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Matrix-assisted laser desorption/ionisation time of flight spectrometry for the fast screening of oxosteroids using aromatic hydrated hydrazines as versatile probes

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ABSTRACT

To make the use of MALDI-based mass spectrometry feasible for the fast analysis of oxosteroids, three new aromatic probes have been designed to be used simultaneously as derivatisation agents and MALDI matrices. This concept brings a number of benefits: the sample handling is reduced, the workflow is less time consuming allowing high throughput and the interferences caused by the MALDI matrix are avoided. Identification was successfully attained for all oxosteroids used in this study. As proof-of-concept, the identification of oxosteroids in urine was performed to evaluate the robustness of the new methodology. The oxosteroids 17-α-methyltestosterone, nandrolone, boldenone, 17-α-Trenbolone, fluoxymesterolone, mesterolone and bolasterone were identified in human urine at the minimum concentration level recommended by the world anti-doping agency, 2 ng/mL.

1. Introduction

Steroids play an important role at numerous levels in the body. Their function affects both gene expression and metabolism. They include sex hormones, such as testosterone, estrogens and progesterone, and adrenocorticoid hormones, such as cortisol and aldosterone [1]. The steroid hormones generate different responses depending on the organs in which they operate and any alteration of these compounds in the organism will trigger a set of mechanisms that will change the physiological processes of the body.

Since these hormones are bound to highly specific receptors, steroids produce responses in their target cells [1–3] even at low concentration values. As a result, the presence of these compounds in the environment is a matter of serious concern as they can act as endocrine disrupters on both wildlife and human population [4–8].

Their potential use as doping agents to enhance athletic performance, which is a serious problem of ethical and social concern, has more of a popular influence than their effect as endocrine disrupters. Among doping agents, the misuse of androgenic anabolic steroids (AAS) figures in the top of all adverse analytical findings reported by accredited laboratories of the World Anti-Doping Agency [9]. This class of drugs represented 60.8% of all adverse analytical findings in the 2010 statistic report from WADA [9]. This situation has become more complex as the use of doping substances is no longer restricted to professional athletes; their use becoming popular among young sport people and non-competing amateurs [10]. The misuse of AAS has spread in such a way that even outside sport, young people are using them as a way to express a better quality of life compared to the rest of their peers [11].

Oxosteroids are steroids possessing a carbonyl moiety and represent a major group within these hormones. They include some of the most relevant steroids, such as testosterone, androsterone, etiocholanolone, nandrolone, progesterone and estrone.

Currently, most methods for routine detection of these compounds, comprising both screening and confirmatory analysis, are based in chromatographic–spectrometric techniques, mainly GC–MS techniques [12–18]. Although GC–MS is a well-suited technique for the determination of most oxosteroids, the separation time required for each run makes it a time-consuming approach. Thus, introducing matrix-assisted laser desorption/ionisation (MALDI) techniques for the screening of these compounds allows an overcoming of this drawback.

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Although GC–MS and LC–MS are widely employed in the analysis of oxosteroids, few studies have been reported concerning MALDI [19–22].

The application of MALDI techniques for the analysis of small molecules is still scarce due to the presence of matrix ion interferences in the low mass region. In this region is where the masses of most oxosteroids are found. To overcome this drawback different methods for the analysis of small molecules have been reported [23–26]. From these methods the derivatisation of the target analyte and the use of alternative MALDI matrices are the most reported ones.

In a recent investigation developed in our laboratory, we tested the applicability of commercial MALDI matrices for the rapid screening of 15 oxosteroids after derivatisation with reagent Girard T (GT) hydrazine, which is a quaternary ammonium ion that produces an intense [M]+ ion signal in the MALDI mass spectrum and displaces the oxosteroid peaks from the matrix peaks [21]. The positive identification of the characteristic peaks was possible for all the compounds studied from a sample concentration of 10 ng/mL in the MALDI sample plate, using the matrix 2-(4-hydroxyphenylazo)-benzoic acid (HABA). Although these results represented a significant improvement in the analysis of oxosteroids, mainly because of the reduction of the analysis time, there is still an important need to improve and optimise the sample treatment methodology for the analysis of these compounds by MALDI techniques.

The aim of this study was to demonstrate the possibility to use a derivatisation reagent specifically designed for oxosteroids that would be also able to act as a MALDI matrix [27–32]. This would considerably simplify and improve the sample treatment procedure for the analysis of these compounds by MALDI-based mass spectrometry techniques.

2. Material and methods

2.1. Apparatus

Elemental analyses were carried out at the CACTI Service (University of Vigo), on a Thermo Finnigan-CE Flash-EA 1112-CHNS apparatus. Infrared spectra were recorded as KBr discs using a Bio-Rad FTS 175-C spectrophotometer. Proton NMR spectra were recorded using a Bruker WM-400 spectrometer, using $CDCl_3$ solvent. The Fusion points were recorded in a Stuart SPM11 instrument and uncorrected.

Absorption spectra were recorded on a JASCO V-650 spectrophotometer, and fluorescence emission on a HORIBA-JOBIN-IVON Fluoromax 4 spectrofluorimeter. The linearity of the fluorescence emission vs. concentration was checked in the concentration range of 10^{-4}–10^{-6} M. Corrections to the absorbed light and dilutions were performed when necessary.

A minicentrifuge, Spectrafuge-mini model byLabnet (Madrid, Spain), and a minicentrifuge-vortex, Sky Line model by ELMI (Riga, Latvia), were used throughout the sample treatment when deemed necessary. A Millipore (Milan, Italy) Simplicity 185 was used to obtain Milli-Q water throughout the complete process.

An optical microscope (Leica-ATC 2000, Germany), with a Leica Microsystems image system (DMLB model) was used to take images of the MALDI plate spots after application of the active matrix onto the plate.

The mass spectrometric analyses were performed with a model Voyager-DE PRO Biospectrometry Workstation MALDI system (Applied Biosystems, Foster City, CA, USA), equipped with a nitrogen laser operating at 337 nm. MALDI mass spectra were acquired in positive ion reflectron mode, with an accelerating voltage of 20 kV, an ion extraction delay of 80 ns, a grid voltage of 15 kV, and a guide wire voltage of 40 V. The MALDI mass spectra for each sample were based on the average of 600 laser shots per sample. All the data were processed using the Data Explorer software package (Applied Biosystems).

2.2. Standards and reagents

The standards of 17-α-methyltestosterone, 4-androsten-3,17-dione, boldenone and nandrolone were purchased from Riedel-de Haën (Seelze, Germany). The standards 17-α-Trenbolone, fluoxymesterolone, mesterolone and bolasterone were kindly provided by the Portuguese National Anti-Doping Laboratory.

β-glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany). Sodium hydrogen phosphate, methanol (MeOH), acetonitrile (ACN) and the derivatisation reagent, glacial acetic acid (> 99.5%), was from Fluka (Buchs, Switzerland). Diethyl ether was from Riedel-de Haën.

Hydrazine hydrate and anthracene-9-carboxaldehyde were purchased from Sigma-Aldrich (Steinheim, Germany). Pyrene-1-carboxaldehyde and naphthalene-1-carboxaldehyde were bought from Fluka. All standards and reagents were used without further purification. Solvents were purchased in analytical grade and used as received. All reactions were performed under an argon atmosphere.

2.3. Urine samples

First urine of the day was used in this study. Urine samples used in this work were obtained from healthy volunteers from our research team.

2.4. Sample preparation

2.4.1. Standard solutions

Individual stock standard solutions of each anabolic steroid (500 mg/L) were prepared by weighting 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 and were stable for almost 6 months. Working standard solutions were then prepared by diluting the stock standard solutions with the appropriate volume of methanol.

2.4.2. Urine hydrolysis procedure

Urine samples (4 mL) were hydrolysed with 60 μL of β-glucuronidase from *E. coli*, after the addition of 1.5 mL of phosphate buffer (0.8 M, pH 7). The hydrolysis was performed at 55 °C for 60 min.

2.4.3. Liquid–liquid extraction of target analytes from urine

After cooling the urine samples to room temperature, 1 mL of carbonate buffer (pH 9) was added to alkalinise the hydrolysed solution. Liquid–liquid extraction was carried out via agitation with 5 mL of tert-butyl methyl ether for about 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

2.4.4. Derivatisation procedure

The procedure for derivatisation with the synthesised aromatic probes was performed as follows: the collected eluate sample was dried under a gentle nitrogen stream at room temperature. After the addition of 0.1 mL of a previously made solution of L1 aromatic probe (2 mg/mL) in acetonitrile, the vial was closed and the derivatisation reaction was then performed at 60 °C for 30 min. After derivatisation, the sample was directly hand spotted onto the MALDI sample plate and was allowed to dry.

3. Results and discussion

3.1. Design and synthesis of L1-L3 (scheme SM1)

The syntheses of compounds L1–L3 are outlined in Scheme SM1 (Supplementary Information). The obtained yields were of 89% (L1), 82% (L2), and 98% (L3). All compounds were characterised by elemental analysis, ^{1}H NMR, infrared spectroscopy (KBr pellets), MALDI spectrometry, melting points, and UV–vis and emission spectroscopy. The solid-state structures of the compounds L2 and L3 were determined by single-crystal X-ray crystallography. The irradiation of compound L2 by X-Ray diffraction yielded single-crystals of compound L2′. Characterisation data of the aromatic hydrated hydrazine products are presented as Supplementary Material (see Fig. 1).

3.2. MALDI-TOF-MS analysis of steroids: Evaluation of L1-L3 aromatic probes as active matrices

Molecules such as oxosteroids are poorly ionised by MALDI techniques [20,21,33]. This occurs because these compounds are not easily protonated, since they have a lack of functional groups with high proton affinity. To improve the ionisation of steroids and to obtain better detection limits by MALDI, derivatisation is mandatory.

After the synthesis of the aromatic molecular probes, these compounds were tested as active matrices for the analysis of oxosteroids. The first issue to be overcome was to find a solvent in which both, the matrix and the oxosteroids, would dissolve in. A selection was made from the MS grade solvents commonly used with MALDI matrices, such as, water, methanol and acetonitrile. The solvent acetonitrile was found to be the most suitable for the aforementioned purpose, probably due to the non-polar character of the aromatic probes.

Before the derivatisation reaction, a MALDI-TOF-MS spectrum of each active matrix, named as L1, L2 and L3, was acquired to identify their corresponding characteristic peaks. From the spectra depicted in Fig. 2, it is possible to observe the m/z signals corresponding to the molecular ion and to the molecular ion dimer. Dimer formation occurs mainly due to ultraviolet irradiation from the MALDI laser at 337 nm. In the case of the L1 matrix, the dimer ion peak observed at m/z 309.117 exhibits a distinctly higher intensity (approximately ten-fold than the monomer ion, m/z 171.059; Fig. 2, panel a). For the other two active matrices, the ion peak corresponding to the monomer is clearly more intense than the dimer ion peak.

Fig. 2 shows that the spectrum of matrices L2 and L3 have two characteristics peaks. For instance, the spectrum (b) corresponding to the L2 aromatic probe shows two characteristic peaks at 220.101 and another at 221.108.

Before the MALDI analysis, the oxosteroids were mixed with each active matrix in acetonitrile and heated for 30 min at 60 °C. The steroids nandrolone, 4-androsten-3,17-dione and 17-α-methyltestosterone were selected for these studies. Steroid selection was performed according to their availability and stock at the time of this experiment. In order to ensure a good derivatisation yield whilst maintaining a good performance during the MALDI analysis, a set of experiments was devised in which the concentration of the oxosteroids was set at a constant 2 μg/mL while the concentration of the active matrices varied between 10 and 0.01 mg/mL. This was done with the aim to obtain the best steroid/matrix ratio. Aliquots of 1 μL were directly hand-spotted onto the MALDI sample plate, using the dried droplet method. The results obtained were different for the three matrices assessed, the best MALDI signals being obtained with the concentration of 2, 0.5 and 0.1 mg/mL for the matrices L1, L2 and L3, respectively (data not shown). The theoretical m/z values of the oxosteroids after derivatisation with the active matrices are listed in Table 1.

Once the optimum concentrations of the matrices were found, a second set of experiments was devised to find out the lower concentration of oxosteroid detectable through MALDI. For each active matrix, the oxosteroids were derivatised at four concentration levels: 1; 0.5; 0.1 and 0.05 μg/mL.

The data presented in Fig. 3 demonstrates that it was possible to identify the three steroids for concentration of as low as 0.1 μg/mL. At this concentration, the detection of the three steroids analysed was only possible with the L1 and L3 matrices. For the lower concentration of oxosteroids assessed, m/z values with the highest intensity were obtained with the matrix L1.

As it is well known, factors, such as the matrix solvent and the co-crystallisation process greatly influence the ionisation efficiency and, consequently, the quality of the mass spectra acquired in terms of sensitivity and mass resolution. To better evaluate the impact of these factors, light microscopy images of the MALDI plate spots were obtained. Fig. 4 presents the mixture sample/matrix crystallised. As may be seen, the crystallisation obtained is matrix-dependant. Crystallisation with matrix L1 fulfils the main requirement for higher mass data quality and reproducibility,

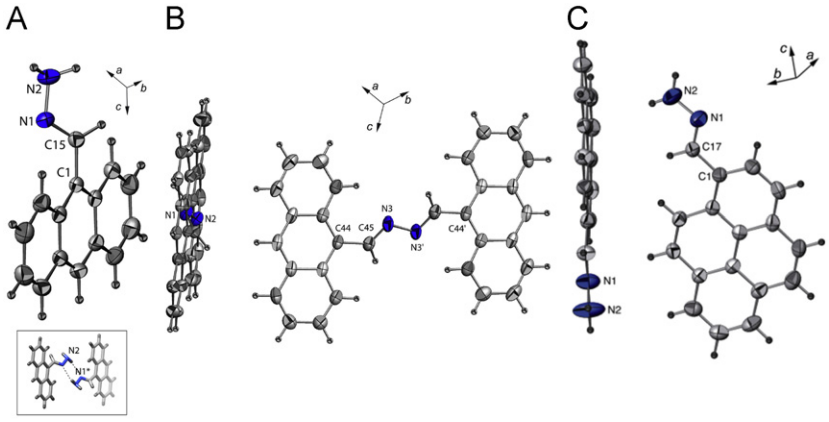

Fig. 1. ORTEP view of the compounds L2 (a), L2′ (b) and L3 (c) with partial labelling scheme. Selected distances (Å) and angles (degree) of the compounds L2: N1-N2: 1.3918(15), N1-C15: 1.274(15), C15-C1: 1.478(16), N1-N2-C15: 116.7(12), N1-C15-C1: 123.9(12), N2-H16…N1*: 0.89(2), 2.468(17), 145.9(13), 3.243(2). Selected distances (Å) and angles (degree) of the compound L2′: N1-N2: 1.418(3), N3-N3′: 1.415(3), N3-C45: 1.263(3), C45-C44: 1.467(3), N3′–N3-C45: 112.2(3), N3-C45-C44: 125.9(2). Selected distances (Å) and angles (degree) of the compound L3: N1-N2: 1.362(5), N1-C17: 1.284(6), C1-C17: 1.457(6), N2-N1-C17: 116.9(4), N1-C17-C1: 121.7(4).

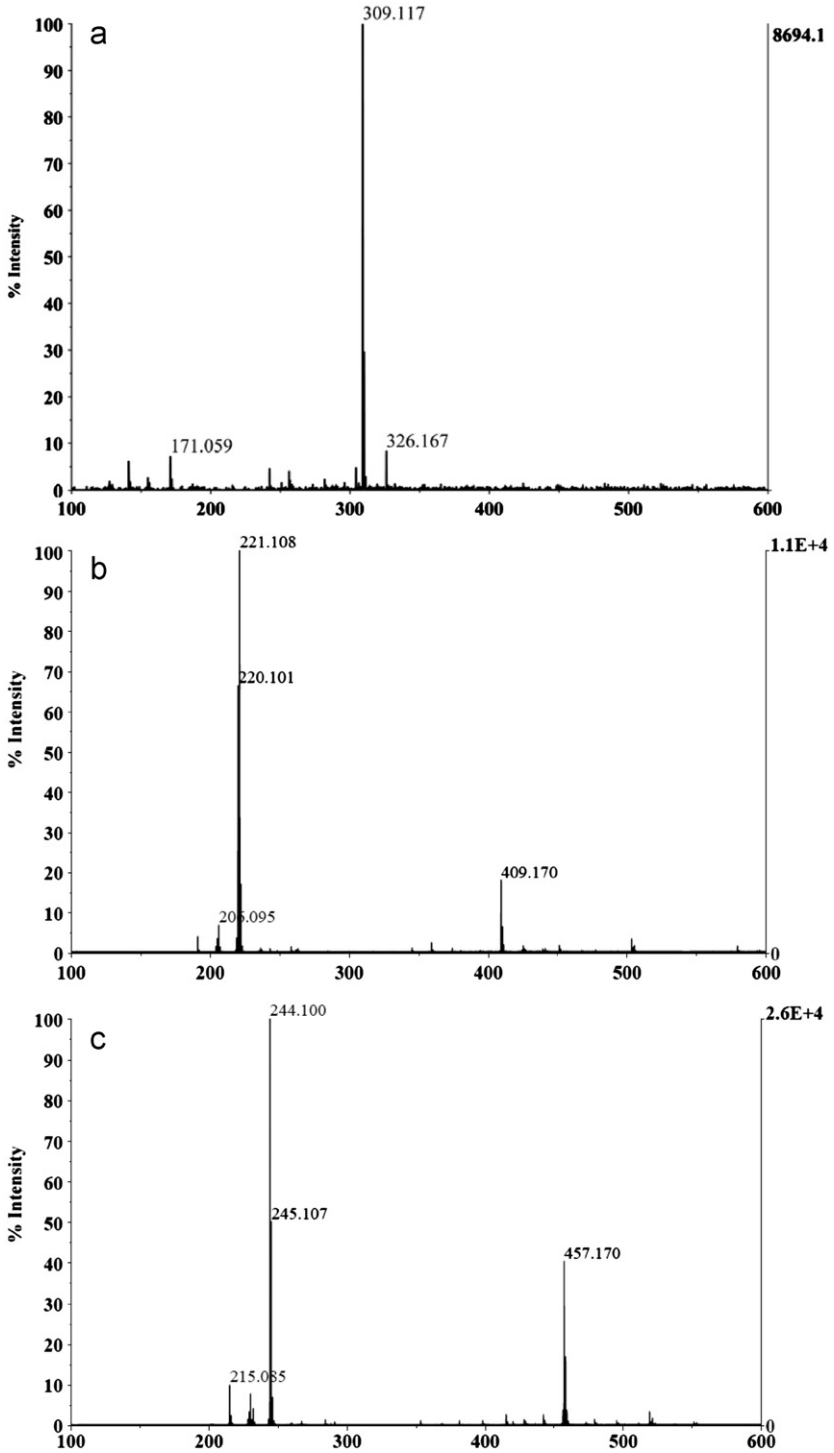

Fig. 2. MALDI mass spectra of (a) the emissive probe L1; (b) the emissive probe L2; (c) the emissive probe L3. Each matrix was at a concentration value of 5 mg/mL, horizontal axis: *m*/*z*.

Table 1
Molecular weights of the free steroids and MALDI-TOF-MS *m*/*z* signal after steroids derivatisation with L1, L2 and L3 active matrices.

AAS	Molecular mass (Da)	*m*/*z* after derivatisation		
		L1	L2	L3
Nandrolone	274.193	427.275	477.291	500.283
4-Androsten-3,17-dione	286.193	439.275	489.291	512.283
17-α-methystestosterone	302.225	455.306	505.323	528.314

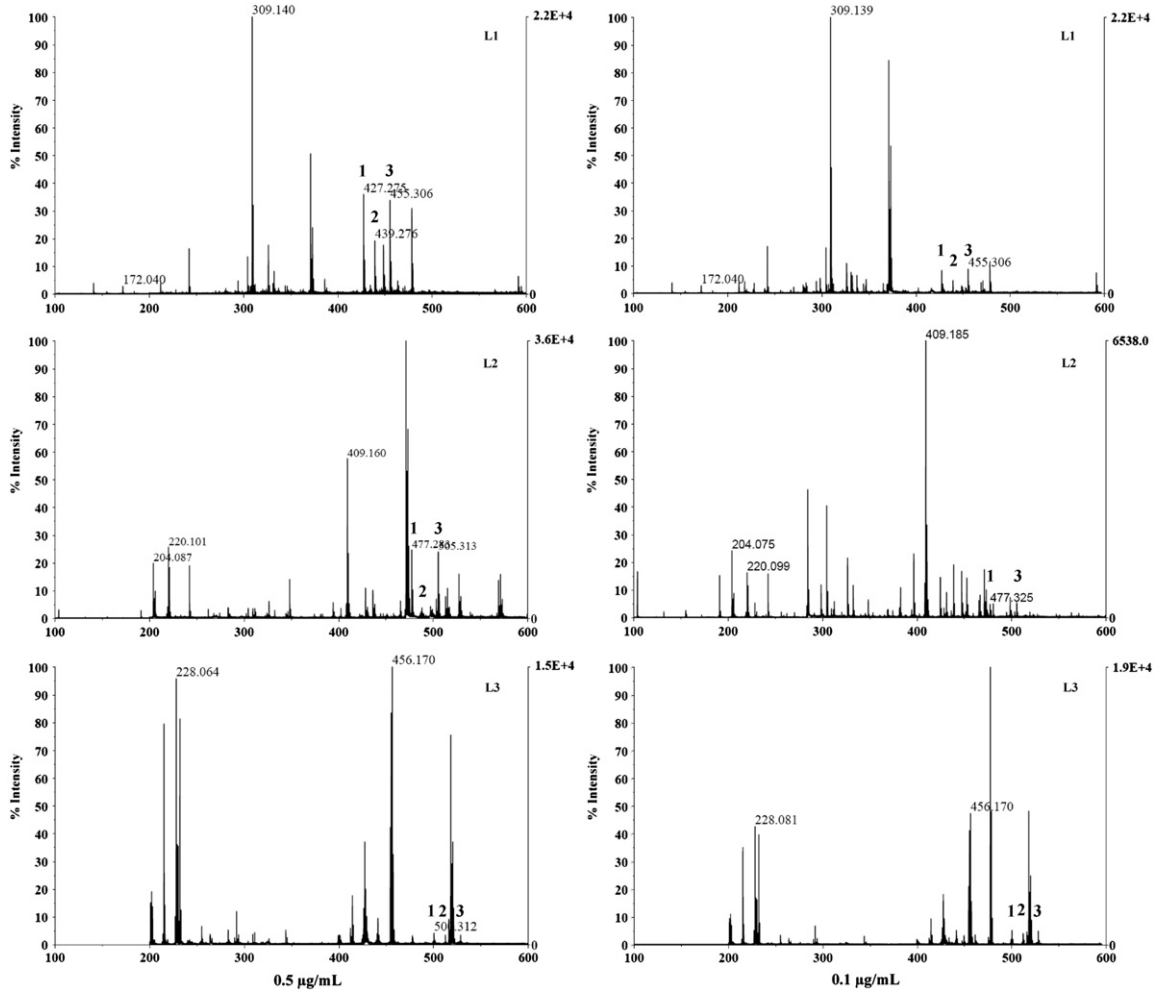

Fig. 3. MALDI mass spectra of the target steroids, at concentrations of 0.5 and 0.1 µg/mL in the MALDI sample plate, obtained with L1, L2 and L3 active matrices. Peak identification: 1: nandrolone; 2: 4-androsten-3,17-dione; 3: 17-α-methyltestosterone, horizontal axis: *m*/*z*.

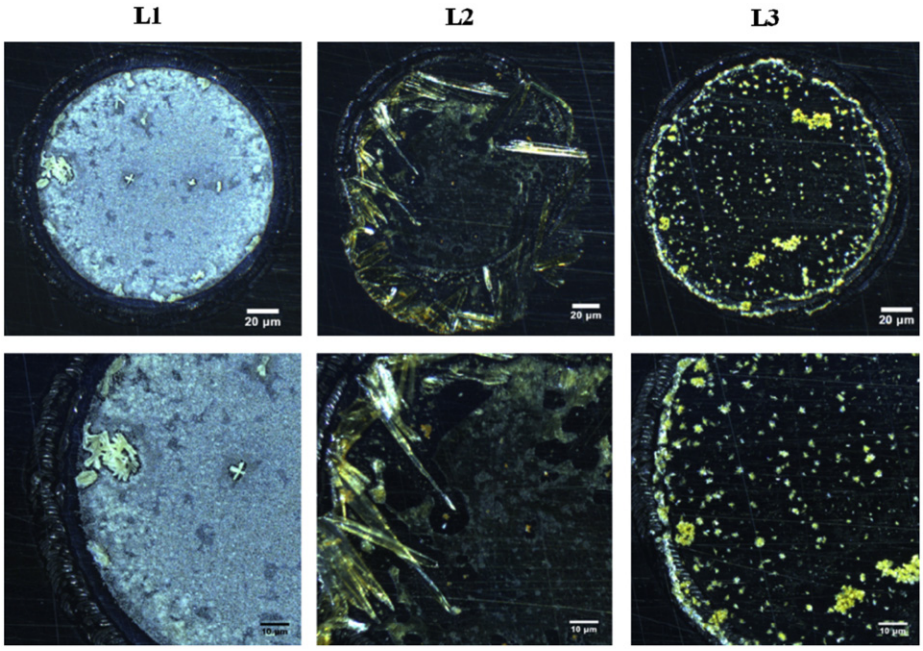

Fig. 4. Optical microscopic images, at two levels of magnification, of L1, L2 and L3 active matrix-sample crystals formed after application onto the MALDI sample plate.

which is the formation of a microcrystalline layer uniformly distributed within the spot. This factor may be the reason why L1 showed the better performance.

3.3. Proof-of-concept: Determination of oxosteroids in human urine

As proof-of-concept, human urine samples were spiked with oxosteroids, with the aim to demonstrate that this methodology can be used as a fast and robust screening method for oxosteroids, mainly exogenous oxosteroids. Table 2 shows the theoretical *m*/*z* values of the oxosteroids used in this study after derivatisation with the L1 active matrix.

The present analytical method was developed and tested in agreement with international guidelines for qualitative methods and within the requirements established by anti-doping agencies for the analysis of these compounds (e.g. minimum required performance levels) [34–36]. The parameters used to assess the method were:

(a) *Specificity*. The ability of the assay to identify negative samples as truly negative and to discriminate between compounds possessing related structures.
(b) *Sensitivity and identification capability*. The ability of the assay to identify positive samples as truly positive. This parameter depends on the amount of analyte, but the test should be capable of identifying the analyte as present in the sample even the minimum required performance levels (MRPL).
(c) *Robustness*. The ability of the method to produce similar results in spite of minor variations to the analytical conditions.
(d) *False negative rate*. The probability that the method identifies positive samples incorrectly as negative.
(e) *False positive rate*. The probability that the method identifies negative samples incorrectly as positive.

Assesment was performed through the analysis of 6 urine samples, by triplicate, spiked with the oxosteroids listed in Table 2. The oxosteroid concentration values were randomly chosen and were in the range of routine reporting results by WADA laboratories (see Table 2). The urinary concentration of the compounds nandrolone and bolasterone were set to their MRPL to evaluate the identification capability of the method. The analysis of the 6 blank urine samples was also performed.

Table 3 summarises the quality parameters assessed. Specificity was defined as the probability to obtain a negative identification given that no analyte was present [35]. The specificity achieved was 100%.

Sensitivity was assessed by analysing the 6 urine samples spiked with the oxosteroids presented in Table 2. Sensitivity of the methodology was estimated from the ratio between the number of samples correctly identified as positives and the total number of samples known to be positives. For the evaluation of this parameter, urine samples were considered positives when the analyte was present at a concentration value equal or in excess to the MRPL. All the oxosteroids spiked in the 6 urine samples used in this study were identified. Accordingly, the level of sensitivity achieved was 100%.

From the values of specificity and sensitivity, it is easy to conclude that the rate of false positives and false negatives accomplished by the developed method is 0%.

Regarding the robustness of the methodology, small modifications to the analytical procedure were made, such as using different volumes of extraction solvent and applying slight variations to the derivatisation temperature and time within the 12 urine samples employed in this study (6 blanks and 6 spiked urine samples). For all the modifications assessed sensitivity and selectivity was always 100%.

Fig. 5 presents the MALDI-MS data obtained for the analysis of blank and spiked urine samples. As it is shown, all oxosteroids spiked in the urine were detected. It is important to highlight that the other peaks that can be noted in the MALDI mass spectra correspond to the endogenous steroids. For instance, the most intense peak at *m*/*z* 443.302 is the characteristic peak of androsterone and etiocholanolone, while the peak at *m*/*z* 441.287 is characteristic for testosterone, epitestosterone and DHEA. Moreover, the peak at 457.322 is a characteristic peak of 11-ketoetiocholanolone and the one at 459.301 is characteristic of both 11-hydroxyandrosterone and 11-hydroxyetiocholanolone.

The use of this new type of MALDI matrices allows the considerably reduction of the sample handling and usual required time in the derivatisation protocol using Girad T reagent, not to mention the great improvement when compared to GC–MS strategies [21].

It is important to stress that although the identification of isobaric compounds by MALDI-TOF-MS is not possible without a separation procedure prior to the analysis or by the use of post-source decay (PSD) product-ion analysis, the study presented in this report deals with the synthesis of aromatic probes that can act as active matrices for the detection of small molecules, such as oxosteroids, by MALDI techniques. This is of great importance, since the results achieved can potentially also be applied to MALDI-TOF-TOF-MS.

Table 2
Molecular weights of the free steroids and MALDI-TOF-MS *m*/*z* signal after steroids derivatisation with L1 active matrix and concentration of the oxosteroids in urine.

AAS	Molecular mass (Da)	*m*/*z* after derivatisation	Concentration in urine (ng/mL)
Nandrolone	274.193	427.275	2
17-α-methyltestosterone	455.306	50	302.225
Boldenone	286.193	439.275	100
Trenbolone	270.162	423.244	200
Fluoxymesterone	336.210	489.292	100
Mesterolone	304.240	457.322	75
Bolasterone	316.240	469.322	10

Table 3
Qualitative performance parameters and validation parameters rate expression and values.

Parameter	Expression	Result
Specificity	$TN/TN+FP$	100%
Sensitivity	$TP/TP+FN$	100%
False positive	$FP/TN+FP$	0%
False negative	$FN/TP+FN$	0%
Robustness	–	Robust

4. Conclusion

Of the three aromatic probes designed and tested is this study, the compound L1, was found to be the most suitable for the fast screening of oxosteroids. L1 promotes the formation of homogeneous and regularly distributed crystals, which is essential for a good result in MALDI-analysis. Furthermore, the presence of interfering peaks at the characteristic *m*/*z* of most oxosteroid derivatives is avoided. The method proposed has a sensitivity and specificity of 100%, matching the criteria of the WADA agency. In

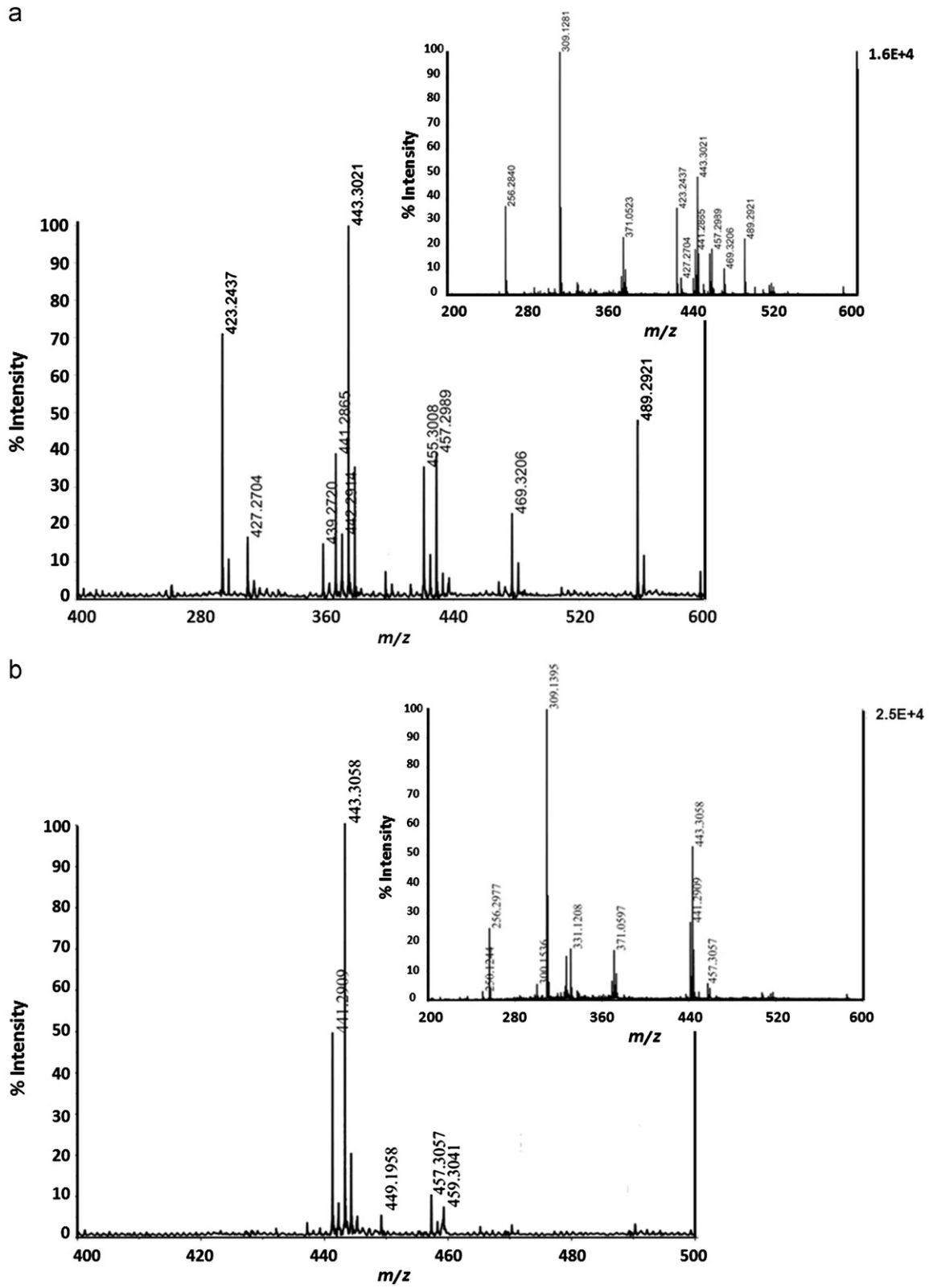

Fig. 5. (a) MALDI mass spectra of a spiked urine after derivatisation with L1 active matrix. Peak identification: *m*/*z* 423.244: 17-α-trenbolone; *m*/*z* 427.270: nandrolone; *m*/*z* 439.272: Boldenone; *m*/*z* 455.310: 17-α-methyltestosterone; *m*/*z* 469.321: Bolasterone; *m*/*z* 489.292: fluoxymesterolone. (b) MALDI mass spectra of a blank urine after derivatisation with L1 active matrix.

addition the robustness of the method was demonstrated. It was also possible to detect all compounds used in this study at their minimum required performance levels (MRPL) in urine.

This new approach greatly simplifies sample handling and is less time consuming than other procedures, such as the one using the derivatisation reagent Girard T and GC–MS-based methods.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.07.013.

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