T. Piper, V. Gougoulidis, U. Flenker, W. Schänzer

Presentation of the IRMS results of suspicious samples with elevated T/EpiT ratios in 2005

Institute of Biochemistry, German Sport University Cologne, Germany

Abstract

Athletes with a T/EpiT ratio higher than 4 are suspicious for the intake of synthetic anabolic androgenous steroids. In order to find out whether the T/EpiT ratio occurs naturally or is due to a doping offence, the urine samples are measured by GC/C/IRMS. The δ^{13}C-values of the main testosterone metabolites and the endogenous reference compounds are compared. If any measured $\Delta\delta$ value between an endogenous reference and a target compound is not larger than 3‰ a naturally elevated T/EpiT ratio is assumed.

After lowering the critical threshold from T/EpiT > 6 to T/EpiT > 4 by WADA in 2004 the number of samples measured by GC/C/IRMS in Cologne increased more than two-fold. The presented dataset consists of n = 331 samples from Cologne and n = 246 samples from other laboratories, mainly from out of Europe. The total number of doping control samples confirmed by IRMS was n = 603 with n = 34 IRMS–positive results.

Although the dietary status throughout Europe is assumed to be quite similar, it is possible to set up groups of related δ^{13}C-values depending on the geographical origin of the sample. They were sorted by the laboratories which sent the samples for IRMS–analysis to Cologne. These groups have different mean values but statistically are not significantly different whereas samples from South Africa or South America exhibit obviously different values.

1. Introduction

The abuse of endogenous anabolic steroids like testosterone (T) or dehydroepiandrosterone (DHEA) is an important issue in sports and its detection a challenge for doping control laboratories. The misuse of anabolic steroids was banned by the International Olympic Committee (IOC) in 1976 for the first time and in order to detect the abuse of especially T or testosterone prohormones, in 1982 the Medical Commission of the IOC established a

threshold value for the ratio of T/EpiT (epitestosterone).[1)] According to the work by Donike et al.[2)] a cut-off point of 6 was adopted. Although in a healthy reference population the average T/EpiT ratio ranges from 0.3 to 3.3, in some rare cases the ratio exceeds the cut-off criteria without an application of exogenous T.[2-7)] Since the 1990´s gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been established as the method of choice to distinguish between endogenous or exogenous T.[8-12)] By this method the $^{13}C/^{12}C$-ratios of different urinary steroids can be measured and compared among each other. $^{13}C/^{12}C$-ratios are expressed as $\delta^{13}C$-values:

$$\delta^{13}C_{VPDB} = \left(\frac{R_{SPL} - R_{VPDB}}{R_{VPDB}} \right) * 10^3 \qquad \text{where} \qquad R = {}^{13}C / {}^{12}C$$

Differences between compounds are expressed as Δδ-values:

$$\Delta\delta = \delta^{13}C_{TC} - \delta^{13}C_{ERC}$$

On the one hand endogenous reference compounds (ERC) like 11β-OH-androsterone (11OHA) or pregnanediol (PD) are determined, on the other hand T or T-metabolites like androsterone (A) or etiocholanolone (E) are measured. The difference between the $\delta^{13}C$-values of the ERC and the target compounds (TC) must not exceed 3‰.[13)] While the $\delta^{13}C$-values of exogenous steroids available on the market usually are ≤ -26‰, the values of the ERC and the TC range from -24 to -17‰, depending on the individual diet.[14-17)] Following the intake of exogenous steroids the $\delta^{13}C$-values of the TC are depleted while the ERC remain unchanged – so that the Δδ value may exceed the threshold value. The curve progression of the Δδ values plotted against the T/EpiT ratios will be the first part of this article.

In the second part there will be a closer look at the distribution of the $\delta^{13}C$-values within Europe. As the $^{13}C/^{12}C$-ratio of all endogenous steroids finally depends on the individual diet and as there are slightly differences in the habit of eating throughout Europe, it should be possible do assess the provenience of an athlete by means of his urinary steroid $\delta^{13}C$-values.

2. Method

Sample preparation for a GC/C/IRMS measurement is extraordinary time-consuming because of different peculiarities of this method. Analytes have to be cleaned up carefully before

measurement in order to avoid co-elution of confounding compounds and to keep in readiness the ability to measure differently concentrated urinary steroids in a comparable amount. Both requirements have to be met for a valid $^{13}C/^{12}C$ determination.[18-19)]

Hence sample preparation encompasses the following steps:

1) Solid phase extraction with an C^{18}-Cartridge, conditioned and eluted with methanol
2) Clean up and extraction of the free steroids with tert.-buthylmethylether (TBME) at pH = 7
3) Enzymatic hydrolysis of glucuronized steroids by β-glucuronidase
4) Liquid-liquid-extraction with TBME at pH = 9.6
5) Purification and separation of the different steroids by HPLC on a RP^{18}-column, solvent gradient water/acetonitril 70/30 to 100% acetonitrile within 20 min (see figure 1)
6) Evaporation of the different HPLC fractions and dissolving the analytes for GC/C/IRMS in acetone containing 100mg/ml androstanol (see figures 2 and 3)

Detailed description of the method can be found elsewhere.[20)]

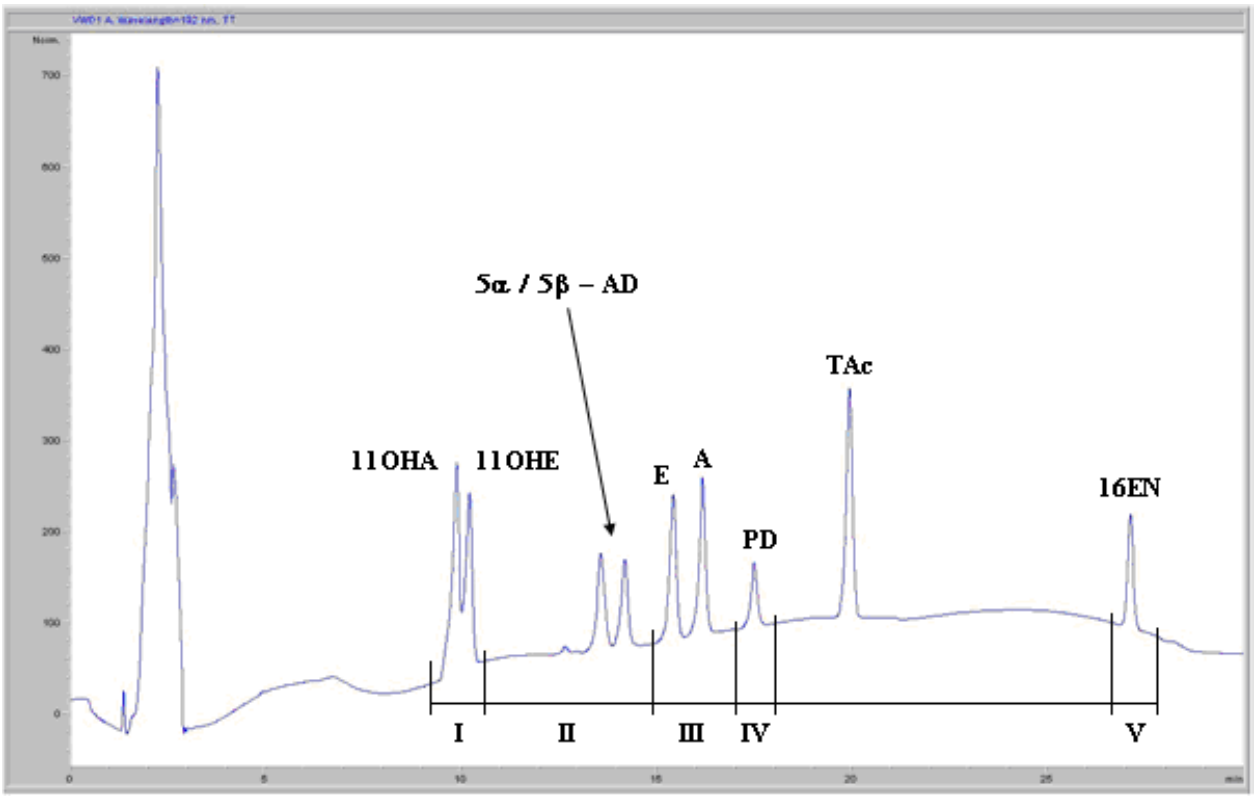

Figure 1: HPLC-UV chromatogram of a standard mixture measured to ascertain the retention times of different steroids. Five fractions are collected: I: 11OHA and 11β-OH-etiocholanolone (ERC); II: androstanediols, T, EpiT (TC); III: E,A (TC); IV: PD (ERC); V: 16-androstenol (ERC); testosterone acetate (TAc) as reference standard.

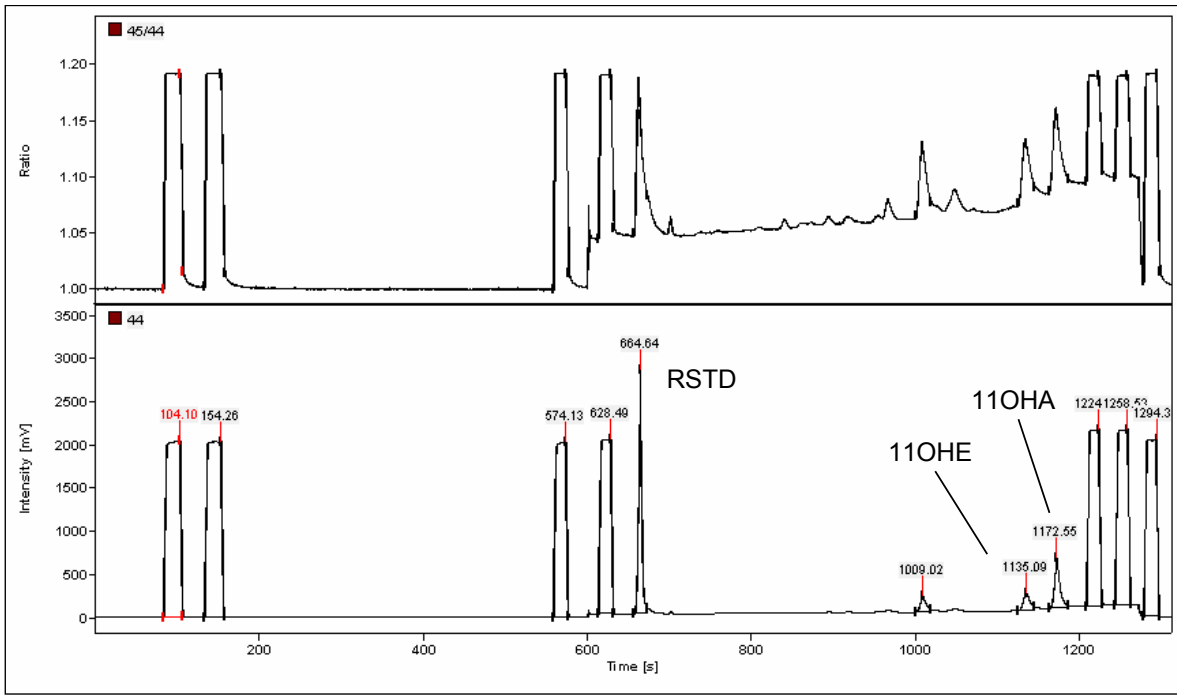

Figure 2: GC/C/IRMS chromatogram of HPLC fraction I. The rectangular peaks are obtained by CO_2 pulses added to the analysis as reference gas. The lower panel shows the m/z 44 trace while the upper panel shows the ratio of m/z 45 to 44. Androstanol (RSTD) serves as reference standard.

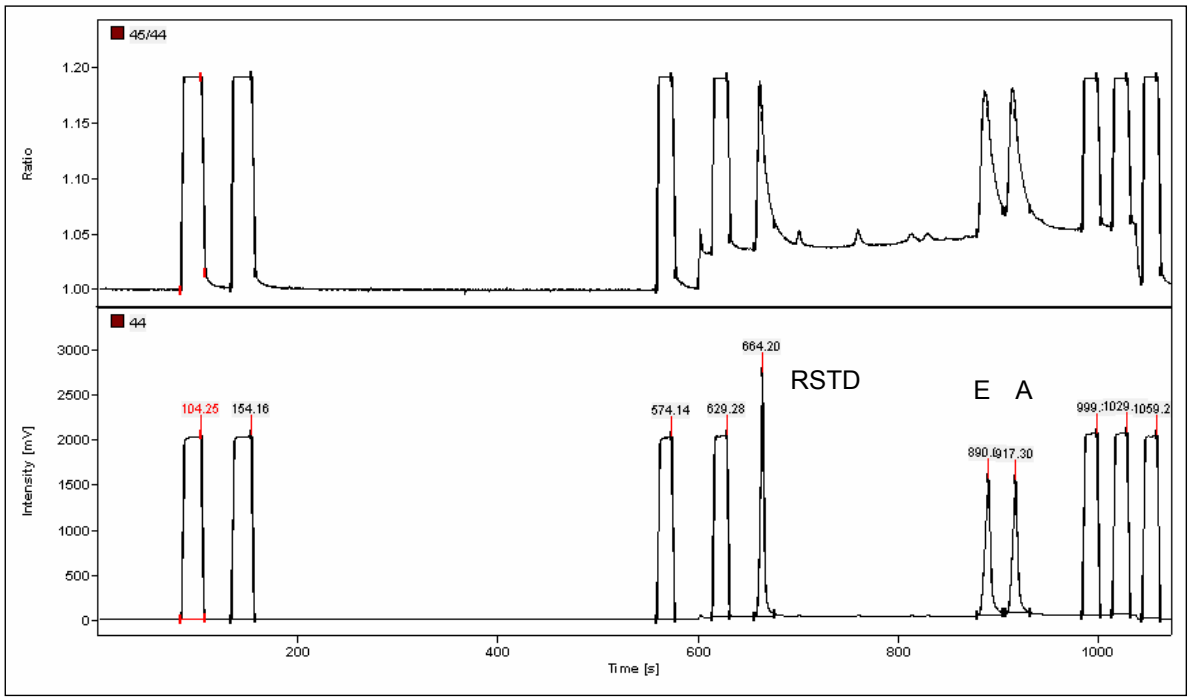

Figure 3: GC/C/IRMS chromatogram of HPLC fraction III.

3. Results

3.1 T/EpiT samples

Figure 4 shows the distribution of the measured routine samples, suspicious for the intake of T or T-prohormones indicated by an elevated T/EpiT ratio or suspicious because of other endogenous steroid concentrations like E, A or DHEA above the defined thresholds.[13)]
Out of the 603 samples 100 could not be utilized in this article because of missing data concerning the T/EpiT ratio as steroid profiles were not at hand. There were 172 samples left from different laboratories plus 331 samples from Cologne routine analyses.
As expected, for positive samples there is an increase in the Δδ-values of 11OHA minus A with an increase in the T/EpiT ratio. The higher the T/EpiT value is, the larger the amount of exogenous T in the athlete's body should be and therefore the more depleted is his $^{13}C/^{12}C$ value of A - resulting in a large difference. Although the data are in accordance with theory, there are some interesting outliers:

a) Two samples with an urinary concentration of E and A > 10.000 ng/ml
b) A DHEA case with a 3‰ difference between E and A according to the literature[21)]
c) One female athlete suffering from andrenogenital syndrome perhaps treated with corticosteroids resulting in this abnormal Δδ−value
d) Three routine samples with a very low concentration of EpiT resulting in this high T/EpiT ratio but with no hint for intake of exogenous steroid
e) A sample from Stockholm with ^{13}C depleted 11OHA, for further information see 3.2

The mean Δδ value of all measured samples assumed to be negative was -0.36‰ with a standard deviation of ±0.61‰ while the precision referring to the RSTD was -32.8 ± 0.40‰. The fourfold standard deviation used in figure 5 represents a confidence level of 99.997% to avoid a false positive finding. The resulting threshold value of -2.8‰ fits well with the WADA criteria. Furthermore figure 5 is a more detailed view of the results. Up to a T/EpiT ratio of 10 the Δδ values show an inconspicuous distribution. This supports the hypothesis of the existence of naturally elevated T/EpiT ratios.

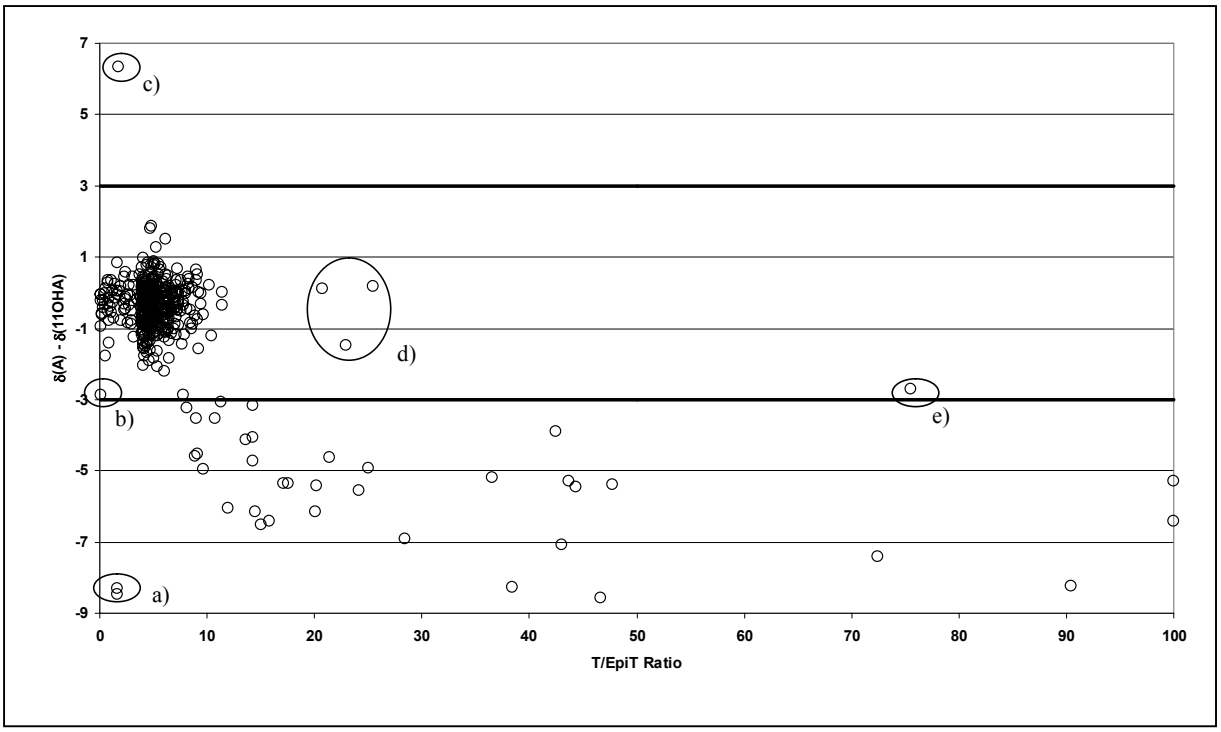

Figure 4: Scatter plot of the measured routine samples, Δδ-values in ‰ . Two samples with T/EpiT = 100 were out of the measurable range and arbitrarily set to this value. Bold lines represent the WADA threshold. a)-e) see text.

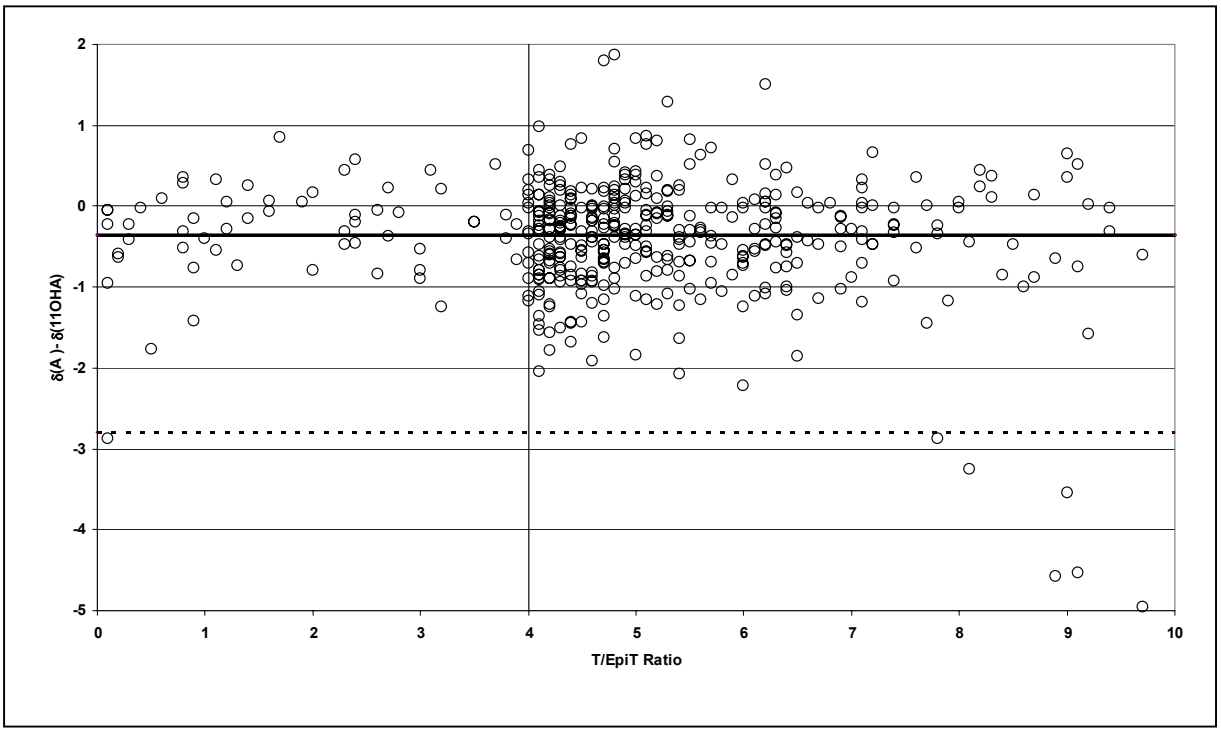

Figure 5: Enlargement of the area from 0 to 10 for the T/EpiT ratio. The bold line represents the mean value of all samples assumed to be negative, the dashed line the fourfold standard deviation.

3.2 $\delta^{13}C$ -Values referring to the origin

In spite of the quite similar dietary status throughout Europe it is possible to establish groups of different $\delta^{13}C$-values according to the samples` origin. As can be taken from Figure 6 there is a shift towards lighter $\delta^{13}C$-values the more to the north of Europe the athlete comes here. There were 66 samples available from Helsinki and Stockholm, 37 from Ghent representing Central Europe and 96 from Seibersdorf and Rome. Another 10 samples have been provided by laboratories from South America and by Bloemfontein. With values around -17.8‰ for 11OHA and -18.2‰ for A they were even more enriched than the samples from Southern Europe.

After these considerations the above mentioned problem with the denounced sample from Stockholm (sample e) in figure 4) becomes obvious. If the ERC–value is close to -25‰, the sample won't necessary reach the 3‰ criteria after administration of exogenous steroids which may have a $\delta^{13}C$-value of -27‰. Due to this the sample shows a relatively small $\Delta\delta$–value despite a large T/EpiT ratio. However by measuring the $\delta^{13}C$-value of T it was possible to convict the athlete of doping.

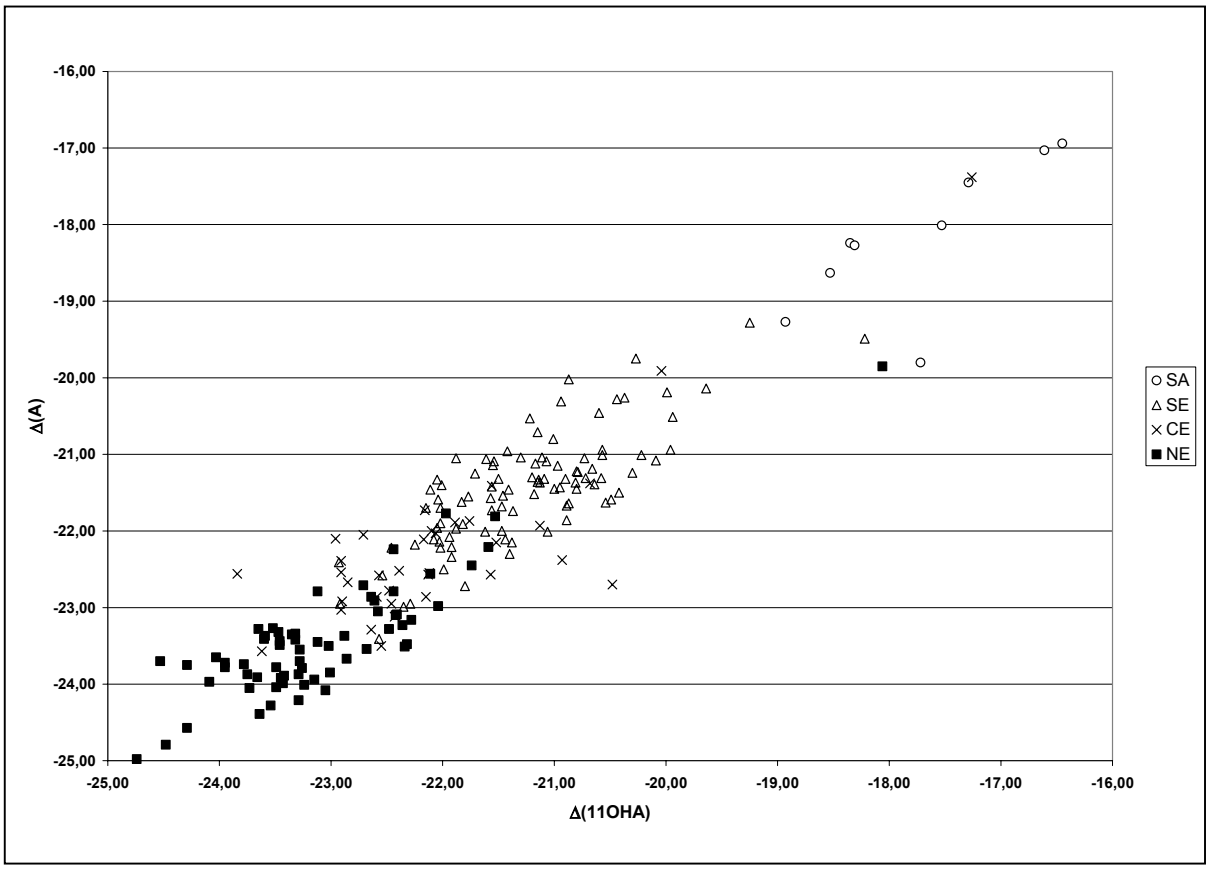

Figure 6: Scatter plot of 11OHA– vs. A-values. Filled squares: Northern Europe; Crosses: Central Europe; Open triangles: Southern Europe and Open circles: South America and South Africa.

Table 1 summarizes the different mean values and the corresponding standard deviations again showing the shift towards smaller $\delta^{13}C$-values for athletes from Northern Europe. As there is a large inter-individual variation within the groups, indicated by the large standard deviation, it is not possible to clearly separate one from another. The sole exception is the first group due to their different diet utilising more C4-plants.

Origin	11OHA [‰]	SD[‰]	A [‰]	SD [‰]
South America + Bloemfontein	-17.75	± 0.81	-18.18	± 0.91
Southern Europe	-21.25	± 0.78	-21.44	± 0.73
Central Europe	-22.01	± 1.16	-22.24	± 1.06
Northern Europe	-23.08	± 0.95	-23.43	± 0.77

Table 1: Summary of the different $\delta^{13}C$ – values and their standard deviations (SD)

4. Discussion

GC/C/IRMS is a useful tool to discriminate between endogenous and exogenous urinary steroids. The threshold value established by WADA of 3‰ is well in accordance with our results and should be permissive enough to avoid false positive cases. By application of this method we will have to face two major problems in the future: On the one hand it is difficult to evaluate samples from athletes with light ERC ($\delta^{13}C$-values $\leq$ -24‰). On the other hand synthetic steroids may appear on the market showing $^{13}C/^{12}C$ ratios close to physiological ERC values. This can result in a false negative case. Under both circumstances it may be helpful to develop additional methods like IRMS of the $^{2}H/^{1}H$ ratio or to employ other criteria such as the difference between E and A as has been suggested for DHEA abuse.

5. References

1) List of Doping Classes and Methods. International Olympic Committee Medical Commission, Lausanne (1982)
2) Donike M, Bärwald KR, Klostermann K, Schänzer W, Zimmermann J. Nachweis von exogenem Testosteron. In: Heck H, Hollmann W, Liesen H, Rost R, eds. Sport: Leistung und Gesundheit. Köln: Deutscher Ärtze-Verlag, 1983, 293-300
3) Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, Wheeler M. Criteria to Indicate Testosterone Administration. Br J Sports Med, 1990, 24, 253-264

4) Rauth S. Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten. PhD. thesis, German Sport University, 1994, 29-30
5) de la Torre X, Pascual JA, Ortuno J, Segura J. Steroid Profile and Sports. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent Advances in Doping Analysis (4). Köln: Sport und Buch Strauß, 1997, 59-69
6) Kicman AT, Oftebro H, Walker C, Norman N, Cowan A. Potential Use of Ketoconazole in a Dynamic Endocrine Test to Differtiate between Biological Outliers and Testosterone Use by Athletes. Clin Chem, 1993, 39, 1798-1803
7) Catlin DH, Hatton CK, Starcevic SH. Issue in Detection Abuse of Xenobiotic Anabolic Steroids and Testosterone by Analysis of Athletes´ Urine. Clin Chem, 1997, 43, 1280-1288
8) Becchi M, Aguilera R, Farizon Y, Flament M-M, Casabianca H, James P. Gas Chromatograhy/Combustion/Isotope Ratio Mass Spectrometry Analysis of Urinary Steroids to Detect Misuse of Testosterone in Sport. Rapid Commun Mass Spectrom, 1994, 8, 304-308
9) Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG Jr. Improved Method of Detection of Testosterone Abuse by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry Analysis of Urinary Steroids. J Mass Spectrom, 1996, 31, 169-176
10) Horning S, Geyer H, Machnik M, Schänzer W, Hilkert A, Oeßelmann J. Detection of Exogenous Testosterone by 13C/12C Analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent Advances in Doping Analysis (4). Köln: Sport und Buch Strauß, 1997, 275-283
11) Flenker U, Horning S, Nolteernsting E, Geyer H, Schänzer W. Measurement of 13C/12C-Ratios to Confirm Misuse of Endogenous Steroids. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, eds. Recent Advances in Doping Analysis (6). Köln: Sport und Buch Strauß, 1999, 243-256
12) Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance Charakteristics of a Carbon Isotope Ratio Method for Detecting Doping with Testosterone Based on Urine Diols: Controls and Athletes with Elevated Testosterone/Epitestosterone Ratios. Clin Chem, 2001, 47, 292-300
13) WADA Laboratory Comittee. Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio and Other Endogenous Steroids. World Anti-Doping Agency, Montreal, 2004. WADA Technical Document – TD2004EAAS

14) Farquhar GD, Ehleringer JR, Hubick KT. Carbon Isotope Discrimination and Photosynthesis. Annu Rev Plant Physiol Plant Mol Biol, 1989, 40, 503-537

15) de la Torre X, Gonzáles JC, Pichini S, Pascual JA, Segura J. $^{13}C/^{12}C$ Isotope Ratio MS analysis of testosterone, in chemicals and pharmaceutical peparations. J Pharm Biomed Anal 2001, 24, 645-650

16) Gaffney JS, Irsa AP, Friedman L, Slatkin DN. Narural $^{13}C/^{12}C$ Ratio Variations in Human Populations. Biomed Mass Spectrom 1978, 5, 495-497

17) Flenker U, von Kuk C, Güntner U, Hülsemann F, Gougoulidis V, Schänzer W. Influence of Changes in Diet on the Dynamics of $^{13}C/^{12}C$ in Selected Urinary Steroids: Diet free from Cholesterol. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, eds. Recent Advances in Doping Analysis (13). Köln: Sportverlag Strauß, 2005, 227-233

18) Goodmann KJ, Brenna JT. Curve Fitting for Restoration of Accuracy for Overlapping Peaks in Gas Chromatography/ Combustion Isotope Ratio Mass Spectrometry. Anal Chem, 1994, 66, 1294-1301

19) Meier-Augenstein W. Applied Gas Chromatography Coupled to Isotope Ratio Mass Spectrometry. J Chromatogr A, 1999, 842, 351-371

20) Flenker U, Güntner U, Schänzer W. $^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport. In: Bowers LD, Hilderbrand RL, Symanski EJ, eds. Application of Gas Chromatography - Combustion - Isotope Ratio Mass Spectrometry to Doping Control. USADA, Colorado Springs, 2003, 29-33

21) Cawley AT, Kazlauskas R, Trout GJ, Rogerson JH, George AV. Isotopic Fractionation of Endogenous Anabolic Androgenic Steroids and ist Relationship to Doping Control in Sports. J Chrom Sci, 2005, 43, 32-38