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Statistical analysis of fragmentation patterns of electron ionization mass spectra of enolized-trimethylsilylated anabolic androgenic steroids

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ABSTRACT

Anabolic androgenic steroids (AAS) are included in the List of prohibited substances of the World Anti-Doping Agency (WADA) as substances abused to enhance athletic performance. Gas chromatography coupled to mass spectrometry (GC–MS) plays an important role in doping control analyses identifying AAS as their enolized-trimethylsilyl (TMS)-derivatives using the electron ionization (EI) mode. This paper explores the suitability of complementary GC–MS mass spectra and statistical analysis (principal component analysis, PCA and partial least squares-discriminant analysis, PLS-DA) to differentiate AAS as a function of their structural and conformational features expressed by their fragment ions. The results obtained showed that the application of PCA yielded a classification among the AAS molecules which became more apparent after applying PLS-DA to the dataset. The application of PLS-DA yielded a clear separation among the AAS molecules which were, thus, classified as: 1-ene-3-keto, 3-hydroxyl with saturated A-ring, 1-ene-3-hydroxyl, 4-ene-3-keto, 1,4-diene-3-keto and 3-keto with saturated A-ring anabolic steroids. The study of this paper also presents structurally diagnostic fragment ions and dissociation routes providing evidence for the presence of unknown AAS or chemically modified molecules known as designer steroids.

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1. Introduction

Anabolic androgenic steroids (AAS) are the most frequently abused substances in sports [1] and are included in the World Anti-Doping Agency (WADA) List of prohibited substances as performance promoters [2–4]. Chromatographic techniques interfaced to various kinds of mass spectrometers are a well-known strategy for sensitive and selective AAS identification in human and racing animal urine doping control samples. Gas chromatography coupled to mass spectrometry (GC–MS) remains one of the preferred techniques for AAS detection [5] and its use is more significant for analytes with poor ionization behavior under atmospheric pressure liquid chromatography–mass spectrometry (LC–MS) interfaces [6]. The common analytical approach, before the GC–MS analysis, employs enzymatic hydrolysis of urine, followed by liquid–liquid (LLE) or solid-phase extraction (SPE), solvent evaporation and, finally, derivatization. Derivatization is used to improve the chromatographic and mass spectrometric characteristics of AAS and has become common practice in doping control laboratories. AAS can be identified as trimethylsilyl (TMS)-derivatives through their reaction with silylation agents like the mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with ammonium iodide (NH_4I) and ethanethiol or dithioerythritol, leading to the formation of the highly reactive trimethyliodosilane (TMIS) which is formed in situ by reaction of iodide with MSTFA [7,8].

GC–MS and LC–MS methods are based on the target analysis of ions originated from known compounds. However, unknown designer steroids have been found to circulate illegally (e.g., norbolethone [9] and madol [10]) in order to be abused by cheating athletes wishing to avoid detection. Since GC–MS and LC–MS identification remains the basic analytical tool, understanding of fragmentation pathways of AAS and their metabolites is important in the detection of the unknown molecules [11]. Mass spectrometric studies on fragmentation of underivatized steroids using electron ionization (EI) have been performed [12–15], while an attempt to compile fundamental information on dissociation pathways of trimethylsilyl (TMS)-derivatized anabolic steroids has been presented [16]. Characteristic fragmentation patterns of distinct steroid nuclei from collision-induced dissociation (CID) after electrospray ionization (ESI) using liquid chromatography tandem mass spectrometry (LC–MS/MS) have been also presented [11,17,18].

A useful approach for the GC–MS or LC–MS detection of the designer steroids would be the prediction of their mass spectra. However, for nonpeptidic molecules, algorithms that successfully

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predict MS spectra do not exist. An attempt to develop algorithms for interpreting MS/MS data for pharmaceuticals by evaluating MS/MS spectra and fragmentation trends via statistical methods resulted in flaws [19].

The method described herein proceeds a step forward from the study of Thevis et al. [16] and is used to develop predictive rules for EI MS data of designer steroids and to extract fragmentation patterns based on fragmentation data of TMS-derivatives of known AAS molecules with similar steroid nuclei structures. The application of principal component analysis (PCA) to fragmentation data of TMS-derivatized AAS molecules led to the AAS classification related to their structural features, and proved that similar steroidal structures show similarities to their fragmentation routes. The application of partial least squares-discriminant analysis (PLS-DA) confirms the grouping of the AAS molecules. The rule-based approach of this paper is intended to lead to the elucidation of diagnostically useful fragmentation pathways of chemically modified anabolic steroids and their metabolites for which mass spectra may be unavailable.

2. Experimental

2.1. Organization of the AAS fragmentation dataset

The mass spectra of enol-TMS derivatives of 18 parent AAS and 51 metabolites, included in the WADA List of prohibited substances [2], recorded in EI quadrupole full scan GC–MS analysis described elsewhere [20], constituted the dataset. AAS with conjugated double bonds (e.g., gestrinone, tetrahydrogestrinone, methyltrienolone, methyldienolone, trenbolone) were excluded from the study due to their inability to undergo thermal enol-TMS derivatization to a satisfactory extent using the preparative protocols for GC–MS analysis [21].

TMS-derivatization of AAS molecules can be achieved following a two-step procedure:

- addition of 50 μl MSTFA and 25 μl acetonitrile (ACN) at 80 °C for 30 min and, then,
- addition of 50 μl MSTFA/NH_4I/propanethiol 1000:4:7 (v:w:v) at 80 °C for 30 min.

Structural diagnostic ions of the AAS from the laboratory mass spectra library with abundance higher than 5% were included in the study, ignoring the ion at *m/z* 73 which is common ion in AAS TMS-derivatized mass spectra due to the $[TMS]^+$ fragment and has no diagnostic significance [22]. Fragmentation similarities among the spectra of the dataset were sought in the following ion types: molecular ions ($[M]^+$), ions derived after neutral or radical losses, such as a methyl or ethyl groups, TMSOH, halogen and ions derived from fragments at specific sites in the steroidal nucleus. For the sake of the statistical analyses, ion relative abundances were converted to values 1, 2 or 3, as following; ions with relative abundance <25% were given the value 1, ions with relative abundance 25% to 50% were given the value 2, and ions with relative abundance >50% were given the value 3 [23].

2.2. Significant fragment ions as variables for TMS-derivatized AAS classification

The ions included in the final dataset as variables with diagnostic significance are presented in the sections below.

2.2.1. The molecular ion

The molecular ion ($[M]^+$) along with the ions $[M-15]^+$ and $[M-29]^+$, derived from the molecular ion with the loss of a methyl or an ethyl radical, respectively, are valuable for TMS-derivatized AAS mass spectra interpretation [24]. Moreover, the stepwise elimination of either trimethylsilanol $[TMSOH]^+$ (90 Da), $[TMSOH-15]^+$ or $[TMSOH-29]^+$ ions of trimethylsilyloxy steroidal ethers is a feature which can be diagnostic in nature as it indicates the extent of hydroxylation of steroids and their metabolites [25].

2.2.2. Ion at m/z 103

The ion at *m/z* 103 is derived from the loss of a $[TMSOCH_2]^+$ fragment [22]. Ions of type $[M-103]^+$ are typical of TMS ether derivatives of primary aliphatic alcohols [26] and of other primary carbinols such as 21-hydroxyl steroids [27]. The $[M-103]^+$ ion can be more prominent than the ion at *m/z* 103. Moreover, the stepwise elimination of $[M-TMSOH-103]^+$ ions can be also of diagnostic significance.

2.2.3. Ion at m/z 129

The ion at *m/z* 129 is indicative of a derivatized 3- or 17-hydroxyl function of steroids from A- or D-ring cleavage, respectively (Fig. 1a). The origin of such fragments has been investigated by analyses of structurally related or stably deuterated compounds [28,29].

The existence of an intense peak at *m/z* 129 has been also used as conclusive evidence for the identification of the TMS-derivatives of Δ5-3-hydroxyl steroids [22,30]. The exact composition of this fragment ion, utilizing deuterium labeling, verified that *m/z* 129 substantially decreases in intensity, while the ion at $[M-129]^+$ becomes the base peak in spectra of 4-methyl substituted steroids [28].

2.2.4. Ions at m/z 130 and m/z 143

The ions at *m/z* 130 and *m/z* 143, resulting from D-ring cleavage, are diagnostic for the presence of a 17α-methyl group in trimethylsilylated 17β-hydroxyl steroids (Fig. 1b) [31]. The formation of *m/z* 143 is due to the fission of the bonds between C-13/C-17 and C-14/C-15, while the ion at *m/z* 130 originates from the fission of the bonds between C-13/C-17 and C-15/C-16. The mechanisms of formation of ions at *m/z* 130 and *m/z* 143 are available in the literature ([16] and [30,32], respectively). The ion at *m/z* 143 is considered as a biomarker for the presence of 17α-alkylated synthetic steroids that have been developed for oral administration to delay hepatic metabolism [3]: abnormal and intense chromatographic peaks in the *m/z* 143 extracted ion chromatogram of a urine sample are typically subjected to further investigation.

However, under TMS-derivatized EI conditions, an ion at *m/z* 143 with the same elemental composition is produced from the A-ring of 3-keto-enol steroids. Additional data also demonstrate the generation of an ion (in low abundance) with *m/z* 143 from 3-hydroxyl-4-ene steroids [32]. Finally, an ion at *m/z* 143 with the same elemental composition, but different structure than the ion derived from 17α-methyl,17β-hydroxyl steroids, arises from a variety of 11-trimethylsiloxy steroids.

2.2.5. Ions at m/z 144 and m/z 157

Similarly with the fissions at *m/z* 130 and *m/z* 143 of 17α-methyl,17β-hydroxyl steroids, the corresponding fragment ions of 17α-ethyl,17β-hydroxyl steroids are the ions at *m/z* 144 and *m/z* 157 (Fig. 1c).

2.2.6. Ion at m/z 169

17-Keto steroids show a characteristic ion at *m/z* 169 when the 17-keto function is derivatized to the corresponding enol-TMS ether (Fig. 1d) [33]. The ion is derived from C-ring dissociation of the bonds between C-8/C-14 and C-12/C-13 including the angular C-18 methyl function [34].

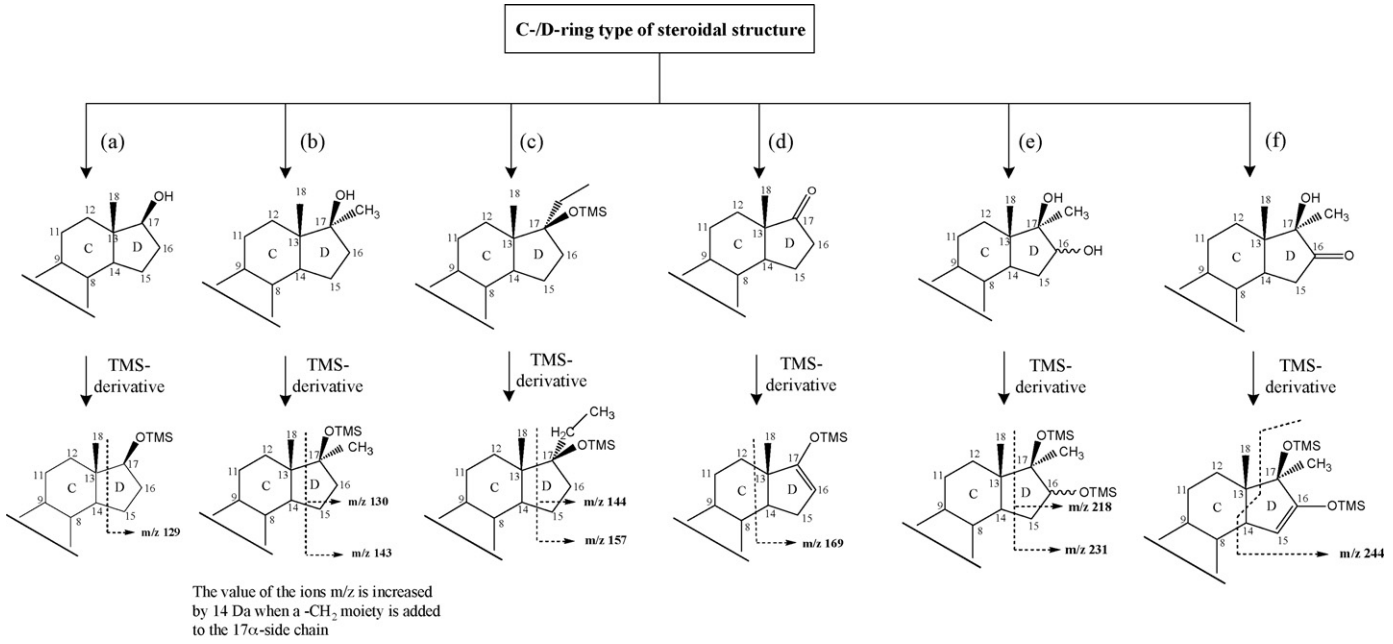


Fig. 1. Characteristic C-/D-ring fragment ions for the TMS-derivatives of anabolic androgenic steroids.

2.2.7. Ions at m/z 218 and m/z 231

The ions at *m*/*z* 218 and *m*/*z* 231 are typical of 16-hydroxylated 17α-methyl,17β-hydroxyl steroids (Fig. 1e) and are the corresponding 16-OTMS analogs (+88 Da) of the ions at *m*/*z* 130 and *m*/*z* 143 of the 17α-methyl,17β-hydroxyl steroids, as described in Section 2.2.4 [35].

2.2.8. Ions at m/z 244

The fragment ion at *m*/*z* 244 is present in the EI mass spectra of the TMS enolized derivatives of 17α-methyl,17β-hydroxyl steroids bearing a 16-keto group. The dissociation pathway includes the fission of bonds in C- and D-rings (Fig. 1f) [36].

2.2.9. Fragment ions of type F1, F2, F3, F4, F5

Fragment ions at specific sites of the steroidal structure (mainly at rings A and B of the steroidal structure) are symbolized by F1 to F5 as they depend, arithmetically, on the exact position of the steroid substituents (Table 1, top scheme).

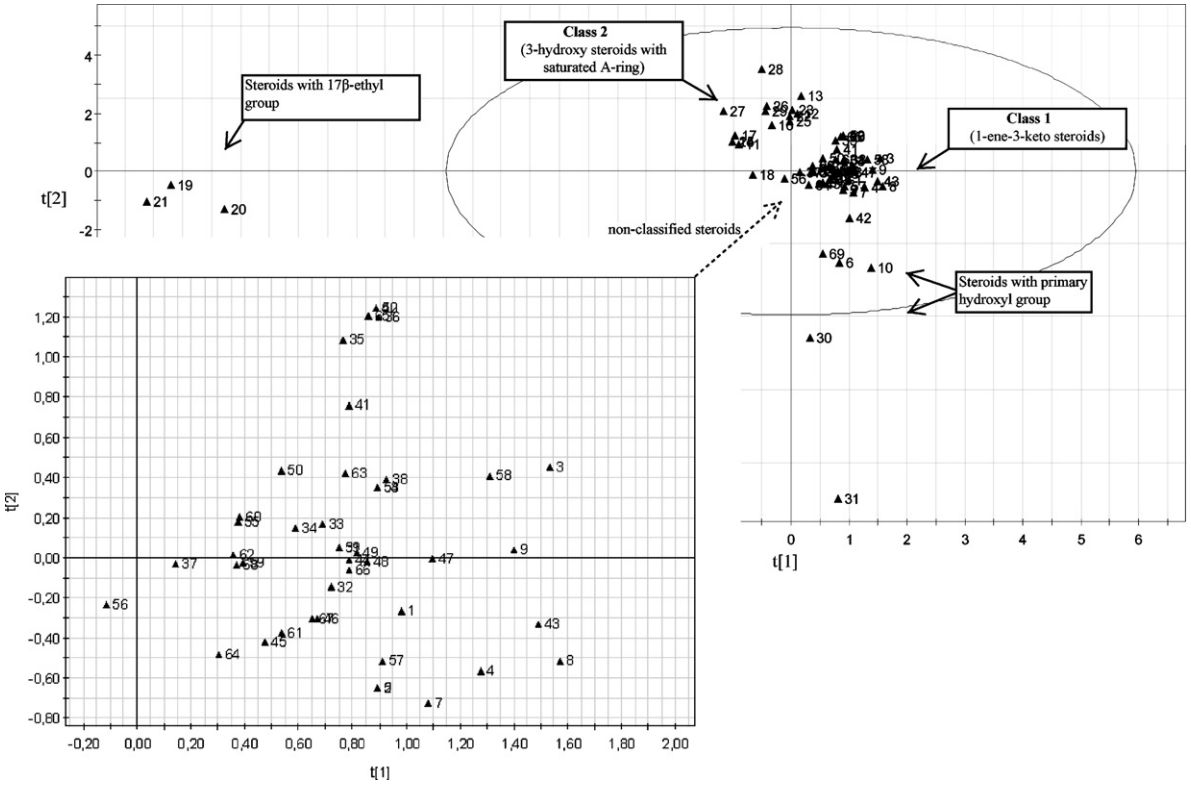
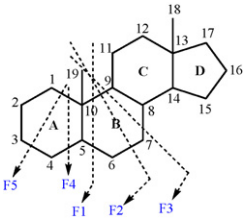


Fig. 2. Scores plot from the principal component analysis of the AAS and their metabolites included in the study. Analytes are numbered as in Table 1.

Table 1
Characteristic fragment ions (m/z), relative abundancies[a] (%, inside the parentheses)/WADA range[b] (W1, 2 or 3) of the ions of trimethylsilylated anabolic androgenic steroids identified by EI GC–MS.



Class 1: 1-ene-3-keto steroids

No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-90-15]^+$	$[M-103]^+$	$[M-103-90]^+$	F1	F2	F3	D-ring fragment
1	Methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-androst-1-ene-3-one)	bis-TMS	446 (30)/W2	431 (12)/W1	356 (9)/W1	341 (4)/W1			179 (17)/W1	194 (35)/W2	206 (30)/W2	143 (100)/W3
2	Boldenone metabolite (5β-androst-1-ene-17β-ol-3-one)	bis-TMS	432 (20)/W1	417 (22)/W1	342 (6)/W1	327 (4)/W1			179 (18)/W1	194 (100)/W3	206 (26)/W2	129 (14)/W1
3	Boldenone metabolite (5β-androst-1-ene-3,17-dione)	bis-TMS	430 (53)/W3	415 (95)/W3	340 (11)/W1	325 (11)/W1			179 (68)/W3	194 (100)/W3	206 (21)/W1	169 (32)/W2
4	1-Testosterone (5β-androst-1-ene-17β-ol-3-one)	bis-TMS	432 (47)/W2	417 (19)/W1	342 (5)/W1	327 (5)/W1			179 (42)/W2	194 (100)/W3	206 (38)/W2	129 (38)/W2
5	Methenolone (1-methyl-5α-androst-1-ene-17β-ol-3-one)	bis-TMS	446 (16)/W1	431 (5)/W1	356 (2)/W1	341 (2)/W1			179 (25)/W2	195 (100)/W3	206 (44)/W2	129 (5)/W1
6	Methenolone metabolite (18-hydroxy-1-methyl-5α-androst-1-ene-3,17-dione)	tris-TMS	532 (10)/W1	517 (4)/W1	442 (7)/W1		429 (100)/W3	339 (14)/W1	179 (13)/W1	195 (38)/W2	208 (10)/W1	169 (7)/W1
7	Methenolone metabolite (17-epimethenolone)	bis-TMS	446 (21)/W1	431 (17)/W1	356 (4)/W1	341 (4)/W1			179 (22)/W1	195 (100)/W3	206 (52)/W3	129 (35)/W2
8	Stenbolone (2-methyl-5α-androst-1-ene-17β-ol-3-one)	bis-TMS	446 (100)/W3	431 (25)/W2	356 (4)/W1	341 (7)/W1			193 (61)/W3	208 (71)/W3	221 (29)/W2	129 (14)/W1
9	Stenbolone metabolite (2-methyl-5α-androst-1-ene-16z,17β-diol-3-one)	tris-TMS	534 (50)/W3	519 (100)/W3	444 (8)/W1	429 (8)/W1			193 (42)/W2	208 (42)/W2	221 (33)/W2	
10	Stenbolone metabolite (18-hydroxy-2-methyl-5α-androst-1-ene-3,17-dione)	tris-TMS	532 (73)/W3	517 (13)/W1			429 (100)/W3	339 (13)/W1	193 (27)/W2	208 (33)/W2	221 (40)/W2	

Class 2: 3-hydroxy steroids with saturated A-ring

No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-29]^+$	$[M-90]^+$	$[M-2·90]^+$	$[M-90-15]^+$	$[M-2·90-15]^+$	$[M-103]^+$	$[M-103-90]^+$	F5	D-ring fragment	
11	Methasterone metabolite (2α,17α-dimethyl-5α-androstane-3α,17β-diol)	bis-TMS	464 (4)/W1	449 (4)/W1		374 (4)/W1	284 (4)/W1	359 (4)/W1	269 (4)/W1				143 (100)/W3	
12	Androsterone (5α-androstan-3α-ol-17-one)	bis-TMS	434 (79)/W3	419 (100)/W3		344 (3)/W1		329 (41)/W2	239 (14)/W1			129 (6)/W1	169 (23)/W1	
13	Etiocholanolone (5β-androstan-3α-ol-17-one)	bis-TMS	434 (95)/W3	419 (100)/W3		344 (3)/W1		329 (58)/W3	239 (15)/W1			129 (6)/W1	169 (25)/W2	
14	Bolasterone metabolite (7α,17α-dimethyl-5β-androstane-3α,17β-diol)	bis-TMS	464 (1)/W1	449 (4)/W1		374 (9)/W1	284 (15)/W1	359 (1)/W1	269 (9)/W1			129 (9)/W1	143 (100)/W3	
15	Calusterone metabolite (7β,17α-dimethyl-5β-androstane-3α,17β-diol)	bis-TMS	464 (1)/W1	449 (3)/W1		374 (7)/W1	284 (11)/W1	359 (1)/W1	269 (5)/W1			129 (9)/W1	143 (100)/W3	
16	Clostebol metabolite (4z-chloro-3α-hydroxy-5α-androstan-17-one)	bis-TMS	468 (53)/W3	453 (100)/W3								129 (35)/W2	169 (91)/W3	
17	Oxymetholone metabolite (17α-methyl-5α-androstan-3α,17β-diol)	bis-TMS	450 (6)/W1	435 (34)/W2		360 (6)/W1	270 (5)/W1	345 (5)/W1	250 (12)/W1			129 (8)/W1	143 (100)/W3	
18	Oxymetholone metabolite (2z-hydroxymethyl-17α-methyl-5α-androstan-3z,6z,17β-triol)	tetrakis-TMS				550 (4)/W1	460 (4)/W1						143 (100)/W3	
19	Norethandrolone metabolite (17α-ethyl-5α-estrane-3α,17β-diol)	bis-TMS		435 (2)/W1	421 (51)/W3	360 (2)/W1	270 (2)/W1	345 (2)/W1 {[M-90-29]$^+$}: 331 (2)/W1	255 (2)/W1 {[M-2·90-29]$^+$}: 341 (9)/W1			129 (8)/W1	157 (100)/W3	144 (55)/W3
20	Norethandrolone metabolite (17α-ethyl-5β-estrane-3α,17β-diol)	bis-TMS			421 (22)/W1		270 (3)/W1	{[M-90-29]$^+$}: 331 (17)/W1	{[M-2·90-29]$^+$}: 241 (11)/W1			129 (11)/W1	157 (100)	144 (58)/W3
21	Norbolethone metabolite (18β,17α-diethyl-5β-estrane-3α,17β-diol)	bis-TMS		449 (4)/W1	435 (27)/W2	374 (4)/W1		359 (2)/W1 {[M-90-29]$^+$}: 345 (27)/W2	{[M-2·90-29]$^+$}: 255 (22)/W1			129 (6)/W1	157 (100)/W3	144 (46)/W2

Table 1 (*Continued*)

Class 2: 3-hydroxy steroids with saturated A-ring

No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-29]^+$	$[M-90]^+$	$[M-2·90]^+$	$[M-90-15]^+$	$[M-2·90-15]^+$	$[M-103]^+$	$[M-103-90]^+$	F5	D-ring fragment
22	19-Nor-androsterone (5α-estrane-3α-ol-17-one)	bis-TMS	420 (63)/W3	405 (100)/W3				315 (31)/W2	225 (15)/W1			129 (6)/W1	169 (19)/W1
23	19-Nor-etiocholanolone (5β-estrane-3α-ol-17-one)	bis-TMS	420 (77)/W3	405 (100)/W3				315 (46)/W2	225 (17)/W1			129 (6)/W1	169 (30)/W2
24	Methyl-testosterone metabolite (17α-methyl-5α-androstan-3α,17β-diol)	bis-TMS	450 (2)/W1	435 (11)/W1		360 (4)/W1	270 (4)/W1	345 (4)/W1	255 (7)/W1			129 (5)/W1	143 (100)/W3
25	Methenolone metabolite (1-methylen-5α-androstan-3α-ol-17-one)	bis-TMS	446 (82)/W3	431 (100)/W3				341 (22)/W1	251 (11)/W1			144 (11)/W1	169 (37)/W2
26	Methenolone metabolite (3α-hydroxy-1α-methyl-5α-androstan-17-one)	bis-TMS	448 (80)/W3	433 (100)/W3		358 (9)/W1	268 (17)/W1	343 (26)/W2	253 (17)/W1			144 (9)/W1	169 (23)/W1
27	Drostanolone metabolite (2α-methyl-5α-androstan-3α,17β-diol)	bis-TMS	450 (22)/W1	435 (17)/W1		360 (28)/W2	270 (33)/W2	345 (39)/W2	255 (61)/W3			144 (8)/W1	129 (100)/W3
28	Drostanolone metabolite (2α-methyl-5α-androstan-3α-ol-17-one)	bis-TMS	448 (91)/W3	433 (100)/W3		358 (4)/W1	268 (4)/W1	343 (56)/W3	253 (33)/W2			144 (5)/W1	169 (80)/W3
29	Mesterolone metabolite (1α-methyl-5α-androstan-3α-ol-17-one)	bis-TMS	448 (78)/W3	433 (100)/W3		358 (2)/W1	268 (2)/W1	343 (22)/W1	253 (9)/W1			144 (11)/W1	169 (38)/W2
30	Mesterolone metabolite (3α,18-dihydroxy-1α-methyl-5α-androstan-17-one)	bis-TMS	536 (14)/W1	521 (5)/W1						433 (100)/W3	343 (14)/W1		
31	Mesterolone metabolite (3α,6z,18-trihydroxy-1α-methyl-5α-androstan-17-one)	tetrakis-TMS	624 (20)/W1	609 (5)/W1						521 (100)/W3	431 (50)/W3		

Class 3: 1-ene-3-hydroxy steroids

No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-90-15]^+$	F4	F4-15	D-ring fragment
32	Methyl-1-testosterone metabolite (17α-methyl-5α-androstan-1-ene-3α,17β-diol)	bis-TMS	448 (25)/W2	419 (15)/W1	358 (5)/W1				143 (100)/W3
33	Oral turinabol metabolite (4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-ene-16-one)	tetrakis-TMS	656 (100)/W3	641 (11)/W1	566 (5)/W1	551 (7)/W1			244 (30)/W2
34	Boldenone metabolite (5β-androst-1-ene-3α-17β-diol)	bis-TMS	434 (41)/W2	419 (4)/W1	344 (7)/W1	329 (4)/W1	142 (48)/W2	127 (40)/W2	143 (100)/W3
35	Boldenone metabolite (3α-hydroxy-5β-androst-1-ene-17-one)	bis-TMS	432 (44)/W2	417 (40)/W2	342 (4)/W1	327 (28)/W2	290 (48)/W2	275 (100)/W3	169 (32)/W2
36	1-Testosterone metabolite (3α-hydroxy-5α-androst-1-ene-17-one)	bis-TMS	432 (100)/W3	417 (92)/W3	342 (6)/W1	327 (27)/W2	290 (23)/W1	275 (48)/W2	169 (23)/W1
37	Methandienone metabolite (17β-methyl-5β-androst-1-ene-3α,17β-diol)	bis-TMS	448 (8)/W1	433 (3)/W1	358 (23)/W1	343 (5)/W1			143 (100)/W3
38	Stenbolone metabolite (2-methyl-5α-androst-1-ene-3α-ol-17-one)	bis-TMS	446 (78)/W3	431 (22)/W1	356 (4)/W1	341 (4)/W1	290 (100)/W3	275 (83)/W3	169 (17)/W1

Table 1 (*Continued*)

Class 4: 4-ene-3-keto steroids													
No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-90-15]^+$	$[M-103]^+$	$[M-103-90]^+$	F1	F2	F3	D-ring fragment	
39	Testosterone (17β-hydroxy-androst-4-ene-3-one)	bis-TMS	432 (100)/W3	417 (11)/W1	342 (1)/W1	327 (3)/W1					209 (6)/W1	129 (3)/W1	
40	Bolasterone (7α,17α-dimethyl-17β-hydroxyandrost-4-ene-3-one)	bis-TMS	460 (57)/W3	445 (100)/W3	370 (1)/W1	355 (26)/W2						143 (10)/W1	$[M-143]^+$ (69)/W3
41	Calusterone (7β,17α-dimethyl-17β-hydroxyandrost-4-ene-3-one)	bis-TMS	460 (63)/W3	445 (100)/W3	370 (1)/W1	355 (15)/W1						143 (11)/W1	$[M-143]^+$ (49)/W2
42	Danazol metabolite (17-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-ene-20-yn-3-one)	tris-TMS	558 (100)/W3	543 (9)/W1	468 (3)/W1	455 (11)/W1	455 (11)/W1	365 (3)/W1				$[M-140]^+$ (6)/W1	$[M-155]^+$ (6)/W1
43	Danazol metabolite (17-hydroxy-17α-pregn-4-ene-20-yn-3-one, ethisterone)	tris-TMS	456 (100)/W3	441 (33)/W2	466 (1)/W1	351 (1)/W1					208 (28)/W2	$[M-140]^+$ (27)/W2	$[M-155]^+$ (42)/W2
44	Fluoxymesterone (11β,17β-dihydroxy-9α-fluoro-17α-methyl-4-androsten-3-one)	tris-TMS	552 (100)/W3		462 (38)/W2	447 (9)/W1					208 (6)/W1	143 (15)/W1	
45	Fluoxymesterone metabolite (6β-hydroxy-fluoxymesterone)	tetrakis-TMS	640 (100)/W3	625 (4)/W1	550 (2)/W1							143 (6)/W1	
46	Formebolone metabolite (11α,17β-dihydroxy-17α-methyl-androst-4-ene-3-one)	tris-TMS	534 (100)/W3	519 (5)/W1	444 (8)/W1						208 (3)/W1	143 (3)/W1	
47	4-Hydroxy-testosterone (isomer 1)	tris-TMS	520 (44)/W2	505 (100)/W3	430 (3)/W1	415 (9)/W1			267 (76)/W3	281 (31)/W2		129 (38)/W2	
48	4-Hydroxy-testosterone (isomer 2)	tris-TMS	520 (100)/W3	505 (6)/W1	430 (3)/W1	415 (1)/W1				281 (1)/W1	296 (4)/W1	129 (11)/W1	
49	Oxymesterone (17α-methyl-4,17β-dihydroxy-androst-4-ene-3-one)	tris-TMS	534 (100)/W3	519 (5)/W1	444 (2)/W1	429 (1)/W1				281 (2)/W1	296 (5)/W1	143 (17)/W1	
50	Nor-clostebol (4-chloro-17β-hydroxyestr-4-ene-3-one)	bis-TMS	452 (78)/W3	437 (100)/W3		347 (16)/W1							
51	19-Nor-testosterone (17β-hydroxy-4-estren-3-one)	bis-TMS	418 (100)/W3	403 (14)/W1	328 (1)/W1	313 (1)/W1					194 (21)/W1	129 (6)/W1	
52	Mibolerone (17β-hydroxy-7α,17α-dimethylestr-4-ene-3-one)	bis-TMS	446 (75)/W3	431 (93)/W3	356 (2)/W1	341 (40)/W2						143 (12)/W1	$[M-143]^+$ (100)/W3
53	Methyl-testosterone (17β-hydroxy-17α-methyl-androst-4-ene-3-one)	bis-TMS	446 (100)/W3	431 (8)/W1	356 (18)/W1	341 (9)/W1					208 (5)/W1	143 (9)/W1	$[M-143]^+$ (76)/W3
54	Methyl-testosterone metabolite (17-epimethyltestosterone)	bis-TMS	446 (77)/W3	431 (5)/W1	356 (11)/W1	341 (9)/W1					208 (7)/W1	143 (7)/W1	$[M-143]^+$ (100)/W3

Class 5: 1,4-diene-3-keto steroids										
No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-90-15]^+$	F2	F3	D-ring fragment	
55	Oral turinabol metabolite (6β-hydroxy-dehydro-chlormethyltestosterone)	tris-TMS	566 (5)/W1	551 (100)/W3	476 (1)/W1	461 (3)/W1		328 (3)/W1	143 (3)/W1	
56	Oral turinabol metabolite (6β,16β-dihydroxy-dehydrochlormethyltestosterone)	tris-TMS	582 (38)/W2	567 (4)/W1	492 (9)/W1				218 (100)/W3	231 (47)/W2
57	Boldenone (androsta-1,4-diene-17β-ol-3-one)	bis-TMS	430 (38)/W2	415 (13)/W1		325 (24)/W1	191 (25)/W2	206 (100)/W3	129 (5)/W1	
58	Boldenone metabolite (androsta-1,4-diene-3,17-dione)	bis-TMS	428 (65)/W3	413 (31)/W2		323 (31)/W2	191 (58)/W3	206 (100)/W3	169 (23)/W1	
59	Boldenone metabolite (androsta-1,4-diene-6β,17β-diol-3-one)	tris-TMS	518 (4)/W1	503 (100)/W3		413 (2)/W1	279 (9)/W1	294 (11)/W1	129 (4)/W1	
60	Boldenone metabolite (androsta-1,4-diene-6β-ol-3,17-dione)	tris-TMS	516 (5)/W1	501 (100)/W3		411 (1)/W1	279 (5)/W1	294 (4)/W1	169 (2)/W1	
61	Formebolone metabolite (Methylenehydroxy-6β-hydroxy-formebolone)	pentakis-TMS	722 (4)/W1	707 (100)/W3	632 (1)/W1	617 (3)/W1	366 (4)/W1	381 (3)/W1	143 (9)/W1	
62	Methandienone metabolite (6β-hydroxymethandienone)	tris-TMS	532 (4)/W1	517 (100)/W3		427 (2)/W1	279 (4)/W1	294 (4)/W1	143 (2)/W1	
63	Methandienone metabolite (17-epimethandienone)	bis-TMS	444 (40)/W2	429 (2)/W1		339 (56)/W3	191 (100)/W3	206 (18)/W1	143 (13)/W1	

Table 1 (*Continued*)

No.	Steroid	Derivative	$[M]^+$	$[M-15]^+$	$[M-90]^+$	$[M-90-15]^+$	$[M-103]^+$	$[M-103-90]^+$	F4	F4-15	D-ring fragment	
Class 6: 3-keto steroids with saturated A-ring												
64	Methasterone (17β-hydroxy-2α,17α-dimethyl-5α-androstane-3-one)	bis-TMS	462 (35)/W2	447 (3)/W1							143 (100)/W3	
65	Mibolerone metabolite (7α, 17α-dimethyl-5β-estrane-17β-ol-3-one)	bis-TMS	448 (33)/W2	433 (16)/W1	358 (56)/W3	343 (100)/W3					$[M-143]^+$ (83)/W3	
66	Mibolerone metabolite (7α, 17α-dimethyl-6β, 16β,17β-trihydroxy-5β-estrane -3-one)	tetrakis-TMS	624 (70)/W3		534 (30)/W2	519 (3)/W1					218 (100)/W3	231 (65)/W3
67	Drostanolone (2α-methyl-17β-hydroxy-5α-androstan-3-one)	bis-TMS	448 (100)/W3	433 (10)/W1					156 (16)/W1	141 (58)/W3	129 (12)/W1	
68	Mesterolone (1α-methyl-5α-androstan-17β-ol-3-one)	bis-TMS	448 (19)/W1	433 (25)/W2		343 (1)/W1			156 (40)/W2	141 (100)/W3	129 (10)/W1	
69	Mesterolone metabolite (18-hydroxy-1α-methyl-5α-androstane-3,17-dione)	tris-TMS	534 (9)/W1	519 (5)/W1	444 (9)/W1		431 (100)/W3	341 (9)/W1	156 (46)/W2	141 (23)/W1	169 (13)/W1	

[a] With regard to the ion of the highest abundance except of that at *m/z* 73 which is of no diagnostic significance.
[b] Ranges referred in the WADA technical document about the identification criteria for qualitative assays incorporating chromatography and mass spectrometry [23].

2.3. Classification of the AAS fragmentation data

The elucidation of trends, similarities and dissimilarities concerning the fragmentation behavior of the analytes, as well as, the detection of outliers were carried out using PCA [37]. According to PCA, the data (objects), represented in *K*-dimensional space (where *K* is equal to the number of variables), are projected into a few principal components, which are linear combinations of the original variables and describe the maximum variation within the data. Each principal component (PC) is characterized by scores which are the new coordinates of the objects projected, and by loadings which reflect the direction with respect to the original variables. The scores plot of the first two PCs is a two-dimensional map which provides a data overview, displaying patterns or groupings within the data and can also be useful in highlighting outliers (data lying outside the Hotelling T^2 ellipse). The corresponding loadings plots display relationships between variables and can be used to identify which variables (fragment ions in this study) contribute to the positioning of the objects on the scores plot and hence influence any observed separation in the dataset. PCA is an unsupervised classification method and any prior knowledge relating to the class membership is not used. On the other hand, in the PLS-DA method objects are assigned to certain groups prior to the analysis, not more than 3 simultaneously, and this knowledge is used to derive models for further predictions [38].

SIMCA-P 10.0 (Umeå, Sweden) statistical program was used to perform the PCA and PLS-DA analyses [39]. The dimensions of the dataset were $N \times K$ (69×32), where N is the number of observations (parent molecules and their metabolites) and K is the number of variables (ions with diagnostic significance whose relative abundances were converted to values 1, 2 or 3, Sections 2.1 and 2.2). Before the analyses the data were mean-centered and scaled to unit variance. The following statistics of the derived models are discussed; R^2X is the cumulative modeled variation in the variables, R^2Y is the cumulative modeled variation in the observations variables and Q^2Y is the cumulative predicted variation in Y, according to cross-validation.

3. Results and discussion

3.1. Classification of the AAS fragmentation data according to PCA and PLS-DA analyses

The application of PCA to the TMS-derivatives of the AAS molecules revealed a poor differentiation of the fragmentation data, but still offers valuable evidence for potential AAS classification (Fig. 2). A four PCs model was accompanied with $R^2X = 50.1\%$, while the first two PCs cumulatively accounted for R^2X 31.4% of the data variance. Two classes of steroids are clearly separated, namely class 1 (1-ene-3-keto steroids) at the bottom right quartile of the Hotelling T^2 ellipse and class 2 (3-hydroxyl steroids with saturated A-ring) positioned at the upper left and right quartile of the ellipse. The majority of the AAS molecules (non-classified) were positioned between classes 1 and 2, near the center of the PCA axes. The discrimination of more classes within class 1 seems to be difficult indicating similarities in the mass spectra of these molecules, although belonging to structurally dissimilar steroids. It is also worth mentioning that steroids with a primary hydroxyl group, such as the C-18 hydroxylated metabolites of methenolone (Fig. 2, analyte 6), stenbolone (Fig. 2, analyte 10) and mesterolone (Fig. 2, analytes 30, 31 and 69), as well as, the steroid 17-hydroxyl-2α-hydroxylmethyl-17α-pregn-4-ene-20-yn-3-one (danazol metabolite, Fig. 2, analyte 42) are positioned far from the majority of the analytes (bottom right quartile) (see Table 1 for exact names of the analytes). The ions $[M-103]^+$ and $[M-103-90]^+$ proved to be crucial for their positioning. Another group of outliers

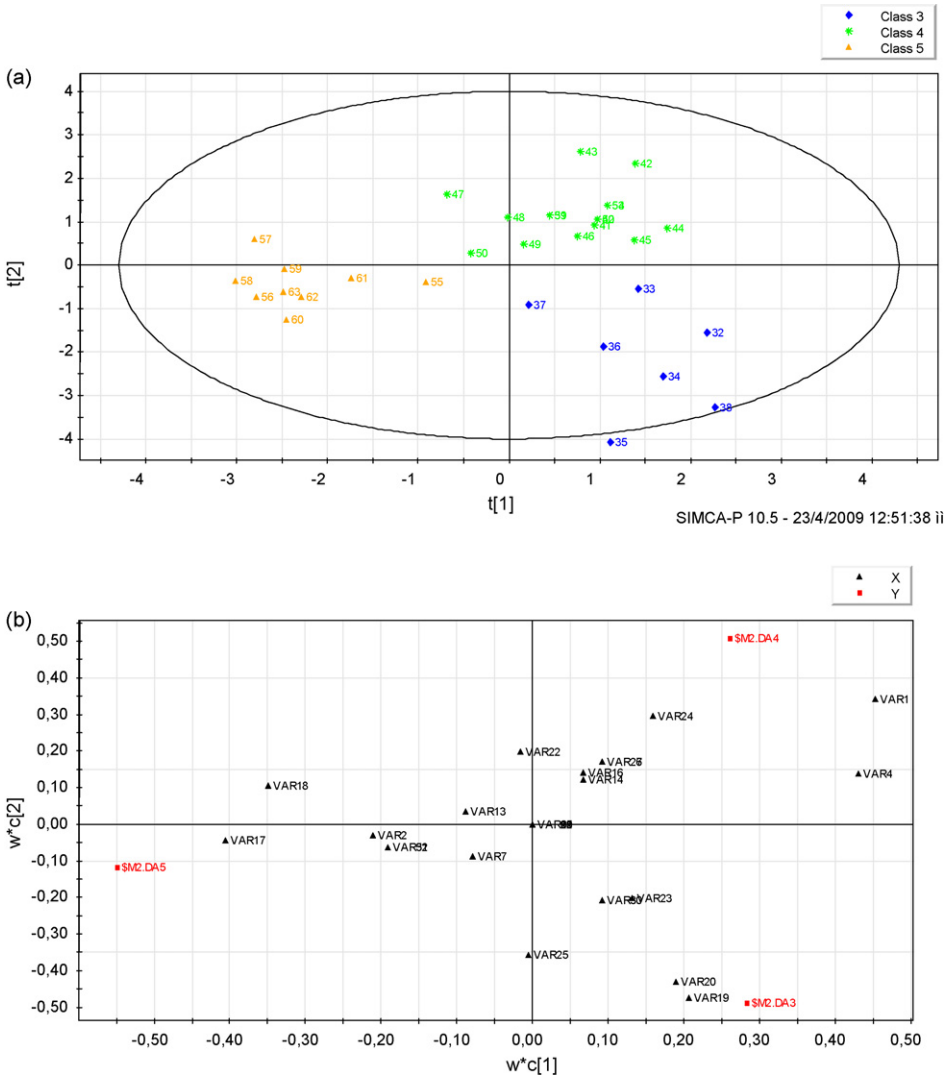


Fig. 3. PLS-DA model (a; scores plot and b; loadings plot) of the TMS-derivatized AAS molecules belonging to classes 3, 4 and 5.
Abbreviations: VAR1: $[M]^+$, VAR2: $[M\text{-}15]^+$, VAR3: $[M\text{-}29]^+$, VAR4: $[M\text{-}90]^+$, VAR5: $[M\text{-}2{\cdot}90]^+$, VAR6: $[M\text{-}3{\cdot}90]^+$, VAR7: $[M\text{-}90\text{-}15]^+$, VAR8: $[M\text{-}2{\cdot}90\text{-}15]^+$, VAR9: $[M\text{-}3{\cdot}90\text{-}15]^+$, VAR10: $[M\text{-}90\text{-}29]^+$, VAR11: $[M\text{-}2{\cdot}90\text{-}29]^+$, VAR12: m/z 103, VAR13: $[M\text{-}103]^+$, VAR14: $[M\text{-}103\text{-}90]^+$, VAR15: $[M\text{-}103\text{-}2{\cdot}90]^+$, VAR16: F1, VAR17: F2, VAR18: F3, VAR19: F4, VAR20: F4-15, VAR21: F5, VAR22: m/z 129, VAR23: m/z 143, VAR24: $[M\text{-}143]^+$, VAR25: m/z 169, VAR26: $[M\text{-}155]^+$, VAR27: $[M\text{-}140]^+$, VAR28: $[M\text{-}157]^+$, VAR29: $[M\text{-}144]^+$, VAR30: m/z 244, VAR31: m/z 218, VAR32: m/z 231.

are positioned outside the ellipse (left part) and comprises 19-nor steroids with a C-17 ethyl group (Fig. 2, analytes 19–21) due to the ions $[M\text{-}29]^+$, $[M\text{-}90\text{-}29]^+$, $[M\text{-}144]^+$ and $[M\text{-}157]^+$ derived mainly from ion fragments characteristic of the 17-ethyl group.

Further discrimination was achieved by applying PLS-DA statistical analysis to the fragmentation data, dividing the AAS derivatives into groups according to their structural similarities. Several PLS-DA models were derived using three different groups each time. In this way, four more AAS classes were distinguished besides classes 1 and 2. The AAS classes along with the significant fragment ions responsible for their classification are summarized in Table 1, and are described as follows:

- Class 1; 1-ene-3-keto steroids,
- Class 2; 3-hydroxyl steroids with saturated A-ring,
- Class 3; 1-ene-3-hydroxyl steroids,
- Class 4; 4-ene-3-keto steroids,
- Class 5; 1,4-diene-3-keto steroids,
- Class 6; 3-keto steroids with saturated A-ring.

The three principal components of the PLS-DA model derived for classes 3, 4 and 5 explained a very high percentage of the cumulative variance (R^2Y 85.8%) and a satisfactory predicted variance (Q^2Y 67.5%). Fig. 3a illustrated the clear discrimination of the three classes according to the scores plot of the first two PCs. The corresponding loadings plot in Fig. 3b reveals that fragment ions of type F4 and F4-15 (schematically shown in Table 1, top) are significant for positioning of the analytes of class 3, while ions $[M]^+$, $[M\text{-}143]^+$ and F2, F3 are important for the positioning of the analytes of classes 4 and 5, respectively.

The "worst" PLS-DA statistical analysis was achieved for classes 3, 4 and 6 (with R^2Y 75.2% and Q^2Y 17.0%), but still their discrimination is apparent (Fig. 4a). The corresponding loadings plot reveals that the ions at m/z 143 and m/z 169 are significant for the positioning of the analytes of class 3, while that ions $[M]^+$, F3 and m/z 129 are important for the positioning of the classes 4 and 6, respectively (Fig. 4b).

The fragmentation patterns of the above mentioned AAS classes are more thoroughly discussed in Section 3.2.

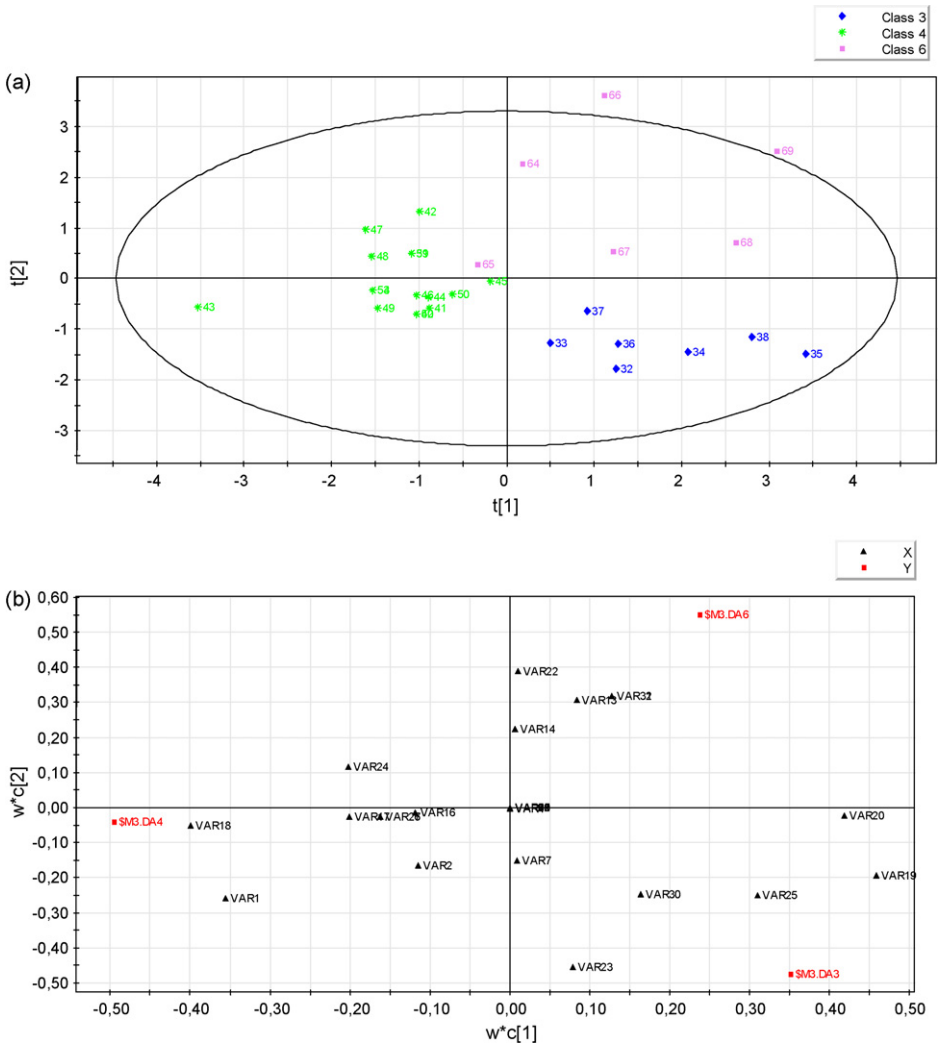


Fig. 4. PLS-DA model (a; scores plot and b; loadings plot) of the TMS-derivatized AAS molecules belonging to classes 3, 4 and 6. Abbreviations as in Fig. 3.

3.2. Dissociation routes of AAS EI mass spectra

3.2.1. Class 1: 1-ene-3-keto steroids

The TMS-derivatives of anabolic steroids with 1-ene-3-keto structure are dominated by fragmentation patterns resulting in three B-ring bond fissions, depicted as fragments F1, F2 and F3 (Fig. 5a).

In the case of methyl-1-testosterone (Table 1, analyte 1) and 1-testosterone (Table 1, analyte 4) these fragments are common and observed at values of *m/z* 179, 194 and 206, respectively [40,41]. The presence of a C-1 or a C-2 methyl substituent, as in the case of methenolone (Table 1, analyte 6) and stenbolone (Table 1, analyte 8), respectively, does not alter this general dissociation route; the fragments F1, F2 and F3 are still observed [42,43].

The high abundance of $[M]^+$ and $[M\text{-}15]^+$ ions are also common characteristics in the mass spectra of the TMS-derivatized steroids with 1-ene-3-keto structure. The ions at $[M\text{-}90]^+$ and $[M\text{-}90\text{-}15]^+$ are of very low abundance but still present and indicative in the TMS mass spectra of this class of compounds.

3.2.2. Class 2: 3-hydroxyl steroids with saturated A-ring

The main common feature of this class of steroids is the substantial abundance of the $[M]^+$ and/or $[M\text{-}15]^+$ ions and the existence of a low abundance ion at *m/z* 129 (fragment F5, Fig. 5b), as occurs for, e.g., androsterone (Table 1, analyte 12). However, in steroids with a C-17 methyl group the characteristic D-ring fragment ions (*m/z* 143, *m/z* 130) become the significant ones, as occurs for bolasterone metabolite (Table 1, analyte 14) and oxymetholone metabolite (Table 1, analyte 17).

The existence of a C-17 ethyl group gives rise to prominent D-ring fragment ions at *m/z* 144 and *m/z* 157, while the ions at $[M\text{-}29]^+$ and $[M\text{-}n{\cdot}90\text{-}29]^+$ (*n*, the number of TMSOH groups in the steroidal molecule) are also of considerable abundance, as occurs for norethandrolone metabolites (Table 1, analytes 19–20).

The existence of a primary hydroxyl group leads to a low abundance ion at *m/z* 103 and to an intense fragment ion at $[M\text{-}103]^+$, while the fragment ion F5 and the characteristic D-ring fragments become of insignificant abundance, as occurs for mesterolone metabolites (Table 1, analytes 30–31).

3.2.3. Class 3: 1-ene-3-hydroxyl steroids

The common dissociation pathway of this class of steroids results in A-ring cleavage with fission of bonds C-1/C-10 and C-4/C-5 (fragment F4, Fig. 5c). Another fragment ion with the loss of 15 Da (F4-15) is also commonly observed.

The presence of a C-2 methyl substituent does not alter this fragmentation pathway, but also one more A-ring fragment of low abundance may be observed from cleavage of bonds C-1/C-10 and C-3/C-4 (fragment F5), as in the case of stenbolone metabolite (Table 1, analyte 38) [42].

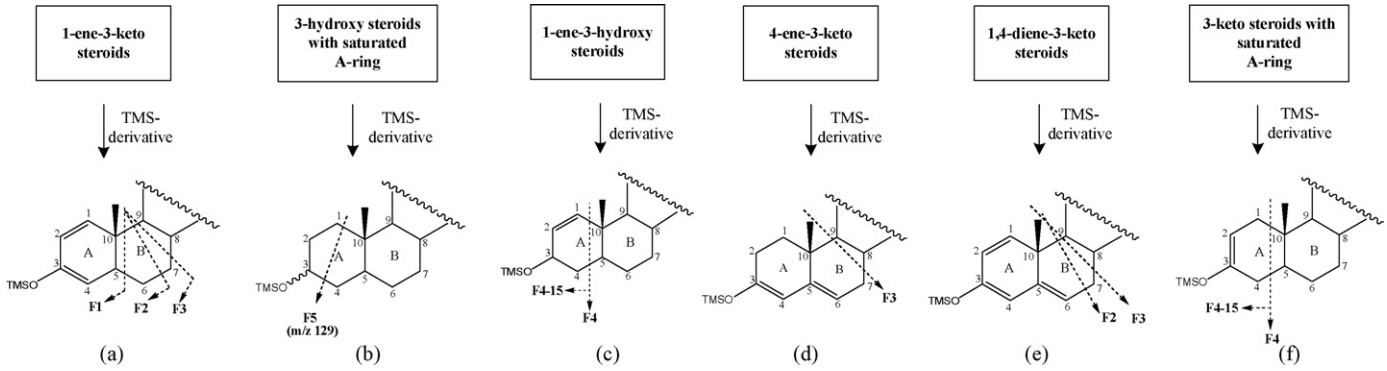


Fig. 5. Characteristic A-/B-ring fragment ions for the TMS-derivatives of anabolic androgenic steroids.

Other characteristic fragment ions, as the $[M-90]^+$ and $[M-90-15]^+$, are also observed but are of very low abundance. The abundance of the $[M]^+$ ion in the mass spectra of the TMS-derivatized 1-ene-3-hydroxyl anabolic steroids is substantial as occurs for the oral turinabol metabolite (Table 1, analyte 33), except in the corresponding 17β-hydroxyl,17α-methyl steroids where the D-ring fragment ion (m/z 143) becomes the prominent one, as observed for methyl-1-testosterone metabolite (Table 1, analyte 32).

3.2.4. Class 4: 4-ene-3-keto steroids

The common fragment ion for the TMS-derivatized 3-keto-4-ene steroids results in B-ring cleavage giving rise to fragment F3 (Fig. 5d) despite the fact that it is generally of low abundance. The abundance of the F3 characteristic fragment ion becomes even lower in the TMS mass spectra of:

- C4-substituted steroids, e.g., 4-hydroxyl-testosterone, oxymesterone and nor-clostebol (Table 1, analytes 48–50, respectively) and
- C6- or C7-substituted steroids (e.g., methyl- or hydroxyl-), e.g., bolasterone, calusterone and mibolerone (Table 1, analytes 40, 41 and 52, respectively).

Another common feature of this class of steroids is the high abundance observed for the $[M]^+$ and/or $[M-15]^+$, as well as for $[M-143]^+$ ion (the last ion concerns 17β-hydroxyl,17α-methyl steroids). Representative examples of TMS-mass spectra of 3-keto-4-ene steroids are those of testosterone, oxymesterone and methyl-testosterone (Table 1, analytes 39, 49 and 53, respectively).

However, in the TMS-derivatized mass spectrum of fluoxymesterone, the low abundance fragment F3 and the m/z 143 are observed, instead of the $[M-143]^+$ ion mentioned above (Table 1, analyte 44).

3.2.5. Class 5: 1,4-diene-3-keto steroids

The main fragmentation pattern in TMS-derivatized 1,4-diene-3-keto steroids is the prominent B-ring fragment F3 (Fig. 5e), as occurs in the mass spectra of the TMS-derivatized boldenone and its metabolite (Table 1, analytes 57–58), which both give a dominant ion at m/z 206. Another B-ring characteristic fragment ion, F2, and the $[M-90-15]^+$ ion are also of significant abundance.

The presence of a C6-hydroxyl function in 1,4-diene-3-keto steroids, mainly after biotransformation processes, lowers the abundance of the characteristic fragment ions F2 and F3. The TMS mass spectra of the C6-hydroxylated metabolites of oral turinabol, boldenone and methandienone show the $[M-15]^+$ ion as the dominant one, while their D-ring characteristic fragments and the $[M]^+$ ion are of low abundance (Table 1, analytes 55, 59–60, 62, respectively).

3.2.6. Class 6: 3-keto steroids with saturated A-ring

The common fragment ion for the TMS-derivatized steroids with a 3-keto group and saturated A-ring, results in A-ring cleavage giving rise to fragments F4 and F4-15, as shown in Fig. 5f. Other characteristic fragment ions of considerable abundance are the $[M]^+$, $[M-15]^+$, $[M-90]^+$ and $[M-90-15]^+$. Fragments ions derived from D-ring cleavage are also of significant abundance, especially for C-17 alkylated steroids. Representative examples are the TMS-derivatized mass spectra of mibolerone metabolites, drostanolone and mesterolone (Table 1, analytes 65–66, 67 and 68, respectively).

3.3. Case studies

3.3.1. Case study 1: estimation of fragmentation patterns of 17β-hydroxylandrosta-4,6-dien-3-one bis-TMS derivative

17β-Hydroxylandrosta-4,6-dien-3-one (Cs-1) has been detected in human urine after the administration of androsta-1,4,6-triene-3,17-dione and its mass spectrum, as bis-TMS derivative, is shown in Fig. 6 [44]. According to Section 3.2.4 of the present study, Cs-1, as a 4-ene-3-keto TMS-derivatized steroid, is expected to give a B-ring fragment ion (F3) and high abundance $[M]^+$ and/or $[M-15]^+$ ions. Other possible fragment ions are the $[M-TMSOH]^+$ and $[M-TMSOH-15]^+$ (Fig. 6).

3.3.2. Case study 2: estimation of fragmentation patterns of 6z-hydroxyl-androst-4-ene-dione tris-TMS derivative

6z-Hydroxyl-androst-4-ene-dione (Cs-2) was detected during the GC–MS analysis of the nutritional supplement 6-OXO® and as metabolite of 4-androstenedione [45,46]. According to Section 3.2.4 of the present study, Cs-2, as C6-hydroxyl substituted steroid is expected to give a very low abundance fragment ion of type

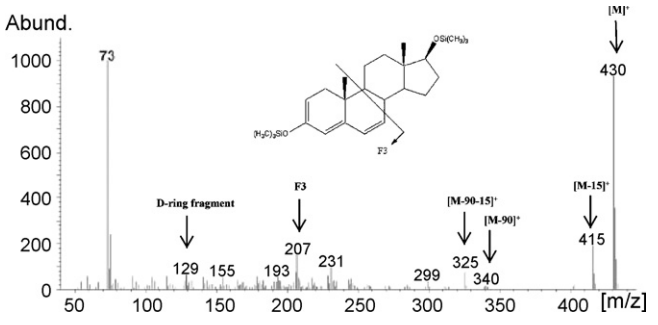


Fig. 6. Mass spectrum of 17β-hydroxy-androsta-4,6-dien-3-one bis-TMS derivative.

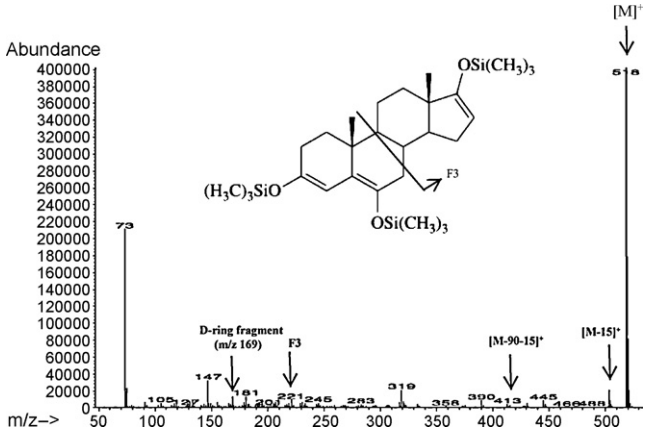


Fig. 7. Mass spectrum of 6z-hydroxy-androst-4-ene-dione tris-TMS derivative.

F3, while the ions $[M]^+$ and/or $[M-15]^+$ are estimated to be the prominent ones. Fragment ions of type $[M\text{-}n\cdot TMSOH]^+$ and $[M\text{-}n\cdot TMSOH\text{-}15]^+$, as well as the corresponding D-ring fragment ion (m/z 169), are expected to be of insignificant abundance. These, indeed, happen as reveals the mass spectrum of the Cs-2 TMS-derivative (Fig. 7).

4. Conclusions

In consequence, the study indicates that the complementary use of PCA and PLS-DA for the analysis of mass spectra permits a differentiation among groups of TMS-derivatized AAS molecules. The results show that their mass spectra contain ions that are characteristic giving information as a "fingerprint" according to their different structural features. Characteristic fragmentation patterns of TMS-derivatized AAS were elucidated considering their EI full scan mass spectra which can be also useful for the estimation of expected fragment ions of chemically modified steroids and their metabolites that could be inserted in SIM or full scan screening methods of anti-doping laboratories. The study can be considered as a useful tool for the estimation of fragmentation pathways when unknown or chemically modified steroids are detected during the doping control analyses of human urines using the EI GC–MS technique.

Acknowledgements

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