Analytica Chimica Acta 1175 (2021) 338739

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Ion mobility-high resolution mass spectrometry in doping control analysis. Part II: Comparison of acquisition modes with and without ion mobility

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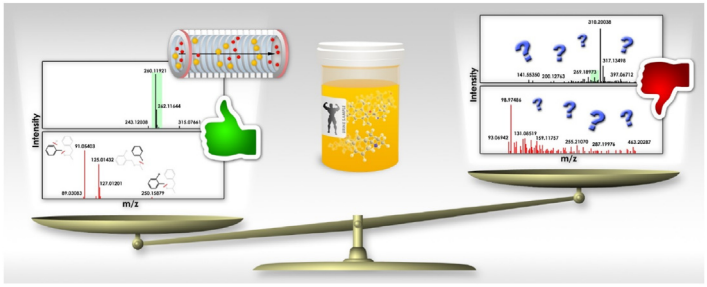
HIGHLIGHTS

- A comparison of fragmentation in the MS/MS spectra of DIA-IMS and DIA approaches was demonstrated.
- Differences in the sensitivity between DIA-IMS and DIA HRMS were determined.
- The possibility to reduce false positive screening results using IM-HRMS was evaluated.
- The processing method was optimized in terms of selectivity and sensitivity.
- Analysis of urine samples from administration studies was carried out.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 February 2021
Received in revised form
3 May 2021
Accepted 19 May 2021
Available online 7 June 2021

Keywords:
Ion mobility spectrometry
Ultra-high performance liquid chromatography
Anti-Doping analysis
Collision cross section
High resolution mass spectrometry

ABSTRACT

In the second part of this study, a systematic comparison was made between two ion fragmentation acquisition modes, namely data-independent acquisition (DIA) and DIA with ion mobility spectrometry (IMS) technology. These two approaches were applied to the analysis of 192 doping agents in urine. Group I included 102 compounds such as stimulants, diuretics, narcotics, and β2-agonists, while Group II contained 90 compounds included steroids, glucocorticoids, and hormone and metabolic modulators. Important method parameters were examined and compared, including the fragmentation, sensitivity, and assignment capability with the minimum occurrence of false positive hits.

The results differed between Group I and II in number of detected fragments when exploring the MS/MS spectra. In Group I only 13%, while in the Group II 64% of the substances had a higher number of fragments in DIA-IMS mode vs. DIA. In terms of sensitivity, the performance of the two modes with and without activated IMS dimension was identical for about 50% of the doping agents. The sensitivity was higher without IMS, i.e. in simple DIA mode, for 20–40% of remaining doping agents. Despite this sensitivity reduction with IMS, 82% of compounds from both Groups met the minimum required

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https://doi.org/10.1016/j.aca.2021.338739

performance level (MRPL) criteria of the World Anti-Doping Agency (WADA) when the DIA-IMS mode was applied.

Automated data processing is important in routine doping analysis. Therefore, processing methods were optimized and evaluated for the prevalence of false peak assignments by analysing the target substances at different concentrations in urine samples. Overall, a significantly higher number of misidentified compounds was observed in Group II, with an almost 2-fold higher number of misidentifications in DIA compared to DIA-IMS. This result highlights the benefit of the IMS dimension to reduce the rate of false positive in screening analysis. The optimized UHPLC-IM-HRMS method was finally applied to the analysis of urine samples from administration studies including nine doping agents from both Groups. However, to limit the number of interferences from the biological matrix, an emphasis is needed on the adequate settings of the data processing method.

1. Introduction

The list of substances prohibited by the World Anti-Doping Agency (WADA) is updated yearly, rendering anti-doping analysis challenging in different aspects [1,2]. Anti-doping analyses are carried out by an initial testing procedure (ITP, "screening"), followed by confirmatory analyses of the suspicious samples. During the ITP, analytical methods with high throughput, excellent selectivity, and adequate sensitivity are required to increase productivity and minimise the number of unnecessary confirmatory analyses caused by false alarms. In terms of selectivity, the use of ultra-high performance liquid chromatography (UHPLC) hyphenated to ion mobility-high resolution mass spectrometry (IM-HRMS) is a particularly attractive alternative for ITP that could meet most of the current challenges in anti-doping analysis.

In the first article of this series [3], a large database including retention times and traveling-wave collision cross section acquired on N_2 ($^{TW}CCS_{N2}$) values for almost 200 target compounds across the various classes of substances on the prohibited list of WADA, including stimulants, narcotics, cannabinoids, diuretics, β_2-agonists, β-blockers, anabolic agents, and hormone and metabolic modulators, was presented. The UHPLC-IM-HRMS method has demonstrated good performance and excellent stability in terms of intraday, interday, and interweek variability of retention times and $^{TW}CCS_{N2}$ values for all compounds at different concentrations in mixtures of standard solutions and in human urine samples. We have shown that a complex biological matrix, such as urine, had an extremely small effect on the stability of retention times and variation of $^{TW}CCS_{N2}$ values. Finally, IM technology was demonstrated to be a very promising tool to filter the data and remove interferences for result interpretation from MS spectra [3].

With hundreds of compounds and thousands of samples being screened, automated data processing and ease of data interpretation play a significant role [4,5]. Therefore, several parameters such as mass accuracy, intensity thresholds, and use of compound specific fragments, are optimized settings of most HRMS processing methods. Compound identification and annotation is then based on accurate mass, but additional data such as retention time, MS/MS fragmentation, and UV/Vis spectra are usually used for unambiguous identification [6–8]. Moreover, theoretically predicted (*in silico)* fragmentation can be used to support the workflow with MS/MS spectra. It was successfully used in several studies for the annotation of unknown peaks [9] or as a proposed screening strategy [10]. There are several approaches of mining molecular structure databases to identify the compounds [11–13], including rule-based fragmentation spectrum prediction [14], combinatorial fragmentation [15,16], fragmentation trees, machine learning, and fingerprint prediction [10,17]. Nowadays, these algorithms are available as both platform-independent software and vendor instrument operating software. Usually, vendor proposed solutions are easier to implement in routine laboratories. In the present work, the UNIFI (Waters) software with integrated MassFragment, i.e. rule-based fragmentation algorithm, was used.

The aim of this second part was to compare and evaluate the differences between two data-independent acquisition modes (DIA), i.e. DIA without IM technology (designated as MS^E in the case of our particular instrumentation) and DIA-IMS using IM technology (designated as high-definition MS^E, $HDMS^E$), in order to identify and characterize prohibited substances and their metabolites in human urine. Moreover, a careful and systematic evaluation of the fragments detected, i.e. the number of theoretical fragments found, sensitivities, and the assignment capability with the minimum occurrence of false positive hits using the two methodologies are presented. Finally, the method setup for automated data processing using different parameters was optimized and discussed in terms of the number of misidentifications. The applicability of the method was also demonstrated by analysing urine samples obtained from administration studies.

2. Experimental part

2.1. Reagents and analytes

The list of all analysed compounds can be found in the first part of this study [3]. Group I substances included mainly stimulants, diuretics, narcotics, and β2-agonists, while Group II contained especially steroids, glucocorticoids, and hormone and metabolite modulators. The standards of the analytes and β-glucuronidase from *Escherichia coli* were kindly provided by the Swiss Laboratory for Doping Analyses (Epalinges, Switzerland). Acetonitrile (ACN) and water of UHPLC/MS grade were obtained from Fisher (FisherScientific, Loughborough, UK). UPLC-MS grade formic acid was supplied by Biosolve (Valkenswaard, Netherlands). Monopotassium phosphate, dipotassium phosphate, and methyl *tert*-butyl ether were purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2. Sample preparation of biological samples

The preparation of biological samples was previously described in the first part of this study [3]. Briefly, 1 mL of blank urine, i.e. a pool of urines from 6 healthy volunteers, was spiked with mixtures of doping agents in water. Compounds of Group I were prepared at 0.1, 1, 5, 10, 50, and 100 ppb, while Group II compounds at 1, 5, 10, 50, 100, and 500 ppb. Different sample preparation methods were used for Group I and Group II due to differences in analytical response, sensitivity criteria, and metabolism. Group I samples were prepared by a 5x dilution by water, while enzymatic hydrolysis and supported liquid-liquid extraction (SLE) with a 10-fold

preconcentration were applied to the processing of Group II compounds.

Finally, several urine samples from administration studies were analysed to demonstrate the fitness for the purpose of the developed method. These samples were collected before administration (T0) and/or at different time points (Tx) after administration and involved nine representative target compounds. The six selected compounds from Group I were ephedrine (T0 and T5), atenolol (T0 and T3), MDMA (collection time not defined), nikethamide metabolite (collection time not defined), methadone (collection time not defined), and cocaine (T0 and T9). Due to expected high concentrations, these urine samples were diluted 100x instead of 5x. Three compounds from Group II were stanozolol (T10 and T17), methylprednisolone (T2), and clenbuterol (T0 and T8).

2.3. Ultra-high performance liquid chromatography ion mobility-high resolution mass spectrometry

An Acquity Ultra Performance Liquid Chromatography (UPLC) system from Waters (Milford, MA, USA) hyphenated to a Waters Vion quadrupole-time of flight (Q-ToF) mass spectrometer (Wilmslow, UK) with travelling wave ion mobility (TWIMS) was used for the analyses. The UPLC was composed of a binary solvent manager, an autosampler, and a column manager including a pre-column eluent heater and a column oven set at 40 °C. The separation was carried out using a Waters Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) and the corresponding VanGuard pre-column. Solvent A, H_2O, and solvent B, ACN, both containing 0.1% formic acid, were used as mobile phases. The gradient profile started at 2% of B and increased up to 98% of B in 6 min and then decreased back to initial conditions of 2% B in 0.1 min followed by 4 min equilibration of the column. The flow rate was 0.4 mL/min and the injection volume 5 μL.

The UHPLC was interfaced with a high-resolution mass spectrometer (HRMS) with positive mode ESI. The analyses were carried out with and without activated IMS mode, and data-independent scan MS^E was applied to the data acquisition. The source temperature was 120 °C, the capillary voltage 1.5 kV, and the cone voltage 30 V. Nitrogen was used as the desolvation gas at 1000 L/h and 500 °C, and as the cone gas at 50 L/h. The continuum spectra were acquired in the mass range of 50–700 *m/z*, with scan time 0.15 s, low energy 6 eV for MS spectra, and high energy ramp from 28 to 56 eV for MS/MS spectra using nitrogen as collision gas.

The settings for travelling wave ion mobility were the following: Stepwave; SW 1 Offset 5 V, SW 1 Velocity 300 m/s, SW 1 Pulse Height 5.0 V, SW 2 Offset 30 V, SW 2 Velocity 200 m/s, SW 2 Pulse Height 15.0 V. Trap; Trap Entrance 0 V, Trap Stopper 0 V, Stopper Height 40 V, Trap Bias 40 V, Gate Offset 0 V, Gate Height 40 V, Aperture 1 0.0 V, Trap Wave Velocity 100 m/s, Trap Pulse Height 'A' 10.0 V, Trap Pulse Height 'B' 5.0 V, IMS Wave Velocity 250 m/s, IMS Pulse Height 45.0 V, Gate Release 2.00 ms, Gate Delay 0 ms, Wave Delay 20 # pushes. Cell 1; Entrance 2 V, Exit 5 V, CE2 5 V, Hex DC 2 V, Wave Velocity 150 m/s, Pulse Height 1.0 V. Cell 2; Entrance −10 V, Gradient 3.0 V, Static Offset 180 V, Offset B 0.0 V, Offset C 0.5 V, Exit 15 V, Exit Trap 4 V, Exit Extract 15 V.

Internal calibration was carried out using 80 ng/mL leucine-enkephalin. External and $^{TW}CCS_{N2}$ calibration was run using Major Mix IMS/ToF Calibration Kit from Waters (Milford, MA, USA). UNIFI software v1.9.3 was used for instrument control, data acquisition, and data processing.

2.4. Study design

Analyses of Group I and Group II compounds were carried out within one sequence with both standard solutions and urine samples measured consecutively. The results of the first part of this study focus on IMS features [3], whereas the emphasis of this paper is on data obtained from the analysis of samples measured first in DIA with IMS mode, followed by the analysis of the same samples in DIA without IMS mode. As no modifications were made in the instrument between these two types of measurements (no maintenance, no re-calibration), the obtained data allowed for direct comparison of selected parameters of DIA and DIA-IMS modes, including fragmentation and sensitivity. The data acquisition settings were exactly the same in both modes (DIA and DIA-IMS) to enable direct comparison. The only exception was the option of ion mobility function turned on/off.

2.5. Processing method

A library was created of each group of target compounds, based on the experimental data of *m/z*, retention times, and $^{TW}CCS_{N2}$ values [3]. All sequences were evaluated using a processing method with this library and the following setup: mass accuracy tolerance 5 ppm, intensity threshold for MS/MS spectra 50 counts, intensity threshold for MS spectra 100 counts, $^{TW}CCS_{N2}$ tolerance <2%, and absolute retention time identification tolerance <0.1 min. Moreover, additional settings for mass accuracy tolerance and MS and MS/MS spectra intensity thresholds were evaluated to examine the effect of the selected parameters on the selectivity and sensitivity of the method. The possibility to obtain correct assignments based on expected fragments was also examined.

UNIFI software with integrated MassFragmenter algorithm is able to predict possible fragmentation of the analytes based on the structure of the compounds, returning a list of theoretical (or *in-silico*) fragments. The software is also capable of searching the measured MS/MS spectrum and identifying those fragments that would match with a theoretical fragment. Therefore, the number of fragments identified in MS/MS spectra and matching the theoretically predicted fragments was used to compare the spectra obtained with and without ion mobility.

3. Results and discussion

The addition of ion mobility spectrometry to the UHPLC-HRMS method provides an additional dimension for separation, based on the collision cross section of the molecule of interest in the gas phase. The most interesting field of application is certainly the separation of isomeric compounds that would otherwise be challenging, if not impossible, using only chromatographic and mass spectrometric methods. The separation of isobaric and isomeric compounds in doping control analysis with ion mobility has been described and discussed in the context of available scientific literature in the first part of this series [3]. Here, an in-depth investigation of the effect of ion mobility on the fragmentation MS/MS spectra is performed. Data obtained from the experiments with and without ion mobility in urine and reference standard mixtures were compared in terms of the number of fragment ions recorded in the MS/MS spectra.

3.1. Comparison of MS/MS spectra obtained with and without IMS dimension

The same samples were analysed in DIA-IMS and DIA on consecutive days to limit as much as possible the instrumental variability. Subsequently, a comparison was made between the MS/MS spectra with and without IMS. Due to differences in sensitivity between modes, only the three highest concentration levels were used for the following evaluation.

The use of the data-independent acquisition mode can result in

very complex MS/MS spectra due to non-specific fragments and biological background. Examples of spectra corresponding to fencamine and bambuterol, representatives for Group I doping agents, and fluocortolone and fluoxymesterone metabolite M2 (9α-fluoro-17,17-dimethyl-18-nor-androstan-4,13-diene-11β-ol-3-one) from Group II are shown in Figs. 1 and 2, respectively. These figures show MS and MS/MS spectra obtained in DIA and DIA-IMS modes.

Theoretically *(in-silico)* predicted fragments can serve as one of the confirmatory features for peak assignment. Therefore, the software in this study predicted theoretical (*in-silico*) fragments based on the structure of the analyte. In the next step, the MS/MS spectrum of the analyte was measured and fragments matching the theoretically predicted ones were identified. Lastly, the number of these corresponding fragments was determined for all doping agents and used to compare data in DIA-IMS *vs.* DIA. The used workflow is shown in Fig. 3. The fragments corresponding to theoretically predicted fragments are marked with a blue symbol in the presented figures (Figs. 1 and 2). In order to maintain the readability of the figures, not all fragments (marked as theoretical or not) are visible in the figures due to the lower intensity of some fragments. However, the software goes through all the measured fragments and returns a number of the fragments corresponding to the theoretical fragments in a complete results table. This number in DIA mode was then considered 100% for each analyte. Subsequently, the corresponding parameter in DIA-IMS mode was classified into one of the seven categories (Fig. 4), i.e. lower: (i) < 10%, (ii) 10−50%, and (iii) 51−80% shown in red colours, equal (iv) 100 ± 20% shown in yellow colour, and higher (v) 121−150%, (vi) 151−200% and (vii) > 200% shown in blue colours.

A difference was observed in fragmentation behaviour between Groups I and II. In Group I, a similar fragmentation behaviour was noticed in both modes of acquisition, especially in standard solutions where equal, i.e. ± 20%, number of theoretical fragments was detected for 70% of compounds. On average, 89% of DIA fragments were detected in DIA-IMS mode in standards and 73% in urine samples. As an example, fencamine exhibiting similar fragmentation in both modes is shown in Fig. 1A. On the other hand, the more pronounced filtering effect of IMS is visible in the spectra of the second example, bambuterol, measured in urine (Fig. 1B). As there were fewer interferences in DIA-IMS mode, it was possible to determine theoretical fragments more efficiently in this case. Fig. 4 summarizes the representation of each classification category, i.e., what percentage of analytes showed the particular behaviour. Overall, only 8% and 6% of the compounds had a higher number of theoretical fragments in DIA-IMS than in DIA in standard and urine samples, respectively. In contrast, substantially lower number, i.e., ≤50%, of theoretical fragments in DIA-IMS contrary to DIA mode was detected for 9 and 30% of Group I compounds in standard and urine samples, respectively. Cleaner MS spectra enable more precise and reliable identification, and by offering a lower rate of false hits in the initial testing procedure, it is of great interest in various fields of targeted routine batch analysis, including anti-doping analysis. On the other hand, the number of fragments applicable for annotation and identification should be preserved to prevent the loss of any relevant structural information for the compounds of interest.

The loss of theoretical fragments in DIA-IMS could generally be attributed to several causes. The CCS values attributed to each compound correspond to the most intense ion in MS spectra based on the measurement of standard solutions. In most cases, the selected parent ion was $[M + H]^+$, while in some cases, a different parent ion, e.g. sodium adduct or protonated molecule after loss of water, was selected. Indeed, the mobility cell is placed before the fragmentation cell in the construction of the instrumentation used in this study. Therefore, different parent ions, for example $[M + H]^+$ and $[M + Na]^+$, have different ion mobility. This difference is highly dependent on the compound and in our case, expressed as percentage deviation, varying from 0.4 to 9.7%. Consequently, these two ions are separated and only fragments with the same ion mobility as the parent will be present in MS/MS spectra. Contrary to that, MS/MS spectra in DIA mode show fragments of all present parent ions. A typical example of such behaviour is oxycodone from Group I for which sodium adduct was selected in DIA-IMS, showing only one theoretical fragment in MS/MS contrary to DIA mode where $[M + H]^+$, $[M + Na]^+$, and $[M + H-H_2O]^+$ resulted in 16 theoretical fragments. Similarly, sodium adduct was selected for dexamethasone from Group II leading to only 3 theoretical fragments in DIA-IMS contrary to DIA mode with 67 theoretical fragments from both $[M + H]^+$ and $[M + Na]^+$. Indeed, sodium adducts do not fragment easily, thus extreme loss (<10%) of theoretical fragments of the compound will be observed in cases where only this type of adduct will be selected in DIA-IMS. In another example, protonated molecule after loss of water selected in DIA-IMS for metenolone enabled detection of 5 theoretical fragments as opposed to DIA where $[M + H]^+$, $[M - H_2O]^+$, and $[M + H-2H_2O]^+$ creating 34 theoretical fragments.

With Group II substances, more pronounced advantage of DIA-IMS mode was observed, as a significantly higher number of theoretical fragments was found in DIA-IMS mode than in DIA, with average values of 240% of fragments for standards and 141% for urine samples. A higher number of theoretical fragments was detected in DIA-IMS for more than 78% of compounds in standards and 55% in urine. The spectrum of fluoxymesterone metabolite M2 (Fig. 2A) is a typical example of this behaviour. The interfering ion at *m/z* 353.1124 observed in the DIA MS spectra was filtered by ion mobility leading to a much cleaner and easier spectrum to interpret. The higher number of peaks observed in DIA-IMS in the MS/MS trace is only apparent as evident from the different scales of spectra in both modes. The interfering ions at *m/z* 222.0907, 250.0855, and 278.0803 were filtered, enabling the detection of the lower intensity ions. Therefore, in this case, the number of assigned theoretical fragments was much higher in DIA-IMS, i.e. 67 *vs.* 51.

Overall, the higher number of fragments detected in DIA-IMS could be due to several reasons. First, the cleanliness of the obtained spectra could be a contributing factor, as discussed in the spectra of fluoxymesterone metabolite M2 (Fig. 2B) and bambuterol (Fig. 1B). Indeed, in simple DIA MS/MS spectra, fragments can be covered by the background noise contrary to fragments in the MS/MS spectra of the DIA-IMS mode which could be more easily observed due to the absence of interfering ions. Secondly, fragmentation in TWIMS cell has already been reported to occur for small molecules due to the heating and collisions with the gas in the IMS cell [18,19]. This fragmentation can occur in any part of the IMS cell. In case that the ion is fragmented in the beginning of the IMS cell, the fragments should have different drift times and thus different CCS values than the parent ion. However, the fragmentation of the parent ion is more probable with an increasing number of collisions and thus, at the end of IMS cell. Here, the drift times of the parent ion and resulting fragments should be quite similar, enabling the processing software to line them up within one compound with the same CCS. This phenomenon could also contribute to the higher amount of fragmentation in DIA-IMS we observed in this study. One way to show whether the fragmentation in IMS cell occurs or not, is to compare the intensity of the parent ion between the MS trace in DIA and DIA-IMS mode. As also presented in Figs. 1 and 2, the intensity of the parent ion of the two

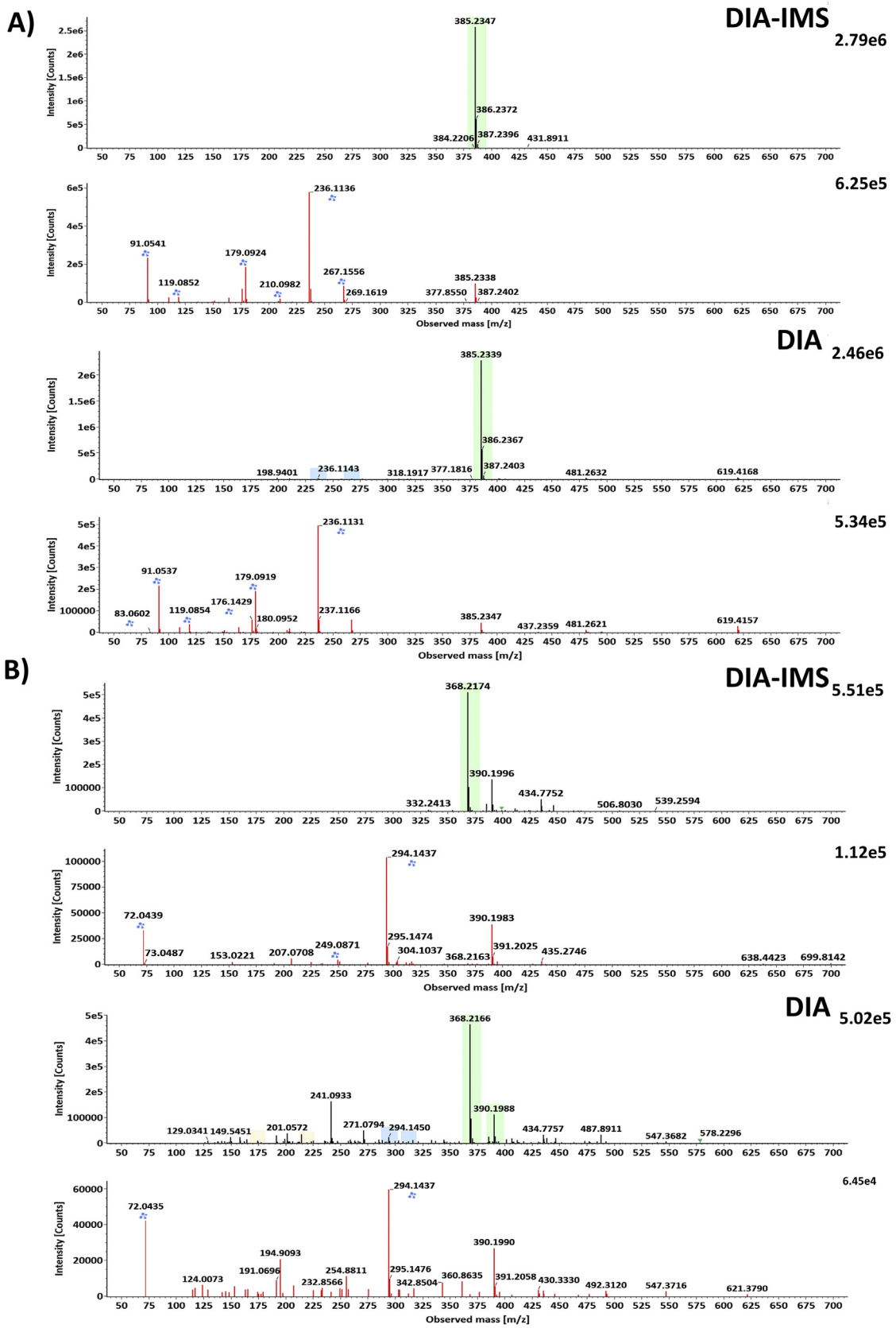

Fig. 1. Comparison of HRMS MS spectra (in black) and MS/MS spectra (in red) of (A) fencamine measured in standard sample and (B) bambuterol measured in urine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

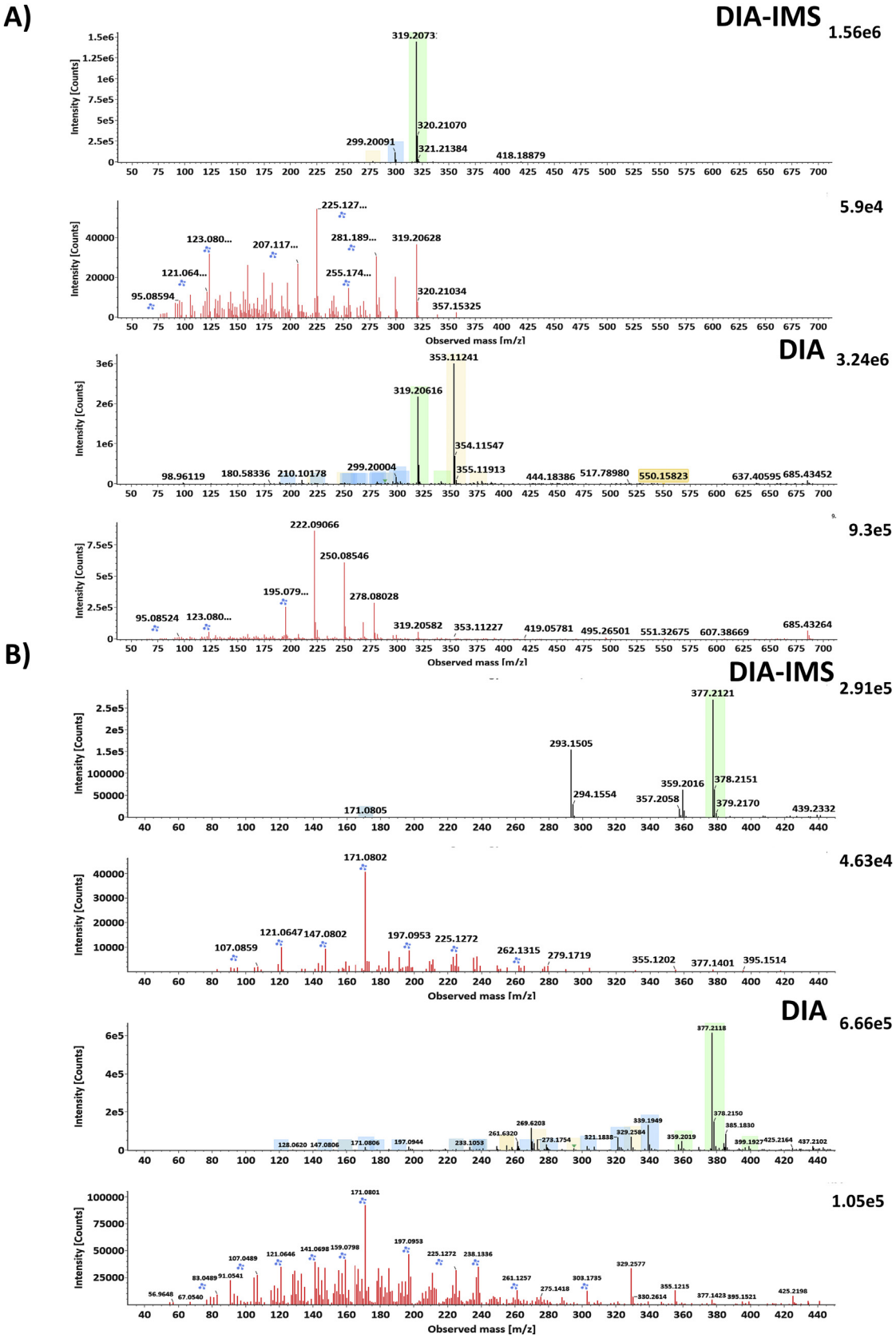

Fig. 2. Comparison of HRMS MS spectra (in black) and MS/MS spectra (in red) of (A) fluocortolone measured in standard sample and (B) fluoxymesterone metabolite M2 measured in urine sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

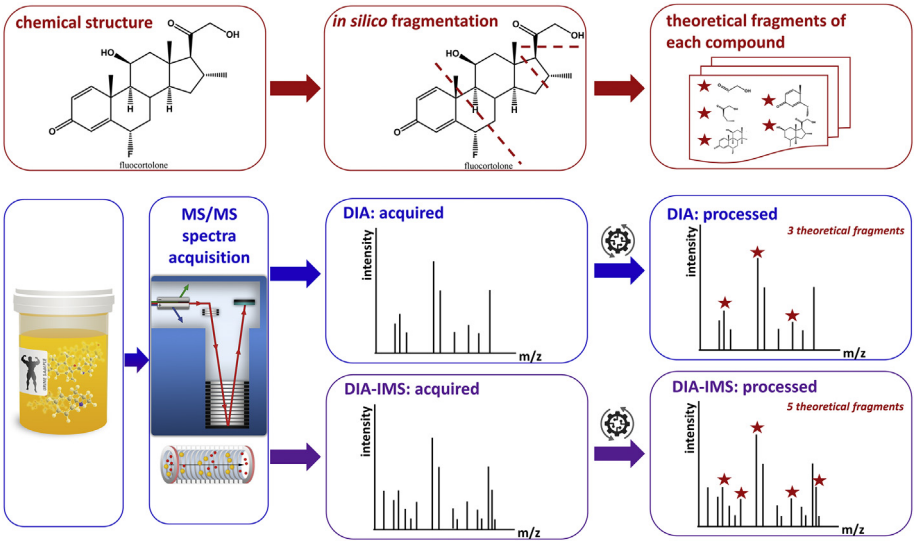

Fig. 3. The workflow used in this study for the evaluation of fragmentation in DIA-IMS and DIA modes using theoretically predicted fragments.

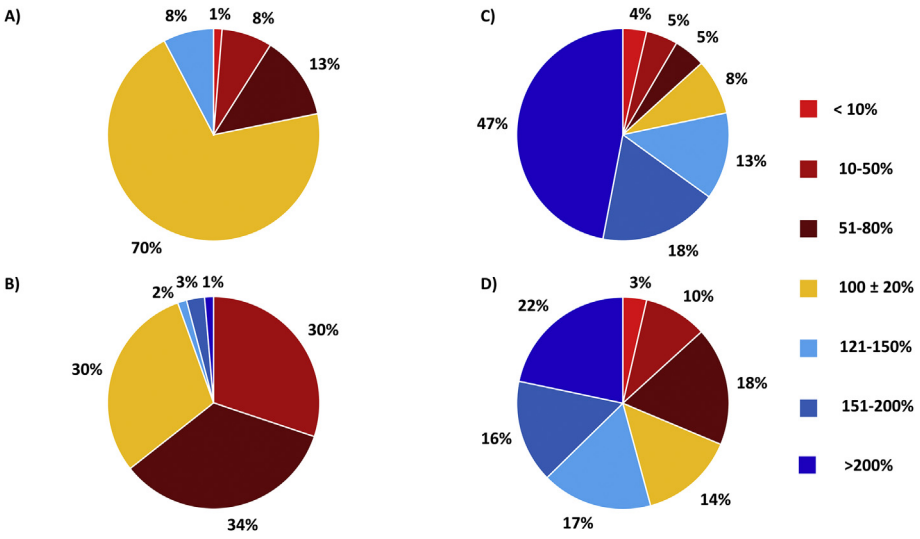

Fig. 4. Comparison of fragmentation in DIA-IMS and DIA modes using the parameter "Theoretical fragments found in High Energy Spectra". The values reported here were calculated as a percentage of fragments found in DIA-IMS MS/MS spectra compared to DIA MS/MS spectra measured from the same samples for Group I doping agents in (A) standards and (B) urine samples and for Group II substances in (C) standards and (D) urine samples.

examples from Group I (Fig. 1) is basically the same in both modes (±15%). On the other hand, examples from Group II (Fig. 2) showed more than 2-times lower intensity of parent ion in DIA-IMS, suggesting that fragmentation in the TWIMS cell might indeed occur for some compounds.

Finally, higher fragmentation in DIA was also observed for Group II compounds and an example of this behaviour is shown in the spectrum of fluocortolone (Fig. 2B). In this case, only 9 and 13% of Group II compounds had substantially lower number of theoretical fragments (<50%) in DIA-IMS in standard and urine samples, respectively (Fig. 4).

3.2. Comparison of sensitivity in DIA-IMS and DIA modes and compliance with MRPL

The sensitivity of DIA-IMS and DIA modes was also systematically compared, with the same dataset, processing method, and the settings described in Section 2.5. The lowest detected concentrations (LDC) from tested concentration levels of all compounds, i.e. 0.1–100 ppb for Group I and 1–500 ppb for Group II substances, were determined in each mode and subsequently compared in Fig. 5. The LCD was determined as the lowest concentration where the analyte's peak was detected at all 3 subsequent injections and the signal to noise ratio was ≥30. As only LCDs were compared and not the absolute intensity, the sensitivity was entitled comparable if the same LDCs were determined for both modes.

For 62% of Group I compounds in standards samples, LDC was comparable between DIA-IMS and DIA. Among these compounds, in 45% of these cases, LDC corresponded to the lowest measured concentration. Therefore, a comparison of the peak area values measured as well as signal to noise ratios (S/N) was carried out. This comparison showed that lower concentrations would probably not be detected. DIA had higher sensitivity for 32% of compounds, and a gain in sensitivity by a factor of 10 for 20 compounds. The highest

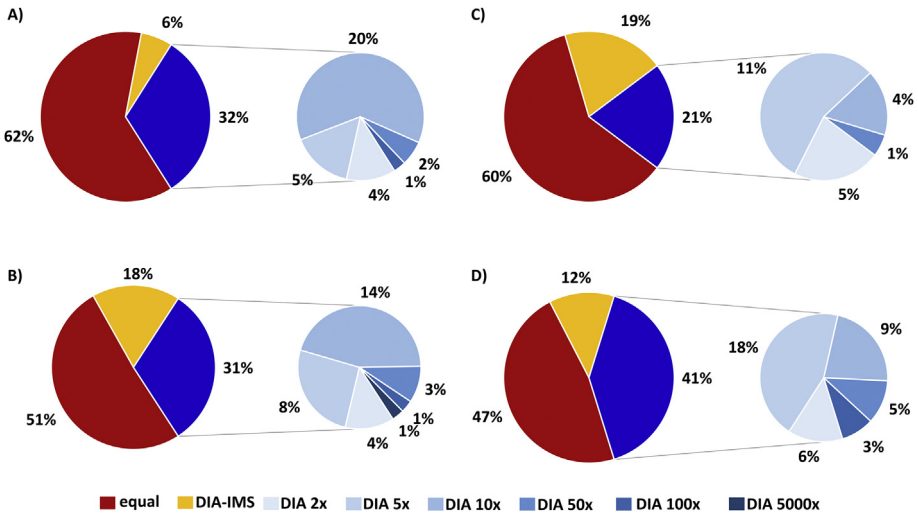

Fig. 5. Comparison of sensitivity in DIA-IMS and DIA modes for Group I substances in (A) standards and (B) urine samples and for Group II doping agents in (C) standards and (D) urine samples. The number indicated represents the percentage of analytes showing higher sensitivity in either DIA-IMS or DIA mode.

factor was 100 (mephetermine). DIA-IMS mode was 2–10 times more sensitive than DIA only for six compounds (i.e. terbutaline, ortetamine, fenetylline, pemoline, fenbutrazate, and furfenorex).

Similar results were also obtained for urine samples. Indeed, comparable LDC values were found in both modes for 51% of compounds. DIA had higher sensitivity for 32% of substances, up to a factor of 10 for 14 compounds, and with the highest factor equal to 5000 for octopamine. In this case, loss of water molecule was observed when the TWIMS cell was activated, suggesting that fragmentation in the mobility cell might explain this sensitivity loss. On the other hand, DIA-IMS was more sensitive for 17 compounds, usually by factor 5–10.

Method performance of Group II doping agents showed comparable results to Group I substances. Equal sensitivity was observed for 60% and 47% of the compounds in standards and urine samples, respectively. Lower concentrations were detected in DIA in 20% and 41% of the cases in standard and urine samples, respectively. DIA-IMS was more sensitive for 17 compounds in standard samples and for 11 compounds in urine, as shown in Fig. 5.

The MRPL is the minimum concentration of prohibited non-threshold substances, metabolites and/or markers that laboratories should be able to reliably detect and identify [20]. The MRPL criteria are established by WADA to harmonize the performance of accredited anti-doping laboratories. Therefore, the method developed for the determination of doping agents in urine samples was finally assessed for compliance with these criteria. For our instrument, 82% of compounds from both Groups of doping agents met the MRPL in the DIA-IMS mode. Only a slightly higher number of compounds complied with MRPL in DIA mode, i.e. 85% and 89% of compounds from Groups I and II, respectively. Since both modes showed similar results, DIA-IMS could be used instead of DIA for initial testing procedure purposes in the anti-doping context, enabling a higher degree of confidence in targeting the substances of interest by having access to the CCS values as an additional parameter for result interpretation.

3.3. *Effect of the processing method*

The original processing method used for the evaluation of all doping agents contained the library of all Group I or II compounds and used the following settings: 5 ppm, 50, and 100 counts for intensity in MS/MS and MS spectra, respectively. In some cases, incorrect compound annotations (i.e. misidentifications) were observed using this processing method and some samples returned positive hits for analytes that were not present in the sample.

The number of misidentifications (NoM) was determined as the number of compounds incorrectly identified as positive. For example, a sample was spiked with 30 compounds from Group II, but 35 compounds were identified in this sample, so the NoM is 5. Some of these wrong assignments were observed only at the high concentration levels of the spiked compounds. In some cases, these misidentified substances were chemically related to the analysed compounds, such as metabolites or derivatives. On the other hand, most misidentifications were completely different compounds originating from interfering signals and found at any concentration. Fig. 6 summarizes the comparison of the number of misidentified compounds in each Group when measured in DIA-IMS and DIA modes. Overall, a significantly higher number of misidentified compounds was detected in Group II, especially in urine samples with an almost 2-fold higher number of misidentifications in DIA. These experimental data highlight the potential of IMS in reducing the prevalence of false positive hits. Despite this, the number of incorrectly identified compounds was still significantly high, especially in Group II with around 20 misidentifications for analyses of each mixture. To improve the applicability of the approach and reduce the NoM, the settings of the processing method were further investigated. A list of the parameters that were evaluated in a systematic way is presented in Table 1.

Misidentified compounds were found in both standard and urine samples. As expected, due to the complexity of the biological matrix, they were present in a higher number in urine samples by a factor of 2–5, depending on the analyte of interest. Due to the application-oriented interest of the method, only results obtained with urine samples will be shown and discussed here. The number of misidentifications using the tested processing methods are presented in Fig. 7. Since similar trends were observed at all concentration levels, only the results obtained with samples spiked at 10 ppb are shown for simplicity.

Generally, the same compounds erroneously identified in DIA-IMS were also incorrect in DIA mode. Additional compounds were also incorrectly identified in DIA mode. Thus, the number of misidentifications in DIA mode was 2 times higher than in DIA-IMS mode, whatever the concentration levels demonstrating the benefit of considering the CCS values to increase selectivity and

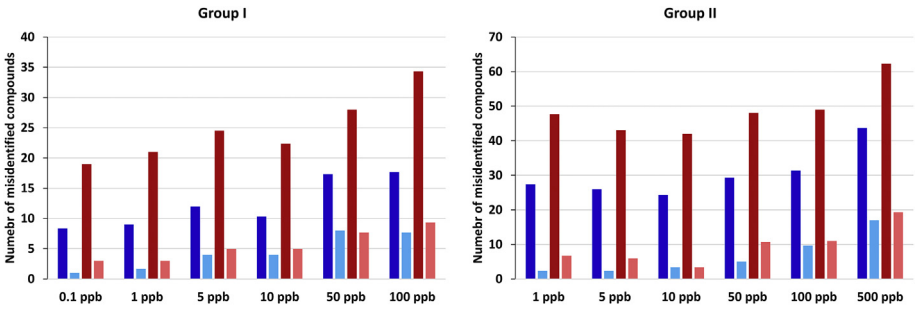

Fig. 6. Comparison of the number of misidentified compounds for Group I and Group II compounds in standard (lighter colours) and urine samples (darker colours), using DIA-IMS (blue) or DIA (red) modes, at different concentration levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Settings of the different processing methods.

Method name	mass accuracy (ppm)	MS/MS intensity threshold (counts)	MS intensity threshold (counts)	expected fragments
5 ppm	5	50	50	–
expected fragments	5	50	50	**min 2 out of 3**
3 ppm	**3**	50	50	–
T 2x	5	**100**	**200**	–
T 10x	5	**500**	**1000**	–

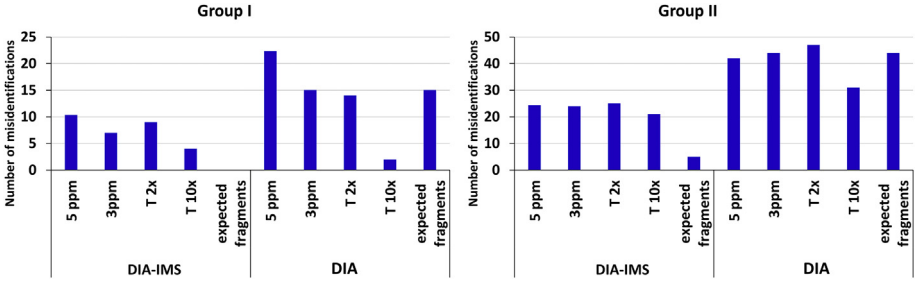

Fig. 7. Comparison of the number of misidentified compounds in (A) Group I and (B) Group II doping agents measured at 10 ppb in urine samples using 5 different processing methods.

significantly reduce the rate of false positive screening hits. In Group II doping agents, several compounds were detected as false alarms in most of the urine samples, i.e. boldenone, methyldienolone, calusterone and its metabolites, and metabolites of nandrolone-18-methyl, norbolethone, and norethandrolone, mainly due to the structural similarity and high abundance of diverse endogenous steroids in human urine.

The mass accuracy tolerance and the signal intensity threshold (minimum signal value to discriminate between “noise” and “peak”) were then investigated. Changing the mass accuracy tolerance setting from 5 to 3 ppm improved the selectivity of both compound Groups, but especially for Group I compounds measured in DIA mode. Similar results were obtained by increasing the signal intensity threshold 10 times, but in this case, the effect was more visible for Group II substances in both modes. On the other hand, increasing the intensity threshold by factor 2 instead exhibited almost no effect on method selectivity.

Among the investigated method parameters, the application of expected fragments as an additional assignment feature proved to be very useful in enhancing selectivity among the tested methods. A list of so-called expected fragments for each analyte was specified containing *m/z* values of fragments that the scientist assigned as characteristic for each specific analyte. Usually, the most abundant fragments in MS/MS spectra of reference standards are used as expected fragments. However, when reference standards are not available, the usage of theoretical fragments is advantageous as the most abundant theoretical fragments of each compound can be used as expected fragments. To obtain the list of expected fragments in this study, the combination of these two approaches was used. The three highest abundance theoretical fragments generated as described in section 2.5 and detected in standard solutions were selected and then included in the processing method. For compounds where three expected fragments could be included in the list, a minimum match of two out of the three was set as the criterion for the correct assignment of doping agents. For the compounds where only one or two expected fragments could be included in the library, a single expected fragment match was considered sufficient for confirmation. In this case, the number of misidentifications was close to 0 for Group I and lower than 10 for Group II. Unfortunately, no fragments could be detected at the lowest concentration levels for most compounds, and thus, the positive effect on method selectivity by their inclusion in the processing parameters was significantly reduced at these concentrations.

The effect of the processing method settings on the detection limits (LDC) was also investigated. Decreasing the mass accuracy tolerance from 5 to 3 ppm led to filtering of approximately 20% of the compounds at their lowest concentrations, particularly for

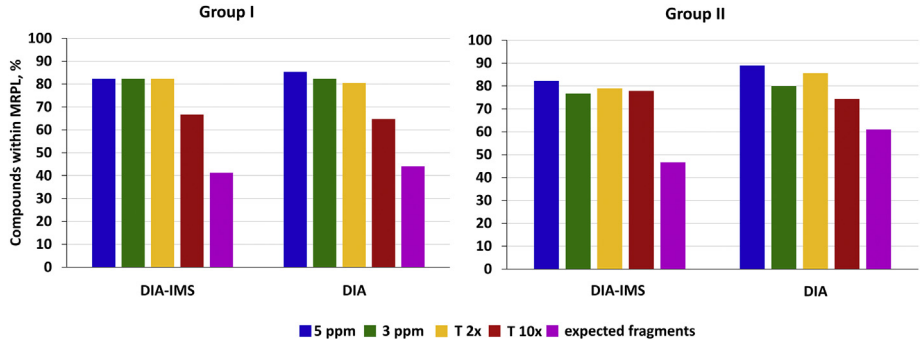

Fig. 8. Comparison of the number of compounds detected at the WADA MRPL (expressed as a percentage) as a function of the different processing methods.

Group II compounds in DIA mode. Increasing the intensity thresholds by a factor 2 had similar effects, filtering out 15% of the compounds. Opposite to narrowing the mass accuracy tolerance, the increase in intensity threshold setting had a more pronounced effect on Group I compounds. A 10 times higher intensity thresholds (T 10x) led to higher LDC for 75% of Group I compounds and 50% of Group II compounds. The applications of expected fragments as confirmation feature for correct assignment caused an even more critical drop with higher LDC for almost 90% of the compounds.

In anti-doping testing, the compromise between sensitivity and selectivity needs to be linked to the WADA MRPL requirements in the ITP ("screening"). The objective is to obtain sensitivity, which is sufficient for targeting all the "true" positive samples from the large population of samples with a manageable number of "false" positive alarms brought forward to the confirmation procedure. Fig. 8 shows the percentage of compounds detected at the MRPL as a function of the processing method used. Similarly to the previously described effects of each processing method, the most significant drop was observed for the processing method that included expected fragments as the assignment criteria. However, using a 3 ppm mass accuracy tolerance showed almost the same MRPL compliance as the original method with a 5 ppm tolerance, while the 2 times higher threshold settings (T 2x) led to only a slight decrease. Therefore, the combinations of the latter two approaches should be optimal, considering both the number of misidentifications and sensitivity using Q-ToF instrumentation in this study with urine samples.

3.4. Analysis of urine samples from administration studies

Excretion study samples were analysed to demonstrate the fitness for purpose of the developed UHPLC-IM-HRMS method for the detection of the target analytes in authentic urine samples. The specificity of the method was tested using samples collected at T0, i.e. before administration, that were available for several doping agents. In the collected excretion samples, target analytes and representative metabolites were determined in both DIA-IMS and DIA modes. All pre-administration samples were tested negative for the target analyte (Table 2), whereas in post-administration samples, ephedrine, methylephedrine, methadone (parent and its metabolite EDDP), metabolites of cocaine (benzoylecgonine and methylecgonine), and stanozolol metabolites (16β-hydroxystanozolol and 4β-hydroxystanozolol) were detected by both modes of analysis. However, a significant number of other compounds (up to 40) were identified in addition to the target analytes, especially in samples containing Group II substances, depending on the processing method. Table 2 shows the corresponding results for all tested processing methods. As these misidentifications could lead to false positive screening results in doping control, correct settings of the processing method are crucial for efficient routine analysis.

The addition of expected fragments feature in the processing method led to the lowest number of misidentifications. However, it also caused a significant decrease in sensitivity for some compounds corresponding to non-compliance with MRPL, as discussed in section 3.3. Indeed, nikethamide metabolite and cocaine could not be detected in the excretion samples when the expected fragments were included in the processing method, even though they were detected using the other processing methods in both DIA-IMS and DIA mode. The same applies for the T 10x processing method in DIA-IMS mode. In DIA, the 10 times higher intensity thresholds processing method did not permit the detection of MDMA and cocaine. Except for these compounds, the other target analytes were always detected in the administration samples regardless of the processing method used. Similarly to results previously discussed in Section 3.3, 3 ppm and T 2x processing methods enabled the correct identification of all target analytes and a significant decrease of misidentified compounds. Therefore, the obtained results were evaluated using a combination of these two approaches in the last step. The number of misidentifications was indeed lower. However, stanozolol could not be detected in DIA mode as well as ephedrine, MDMA, and one of cocaine metabolites in DIA-IMS mode. For Group I compounds, lowering the dilution factor during the sample preparation could enable the detection of these compounds with the proposed processing method and adequate selectivity.

4. Conclusion

In this work, two HRMS acquisition modes were compared, namely DIA and DIA-IMS, which added an IMS dimension to the acquisition method using TWIMS technology. The applicability of these two strategies was evaluated for a set of 192 representative substances prohibited in sports and their metabolites in both standard solutions and urine.

Firstly, the effect of activating the IMS cell on fragmentation was investigated, and the ratio of detected *vs.* theoretical fragments was assessed for each compound. It appeared that the fragmentation of a wide range of Group I doping agents, i.e. 102 compounds, stimulants, diuretics, narcotics, and β2-agonists, was similar in DIA-IMS *vs.* DIA, with 73–89% of theoretical fragments observed. On the other hand, the rate of detected fragments increased almost by a factor 2 for Group II doping agents, i.e. steroids, glucocorticoids, and hormone and metabolic modulators. This could be caused by several factors such as filtration of interferences and a subsequently increased sensitivity and/or by induced fragmentation in the ion mobility cell due to heating. The MS sensitivity was also evaluated

Table 2
Target analytes in administration samples with the number of misidentified compounds in each sample evaluated by different processing methods. For the description of the processing methods, see Table 1. DA – target doping agent, NoM – number of misidentifications, ✓ - target doping agent detected in the sample, x - target doping agent not detected in the sample.

Compound	sample	DIA-IMS								DIA											
		5 ppm		frag		3 ppm		T 2x		T 10x		3 ppm + T 2x		5 ppm		frag		3 ppm		T 2x		T 10x		3 ppm + T 2x	
		DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM	DA	NoM
ephedrine + methylephedrine	T0	x	0	x	0	x	0	x	0	X	0	x	0	x	6	x	1	x	3	x	4	x	0	x	1
	T5	✓✓	1	✓✓	0	✓✓	1	✓✓	0	✓✓	0	✓x	0	✓✓	4	✓✓	2	✓✓	3	✓✓	3	✓✓	1	✓✓	3
atenolol	T0	x	0	x	0	x	0	x	0	X	0	x	0	x	4	x	0	x	4	x	4	x	0	x	4
	T3	✓	1	✓	0	✓	1	✓	1	✓	0	x	0	✓	7	✓	2	✓	7	✓	3	✓	1	✓	2
MDMA	TX	✓	0	✓	0	✓	0	✓	0	✓	0	✓	0	✓	5	x	1	✓	4	✓	3	x	2	✓	1
niketamide metabolite	TX	✓	0	x	0	✓	0	✓	0	X	0	✓	0	✓	4	✓	1	✓	3	✓	1	✓	0	✓	1
methadone + EDDP	TX	✓	0	✓	0	✓	0	✓	0	✓	0	✓	0	✓	2	✓	0	✓	1	✓	0	✓	0	✓	0
cocaine +2 metabolites	T0	x	0	x	0	x	0	x	0	X	0	x	0	x	4	x	0	x	2	x	1	x	0	x	0
	T9	✓✓✓	0	✓✓x	0	✓✓✓	0	✓✓✓	0	✓✓x	0	✓✓x	0	✓✓✓	3	✓✓x	0	✓✓✓	2	✓✓✓	2	✓✓x	0	✓✓✓	1
stanozolol +2 metabolites	T10	✓✓✓	12	✓✓✓	2	✓✓✓	7	✓✓✓	11	✓✓✓	9	✓✓✓	7	✓✓✓	38	✓✓✓	14	✓✓✓	29	✓✓✓	31	✓✓✓	20	✓✓✓	25
	T17	✓✓✓	7	✓✓✓	1	✓✓✓	4	✓✓✓	6	✓✓✓	6	✓✓✓	4	✓✓✓	32	✓✓✓	11	✓✓✓	28	✓✓✓	30	✓✓✓	18	✓✓x	21
methylprednisolone	T2	✓	11	✓	2	✓	7	✓	11	✓	8	✓	6	✓	33	✓	9	✓	31	✓	29	✓	16	✓	26
clenbuterol	T0	x	15	x	3	x	11	x	15	X	10	x	11	x	36	x	9	x	32	x	31	x	20	x	27
	T8	✓	14	✓	5	✓	9	✓	14	✓	10	✓	8	✓	40	✓	11	✓	33	✓	35	✓	24	✓	28

with and without IMS, and comparable results were obtained for about 50% of the tested substances. For the rest of the compounds, sensitivity was slightly reduced with IMS dimension with only a few exceptions. More importantly, the MRPL criteria required by WADA were met for 82% of doping agents in urine from both Groups in the IMS mode. In terms of number of false alarms, a relatively high number of substances from Group II were problematic, particularly when the IMS cell was not activated which led to an almost two-times larger number of incorrectly assigned substances. This demonstrates the benefit of IMS in reducing false positives in routine doping control analysis. Finally, the method was applied to the analysis of several excretion urine samples obtained from administration studies. The results showed the importance of an adequately selected processing method for correct analyte recognition to reduce incorrect assignments.

CRediT authorship contribution statement

Kateřina Plachká: Writing – original draft, Methodology, Investigation. **Julian Pezzatti:** Methodology, Investigation, Writing – review & editing. **Alessandro Musenga:** Resources, Writing – review & editing. **Raul Nicoli:** Resources, Writing – review & editing. **Tiia Kuuranne:** Resources, Writing – review & editing. **Serge Rudaz:** Resources, Writing – review & editing. **Lucie Nováková:** Writing – original draft, Methodology, Investigation. **Davy Guillarme:** Supervision, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the STARSS project (Reg. No. CZ.02.1.01/0.0/0.0/15_003/0000465) co-funded by ERDF. The authors wish to thank Prof. Jean-Luc Veuthey from the University of Geneva for his fruitful comments and discussions.

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