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The Analysis of Nutritional Supplements for Anabolic-Androgenic Steroids

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Summary

Nutritional supplements, which should not contain prohibited doping substances and were produced in the USA, were analysed for anabolic-androgenic steroids by gas-chromatography/ mass spectrometry. Additionally these supplements were administered to volunteers. Their urine samples were collected before and after the application of the products and were analysed with a screening method for anabolic steroids with gas-chromatography/mass spectrometry.

In a Chrysin-, a Tribulus Terrestris and a Guarana-product, anabolic-androgenic steroids were detected, which were not declared on the label. The steroids could be detected in three different charges of each product. The total amount of steroids and the profile of the steroids varied from charge to charge and from capsule to capsule within one charge. The total amount of steroids varied between 0,3 µg and 5000 µg per capsule.

After application of one capsule of the above mentioned nutritional supplements, all volunteers showed positive results for the nandrolone metabolite norandrosterone. 3-4 hours after application of the capsules, urinary concentrations of norandrosterone between 4 and 623 ng/ml were estimated.

Introduction

Several studies have shown, that prohormone products, sold as nutritional or dietary supplements, do not always contain what is declared on the label. The analyses of 16 different DHEA preparations resulted in detected contents of this steroid from 0% to 150% of the dosage, indicated on the label (1). In androstenediol preparations were found amounts of not declared testosterone, androstenediol and epitestosterone. In preparations labelled as androstendione and androstendione + DHEA “complex” were found minor amounts of testosterone (2). The reason for these findings are, that the prohormones, available for self-administration, are not evaluated for their safety and efficacy. Their production and manufacturing do not have to be made in compliance with GMP (Good Manufacturing Practices)- regulations as the production of approved medications (1, 2).

With the knowledge of a missing quality control of nutritional supplements, we started to analyse also non-hormonal nutritional supplements for anabolic-androgenic steroids. These

analyses we performed within follow up studies of positive norandrosterone cases in doping control.

Experimental

Analysis of the nutritional supplements

From a Chrysin-, a Tribulus Terrestris-and a Guarana product three different charges were analysed. One charge of each product was from the possession of athletes involved in positive doping cases and two charges of each product were bought via Internet from companies in the USA and in Belgium between May and November 1999.

The samples were prepared according to the flow scheme in figure 1. The gas-chromatographic/ mass-spectrometric data of the analysed steroids are presented in table 1. Some spectras are presented in the figures 2-5.

The samples were screened for 9 anabolic-androgenic steroids. The following reference standards were used: DHEA, testosterone and 19-nor-4-androstene-3,17-dione from Sigma, St Louis USA, 5-androstene-3 β ,17 β -diol and 19-nortestosterone from Serva, Heidelberg, Germany, 4-androstene-3 β ,17 β -diol from Steraloids, Wilton, USA, 4-androstene-3,17-dione from Schering, Berlin, Germany. The reference standards 19-nor-4-androstene-3 β ,17 β -diol and 19-Nor-5-androstene-3 β ,17 β -diol were synthesized in our laboratory. Because of an insufficient purity, the 19-nor-5-androstene-3 β ,17 β -diol was only used for qualitative analyses. As internal standard was used d3-19-noretiocholanolone from Steraloids, Wilton, USA.

The detection limit (peak to noise ratio of at least 3:1 for 1 characteristic ion) for all steroids was estimated at about 1 – 2 ng/g.

Excretion studies with the nutritional supplements

5 male volunteers (VP1-VP3; VP5-VP6) and 1 female volunteer (VP4) participated at the excretion studies. VP1-VP3 took one capsule of the Chrysin product, VP4-VP6 took one capsule of the Tribulus-Terrestris product and VP1 took one capsule of the Guarana product. Before the application and 3-4 hours after the application of the capsules, urine samples were collected and analysed by gas-chromatography/mass-spectrometry for the glucuronides of norandrosterone, testosterone and epitestosterone according to the screening procedure for anabolic steroids (3, 4)

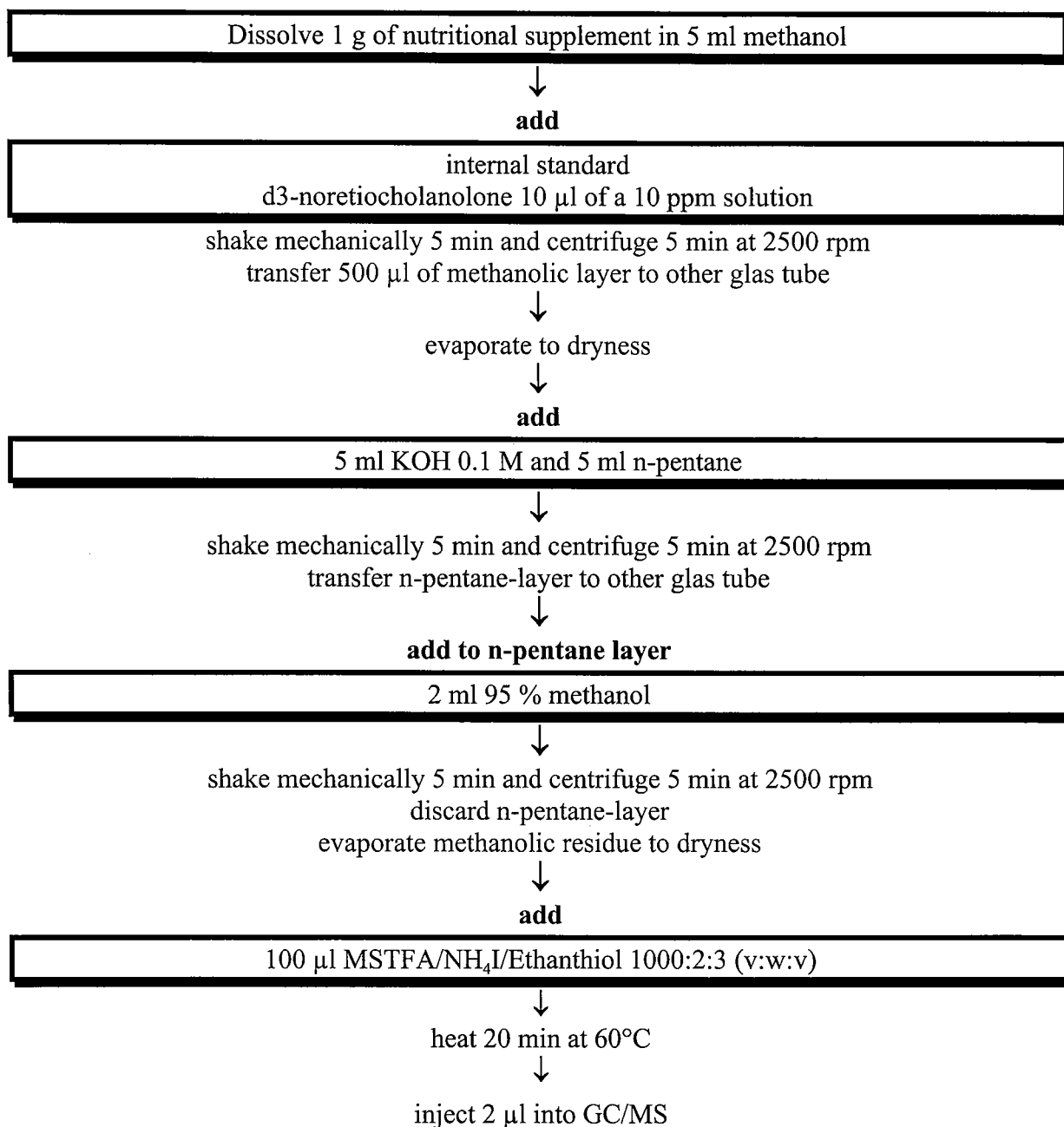


Fig. 1: Flow sheme for the sample preparation for the analysis of nutritional supplements for anabolic-androgenic steroids.

Results and discussion

The analyses of the nutritional supplements showed the following results: In the three nutritional supplements, anabolic-androgenic steroids were detected, which were not declared on the label. In the Chrysin product the anabolic-androgenic steroids 19-nor-4- androstene-3,17-dione and 19-nor-4-androstene-3 β ,17 β -diol were found.

Tab. 1 : Retention times and characteristic ions of the bis-TMS derivatives of the analysed prohormones and the internal standard (ISTD).

GC/MS conditions: Injection mode: 2 µl splitless; carrier gas: Helium; column: 16,5m

SE 54 (HP5 MS) i.d. 0.25 mm, filmthickness 0,25 µm; temperature-program: 100° - 40°/min - 190° - 5°/min - 240° - 40°/min - 320° - 3 min; analysis in SIM-mode

No	substance	RT [min]	M+	Base	other characterisic ions
1	d3-noretiocholanolone (ISTD)	9.50	423	408	318
2	19-nor-5-androstene-3β,17β-diol	10.34	420	330	225, 240, 405
3	19-nor-4-androstene-3β,17β-diol	10.46	420	420	405, 330, 240
4	dehydroepiandrosterone (DHEA)	10.91	432	432	417, 327
5	4-androstene-3β,17β-diol	11.01	434	434	419, 405, 344, 239, 143
6	19-nor-4-androstene-3,17-dione	11.10	416	416	401, 194
7	5-androstene-3β,17β-diol	11.12	434	239	434, 344, 305, 329
8	19-nortestosterone	11.34	418	418	403
9	4-androsten-3,17-dione	11.69	430	430	415, 169
10	testosterone	11.89	432	432	432, 417

In the Tribulus Terrestris product the anabolic-androgenic steroids 4-androstene-3,17-dione, 4-androstene-3β,17β-diol, 5-androstene-3β,17β-diol, 19-nor-4-androstene-3,17-dione and 19-nor-4-androstene-3β,17β-diol were detected. The Guarana product contained the steroids 4-androstene-3,17-dione, 5-androsten-3β,17β-diol, testosterone and 19-nor-4-androstene-3,17-dione.

Tab. 2: Amount of not-declared anabolic-androgenic steroids (in microgramm per capsule) in capsules (Kap) of three different charges of the Chrysin product.

CHRY SIN	Charge 1			Charge 2			Charge 3		
	Kap1	Kap2	Kap3	Kap1	Kap2	Kap3	Kap1	Kap2	Kap3
4-androstendione	0,37	0,68	0,15	0,82	0,07	0,18	0,28	0,04	0,01
4-norandrostendione	5,7	24,9	77,7	381,3	26,0	89,3	5,9	6,7	1,6
4-norandrostendiol	2,5	0,7	1,4	1,3	1,4	1,5	14,1	21,4	22,9
total amount of steroids	8,6	26,3	79,3	383,4	27,5	91,0	20,3	28,1	24,5
amount of 19-norsteroids	8,2	25,6	79,1	382,6	27,4	90,8	20,0	28,1	24,5

The steroids could be detected in three different charges of each product. The total amount of steroids and the profile of the steroids varied from charge to charge and from capsule to capsule within one charge. The results are presented in table 2-4. The total amount of steroids varied between 0,3 µg and 5000 µg per capsule. These amounts are 5 to 100 000 fold lower than the amount of steroids in the lowest concentrated prohormone products of the nutritional supplement market (25 000 µg per capsule or tablet).

Tab. 3: Amount of not-declared anabolic-androgenic steroids (in microgramm per capsule) in capsules (Kap) of three different charges of the Tribulus Terrestris product.

TRIBULUS TERRESTRIS	Charge 1			Charge 2			Charge 3		
	Kap1	Kap2	Kap3	Kap1	Kap2	Kap3	Kap1	Kap2	Kap3
4-androstendione	0,3	1,3	76,3	0,6	2,6	0,9	1,3	1,4	0,8
5-androstendiol	-	-	-	-	-	-	16,6	30,1	5,6
4-androstendiol	-	0,7	1,5	0,3	1,4	0,8	1,8	3,8	0,8
4-norandrostendione	0,04	0,13	0,64	0,08	0,12	0,17	0,48	0,51	0,22
4-norandrostendiol	-	0,74	1,36	-	1,02	0,51	3,7	15,1	1,6
total amount of steroids	0,3	2,9	79,8	1,0	5,1	2,4	23,9	50,9	9,0
amount of 19-norsteroids	0,04	0,9	2,0	0,08	1,1	0,7	4,2	15,6	1,8

Tab. 4: Amount of not-declared anabolic-androgenic steroids (in microgramm per capsule) in capsules (Kap) of three different charges of the Guarana product.

GUARANA	Charge1	Charge 2					Charge 3	
	Kap1	Kap1	Kap2	Kap3	Kap4	Kap5	Kap1	Kap2
4-androstendione	822	310	51,2	19,6	84,8	3335	290	44,8
5-androstendiol	109	30,8	0,5	1,0	6,7	449	30,7	1,1
testosterone	4,5	1,5	-	-	-	20,1	1,4	-
4-norandrostendione	355	142	63,2	32,1	50,7	1336	151	60,8
total amount of steroids	1291	484	115	52,7	142	5140	473	107
amount of 19-norsteroids	355	142	63,2	32,1	50,7	1336	151	60,8

The highest amount of 19-norsteroids (norandrostendione, norandrostendiol) were detected in the Guarana capsules (tab.4). The concentrations varied between 32 µg and 1336 µg per capsule. Lower concentrations were found in the Chrysin product (tab. 2; 8,2 –382 µg/capsule) and the Tribulus Terrestris product (tab. 3; 0,04-15, 6 µg per capsule). Compared to the lowest concentrated commercial prohormone products with 19-norsteroids, these amounts are 20-600000 fold lower.

According to studies with androstendione (5 -7) a physiological effect after the oral application of such low amount of steroids is improbably.

Tab. 5: Urinary concentrations of norandrosterone 3 to 4 hours after the application of one capsule of the nutritional supplements.

Supplement	volunteer	norandrosterone [ng/ml]
Chrysin	VP1	623
Chrysin	VP2	34
Chrysin	VP3	157
Tribulus Terrestris	VP4	16
Tribulus Terrestris	VP5	5,5
Tribulus Terrestris	VP6	3,8
Guarana	VP1	360

Because of the strong variation and the low concentration of the steroids in the capsules it is in all probability that the steroids in the non-hormonal products are not intentional counterfeits but so called cross-contaminations. We suppose, that the contaminations occur preferably in companies, which process and transport prohormones. If the same machines and vessels are used in the processing of other nutritional supplements, contaminations are the consequence of an insufficient cleaning of the processing tools and vessels. These so called cross-contaminations are possible, because the nutritional supplement industry has not to fullfill GMP (Good Manufacturing Practices) conditions as the pharmaceutical industry.

The application of 1 capsule of each product resulted in positive results for the common 19-norsteroid metabolite norandrosterone. 3-4 hours after application of the capsules the urinary concentrations of norandrosterone were in all volunteers above the IOC limit for male athletes (2 ng/ml) and varied between 3,8 and 623 ng/ml (tab. 5).

Tab. 6: Urinary testosterone/epitestosterone-ratios (TEST/EPI) before (0 h) and after (3-4 h) the application of one capsule of the nutritional supplements. VP4 is a female volunteer.

Supplement	volunteer	TEST/EPI 0 h	TEST/EPI 3-4 h
Chrysin	VP1	0,8	1,0
Chrysin	VP2	2,2	3,1
Chrysin	VP3	1,0	1,1
Tribulus Terrestris	VP4	0,6	4,2
Tribulus Terrestris	VP5	0,5	0,4
Tribulus Terrestris	VP6	0,7	0,7
Guarana	VP1	1,2	1,4

As it is known from former studies, the application of 20 µg 19-nortestosterone can lead to positive results for urinary norandrosterone (> 2 ng/ml) for more than 10 hours (8). From these results can be concluded, that the application of contaminated nutritional supplements with maximal amounts of 19-norsteroids of more than 1000 µg/capsule, as in the Guarana product (tab. 4), can lead to positive results for a longer time period, especially if the recommended dose –several capsules per day- is taken. It is difficult to predict the expected maximum concentrations of norandrosterone, because of the interindividual differences in metabolism and the strong variation of the concentrations of the steroids in the capsules. Urinary values of norandrosterone of more than 1000 ng/ml are possible with the strongest contaminated supplements.

Beside the 19-norsteroids the prohormones 4-androstendione, 4- and 5-androstenediol and testosterone were found. The application of high amounts of these steroids normally can be detected in doping analysis by an increase of the testosterone/epitestosterone ratio. As it is shown in table 6 the low amounts of these steroids in the capsules did not increase the testosterone/epitestosterone ratio in the male volunteers. In contrary to this, the female volunteer (VP 4), with natural low concentrations of testosterone and epitestosterone, showed an increase in the testosterone/epitestosterone ratio from 0.6 to 4.2. This result was only obtained in one person and should be confirmed in further studies.

In the positive doping cases connected with the above mentioned 3 supplements, the anabolic steroids could not only be detected in the product of the athlete`s possession but also in new original packed products. So manipulation of the products by the athletes could be excluded.

The conclusions of the results are:

Athletes should be very careful with the use of nutritional supplements, especially if they are from companies, which also sell or produce prohormones. Athletes or their medical attendants should ask the supplier for a certification about a quality control of the supplements for anabolic-androgenic steroids. If such a certification is not available, the supplement should not be used.

Prohormones should be treated as medicaments and not as nutritional supplements. For medicaments GMP conditions are mandatory and cross-contaminations are no longer possible.

In principal, the sense of the administration of nutritional supplements like Chrysin, Guarana, Tribulus Terrestris etc. should critically be discussed by sports scientists.

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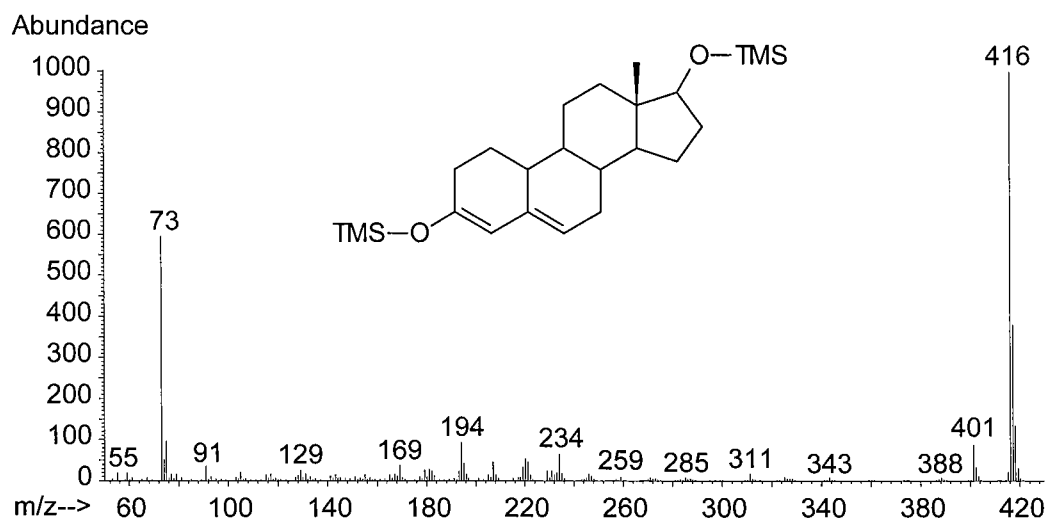


Fig. 2: EI mass spectrum of 19-Nor-4-androsten-3,17-dione bis-TMS (M^+ 416)

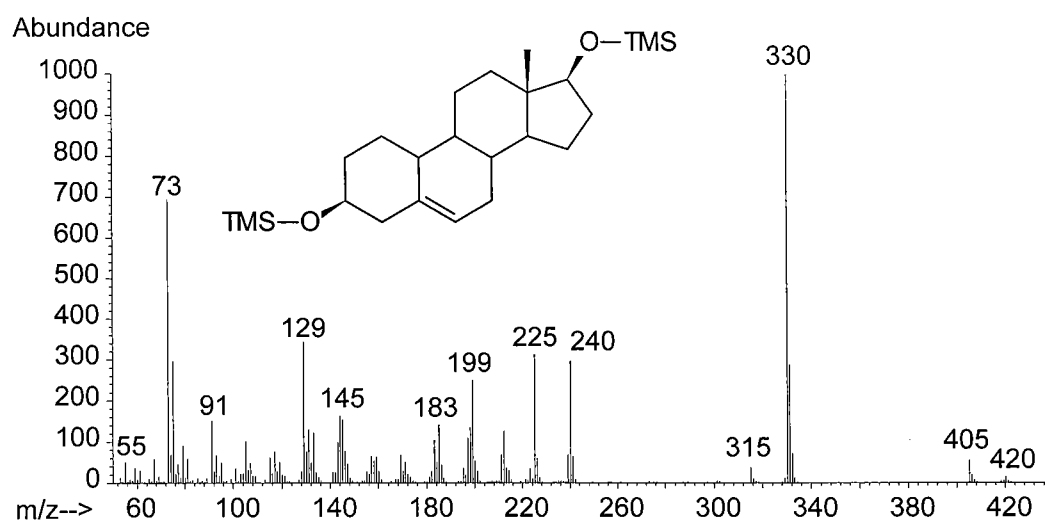


Fig. 3: EI mass spectrum of 19-Nor-5-androsten-3 β ,17 β -diol bis-TMS (M^+ 420)

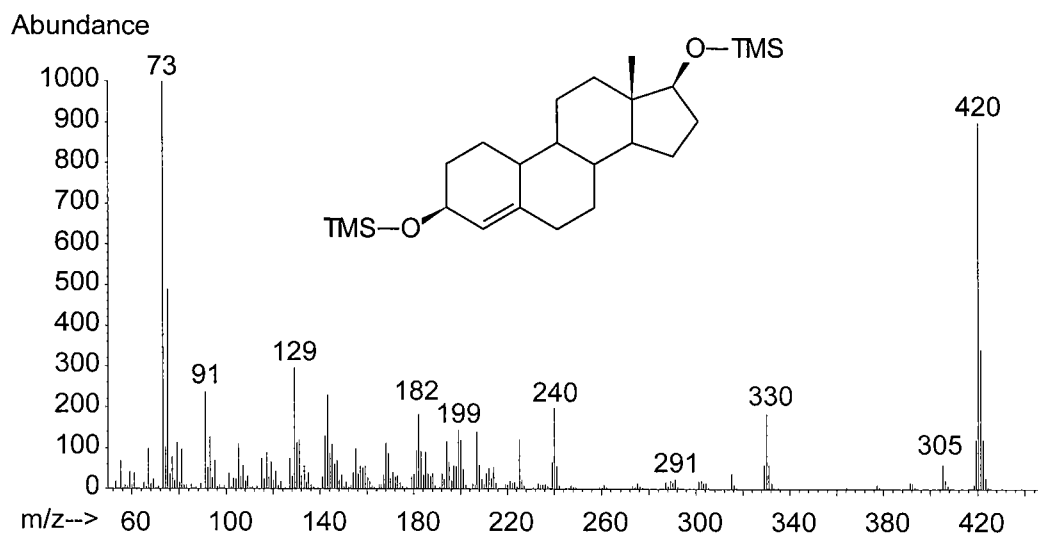


Fig. 4: EI mass spectrum of 19-Nor-4-androsten-3 β ,17 β -diol, bis-TMS (M+ 420)

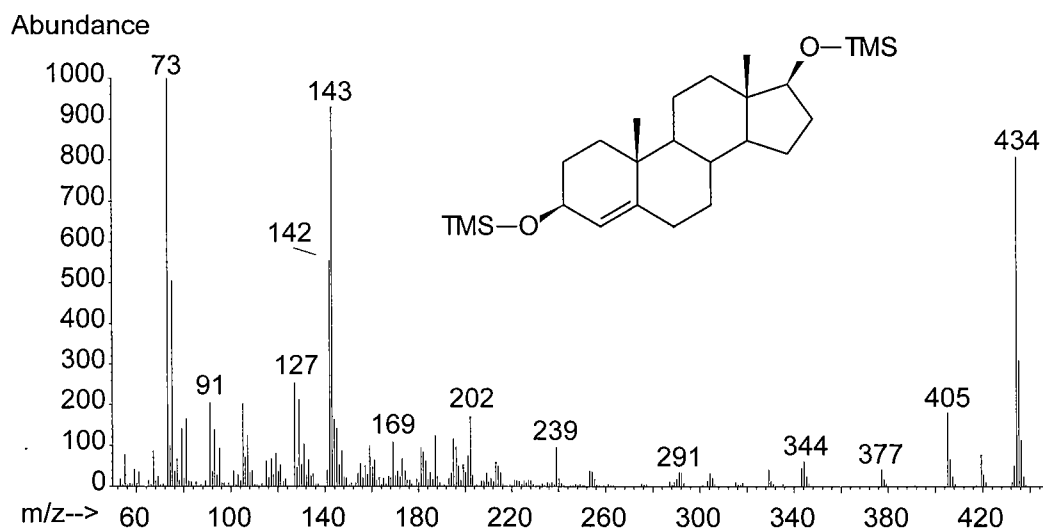


Fig. 5: EI mass spectrum of 4-Androsten-3 β ,17 β -diol, bis-TMS (M+ 434)