

## Foreword

So far doping control procedures have focussed on the detection of doping substances or their signal metabolites in urine. However, since a method was developed to produce peptide hormones by genetic engineering which subsequently led to an extensive marketing of these hormones, blood tests have been increasingly discussed as a supplementary method for doping detection. However, from a legal point of view, blood tests must be judged differently from urine tests.

Apart from urine and blood, specialists are discussing other endogenous excretions or parts (e.g. saliva, sweat, hair, nails) as being suitable for the detection of doping substances.

Methods of analysing the contents of hair have been successfully used in forensic medicine for years. It is for example possible to verify the misuse of drugs in this matrix.

For this reason, the Federal Institute of Sport Science has supported research on detecting doping substances in human hair. First positive results were achieved by Sauerwein and Meyer who developed a method for the detection of clenbuterol in human scalp hair. Although clenbuterol is used for therapeutic purposes in medicine, it is a banned substance according to the list of the IOC. Currently, additional methods are being developed in cooperation with the Forensic Institute of the University of Munich and the Institute for Doping Analytics and Sports Biochemistry (IDAS) in Kreischa. The connection between scientific know-how and instrumental analytics on the one hand and the chemistry of hair and its contents on the other hand can give new impetus to doping analytics.

I am glad that the workshop in Kreischa has also led to a cooperation between the Federal Institute of Sports Science and the International Society for Hair Analytics. It was only last year that this society at its congress in Martigny stated clearly in a resolution that it considered hair analytics as an important additional contribution to doping analytics.

I would like to thank the organizers of this workshop for paying attention to hair analytics and hope that there will be fruitful discussions among the participants.

Dr. Martin-Peter Büch

Head of the Federal Institute of Sports Science



<b>Contents</b>	<b>Page</b>
Foreword..... <i>Martin-Peter Büch</i>	1
Preface..... <i>Hans Sachs</i>	5
Introduction to Hair Analysis..... <i>Hans Sachs</i>	7
Hair Sample Collecting Kit for the Police Forces..... <i>Werner Bernhard, Beat Aebi, Christian Staub, Hans Sachs</i>	11
 <b>Workshop Stations</b>	
A General Solid-Phase Extraction Procedure for Drug Testing in Hair..... <i>Christian Staub, Christèle Girod</i>	15
Determination of Benzodiazepines in Hair by GC/MS-NCI..... <i>Susanna Fehn, Hans Sachs</i>	23
Specificity and Sensitivity Requirements in Hair Analysis..... <i>Detlef Thieme</i>	35
Identification of Buprenorphine and Methadone in Hair by HPLC/MS..... <i>Vincent Cirimele, Pascal Kintz, Bertrand Ludes</i>	49
Interpretation of Quantitative Findings and Data Evaluation in Hair Analysis..... <i>Michael Uhl</i>	61
Criteria that Can Affect the Detection of Doping Agents in Hair..... <i>Pascal Kintz</i>	79
 <b>Reviews on Detection of Anabolic Agents in Hair</b>	
Analysis of Hair Samples for $\beta_2$ -Agonists..... <i>Kurt Einhellig, Michael Uhl</i>	91
Testing Endogenous Steroids in Hair..... <i>Hans Sachs</i>	97
Detection of Exogenous Steroids in Hair..... <i>Detlef Thieme, Joachim Grosse and R. Klaus Mueller</i>	103
Testing for 19-Norsteroids in Hair..... <i>Pascal Kintz, Vincent Cirimele, Bertrand Ludes</i>	111



## Preface

The *Society of Hair Testing* feels delighted being able to continue the tradition of conferences and workshops concerning analytic methods for drugs in human hair.

There are two special reasons for holding the workshop 2000 at the *Institute of Doping Analysis and Sports Biochemistry*: The conference in Martigny in 1999 has already shown that hair analysis is expanding to the field of doping analysis. Secondly, there is an excellent equipment for trace analysis in Kreischa, and the scientists of the institute, especially Detlef Thieme and Joachim Grosse, supported by the director of the institute, Klaus Müller, have already published well recognized contributions concerning the analysis of doping agents in hair. The preparation of the workshop is showing that the whole institute staff is supporting this workshop, and obviously enjoys hosting the participants from all over the world.

This workshop will not only introduce to fields which are new to some of the hair specialists but also give a survey on routine methods and the evaluation of their results as well as on the acceptance of hair analysis by the courts, traffic authorities and medical doctors, especially psychiatrists.

This booklet does not only intend guiding the participants through the different stations of the workshop, but also giving a survey on the modern field of hair analysis, including just recently developed methods, to the members of the Society and to other interested scientists. As a major part of the book is dealing with doping agents, it might also be interesting to authorities and sports associations responsible for doping control.

The workshop would not have been possible without the support of the director of the *Bundesinstitut für Sportwissenschaft* (Federal Institute of Sports Science) Dr. Peter Büch and the special promotion of Dr. Carl Müller-Platz. Their interest in new fields of modern toxicological and biochemical analysis have encouraged us to provide the workshop in Kreischa.

Dr. Hans Sachs

President of the Society of Hair Testing



# Introduction to Hair Analysis

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Regular control of drug consumers by examining their hair starts with the publication of Baumgartner 1979. He extracted opiates with methanol by heating for 2 hrs, then evaporating and reconstituting in buffer and examining with Abuscreen RIA. In Germany this method was introduced by Arnold in 1980. With the introduction of the new desktop GC/MS, the mass selective detector, which lead the capillary directly to the ion source of the MSD, a new era began because of the improved sensitivity. After morphine and codeine could be determined separately in hair of a heroin abuser, the other drugs were also soon detected and a regular drug consumption could be proved. Although possible contamination, cosmetic hair treatment, and the irregular growth speed of the hair restrict the usefulness of hair examination, hair analysis is used today for workplace testing in the United States in thousands of cases per day. In Europe, where workplace tests on drug consumption are not performed consequently, hair analysis is used for forensic and clinical applications.

The general fields of hair analyses are:

- Criminal court proceedings
  - Reliability of the defendant
  - Question of chronic drug abuse by the victim (including hair of exhumed bodies)
- Clinical purposes
  - Detection of psychiatric patients with chronic drug abuse
  - Explanation of withdrawal symptoms of neonates.
- Driving ability control
- Doping control?

Prior to the evaluation of the results of hair analyses it has to be considered that hair is not a homogenous matrix like blood or urine, and it can only be homogenised to a certain extent before examination.

Hair has a cylindrical fibre structure consisting of mainly three chemically different layers:

- *Cuticle*  
Resistant shield of keratinized cells of fish scale-like appearance with 4  $\mu\text{m}$  of thickness.
- *Cortex*  
The main layer of the hair fibre containing melanin granules which are responsible for the colour of the hair. Strong racial influence leads to differences in hair thickness of Caucasians (40 – 75  $\mu\text{m}$ ), Afro-Americans (55 – 75  $\mu\text{m}$ ), Spanish (65 – 80  $\mu\text{m}$ ), and Asians (65 – 95  $\mu\text{m}$ ).
- *Medulla*  
If present, in the centre of the hair, rich in protein and lipids, with insoluble fractions.

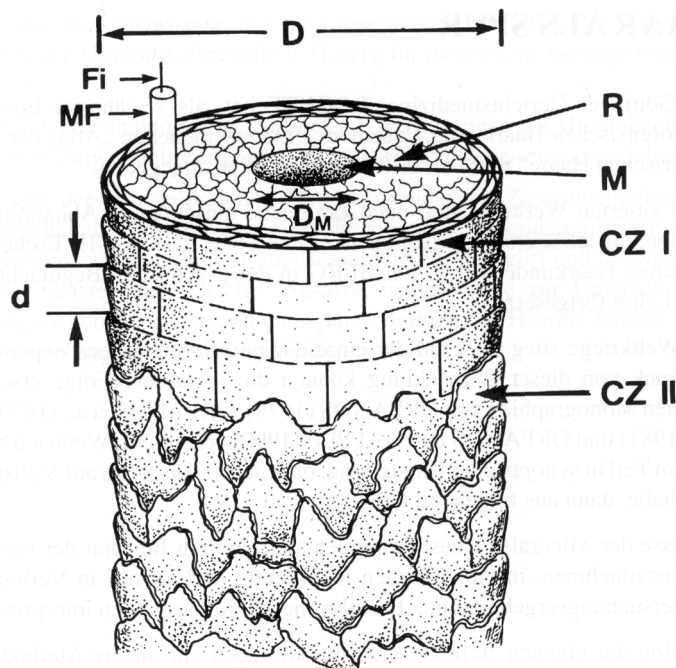


Fig.1: CZ= cuticle cells; R = Cortex; M= medulla

The possible differences in races lead to juridical problems in multi-ethnic societies. Hair of Afro-Americans could contain higher drug concentrations than hair of blond Caucasians after the same drug consumption.

Today, drugs in hair are analyzed in forensic cases by GC/MS. Immunochemical methods can be taken as a pre-test. In special cases when higher sensitivity and specificity are demanded, high resolution MS or LC/MS are used.

Table 1  
Screening Procedures for the Detection of Illegal Drugs in Hair

Reference s. chapter: Legal aspects and data evaluation.....	Kauert and Röhrich	Moeller et al.	Kintz and Mangin
Analytes	heroin, 6-MAM, dihydrocodeine, codeine, methadone, THC, cocaine, amphetamine, MDMA, MDEA, MDA	heroin, 6-MAM, dihydrocodeine, codeine, methadone, THC, cocaine, amphetamine, MDMA, MDEA, MDA	heroin, 6-MAM, dihydrocodeine, codeine, methadone, THC, cocaine, amphetamine, MDMA, MDEA, MDA
Decontamination step	Ultrasonic bath 5 min each 5 ml H <sub>2</sub> O 5 ml acetone 5 ml petrolether	20 ml H <sub>2</sub> O (2x) 20 ml acetone	5 ml Cl <sub>2</sub> CH <sub>2</sub> (2 x 5 min)
Homogenization	100 mg hair cut into small sections in a 30 ml vial	ball mill	ball mill
Extraction	4 ml methanol ultrasonic bath 5 h, 50°C	20-30 mg powdered hair, 2 ml acetate buffer + β- glucuronidase/aryl-sulfatase, 90 min/ 40°C	50 mg powdered hair, 1 ml 0,1 M HCl, 16 h / 56 °C
Clean-up	none	NaHCO <sub>3</sub> ; SPE (C18) , elution with 2 ml acetone/CH <sub>2</sub> Cl <sub>2</sub> (3:1)	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> ; extraction 10 ml CHCl <sub>3</sub> /2-propanol/n-heptane (50:17:33); organic phase purified with 0.2 M HCl; HCl phase to pH 8.4; re-extraction with CHCl <sub>3</sub>
Derivatization	propionic acid anhydride	1000 µL PFPA/75 µL PF-n- propanol; 30 min/60°C; N <sub>2</sub> /60°C; 50 µL ethyl acetate	40 µL BSTFA/1% TMS; 20 min/70°C
GC conditions	Column: 20m x 0.25 mm x 0.25 µm methyl silicone; inj. temp.: 280 °C; temp. prog. 140 °C, 20 °C/min to 300 °C, 8 min	Column: 12m x 0.2 mm x 0.33 µm phenyl methyl silicone; inj. temp.: 260 °C; temp. prog. 70 °C, 30 °C/min to 155 °C, 10 °C/min to 240, 1 min	
MS conditions	EI 70 eV; SIM at m/z 297, 313, 370 (THC) 82,182, 303 (cocaine) 268, 310, 327, 341, 369, 383, 397 (opiates) 235, 250 (methaqualone)	EI 70 eV; SIM at m/z 118, 190 (amphetamines) 421, 300, 182, 303 (BZE & cocaine) 282, 284, 390, 414, 444, 447, 473, 577 (opiates)	

The development of the methods used for opiates in the past 5 years is combined with the development of screening methods for opiates, cocaine, cannabinoids, and amphetamine including its derivatives. Three methods are dominating in the literature, as it is briefly described in Tab. 1. The liquid-liquid extraction after HCl-hydrolysis introduced by Kintz et al. and the solid-phase extraction after enzymatic hydrolysis with β-glucuronidase/sulfatase lead to similar results, both with the disadvantage, that heroin and 6-O-acetylmorphine (MAM) might be hydrolyzed to morphine. The methanol extraction and direct detection with GC/MS is known

since the early 90s, but published in detail by Kauert and Röhrich not before 1996. It is undoubtedly the simplest method with high sensitivity for heroin, cocaine, and THC, but a poor sensitivity for their metabolites morphine, benzoylecgonine, and THC-COOH.

## **Hair Sample Collecting Kit for the Police Forces**

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In 1997 the Society of Hair Testing published a general statement concerning the examination of drugs in human hair. This statement made the following demands on the collection of hair samples (1):

*Sample collection should be performed by a responsible authority respecting the legal, ethical and human rights of the person to be tested for drugs of abuse. Hair samples should be obtained in a non drug contaminated environment by an appropriately trained individual, not necessarily a physician. A sufficient amount of sample should be collected so that a repeat analysis or a confirmation analysis by another laboratory can be performed should it be needed.*

To fulfill these demands, hair collection is performed obeying the following rules (2,3):

1. Hair sampling must not be performed in the neighbourhood of street drugs and not by persons who were in contact to those drugs.
2. To obtain comparable results, hair should be collected from the posterior vertex region of the scalp. If this is not possible, the place from which the sample was taken, has to be documented.
3. In order to maintain a statistical significance of the sample and/or to perform a screening test as well as a confirmation test, the weight of the specimen should aim to be approximately 200 mg (a suitable amount of sample could be estimated by comparison with the diameter of a pencil).
4. The hair strand has to be cut close to the skin. The length of remaining hair has to be documented.
5. For shipment and storage, the hair sample should be wrapped in aluminum foil to maintain integrity and to avoid contamination. (Long hair strands can be tied together with a piece of string or of dental floss before being cut.)

6. Hair tip and the cut end of the samples have to be labelled on the aluminum foil.
7. Specimens can be stored under dry conditions at room temperature

In 1997, a hair sample collecting kit was introduced and training in hair sample taking was given to police officers in Switzerland. The training course for the police forces was effective. The content of the kit and the chain of custody form permitted a correct sampling avoiding contamination and ensuring the identity of the specimens. The kit provided to the officers is shown on the photograph on the next page.

References:

1. Statement of the Society of Hair Testing concerning the examination of drugs in human hair, *Forensic Sci. Int.* 84 (1997) 3-6
2. R. Denk, I. Raff, H. Sachs, *Haaranalysen bei Betäubungsmittelkonsum*, *Kriminalistik* 4 (1992) 253-255
3. United Nations, *Guidelines for Testing Drugs under International Control in Hair, Sweat, and Saliva*, United Nations Publication, 1999, Sales No. E.99.XI.14





# A General Solid-Phase Extraction Procedure for Drug Testing in Hair

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## 1. Introduction

Different methodological approaches have been proposed for drug testing in hair. These include pretreatment, ranging from simple elution with solvents to complete dissolution of the protein structure by deep alkaline hydrolysis. After this pretreatments, different extraction techniques are described ranging from liquid-liquid extraction to the so called solid-phase extraction.

The analytical procedures generally involve the following steps:

1. Decontamination of the specimen
2. Preparation (such pulverization or segmentation in 2-3 mm)
3. Hydrolysis (acid, alkaline or enzymatic...)
4. Purification (extraction, concentration and derivatization)
5. Analysis by chromatography

In our laboratory in Geneva (Switzerland), analysis of human hair for the detection of drugs of abuse was first performed in 1995. Initially, request for hair analysis were few, and it is only since 1997 that these analyses have become routine. As demand grew, we developed a solid-phase extraction (SPE) method for the following drugs:

- codeine
- 6-monoacetylmorphine (6-MAM)
- morphine
- cocaine
- methadone
- ecstasy (MDMA)
- eve (MDEA)

Subsequently, the use of a robot ASPEC (Gilson Medical Electronics, Villiers-le-Bel, France) allows to drop certain fastidious manipulations, and to treat a large number of samples at the same time.

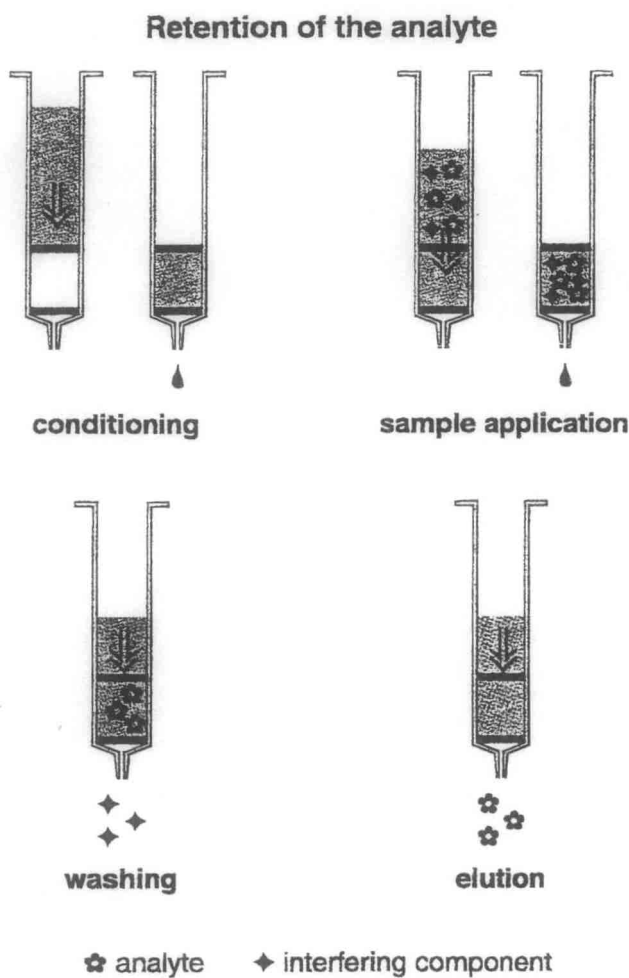
## 2. Basic principles of solid-phase extraction (SPE).

In general SPE can be used for three important purposes in up-to-date analyses:

- concentration of the analyte
- removal of interfering substances
- changing the matrix of the analyte as needed for subsequent analyses

In most cases these three effects occur together. Since analytes can be either adsorbed on the SPE packing material or directly flow through while the interfering substances are retained.

The first case is shown in the figure below :



The sample is drawn through the solid phase, and the analyte molecules are enriched on the adsorbent. Some interfering components and solvent molecules are not retained. Then remaining interfering components are washed from the adsorbent with a suitable washing solution. Finally, the analyte is removed from the adsorbent by elution with a suitable solvent.

The consideration made above indicate that an optimum SPE presents a poor column-chromatographic separation. However, this method can be utilised efficiently for a pre-separation of groups of compounds or a single analyte from the matrix. It is extensively used for clean-up by solid phase extraction.

### **3. Analytical Procedure**

#### **3.1. Decontamination and pulverization**

At the beginning, the hair is washed successively by percolation with 5 ml of methylene chloride, 5 ml of water and finally 5 ml of methanol. This step is very important to eliminate possible external contamination. Then the hair is dried at 60 °C for 30 min and pulverised for 10 min at 70 s<sup>-1</sup>.

#### **3.2. Hydrolysis**

Drugs are fixed inside the hair matrix, therefore a digestion procedure is necessary before the extraction. About 50 mg of powdered hair are placed in a tube and 1 ml of hydrochloric acid 0.01 M is added. After incubation at 60 °C for 12 hours, the solution is then neutralised with 1 ml of NaOH 0.01 M and buffered with 1 ml of phosphate buffer pH 7.0. After centrifugation at 4000 r.p.m. for 5 min, the supernatant was removed into a special tube for the extraction.

#### **3.3. Extraction**

Isolute HCX cartridges, provided by IST (Hengoed, UK), or Bond Elut Certify cartridges, provided by Varian (CA, USA), could be used for the extractions.

The samples are extracted in the following ten steps:

1. Column conditioning with methanol (2 ml)
2. Column conditioning with water (2 ml)
3. Dispensing sample on the column
4. Rinsing with water (2 ml)

5. Rinsing column with acetate buffer pH 4 (1 ml)
6. Rinsing column with methanol (2 ml)
7. Drying column at 10 in. Hg for 5 min
8. Elution with methylene chloride/isopropanol (80/20) + 2% ammonia hydroxide (2 ml)
9. Addition of the standard nalorphine
10. The eluent is dried under nitrogen

This extract could be analysed by GC/MS or other chromatographic methods.

### 3.4. Derivatization

GC/MS analysis requires a procedure of derivatization, for example by propionylation. 100  $\mu$ l of pyridine and 100  $\mu$ l of propionic anhydride (PA) is added to the extract and incubated for 30 min at 60 °C. After evaporation under a stream of nitrogen, the extract is reconstituted in 50  $\mu$ l of ethyl acetate.

### 3.5. Gas chromatography - mass spectrometry method

Helium is used as carrier gas with the following capillary column (DB-5MS, 15 m x 0.25 mm x 0.25  $\mu$ m, J&W Scientifics (Folsom, USA).

Temperatures: 85°C maintained for 1 min to 190°C at 15°C / min and maintained for 0.5 min, to 210°C at 2 °C/min maintained for 1 min, to 270°C at 20°C/min for the final 8 min. Injector temperature is 270°C and injection is made in splitless mode.

Three  $\mu$ l of the sample are injected into the GC/MS system operating in selected ion monitoring mode (SIM). The source and interface temperatures are 200 and 280°C respectively.

The detection is performed with the following ions: codeine m/z = 355 and 282, 6-MAM m/z = 383 and 327, morphine m/z = 397 and 341, cocaine m/z = 303 and 182, methadone m/z = 294 and 72, MDMA m/z = 114 and 162, MDE m/z = 162 and 72, nalorphine (chromatographic standard) m/z = 423 and 367.

## 4. Validation data

Soaked standard hair is used for the validation process. This hair is prepared by adding an aqueous standard opiate solution to the pulverized hair. The mixture is submitted to magnetic stirring for at least five hours, then filtered and the hair is finally washed (see procedure described in section 3.1) in order to retain only the most strongly bound drug fraction.

In these conditions following validation data could be obtained.

### 4.1. Linearity

The following calibration curves and correlation coefficients are obtained for a range of 1 to 20 ng/mg.

Compound	Target ion	Equation	R
codeine	355	$y = 0.00286x - 0.03355$	0.9987
6-MAM	383	$y = 0.00146x - 0.02924$	0.9979
morphine	397	$y = 0.00127x - 0.00468$	0.9999
cocaine	303	$y = 0.00055x - 0.00071$	0.9984
methadone	294	$y = 0.00067x - 0.00487$	0.9963
MDMA	114	$y = 0.00510x - 0.16091$	0.9969
MDEA	162	$y = 0.03426x + 1.52329$	0.9958

### 4.2. Extraction recovery and limits of detection

Extraction recovery is determined by comparing the peak areas of an extracted hydrochloric acid solution with the peak areas of methanolic solutions both at the same concentration. A post-hydrolysis recovery is measured in these conditions.

The limit of detection (LOD) is evaluated as the lowest concentration giving a chromatographic peak with the signal to noise ratio  $S/N = 3$ .

Compound (n = 6)	Recovery (%)	LOD (ng/mg)
codeine	95	0.05
6-MAM	80	0.05
morphine	103	0.05
cocaine	79	0.15
methadone	94	0.10
MDMA	81	0.10
MDE	71	0.05

The recovery of 103% obtained for morphine is probably due to a slight hydrolysis of 6-MAM.

#### 4.3. Repeatability

Six replicates of soaked hair of low concentration and high concentration are analysed through the complete procedure. The relative standard deviation (RSD) obtained is generally inferior to 10% for the two concentrations studied.

Compound (n = 6)	High concentration		Low concentration	
	(ng/mg)	Cv(%)	(ng/mg)	Cv(%)
codeine	31.9	7.0	4.2	7.5
6-MAM	26.6	2.4	3.5	7.9
morphine	32.9	3.8	3.5	6.4
cocaine	41.1	6.2	5.5	11.6
methadone	31.9	6.8	3.5	9.7
MDMA	27.7	5.0	7.9	6.1
MDEA	37.1	5.2	10.9	8.0

## 5. Concentrations of drugs in hair.

This analytical technique was used in routine in our laboratory for two years.

The table below summarises the various concentrations of drugs in hair.

Compound	n	mean (ng/mg)	median(ng/mg)	range(ng/mg)
codeine	32	0.9	0.5	0.1 - 5.6
6-MAM	39	4.0	1.7	0.1 - 45
morphine	36	1.1	0.7	0.1 - 6.0
cocaine	101	35.5	6.3	0.2 - 370
methadone	34	4.4	3.0	0.1 - 15
MDMA	9	3.8	1.1	0.6 - 14

## 6. References

- (1) P. Zimmerli, Ch. Staub, Analyse de drogues dans les cheveux par extraction automatisée en phase solide, *Rev. Fr. Labor.* 282 (1996) 51-55.
- (2) Ch. Girod, F. De Dominicis, R. Giovannini, Ch. Staub, Analyse de drogues dans les cheveux par extraction automatisée en phase solide: validation et utilisation dans la routine, *Toxicorama* 1 (1999) 46-50.
- (3) Ch. Girod, Ch. Staub, Analysis of drugs of abuse in hair by automated solid-phase extraction, *GC/EI/MS and GC/ion trap/CI/MS*, *Forensic Sci. Int.* 107 (2000) 261-271.



# Determination of Benzodiazepines in Hair by GC/MS-NCI

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## 1. Target

The described method enables the qualitative detection and quantitative determination of Diazepam, Nordazepam, Oxazepam, Flunitrazepam, Lorazepam, Lormetazepam, Prazepam, Flurazepam, Midazolam and Triazolam in hair.

## 2. Working Principle

After addition of the deuterated standard flunitrazepam-d<sub>7</sub> to the hair which is cut into small pieces the sample are extracted with Sørensen buffer followed by liquid-liquid extraction with 1-chlorobutane. The extract is examined by GC/MS in SIM mode and the evaluation is made by HP software and EXCEL data sheets.

## 3. Field of Application

This method is created for the determination and quantification of

Diazepam

Nordazepam

Oxazepam

Flunitrazepam

Lorazepam

Lormetazepam

Prazepam

Flurazepam

Midazolam

Triazolam

in hair. About 50-150 mg of hair, cut into small pieces, are examined.

## Precaution and Safety Regulations

- The valid safety handling instructions informs about outgoing dangers.
- Danger of infection: human material has to be regarded as potentially infectious.

## 4. Reagents and Supporting Material

For best results, the below mentioned chemicals should be used. These chemicals seem to be optimal and cause no interference with extraction, chromatography and detection. Other reagents and supporting material can be used after testing.

### 4.1. Chemicals and Reagents

- 4.1.1. Water (Braun Melsungen, Aqua ad injectabilia)
- 4.1.2. Acetone (Merck p.a. 14)
- 4.1.3. Petroleum ether (Merck 1.00915)
- 4.1.4. Disodium hydrogen phosphate (Merck p.a. 4873)
- 4.1.5. Potassium dihydrogen phosphate (Merck p.a. 6580)
- 4.1.6. Chlorobutane (Fisher Scientific HPLC C/4765/17)
- 4.1.7. Ethyl acetate (Merck Licrosolv 1.00868)

### 4.2. Solutions

- 4.2.1. Solution A:  $\text{KH}_2\text{PO}_4$  9,07g/L pH 4,5
- 4.2.2. Solution B:  $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$  11,83g/L pH 9,3
- 4.2.3. Söerensen buffer: 19,6 ml Solution A + 80,4 ml Solution B (pH 7,4)

#### 4.2.1 Stock solution 1g/l [see 4.3.1]

10mg of each substance are dissolved in a graduated flask in 10 ml of methanol [4.1.5.] and an stored in a glas tube with a teflon screw cap.

storage: refridgerator

- 4.2.2 purchasable ampoule:  
flunitrazepam-d<sub>7</sub> (100 mg/l)

### 4.3 Stock Solutions and Calibrators

#### 4.3.1 Stock solutions

- concentration 1g/L of each substance :

Diazepam

Nordazepam

Oxazepam

Flunitrazepam

Lorazepam

Lormetazepam

Prazepam

Flurazepam

Midazolam

Triazolam

Dilutions:

- concentration 1mg/l:

Oxazepam

Lorazepam

Midazolam

- concentration 0,5mg/l:

Diazepam

Nordazepam

Flunitrazepam

Lormetazepam

Prazepam

Flurazepam

Triazolam

#### 4.3.2. Calibrators

The solutions for calibration were made straight before use.

A mixture of Oxazepam, Lorazepam, Midazolam (1 mg/l each) and Diazepam, Nordazepam, Flunitrazepam, Lormetazepam, Prazepam, Flurazepam, Triazolam (0.5 mg/l each) is made.

The calibrators are prepared by adding 5  $\mu$ l, 10  $\mu$ l and 20  $\mu$ l of the calibrator mixture to drug-free hair samples.

Calibrator I: 50 pg/mg Diazepam, Nordazepam, Flunitrazepam, Lormetazepam, Prazepam, Flurazepam, Triazolam, and 100 pg/mg Oxazepam, Lorazepam, Midazolam

Calibrator II: 100 pg/mg Diazepam, Nordazepam, Flunitrazepam, Lormetazepam, Prazepam, Flurazepam, Triazolam, and 200 pg/mg Oxazepam, Lorazepam, Midazolam

Calibrator III: 200 pg/mg Diazepam, Nordazepam, Flunitrazepam, Lormetazepam, Prazepam, Flurazepam, Triazolam, and 400 pg/mg Oxazepam, Lorazepam, Midazolam

#### 4.3.2.2. Internal Standard (IS)

1  $\mu$ l flunitrazepam- $d_7$  (10 mg/l) is added to the calibrators, to the samples as well as to one drug free hair sample.

## 5. Instrumentation and Supporting Material

(Other instrumentation and supporting material could be used after testing)

### 5.1 Instrumentation:

5.1.1. Vortex (Scientific Industries Vortex-Genie 2TM)

5.1.2. Shaker KS 10 (Laborgerätebau Bühler, Tübingen, Germany)

5.1.3. Heating block and evaporation station (ReactiTherm III Heating Modul and ReactiVap III, Pierce Rockford, USA)

5.1.4. Ultrasonic bath Transsonic Digital (Elma, Singen Germany)

5.1.5. GC/MS:

- Gaschromatograph HP 6890 (Hewlett Packard, Böblingen, Germany)
- Mass Selektive Detector HP 5973 (Hewlett Packard, Böblingen, Germany)
- GC column J&W DB 5 MS, 30m x 0,25mm i.d. x 0,25 $\mu$ m thickness (Chromatographiehandel Müller, Fridolfing, Germany)

5.1.6. Centrifuge

## 5.2 Supporting Material

5.2.1 Graduated flasks 10 ml

5.5.2 Polypropylene reagent vials 30 ml (Sarstedt, Nürnberg, Germany Nr.60.543)

5.5.3 Pipetten:

Gilson Microman M10

Gilson Distriman

Eppendorf 100 µl

5.5.4 Glas tube 10 ml: Duran-Glas, Schliff NS 14, volume 10ml

5.2.5 Glas stopper: Duran-Glas mit Schliff NS 14

5.2.6 Glas tube 7 ml

5.2.7 GC vials:

Glas injections vials including 100 µl inserts, (Chromatographiehandel Müller, Fridolfing, Germany no.610025 and no.610261, respectively)

Fitting caps N 11 TB/aA (Chromatographiehandel Müller, Fridolfing, Germany)

## 6. Preparation prior to Analysis

Every vial to be used has to be signed clearly with the toxicological registration number, internal calibration or control mark with a waterproof marker.

## 7. Experimental

### 7.1. General

Under normal conditions 50 – 150 mg of hair material are used for one hair analysis. Regarding this amount, the findings have to be re-calculated.

Normally a strand of hair of 6 cm beginning at the head skin is to be examined. Samples referring to police are divided up into three segments 3 cm each if possible.

### 7.2. Sample Preparation and Extraction

1. The hair samples are cut as close as possible to the skin from the posterior vertex with a scissor and are stored in a piece of aluminium foil at room temperature. The hair length is

documented and the needed segments are prepared. 50 – 150 mg of each segment is weighed into a polypropylene vial [5.2.2] and cut into small pieces with a scissor.

2. The sample is washed with 5 ml of deionized water [4.1.1], 5 ml of acetone [4.1.2] and 5 ml of petroleum ether [4.1.3], respectively. The hair sample is rocked for 5 min at each washing step and the supernatant is removed and disposed. After addition of 4 ml Sørensen buffer and 5 µl of flunitrazepam-d<sub>7</sub> (conc. 1mg/l) as internal standard, the sample is incubated for 5 h at 50°C in an ultrasonic bath.
3. The buffer phase is put into a 10 ml glass tube and is extracted by liquid/liquid extraction with 6 ml chlorobutane (rocking the sample for 1 min and centrifugation at 3000 rpm for 3 min). The organic layer (upper phase) is pipetted in another glass tube (7ml) and is evaporated to dryness under a stream of nitrogen.
4. The residue is reconstituted in 50 µl ethyl acetate [4.1.7] and filled in a GC-vial [5.2.7].

The drug free hair sample and the calibrators are handled the same way.

### 7.3. GC/MS - Analysis

#### 7.3.1. General

The extract is analysed in the SIM-Mode especially for

- A) Diazepam, Nordazepam, Flunitrazepam, Lormetazepam, Prazepam, Flurazepam, Triazolam (Temperature Program A, see 7.3.2)
- B) Oxazepam, Midazolam, Lorazepam (Temperature Program B, see 7.3.2)

For both methods 1 µl is injected. The syringe of the autosampler is rinsed with methanol and chloroform/ethyl acetate (1:1).

#### 7.3.2. GC-conditions (see [5.1.5]):

Column J&W DB 5-ms (30 m x 0.25 mm i.d. x 0.25 µm thickness)

Carrier gas: helium (flow 1,2 ml/min)

Injection volume: 1µl (splitless)

Injector temperature: 250°C

## A Temperature program A:

150°C/ 1min → 280°C/9.5 min (rate 20°C/min)

duration of analysis: 17,00 min

## B Temperature program B:

150°C/ 1min → 280°C/5.5 min (rate 20°C/min)

duration of analysis: 13,00 min

## 7.3.3. MS-conditions (see [5.1.5]):

The mass spectrometer is operated in the negative chemical ionization detection mode (+ 400 eV above NCI autotune).

Methane is used as reagent gas and its flow is set 40% of max. flow with a pressure of  $2.0 \times 10^{-4}$  torr in the ion source. The ion source temperature and quadrupole temperature are 150°C and 106°C, respectively.

## A Temperature program A:

Solvent-delay: 7.00 min

Following masses are recorded:

Lorazepam m/z 302,304,306Diazepam m/z 284,286,285Midazolam m/z 325,327,326Prazepam m/z 324,325,326Lormetazepam m/z 304,306,308Triazolam m/z 306,308,307Flunitrazepam-d<sub>7</sub> (IS) m/z 320,321

## B Temperature program B:

Solvent-delay: 7.00 min

Following masses are recorded:

Oxazepam m/z 268,270,269Nordazepam m/z 270,272,271Flunitrazepam m/z 313,314,315

Flurazepam                      m/z 387,389,388

Flunitrazepam-d<sub>7</sub> (IS)              m/z 320,321

#### 7.3.4. Recording

Data recording and evaluation is made with HP Chemstation software for GC

Recording methods : BENZO3.M und BENZO4.M.

The elution of the substances takes place in the following order:

##### A) BENZO3.M

Lorazepam, Diazepam, Midazolam, Prazepam, Lormetazepam, Triazolam

##### B) BENZO4.M.

Oxazepam, Nordazepam, Flunitrazepam, Flurazepam

#### 7.3.5. Evaluation

The integration of the peaks is made manually. Through the standard of calibration the concentration of the substances is calculated with an EXCEL datasheet (1 point calibration).

For quantitation the so called Target-Ions are used [siehe 7.3.3]. The other ions are qualifier (+/- 20% range is allowed). To get peaks as qualitative positive the signal to noise ratio has to be >3. The signal to noise ratio has to be >10 to quantize the peaks.

## 8. Documentation and Archivation

### • Internal Documentation

- The printed chromatograms of each sample and the values which are calculated with the EXCEL datasheet are attached to laboratory concerning identification sheets.
- The prints of the calibrators are kept in special archive boxes.
- The chronological order of the injections to the GS/MS is noted in a special logbook referring to the gaschromatograph.
- The used software automatically documents the recording date and the operator.

- The results are transferred into an EXCEL sheet with the description of the used method and operator. For result documentation an ACCESS database is used (Hair(year)Off97).

## **8.2. Storage of Data, Samples and Hair Material**

- The files of the sequence runs and the single runs of the GC/MS are saved first on harddisk later on CD to clear harddisk space.
- The hair samples are archived for another two years.
- The autosampler vials with the extraction residues and the examined hair samples are put to the laboratory waste.

Reference	A. Negrusz et al, J. Anal. Toxicol. 23/6 (1999) 429-435	V. Cirimele et al, J. Anal. Toxicol. 20 (1996) 596-598	V. Cirimele et al, Forensic Sci. Int. 84/1-3 (1997) 211-218	V. Cirimele et al, J. Leg. Med. 108/5 (1996) 265-267
Analytes	flunitrazepam, 7-aminoflunitrazepam	flunitrazepam, 7-aminoflunitrazepam	flunitrazepam, 7-aminoflunitrazepam	lorazepam
Homogenisation	50 mg of hair are pulverized	50 mg of hair are pulverized	50 mg of hair are pulverized	approximately 50 mg of hair are pulverized
Extraction	3 ml Methanol + 5 ng flunitrazepam-d <sub>7</sub> and 5 ng 7-aminoflunitrazepam-d <sub>7</sub> , sonication for 1h, supernatant stored in refrigerator, 3 ml HCl (0,1N) incubation overnight at 55°C, combined extracts → SPE (CH <sub>2</sub> Cl <sub>2</sub> /isopropanol/ammonium hydroxide 78:20:2)	1 ml Sörensen buffer (pH 7.6) + 20ng diazepam-d <sub>5</sub> , incubation 20h/40°C, LLE (diethylether/CHCl <sub>3</sub> 80/20)	1 ml Sörensen buffer (pH 7.6) + 20ng diazepam-d <sub>5</sub> , incubation 20h/40°C, LLE (diethylether/CHCl <sub>3</sub> 80/20)	Sörensen buffer (pH 7.6) + 5ng lorazepam-d <sub>4</sub> , incubation 2h/40°C, LLE (diethylether/CHCl <sub>3</sub> 80/20)
Derivatisation	50 µl HFBA 60°C/30 min, evaporation, 25 µl ethyl acetate	150 µl HFBA/ethyl acetate (2:1) 60°C/30 min, evaporation, 25 µl ethyl acetate	150 µl HFBA 60°C/30 min, 25 µl ethyl acetate	35 µl BSTFA + 1%TMCS 60°C/20 min
GC-conditions	Column 30 m x 0.25 mm x 0.25 µm Inj. tem. 240°C (splitless), 1µl inj. Tem. progr.: 60°C/1min → 310°C/3min at a rate of 30°C/min	Column 30m x 0.25 mm Inj. tem. 240°C (splitless), 1.5µl inj. Tem. progr.: 60°C/1min → 295°C/6min at a rate of 30°C/min	Column 30m x 0.25 mm x 0.25 µm Inj. tem. 240°C (splitless), 1µl inj. Tem. progr.: 60°C/1min → 295°C/6min at a rate of 30°C/min	Column 30m x 0.25 mm x 0.25 µm Inj. tem. 250°C (splitless), 1µl inj. Tem. progr.: 60°C/1min → 295°C/5min at a rate of 30°C/min
MS-conditions	NCI (+ 400 eV above autotune), methane (3.7 x 10 <sup>-4</sup> torr), ion source 250°C, quadrupole 106°C	NCI (+ 400 eV above autotune), methane (1.3 torr), ion source 200°C, quadrupole 100°C	NCI (+ 400 eV above autotune), methane (1.3 torr), ion source 200°C, quadrupole 100°C	NCI (+ 500 eV above autotune), methane (1.4 torr), ion source 200°C, quadrupole 100°C

**Table 1: Comparison of described methods for the determination of benzodiazepines in hair**

Reference	M. Yegles et al, Forensic Sci. Int. 84/1-3 (1997) 211-218	Institute of Legal Medicine Munich (2000)	K. M. Höld et al, Forensic Sci. Int. 84 (1997) 201-209	P.Kintz et al, J. Chromatogr. B: Biomed. Appl. 677 (1996) 241-244
Analytes	diazepam, nordazepam, oxazepam, lorazepam, lormetazepam, flunitrazepam, 7-aminoflunitrazepam	diazepam, nordazepam, oxazepam, midazolam, lorazepam, prazepam, lormetazepam, flunitrazepam, flurazepam, triazolam	alprazolam	nordiazepam, oxazepam
Homogenisation	3050 mg of hair are pulverized	about 100 mg of hair are cut up into small pieces	10-20 mg of rat hair are cut up into small pieces	approximately 50 mg of hair are pulverised
Extraction	2 ml acetate buffer (pH 4)+ deuterated standards, hydrolyzation with 70 µl of β-glucuronidase/arylsulfatase for 2h/40°C, supernatant was removed, 2 ml distilled H <sub>2</sub> O were added and after shaking also removed, SPE of the buffer fractions (acetone/CH <sub>2</sub> Cl <sub>2</sub> 3:1), 50 µl ethyl acetate	4 ml Söerensen buffer (pH 7.4) + 5ng flunitrazepam-d <sub>7</sub> , 5h/50°C ultrasonic bath, LLE (Chlorobutane), evaporation of chlorobutane, 50 µl ethyl acetate	Digestion with 2 ml 1N NaOH + 5ng triazolam-d <sub>4</sub> , 40°C/overnight, LLE (toluene/CH <sub>2</sub> Cl <sub>2</sub> 7/3)	Söerensen buffer (pH 7.6) + 200ng nordiazepam-d <sub>5</sub> + 200ng oxazepam-d <sub>5</sub> , incubation 20h/40°C, LLE (diethylether/CHCl <sub>3</sub> 80/20)
Derivatisation			25 µl ethyl acetate + 25 µl BSTFA + 1% TMCS 80°C/30 min	35 µl BSTFA + 1% TMCS 90°C/20 min
GC-conditions	Column 12 m x 0.2 mm x 0.33 µm Inj. tem. 260°C (splitless), 2µl inj. Tem. progr. 70°C/2min → 220°C/9.5min at a rate of 25°C/min → 255°C at 5°C/min → 300°C/7min	Column 30m x 0.25 mm x 0.25 µm Inj. tem. 250°C (splitless), 1µl inj. Tem. progr. A: 150°C/1min → 280°C/9.5min at a rate of 20°C/min Tem. progr. B: 150°C/1min → 280°C/5.5min at a rate of 20°C/min	Column 15 m x 0.25 mm x 0.25 µm Inj. tem. 275°C (splitless), 1µl inj. Tem. progr.: 190°C/1min → 320°C/2min at a rate of 20°C/min interface 310°C, transferline 300°C, ionizer 130°C	Column 30m x 0.25 mm x 0.25 µm Inj. tem. 250°C (splitless), 1µl inj. Tem. progr.: 60°C/1min → 290°C/2min at a rate of 30°C/min
MS-conditions	EI (70 eV) , interface 280°C	NCI (+ 400 eV above autotune), methane (2.0 x 10 <sup>-4</sup> torr), ion source 150°C, quadrupole 106°C	NCI, methane (0.6 torr),	NCI (+ 400 eV above autotune), methane (0.2 kPa), ion source 200°C

**Table 1: Comparison of described methods for the determination of benzodiazepines in hair (continued)**

## Literature

- [1] M. Yegles, Y. Marson, R. Wennig, Influence of bleaching on stability of benzodiazepines in hair. *Forensic Sci. Int.* 107/1-3 (2000) 87-92
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- [3] K. B. Van-Loon, Haaranalyseresultaten benzodiazepinen roepen vragen op (Results of hair analysis for benzodiazepines raise questions). *Pharm. Weekbl.* 133/8 (1998) 348-349
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- [5] J. Segura, A. Redon, G. Gonzalea, C. J. Sanchez, L. San, Deteccion del consumo de benzodiazepinas mediante analisis de pelo por metodos inmunologicos (Immunological analysis of hair to detect benzodiazepines consumption). *Rev. Toxicol.* 14/1 (1997) 30-35
- [6] M. Yegles, F. Mersch, R. Wennig, Detection of benzodiazepines and other psychotropic drugs in human hair by GC/MS. *Forensic Sci. Int.* 84/1-3 (1997) 211-218
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- [9] V. Cirimele, P. Kintz, P. Mangin, Determination of chronic flunitrazepam abuse by hair analysis using GC-MS-NCI. *J. Anal. Toxicol.* 20 (1996) 596-598
- [10] V. Cirimele, P. Kintz, P. Mangin, Detection and quantification of Lorazepam in human hair by GC-MS/NCI. *Int. J. Leg. Med.* 108/5 (1996) 265-267
- [11] P. Kintz, V. Cirimele, F. Vayssette, P. Mangin, Hair analysis for nordiazepam and oxazepam by gas chromatography-negative ion chemical ionization-mass spectrometry. *J. Chromatogr. B: Biomed. Appl.* 677 (1996) 241-244
- [12] P. Kintz, P. Mangin, Determination of gestational opiate, nicotine, benzodiazepine, cocaine and amphetamine exposure by hair analysis. *J. Forensic Sci. Soc.* 33/3 (1993) 139-142
- [13] J. J. Sramek, W. A. Baumgartner, T. N. Ahrens, V. A. Hill, N. R. Cutler, Detection of benzodiazepines in human hair by radioimmunoassay. *Ann. Pharmacother.* 26/4 (1992) 469-472

Specificity and Sensitivity Requirements in Hair Analysis

**Application of High Resolution and Tandem Mass Spectrometry to the  
Identification of Anabolic Steroids (stanozolol) or Benzodiazepines (oxazepam)  
in Complex Hair Matrices**

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**Abstract**

Hair analysis includes all analytical problems associated with the presence of low substance concentrations in a dirty matrix. Extensive clean-up procedures and specific identification techniques are analytical requirements in hair testing. The implication of this workshop station is to demonstrate the capability and limitations of different sophisticated mass spectrometric detection techniques, whilst all other influences (matrix, extraction, derivatisation, chromatography and ionisation) remain unchanged.

**1 Introduction**

The requirement of sensitive and selective identification techniques is a logical consequence in hair analysis, because the analytes potentially present in a complex matrix are typically characterised by low concentrations and high lipophilicity.

Clean-up procedures to increase the concentration of the substances in question and to suppress the background are mandatory but often not sufficient for an unequivocal identification. Simple routine procedures like solid phase extraction or liquid-liquid extraction have often failed to separate the lipophilic target compounds from a fatty matrix, and more sophisticated clean-up techniques had to be applied.

Nevertheless, requirements to specificity and sensitivity of the detection techniques are still high. Both aspects have to be adjusted according to the analytes properties, to optimise separation (chromatography) and detection (mass spectrometry). This does not necessarily mean the application of expensive equipment - specific derivatisation and/or ionisation techniques may be

effective as well but access to robust screening procedures applicable to larger groups of substances or even to different substance classes is difficult. Oxazepam and stanozolol were chosen as model substances to outline the influence of enhanced specificity in hair analysis and to demonstrate, that false negative as well as false positive results can be prevented by sophisticated techniques.

## 2 Instrumentation and Analytical Principles

Resolution in terms of mass spectrometry defines the capability of an instrument to differentiate between adjacent masses. High resolution mass spectrometry is mainly associated with application of sectorfield instruments, although the resolution of other types of mass spectrometers has recently improved considerably (time of flight).

In contrast to the high technical standard (and costs) of HRMS instruments, the basic scientific background of sectorfield instruments is straight forward and easy to understand. The mass specific separation of ions is based on a balance between Lorentz and centrifugal forces in a magnetic field and the resolution of the instrument is controlled by the width of the ion beam (fig 1).

Substances which appear to be identical at unit mass resolution may be distinguished in principle by HRMS, if their elemental composition is different. The most efficient applications are directed to substances containing elements with negative mass defects (dioxins, benzodiazepines, clenbuterol etc.) whereas substances with 'common' composition (hydrocarbons) are less specific.

Tandem mass spectrometry is based on combinations of at least two mass spectrometers. Almost any type of spectrometer is suitable for such a 'coupling in space', whereas the 'coupling in time' means a subsequent application of the same device (ion trap) for several separation steps. The most common instruments are triple stage quadrupoles but there are new upcoming techniques like quadrupole / time-of-flight coupling [1].

The general intention consists in the separation of one specific fragment in the first stage. This 'precursor' ion is then fragmented in a collision cell and the resulting 'product ions' are analysed in a second mass spectrometer.

The type of experiments practically employed in this workshop-demonstrations is Multiple Reaction Monitoring (MRM) where the resulting information is two-dimensional and

interference is only possible, if both masses (precursor and product ions) would match (as well as the retention time). This means: only ions of the same mass, which undergo the same fragmentation process could produce identical signals. This is by far less likely than coincidence of masses in conventional MS.

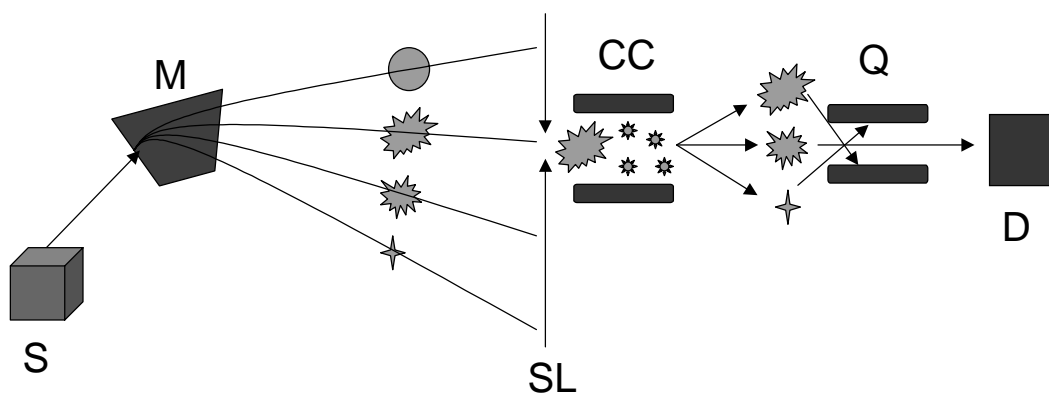


Fig.1. Schematics of a hybrid sectorfield / quadrupole mass spectrometer: Ions are formed by electron impact in the source [S] and separated in a magnetic field [M]. The mass spectrometric resolution of the first stage is controlled by a slit [SL] limiting the width of the beam. Selected ions enter the collision cell [CC], where product ions are formed and analysed in the second stage (quad [Q]).

The instrument used at the workshop is the hybrid version of the mass spectrometer AutoSpec (Micromass), which is a combination of a high resolution sectorfield instrument with a quadrupole.

This instruments permit a realistic comparison of different techniques, because GC-separation and ionisation is identical and the differences between low resolution MS, high resolution MS and tandem MS can be compared immediately by a variation of method parameters.

### 3 Experimental

#### 3.1 Sample Preparation

##### 3.1.1 Extraction

There is no optimum standard hair sample preparation procedure for anabolic steroids, which covers the requirements of all substances with adequate recovery.

We have always used an optimised procedure rather than a general unknown screening. An overview of different approaches is summarised in figure 2. The main difference between methanol or sodium hydroxide extraction depends on the target substances, many of the steroids –especially their esters- are not stable under conditions of a hydroxide disintegration, whereas the methanol extraction has insufficient recovery for some analytes (stanozolol). Clean-up and derivatisation procedures are substance-specific too. The most effective and versatile procedure was LC cleanup, which was applicable to most of the analytes and permits a simultaneous separation / enrichment of different substances.

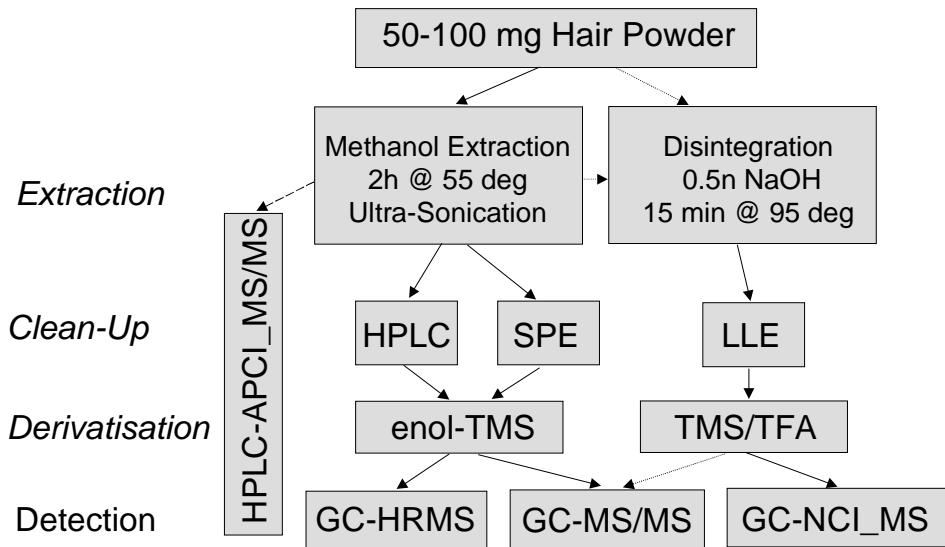


Fig. 2. Scheme for hair sample preparation

### 3.1.2 Derivatisation

The mobile phase / organic solvent is removed at 80°C in a nitrogen stream. The residue is then desiccated and derivatized 30 min at 60°C using the following reagent mixture to yield enol-TMS derivatives:

MSTFA + NH<sub>4</sub>I + dithioerythritol (15 ml + 100 mg + 10 mg).

## 3.2 Experimental Parameters

### 3.2.1 GC – separation

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

### 3.2.2 MS – detection

- ionisation mode: EI, 38 eV

- trap current 500 µA

- source temperature: 250 deg C

*HRMS parameter*

- acquisition mode selected ion recording (SIR),

- resolution 1.000 compared to 10.000

- ions detected

stanozolol 472.3305,

3'OH-stanozolol 545.3415, 560.3650

oxazepam 401.1271, 415.1065, 429.1221, 430.1300

*MS-MS parameters*

- acquisition mode	multiple reaction monitoring (MRM-Q),		
- collision energy	100 eV		
- resolution	1000 (magnet), 2 amu (quad)		
- fragmentation reactions			
stanozolol	472→143,	472→457	
oxazepam	430→267,	430→341	430→401

### 3.3 Experimental demonstration

#### 3.3.1 Model Substances

The specificity of a high resolution or tandem-mass spectrometric detection depends on the target substance's properties and the experimental conditions.

Tandem mass spectrometry is not very effective, if the fragmentation pattern of a molecule is very poor (for instance molecular ion only) or too complex (high number of low abundant peaks in unspecific regions).

Fragmentation reactions should be substance- or substance-class specific, whereas reactions like loss of water, of trimethylsilanol or methylgroups in a TMS derivative are not very specific.

The suitability of high resolution techniques depends on the presence of hetero-atoms in the molecule. Halogens in particular reduce the accurate mass of the fragments compared to the average mass of the background.

This means on the other hand, that increase of mass spectrometric resolution will not improve the identification of a substance, coeluted by a interference, which is identical in chemical composition or produces isomeric fragments. The model substances for the experiments are chosen according to their relevance in hair analysis [2, 3] as well as to their chemical properties.

### 3.3.2 Oxazepam

Oxazepam is a benzodiazepine containing chlorine with several diagnostic fragments in the mass spectrum (fig. 3). It carries polar amino and hydroxy groups and is therefore critical regarding impurities in the gas chromatographic systems. The formation of a bis TMS derivative proved to be helpful if it should be identified under routine conditions in an instrument which is used for injections of MSTFA (fig. 4).

Both techniques (HRMS and MS/MS) are suitable for a sensitive identification, although more classical options (negative CI [4], and station 2 of this workshop) are applicable too.

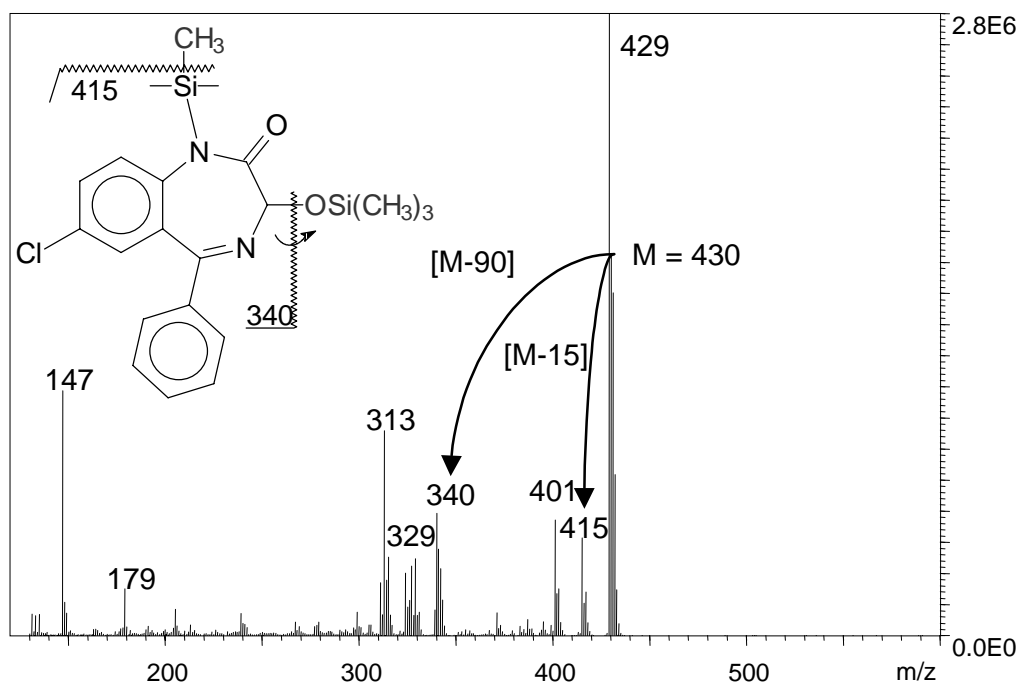


Fig.3. Structure and mass spectrum of oxazepam-bis-TMS

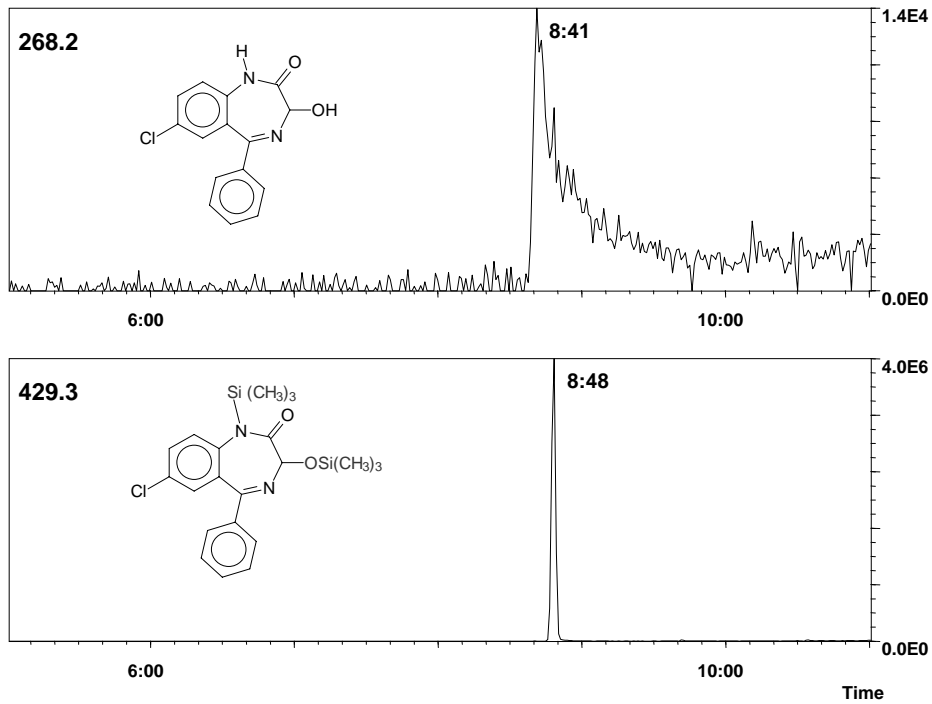


Fig.4. Structure and selected ion recording chromatograms of oxazepam. Comparison of intensity and peak shape of the oxazepam standard with the bis-TMS-derivative.

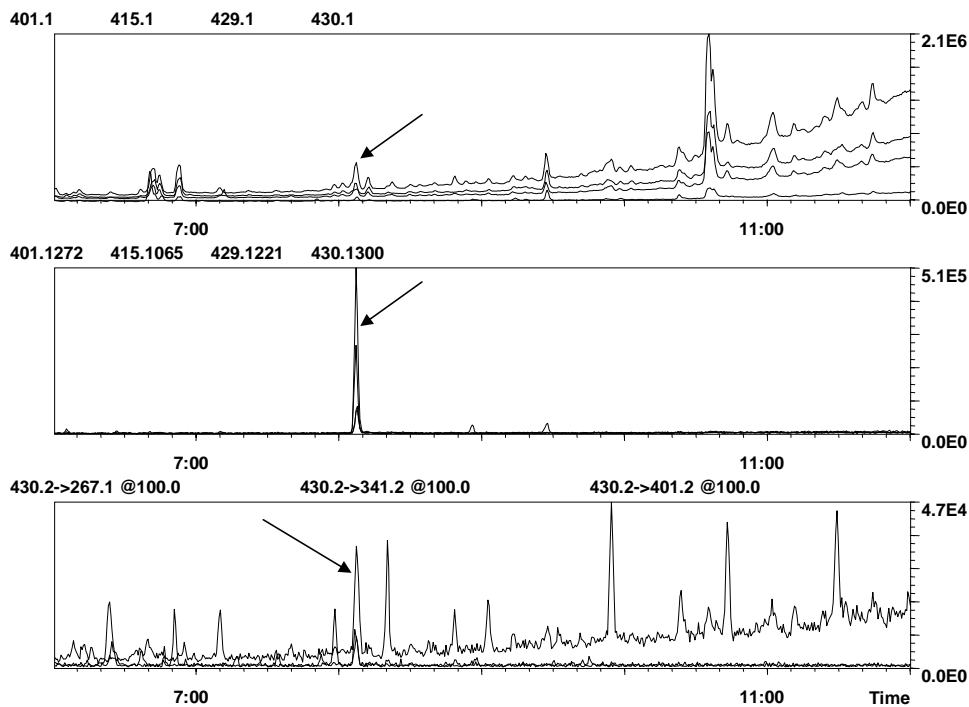


Fig. 5. Comparison of low resolution (top), high resolution (middle) and tandem mass spectrometry (bottom), applied to the identification of oxazepam in a spiked hair sample.

The comparison between the techniques (fig. 5) demonstrates, that concentrations equivalent to 10pg/mg can hardly be detected with conventional MS after derivatisation, but the detection limit will be about 100 times higher, if a resolution of 10.000 is applied. Tandem mass spectrometry was less successful in this application.

### 3.3.3 Stanozolol

Stanozolol was a very popular anabolic steroid, frequently abused by athletes and bodybuilders in the past. The dependency of the detection limit on the stability of the chromatographic system, caused by the amino group in the A'-pyrazol-ring was a crucial problem for a long period of time. The formation of mixed O-TMS / N-perfluoroalcyamide derivatives was attempted to improve the chromatographic stability. Moreover, this permits a very specific NCI detection of stanozolol in complex matrices [2], because no analogue derivatives of endogenous steroids exist.

The situation has improved considerably due to the refined passivation of GC columns and detection of stanozolol and its metabolites is usually carried out based on their per-TMS-derivatives. Fig. 6 shows the successful identification of stanozolol in a hair sample of a bodybuilder by MS/MS.

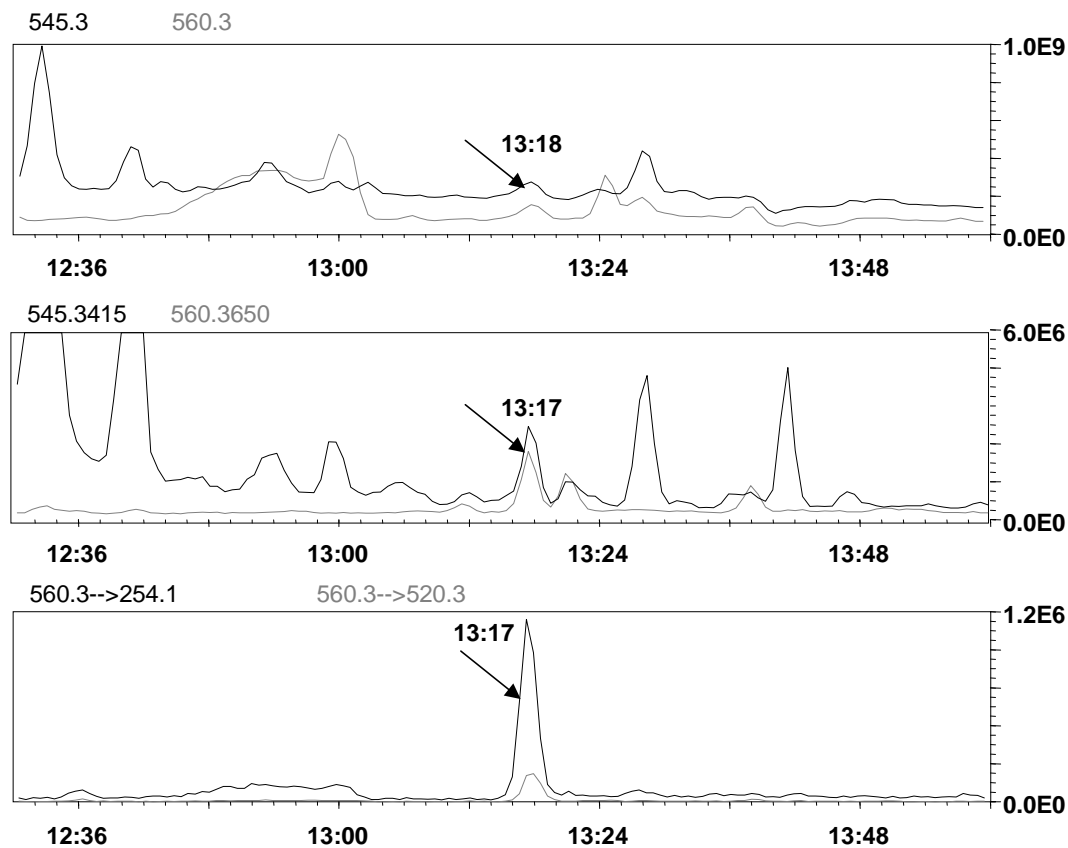


Fig. 6. Comparison of low resolution (top), high resolution (middle) and tandem mass spectrometry (bottom), applied to the identification of 3'OH-stanozolol in a hair sample of a bodybuilder.

The 3'OH-stanozolol metabolite was chosen as sensitive criterion for the comparison of techniques because of its lower concentrations, although the major product incorporated into hair was the parent compound stanozolol (fig. 7).

The required sensitivity for stanozolol identification is relatively high, and conventional mass spectra at unit resolution clearly exhibit lower detection limits. A comparison of identifications using different detection techniques demonstrates the problem of high amounts of background interfering the signals at low resolution. Sophisticated techniques have to be applied to identify

stanozolol (per-TMS) at appropriate peak to noise ratios. Tandem mass spectrometry is more effective than HRMS in this example.

Starting to deal with identification of steroids in hair matrix, stanozolol was a helpful tool for understanding of principles of incorporation into hair, because the parent compound was detectable in hair at higher amounts than their typical urinary metabolites 3'-hydroxy-, 4 $\beta$ -hydroxy and epi-stanozolol. In a fatal case, the concentration of the parent compound detected in hair was 30 times higher than the concentration of the corresponding metabolite (fig. 7).

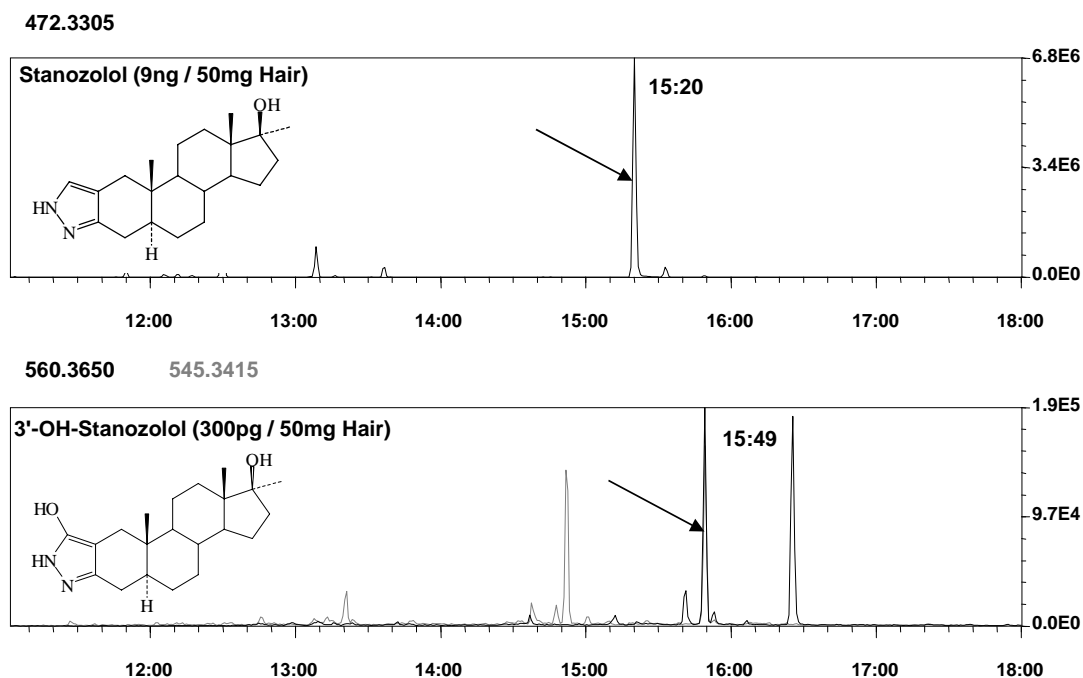


Fig. 7. Identification of stanozolol and 3'hydroxy-stanozolol.

### 3.3.4 Miscellaneous Results

The spectrum of anabolic substances we have detected so far in hair is rather wide [5]. Based on forensic cases of steroid trafficking, diminished responsibility or fatalities amongst bodybuilders, the random collection of substances comprises

- Clenbuterol

- Stanozolol (3'OH metabolite)
- Metandienone (epi-metandienone, 6 $\beta$ -OH-metabolite)
- Mesterolone
- Metenolone enantate
- Nandrolone decanoate and laureate
- Testosterone phenylpropionate, propionate, decanoate, enantate and isocaproate

The most valuable result was the identification of testosterone esters in hair (fig. 8), because the differentiation of the endogenous steroid testosterone in urine is a crucial problem. The identification in hair of the injected esters (which are neither endogenous, nor excreted in urine) is a unique marker of testosterone abuse.

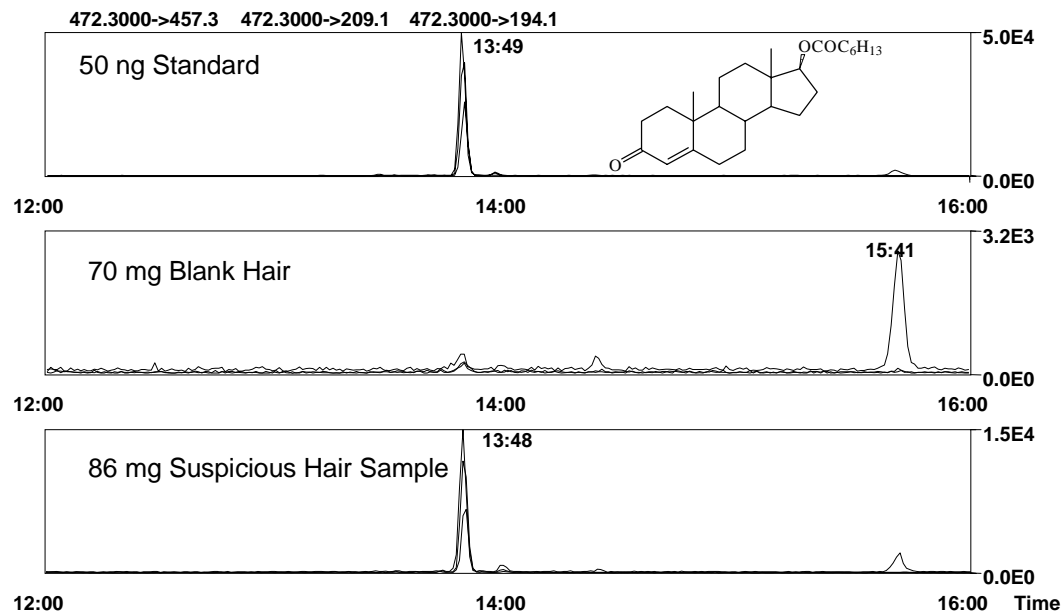


Fig. 8. Identification of testosterone enantate in hair of a bodybuilder by tandem mass spectrometry.

## 4 Conclusions and Forensic Considerations

- Specificity differences between GC/MS, GC/HRMS and GC/MS-MS are studied using the identical chromatographic and ionisation techniques. The improvement of specificity by application of HRMS and MS-MS is substance dependent. The model substances applied were easily detectable by both techniques. Detection limits were improved considerably compared to low resolution MS.
- The identification criteria defined for low resolution MS (three diagnostic ions must be detected, signal/noise and relative abundances below cut-off) are applied to high resolution and tandem MS as minimum requirements.
- Parent compounds are often incorporated into hair although they are (practically) not excreted in urine. This offers the opportunity to identify diagnostic key substances for longer periods of time. Especially precursors of endogenous steroids are attractive targets of hair analyses.
- Dealing with steroids there are individual variations of endogenous steroid concentrations depending on body site, scalp region, health, age and sex, extra to the usual restrictions in hair analysis (hair pigmentation, treatment etc.) to consider.
- Practical cases of identification of steroids in hair were devoted to forensic cases, (trafficking of steroids, diminished responsibility, steroid related fatalities) where the number of possible analytes was reduced and analyses could be optimised in advance. A general unknown screening of 'all anabolic agents' seems to be too complex to be effective.

### Acknowledgement

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# Identification of Buprenorphine and Methadone in Hair by HPLC/MS

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## 1. HPLC/MS determination of buprenorphine and norbuprenorphine in hair samples

### Introduction

Buprenorphine (BUP) is an hemi-synthetic opioid derivative, closely related to morphine and congener alkaloids, which is obtained from thebaine after a 7-step chemical procedure. BUP is a powerful analgesic (25 to 40 times more potent than morphine) that exhibits both partial agonist activity at the  $\mu$ -opiate receptor and antagonist activity at the  $\kappa$ -opiate receptor [1,2]. This drug has been initially developed for the treatment of acute and chronic pain, especially of surgical or neoplastic origin. Its main advantages over morphine are a poor respiratory depressant activity and a lack of significant withdrawal symptoms. A high-dosage, sublingual formulation of buprenorphine is available in France since February 1996 (Subutex®, Schering-Plough Labs.) for the management of heroin addicts. As it may be ordered by any physician and is delivered under limited control, this alternative to the methadone substitution previously organized in specific detoxication centers only has led to some deviations (appearance of a black market, intravenous injection of crushed tablets). Some addiction potential, cases of abuse and fatalities attributable to the misuse of Subutex® associated to benzodiazepines have been reported in France and other countries (3).

As a consequence, it has become necessary for every forensic laboratory to be able to assay BUP in biological samples and in hair specimens. For the past twenty years, hair analysis has been proposed for identifying chronic drug abusers in forensic science. Drug concentration along the hair shaft can be correlated with the time of drug use. Hair is known to allow a drug administration to be tracked back for months, and thus offers the possibility of determining long-term drug exposure (4).

This paper describes the procedure based upon HPLC hyphenated to ionspray-mass spectrometry (HPLC/ISP-MS) for the sensitive and specific determination of BUP and norBUP in human hair.

## Material and Methods

### *Liquid Chromatography*

The HPLC separations were performed at ambient temperature on a 4- $\mu$ m NovaPak (Waters) C18 column (150 x 2.0 mm, i.d.) protected by a 5- $\mu$ m Opti-Guard™ (Interchim) C18 guard cartridge (15 x 1.0 mm, i.d.). The mobile phase was acetonitrile/2 mM NH<sub>4</sub>COOH buffer, pH 3.0. A 20-ml, dual syringe HPLC pump (Applied Biosystems 140 B) was employed to deliver a continuous flow of 200  $\mu$ L/min. A post-column split of 1 : 3 was used to reduce at 50  $\mu$ L/min the flow rate infused into the HPLC/MS interface.

### *Mass Spectrometry*

The MS detection was carried out on a Perkin-Elmer Sciex API-100 apparatus equipped with an Ionspray™ (= pneumatically assisted electrospray) interface. Nitrogen (99.95 %, 40 psi) was employed as both the nebulizing gas (flow rate 1.16 L/min) and the 'curtain gas' (flow rate 1.08 L/min) that prevents solvent vapours and solid contaminants from entering the vacuum chamber. The ion sampling orifice was held at a potential of + 50 V, and the electron multiplier at + 2400 V. MS data were collected as either 1) total ion chromatograms (TIC) by monitoring the signal over the mass range  $m/z$  260-475 for drug identification, or 2) multiple ion monitoring (MIM) at  $m/z$  414 (norBUP), 417 (norBUP-d<sub>3</sub>), 468 (BUP), and 472 (BUP-d<sub>4</sub>).

### *Specimens and BUP/norBUP Extraction Procedure*

Hair samples (at least 50 mg cut close to the scalp at the posterior vertex) were decontaminated by two CH<sub>2</sub>Cl<sub>2</sub> washes (5 mL, 2 min) then pulverized (Retsch® MM2 ball mill, 5-10 min); 50 mg of the resulting powder were then incubated overnight at 56 °C in 1 mL of 0.1 N HCl, after addition of 15 ng of deuterated BUP and norBUP (Radian).

After neutralization (using 0.1 N NaOH, 1 mL) of the incubation medium, 2 mL of a saturated, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> buffer, pH 8.4, and 5 mL of CHCl<sub>3</sub>/2-propanol/*n*-heptane (25 : 10 : 65, v/v) were added. After agitation and centrifugation (3500 g, 10 min), the organic phase was evaporated (Speed Vac Concentrator, 45 °C, 30 min); the dry extract was resuspended in 40  $\mu$ L of the mobile phase, and after a final centrifugation (10,000 g, 5 min) 25  $\mu$ L of the supernatant were removed, from which 5  $\mu$ L were injected onto the column.

## Results and Discussion

The ISP mass spectra of BUP, norBUP are quite simple since they exhibit one unique peak corresponding to the protonated molecular ion  $[M + H]^+$  at  $m/z$  468 and 414, respectively (fig 1 and 2). This low abundance or absence of fragmentation is a typical character common to mass spectra generated by the different atmospheric pressure ionization (API) HPLC/MS interfaces.

Retention times and  $m/z$  values for norBUP, norBUP- $d_3$ , BUP, and BUP- $d_4$  are listed in the following table.

Analyte	Retention time (min)	Ions ( $m/z$ )
Norbuprenorphine	6.33	414 ( $d_3$ : 417)
Buprenorphine	9.17	468 ( $d_4$ : 472)

Results of the analytical validation are summarized in this second table.

	Buprenorphine	Norbuprenorphine
Linearity	0.02 to 5 ng/mg (r 0.993)	0.01 to 3 ng/mg (r 0.998)
Recovery	62 %	54 %
Repetability (0.2 ng/mg)	8.8 %	7,5 %
LOD	4 pg/mg	2 pg/mg

Hair concentrations for BUP and norBUP measured by HPLC/ISP-MS in the hair of 71 chronic users ranged from 0.01 to 8.16 ng/mg and from not detected to 1.47 ng/mg, respectively.

For Vincent and colleagues (5), BUP ranged from 60 to 361 pg/mg and norBUP from 29 to 785 pg/mg in the hair of 5 subjects.

For Wilkins and colleagues (6), BUP ranged from 4.5 to 156.8 pg/mg and norBUP from 4.8 to 1438.5 pg/mg in the hair of 4 subjects.







As an example, fig. 3 shows the SIM chromatogram obtained by extracting a hair sample from a 25-year-old addict. BUP and norBUP concentrations were 3.15 and 0.64 ng/mg, respectively.

BUP and norBUP have been also reported to be detectable in hair from treated subjects by means of RIA or HPLC with coulometric detection [7]. For this application HPLC/ISP-MS appeared at least as sensitive (LODs in drug-free, spiked hair powder about 4 pg/mg for BUP, and 2 pg/mg for norBUP), and much more specific due to the selected-ion detection.

In conclusion, the coupling of HPLC to mass spectrometry via API interfaces, that has been already presented as a complement of choice to GC/MS for the determination of nonvolatile and/or thermolabile substances with high sensitivity and specificity, thereby appears as an interesting alternative in the case of BUP and norBUP dosage in human hair.

## **Dosage of buprenorphine and norbuprenorphine in human hair**

### **Extraction of hair specimen**

#### *1. Decontamination*

Two successive washes of the hair strand (100 mg approximately) in 5 ml of methylene chloride for 2 min.

Pulverization in a ball mill

#### *2. Hydrolysis*

50 mg of powdered hair

15 ng of deuterated internal standards (buprenorphine-d<sub>4</sub> and norbuprenorphine-d<sub>3</sub>)

1 ml of HCl 0.1M

Incubation overnight at 56 °C

### 3. Extraction

1 ml NaOH 0.1 M

2 ml of NH<sub>4</sub>Cl buffer pH 9.5

5 ml chloroforme/2-propanol/*n*-heptane (25:10:65, v/v/v)

Agitation (15 min), centrifugation (15 min at 3000 rpm)

Organic phase removed and evaporated to dryness

Dry extract reconstituted in 35 ml of methanol

### Analyses by HPLC-ISP-MS

Injection volume : 2 ml

Column : 5-mm Novapak C<sub>18</sub> Waters (150 x 2.0 mm i.d.)

Binary mobile phase of ACN-TMA 50 mg/L/2mM NH<sub>4</sub>COOH pH 3.0 buffer

ACN : 35 %, 70 % at 9 min

Flow rate : 200 ml/min (50 ml/min infused)

Detection carried out using positive ionization mode

IS + 4500 V; OR +75 V; RNG + 350 V; Qo -10 V

SIM acquisition

Analytical parameters (tab. 1)

Analyte	Retention time (min)	Ions (m/z)
Norbuprenorphine	6.33	414 (d <sub>3</sub> : 417)
Buprenorphine	9.17	468 (d <sub>4</sub> : 472)

## Analytical validation

	Buprenorphine	Norbuprenorphine
Linearity	0.02 to 5 ng/mg (r 0.993)	0.01 to 3 ng/mg (r 0.998)
Recovery	62 %	54 %
Repetability (0.2 ng/mg)	8.8 %	7,5 %
LOD	4 pg/mg	2 pg/mg

## Positive hair specimen (tab. 2)

Analyte	Concentrations
Norbuprenorphine	0.64 ng/mg
Buprenorphine	3.15 ng/mg

**2. Chiral separation of methadone and EDDP extracted from human hair****Extraction of hair specimen***1. Decontamination*

Two successive washes of the hair strand (100 mg approximately) in 5 ml of methylene chloride for 2 min.

Pulverization in a ball mill

*2. Hydrolysis*

50 mg of powdered hair

200 ng of deuterated internal standards (methadone-d<sub>3</sub> and EDDP-d<sub>3</sub>)

0.5 ml  $\beta$ -glucuronidase from bovine liver (20 000 units prepared in water)

1 ml of NH<sub>4</sub>Cl buffer pH 9.5

Incubation overnight at 40 °C

### 3. Extraction

5 ml chloroforme/2-propanol/*n*-heptane (25:10:65, v/v/v)

Agitation (15 min), centrifugation (15 min at 3000 rpm)

Organic phase removed and evaporated to dryness

Dry extract reconstituted in 35 ml of methanol

### Analyses by HPLC-IS-MS

Injection volume : 2 ml

Column : 5-mm CHIRAL-AGP Chromtech (100 x 4.0 mm i.d.)

Binary mobile phase of 2-propanol/2mM NH<sub>4</sub>COOH pH 5.8 buffer

2-propanol : 8 % for 8 min, 20 % at 9 min, 20 % at 16 min

Flow rate : 500 ml/min (50 ml/min infused)

Detection carried out using positive ionization mode

IS + 4500 V; OR +30 V; RNG + 375 V; Q<sub>o</sub> -10 V

SIM acquisition

Analytical parameters (tab. 1)

Analyte	Retention time (min)	Ions (m/z)
(R)-Methadone	14.5	310-265 (d <sub>3</sub> : 313-268)
(S)-Methadone	17.0	310-265 (d <sub>3</sub> : 313-268)
(R)-EDDP	12.5	278 (d <sub>3</sub> : 281)
(S)-EDDP	13.5	278 (d <sub>3</sub> : 281)

## Analytical validation

	Methadone	EDDP
Linearity	0.5 to 20 ng/mg (r 0.997)	0.2 to 10 ng/mg (r 0.995)
Recovery	86 %	82 %
Repetability (1 ng/mg)	9.5 %	8.3 %
LOD	0.2 ng/mg	0.1 ng/mg

## Positive hair specimen (tab. 2)

Analyte	Concentrations
(R)-Methadone	10.22 ng/mg
(S)-Methadone	5.93 ng/mg
(R)-EDDP	1.38 ng/mg
(S)-EDDP	1.38 ng/mg

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# **Interpretation of Quantitative Findings and Data Evaluation in Hair Analyses**

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## **Abstract**

Hair analysis for illegal and therapeutic drugs provides a long-term information on an individual's drug use. Hair retains drugs in a relatively inert matrix, its surveillance window is limited by the type and length of hair. The first steps of toxicological hair analysis are sample collection followed by drug isolation from the hair structure. Drug identification and quantification will supply numerical data, but the question is: how could a simple information about a concentration of a drug or a metabolite be evaluated? Some biochemical, mechanical and empirical aspects are assumed to assist the task of interpretation of quantitative findings.

## **1. Introduction**

A pioneer in hair analysis is Werner Baumgartner who decided 1979 to apply immunoassays to the detection of morphine in hair [1]. His efforts led to great interest and activity by many scientists entering the unknown and fascinating territory hair analysis. Later, gas chromatography coupled with mass spectrometry (GC/MS) or coupled with tandem mass spectrometry (GC/MSMS) were introduced as important analytic tools for hair testing.

An early period of uncritical euphoria was discontinued when simple mechanistic models for drug incorporation into the hair matrix were replaced by more complex considerations. Modern concepts about environmental exposure [2], passive contamination, multi-compartment model for drug incorporation [3], irregular hair growth, cosmetical treatment of hair [4], and ethnic head hair types [5] were published.

An understanding of these new data lead in the history of hair analysis to the present period of critical evaluation.

In addition to determination of illegal and therapeutic drugs, hair testing was utilized for different goals also:

- Determination of heavy metals [6], hair as a mirror of the environment
- In diagnosis of disease and assessment of nutritional status [6]
- For doping control in special cases, in addition to urine testing [7]

Different legal situations in different countries lead to different questions. Orders for hair analyses for drugs of abuse come from a various kind of mandators: coming from the court, from the prosecutor`s office, from the police, from the army, from employers, or from administration authorities. Applications of hair analysis for illegal and therapeutic drugs fall into the following categories:

In criminal court proceedings:

- Confirmation of chronic abuse to order a medical and physiological examination.
- Examination of the reliability of an accused or of a witness.
- Testing a supposed victim`s hair sample, intoxication over a long period?
- Detoxification control in addicts

General and special workplace testing:

- Preemployment and workplace testing (e.g. police candidates or officers)
- Drug testing in military court-martials

Additional scopes of application are:

- Drug related fatalities
- Examination of the reliability of a father or a mother in child custody and adoption cases, prenatal and neonatal hair analysis
- Testing a hair sample of a former chronic drug user for restoration of his driving licence

These heterogenous orders require individual evaluations, exceeding the simple statement “yes“ or “no“ to a question like: “was this person exposed to drugs“ or “did this person use drugs?“

## **2. Basic aspects**

Hair is an unique matrix because no active metabolism and excretion is present to remove drugs once deposited.

The first steps of toxicological hair analysis are sample collection followed by drug isolation from the hair structure. Drug identification and quantification will mostly be performed by

chromatographic techniques. After determination of a correct concentration there is still a question:

How could a simple information about a concentration of a drug or a metabolite be evaluated? Is it responsible that the same numerical data about a certain illegal drug always conducts to the same conclusion? Is 1,0 ng benzoylecgonine per mg hair determined in different samples always due to a similar drug history? It is likely a difference if this concentration of benzoylecgonine was detected in a proximal 6 cm segment, in a proximal 1 cm segment, in a proximal 1 cm segment of a dyed strand or in a distal (14-15 cm) 1 cm segment.

For careful interpretation of quantitative findings and data evaluation several important aspects should be considered.

### **2.1 Concentration decrease in normally treated hair**

Washing the hair several times per week could be seen as normal treatment. Repeated shampoos have no significant influence on drug content in hair [8]. It was found in a controlled study [9] that a single dose of deuterated cocaine could be detected for 2-6 months and it could be detected longer the higher the dose was applied.

With increasing distance from the root the cuticula will be more and more damaged, and the natural barrier against the loss of entrapped drugs is broken. In experiments with the model compound methoxyamphetamine a decrease of about 50% in drug concentration was observed after five months [10].

Polar substances with a tight affinity to the hair matrix are extracted more slowly than unpolar substances [11]. In segmental analysis the concentration ratio of the polar metabolite to the parent drug increases with growing distance from the root.

According to personal experience with the detection of the acidic and polar substance 9-carboxy-THC this specific, low concentrated metabolite is washed out and decomposed quickly. In the great majority of cases the highest concentration of 9-carboxy-THC will rather be in the proximal than in the distal segment [12].

## 2.2 Effects of cosmetically treated hair

Every strong chemical, physical and mechanical influence could have harmful effects on the cuticula: perming, straightening, dyeing, bleaching, excessive washing, intensive illumination with ultraviolet radiation, unusual exposure to the sunlight.

Bleaching, blinding or lightening involve the irreversible destruction of melanin by oxidation, a partial or even complete degradation of melanin is possible. When strong bleach is used the physical properties of hair (e.g. a higher porosity) will be altered.

It is important to know that there are different techniques to modify hair color or to mask white hair. Hair dyeing includes the use of permanent, semipermanent, and temporary dyes. A permanent dye lasts through any number of washings (as well as permanent perming). A semipermanent dye is removed after 2-10 washings, and a temporary dye is largely eliminated after 1 washing [13].

The best method of achieving a permanent hair color is the use of oxidation hair dyes. This drastic method seems to have a harming influence on the drug molecules entrapped within the hair. Direct dyes impart a semipermanent or temporary color that lasts a variable time. A historically and well known representative of this class is henna.

Commercially available bleaching and perming formulas were applied in vitro to the hair strands, and a decrease in opiate concentration was observed [4]. In authentic hair samples, the drug levels of the formerly positive hair fibers had been reduced but distinct tendencies couldn't be scrutinized.

The concentration of drugs in hair of an addict with bleached and natural colored hair was investigated [14]. The concentrations found in bleached compared with natural hair were about 33-34 % for cocaine, benzoylecgonine, and methylecgonine, for codeine 25 %, for morphine 11 %, and for 6-MAM 14 %.

## 2.3 Different specificities of head hair and nonhead hair

The growth rate of head hair could range widely from 0,2 mm/d-1,12 mm/d [15], about 80-95% of the follicles remain in anagen stage. The smallest part of resting hair is found in the vertex region. Growth rate could be influenced by therapeutic drugs and is also depending on age, sex, race and even on seasonal fluctuation [16,17]. In many cases a medium head hair growth rate of 1

cm/month was taken. For daily routine we recommend to assume a growth rate of 0,8-1,5 cm/month.

Nonhead hair has a quite similar growth rate (0,9-1,1 cm/month [17]) but a different growth cycle in comparison to scalp hair. In beard, axillary and pubic hair 40-60% remains in the resting phase. Therefore, nonhead hair are not suitable for segmental analysis. In case of a positive result no realistic decision on the period of drug exposure (use, consumption) is possible: maybe just the last month before sample collection, maybe for a whole year, maybe for a short period, but nearly 1 year in the past.

## **2.4 Drug binding to hair**

Hair may be thought as an ion exchange resin, it contains three major functionalities whose characteristics can vary as a function of pH. Acidic groups will be produced by oxidation of cysteine [2]. Sulfate groups will be expected to be more prevalent in damaged or cosmetically, chemically treated hair. The isoelectric point of hair decreases when the hair is damaged or when hair ages. Negatively charged drugs or metabolites like an acidic compound should bind poorly to the hair matrix.

## **2.5 Metabolites, pseudometabolites, quality of drugs in street samples**

There are three different kind of drugs suitable for the determination in hair samples:

- -Drugs without a specific metabolite:

-amphetamine

- -Drugs with nonspecific metabolites:

-Cocaine

For cocaine, the metabolites benzoylecgonine (BE), methylecgonine (ME) and ecgonine are also products of hydrolysis. It is published in literature [2] that in USA the ratio of BE/cocaine determined in street samples of cocaine-hydrochloride could be 0,86 and even 2,39. On the other hand, concentrations of these products of hydrolysis (pseudometabolites) found in Germany are different. According to data of several thousand street samples of cocaine-hydrochloride seized in Bavaria more than 99,9% have an absolute concentration of BE considerably less than 5% [18]. Data of drug concentrations must be transformed to the local situation of each country.

### -Heroin

In addition to 6-monoacetylmorphine and morphine acetylcodeine could be utilized as additional marker for the use of illicit heroin [19].

### -Methylenedioxyamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA)

The detection of methylenedioxyamphetamine (MDA) could be due to hydrolysis and metabolism of MDMA or MDEA or use of MDA

### - Drugs with specific metabolites:

#### -Hashish and marijuana

11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid is a metabolite of THC [12,20] and will not be found in smoke of hashish or marijuana.

## **2.6 What is a drug positive hair? What indicates a negative result? Relation between drug dose and hair concentration**

Positive test result demonstrates that the individual has used/consumed drugs during the time period represented by the hair length analyzed. The additional detection of specific metabolites provides confidence in the evaluation of the findings. The combination of washing techniques, cut off levels and specific metabolites is recommended.

So far, there are two important statements dealing with the basis issue: what are criteria for obtaining a positive result:

A consensus initiated by the Society of Hair Testing (SHT) obtained during the 1st European Meeting on Hair Analysis in Genoa, Italy in June 1996 and a final consensus of the Hair Testing Working Group (HTWG) documented after its third meeting in Texas, May, 1999.

These two statements will be cited partly.

### - **Statement of the Society of Hair Testing concerning the examination of drugs in human hair [21].**

#### Criteria for obtaining a positive hair test result

- 1) In order to evaluate the possibility of passive contamination, four criteria are recommended:
  - . The identification of metabolites
  - . The use of metabolite-to-parent drug ratios
  - . The assay values of the decontamination washes
  - . Threshold values

2) The determination of the following metabolites can be recommended:

- . Cocaine: Benzoylecgonine and Cocaethylene;
- . Heroin: 6-monoacetylmorphine and morphine;
- . THC: Carboxy-THC;
- . Amphetamines: none

3) The following metabolite-to-parent drug ratios may be used:

- Cocaine: benzoylecgonine/cocaine  $>0.05$  (since BE is not always present, hydrolysis controls should be used)
- . Heroin: 6-monoacetylmorphine / morphine  $>1.3$  (corrected for hydrolysis)

The positive result of a hair analysis may be used to confirm if a person has used or was exposed to a drug.

#### What indicates a negative result?

A negative result does not refute use of or exposure to the drug.

#### Relation between drug dose and hair concentration

To ascertain the time of exposure and the extent of drug use is difficult and needs further research. For some drugs, data now exist which indicate that, in very controlled clinical studies, a positive dose-concentration relationship exists.

The most extensively discussed point was the introduction of threshold values or cut-offs. Different legal situations in the different countries lead to different questions in court proceedings which require different cut-offs. The society will therefore set up a committee in which every concerned country will be represented by one member. This committee will have the task to develop a list of cut-offs with respect to:

- country
- purpose of hair analysis
- substance of abuse
- GC/MS method

**A final consensus of the Hair Testing Working Group documented after its third meeting in Texas, May, 1999.**

For confirmation, the the following target analytes and cutoff levels are recommended:

<b>Drug Class</b>	<b>Target analyte</b>	<b>Cutoff</b>
-		
- <b>THC</b>	9-Carboxy-THC	0,05 pg/mg hair
-		
- <b>Cocaine</b>	cocaine	1,0 ng/mg hair
	and benzoylecgonine (BE)	0,1 ng/mg hair
	and BE/cocaine ratio 0,1	
	<i>cocaine shall not be reported unless BE</i>	<i>0,1 ng/mg hair</i>
	or	
-	cocaine	0,5 ng/mg hair
	and cocaethylene (CE)	0,1 ng/mg hair
	or norcocaine (NC)	0,1 ng/mg hair
	<i>cocaine shall not be reported unless CE or NC</i>	<i>0,1 ng/mg hair</i>
- <b>Amphetamines</b>	methamphetamine	0,3 ng/mg hair
	amphetamine	0,3 ng/mg hair
	<i>Amphetamine must be present</i>	<i>0,05 ng/mg hair to report methamphetamine</i>
- <b>Opiates</b>	morphine	0,2 ng/mg hair
	codeine	0,2 ng/mg hair
	6-MAM	0,2 ng/mg hair
	<i>6-MAM cannot be reported unless another analyte in the opiate class is present at a concentration above its appropriate cutoff</i>	
- <b>PCP</b>	PCP	0,3 ng/mg hair

### 3. Case examples

Most of the institutes experienced in hair testing will use GC/MS as analytical instrument for the detection of drugs in hair. For determination of 9-carboxy-THC it is necessary to enhance the capabilities of a conventional instrumentation by using GC/MS/MS .

Some of the following authentic case examples should demonstrate that the capability of a good analytical instrument like a tandem mass spectrometer can be a helpful tool for a rendering of an expertise.

#### 3.1 Case 1: Testing of pubic hair as adjunctive specimen

A man with short and bleached head hair was seized with a lot of ecstasy (MDMA) tablets and 10 g of cocaine. Only a trace of cocaine (0,35 ng/mg hair), but no benzoylecgonine was detectable in the head hair. According to the definition of the SHT and HTWG this sample could not be valued as positive.

The pubic hair of the same person (fig.1) was tested positive for MDMA (1,3 ng/mg hair), its potential metabolite MDA (0,35 ng/mg hair), cocaine (4,1 ng/mg hair), benzoylecgonine (0,3 ng/mg hair) and amphetamine (1,1 ng/mg hair).

No statement should be made about the overall dosage and and time of drug use/exposure.

#### 3.2 Case 2: unusual ratio of cocaine/benzoylecgonine

In cases where the time of chronic abuse lies more than several months in the past, the evaluation of the results will be more and more complicated. According to the court it was to be proven that a defendant with 43 cm long hair had only abused cocaine during a time period earlier than 15 month in the past. The sample, a 43 cm long strand of hair was cut into four segments of equal length.

Two proximal segments were tested negative for cocaine and metabolites. As presented in Fig.2 two distal segments had a concentration of 0,2 ng/mg cocaine and 1,5 and 4,3 ng/mg benzoylecgonine. A cocaine/benzoylecgonine ratio of that kind was found so far in the hair of ancient peruvian coca leaf chewers [22].

### 3.3 Case 3: detection of acetylcodeine as additional marker for heroin abuse

A person taken into custody for possession of 100 grams of heroin claimed severe addiction to this opiate in order to attenuate penalty. Chronic heroin abuse could be confirmed by the result of a hair analysis (Fig.3). Concentrations of the parent drug, metabolites and of an impurity due to manufacturing procedure [7] were high and might be due to heroin addiction: heroin: 8,2 ng/mg; 6-mono-acetylmorphine: 36 ng/mg; morphine: 1,4 ng/mg and acetylcodeine 3,6 ng/mg hair.

### 3.4 Case 4: detection of 9-carboxy-THC

Two examples should demonstrate capabilities and limits of GC/MS/MS. The scalp hair of a supposed hashish dealer was to be tested for cannabinoids. According to the dealer's self admitted use he characterizes himself as a moderate cannabis smoker. The sample was cut into a 3 cm root and a 3 cm tip segment: the concentration of 11-nor-delta-9-THC-9-carboxylic-acid in the root segment was very close to the LOQ of 0.16 pg (fig.4)

However, the result for this metabolite was not positive in the tip segment. (fig.5)

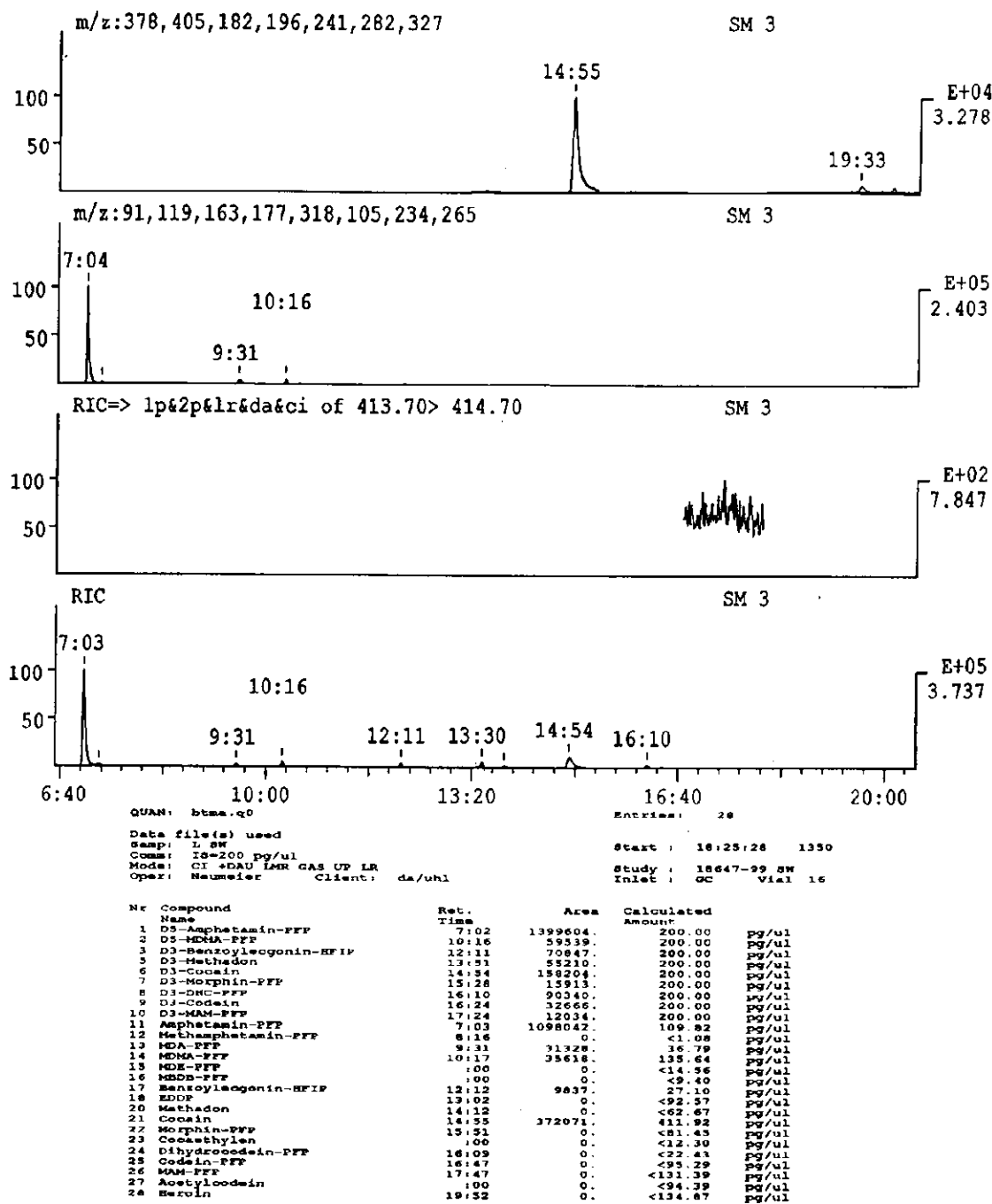
According to our available data we don't realize a significant correlation between the concentration of THC and 9-carboxy-THC. It is a preliminary observation that in segmental hair analysis the highest concentration of 9-carboxy-THC is detected in the root segment, a lower concentration in the middle segment (if available), the minimum concentration in the tip segment.

### 3.5 Case 5: hair testing of a sample coming from cosmetically untreated hair

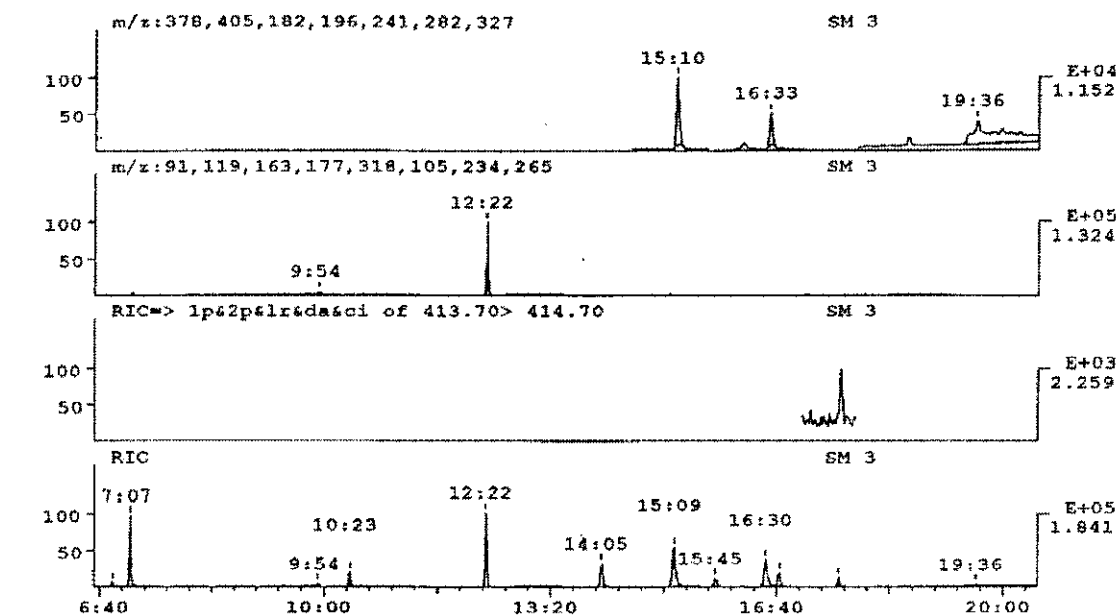
A man already imprisoned for 11 months being a trafficker of cannabis claimed that he consumed a lot of marijuana in a period more than one year ago. The hair should be tested for 9-carboxy-THC. He expressed his sympathy for the jamaican religious community rastafari by presenting a special hair-dress. Because he never combed his hair and used shampoo the hair matted completely. A distal segment (12-16 cm) of the dreadlock should correspond with that past period 1 year before sample collection. However, due to the helical appearance the actual length of every single hair within this lock is longer. The right choice of segmental length was not possible. The result for 9-carboxy-THC in the distal segment was negative. So, the rastafari's affirmation couldn't be refuted.

### 3.6 Case 6: sample of a robber with repeatedly dyed hair.

A female haircutter and pretended addict to opiates tried to improve her financial situation by committing a bank robbery. She affirmed during her interrogation that she consumed in this past period of this crime about 0,3 g dihydrocodeine and 2-3 g heroin in normal street quality (5-15 %) daily. Furthermore, she claimed that she bleached and dyed her hair every week. Her hair sample was negative for 6-monoacetylmorphine, morphine, heroin, and acetylcodeine. Only traces of dihydrocodeine (0.9 ng/mg hair) were detected. It is hard to imagine that there was only a selective oxidation of heroin, its metabolites, and of acetylcodeine. Therefore, the extent of her heroin addiction seemed to be exaggerated.



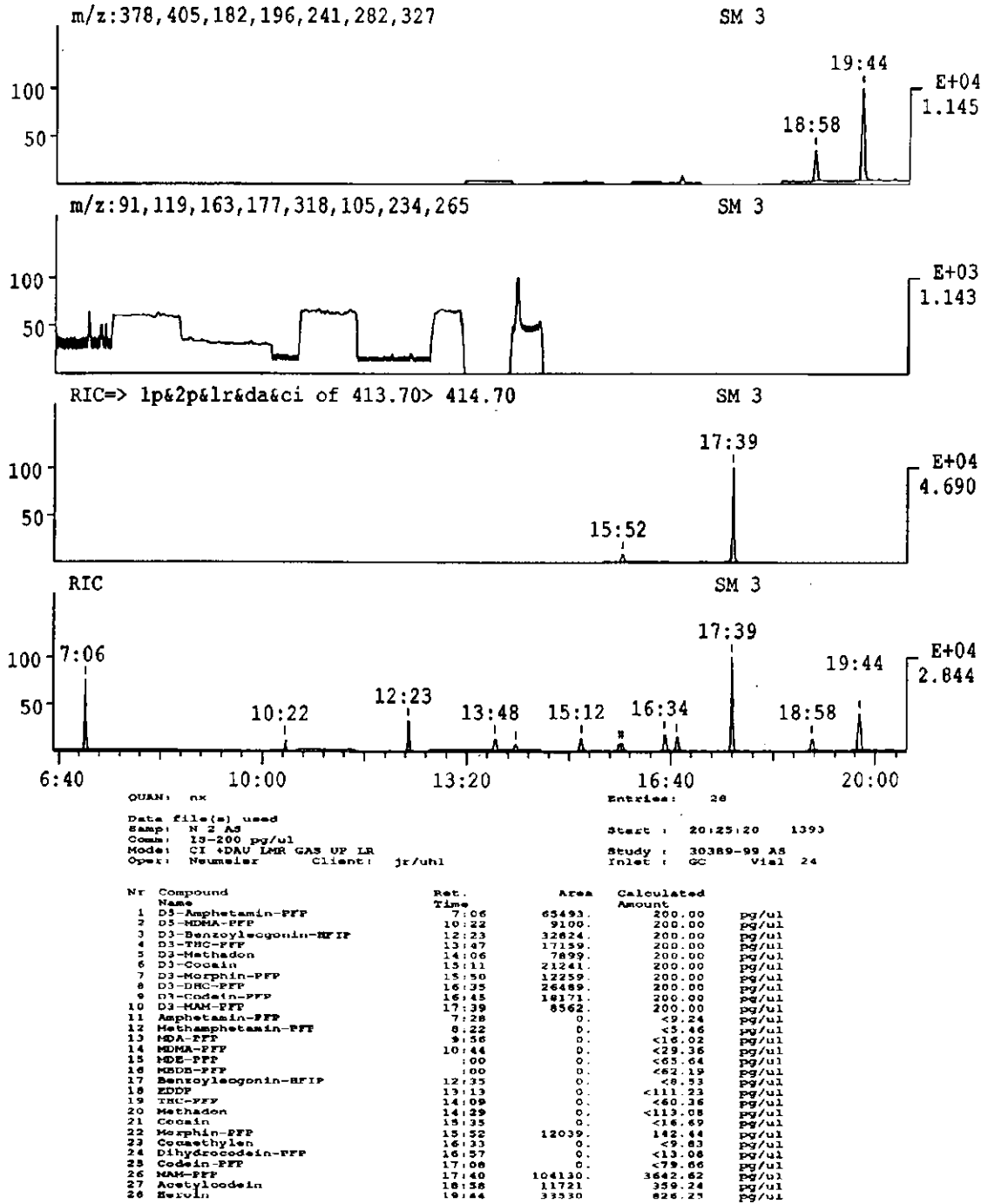
**Fig 1:** Testing of pubic hair as adjunctive specimen: MDMA (1,3 ng/mg hair), MDA (0,35 ng/mg hair), cocaine (4,1 ng/mg hair), benzoyllecgonine (0,3 ng/mg hair) and amphetamine (1,1 ng/mg hair).



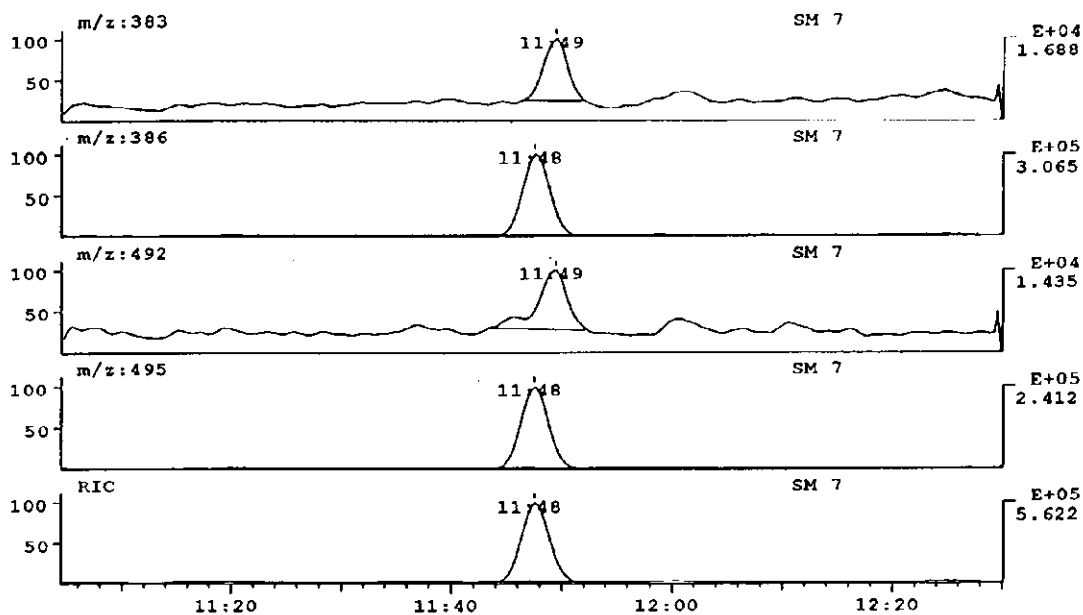
QUAN: btma.q0 Entries: 28  
 Data file(s) used  
 Samp: S 3 RS Start : 05:00:46 1456  
 Comm: IS=200 pg/ul  
 Mode: CI +DAU LMR GAS UP LR Study : 11961-97 RS  
 Oper: Neumeier Client: ai/uhl Inlet : GC Vial 32

Nr	Compound Name	Ret. Time	Area	Calculated Amount	
1	D5-Amphetamin-PFP	7:07	525185.	200.00	pg/ul
2	D5-MDMA-PFP	10:22	123373.	200.00	pg/ul
3	D3-Benzoylecgonin-HFIP	12:22	235400.	200.00	pg/ul
5	D3-Methadon	14:05	161093.	200.00	pg/ul
6	D3-Cocain	15:09	597550.	200.00	pg/ul
7	D3-Morphin-PFP	15:46	115300.	200.00	pg/ul
8	D3-DHC-PFP	16:31	408628.	200.00	pg/ul
9	D3-Codein	16:42	190968.	200.00	pg/ul
10	D3-MAM-PFP	17:34	95897.	200.00	pg/ul
11	Amphetamin-PFP	7:08	9978.	3.80	pg/ul
12	Methamphetamin-PFP	8:22	0.	<1.56	pg/ul
13	MDA-PFP	9:37	0.	<3.00	pg/ul
14	MDMA-PFP	10:44	0.	<8.32	pg/ul
15	MDE-PFP	10:57	0.	<8.05	pg/ul
17	Benzoylecgonin-HFIP	12:23	504336.	428.49	pg/ul
18	EDDP	13:14	0.	<12.95	pg/ul
20	Methadon	14:27	0.	<7.37	pg/ul
21	Cocain	15:10	68903.	23.06	pg/ul
22	Morphin-PFP	16:08	0.	<10.18	pg/ul
23	Cocaethylen	16:09	2612.	0.87	pg/ul
24	Dihydrocodein-PFP	16:32	34327.	16.80	pg/ul
25	Codein-PFP	17:04	0.	<9.28	pg/ul
26	MAM-PFP	17:58	0.	<21.47	pg/ul
27	Acetylcodein	19:28	0.	<24.80	pg/ul
28	Heroin	20:04	0.	<40.91	pg/ul

**Fig 2:** Unusual cocaine/benzoylecgonine ratio determined in a distal segment: cocaine 0,2 ng/mg hair, benzoylecgonine 4,3 ng/mg hair.



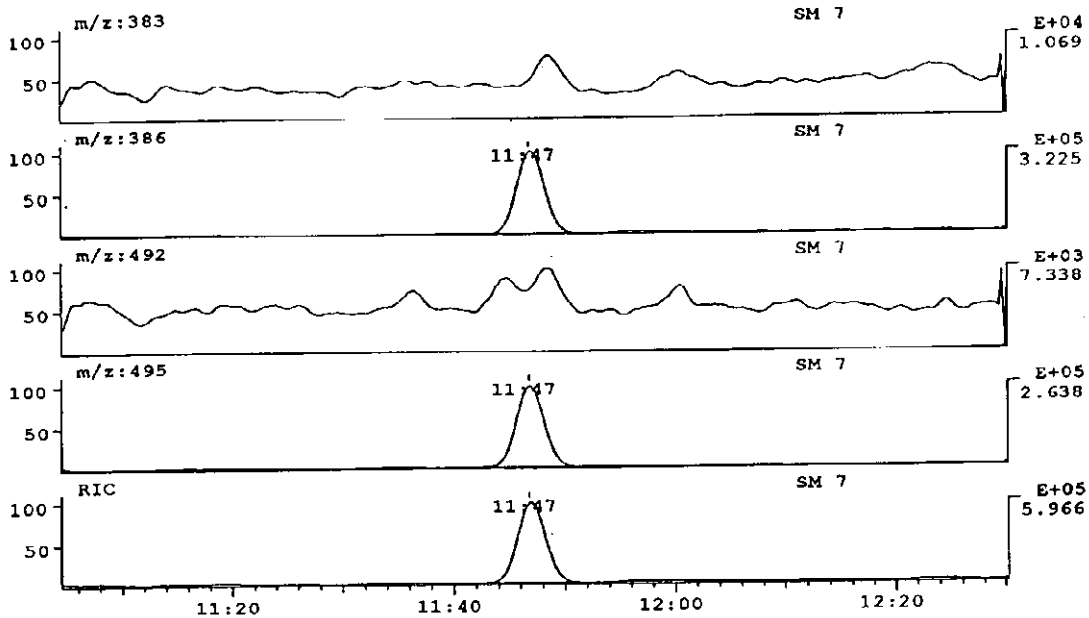
**Fig 3:** Confirmation of a claimed and confirmed addiction to heroin: 6-mono-acetylmorphine: 36 ng/mg; morphine: 1,4 ng/mg, heroin: 8,2 ng/mg and acetylcodeine 3,6 ng/mg hair.



QUAN: thca.q0 Entries: 2  
 Data file(s) used Start : 15:09:44 314  
 Samp: C 1 EL  
 Comm: IS=1 pg/Inj Study : 18078-99 EL  
 Mode: CI -DAU LMR GAS UP LR Inlet : GC Vial 5  
 Oper: Neumeier Client: si/uhl

Nr	Compound Name	Ret. Time	Area	Calculated Amount	
1	D3-THCA-Der.	11:48	3061870.	1.00	pg/Inj.
2	THCA-Der.	11:49	143124.	0.05	pg/Inj.

Fig. 4: Detection of 9-carboxy-THC in low concentration 0,16 pg/mg hair



QUAN: thca.q0 Entries: 2

Data file(s) used Start : 15:37:29 314  
 Samp: C 2 EL  
 Comm: IS=1 pg/Inj Study : 18078-99 EL  
 Mode: CI -DAU LMR GAS UP LR Inlet : GC Vial 6  
 Oper: Neumeier Client: si/uhl

Nr	Compound Name	Ret. Time	Area	Calculated Amount
1	D3-THCA-Der.	11:47	3329912.	1.00 pg/Inj.
2	THCA-Der.	11:48	20114.	0.01 pg/Inj.

Fig. 5: Futile detection of 9-carboxy-THC: an attempt exceeding the capabilities

#### 4. Conclusion

Each analytical toxicologist will appreciate the solid bases of reliable data. He will probably prefer two, three or even more data in order to evaluate the drug history of a certain person.

Generally, a particular information from the mandator about the individual subject can be important for a rendering of an expertise. Aspects that could be considered are for example: When is the person taken into custody? What are the special allegations? Is there a self reported use?

The intended application of the data must be carefully considered. Mostly, a high concentration of metabolites will mostly harmonize with use of a great amount of drugs. As seen in chapter 2 a low concentration could have different reasons.

**Possible criteria for obtaining a positive hair result are recommended by the SHT and HTWG.**

The whole pattern of metabolites should be evaluated in order to diagnose drug consumption. It is essential that all these analytical results are in good harmony.

For rendering an expertise based on hair analysis an analytic expert with diligence, experience and the capabilities of a excellent analytical instrument is a reliable combination. Many unanswered questions remain. The qualitative results are valid, but the interpretation of these results and the quantitative data is still debatable.

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# Criteria that Can Affect the Detection of Doping Agents in Hair

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## 1. Introduction

During the last two years in France, several forensic cases involving doping agents were reported in the popular press, including the nandrolone use by of Djamel Bouras (a judo olympic winner), use by the cyclists participating in the Tour de France, unapproved use in animals slated for food consumption, and several trafficking cases in humans. To document the pattern of drug use, the judges in charge of these cases requested toxicological analyses based on blood, urine, and hair tests (1-3). Although hair is not yet a valid specimen for the International Olympic Committee (IOC), it is accepted in most courts of justice. During the same period, some conflicting results were observed, all involving athletes that tested positive in urine in accredited IOC laboratories and negative in hair in forensic certified laboratories.

A lot of experience has been acquired in the detection of opiates and cocaine in hair. In contrast, there is a serious lack of suitable references to interpret the analytical findings for doping agents. In hair, doping agents concentrations, such as anabolic steroids, corticosteroids, or  $\beta$ 2-agonists, are in the range of picograms per milligram, whereas cocaine, amphetamines or opiates are generally found in the range of several nanograms per milligram (4-7). Therefore, it was the feeling of the Society of Hair Testing to obtain a consensus on hair testing for doping agents (8).

It is clear that there is a great deal of research to be performed before the scientific questions and curiosity surrounding hair drug testing is satisfied. Some of this is due to a lack of consensus among the active investigators on how to interpret the results on an analysis of hair. Among the unanswered questions, five are of critical importance: 1. What is the minimal amount of drug detectable in hair after administration? 2. What is the relationship between the amount of the drug used and the concentration of the drug or its metabolites in hair? 3. What is the influence of hair color? 4. Is there any racial bias in hair testing? 5. What is the influence of cosmetic treatments?

The present report documents scientific findings on these questions, with particular attention to the applications of hair in doping control.

## **2. What is the minimal amount of drug detectable in hair ?**

When using hair in a suspected doping case, particularly when urine of the athlete was positive and hair negative (several cases were reported during the past two years), the question of importance is to know whether the analytical procedure was sensitive enough to identify traces of drugs. It has been always accepted in the forensic community that a negative hair result cannot exclude the administration of the detected drug or one of its precursors (such as norandrosterone or norandrosterone for the metabolites of nandrolone) and should not overrule a positive urine result. Nevertheless, the negative hair findings lends enough ambiguity to the positive urine result, coupled with the sporting consequences for the athlete, that substantial justice refereeing occurs.

For drugs of abuse like cocaine and opiates, several controlled studies were conducted during the past years to evaluate the threshold dose. According to Henderson, et al. (9), the threshold dose for detecting cocaine in hair appears to be approximately 25-35 mg cocaine, administered intravenously. Once incorporated into hair, a single dose of cocaine can be detected for 2 to 6 months. Codeine was detected in hair for 8 weeks after a single oral dose of 60 mg (10). In contrast, with anabolic steroids we were not able to identify nandrolone nor nandrolone undecanoate in the hair of a 37-year old man, receiving a single intramuscular injection of 50 mg nandrolone undecanoate, although his urine remained positive for norandrosterone and noretiocholanolone, the nandrolone metabolites, for at least 8 months. Hair was tested 2 and 6 months after administration, and the limit of detection for nandrolone in hair was 1 pg/mg. The same observations were recently made by Segura, et al. (11) who did not detect nandrolone, nandrolone decanoate nor testosterone esters (enanthate, propionate, undecanoate) after a single dose administration. However, in the same study, hair analysis revealed an increase of more than 2 fold in testosterone concentration in hair from subjects treated with the enanthate and propionate esters as compared with the pre-dose samples.

The physiochemical properties of drugs on the incorporation rates into hair are of paramount importance. The influence on drug incorporation of melanin affinity, lipophilicity, and membrane permeability was evaluated by Nakahara, et al. (12). Despite their high lipophilicity, steroids have quite low incorporation rate into hair, suggesting that the higher incorporation rates of basic drugs (cocaine, amphetamines ...) than neutral (steroids, benzodiazepines, cannabinoids ...) or acidic ones are strongly related to the membrane permeability of the drug based on the pH gradient between blood and the acidic hair matrix. This can perhaps explain the low steroids concentrations that are reported in hair, and therefore, it is not surprising that a single exposure cannot be demonstrated. When compared, clenbuterol (with a primary amine function) and salbutamol, two  $\beta_2$ -agonists, showed 2 different pattern of accumulation in guinea-pigs hair, largely in favour of clenbuterol, even with a 10 times lower dose (13).

Therefore, until laboratories will have sensitive enough methodologies to detect a single use of steroids, care should be taken to compare urine and hair findings. For anabolic steroids to have an appreciable performance enhancing effect, they must be chronically administered, in contrast to the immediate stimulant properties of cocaine or amphetamine. Repeated use would favor identification by hair analysis.

The knowledge of the minimal quantity that can be identified in hair is useful when interpreting the analytical results. Although the following example was reported in newspapers, it is indicative of the differences between sport rules and forensic cases and the place that hair has in such situations. During the summer 1999, the Cuban high jumper Javier Sotomayor tested positive in urine for cocaine. The athlete claimed that he only drunk one cup of tea coming from Peru (Reuters agency, Wednesday August 4, 1999). For the International Athletic Federation and IOC, the presence of cocaine or metabolites in urine is enough to be considered as doping, irrespective of the manner of use. Our laboratory was involved in a similar case. The judge asked if it is possible to find cocaine metabolites in urine after tea consumption. It was necessary to establish the urinary excretion of cocaine and metabolites after oral administration of tea (Mate de Coca, Zurit, ASA Alimentos) containing 3.9 mg of cocaine. The hair of the same subject was tested 1 month later (first 1-cm section) and was negative (LOD: 10 pg/mg), suggesting that the minimal detectable dose in hair is higher than 4 mg of cocaine. A hair test of Sotomayor would aid in confirming his claim (no cocaine abuse and negative hair test) or, at the opposite (positive

hair test) would demonstrate cocaine abuse, as a single "line" of street cocaine (e.g., 50-100 mg) would be detectable.

### **3. What is the relationship between the amount of the drug used and the concentration of the drug or its metabolites in hair ?**

A common interpretative question posed when positive drug test results are reported is if it is possible to put a quantitative finding into context. In other words, "Is this a lot of drug?" or " Did this person use a lot of drug or just a little ? " A standard answer to either of these inquiries is that a positive hair test result can be interpreted as meaning that the donor has chronically or repetitively used the drug identified in the hair; however, chronic or repetitive are not defined in the same way by all individuals.

To document hair findings, in term of amount and frequency of consumption, essentially two approaches have been developed. One (14) is to compare the measured concentration with a level (low, medium or high) of consumption obtained from the habitual use declared by a large number of addicts. For example, according to Pépin and Gaillard (14), a cocaine concentration in hair lower than 4 ng/mg indicates a low cocaine consumption. The second method, used in our laboratory, is to compare the positive result with the distribution of concentrations for the drug in positive hair that are regularly tested. However, this can only be done for drugs that are tested in a routine basis (more than 100 samples per year).

In case of doping agents, historical data are not available, and therefore its not possible to estimate the pattern of drug use. At this time, only data for testosterone (5, 15-17) and dehydroepiandrosterone, DHEA (18), two endogenous substances for which physiological concentration ranges in human hair are available in the literature. These concentrations are generally in the range 1-10 pg/mg, including hair specimens obtained from high level (Olympic) athletes. A higher testosterone concentration can be indicative of use, and further investigations are necessary to identify in hair the esters of testosterone (2, 4, 11, 17).

There is a need to put a quantitative interpretation into context, particularly for forensic applications. In a case of suspected anabolic trafficking by bodybuilders (3), the judge asked us if the seized drugs (tablets and ampoules) supported personal use or if it was for resale. Hair analysis was used to demonstrate abuse by the bodybuilders, without the possibility of indicating the frequency of exposure and the dosage. Another example of the medico-legal aspects of doping (19, 20) is cardiovascular complications, that can be fatal, or psychiatric problems associated with enhanced aggressivity. In such cases, toxicological investigations are necessary to document the side-effects of steroids, and the pattern of exposure is often requested. The quantitative findings are of less importance in comparison with qualitative identification in doping control. However, it can be useful to estimate the habit of consumption.

Some papers reported for drugs of abuse or pharmaceuticals, that there is a significant dose-concentration relationship between dosage and concentration in hair, while some other failed (21). The lack of correlation can be explained by the following factors : variability of hair-growth cycle, influence of cosmetic treatments and hygienic practices, uncertainty of dosages ingested by abusers, typical underestimation of self-reported doses, unknown purity of compounds, and considerable variation in uptake of drug from blood to hair, rate of sweating and amount of apocrine and sebaceous gland secretions between individuals. Until controlled studies are done for doping agents (is this possible?), it seems difficult to use hair analysis to calculate the dose, time or duration of drug use.

#### **4. What is the influence of hair color?**

Melanins are responsible for the color of hair, as determined by the quantity of pheomelanin and eumelanin present in hair. Black and brown hair contain more eumelanin than red and blond hair. Current evidence suggests that melanin is the principal component of binding of drugs. Several studies performed with animals and humans reported that organic compounds are excreted preferentially in dark hair and suggested that excretion of drug is closely linked to the presence of melanin (22).

In 1996, Höld et al (23) demonstrated that stanozolol is incorporated preferentially into rat pigmented hair. This was also noticed for nandrolone in guinea pigs (13) or clenbuterol in calves (24). After administration of the same daily dose of 10 µg clenbuterol during 25 days to 9 volunteers, Gleixner et al (25) observed that the clenbuterol residues were lower in light hair (blond, grey) than in dark hair (black, brown).

More recently, Kronstrand et al (26), after single oral administration of 100 mg of codeine to 9 subjects, concluded that the incorporation of codeine into hair is affected by its melanin content and that the relationship is exponential.

All these studies suggest that the hair color or melanin content may be the major determinant of drug binding and, consequently, may result in color bias in hair testing.

#### **5. Is there any racial bias in hair testing ?**

Several researchers have demonstrated that different hair types incorporate differing amounts of drugs when exposed under identical conditions (27-29). These studies have suggested that coarse, dark hair may incorporate more drug than fine brown or blond hair. Henderson et al (27) showed that non-Caucasian subjects (n=9) incorporate 2.9 (in average) times more deuterated cocaine into their hair as did the Caucasian subjects (n=6), in equivalent experimental conditions. These findings were consistent with those of Cone et al (30), who found that cocaine concentrations in both head and arm hair of African-American subjects were significantly higher ( $p < 0.05$ ) than those found in Caucasian subjects.

Bias in drug testing can be defined as an increased likelihood of detecting one group of individuals over another when both have comparable use. There is a debate (31, 32) among the forensic community about racial bias as a problem may arise in the use of hair analysis procedures that identify more African-American drug users than Caucasian drug users. Thus, for a low-use population, such as athletes, there is a potential risk to identify more frequently a population than another. The introduction of positive cut-offs concentrations will not solve this problem. Kidwell (31) has recently proposed that the uptake and retention of drugs into hair is culturally (cosmetic hair treatments, hair care habits, removal by personal hygiene) rather than genetically (porosity of hair) influenced. In consequence, the author has suggested methods that use either the incorporation of dyes or of drug homologs to reduce bias in hair testing. This means that a correction for potential bias can be achieved in a special requested situation.

In conclusion, for the interpretation of results, the higher accumulation of several substances in black hair as compared with blond hair will need to be addressed in order not to discriminate specific populations due to their hair color, resulting in inequity during doping control (33).

## **6. What is the influence of cosmetic treatments?**

Interpreting the results from hair analysis for drugs can be difficult, because this is a relatively new methodology and there are many issues that still remain to be answered. One important issue is the stability of drugs in human hair. Many factors are likely to affect the concentration of drugs in hair and thus complicate the interpretation of results from the analyses. These factors include cosmetic treatments of hair (34, 35). In all the studies, the authors observed that drug concentrations declined dramatically after cosmetic treatment (bleaching or permanent waving) and UV exposure, concluding that both the chemical composition of different cosmetic agents and the methods applied in hair treatment affect drug stability. When comparing treated hair by bleaching or dyeing before sample collection with the corresponding non-treated hair strand, the mean differences in drug concentrations were in the range 30-80 % for opiates, cocaine, cannabis and nicotine. These differences depended not only on the type of cosmetic treatment, as bleaching produced higher decreases than dyeing, but also on the degree of hair damage, i.e. the more damaged the hair, the larger the differences in the concentration levels of drugs.

In case of cosmetic treatment, the hair analysis results appear to be biased rather towards false negatives than towards false positives. A recent study performed by Cirimele, et al. (36) demonstrated that the daily use of a shampoo containing cannabinoids, including THC, would not lead to a positive hair test.

During the past years, numerous athletes, particularly cyclists, have colored their hair, but it was rather considered as an aesthetic approach rather than an evasive procedure. However, these treatments have consequences on the hair test results. Gaillard and coworkers (2) only found positives for anabolic agents (nandrolone, testosterone undecanoate) in two non-bleached hair samples. Our laboratory, missed identifying testosterone (LOD 0.5 pg/mg) in several specimens bleached with hydrogen peroxide, and therefore we recommend the collection of pubic hair in case of bleached or colored head hair. However, care should be taken when sampling hair from other anatomic regions, as the concentrations can be highly variable according to the specimens. We were very surprised when comparing the physiological concentrations of DHEA and testosterone in hair collected from the head, pubis, and axillae. An unusual high amount of DHEA was sequestered in axillary hair. Generally, the overall pattern of drug is similar in head and non-head hair, and with some exceptions, the highest drug and metabolite concentrations are observed in pubic hair, while the lowest are found in axillary hair (37).

## **7. Perspectives and conclusion**

In case of doping control, drugs are screened in urine specimens according to validated standard operating procedures in accredited laboratories. As forensic laboratories can be involved in testimony dealing with doping agents, the idea of using hair for doping control has emerged as hair analysis has been accepted in court in other cases. Courts can request additional informations on the pattern of use of doping substances, such as during the 1998 cycling Tour de France where blood, urine, and hair were simultaneously collected. Hair can both confirm repetitive abuse and identify the exact nature of the parent compound (e.g., nandrolone, norandrosterone or norandrosterone, in case of positive urine for norandrosterone). Moreover, long-term use (over several months) of restricted compounds (only authorized under specific conditions and for a short period), such as salbutamol or corticoids, can be documented through hair analysis. The

determination of testosterone esters in hair should allow a definitive unambiguous confirmation of the administration of exogenous testosterone.

However, some issues have to be discussed before considering hair as a valid specimen by the I.O.C. and the International Sport Federations. The relationship between urine and hair results is not yet established and negative hair result does not mean "no doping". The potential ethnic discrimination must be evaluated to avoid inequality during doping control.

In contrast with the problems associated with cosmetic treatments or the absence of specimen (bald or fully shaved subject), external contamination does not constitute a major trouble when testing for doping agents.

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# Analysis of Hair Samples for $\beta_2$ -Agonists

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## Introduction

The International Olympic Committee's Medical Code outlines classes of banned substances that are subject to restrictions and provides a list for each category.  $\beta_2$ -agonists are listed in the category anabolic agents.

Associated with the name of the former world champion in athletics Kathrin Krabbe clenbuterol became wellknown in the public. Despite her urine sample was positive for clenbuterol she was not banned for a doping offence but she was convicted for a violation against the German drug law.

The potent  $\beta_2$ -agonist clenbuterol (4-amino- -[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol; or 1-(4-amino-3,5-dichlorophenyl)-2-(tert-butylamino)-1-ethanol,) is used as a sympathomimetic bronchodilator in the management of asthma and obstructive lung disease. Proprietary names are Broncodil, Ventolase, Spiropent.

Moreover, when used at doses being several times higher than the therapeutic application, clenbuterol has anabolic effects stimulating protein deposition in striated muscle (20% increase). The  $\beta_2$ -agonist increases simultaneously the energy expenditure hence reducing muscle glycogen and body fat deposition (20% decrease) [1,2]. Clenbuterol induces a true hypertrophy of muscle (type II fibers) and an increase in the 1-glycolytic capacity of a muscle in a whole, giving rise to an increase in force and a reduction in relaxation time [3].

Administration of this  $\beta_2$ -agonist in veterinary therapy was allowed in Germany until June 1997.

The brominated analog of clenbuterol is brombuterol, being an illegal substance in Germany.

## Detection of clenbuterol in hair

### 2.1 Detection of clenbuterol in human hair samples [4].

Hair samples from 67 volunteers were analyzed for clenbuterol by enzyme immunoassay (EIA) and by HPLC. Clenbuterol residues could be detected in hair from 9 volunteers who took a known therapeutic dosage. The concentrations varied from 25-160 pg/mg hair. The accumulation in dark hair (150 pg/mg hair) was markedly higher than in light hair (30 pg/mg hair), demonstrating the importance of hair pigmentation of the binding of clenbuterol to the hair matrix.

### 2.2 Detection of clenbuterol in hair samples of calves [5,6]

9 calves received oral doses of clenbuterol. 6.7 mg of the  $\beta_2$ -agonist was administered daily for a period of 15 days, the overall dose being 100 mg [5]. The hair samples were pulverized, after clean-up by solid phase extraction the detection was performed by GC/MS. The concentrations were in the range of 20-4372 pg/mg hair.

In another study [6] 3 calves were fed with clenbuterol in doses of 5  $\mu$ g/kg body weight twice daily during 3 weeks. The hair samples were analyzed using GC/MS, the concentrations ranged between 135-1133 pg/mg hair.

### 2.3 Detection of clenbuterol in hair samples of guinea pigs [7]

Guinea pigs received a dose of 120  $\mu$ g/kg clenbuterol intraperitoneally for 15 days. Analysis of the collected hair samples were performed by GC/MS.

## Detection of brombuterol in hair

An authentic case example will demonstrate the detection of brombuterol in hair [8].

The owner of a big calf breeding farm was suspected by the prosecutor of using brombuterol (Fig.1) illicitly to increase growth of calves. Several seized samples were to be analyzed in the laboratories of the Bavarian State Bureau of Investigation (Bayerisches Landeskriminalamt) to ensure that these animals were treated with brombuterol. Among the seized items were some vials containing a solution, some used syringes and hair samples taken from 61 calves.

The results will be discussed.

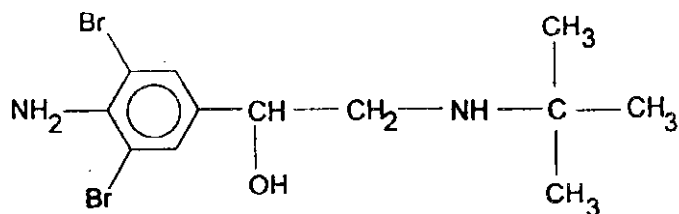


Fig.1: Molecular structure of brombuterol

### 3.1 Experimental

#### 3.1.1 Preparation of hair samples

The hair samples were placed into a Eppendorf microtube and washed by vortexing in an ultrasonic bath twice with 1ml n-hexane (60 s), twice with 1 ml acetone (60 s). The prewashed samples were transferred into a folded filter, remaining residues of solvents evaporated at room temperature. Each specimen was then cut into 1-2 mm sections, and a 15 mg quantity was weighed into an Eppendorf microtube. After addition of 0,5 ml methanol, the mixtures were vortexed in an ultrasonic bath (40°C) for 60 min and following centrifugation for 15 min. The mixtures were allowed to stand overnight. The methanolic phase was decanted, transferred into a vial and evaporated at 40°C under a gentle stream of nitrogen.

The residues obtained were then derivatized with 100 $\mu$ l of a mixture (1:1, v/v) of acetonitrile/hexamethyldisilazane (HMDS) for 45 min at 75°C. The mixture was dried again under nitrogen at 25°C and reconstituted in 100 $\mu$ l methylenechloride/HMDS (95:5, v/v). A 1 $\mu$ l quantity of the derivatized samples were then injected into the GC/MS/MS system.

#### 3.1.2 Instrumentation

The analyses were performed with a Finnigan triple stage quadrupole mass spectrometer (TSQ 700) coupled with a DEC station 2100 (GC/MS/MS). The tandem mass spectrometer is linked to a Varian 3400 gas chromatograph equipped with an A 200 S autosampler.

A fused-silica capillary column (J&W, DB-5, 30 m x 0,32 mm i.d., 0,25  $\mu$ m film thickness) was used for GC separation.

The injector was controlled with a cold injection system and with solvent purging (Gerstel, Mühlheim, Germany). Injection temperature was 40°C, 40-60°C at 2°C/min, 60s at 60°, 60-

280°C at 12°C/s and temperature was kept at 280°C for 60s. The interface was maintained at 280°C. Helium was used as carrier gas with a flow rate of 1,0 ml/min.

The mass spectrometer was operated in the negative ionisation mode (NCI) using methane as the reagent gas set at 220 000 millitorr. The electron multiplier was operated at 2000 V.

### 3.2 Discussion of the results

Among the seized items from the calf breeding farm were vials containing a solution and syringes contaminated with unknown material. Analyses of these items were conducted with thin-layer chromatography, gas chromatography (ECD) and gas chromatography/mass spectrometry. The component identified was brombuterol.

Testing of the hair samples collected from 61 calves was utilized as a corroborative evidence. A positive result for residues of brombuterol would be a sufficient evidence.

After derivatization by a silylating compound (HMDS) brombuterol could be detected in 13 hair samples in the SIM mode using negative chemical ionisation (Fig.2). The semiquantitative determination of the analyte is conducted with external reference standard brombuterol. The concentrations ranged between 10 pg and 100 pg/mg hair. These findings are of the same magnitude comparable with data determined in hair specimens of calves being subjected illegally clenbuterol administration [5,6]. It is about 5-10 fold above the dosage used for therapeutical application.

Human consumption of the  $\beta_2$ -agonist brombuterol from treated animals is presumed to be a significant health issue [9].

It should be mentioned that there is a difference in reactivity between clenbuterol and brombuterol. Unlike its chlorinated analog clenbuterol producing a bistrimethylsilyl substituted derivate, only a single trimethylsilyl group is attached to the brombuterol moiety.

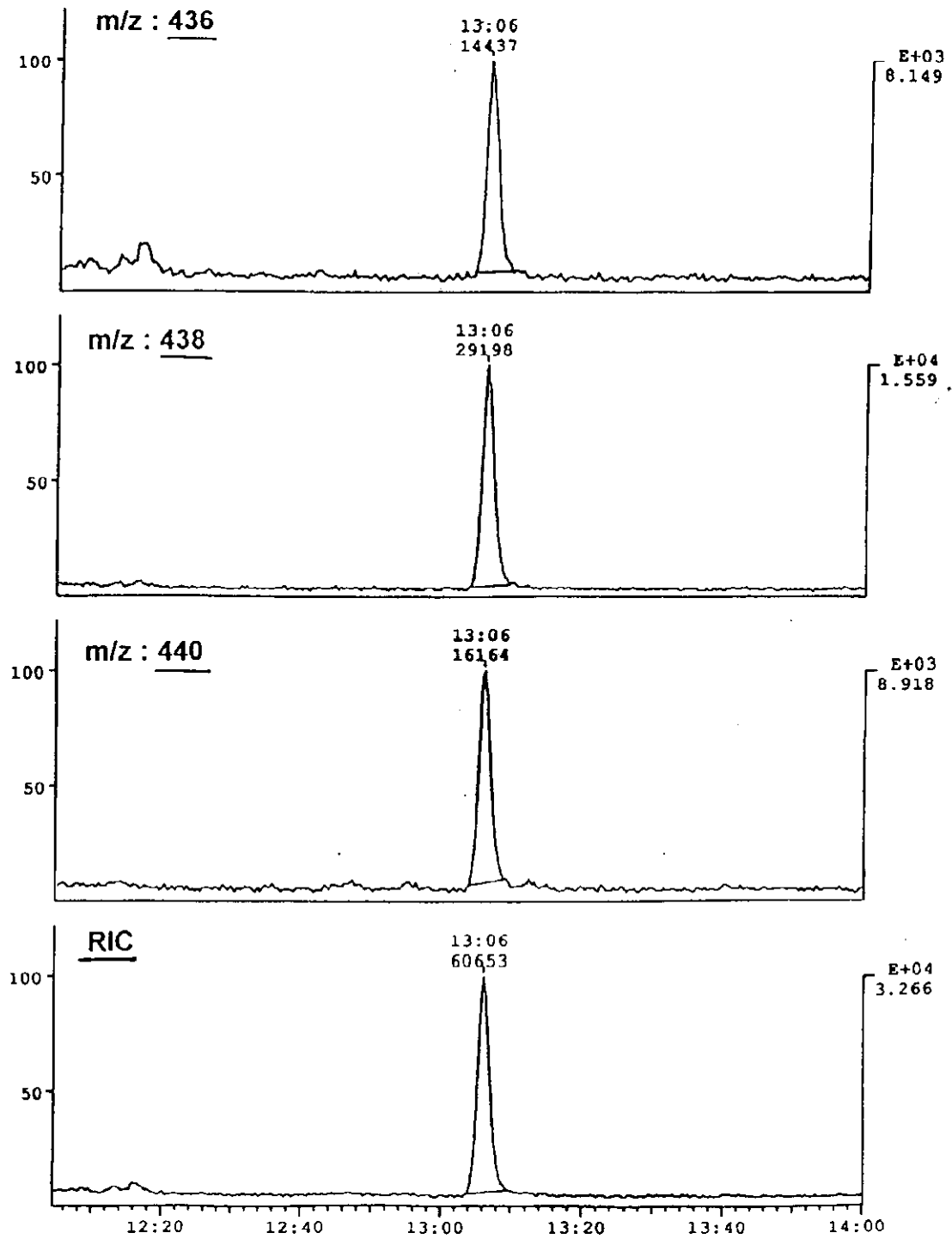


Fig.2: SIM chromatograms and reconstructed ion chromatogram (RIC) of a hair sample collected from a calf positive for brombuterol.

Despite the opportunity of utilizing a GC/MS/MS (tandem mass spectrometry) and selected reaction (SRM) monitoring analyses were conducted by GC/MS in the SIM mode.

These results show that also hair analysis can be utilized for the detection of  $\beta_2$ -agonists in sports doping.

### Acknowledgement

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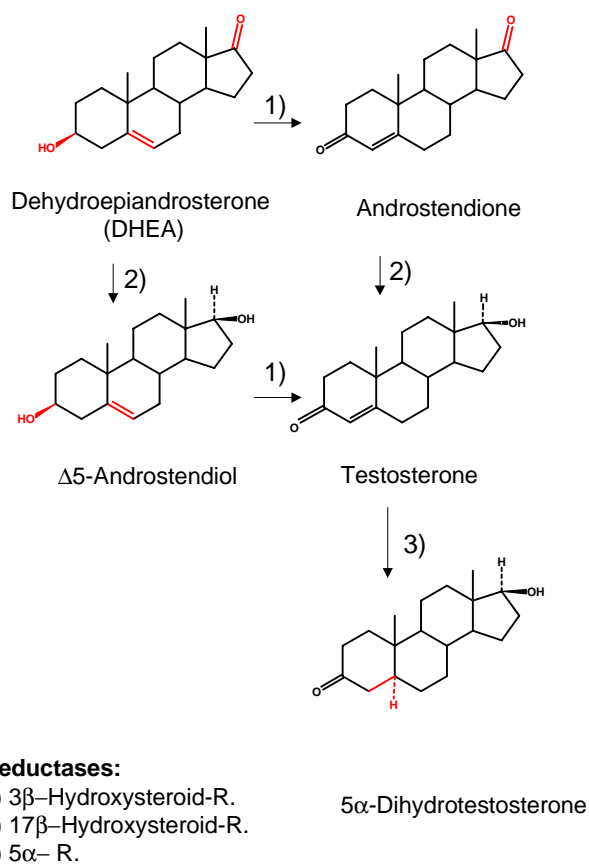
# Testing Endogeneous Steroids in Hair

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During the examining of human hair on illegal drugs, some testosterone metabolites like androsterone and epi-androsterone could also be detected by gaschromatography/mass spectrometry (1). In 1989 it did not seem promising to use the quantification of hormones in hair to prove the additional application of testosterone or its derivatives as anabolic agent.

Many steroids which are close to testosterone in the biosynthetic pathway of testosterone (fig.1) are commercially available and abused as anabolics (2).



In 1995 Scherer (3) determined the androgens androstenediol, testosterone, androstenedione, dehydro-epiandrosterone, and dihydro-testosterone as well 17 $\alpha$ -hydroxy-progesterone, a precursor of the androgens. After enzymatic digestion the solution was extracted on Sephadex LH-20. The extracts were derivatized with HFBA and determined by GC/MS. The following results were obtained:

Androstenediol	9.1 - 18.7 ng/g
Testosterone	12.9 - 24.3 ng/g
Androstenedione	4.9 - 14.6 ng/g
Dehydro-epiandrosterone	21.3 - 56.0 ng/g
Dihydro-testosterone	2.1 - 7.9 ng/g
17 $\alpha$ -hydroxy-progesterone	1.3 - 6.9 ng/g

These preliminary studies did not lead to a procedure to distinguish between hair of male and female subjects, or of children before and after puberty. In a subsequent study the author found a significant sex difference with 2.7 ng/g (2.5 - 4.2) in male and 1.7 ng/g (1.0 - 3.4) in female hair (4).

In 1998 Wheeler et al. (5) determined testosterone levels in the hair of 22 male and 19 female subjects. The sample was digested in sodium hydroxide. The solution was cleaned up by HPLC, which provided a separation between testosterone, androstenedione, and dihydrotestosterone, before the analytes were quantified by radioimmunoassay. The authors found a clear difference between testosterone concentrations found in hair collected from men (12.9 - 77.7 pmol/g) and those found in hair from women (<0.9 - 10.8 pmol/g). The concentrations of children lay in the range of the female subjects.

Deng et al. (6) published results of anabolic steroids including some endogenous compounds in head hair of 7 adult white male abusers. The hair was digested in 1 N NaOH, the pH adjusted to 5.6 with 6 N HCl and extracted over a C18 Isolute SPE cartridge. The compounds were quantified by GC/MS after derivatization with BSTFA/1% TMCS. Among other compounds the following steroids were detected:

Dehydromethyltestosterone	up to 0.02 ng/mg
Testosterone	up to 0.15 ng/mg
Progesterone	Up to 0.04 ng/mg
Epi-testosterone	0.05 in 1 case
Methyltestosterone	Up to 0.17 ng/mg
Dihydromethyltestosterone	In traces
Estradiol	Up to 0.11 ng/mg

The authors stated that the testosterone concentration of 0.15 ng/mg clearly reflects abuse.

Hair samples of numerous steers ("Fleckvieh" with white and black hair), cows, bulls, female and male calves were examined for estradiol and testosterone by Gleixner et al. (7). The compounds were extracted from hair with liquid-liquid and solid-phase extraction procedures. The extracts were cleaned up by HPLC and estradiol and testosterone were quantified by an enzyme immunoassay. The estradiol concentrations in the hair of steers, cows, and bulls lay in the range of 1 ng/g or below. The testosterone concentrations in the hair of steers was about 3 ng/g, in hair of cows 6 ng/g, and in the hair of bulls in average 15 ng/g. Significant differences were found in white hair (8 ng/g) and black hair (33 ng/g). There was no significant difference between the concentrations in hair of male or female calves, or between the concentrations of hair with different colours. The estradiol concentrations as well as the testosterone concentrations lay below 10 ng/g.

Together with testosterone-esters Kintz et al. (8) determined the testosterone concentrations of 26 control subjects. After decontamination with dichloromethane, 100 mg of hair was incubated in 1M NaOH in the presence of 1 ng of testosterone-d-3. After neutralization, the extract was purified using solid-phase extraction with isolute C-18 columns followed by liquid-liquid extraction with pentane. After silylation, testosterone was analyzed by gas chromatography-mass spectrometry. Concentrations were in the range 1.2 to 11.4 pg/mg with a mean value of 3.8 pg/mg.

Dehydroepiandrosterone (DHEA) is a steroid hormone naturally produced by the adrenal glands and by the ovaries. DHEA can be converted into other hormones, including estrogen and

testosterone (fig. 1). In the United States, DHEA is classified as a nutritional supplement. This is not the case in France, where the drug is listed as a doping agent. As athletes can abuse DHEA to benefit from its conversion to testosterone, there is a need to establish the physiological range of DHEA concentrations in human hair. DHEA was investigated by Kintz et al. (9) in hair obtained from 27 control subjects, including 15 males and 12 females aged 17-42 years. After decontamination with dichloromethane, 100 mg of hair was incubated in 1 M NaOH in presence of 1 ng of testosterone-d<sub>3</sub>. After neutralization, the extract was purified using solid-phase extraction with Isolute C18 columns and subsequent liquid-liquid extraction with pentane. After silylation, DHEA was analyzed by gas chromatography/mass spectrometry. Results were linear in the range 1-20 pg/mg. Relative extraction recovery was 91.6% with a limit of detection of 0.5 pg/mg. Concentrations were in the range 1.2-6.7 pg/mg (mean value of 4.3 pg/mg) and 0.5 to 10.6 pg/mg (mean value of 5.3 pg/mg) for the males and females, respectively.

In a further study, Kintz et al. (10) determined the testosterone and DHEA levels in hair of different origin, head, axillary, and pubis. In accordance with results of hair examinations after drug abuse, axillary and pubic hair show often higher concentrations than head hair, as shown in the following table.

Subject	Testosterone			DHEA		
	Head	Axillary	Pubis	Head	Axillary	Pubis
1	4.1	182	3.9	3.2	1984	1363
2	3.3	39	3.5	6.7	719	90
3	2.2	17	4.7	4.9	2735	60

Table Ref (10)

## Conclusion

Endogenous steroids, including precursors and metabolites of testosterone can be detected in human hair. The concentrations lay in the range up to 100 pg/mg in head hair, but different examiners found different levels of testosterone in control groups. Testosterone concentrations of more than 100 pg/mg indicate an abuse of testosterone-derivatives, but more experience is needed to prove exogenous testosterone. The introduction of a ratio-limit of testosterone/epitestosterone has failed until now. The most elegant way to prove an intake of substances which are also endogenous, is to detect the parent drug, e.g. the testosterone esters.

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# Detection of Exogenous Anabolic Steroids in Hair

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## Introduction

The definition of exogenous steroids is certainly arbitrary. Typical exogenous steroids of abuse may be considered endogenous in trace amounts in certain individuals (boldenone) or under specific circumstances (nandrolone in pregnancy), and the endogenous character of anabolic steroids is doubtful, if they are abused in excessive overdose amounts. However any possible chemical modification (incl. esterification) of endogenous substances outside the biosynthetic pathways of steroids was included into the considerations.

The purpose of this review was to summarize and compare technical aspects, analytical results, data evaluations and resulting conclusions published in recent papers.

## Extraction

There is a common agreement that extraction of hair sample is done by

- Sodium hydroxide disintegration for all steroids with sufficient chemical stability. Hydroxide concentration, temperature and digestion time applied to enable complete homogenisation of hair sample differ considerably.
- Methanol extraction for all steroids, which may undergo hydrolysis if treated with hydroxide, especially steroid esters. The procedures were adapted by elevation of temperature, ultrasonication and addition of modifiers (trifluoroacetic acid [1]).

Sample pre-treatment was applied in some cases although a passive contamination is less probable. Considerable wash-out effects have been observed after sample washing with aqueous dodecyl sulfate [2] diminishing the concentration of the steroid in hair.

## Clean-Up Procedure

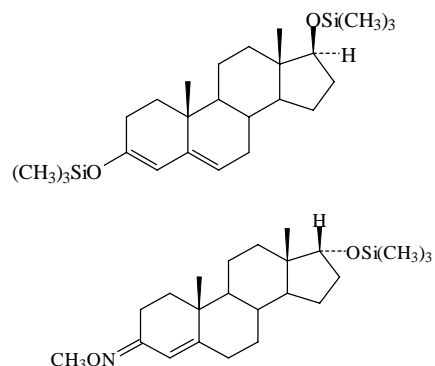
The application of clean-up procedures includes

- liquid-liquid extraction, (organic solvents: ethyl acetate, ether, hexane)
- solid phase extraction, using
  - C18-silica – as most versatile technique for routine purposes, [3], [4],
  - NH<sub>2</sub> - for fixation of polar impurities, [5]
  - XAD2 – unspecific removal of polar substances, [6]
  - sephadex – to clean the extract
  - lipidex - enrichment and clean-up of derivatives [7]
- HPLC clean-up procedures using reverse phase liquid chromatography [6] permits to obtain clean fractions which may be analysed independently.

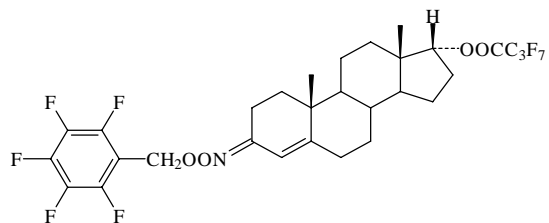
## Derivatisation

There is a wide spectrum of derivatisation techniques [8] which may be summarised with respect to steroids analysis in hair to the classes:

- per-TMS derivatives of 17 $\beta$ -hydroxy,3-keto-steroids (incl. stanozolol) and mono TMS derivatives of the respective esters, [3] [1] [9]
- MO-TMS derivatives 17 $\beta$ -hydroxy,3-keto-steroids [2], [7], [4]
- O-TMS,N-HFB-derivative of stanozolol, [10] [4]



bis-HFB or HFB-PFBO esters of 3-keto-steroids and mono PFBO derivatives of the respective esters [11] applied to enable NCI-MS.



The advantages of per-TMS derivatives are fast and robust reactions with single reactions resulting in uniform products (only few exceptions forming isomers: mesterolone [9], oxymetolone, mestanolone, androstnolone [4]). The mass spectrum is often poor and characterised by intense molecular ions. Methoxime or oxime derivatives are less stable and form two isomers (syn and anti), but their MS/MS sensitivity is often improved due to diagnostic fragmentation patterns and combined with perfluoroacylic-derivatisations they are suitable for NCI/MS.

Abbreviations of derivatives:

TMS	trimethylsilyl
MO	methoxime
HFB	heptafluorobutyryl
PFBO	pentafluorobenzyl oxime

## Detection Techniques

Early attempts of identification of steroids in hair were based on immunoanalysis techniques like ELISA [12] or EIA after HPLC separation [13]. The combination of HPLC with scintillation detection of radio-labelled compounds is probably exceptional, [2] whereas the combination of GC with conventional (unit resolution) mass spectrometry appeared to be the most frequent detection technique. Electron impact ionisation was applied as versatile principle for detection of many exogenous steroids including steroid esters [3], [7], [2]. Detection based on chemical ionisation was applied in positive (steroid esters [11]) and negative mode (steroids especially stanozolol [11]). High resolution mass spectrometry in EI ionisation mode was employed to identify anabolic steroids with improved sensitivity and specificity [6, 9]. The application of tandem mass spectrometry is another option to improve detection limits, were all kind of steroids were investigated in EI ionisation mode with triple stage quadrupole [5], sectorfield-quadrupole [6], or ion trap [1] coupling.

## Qualitative Results

There are many anabolic steroids included into screening experiments and detectable at adequate detection limits, like

- 17 $\alpha$ -alkyl-derivatives (bolasterone, metandienone, methyltestosterone, stanozolol, mestanolone etc.)
- 17 $\beta$ -esters (acetates, propionates, isocaproates, cypionates, phenylpropionates, enantates, decanoates, undecanoates and dodecanoates of testosterone, nandrolone and metenolone)
- A-ring modifications of testosterone (clostebol, mesterolone, metenolone, oxymetolone etc.)
- Others (nandrolone, trenbolone).

Compared to these exogenous steroids, which are potentially detectable, there are only some of them identified in real cases (humans, usually bodybuilders). The spectrum of these depends mainly on the popularity of / access to the steroids and occurrence in 'real' cases is more or less by chance:

- Methandienone [7]
- methyltestosterone and metandienone [4]
- stanozolol and nandrolone [14]
- metandienone, stanozolol, metenolone enantate, nandrolone decanoate, testosterone enantate, propionate, isocaproate, phenylpropionate and decanoate [6]
- testosterone undecanoate [5]

Few studies to control the incorporation of steroids into hair were directed to stanozolol, nandrolone and testosterone esters. [1, 2, 10-12]

Little is known about the identification of metabolites in hair. 3'-OH-stanozolol was detected in concentrations 30 times lower than stanozolol and epi-methandienone and 6 $\beta$ -OH-methandienone were detected in trace amounts of a bodybuilder's hair sample which was clearly metandienone-positive. [6]

## Quantification

Quantitation limits examined in systematic studies are in the low pg/ml range. The published numbers vary between 0.08 pg/mg for nandrolone GC/MS-MS and 200 pg/mg, logically correlated to sample preparation, derivatisation and especially to the mass spectrometric detection method.

The concentration of the steroids detected range from 13.4 pmol/g up to several ng/mg. However they are in general in the order of magnitude of the detection limits. For the random findings in forensic cases, the concentration of exogenous steroids were in the low pg/mg concentration range too.

There is a general tendency that concentration in steroid esters are higher than the corresponding concentrations in the hydrolysed steroid. Moreover the fact that concentrations in pigmented hair are higher than unpigmented samples is undisputed. There are clear experimental evidences for an accumulation of stanozolol [10], nandrolone-laurate[2] and nandrolone [11] in pigmented tissues.

## Species and Dosages

The major part of human studies was based on forensic cases, abusers or volunteers amongst bodybuilders. Typical dosage recommendations as well as urinary concentrations of positive cases proved to be much higher (at least by factor 10) in this group compared to other populations. Therefore it is doubtful that this group represents a reliable model population, for clinical consideration or doping control.

Controlled animal studies under defined conditions had been carried out with guinea pig (single dosage of 10 mg/kg or sequence of 20 mg/kg intramuscular application of nandrolone [1, 8]), Long-Ewans rat (20 mg/kg intraperitoneal ingestion of stanozolol, 60 mg/kg daily intraperitoneal ingestion on 10 subsequent days, [10, 11] and steer (2 mg/kg intramuscular injection of nandrolone laurate [2]).

Most of the applied dosages were far beyond the therapeutic ranges; only in one study practically relevant dosages of a radio-labelled steroids were administered to steer [2], administration of single dosages of steroids were not detectable in hair [1] .

## Conclusions

The identification of anabolic steroids in hair is a well established experimental procedure. There is no obvious qualitative limitation for an incorporation of exogenous steroids into hair, if they are administered at high concentrations. Application in forensic cases, like

- evaluation of self-consumption in case of possession or trafficking of anabolics
- diminished responsibility of steroid abuser
- unexpected fatalities amongst bodybuilders

are realistic because the presumptive dosages are high and target substances are known.

On the other hand, the similarity of detection limits of procedures with realistic target concentrations demonstrates the 'general unknown dilemma':

The detection and quantitation of anabolic steroids in hair required hyphenated techniques, in spite of high dosages and methods optimised to few substances only. Screening procedures covering hundreds of potential steroids with adequate sensitivity are not available.

Many phenomena are not yet examined very well and cannot be explained conclusively.

Hair concentrations of esters are expected to be higher than these of corresponding free steroids.

This is described in some cases [1, 6, 11] whereas an increase of testosterone was described in parallel with undetectable testosterone esters [1]. Controversial results like these are clearly due to technical limitations.

The knowledge about the influence of metabolism of steroids is still unclear. Hair growth is primarily controlled by activity of endogenous steroids, the presence of receptors, activities of enzymes, (especially 5 $\alpha$ -steroid reductase) and corresponding blockers. These mechanisms depend at least on age, race, sex, health condition and body site (even regions of scalp hair) [15, 16] and are therefore expected to exhibit a great biovariability.

Nevertheless, suggestions to apply hair analysis range from a complementary method to its application in routine analysis, even replacement of urine testing. Expectations of an application to problems like testosterone doping in sports are high, although the knowledge of basal values in hair are based on some hundred evaluations (compared to a database in urine of several hundred thousands).

An attempt to establish a harmonisation was made by the Society of Hair Testing in 1999 [17].

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## Testing for 19-Norsteroids in Hair

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### Introduction

Androgen use was widespread among athletes at the time of the 1964 Olympic Games, and, despite a ban by the International Olympic Committee (IOC) in 1974, the use of anabolic steroids increased during the last decade.

Nandrolone or 19-nortestosterone, or 17 $\beta$ -hydroxy-19-nor-4-androsten-3-one, has been one of the most abused anabolic steroids and doping practices are increasing, as revealed by numerous positive cases during the last 3 years in judo, tennis, football or athleticism. The drug accelerates muscle growth by anabolic effects. Athletes use nandrolone because it has been claimed that it increases lean body mass, increases strength, increases aggressiveness and leads to a shorter recovery time between workouts.

Nandrolone is metabolized into norandrosterone (NA) and noretiocholanolone (NE) (1). Other 19-norsteroids, such as norandrostenedione or norandrostenediol, classified as dietary supplements, are available over-the-counter or through the Internet and have the same metabolites as nandrolone (1-3).

Long-term effects (severe cardio-vascular side-effects, liver diseases, etc.) and fatalities have been reported in young steroid abusers (4, 5).

The standard of testing for anabolic steroids for doping control is gas chromatography coupled to mass spectrometry conducted on an urine sample, and performed in accredited laboratories.

Nandrolone doping is claimed when norandrosterone is detected in urine with a chromatographic

response higher than that obtained with a standard spiked at 2 or 5 ng/ml for men or women, respectively (6). Even if norandrostenediol and norandrostenedione are banned by the IOC, there is an great interest, at least in forensic science and for survey of the athletes, to discriminate nandrolone abuse from other 19-norsteroids. This is obviously not possible in urine, as the metabolites are common. The ratio between NA and NE has been recently proposed to differentiate the nature of the doping agent, but this seems extremely cautious, as mixtures can be ingested (3).

As forensic laboratories can be involved in testimony dealing with doping agents, the idea of using hair for doping control has emerged as hair analysis has been accepted in court in other cases. Courts can request additional informations on the pattern of use of doping substances, such as during the 1998 cycling Tour de France where blood, urine, and hair were simultaneously collected. Hair can both confirm repetitive abuse and identify the exact nature of the parent compound (e.g., nandrolone, norandrostenediol or norandrostenedione, in case of positive urine for norandrosterone), as it has been accepted by the scientific community that the parent compound is the major analyte that is incorporated in hair (7, 8). Thus, hair analysis would discriminate nandrolone abuse from over-the-counter preparations containing 19-norsteroids.

### **Case reports**

Recently, our laboratory was requested by an attorney to evaluate potential doping practices from an athlete. The subject was 30-year old and tested positive for norandrosterone in urine at 230 ng/ml. The analysis was done in an accredited laboratory, but the athlete denied the result. Nandrolone, but also norandrostenediol and norandrostenedione were then tested in hair, according some modifications of our previous procedure for testosterone (9). Full-length hair

samples (12 cm long) were taken at the surface of the skin from the vertex of the athlete and stored in plastic tubes at room temperature. Only the first 6-cm from the root were analyzed.

Hair strands were obtained from 3 bodybuilders who were arrested by the French customs, accused of trafficking. In their luggage, the officers discovered a large amount of anabolics in ampoules. Full-length hair samples (3 to 5-cm long) were taken at the surface of the skin from the vertex and stored in plastic tubes at room temperature.

Controlled hair specimens were obtained from laboratory personal.

### **Analytical procedure**

One hundred milligrams of decontaminated hair (with dichloromethane) were incubated in 1 ml 1N NaOH, 15 min at 95°C, in presence of 10 ng of nandrolone-d<sub>3</sub> used as internal standard. After cooling, the homogenate was neutralized with 1 ml 1M HCl, and 2 ml of 0.2 M phosphate buffer (pH 7.0) were added. The drugs were extracted by solid-phase extraction. The Isolute C18 columns were conditioned with 3 ml of methanol, followed by 2 ml of deionized water. After sample addition, the columns were washed twice with 1 ml of deionized water. After column drying, analytes elution occurred with the addition of 3 aliquots of 0.5 ml of methanol. The eluant was evaporated to dryness under nitrogen flow, and the residue reconstituted in 1 ml of 0.2 M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (1:10, w/w) and 2 ml of pentane. After agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatized by adding 50 µl MSTFA/NH<sub>4</sub>I/2-mercaptoethanol (1000 : 2 : 5 ; v/v/v), then incubated for 20 min at 60 °C.

A 4-µl aliquot of the derivatized extract was injected into the column of a Hewlett Packard (Palo Alto, CA) gas chromatograph (6890 Series) via a Hewlett Packard (7673) autosampler. The flow

of carrier gas (helium, purity grade N 55) through the column (HP5-MS capillary column, 5 % phenyl-95 % methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm film thickness) was 1.0 ml/min. The injector temperature was 270 °C and splitless injection was employed with a split valve off-time of 1.0 min, using the pulsed mode. The column oven temperature was programmed to rise from an initial temperature of 150 °C, maintained for 1 min, to 295 °C at 30 °C/min and maintained at 295 °C for the final 8 min.

The detector was a Hewlett Packard 5973 operated in the electron impact mode. The electron multiplier voltage was set at 600 V above the EI-tune voltage.

## Results and discussion

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in hair. Selected ions, retention times and analytical parameters of the drugs are reported in Table 1. Analytes were identified and quantified on the basis of their retention times and the abundance of three confirming ions.

Responses for the 3 norsteroids were linear in the range 1 to 50 pg/mg, with a correlation coefficient in the range of 0.992 to 0.997.

The within-run precisions were in the range 11.2 to 17.6 %, as determined by analyzing 6 replicates of 100 mg of drug-free hair obtained from the same subject and spiked with a final concentration at 10 pg/mg. The extraction recovery ( $n = 3$ ) was determined to be in the range 76.1 to 86.4 %. The limits of detection ( $S/N > 3$ ) of the doping agents were in the range 0.5 to 1 pg/mg.

The analysis of a strand of hair obtained from the athlete revealed the presence of 19-norandrostenedione at the concentration of 7 pg/mg. Extensive chromatographic procedures (2 purification steps by solid phase and liquid-liquid extraction, combined with injection of 4  $\mu$ l

through the column in pulsed mode) were analytical prerequisites for successful identification of norandrostenedione in hair due to the low target concentration. The analysis of 3 strands of hair, obtained from 3 bodybuilders revealed the presence of nandrolone at the concentrations of 1, 3.5 and 7.5 pg/mg.

The sensitive, specific and reproducible method developed seems to be suitable for the detection and quantification of 19-norsteroids in human hair. The determination of one 19-norsteroid in hair should allow a definitive unambiguous confirmation of the exact nature of the abused agent. This seems one of the major advantage of hair over urine, as it was also clearly demonstrated that the identification of testosterone esters in hair can confirm the administration of exogenous testosterone (9, 10).

The international literature is very poor in papers dealing with the identification of nandrolone in hair. One paper, published in 1995, has been focused on guinea pigs (11) with unrealistic nandrolone dosages, 10 or 20 mg/kg. In 1999, Höld et al (12) demonstrated that it is possible to detect nandrolone in rat hair after systemic administration. However, the growing interest of scientists to the detection of anabolic drugs in human was observed in the late 1998.

In their early work, Cirimele et al (13), published results from 2 bodybuilders preparing the next world championship, with positive hair for nandrolone at 196 and 260 pg/mg. Deng et al (14), among other anabolics, identified nandrolone in the hair of a steroid abuser at 20 pg/mg. Gaillard et al (15) detected nandrolone in the hair of a cyclist at 5.1 pg/mg.

When using hair in a suspected doping case, particularly when urine of the athlete was positive and hair negative (several cases were reported during the past 3 years), the question of importance is to know whether the analytical procedure was sensitive enough to identify traces of drugs. It has been always accepted in the forensic community that a negative hair result cannot

exclude the administration of the detected drug or one of its precursors (such as norandrostendiol or norandrostendione for the metabolites of nandrolone) and should not overrule a positive urine result. Nevertheless, the negative hair findings lends enough ambiguity to the positive urine result, coupled with the sporting consequences for the athlete, that substantial justice refereeing occurs. This laboratory was not able to identify nandrolone in the hair of a 37-year old man, receiving a single intramuscular injection of 50 mg nandrolone undecanoate, although his urine remained positive for norandrosterone and noretiocholanolone, the nandrolone metabolites, for at least 8 months. Hair was tested 2 and 6 months after administration (16). The same observations were recently made by Segura, et al (17) who did not detect nandrolone after a single dose administration. Therefore, until laboratories will have sensitive enough methodologies to detect a single use of steroids, care should be taken to compare urine and hair findings. For anabolic steroids to have an appreciable performance enhancing effect, they must be chronically administered, in contrast to the immediate stimulant properties of cocaine or amphetamine. Repeated amount of drug used per hair growth length would favor identification by hair analysis.

## **Conclusion**

The sensitive, specific and reproducible method developed seems to be suitable for the detection and quantification of nandrolone in human hair.

Hair analysis may be a useful adjunct to conventional drug testing in sports. Methods for evading urine analysis do not affect the drug concentrations in hair. Specimens can be more easily obtained with less embarrassment, and hair can provide a more accurate history of drug use. This technology may find useful applications in doping control, if accepted by the International Olympic Committee (18).

**Table 1** Selected ions, retention times and analytical parameters for nandrolone, norandrostenediol and norandrostedione in human hair

Parameters	norandrostenediol	norandrostedione	nandrolone
Retention time (min)	6.99	7.19	7.27
Ions (m/z)	330, 405, <u>420</u>	344, 401, <u>416</u>	287, 403, <u>418</u>
Linearity (1-50 pg/mg)	0.992	0.995	0.997
Within run precision (%)	17.6	14.3	11.2
Recovery (%)	76.1	86.4	81.7
LOD (pg/mg)	1	0.5	0.5

The underlined ions were used for quantification.

nandrolone-d<sub>3</sub> was eluted at 7.26 min, and the ion (m/z) 421 was used for quantification.

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