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Ultra-fast retroactive processing by MetAlign of liquid chromatography/high-resolution full-scan Orbitrap mass spectrometry data in WADA Human Urine Sample Monitoring Program

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Rationale: The World Antidoping Agency (WADA) Monitoring program concentrates analytical data from the WADA Accredited Laboratories for substances which are not prohibited but whose potential misuse must be known. The WADA List of Monitoring substances is updated annually, where substances may be removed, introduced or transferred to the Prohibited List, depending on the prevalence of their use. Retroactive processing of old sample datafiles has the potential to create information for the prevalence of use of candidate substances for the Monitoring List in previous years. MetAlign is a freeware software with functionality to reduce the size of liquid chromatography (LC)/high-resolution (HR) full-scan (FS) mass spectrometry (MS) datafiles and to perform a fast search for the presence of substances in thousands of reduced datafiles.

Methods: Validation was performed to the search procedure of MetAlign applied to Anti-Doping Lab Qatar (ADLQ)-screened LC/HR-FS-MS reduced datafiles originated from antidoping samples for tramadol (TRA), ecdysterone (ECDY) and the ECDY metabolite 14-desoxyecdysterone (DESECDY) of the WADA Monitoring List. Searching parameters were related to combinations of accurate masses and retention times (RTs).

Results: MetAlign search validation criteria were based on the creation of correct identifications, false positives (FPs) and false negatives (FNs). The search for TRA in 7410 ADLQ routine LC/HR-FS-MS datafiles from the years 2017 to 2020 revealed no false identification (FPs and FNs) compared with the ADLQ WADA reports. ECDY

Safa Khelifi and Khadija Saad contributed equally to this work.

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and DESECDY were detected by MetAlign search in approximately 5% of the same cohort of antidoping samples.

Conclusions: MetAlign is a powerful tool for the fast retroactive processing of old reduced datafiles collected in screening by LC/HR-FS-MS to reveal the prevalence of use of antidoping substances. The current study proposed the validation scheme of the MetAlign search procedure, to be implemented per individual substance in the WADA Monitoring program, for the elimination of FNs and FPs.

1 | INTRODUCTION

The World Antidoping Agency (WADA) is the leading international organization with the aim to fight doping in sports. The World Anti-Doping Code (Code)¹ is a UNESCO International Convention against Doping in Sport and the highest in WADA's rank of documents, that harmonizes antidoping policies and regulations for sports stakeholders. The International Standard of Prohibited List (List)² is one of the pillars of the Code, because it contains the definition of what it is prohibited in sports antidoping. The List is updated annually. Article 4.5 of the Code states: "WADA, in consultation with Signatories and governments, shall establish a monitoring program regarding substances which are not on the Prohibited List, but which WADA wishes to monitor in order to detect patterns of misuse in sport". Based on this Article, WADA established the Monitoring program,³ which is also updated annually. WADA is responsible for the management and publication of both, the List and the Monitoring substances. Another cornerstone of the WADA system is the WADA Accredited Laboratories, which have the role of detecting the substances defined in the List and Monitoring in the athletes' biological samples, mainly urine and blood.⁴ The Accredited Laboratories report their analytical findings electronically to the testing authorities and WADA through the database of the Anti-Doping Administration & Management System (ADAMS).⁵ The analytical findings are characterized either as adverse, in the case of a List related finding that may result in an antidoping rule violation, or as Monitoring program findings without any consequence to the athlete. The Monitoring program findings' statistics are communicated to the WADA Prohibited List Expert Group⁶ for evaluation of which substances of the Monitoring program have to be introduced to the List, which may be removed or continue being monitored.

The Accredited Laboratories perform the Initial Testing Procedure (ITP) or screening of small molecules using Gas Chromatography (GC) and Liquid Chromatography (LC) coupled with Mass Spectrometry (MS) to fulfill List and Monitoring substances analytical specifications. In the case of a List analytical finding in the ITP, the laboratory repeats the analysis using a specific method for the substance confirmatory procedure (CP) in a new aliquot from the original sample. In the case of substance finding from the Monitoring program, a CP is not required.

MetAlign is a software program for the processing and comparison of single-stage full-scan nominal or accurate mass LC/MS and GC/MS data developed by Wageningen Food Safety Research.^{7–10} MetAlign has been studied in Anti-Doping Lab Qatar (ADLQ) for Orbitrap LC/HR-FS-MS data processing.¹¹ MetAlign performs datafile digital information reduction and subsequently searches for substances using the HR_MS_search module. MetAlign has the potential to be used as an antidoping tool for preventive and fast retroactive reprocessing of old datafiles for the discovery of designer drugs or new long-term metabolites for prohibited substances without reporting limits, like anabolic androgenic steroids (AAS), as defined in the WADA Technical Document for the Minimum Required Performance Limits (MRPL).¹² MetAlign searching procedure evaluation criteria are based on correct identification of substances, the false negative (FN) and the false positive (FP) searching results. The FN reports are created when the substances exist in the sample/datafile but they are not detected by the MetAlign HR_MS_search module. The FP reports are created for substances that do not exist in the sample/datafile and are included in the MetAlign/HR_MS_search output file.

The importance of long-term storage of samples and retroactive analysis as doping prevention has been intensified by WADA and International Olympic Committee (IOC).¹³ MetAlign is a tool for the retroactive reprocessing of data to select suspicious samples for the retroactive analysis. FS-HR-MS ITP data, acquired either with Orbitrap^{14,15} or time-of-flight (TOF) mass analyzers,^{16–18} is the requisition of the retroactive data reprocessing. The current study examined the performance of MetAlign as an alternative tool of the WADA Monitoring program data collection. The application was performed on randomly selected official ADLQ samples from 2017 to 2020. Two model substances of the Monitoring program were evaluated in the current study of MetAlign. The first was the narcotic tramadol (TRA), which has been part of the Monitoring program since 2012 (Table 1). The second substance was the steroid ecdysterone (ECDY) with probable AAS activity, which was introduced into the Monitoring program in 2020. MetAlign searching operation was validated for correct identifications using specificity (FP) and sensitivity (FN) of compound detection in two ways: (a) the TRA findings reported in ADAMS by ADLQ during 2017–2020 were compared with MetAlign search reports and (b) the prevalence of the use of ECDY for the years 2017–2020, as determined by MetAlign.

TABLE 1 Summary of WADA Monitoring program 2009–2021

Class	Target	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
S.O. I/O ^a	Bemtil	✓										✓	✓	✓
Anabolic agents I/O	Ecdysterone							✓					✓	✓
Beta-2-agonists I/O	Any combination of beta-2-agonists	✓												
	Salmeterol below min reporting level													✓
	Vilanterol below min reporting level													✓
Stimulants I	Bupropion	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Caffeine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Nicotine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Phenylephrine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Phenylpropanolamine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pipradrol	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Synephrine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pseudoephedrine					✓ ^b								✓
Narcotics I	Codeine	✓								✓	✓	✓	✓	✓
	Hydrocodone	✓				✓	✓	✓			✓	✓	✓	✓
	Tramadol	✓				✓	✓	✓	✓	✓	✓	✓	✓	✓
	Mitragynine			✓					✓					
	Morphine/codeine ratio						✓	✓ ^b						✓
	Tapentadol													
Glucocorticoids I	By routes of administration other than oral, intravenous, intramuscular or rectal	✓						✓	✓	✓	✓	✓	✓	✓
Glucocorticoids O	All routes of administration	✓						✓	✓	✓	✓	✓	✓	✓
Metabolic modulator I/O	Telmisartan							✓ ^b	✓	✓	✓			
	Meldonium													

^aI: In, O: Out of Competition.
^bIntroduction to the List.

2 | MATERIALS AND METHODS

2.1 | Study design

The substances examined in the current study were the narcotic TRA and the steroid ECDY together with its metabolite DESECDY. Between 2017 and 2020, ADLQ performed analysis for more than 18,000 urine samples, originating from countries in Asia, Africa, Europe and Oceania continents. All samples were subjected for the routine ITP, part of which is analysis by LC/HR-FS-MS.^{14,15} In the current study, 7410 LC/HR-FS-MS datafiles from ADLQ ITP samples were randomly selected out of the total 18,000 available and used for searching. After reduction and searching of datafiles in MetAlign, the laboratory sample code identifications were removed to ensure that any result could not be traced back to any athlete. Athlete's consent was not considered mandatory for the inclusion of the samples, as the current study used the datafiles for additional evaluation of analytical testing procedures.

2.2 | Urine sample analysis

TRA and ECDY were provided by Toronto Research Chemicals (TRC, Toronto, Canada) and Steraloids (Newport, RI, USA), respectively. The DESECDY reference urine sample was provided by ADLQ¹⁹ through the World Association of Antidoping Scientists (WAADS) as part of the 2020 proficiency testing scheme. The reference materials, preparation and instrumental ITP LC/MS analytical procedure are described in detail elsewhere.^{14,15} The internal standards used were d3-mefruside, morphine-d3-3 β -D-glucuronide, d3-phendimetrazine and d3-epiandrosterone sulfate (TRC, Toronto, Canada). Briefly, the analytes were extracted from the urine matrix by applying the following procedure: after spiking 5 mL urine with internal standards solution, deconjugation of phase II metabolites by enzymatic hydrolysis of β -glucuronidase from *E. coli* at pH 7 was performed in urine and the pH was adjusted by phosphate buffer for 1.5 h at 50° C. Ethyl acetate extraction of analytes was the next performed step at pH 9–10 adjusted by NaHCO₃:Na₂CO₃ solid salts. After extraction and centrifugation, the organic layer was ice separated, evaporated under a nitrogen stream and reconstituted with 200 μ L of reconstitution solvent, which was mixed with 20 μ L of the non-processed original human urine to facilitate detection of polar compounds like ethylglucuronide and meldonium. Finally, 5 μ L of the mixture were injected into the LC/MS system.

The LC/HR-FS-MS analysis was performed using a Dionex UHPLC system (Thermo Scientific, Bremen, Germany) equipped with a Zorbax Eclipse Plus C18 column (100 \times 2.1 mm i.d., 1.8 μ m particle size; Agilent Technologies, Santa Clara, CA, USA) coupled with a QExactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany). Mobile phase A was water containing 5 mM HCOONH₄ and 0.02% (v/v) HCOOH, whereas mobile phase B was a mixture of acetonitrile/water (90:10 v/v) containing 5 mM

HCOONH₄ and 0.02% formic acid. The total chromatographic run time was 20 min with a constant flow rate at 0.2 mL min⁻¹ in gradient conditions, set to 95% A and 5% B for the first minute, then solvent B was changed to 90% on the 9th minute, followed up to 100% on the 11th minute to remain constant for 3 min. Post-run equilibrium after the 14th minute of the run for the remaining run time was applied. The electrospray ionization (ESI) source was heated under nitrogen 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, whereas the mass spectrometer was operated with positive-negative polarity switching in the FS acquisition mode range of 100–1000 *m/z* at 17,500 MS resolution.

2.3 | Analytical validation

The ITP analysis of the three analytes was validated according to qualitative specifications of the International Standards of Laboratories (ISL).²⁰ Initial FS-MS acquisition optimization was conducted. The validation parameters for TRA comprised selectivity, carryover and reliability of detection. TRA has a reporting limit of 50 ng mL⁻¹, which prevents the estimation of the Limit of Detection (LOD), since the approximate LOD is two levels of magnitude lower than the reporting limit. ECDY was validated in addition to the previous ITP parameters of TRA in 50% of MRPL at 2.5 ng mL⁻¹ and for the LOD. DESECDY was validated for selectivity and carryover only, since the reference material was not available. CP validation for TRA and ECDY was conducted additionally to ITP, comprising optimizations of MS² acquisition, selectivity, carryover, reliability of detection at 50% or less of the MRPL, LOD, ion suppression, matrix effects and robustness of detection.

2.4 | MetAlign/HR_MS_search search module validation protocol

MetAlign algorithmic and usability description details can be found elsewhere.^{7–11} MetAlign was applied in two steps. In the first step, data reduction was applied to the 7410 LC/HR-FS-MS datafiles. One original datafile can generate two reduced datafiles, one for positive and one for negative ionization mode, with approximately a 100-times reduction in volume. For the current study, only the positive mode reduced datafiles were used, since all analytes were detected in positive ion mode.

In the second step, the searching parameters for the three analytes were optimized and validated based on the reduced positive mode datafiles. Figure 1 shows the scheme of the searching validation. The following searching parameters were applied: accurate *m/z*, abundance, maximum mass error in parts per million ($\{E6 \times \text{mass error/exact mass}\}$ in ppm), RT and relative RT (rRT) showing the retention time difference relative to the RT of the internal standard. Searching optimization was assessed based on the correct

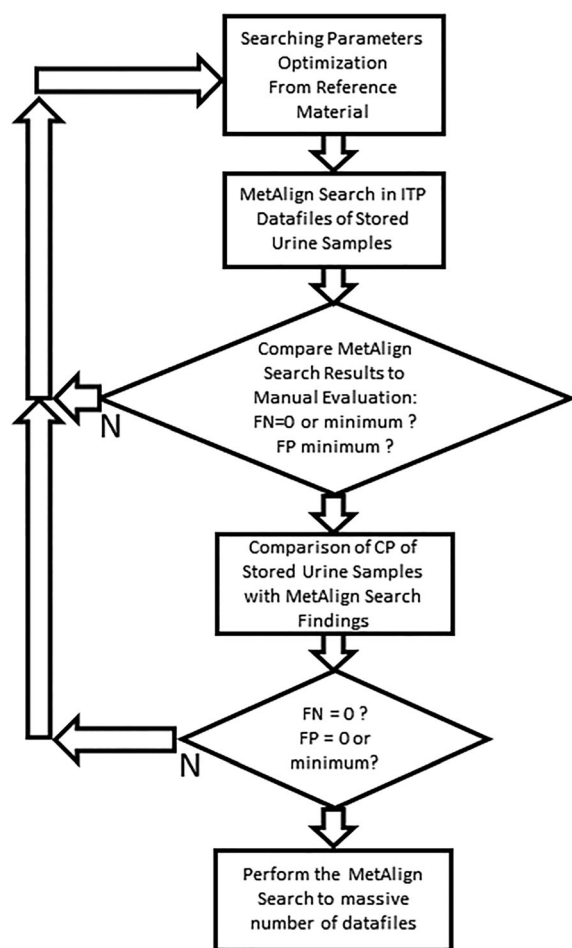


FIGURE 1

FIGURE 1 The MetAlign search validation scheme developed in the current study for the WADA Monitoring program retroactive processing

identifications, the FP and FN criteria. Optimization and validation of searching for ECDY and DESECDY was applied on a validation set of 280 datafiles selected from the group of a total of 7410 datafiles analyzed, originating from seven 2020 routine ITP batches. All 280 datafiles were acquired from samples reported to be negative in ADAMS; these samples had been stored at under -20°C . All MetAlign searching reports were compared with manual assessment of the presence of the target compounds in the printouts of the extracted ion chromatograms generated by Thermo Xcalibur software according to Fragkaki et al.²¹ Several rounds of searching parameters optimization were planned to reduce FPs and FNs to zero. For the final set of optimized parameters of ECDY and DESECDY, CPs were applied on new aliquots of the 280 samples, for which MetAlign and manual evaluation detected ECDY and/or DESECDY. TRA was included in the current study as an additional MetAlign search validation, since the ADLQ CP(s) had already been conducted at the time of routine analysis and reporting.

3 | RESULTS AND DISCUSSION

3.1 | WADA monitoring program

According to the Code,¹ Article 4.3, the criteria of inclusion of one substance in the List are the following: (a) it has the potential to enhance sport performance, (b) its use is a potential health risk to the athlete, and (c) its use is contrary to the spirit of sport. The WADA List Expert Group (ListEG) proposes and the WADA Executive Committee takes the decision for the List content; a similar procedure is followed for the WADA Monitoring program.³ Table 1 presents since the start of the program the summary of the Monitored substances showing the years of introduction, removal or transfer to the Prohibited List. Three examples of monitored substances from Table 1 are developed with more detail in this section. The stimulant pseudoephedrine is a sinus/nasal decongestant and can be supplied as an over-the-counter medication.²² Pseudoephedrine at supratherapeutic doses has ergogenic effects and it was included in the International Olympic Committee Prohibited Lists. WADA removed pseudoephedrine from the Prohibited List and introduced it into the Monitoring program in 2009 for the In-Competition sample collection. However, it was removed from the Monitoring program and reintroduced to the Prohibited List at urinary threshold levels above $150\text{ }\mu\text{g mL}^{-1}$ in 2015. Another Monitoring substance example is TRA.²³ TRA increases pain tolerance, euphoria during exercise, and mood or pain perception. TRA presents addictive effects within therapeutic dose range and can create side effects like agitation, confusion, hypertension, and tachycardia. The sedative effects can pose risks to other athletes, in sports like boxing, where body pain plays an important repressing role during competition. TRA is under further evaluation for ergogenic effects and is included in Monitoring for the In-Competition sample collection when detected above 50 ng mL^{-1} in urine, similar to the S7.Narcotics category of the Prohibited List (MRPL¹²). The final example of a Monitoring substance is ECDY²⁴ which is often labelled as the food supplement “natural anabolic agent”. Several studies have reported it to increase strength and muscle mass, to reduce fatigue and to ease recovery.^{25,26} ECDY can be administered from plant extracts, such as spinach. As a steroid, ECDY and/or its metabolite DESECDY are Monitored in both In- and Out-Of-Competition sample collections without reporting limit of concentrations.

The 2021 Monitoring program has two categories. The active Monitoring refers to substances that are reported by Accredited Laboratories to WADA in the ordinary reporting of routine sample analysis in ADAMS. In the passive Monitoring, Laboratories analyze for substances of the Monitoring program, but report to WADA in relation to Laboratories' evaluation of increased use detection. Moreover, detection of TRA is mandatory for the Accredited Laboratories, while detection of ECDY and DESECDY is conducted involuntarily.

3.2 | Optimization and validation

The substances of the Monitoring program need only to be reported in ADAMS by the Accredited Laboratories from ITP data and do not require a CP. However, acquisition of ITP and CP validation data for TRA and ECDY has been conducted according to ISL.²⁰ The assessment of MetAlign for massive ITP datafiles reprocessing-identification of substances in the WADA Monitoring program and to validate the optimized MetAlign searching parameters was the aim of this work. The MetAlign validation was implemented in this study only for the compound searching and not the datafile information reduction, which has been validated by Wageningen Food Safety Research in several previous projects over a period of more than a decade by adding and tracing reference compounds in matrices at different levels.⁹ The searching validation plan shown in Figure 1 was the following: optimized searching parameters of accurate m/z (s) and RTs and their tolerance ranges for each substance validated based on the correct/incorrect identifications. FPs and FNs were evaluated after comparison of MetAlign searching reports versus the manual routine ITP ion chromatograms evaluation generated by the Thermo Xcalibur software. This process was complemented by selected routine CPs for samples stored in ADLQ, where the MetAlign searching procedure detected the target substances. The final set of searching parameters, m/z (s), RTs and tolerance ranges, was used to search compounds retrospectively on reduced datafiles collected in ADLQ within the Monitoring program as presented in section 3.3. To the authors' knowledge, this is the first study in the antidoping field which has used an automated software to identify compounds in the Monitoring list with retrospective analysis of collected antidoping data. For that reason, validation and searching was applied not only for ECDY and DESECDY, but also for TRA, for which ADLQ had already Monitoring reporting records in ADAMS for the period of 2017–2020.

3.2.1 | Optimization of search parameters for ECDY and DESECDY

ECDY and DESECDY were detected as the protonated molecule $[M + H]^+$: m/z 481.3165 and 465.3211, respectively. The LOD for ECDY was 0.5 ng mL⁻¹ from the ITP analytical test data. The initial searching parameters of ECDY and DESECDY were applied to the ADLQ/WAADS reference urine sample using mass and RT tolerance windows of 7.5 ppm and 0.2 min, respectively. These parameters were used to generate extracted ion chromatograms manually with the Thermo Xcalibur program. Subsequently, the searching optimized parameters were applied on a validation test set of 280 ITP datafiles. The MetAlign searching module identified the target compounds using the RT relative to the RT of the internal standard to compensate for any potential shift in RT. The d3-mefruside deuterated internal standard eluting close to the ecdysteroids was used for correction of the RT. The searching criteria to determine FPs and FNs in the MetAlign report are shown in Table 2.

TABLE 2 The searching criteria in the ITP for ECDY and DESECDY

	Column number	1	2	3	4
Reported identifications	MetAlign ITP	–	–	+	+
	Xcalibur ITP	+	–	+	–
MetAlign evaluation criteria	FP				X
	FN	X			
	Correct		X	X	

– = Substance not detected; + = Substance detected; X = MetAlign evaluation criterion for the particular combination of reported identifications.

For ECDY, the influence of mass tolerance window of $[M + H]^+$ was examined at three levels using 3, 5 and 7.5 ppm with a RT tolerance of 0.3 min. The number of FNs obtained using the above parameters are presented in Table S1 (supporting information; rows 1, 2, and 3 respectively), while with these parameters the number of FPs was zero. Then, the influence of the RT tolerance window was examined at 0.2 and 0.3 min in combination with mass errors of 3 and 5 ppm (Table S1, rows 1, 2, 4, 5; supporting information). The minimum number of FNs was generated with the combination of 5 ppm and 0.3 min (Table S1, row 2). In addition, ECDY showed another ion resulting from the loss of two molecules of water from $[M + H]^+$, i.e. $[M + H - 2H_2O]^+ = C_{27}H_{41}O_5^+$, m/z 445.2949. The inclusion of the second m/z value in the MetAlign search using 7.5 ppm tolerance eliminated all FNs (Table S1, row 6). The final searching parameters set for ECDY were: m/z 481.3165 and 445.2949 applied with 7.5 ppm m/z and 0.3 min RT tolerance window, which resulted in no observable FNs and FPs in the validation test set of 280 datafiles analyzed for ECDY.

For DESECDY MetAlign searching in the validation test set of 280 samples, the optimization of the RT tolerance window was tested with 0.3 and 0.2 in 7.5 ppm mass tolerance (Table S2, rows 1 and 2, respectively, supporting information). The number of FPs reached zero, showing the elimination from reporting of an interference peak eluting 0.21 min after DESECDY (detected at 6.7 min) after reducing the RT window from 0.3 to 0.2 min. In addition, the results showed a significant decrease in the number of FNs from 22 to 3, as the interfering peak was not detected by Xcalibur at a 0.2 min RT window. For the mass tolerance window 7.5 and 5 ppm were applied for the $[M + H]^+$ ion with an RT tolerance of 0.2 min. The number of FNs obtained with these parameters are presented in rows 2 and 3, respectively in Table S2 (supporting information) and show a reduction in the number of FNs from 3 to 2 for the 7.5 to 5 ppm tolerance window. The final optimized MetAlign searching parameters for DESECDY were: m/z 465.3211, 5 ppm mass and 0.2 min RT tolerance windows (row 3) resulting in 2 FNs and no FPs.

The samples that were classified in columns 1 or 3 of Table 2 for ECDY and DESECDY were selected for the CP. The CP comprised repeated analysis on a new aliquot from the original urine sample. All

selected samples were processed together with blank urine, positive control spiked with ECDY at a concentration of 5 ng mL⁻¹ and the reference urine sample of ADLQ/WAADS for DESECDY.¹⁹ The MS/MS transitions from [M + H]⁺ for the CP were for ECDY *m/z* 165.1274, 371.2216, 445.2949, 427.2840, 125.0962, 69.0706 and for DESECDY 285.1849, 303.1957, 81.0704, 125.0962, 99.0808, 267.1743, 191.1067. Table 3 shows the searching criteria, similar to Table 2, with the addition of the CP. The CP results showed that one sample for ECDY and 5 samples for DESECDY were detected with both MetAlign ITP and manual Xcalibur ITP assessment (column 3 of Table 2), but were negatives based on WADA MS identification criteria.²⁷ The two FN samples for DESECDY, that were not identified by MetAlign ITP, were confirmed as negatives with the CP. Therefore, the number of FNs validated for DESECDY was zero. Figures S1 and S2 (supporting information) show typical extracted ion chromatograms for ECDY and DESECDY in ITP and CP. ECDY was confirmed with and without the presence of DESECDY in the concentration range 2 to 254 ng mL⁻¹. DESECDY was detected in all the samples that were confirmed for ECDY with concentration approximately greater than 7 ng mL⁻¹, except for one sample. DESECDY was not detected in the samples where ECDY had been confirmed with a concentration below 5 ng mL⁻¹. The CP findings showed a high concentration of DESECDY along with or without ECDY. In conclusion, ECDY and DESECDY could be both detected and the one without the other in the same sample. This complicated metabolic profile of human urine is probably due to the variety of origins of ECDY from food supplements, ordinary food or other substances of the ECDY family from natural sources.

3.2.2 | Optimization of search parameters of TRA

The MetAlign search parameters of TRA were optimized and tested in 3018 ITP datafiles from 2017 and 2018. TRA was detected in ITP positive mode as [M + H]⁺ with *m/z* 264.1958. All datafiles were searched for TRA using a mass tolerance of 7.5, 5 and 3 ppm and RT tolerance of 0.2 and 0.3 min (Table S3, supporting information). The findings from the MetAlign search were compared with the TRA results reported by ADLQ after a CP. TRA CP MS/MS transitions from [M + H]⁺ were *m/z* 58.0660, 264.1958 and 246.1847. Based on

the reporting limit for TRA of 50 ng mL⁻¹,¹² the abundance searching filter was used as 10% of the average 25 ng mL⁻¹ QC intensity ($5 \cdot 10^7 \approx 2.5$ ng mL⁻¹ of TRA). Due to the broad TRA peak shape detected whenever the signal intensity was high (Figure 2), three RTs at 6.68, 6.50 and 6.60 min were tested. On the other hand, the isotopic ¹³C of [M + H]⁺ with *m/z* 265.1992 was also used as an additional *m/z* searching parameter. Searching results using only [M + H]⁺ are shown in rows 1 and 2 of Table S3 (supporting information). The results obtained with the additional searching parameters for the isotopic ¹³C of [M + H]⁺ are presented in rows 3–7 of Table S3 (supporting information). The inclusion of the second *m/z* value in the search with 3 ppm mass tolerance and 0.2 min RT tolerance window at RT 6.5 min reduced the number of FPs from 934 to 75 (rows 2 and 3 of Table S3, min, 264.1958 and 265.1992 in 5 ppm mass and 0.2 min RT tolerance, respectively (row 7, Table S3, supporting information). The search results in the test set of 3018 datafiles for TRA of row 7 compared with ADLQ reports showed no FNs and 24 FPs. However, 15 out of 24 were In-Competition samples where TRA was detected below the reporting limit. The remaining 9 out of the 24 were Out-of-Competition samples, where the Monitoring program for TRA is not applied. Therefore, the total number of FPs was also zero.

3.3 | Application of search parameters in the final reduced datafile set of 7410 samples

The final searching parameters of TRA (row 7, Tables S3, 4 summarizes the search results showing no FNs and 41 FPs. However, a total of 21 out of 41 samples were In-Competition samples with TRA detected below the reporting limit; the rest of the samples were Out-of-Competition samples. Therefore, TRA searching generated zero FPs. In conclusion, the search proved that MetAlign and ADLQ reporting were identical, even if some samples, like those shown in Figure 2B, showed chromatographic peaks distorted due to saturation.

The search results of ECDY using the parameters of Table S1, row 6 (supporting information) are summarized in Table 5. Estimation of the FN search in the 7410 samples was not performed, because this is beyond the scope of the use of MetAlign searching in thousands of datafiles. All MetAlign findings for ECDY and DESECDY

TABLE 3 The searching criteria in the CP for ECDY and DESECDY

	Column number	1	2	3	4	5	6
Reported identifications	MetAlign ITP	–	–	+	+	–	+
	Xcalibur ITP	+	–	+	+	+	–
	CP	–	–	+	–	+	–
MetAlign evaluation criteria	FP				X		X
	FN					X	
	Correct	X	X	X			

– = Substance not detected; + = Substance detected; X = MetAlign evaluation criterion for the particular combination of reported identifications.

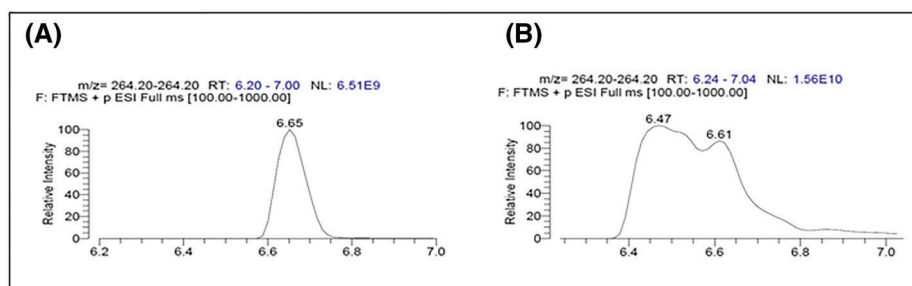


FIGURE 2 Examples of ion chromatograms of two TRA cases with different peak shapes due to the signal intensity of $[M + H]^+$ m/z 264.1958 of: A, Normal peak shape and B, broad peak shape due to instrument saturation

TABLE 4 Search results in the validation set of 7410 datafiles for TRA

Search parameters				Without filter				Filter: Second ion				Filter: 10% QC + second ion			
Error (ppm)	RT window	m/z	RT	Auto	Positives	FN	FP	Auto	Positives	FN	FP	Auto	Positives	FN	FP
5	0.2	2	6.6	2074	25\25	0	2049	285	25\25	0	260	66	25\25	0	41

Auto = automatic; No. of samples that were detected by MetAlign; Positives = No. of positive samples that were detected by MetAlign; FN = No. of false negatives; FP = No. of false positives; Second ion = second ion criterion (see text); 10% QC = signals >10% QC.

TABLE 5 Search results in the validation set of 7410 datafiles for ECDY

Search parameters			Auto			Xcalibur	
Error (ppm)	RT window	m/z	Without filter	Filter: Second ion	Filter: 1E+05 + second ion	Detected	FP
7.5	0.3	2	1933	236	212	177	35

Auto = automatic; No. of samples that were detected by MetAlign; second ion = second ion criterion (see text); 1E+05 = abundance cut-off filter (see text); Xcalibur = No. of samples that were detected in Xcalibur; Detected = No. of samples that were detected in Xcalibur and MetAlign; FP = No. of false positives.

TABLE 6 Search results in the validation set of 7410 datafiles for DESECDY

Search parameters			Auto			Xcalibur	
Error (ppm)	RT window	m/z	Without filter	Filter: 1E+05	Filter: RT window (0.2)	Detected	FP
5	0.2	1	519	300	277	270	7

Auto = automatic; No. of samples that were detected by MetAlign; 1E+05 = abundance cut-off filter (see text); Xcalibur = No. of samples that were detected in Xcalibur; Detected = No. of samples that were detected in Xcalibur and MetAlign; FP = No. of false positives.

were compared with manually assessed extracted ion chromatograms generated with Thermo Xcalibur to evaluate the number of FPs. Without any filter applied, 1933 samples were shown to contain ECDY by MetAlign. After the application of the second ion filter and the $1 \cdot 10^5$ abundance cut-off (corresponding to 0.5 ng mL^{-1} urine concentration of ECDY), ECDY was detected in 212 samples. However, only 177 of these were identified as ECDY-positive samples after manual evaluation and the other 35 as FPs. For DESECDY, the searching parameters of row 3 in Table S2 (supporting information) were applied to the 7410 ITP datafiles and the search results are presented in Table 6 showing 7 FPs and 270 positives.

ECDY and DESECDY together were detected in 79 samples. DESECDY was detected alone without ECDY in 71% of the samples. DESECDY was not detected in samples with FP ECDY, similarly ECDY

was not detected in samples with FP DESECDY. ECDY and DESECDY were detected in 368 of the 7410 samples.

4 | CONCLUSIONS

MetAlign was proposed as an additional tool to retrospectively collect Monitoring data from ITP LC/HR-FS-MS datafiles. MetAlign is applied in two steps. In the first step, the ITP LC/HR-FS-MS datafiles are reduced in size by approximately 100-times. In the second step, the reduced datafiles are searched for target substances, e.g. compounds from the Monitoring program, which is fast and can be performed using an ordinary computer. The MetAlign search can be easily conducted for thousands of reduced

datafiles collected over years providing WADA with statistics of prevalence. MetAlign uses searching parameters such as accurate mass, mass tolerance window, relative or absolute RT, RT tolerance window, additional accurate m/z (s) and mass abundances. Searching parameters are not universal; the MetAlign search module requires optimization and validation of searching parameters for each Monitoring substance and for each applied analytical methodology to minimize FNs and FPs. In the current study, zero FNs was one of the targets. Manual assessment of the performance of optimal parameters can be performed by comparing the automatic MetAlign searching results with extracted ion chromatograms generated by the instrument software and a CP. In the current study, the MetAlign search with optimized and validated parameters was applied to ECDY, DESECDY and TRA. In the case of TRA, the study proved that MetAlign and ADLQ reports were identical. For ECDY and DESECDY, the MetAlign search results identified the presence of ECDY in approximately 5% of the samples from years 2017–2020, and indicated a complex metabolic profile, most probably due to the various sources of ecdysterone from food, food supplements or other related ecdysteroids.

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PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/rcm.9141>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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