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Speeding up the screening of steroids in urine: Development of a user-friendly library

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ABSTRACT

This work presents a novel database search engine – MLibrary – designed to assist the user in the detection and identification of androgenic anabolic steroids (AAS) and its metabolites by matrix assisted laser desorption/ionization (MALDI) and mass spectrometry-based strategies. The detection of the AAS in the samples was accomplished by searching (*i*) the mass spectrometric (MS) spectra against the library developed to identify possible positives and (*ii*) by comparison of the tandem mass spectrometric (MS/MS) spectra produced after fragmentation of the possible positives with a complete set of spectra that have previously been assigned to the software. The urinary screening for anabolic agents plays a major role in anti-doping laboratories as they represent the most abused drug class in sports. With the help of the MLibrary software application, the use of MALDI techniques for doping control is simplified and the time for evaluation and interpretation of the results is reduced. To do so, the search engine takes as input several MALDI-TOF–MS and MALDI-TOF–MS/MS spectra. It aids the researcher in an automatic mode by identifying possible positives in a single MS analysis and then confirming their presence in tandem MS analysis by comparing the experimental tandem mass spectrometric data with the database. Furthermore, the search engine can, potentially, be further expanded to other compounds in addition to AASs. The applicability of the MLibrary tool is shown through the analysis of spiked urine samples.

1. Introduction

The use of androgenic anabolic steroids (AAS) and hormones to enhance athletic performance has important health and social implications. Their use was first introduced in sports as agents supporting the athlete recuperation after extreme stress and fatigue, but rapidly became the main agents used in doping abuse [1].

Nowadays, this class of drugs is a major group included in the prohibited list of the world anti-doping agency (WADA) as well as of major sports authorities [2–5]. In the WADA statistic report for 2011, the AAS represented 59.4% of all adverse analytical findings reported by WADA accredited laboratories [6]. Although this data may not reflect the real doping abuse statistical status, because of the well-known problems in the detectability of clandestinely designed AAS, micro dosages of endogenous AAS and "modern" doping agents (e.g., peptide hormones) [1,7–9].

The use of AAS to increase muscle mass and strength is not a behaviour strictly related to elite athletes, as their use is increasing amongst amateur athletes as well as outside sports as an expression of an improved life style [10,11]. The illicit AAS use is an increasing trend in western societies and the emergent AAS dependence is a matter of growing public health concern [12].

Quickly following the development of mass spectrometry (MS) detectors, its use coupled to gas chromatography (GC) has become the standard technique for AAS control. Currently, most methods for routine detection of these compounds and their metabolites, comprising both screening and confirmatory analysis, are still based in GC–MS techniques [13–17]. More recently, due to the increasing complexity of doping analyses and in order to enhance the detection of this group, liquid chromatography coupled with MS/MS is gaining ground within anti-doping laboratories [18–20]. In particular for the detection of thermo-labile and polar steroids, such as Trenbolone. It avoids the derivatisation step required by GC–MS and provides good sensitivity for the determination of these compounds [21,22]. Moreover, the low throughput provided by LC–MS techniques are to some extent being surpassed by the introduction of uHPLC systems coupled with LC-columns containing solid core particles that allows high speed and high efficiency

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separations. Unfortunately, for the majority of the AAS, due to their poor ionisation efficiencies, their determination by LC–MS may lead to losses in sensitivity.

The combination of these two factors, the long separation times of gas chromatographic techniques and the increasing workloads within anti-doping laboratories, expose an urgent need for an analytical technique allowing simplicity, speed and high throughput for the screening of the huge number of banned compounds, particularly the AAS.

Recently, the use of matrix-assisted laser desorption/ionization (MALDI) for the analysis of small molecules, has grown as a potential technique, which is reflected by the increasing number of studies reported in literature [23–27]. Moreover, it appears extremely promising for high-throughput, which is a major demand for future anti-doping methods.

In light of the latest technological improvements of this analytical technique we have recently study the applicability of a wide variety of commercial MALDI matrices for the rapid screening of AAS [28]. The matrix 2-(4-hydroxyphenylazo)-benzoic acid (HABA) was found to be the most robust for the analysis of anabolic steroids after a derivatisation step with the reagent Girard T hydrazine. The Girard T hydrazone derivative produced after derivatisation is a quaternary ammonium ion that originates a strong $[M]^+$ ion signal in the MALDI mass spectrum, as a result it increases the intensity of the steroid signal. In the aforementioned work it was demonstrated that positive identification of the characteristic peaks for all the compounds studied is possible for a sample concentration of 10 ng/mL in the MALDI sample plate. The sensitivity achieved with the HABA matrix after derivatisation was similar to that achieved by GC/MS – around 4–10 ng/mL in the single ion monitoring mode.

In the present work it is presented a step forward in simplifying AAS control through the use of and easy sample treatment and friendly software. The software is freely source code available, and it can be run as a multiple platform. As a proof-of-concept, the rapid screening of AAS in urine is reported using a sample treatment previously published by our team [28].

2. Material and methods

2.1. Chemicals

Standards of 17-α-methyltestosterone and nandrolone were purchased from Riedel-de Haën (Seelze, Germany). The standards 17α-trenbolone, 2α-methyl-5β-androstane-3α-ol-17-one, mesterolone, methandienone, calusterone, fluoxymesterone, ethisterone and mibolerone were kindly provided by the Portuguese National Anti-doping Laboratory and the Italian National Anti-doping Laboratory. A solution of β-glucuronidase from *Escherichia coli K12* with a specific activity approximately of 140 U/mg at 37 °C and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140 U) was purchased from Roche Diagnostic (Mannheim, Germany). Sodium hydrogen phosphate, sodium phosphate dibasic, tert-butylmethyl ether, methanol (MeOH), acetonitrile (ACN) and the derivatisation reagent, Girard T (GT) hydrazine, used for sample and matrix preparation were purchased from Sigma (Steinheim, Germany); glacial acetic acid (>99.5%), matrices αCHCA and HABA were purchased from Fluka (Buchs, Switzerland); trifluoroacetic acid (TFA, 99%) was from Riedel-de Haën.

Urine samples used in this work were obtained from healthy volunteers from the research team. Volunteer's age ranged between 22–30 years, including both male and female. The research ethical committee from the Science Faculty of Ourense approved the study protocol and all the volunteers gave their consent.

2.2. Apparatus

A model UNIVAPO 100H vacuum concentrator centrifuge (UniEquip, Martinsried, Germany) with a model Unijet II refrigerated aspirator vacuum pump (UniEquip) was used for (i) sample drying and (ii) sample pre-concentration. A Spectrafuge-mini minicentrifuge (Labnet, Madrid, Spain) and a Sky Line minicentrifuge-vortex (ELMI, Riga, Latvia) were used throughout the sample treatment, when necessary. A Simplicity 185 system (Millipore, Milan, Italy) was used to obtain Milli-Q ultrapure water throughout all the experiments. The derivatisation procedure was performed in a 1.5 mL microtube flat cap from Delta Lab (Barcelona, Spain). Separation of the steroid Girard T (GT) hydrazones from the unreacted Girard T reagent was carried out in a 2 mL empty reversible solid-phase extraction (SPE) cartridge from Supelco (Belefonte, PA, USA) packed with a preparative C18 resin (125 Å, 55–105 μm; Waters, Barcelona, Spain).

2.3. Sample preparation

2.3.1. Standard solutions

Individual stock standard solutions of each compound (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25 mL volumetric flask and making it to volume with methanol. These standard solutions were stored in the dark at −20 °C. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

2.3.2. Urine hydrolysis procedure

Urine samples (2 mL) were hydrolysed with 50 μL of the commercial solution of β-glucuronidase, after the addition of 0.750 mL of phosphate buffer (0.8 M, pH 7). The hydrolysis was performed at 55 °C during 60 min.

2.3.3. Liquid–liquid extraction of target analytes

After cooling to room temperature, 0.5 mL of carbonate buffer (pH 9) was added to alkalinize the hydrolyzed solution. Liquid–liquid extraction was carried out by agitation with 5 mL of tert-butyl methyl ether for around 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

2.3.4. Derivatisation procedure

The procedure for derivatisation with Girard T hydrazine was performed based on the protocol described by Wheeler [29], as follows: The collected organic phase was dried under a gentle nitrogen stream at 40 °C. After the addition of 500 μL of a methanolic solution with 10% glacial acetic acid and 4 mg of Girard T hydrazine, the vial was closed and the derivatisation reaction was then performed at 60 °C during 30 min. After cooling, the solution was evaporated to dryness in a vacuum concentrator centrifuge and then reconstituted with 1 mL of methanol/water (10:90, v/v).

2.3.5. SPE clean-up

After derivatisation, the steroid GT hydrazones were separated from un-reacted GT hydrazine reagent by SPE in a C18 cartridge, according to the protocols described by Khan et al. [24] and Griffiths et al. [25]. Briefly, before use, the cartridges were conditioned with 5 mL of methanol plus 10 mL of MilliQ-water without allowing the cartridges to dry out. After loading the sample, the cartridge was washed with 2 mL of methanol/water (10:90, v/v) in order to remove impurities from the cartridge and, finally, the steroid GT hydrazones were eluted from the cartridge with 1 mL of methanol.

2.4. Experimental design

To assess the use of the MLibrary software for the analysis of AAS present in human urine, five urine samples were spiked with different AAS at different concentration levels. The five urine samples were designated as Urine 1, Urine 2, Urine 3, Urine 4 and Urine 5. Urine 1 was spiked with 17α-methyltestosterone (100 ng/mL). Urine 2 was spiked with calusterone (250 ng/mL). Urine 3 was spiked with nandrolone (10 ng/mL). Urine 4 was spiked with fluoxymesterone (200 ng/mL) and ethisterone (150 ng/mL). Urine 5 was spiked with 17α-trenbolone (25 ng/mL) and mesterolone (300 ng/mL).

2.5. MALDI-TOF–MS and MALDI-TOF–TOF–MS analysis

The mass spectrometric analyses were performed on Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF™ Optics system (Applied Biosystems, Foster City, CA, USA) equipped with and a diode pumped Nd:YAG laser with 200 Hz repetition rate. The instrument was operated for detection in positive ion reflectron mode. The MS spectrum for each sample was based on the average of 1000 laser shots; for the MS/MS up to 4000 shots were accumulated. MS/MS mode was operated with 1 kV collision energy; air was used as the collision gas such that nominally single collision conditions were achieved. For MS analysis, laser desorbed ions were accelerated from the source at 20 kV. For MS/MS analysis, ions were accelerated from the source at 8.0 kV. Both modes employed delayed ion extraction for improved ion focusing. The MS/MS data was acquired using the instrument default calibration, whilst the MS was acquired using the peaks from the HABA matrix for internal calibration. Prior to MALDI analysis, the sample was mixed with an equal volume of the MALDI matrix solution and homogenised in a vortex instrument. The HABA MALDI matrix used in this work was prepared according to the developed method, 0.52 mg in 1 mL of a solution of ACN/H2O/MeOH (40/40/20, v/v/v) [28]. The matrix αCHCA was prepared by dissolving 10 mg in 1 mL of a solution ACN/H2O/TFA (50/49.9/0.1, v/v/v). An aliquot of the sample/matrix solution (0.5 μL) was hand-spotted onto the MALDI sample plate and the sample was allowed to dry. Fig. 1 presents a scheme of the sample treatment followed in this study.

2.6. MLibrary software

Current versions of the software and their supporting user manuals are freely available for download and use, without restriction, via Internet at http://sing.ei.uvigo.es/MLibrary. The program was developed based on previous work on a tool for accurate protein quantification [30].

MLibrary operates on comma-separated-values (CSV) files with centroid mass and relative intensity data extracted from the instrument software (Data ExplorerTM Software, version 4.5). This data can be analysed and compared with the compound data stored in the MLibrary repository, which contains the characteristic mass values of the molecular ion and the fragmentation ions for each target AAS in separated databases. For each database created and stored in the MLibrary repository, the user may include standard modification mass variations corresponding to specific derivatisation reagents and the consequent MS/MS spectra for each AAS presenting that particular modification. The MLibrary repository comprises also MS/MS compound markers. The MLibrary repository is stored in a single standard XML file, which can be easily modified with any plain text editor. More information about how to edit this file can be found in the software web page.

The installation wizard is available from the MLibrary web site as an executable file that depends on the final user operating system: Windows, Linux or MAC. By executing the setup file, the installation wizard will be automatically launched. The user has to simply follow the instructions on the screen to successfully complete the installation.

3. Results and discussion

The MLibrary software is explained in detail in the following sections. First we describe the use of MLibrary to detect the presence of possible AASs in the target samples (MS mode) and then its use to confirm the identity of the compound (MS/MS mode).

3.1. MS mode

3.1.1. Construction of MS database

The MS database in the MLibrary software contains the characteristic mass values for each target AAS. Additionally, the mass values of AAS glucuronides, which are the main excretion metabolites of AAS in the human body, were also introduced in the MLibrary database. In our previous work, the analysis of AAS by MALDI techniques was performed after derivatisation with Girard T hydrazine and therefore only this specific modification was introduced in the repository. Nevertheless, the introduction of specific modifications to the database is easily performed by typing the name and the mass variation in the modifications correspondent line within the XML file.

3.1.2. Detecting the presence of AASs

After the SPE clean-up procedure, as explained in the experimental section, the collected sample solution was mixed with the MALDI matrix HABA and spotted onto the MALDI sample plate. The MALDI ion source is a soft ionisation technique and therefore the MALDI-TOF–MS analysis measure, primarily, singly charged ions that correspond to the molecular ions of the sample solution species as well as to the MALDI matrix characteristic ions. The mass spectrum obtained after the MALDI-TOF–MS analysis is exported as a list of peaks to a CSV file. Only the centroid mass and relative intensity of each peak is used by the MLibrary software (see Fig. 1 Supplementary material Fig. 1SM).

The AASs detection process in MLibrary starts with the MS data loading. Through the "Load MS Data" operation (see Fig. 2SM), the user can input the CSV file and filter peaks by their intensity, avoiding the load of peaks with lower intensities. Alternatively, the spectrum can be previously processed using the respective MS data software and only then transferred to a CSV file. It is important to stress that the MLibrary software permits the loading of multiple CSV files corresponding to several spectra.

After loading the MS data, the user can perform a compound search, in order to identify which AAS are present in the loaded data. The "MS Analysis" operation (see Fig. 4) determines if a specific AAS is present in the MS spectrum. This operation contemplates several parameters that provide flexibility to the software. It takes into account if the compound is in its "conjugated" or "free" form and also allows the user to select the derivatisation agent employed. It is important to stress that the "conjugated" parameter only takes into account the glucuronide conjugates, which are the main excretion metabolites of AAS in the human body. The search is performed taking into account the error between the database and the experimental mass values. This error value can be selected as percentage, parts per million (ppm) or absolute mass units (amu) (see Fig. 3SM). Then, the search retrieves the mass values that matched between the experimental data and the database values, showing both experimental and theoretic values as well as the name of the compound and the experimental peak intensity (see Fig. 2). MLibrary also provides an additional operation named "MS Full Analysis", which performs a

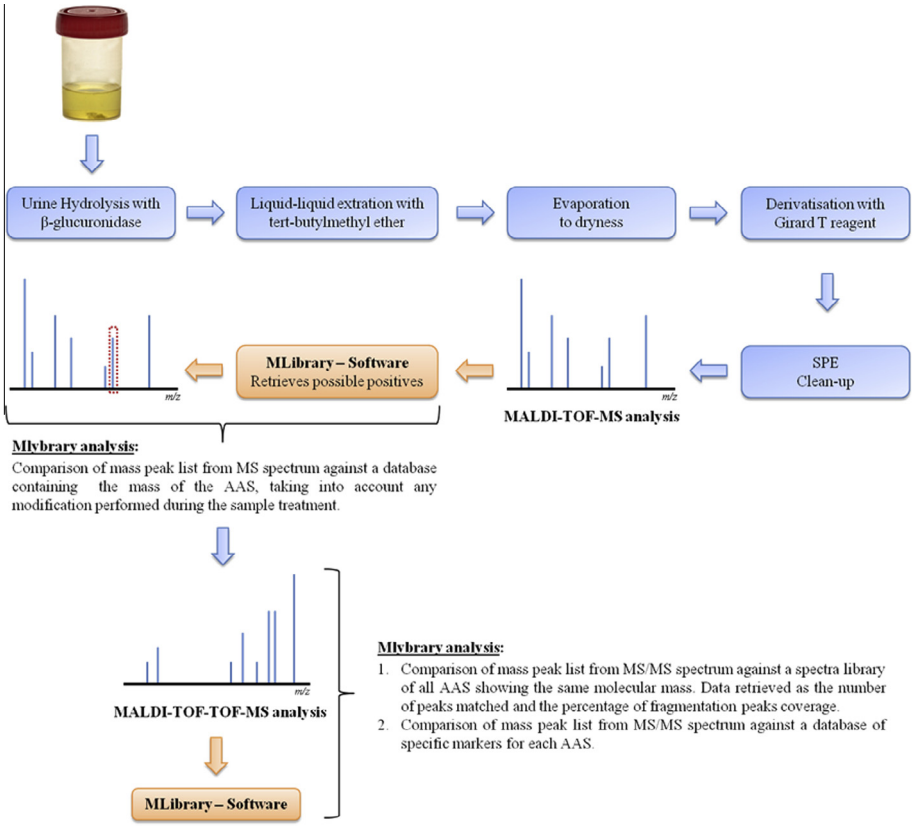

Fig. 1. Schematic diagram of the MALDI–MS(/MS) strategy to analyse AAS using the MLibrary software.

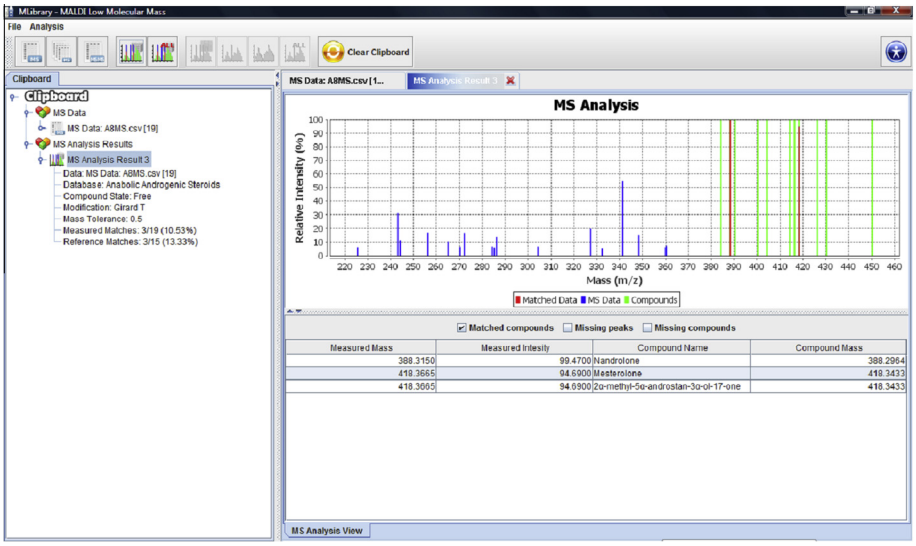

Fig. 2. MLibrary MS spectrum analysis display window. Data identification: Blue line: experimental spectrum mass peaks; Green line: database reference masses; Red line: experimental mass that matched with the reference mass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

search within all the MS databases present in the MLibrary repository.

Since the AAS mass values are close to the matrix characteristic ion mass ones, it is possible to perform an internal calibration for each MALDI plate spot using the matrix peaks as reference. Consequently, the experimental mass values obtained are very accurate and precise.

3.2. MS/MS mode

In the first step of the analysis, the Mlibrary software detected the ions that matched with the theoretic mass values of AAS within the MS database. In the second step, the ions detected by the MLibrary are selected for fragmentation in a second round of MS analysis, in which MS/MS spectra are acquire. Each AAS compound

presents a characteristic MS/MS fragmentation pattern that will be used by the MLibrary as a signature of that compound. Likewise the MS mode, the mass spectrum obtained after the MALDI-TOF–TOF–MS analysis is exported as a list of peak to a CSV file.

3.2.1. Construction of MS/MS spectral library

The MS databases in the MLibrary contain the characteristic fragmentation ions for each associated compound. The construction of the database can be easily performed using standard solutions of AAS. To ensure reliable spectral data, the software allows the input of several replicates corresponding to each standard. With these data, the MLibrary software generates a list of common fragmentation mass values for all replicates, along with their average relative intensities. In addition, when comparing with loaded data, the software allows the user to choose the discriminate power of the generated list, meaning that the user can decide to include within this list, the mass values that are present, for instance, at least in 95% percent of the spectral mass data inputted for each compound.

The construction of the mass spectral library in the MLibrary software is an ongoing process that is simple to achieve and updated by any qualified user, with the advantage that it can be easily adapted to numerous experimental conditions and compounds.

3.2.2. Confirming the presence of AASs

This process is similar to the AAS detection process except that, in this case, the user can perform two additional analysis operations. As before, the process starts with the data loading, in this case, through the "Load MS/MS Data" operation (see Fig. 4SM). This operation also provides the "Peak Intensity" parameter for peak filtering.

After loading the sample CSV file, the user can use the "MS/MS Library Analysis" operation (see Fig. 5SM) to compare it with the characteristic MS/MS spectra of the AAS compounds stored in the MLibrary databases. At this stage the user has to select the database, the modifications performed in the sample treatment procedure, the mass value of the precursor molecular mass and the mass tolerance permitted for each mass peak. Additionally, the user has to select the mass tolerance within the database mass spectra and its discriminate power. This operation retrieves a list of all compounds within the MS/MS library that fit the search criteria, ranked by similarity to the inputted file. The similarity is attained regarding the number of mass values that matched and the relative intensity of all peaks. To ensure reliable results, the search score concerns only to a limited number of mass values, defined previously by the user. For instance, the user may limit the search to the 10 most intense peaks within the inputted file and the library data (see Fig. 6SM). This tool is essential to avoid misinterpretation of the results due to the fact that different spectra of the same sample, generally present distinct overall number of mass peaks.

Another important tool within the MLibrary software is the "MS/MS Std. Match Analysis" operation. With this tool the user can compare two experimental spectra, which is extremely important if we are working at different conditions than the one recorded in the MLibrary MS/MS database. By adding a standard solution of a specific compound to the MALDI analysis and comparing the two spectra using the MLibrary (see Fig. 7SM), the user can confirm the identity of this compound. The results appear in the same way that showed in Fig. 6SM.

A third MS/MS Analysis tool is available in the MLibrary software. The "MS/MS Marker Analysis" operation allows the user to locate concrete biomarkers into the loaded MS/MS data (see Fig. 8SM). This feature is particularly important for the analysis of isobaric compounds having very similar MS/MS fragments or compounds having poor fragmentation pattern. For isobaric compounds the MS/MS Library analysis does not allow the differentiation between the two species, since it will retrieve very close results. For this reason, the identification of exclusive fragments corresponding to each compound is essential for the interpretation of the spectra and it will become essential for their identification. Additionally, the user can use the "MS/MS Full Marker Analysis" in order to search for all the biomarkers stored in the MLibrary repository.

4. Case study

Table 1 presents the results obtained with the data acquired from five urine samples spiked with different AAS at different concentration levels as described in the sample preparation section. All spiked compounds were detected by single MS as possible positives and their identity was confirmed by MS/MS. In the MS mode, besides the spiked compounds, the MLibrary retrieved other AAS as possible positives. This result occurs due to the fact that some synthetic AAS possesses the same molecular mass than some endogenous AAS, which are present in urine at low concentration values. For instance, 4-androsten-3,17-dione is a minor AAS metabolite that possesses the same mass than boldenone.

Fig. 9SM presents the results retrieved by the MLibrary for the sample Urine 1. As it may be seen in Fig. 9ASM, in the MS mode, the software identified three peaks corresponding to four possible positives: boldenone, 17α-methyltestosterone, mibolerone, which has the same molecular mass that 17α-methyltestosterone, and mesterolone. As mentioned above, following the detection of the possible presence of specific AASs in the urine sample, a MALDI-TOF–TOF–MS analysis was performed to the ions retrieved as possible positives. The MLibrary software was used to confirm the identity of the compounds. Figs. 9BSM, 9CSM, 9DSM and 9CSMe shows the results obtained for the four compounds. The presence of both boldenone and mesterolone is easily proved false by MLibrary MS/MS data analysis, showing less than 10% of positive match to the reference database spectra within the 10 most intense peaks.

Regarding 17α-methyltestosterone and mibolerone, the MLibrary MS/MS data analysis clearly confirms the presence of 17α-methyltestosterone. MLibrary software retrieves a higher percentage of mass values matching with 17α-methyltestosterone reference data within the 10 more intense peaks; 70% against 50% for mibolerone. Furthermore, the peak intensity of all matched mass ions present in the MS/MS spectrum is similar to the MLibrary reference data for 17α-methyltestosterone.

Figs. 1–4 of the Appendix A present the results retrieved by MLibrary for the samples Urine 2, Urine 3, Urine 4 and Urine 5, respectively.

In sample Urine 2, the presence of the isobaric compounds calusterone and bolasterone are detected by the MS mode (see Fig. 1 of the Appendix A). Although both calusterone and bolasterone present similar fragmentation patterns, the intensity of the peaks are different. For this reason, despite the similarity of the matched peaks between the sample compound and the reference data for calusterone and bolasterone, the MLibrary software easily confirms the identity of calusterone. It retrieves a higher percentage of mass values matching with calusterone within the 10 more intense peaks; 80% against 40%.

In sample Urine 3, the presence of nandrolone was confirmed using the MS/MS Marker Analysis tool (see Fig. 2 of the Appendix A). As it was mentioned above for poor fragmentation pattern compounds, such as nandrolone, the detection of specific markers, previously identified by the user, is of major importance.

Fig. 3 of the Appendix A shows the results retrieved by MLibrary that allowed the identification of both fluoxymesterolone and ethisterone in sample Urine 4. The identification of ethisterone

Table 1
Urine samples data analysis by the MLibrary software.

		MLibrary data analysis	
	AAS compounds spiked into urine	AAS identified by MS mode	AAS confirmed by MS/MS mode
Urine 1	17α-methyltestosterone	Boldenone	No
		17α-methyltestosterone	Yes
		Mibolerone	No
		Mesterolone	No
Urine 2	Calusterone	Calusterone	Yes
		Bolasterone	No
Urine 3	Nadrolone	Nadrolone	Yes
		Boldenone	no
		Mesterolone	no
Urine 4	Ethisterone	Mesterolone	No
	Fluoxymesterone	Ethisterone	yes
		Fluoxymesterone	yes
Urine 5	Trenbolone	Trenbolone	Yes
	Mesterolone	Boldenone	No
		Mesterolone	Yes

was performed in the same manner than for nandrolone by using the MS/MS Marker Analysis Tool. The presence of mesterolone was also proved false, showing 0% of matching within the most intense peaks.

In sample Urine 5, the MLibrary identified the presence of 17α-trenbolone and mesterolone. The identification of 17α-trenbolone was performed using the tool MS/MS Standard Mach Analysis. The MS/MS identification of 17α-trenbolone was performed, in a first approach, using the Library analysis tool, however only 60% of matching was achieved. The MS/MS Standard Mach Analysis tool allowed the unequivocal confirmation of this compound by comparing it with a standard solution analysed in the same experimental conditions.

5. Conclusions

We have developed friendly software to help in an automated mode to detect and identify the presence of AAS in urine samples by MALDI-TOF–TOF–MS. MLibrary software allows the user to perform robust and accurate screening and confirmation for AAS in both MS and MS/MS mode. As a proof-of-concept, when applied to five urine samples spiked with AAS, the software was able to successfully identify all spiked compounds. The software presented here is a very versatile tool that can be simply adapted to any future modifications carried out on the sample treatment procedure, as well as, be easily applied to other compounds. The MLibrary software saves times and it is a simple tool to work with. Additionally, MLibrary software has a wizard easy to follow for its installation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.08.014.

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