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## **The Netherlands Security System Nutritional Supplements Elite Sports - Stimulants analysis in Food Supplements (NZVT)**

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### *Introduction*

Both competitive and recreational athletes are often in search of a nutrition program to improve their performance level. They often are prone to experiment with vitamins and other supplements, protein powders, and other pills to improve their athletic abilities and recuperate faster from an intensive training session. An appropriate sports diet will help to support both athletic training and competition. But occasionally athletes are found positive on the use of illegal substances from the WADA doping list although they claim not to have used anything. To limit the risk of unintentional intake of forbidden substances the Dutch Government requested the RIVM to analyze food supplements used by the Dutch athletes competing in the Salt Lake City Winter Olympics, 2002, for the presence of (forbidden) stimulants a.o. In order to fulfil this request an analytical procedure was developed for the determination of a group of stimulants in food supplements.

This method has proven reliable for a large variety of matrices at medium to low concentration levels.

After the 2002 Winter Olympics the screening of the supplements was continued at a regular basis. Approximately 200 samples have been analyzed during the last years and no “non-results” (meaning all supplements could be analyzed) and only one “non-compliant” result were obtained.

### *Materials and methods:*

#### Standards, Reagents, and Chemicals:

All standards and internal standards are from Sigma, other chemicals are from Merck, and Biosolve.

In table1 the analytes are shown which were analysed.

Table 1: Analytes analysed for and SRM (Parent ion and Daughter ion)

Analyte	Parent ion	Collision Energy	Daughter ion
Amphetamine	136.1	30	119.0
Methyl – amphetamine	150.2	32	119.0
Nor-ephedrine	152.1	30	134.2
Nor-pseudo-ephedrine	152.1	30	134.2
Ephedrine	166.1	32	148.2
Pseudo-ephedrine	166.1	32	148.2
Methyl-ephedrine	180.2	30	162.2
MDA (love)	180.1	36	163.0
MDMA (ecstasy, XTC)	194.1	32	163.0
Caffeine **	195.1	36	138.1
MDE (eve)	208.2	32	163.0
Strychnine	335.4	36	264.1
Fenfluramine	232.1	34	187.0
<b>Internal standards:</b>			
Amphetamine-d3	139.1	28	122.0
Methylamphetamine-d5	155.3	32	121.0
Efedrine-d3	169.1	33	151.1

\*\* Caffeine is not on the WADA prohibited list anymore since 1<sup>st</sup> January 2005

#### Sample preparation, extraction and cleanup

All solid materials (tablets, pills, capsules, powders, candy bars etc.) are homogenized and milled using a waring blender. Liquid materials (oils, energy drinks etc.) are thoroughly mixed.

From these mixed materials 0.5 g is taken and extracted with 5 ml of 0.1 M HCl using successive a shaker for 30 min. and an ultrasonic bath for 10 min. Then the samples are centrifuged for 15 min at 3000 rpm (2000g).

From this primary extract, two 0.5 ml aliquots are used for further liquid/liquid extraction with 4 ml TBME at basic conditions. The duplicate sample is spiked at a level of 100 µg/kg (or l). Then the analytes are back-extracted from the TBME layer into 100 µl 1 % acetic acid. After a washing step with 1 ml TBME the acetic acid phase is diluted with 100 µl eluent A from which 40 µl is injected.

#### LC-MS<sup>n</sup> analysis

Chromatographic separation was achieved using a Phenomenex Synergy Hydro column (4 µm, 80 Å, 150 x 2 mm). The mobile phase consisted of a mixture of Acetonitrile, water and acetic acid (eluent A= 3/97/0.2, eluent B = 95/5/0.2 (v/v)) at a flow rate of 0.2 ml/min. A linear gradient was used: the first 3 min at 100 % A, followed by an increase to 15 % B in 14 min, then an increase to 90% B in 3 min. In between samples was an equilibration time of 10

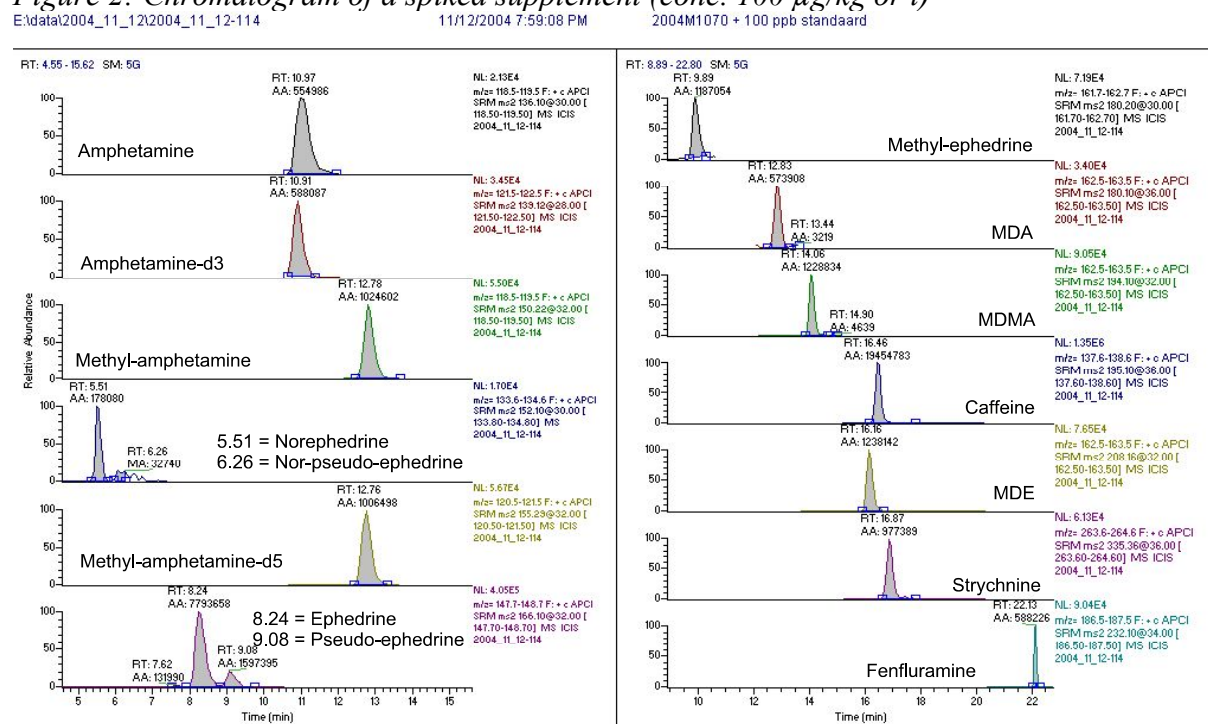
min at initial conditions. A Thermo Finnigan P4000 quaternary pump and an AS 3000 autosampler are used. A LCQ classic ion trap (Finnigan) was used for screening in APCI pos MS<sup>2</sup> mode.

Confirmation was carried out under the same chromatographic conditions but here a Micromass Ultima PT was used. The ion trap only produces one stable daughter ion, whereas the triple quadrupole instrument produces multiple stable daughter ions.

### Results

In the last years, more than 200 samples have been analyzed and only one “non-compliant” results was obtained. In a few samples low levels (< 50 ppb) of stimulants were detected, probably due to the use of herbal ingredients in the preparation of the supplements. The “non-compliant” sample contained ephedrine and pseudo-ephedrine. The components could be confirmed according to the EU criteria (2002/657/EU). Ephedrine and Pseudo-ephedrine were confirmed on three daughter ions and two ratios. The concentration levels were estimated at 850 ppb ephedrine and 170 ppb pseudo ephedrine.

Figure 2: Chromatogram of a spiked supplement (conc. 100 µg/kg or l)



### Discussion

The use of an ion trap in APCI mode for the screening and confirmation of stimulants has only limited value. In most cases the ion trap produces only one daughter ion, being the [M+H-18]<sup>+</sup> ion. This ion is very stable and intense, making it useful for the screening of

samples for stimulants. When trying to produce multiple daughter ions on the LCQ the resulting daughter ions are often unstable and not reproducible.

The triple quadrupole mass spectrometer produces multiple daughter ions. These ions are more specific than the observed loss of water on the iontrap LC-MS. These daughter ions are very stable under mild fragmentation settings, making confirmation according to the EU legislation possible. The use of internal standards is mandatory due to the enormous variation in matrices which can influence the extraction recovery of the stimulants. Fortunately there are a lot of deuterated internal standards commercially available.

Validation of the procedure is done by spiking every supplement with the analytes at the action level of the NZVT system. This provides proof for every supplement the extraction and recovery is sufficient. Due to the enormous variability in matrices for the supplements a validation of every matrix is not possible.

### *Conclusions*

The method has proven to be reliable for a number of stimulants. In the last years all supplements could be analysed. No non-results were produced. To further optimize the method some extra internal standards have to be incorporated in the method. As the clean-up is very general, the method is flexible of incorporation other or more analytes into it. Example given, THG is also incorporated in the method.

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