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Investigation of the silylation of ephedrines using *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide

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Abstract

This paper describes a gas chromatography (GC)–mass spectrometry (MS) method for the simultaneous quantification of ephedrine, pseudoephedrine, cathine, norephedrine and methylephedrine in urine. The sample preparation step includes solid phase extraction and derivatisation with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). An evaluation of various silylation conditions compatible with screening methods in doping control analysis is presented. The method was found to be well suited for quantification of ephedrines in doping control.

Keywords: Ephedrine; Pseudoephedrine; Cathine; Norephedrine; Methylephedrine; Derivatisation; Doping; Silylation; *N*-Methyl-*N*-timethylsilyltrifluoroacetamide

1. Introduction

Ephedrine, pseudoephedrine, norephedrine, cathine (norpseudoephedrine) and methylephedrine are potential central nervous system stimulants. They are included in the 2003 International Olympic Committee (IOC) list of banned substances because of their simulating properties, for instance, reduced tiredness and increased alertness [1]. These substances, however, are commonly used in the treatment of colds and allergy. In addition, some of them are often ingredients of dietary and sports nutritional supplements [2]. Because of their wide use, the IOC medical commission has defined concentrations above which a urine sample is considered positive. The threshold levels are 5 μg/ml for cathine, 10 μg/ml for ephedrine and methylephedrine, and 25 μg/ml for pseudoephedrine and norephedrine [3]. The chemical structure of the ephedrines is shown in Fig. 1.

Various methods for the simultaneous quantification of ephedrines in urine have been described earlier. These methods include HPLC [4–7], LC–MS [8] and CE [9,10]. GC methods include either NPD detection [2] or MS detection [11]. In doping control analysis, the use of mass spectrometry for the final confirmation of a compound in the lower molecular weight range is mandatory [12].

In the current work, the potential of silylation with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) for the quantitative determination of ephedrines in urine is evaluated. Contrary to methods like acetylation [11], more then one derivative is formed with some of the compounds. In return, however, compatibility with doping screening procedures using solely silylation as derivatisation step, in addition to reduced sample handling, is achieved. Moreover, column damage caused by acetylation reagents is avoided. The method described was validated and successfully applied to quantification and identification of ephedrines in urine, using the same instrumentation as for several other screening and confirmation analysis in the laboratory.

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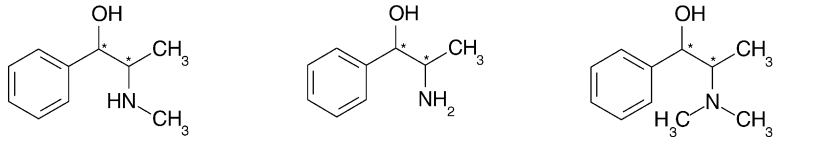

(1R, 2S)(1S, 2R)-Ephedrine
(1R, 2R)(1S, 2S)-Pseudoephedrine

(1R, 2S)(1S, 2R)-Norephedrine
(1R, 2R)(1S, 2S)-Cathine

(1R, 2S)(1S, 2R)-Methylephedrine

Fig. 1. Chemical structure of ephedrine, pseudoephedrine, cathine, norephedrine and methylephedrine.

2. Experimental

2.1. Chemicals and reagents

Ephedrine hydrochloride and cathine (norpseudoephedrine) hydrochloride were kindly provided by Knoll AG (Ludwigshafen, Germany). Methylephedrine hydrochloride was purchased from Klinge Pharma (Munich, Germany) and pseudoephedrine hydrochloride was provided by Glaxo Welcome (Greenford, United Kingdom). Norephedrine hydrochloride was obtained from Sigma (St. Louis, MO, USA) and etilefrine hydrochloride from Boehringer Ingelheim (Vienna, Austria). Etafedrine hydrochloride was a generous gift from the Institute of Biochemistry, Deutsche Sporthochschule Köln, Germany. d3-Ephedrine hydrochloride was provided by Campro Scientific (Berlin, Germany), and d10-pyren was purchased from Cambridge Isotope Laboratories (Wesel, Germany). All reference substances were certified and had more than 99% purity. Chloroform, 2-propanol, hydrochloric acid, sodium acetate, potassium hydroxide, anhydrous sodium acetate (all analytical grade), 32% ammonia (extra pure) and di-phosphorus pentoxide (P_2O_5) (extra pure) were purchased from Merck (Darmstadt, Germany). Methanol (analytical grade) was supplied by Scharlau (Barcelona, Spain). MSTFA was provided by Machery and Nagel (Düren, Germany) and MSTFA 2 by the Institute of Organic Chemistry at the Vienna University of Technology. *tert*-Butyl-methyl-ether (MTBE) was purchased from Aldrich (Vienna, Austria) and purified water was obtained by a milli-Q-reagent-grade water system (Millipore, Bedford, MA, USA). Oasis MCX columns were provided by Waters (Milford, MA, USA).

2.2. Reagent and solutions

Stock standard solutions of ephedrines (10 mg/ml) and the internal standards d3-ephedrine (1 mg/ml), etafedrine (1 mg/ml) and etilefrine (10 mg/ml) were prepared in methanol. Working solutions were prepared by diluting stock solutions. All solutions were stored at −20 °C.

2.3. Instrumental analysis

GC analysis was performed on a Trace GC 2000 gas chromatograph equipped with a Voyager mass spectrometer (Thermo Quest, Austin, USA). GC separation was achieved on a Rtx-1MS fused silica capillary column, 15 m × 0.25 mm i.d., 0.1 μm film thickness (Restek, CP-Analytica, Mistelbach, Austria). Helium was used as a carrier gas and the inlet pressure was set to 65 kPa. The injections were done at 270 °C in the split mode (20:1 spilt ratio). The GC oven temperature program was as follows: 73 °C initial column temperature, 25 °C/min to 180 °C, 45 °C/min to 310 °C, held for 2 min.

The analysis was performed in the electron impact (EI) mode at an ionisation energy of 70 eV. The MS acquisition was carried out in the selected ion monitoring (SIM) mode by monitoring at least three characteristic ions for each compound. The following ions were measured: retention window 1 (2.30–2.70 min): 102 *m/z*, 146 *m/z*, 163 *m/z*, 191 *m/z* and 236 *m/z*; retention window 2 (2.70–3.00 min): 86 *m/z*, 100 *m/z*, 116 *m/z*, 179 *m/z*, 206 *m/z* and 280 *m/z*; retention window 3 (3.00–4.00 min): 130 *m/z*, 133 *m/z*, 163 *m/z*, 166 *m/z*, 179 *m/z*, 182 *m/z*, 220 *m/z*, 294 *m/z* and 297 *m/z*; retention window 4 (4.00–5.50 min): 130 *m/z*, 237 *m/z* and 382 *m/z*. In addition to SIM mode, scan mode was performed in the mass range 40–500 *m/z*. The ions monitored in the SIM mode were selected from the full scan spectra shown in Fig. 2. For the quantification, the following ions were used: 116 *m/z* (cathine and norephedrine), 130 *m/z* (ephedrine, pseudoephedrine and etilefrine), 133 *m/z* (d3-ephedrine), 176 *m/z* (etafedrine) and 236 *m/z* (methylephedrine).

2.4. Extraction

Extractions were performed by adding an internal standard and 1 ml sodium acetate buffer pH 4.8 to 1 ml urine samples. The following automated extraction was thereafter performed on an ASPEC XL (Gilson, France):

- conditioning with 2 ml methanol followed by 2 ml 0.1 M HCl;
- the samples were passed through the cartridges (rate: 3 ml/min);
- the cartridges were rinsed with 2 ml 0.1 M HCl and 2 ml methanol;
- elution was performed with 4 ml chloroform/2-propanol/ammonia 32% (80:20:2, v/v/v) (rate 4 ml/min).

The extracts were evaporated to dryness and dried for 15 min over P_2O_5 under rough vacuum. Derivatisation was performed with 100 μl MSTFA, and thereafter the samples

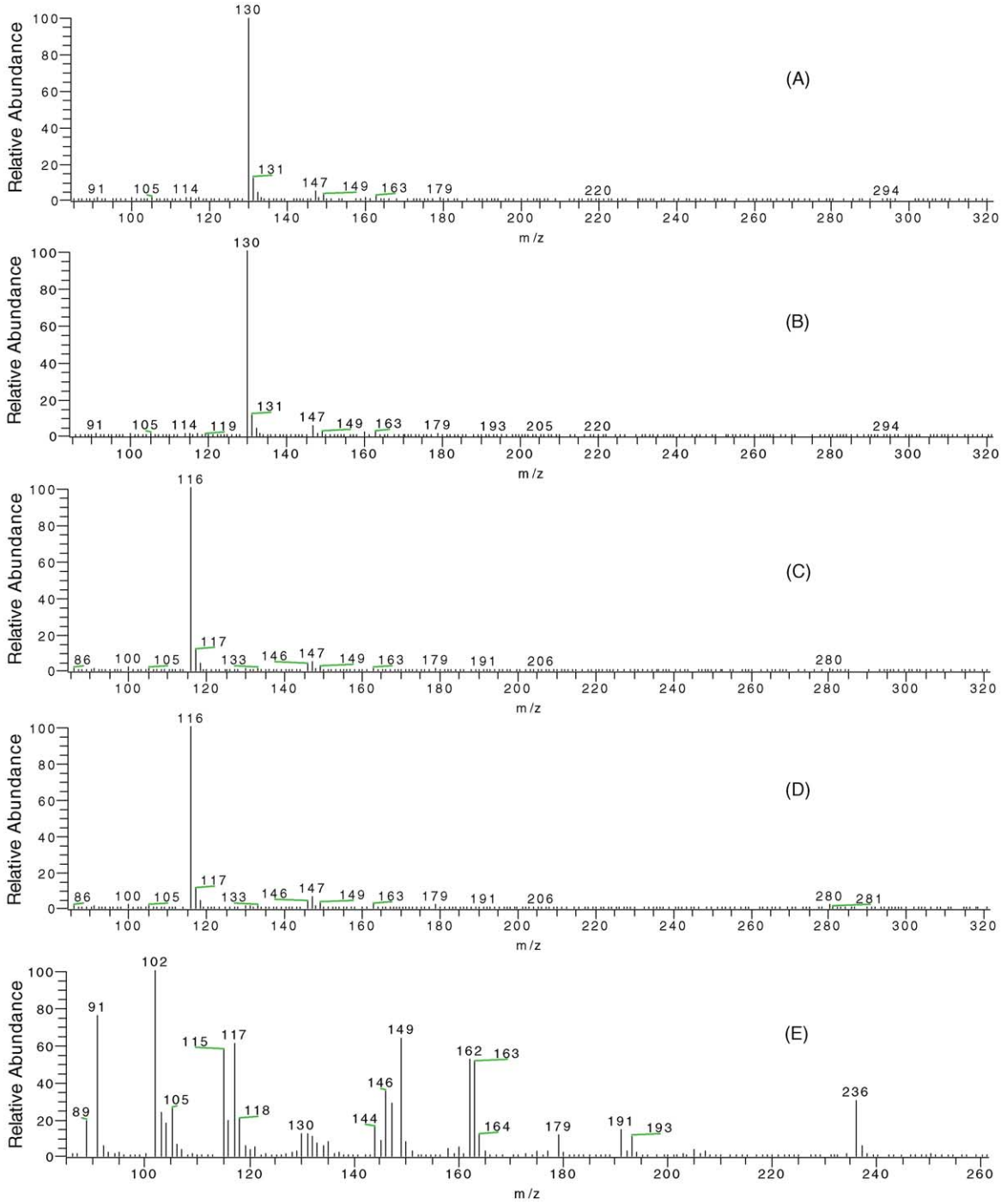

Fig. 2. Full scan spectra for (A) ephedrine bis-TMS, (B) pseudoephedrine bis-TMS, (C) norephedrine bis-TMS, (D) cathine bis-TMS and (E) methylephedrine mono-TMS. For detailed analytical conditions, see text.

were heated at 60 °C for 30 min. In order to prevent an overloading of the GC-column, samples containing ephedrine, pseudoephedrine and norephedrine were diluted 1:10 before extraction. Internal standards were chosen from intra-assay precision studies. The intra-assay precision was assessed after analysis of 10 independent extracted spiked urine samples on the same day. The urine samples were spiked with ephedrines at the threshold value concentrations. Before the extraction, 50 μl of an internal standard solution containing d3-ephedrine (0.02 mg/ml), etafedrine (0.2 mg/ml) and etilefrine (0.02 mg/ml) was added to the samples.

2.5. Derivatisation conditions

Various derivatisation conditions were evaluated, measuring the amount of the different derivatives. All samples were

derivatised with MSTFA, and analysed as described in Section 2.3, using the scan mode.

A No extraction was performed. One hundred microlitres stock solutions (10 μg of substance) were dried for 15 min over P_2O_5 in vacuum and derivatised as described in Section 2.4.

B Spiked urine samples (concentration of substances = threshold values) were extracted and derivatised as described in Section 2.4.

C The experiment was performed as in B, but at different derivatisation temperatures (40 °C, 60 °C and 80 °C).

D Matrix was diluted at different levels before the samples were spiked. Thereafter, the experiment was performed as in B.

E The experiment was performed as in B, except for various derivatisation times (15 min, 30 min and 1 h).

F The extraction was performed as in B. The eluent was divided in two parts (each part exactly 1 ml). To one part, 100 μl MSTFA was added, and to the other part, 200 μl MSTFA. The samples were derivatised for 30 min at 60 °C.

G The experiment was performed as in B, except for adding one drop concentrated HCL before evaporation and derivatisation.

H MSTFA from two different sources (MSTFA and MSTFA 2) were tested using the same extraction and derivatisation conditions as in B. The eluent was divided in two parts (each part exactly 1 ml) prior to drying and derivatisation.

I The extraction was performed as in B, and the eluate was divided in two parts (each part exactly 1 ml). One part was dried for 15 min, while the other part was dried over night. After derivatisation, 50 μl of a mixture of d10-pyren and MSTFA were added (0.02 mg d10-pyren/ml MSTFA), in order to be able to evaluate the stability of the ephedrines during the long drying time.

2.6. Assay validation

The final method was validated by determining its specificity, limit of quantification (LOQ), linearity, recovery, intra-assay precision (see Section 2.4) and inter-assay precision. In addition, the method was tested for matrix effects.

In order to study the linearity, a calibration graph was prepared, covering the expected concentration rage. Urine samples were spiked with four different concentrations of the ephedrines in the range 0.5 × IOC threshold value–2 × IOC threshold value. LOQ and standard deviations of the overall procedure were calculated according to the standard DIN 38402, Teil 51 [13].

To exclude interfering substances, specificity was evaluated analysing seven blank urines. Additionally, urine samples spiked with the structurally related compounds amphetamine and methamphetamine were analysed.

The extraction recoveries of the ephedrines were calculated by analysis of spiked urine samples at the following concentrations: 0.5 × IOC threshold value, 1 × IOC threshold value and 2 × IOC threshold value. The internal standards were added after the extraction, and the peak area ratios were compared to peak area ratios obtained when pure standards were derivatised.

Inter-assay precision was determined after extraction and analysis of independent spiked urine samples on three different days by different analysts.

Matrix effects were evaluated by preparing calibration graphs using spiked urine and water samples. The concentration range was 0.5 × IOC threshold value–2 × IOC threshold value.

3. Results and discussion

3.1. Extraction

A solid phase extraction was chosen for the sample preparation. A solid phase extraction represents several advantages compared to LLE, like easy automatisation and the use of a less amount of solvents. The solid phase extraction columns used in these experiments are so-called mixed phase extraction columns, which possess both lipophilic and ion-exchange properties.

Table 1
Inter-assay precision calculated for ephedrine, pseudoephedrine, cathine, norephedrine and methylephedrine using three different internal standards

	Intra-assay precision		
	Internal standard: d3-ephedrine (%)	Internal standard: etilefrine (%)	Internal standard: etafedrine (%)
Ephedrine	2	4	16
Pseudoephedrine	4	6	14
Cathine	5	1	18
Norephedrine	3	3	20
Methylephedrine	25	23	5

Table 2
Amount of mono/bis TMS derivatives after derivatisation of clean standards compared to derivatisation of extracted samples

Compound	No extraction (%)	Extraction (%)
Ephedrine	1	6
Pseudoephedrine	2	2
Cathine	–	–
Norephedrine	–	–
d3-Ephedrine	1	7
Etilefrine	–	4

The signal areas are expressed as a percentage ratio defined as the area of the mono(bis)-TMS derivative divided through the area of the bis(tris)-TMS derivative, multiplied by 100%.

Table 3
Amount of mono/bis TMS derivatives after various derivatisation times

Compound	15 min, 60 °C (%)	30 min, 60 °C (%)	1 h, 60 °C (%)
Ephedrine	1	8	8
Pseudoephedrine	4	3	2
Cathine	–	–	–
Norephedrine	–	–	–
d3-Ephedrine	11	6	5
Etilefrine	5	2	2

The signal areas are expressed as a percentage ratio defined as the area of the mono(bis)-TMS derivative divided through the area of the bis(tris)-TMS derivative, multiplied by 100%.

The intra-assay precisions calculated for the ephedrines using different internal standards are summarised in Table 1. As shown, the lowest standard deviations were achieved by using d3-ephedrine as an internal standard for ephedrine and pseudoephedrine, etilefrine as an internal standard for cathine and norephedrine, and etafedrine as an internal standard for methylephedrine.

3.2. Derivatisation conditions

Derivatisation is a critical step in the analysis of ephedrines. The formation of a single derivative is preferred in order to obtain better sensitivity and optimal quantification conditions. Previous studies have shown the possibility to obtain only one derivative for each ephedrine by combining silylation and acetylation, giving 0-TMS (trimethylsilyl) and *N*-TFA (trifluoroacetyl) derivatives [11]. In our laboratory, however, it was not desirable to perform an acetylation. According to our own experience, acetylation agents are causing damage to the GC column, and the column cannot be used for the detection of silylated metabolites of anabolic steroids. By performing solely silylation, the quantification procedure is better compatible to doping screening. No additional instrument is required, and moreover, it is not necessary to change chromatographic conditions, for instance, exchange of GC column and injection parameters.

Optimum conditions for the derivatisation step are studied by subjecting the samples containing the ephedrines and the internal standards to several variations during derivatisation. In all cases, one significant derivative was formed for each compound. For ephedrine, d3-ephedrine, pseudoephedrine, cathine and norephedrine, the main derivative formed is the bis-TMS derivative, while the tris–TMS derivative is the most abundant for etilefrine. In the case of etafedrine and methylephedrine, mono-TMS derivatives are obtained. The results are summarised in Tables 2–5. The amounts of mono/bis derivatives are expressed as a ratio percentage. For ephedrine, pseudoephedrine, cathine and norephedrine, the ratio percentage is defined as the area of the mono-TMS derivative divided through the area of the bis-TMS derivative, multiplied by 100%. For etilefrine, the ratio percentage is defined as the area of the bis-TMS derivative divided through the area of the tris–TMS derivative, multiplied by 100%. Etafedrine and methylephedrine are not included in the tables, since the mono-TMS derivatives are the only possible derivatives to be formed during silylation.

Only one derivative (the bis-TMS derivative) is obtained for cathine and norephedrine, independent of the derivatisation conditions tested.

In the case of ephedrine, pseudoephedrine and etilefrine, less mono-TMS derivatives (ephedrine, d3-ephedrine and pseudoephedrine) and bis-TMS (etilefrine) are obtained when no matrix is present compared to extracted samples. The results are shown in Table 2. Dilution of the matrix before addition of analytes and extraction, however, showed no conclusive effects.

By varying the temperature, some variations in the derivative ratios were observed, but no significant increase in silylation efficiency with elevated temperature.

The derivatisation time appeared to have some influence on the derivative ratio percentages. In the case of d3-ephedrine and etilefine, the silylation efficiency significantly increased by prolonging the derivatisation time from 15 min to 30 min. In regards of ephedrine, however, the opposite effect could be observed. The results are summarised in Table 3.

By increasing the amount of derivatisation agent, and by adding HCl to the samples before evaporation and derivatisation (to obtain the hydrochlorides), no significant effects on the derivative ratio percentages were achieved. In both cases, however, the absolute areas of all derivatives decreased.

Derivatisation agents from two different sources were evaluated, labeled as MSTFA and MSTFA 2. As shown in Table 4, less mono-TMS derivatives could be observed

Table 4
Amount of mono/bis TMS derivatives after derivatisation with agents of different quality

Compound	MSTFA (%)	MSTFA 2 (%)
Ephedrine	4	7
Pseudoephedrine	4	12
Cathine	–	–
Norephedrine	–	–
d3-Ephedrine	3	5
Etilefrine	–	–

The signal areas are expressed as a percentage ratio defined as the area of the mono(bis)-TMS derivative divided through the area of the bis(tris)-TMS derivative, multiplied by 100%.

Table 5
IOC threshold level, LOQ, method standard deviation, recovery, correlation coefficient, intra-assay precision and inter-assay precision obtained for ephedrines according to the described procedure

	Compound				
	Ephedrine	Pseudoephedrine	Cathine	Norephedrine	Methylephedrine
IOC cut-off (μg/ml)	10	25	5	25	10
LOQ (μg/ml)	1.7	6.9	1.1	7.4	2.5
Method standard deviation (%)	2.4	4.0	3.3	4.4	4.4
Recovery (%)	85 ± 4	84 ± 3	91 ± 7	93 ± 7	103 ± 5
Correlation coefficient	0.999	0.997	0.998	0.996	0.997
Intra-assay precision (%)	2.3	4.1	1.2	3.2	5.4
Inter-assay precision (%)	5.7	7.6	10.6	10.7	7.0

for ephedrine, pseudoephedrine and d3-ephedrine, using MSTFA compared to MSTFA 2. Conclusively, the quality of the derivatisation agent has an effect on the derivatives formed.

The drying time only slightly influenced the derivative ratios, and for ephedrine, pseudoephedrine and d3-ephedrine, a slightly higher derivative ratio percentage could be observed with longer drying time. Moreover, a significant less amount of ephedrine bis-TMS, pseudoephedrine bis-TMS, d3-ephedrine bis-TMS, cathine bis-TMS, norephedrine bis-TMS and methylephedrine mono-TMS could be observed in the samples dried over night, compared to the samples dried for 15 min. The amounts of the main derivatives were reduced as much as 25–50%. This indicates an evaporation of the substances during the long drying step.

3.3. Figures of merit

The final optimised assay includes SPE performed at pH 4.5, drying in vacuum over P_2O_5 for 15 min, derivatisation with MSTFA at 60 °C for 30 min and MS-detection in SIM mode. Fig. 3 shows ion chromatograms for the ephedrines and the internal standards (spiked urine sample: 1 μg/ml (ephedrine and d3-ephedrine), 2.5 μg/ml (pseudoephedrine and norephedrine), 5 μg/ml (norpseudoephedrine) and 10 μg/ml (methylephedrine, etafedrine and etilefrine)).

In Table 5, LOQ, the standard deviation of the overall procedure, correlation coefficient, recovery and inter-assay precision are given for the ephedrines studied.

All substances showed linear behaviour within the analysed concentration range. The LOQ is well beyond the IOC cut-off limits for all compounds, and the standard deviations of the entire procedure are all lower then 5%. The method reported showed satisfactory extraction recoveries between 85% and 103%. The inter-assay precision proved to be acceptable, and varied between 5.7% and 10.7%.

No interferences were detected in the blank urine samples analysed for testing specificity of the method. However, a peak containing the fragment ion 130 was observed close to the ephedrine peak in some of the samples. This peak could be identified as creatinine TMS derivative. The related compounds amphetamine and methamphetamine did not interfere with the analysis of the ephedrines. Additionally, no matrix effects could be detected, comparing the calibration graphs generated for urine and water samples.

3.4. Qualitative confirmation

In order to assure a reliable confirmation of the identity of a substance, the World Anti-Doping Agency (WADA) has established a number of identification criteria when using chromatography and MS. In cases where the SIM mode is used, a minimum of three diagnostic ions must be acquired. The relative intensities of any of the ions must be within certain range limits depending on the relative abundances of the ions [12]. In Table 6, an example is shown with a sample from an athlete who tested positive for ephedrine. The areas of three diagnostic ions are compared to those in a urine sample spiked with ephedrine. As can be seen, the deviations are well beyond the range limits given by the WADA.

Table 6
Relative abundances of three ions in an athlete sample containing ephedrine are compared to the relative abundances of the same three ions in a urine sample spiked with ephedrine

m/z	Ephedrine standard		Athlete sample			Range limits	
	Area	Relative abundance (%)	Area	Relative abundance (%)	Difference (%)	Lower (%)	Upper (%)
294	8837	63.9	8523	64.5	0.6	53.9	73.9
220	9045	65.5	8889	67.3	1.8	55.5	75.5
179	13819	100.0	13213	100.0	0.0	90.0	110.0

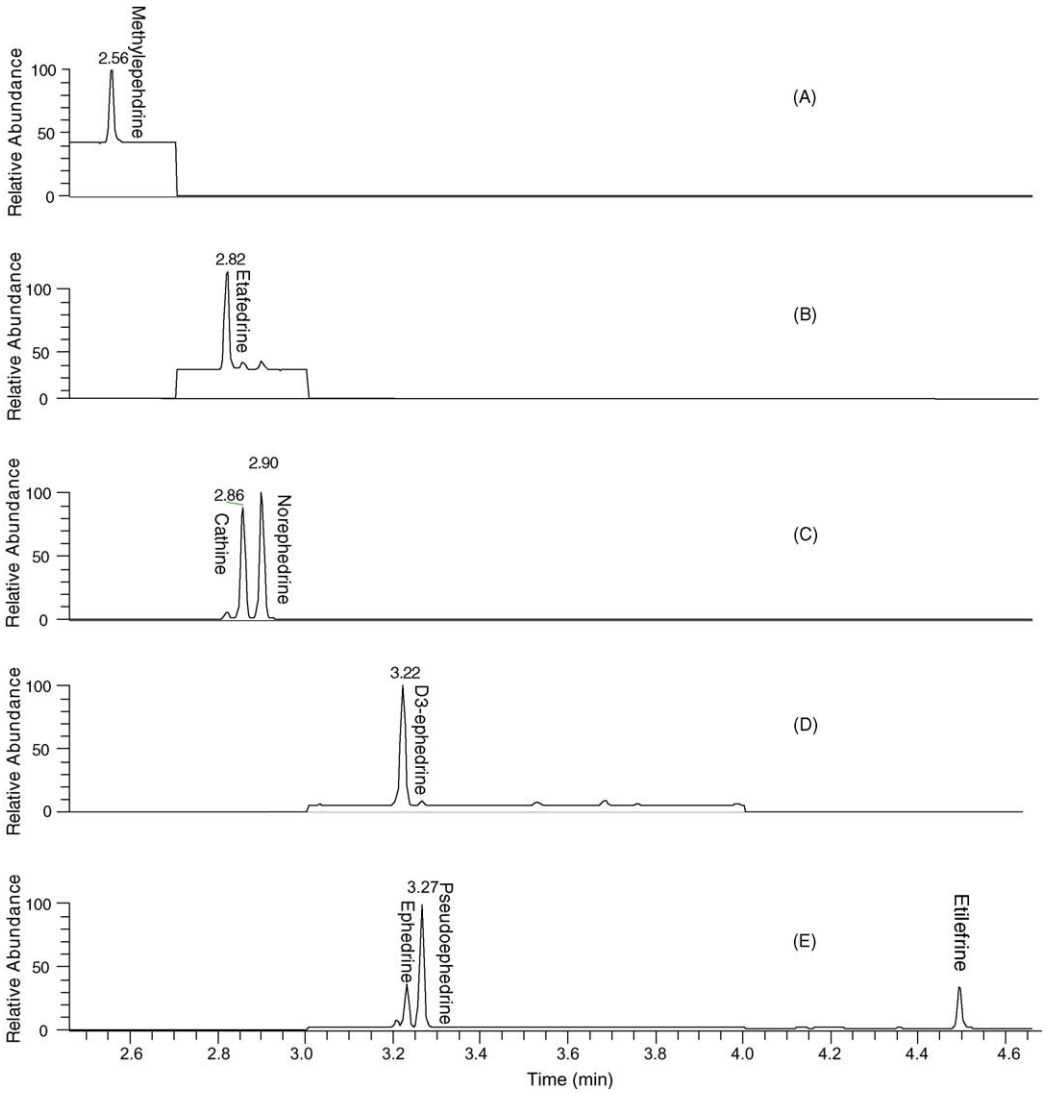

Fig. 3. Ion chromatograms of ephedrines and internal standards: (A) 236 *m*/*z* extracted, (B) 179 *m*/*z* extracted, (C) 116 *m*/*z* extracted, (D) 133 *m*/*z* extracted, (E) 130 *m*/*z* extracted. For detailed analytical conditions, see text.

4. Conclusion

The presented assay allows simultaneous quantification of ephedrines in urine subjected to doping control analysis. The derivatisation step includes solely silylation with MSTFA, leading to the formation of a suitable derivative for each ephedrine. By avoiding the use of an acetylation agent, GC column damage could be prevented. The method proved to be rapid and sensitive, showing limits of quantification well below the IOC threshold levels.

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