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Confirmatory procedure of ephedrines by gas chromatography-mass spectrometry. Migration of the trimethylsilyl group in the N-acetyl-O-trimethylsilyl derivatives

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1. INTRODUCTION

Ephedrines are sympathomimetic amines known to have central nervous system stimulating properties[1]. For this reason some of them are classified as prohibited substances according to the list of forbidden substances in sports by the World Antidoping Agency (WADA) [2]. As many pharmaceutical preparations commonly used for asthma, colds, sinusitis, rhinitis, and appetite suppressants contain ephedrines[3], the WADA has set threshold levels for these substances above which a doping sample is considered positive[1]. Ephedrines have also been the ingredients of several dietary supplements found in sports nutritional supplements for boosting energy during exercise or as weight reduction aids[4-6]. Adverse effects of ephedrine-related preparations include tremor, hypertension, cardiac arrhythmia, psychosis, seizure, myocardial infraction, intracranial haemorrhage and even death[7-10].

Numerous methods including gas chromatography coupled with nitrogen phosphorous detection (GC-NPD) or mass selective detectors to determine ephedrines in doping control and toxicological analysis have been published[11-16]. Derivatization as trimethylsilyl or fluoracetyl derivatives after extractive clean up leads to excellent separation and reliable identification and quantitation results[17, 18].

Nevertheless, these procedures are not suitable for confirmation purposes because of some diagnostic ions have not a relative abundance greater than 5 % as it is required by the WADA technical document (TD2003IDCR). Also, the mass spectra obtained by the methods mentioned above show little structural information. Following TD2003IDCR in these cases a

second derivative shall be prepared or a second ionization or fragmentation technique shall be used[19].

In this paper, a simple and easy confirmatory procedure based on gas chromatography-mass spectrometry for ephedrines including norpseudoephedrine (NORP), norephedrine (NORE), ephedrine (EPHE) and pseudoephedrine (PSEU) in human urine is presented.

2. MATERIALS AND METHODS.

Chemicals

All reagents and solvents used were of analytical quality and obtained from Aldrich (Milwaukee, USA), The N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrous sodium sulfate (p.a) and potassium hydroxide (KOH) (p.a) were purchased from Merck (Germany). The acetic acid anhydride was obtained by Sigma (Germany). All reference substances were purchased from Sigma-Aldrich (Steinheim, Germany).

Instrumentation

The analysis was carried out using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometer detection system (GC/MS).

Analytes separation was achieved on a fused silica capillary column (Ultra-2, 12m x 0.20 mm i.d., film thickness 0.33 μm, Agilent Technology). The oven temperature was programmed at 95 °C increased to 270 °C at 10 °C/min and held for 2.5 min. Split injection mode (10:1) was used. Helium with a flow of 1ml/min was used as carrier gas. Constant flow mode was used. The injection port, ion source and interface temperature were: 280, 230 and 280 °C respectively. The mass spectrometer was operated in electron ionization mode at 70 eV and to study mass fragmentation, experiments at 12 eV was made. The mass spectra were obtained in Full Scan mode with a scan range of 40-360 amu. For GC-MS analysis, 1μL amount was injected.

Urine samples preparation

A 25 μL volume of the internal standard (levallorphane at 100 μg/mL in methanol) was added to 2.5 mL of urine. Then 0.5 mL KOH 5M, 2 mL *tert*-butylmethyl ether and approximately 3 g sodium sulphate were added and the mixture was briefly vortex-mixed. After 20 min of mechanical shaking and centrifugation for 5 min at 1200 g, the ether layer was then evaporated to dryness under nitrogen stream at 40°C. Before derivatization the samples were dried in a desiccator for 1h.The dried residue was derivatized as follow:

The dry residue was derivatized with 50 µL of acetic acid anhydride (20 min at 60°C). Afterwards the acetic acid anhydride was evaporated to dryness under nitrogen at 40°C and 50 µL of MSTFA was added to the residued. Derivatization was at 60°C for 10 min.

The O-trimethylsilyl-N-trifluoroacetyl derivative was obtained using a previously described method.[14]

3. RESULTS AND DISCUSSION.

All samples were spiked with one substance at the threshold level established by WADA. In the cases of pseudoephedrine and norephedrine, which were removed from the list of prohibited substances since 2004, the concentrations used were the 2003 WADA cut-off limits. The concentrations of each compound, retention time, relative retention time, diagnostics ions and its relative abundances are shown in Table 1.

Compound	Method Parameters			Concentration (µg/ml)
	RT	RRT	m/z (relative abundance)	
Norpseudoephedrine	6.420	0.510	179 (100 %), 159 (49 %), 116 (14 %), 44 (16 %)	5
Norephedrine	6.504	0.517	179 (100 %), 159 (51 %), 116 (14 %), 44 (14 %)	25
Ephedrine	7.062	0.561	100 (100 %), 58 (97 %), 179 (52 %), 173 (38 %)	10
Pseudoephedrine	7.259	0.577	100 (100 %), 58 (93 %), 179 (56 %), 173 (40 %)	25
Levallorphane	12.573	-	355 (100%)	1

Table 1. Concentrations of each compound, retention time, relative retention time, diagnostics ions and relative abundances.

A representative ion chromatogram (m/z 179) obtained from urine extracts is shown in figure1. The blank urine sample showed no interfering endogenous compounds. An excretion urine containing pseudoephedrine at 20 µg/mL is also shown in figure 1. In this chromatogram the metabolite norpseudoephedrine[16] is also detected.

All four ephedrines (NORE, NORP, EPHE, and PSEU) were reasonably well separated by GC/MS and only one peak per compound was obtained. Recently; several studies showed that these ephedrines were separated by HPLC[20] and GC-MS[13, 18]. For separating two pairs of diasteroisomers (NORE/NORP and EPHE/PSEU) GC-NPD is used in our laboratory, therefore this method is to confirmate the previously suspected samples.

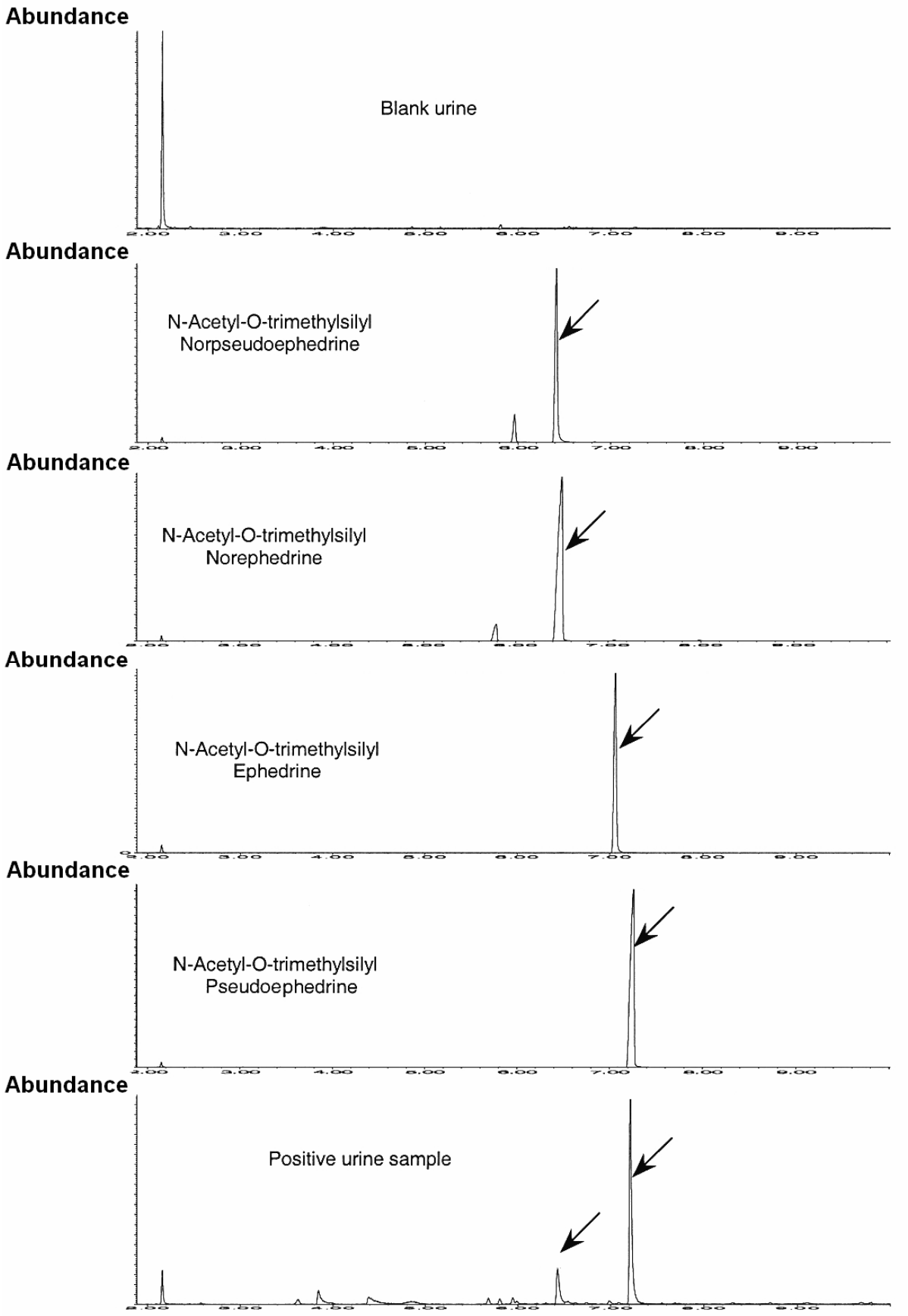

Figure 1. EI Ion Chromatogram for m/z 179 (common ion) obtained from urine sample extracts.

With this method it was possible to obtain spectra, which have several diagnostics ions with a relative abundance exceeding, the 5% WADA requirement. Also, the mass spectra have many characteristics fragments that show great structural information content.

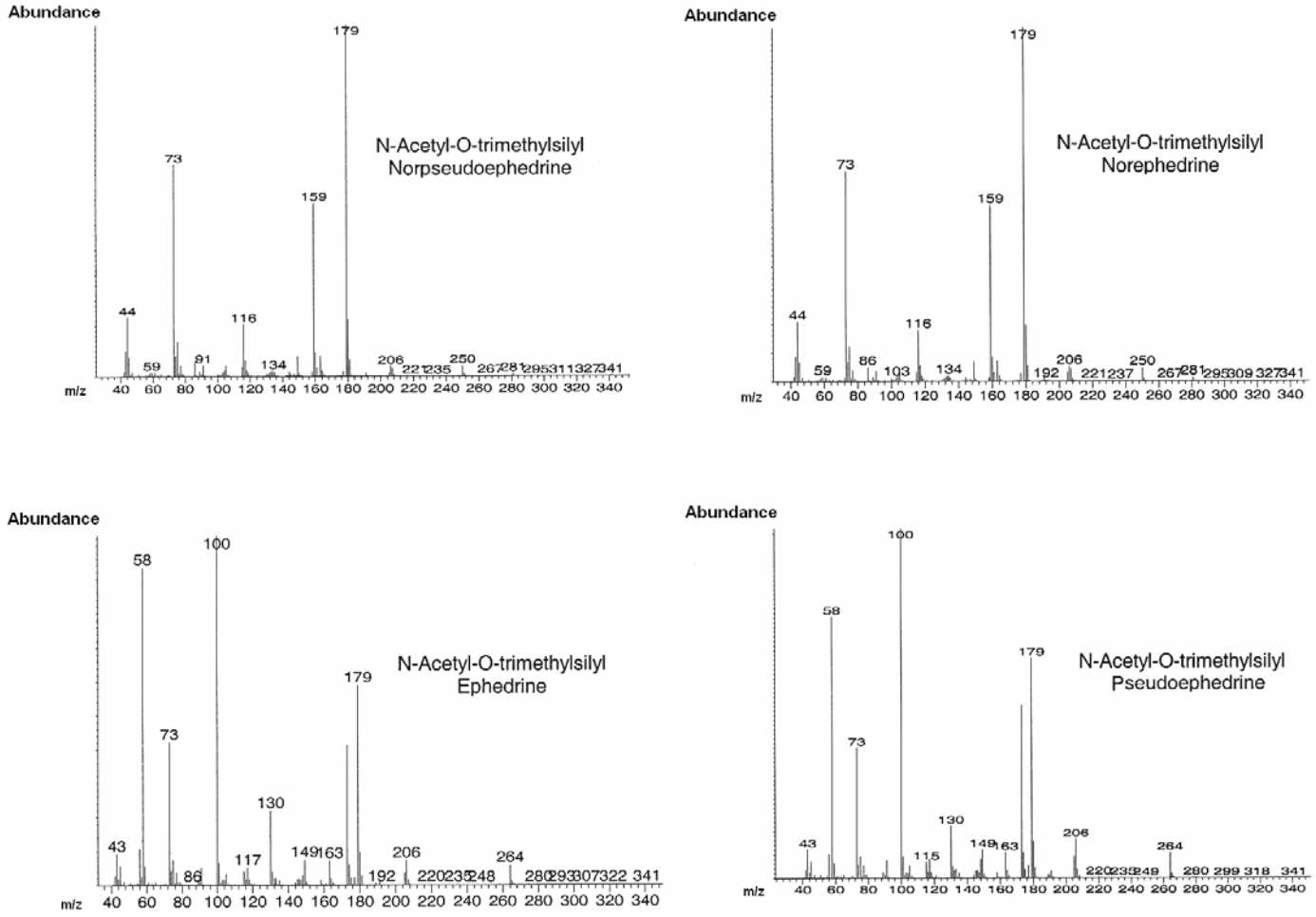

Figure 2. Mass spectra obtained from N-acetyl-O-trimethylsilyl derivatives of ephedrines.

In all ephedrines the molecular ions are absent but it M^+-15 was observed. The most important process in the fragmentation in these molecules is the α cleavage with the formation of the common ion m/z 179, in high abundances. Also, this fragmentation leads to the formation of the ions m/z 100 and m/z 86 for EPHE/PSEU and NORE/NORP respectively. However, there is a significant difference in the spectra. Meanwhile the ion m/z 100 for EPHE/PSEU is the base peak, its analogue m/z 86 for NORE/NORP exhibits low abundancy. This can be explained by the stabilizing effect of the methyl group present in EPHE/PSEU on the imonium ion formed during the α cleavage. The fragmentation sequence yields the ions m/z 58 and 44 further by the C-N cleavage with hydrogen rearrangement and loss of ketene (Fig. 3).

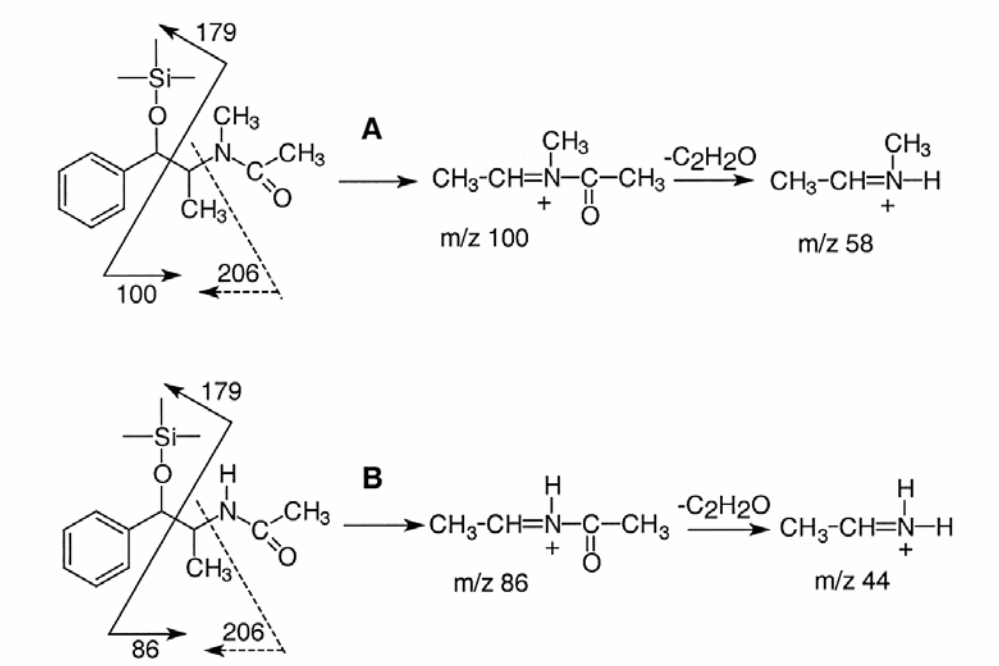

Figure 3. Fragmentation proposed for ephedrines. (A): EPHE/PSEU. (B): NORE/NORP.

Equally important are the ions m/z 173 and m/z 159 for EPHE/PSEU and NORE/NORP respectively. The origin of these ions could be explained by migration of the trimethylsilyl moiety to the N atom with the involvement of a five-member intermediate. Such transition states are not well established in mass spectrometry. The elimination of the stable molecule of benzaldehyde from the M^+ could be considered as the driving force of this process (Fig. 4).

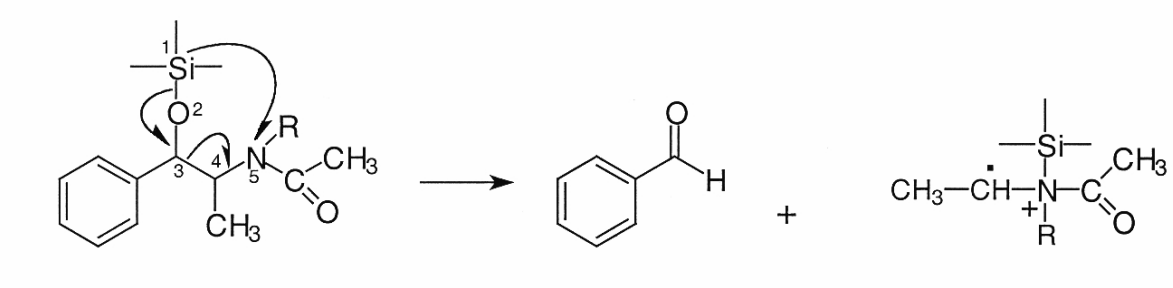

Figure 4. Rearrangement proposed for EPHE/PSEU (R=CH_3 m/z 173) and NORE/NORP (R=H m/z 159).

Evidence for this mechanism is the mass displacement provoked by CF_3 in the O-trimethylsilyl-N-trifluoroacetyl derivatives of ephedrines. This derivative of EPHE/PSEU and NORE/NORP shows the corresponding rearrangement with the formation of the ion at m/z 227 and m/z 213 respectively.

Two other facts can be considered: In the N, O-bis-trimethylsilyl-N-trifluoroacetyl derivatives of NORE/NORP this rearrangement can not take place due to steric hindrance and in the mass spectra obtained at 12 eV (experience at low voltage), the intensity of the ions m/z 159 and

m/z 173 is increased evidencing that this rearrangement is favored over simple rupture at low ionization energy (Fig.5).

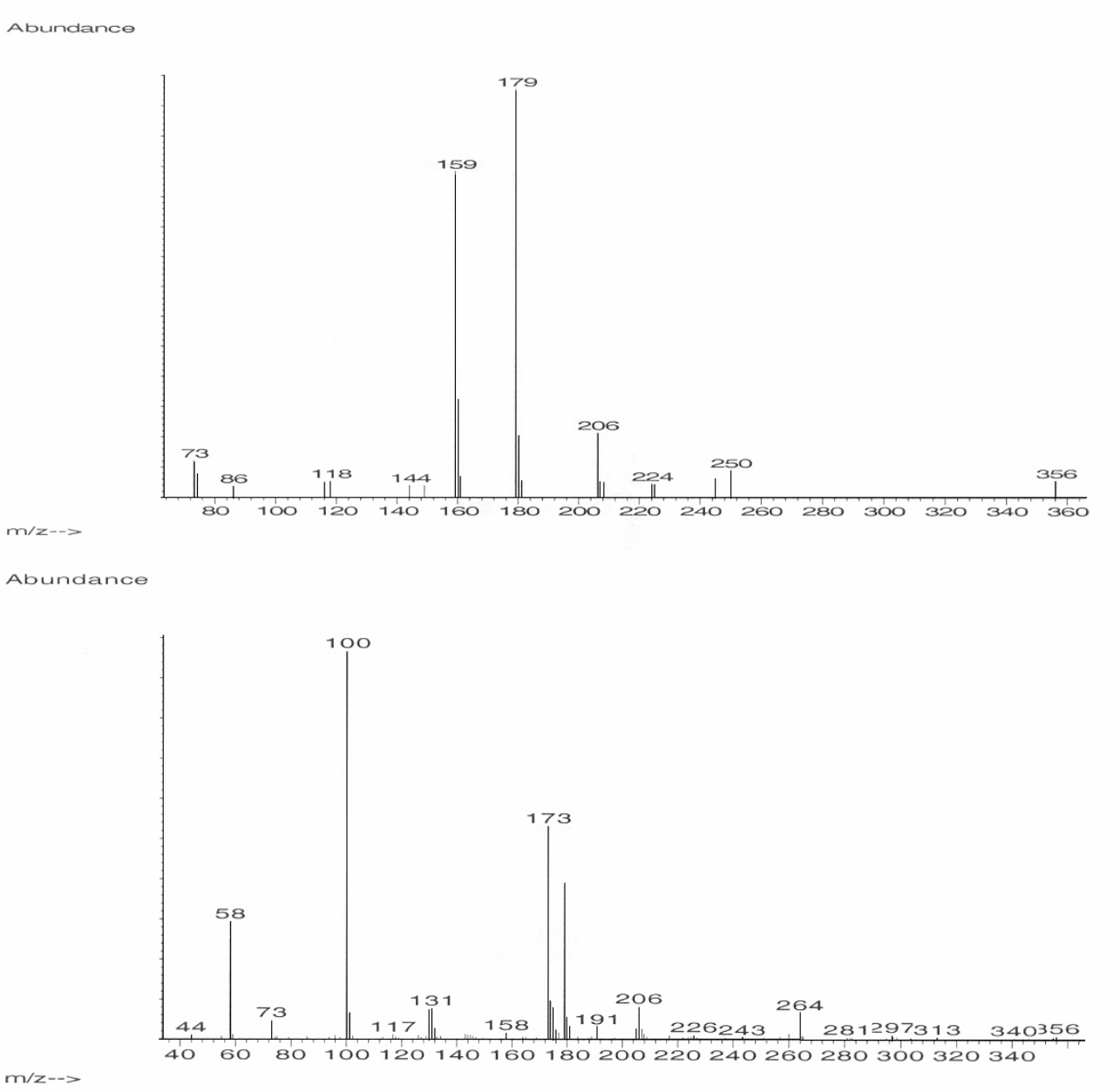

Figure 5. Mass spectra obtained at 12 eV from N-acetyl-O-trimethylsilyl derivatives of norephedrine and ephedrine.

In the four spectra obtained appears the ion m/z 206 corresponding to rupture of C-N bonds with the loss of 59 amu and 73 amu from M^+ of NORE/NORP and EPHE/PSEU respectively. Considering the initial charge of the molecular ion on the oxygen atom of the acetyl group, formation of the ion m/z 206 can be proposed as a Mc Lafferty's rearrangement with formation of a neutral nitrogen particle (Fig. 6).

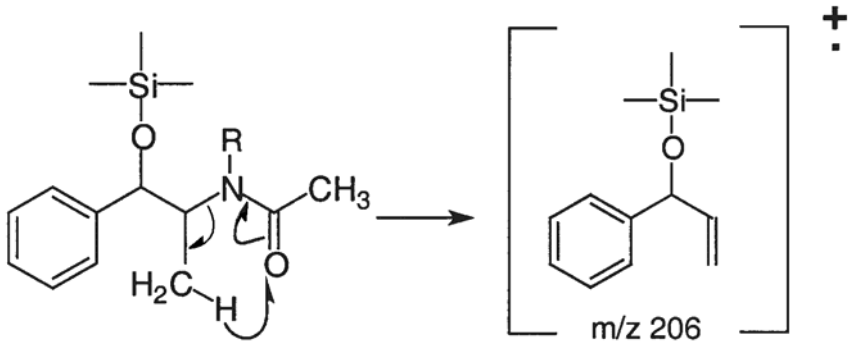

Figure 6. Proposed mechanism for the formation of the common ion m/z 206.

As general rule in doping control, when a substance is detected in a sample a second independent analysis must be done to confirm unequivocally its presence. This second analysis consists of a reextraction of the presumptive positive sample in a batch which also includes a urine obtained after a real excretion study of the banned substance and other quality control samples. The batch can be processed with another suitable analytical method [21]. In this case a suitable derivative has been designed to achieve unequivocal identification of ephedrines.

4. CONCLUSIONS.

The qualitative confirmatory procedure reported in this article allows the confirmation of norpseudoephedrine, norephedrine, ephedrine and pseudoephedrine simply and easily. With the formation of N-acetyl-O-trimethylsilyl derivatives of ephedrines, we obtained mass spectra with many diagnostic ions, which have relative abundances in accordance with the WADA requirements and show great structural information content compared to other previously reported methods. For EPHE and PSEU the ions m/z 100, m/z 58 and m/z 179, can be used as diagnostics ions together with the m/z 173. For NORP and NORE the ions m/z 179, m/z 159 and m/z 116 can be used as diagnostics ions together with the m/z 44.

The fragmentation of these derivatives has been discussed with a curious migration of the trimethylsilyl group being proposed.

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