Removal of the Interferences in the Norandrosterone Analysis at Trace Level in Complex Matrix

ILEANA VAJIALA*, MIRELA ZORIO
National Anti-Doping Agency, Doping Control Laboratory, 37-39 Basarabia Bvd., 022103, Bucharest, Romania

Reporting a positive doping case requires appropriate methods of urine sample purification and selective analytical techniques fit to provide unequivocal evidence of doping agent abuse. The detection of Norandrosterone at the threshold of 2ng/mL by liquid-liquid extraction followed by gas chromatography coupled to mass spectrometry with quadrupol filter (GC/MS) is affected by interferences caused by urine biological matrix and by medication intake. This paper introduces the practical application on real urine samples of two methods for Norandrosterone determination at trace level, in a complex biological matrix, using LLE followed by GC-HRMS analysis and SPE with GC-MS respectively. The comparative data presented herein emphasize the benefits of each method for doping control purposes.

Key words: Norandrosterone, GC/MS, GC/HRMS

Given the increase in muscular tissue during intense and prolonged physical exercise, the anabolic agents remain the most commonly used class of abuse substances in sports, accounting for more than 90% of the positive cases reported by the doping control laboratories. The technical guidelines [1] which regulate the control of banned substances in sport require special detection features for the anabolic agents, namely detection at the level of 2ng/mL of urine for 5 particular substances (anabolic agents or their metabolites): Methandienone [2-5], Stanozolol [2,5-8], Norandrosterone [9,10], Methyltestosterone [11] and Clenbuterol [7,12].

In addition to the low concentration rate at which these are eliminated through urine, the interferences owed to the complex biological matrix strongly affect the detection of these components. As such, the labeling of a doping control sample as positive requires a selective isolation of the analytes and a highly specific and sensitive instrument-based technique to ensure the unequivocal identification of banned substances and to ensure that the specific criteria are met in relation to the witness sample. The general approach consists in pre-treating the urine test sample by chromatographic methods coupled to mass spectrometry [1,8,13].

Excreted in urine as conjugated with glucuronic acid, Norandrosterone (5α–estran-3α ol-17-one), is determined by gas chromatography coupled with low-resolution mass spectrometry (GC-MS) after its isolation by liquid-liquid extraction LLE [14,15]. Upon re-testing of the sample for confirmation, the method does not ensure that the requirements for the relative abundance of mass fragments are met, due to the background noise and the coelutive compounds, such as vitamin E metabolites.

This paper presents the results obtained in the laboratory after the application of two methods for eliminating the interferences in the confirmation test for low concentration of Norandrosterone in urine samples: respectively the association of the LLE with high resolution mass spectrometry (GC-HRMS) and the purification of the urine matrix by SPE, the removal of the free fraction, followed by the determination via low resolution mass spectrometry (GC-MS).

The performance characteristics of both methods were also investigated and the results obtained by the practical application of the methods on real urine samples, collected in doping control, are presented.

Experimental part

Materials

The reference substance for Norandrosterone was obtained from the National Analytical Reference Laboratory (NARL, Australia). The derivatization agent N-methyl-N-trimethylsilyltrifluoroacetamide MSTFA (for gas chromatography) is manufactured by Merck, the β-glucuronidase from *E.coli* is manufactured by Roche Diagnostics Manheim and the resin type Amberlite XAD_2 is obtained from Supelco U.S.A. All the other reagents and solvents were of analytical and chromatographic grade and were purchased from Sigma or Merck.

The methods developed herein were checked up with positive control samples obtained from fortifying blank steroid-free urine with reference Norandrosterone solution at critical concentration of 2ng/mL. The blank urine sample was provided by a volunteer during a vitamin E cure – *alpha*-tocopheryl acetate, 100mg daily, for 10 days. For the practical application of the methods, current doping control samples were tested in compliance with the principles of bioethics and confidentiality of the athlete's identity. For stopping the bacterial activity all the samples were added 1g/L NaN_3 and were stored frozen at -24°C.

Isolation of Norandrosterone from urine by liquid-liquid extraction

Considering the data published in the domain literature [15], a LLE method of Norandrosterone separation from urine was developed within Doping Control Laboratory [14]. After enzymatic hydrolysis with β-Glucuronidase from *E.coli* at neutral *p*H, free Norandrosterone was extracted in *tert*-butyl methyl ether (TBME) at a *p*H value of about 9, following the evaporation of the organic phase to dryness under reduced pressure, the extracts were derivatized with 100µL mix MSTFA-NH_4I-ethaniol (100:0.2:0.6 v/w/v); trimethylsilyl derivatives were yielded by heating the mixture for 20 min at 60°C.

Isolation of Norandrosterone from urine by solid-liquid extraction

The method described in [9] was used. After the application of the urine sample on a glass column

* email: i.vajiala@anad.gov.ro; Tel.: 0726 48 50 29

containing Amberlite XAD_2 resin, the Norandrosterone glucuronide was eluted with freshly distilled methanol. After evaporation to dryness of the methanol, the sample is retrieved with buffer phosphate of *p*H 7 and the steroids excreted in free forms are extracted in *tert*-butyl methyl ether (TBME). The ether layer is removed through vacuum aspiration and the aqueous phase is hydrolyzed with β-Glucuronidase from *E.coli*. After the hydrolysis is stopped by adding a buffer of K_2CO_3-$KHCO_3$, up to a *p*H value of about 9, n-pentane is added to extract the Norandrosterone; finally the sample is derivatized with 100µL mix MSTFA-NH_4I-ethaniol (100:0.2:0.6 v/w/v); trimethylsilyl derivatives were yielded by heating the mixture for 20 min at 60°C.

Determination of Norandrosterone by GC-MS

The analysis was conducted on a laboratory equipment consisting in a gas chromatograph Agilent Technologies 6890N coupled to a quadrupol mass spectrometer 5973 Network, according to the published methods [14,15]. On a capillary chromatographic column HP-ULTRA 1 (J&W Scientific) with stationary phase 100% dimethyl-polisyloxane, 17m length, 0.2mm inner diameter, 0.11µm film thickness, at a constant flow of 0.8mL/min carrier gas helium, 3µL of derivatized extracts were injected with a split rate 10:1. The injector and the transfer line were set at 300°C, and the GC oven on the following temperature ramps: 160°C (2 min), with 5°C /min up to 255°C (0 min), with 30°C /min up to 285°C (5 min), with 60°C/min up to 300°C (3.75 min). The trimethylsilyl derivative, Norandrosterone bis TMS was monitored in SIM (selected ion monitoring) acquisition mode, after electronic impact ionization at 70 eV.

Determination of Norandrosterone by GC-HRMS

The high resolution mass spectrometry was processed on a mass spectrometer with double focusing and reversed geometry MAT 95 XP ThermoFinnigan, coupled to a gas chromatograph Agilent Technologies 6890N, according to the established method [2]. On a capillary chromatographic column HP-ULTRA 1 (J&W Scientific) with stationary phase 100% dimethylpolisyloxane, cross-linked, 17m length, 0.2mm inner diameter, 0.11µm film thickness, at a constant flow of 0.7mL/min carrier gas helium, 2µL of derivatized extracts were injected with a split rate of 10:1. The injector and the transfer line were set at 300°C and the GC oven on the following temperature ramps: 175°C (0 min), with 5°C/min up to 235°C (0 min), 20°C/min up to 310°C (2 min). The ions were formed in the ion source through electronic impact EI ionization at 70eV (1mA emission current). The temperature of the ionization source was maintained at 230°C. High resolution monitoring of the selected ions (MID mode) was done by electric field scanning using a fluorocarbon reference compound (fc_5311) to calibrate and fix the magnet. The processing was done for a mass resolution of 5,000 at 1.8kV tension on the electrons multiplier.

Results and discussions

LLE and GC-HRMS analysis

The performance characteristics

Within-day precision was examined by analyzing six different control samples (blank urines spiked with Norandrosterone at a concentration of 2ng/mL), which were prepared and analyzed according to the described method. Reproducibility (between-day precision) was investigated using six replicates of the control sample of 2ng/mL extracted and analyzed by different analysts on different times. The calculated relative standard deviations RSD were 3.7% for within-day precision and 7.7% for reproducibility, values situated below 25%, the doping criteria for the low concentration sample. The detection limit was determined by analysis of samples spiked at lower and lower concentrations; 0.5ng/mL was declared as LOD of the method, as being the lowest concentration at which Norandrosterone could be detected with a signal to noise ratio greater than 3 (S/N>3), for each of its characteristic ions. The linearity was investigated in the range of 1-5ng/mL; the calibration curve was described by a linear model $y = 0.0407x + 0.0093$ and the correlation coefficient was 0.998.

Calculating the average of sound to noise ratios of Norandrosterone characteristic signals in control samples at 2ng/mL, the values were 93.1 for GC-HRMS analysis (RSD 9.37%) and 3.2 for GC-MS (RSD 21.83%) respectively. It is noticed that whilst the GC-MS values represent the background noise due to urine matrix, the higher values obtained on GC-HRMS and the RSD < 20% demonstrate that the limit of detection for Norandrosterone could be significantly below 2ng/mL.

Norandrosterone is excreted in urine as glucuronide conjugate. Its release from the conjugated forms is achieved by hydrolysis with β -Glucuronidase from *E.coli*, an enzyme which performs optimally at 50°C. The free Norandrosterone (fig.1) was extracted in *tert*-butyl methyl ether, in basic conditions, after adding carbonate-bicarbonate buffer to a *p*H value of aprox. 9. Thus, the polar and less polar steroid components are isolated from urine together with a large number of natural substances, which leads to the increase of background noise.

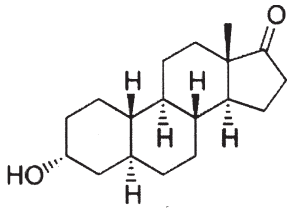

Fig. 1. Structure of Norandrosterone

The derivatization of the functional groups is required to form the more volatile trimethylsilyl ethers (Norandrosterone bis TMS), with reduced polarity and easy to separate by chromatography. The derivatization mix which was employed is efficient for the hydroxy- and keto- groups which are tied to the steroid nucleus.

To obtain comparative data, the extracted samples were analyzed in parallel by low resolution mass spectrometry with quadrupol filter and by high resolution mass spectrometry with double sector, magnetic and electrostatic. In the case of Norandrosterone testing by GC/MS, SIM mode, the following mass fragments are monitored: m/z 405, 420, 315, the characteristic ratio applicable to the intensities of these fragments being around 100:60:60 [16]. In the MID method processed on the high resolution mass spectrometry GC/HRMS, Norandrosterone is detected through the mass fragments m/z 405.2645 and 420.2879, the ratio applicable to the signal intensity being 100:50 [10].

The detection selectivity results from the comparison of the chromatograms of the suspicious sample on Norandrosterone, recorded in the two analysis systems, through the protocols described above. In figure 2, *a* it is noted that for the actual doping control sample analyzed by low resolution method GC/MS, the data is equivocal, the noise background is elevated, and due to the abundance of fragment m/z 315, higher than the base peak

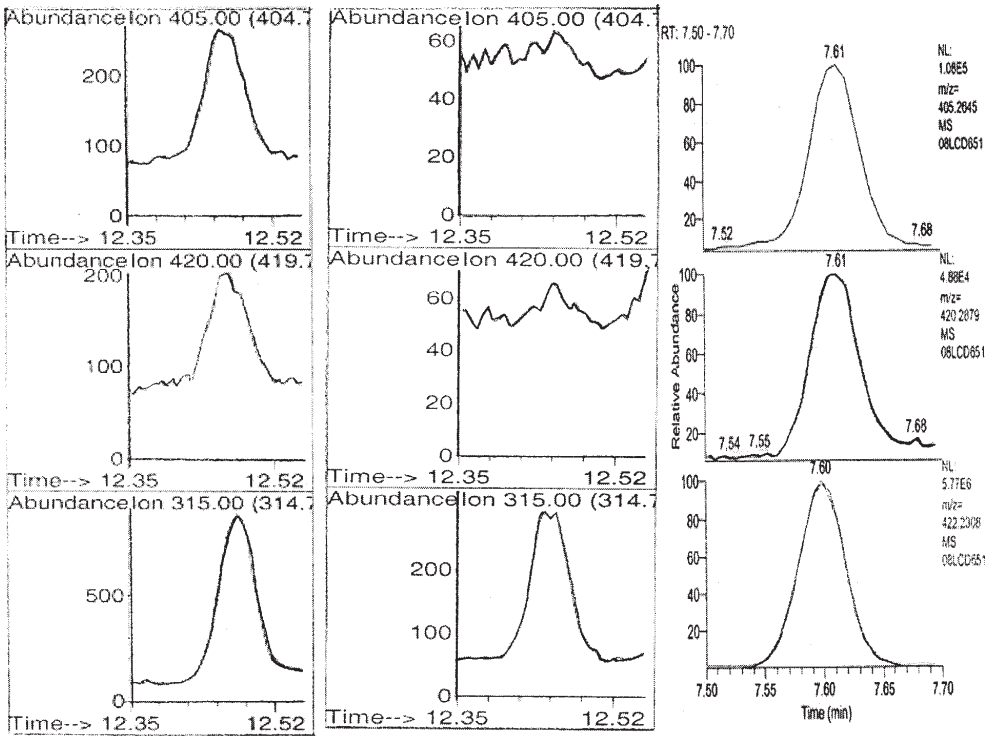

Fig. 2. The chromatogram of Norandrosterone specific ions in the suspicious (*a*) and blank sample (*b*) seen in GC/MS analysis; suspicious sample analyzed in GC/HRMS (*c*)

m/z 420, the characteristic ratio of the relative intensities is not observed. The weak, hard to integrate signals do not match the criteria required to confirm the presence of Norandrosterone in the suspicious sample (Tr 12.40 min). The abundant signal of the ion m/z 315 is due to the interference with the fragments of the vitamin E metabolites (m/s 422) – even if there is a difference in the masses of the bis-TMS derivatives. The contribution of this fragment is also noted in the blank urine sample collected after the vitamin E cure (fig.2, b).

The same sample analyzed by high resolution system GC/HRMS (fig.2, c) presents clear signals, reduced background noise and high intensity. At a mass resolution of 5,000, the signals are better defined and more intense than in the quadrupol system, and the characteristic ratio between the height of the peaks is maintained (100:50), which thus confirm the presence of Norandrosterone in the suspicious sample. At the same time, by using GC/HRMS, MID mode, the molecular ion of Norandrosterone bis-TMS, 420.2879 (Tr 7.61 min) and the mass fragment obtained from the vitamin E bis-TMS metabolite, 422.2308 (Tr 7.60 min) do not influence each other at a resolution of 5,000.

SPE and GC-MS analysis

The performance characteristics

Within-day precision and the reproducibility were investigated following the protocol previously described. The calculated RSD were 4.7% for within-day precision and 5.6% for reproducibility. By subsequent dilutions of the control sample of 2ng/mL, it was demonstrated that at 1ng/mL Norandrosterone could be detected with S/N>3, for each of its monitored ions. The calibration curve was found to be linear over the range of 1 to 5ng/mL, with a correlation coefficient of 0.998 (y = 0.1355x + 0.0177).

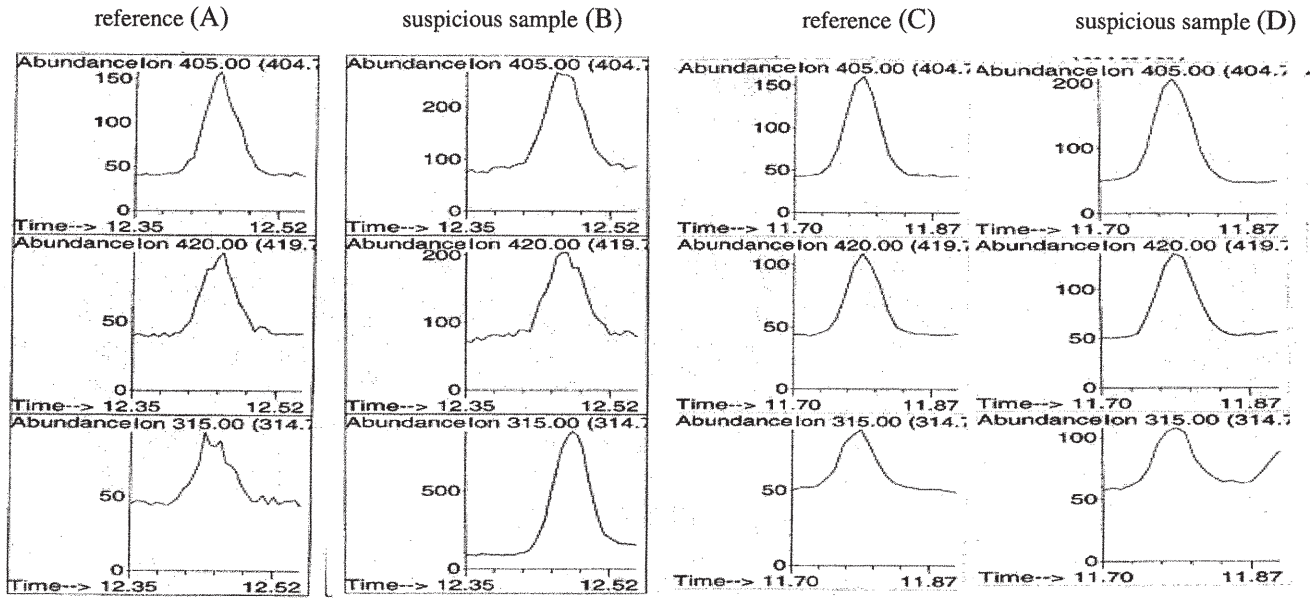

Fig. 3. GC/MS chromatogram of Norandrosterone specific ions in reference sample (2ng/mL) and in the suspicious sample: A, B after L-L extraction; C, D after S-L extraction and selective isolation in n-pentane

Table 1
GC-MS DETERMINATION OF NORANDROSTERONE IN REAL POSITIVE SAMPLE

Code of real urine sample analyzed	LLE + TBME				SPE + n-pentane			
	Characteristic ions's area (cts)			Estimated concentration (ng/mL)	Characteristic ions's area (cts)			Calculated concentration (ng/mL)
	m/z 405	m/z 420	m/z 315		m/z 405	m/z 420	m/z 315	
xxx81	693	422	1752	5.3	704	385	249	3.48 ± 0.94
xxx30	719	430	2713	4.2	733	447	247	4.43 ± 1.40
xxx92	667	414	816	3.9	658	375	234	4.62 ± 1.46
xxx61	3674	2210	5140	16.2	2482	1361	800	15.8 ± 4.27
xxx64	1206	796	2600	7.8	1727	839	708	7.05 ± 1.99
xxx08	12732	7028	4667	85.3	17926	8068	6566	82.39±19.04
xxx19	33803	18206	13016	160.4	29455	13280	10855	170.9±39.58

The elevated background noise observed after LLE in *tert*-butyl methyl ether can be reduced through a procedure of purification via polystyrene-divinylbenzene resin XAD_2, with size particles of 0.1–0.2μm. The isolation of the steroid components by SPE is applied to separate small molecules (inorganic ions) which inhibit the enzymatic hydrolysis, as well as components resulted from a possible bacterial activity traced in samples with elevated *p*H (>8).

The steroid substances, free and conjugated, are adsorbed on the active centers of the resin and eluted later with methanol; after the resin-based processing, the free forms are separated and they are removed through TBME extraction. After release through enzymatic hydrolysis, the free Norandrosterone is selectively extracted in n-pentane being thus separated from the coelutive vitamin E. The outcome of the purification stages both on the actual doping control sample and on the reference sample is depicted in figure 3. The abundance of m/z 315 fragment decreases considerably in the suspicious sample and the ratio of abundance for the mass fragments in the real sample matches the reference sample, which ensures compliance with the identification criteria [8]. The chromatographic peaks display a far better symmetry which allows an appropriate integration of the signal and the quantitative determination via linear regression of Norandrosterone, through the GC/MS technique.

In table 1 a summary is given of the results of the GC-MS analysis of Norandrosterone positive cases during one year period of time. To ensure the monitoring of a large number of steroidic compounds, the current analysis of the doping samples was performed by LLE and the estimation of concentration was made by direct comparison of peak area between the suspicious and reference samples. The removal of the interferences by SPE reduces the contribution of the mass fragment m/z 315 and allows the calculation of Norandrosterone concentration by linear regression around the estimated value. The expanded uncertainty was evaluated in the range 23.2 to 31.5% (95% confidence level, k = 2). The results show that, in both extraction methods, the additional contribution of m/z 315 is no longer noticed at concentration levels higher than 50ng/mL.

Conclusions

In HRMS technique, by the exact measurement of masses, it is possible to discriminate the signal of the background noise or of the coeluents from the fragments of target analytes. The electronic stability of the analysis system and the mass resolution reduce the overall noise, which constitute a sum of the chemical and electrical noise, leading to an increased sensitivity of detection.

The trimethylsilyl derivatives of Norandrosterone and of the vitamin E metabolites, fully co-elutive in the low resolution analysis, can be separated using GC-HRMS at resolutions of 5,000. High resolution mass spectrometry can thus detect the possible attempts to conceal the presence of Norandrosterone through ingestion of vitamins in high dosage.

The processing based on ion exchange polystirenic resin Amberlite XAD_2 and the removal of the ether layer after its separation from the aqueous phase constitute additional steps in the sample purification. The discarded organic phase inorporates the free components excreted in urine and leads to a decreased biological noise.

The use of the non polar solvent n-pentane (instead of ether) ensures the selective extraction of Norandrosterone which leads to an increased sensitivity and selectivity of the separation and determination method.

As a consequence of the comparative data obtained, we considered more efficient to apply LLE and GC-HRMS analysis in current control procedures, in order to assess the doping samples for the presence of forbidden steroidic compounds, well separated from the potential interferences. SPE coupled to GC-MS proved to be more suitable in confirmatory analysis and quantitative determination of traces of Norandrosterone in urine matrix.

Acknowledgements: The authors wish to thank to the Romanian National Anti-Doping Agency for support.

References

1. *** WADA, The World Anti-Doping Agency, International standard for laboratories, version 6.0. (2009) http://www.wada-ama.org/.
2. SCHÄNZER, W., DELAHAUT, P., GEYER, H., MACHNIK, M., HORNING, S., J.Chromatography B, **687**,1996, p.93
3. GOTZMANN, A., GEYER, H., SCHÄNZER, W., Recent Advances in Doping Analysis, **4**, Ed. Sport und Buch Straub, Köln, 1997, p.239
4. SCHÄNZER, W., GEYER, H., HORNING, S., Recent Advances in Doping Analysis, **5**, Ed. Sport und Buch Straub, Köln, 1998, p.13
5. HORNING, S., HÖHN, M., MÜNSTER, H., GEYER, H., SCHÄNZER, W., Recent Advances in Doping Analysis, **6**, Ed. Sport und Buch Straub, Köln, 1999, p.277
6. MACHNIK, M., DELAHAUT, P., HORNING, S., SCHÄNZER, W., Recent Advances in Doping Analysis, **4**, Ed. Sport und Buch Straub, Köln, 1997, p.223
7. VÂJIALĂ, I., RADU, G., L., Tehnici experimentale în bioanaliză, coordinator G. L. Radu, Ed. Printech, Bucharest, 2005, p.1

8. VÂJIALĂ, I., SUBAŞU, R., ZORIO, M., PICU, R., Rev. Chim.(Bucharest), **59**, no. 7, 2008, p.748
9. MAREK-ENGELKE, U., GEYER, H., SCHÄNZER, W., Recent Advances in Doping Analysis, **6**, Ed. Sport und Buch Straub, Köln, 1999, p.119
10. HORNING,S., SCHÄNZER,W., Recent Advances in Doping Analysis, **4**, Ed. Sport und Buch Straub, Köln,1997, p.261
11. TROUT, G., MURBY, J., KAZLAUSKAS, R., Recent Advances in Doping Analysis, **6**, Ed. Sport und Buch Straub, Köln, 1999, p.269
12. AYOTTE, C., 10th Cologne Workshop on Dope Analysis-Proceedings, Ed. Sport und Buch Straub, Köln, 1993, p.185
13. TROUT, G., SOO, S., KAZLAUSKAS, R., Recent Advances in Doping Analysis, **7**, Ed. Sport und Buch Straub, Köln, 1999, p.249
14. VÂJIALĂ, G., LAMOR, M., CHIŢIMIA, B., Recent Advances in Doping Analysis, **9**, Ed. Sport und Buch Straub, Köln, 2001, p.233
15. DÖNIKE,M., GEYER,H., GOTZMANN,A., KRAFT,M., MANDEL,F., NOLTEERNSTING,E., OPFERMANN,G., SIGMUND,G., SCHÄNZER,W., ZIMMERMANN,J., Dope Analysis. În: P.Belloti, G. Benzi, A. Ljungquist (eds.), Official Proceedings of the International Athletic Foundation Word Symposium on Doping in Sport**,** Florenz, 1987, International Athletic Foundation, Monte Carlo (1988), p.53
16. SCHÄNZER, W., DÖNIKE, M., Anal. Chim. Acta, **275,** 1993, p.23

Manuscript received: 12.04.2009