Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (11)

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

Sport und Buch Strauß, Köln, 2003

M.K. PARR, H. GEYER, G. SIGMUND, K. KÖHLER, W. SCHÄNZER:
Screening of Nutritional Supplements for Stimulants and other Drugs
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (11). Sport und Buch Strauß, Köln, (2003) 67-75

Maria Kristina Parr, Hans Geyer, Gerd Sigmund, Karsten Köhler, Wilhelm Schänzer

Screening of Nutritional Supplements for Stimulants and other Drugs

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

Introduction

In the last few years several studies have proven that the production of nutritional supplements can result in intentional or unintentional contamination with anabolic steroids. These contaminations may lead to positive tests in doping control [1-5].

Recently the findings of several other pharmaceutical active compounds have been reported: a US based working group reported the finding of warfarin and nutritional supplements containing amphetamines resp. fenfluramine have been taken from the market by governmental authorities [6].

The Netherlands Centre for Doping Affairs (NeCeDo) reported the findings of non-declared caffeine and ephedrines in several nutritional supplements of top level athletes. Stimulants as well as narcotics belong to the prohibited classes of substances [7] of the International Olympic Committee (IOC), and the ingestion of such supplements may also lead to unintentional use of doping substances.

Based on this knowledge a screening method with GC-MS and GC-NPD for stimulants and other N-containing doping relevant drugs has been adjusted for the analysis of nutritional supplements. During the analysis of 110 nutritional supplements from the international market first positive results with this procedure have been proven.

Experimental

Supplements

The method was tested with 15 different nutritional supplements containing caffeine and/or ephedrines as declared ingredients. Then this method was applied to 110 supplements from nine different countries with neither caffeine nor ephedrine content declared.

Sample preparation

To 1 g of the pulverized supplement 20 µg of 1-N,N-diisopropylaminododecan (DIPA-12) as internal standard and 5 ml of hydrochloric acid (HCl, 0.06 N) are added. After adjusting pH < 5 the mixture is extracted with 5 ml of n-pentane while shaking for one hour. The n-pentane layer is then discarded. To the aqueous layer 0.5 ml of KOH (5 M) are added to adjust pH ≥ 13. The mixture is extracted with 1.8 ml of t-butylmethyl ether (TBME) under addition of sodium sulphate (saturation). The organic layer is injected onto a GC-MS/NPD system. The flow scheme of the standard sample preparation is shown in Fig. 1.

The identification of the analytes is performed according to the recommendations of the IOC [8].

For the confirmation of the ephedrines the final extract is evaporated to dryness and derivatised with 100 µl of MSTFA/MBTFA at 80°C for 30 min [9]. The residue is then reanalysed by means of GC-MS.

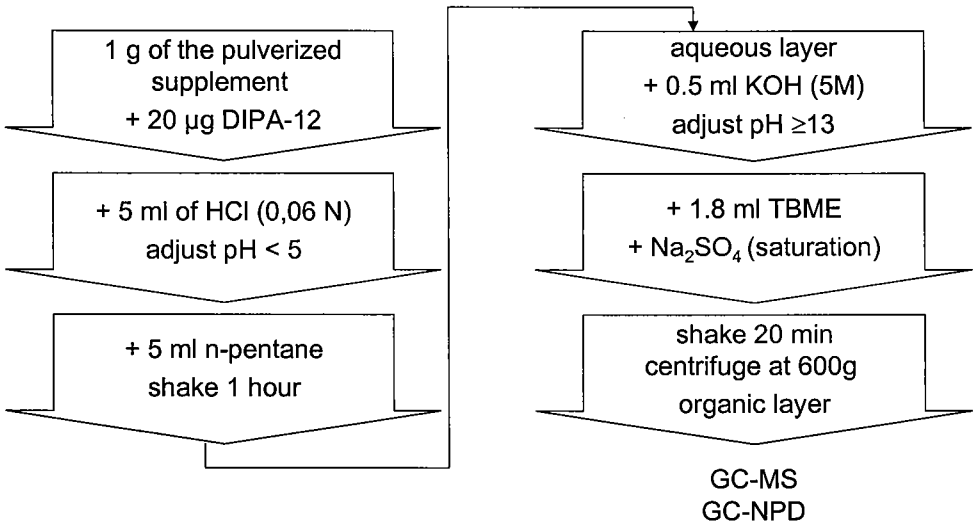


Fig. 1: Flow scheme of the standard sample preparation for the analysis of nutritional supplements for stimulants and other drugs

Instrumentation

GC/MS analyses

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

Injection parameters:	Volume: 5 µl, split mode (1:5), Temp.: 300°C
Column:	MSD: HP-5MS, 24m, 0.25mm i.d., 0.25µm film thickness NPD: HP-5MS, 19m, 0.25mm i.d., 0.25µm film thickness
Carrier gas:	Helium, head pressure 12 psi
Oven temp.:	100°C with 22°C/min to 330°C, 2.5 min hold
MS Parameters:	Electron impact ionisation (EI) 70 eV, data aquisition SCAN 40-400
NPD:	Aux+carrier (const.): 20ml/min, hydrogen (2ml/min), air (60ml/min), temp: 290°C

For the confirmation of the ephedrines the N-TFA,O-TMS derivatives are analysed on a Hewlett Packard (HP) 6890 plus gas chromatograph coupled to a HP 5973 mass selective detector (MSD) and a NPD detector with the following parameters:

Injection parameters:	Volume: 1 µl, split mode (1:5), Temp.: 290°C
Column:	HP-5MS, 18m, 0.25mm i.d., 0.25µm film thickness
Carrier gas:	Helium, head pressure 9 psi
Oven temp.:	100°C with 22°C/min to 330°C, 3 min hold
Ionisation:	Electron impact (EI) 70 eV
Data aquisition:	SCAN 40-500

Characteristics of the method

The lower limit of detection (LOD) is determined as the concentration where two diagnostic ions with a signal-to-noise ratio > 3 are obtained in the chromatograms.

The recovery is calculated from the areas obtained for the analytes after analyses of a six times replicate of a spiked creatine sample with the areas of a spiked final extract of a blank sample.

Results and Discussion

GC/MS characteristics of the analytes

The GC/MS data of some analysed drugs are listed in Tab. 1. The electron impact (EI) mass spectra of ephedrine as underivatised substance and its N-TFA,O-TMS derivative are shown in Fig 2 and Fig. 3.

The combination of the detection with MSD and NPD opens the possibility to detect and identify other N-containing bases in the final extract. These compounds may also be of relevance for doping issues.

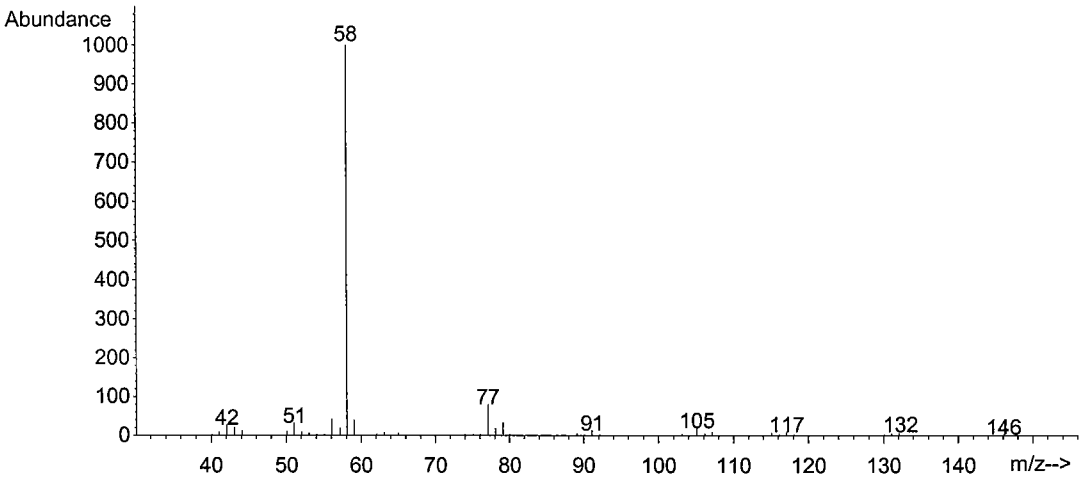


Fig. 2: EI mass spectrum of ephedrine, M^+=165

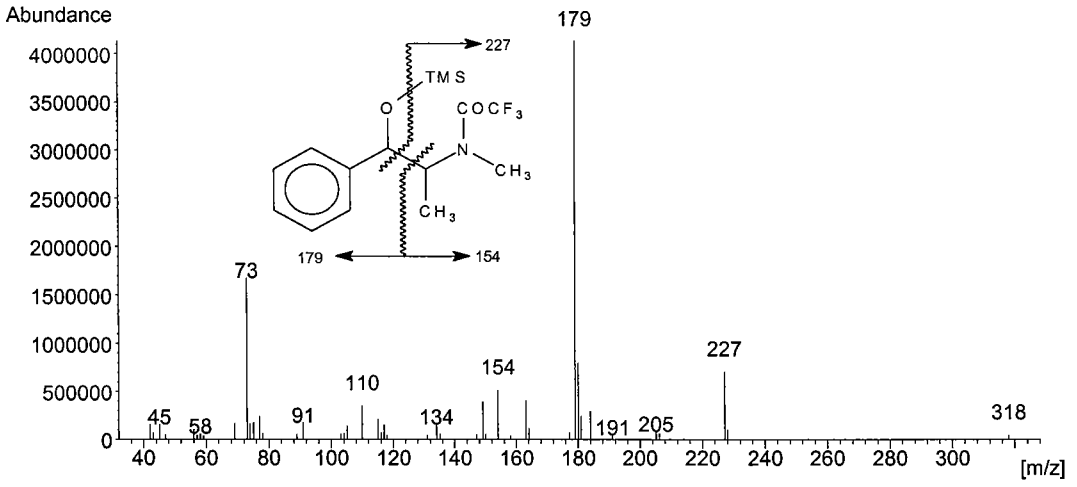


Fig. 3: EI mass spectrum of ephedrine N-TFA, O-TMS, M^+=333

Tab. 1: Retention times and characteristic ions of some drugs monitored and the internal standard

Substance	RT [min]	M^+	Base peak	other characteristic ions
Heptaminol	1.85	145	44	59, 69, 113, 95
Amphetamine	1.97	135	44	91, 65, 120
Phentermine	2.14	149	58	91, 134, 65
Metamphetamine	2.24	149	58	91, 65, 134
Fenfluramine	2.51	231	72	44, 159, 109, 216
Dimetamphetamine	2.58	163	72	91, 65, 56
Cathine	3.06	151	44	77, 105, 51
Phenylpropanolamine	3.06	151	44	77, 105, 51
Nicotine	3.17	162	84	133, 162, 161, 119
Ephedrine	3.33	165	58	77, 105
Pseudoephedrine	3.33	165	58	77, 105
Methoxyphenamine	3.37	179	58	91, 77
Methylephedrine	3.58	179	72	77, 56, 91, 105
Phenmetrazine	3.78	177	71	56, 77, 105, 177, 117, 91
MDA	3.95	179	44	136, 77, 51, 179
Pholedrine	3.97	165	58	77, 107
Methylecgonine	4.01	199	82	96, 199, 168, 182
Amfepramone	4.09	205	100	44, 72, 77
MDMA	4.23	193	58	135, 77, 105, 89
Nikethamide	4.31	178	106	78, 177, 178, 149, 163
Pentylenetrazole	4.51	138	55	82, 109, 138
MDEA	4.66	207	72	44, 135, 77,105
Fenproporex	4.71	188	97	56, 91, 68
Prolintane	4.91	217	126	91, 174
Fencamfamine	5.16	215	98	215, 84, 58, 91, 186, 158, 115, 124
Crotethamide	5.22	226	86	154, 69, 181, 115, 100, 129
Cropropamide	5.50	240	100	168, 69, 195, 115, 143
Pethidine	5.68	247	247	71, 172, 218, 103, 91, 96, 115, 131, 232
Methylphenidate	5.86	233	84	56, 91, 115, 130
Caffeine	6.03	194	194	109, 67, 55, 82, 165, 136
Benzphetamine	6.08	239	91	148, 65
ISTD (DIPA-12)	6.68	269	114	
Clenbuterol	7.22	276	86	127, 190, 243, 245
Methadone	7.45	309	72	165, 178, 223, 91
Pipradol	7.71	267	84	77, 56, 105, 165
Pentazocine	8.45	285	217	202, 110, 285, 270, 159, 173, 146, 230
Fenethylline	10.52	341	250	207, 91, 70
Strychnine	11.93	334	334	130, 120, 143, 107, 162, 77

Extraction from the matrix

During the development of the method 52 nutritional supplements were analysed by a simple extraction with 5 ml of methanol as well as with the method described in Fig. 1.

Sample clean-up can be improved by several steps of extraction as demonstrated in Fig. 4 and Fig. 5. This is important especially for supplements containing amino acids, creatine etc. The first step of extraction at acidic pH is necessary especially for the analysis of supplements containing higher amounts of fatty ingredients. During this step the supplements are degreased allowing a better wetting during the final extraction and cleaner residues.

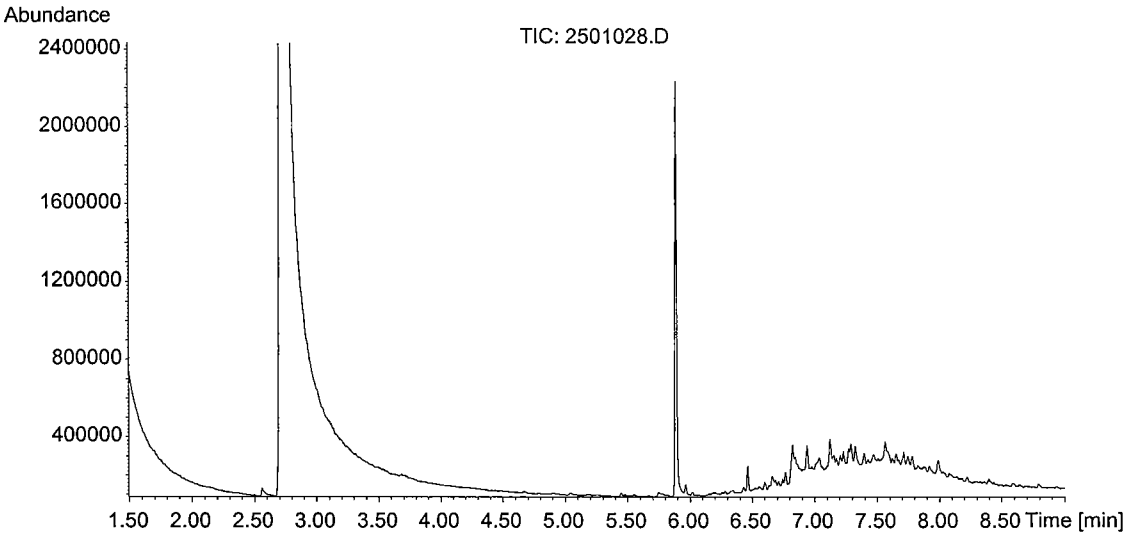


Fig. 4: Chromatogram of a supplement containing zincpicolinate and -histidinate after methanolic extraction and direct injection of the methanolic residue

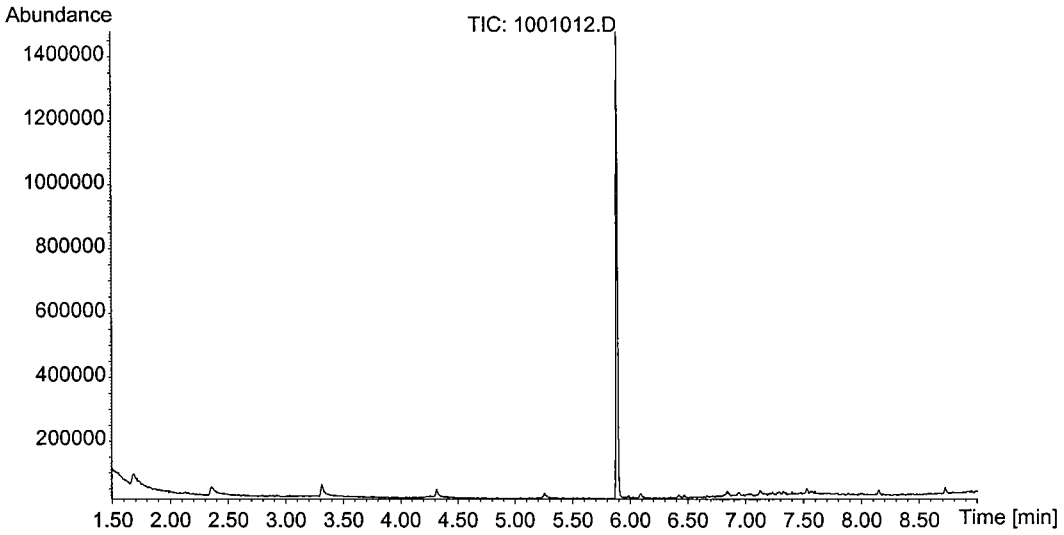
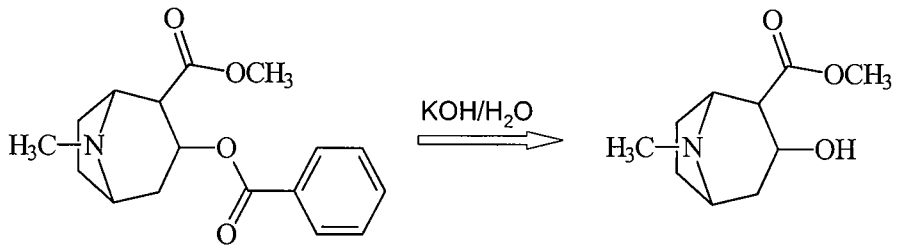


Fig. 5: Chromatogram of the same supplement (Zincpicolinate and –histidinate) as in Fig. 4 but after sample preparation according to Fig. 1

Detection of cocaine

During the sample preparation the benzoic ester in cocaine is cleaved to methylecgonine. Methylecgonine is easily extracted and can be detected in the chromatograms as a marker for the presence of cocaine in the supplement. A chromatogram of a sample spiked with several compounds including cocaine is presented in Fig. 7.



Cocaine Methylecgonine

Fig. 6: Cleavage of cocaine at alkaline conditions

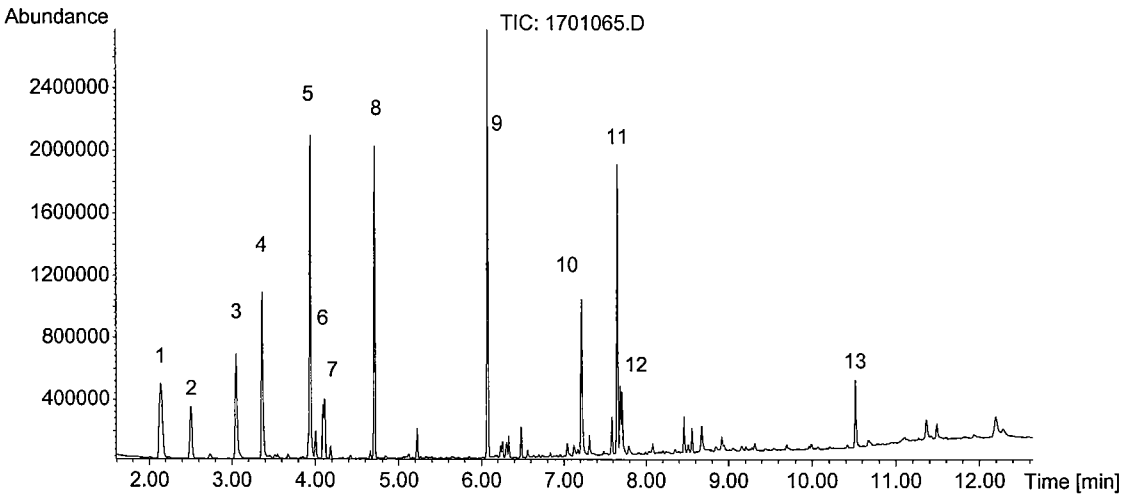


Fig. 7: Chromatogram (TIC) of a sample spiked with 1. phentermine, 2. fenfluramine, 3. norephedrine, 4. methoxyphenamine, 5. MDA, 6. cocaine (detected as methylecgonine), 7. amfepramone, 8. fenproporex, 9. benzphetamine, 10. pipradol, 11. clenbuterol, 12. methadone, 13. fenethylline

Characteristics of the method

In a creatine matrix LODs of 0.1 to 1 µg/g are found for most of the stimulants. Only for MDA, benzphetamine, cocaine, fenetylline and strychnine LODs of 2 µg/g and for pholedrine of 5 µg/g were determined. The recovery ranged between 65 to 100 %.

Screening of market samples

The method was tested with 15 supplements with caffeine and/or ephedrine as declared ingredients. In case of caffeine or ephedrine containing herbs according to European legislation labelling of the specific herb is sufficient. In this case, labelling of the pharmaceutically active compounds themselves is not necessary. If the caffeine is added from synthetic sources, the amount of caffeine has to be declared.

Natural caffeine containing products, which can be found in nutritional supplements, are coffee, tea, guarana, yerba mate and others.

Herbal products of *Ma Huang* contain parts of the ephedra plant (*ephedra sinica, ephedra intermedia, ephedra equisetina*) a natural source of ephedrine and ephedrine derivatives [10]. They are often part of nutritional supplements marketed as fat burner or as generally activating products.

In all these supplements caffeine and/or ephedrines could be detected after the analysis according to the presented method.

In addition 110 nutritional supplements from the international market with neither caffeine nor ephedrine as declared content were analysed with this method.

Out of them 14 supplements (12.7 %) contained caffeine in concentrations up to 10 mg/g. One of these samples was additionally labelled with "pharmaceutical quality".

In one sample ephedrine could be detected in a low concentration of about 8 µg/g and one supplement contained different ephedrines (ephedrine, pseudoephedrine and methylephedrine). The sum of the ephedrine and pseudoephedrine amount was estimated at 150 µg/g. For the separation of the diastereomers selective derivatisation was performed.

The pattern of the ephedrine derivatives is characteristic for Ma Huang products [10].

Summary

A sensitive analytical method for screening of nutritional supplements for N-containing drugs is presented. It is tested with 15 caffeine and/or ephedrine containing supplements.

Caffeine and/or ephedrines can be declared on the label as contents of different herbal preparations. In this case neither caffeine nor ephedrine themselves have to be labelled.

Out of 110 supplements from the international market more than 10 % contained non-declared caffeine or ephedrines. The highest concentrations were estimated at 10 mg/g of caffeine and 150 µg/g of ephedrines. Ingestion of the recommended dose of the supplement results in a total uptake of 200 mg of caffeine and 3 mg of ephedrine per day.

Athletes have to be informed about the risk of unintentional doping not only with prohormones but also with stimulants by using nutritional supplements. In addition companies selling nutritional supplements should be forced to install a Good Manufacturing Practice (GMP) system.

Acknowledgements

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

References

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

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

[3] Gmeiner G, Hofer H. Untersuchung auf mögliche Verunreinigungen von Nahrungsergänzungsmitteln mit anabolen Steroiden. Forschungsberichte des Österreichischen Bundesministeriums für soziale Sicherheit und Generationen. ARC Seibersdorf research GmbH, Seibersdorf, 2 (2002)

[4] Geyer H, Parr MK, Reinhart U, Schrader Y, Mareck U, Schänzer W. Analysis of Non-Hormonal Nutritional Supplements for Anabolic Androgenic Steroids - an International Study -. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (10). Sport und Buch Strauß, Köln (2002) 83-85

[5] Edelhäuser, M. Vortrag über die Ergebnisse der Analyse von Nahrungsergänzungsmitteln im Ministerium für Ernährung und ländlicher Raum Baden-Württemberg, Pressekonferenz Bundesinstitut für Sportwissenschaften (BISP), Köln, 08.02.01.

[6] U.S. Food and Drug Administration. FDA warns public about Chinese diet pills containing fenfluramine, FDA News P 02-26 (2002), http://www.fda.gov/opacom/hpwhats.html

[7] International Olympic Committee, Medical Commission. List of prohibited classes of substances and methods of doping (2001)

[8] International Olympic Committee. Criteria for reporting low concentrations of anabolic steroids (1998)

[9] Donike, M, Kaiser, C. Moderne Methoden der Doping-Analyse. Sportarzt und Sportmedizin, 22 (1971) 32

[10] Gurley BJ, Gardner SF, Hubbard MA. Content versus label claims in ephedra-containing dietary supplements. Am J Health Syst Pharm. 57 (2000) 963-969.