

# HR-MS/MS EAD and CID fragmentation leads to selective, sensitive and reliable quantitation of anabolic steroids in urine.

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The authors declare no competing financial interest.

## INTRODUCTION

Anabolic androgenic steroids (AAS) are a group of natural and synthetic compounds that are chemically similar to endogenous testosterone. They stimulate increase in muscle mass, strength and reduction of body fat. The use of AAS in sports is banned, however they are still the most abused class of drugs in sports. High-resolution mass spectrometry (HRMS) offers analytical laboratories a powerful tool to efficiently and confidently detect and identify drugs using mass defect data on a precursor and quantifier ion with parallel spectral library matching followed by quantitative analysis at low levels.

## MATERIALS AND METHODS

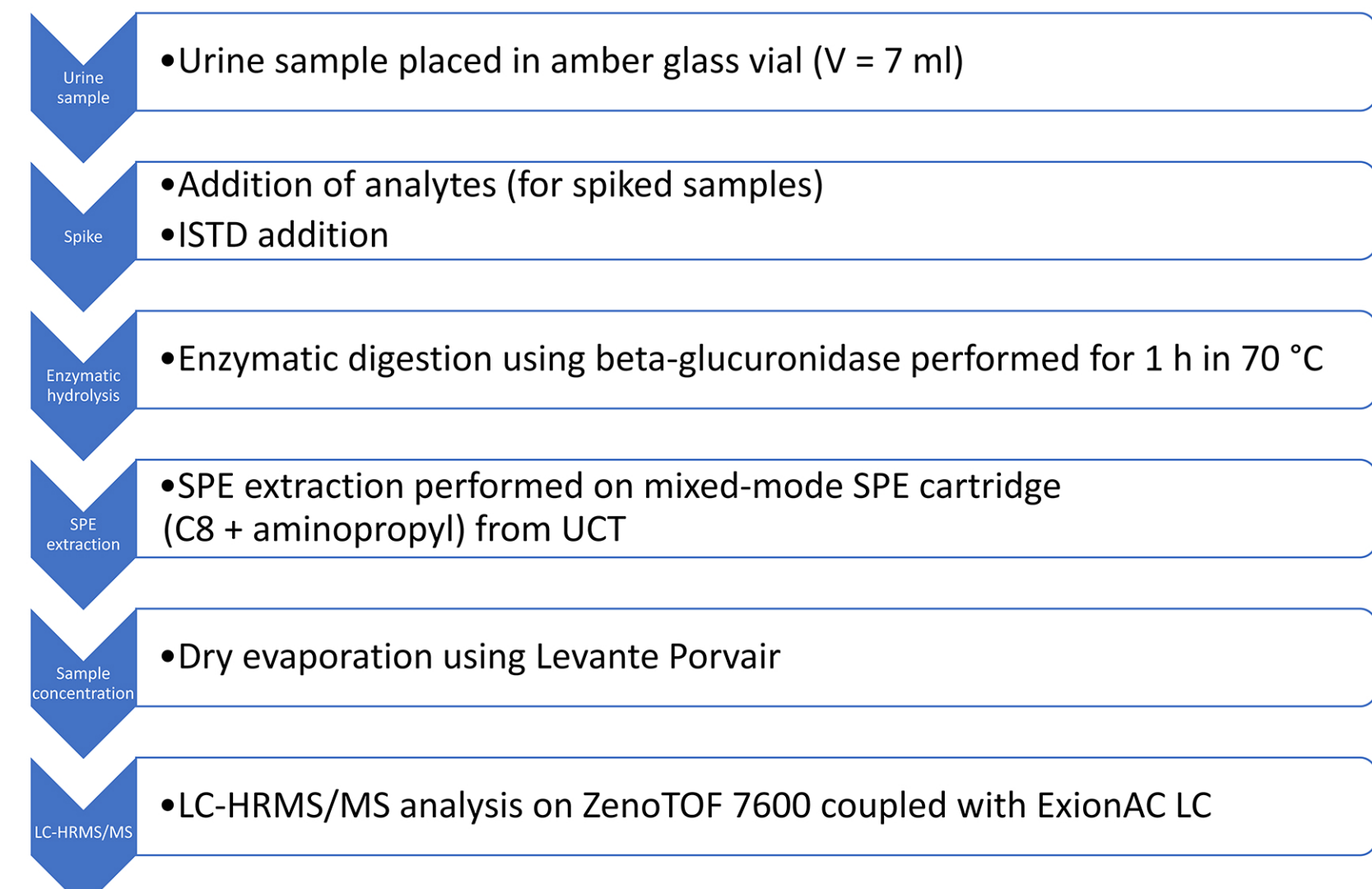


Figure 1. Workflow of sample pretreatment.

Samples were separated using reversed phase chromatography with ExionAC system on a Kinetex 2.6 μm XB-C18 (100 Å; 100 x 2.1 mm) column at 40°C with 500 μl/min flow rate. The mobile phases used were water and 50% acetonitrile in methanol (Table 1).

Table 1. Mobile phase gradients used for the LC separation.

Time [min]	B [%]
0	30
1	30
6	95
10	100
10.5	30

MS and MS/MS detection was conducted on a ZenoTOF 7600 system operated in positive electrospray ionization (ESI) mode. MRM<sup>HR</sup> workflow was used to obtain maximum sensitivity. CID and novel EAD fragmentation modes were applied for the identification and quantification of compounds in the matrix.

## RESULTS

Table 2. Compound settings in MRM<sup>HR</sup> (CID) method.

Compound ID	Group Name	Precursor Ion (Da)	TOF Start Mass (Da)	TOF Stop Mass (Da)	Accumulation Time (sec)	DP (V)	CE (V)	CE Spread (V)	Retention Time (min)	Retention Time Tolerance (+/- sec)
1	6-beta-hydroxymethandienone	317.21	50.00000	320.00000	0.1000	80	17	0	3.04	15
2	3-hydroxystanozolol	345.25	50.00000	350.00000	0.0500	120	62	0	4.23	15
3	4beta-hydroxystanozolol	345.25	50.00000	350.00000	0.0500	100	24	0	4.23	15
4	17-alpha-trenbolone	271.18	50.00000	275.00000	0.0500	120	107	0	4.26	15

Table 3. Compounds settings in MRM<sup>HR</sup> (EAD) method. Note, that it possible to performed analysis both in CID or EAD fragmentation in one method.

Compound	Group Name	Precursor Ion (Da)	TOF Start Mass (Da)	TOF Stop Mass (Da)	Accumulation Time (sec)	DP (V)	CE (V)	CE Spread (V)	Fragmentation mode	Electron KE (eV)	ETC (%)	Retention Time (min)	Retention Time Tolerance (+/- sec)
1	6-beta-hydroxymethandienone	317.21	50.00000	320.00000	0.1000	80	17	0	EAD	15.000	100.0	3.10	15
2	3-hydroxystanozolol	345.25	50.00000	350.00000	0.0500	120	62	0	CID	15.000	100.0	4.21	15
3	4beta-hydroxystanozolol	345.25	50.00000	350.00000	0.0500	100	24	0	EAD	15.000	100.0	4.23	15
4	17-alpha-trenbolone	271.18	50.00000	275.00000	0.0500	120	107	0	EAD	15.000	100.0	4.23	15

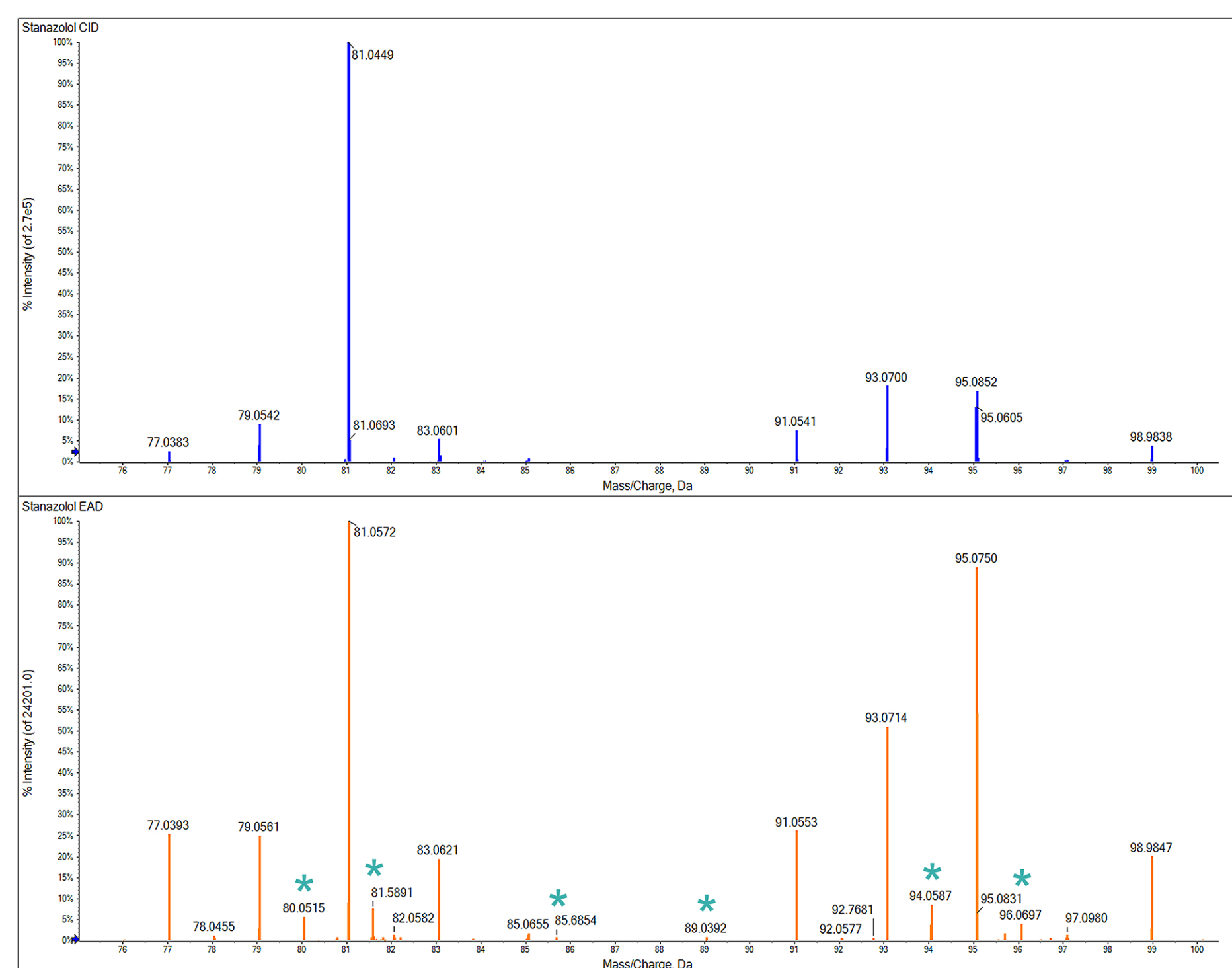


Figure 2. An example comparison of the stanazolol fragmentation spectrum obtained with the CID (top) and EAD (bottom) fragmentation in the range of 75-100 m/z. EAD fragmentation provides unique fragmentation ions.

ZenoTOF 7600 LC-MS/MS system was used to establish sensitive, selective, qualitative, and quantitative method to determine 28 AAS in urine matrix. The matrix-matched calibration curves were prepared in the range of 0.01 - 100 ng/ml. The linearity range ( $r \geq 0.995$ ) of the curves were analyte dependent. The library created by our team collects the fragmentation spectra after optimization of CE per analyte in matrix from both CID and EAD fragmentation methods as there are no commercial databases for EAD fragmentation data. The HR-MS/MS enables determination of additional factors confirming the presence of the compound including mass defect for precursor and quantifier ion, isotope distribution and library matching. In addition, the presence of Zeno ion trap gives better quality and sensitivity of the fragmentation spectrum.

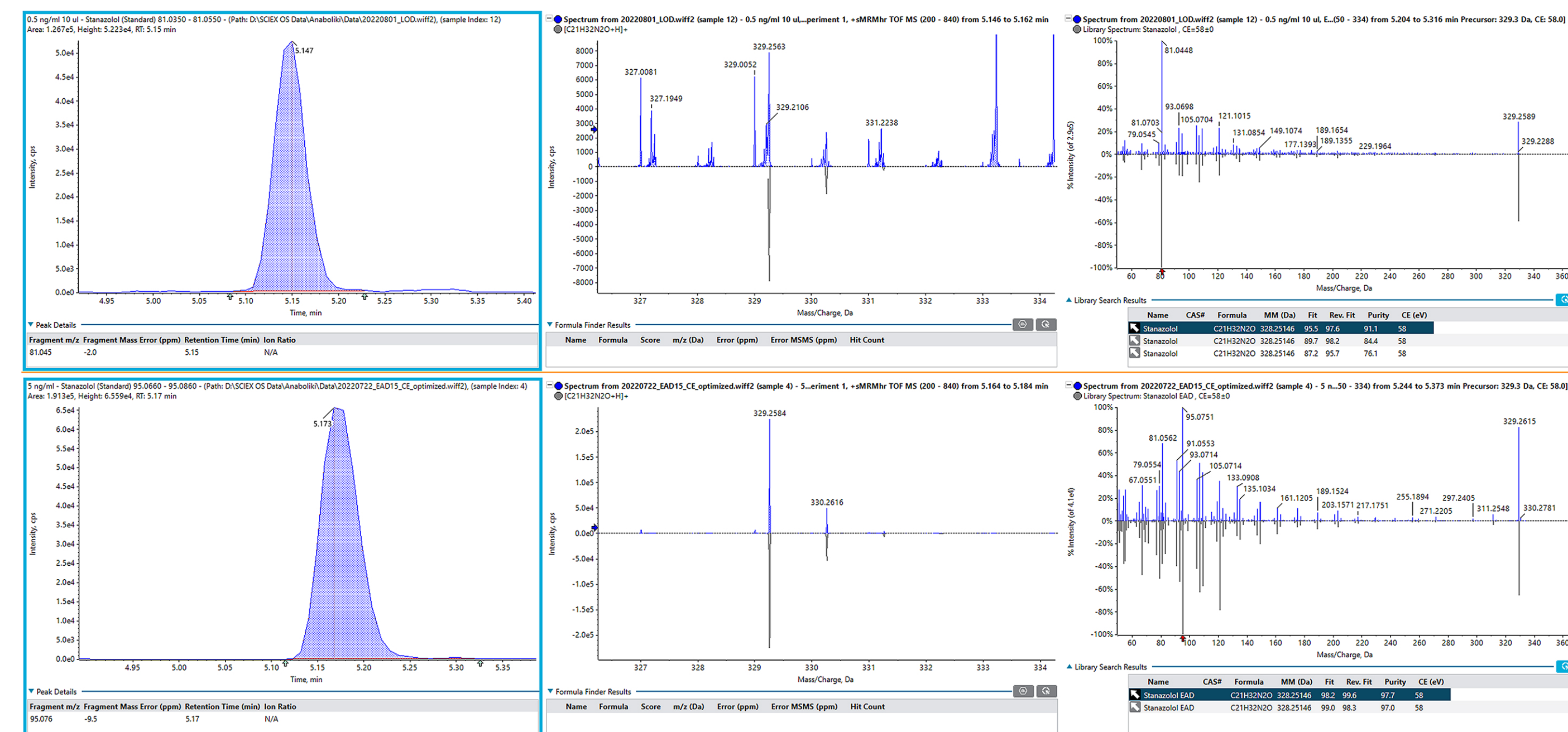


Figure 3. Example data obtained using CID (up) and EAD (down) fragmentation mode.

Table 4. Example of multi-level confirmation and scoring by the anabolic AAS method in urine using Zeno TOF 7600 LC-MS/MS system.

Component Name	Formula	Precursor Mass	Found At Mass	Mass Error (ppm)	Fragment Mass	Found At Fra.	Fragment Mass Error	Mass Error	Frag. Mass	RT Conf.	Isotope Conf.	Library Conf.	Retention Time	Library HR	Library Score	Comb. Score	Isotope Ratio Difference
6-beta-hydroxymethandienone	C20H28O3	317.211	317.2113	0.5	299.201	299.2016	2.5	✓	✓	✓	✓	✓	3.02	6-beta-hydroxymethandienone	97.4	89.952	8189.8
3-hydroxystanozolol	C21H32N2O2	345.254	345.2539	0.7	97.040	97.0399	0.4	✓	✓	✓	✓	✓	4.21	3-hydroxystanozolol	94.6	94.198	4.7
4beta-hydroxystanozolol	C21H32N2O2	345.254	345.2539	0.7	309.233	309.2364	11.1	✓	✓	✓	✓	✓	4.21	3-hydroxystanozolol (Smart Confirmation)	94.6	83.534	4.8
17-alpha-trenbolone	C18H22O2	271.169	271.1695	0.7	115.054	115.0545	1.8	✓	✓	✓	✓	✓	4.23	17-alpha-trenbolone	100.0	93.984	2.3
16-beta-hydroxystanozolol	C21H32N2O2	345.254	345.2539	0.8	81.045	81.0452	2.0	✓	✓	✓	✓	✓	4.30	16-beta-hydroxystanozolol	85.0	87.395	5.5
Chandrolone	C19H30O3	307.227	307.2268	0.2	229.195	229.1955	2.3	✓	✓	✓	✓	✓	4.42	Chandrolone	76.4	83.232	8.4
Methandienone	C20H28O2	301.216	301.2158	-1.5	121.065	121.0651	1.7	✓	✓	✓	✓	✓	4.44	Methandienone	92.9	88.113	14.3
Testosterone-D3	C19H25.2H3 O2	292.235	292.2348	-0.9	97.065	97.0649	0.3	✓	✓	✓	✓	✓	4.54	Testosterone-D3	99.6	96.907	2.4
Testosterone	C19H28O2	288.216	288.2159	-1.0	97.065	97.0652	3.1	✓	✓	✓	✓	✓	4.55	Testosterone [2]	99.0	93.349	3.7
Epitestosterone	C19H28O2	288.216	288.2165	1.0	97.065	97.0652	3.0	✓	✓	✓	✓	✓	4.57	Epitestosterone	97.2	92.328	4.6
Turinabol	C20H27O2	335.177	335.1776	1.1	155.026	155.0262	0.1	✓	✓	✓	✓	✓	5.00	Turinabol	97.4	93.966	9.5
5-alpha-dihydrotestosterone	C19H30O2	291.232	291.2322	1.2	255.211	255.2115	2.5	✓	✓	✓	✓	✓	5.04	5-alpha-dihydrotestosterone	90.8	85.792	17.9
Stanozolol	C21H32N2O	329.259	329.2590	0.9	81.043	81.0433	3.7	✓	✓	✓	✓	✓	5.15	Stanozolol	94.5	92.198	2.0
19-nandrolone	C18H28O2	277.216	277.2156	-2.1	241.195	241.1954	1.8	✓	✓	✓	✓	✓	5.19	19-nandrolone	96.5	87.107	21.6
Trenbolone acetate	C22H40O3	313.180	313.1800	0.5	115.054	115.0544	1.8	✓	✓	✓	✓	✓	5.39	Trenbolone acetate	98.3	95.582	3.3
Methenolone acetate	C22H40O3	345.242	345.2425	0.1	83.049	83.0493	1.5	✓	✓	✓	✓	✓	6.10	Methenolone acetate	100.0	97.268	3.9
Testosterone propionate	C22H38O3	345.242	345.2428	1.2	97.065	97.0652	2.9	✓	✓	✓	✓	✓	6.28	Testosterone propionate	98.8	91.685	5.0
Methandienone phenylpropionate	C27H40O3	407.258	407.2582	0.3	105.070	105.0701	1.0	✓	✓	✓	✓	✓	6.67	Methandienone phenylpropionate	99.3	96.344	5.3
Testosterone phenylpropionate	C28H40O3	421.274	421.2736	-0.3	105.070	105.0699	-0.8	✓	✓	✓	✓	✓	6.81	Testosterone phenylpropionate	98.1	93.238	5.7
Drostanolone propionate	C28H40O3	361.274	361.2734	-0.9	269.226	269.2271	1.7	✓	✓	✓	✓	✓	6.94	Drostanolone propionate	96.9	92.568	4.7
Testosterone caproate-isoacipate	C28H40O3	387.289	387.2893	-0.2	97.065	97.0648	-1.4	✓	✓	✓	✓	✓	7.04	Testosterone caproate-isoacipate	97.8	96.402	2.1
Testosterone enanthate	C28H40O3	401.305	401.3050	0.0	97.065	97.0650	1.2	✓	✓	✓	✓	✓	7.30	Testosterone enanthate	98.9	97.247	3.3
Testosterone cypionate	C27H40O3	413.305	413.3052	0.5	97.065	97.0649	0.4	✓	✓	✓	✓	✓	7.38	Testosterone cypionate	99.4	96.866	5.0
Methenolone enanthate	C27H40O3	413.321	413.3209	0.5	83.049	83.0491	-0.1	✓	✓	✓	✓	✓	7.45	Methenolone enanthate	93.6	94.587	3.7
Boldenone undecanoate	C30H44O3	453.336	453.3368	1.0	121.065	121.0651	1.5	✓	✓	✓	✓	✓	7.65	Boldenone undecanoate	98.6	96.428	5.9
Drostanolone enanthate	C27H40O3	417.316	417.3165	0.5	269.226	269.2268	1.5	✓	✓	✓	✓	✓	7.90	Drostanolone enanthate	94.8	93.266	10.1
Testosterone decanoate	C29H48O3	443.352	443.3520	0.0	97.065	97.0647	-1.7	✓	✓	✓	✓	✓	8.10	Testosterone decanoate	97.7	96.500	1.6
Testosterone undecanoate	C30H48O3	457.368	457.3679	0.6	97.065	97.0650	0.7	✓	✓	✓	✓	✓	8.45	Testosterone undecanoate	99.0	96.970	2.6

## CONCLUSIONS

**The developed LC-HR-MS/MS method is characterized by a way better specificity when compared to the analyzes performed on any LR device. EAD gives complementary diagnostic fragment ions, enabling more definitive characterization and confirmation of compound presence.**