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GC/MS SEPARATION AND QUANTIFICATION OF EPHEDRINES

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INTRODUCTION

The 2004 WADA prohibited list specifies cut-off levels for the concentration of cathine, ephedrine and methylephedrine. Therefore a method for their quantitative determination is required. This method should include also the quantitative determination of the diastereoisomers of the ephedrine and cathine, the pseudoephedrine and, respectively, the phenylpropanolamine (norephedrine) for two reasons:

1. Pseudoephedrine and norephedrine have the same characteristic ions in their mass-spectra and retention times close to those of ephedrine and, respectively, cathine; therefore, they can be misidentified, leading to false positives. In each case, care for clear chromatographic resolution of the two diastereoisomers is to be taken [1], in order to avoid any contribution of the pseudoephedrine and norephedrine in the chromatographic peak of the ephedrine and, respectively, cathine.
2. Although the pseudoephedrine and the norephedrine were withdrawn from the WADA Prohibited List, they are nevertheless on the WADA Monitoring List; therefore a quantitative evaluation of these two substances is still mandatory.

The methods proposed for the quantification of the ephedrines include GC/MS [2,3,4], GC/NPD [5] and LC/MS [2]. Usually, the GC/MS methods involve a two-step derivatization: a derivatization with MSTFA, followed by a derivatization with MBTFA [2,3]. However, the products of the MBTFA derivatization cause column damages. In the current work, is described a method that obtains a good gas-chromatographic separation and quantification of the ephedrines through a one-step derivatization, using solely MSTFA.

MATERIAL AND METHODS

REFERENCE SUBSTANCES

Ephedrine hydrochloride was purchased from Sigma-Aldrich (Germany) and methylephedrine hydrochloride, cathine, pseudoephedrine and norephedrine from AGAL-NARL (Australia). D₃-ephedrine (internal standard) was obtained from the Cologne Doping Control Laboratory. Potassium hydroxide, sodium sulphate anhydrous, tert.-butyl-methyl-ether (all p.a.) and N-methyl-N-trimethylsilyltrifluoroacetamide were purchased from Merck (Germany).

SAMPLE PREPARATION

25µl d₃-ephedrine 1mg/ml (internal standard) were added to 2.5ml urine in a 10ml screw-cap vial and the pH adjusted at 9-10 with approximately 0.1ml KOH 5N. Then 1g Na₂SO₄ anhydrous and 2ml tert.-butyl-methyl-ether were added and the mixture was mechanically shaken for 20min and centrifuged at 3000g for 5min.

The organic phase, was transferred in a 5ml screw-cap vial and evaporated to dryness, at room temperature, under nitrogen flow, then further dried, 1h, in a vacuum dessicator, over P₂O₅/KOH [6,7].

The residue was derivatized with 100µl MSTFA, at 60⁰C, for 30min.

1µl of the resulting solution was injected in GC/MS.

GC/MS ANALYSIS

- System: Hewlett-Packard GC 6890 / MS 5972;
- Column: HP-5, 25m length, i.d. 0.25mm, film thickness 0.25µm;
- Carrier gas: Helium 0.8ml/min;
- Injector temperature: 300°C, injection mode-split 1:10;
- Oven: 100°C, 20°C/min, 320°C (2min);
- Acquisition mode: SIM, retention times and monitored ions are presented in Table 1.

Table 1. Method parameters for the analyzed ephedrines

Compound	Method parameters		
	RT (min)	RRT	m/z* (relative abundance)
Methylephedrine	4.43	0.849	72 (100%), 149 (6.42%), 163 (5.85%)
Cathine	4.73	0.906	116 (100%), 149 (8.26%), 163 (6.62%)
Norephedrine	4.78	0.916	116 (100%), 149(10.22%), 163 (7.32%)
IS-D ₃ -Ephedrine	5.22	1.000	133
Ephedrine	5.24	1.004	130 (100%), 149(10.29%), 163 (7.40%)
Pseudoephedrine	5.29	1.013	130 (100%), 149 (9.14%), 163 (6.32%)

* the characteristic ions of the bis-TMS derivatives (mono-TMS derivative for methylephedrine), monitored in SIM acquisition mode; ions for quantification are bolded;

QUANTIFICATION

Method: internal standard (d3-ephedrine) calibration curves.

In order to prepare a calibration solution, 500 μ l solution cathine 1mg/ml, 1ml solution ephedrine 1mg/ml, 1ml solution methylephedrine 1mg/ml, 2.5ml solution norephedrine 1mg/ml and 2.5ml solution pseudoephedrine 1mg/ml are brought to 10ml with methanol.

The calibration solution therefore obtained contains 50 μ g/ml cathine, 100 μ g/ml ephedrine, 100 μ g/ml methylephedrine, 250 μ g/ml norephedrine and 250 μ g/ml pseudoephedrine.

50-400 μ l calibration solution (corresponding, for a 2.5ml urine sample, to the calibration levels shown in Table2) and 25 μ l solution d3-ephedrine 1mg/ml (corresponding, for a 2.5ml urine sample, to 10 μ g/ml) are evaporated and derivatized with 100 μ l MSTFA; then 1 μ l is injected in the GC/MS system.

Table 2. Calibration levels (μ g/ml urine sample)

Compound	Compound concentrations (μ g/ml) in a sample spiked with the following volume of calibration solution (μ l):							
	50	100	150	200	250	300	350	400
Cathine	1	2	3	4	5*	6	7	8
Methylephedrine	2	4	6	8	10*	12	14	16
Ephedrine	2	4	6	8	10*	12	14	16
Norephedrine	5	10	15	20	25*	30	35	40
Pseudoephedrine	5	10	15	20	25*	30	35	40

* 2004 WADA cut-off limits (for norephedrine and pseudoephedrine, which are, at the present, withdrawn from the prohibited list, are used the 2003 IOC cut-off limits)

RESULTS

All 5 ephedrines were adequately separated by GC/MS as shown in Fig.1 – the retention times are close, but the peaks of the two pairs of diastereoisomers are well delimited. Although the GC/MS spectra of the ephedrines are relatively poor, 3 ions with a relative abundance exceeding 5% have been found for each ephedrine (see table 1), therefore complying with the WADA prerequisite.

The calibration curves are linear within the selected range. The linear correlation coefficients were: 0.993 for methylephedrine, 0.997 for cathine, 0.999 for norephedrine, 0.999 for ephedrine and 0.992 for pseudoephedrine. Examples of calibration curves for ephedrine and cathine are shown in Fig.2 and Fig.3, respectively.

CONCLUSIONS

- The MSTFA-derivatization of ephedrines allowed the chromatographic resolution of the two pairs of diastereoisomers: cathine and norephedrine, ephedrine and pseudoephedrine, in a fast and unsophisticated GC-temperature program.
- Although there are big differences between the aimed concentrations (from 5µg/ml for cathine to 25µg/ml for norephedrine and pseudoephedrine), simultaneous quantification of all the 5 ephedrines was achieved.
- Calibration curves, of linear curve fit, with correlation coefficients between 0.992-0.999, were obtained for the 5 ephedrines that require quantification.

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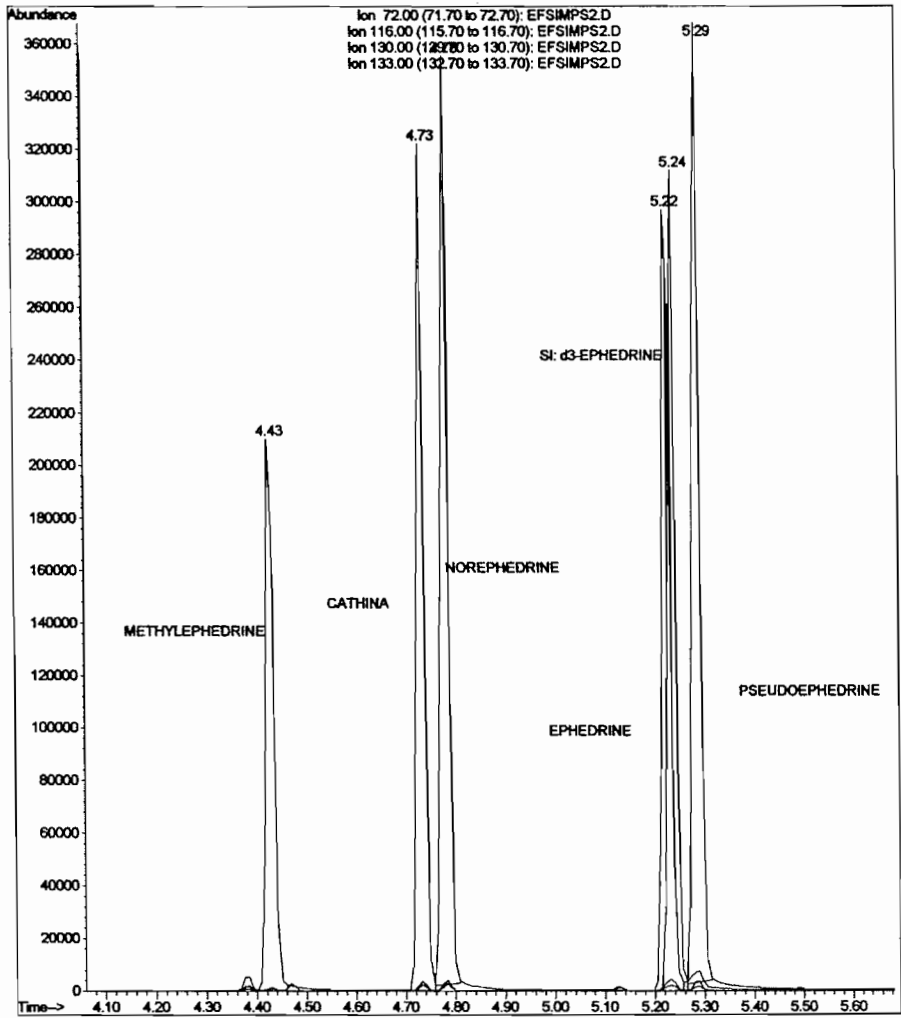
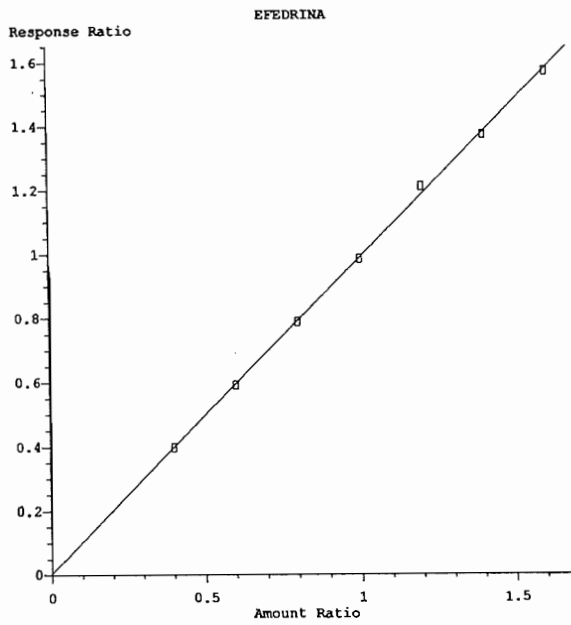
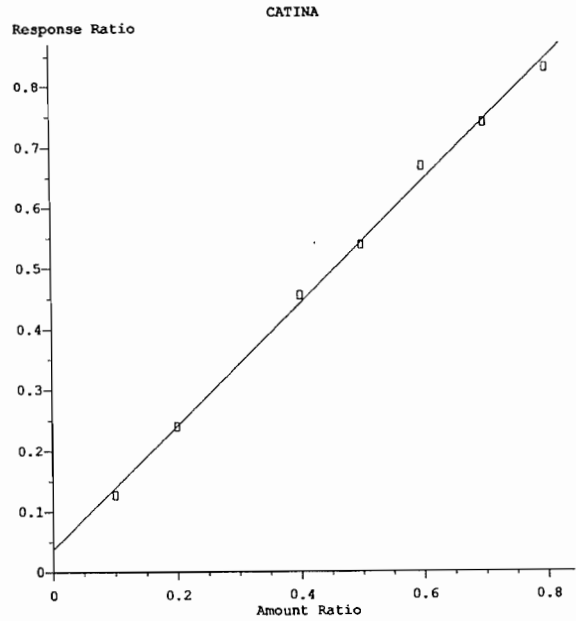


Figure 1. Identification and GC resolution of ephedrine



$$\text{Resp Ratio} = 9.84e-001 * \text{Amt} + 4.84e-003$$
 Coef of Det (r^2) = 0.999 Curve Fit: Linear

Figure 2. Calibration curve for ephedrine



$$\text{Resp Ratio} = 1.01e+000 * \text{Amt} + 3.82e-002$$
 Coef of Det (r^2) = 0.997 Curve Fit: Linear

Figure 3. Calibration curve for cathine