APPLICATION NOTE

# Detection of steroids and SARMs

A-105.1

## Keywords

Selective Androgen Receptor Modulators, endogenous hormones, testosterone, anabolic, androgen

## Introduction

Steroids are used since the 1950's in competitive sports and in personal attempts to improve the body shape. Today, in addition to the steroids, SARMs (Selective Androgen Receptor Modulators) are increasingly developed and marketed as Dietary supplements. Most of these novel SARM structures are derived from the antiandrogen bicalutamide, and their properties and long term side effects have not been assessed. Due to the ever-growing demand for "new miracle drugs" not commercialized candidates from almost forgotten data bases of many pharmaceutical companies are appearing on a regular basis in the online market, despite the obvious risks. HPTLC offers a rapid way of screening for these substances. Following a simple sample preparation step, several samples can be analyzed in parallel for the presence or absence of steroids and SARMS.

## Scope

This method is suitable for detection of steroids and SARMs in fitness boosters and dietary supplements. Both substance classes can be analyzed by scanning densitometry and visual inspection after selective derivatization. Quantitation is done by scanning densitometry with the TLC Scanner. The CAMAG TLC-MS Interface 2 is used to directly elute target zones from the HPTLC plate into the Waters ACQUITY QDa® for mass detection. A second confirmation can be achieved by recording the UV spectra with the TLC Scanner. A fast screening method is shown below.

## Required or recommended devices

Automatic TLC Sampler (ATS 4), Automatic Developing Chamber (ADC 2), Chromatogram Immersion Device 3 or Derivatizer, TLC Plate Heater 3, TLC Visualizer or TLC Visualizer 2, visionCATS, TLC Scanner 3 or 4, UV Cabinet 4, TLC-MS Interface 2, Waters ACQUITY QDa Detector (Performance), Empower® or MassLynx® software

## Samples

Samples were purchased from online stores and extracted with dichloromethane (provided by Lipomed AG, Arlesheim, Switzerland). For the quantification of methadienone in a tablet methyltestosterone was added as internal standard (same amount as claimed for the steroid). To confirm the presence of ostarine and ibutamoren in capsules the UV and mass spectra were recorded.

## Standards

Standards were dissolved in dichloromethane (0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL). Standards were provided by Lipomed AG (Arlesheim, Switzerland).

## Chromatography

| | |
|---|---|
| Stationary phase | HPTLC Si 60 $F_{254}$, 20 x 10 cm (Merck). |
| Sample application | Bandwise application with ATS 4, 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume between 2-10 µL for sample and standard solutions. |
| Developing solvent | Steroids: heptane – ethyl acetate 1:1 (v/v)<br>SARMs: dichloromethane – methanol 9:1 (v/v) |
| Development | In the ADC 2 with chamber saturation (with filter paper) 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride. |
| Developing distance | 70 mm (from the lower edge) |
| Plate drying | Drying 5 min in the ADC 2 |
| Documentation | With the TLC Visualizer 2 under UV 254 prior derivatization and UV 366 nm and white light after derivatization. |
| Densitometry | Densitometric analyses are performed at 254 nm and several wavelengths for quantification (multi-wavelength scan), slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, spectra recording 190 to 450 nm. |
| Derivatization | (1) Reagent name: Seebach reagent<br>Reagent preparation: 5 g of phosphomolybdic acid, 2 g of cerium (IV) sulfate, and 12 mL of $H_2SO_4$ are dissolved in 190 mL of water and filled up to 200 mL.<br>Reagent use (dipping): The plate is immersed into 200 mL of Seebach reagent using the Chromatogram Immersion Device, immersion time 1 s and immersion speed 3 cm/s and then heated on the TLC Plate Heater at 110°C for 5 min.<br>Reagent use (spraying): The plate is sprayed with 3 mL of Seebach reagent with the Derivatizer, red nozzle, spraying level 2 and then heated on the TLC Plate Heater at 110°C for 5 min.<br>(2) Reagent name: Toluene sulfonic acid reagent<br>Reagent preparation: 10% in ethanol<br>Reagent use (dipping): The plate is immersed into 200 mL of Toluene sulfonic acid reagent using the Chromatogram Immersion Device, immersion time 1 s and immersion speed 3 cm/s and then heated on the TLC Plate Heater at 150°C for 3 min.<br>Reagent use (spraying): The plate is sprayed with 3 mL of Seebach reagent with the Derivatizer, blue nozzle, spraying level 3 and then heated on the TLC Plate Heater at 110°C for 5 min. |
| MS confirmation | The zones to be eluted are marked with a soft pencil under UV 254 nm using the UV Cabinet or TLC Visualizer 2. For non-UV-active compounds: standards or samples are applied twice. One part of the plate is derivatized for localizing the corresponding zones on the non-derivatized part of the plate.<br>Target zones are directly eluted using the TLC-MS Interface 2 with oval elution head into the ACQUITY QDa Detector at a flow rate of 0.5 mL/min with methanol (with 0.1% formic acid). For a full scan spectrum it is recommended to first elute a blank, which can be subtracted from the spectra of the target zones. For confirmation of substances between 50 and 500 ng per zone are required. |

MS parameter

The ACQUITY QDa Detector is operated in $ESI^+$ mode using default parameters. The ESI capillary is set to 0.8 kV, cone voltage to 15 V, and desolvation temperature at 600 °C. A full scan mass spectrum between m/z 200 and 600 is acquired at a sampling rate of 10.0 points/sec (continuum). Data processing and evaluation of mass spectra are performed with Empower. For routine use in quality control Single Ion Recording (SIR) can be performed.

## Results

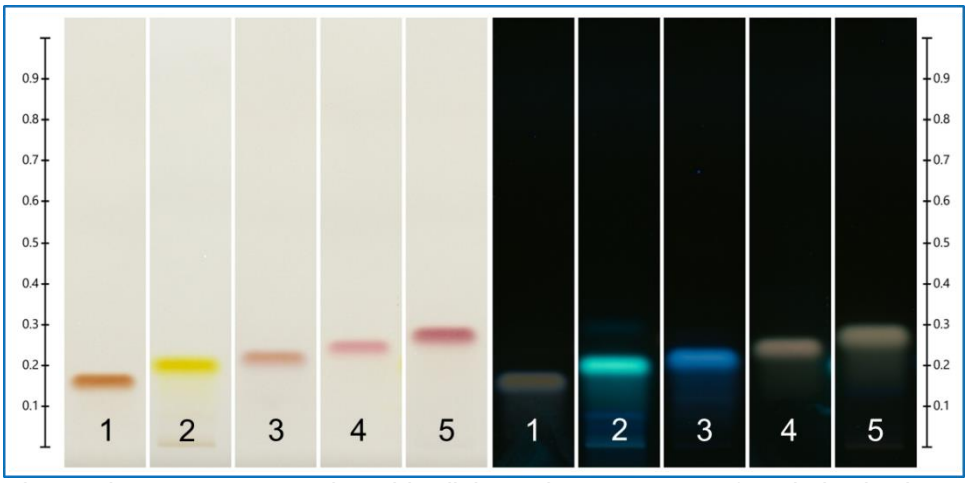


*Fig. 1 Chromatograms under white light and UV 366 nm after derivatization with toluene sulfonic acid, 1: boldenone, 2: trenbolone, 3: nandrolone, 4: testosterone, 5: clostebole*

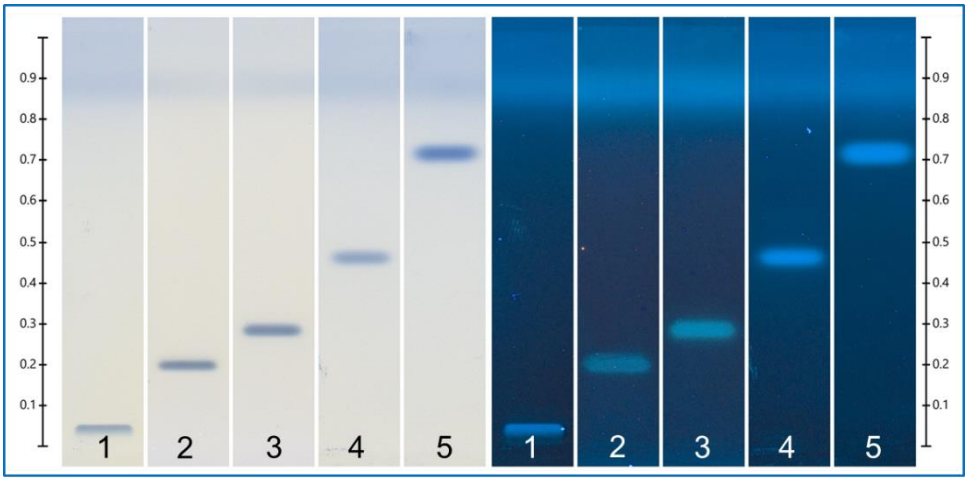


*Fig. 2 Chromatograms under white light and UV 366 nm after derivatization with Seebach reagent, 1: stanozolole, 2: methandienone, 3: methyltestosterone, 4: drostanolone, 5: clostebole cypionat*

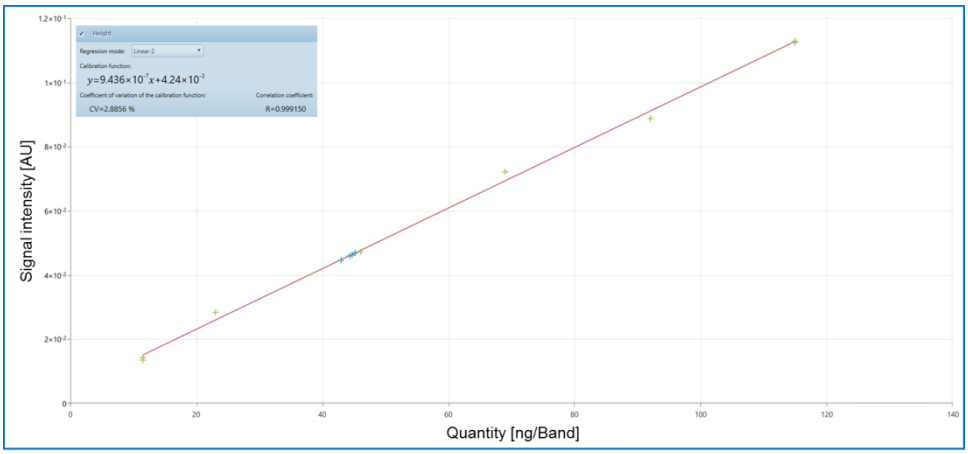


*Fig. 3 Calibration curve for methandienone (green standards and blue replicates of the tablet sample), scanned at 250 nm, corrected by internal standard (methyltestosterone, linear working range between 10-100 ng*

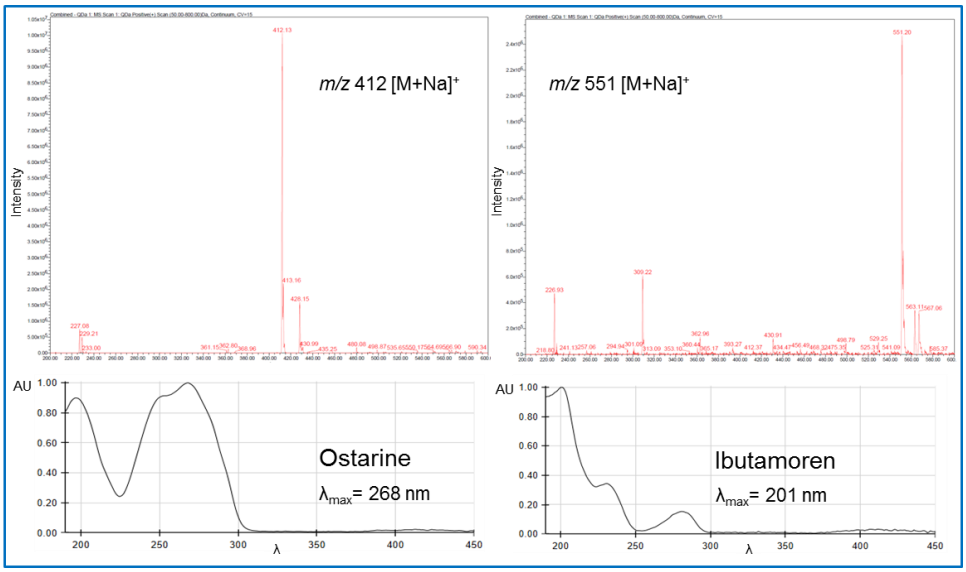


*Fig. 4 UV spectra and HPTLC-MS mass spectra of ostarine and ibutamoren (extracted from capsules), displayed range m/z 200 to 600 and 190 to 450 nm*

*Table 1*

| Steroid | $hR_F$ | UV 254 | UV 366 | Seebach | | Toluen sulfonic acid | | Absorption max. | *m/z* [M+Na]$^+$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | UV 366 | White light | UV 366 | White light | | |
| Stanozolole | 5 | - | - | +* | +* | + | - | - | 329 |
| Boldenone | 16 | + | - | + | + | +* (brown) | +* (orange-brown) | 251 | 309 |
| Methandienone | 20 | + | - | + | + | +* | +* | 251 nm | 323 |
| Trenbolone | 21 | + | + | + | + | +* (green) | +* (yellow) | 352 nm | 293 |
| Nandrolone | 22 | + | - | + | + | +* (blue) | +* (brown) | 248 nm | 297 |
| Chlordehydro-methyltestosterone | 23 | + | - | + | + | +* | + | 253 nm | 357 |
| Oxandrolone | 23 | - | - | +* | +* | + | - | - | 329 |
| Testosterone | 24 | + | - | + | + | +* (brown) | +* (pink) | 250 nm | 311 |
| Clostebole | 27 | + | - | + | + | +* (green-brown) | +* (red) | 264 nm | 345 |
| Methyltestosterone | 29 | + | - | + | + | +* (blue) | + | 250 nm | 325 |
| Oxymetholone | 37 | + | - | +* | - | + | - | 295 nm | 333** |
| Oxystanolone | 39 | - | - | +* | - | + | - | 300 nm | 319** |
| Trenbolone acetate | 42 | + | + | +* | +* | + | + | 351 nm | 335 |
| Mestanolone | 44 | - | - | + | + | +* | + | - | 327 |
| Drostanolone | 50 | - | - | + | + | +* | + | - | 327 |
| Methasterone | 50 | - | - | + | + | +* | + | - | 341 |
| Clostebole cypionate | 74 | + | - | + | + | +* | + | 264 nm | 469 |
| SARMs (no reaction with Seebach and Toluene sulfonic acid reagent) | | | | | | | | | |
| Ibutamoren | 43 | +* | - | | | | | 201 nm | 551 |
| LGD-2226 | 61 | +* | +* | | | | | 248 nm | 415 |
| Ostarine | 72 | +* | - | | | | | 268 nm | 412 |
| LGD-4033 (II) | 78 | +* | + | | | | | 299 nm | 361 |
| LGD-4033 (I) | 80 | +* | + | | | | | 299 nm | 361 |

**Recommended detection for visual inspection; **[M+H]$^+$*

## Acknowledgement

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## Literature

[1] Dr. Matthias Grill and Dr. Melanie Broszat, CAMAG Bibliography Service CBS 118 (2017) 2-4