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**Validation of a LC-MS-MS method for anabolic steroids in nutritional supplements**

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Methods/Techniques:	Chromatographic analysis, Chromatography - LC/MS
Additives/Contaminants:	Hormones
Food Types:	Dietary supplements, Nutritional supplements
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Table III.doc	

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Manuscripts

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7 **A survey of nutritional supplements for selected illegal anabolic steroids and**  
8 **ephedrine using LC/MS/MS and GC/MS methods respectively**  
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14 **Abstract**  
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16  
17 Several studies have highlighted that nutritional supplements may contain undeclared substances  
18 that are banned by the International Olympic Committee/World Anti-Doping Agency. This paper  
19 describes a qualitative LC-MS-MS method to detect anabolic androgenic steroids (4-androsten-  
20 3,17-dion, 4-estren-3,17-dion, 5 $\alpha$ -androsten-17 $\beta$ -ol-3-one, boldenone, nandrolone, nandrolone  
21 decanoate, testosterone, testosterone decanoate) and ephedrine in food supplements. The products  
22 are dissolved in methanol and analysed by GC/MS. The methanolic solution was added with  
23 testosterone-d3, evaporated to dryness, mixed with NaOH and extracted with n-pentane:diethylether  
24 (9:1). LC/MS/MS analyses were performed in SRM on an ion trap equipped with atmospheric  
25 pressure chemical ionisation (APCI) probe operating in positive ion mode. The method was applied  
26 to 64 nutritional supplements. 12.5% of the nutritonal supplements analysed contained banned  
27 substances not declared on the label (anabolic steroids and ephedrine). Detection limits were in the  
28 range 1-25 ng/g.  
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48 **Keywords:** Nutritional supplements; Anabolic steroids; Anabolic steroids esters; Prohormones; LC-  
49 MS-MS analysis  
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## Introduction

Several doping cases have been reported in official doping tests taken during sports competitions worldwide. In some cases athletes denied the use of doping agents and claimed to have inadvertently or passively absorbed the drug, for example by the ingestion of food or products sold as nutritional supplements that contained prohibited substances [?? *et al.* 2004]. Nutritional supplements seem to modify the body appearance and to promote more consistent and intensive training for athletes by allowing a speedy recovery between training sessions and by enhancing competitive performance. Most of these products do not contain doping agents, but studies have demonstrated that some dietary supplements may contain substances that are not declared on the label, and have been prohibited by the International Olympic Committee/World Anti-Doping Agency [Parr *et al.* 2004, De Cock *et al.* 2001, Delbeke *et al.* 2003., Pipe, Ayotte 2002, Ayotte *et al.* 2001]. Banned substances such as ephedrine, caffeine, steroids and prohormones were found in some “non-hormonal” nutritional supplements [Geyer *et al.* 2004]. It is not known whether the reason is faulty production control or intentional addition [Maughan *et al.* 2004, Ziegenfuss *et al.* 2002, Broeder 2003, Millman, Ross 2003].

In spite of the widespread use of nutritional supplements among sportsmen, the European Community promulgated the first Council Directive on dietary supplements (2002/46/CE), but supplements commonly used by sportsmen (BCCA, amino acids, proteins, etc) are not mentioned. In doping control, present testing methods for anabolic androgenic steroids in nutritional supplements rely on GC/MS [Parr *et al.* 2004, De Cock *et al.* 2001, Delbeke *et al.* 2003] or tandem mass spectrometry [De Cock *et al.* 2001, Van Thuyne, Delbeke 2004]. The methods developed using LC-MS [Joos, Van Ryckeghem 1999] and LC-MS-MS [Leinonen *et al.* 2002, Ho *et al.* 2006, Guan *et al.* 2005, Devebter *et al.* 2006, Politi *et al.* 2006] have not been applied to nutritional supplements. Only Reilly and Crouch report a LC-MS-MS method applied to 5 $\alpha$ -androst-1-en-3,17-

1  
2 dione and/or 5 $\alpha$ -androst-1-en-3 $\beta$ ,17 $\beta$ -diol (1AD), used as nutritional supplements and analysed in  
3  
4 plasma and urine [Reilly and Crouch 2004].  
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9 This study describes the validation of a qualitative LC-MS-MS method for the determination of 8  
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11 doping substances. The anabolic steroids selected were those most commonly found in nutritional  
12  
13 supplements, as shown by literature data [De Cock *et al.* 2001, Geyer *et al.* 2004]. Moreover in  
14  
15 nutritional supplements ephedrine was detected by GC/MS and was confirmed by LC/MS/MS. The  
16  
17 use of LC-MS-MS provides several advantages over current methods because this technique allows  
18  
19 the direct separation and identification without the need for derivatization, reducing the time of  
20  
21 analysis, eliminating predictable sources of error and decreasing the use of hazardous and expensive  
22  
23 reagents [Leinonen *et al.* 2002].  
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## 30 **Materials and Methods**

### 31 *Nutritional supplements*

32  
33 64 nutritional supplements were purchased from shops and judicial proceeding. The ingredients  
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35 contained in the products has been classified as following: 4 vitamins/minerals, 7  
36  
37 glutamine/creatine, 9 amino acids, 12 proteins, 8 banned substances, 12 herbal extracts, 4 other. The  
38  
39 manufacturer's recommended doses vary according to the supplement.  
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### 47 *Chemicals and reagents*

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49 The following reference standards were used: ephedrine, testosterone, testosterone decanoate,  
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51 nandrolone, nandrolone decanoate, testosterone-d<sub>3</sub> from Sigma (Saint Louis, Missouri USA);  
52  
53 boldenone, 4-androsten-3,17-dione, 4-estren-3,17-dione and 5-androsten-3 $\beta$ -ol-17-one (DHEA),  
54  
55 methyl-testosterone, metandienone, oxandrolone, stanozolol, trenbolone, testosterone propionate,  
56  
57 testosterone acetate, testosterone enanthate, 4-androsten-3 $\beta$ ,17 $\beta$ -diol, 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol, 5 $\alpha$ -  
58  
59 androstan-17 $\beta$ -ol-3-one from Steraloids Inc. (Newport R.I. USA). The stock standard solutions  
60

1  
2 (1mg/ml) were prepared by dissolving 5 mg of each standard in 5 ml of methanol. Working  
3  
4 standard solutions were made by dilution with methanol to the appropriate concentration (10 µg/ml  
5  
6 and 1 µg/ml).  
7

8  
9 All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany); formic acid  
10  
11 (extra pure) was obtained from Sigma-Aldrich-Riedel-de Haen (Seelze, Germany).  
12

### 13 14 15 16 *Materials and apparatus* 17

18  
19 The GC-MS analyses were performed on a Thermo electron gas chromatography coupled to a DSQ  
20  
21 quadrupole mass spectrometer. Each sample was run through the following temperature program:  
22  
23 initial temperature 70° C for 1 minute, then 10° C/min increments to 280° C, followed by a final  
24  
25 isotherm of 5 minutes. The inject port and the source temperature were set at 250° C.  
26  
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28  
29 About LC-MS-MS parameters, the analytes were separated on a Discovery HS C<sub>18</sub> column (150 ×  
30  
31 4.6 mm, 5 µm, Supelco, Bellefonte PA). A Discovery HS C<sub>18</sub> guard column (20 × 4.6 mm, 5 µm)  
32  
33 was used prior to the analytical column. The mobile phase was a mixture of methanol (A) and acetic  
34  
35 acid 0.03% (B) with a flow of 1.2 ml/min. The gradient program of the mobile phase started with  
36  
37 A:B (60:40, v/v), was maintained for 3 minutes, then changed to A:B (95:5, v/v) over 12 min. Then  
38  
39 the column was set back to the initial mobile phase conditions in 10 min. A sample loop of 20 µl  
40  
41 was used.  
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44  
45 A Spectra System P4000 pump coupled with LCQ Advantage ion trap mass spectrometer  
46  
47 (Thermoelectron Corp., San José, CA, USA) was used for the LC-MS-MS analyses. The mass  
48  
49 spectrometer was equipped with an atmospheric pressure chemical ionisation (APCI) probe  
50  
51 operating in positive ion mode. The source parameters were as follows: nitrogen gas used as sheath  
52  
53 and auxiliary gas at flow 1.2 L/min and 6 L/min respectively; vaporizer temperature 450° C; spray  
54  
55 voltage 4500 V; capillary voltage and capillary temperature 5 V and 220° C respectively; and tube  
56  
57 lens offset of 50 V. The mass spectra of the standard compounds were first acquired in full scan  
58  
59 mode (150-460 *m/z*) by infusion of a reference solution of 1 µg/ml. From these spectra the  
60

1 precursor ions were selected and fragmented acquiring the full scan MS-MS spectra. At last the  
2  
3  
4 higher product ions were chosen and each compound was analysed in SRM mode to obtain the best  
5  
6  
7 sensitivity and specificity. Fragmentation of the selected precursor ions was performed by collision-  
8  
9 induced dissociation (CID) with helium, which fills the ion trap. The helium pressure in mass  
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11 analyzer cavity was maintained at approximately 0.1 Pa.  
12

### 13 14 15 16 *Extraction procedure of nutritional supplements*

17  
18 The extraction procedure followed the validated method of Van Thuyne et al. (2004), modified for  
19  
20 liquid chromatography detection [Van Thuyne and Delbeke, 2004]. Nutritional supplement (1 g)  
21  
22 was added with the internal standard (testosterone-d3 100 ng/g). The residue was reconstituted with  
23  
24 5 ml MeOH and shaken by the vortex. 1 µl of the methanolic solution was injected into the GC-MS  
25  
26 to detect the presence of ephedrine. Then the methanol was evaporated to dryness, mixed with 2 ml  
27  
28 NaOH (1 M) and shaken with 5 ml n-pentane:diethylether (9:1) for 1 hr. After centrifugation the  
29  
30 organic layer was separated and evaporated under nitrogen flow at 40°C. The residue was  
31  
32 reconstituted with 50 µl of mobile phase (methanol:water with acetic acid 0.03%, 50:50) and  
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34 injected into the LC-MS-MS system.  
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### 42 43 *Validation Method*

44  
45 The method was validated using a blank nutritional supplement containing branched chain amino  
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47 acids. This nutritional supplement was chosen to represent the spectrum of nutritional supplements  
48  
49 because it is commonly used by body builders. After the validation, the method was applied to 64  
50  
51 unknown nutritional supplements. The analytical method for the determination of 8 anabolic  
52  
53 compounds was validated in accordance to the Eurachem Guide suggestions [Eurachem.1998, 61].  
54  
55 According to Van Thuyne and Delbeke [2004], the method has only been just validated  
56  
57 qualitatively as anabolic androgenic steroids and their esters in nutritional supplements are  
58  
59 absolutely prohibited, with no limits of concentration allowed.  
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4 The Limit of Detection (LoD) was calculated by qualitative measurements spiking a blank  
5 nutritional supplement with a reference mixture at concentrations of 1, 5, 10, 25 and 50 ng/g. Each  
6  
7 level was performed on five different replicates of the blank supplement. Repeatability (intra-assay  
8  
9 precision) for LoDs level was in the range 8-14%. The selected compounds of the reference  
10  
11 mixture were: 4-androsten-3,17-dione, 4-estren-3,17-dione, 5-androsten-3 $\beta$ -ol-17-one (DHEA),  
12  
13 boldenone, nandrolone, nandrolone decanoate, testosterone and testosterone decanoate. The  
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15 specificity was tested during the validation procedure by extracting 10 aliquots of the blank  
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17 nutritional supplement, to look for possible matrix interferences.  
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## 26 **Results and Discussion**

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30 The relative retention time, the precursor ion and the product ions for each standard compound are  
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32 given in Table I. The LC-MS-MS chromatograms of a blank nutritional supplement and a  
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34 nutritional supplement spiked at 50 ng/g, extracted as above, are shown in Figure 1 and 2,  
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36 respectively.  
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42 In order to test the specificity 10 aliquots of the blank supplement were analysed and no matrix  
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44 interference was found at the retention time of the 8 analytes and the internal standard. The relative  
45  
46 retention time of the analyte should correspond to that of the standard analyte, from a spiked  
47  
48 sample, with a tolerance of  $\pm 2\%$ . The relative intensities of the detected ions, expressed as a  
49  
50 percentage of the intensity of the most intense transition, must correspond to those of the standard  
51  
52 analyte, from a spiked sample with the relative tolerance of  $\pm 25\%$ , as suggested by the World Anti-  
53  
54 Doping Agency [The WADA Technical Document – TD2003IDCR]. All these criteria were applied  
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56  
57 to all the spiked samples for the LoD determination.  
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1  
2 According to the Eurachem guidelines [Eurachem 1998, 61] the LoD for qualitative measurements  
3  
4 is a concentration threshold below which specificity becomes unreliable. It was defined as the  
5  
6 concentration level where a compound could be detected in all five of the tested samples. Table II  
7  
8 shows how this threshold concentration was determined for each standard compounds and figure 3  
9  
10 reports the relative LODs chromatograms According to our experimental data six compounds can  
11  
12 be detected at or below 10 ng/g, two at 25 ng/g.  
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18 The validated method was applied to 64 nutritional supplements, containing ingredients as  
19  
20 described above. 12.5% of the nutritional supplements tested contained substances not declared on  
21  
22 the label. The positive nutritional supplements are reported in table III, in which we reported: the  
23  
24 producer, the ingredients declared in the label and the banned substances. Sample 1, 2, 4, 7, 8, 9 and  
25  
26 12 contain banned substances not declared; sample 5 and 6 confirm the presence of ephedrine and  
27  
28 in sample 3 we found ephedrine but the label reports only the plant source. Finally for sample 17 we  
29  
30 could not confirm the presence of ephedrine as reported on the label. In figure 4 a nutritional  
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32 supplement positive to 4-estren-3,17-dione and 4-androsten-3,17-dione is shown.  
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## 40 **Conclusions**

41  
42 The method reported here is sufficiently sensitive, specific and selective for the detection and  
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44 confirmation of 4-androsten-3,17-dione, 4-estren-3,17-dione, 5-androsten-3 $\beta$ -ol-17-one (DHEA),  
45  
46 boldenone, nandrolone, nandrolone decanoate, testosterone and testosterone decanoate in nutritional  
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48 supplements, in accordance to the validation criteria suggested by Eurachem guidelines. This LC-  
49  
50 MS-MS method was applied to 64 nutritional supplements and 12.5% of them were found positive.  
51  
52 The low levels of the compounds found in the samples (in the range of ng/g) may indicate an  
53  
54 accidental contamination and not an intentional admixture. However, athletes should consider only  
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56 purchasing from companies performing quality tests on prohormones and testing for possible  
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58 contamination during production.  
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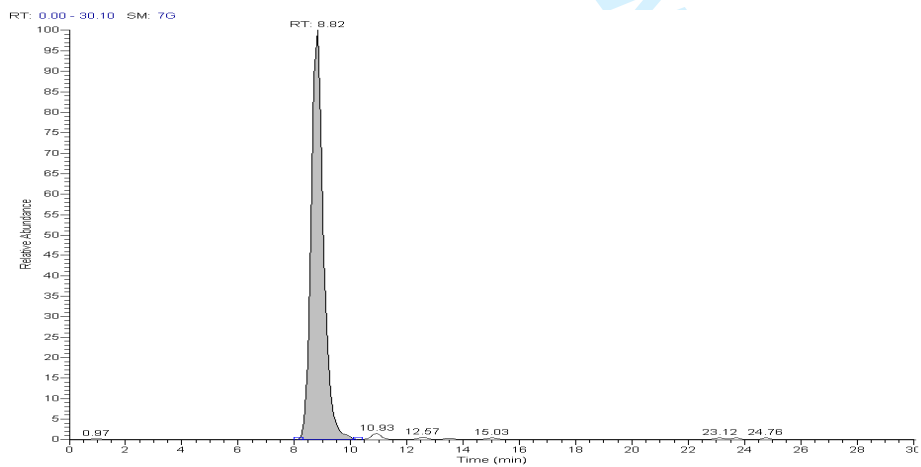
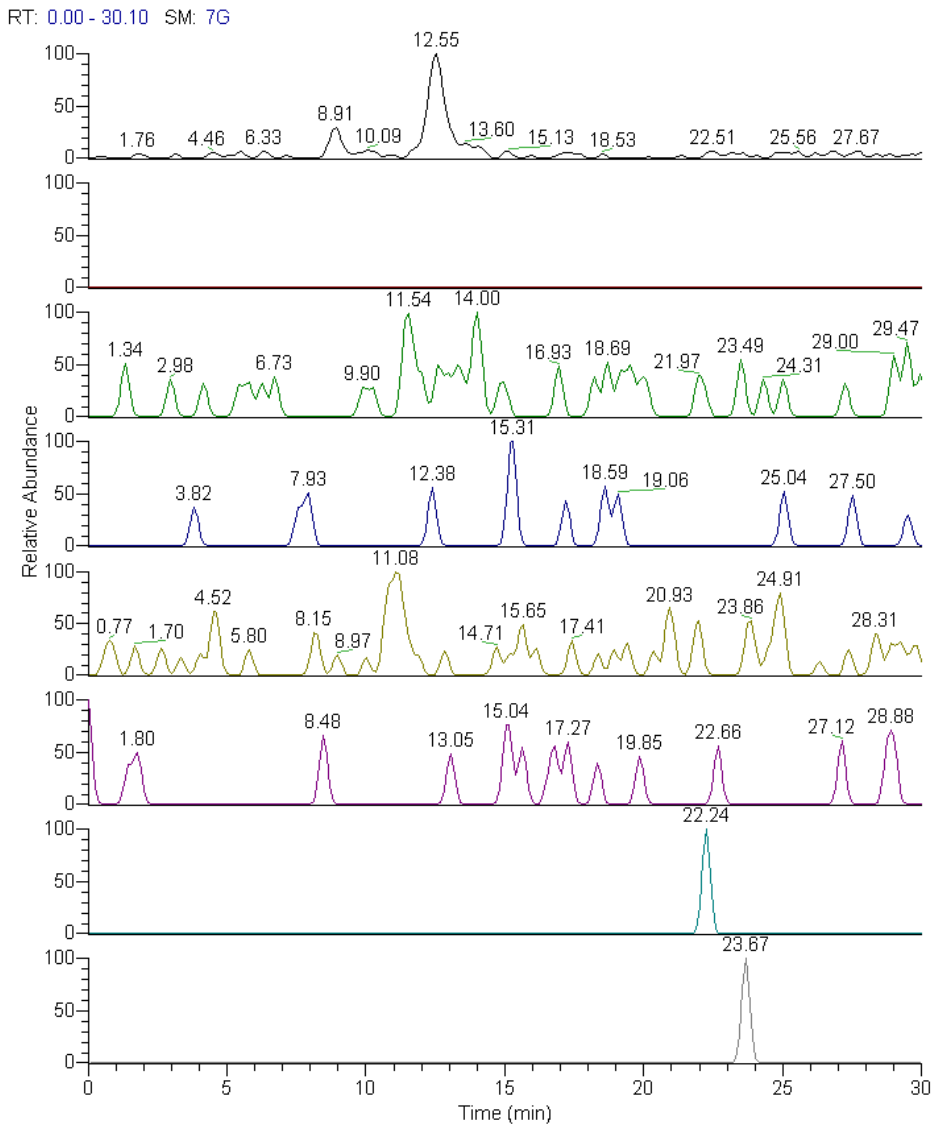


Figure 1

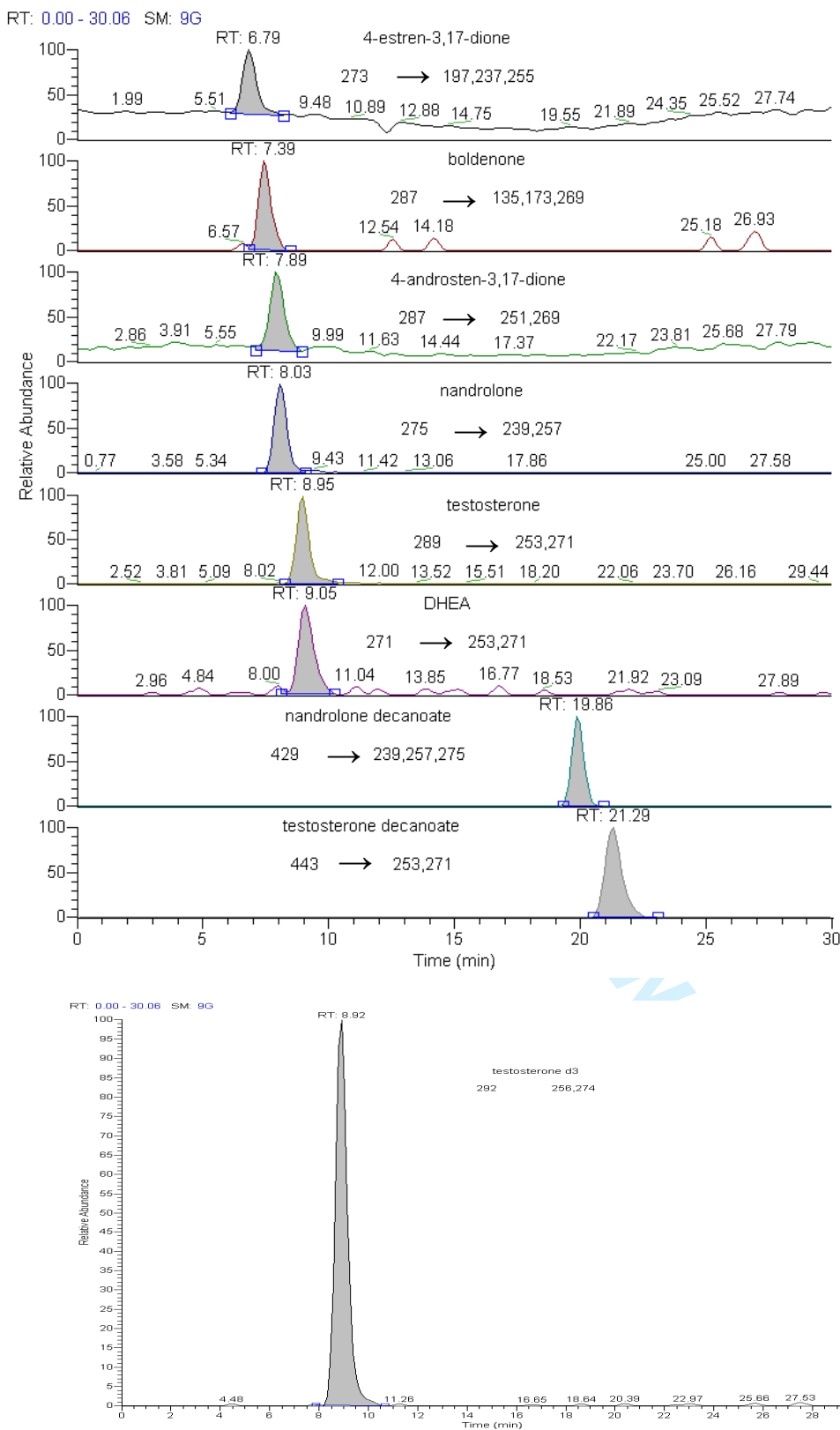


Figure 2

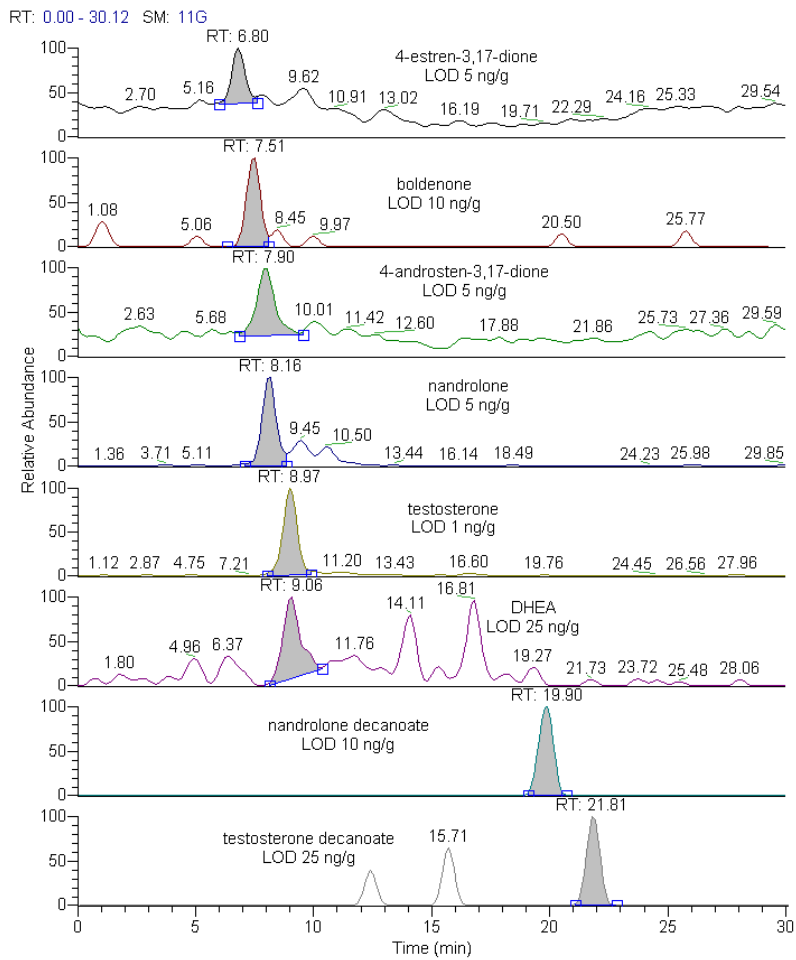


Figure 3

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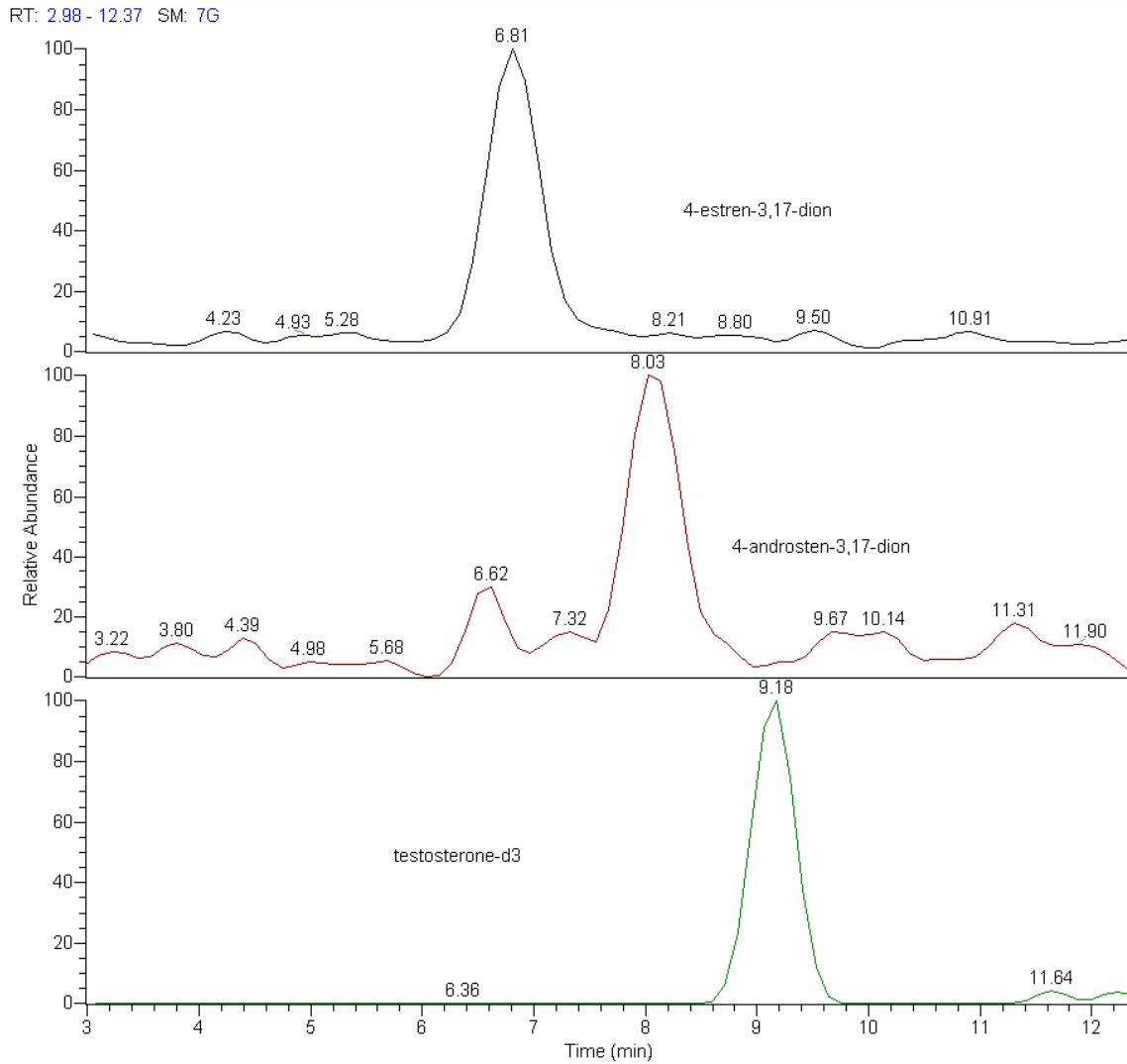


Figure 4

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**Table I:** Relative retention times, precursor ions and product ions of each compound of the standard mixture.

<b>Compound</b>	<b>RRT</b>	<b>Parent Ion (<i>m/z</i>)</b>	<b>Product Ions (<i>m/z</i>)</b>
<b>4-estren-3,17-dione</b>	0.761	273	255, 237, 197
<b>Boldenone</b>	0.828	287	269, 173, 135
<b>4-androsten-3,17-dione</b>	0.884	287	269, 251
<b>Nandrolone</b>	0.900	275	257,239
<b>Testosterone-d3</b>	1.000	292	274, 256
<b>Testosterone</b>	1.003	289	271, 253
<b>5-androsten-3<math>\beta</math>-ol-17-one</b>	1.014	271	271, 253
<b>Nandrolone decanoate</b>	2.226	429	257, 239, 275
<b>Testosterone decanoate</b>	2.387	443	271, 253

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Number of positive and negative findings in spiked samples at different analyte concentrations								
Concentration (ng/g)	4-estren-3,17-dione	Boldenone	4-androsten-3,17-dione	Nandrolone	Testosterone	DHEA	Nandrolone decanoate	Testosterone decanoate
50	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
25	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
10	5/0	5/0	5/0	5/0	5/0	2/3	5/0	3/2
5	5/0	2/3	5/0	5/0	5/0	0/5	3/2	1/4
1	2/3	1/4	2/3	3/2	5/0	0/5	1/4	0/5
LOD	5 ng/g	10 ng/g	5 ng/g	5 ng/g	1 ng/g	25 ng/g	10 ng/g	25 ng/g

Table II

N.	Producer	Ingredients declared on the label	Banned substances
1	A	L-glutamine	4-estren-3,17-dion 4-androsten-3,17-dion
2	A	Proteins, carbohydrates	4-estren-3,17-dion
3	B	Ephedra sinica/Ma Huang; Guaranà; White willow bark; Chromium picolinate	ephedrine
4	B	L-carnitine; Garcinia Cambogia; Vitamin B6; Chromium	ephedrine
5	C	Ma Huang (ephedrine alkaloids); Guaranà extract; Green tea extract; L-phenylalanine; L-tyrosine; White willow bark; Citrus aurantium (sinephrine alkaloids); Herbal blend (ginger, passion flower, yohimbe)	ephedrine
6	C	Ephedrine alkaloids; Guaranà extract; Yerba mate extract; citrimax extract; L-carnitine; L-tyrosine	ephedrine
7	D	Branched-Chain Amino Acids (BCCA)	nandrolone decanoate
8	D	L-carnitine	testosterone decanoate
9	D	Amino acids	testosterone
10	E	Branched-Chain Amino Acids (BCCA)	4-androstendion
11	F	Ephedrine; guaifenesin	ephedrine
12	G	Dehydroepiandrosteron (DHEA)	DHEA testosterone
13	G	Dehydroepiandrosteron (DHEA)	DHEA
14	H	Ephedrine; guaifenesin	ephedrine
15	I	Testosterone decanoate	Testosterone decanoate
16	I	prohormones	4-androstendion 4-estrendion
17	C	Ephedrine (from herbal extract); guarana extract; yerba mate extract; citrimax; white willow bark; yohimbe; bioperine	ephedrine declared on the label not found

Table III