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Detection of selected stimulants as contaminants in solid nutritional supplements by liquid chromatography–mass spectrometry

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Abstract

Nutritional supplements are frequently used by athletes and can contain substances banned by the World Anti Doping Agency (WADA). The aim of this study was to develop a screening method for the detection of selected stimulants in solid nutritional supplements. Analysis of the samples was performed on an LCQ-Deca® instrument after an acidic wash with *n*-pentane and an alkaline liquid/liquid extraction with diethylether. The mass spectrometer was operated in full scan MS^2 using positive ionisation. Detection limits (LODs) were equal or below 50 ng/g for all compounds except strychnine (100 ng/g). The suitability of the method for routine application was illustrated by the analysis of two suspicious samples.

Keywords: Doping; Stimulants; LC–MS; APCI; Nutritional supplements

1. Introduction

As described in the 1994 Dietary Supplement Health and Education Act (DSHEA) (FDA, 1994) and defined by Schröder (Schröder, 2002), nutritional supplements are food supplying in one or more nutrients in a concentrated form, that are theoretically present in a normal balanced diet. As a result herbal preparations, frequently promoted for their alleged preventive and/or therapeutic effects were not regulated as drugs (FDA, 1994). One of the oldest, and best known, medicinal herbs is Ephedra or Ma Huang (*Ephedra sinica*) containing ephedrine, pseudoephedrine and norephedrine (Abourashed, El-Alfy, Khan, & Walker, 2003). Today, herbal preparations containing parts of *E. sinica* are frequently promoted for their performance enhancing effects or their positive influence on weight reduction. Besides these ephedrines other stimulants and anabolising agents have been detected in nutritional supplements as well (Delbeke, 2001; Van Thuyne & Delbeke, 2006).

Stimulants belong to the classes of prohibited substances according to the WADA doping rules (WADA, 2006). Hence athletes using contaminated supplements are at risk of violating the current doping rules. Parr et al. (Parr, Geyer, Sigmund, Kohler, & Schänzer, 2003) described a gas chromatography–mass spectrometric (GC–MS) method for the detection of various stimulating agents in nutritional supplements based upon screening methods routinely used in doping control. Detection of stimulants using GC–MS can only be accomplished after selective derivatisation. Liquid chromatography–mass spectrometry (LC–MS) however, allows for the direct separation and identification of stimulants without derivatisation (Deventer, Van Eenoo, & Delbeke, 2006; Thieme & Sachs, 2003). The aim of this study was to develop a screening method using LC–MS for the detection of 11 selected stimulants in solid nutritional supplements. These 11 stimulants are suggested as target compounds by official authorities involved in testing nutritional supplements (NeCeDo, 2006).

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2. Experimental

2.1. Chemicals and reagents

3-Bromophenethylamine and strychnine were obtained from Sigma (Bornem, Belgium). 3,4-methylenedioxyamphetamine (MDA), 3,4-ethylenedioxyethylamphetamine (MDEA) and 3,4-methylenedioxymethamphetamine (MDMA) were a kind gift from the Portugese doping control laboratory in Lisbon. Norephedrine HCl, norpseudoephedrine HCl, pseudoephedrine HCl and methamphetamine HCl were purchased from Merck (Darmstadt, Germany). Amphetamine sulphate was purchased from Smith-Kline and French Laboratory (Philadelphia, USA). Ephedrine HCl was obtained from Hoechst AG (Frankfurt, Germany) and fenfluramine HCl from Laboratoires Servier (Orleans, France).

Analytical grade hydrochloric acid, potassium hydroxide, acetic acid and diethylether were from Merck (Darmstadt, Germany). *n*-Pentane was obtained from Biosolve (Valkenswaard, Netherlands). HPLC grade methanol (MeOH) was from Acros (Geel, Belgium) and HPLC grade water from Fischer (Loughborough, UK).

Methanolic HCl (1 M) was prepared by the addition of 3.9 g acetylchloride (Sigma) using a dropping funnel during a period of 20 min, with stirring, in 50 ml of methanol p.a. (Acros Organics, Geel, Belgium) cooled to 0 °C. The solution is stored in 0–8 °C.

2.2. Extraction

Extraction was based on a previously described method (Parr et al., 2003). Internal standard solution (50 μl 3-bromophenethylamine, 10 μg/ml) was added to 1 g of grinded supplement. The acidic wash was performed by adding 5 ml of aqueous hydrochlorid acid (1 M) to the grinded sample and rolling for 10 min with 4 ml of *n*-pentane. After centrifugation the organic layer was discarded. To the remaining aqueous layer 2 ml of aqueous potassium hydroxide (5 M) was added and liquid–liquid extraction was performed for 10 min using diethylether. After centrifugation the organic layer was transferred to a new tube and 100 μl of methanolic HCl (1 M) was added to the organic phase before evaporation to avoid undesired loss of the volatile amphetamines (Dallakian, Budzikiewicz, & Brzezinka, 1996). The sample was evaporated until dry under oxygen free nitrogen (OFN) at 40 °C. The remaining residue was dissolved in 200 μl of the mobile phase.

2.3. Validation

The validation was carried out according the Eurachem validation guidelines (Eurachem Working Group, 1998). Ten randomly chosen blank nutritional supplements were spiked at four different levels with the 11 stimulants. Final concentrations were 20, 50, 100 and 200 ng/g. The samples were extracted as described above. The LOD was defined as the lowest level at which a compound could be identified in all 10 samples with a signal to noise (S/N) ratio greater than 3. Repeatability was assessed through the analysis of the samples spiked at different levels during the determination of the LOD. Selectivity was tested by analysing several other doping agents including other stimulants, anabolic agents, beta-blockers, narcotics, diuretics and corticosteroids. Specificity was tested by analysing ten blank nutritional supplements that were used to determine the LOD.

2.4. Apparatus

In LC–MS^2 studies Model P4000 quaternary pump was equipped with Model AS3000 autosampler (sample loop 100 μl) and ThermoFinnigan LCQ-Deca mass spectrometer. All the instruments were from Thermo Separation Products (Thermo, San Jose, CA, USA).

LC-parameters. A Nucleosil C18 column 3 mm × 100 mm, 5 μm (Chrompack, Belgium), protected with a guard column 2 × 10 mm (Chromsep, Antwerp, Belgium), was used for chromatographic separations. 50 μl was injected using push loop injection.

The mobile phase consisted of 1% acetic acid (solution A) and methanol (solution B). Gradient elution at a flow rate of 0.4 ml/min was as follows: 90% A for 2 min, linear to 20% A in 6 min, isocratic for 3 min followed by an increase to 90% A in 0.5 min. The equilibration time before the next injection was 6.5 min and the total run time was 18 min.

MS-parameters. Ionisation of analytes was carried out using atmospheric pressure chemical ionisation (APCI). The capillary temperature and the vaporizer temperature were set to 120 and 350 °C, respectively. The discharge current was set at 5 μA. The sheath gas was maintained at 50 units. No auxillary gas was used.

Tuning of the capillary voltage, tube lens, octapole lenses and entrance lens was done automatically on the protonated molecule of amphetamine.

In MS^2 experiments the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms. The collision energy was set to fully fragment the precursor ion.

3. Results and discussion

3.1. Method development

Mass spectrometry. In order to determine diagnostic ions each compound was infused into the mass spectrometer. Amphetamine-type stimulants contain an amine function which can be easily protonated. Hence very abundant protonated molecular ions $[M+H]^+$were observed. No deprotonated molecules were observed using negative ionisation.

During the infusion experiments both ESI and APCI exhibited good sensitivity. Ultimately APCI was preferred as interface regarding its robustness towards ion

Table 1
Retention time (RT), instrument parameters and LODs

Substance	RT (min)	PI $[M+H]^+$	CE	DI	LOD (ng/g)
Bromophenethylamine[a]	8.13	200	25	183	–
Amphetamine	6.61	136	25	119	20
Ephedrine	5.2	166	30	148	50
Fenfluramine	9.44	232	30	159	50
MDA	7.1	180	25	163	50
MDEA	7.88	208	28	163	50
MDMA	7.52	194	27	163	20
Methamphetamine	7.28	150	30	119	50
Norephedrine	3.79	152	25	134	50
Norpseudoephedrine	4.36	152	25	134	50
Pseudoephedrine	5.67	166	30	148	50
Strychnine	8.23	335	37	264	100

CE: collision energy, PI: precursor ion for MS^2, DI: diagnostic ion.
[a] Internal standard (IS).

suppression caused by matrix interferences (Souverain, Rudaz, & Veuthey, 2004).

Because tandem mass spectrometry (MS^2) often results in improved sensitivity this technique was applied for all compounds. For the four ephedrines the sole ion detected was generated by the loss of H_2O. For the other substances loss of the amine moiety was the most abundant signal. For strychnine, which has a different structure, the most abundant signal originated from the loss of the phenyl ring (Stahl et al., 2004).

Chromatography. Peak tailing in reversed phase HPLC is particularly prevalent when the stationary phase silanol interacts with basic compounds. Since all compounds included in this method posses an amine function, broad, tailing peaks were observed. To reduce this peak tailing a series of eluents containing amines have been suggested

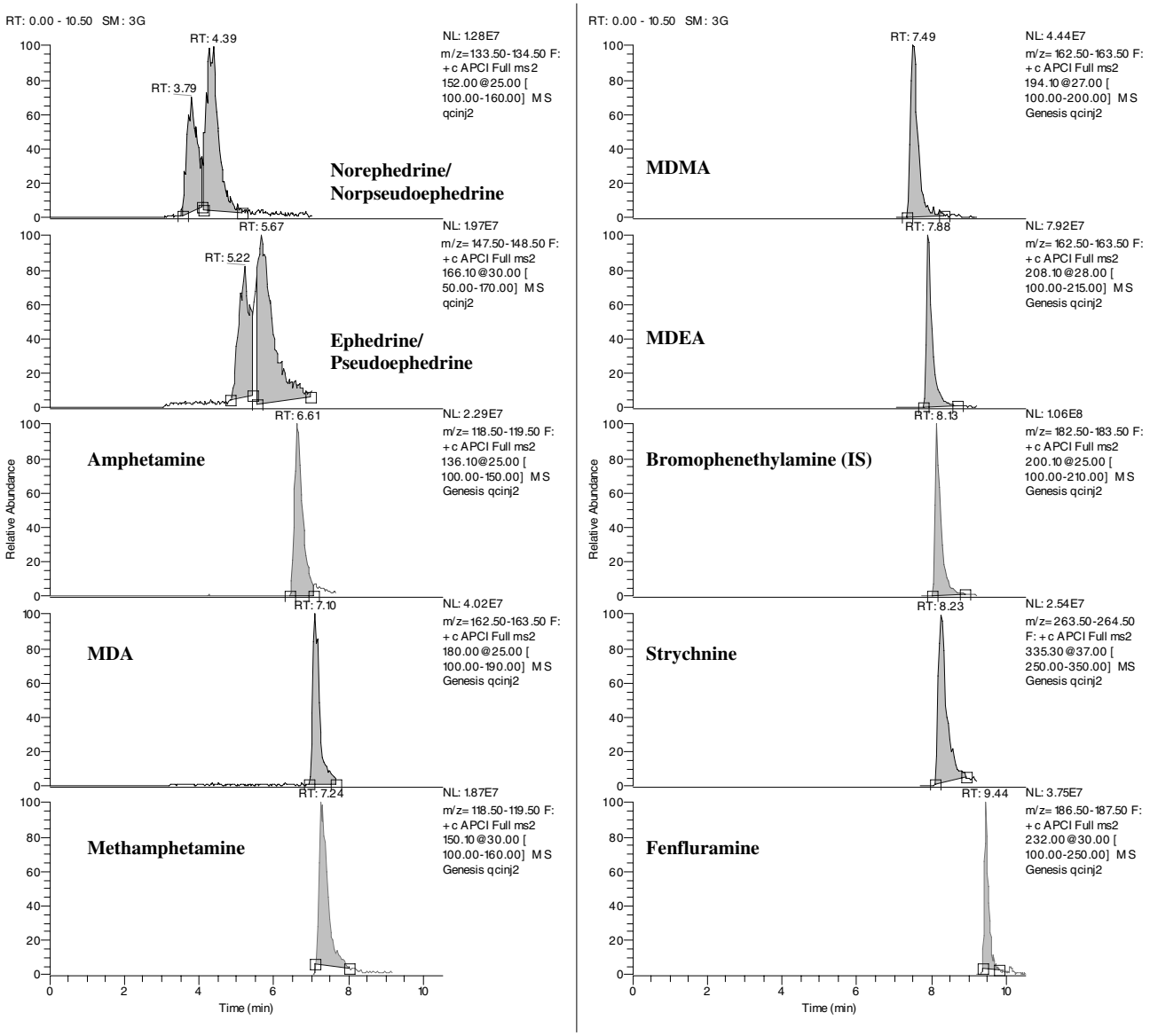

Fig. 1. Quality control sample spiked at 100 ng/g.

(Gill, Alexander, & Moffat, 1982). However these additives are not recommended when mass spectrometrical detection is applied and hence none of these additives were used in this work. Moreover, the analysis of nutritional supplements is not quantitatively and hence peak shape is of minor importance. Both MeOH and acetonitrile were evaluated as organic modifiers. The use of MeOH, instead of acetonitrile was preferred. MeOH allowed the isomers ephedrine–pseudoephedrine and norpseudoephedrine–norephedrine to be partially separated. With acetonitrile no separation of these isomers was observed.

Sample preparation. All stimulants included in this screening method contain a primary, secondary or tertiary amine function and can be extracted with good recoveries at the alkaline pH value used in this method (Deventer, Van Eenoo and Delbeke). Nutritional supplements often consist of complex matrices and are composed of multiple ingredients, including vitamins, amino acids and herbal extracts. Because of the presence of the amine moiety in the chemical structure of the stimulants, a selective acidic wash step could be applied and was consequently used to remove unwanted neutral and acidic compounds.

In doping analysis diphenylamine is commonly used as internal standard (IS) for the analysis of amphetamine-type drugs (Hemmersbach & de laTorre, 1996; Van Eenoo, Delbeke, Roels, & De Backer, 2001). However this substance is lost during the acidic clean up step. Therefore 3-bromophenethylamine, with similar chemical functionalities as the compounds of interest, was used as internal standard. Retention times and diagnostic ions monitored in the procedure are presented in Table 1.

3.2. Validation and routine application

The detection limits are given in Table 1. LODs were equal to or lower then 50 ng/g for all compounds except

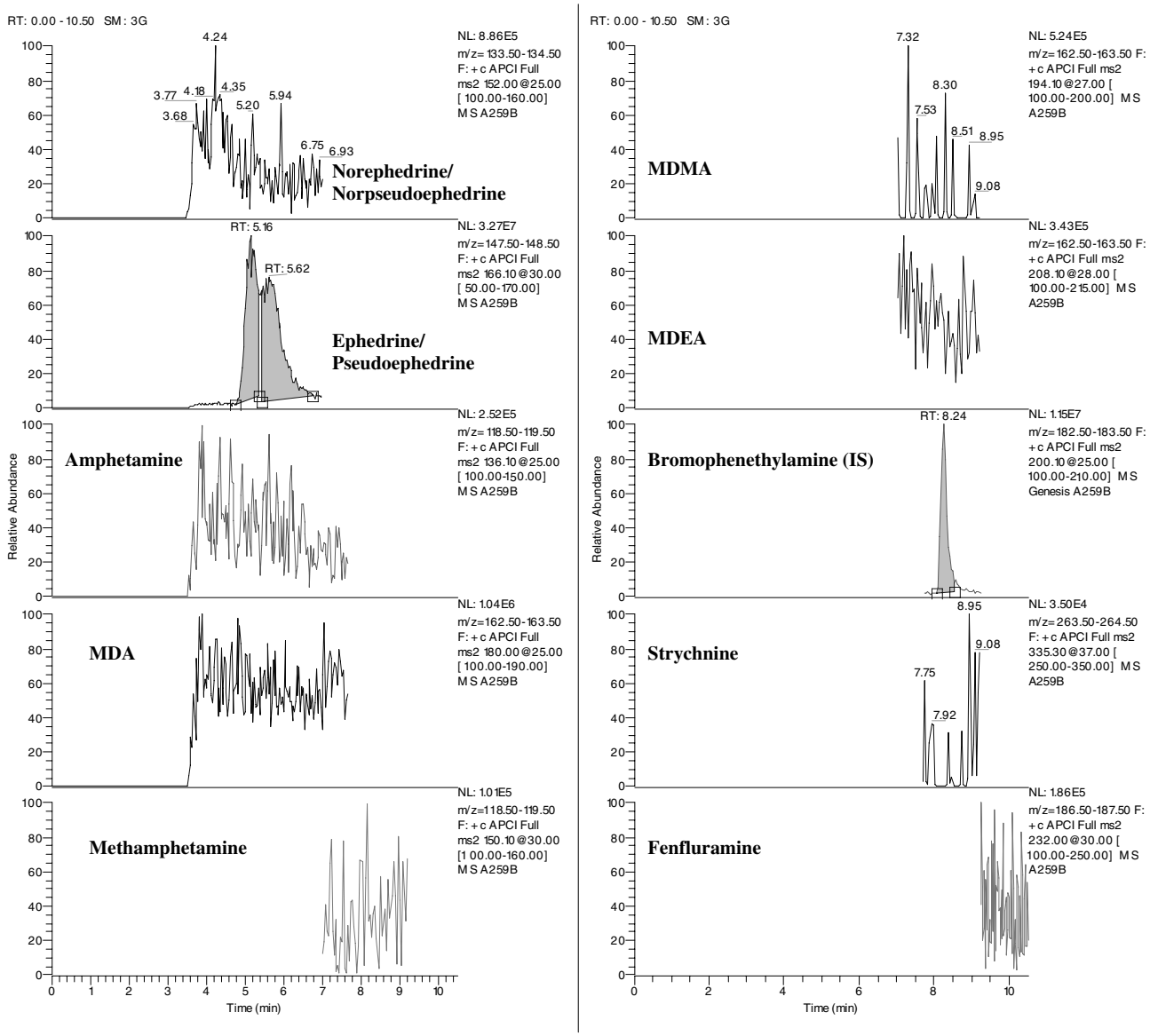

Fig. 2. Analysis of a nutritional supplement containing ephedrine and pseudoephedrine.

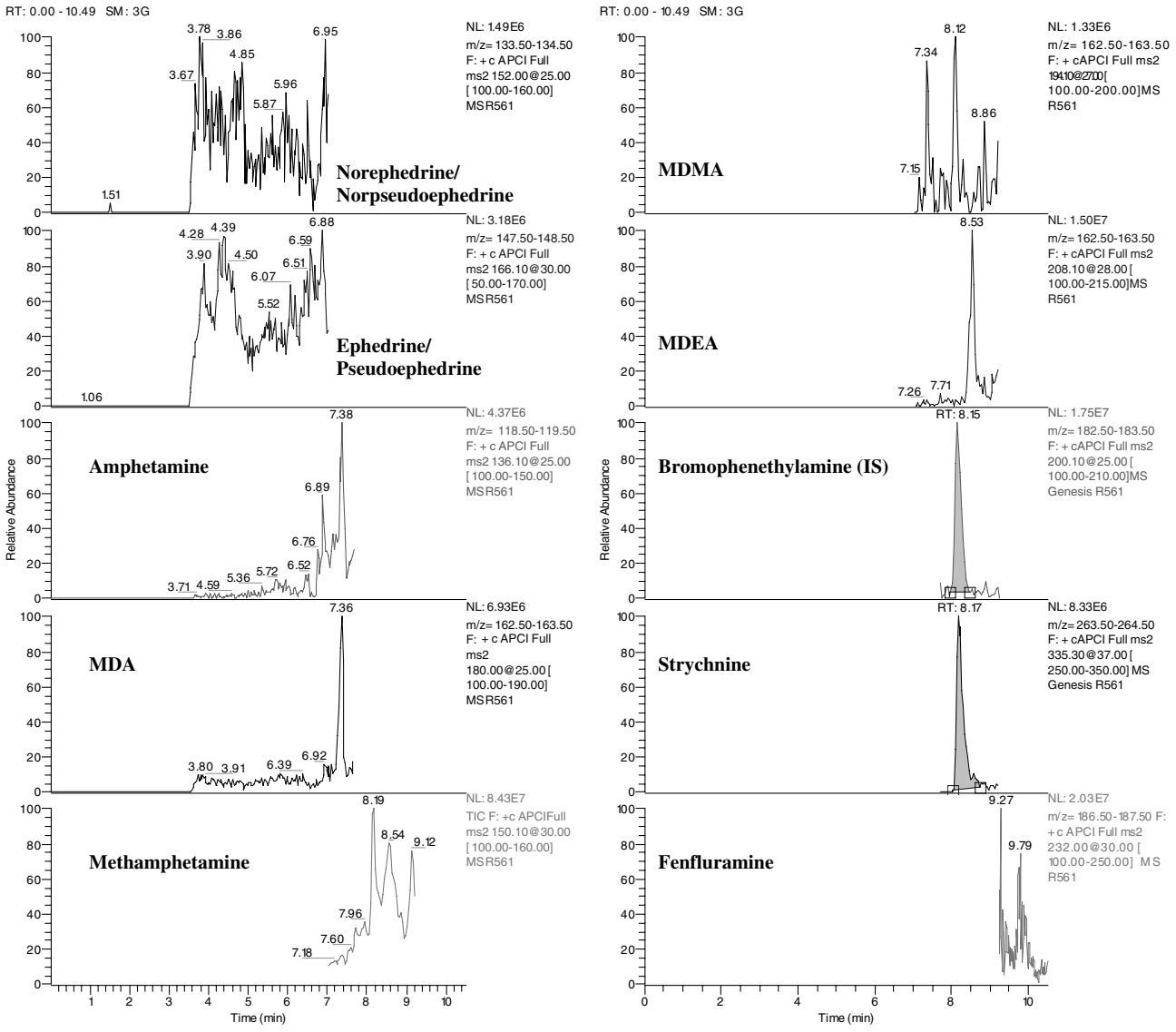

Fig. 3. Analysis of a nutritional supplement containing strychnine.

for strychnine (100 ng/g). Nevertheless, the LOD for strychnine is 20 times better then LODs obtained by GC–MS (Parr et al., 2003). Ion chromatograms obtained after analysis of a quality control sample spiked with all compounds at a concentration of 100 ng/g are presented in Fig. 1.

The described method is very selective as no interferences were detected when other doping products including other stimulants, anabolic agents, beta-blockers, narcotics, diuretics and corticosteroids were analysed. Specificity was satisfactory since no interfering substances at the appropriate retention times were found by analysing 10 blank supplements.

The method was used for the extraction and analysis of two solid supplements.

In the first sample (natural steroid enhancer) ephedrine and pseudoephedrine were detected (Fig. 2). In the second sample (Asian herbal mixture), strychnine was detected (Fig. 3). Extracts from the *Strychnos nux vomica*, a natural source of strychnine, were probably added to this supplement.

4. Conclusions

A sensitive screening method for the detection of 11 selected stimulants using LC–MS^2 was developed and validated. The obtained LODs described are equal or better than the requirements made by official authorities (NeCeDo, 2006) for the detection of stimulants in nutritional supplements. This method allowed for the detection of ephedrines and strychnine in real samples.

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