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# Confirmation of amphetamine, methamphetamine, MDA and MDMA in urine samples using disk solid-phase extraction and gas chromatography–mass spectrometry after immunoassay screening☆

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**Abstract**

A method using mixed phase disk solid-phase extraction (SPE) and gas chromatography–mass spectrometry (GC–MS) was developed for confirmation of amphetamine (AMP), methamphetamine (MET), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in urine samples after immunoassay screening. Disk SPE provided hydrophobic ($C_{18}$) and strong cation-exchange (SCX) interactions. The analytes were retained on SCX functional groups in the disk and eluted with ammoniated ethyl acetate after washed with methanol. Confirmation and quantitation was exercised by selected ion monitoring using nikethamide as chromatographic standard. Recoveries of the amphetamines were between 73.0 and 104.6% with RSDs in range of 2.1–6.4% ($n$=3). The limits of detection were 2 ng/ml for AMP, MET and MDMA, and 4 ng/ml for MDA. Five real urine samples were tested with the method after immunoassay screening, and the results were comparable to those of traditional liquid–liquid extraction (LLE). The method was solvent-saved, simple, rapid and reliable, and the extract was cleaner than that of LLE.
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*Keywords:* Disk solid-phase extraction; Amphetamines; Methamphetamine; 3,4-Methylenedioxyamphetamine; 3,4-Methylenedioxymethamphetamine

## 1. Introduction

Amphetamines are powerful central nervous system (CNS) stimulants [1]. Chronic abuse of amphetamines causes hallucinations and psychosis, in addition to dysphoria and depression upon withdrawal [2]. Abuse of amphetamines remains a serious social problem worldwide, and it has steeply increased in the past decade in recreational places in China. Amphetamine (AMP), methamphetamine (MET), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) are often encountered in forensic toxicological analysis. Amphetamines in urine were commonly screened by immunoassay [3], and positive samples were subsequently confirmed by liquid–liquid extraction (LLE) and gas chromatography–mass spectrometry

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(GC–MS). Solid-phase extraction (SPE) has become popular in sample processing and has been applied for analysis of amphetamines [4–11] and other drugs of abuse [12–14]. Recently, disk SPE was available [14–16]. It has many advantages over conventional particle-loaded SPE. Reduced solvent was needed compared to the particle-loaded SPE due to small volume of the disk. Higher flow-rates can be achieved because of lower resistance and faster mass transfer thanks to a smaller particle size in the disk. Moreover there is no risk of channeling with the disk SPE. Owing to these characteristics, high vicious, particle-laden samples and large volume samples can probably be processed without clogging by the disk SPE. Using SPE with strong cation-exchange (SCX) interaction, most of neutral and acidic impurities are washed away in the washing step, therefore, cleaner extract can be obtained. The investigation described here used the SPEC.PLUS.C18AR/MP3 disk SPE cartridges to extract the amphetamines from urine samples. GC–MS working in the selected ion monitoring (SIM) mode was employed for confirmation and quantitation.

## 2. Experimental

Amphetamine standards of reagent grade, amphetamine sulfate, methamphetamine hydrochloride, MDA hydrochloride and MDMA hydrochloride were obtained from National Narcotics Laboratory (Beijing, China). Stock solutions of 1 mg/ml each drug (in free bases) were prepared separately by diluting appropriate amounts of the amphetamine standards (in salt forms), respectively, in proper volumes of methanol. The composite standard containing 100 μg/ml amphetamines (in free bases) was prepared by diluting 0.1 ml of the 1 mg/ml stock solutions in 0.6 ml methanol. Nikethamide (NIK) was used as chromatographic standard (CS), which original solution containing 250 mg/ml NIK was obtained from a local hospital. NIK stock solution of 1 mg/ml was prepared by dissolving 40 μl of the original solution in methanol and diluting to 10 ml. A working solution of 100 μg/ml NIK was prepared by diluting 0.1 ml of the stock solution in 0.9 ml methanol, and 10 μg/ml of working solution was prepared by diluting 0.1 ml of the 100 μg/ml working solution in 0.9 ml methanol. Phosphate buffer (pH 6.0) of 0.1 *M* was prepared by dissolving 15.6 g sodium dihydrogenphosphate ($NaH_2PO_4 \cdot 2H_2O$) in distilled water, diluting to 1000 ml and adjusting to the specific pH with sodium hydrogenphosphate ($Na_2HPO_4 \cdot 12H_2O$). Carbonate buffer (pH 10.3) of 0.1 *M* was prepared by dissolving 10.6 g sodium carbonate ($Na_2CO_3$) and 8.4 g sodium hydrogencarbonate ($NaHCO_3$) in distilled water and diluting to 1000 ml. All chemicals used in this study were of analytical grade. Real urine samples were collected in recreational places by local policemen. Drug-free urine samples were supplied by volunteers.

The GC–MS system consisted of Agilent 6890 GC instrument, an 7683 autosampler and a 5973N mass-selective detector (Palo Alto, CA, USA). The GC system equipped with a 30 m×0.25 mm, 0.25 μm thickness film capillary column DB-35MS (cross-linked 35% phenyl silicone) was from J&W Scientific (Folsom, CA, USA). The oven temperature was started at 100 °C, held for 2 min, then programmed to 200 °C at 20 °C/min, there it was held for 2 min. The GC inlet and GC–MS interface temperatures were 250 and 280 °C, respectively. Helium was used as carrier gas, and its flow-rate was 1.2 ml/min. The split ratio was 50:1. A sample of 1 μl was injected into the GC inlet.

The mass-selective detector was autotuned with perfluorotributylamide. The electron ionization voltage was set at 70 eV. The ion source and quadrupole temperatures were 230 and 150 °C, respectively. The MS system was operated in the SIM mode for quantitation. All compounds were identified by their retention times ($t_R$) and relative abundances of monitored ions, and quantified by comparing the peak area ratios of analytes to CS to those of spiked sample.

Immunoassay screening was achieved according to the manufacturer's instruction [17] with ACON Laboratories one-step drug of abuse test devices for MET with 1 μg/ml cutoff (San Diego, CA, USA).

The mixed-phase SPE disks, SPEC.PLUS.C18AR/MP3 cartridges, with 70 mg bed mass and 10-ml specimen reservoir, providing hydrophobic ($C_{18}$) and SCX interactions, were purchased from Ansys Technologies (Lake Forest, CA, USA). SPE was complemented with a DL-1 SPE manifold purchased from National Chromatographic Technology Center

(Dalian, China), which was incorporated with a YQ02.30 vacuum pump from Changjian Medical Devices (Shanghai, China). The disk was conditioned with 1 ml methanol and 1 ml of 0.1 *M* phosphate buffer (pH 6), sequentially. After dilution with 6 ml of the phosphate buffer, a sample of 2 ml was applied to the cartridge and passed through the cartridge under slight vacuum. The cartridge was washed with 0.5 ml methanol, and dried under full vacuum for 3 min. Residual liquid droplets inside inner wall and tip of the disk cartridge were wiped with clean filter paper. Analytes were eluted with 2 ml of 2% ammoniated ethyl acetate. After 50 μl of 10 μg/ml CS was added, the eluent was evaporated to 0.2 ml with a gentle nitrogen flow at ambient temperature.

LLE was performed for the real urine samples [18]. Real urine sample of 1 ml was mixed with 1 ml of 0.1 *M* carbonate buffer (pH 10.3), and extracted with 2 ml of ethyl acetate three times. Combined extract was evaporated to 0.2 ml by a gentle flow of nitrogen gas at ambient temperature as well.

## 3. Results and discussion

A total ion chromatogram (TIC) of composite standard containing 10 μg/ml of AMP, MET, MDA and MDMA, and 5 μg/ml of CS, as well as mass spectra of the analytes and CS are illustrated in Fig. 1. Qualitative analysis was performed according to retention times ($t_R$) and relative abundance of monitored ions for the compound. The SIM mode was exercised for quantitation. The monitored ions and retention times for the analytes and CS are given in Table 1. Good peak shape and sensitivity were obtained of the analytes as the mid-polar DB-35MS column was employed. A TIC of 0.5 μg/ml spiked urine extracted with the disk SPE is demonstrated in Fig. 2. Though derivatization of amphetamines can somewhat enhance sensitivity and specificity, we adopted non-derivatization for the consideration of simplifying the method.

The pH and ion strength of the sample are very important factors in SPE, especially in ion-exchange SPE. A 6-ml volume of 0.1 *M* phosphate buffer (pH 6) was added to 2 ml of sample to achieve proper pH and ion strength. Inadequate dilution of the sample can cause low and non-reproducible recoveries (experiment observation).

The analytes were charged at pH 6 because the $pK_a$ values of the amphetamines tested are above 9 [19]. The analytes were retained in the disk by the ion-exchange functional groups. Acidic and/or neutral compounds in the matrices of the sample were mostly washed away from the disk, thus a cleaner extract was obtained (Fig. 3). Although the final desorption volume of 2 ml was adopted, good recoveries can be obtained with only 1 ml of 2% ammoniated ethyl acetate (Fig. 4). Compared with traditional SPE, disk SPE required less solvents not only in conditioning and washing column but also in desorption of analytes (1–2 ml vs. 2–6 ml) owing to lower resistance and faster mass transfer of the disk, which allowed to apply a faster flow-rate (>10 ml/min). The time needed to perform the SPE with an extraction disk is less than that needed with a classical SPE column from conditioning through desorption of extracts (8 min vs. 20 min). However, care should be taken for disk SPE to prevent the column from running to dry before sample application; otherwise, air–water interfaces are formed which may affect recovery of analytes.

It should be mentioned that the eluent was evaporated to ca. 0.2 ml with a very gentle nitrogen flow, otherwise some of more volatile drugs such as amphetamine would be lost in evaporation. The time needed for evaporation was about 20 min.

Recently, solid-phase microextraction (SPME) has been used for extraction of amphetamines in biological fluids [20–24]. Although this new technique is simple, solvent-free and easily automated, it has not been widely used for its high cost, worse precision and more critical optimization procedure.

The linearity of the GC–MS system was up to 50 μg/ml of amphetamines composite standards, which is equivalent to 10 μg/ml of analytes in 2 ml urine samples. The concentrations of amphetamines in urine of abusers usually fell into this range. The calibration graph is shown in Fig. 5. Linear regression equations and correlation coefficients are given in Table 2.

The recoveries were obtained by comparing the peak area ratios of spiked urine with those of 5 μg/ml amphetamines composite standard. Three concentrations were tested (0.2, 0.5 and 1.0 μg/ml

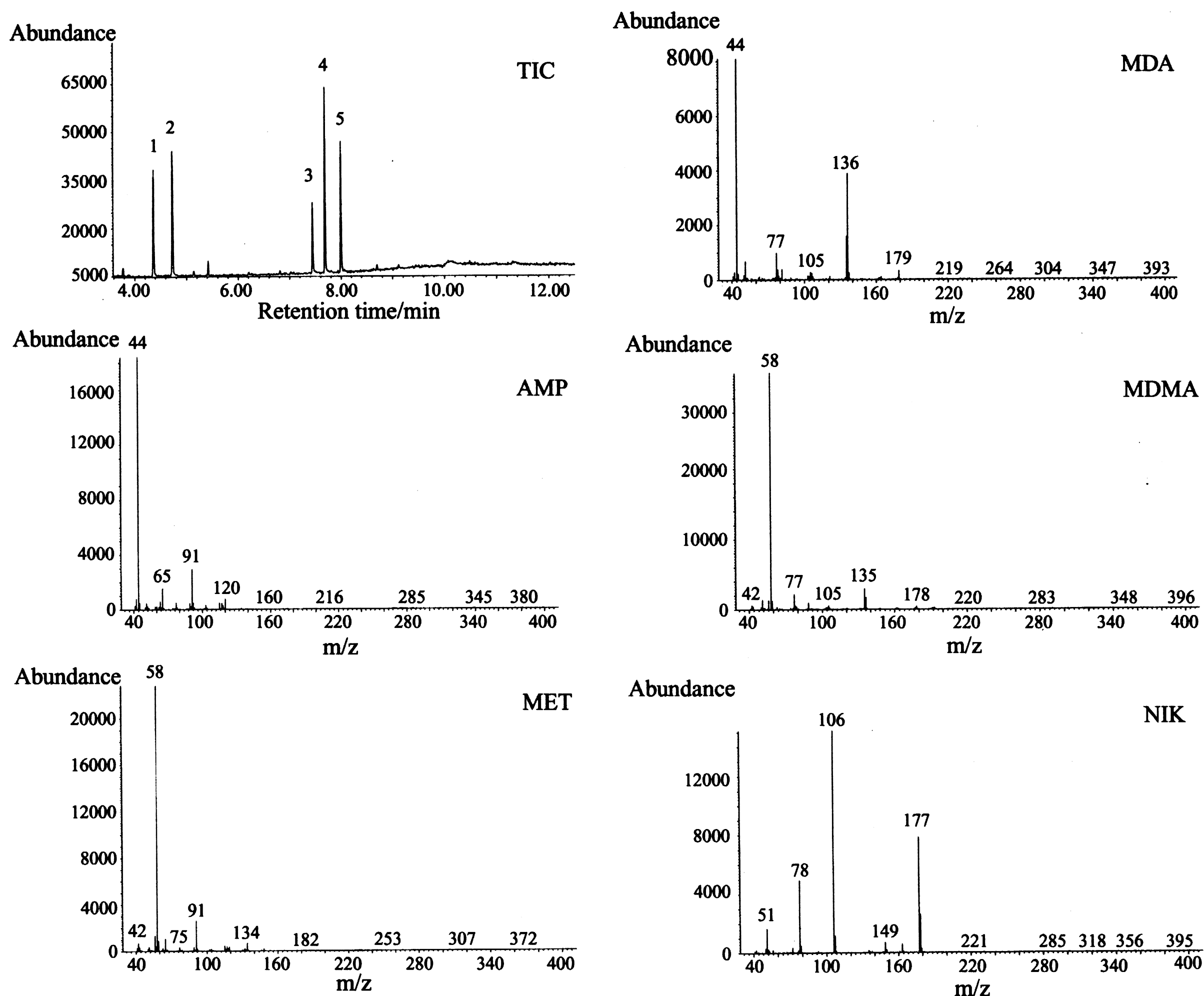



Fig. 1. TIC of amphetamines composite standard of 10 μg/ml added with 0.5 μg/ml NIK (chromatographic standard, CS), and mass spectra of AMP, MET, MDA, MDMA and NIK. Peaks: 1=AMP, retention time ($t_R$) 4.37 min; 2=MET, $t_R$ 4.73 min; 3=MDA, $t_R$ 7.45 min; 4=MDMA, $t_R$ 7.69 min; and 5=NIK, $t_R$ 8.00 min.

Table 1
Retention times ($t_R$) and monitored ions of the amphetamines and chromatographic standard (CS)

| Compound | $t_R$ (min) | Ions[a] |
|---|---|---|
| AMP | 4.37 | **44**, 91 |
| MET | 4.73 | **58**, 91, 134 |
| MDA | 7.45 | **44**, 77, 136 |
| MDMA | 7.69 | **58**, 77, 135 |
| NIK (CS) | 8.00 | 78, **106**, 177 |

[a] Mass-to-charge ratios in bold were for quantitation.

of spiked urine, respectively). Average recoveries of amphetamines in these concentrations for 2 ml of urine were between 73.0 and 104.6%, with RSDs in range of 2.1–6.4% (Table 3).

Limits of detection (LODs, $S/N=3$) and limits of quantitation (LOQs, $S/N=10$) of amphetamines reached the ng/ml level (Table 4). The sensitivity of the method was superior to published results with or without derivatization [8,9]. The LOQs are much lower than routine urine immunoassay cutoffs, i.e., this method was sensitive enough for routine identification.

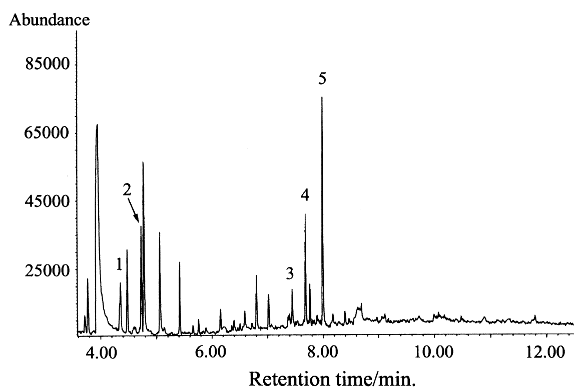
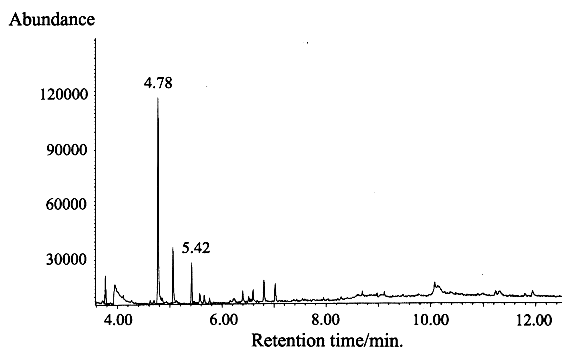



Fig. 2. TIC of spiked sample containing 0.5 μg/ml amphetamines. 0.5 μg NIK was added into the eluent prior to evaporation. Peak identification as in Fig. 1.

Five real urine samples were tested by this method as well as the LLE method. The results are summarized in Table 5. TICs of real urine samples 2 and 5 by the disk SPE are illustrated in Fig. 6. The concentrations of the analytes were calculated by comparing peak area ratios of amphetamines to CS in real sample to those of spiked urine samples in 0.5 μg/ml amphetamines. The results of SPE and LLE agreed well.



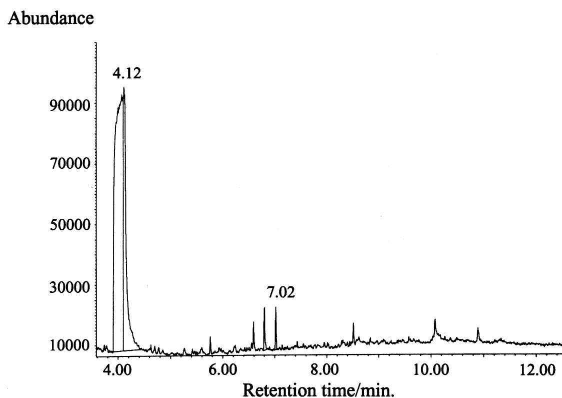




Fig. 3. TICs of blank urine sample by SPE (top) and by LLE (bottom). The numbers shown above some peaks are their retention times.

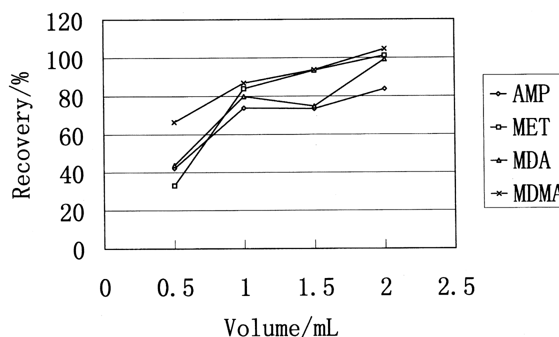



Fig. 4. Recoveries of the amphetamines dependent on the volume of elution solvent.

## 4. Conclusion

Mixed-phase disk SPE and GC–MS in the SIM mode has been developed for confirmation of AMP, MET, MDA and MDMA in urine samples after immunoassay screening. This method was found to be effective and reliable for identification of the amphetamines. Faster flow-rate and less solvent consumption made this novel disk SPE superior to conventional SPE. Good recoveries and cleaner

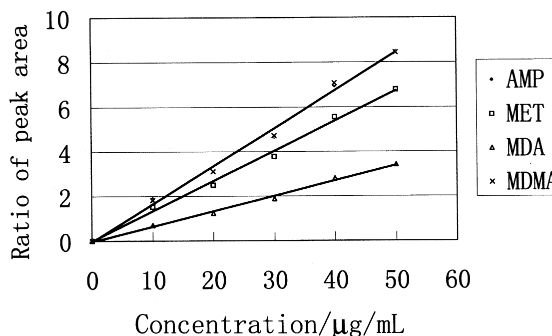



Fig. 5. Calibration curves of amphetamines by SPE–GC–MS.

Table 2
Linear regression equations and correlation coefficients

| Compound | Linear regression equation[a] | Correlation coefficient ($r^2$) |
|---|---|---|
| AMP | $y=0.1691x-0.0456$ | 0.9939 |
| MET | $y=0.1353x-0.0228$ | 0.9948 |
| MDA | $y=0.0689x-0.0510$ | 0.9946 |
| MDMA | $y=0.1702x-0.0609$ | 0.9940 |

[a] $x$, Concentration of analyte; $y$, peak area ratio of analyte to chromatographic standard (CS).

Table 3
Recoveries and relative standard deviations (RSDs) of SPE

| Spiked level (μg/ml) | Recovery (%) | | | | RSD (%, $n=3$) | | | |
|---|---|---|---|---|---|---|---|---|
| | AMP | MET | MDA | MDMA | AMP | MET | MDA | MDMA |
| 0.2 | 73.0 | 85.5 | 73.4 | 79.6 | –[a] | – | – | – |
| 0.5 | 78.7 | 91.1 | 81.2 | 90.4 | 4.9 | 3.1 | 5.1 | 2.2 |
| 1.0 | 83.8 | 101.2 | 99.2 | 104.6 | 2.1 | 6.4 | 5.3 | 5.0 |

[a] –, Not detected.

Table 4
Limits of detection (LODs, $S/N=3$) and limits of quantitation (LOQs, $S/N=10$) (ng/ml)

| | AMP | MET | MDA | MDMA |
|---|---|---|---|---|
| LOD | 2 | 2 | 4 | 2 |
| LOQ | 7 | 7 | 13 | 7 |

extract were obtained with this mixed phase disk. The LODs and LOQs reached the ng/ml level, and LOQs were much less than the cutoffs of routine urine immunoassay. The results of real urine samples by this method were compatible to those of conventional LLE method. Moreover, disk SPE was easy to automate which should be valuable in practical use.

## Acknowledgements

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Table 5
Amphetamine concentrations in real urine samples by disk SPE– and LLE–GC–MS (μg/ml)

| Sample No. | Immunoassay | SPE | | | | LLE | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AMP | MET | MDA | MDMA | AMP | MET | MDA | MDMA |
| 1 | Negative | ND[a] | 0.006 | ND | 0.028 | ND | 0.003 | ND | 0.041 |
| 2 | Positive | 0.140 | 1.962 | 0.061 | 0.015 | 0.071 | 1.805 | 0.018 | 0.002 |
| 3 | Negative | 0.006 | 0.013 | ND | 0.008 | ND | 0.002 | ND | 0.002 |
| 4 | Negative | ND | 0.009 | ND | 0.039 | ND | 0.003 | ND | 0.003 |
| 5 | Positive | 0.482 | 3.765 | 8.405 | 0.019 | 0.333 | 3.277 | 5.042 | 0.007 |

[a] ND: Not detectable.

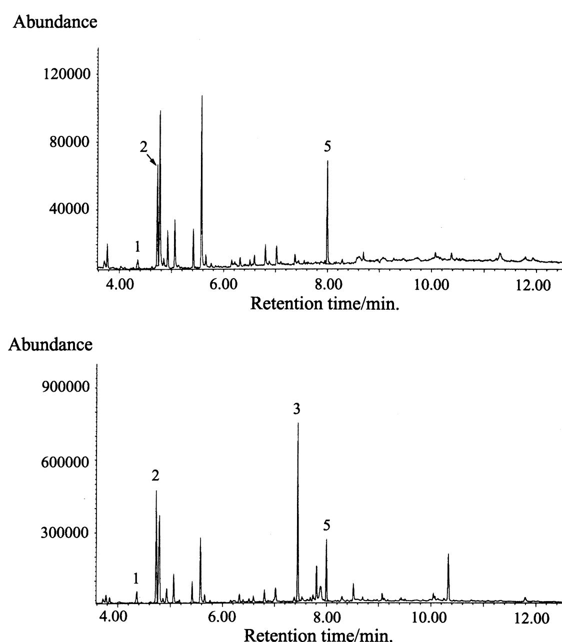


Fig. 6. TICs of real urine samples 2 (top) and 5 (bottom). 0.5 μg NIK (chromatographic standard, CS) was added to the eluents prior to evaporation. Peak identification as in Fig. 1.

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