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E. V. Moroz¹, O. V. Stasevich²¹OJSC “Ferein”²Belarusian State Technological University**VALIDATION OF THE ANABOLIC STEROIDS DETERMINATION METHOD
IN BIOLOGICAL ACTIVE ADDITIVES FOR SPORTSMEN**

The validation of the method of qualitative determination of anabolic steroids in biological active additives for sportsmen by gas chromatography with mass-detection has been carried out in this work. The experimental researches have been carried out at the gas chromatograph Agilent 7890A and mass-spectrometer Agilent 7000 with triple quadrupole at health protection institution “National anti-doping laboratory”. The sample preparation has been done by the extraction of the analyzed compounds and their transformation into volatile trimethylsilyl derivatives. The identification of these received compounds has been carried out by their retention time and their mass-spectra. The determination of validation characteristics of qualitative determination of some anabolic steroids in a biological active additive for sportsmen “Isolife isotonic” has been carried out: 17- α -methyltestosterone, dihydroepiandrosterone, nandrolone, methandienone, testosterone. As a result of validation of this method it has been confirmed that such characteristics as selectivity, limit of detection, sample carrying, robustness are in the accordance with acceptable criteria. Consequently, this method could be used for finding out anabolic steroids in biological active additives and special food for sportsmen.

Key words: anabolic steroids, gas chromatography, mass-detection, validation, biological active additives, selectivity, limit of detection, sample carrying, robustness.

Introduction. Anabolic steroids (AS) are prohibited pharmacologically active compounds that simulate the action of the male sex hormone testosterone. Due to their properties to enhance protein synthesis and thus accelerate all plastic processes in the body, these substances can be used illegally to build muscle in the sport in competitive period as doping. Moreover, the use of anabolic steroids has a negative side-effects on the human body, so control of the content of these substances in the sports nutrition and body fluids of athletes is an urgent task for the Republic of Belarus. Currently, there are health care institution “National anti-doping laboratory”, aiming at an independent objective anti-doping laboratory control in compliance with the Anti-Doping Regulations, as well as the use of modern methods allowing to identify illicit doping substances in samples of body fluids and sports nutrition.

Traditionally, anabolic steroids are analyzed by gas chromatography coupled with mass spectrometry (GC-MS) [1].

One of the ways to confirm the guarantee of the right and produced results is the validation of the test method.

Main part. The aim of this work is the validation methodology of qualitative detection of anabolic steroids in dietary supplements for athletes by gas chromatography-mass spectrometry.

Biologically active additives (BAA) “Isolife isotonic” of the production “BellzoLaif” for athletes was the object of the study.

Testosterone (1), nandrolone (2) dehydroepiandrosterone (3), methandienone (4) 17- α -methyltestosterone-1 (5) were selected as the subject of the experimental survey like the most representa-

tive and often occurring anabolics in falsified sports nutrition and dietary supplements (Fig. 1).

Experimental studies were carried out in the health institution “National Anti-Doping laboratory”.

Five parameters were calculated and analyzed for compliance with criteria of eligibility during the validation procedure: specificity, limit of detection, the degree of extraction, transfer test, robustness. Anabolic steroids were analyzed by gas chromatography coupled with mass spectroscopy meter. Gas chromatograph Agilent 7890A (Agilent Technologies, USA) with mass detector of triple quadrupole (Agilent 7000, Agilent Technologies, USA) type and an automatic entry of liquid samples (Autosampler 7693, Agilent Technologies, USA) were used during the analysis.

Dimethylpolysiloxane deposited in layer thickness 0.25 μ m on the inner walls of the chromatographic capillary column 25 m long with an internal diameter of 0.25 mm was served as a stationary phase. To process the results, the program for working with gas chromatography-mass spectrometer MassHunter GC / MS Acquisition was used. The mobile phase was helium gas. Chromatographic parameters were as follows: the temperature of evaporator – 270°C; sample transfer line temperature – 300°C; mode input sample to the column – splitless; injected sample volume – 1 μ l; flow rate – 0.9 ml/min. Temperature mode of the column: temperature – 300°C, the rate of change of temperature – 8°C/min, while maintaining the temperature achieved – 4.25 min. Mode parameters of mass-spectrometer detector were as follows: ionization conditions – electron impact with ionization energy of 70 eV; ion volume temperature – 230°C; time detection of ion – 15 ms; garbage stream – up to 8 min.

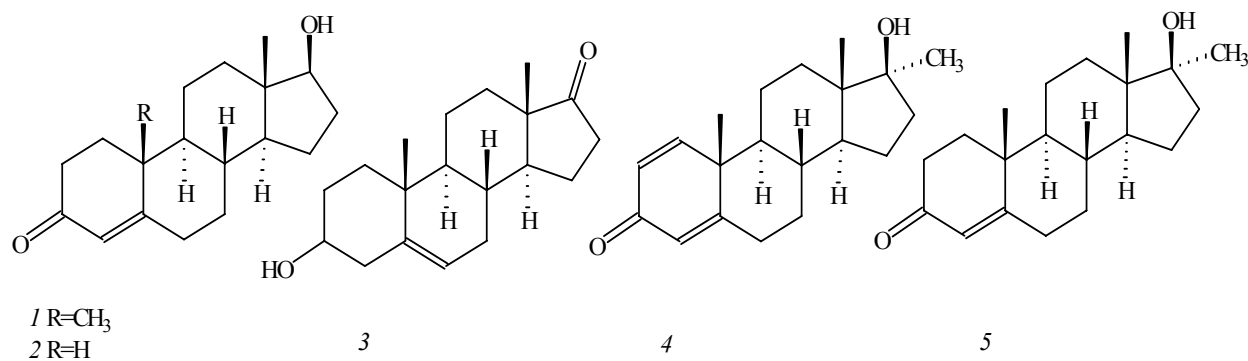


Fig. 1. The structural formulas of analyzed anabolic steroids:

1 – testosterone; 2 – nandrolone; 3 – dehydroepiandrosterone; 4 – methandienone; 5 – 17- α -methyl-1-testosterone

The nitrogen gas served for the secondary ionization.

Sample preparation included liquid extraction and precolumn derivatization of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Carl Roth).

The results of disinfestation were bis-trimethylsilyl (bis-TMC) derivatives having good thermal stability and volatility bis-TMS-testosterone (1a), bis-TMS-nandrolone (2a), bis-TMS-DHEA (3a) bis-TMS-methandienone (4a), 17- α -methyl-testosterone-1 (5a) with a molecular weight of 433.4; 419.4; 433.4; 445.4; 447.5 g/mole, respectively.

Bis-trimethylsilyl derivative of 1-methylene-5 α -androstan-3 α -ol-17-one (National measurement Institute) with a molecular weight of 447.5 g/mole were used as an internal standard.

Identification of the bis-TMS-derivatives of anabolic steroids was performed by retention times and mass spectra.

Retention time and m/z value for the most typical of these compounds the molecular ion $[M-H]^+$ are shown in Table. 1.

Table 1

The absolute retention times and the m/z value for the ion $[M-H]^+$ of bis-TMS-derivatives of AS and internal standard

Designation compound	Time hold, min	The value of m/z for the ion $[M-H]^+$
1a	22.999	432.5
2a	22.015	419.4
3a	21.752	432.4
4a	22.999	444.4
5a	23.144	446.5
6a	21.662	446.5

The mass spectra of compounds containing TMS group was observed in the triplet peaks of molecular ions due to the presence of silicon ²⁹Si and ³⁰Si isotopes. Furthermore, TMS-derivatives were characterized by cleaving particles CH₃ and (CH₃)₃Si (15 and 73 a.e.m. respectively) of the molecular ion [2].

Validation of method for the qualitative detection of anabolic steroids in the dietary supplement was carried out for the all studied AS, but this article will

be presented on the example of the most typical testosterone anabolic, occurred in counterfeit drugs.

Specificity of validation technique was evaluated by analyzing sections of the chromatogram of the standard solution of bis-TMS-of testosterone, the internal standard bis-TMS-1-methylene-5 α -androstan-3 α -ol-17-one and no-load solution supplements containing derivatized mixture with the following eligibility criteria: a) at the chromatograms of standard solutions peaks of defined compounds must be present while the value of signal / noise ratio should be at least 3 : 1; b) peaks should be absent on the chromatogram of blank solution.

The chromatograms of standard solutions bis-TMC-testosterone and bis-TMC-1-methylene-5 α -androstan-3 α -ol-17-one are shown in Fig. 2. Eligibility criteria for specificity were performed: at the chromatograms of standard solutions the peaks of defined compounds were present with retention times for the bis-TMS-testosterone 22.392 min and bis-TMS-1-methylene-5 α -androstan-3 α -ol-17-one 21.662 min, and there were no peaks in the chromatogram of blank solution.

The detection limit was estimated too. For this purpose four supplements solutions were prepared with concentrations of bis-TMC-testosterone including 5, 10, 30, 70 ng/ml and four solutions bis-TMC-1-methylene-5 α -androstan-3 α -ol-17-one having analogous concentrations.

Eligibility criteria were as follows: a) peaks of being identified compounds at the chromatograms of solutions must be present and the value of signal / noise ratio should be at least 3 : 1; b) the absolute retention time of the peak of bis-TMS-1-methylene-5 α -androstan-3 α -ol-17-one should be different from the peak bis-TMS-testosterone of appropriate concentration not more than 0.1 min.

The absolute retention times of the compounds are presented in Table 2.

As it can be seen from table 2, the eligibility criteria for the limit of detection were performed: the absolute retention times of peaks of bis-TMC-1-methylene-5 α -androstan-3 α -ol-17-one differ from the peaks of bis-TMS-testosterone of corresponding concentration not more than by 0.1 min.

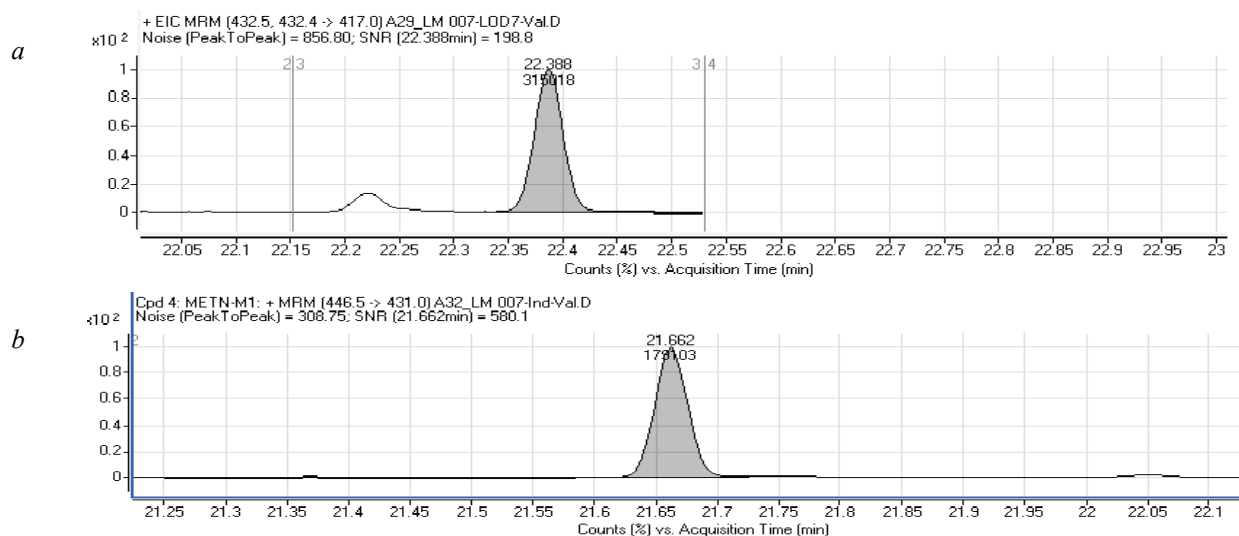


Fig. 2. The chromatograms of standard solutions bis-TMC-testosterone (a) and bis-TMC-1-methylene-5 α -androstan-3 α -ol-17-one (b)

Table 2
The absolute retention times of bis-TMS-testosterone (1a) and bis-TMS-1-methylene-5 α -androstan-3 α -ol-17-one (6a)

Concentration, ng/ml	Retention time, min	
	1a	6a
5	22.386	22.298
10	22.385	22.296
30	22.388	22.295
70	22.390	22.301

To assess the extent of the extraction three extracted solutions of BAA with testosterone content were analyzed as well as three solutions of BAA with testosterone content before extraction. The degree of extraction of steroids from the biologically active additive according to the acceptability criterion should be at least 20%. The degree of extraction was calculated as the ratio of the average value of the peak area being determined at the chromatogram of the compound added prior to extraction to the peak area of the compound being defined added to the solution after the extraction. The degree of extraction was 72.1%, which corresponded to the eligibility criteria. It was also

proved that there is no autosampler transfer by syringe needle of a sample performing sequential chromatographic analyzes from the previous experiments. For this analysis several samples was performed using six idle of six solutions and standard solutions with testosterone alternately. This admissibility criterion was performed. Robustness of validity methodology was evaluated by analyzing the results of tests carried out by two different operators, as well as the extraction of the analyzed compounds by different amounts of solvents (3.0, 3.5 cm³). The evaluation of the results for the robustness research was conducted similarly as for the detection limit. Eligibility criteria for robustness parameter was also performed.

Conclusion. Thus was carried out validation methodology of qualitative detection of anabolic steroids in biologically active additives “Isolife isotonic” by GC analysis, which showed that all eligibility criteria were carried out concerning such validated characteristics as specificity, limit of detection, the degree of extraction, transfer of samples, robustness, and the method can be used for the detection of these compounds in the biologically active additives.

References

1. Aniński P. Hair analysis of anabolic steroids in connection with doping-control results from horse sample. *Journal of Mass Spectrometry*, 2008, no. 43, pp. 1001–1008.
2. Sokolova L. S., Efremov A. A. The appliance of chromato-mass-spectrometry in the research of hormones. *Sorbtsionnye i khromatograficheskie protsessy* [Sorbptional and chromatographic processes], 2012, vol. 12, no. 6, pp. 1033–1041 (In Russian).

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