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Detection of several anabolic steroids of abuse in human hair

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Abstract

In the past, analysis of anabolic agents and steroids in hair was focused on few substances, like

- endogenous steroids, which are present in hair at comparatively high concentrations or
- some special anabolics with a high incorporation rate due to a presumed melanin binding (clenbuterol) or the presence of basic groups in the molecule (stanozolol).

Systematic studies on the incorporation of steroids are difficult, due to the low concentration of the steroids, the interference with similar endogenous matrix constituents and complex metabolic patterns.
A fatal case of a bodybuilder, who abused high amounts of at least 5 anabolic agents (containing 8 steroids) in a period of time of 4 weeks prior to death, is reported.
The intake of the substances was listed in detail in a diary. This provided very helpful information (in spite of the occurrence of counterfeits) about possible target substances.
Notwithstanding this distinct situation – known anabolic substances taken at high dosages - the substance identification required high resolution and tandem mass spectrometry in combination with HPLC clean up.
Various analytical results in urine are compared to corresponding findings in hair to draw conclusions about the tendency of anabolics to be incorporated into hair.

1. Introduction

The analysis of substances with presumed anabolic action in hair was hitherto directed to endogenous substances for diagnostic purposes or to some exogenous substances which are administered in high dosages usually to animals. First examples were the identification of clenbuterol [1, 2] in cattle hair and of stanozolol in rat hair [3]. Both substances appear to have outstanding properties - a high melanin binding of clenbuterol or the combination of lipophilicity and basicity of stanozolol. Therefore, a high incorporation rate into hair can be assumed for both substances which are not very representative for the group of anabolic substances.
Moreover, identifications following administration of very high dosages of anabolic steroids like nandrolone in hair of guinea pig [4] and of methyltestosterone in bodybuilder hair were reported.

The main difficulty for a systematic approach to the analysis of anabolic steroids in hair consists in

- the comparatively low dosages of the steroids
- the complexity of the metabolism and the unknown incorporation rates of all species involved and
- the existence of a variety of endogenous metabolites at much higher concentration levels having close structural and analytical similarity to most of the target substances.

Due to these problems, a systematic approach to the analysis of anabolic steroids in hair should include metabolic as well as kinetic aspects and would require considerable amounts of sample material after incorporation of high dosages of steroids.
As an initial step, hair material obtained from a forensic case with known anabolic drug history was investigated to get an impression, which substances are preferably incorporated into the hair matrix.

2. Experimental

2.1. Chemicals

acetonitrile, gradient grade (Merck)

diethylether, reagent grade (Merck)

dithioerythritol (Serva)

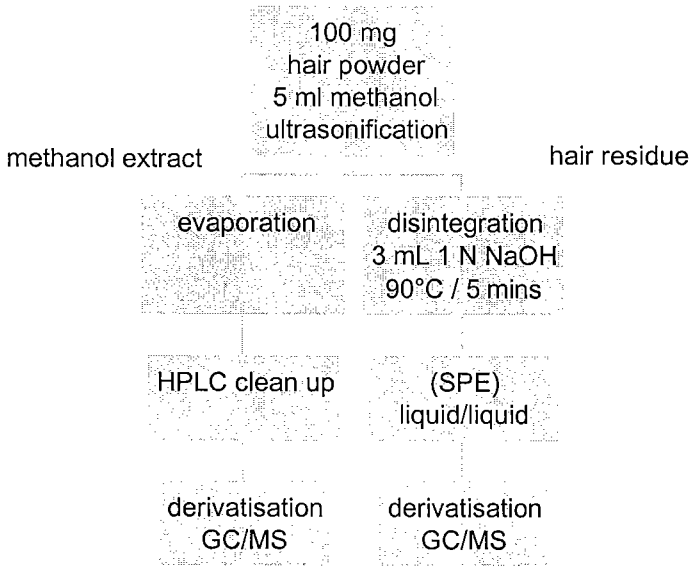
methanol, analytical grade (Merck)

N-methyl-trimethylsilyl-trifluoroacetamide / MSTFA, analytical grade (Macherey-Nagel)

water, gradient grade (Merck)

2.2. Extraction and disintegration of hair samples

The preparation of hair samples was carried out in two steps. The first part consists in an extraction of the hair powder with methanol at 40 deg C in an ultrasonic bath. This method proved to be sufficient for most of the target compounds, i.e. clenbuterol, metandienone testosterone-, nandrolone- and metenolone esters. The recovery of stanozolol could be increased considerably by additional application of a disintegration using 1N sodium hydroxide at 90 deg C.



2.3. Liquid-Liquid-Extraction and HPLC Clean-Up

The purification of methanolic extracts was carried out by liquid/liquid extraction. The methanol was evaporated, the residue was reconstituted with buffer pH9 and extracted with diethyl ether. The ether phase was evaporated to dryness and reconstituted with 40 µl acetonitrile / water (5/95, v/v) solution.

HPLC Parameters

HPLC Chromatograph	HP1090 (Hewlett Packard)
injection volume:	25 µl
detection:	UV-Detector at 240 nm

HPLC - columns

Hypersil ODS (Hewlett Packard)	column	100*2.1 mm
	pre column	20*2.1 mm

Mobile phase and gradient

- mobile phase: water (A) + acetonitile (B)
- flow 0.6 ml/min
- column switch (between HPLC column and pre-column)

	Waste	0-1 min
	Column	1-16min
- gradient	0 % B	0-1 min
	30 % B	2 min
	100 % B	16 min

19-D3-epitestosterone served as time reference standard to control the retention time. The pressure gradient was monitored and examined as additional stability criterion

2.4. Derivatisation

The mobile phase was removed at 80°C in a nitrogen stream. The residue was desiccated and derivatized 30 min at 60°C. The following reagent mixture was used to obtain enol-TMS derivatives:

MSTFA + NH_4I + dithioerythritol (15 ml + 100 mg + 10 mg).

2.5. GC-HRMS and GC-MS/MS analyses

The hybrid version of a high resolution mass spectrometer Autospec M (Micromass) equipped with an HP 5890 II (Hewlett Packard) gas chromatograph and an A 200S (CTC Analytics) autosampler was applied for MS experiments.

GC-conditions

GC - column

12.5 m Optima-5 MS (Macherey-Nagel)

(0.2 mm ID, 0.35 µm film thickness, crosslinked 5% phenyl-methyl-silicone)

injection parameters

- injection mode: splitless
- injection volume: 1.5 µl

oven temperature program

- initial temperature: 150 deg C
- initial time: 0.5 min
- ramp: 12.5 deg C / min to 340 deg C
- final time: 2 min

MS-conditions

- ionisation mode: EI, 38 eV
- trap current 750 µA
- source temperature: 250 deg C

HRMS parameter

- acquisition mode selected ion recording (SIR),
- resolution 3.000 or 10.000

MS-MS parameters

- acquisition mode multiple reaction monitoring (MRM-Q), daughter scan,
- collision energy 150
- resolution 1000 (magnet), 2 amu (quad)

3. Results and discussion

3.1. Case history

A male bodybuilder, who was known to abuse various anabolic steroids regularly, died caused by cardiac infarction at an age of 32. According to his personal diary, he used in the month prior to his death:

- 46 tabl. Ephedrine (30 mg)
- 114 tab. Dianabol (metandienone 5mg)
- 4 amp. Omnadren (mixture of testosterone esters 250mg)
- 4 amp. Primobolan depot (metenolone enantate 100mg)
- 4 amp Winstrol depot (stanozolol 50 mg)
- 4 amp. Parabolane (trenbolone)

A seizure ampoule of Parabolane was identified as fake as it contained nandrolone decanoate instead of trenbolone hexahydrobenzylcarbonate. This was in accordance to the results of the urine analyses, confirming the presence of:

- Metandienone metabolites (6β-hydroxy-metandienone, 17β-methyl-androst-1-ene 3α,17α-ol)
- Nandrolone metabolites (19-nor-androsterone, 19-nor-etiocholanolone)
- Stanozolol metabolites (3'-OH- and 4β-OH-stanozolol)
- Testosterone metabolites (testosterone/epitestosterone = 30),

whereas no trenbolone (epi-trenbolone) could be identified.

3.2. Analytical methods and identification criteria

Highly sensitive detection methods are required to identify anabolic substances in hair, due to their low target concentrations. There is no general preference regarding the analytical technique (high resolution or tandem MS). The final choice depends on the mass spectrometric properties of the target substance:

TMS derivatives of steroid esters are characterized by an abundant molecular ion but weak fragmentation [5] [6]. Therefore, tandem mass spectrometry is not very sensitive in most cases. The only exception was the TMS derivative of metenolone-enantate, which undergoes a ring fragmentation producing A/B ring fragments 194 and 208, which are much more sensitive and selective than typical testosterone ester fragmentation reactions (loss of methyl or TMS). High resolution mass spectrometry enables very sensitive and selective screenings, but the identification certainty is limited.

Most of the other steroid derivatives show characteristic fragmentation patterns, allowing the application of MS/MS as a very specific and flexible analytical method. A lot of fragmentation reactions can be examined, which makes it possible to avoid potential interference's and ensures reliable confirmations of substance identity.
Experimental details (including optimisation of parameters like resolution or collision energy) were always determined for each compound individually.
The correspondence of the HPLC retention times (i.e. the occurrence in the appropriate fractions) is another useful criterion for substance confirmation.
In the most cases, substance identification - based on HPLC and GC retention times, HRMS and MS/MS data - was possible beyond reasonable doubt.
Another potential problem is carry-over in GC and HPLC. A sufficient number of blanks had to be inserted prior to suspicious samples to exclude memory effects, and sensitive parts (GC injector inserts, HPLC columns) were often replaced.

3.3. Analytical results

3.3.1. Metandienone

The presence of comparatively high amounts of metandienone (parent compound) could be demonstrated by HRMS after HPLC clean-up of the sample (fig. 1). The corresponding metabolite epi-metandienone could be detected at about 10-fold lower concentration level (fig. 2) using the same methods. Both results were confirmed by MS/MS data of a second sample aliquot without HPLC clean-up. 6β-hydroxy-metandienone was identified as a second metabolite in this sample (fig.3); its identity was confirmed by recording two fragmentation reactions.

3.3.2. Metenolone

The absence of the parent compound in hair extracts was proved by selected ion recording HR/MS data (10 000 resolution) and by tandem MS. The detection limit was about 1ng/100mg hair (fig. 4).
The substance primarily incorporated was metenolone enantate, which could be detected with tandem MS (two A/B ring fragmentation reactions recorded, fig. 5) as well as with HRMS after HPLC clean-up.

3.3.3. Nandrolone

Similarly to metenolone, the parent compound was not detectable at a reliable concentration level using HR/MS (fig. 6) but the injected precursor substance (nandrolone decanoate) was identified (fig. 7).

3.3.4. Testosterone

No attempt to identify testosterone in hair was made, as a differentiation between endogenous and exogenous seems to be impossible. The injected mixture of four testosterone esters contained propionate, isocaproate, phenylpropionate and decanoate. Each of the substances could be identified in the hair extract in concentration relations, similar to the original mixture. The confirmation of substance identity is based on HPLC and GC retention times and the molecular ion recording by HRMS (10 000 resolution) only (fig. 8).
Surprisingly, another ester (testosterone enantate), which was not mentioned in the diary, was detected and confirmed by three fragmentations in tandem MS and HRMS after HPLC clean–up (fig. 9). This is most likely originated by intake prior to the period covered by in the diary. Unfortunately, segmentation of hair was impossible due to the lack of substance.

3.3.5. Stanozolol

According to our experiences it is not possible to detect stanozolol or its metabolites in the methanol extract. A subsequent disintegration of the hair residue, left over from methanolic extraction, proved to be successful in several cases. A concentration of about 4 ng stanozolol per100mg hair sample could be identified without further cleanup steps by application of HRMS/MS technique (fig. 10).

3.4. Summary of results and conclusions for incorporation of steroids into hair

	Technique		
	High Resolution		MS/MS
	3,000	10,000	
Testosterone	n.e.	n.e.	n.e.
T-Propionate	+	10	n.e.
T-Isocaproate	+	14	+
T-Phenylpropionate	(+)	28	n.d.
T-Decanoate	+	150	n.d.
T-Enantate	+	+	680
Nandrolone	< 5	n.e.	n.d.
N-Decanoate	+	40	n.d.
Metenolone	+	n.d.	n.d.
M-Enantate	n.d.	n.d.	60
Metandienon	58	n.e.	+
Epi-Metandienon	< 5	n.e.	+
6ß-OH-Metandienon	n.e.	n.e.	< 5
Stanozolol	n.e.	+	40

n.e. = not examined **n.d. = not detected**
Concentrations in pg/mg hair

The incorporation of anabolic substances into hair seems to proceed similar to other drugs, i.e. parent compounds and primary metabolites tend to be incorporated rather than typical urinary metabolites. The ratios of quantities of epimetandienon / metandienon, 6β-hydroxymethandienone / metandienone or nandrolone / nandrolonedecanoate in hair indicate, that the administered substances are detectable at >10fold higher concentration levels than corresponding metabolites. This can be due to kinetic effects as well as to the higher lipophilicity of these substances. Slightly different incorporation rates between testosterone esters (decanoate > propionate) are hardly significant.

The comparison of reliability of methodologies shows an increase of specificity and decrease of sensitivity from HRMS (3000 resolution) to MS/MS. There was one false positive detection (metenolone) in HRMS (3000). The identification of some of the esters by MS/MS failed, because detection limits are usually higher, compared to high resolution techniques. The application of HRMS at a sufficient resolution (10 000) appears to be a useful compromise.

5. Conclusion

- The detection of 11 anabolic steroids in hair samples was possible.
- Parent compounds are incorporated into the hair matrix rather than metabolites.
- Useful information about exogenous precursor substances (esters) of endogenous steroids may be available. Therefore, reliable evidences of the intake of endogenous steroids are accessible.
- Low target concentrations and a complex matrix require sophisticated sample preparation procedures, sensitive and specific identification techniques as well as careful interpretations.

Table 1:

HPLC and GC (chromatographic parameters as described in chapter 2.3 and 2.5) retention times of relevant anabolic steroids.

	Retention time (min)	
	GC	HPLC
Testosterone	-	6.90
T-Propionate	11.33	11.21
T-Isocaproate	12.95	13.63
T-Phenylpropionate	16.08	12.91
T-Decanoate	15.73	16.37
T-Enantate	13.77	14.22
Nandrolone	9.93	6.45
N-Decanoate	15.43	16.02
Metenolone	10.40	7.37
M-Enantate	14.02	14.91
Metandienone	10.70	6.66
Epi-Metandienone	10.05	7.91
6ß-OH-Metandienone	11.38	4.71

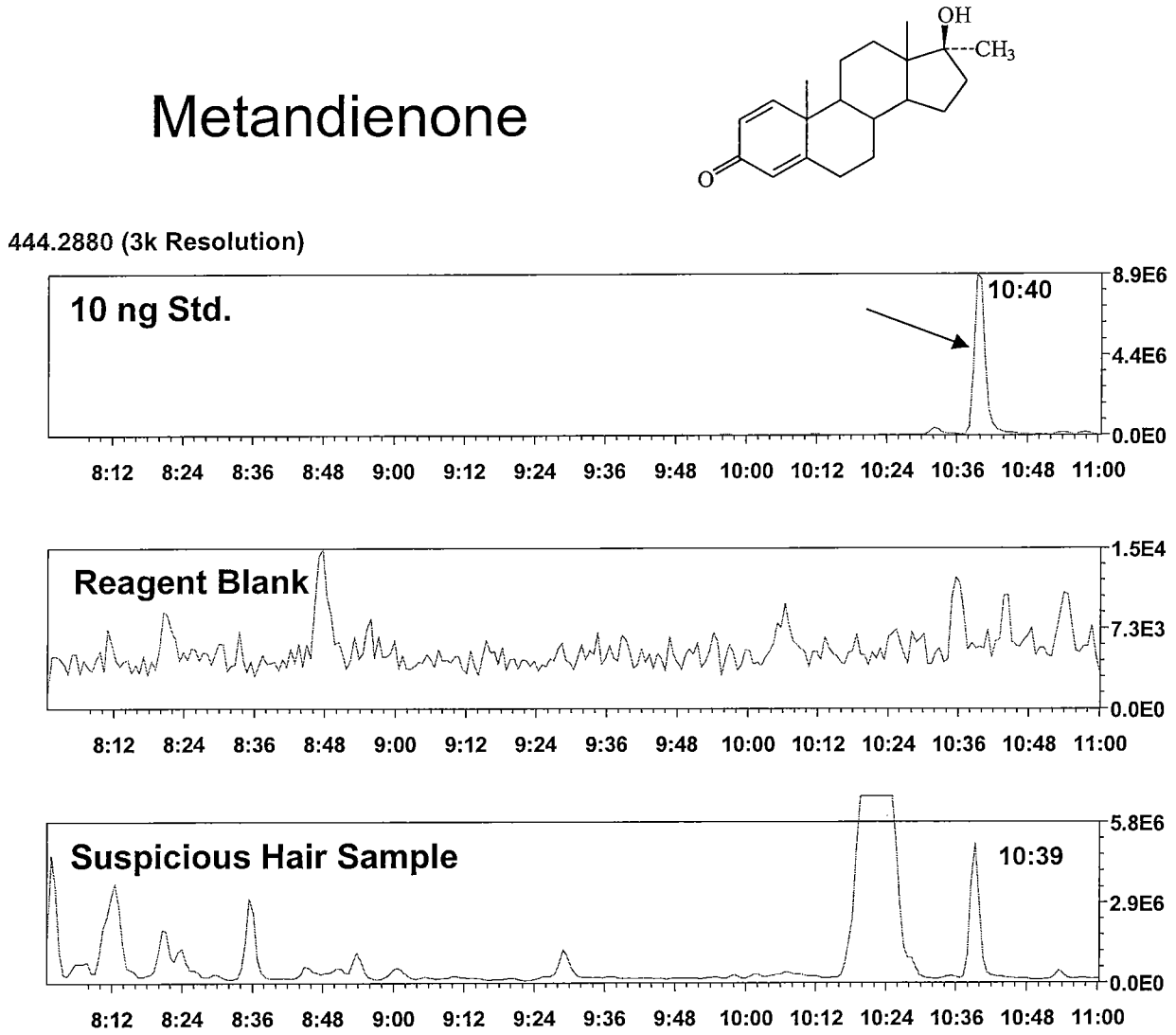


fig. 1: Traces of metandienone, detected in the suspicious hair sample by HRMS (3000 resolution) after HPLC clean-up (fraction 6-7min). The selected ion recording (SIR) of mass 444.2880 (molecular ion of the bis-TMS derivative) is compared to corresponding signals of a standard solution and a blank sample.

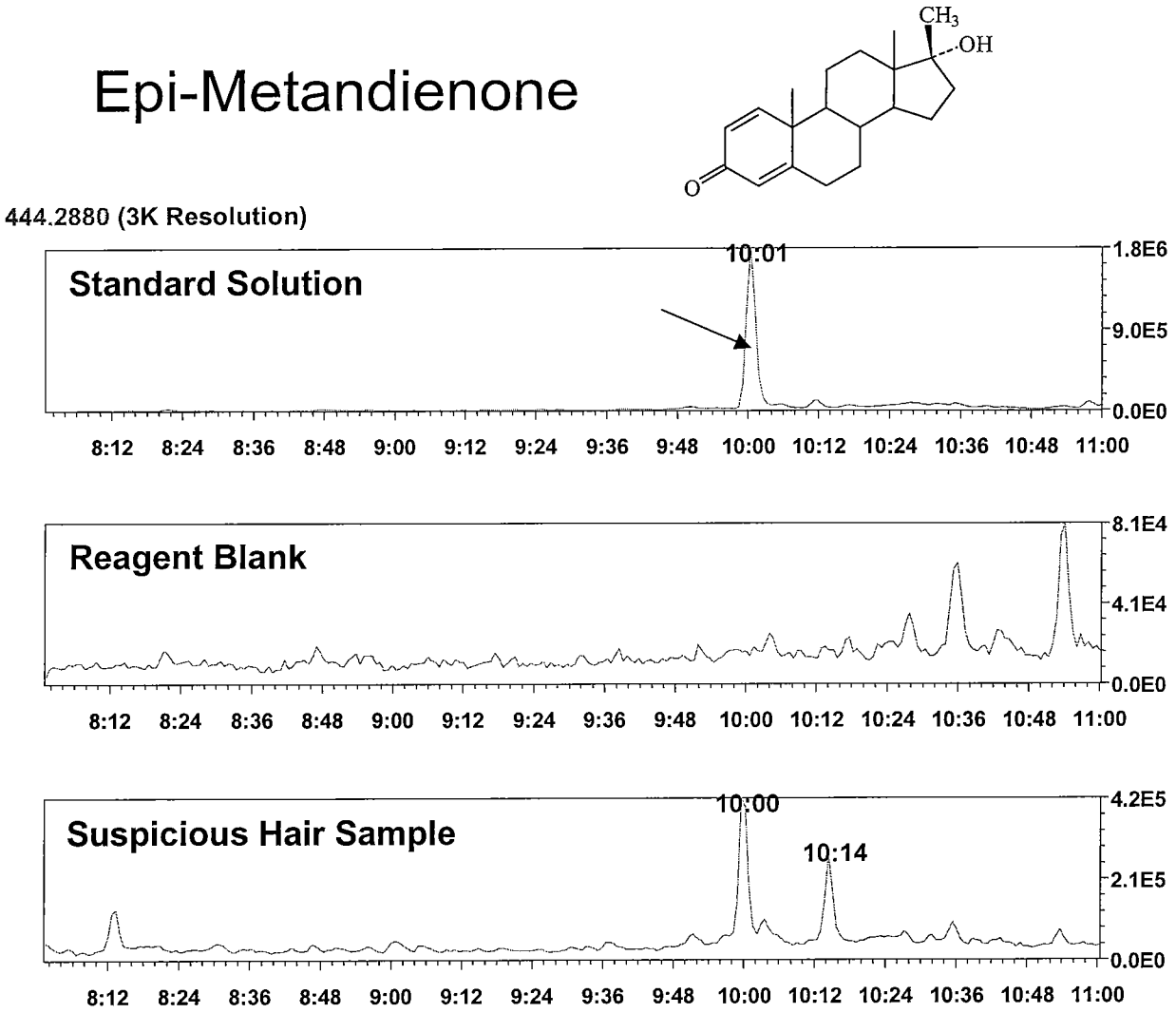


fig. 2: Traces of 17-epi-metandienone, detected in the suspicious hair sample by HRMS (3000 resolution) after HPLC clean-up (fraction 7-8min). The selected ion recording of mass 444.2880 (molecular ion of the bis-TMS derivative) is compared to corresponding signals of a standard solution and a blank sample.

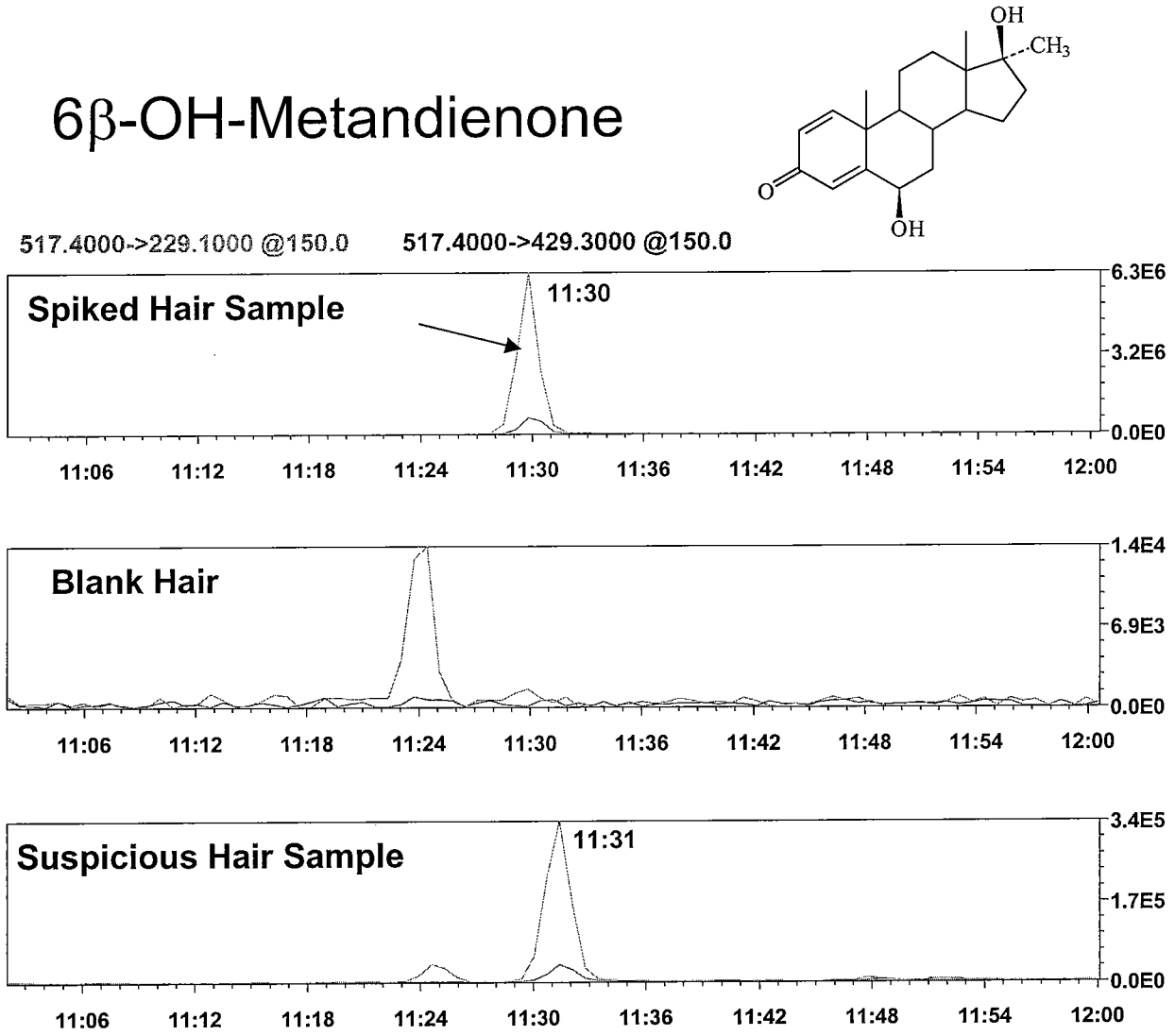


fig. 3: Traces of 6β-hydroxy-metandienone, detected in the suspicious hair sample by MS/MS without HPLC clean-up. Multiple reaction monitorings (MRM-Q) 517→229 and 517→429 are compared to corresponding signals of a spiked hair sample and a blank hair sample.

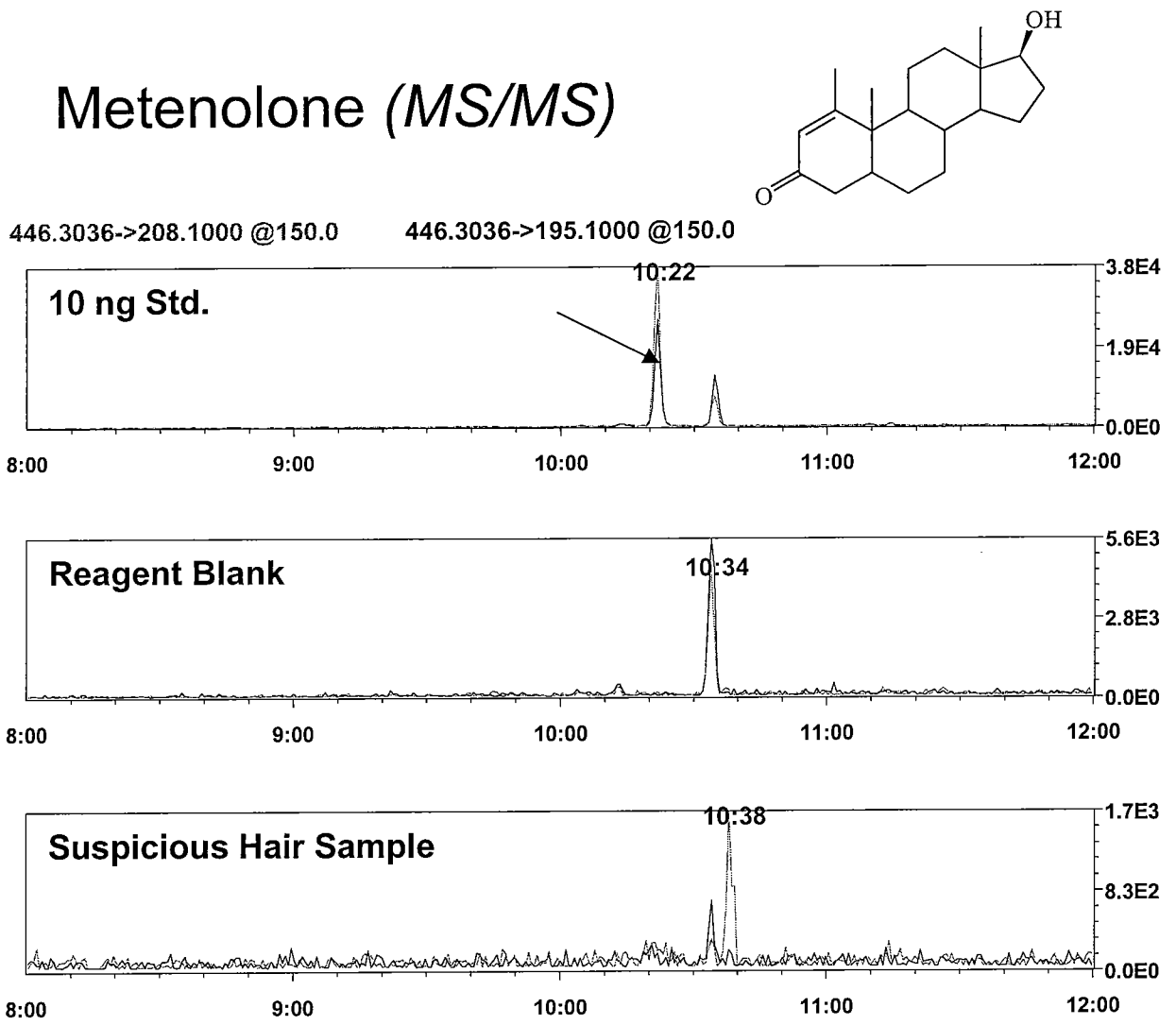


fig. 4: Negative screening for metenolone in the suspicious hair sample by HRMS (10 000 resolution) after HPLC clean-up (fraction 7-8min). The selected ion recording (SIR) of mass 446.3036 (molecular ion of the bis-TMS derivative) is compared to corresponding signals of a standard solution and a blank sample, indicating the absence of metenolone at concentrations above 1ng/100mg.

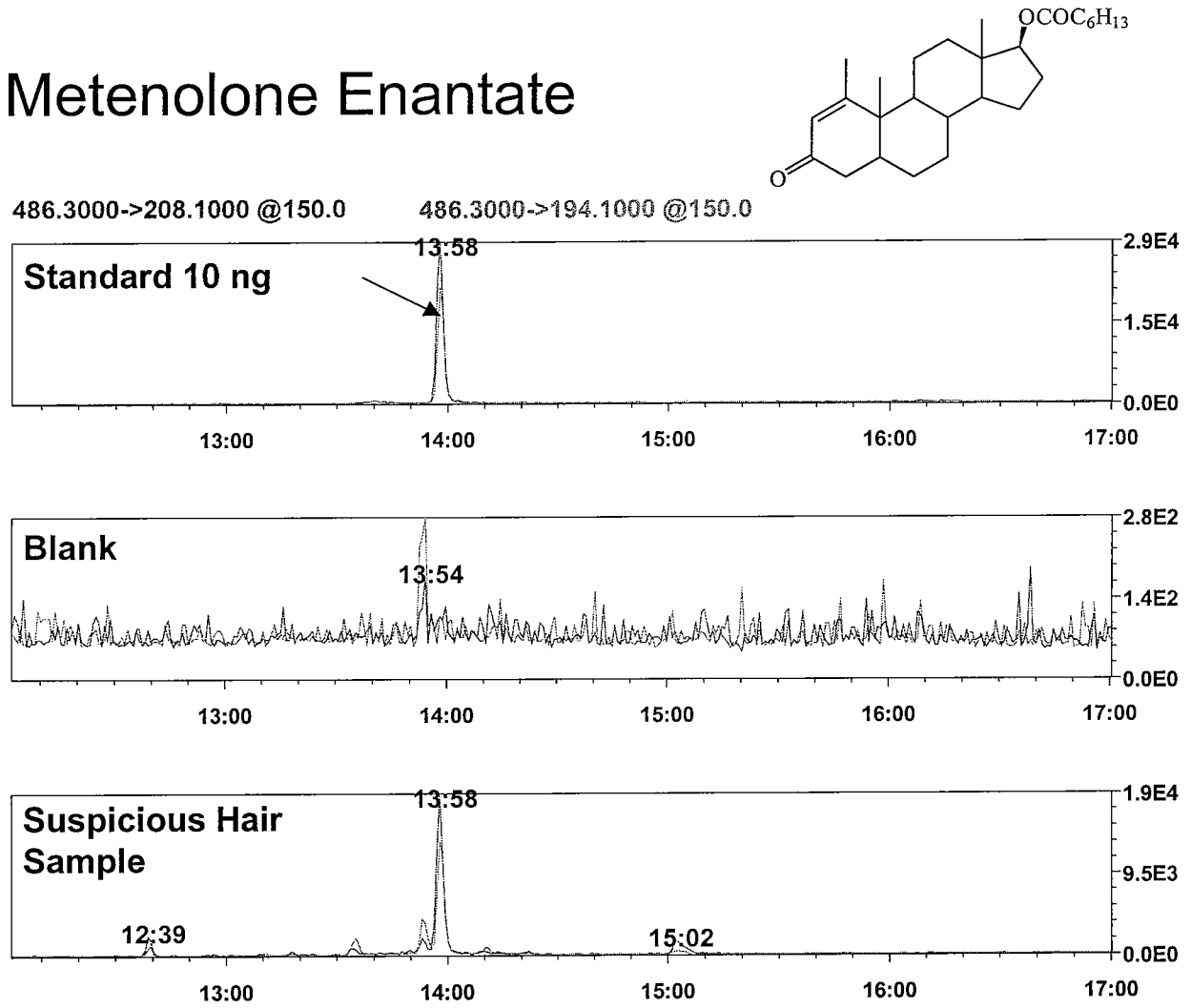


fig. 5: Detection of metenolone enantate in the suspicious hair sample by MS/MS after HPLC clean-up (fraction 14.5-15.5 min). Multiple reaction monitorings (MRM-Q) 486→208 and 486→194 are compared to corresponding signals of a standard and a reagent blank.

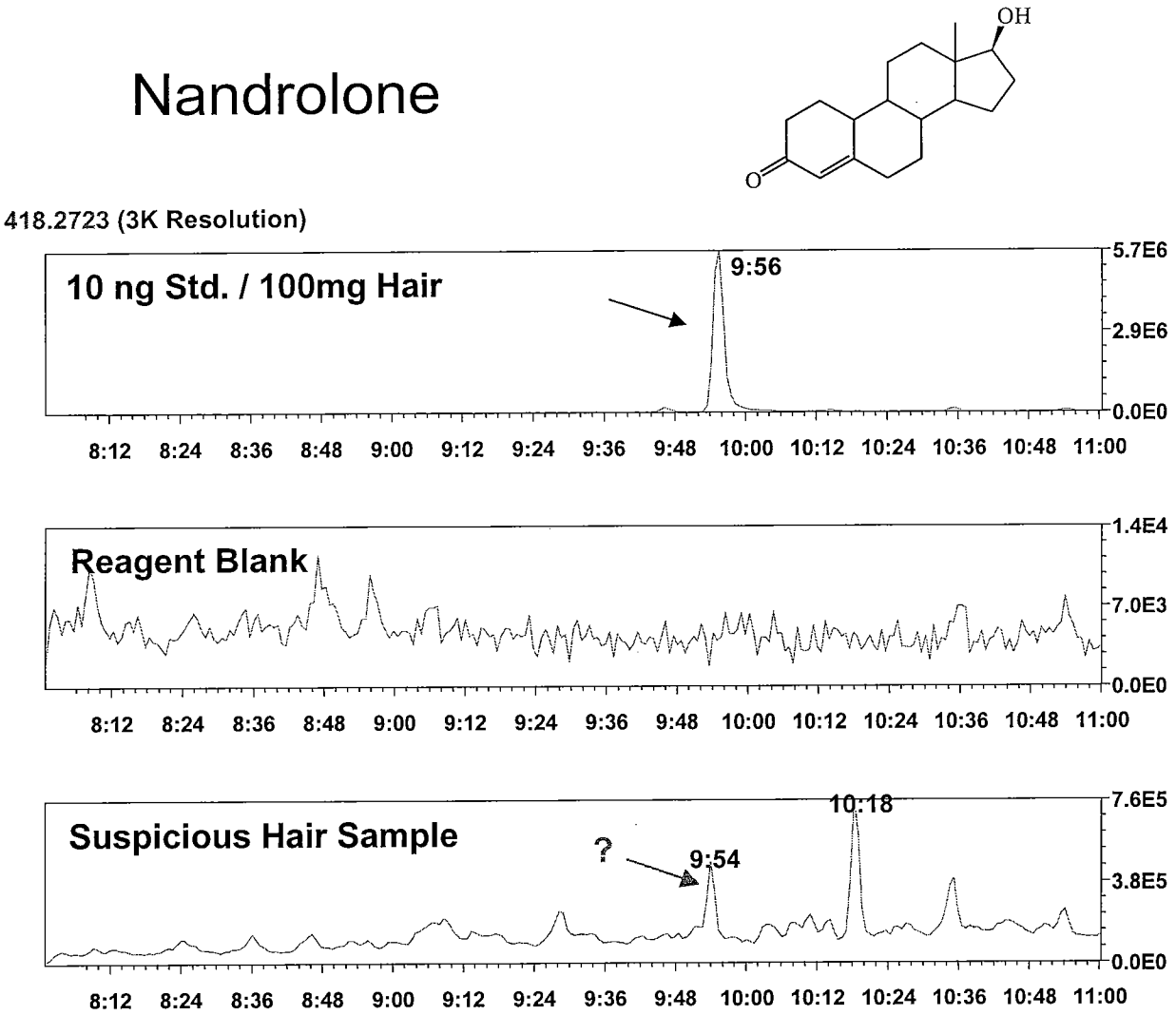


fig. 6: Negative screening for nandrolone in the suspicious hair sample by HRMS (3 000 resolution) after HPLC clean-up (fraction 6-7min). The selected ion recording (SIR) of mass 418.2723 (molecular ion of the bis-TMS derivative) is compared to corresponding signals of a spiked hair sample and a reagent blank, indicating the absence of nandrolone at concentrations above 1ng/100mg.

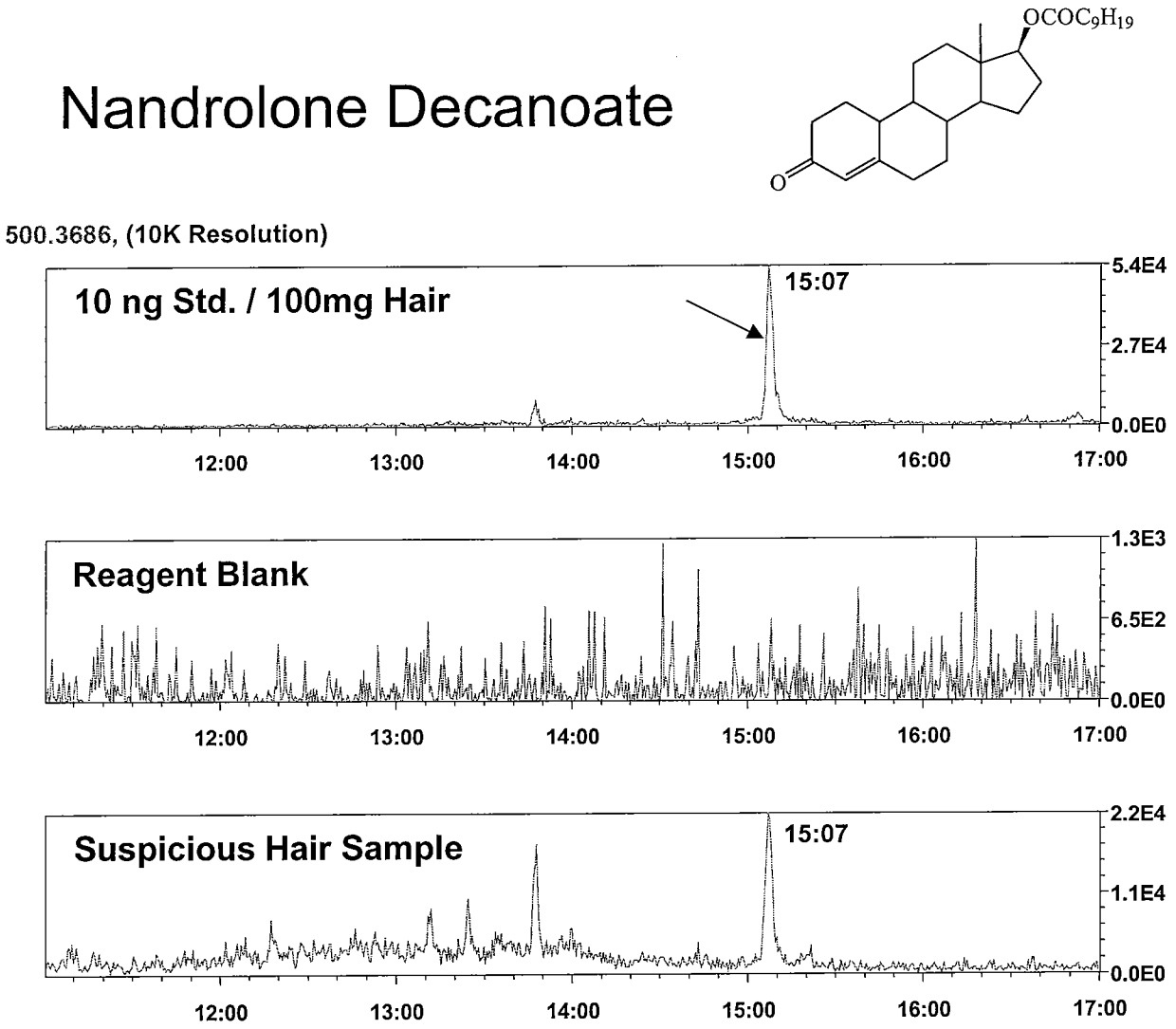


fig. 7: Detection of nandrolone decanoate in the suspicious hair sample by HRMS (10 000 resolution) after HPLC clean-up (fraction 15.5-16.5 min). The selected ion recording (SIR) of mass 500.3686 (molecular ion of the mono-TMS derivative) is compared to corresponding signals of a spiked hair sample and a reagent blank.

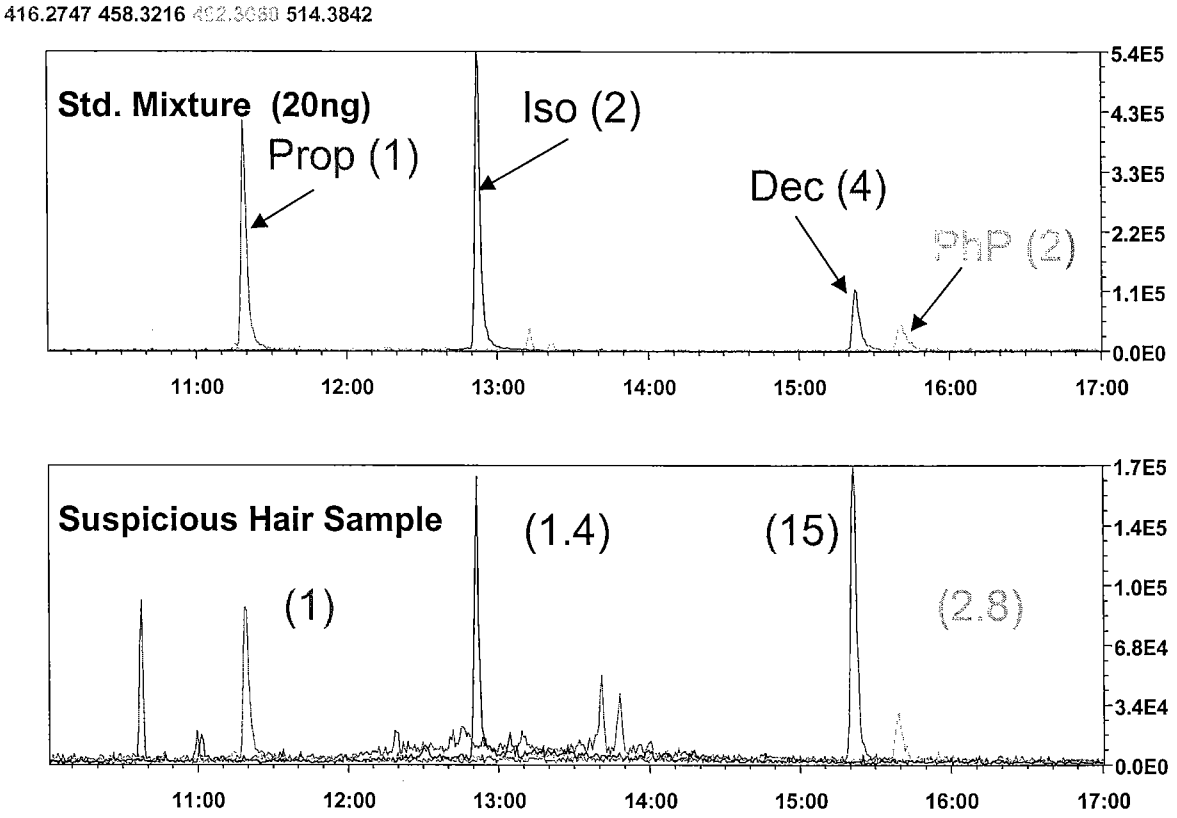


fig. 8: Identification of different testosterone esters in the suspicious hair sample by HRMS (10 000 resolution) after HPLC clean-up. The selected ion recording (SIR) of masses

416.2747 molecular ion of testosterone propionate-mono-TMS (marked as 'Prop')

458.3216 molecular ion of testosterone isocaproate-mono-TMS (marked as 'Iso')

492.3060 molecular ion of testosterone phenylpropionate-mono-TMS (marked as 'PhP')

514.3842 molecular ion of testosterone decanoate-mono-TMS (marked as 'Dec')

are compared to corresponding signals of a standard mixture.

The numbers in brackets indicate the concentration ratios in the hair compared to the quantitative composition of the Omnadren ampoules.

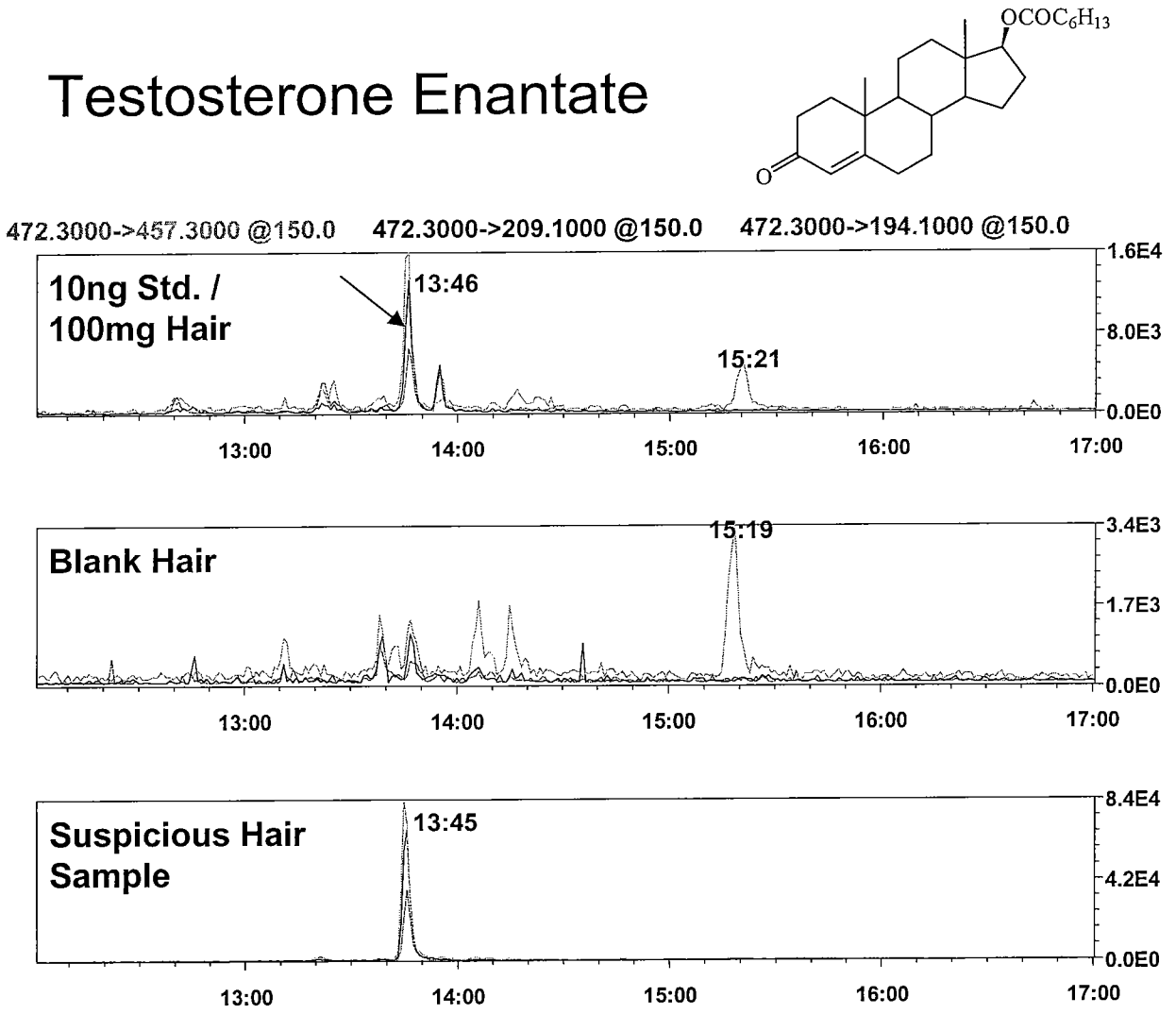


fig. 9: Detection of testosterone enantate in the suspicious hair sample by MS/MS after HPLC clean-up (fraction 13.5-14.5 min). Multiple reaction monitoring (MRM-Q) 472→457, 472→209 and 472→194 are compared to corresponding signals of a spiked hair sample and a blank hair sample, confirming the presence of high amounts of testosterone enantate.

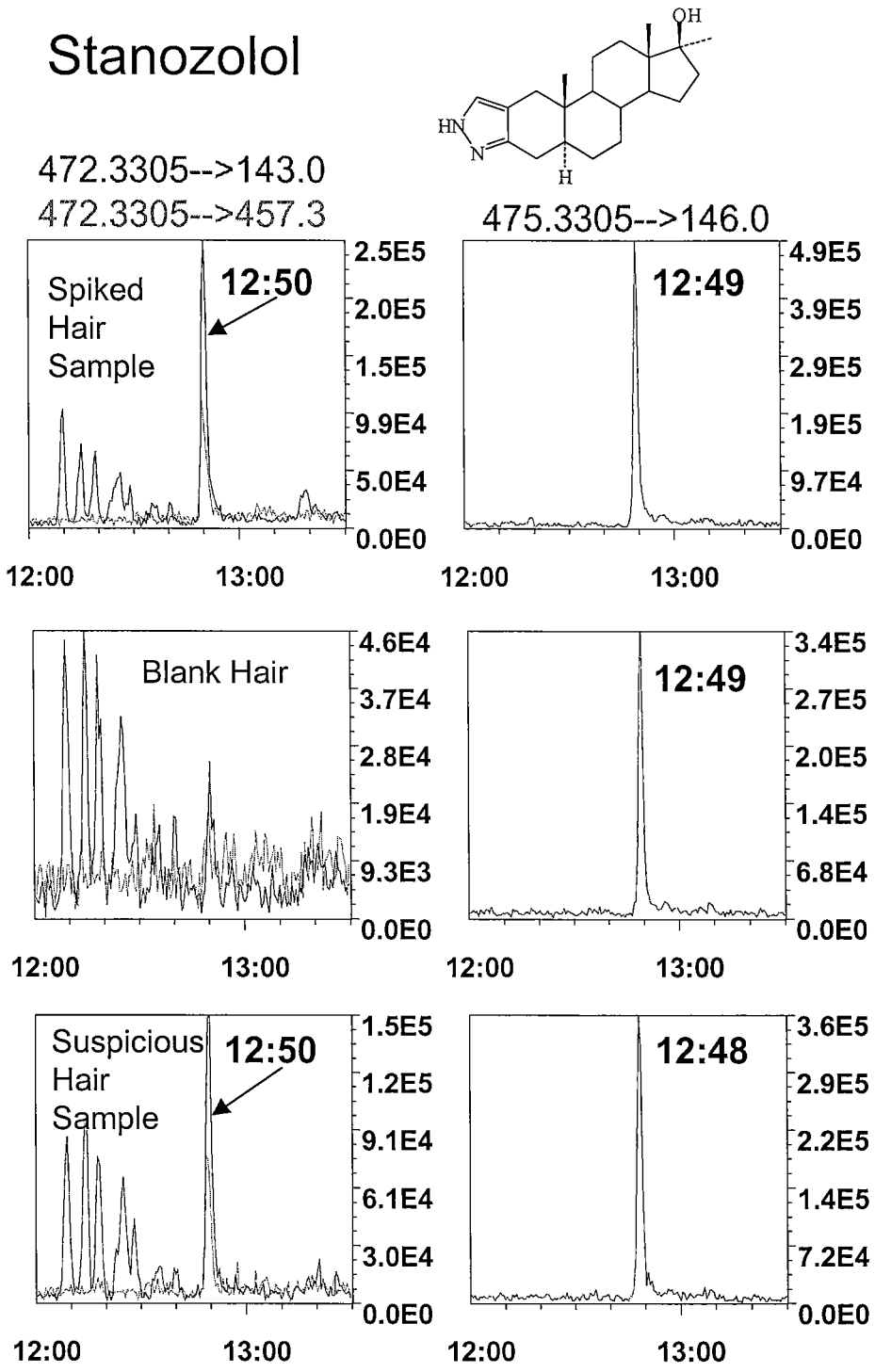


fig. 10: Detection of stanozolol in the suspicious hair sample by MS/MS at 3 000 resolution. Multiple reaction monitoring (MRM-Q) 472→143 and 472→457 (left column) are compared to corresponding signals of a hair sample, spiked with stanozolol to 5ng/100mg hair and a hair blank. Deuterated stanozolol was added as internal standard and identified as 475→146 fragmentation (right column).

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