical food safety, there are currently high expectations about the potential of CCS to be implemented as an identification parameter in the analysis of chemical residues and contaminants in food [3]. The implementation of analytical methods for the determination of chemical residues and contaminants in food products may have legal consequences; therefore, the CCS parameter provides additional evidence to confirm the identity of a compound and increases confidence in the results of analyzes. In this sense, it should be noted that not all IMS technologies allow CCS measurements, with drift-tube ion mobility (DTIM)-MS and traveling wave ion mobility (TWIM)-MS being the most frequently applied technologies for this purpose [2]. The more recent commercialization of trapped ion mobility (TIM)-MS instruments has expanded the options available in the market to implement IM-MS technology in routine analysis and generalize CCS measurements [7].

The CCS represents the averaged momentum transfer impact area of the ion and depends on analyte–buffer gas interactions. It is not strictly an intrinsic molecular property of each compound because it depends on the experimental conditions (i.e., gas nature,

temperature, electric field), but it is a characteristic that provides specific information about the compounds analyzed [8]. This is the reason why the development of CCS databases has recently been encouraged to generate information for using this parameter for identification purposes. Within this framework, a wide range of CCS databases have been published for use in the analysis of pesticides [9], mycotoxins [10], veterinary drugs [11], among others [12]. High reproducibility of CCS measurements performed on different IM-MS instruments working under the same or similar experimental conditions has been observed [13, 14]. Thus, CCS databases can be relied upon for more confident confirmation of the presence or absence of chemical residues and contaminants in food products analyzed by LC-IM-MS. In this sense, it is important to be careful when reporting and using CCS databases since the acquisition conditions must be clearly indicated to be able to compare CCS values [4, 8]. In addition, it is necessary to take into account that the comparison of CCS values obtained by different IMS technologies is not possible or straightforward based on the first studies carried out in this regard [15].

The drift time (previously referred as t_d) is the parameter related to the ion mobility dimension generally measured by IM-MS instruments. However, for example, drift time (or ion mobility) can be directly related to CCS according to Mason-Schamp equation (Eq. 2) in DTIM systems operating under low electric field conditions. The reader is advised to consult specialized literature for more details on the fundamentals of IMS [16].

$$CCS = \frac{3}{16}\left(\frac{2\pi}{\mu k_B T}\right)^{1/2}\frac{ze}{NK} \tag{2}$$

where z and e represent the absolute charge of the ion and the elementary charge, respectively; μ indicates the reduced mass of the ion–neutral drift gas pair (i.e., $\mu = mM/(m + M)$; m and M are the ion and gas-particle masses, respectively); and k_B is the Boltzmann constant. K represents the mobility of the ions (as indicated in Eq. 1) while N refers to the gas number density.

In general, primary methods using mainly DTIM-MS systems allow direct CCS measurements under specific working conditions [13]. However, not all DTIM-MS methods are primary methods, so CCS values must be obtained through an instrumental calibration function. For example, data acquisition by primary methods involves long acquisition times and is not compatible with chromatographic separation methods, so CCS values are generally obtained from CCS calibration curves when using LC-DTIM-MS methods [3]. Calibration curves are built from data of calibrant ions with known CCS values and provide a faster manner to perform CCS measurements. In general, the application of CCS calibration curves is also the normal operating mode of TWIM-MS and

TIM-MS instruments for CCS measurements [4]. Since CCS measurements are typically carried out by secondary methods that require CCS calibration, CCS calibration has become an important step in any laboratory protocol intended to use IMS technology for CCS measurements.

This protocol describes the analytical workflow, including (1) sample preparation, (2) data acquisition, and (3) data analysis, implemented in our research group for the analysis of phase II steroid metabolites in urine samples in a public health context related to chemical food safety. Steroid analysis represents just one of multiple challenges facing chemical food safety and has been chosen as an example to illustrate the implementation of TWIM-MS technology, including CCS measurements, in this field. The administration of steroids to animals intended for human consumption and, more specifically, the application of substances with hormonal actions as growth promoters (e.g., anabolic steroids) has been prohibited in the countries of the European Union (EU) since 1988 [17]. The control of steroid levels in biological matrices of food-producing animals, including metabolites, therefore makes it possible to detect any alteration in the steroid profiles associated with the abuse of hormones to promote growth [18]. In this context, LC-TWIM-MS has recently been applied to the analysis of steroid metabolites in urine samples [19], and large CCS databases for steroids are currently available in the literature [14, 20].

Execution of this protocol involves the specific use of TWIM-MS technology, and more specifically, a Synapt G2-S HDMS instrument from Waters Corporation. In addition, it focuses on the experimental workflow to be followed for CCS measurements, highlighting important technical aspects such as CCS calibration. Data analysis (i.e., generation of CCS values) following this protocol implies the use of Driftscope software, which is also commercially available from Waters Corporation. We suggest consulting the recent book edited by Paglia and Astarita [21] that contains other protocols involving the application of IM-MS systems and alternative software to those used in the protocol described here. Finally, this protocol can be applied to numerous applications in the field of food safety if the same TWIM-MS instrumentation is used. For obvious reasons, sample preparation must be tailored to the food matrix (or related matrix), analytes of interest and associated concentration levels.

2 Materials

2.1 Reagents

1. Acetonitrile (LC-MS Chromasolv® grade, Sigma Aldrich).
2. Propan-2-ol (LC-MS Chromasolv® grade, Sigma Aldrich).

3. Ethanol (for HPLC, Promochem®, LGC Standards).
4. Sodium formate (0.5 mM in 90/10 (%, v/v) propan-2-ol/water) prepared from sodium hydroxide (1 M; Fisher Chemical™, Fisher Scientific) and formic acid (Promochem®, LGC Standards).
5. Formic acid (eluent additive for LC-MS, LGC Standards GmbH).
6. Sodium hydroxide (Puriss. grade, 98–100.5%, pellets, Sigma Aldrich).
7. Major Mix IMS/Calibration Kit (cat. no. 186008113, Waters Corporation).
8. Leucine-enkephalin (cat. no. 186006013, Waters Corporation).
9. Estriol 3-sulfate (cat. no. E2734-000, Steraloids), etiocholanolone glucuronide (cat. no. A3625-000, Steraloids), estradiol diglucuronide (cat. no. E1010-000, Steraloids), estrone 3-sulfate (cat. no. E2335-000, Steraloids), and testosterone glucuronide (cat. no. A6913-000, Steraloids) standard solutions in ethanol (1 mg/mL) were stored in dark at −20 °C.
10. Standard Quality Control (QC) mix containing the following compounds: estriol 3-sulfate, etiocholanolone glucuronide, estradiol diglucuronide, estrone 3-sulfate, and testosterone glucuronide. Prepare an intermediate standard solution in ethanol (100 μg/mL) containing all five steroids from the stock standard solution of each steroid (1 mg/mL). The intermediate standard solution should be stored in dark at −20 °C. Take an aliquot of the intermediate standard solution, transfer it to an HPLC vial and dry it with a stream of nitrogen. Reconstitute with 95/5 (v/v) water/acetonitrile containing 0.1% (v/v) formic acid to obtain a Standard QC mix with a concentration of 1 μg mL^{-1} for each steroid. The Standard QC mix should be prepared each day before running sample analysis.

2.2 Samples

Bovine urine samples kept at LABERCA biobank were used for preparing this protocol. These samples were collected with the authorization for the use of animals for scientific purposes n°02323.01, in accordance with the provisions of article R. 214-124 and R. 214-125 of the French Rural Code.

2.3 Labware

1. 1.5-mL HPLC vials, including caps with polytetrafluoroethylene (PTFE) liner.
2. HPLC vials with inserts (200 μL).
3. VWR® centrifugal tubes (polyethersulfone (PES) membrane, 10 kDa, 500 μL ; cat. no. 82031-350).

4. 4-mL and 40-mL amber vials (for storage of standard solutions).
5. Eppendorf pipettes tips.
6. 20-mL volumetric flasks.
7. Volumetric pipettes (1 ± 0.008 mL; 0.5 ± 0.005 mL).

2.4 Equipment

1. TWIM-MS system: Synapt G2-S HDMS equipped with an ESI source (Waters Corporation).
2. LC system: Acquity UPLC® System (Waters Corporation).
3. LC column: Acquity UPLC® BEH C18, 2.1 × 100 mm, 1.7 μm (Waters Corporation).
4. Corning® LSE™ vortex mixer.
5. Thermo Scientific™ Sorvall™ Legend™ MicroCL 17R microcentrifuge.
6. Eppendorf Research plus single channel pipettes (2–20 μL; 10–100 μL; 20–200 μL; 100–1000 μL).

2.5 Data Generation and Processing

1. MassLynx (version 4.2, Waters®) software, used for data acquisition.
2. Driftscope (version 2.8, Waters®) software, used for data processing and interpretation (i.e., CCS values).

3 Methods

This protocol addresses the UPLC-TWIM-MS workflow applied to the analysis of phase II steroid metabolites in urine samples. This approach is intended to detect the illegal use of steroids in food-producing animals. A similar strategy to that proposed in this protocol can be applied to the targeted or semi-targeted analysis of chemical residues and contaminants in food products and related matrices. In this sense, sample preparation must be adapted to both the matrix to be analyzed and the physicochemical properties and concentration levels of the analytes to be characterized, while data acquisition settings must be optimized for the compounds of interest. On the contrary, general settings must be established in the case of non-targeted analysis, so in this case, for example, LC-TWIM-MS protocols used in metabolomics studies can be applied [22].

3.1 Sample Preparation

1. Store urine samples at −20 °C and thaw at room temperature before sample preparation (*see* **Note 1**).
2. Filter the urine samples by centrifugation at 10,000 × *g* and 15 °C for 10 min using centrifugal filters with a molecular weight cut-off of 10 kDa.

3. Recover the filtered urine sample and transfer 10 μL to a chromatographic vial with insert (*see* **Note 2**).
4. Dilute the sample 10 times with 0.1% (v/v) aqueous formic acid containing internal standards (if necessary for quality assurance (QA) purposes) (*see* **Note 3**).
5. Homogenize the sample by vortex agitation for a few seconds (~10 s) (*see* **Note 4**).
6. Submit the samples for UPLC-TWIM-MS analysis.

3.2 TWIM-MS Parameters: Tuning and Calibration

1. Prepare the sodium formate calibration solution (0.5 mM in 90/10 (%, v/v) propan-2-ol/water) (*see* **Note 5**). To do this, first prepare a 5 mM sodium formate solution in 90/10 (v/v) propan-2-ol/water from a 0.1 mM sodium hydroxide solution in water and formic acid. Then, add 2 mL of 5 mM sodium formate solution to a 20-mL volumetric flask using a 1-mL pipette. Make up to 20 mL with 90/10 (v/v) propan-2-ol/water. Finally, sonicate the solution for 5 min for homogenization.
2. Prepare the leucine-enkephalin "lock mass" solution (2 μg/mL in 50/50 (%, v/v) water/acetonitrile solution with 0.1% (v/v) formic acid). To do this, first prepare 400 μg/mL of leucine-enkephalin in water. Then, transfer 100 μL of the 400 μg/mL leucine-enkephalin solution to a 20-mL volumetric flask using a 200-μL Eppendorf pipette. Make up to 20 mL with 50/50 (v/v) acetonitrile/water containing 0.1% formic acid. Finally, sonicate the solution for 5 min to homogenize it.
3. Startup the instrument according to the supplier's instructions and set the "TOF" and "High Resolution" modes (*see* **Note 6**).
4. Set the ionization source conditions as indicated in Table 1 and keep "default conditions" for other instrument parameters.
5. Infuse the sodium formate calibration solution and the leucine-enkephalin "lock mass" solution directly into the TWIM-MS system at 10 μL/min using the sample and "lock mass" capillaries, respectively.
6. As part of the QA protocol, acquire the leucine-enkephalin mass spectrum twice for 2 min each with a scan time of 0.5 min (*see* **Note 7**). Reference values (i.e., mass-to-charge ratio (m/z) and CCS) for the use of the leucine-enkephalin as "lock mass": m/z 556.2771 and 229.8 Å^2 (ESI+ conditions); m/z 554.2615 and 225.3 Å^2 (ESI-conditions).
7. As part of the QA protocol, verify that the mass spectrum of the sodium formate calibration solution corresponds to the mass spectra commonly observed when performing mass calibration

Table 1
Electrospray ionization (ESI) conditions

Parameter	Set at...
Capillary voltage (sample capillary)	+3.0 kV for ESI+, −2.5 kV for ESI−
Capillary voltage ('lock mass' capillary)	+2.5 kV for ESI+, −2.5 kV for ESI−
Sampling cone	40 V
Source offset	50 V
Source temperature	150 °C
Desolvation temperature	450 °C
Desolvation gas flow	650 L/h
Cone gas flow	50 L/h
Nebulizer gas flow	6 bar

(i.e., *m/z* of detected ions, signal intensity, signal ratio between ions), as well as ensure the stability of the ion beam.

8. Perform the mass calibration in both ionization modes within the *m/z* range of 50–1200, applying the automated calibration mode and with the instrument conditions indicated in the point 4 (Table 1) as tuning setup (*see* **Note 8**). Signal attenuation for calibration and root-mean-square (RMS) residual mass are the parameters to be checked as part of the mass calibration quality control. A threshold of 1.0 ppm for RMS residual mass is set to accept the mass calibration carried out. Also, the entire set of ions included in mass calibration reference file must be found when performing mass calibration to accept it.
9. Switch the instrument from the "TOF" mode to the "Mobility-TOF" mode (also referred to as HDMS mode) and replace the sodium formate calibration solution with the CCS Major Mix (*see* **Note 9**). The ion mobility-mass spectrometry system must remain in "Operate" mode for at least 1 h prior to CCS calibration (or CCS measurements) to ensure system stability.
10. Configure the TWIM-MS settings as indicated in Table 2 for CCS calibration (*see* **Notes 10–12**). The "default" values are kept for other instrument parameters, such as those related to "Step Wave" conditions.
11. As part of the QA protocol, verify that the mass spectrum of the CCS Major Mix solution corresponds to the mass spectra typically observed when performing the CCS calibration (i.e., *m/z* of detected ions, signal intensity, signal ratio between ions) under the established TWIM-MS settings, as well as ensure the stability of the ion beam. Table 3 indicates the

Table 2
TWIM-MS setup for steroid analysis when including LC separation

			ESI+	ESI-
Conditions of the ionization source		Capillary voltage (sample capillary)	+3.0 kV	−2.5 kV
		Capillary voltage ('lock mass' capillary)	+2.5 kV	−2.5 kV
		Sampling cone	31 V	
		Source offset	40 V	
		Source temperature	150 °C	
		Desolvation temperature	350 °C	
		Desolvation gas flow	1000 L/h	
		Cone gas flow	50 L/h	
		Nebulizer gas flow	6 bar	
Gas flow rates		Helium gas	180 mL/min	
		IMS gas	90 mL/min	100 mL/min
		Trap gas	2.0 mL/min	0.4 mL/min
Triwave DC	Trap DC	Entrance	3.0	
		Bias	47.0	
		Trap DC	0.0	
		Exit	0.0	
	IMS DC	Entrance	20.0	
		Helium cell DC	50.0	
		Helium exit	−20.0	
		Bias	3	2
		Exit	0.0	
	Transfer DC	Entrance	5.0	
		Exit	3.0	
Triwave	Trap	Wave velocity	311 m/s	
		Wave height	4.0 V	
	IMS	Wave velocity	1000 m/s	550 m/s
		Wave height	40.0 V	
	Transfer	Wave velocity	219 m/s	
		Wave height	4.0 V	

composition of CCS Major Mix; therefore, the ions to monitor. Mass spectra of CCS Major Mix recorded under ESI+ and ESI- conditions are included in Fig. 1.

12. Perform the CCS calibration within the m/z range of 50–1200 applying the automated calibration mode and selecting the instrument conditions indicated in the point 10 (Table 2) (*see* **Note 13**). CCS calibration can be performed for both ionization modes. Signal attenuation should be noted and monitored as part of the CCS calibration quality control.
13. CCS calibration must be accepted by the user (Fig. 2). Since TWIM-MS settings are generally optimized for the analytes to be further analyzed and not for the CCS calibration mix (*see* **Note 14**), it is important to verify that the proposed calibration curve includes enough calibration points (*see* **Note 15**).

Table 3
Composition of CCS Major Mix, as well as *m/z* and CCS of protonated and deprotonated ions observed within the *m/z* range between 50 and 1200

	$[M+H]^+$ (ESI+ conditions)		$[M-H]^-$ (ESI− conditions)	
Composition of CCS Major Mix	***m/z***	**CCS (Å^2)**	***m/z***	**CCS (Å^2)**
Acetaminophen	152.0712	130.4	150.0555	131.5
Caffeine	195.0882	138.2		
Sulfaguanidine	215.0603	146.8	213.0446	145.2
Sulfadimethoxine	311.0814	168.4	309.0658	170.1
L-Valyl-L-tyrosyl-L-valine	380.2185	191.7	378.2029	192.5
Verapamil	455.2910	208.8		
Terfenadine	472.3216	228.7		
Polyalanine (oligomer; number of repeating units (n) = 7)	516.2782	211.0	514.2631	209.0
Leucine-enkephalin	556.2771	229.8	554.2615	225.3
Polyalanine ($n = 8$)	587.3153	228.0	585.3002	223.0
Reserpine	609.2812	252.3	607.2656	265.2
Polyalanine ($n = 9$)	658.3524	243.0	656.3368	242.1
Polyalanine ($n = 10$)	729.3895	256.0	727.3739	255.9
Polyalanine ($n = 11$)	800.4266	271.0	798.4110	268.5
Polyalanine ($n = 12$)	871.4637	282.0	869.4481	280.2
Polyalanine ($n = 13$)	942.5008	294.0	940.4852	294.6
Polyalanine ($n = 14$)	1013.5379	306.0	1011.5223	308.8

14. The TWIM-MS instrument is now ready for data acquisition. It is important to verify that both the loaded mass and CCS calibration profiles correspond to the calibrations performed previously (Fig. 3). As part of the QA protocol, it is necessary to note the mass and CCS calibration profiles that are used in data acquisition.

3.3 LC System Setup

1. Prepare mobile phases A and B as follows: add 1 mL of formic acid into a bottle containing 999 mL of water (phase A: 0.1% (v/v) aqueous formic acid) and add 1 mL of formic acid into a bottle containing 999 mL of acetonitrile (phase B: acetonitrile containing 0.1% (v/v) formic acid) (*see* **Note 16**).

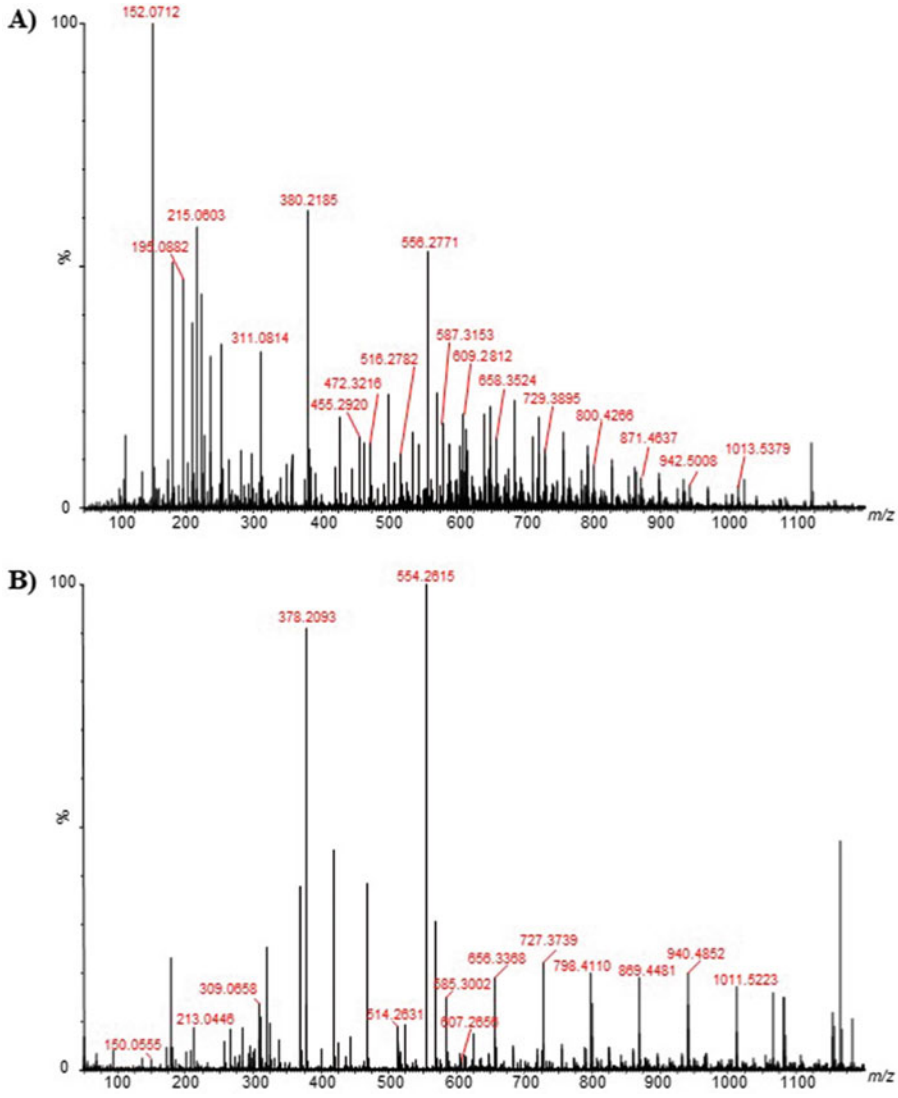

Fig. 1 Mass spectra of CCS Major Mix acquired under ESI+ (**a**) and ESI− (**b**) conditions

2. Cap and shake the mobile phase bottles vigorously to homogenize the mobile phase solutions.
3. Install the mobile phase bottles in the LC system and purge mobile phase tubes to remove air bubbles from the system.
4. Insert the column into the column compartment. An Acquity UPLC® BEH C18 column (2.1 × 100 mm, particle size 1.7 μm) is proposed for the analysis of phase II steroid metabolites.
5. Adjust the column temperature to 50 °C and gradually increase the flow rate of the mobile phase to 0.6 mL/min.

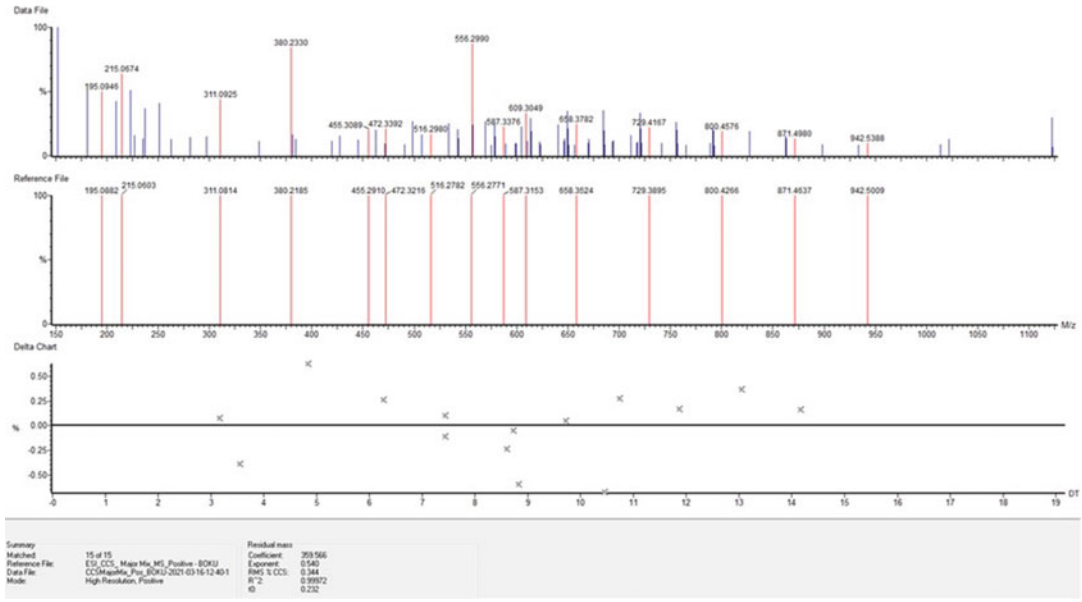

Fig. 2 Screenshot of the report generated after CCS calibration under ESI+ conditions, and which must be accepted by the user before continuing to work with the instrument. This provides information on the detected peaks compared to a set of reference ions, as well as information on the calibration curve created (corrected drift time (t_d') *vs* normalized cross section (Ω'); the calibration curve is a power trend line [23], $y = Ax^b$). A 1% threshold has been set for RMS, as well as a determination coefficient (R^2) > 0.99, to accept the calibration curve

Fig. 3 Partial screenshot of the tuning instrument page showing the mass (**a**) and CCS (**b**) calibration profiles loaded into the system after performing both calibrations and before performing sample acquisition

6. Set the injection volume to 5 μL and the autosampler temperature to 10 °C.
7. Set the elution gradient program as indicated in Table 4.

3.4 Data Acquisition

1. Set ESI-conditions, as negative ionization mode is recommended for analysis of phase II steroid metabolites [24].
2. Prepare the HDMS method (i.e., the acquisition method for TWIM-MS analysis). Set the *m/z* acquisition range to 50–1200 and the scan time to 0.5 s. Make sure the "add drift time function" and "maintain mobility separation" options are checked in the "Mobility" window. In the "Options" window, indicate that the "lock mass" solution must be refilled at the beginning of each run, and set the "lock mass" acquisition to an average of 3 scans (scan time, 0.2 s) every 15 s. Do not select

Table 4
Proposed elution gradient program for analysis of phase II steroid metabolites by LC-TWIM-MS

Time (min)	Mobile phase (%B)
0	5
0.3	5
9.6	43
10.5	100
12.5	100
13	5
16.5	5

"automatic application of 'lock mass' correction" as it will be applied during post-acquisition data processing.

3. Set data acquisition to "continuous" or "profile" mode and not to "centroid" mode.
4. Create the acquisition sequence indicating TWIM-MS settings and the LC and HDMS methods to be used in data acquisition, as indicated in Table 2, Subheading 3.3, and **step 2** of this section, respectively. Important: TWIM-MS settings must be the same as those set for CCS calibration (Table 2) to ensure that CCS measurements are carried out correctly (*see* **Note 17**).
5. It is recommended to include two blank samples at the beginning of the acquisition sequence to ensure that the LC-TWIM-MS system is equilibrated when the sample analysis is performed. Also, run the analysis of the standard QC mix first to evaluate the performance of the LC-TWIM-MS workflow.
6. Run the samples. It is recommended to analyze the samples in triplicate when performing CCS measurements to check the repeatability of the measurement.

3.5 Data Processing

1. Import the CCS calibration data file into the Driftscope software (*see* **Note 18**).
2. Perform peak detection by selecting "non-chromatographic mode." The "Minimum Intensity Threshold" parameter must be adjusted to detect ions related to the calibration solution and avoid detection of peaks associated with the background noise.
3. In the "peak detection" window, select "calibrate peaks" and "new calibration."

4. Open the reference file that contains the information for the ions in the calibration solution (i.e., *m/z* and CCS).
5. In the "peaks" window, select the "auto select..." option to compare "reference" and "measured" peaks (*m/z* tolerance, 0.1 Da; intensity threshold, 5%).
6. Save the calibration profile if the determination coefficient (R^2) > 0.99 in accordance with the results observed in Subheading 3.2, **step 13**. If not, check that the peak selection has been carried out correctly since unsatisfactory calibration curves are often the result of incorrect peak selection.
7. Import the LC-TWIM-MS data into the Driftscope software.
8. Perform peak detection by selecting "chromatographic mode" and applying "Lock Mass" correction (*m/z* 556.2771 for ESI+ conditions, and *m/z* 554.2615 for ESI- conditions) (*see* **Note 19**).
9. Visualize the detected peaks on the 2D plot (*m/z vs* drift time) provided by the Driftscope software (Fig. 4).
10. In the "peak detection" window, select "apply calibration" to apply the previously created CCS calibration curve (point 6 of this section) (*see* **Note 20**).
11. 'Export peak list' to an Excel file.
12. Search the detected peaks in the published CCS databases for steroids [14, 20].

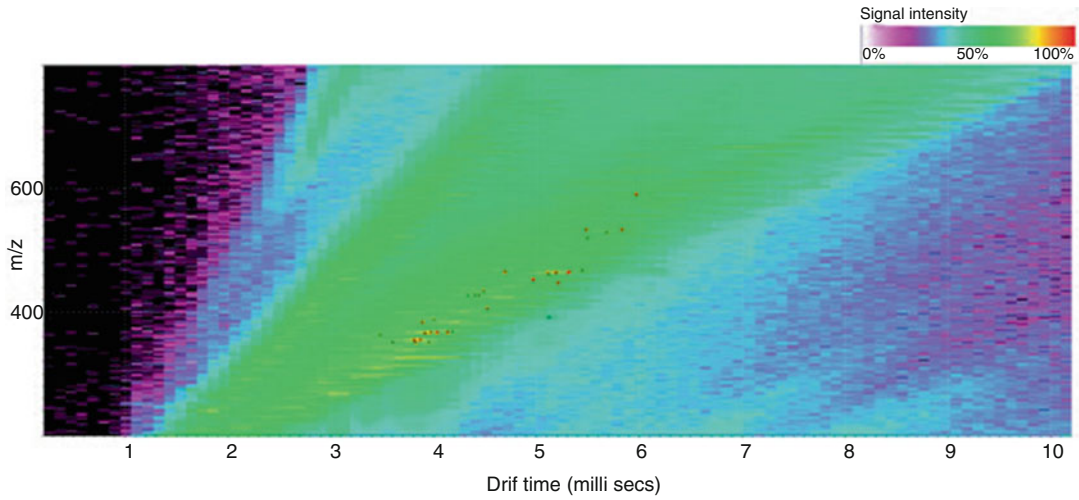

Fig. 4 Representation of *m/z vs* drift time for a bovine urine sample analyzed according to the proposed protocol. The red dots indicate the peaks detected in a retention time range between 3.0 and 9.0 min and a *m/z* range between 350 and 625. The peak detection ranges have been established according to previous knowledge of LC-MS for phase II steroid metabolites [19]

13. In addition, select a region of interest from the mobility spectra (drift time range) on the 2D plot to verify the presence and separation of isomers and isobaric compounds.
14. Export the data to the MassLynx software for better visualization (Fig. 5) (*see* **Note 21**). Separation in the ion mobility dimension indicates differences in the CCS of the related ions (*see* **Note 22**). In TWIM-MS instruments, more compact ions (with smaller CCS) reach the exit of the mobility cell earlier than elongated ions (with greater CCS).
15. Plan fragmentation experiments using LC-TWIM-MS workflows to generate additional information on the analytes of interest and gain more confidence in the identification or confirmation of compounds (*see* **Note 23**). As a starting point, perform $HDMS^E$ experiments by setting 5 eV in the trap cell. Set the transfer cell to 5 eV for low energy fragmentations and a ramp between 20 and 40 eV for high energy fragmentations.

4 Notes

1. Samples must be stored under adequate conditions (−20 °C) to ensure their biological stability over time.
2. It is recommended to use vials with inserts to reduce the sample volume required for analysis. This is especially relevant for those matrices with limited quantities.
3. It is recommended to add isotope-labeled internal standards to samples as a part of the QA protocol, especially for quantification purposes. For phase II steroid metabolites, at least two internal standards representing both steroid sulfates and glucuronides can be added (e.g., epitestosterone glucuronide-d3 and epitestosterone sulfate-d3 at 0.5 μg/mL each one).
4. It is important to check that there are no air bubbles at the bottom of the insert after homogenizing the sample. Air bubbles can prevent proper sample injection into the UPLC-TWIM-MS system.
5. Avoid any contact of the sodium formate calibration solution with the cap of the vial or other non-glass material as it has been observed to increase background noise and decrease signal sensitivity of sodium formate clusters (Fig. 6). Sodium formate solutions expire in 1 week.
6. The "High Resolution" mode is the most appropriate to be used in order to obtain more accurate measurements of the m/z. However, the signal sensitivity that can be reached using this mode can be limited and may compromise the detection of residues and contaminants present at low concentration levels in food matrices. In targeted analysis, where the analytical

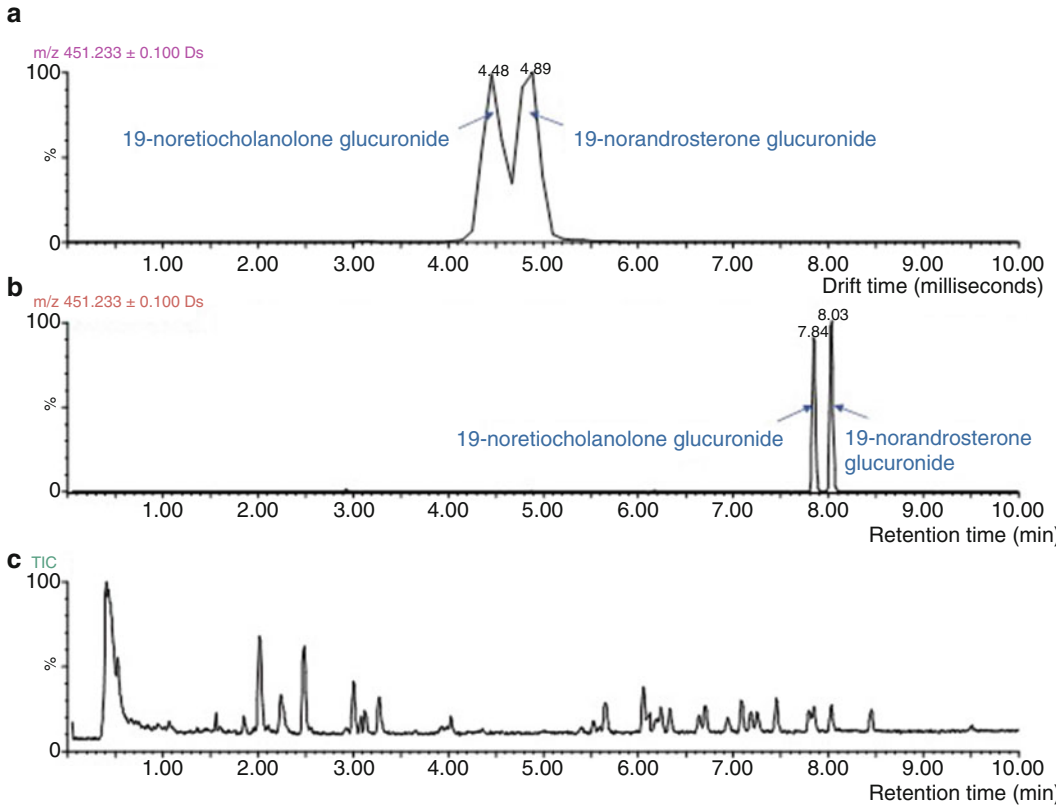

Fig. 5 Separation of two steroid isomers (*m/z* 451.2326) in the ion mobility dimension (**a**) and in the LC dimension (**b**) resulted from the analysis of a spiked (2 μg/mL) bovine urine sample by LC-TWIM-MS. Total ion chromatogram (TIC) observed for the analyzed sample (**c**)

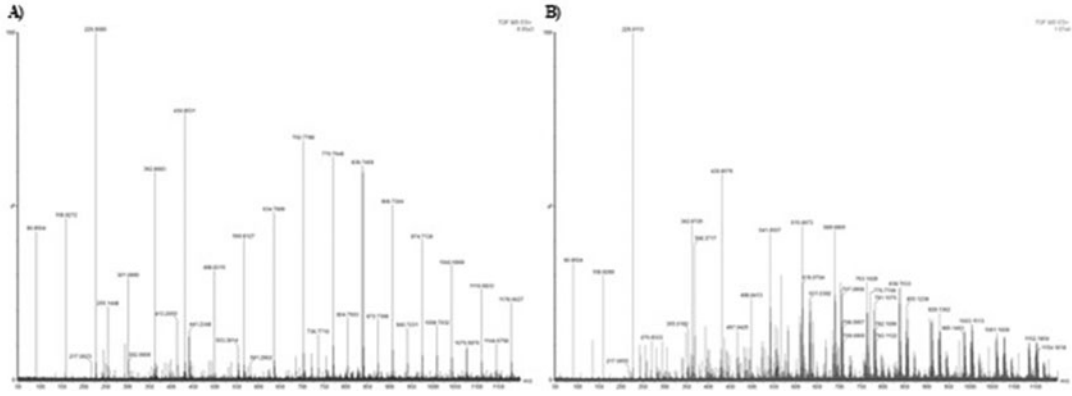

Fig. 6 Mass spectra of the sodium formate calibration solution observed under optimal acquisition conditions (**a**) and negatively affected by improper handling of the calibration solution (**b**)

information on potentially detectable compounds is known, it is recommended to evaluate the performance of the "Sensitivity" working mode to improve sensitivity of the signal to the detriment of mass accuracy.

7. It is recommended to monitor the leucine-enkephalin signal before and after mass and CCS calibration, as well as before running a sample sequence, as part of the QA protocol for IM-MS measurements. This allows the performance of the instrument to be evaluated and recorded over time in terms of signal sensitivity, mass accuracy, and CCS precision.
8. As indicated by the Vendor, the same value for "Transfer DC Exit voltage" must be set for MS and HDMS modes; otherwise, a separate mass calibration is required.
9. The sample capillary should be rinsed with acetonitrile/water (50/50;%, v/v) solution before and after injecting any calibration solution (or other solution or sample) into the instrument using the direct infusion set in order to avoid cross-contamination and obtain correct mass spectra of the infused solution.
10. TWIM-MS settings must be the same for CCS calibration and CCS measurements on samples. These parameters must be optimized for the analytes of interest or generic parameters can be set in the case of non-targeted analysis [22]. The TWIM-MS settings must allow all ions within the m/z acquisition range fit into the TWIM separation window (Fig. 7b). For example, too low wave velocities in the TWIM cell lead to poor mobility separation (Fig. 7a), whereas too high wave velocities cause a "wrapping effect" whereby the slow ions stay for too long in the TWIM cell (more than a 200 bins experiment) and are observed at the beginning of the subsequent mobility separation (Fig. 7c). For a suitable TWIM-MS method (e.g., to avoid "wrapping effects"), it is recommended to ensure that the arrival time distributions of analyte ions are observed within a drift time interval of 20 and 180 bins.
11. TWIM-MS settings can be optimized by direct infusion of a mixture of the analytes of interest (e.g., at 1 μg/mL) in both positive and negative ionization modes [25]. As indicated by the Vendor, it is recommended to set the IMS wave height at 40 V. In the event of unwanted fragmentation of the target analytes, "Trap DC bias" should be adjusted.
12. Parameters such as source temperature, desolvation temperature, and desolvation gas flow rate depend on the flow rate of the mobile phase or infused solution. Check the Vendor's recommendations to establish satisfactory values for these parameters.

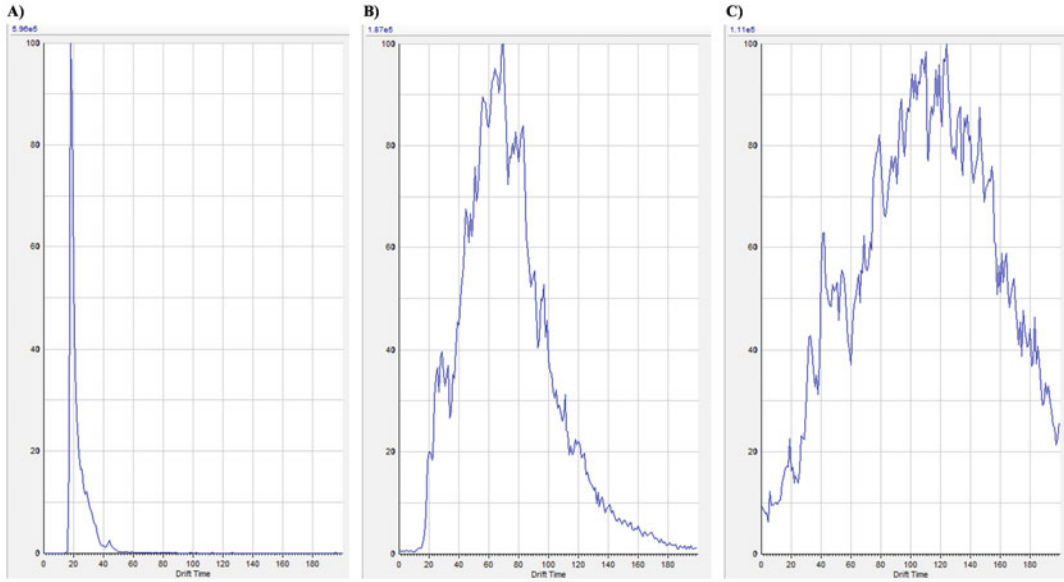

Fig. 7 Mobility spectra of the CCS Major Mix obtained using a wave velocity in the TWIM cell of 250 (**a**), 1000 (**b**) and 1750 m/s (**c**), respectively

13. Mass and CCS calibration are performed sequentially as part of this protocol. Instrument calibration is required to be done weekly. Mass accuracy is the limiting factor that indicates the need to calibrate the system. A mass accuracy deviation of up to 10 ppm can be accepted when working in "High Resolution" mode.
14. It is recommended to use CCS calibration mixtures with physicochemical properties similar to those of the analytes of interest. This ensures that TWIM-MS settings are optimal for the detection and analysis of the molecules either in the calibration mix or in the sample. Furthermore, calibrants with different physicochemical properties compared to the analytes of interest have been found to be unsuitable and can introduce a high bias between the measured CCS and the reference or expected CCS values for these molecules [26].
15. Automated calibration is based on the accumulation of total peak intensity, which can be detrimental to the detection of low intensity peaks if ions with a signal intensity several times higher are present. Therefore, calibrant ions with low intensity (e.g., m/z 1013.5379 in Fig. 2) are not detected with sufficient signal intensity to be used as calibration points in CCS calibration. In this context, it is recommended to perform a manual CCS calibration of the instrument rather than an automatic calibration. For manual calibration, infuse the CCS Major Mix at a flow rate of 10 μL min^{-1} and acquire the signal for at least 2 min (m/z range, 50–1200; scan time, 0.5 s). The

acquired data file must be loaded later in the "Calibration Reference Compound & Raw Data" option in the "Edit Manual Calibration" window when editing the calibration profile.

16. Use glassware and avoid the use of plastic receipts and materials when preparing formic acid solutions. Plasticizers are susceptible to cause unnecessary contamination of solutions and lead to complex mass spectra with unwanted m/z signals.
17. It is recommended to use the same "Tune Page" or "TWIM-MS tuning conditions file" for CCS calibration and CCS measurements to ensure that both processes are performed under the same TWIM-MS conditions.
18. A CCS calibration curve must be created in Driftscope to apply to the sample acquisition files and obtain the CCS values for analytes of interest.
19. Peak detection is time-consuming and computationally intensive, so it is suggested to select retention time and m/z ranges of interest to shorten this automated process.
20. Calibration curves can also be applied directly by replacing the "mob_cal.csv" file in the "samples".*raw* data file with the one generated in the "CCS calibration".*raw* data file after creating a CCS calibration curve in Driftscope. This is a faster way to apply the CCS calibration curve to a wide range of sample data files.
21. Other commercial software such as UNIFI [25] or freely available software such as Skyline [27] can be used for the visualization of TWIM-MS data, as well as for quantification purposes.
22. Separation can be improved by changing the gas in the mobility cell. However, CCS values are highly dependent on the buffer gas used in the mobility cell, so CCS measurements carried out on different neutral gases cannot be directly compared. In addition, it is worth noting the correlation that exists between m/z and CCS parameters (Fig. 8). Despite the correlation between both parameters, CCS has been shown to increase confidence in the determination of residues and contaminants in food products [28].
23. The Synapt G2-S HDMS instrument includes two fragmentation cells: the trap cell placed before the IMS cell and the transfer cell placed after it. Fragmentation in the transfer cell allows alignment during post-acquisition data processing of those ions that show the same drift time, indicating that they are likely related to the same parent ion.

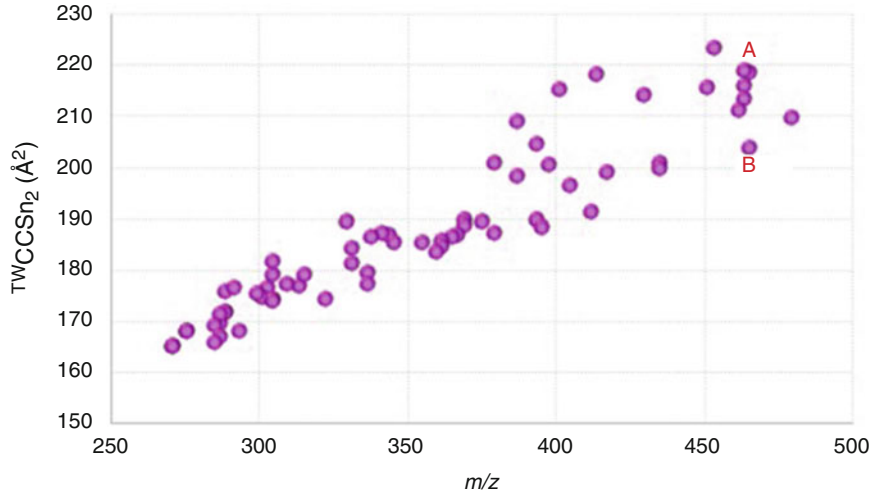

Fig. 8 Graphical representation of CCS *vs* *m/z* for protonated steroid ions. Data extracted from [14]. The differences that exist in the CCS of isomers of phase II steroid metabolites, such as testosterone glucuronide (*m/z* 465.2383; $^{TW}CCSN_2$ = 218.6 Å^2) (**a**) and epitestosterone glucuronide (*m/z* 465.2383; $^{TW}CCSN_2$ = 203.8 Å^2) (**b**), show how the CCS parameter provides additional information to *m/z* and retention indexes for identification

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