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ARTIFACT FORMATION DUE TO ETHYL THIO-INCORPORATION INTO SILYLATED STEROID STRUCTURES

ABSTRACT

For the application of gas chromatography-mass spectrometry (GC-MS) in steroid analysis, trimethylsilylation of target substances in a mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide and ethanethiol is frequently applied. However, using this mixture to silylate the steroids androsterone and etiocholanolone obtained from a urine matrix, the formation of artifacts was established.

The artifacts were identified as ethyl thio-containing products of the respective trimethylsilyl derivatives. The conversion of the studied products increased slowly as a function of time, was dependent on the presence of the urine matrix and was significantly accelerated by adding diethyl disulfide to the reagent before incubation. Also ethyl thio-incorporation into testosterone and epitestosterone was established. A mechanism for ethyl thio-incorporation at the C^{16}-position is proposed.

The conversion that was achieved after 120 hours of sample storage at room temperature was insufficient to significantly influence the analysis of androsterone and etiocholanolone under the studied circumstances. However, the results provide fundamental insight into the mechanism of silylation and the occurring side-reactions. Moreover, when investigating the formation of new metabolites the ethyl thio-incorporation can create false interpretations.

INTRODUCTION

One of the challenging fields in doping analysis has been the mass spectrometric determination of steroids of either exogenous or endogenous origin. Since its introduction in steroid analysis in the eighties by Donike *et al*. [1], *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) has been extensively used as a powerful trimethylsilyl donor in the derivatization procedure. One of the most reported derivatization techniques is the application of a mixture of MSTFA/ammonium iodide/ethanethiol. MSTFA reacts *in situ* with ammonium iodide (NH_4I) to produce trimethyliodosilane (TMSI) that has been reported as the most powerful trimethylsilyl donor available [1]. TMSI reacts with adequate speed to produce both trimethylsilyl (TMS) ether and trimethylsilyl enol (TMS enol) ether derivatives (see Figure 1).

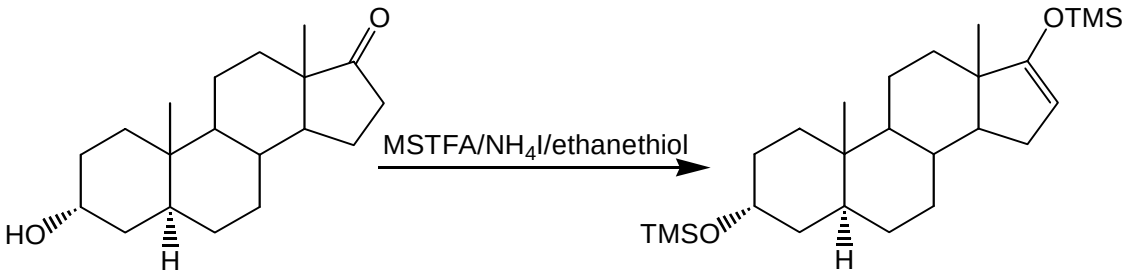

Figure 1: Derivatization of androsterone to its per-TMS ether derivative.

Ethanethiol[1] is added to reduce the formed iodine to hydrogen iodide in order to prevent iodine incorporation into the steroid nucleus. As a result, diethyl disulfide is produced during the derivatization reaction [2]. Diethyl disulfide formation depends on the amount of ammonium iodide and ethanethiol added to the extract and the chosen experimental conditions as reaction time and temperature. Usually a mixture of MSTFA/NH_4I/ethanethiol is used in a ratio of 1000:2:3 (v/w/v).

When applying this procedure, it is assumed that other reactants than TMSI present in the reaction mixture are inert to the steroids to be analyzed. However, as will be described in this paper, incorporation of an ethyl thio-group with steroid structures occurs during the described derivatization procedure.

In this study artifact formation was suspected in derivatized urine sample extracts. An excretion study with [2,2,4,6,6,16,16-$^7H_2$]-androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE) showed that these artifacts were either metabolically or chemically related to Δ4-AEDIONE. Experiments are described to prove incorporation of an ethyl thio-group into the main metabolites of Δ4-AEDIONE: androsterone and etiocholanolone.

[1] Ethanethiol is frequently used as a replacement of dithioerythreitol [2].

As diethyl disulfide is formed as side-product during the described derivatization procedure, an experiment is described to investigate the role of ethanethiol and diethyl disulfide as reagent in ethyl thio-incorporation. Repeated measurements over time give insight into the significance of the side-reactions as compared to the desired derivatization reactions. Also ethyl thio-incorporation into testosterone and epitestosterone was established.

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone), androst-4-en-17α-ol-3-one (epitestosterone), 5α–androstan-3α-ol-17-one (androsterone), 5β–androstan-3α-ol-17-one (etiocholanolone), 5α-androstane-3α,17β-diol, androst-4-ene-3,17-dione and androst-5-ene-3β,17β-diol, 17α-methylandrost-4-ene-11α,17β-diol-3-one (11α-hydroxy-methyltestosterone), and diethyl disulfide were obtained from Sigma, St. Louis, Missouri, USA. [2,2,4,6,6,16,16-$^{7}H_2$]--Androst-4-ene-3,17-dione (purity 98.4%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

N-Methyl-*N*-trimethylsilyltrifluoroacetamide was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, USA. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Ethanethiol was obtained from Acros Organics, New Jersey, USA. Diethyl ether was obtained from Merck, Darmstadt, Germany.

A crude solution of Helix pomatia (type HP-2, containing 110.000 IU/ml of β–glucuronidase and 1000-5000 IU/ml of arylsulfatase) was obtained from Sigma, St. Louis, Missouri, USA. Columns for solid phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Gas chromatography and mass spectrometry

GC-MS analysis was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in the electron ionization mode at 70 eV. Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 μm). Through electronic pressure control the column flow (helium) was constant: 1 ml/min.

Sample injection of 1 µl was performed in split mode (ratio 1:10). A Hewlett Packard autosampler (Model 7673) was used for auto-injection. The injector temperature was set at 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 minutes. The interface temperature was set at 280°C.

Confirmation of artifact formation

Two products (X, Y) that were suspected to be analytical artifacts, were discovered during the full scan mode analyses of urine samples obtained from an excretion study with d_7-Δ4-AEDIONE. In this study 50 mg of d_7-Δ4-AEDIONE was administered to a healthy male subject (age: 29 years). Urine samples were collected during 24 hours before and 24 hours after administration.

11α-Hydroxy-methyltestosterone (5 µg) was added as internal standard to 4 ml of urine. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrating with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The organic material was collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) was added to the extract. Hydrolysis was performed by adding 100 µl of Helix pomatia and incubating for 1 hour at 55°C. Steroids were isolated from the buffer solution by extracting with 5 ml of diethyl ether (twice). After extraction the phase separation was stimulated by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a gentle stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. One µl of the incubation mixture was directly injected.

Screening of the urine extracts was performed in selected ion monitoring (SIM) mode. Δ4-AEDIONE was monitored at m/z 430 representing $[M]^{\bullet+}$. AO and EO were monitored at m/z 434 and 419, representing $[M]^{\bullet+}$ and $[M-15]^{+}$, respectively. The suspected artifacts X and Y were monitored at m/z 494 and 479. The monitored m/z values corresponding to labeled AO and EO (m/z 440 and 425) and artifacts X and Y (m/z 499 and 484), were chosen on the basis of the maximum area response. These m/z values were established by SIM monitoring of the m/z range 434 to 441 for AO and EO and m/z 494 to 501 for X and Y, respectively.

Derivatization of synthetic steroids

To study the source of formation of X and Y as analytical artifacts, synthetic steroids (25 µg) were derivatized as described above and analyzed for X and Y. Some of the

most relevant endogenous steroids in relation to doping analysis were selected: AO, EO, testosterone (T), epitestosterone (E), Δ4-AEDIONE, dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (Δ5-AEDIOL).

Influence of diethyl disulfide on ethyl thio-incorporation with AO and EO

To prove the reactivity of diethyl disulfide in the incorporation of an ethyl thio-group, the derivatization of synthetic AO and EO was performed under the following conditions:

1. 25 μg of AO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
2. 25 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
3. 25 μg of AO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:3; v/w/v/v)
4. 25 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:3; v/w/v/v)

To every sample 25 μg of 5α-androstane-3α,17β-diol (ADIOL) was added as internal standard. Incubation was performed at 80°C for 30 min. The derivatization mixtures were analyzed on day 0 (immediately after derivatization), day 7 and day 12 (the samples were kept at room temperature between the analyses). The area ratios, Y/AO and X/EO were determined by monitoring ions of the derivatives at m/z 494 (X and Y) and m/z 434 (AO and EO).

Time dependence of ethyl thio-derivative formation

To study the time dependent ethyl thio-derivative production in more detail, the following samples were prepared for analysis and analyzed repeatedly for 120 hours (once every 4 hours):

1. 10 μg of AO and 10 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
2. 10 μg of AO and 10 μg of EO in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v)
3. Extract of 3 ml of blank urine in 100 μl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)
4. Extract of 3 ml of blank urine in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v)

ADIOL (10 μg) was used as internal standard. Urine sample cleanup was performed as described above. GC-MS analysis of the per-TMS derivatives was performed by selected ion monitoring of the ions at m/z 434 (AO and EO), 494 (X and Y) and 436

(ADIOL). The area ratios of AO, EO, X and Y vs. ADIOL, respectively, were calculated and graphically presented as a function of time of analysis.

Ethyl thio-incorporation with T and E

T and E (25 μg) were derivatized in 100 μl of MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v) for 30 minutes at 80°C. After 12 days of storage in glass vials at room temperature, full scan mass spectra were recorded.

Statistics

Confidence intervals of 95% were calculated for the regression coefficient 'a' (slope of the linear regression line for the area ratio as a function of time). Significance of production of X and Y and loss of AO and EO were tested using the hypothesis H_0: a=0 and H1: a>0 or H_1:a<0. Statistical analysis was performed with SPSS 9.0.

RESULTS

Confirmation of artifact formation

In all urine samples two unknown compounds (X and Y) were detected at m/z 494 and m/z 479 (see Figure 2). The identity and origin of these products were unknown. After administration of d_7-Δ4-AEDIONE and at the retention times of X and Y (16.40 and 16.55, respectively), a signal at m/z 499 and 484 was also detected. These were suspected to be labeled derivatives of X and Y. The signals of X and Y could also be observed in full scan mode, but concentrations of the two products were too low to obtain representative full scan mass spectra.

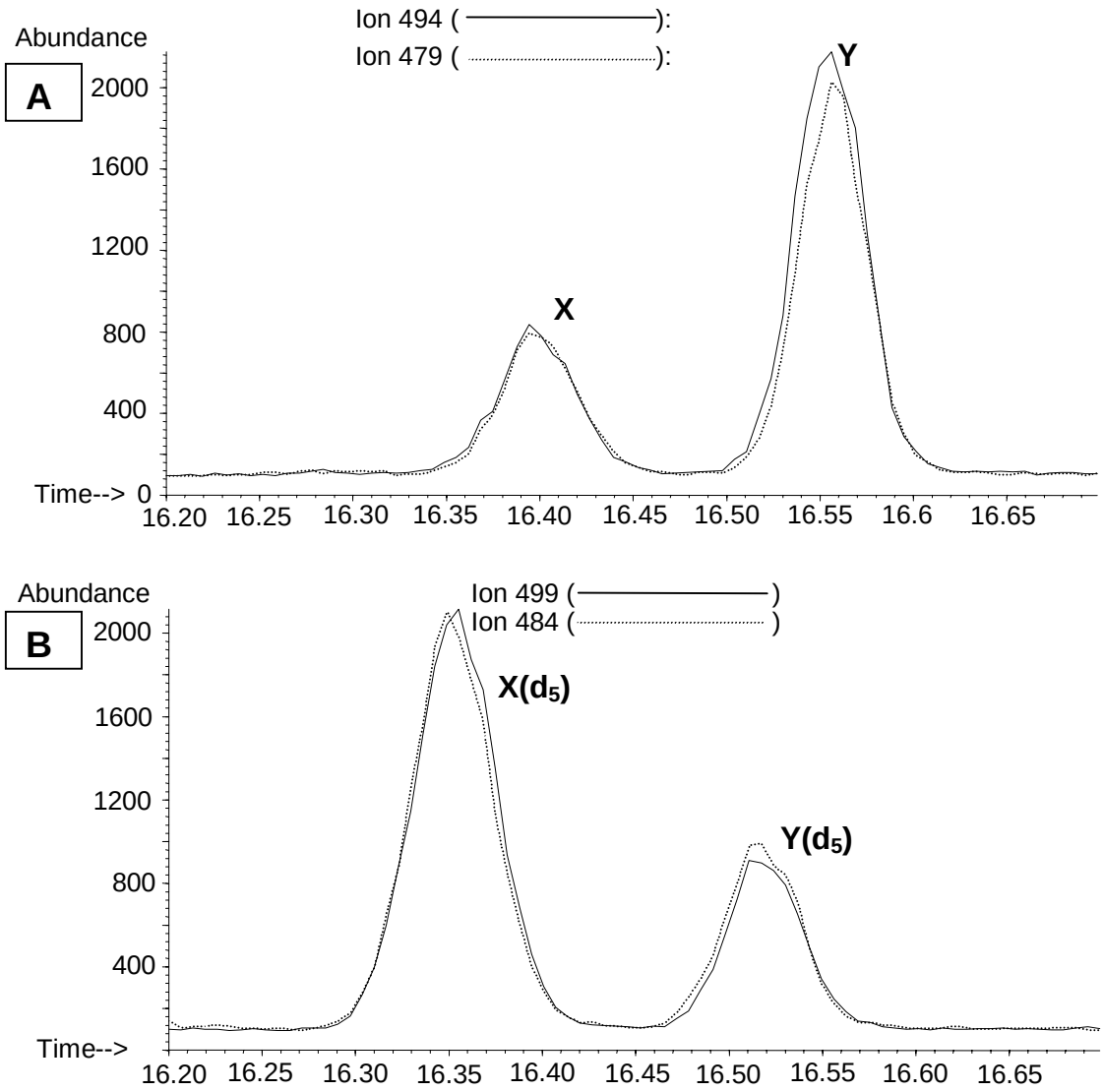

Figure 2: Selected ion chromatograms of non-deuterated (chromatogram A: m/z 494 and 479) and deuterated (chromatogram B: m/z 499 and 484) products X and Y

Derivatization of synthetic steroids

In the derivatization mixtures containing the per-TMS derivatives of AO and EO, minor quantities were detected of respectively Y and X. Other steroids studied did not result in product formation, as determined by the analysis of the selected ions at m/z 494 and 479, respectively. Concentrations of X and Y were too low for obtaining representative full scan mass spectra.

Influence of diethyl disulfide on ethyl thio-incorporation

The results are summarized in Table 1. After 12 days incubation it was possible to obtain representative full scan spectra for the derivatives of X and Y (Figure 3).

Table 1: *Area ratio of ions at m/z 494 and 434 of 4 mixtures over 12 days.*

	Mixture	Day 0	Day 7	Day 12
1	AO in MSTFA/NH_4I/ethanethiol	0.0111	0.0416	NA*
2	EO in MSTFA/NH_4I/ethanethiol	0.0068	0.0357	NA*
3	AO in MSTFA/NH_4I/ethanethiol/diethyl disulfide	0.140	0.927	2.17
4	EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide	0.104	0.694	1.48

* NA = not analyzed

Time dependence of ethyl thio-derivative formation

A significant ($p<0.05$) and approximate constant production rate of X and Y occurred in all four reaction mixtures (Figure 4). However, when MSTFA/NH_4I/ethanethiol was applied, the area of X and Y remained smaller than 1 percent compared to the derivatives of AO and EO (Figure 5) after 120 hours. In case of MSTFA/NH_4I/ethanethiol/diethyl disulfide derivatization of a urine sample extract, the areas of X and Y approached 5 percent of the areas of respectively per-TMS EO and AO after 120 hours incubation at room temperature. Also in this case the concentrations of silylated AO and EO decreased significantly ($p<0.05$).

When the two studied derivatization methods were applied to one urine sample extract that was split into two separate fractions, different recoveries of derivatization were obtained. MSTFA/NH_4I/ethanethiol derivatization of AO and EO led to significantly smaller recoveries of the silylated products compared to the MSTFA/NH_4I/ethanethiol/diethyl disulfide derivatization (approximately 70%). As can be observed in Figure 5, these recoveries showed an approximate linear increase with increasing incubation time, to 85-90% of the recovery obtained with the MSTFA/NH_4I/ethanethiol/diethyl disulfide method. Differences in conversion were not present in case of derivatization of synthetic AO and EO.

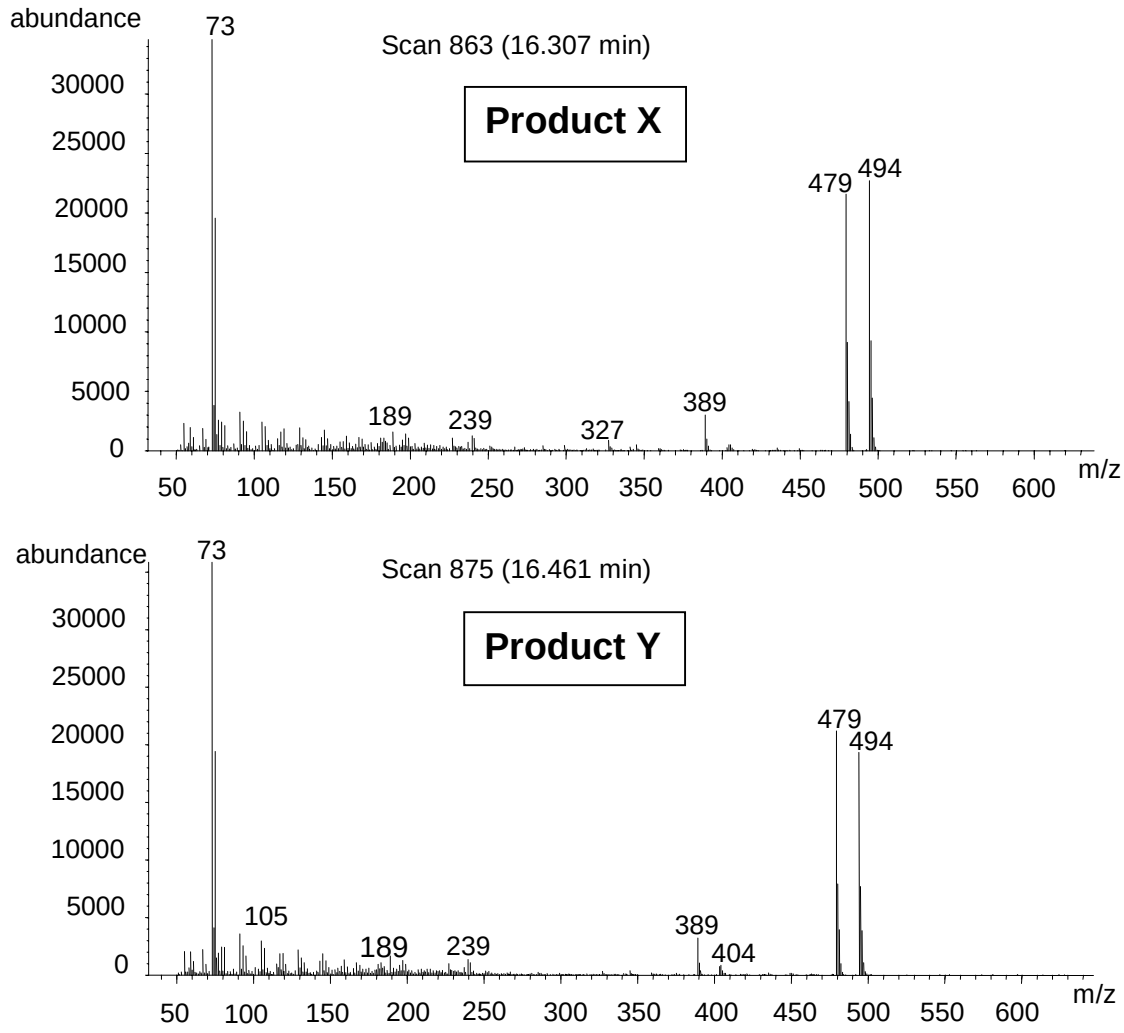

Figure 3: Full scan mass spectra of products X and Y as determined in a urine sample extract incubated in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).

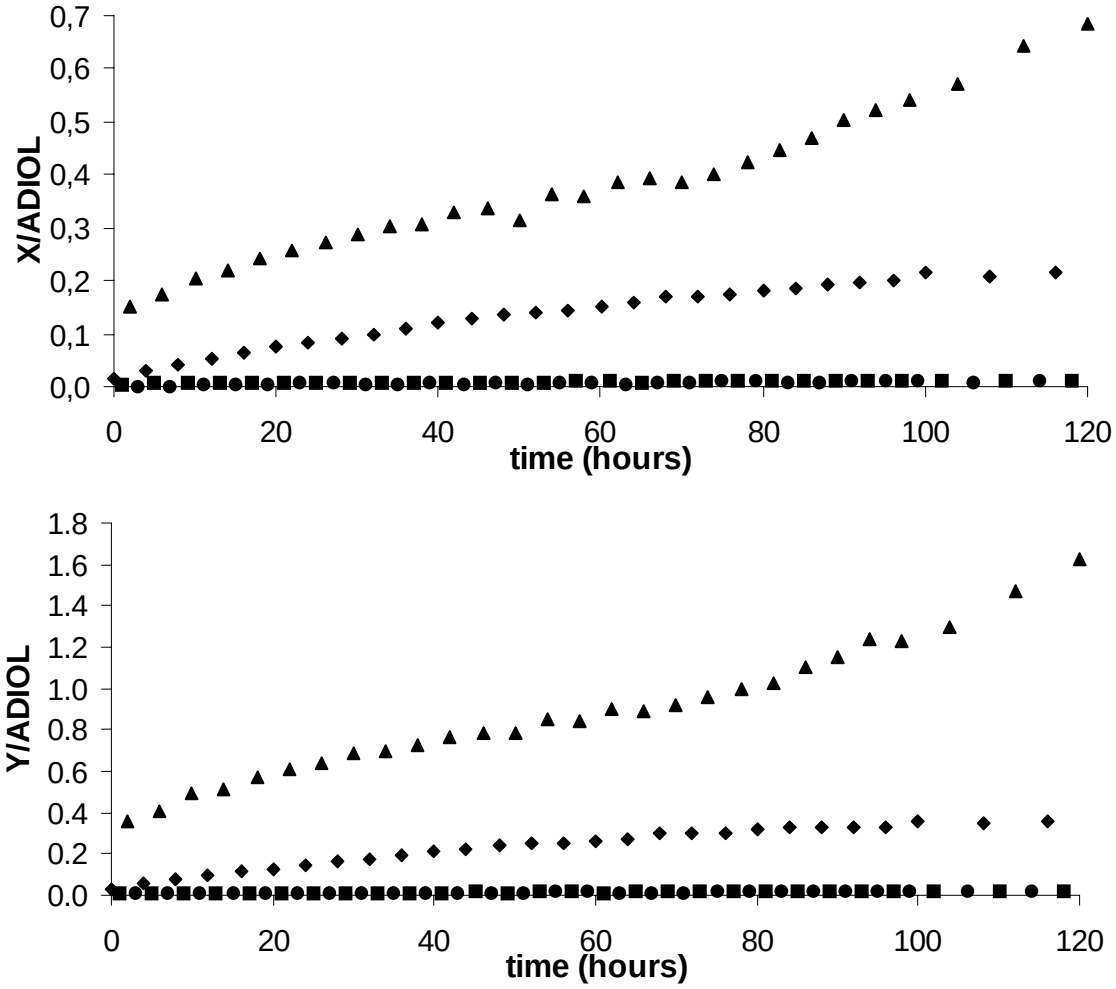

Figure 4: Ratios of X/ADIOL and Y/ADIOL as a function of time after derivatization.

- ▲ - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ● - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)).
- ♦ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).
- ■ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

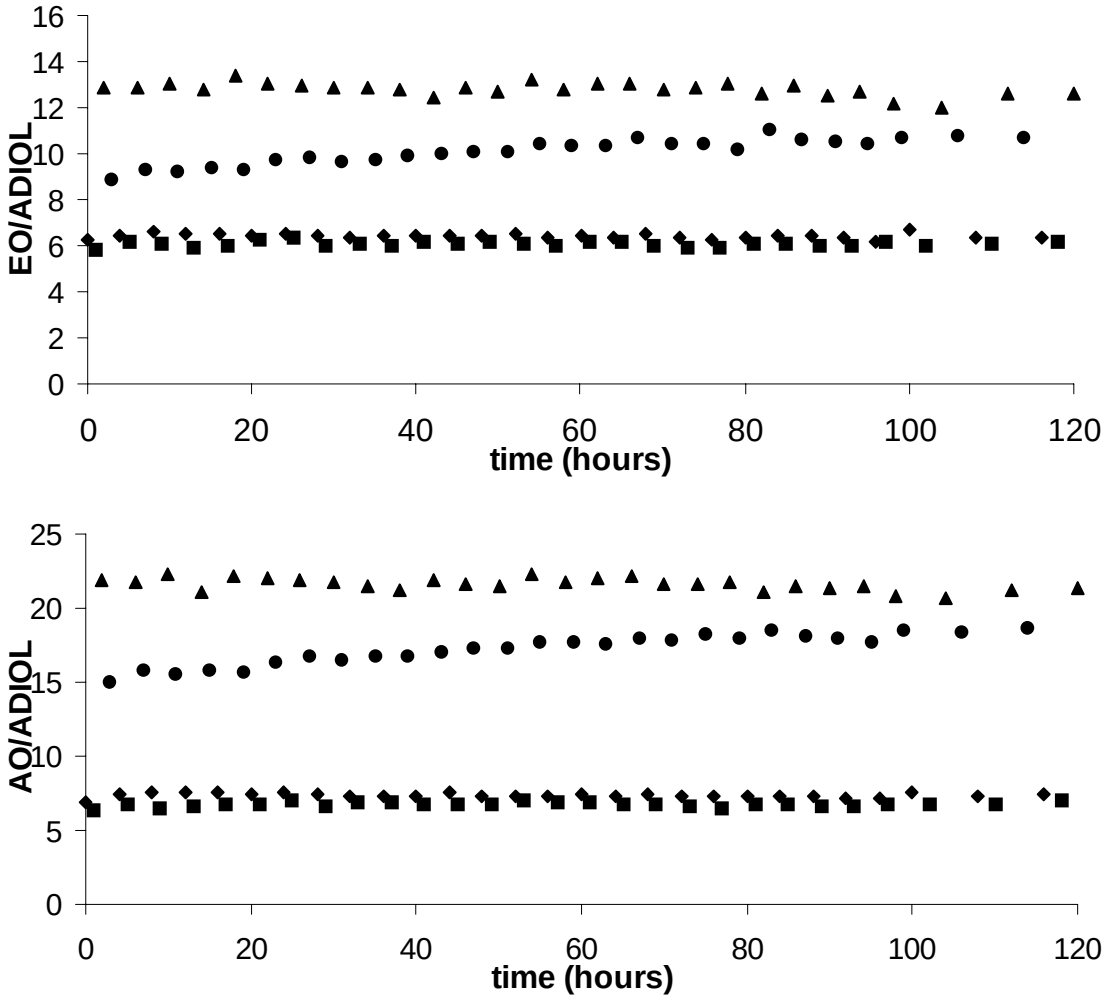

Figure 5: Ratios of AO/ADIOL and EO/ADIOL as a function of time after derivatization.

▲ - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).

● - Incubation of a urine extract in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v)).

♦ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol/diethyl disulfide (1000:2:3:50; v/w/v/v).

■ - Incubation of synthetic AO and EO in MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

Ethyl thio-incorporation with T and E

Total ion current chromatograms of the derivatization mixtures and the mass spectra of the ethyl thio-products of T and E, are shown in Figures 6 and 7.

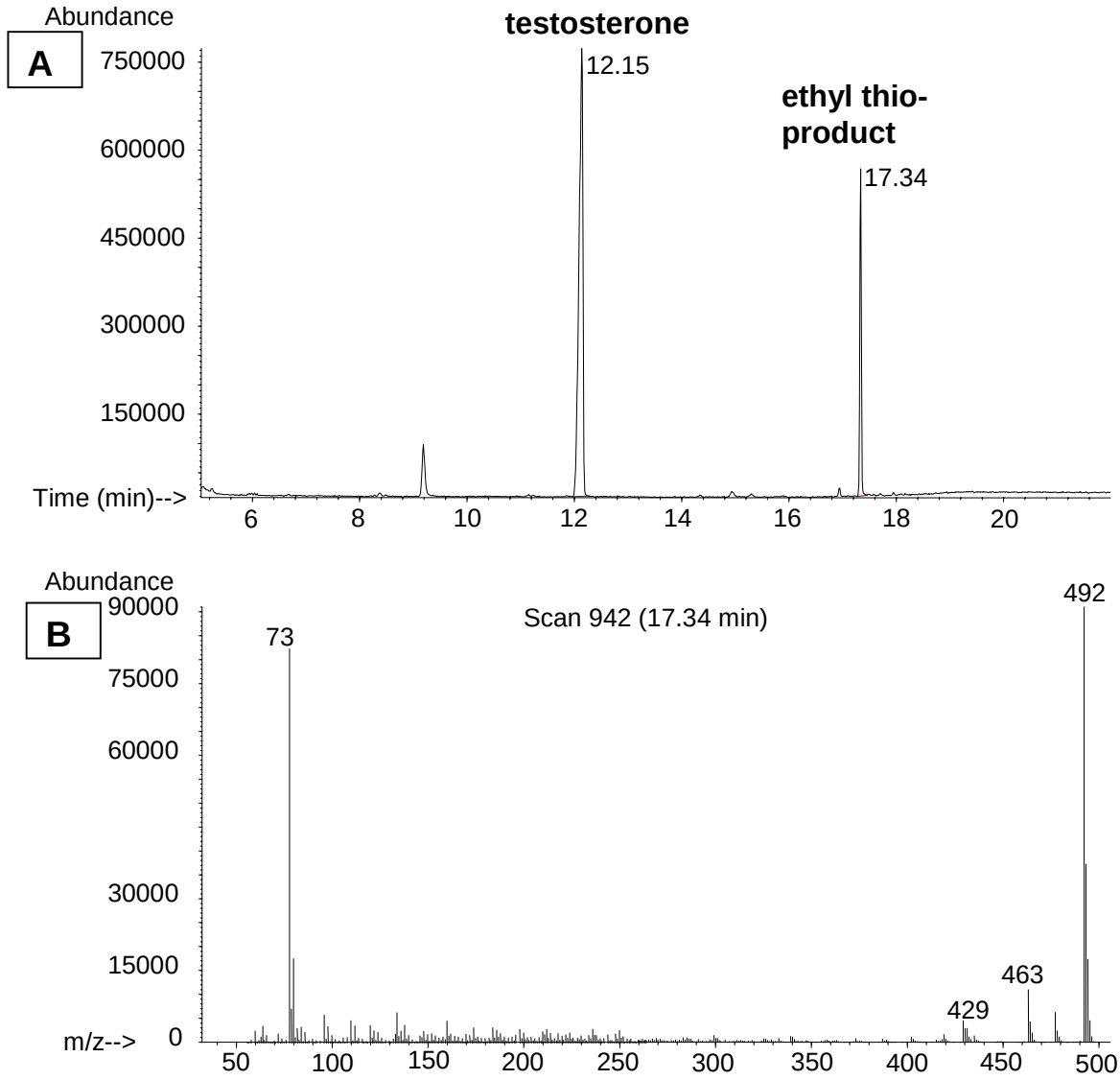

Figure 6: **A**.Total ion current chromatogram of silylated synthetic testosterone (12 days after derivatization). **B**.Full scan mass spectrum of the obtained ethyl thio-derivative.

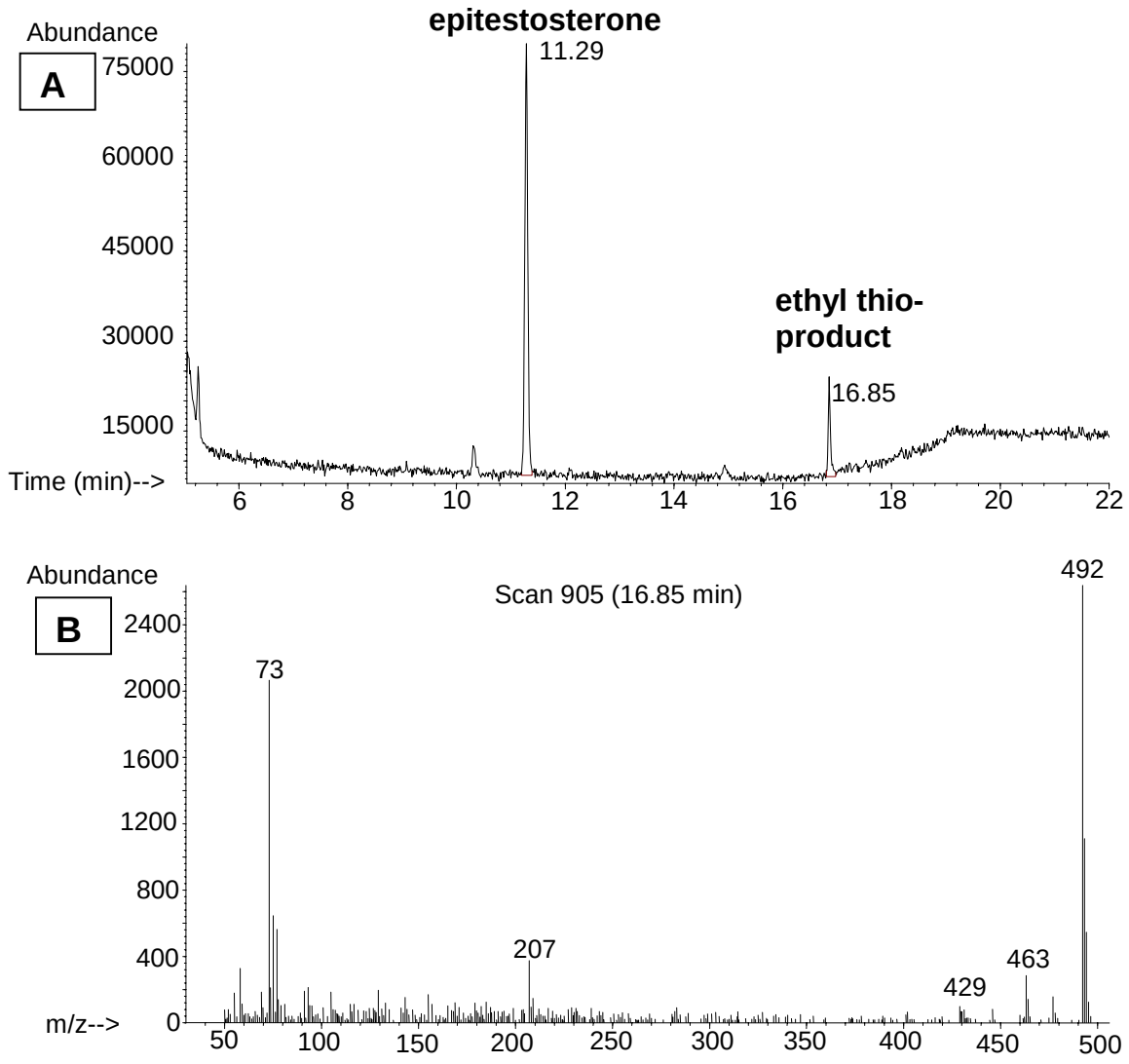

Figure 7: **A**.Total ion current chromatogram of silylated synthetic epitestosterone (12 days after derivatization). **B**.Full scan mass spectrum of the obtained ethyl thio-derivative.

DISCUSSION

X and Y were proven to be analytical artifacts, formed during the derivatization reaction in the MSTFA/NH_4I/ethanethiol (1000:2:3;v/w/v) medium. X and Y were initially observed in blank urine samples. The response of X and Y in the steroid profile increased after administration Δ4-AEDIONE or DHEA (data not shown). When d_7-Δ4-AEDIONE was administered in the described excretion study, besides the signal at m/z 494 and 479 also the signals at m/z 499 and 484 increased. These were suspected to be X and Y containing 5 deuterium atoms, which matched either a metabolically or chemically relation of X and Y to the administered labeled Δ4-AEDIONE. When the observed signal at m/z 494 is assumed to be corresponding to the molecular mass of X and Y, the product formation could not be explained by metabolism, as androgens have a molecular mass range of 430-436 Da and hydroxylated androgen metabolites a range of 518-524 Da.

When chemical side-reactions are considered, a mass of 494 Da could represent the incorporation of an ethyl thio-group into the steroid structure of AO and EO, causing a shift in molecular mass of 60 Da. The increasing signal during repeated GC-MS analysis of one urine sample, illustrated the production of X and Y as a function of time in the derivatization mixture and made the optional source metabolism a less likely explanation. The detection of a significant signal at m/z 494 after derivatization of synthetic AO and EO proved these steroids to be the source of respectively Y and X. Additional proof for ethyl thio-incorporation as the source of the artifact formation was obtained by the extensive acceleration of incorporation after diethyl disulfide was added to the derivatization medium.

The obtained spectra of X and Y show little specific fragmentation. Significant ions in the spectra represent losses usually observed in TMS-derivatization (Table 2).

***Table 2**: Suggested fragmentation for X and Y (see Figure 3).*

m/z	Relative signal X/Y (%)	Loss	Fragmentation
494	100.0/91.1	$[M]^{\bullet+}$	$[M]^{\bullet+}$
479	95.0/100.0	$[M-15]^+$	$[M-CH_3]^+$
404	2.2/4.1	$[M-90]^{\bullet+}$	$[M-TMSOH]^{\bullet+}$
389	13.2/15.1	$[M-90-15]^+$	$[M-TMSOH-CH_3]^+$
327	3.9/1.5	$[M-90-15-62]^+$	$[M-TMSOH-CH_3-CH_3CH_2SH]^+$

The reagent MSTFA/NH_4I/dithioerythreitol was established by Donike *et al.* [1] for application in doping analysis of steroids. Nowadays dithioerythreitol is frequently replaced by ethanethiol to prevent chromatographic interference. The essence of

ethanethiol is preventing iodine incorporation into the steroid nucleus [2]. Upon decomposition of TMSI, iodine is formed that can add to the steroid nucleus. To prevent this side-reaction to occur, iodine is reduced by ethanethiol to form hydrogen iodide and diethyl disulfide.

From Table 1 and Figures 4 and 5 can be concluded that diethyl disulfide is more reactive towards ethyl thio-incorporation than ethanethiol. This could explain the relatively extensive ethyl thio-incorporation that occurs during derivatization of urine extracts compared to synthetic substance derivatization, as the total concentration of target compounds for derivatization is much higher in urine extracts than in that case of synthetic substances. That results in higher diethyl disulfide levels and therefore higher ethyl thio-derivative formation. Consequently, a matrix effect can be defined for trimethylsilylation with the described method, corresponding to the scientifically established matrix effect in steroid analysis [3].

As the mass of the molecular ion increases by 60 Da, it must be expected that a proton is replaced by a $-SCH_2CH_3$ group, leaving the double bond at C^{16}-C^{17} intact. A hypothetical mechanism for such incorporation is proposed in Figure 8. According to this mechanism, the TMS-moiety from the derivatized AO or EO is removed from the steroid by nucleophilic attack of diethyl disulfide on the silicon atom under simultaneous addition of an ethyl thio-group with C^{16}. Subsequently, the generated 17-ketogroup is silylated again. Unfortunately, due to the lack of specific fragmentation of X and Y it was impossible to confirm the structure as proposed in Figure 8.

When Δ4-AEDIONE is orally administered, it is rapidly metabolized, showing AO and EO as the main metabolites [4]. Because AO and EO are present in the urine matrix in relatively high concentrations as compared to other androgens as T, E and Δ4-AEDIONE, the studied derivatives were presumable products of those steroids and could easily be detected. This does however not imply that no ethyl thio-incorporation occurs with other steroids present in the matrix. For example, T and E are interesting compounds to study regarding ethyl thio-incorporation for two reasons. First, the α,β–unsaturated 3-keto moiety could result in different quantities of product formation. Second, as the urinary testosterone/epitestosterone ratio (T/E ratio) is applied to establish use of testosterone, this ratio could be affected by significant ethyl thio-incorporation.

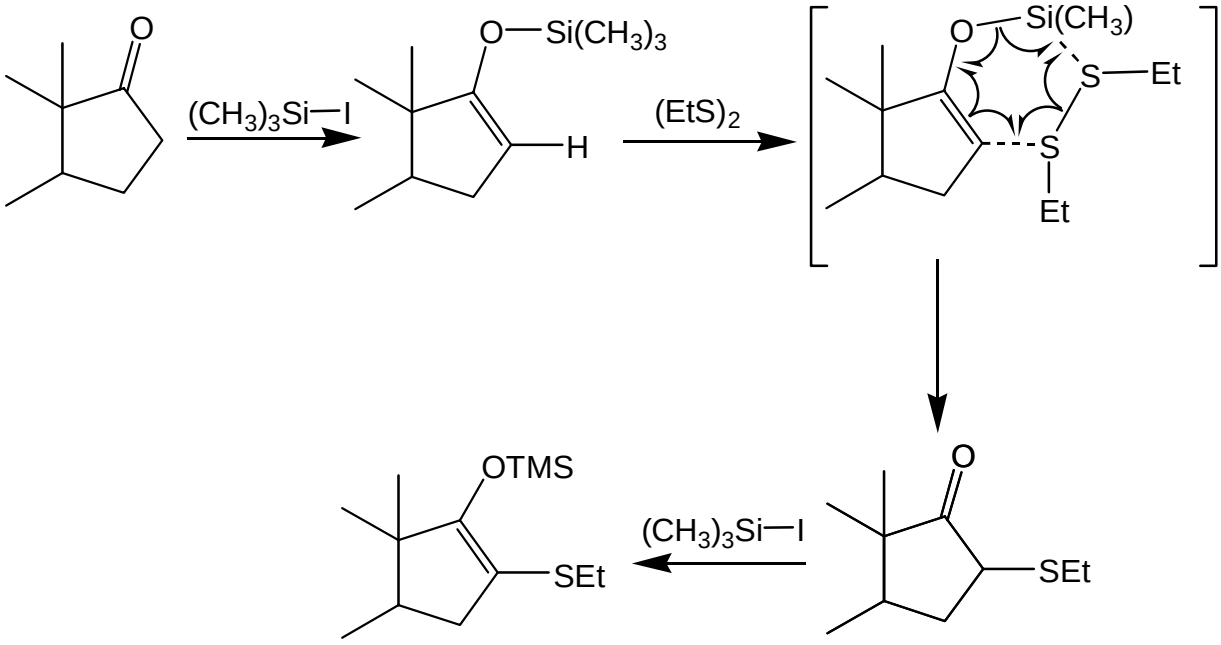

Figure 8: Hypothetical mechanism for ethyl thio-incorporation with AO and EO TMS-derivatives. Step 1: AO/EO are silylated to their per-TMS derivatives. Step 2: The TMS-moiety is lost by nucleophilic attack of EtSSEt on the silicon atom under simultaneous incorporation of the ethyl thio-group with C^{16}. Step 3: The keto group is silylated again by *in situ* formed TMSI.

As shown in Figures 4 and 5, significant ethyl thio-incorporation will only affect the analysis of per-TMS AO and EO, when extra diethyl disulfide is added to accelerate the incorporation. The application of MSTFA/NH_4I/ethanethiol (1000:2:3;v/w/v) did not result in a significant change in the area of AO and EO after 120 hours. This is in agreement with the reported 48 hour stability (at room temperature) or 5-6 days stability (at 4°C) of per-TMS derivatives of steroids in general [5]. The stability estimate of days to weeks reported as by Donike [1] should be considered as an overestimation.

In this case, the role of ethyl thio-incorporation was of purely theoretical interest. However, the presented results give theoretical insight into less accessible aspects of a derivatization procedure that is often applied in steroid analysis. Although the results described in this paper are incomplete to obtain a fully detailed mechanistic overview on the ethyl thio-incorporation in steroid analysis, it illustrates the still insufficient knowledge of the silylation mechanism. In particular, the role of diethyl disulfide has been considered insufficiently. Moreover, when investigating the

formation of new metabolites the ethyl thio-incorporation can create misinterpretations.

The ethyl thio-incorporation could become of practical relevance when derivatized samples are re-analyzed that were stored at room temperature for several days. Also the influence of ethyl thio-incorporation on the quantitative analysis of T and E and the analysis of anabolic steroids at ppb level should be considered. To avoid significant differences in steroid quantification due to artifact formation, deuterated internal standards should be considered as a necessity.

LITERATURE

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