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The Netherlands Security System Nutritional Supplements Elite Sports (NZVT)

(Pro)-Hormone analysis in Food Supplements for the NZVT.

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Introduction

Foodsupplements can be "contaminated" with doping substances (Catlin, Geyer, Ayotte). To limit the risk of unintentional intake of forbidden substances, a method of analysis was required to test food supplements.

An analytical procedure for the determination of a number of (pro-)hormones in food supplements, based on automated sample preparation (SPE) combined with coupled-column HPLC and GC-MS was developed. The method is reliable for the screening of the compounds mentioned in table 1.

Table 1. Analytes, molecular formulas, mass fragments used in GC-MS. Underlined m/z value is used for screening and quantification.

Analytes	formula	Mwt	Derivative	m/z measured			
17α-19-Nortestosterone	$C_{18}H_{26}O_2$	274.3	HFBA	**666**	453	306	133
Nandrolone	$C_{18}H_{26}O_2$	274.3	HFBA	**666**	453	306	133
17α-Testosterone	$C_{19}H_{28}O_2$	288.4	HFBA	**680**	467	355	320
17ß-Testosterone	$C_{19}H_{28}O_2$	288.4	HFBA	**680**	467	355	320
19-Nor-4-androstene-3ß,17ß-diol	$C_{18}H_{28}O_2$	276.4	HFBA	**454**	439	425	241
19-Nor-5-androstene-3ß,17ß-diol	$C_{18}H_{28}O_2$	276.4	HFBA	**454**	439	425	241
19-Nor-5-androstene-3,17-dione	$C_{18}H_{28}O_2$	272.4	HFBA	**468**	411	319	255
4-Androstene-3ß, 17ß-diol	$C_{19}H_{30}O_2$	290.4	HFBA	**468**	453	425	239
5-Androstene-3ß, 17ß-diol	$C_{19}H_{30}O_2$	290.4	HFBA	**468**	453	425	239
4-Androstene-3, 17-dione (AED)	$C_{19}H_{26}O_2$	286.4	HFBA	**482**	467	449	425
1,4-androstediendione (ADD)	$C_{19}H_{24}O_2$	284.4	HFBA	**480**	436	369	267
1-Androstenedione	$C_{19}H_{26}O_2$	286.4	HFBA	**482**	467	318	303
1-Testosterone	$C_{19}H_{28}O_2$	288.4	HFBA	484	**442**	427	400
DHEA,	$C_{19}H_{28}O_2$	288.4	MSTFA++	**432**	417	327	303
Epiandrosterone	$C_{19}H_{30}O_2$	290.4	HFBA	**486**	468	442	427
Methyl-Boldenone	$C_{20}H_{28}O_2$	300.4	HFBA	**478**	463	435	367

Experimental

SPE C18 and, SPE NH_2 extraction columns (Alltech). All chemicals used were of analytical grade. Standards from Steraloids and Sigma.

The column-switching set-up using Aspec® (=Automatic Sample Preparation with Extraction Columns). The other system consisted of a gradient HPLC pump, a Rheodyne injection valve, a 25 mm x4 mm i.d. Ramm (Alkyl diol silica -Restricted Access Materials) column packed with 25 µm LiChrospher ADS (Merck) and an analytical column LiChrospher 100 EcoCart 125-4 RP-18 (5 mµ). The two systems are connected via an automated MUST-IET (Multi-port Stream switch, Spark Separation) switching valve. The chromatographic analysis of the extracts is performed on a GC-MSD, Agilent, 6890 Series equipped with Mass Selective Detector Agilent 5973, an automatic sample injector and a fused silica capillary column ZB-35 (Zebron) Low Bleeding/non polar (30 m, 0.25 mm i.d. 0.2 µm film).

Analytical method

A test portion of one gram is weighed into a 50 ml plastic test tube and spiked with a mixture of deuterated internal standards (Nortestosterine-d3 (=NT-d3), Testosterone-d2 (=T-d2)) (20 ng of each compound), then 5 ml of water is added. The mixture is vortexed for 30 seconds after which the sample is incubated during one hour at 20°C. 10 ml of TBME is added, and shaken during three hours, after that centrifuged for 3 minutes at 3600 rpm.

The organic layer is transferred to a glass tube. This extraction is repeated (5 ml TBME). The organic layers are collected and the TBME is evaporated under N_2. The dry residue is dissolved in 40/60 v/v % MEOH/water, washed with n-heptane and centrifuged for 1 minute. The n-heptane layer is separated from the MEOH-water layer and discarded. The MEOH-water layer in the tube is further processed according to the procedure described below.

The ASPEC® system is coupled to the HPLC system using a column-switching system. The coupled-column system consist of two HPLC columns, first a RAM-column (Restricted Access Materials) and second a RP-18 analytical column. The ADS-Ram column has been specifically designed for the fractionation of sample components into macromolecular compounds and low molecular weight analytes. After sample preparation, a single fraction is collected containing all the analytes.

The fraction is evaporated and the residue is dissolved, transferred to a derivatisation-vial and evaporated to dryness. The residue is derivatised with a mixture of Heptafluorobutyric Acid Anhydride:acetone (1:4) or in case of DHEA with a mixture of MSTFA++ (MSTFA-ammoniumiodide-dithioerythritol(1000:2:4,v/w/w).

The final analysis is performed using GC-MSD. The automatic sampler injects 1 µl of these extracts into the GC-MSD, split/splitless, constant flow, oven temp. 80° C for 1 min, increased at 30° C/min. to 300° C and held at this temperature for 10 min.

Results and discussion

To determine the accuracy of the procedure, a serie of fortified samples of vitamin tablets at 2, 4, 6 and 8 ppb was analyzed (Table 2), in triplicate on three different days.
Preliminary results before validation according the EU Commission Decision 2002/657/EC (comparable to WADA requirements), showed that good and reproducible recoveries were obtained for all the analytes at the different fortification levels (see table 2).

Table 2. Accuracy (%) and repeatability (CV in %).
(at different concentration levels, average over three days).

Analytes	Levels			
	2 ppb	4 ppb	6 ppb	8 ppb
17α-19-Nortestosterone	69 ± 10	83 ± 15	72 ± 3	74 ± 7
Nandrolone	106 ± 10	137 ± 3	121 ± 15	117 ± 12
α-Testosterone	63 ± 13	60 ± 15	89 ± 21	53 ± 12
ß-Testosterone	73 ± 17	75 ± 15	103 ± 19	78 ± 16
19-Nor-4-androstene-3ß,17ß-diol	102 ± 15	135 ± 3	137 ± 9	125 ± 14
19-Nor-5-androstene-3ß,17ß-diol	104 ± 12	155 ± 3	107 ± 14	155 ± 11
19-Nor-4-androstene-3,17-dione	92 ± 10	120 ± 16	80 ± 9	90 ± 8
4-Androstene-3ß, 17ß-diol	101 ± 12	122 ± 4	101 ± 6	128 ± 5
5-Androstene-3ß, 17ß-diol	103 ± 14	121 ± 3	85 ± 5	118 ± 2
4-Androstene-3,17-dione(AED)	96 ± 17	126 ± 14	93 ± 14	111 ± 12
1,4-Androstedienedione (ADD)	70 ± 10	82 ± 15	75 ± 3	75 ± 7
1-Dehydrotestosterone	104 ± 10	107 ± 3	101 ± 15	117 ± 12
1-Testosterone	95 ± 17	120 ± 14	97 ± 14	110 ± 12
DHEA	99 ± 38	73± 50	130 ± 39	70 ± 32
5α-Androstane-3ß-ol-17-one	96 ± 17	126 ± 14	93 ± 14	111 ± 12
1-Dehydromethyltestosterone	70 ± 10	82 ± 15	75 ± 3	75 ± 7

Figure 1 illustrates a chromatogram for 19-Nor-4-androst-3,17-dione of a confirmatory analysis in a food supplement (anti-oxidant tablets), measured at 4 diagnostic ions. (m/z 468, 411, 319, 255).

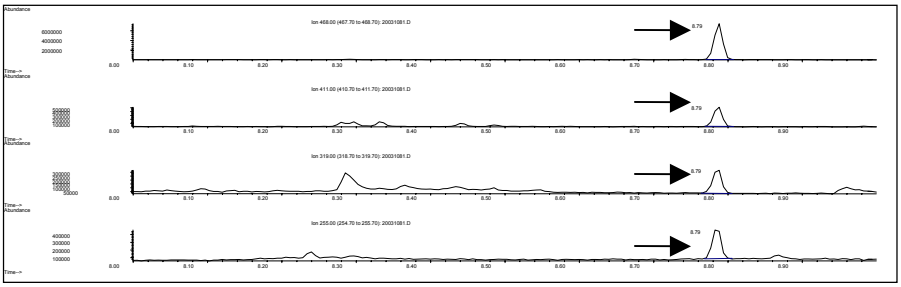

Figure 1. Ion chromatograms of a food supplement, positive for 19-Nor-4-androst-3,17-dione (Conc. ± 1 mg/kg)

Analysis over two years resulted in several samples of food supplements containing (pro-)hormones at levels > 10 ppb. In 12 of the 207 samples 1-Testosterone, 4-Androstene-3ß, 17ß-diol, 19-Nor-4-androstene-3, 17-dione and DHEA were detected and confirmed (Table 3).

Table 3. Analytes found in food supplements within NZVT.

Analytes	Concentration range	Confirmation 2002/657/EC
1-Testosterone	>98 ppb	+
4-Androstene-3ß, 17ß-diol	>40 ppb	+
19-Nor-4-androstene-3,17-dione	16 ppb - 1000 ppb	+
DHEA	10 ppb -137 ppb	+
4-Androstene-3, 17-dione(AED)	15 ppb - 17 ppb	+

Conclusion

The developed procedure is applicable for all relevant types of supplements. The sample preparation and extraction procedure is rapid and quantitative. The method has proved to be suitable also for the confirmation of the identity of the above mentioned (pro-)hormones in food supplements. No non-results were obtained when approximately 200 supplements were analyzed over a 2 year period. The samples included powders, drinks, emulsions, tablets, herbs, minerals, vitamins, oily capsules, kreatine etc. In approximately 6% of all samples (pro)hormones were detected with levels exceeding 10 µg/kg or l.

References

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