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RESEARCH ARTICLE

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Ultra-fast retroactive processing of liquid chromatography high-resolution full-scan Orbitrap mass spectrometry data in anti-doping screening of human urine

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Rationale: Retroactive analysis of previously tested urine samples has become an important sports anti-doping tool. Retroactive reprocessing of old data files acquired from a generic screening procedure can reveal detection of initially unknown substances, like illegal drugs and newly identified metabolites.

Methods: To be able to efficiently search through hundreds to thousands of liquid chromatography high-resolution full-scan Orbitrap mass spectrometry data files of anti-doping samples, a combination of MetAlign and HR_MS_Search software has been developed. MetAlign reduced the data size *ca* 100-fold making possible local storage of a massive volume of data.

Results: The newly developed HR_MS_Search module can search through the reduced data files for new compounds (mass or isotope pattern) defined by mass windows and retention time windows. A search for 33 analytes in 940 reduced data files lasted 10 s. The output of the automatic search was compared to the standard manual routine evaluation. The results of searching were evaluated in terms of false negatives and false positives. The newly banned b2-agonist higenamine and its metabolite coclaurine were successfully searched in reduced data files originating from a testing period for which these substances were not banned, as an example of retroactive analysis.

Conclusions: The freeware MetAlign software and its automatic searching module HR_MS_Search facilitated the retroactive reprocessing of reduced full-scan high-resolution liquid chromatography/mass spectrometry screening data files and created a new tool in anti-doping laboratories' network.

1 | INTRODUCTION

The World Anti-Doping Agency (WADA) is the worldwide leading body in human sports anti-doping control. Doping in sports is defined by the List of Prohibited Substances with Performance Enhancement Capability, published annually by WADA.[1] The List makes reference to prohibited pharmacological actions related to pharmacological classes, while the prohibited drugs named therein are included as examples and it does not constitute an exhaustive list. The drugs of the List can be characterized as peptides or small molecules. WADA Accredited Laboratories perform the initial testing procedure (ITP) or screening of small molecules using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS), in parallel for all urine samples, to comply with the analytical needs

created by the List. For the LC/MS part of ITP, the analysis performed using Orbitrap high-resolution full-scan LC/MS (LC/HR-FS-MS) has particular advantages, because (1) of the sensitive qualitative detection of the analytes, (2) the full-scan MS acquisition can be conducted in both positive and negative ionization polarity within a single analysis for all analytes, and (3) it can be combined in future with quantitative intact Phase II metabolite analysis.[2-4] Because of its untargeted nature, this technology may not only help in ITP of a large list of known doping substances, but also acquire MS signals from doping substances that are not known yet, but belong to the prohibited pharmacological classes of the List.

Due to the technological advances and increasing knowledge of metabolism, new slowly excreted metabolites of known anabolic androgenic steroids (AAS) were discovered; these long-term metabolites allow the possible detection of AAS abuse for a longer time after the administration has been stopped, compared to the previously known metabolites.[5-8] Furthermore, new designer drugs – molecules outside the official pharmaceutical system – have been produced and marketed escaping national legislations.[9-11] It has been proven that the reanalysis of old samples previously reported as negatives has resulted in reporting more positive cases for doping use than the initial testing.[12,13]

Consequently, the possibility of retesting stored negative samples, when new pharmaceutical and metabolic knowledge is available, is an important anti-doping tool that may act as a deterrent against doping. However, this creates a logistical problem, since the reanalysis (from sample preparation and instrument analysis to data review and detection) of all the available stored urine samples from the past for each new substance is virtually impossible, because the sample urine volume is limited and the needed human, material and instrumental resources are important.[3,14] In practice, only specifically selected samples can be retested.[12,13] If, however, initially the data have been acquired in full-scan high-resolution mode, it might suffice to only reprocess the previously acquired LC/HR-FS-MS data files, since they contain all non-fragmented compound information that MS can acquire.

The use of HR-FS-MS with LC and GC in anti-doping has been reported and applied recently.[2,3,15-19] However reprocessing of thousands of data files is also a logistically difficult task, because the size of high-resolution MS data files per sample is in the range 100–500 MB. This task requires a strong information technology (IT) infrastructure, which is not usually available to the WADA Accredited Laboratories. In addition, conventional manual processing of thousands of data files requires substantial human resources for the creation of ion chromatograms and visual evaluation.

A solution for performing fast reanalysis of large numbers of data files is to use preprocessed size-reduced data files in which all essential analytical information of substances is still available. This size reduction can be obtained by eliminating noise and baselines from chemical background followed by peak-picking. The previously described MetAlign software, which has a long history in the analysis of metabolomics LC and GC MS/MS data, can perform this task as part of its use in processing untargeted metabolomics data of several formats.[20-22] Herein, its use to obtain a 100- to 1000-fold data reduction of raw data files is presented. Obtaining such size-reduced data makes feasible local storage of thousands of LC/HR-FS-MS files on a solid-state drive (SSD). Because the size-reduced files do not need any further preprocessing, retrieving information – such as mass, intensity, retention time – from hundreds to thousands of files is a very fast exercise.[23] A new module called HR_MS_Search was developed in the study reported here, which allows one to search for isotope pattern matches for multiple substances simultaneously in hundreds to thousands of reduced data files in the redms_acc format, which is the output format of MetAlign of the size-reduced pre-processed LC/MS data. This HR_MS_Search software is now part of the MetAlign software suite.

The study reported here examined the performance of MetAlign data reduction and subsequent searching by the HR_MS_Search module for LC/MS ITP retroactive reprocessing. The goal was the use of this software for searching of large numbers of reduced data files from ITP LC/HR-FS-MS, in order to prioritize samples for confirmation. The study used existing data files from routine ITP analysis at ADLQ. The searching performance was evaluated using the risk rate of false negatives and false positives as main criteria. In ITP, the main interest of the WADA Accredited Laboratories is to eliminate false negatives (i.e. evaluating positive samples as negative). On the other hand, too many ITP false positive samples (i.e. evaluating negative samples as positive) creates an additional unnecessary workload of additional investigation analyses and a waste of laboratory resources to prove that a sample was negative. Therefore, the study focused not only on the speed and the ease-of-use of the HR_MS_Search module, but also on how to minimize ITP false negatives and false positives when using automatic searching, in order to assess this retroactive analysis tool in ITP. The overall MetAlign evaluation was conducted as a new anti-doping deterrent tool.

2 | MATERIALS AND METHODS

2.1 | Samples, materials, instruments and data acquisition

ITP sample data files after LC/HR-FS-MS analysis were used in the current study. They were originated from the routine screening analytical data of ADLQ. The detailed sample preparation, reference materials and instrumental LC/MS analytical procedure are described elsewhere.[2] Sample preparation was conducted as follows: 5 mL of urine aliquots, spiked with internal standard solution, were applied for enzymatic hydrolysis using 100 µL of β-glucuronidase from *E. coli* at pH 7, adjusted by addition of 1 mL of phosphate buffer. The hydrolysis was conducted at 50°C for a duration of 1.5 h. After cooling, the aliquots were extracted with 5 mL of ethyl acetate at pH 9–10 adjusted by a solid mixture $NaHCO_3$–Na_2CO_3 (10:1 w/w). After extraction, centrifugation and the separation of the organic from the aqueous phase, the organic layer was evaporated under a stream of nitrogen at 40°C, reconstituted with 200 µL of reconstitution solvent (mobile phase A/B 80:20 v/v) and mixed with 20 µL of the

non-processed original urine to the final extract. An amount of 5 µL of the mixture was injected into the LC/MS system. The addition of the non-extracted urine part to the reconstituted extract was important for the detection of small molecules that are not extracted with the applied extraction protocol, such as melidonium, ethylglycuronide, FG4592 and other HIF stabilizers, AICAR, finasteride carboxylic acid metabolite, ritalinic acid, dextran and hydroxyethylstarch.

The LC/MS analysis[2] was performed using a Dionex UHPLC system (Thermo Scientific, Bremen, Germany) equipped with a Q Exactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific). The chromatographic separation was performed using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm i.d., 1.8 µm particle size; Agilent Technologies, Santa Clara, CA, USA). Water containing 5 mM $HCOONH_4$ and 0.02% (v/v) HCOOH (solvent A) and a mixture of acetonitrile–water (90:10 v/v) containing 5 mM $HCOONH_4$ and 0.02% formic acid (solvent B) were used as mobile phase solvents. The chromatographic programme was modified from Abushareeda et al[2] as follows. The total analysis run time per sample was 20 min with a constant flow rate set at 0.2 mL min^{-1} throughout the entire run. The initial conditions were set to 95% A and 5% B for the first minute of the run. Solvent B was then increased to 90% at 9 min, followed up to 100% at 11 min after which it remained constant for 3 min. After 14 min, solvent B was reduced to 5% within 30 s to start the post-run equilibrium for the remaining duration of the 20 min run.

The mass spectrometer was equipped with a heated electrospray ionization source (HESI2) and operated with positive–negative polarity switching full-scan MS acquisition mode.[2] Nitrogen was used as sheath gas, ion sweep gas and auxiliary gas. The ion spray voltages were set to 4000 V in positive mode and 3800 V in negative mode. The settings of the Orbitrap for the FS acquisition were as follows: scan range *m/z* from 100 to 1000 at 17 500 resolving power, automatic gain control target set at 10^6 and duty cycle at 100 ms.

In Table 1, the 33 analytes examined in the current study are shown together with their preferred detection polarity ionization mode. Those analytes were incorporated by spiking in the positive quality control (QC) samples prepared in each analytical batch at a concentration level corresponding to 50% of the minimum required performance limit (MRPL) level (see also Table 1).[4] The analytes included herein were considered representative for the more than 300 analytes included in the LC/MS ITP at ADLQ and belong to various drug classes, such as AAS, b2-agonists, narcotics, diuretics, stimulants, and others.[2]

In this study a total of 940 data files were processed, which originated from 20 ITP analytical batches of ADLQ. The samples were analysed over a period of several months using two instruments with identical LC/MS configuration and method settings (discussed in paragraphs above).[2] Retention time differences between the two instruments were less than 0.1 min. Of the total 940 data files, 860 were routine athlete urine samples, 20 were blank negative QC urine samples and 60 were positive QC spiked urine samples. Fifty-three of the 860 urine samples were manually evaluated as containing one or more of the substances of Table 1 (also present in the QC samples).

TABLE 1 List of substances used in this evaluation study

Substance	QC (ng ml^{-1})	Polarity
3′-Hydroxystanozolol	1	+
4-Hydroxystanozolol	1	+
16-Hydroxystanozolol	1	+
4-Methylhexaneamine	50	+
Thiazides ACB artefact[a]	100	–
Amphetamine	50	+
Benzoylecgonine	50	+
Boldenone	2.5	+
Cannabis (THCCOOH)	75	–
Canrenone	50	+
Chlorothiazide	50	–
4-Hydroxyclomiphene	10	+
Codeine	5	+
Ephedrine	50	+
Furosemide	50	–
Gestrinone	2.5	+
Hydrochlorothiazide	50	–
Indapamide	50	+
Letrozole metabolite[b]	10	–
Methylphenidate	50	+
Methandienone long-term metabolite[c]	1	+
Pemoline	50	+
Pentazocine	25	+
Raloxifene	10	+
Ritalinic acid	50	+
Salbutamol	10	+
Tamoxifene metabolite[d]	10	+
Testolactone	10	+
THG[e]	2.5	+
Tramadol	25	+
Epitrenbolone	2.5	+
Trenbolone	2.5	+
Trimetazidine	10	+

QC = concentration in QC spiked sample; polarity = polarity used for substance detection.

[a]4-Amino-6-chloro-1,3-benzenedisulfonamide.

[b]Bis(4-cyanophenyl)methanol.

[c]18-Nor-17B-hydroxymethyl,17A-methylandrost-1,4,13-trien-3-one.

[d]3-Hydroxy-4-methoxytamoxifen.

[e]Tetrahydrogestrinone: 17-hydroxy-18A-homo-19-nor-17A-pregna-4,9,11-trien-3-one.

2.2 | Standard ADLQ LC/MS data processing setup for ITP

For each routine ITP analytical batch, the generated ion chromatograms were manually reviewed by two analysts, after grouping printouts per analyte[24] for all the samples of the batch (maximum 60 samples including QC). The printouts were generated by applying an extraction mass window of ±5 ppm. The reviewing

was based on experience in evaluating criteria for analyte detection by direct comparison to QC signals. The applied criteria were based on: (a) peak abundance sufficiently above the noise and background to facilitate a probable confirmation follow-up procedure, (b) the retention time to match with the QC sample and (c) applicable ion ratios within analyte to match. Data acquisition files were stored on instrument computers, until backed up on a weekly basis by the EMC Avamar server located in the ADLQ data centre. Backups were kept on hard drives and off-loaded to tapes on an annual basis. The retrieval of old backed-up data files was accomplished through the same EMC Avamar software.

2.3 | Data analysis protocol and hardware for MetAlign software suite

2.3.1 | Hardware

Although MetAlign runs on any modern PC with a Windows 7 64-bit platform or better,[21] here it was run on a hyper-threaded 16-core PC (32 virtual cores; 3 GHz; 64 GB RAM) equipped with a solid state disc under a Windows 7 64-bit operating system. HR_MS_Search was run on a PC (W7 64-bit) equipped with a SSD.

2.3.2 | MetAlign software and settings

The MetAlign software used in this study is freeware. MetAlign settings specific for ADLQ data were used for the size reduction of the positive and negative mode data. Positive and negative mode data within the same original data file were processed separately. Currently up to 1000 data sets can be processed per batch; 940 ADLQ files in the present configuration took *ca* 4 h per polarity mode to process for the size reduction. The size reduced data files were stored on a SSD in a folder structure in a way comparable to how the original raw data files were stored by date. A thorough description of MetAlign software data reduction algorithm can be found elsewhere.[20]

2.3.3 | HR_MS_Search

HR_MS_Search was used as freeware searching module together with typical Excel-compatible input and output sheets. An Excel-compatible search template contained information per substance to be searched, i.e. accurate masses, retention times, mass error window (7.5 ppm), retention time window (± 0.1 min). It was therefore possible to define a search for isotope patterns. The Excel-compatible output provided all the information present in all the searched size-reduced data files. The output for each data file, where the analyte was detected, comprised delta retention, ppm errors for each defined mass, the number of masses including the molecular ion, as well as a match factor for the isotope pattern, in the case of an isotope pattern for a substance being found. Searching 940 size-reduced data files for multiple masses of one substance took approximately 10 s.

2.4 | MetAlign/HR_MS_Search software evaluation protocol

In order to evaluate the performance of the MetAlign approach (Sections 2.3.2 and 2.3.3), the searching output had to be compared to the manual evaluation outlined in Section 2.2 comprising the following parameters: (a) number of QC and routine sample positives detected by MetAlign/HR_MS_Search, where detection was true, i.e. the analyte was detected in the data file and existed in the sample, (b) number of false negatives reported by MetAlign compared to the ADLQ manual evaluation, i.e. the substances existed in the sample/data file but is not detected by MetAlign/HR_MS_Search, and (c) number of false positives compared to the ADLQ manual evaluation, i.e. the substances did not exist in the sample/data file and were included in the MetAlign/HR_MS_Search output file.

Subsequently, the influence of additional filter criteria on false positive and false negative rates was examined. The following filters were individually applied to examine their influence: (a) filter on mass accuracy error smaller than 3 ppm, (b) filter on the existence of a second mass signal where applicable, with the printout accuracy and mass error parameters the same as of the base peak and (c) filter on abundance of signals higher than the 10% of the average QC peak abundance of the same substance.

2.5 | Search for new substance and metabolite (higenamine and coclaurine: added to the WADA List in 2017)

The retroactive reprocessing capabilities of MetAlign were tested with higenamine, which was newly identified as a prohibited b2-agonist substance introduced to the List in 2017. Higenamine and its metabolite coclaurine (methylated higenamine) were obtained as reference standards (TRC, Toronto, Canada) and their solutions were injected into the chromatographic system as described in Section 2.1 to obtain their retention times and detection ions. Both substances were detected in positive mode as protonated molecules (*m/z* 272.1281 for higenamine and *m/z* 286.1438 for coclaurine). All data files were searched for these substances (mass error window of ±5 ppm; retention time window of ±0.1 min); samples containing possible signals only for both substances were selected for follow-up. The selected samples underwent confirmatory analysis, which comprised repetition of the entire confirmatory analysis from the original urine sample, as described in Section 2.1. The LC/MS confirmatory analysis was conducted in both full-scan and targeted MS^2 acquisition mode: for higenamine the precursor ion *m/z* was 272.1281 and the product ions *m/z* were 107.0493, 255.1010, 161.0594, 272.1281, 123.0441, 145.0646; for coclaurine the precursor ion *m/z* was 286.1438 and the product ions *m/z* were 269.1166, 175.0751, 237.0906, 286.1438, 209.0958, 137.0595. The collision energy was 35 eV. The WADA Technical Document for Identification Criteria[25] was applied to confirm the detection or the absence of both substances.

3 | RESULTS AND DISCUSSION

3.1 | General

For the MetAlign/HR_MS_Search approach, the evaluation comprised the following parameters: the required IT infrastructure for the application, the processing speed, the processing performance scores and the reliability of the automatic processing.

3.2 | Comparison of hardware and processing time

3.2.1 | Analysis by normal manual routine

The normal manual ITP (see Section 2.2) for a batch of 40 data files and the usual 300 analytes resulted in the generation of 12 000 ion chromatograms. Careful visual evaluation was needed to evaluate all suspicious signals to minimize false negatives prior to deciding for confirmation by a subsequent independent analysis on a new sample aliquot. The evaluation of 940 data files manually for all 33 analytes using this method herein needed not only the retrieval of the data files from backup media, but also 12 working hours for two senior analysts to evaluate the generated ion chromatograms.

3.2.2 | Analysis by MetAlign/HR_MS_Search (automatic approach)

Data size reduction for local storage on SSD

The size reduction process was a one-time event done in full automation (easily done overnight) and therefore required very minimal human resources. Once the size-reduced data files had been

TABLE 2 List of results based on the manual and automatic (MetAlign\HR_MS_Search) evaluation approach

					Filter: 3 ppm		Filter: second ion		Filter: 10% QC	
Substance	Manual	Auto	FN	FP	FN	FP	FN	FP	FN	FP
3′-Hydroxystanozolol	3	23	0	20	0	8	1	0	0	11
4-Hydroxystanozolol	3	44	0	41	1	14	2	0	0	14
16-Hydroxystanozolol	1	22	0	21	0	6	0	2	0	2
4-Methylhexaneamine	1	36	0	35	0	20	0	0	0	0
Thiazides ACB artefact	2	6	0	4	0	3	0	1	0	0
Amphetamine	3	50	0	47	0	8	0	10	0	1
Benzoylecgonine	1	787	0	786	0	479	0	44	0	233
Boldenone	1	632	0	631	0	457	0	40	0	248
Cannabis (THCCOOH)	4	73	0	69	0	45	0	42	0	9
Canrenone	1	292	0	291	0	135	0	22	0	11
Chlorothiazide	1	31	0	30	0	22	0	12	0	2
4-Hydroxyclomiphene	1	7	0	6	0	2	0	6	0	2
Codeine	19	235	0	216	0	106	0	46	0	13
Ephedrine	13	858	0	845	0	808	0	172	0	82
Furosemide	1	3	0	2	0	1	0	1	0	0
Gestrinone	0	1	0	1	0	1	0	0	0	1
Hydrochlorothiazide	1	44	0	43	0	40	0	40	0	12
Indapamide	1	3	0	2	0	2	0	2	0	0
Letrozole metabolite	1	1	0	0	1	0	0	0	0	0
Methylphenidate	2	450	0	448	0	176	0	6	0	0
Methandienone long-term metabolite	1	585	0	584	0	266	1	17	0	7
Pemoline	0	101	0	101	0	38	0	3	0	0
Pentazocine	0	0	0	0	0	0	0	0	0	0
Raloxifene	0	12	0	12	0	4	0	1	0	1
Ritalinic acid	11	548	0	537	0	250	2	10	0	395
Salbutamol	9	841	0	832	0	638	0	227	0	40
Tamoxifene metabolite	1	7	0	6	0	3	0	1	0	1
Testolactone	16	157	0	141	11	48	0	38	0	45
THG	1	30	0	29	0	7	0	6	0	11
Tramadol	13	745	0	732	0	526	0	117	0	6
Epitrenbolone	0	46	0	46	0	32	0	1	0	1
Trenbolone	0	46	0	46	0	28	0	0	0	2
Trimetazidine	1	88	0	87	0	42	0	1	0	7

Auto = automatic; FN = number of false negatives; FP = number of false positives; 3 ppm = 3 ppm criterion (see text); second ion = second ion criterion (see text); 10% QC = signals >10% QC average (see text).

made, they could be stored in a ready-to-search folder structure. In the current study, 940 data files (20 batches) needed separate size-reduction processing for the positive and negative polarities, which lasted together *ca* 8 h on the hardware configuration described in Section 2.3.1. On average a typical original data file here was 115 MB, while a positive polarity size-reduced file was *ca* 900 KB and a negative one was *ca* 600 KB. Once stored locally in a size-reduced format, the data files can be searched over and over without further processing needs.

Searching database of size-reduced data files

Performing a search with HR_MS_Search for multiple masses from one substance as described in Section 2.3.2 on 940 size-reduced files took about 10 s. This search time scales with the number of files and the number of substances. For example, a search on 10 000 size-reduced files (together *ca* 10 GB) lasted *ca* 100 s.

3.3 | Comparison of automatic and manual approaches

The manual evaluation by two senior analysts was a visually demanding task comparing multiple ion traces simultaneously. The reviewing was based on experience in applying criteria (see Section2.2). The analyst decided if a peak was enough above noise and background to facilitate a subsequent confirmation; furthermore the retention was recorded and, if applicable, ion ratios were taken into account.

The routine ITP manual evaluation was used as reference method. Comparing automatic searching outcome with manual evaluation was therefore biased towards the manual approach. In Table 2, a comparison of results is presented between manual and automatic approaches. First, it was checked if the substances of Table 1 could be found in the QC samples. The MetAlign performance was evaluated by the number of spiked QCs detected in comparison to those found

TABLE 3 Numbers of table in percentages (from Table 2)

	Without filter		Filter: 3 ppm		Filter: second ion		Filter: 10% QC	
Substance	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
3′-Hydroxystanozolol	100	97.9	100	99.1	99.9	100	100	98.8
4-Hydroxystanozolol	100	95.6	99.9	98.5	99.8	100	100	98.5
16-Hydroxystanozolol	100	97.8	100	99.4	100	99.8	100	99.8
4-Methylhexaneamine	100	96.3	100	97.9	100	100	100	100
Thiazides ACB artefact	100	99.6	100	99.7	100	99.9	100	100
Amphetamine	100	95.0	100	99.1	100	98.9	100	99.9
Benzoylecgonine	100	16.4	100	49.0	100	95.3	100	75.2
Boldenone	100	32.9	100	51.4	100	95.7	100	73.6
Cannabis (THCCOOH)	100	92.7	100	95.2	100	95.5	100	99.0
Canrenone	100	69.0	100	85.6	100	97.7	100	98.8
Chlorothiazide	100	96.8	100	97.7	100	98.7	100	99.8
4-Hydroxyclomiphene	100	99.4	100	99.8	100	99.4	100	99.8
Codeine	100	77.0	100	88.7	100	95.1	100	98.6
Ephedrine	100	10.1	100	14.0	100	81.7	100	91.3
Furosemide	100	99.8	100	99.9	100	99.9	100	100
Gestrinone	100	99.9	100	99.9	100	100	100	99.9
Hydrochlorothiazide	100	95.4	100	95.7	100	95.7	100	98.7
Indapamide	100	99.8	100	99.8	100	99.8	100	100
Letrozole metabolite	100	100	99.9	100	100	100	100	100
Methylphenidate	100	52.3	100	81.3	100	99.4	100	100
Methandienone long-term metabolite	100	37.9	100	71.7	99.9	98.2	100	99.3
Pemoline	100	89.3	100	96.0	100	99.7	100	100
Pentazocine	100	100	100	100	100	100	100	100
Raloxifene	100	98.7	100	99.6	100	99.9	100	99.9
Ritalinic acid	100	42.9	100	73.4	99.8	98.9	100	58.0
Salbutamol	100	11.5	100	32.1	100	75.9	100	95.7
Tamoxifene metabolite	100	99.4	100	99.7	100	99.9	100	99.9
Testolactone	100	85.0	98.8	94.9	100	96.0	100	95.2
THG	100	96.9	100	99.3	100	99.4	100	98.8
Tramadol	100	22.1	100	44.0	100	87.6	100	99.4
Epitrenbolone	100	95.1	100	96.6	100	99.9	100	99.9
Trenbolone	100	95.1	100	97.0	100	100	100	99.8
Trimetazidine	100	90.7	100	95.5	100	99.9	100	99.3

in the instrument's reports processed by analysts. In Table 3, a summary of the MetAlign software searching performance of Table 2 can be found in relation to the sensitivity and specificity validation parameters. A total of 714 out of 717 spiked analytes peaks present in the QCs across the batches were detected and only 3 peaks were missed. Investigation of the 3 false negatives in QCs showed that the missed signals were of very low intensities (just above noise levels) for ritalinic acid and clomiphene 4-OH metabolite (4-hydroxy metabolite of clomiphene); consequently they were not considered as false negatives. In Figure 1A, the example of the missed signal of ritalinic acid is presented, which was due to the low peak intensity from material deterioration in dilution condition. Ritalinic acid is a relatively unstable substance in the solution conditions of ADLQ.

In all other non-QC data files, where substances were detected by the manual approach and analytically confirmed, the automatic approach found them too. Therefore the false negative rate in this study was zero (FN in Tables 2 and 3). However, the automatic approach found many more signals that fitted the search profile (mass error window of 7.5 ppm and retention time window of ±0.1 min). These were referred to as ITP false positives (FP in Tables 2 and 3) and were part of the low-intensity background in the data files. In Figure 1 B, the example of the benzoylecgonine signals, which generated a high rate of FP, is presented. The QC blank urine sample in Figure 1B showed a signal in the retention time of benzoylecgonine. For this signal, the analyst was alerted by MetAlign to create a human-driven decision, i.e. whether this signal needed further investigation as suspicious, or could be neglected for reasons outside the automatic searching (see application of filters below).

To see if the number of false positives in the automatic approach could be reduced without increasing false negatives, some extra filters in the Excel-compatible results were applied. When decreasing the mass error window to 3 ppm, the number of false positives was decreased by 37%, but this in turn caused 13 false negatives to appear (from 0 up to 12%). Most false negatives were for testolactone. Careful inspection of original data showed that a near isobaric compound co-eluted with testolactone, which evidently mixed their masses at the given resolution, increasing mass error. A second option was to include signals only having a second isotope present. This approach decreased the number of false positives by 87%, but 6 false negatives then occurred (from 0 up to 5%). These false negatives were from smaller signals for which the second isotope was absent or too close to noise to have a detectable accurate mass signal. The third option was the introduction of an intensity limit based on a reference standard in the QC samples. The cut-off value at 10% of the average signal in the QC samples resulted in an 83% reduction of false positives without any new false negatives occurring (stayed at 0%).

The output spreadsheets could be sorted on hits based on intensities. Therefore, after performing the search on a database of reduced data files, any intensity threshold could be applied. Although the best selection method was based on the relative intensity to QC, additional filtering of the output by taking into account the presence of a second isotope and a more precise mass could also help in narrowing down potential candidate samples for confirmation analysis.

An additional way to select signals was by changing the retention time window. In the automatic approach, the window can be defined

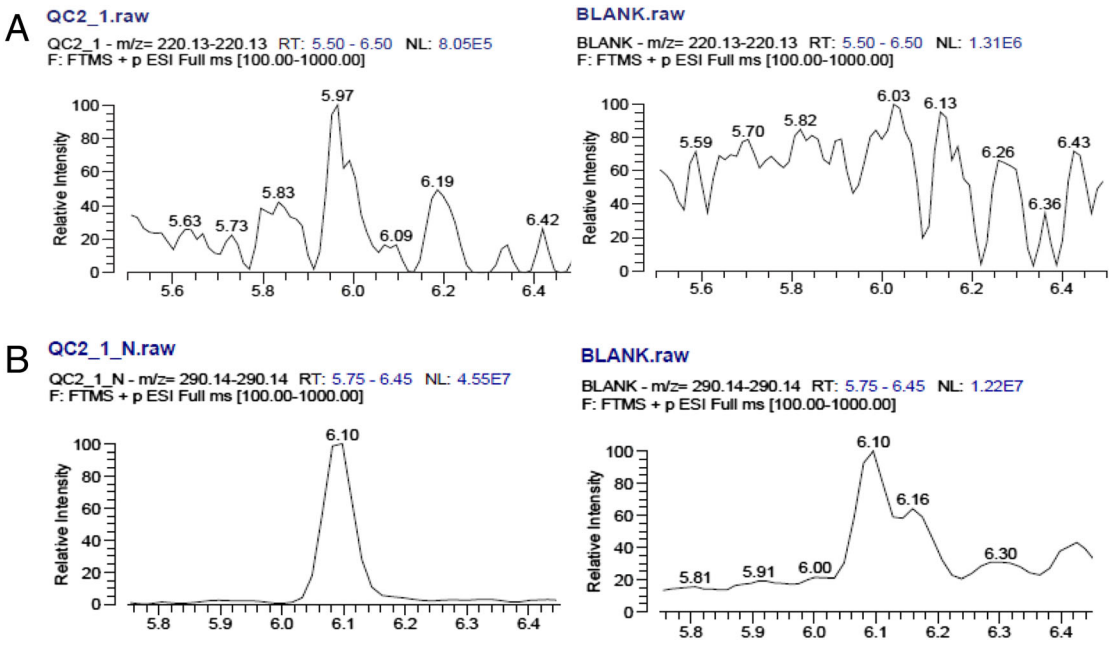

FIGURE 1 A, An example of quality control urine sample spiked with ritalinic acid at 50 ng mL^{-1} (compound with stability problems in solution) in the left ion chromatogram at time of 5.97 min to be compared with a blank urine sample in the right ion chromatogram. B, An example of quality control urine sample spiked with benzoylecgonine at 50 ng mL^{-1} in the left ion chromatogram at 6.10 min to be compared with a blank urine sample provided voluntarily by an ADLQ staff member in the right ion chromatogram, which also comprised a signal at 6.10 min originating from the matrix [Color figure can be viewed at wileyonlinelibrary.com]

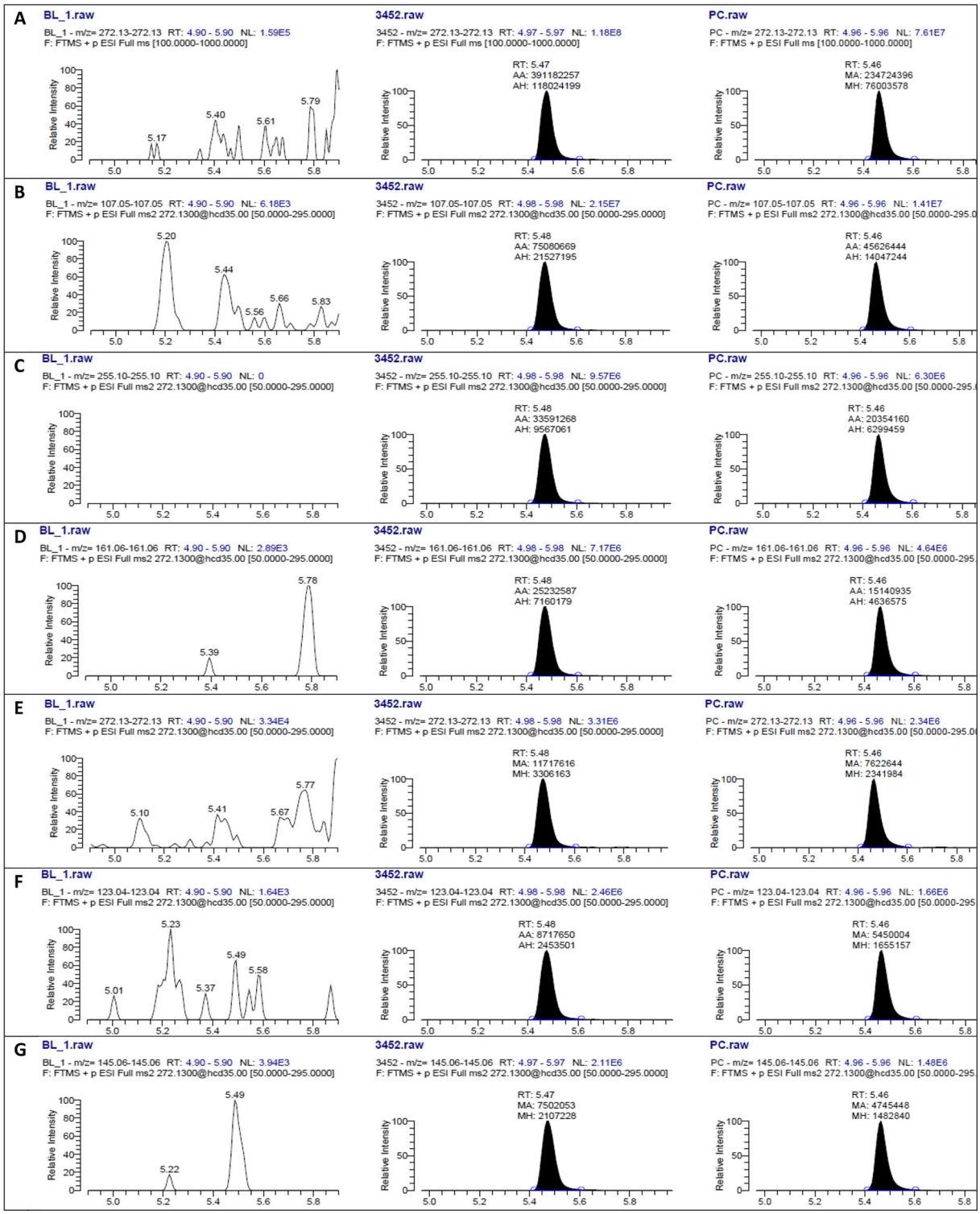

FIGURE 2 Extracted ion chromatograms from the confirmatory procedure of the full scan and the product ions used for the identification of higenamine obtained for a blank urine specimen, the positive case urine sample and the spiked (100 ng mL^{-1}) positive control urine: A, full-scan acquisition ion *m/z* 272.1272, B, ion 1 *m/z* 272.13 to 107.0493, C, ion 2 *m/z* 272.13 to 255.1010, D, ion 3 *m/z* 272.13 to 161.0594, E, ion 4 *m/z* 272.13 to 272.1272, F, ion5 *m/z* 272.13 to 123.0441, G, ion 6 *m/z* 272.13 to 145.0646. Percentages of ion ratios at B–G are used in comparison for compliance to the identification and acceptance criteria of Fragkaki et al[24] between the suspicious sample (first percentage in parentheses) and the positive control urine (second percentage in parentheses): B, (100%, 100%), C, (44.4%, 44.8%), D, (33.3%, 33.0%), E, (15.4%, 16.7%), F, (11.4%, 11.8%), G, (9.8%, 10.6%) [Color figure can be viewed at wileyonlinelibrary.com]

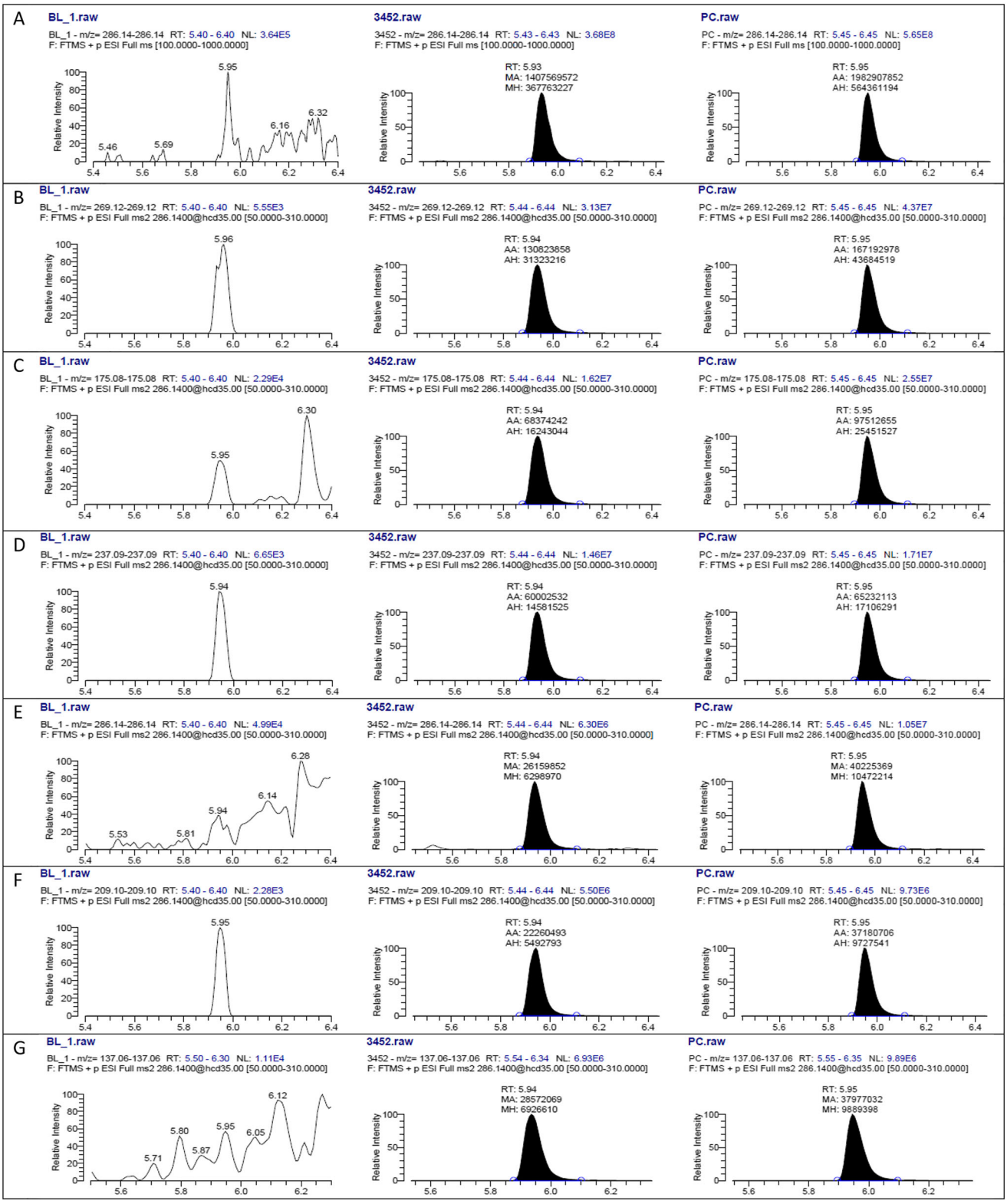

FIGURE 3 Extracted ion chromatograms from the confirmatory procedure of the full scan and the product ions used for the identification of coclaurine obtained for a blank urine specimen, the positive case urine sample and the spiked (100 ng mL^{-1}) positive control urine. The low intensity ion signal of coclaurine detected in the blank urine sample is due to the presence of traces of the compound in almost all urine, as coclaurine can be found in plenty of plant sources: A, full-scan acquisition ion *m/z* 286.1438, B, ion 1 *m/z* 286.14 to 269.1166, C, ion 2 *m/z* 286.14 to 175.0751, D, ion 3 *m/z* 286.14 to 237.0906, E, ion 4 *m/z* 286.14 to 286.1438, F, ion 5 *m/z* 286.14 to 209.0958, G, ion 6 *m/z* 286.14 to 137.0595. Percentages of ion ratios at B–G are used for compliance to the identification criteria of Fragkaki et al[24] between the suspicious sample (first percentage in parentheses) and the positive control urine (second percentage in parentheses): B, (100%, 100%), C, (51.9%, 58.3%), D, (46.6%, 39.2%), E, (20.1%, 24.0%), F, (17.5%, 22.3%), G, (22.1%, 22.6%) [Color figure can be viewed at wileyonlinelibrary.com]

separately for each compound. Care must be taken not to apply retention time windows that are too small, because: (1) pH-dependent shifts in retention time might occur for some compounds that have a pK_a close to the on column pH, (2) some compounds may be so high in concentration that saturation of the column may occur which alters peak shape and may result in peaks broader than the retention time window and with apexes outside the window and (3) retention time may vary up to 0.1 min over time in applied batches. A retention time window of ±0.1 min was a good value for those data files.

3.4 | Real retroactive example: higenamine and coclaurine (see section 2.5)

The HR_MS_Search software was used in order to examine the practicality of the approach to real anti-doping conditions, where retroactive reprocessing was needed. The WADA specifications take into account the protection of clean athletes from cheating athletes and from accusation of a clean athlete for a false doping offense (FP). This aim has formulated the WADA analytical framework to become simultaneously sensitive in detection of doping substances and without any doubt when a doping substance has to be reported. In the retroactive reprocessing aiming to detect new substances, probably under lack of complete human metabolic profile information, the anti-doping laboratories maximize the strictness of the conditions of reporting a positive result. Such a strict approach was followed in case described here, requiring the detection of both parent compound and metabolite in the same sample. Higenamine and coclaurine standards were injected to obtain retention times. Then 940 data files were reprocessed both by HR_MS_Search software and manually via the LCQUAN Thermo software procedure aiming to identify the co-presence of higenamine and coclaurine. The HR_MS_Search reprocessing resulted in 25 findings. Four (of 25) samples were considered as real suspects, since both substances were also found by the manual routine search and for the reasons referred to above in this paragraph. A second sample preparation of the above four suspect samples was performed together with a blank urine sample and a positive control urine sample spiked with higenamine and its metabolite at a concentration of 100 ng mL^{-1}. The reanalysis of the four suspicious samples resulted in the confirmation of the presence of higenamine and its metabolite in one urine in compliance with WADA identification and MRPL criteria[4,25] of reporting. In Figures 2 and 3, the full descriptions of the higenamine and coclaurine confirmatory MS data are found respectively.

3.5 | MetAlign/HR_MS_Search software as quality tool

The ability of HR_MS_Search software to produce a summary from the reduced QC sample data files is also of interest as part of a laboratory quality control scheme. Fast searching could output data from all spiked compounds in QC samples over an extended time period.

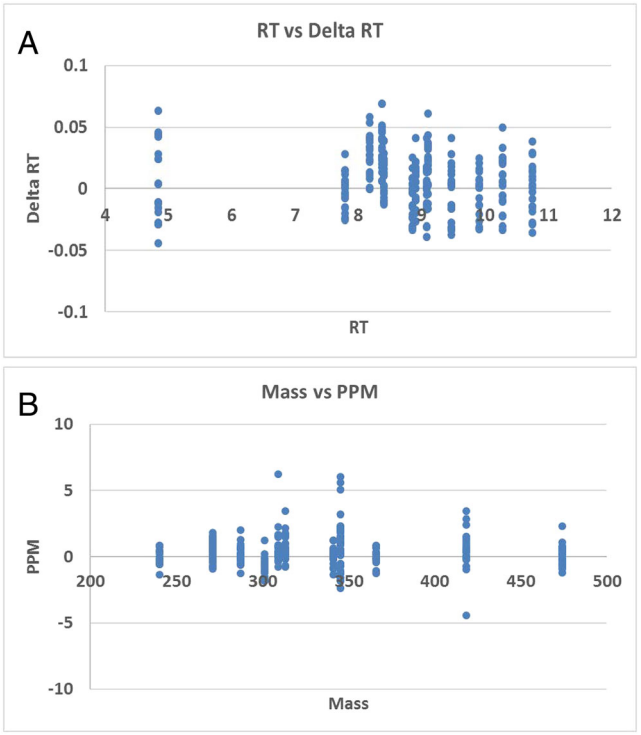

FIGURE 4 Output data from spiked compounds in QC samples over an extended time period using MetAlign/HR_MS_Search. A, Delta retention time versus retention time in minutes. B, Mass error in ppm versus mass range in *m/z* [Color figure can be viewed at wileyonlinelibrary.com]

From this output the extraction of the necessary data to create graphs of delta retention time versus retention time range presented in Figure 4A, as well as mass error in ppm versus mass range of the analytes (Figure 4B) for all the QC compounds, was feasible.

4 | CONCLUSIONS

The present study describes the use of MetAlign/HR_MS_Search software as a tool for retroactive reprocessing in anti-doping analysis for data files acquired in full-scan MS mode by Thermo Orbitrap LC/MS. MetaAlign processing consisting of search of thousands of data files of urine samples can be performed in seconds. This capability, focused on WADA ITP procedures on samples from the past that could contain suspicious signals (of unknown illegal substances or new metabolites), created an important additional value of the old analytical data. Anti-doping laboratories could consider this as a useful tool for selecting samples for a follow-up confirmation analysis. This automated approach performed satisfactorily with regard to having no false negatives compared to the manual routine approach. The searching module created a number of false positives, a usual fact originating from urine background peaks. The rate of false positives could be reduced after application of specific per-substance filter parameters such as mass error ranges, abundance thresholds in the case of signals corresponding to low initial concentration for the substance,

existence of more than one *m/z*, or parameters outside the substance, such as the existence of an additional relevant metabolite. Consequently, the searching parameters could be adjusted per analyte, in order to optimize case-by-case the searching results.

The HR_MS_Search software is run on a standard IT infrastructure and could be used to generate laboratory quality control charts with regard to retention time and mass error precision over time. The wide application of the current approach could result in a substantial improvement of the deterrence in the anti-doping system, in combination with the already applied WADA long-term sample storage policy.

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