Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (10)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2002

M.K. PARR, H. GEYER, U. REINHART, W. SCHÄNZER:
Approaches towards an Improved Analysis of Nutritional Supplements for Anabolic Androgenic Steroids
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (10). Sport und Buch Strauß, Köln, (2002) 87-96

Maria Kristina Parr, Hans Geyer, Ulf Reinhart, Wilhelm Schänzer

Approaches towards an improved analysis of nutritional supplements for anabolic androgenic steroids

Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Introduction

Since 1996 prohormones are available on the US sports nutrition market. According to the doping regulations of the IOC they belong to the prohibited class of anabolic agents [1].

Recent studies have shown that "non-hormonal" supplements such as vitamins, minerals, amino acids, etc. can contain prohormones not declared on the label [2-5]. These prohormones may lead to positive results in doping tests, especially for the nandrolone metabolite norandrosterone [2-4]. Based on this knowledge, a broad-based investigation of the international nutritional supplement market was conducted.

Approximately 600 nutritional supplements were analysed with gas chromatography / mass spectrometry (GC/MS) for the following anabolic androgenic steroids: estr-4-ene-3,17-dione (4-norandrostene-3,17-dione), estr-4-ene-3β,17β-diol (4-norandrostene-3β,17β-diol, estr-5-ene-3β,17β-diol (5-norandrostene-3β,17β-diol), 19-nortestosterone (nandrolone), androst-4-ene-3,17-dione, androst-5-ene-3β,17β-diol, androst-4-ene-3β,17β-diol, dehydroepi-androsterone (DHEA), testosterone, 5α-androstane-3β,17β-diol and androsta-1,4-diene-3,17-dione.

During this investigation analytical difficulties occurred due to the different matrices in the various products. For most of the products minor modifications from a standard method yielded suitable results. The standard method as well as the modifications applied for the critical steps during the sample preparation are worked out.

Experimental

Supplements

From October 2000 until November 2001 634 non-hormonal nutritional supplements were bought in 13 different countries. The nutritional supplements were not prohormone products.

Chemicals

DHEA, testosterone, 5α-androstane-3β,17β-diol and estr-4-ene-3,17-dione were purchased from Sigma (St Louis, USA), androst-5-ene-3β,17β-diol and 19-nortestosterone from Serva (Heidelberg, Germany), androst-4-ene-3,17-dione from Schering (Berlin, Germany), androst-4-ene-3β,17β-diol, estr-5-ene-3β,17β-diol and androsta-1,4-diene-3,17-dione from Steraloids (Wilton, USA) and 3,4,5-d_3-5β-estran-3α-ol-17-one (d_3-noretiocholanolone) from Radian International (Austin, USA). Estr-4-ene-3β,17β-diol, 16,16,17-d_3-testosterone, 16,16,17-d_3-nortestosterone and the 1-N,N-diisopropylamino-n-alkanes (DIPA 14-23) were synthesized in our laboratory. N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Other reagents and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Sample preparation

The flow scheme of the standard sample preparation is shown in Fig. 1.

In case of analytical difficulties several minor modifications, which are described later, may lead to suitable results.

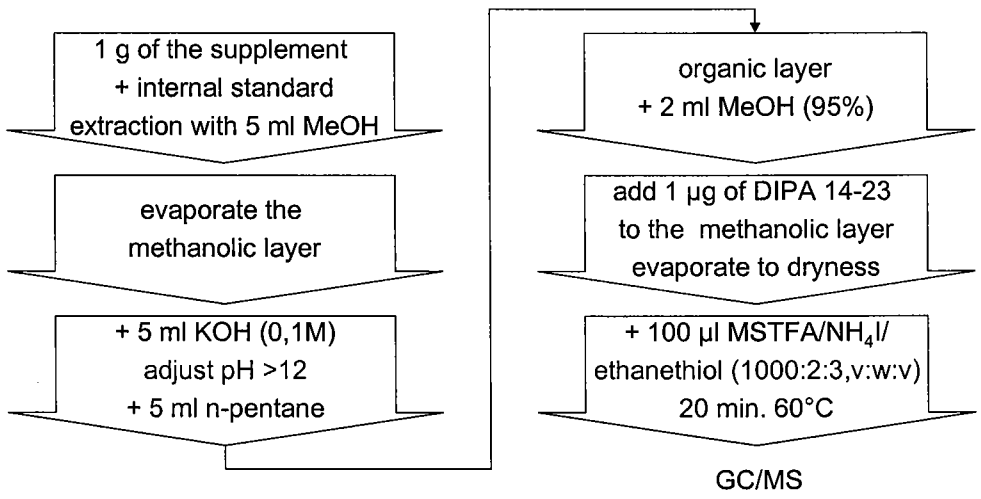

Fig. 1: Flow scheme of the standard sample preparation for the analysis of anabolic steroids in non-hormonal nutritional supplements, internal standard: mixture of 16,16,17-d_3-testosterone, 16,16,17-d_3-nortestosterone and 3,4,5-d_3-19-noretiocholanolone, 50 ng each

Instrumentation

GC/MS analyses

The GC/MS analyses were performed on a Hewlett Packard (HP) 6890 gas chromatograph coupled to a HP 5973 mass selective detector (MSD) with the following parameters:

Injection parameters:	Volume: 2 µl, Temp.: 300°C
Column:	HP 5 MS; 16.5 m; 0.25 mm i.d.; 0.25 µm film thickness
Carrier gas:	Helium, splitless, head pressure 13 psi
Oven temp.:	100°C with 40°C/min to 190°C, with 5°C/min to 240°C, with 40°C/min to 320°C, 3 min hold
Ionisation:	70 eV, electron impact (EI)
Data aquisition:	SIM

The GC/MS data of the analysed steroids are shown in Tab. 1. The identification was performed according to the recommendations of the IOC [6].

Tab. 1: Retention times and characteristic ions of the bis-TMS derivatives of the anabolic androgenic steroids monitored and the internal standards (ISTD)

	Substance	RT [min]	M+	Base peak	other characteristic ions
ISTD1	d_3-Noretiocholanolone	9.68	423	408	318
	Estr-5-ene-3β, 17β-diol	10.49	420	330	225, 240, 405
	Estr-4-ene-3β, 17β-diol	10.66	420	420	405, 330, 240
	Dehydroepiandrosterone	11.11	432	432	417, 327
	Androst-4-ene-3β,17β-diol	11.20	434	434	419, 405, 344, 239, 143
	Estr-4-ene-3,17-dione	11.31	416	416	401, 194
	Androst-5-ene-3β,17β-diol	11.32	434	239	434, 344, 305, 329
	5α-Androstane-3β,17β-diol	11.37	436	421	241, 215, 346, 256, 331
ISTD2	d_3-Nortestosterone	11.56	421	421	406
	19-Nortestosterone	11.56	418	418	403
	Androst-4-ene-3,17-dione	11.90	430	430	415, 169
ISTD3	d_3-Testosterone	12.11	435	435	420
	Testosterone	12.12	432	432	432, 417
	Androsta-1,4-diene-3,17-dione	12.72	428	413	323, 206

GC/MS/MS analyses

For confirmation of testosterone, 19-nortestosterone, estr-4-ene-3,17-dione and androst-4-ene-3,17-dione the following GC/MS/MS conditions were used:

GC/MS/MS system:	GC Finnigan, GCQ
Injection parameters:	Volume: 2 µl, Temp.: 325°C
Column:	HP Ultra-1 (OV 1); 14 m; 0.25 mm i.d.; 0.11 µm film thickness
Carrier gas:	Helium, split 1:10, head pressure 10 psi
Oven temp.:	100°C with 40°C/min to 190°C, with 5°C/min to 240°C, with 40°C/min to 320°C, 3 min hold
Ionisation:	70 eV, electron impact (EI)

Results and Discussion

Influence of the matrix on the result of the analyses

Addition of DIPAs

As already described by Geyer et al. [7] the addition of a mixture of DIPAs leads to higher peak areas in the GC/MS analyses of anabolic steroids compared to pure standard solutions. Also for the analyses of anabolic steroids in different nutritional supplement matrices these increased peak areas are found after the addition of DIPAs to the final extract. This leads to an improved limit of detection for the steroids in nutritional supplements. The addition of two different amounts (1 µg and 10 µg) of DIPAs was tested. A higher amount of DIPAs leads to higher peak areas, corresponding to higher S/N-ratios and therefore lower limits of detection. As an example the S/N-ratios obtained for androst-4-ene-3,17-dione and testosterone at different concentrations are shown in Fig. 2.

The extent of the effect differs for the specific steroids. For the analyses of nutritional supplements the addition of 1 µg of DIPAs to the final extract leads to sufficient limits of detection (for all steroids analysed lower than 5 ng/g in creatine) and allows to reduce the amount of DIPAs.

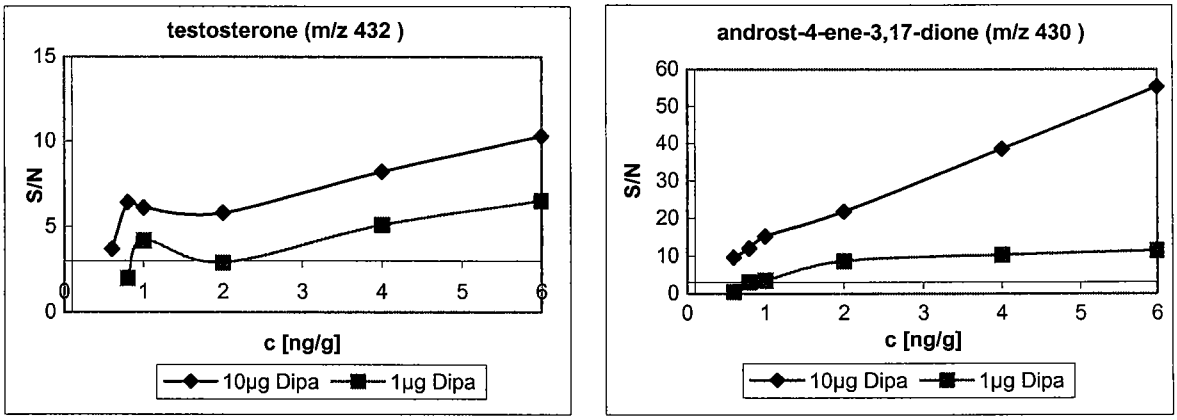

Fig. 2: Effects of DIPAs addition on the S/N-ratio of anabolic steroids

GC/MS analyses

The GC/MS analyses following the standard sample preparation cause in several cases difficulties:

Fig. 3 shows a chromatogram of the derivatisation reagent injected after a sample with albumin, vitamins, minerals, and amino acids as the declared ingredients. Several peaks occurred which of course do not origin from the derivatisation reagent. The corresponding substances can be addressed to matrix compounds of the sample injected before. If the same

derivatisation mixture is injected after pure creatine matrix (as e.g. used in our laboratory for the positive and negative control sample) no peaks occur in the chromatogram (Fig. 4). Also after the injection of samples containing several other matrix compounds no peaks are obtained in the chromatograms of the derivatisation reagent. Unfortunately it is still impossible to predict from the list of ingredients, which samples may cause problems and which do not. To avoid possible problems in the measurement of the next sample, the sequence of injection for the analyses of nutritional supplements should include the injection of 2 µl of the derivatisation reagent and running the entire method following each unknown sample (Fig. 5).

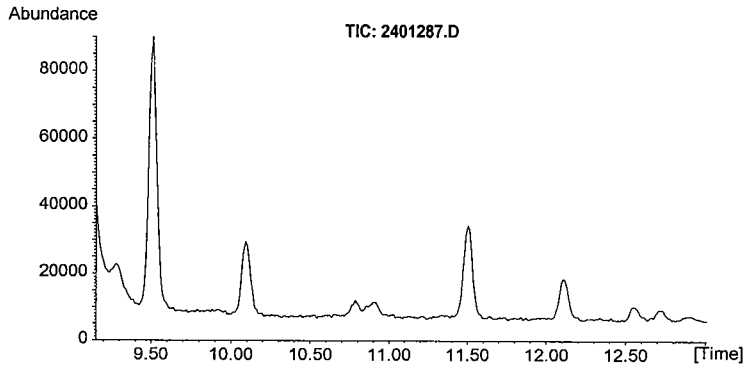

Fig. 3: Chromatogram of the derivatisation reagent injected after a supplement with the declared ingredients albumin, vitamins, minerals and amino acids

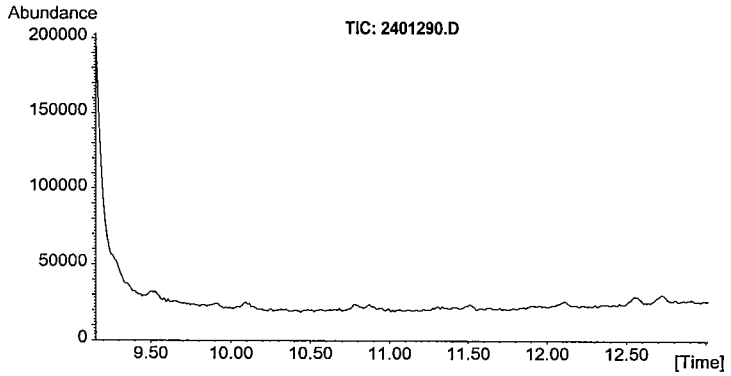

Fig. 4: Chromatogram of the pure derivatisation reagent (injected after a sample with a pure creatine matrix)

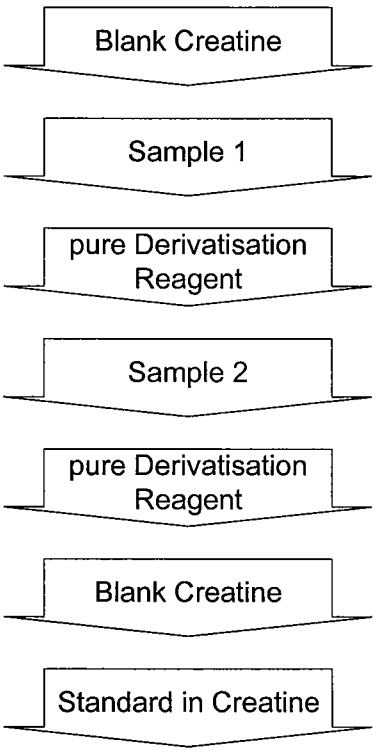

Fig. 5: Sequence of injection

For confirmation of the specific anabolic androgenic steroids three diagnostic ions were recorded [6]. For the per-TMS derivatives of androst-4-ene-3,17-dione, estr-4-ene-3,17-dione, testosterone and 19-nortestosterone only one abundant diagnostic ion (M^+) can be obtained. For those steroids GC/MS/MS was applied to obtain sufficient diagnostic ions.

As an example the EI mass spectrum and the corresponding daughter ion spectrum of the molecular ion of estr-4-ene-3,17-dione bis-TMS are shown in Fig. 6 and Fig. 7.

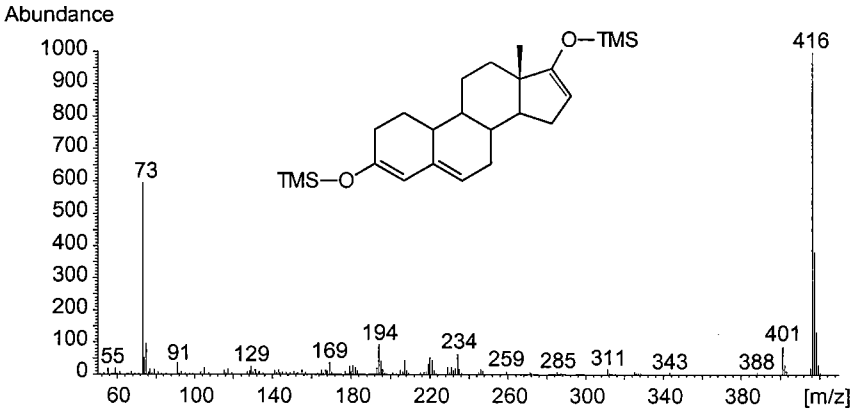

Fig. 6: EI mass spectrum of estr-4-ene-3,17-dione bis-TMS

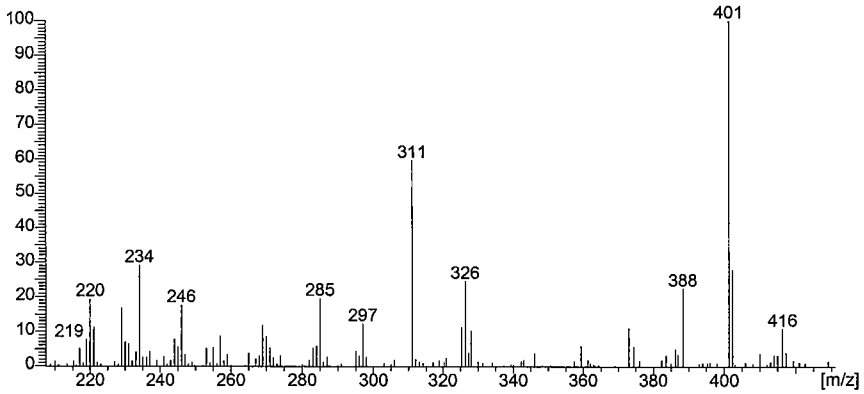

Fig. 7: Daughter ion spectrum of m/z 416 of estr-4-ene-3,17-dione bis-TMS

Homogenisation

As presented by Geyer et al. [2] the concentrations of prohormones in contaminated supplements can vary a lot between different capsules of the same lot number as well as between different lot numbers of the same product. For a representative analysis of the products 10 capsules or tablets have to be pulverised and homogenised in a mortar. In case of powders special care has to be taken on the homogenisation, which is done by stirring. After this the aliquot of 1 g of the supplement is taken from this mixture. Liquid samples are easily homogenised by shaking.

Extraction

During the standard procedure (Fig. 1) the extraction of the anabolic steroids is performed by liquid-liquid-extraction of the dried methanolic extract at pH 12 with n-pentane. It is followed by a re-extraction from the n-pentane with methanol (95 %). If the final methanolic extract is intensively coloured, often overloaded and/or not evaluable chromatograms are obtained because of the extraction of too many matrix compounds. Washing of the methanolic residue with n-pentane may lead to better results. For samples containing high amounts of fat or Vit. E or A this procedure also seems to be the method of choice.

Separation

For some nutritional supplements the separation of the aqueous and the n-pentane layer after the extraction is very difficult. The addition of sodium sulphate to the mixture after centrifugation and additional centrifuging without further shaking solves this problem.

Derivatisation

Depending on the matrix of the supplements in some cases insufficient derivatisation occurs. Sometimes the final residue in the autosampler vials is totally solid due to the crystallised N-methyltrifluoroacetamide (MTFA), indicating that the amount of MSTFA added was not sufficient. Also in cases where the derivatisation mixture remains fluid insufficient derivatisation can occur and be detected in the chromatograms of the GC/MS analyses (Fig. 8). Following a second derivatisation with additional 100 µl of the derivatisation reagent at 60°C for 20 min. suitable results can be obtained for most of the samples. Fig. 9 shows the chromatogram of the same sample as shown in Fig. 8 but after the second derivatisation. Until now it was not possible to identify any compounds of the matrix causing this effect.

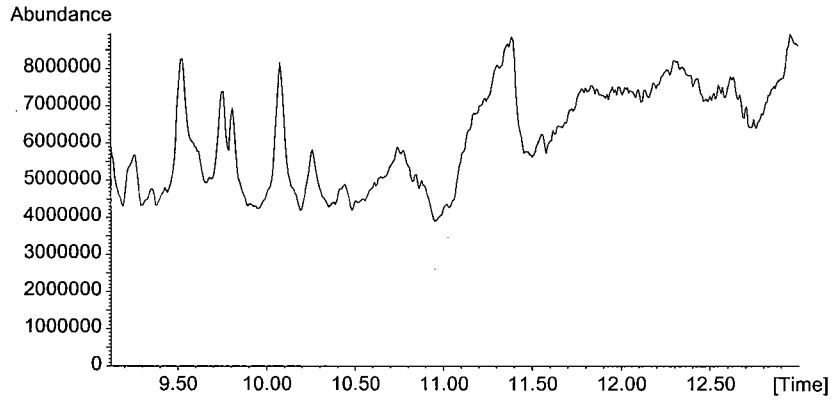

Fig. 8: Chromatogram (TIC) of a sample after insufficient derivatisation

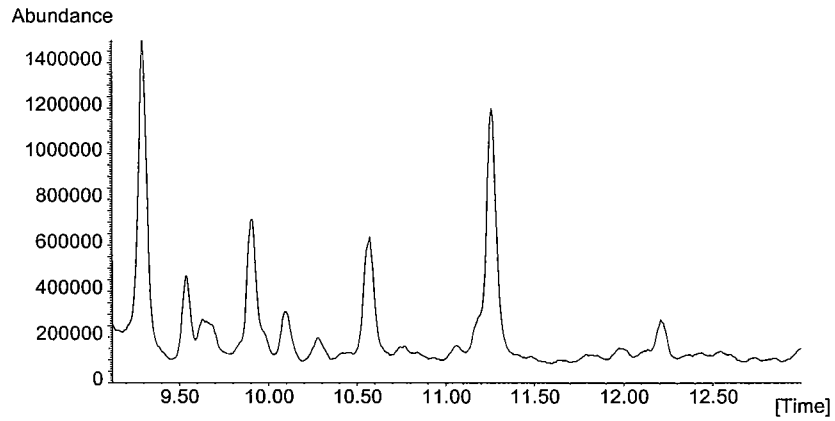

Fig. 9: Chromatogram (TIC) of the same sample as in Fig. 8 after a second derivatisation

Oily supplements

For most of the oily samples a reduction of the amount of the supplement analysed is a possibility to solve the problems caused by matrix compounds. Only an aliquot of 500 µl of the methanolic solution (5 ml) of the supplement (corresponding 100 mg/500 µl) is used for further sample preparation. The following steps of sample preparation are carried out as described in Fig. 1 but with an extra-washing of the final methanolic extract with n-pentane. By this all compounds are reduced and most of the supplements are successfully analysed.

Quantification

Matrix compounds strongly influence the analyses of anabolic steroids in nutritional supplements. Therefore they cause problems in quantification. An external standard can only be used for a rough estimation of the concentration. Deuterated internal standards of the specific steroids should be used for the determination of the concentration. Where no corresponding deuterated steroids are available the method of standard addition is necessary to compensate for matrix effects. But because of small concentration ranges of linear calibration curves also quantification by standard addition is difficult to perform. In many cases also the standard addition leads only to estimates of the concentration and a second standard addition analysis is needed to finally determine the concentration in the supplements with linear correlations for the standard addition.

Characteristics of the standard method

The characteristics of the method vary in wide ranges with the matrices of the samples. As an example the characteristics determined in a creatine monohydrate powder are presented. For screening purposes this matrix was chosen for the negative and positive control because it is easily available in a constant quality and does not cause any problems in sample preparation and measurements.

The limit of detection (S/N-ratio >3) could be determined at 0.1 ng/g for DHEA and estr-4-ene-3β,17β-diol, at 0.7 ng/g for 5α-androstane-3β,17β-diol and androsta-1,4-diene-3,17-dione, at 1 ng/g for estr-5-ene-3β,17β-diol, estr-4-ene-3,17-dione, 19-nortestosterone, androst-4-ene-3,17-dione and testosterone, and 2 ng/g for androst-4-ene-3β,17β-diol and androst-5-ene-3β,17β-diol. A chromatogram of a positive control sample with 0.1 ng/g of DHEA is presented in Fig. 10.

The recovery (determined at 200 ng/g) differed from 32 % for 19-nortestosterone to 92 % for androst-5-ene-3β,17β-diol. Linearity could be obtained in small concentration ranges e.g. in a

range of 40 to 200 ng/g. Hence, for the estimation of the concentration a standard in a concentration very similar to the concentration in the supplement is needed.

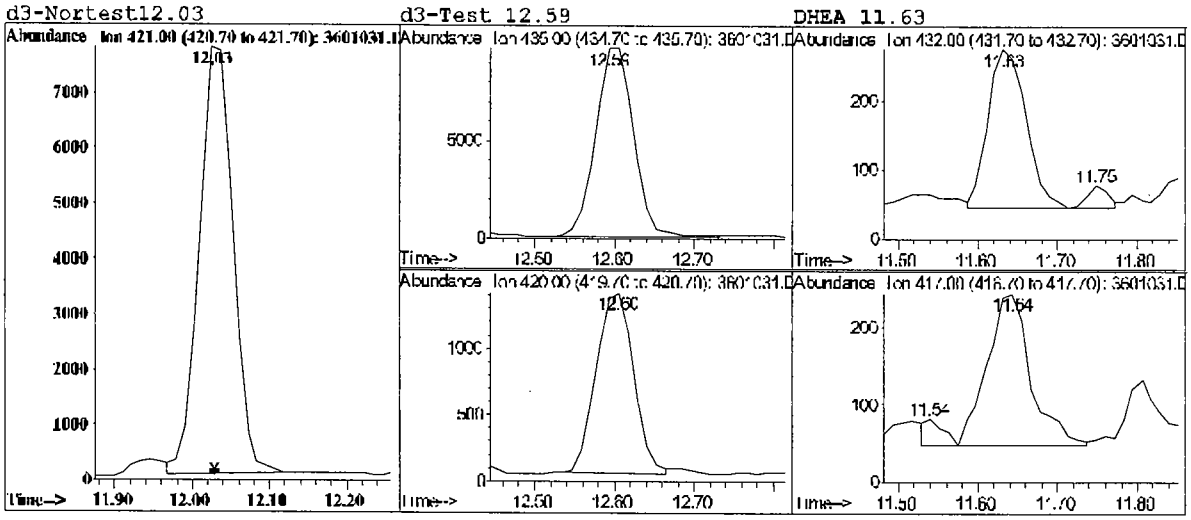

Fig. 10: Chromatogram of a positive control with 0.1 ng/g DHEA in creatine

Results of the analyses of the nutritional supplements

Out of the 634 samples 445 supplements (70.2 %) could be analysed with the standard method without any modification. Four further samples could be analysed with this method but after the addition of sodium sulphate for the separation of the aqueous and the n-pentane layer. The second derivatisation led to suitable results for 37 supplements, additional washing of the final extract with n-pentane for 12 supplements and both, second derivatisation and extra-washing, for three samples. Skipping the first extraction of the supplement with methanol (starting directly with the distribution between 0.1 M KOH and n-pentane) allowed the analyses of further 48 samples (7.6 %). Out of those 21 had to be extra-derivatised, two extra-washed and one supplement extra-washed and extra-derivatised.

The special sample preparation for oily supplements led to reliable data for 19 oily supplements (3.0 %). For two of them a second derivatisation was needed. For 66 supplements (10.4 %) with none of these modifications suitable results could be obtained.

Positive results for anabolic steroids were obtained in 94 supplements (14.8 %). Out of all positive supplements 23 samples (24.5 %) contained prohormones of nandrolone and testosterone, 64 samples (68.1 %) contained only prohormones of testosterone, 7 samples (7.5 %) contained only prohormones of nandrolone. One sample contained the prohormone of dihydrotestosterone, none the prohormone of boldenone.

The detailed results of this investigation will be presented elsewhere.

Conclusion

The analysis of the nutritional supplements has proven rather difficult due to the different matrices in the various products available. Anyway a standard sample preparation procedure can be used for most matrices, which includes extraction and purification of the analytes followed by GC/MS measurements of the TMS derivatives of the steroids. The addition of a mixture of diisopropylaminoalkanes (DIPAs) to the final extract leads to an improved sensitivity of the analyses. Several modifications are necessary to obtain suitable results in case of matrix problems. The most useful modifications are a second derivatisation of the final residue, extra-washing of the final methanolic extract with n-pentane and skipping of the first methanolic extraction of the supplement. In some cases combining these modifications has led to suitable results. Yet for approximately 10 % of the supplements (66 out of 634) no reliable data could be obtained.

Because of matrix effects quantification by the use of an external standard can only be a rough estimate. Deuterated internal standards of the specific steroids or standard addition lead to better results because of the compensation of matrix effects. Especially because of the limited range of concentration where linearity is obtained the quantification by standard addition is very work intensive. Often two analyses with standard addition are necessary to obtain reliable data.

Acknowledgements

The Manfred-Donike-Society, Cologne, the International Olympic Committee and the Bundesinstitut für Sportwissenschaften, Köln are acknowledged for supporting the study.

References

[1] International Olympic Committee: List of prohibited classes of substances and methods of doping. International Olympic Committee, Medical Commission, Lausanne 2001

[2] Geyer, H., Mareck-Engelke, U., Reinhart, U., Thevis, M., Schänzer, W.: Positive Dopingfälle mit Norandrosteron durch verunreinigte Nahrungsergänzungsmittel. Dtsch. Z. Sportmed. 51, 11 (2000) 378-382

[3] Geyer, H., Mareck-Engelke, U., Wagner, A., Schänzer, W.: Analysis of "non -hormonal" nutritional supplements for prohormones In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln (2001), 63-72

[4] De Cock, K.J.S., Delbeke, F.T., Van Eenoo, P., Desmet, N., Roels, K., De Backer, P., Detection and determination of anabolic steroids in nutritional supplements, J. Pharm Biomed Anal 25 (2001) 843-852

[5] Ministry for food and rural area, Baden-Württemberg, Germany (Edelhäuser, M): Lecture about results of the analyses of nutritional supplements for anabolic steroids. Press conference BISP, Cologne 08.02.01

[6] IOC-Document, Criteria for reporting low concentrations of anabolic steroids (1998)

[7] Geyer, H., Mareck-Engelke, U., Nolteernsting, E., Opfermann, G., Donike, M.: The matrix Problem in calibration of the GC/MS for Endogenous Steroids. The search for an artificial urine In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995), 199-209