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

Adrian Soboń[1,2], Julia Mironenka[1], Anna Lenartowicz[1], Rafał Szewczyk[1,2], Katarzyna Krupczyńska-Stopa[1,2], Maciej Stopa[1,2], Andrzej Kwaśnica[3]

1. LabExperts sp. z o. o., Gdańsk, Poland; 2. Bioanalytic sp. z o. o., Gdańsk, Poland; 3. Lab4Tox sp. z o. o., Wrocław, Poland
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.
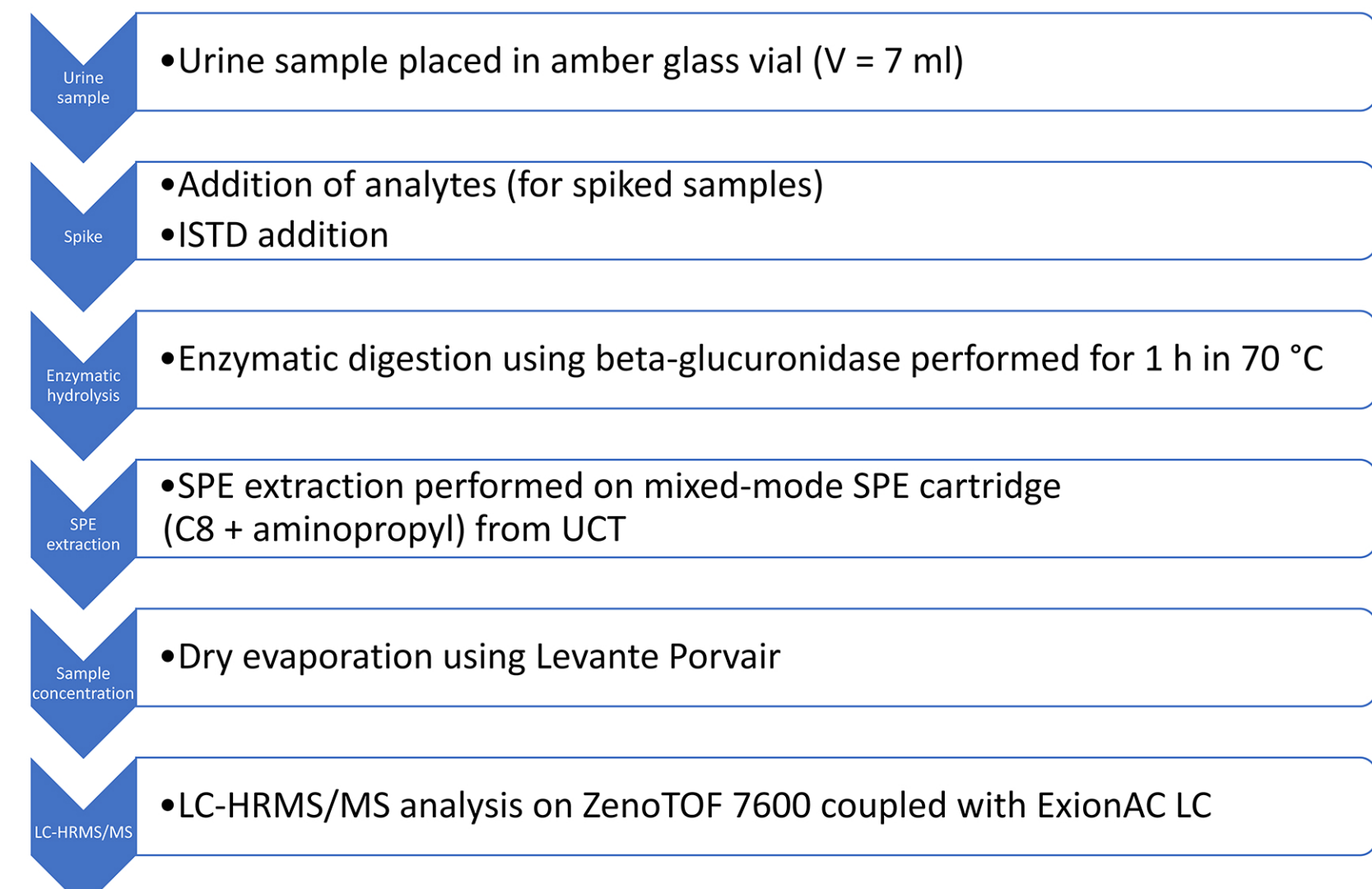
MATERIALS AND METHODS

- **Urine sample**: • Urine sample placed in amber glass vial (V = 7 ml)
- **Spike**: • Addition of analytes (for spiked samples) • ISTD addition
- **Enzymatic hydrolysis**: • Enzymatic digestion using beta-glucuronidase performed for 1 h in 70 °C
- **SPE extraction**: • SPE extraction performed on mixed-mode SPE cartridge (C8 + aminopropyl) from UCT
- **Sample concentration**: • Dry evaporation using Levante Porvair
- **LC-HRMS/MS**: • LC-HRMS/MS analysis on ZenoTOF 7600 coupled with ExionAC LC

Figure 1. Workflow of sample pretreatment.

Samples were separated using reversed phase chromatography with ExionLC AC system on a Kinetex 2.6 µm XB-C18 (100 A; 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[HR] 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[HR] (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	Group 01	317.21	50.00000	320.00000	0.1000	80	17	0	3.04	15
2	3-hydroxystanozolol	Group 02	345.25	50.00000	350.00000	0.0500	120	62	0	4.23	15
3	4beta-hydroxystanozolol	Group 03	345.25	50.00000	350.00000	0.0500	100	24	0	4.23	15
4	17-alpha-trenbolone	Group 04	271.18	50.00000	275.00000	0.0500	120	107	0	4.26	15

Table 3. Compounds settings in MRM[HR] (EAD) method. Note, that it possible to performed analysis both in CID or EAD fragmentation in one 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	Fragmentation mode	Electron KE (eV)	ETC (%)	Retention Time (min)	Retention Time Tolerance (+/- sec)
1	6-beta-hydroxymethandienone	Group 01	317.21	50.00000	320.00000	0.1000	80	17	0	EAD	15.000	100.0	3.10	15
2	3-hydroxystanozolol	Group 02	345.25	50.00000	350.00000	0.0500	120	62	0	EAD	15.000	100.0	4.21	15
3	4beta-hydroxystanozolol	Group 03	345.25	50.00000	350.00000	0.0500	100	24	0	CID	15.000	100.0	4.21	15
4	17-alpha-trenbolone	Group 04	271.18	50.00000	275.00000	0.0500	120	107	0	EAD	15.000	100.0	4.23	15
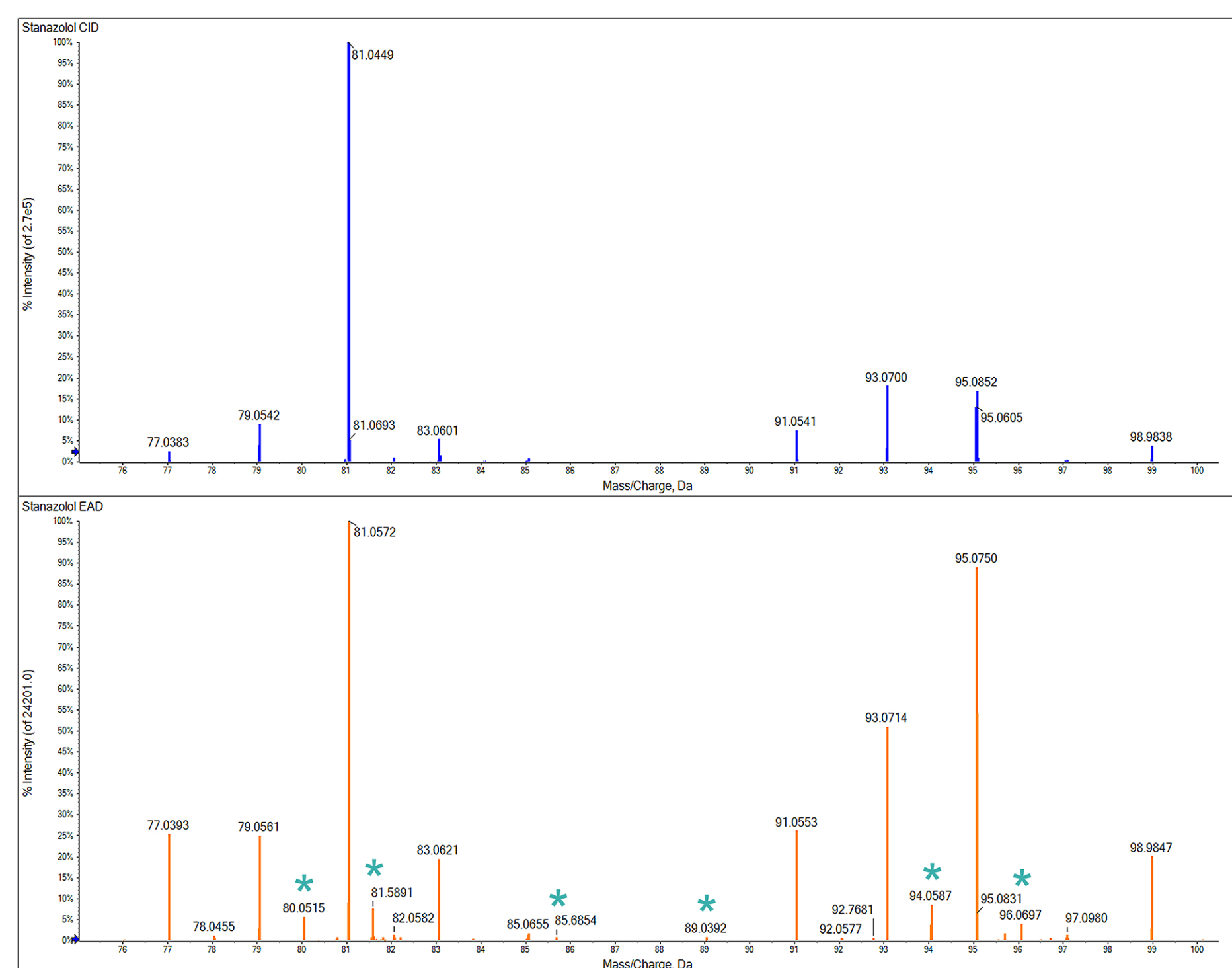
Figure 2. An example comparison of the stanazolol fragmentation spectrum obtained with the CID (top) and EAD (bottom) fragmentation in the range of 75-100 m/z. EAD fragmentation provides unique fragmentation ions.

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 ≥ 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.
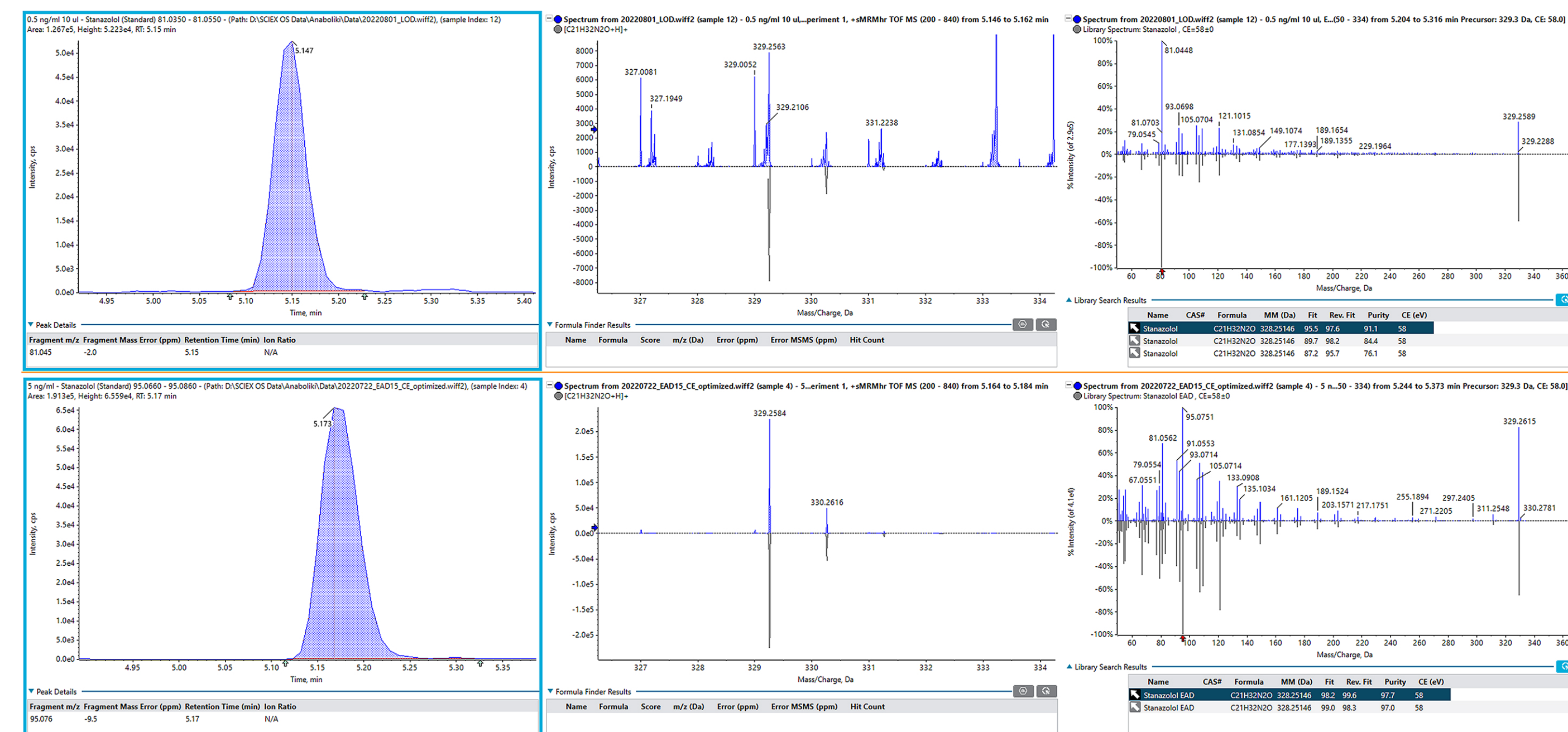

Figure 3. Example data obtained using CID (up) and EAD (down) fragmentation mode.

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

Developed processing method use multi-level confirmation and combined scoring based on HR MS and MS/MS data. The cut-off point of combined score was estimated at 70%.

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.