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# Method of Test for Synthetic Phenethylamines in Urine (1)

## 1. Scope

This method is applicable to the determination of 74 phenethylamines (amphetamine etc. listed as the attached table) in urine.

## 2. Method

After dilution, analytes are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

**2.1.** Equipment

**2.1.1.** Liquid chromatograph/tandem mass spectrometer

**2.1.1.1.** Ion source: electrospray ionization (ESI).

**2.1.1.2.** Column: Kinetex® Phenyl-hexyl, 1.7 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

**2.1.2.** Vortex mixer.

**2.2.** Chemicals

Methanol, HPLC grade;

Formic acid and ammonium acetate, reagent grade;

Artificial urine (UTAK 88121-CDF(L) or an equivalent product), reagent grade;

Deionized water, resistivity ≥ 18 MΩ·cm (at 25℃);

Amphetamine etc. listed in the attached table, reference standards;

Amphetamine-$d_8$ and other isotope-labeled internal standards (listed in the attached table).

**2.3.** Apparatus

**2.3.1.** Volumetric flask: 1 mL and 10 mL.

**2.3.2.** Membrane filter: 0.22 μm, PVDF.

**2.4.** 50% Methanol:

Mix methanol and deionized water at the ratio of 1:1 (v/v).

**2.5.** Mobile phase

**2.5.1.** Solvent A

Dissolve and dilute 0.4 g of ammonium acetate with deionized water to 1000 mL, add 1 mL of formic acid, and filter with a membrane filter.

**2.5.2.** Solvent B:

Dilute 1 mL of formic acid with methanol to 1000 mL, and filter with a membrane filter.

**2.6.** Internal standard solution preparation

Transfer 1 mg of internal standards accurately weighed into each 10-mL

volumetric flask, dissolve and dilute with methanol to volume as the internal standard stock solutions. Store at -20℃ in the dark. Prior to use, mix appropriate volume of the internal standard stock solutions, and dilute with 50% methanol to 500 ng/mL as the internal standard solution.

**2.7.** Standard solution preparation

Transfer 1 mg of reference standards accurately weighed into each 10-mL volumetric flask, dissolve and dilute with methanol to volume as the standard stock solution. Store at -20℃ in the dark. Prior to use, mix appropriate volume of the standard stock solutions, and dilute with 50% methanol to 500 ng/mL as the standard solution.

**2.8.** Sample solution preparation

Transfer 20 µL of the homogenized sample and 20 µL of the internal standard solution into a 1-mL volumetric flask, and dilute to volume with 50% methanol. Filter with a membrane filter, and take the filtrate as the sample solution.

**2.9.** Calibration curve

Use the artificial urine as the blank sample. Separately take 20 µL of the artificial urine, add 2-100 µL of the standard solution and 20 µL of the internal standard solution, dilute with 50% methanol to 1 mL, and filter with a membrane filter. Take the filtrates as the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration curve of each phenethylamine by the ratios of the peak area of each phenethylamine to that of the respective internal standard vs. the added concentrations (1-50 ng/mL).

LC-MS/MS operating conditions(note):

Column: Kinetex® Phenyl-hexyl, 1.7 µm, 2.1 mm i.d. × 10 cm.

Column temperature: 40℃.

Injection volume: 3 µL.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0.0 → 0.5 | 95 → 95 | 5 → 5 |
| 0.5 → 1.0 | 95 → 70 | 5 → 30 |
| 1.0 → 1.5 | 70 → 70 | 30 → 30 |
| 1.5 → 2.0 | 70 → 63 | 30 → 37 |
| 2.0 → 2.5 | 63 → 63 | 37 → 37 |
| 2.5 → 2.6 | 63 → 60 | 37 → 40 |

| | | |
|---|---|---|
| 2.6 → 3.0 | 60 → 60 | 40 → 40 |
| 3.0 → 5.0 | 60 → 54 | 40 → 46 |
| 5.0 → 5.5 | 54 → 54 | 46 → 46 |
| 5.5 → 8.0 | 54 → 50 | 46 → 50 |
| 8.0 → 9.5 | 50 → 35 | 50 → 65 |
| 9.5 → 10.5 | 35 → 0 | 65 → 100 |
| 10.5 → 11.0 | 0 → 0 | 100 → 100 |
| 11.0 → 11.1 | 0 → 95 | 100 → 5 |
| 11.1 → 14.0 | 95 → 95 | 5 → 5 |

Flow rate: 0.3 mL/min.

Ion spray voltage: 5.5 kV.

Ionization mode: $ESI^+$.

Turbo heater temperature: 550℃.

Nebulizer gas, GS1: 50 psi.

Heated gas, GS2, 60 psi.

Curtain gas: 30 psi.

Collision gas: Medium.

Detection mode: multiple reaction monitoring (MRM). Detection of ion pair, declustering potential and collision energy are shown in the attached table.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

**2.10.** Identification and quantification

Accurately inject 3 μL of the sample solution and the calibration standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.9. Identify each phenethylamine based on the retention time and the relative ion intensities(note). Calculate the amount of each phenethylamine in the sample by the following formula:

$$\text{The amount of each phenethylamine in the sample (ng/mL)} = \frac{C \times V}{M}$$

Where,

C: the concentration of each phenethylamine in the sample solution calculated by the calibration curve (ng/mL)

V: the final make-up volume of the sample (mL)

M: the volume of the sample (mL)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities are as the following:

| Relative ion intensity (%) | Tolerance (%) |
|---|---|
| > 50 | ± 20 |
| > 20～50 | ± 25 |
| > 10～20 | ± 30 |
| ≤ 10 | ± 50 |

**Remark**

1. Limit of quantification (LOQ) for each phenethylamine is 50 ng/mL.
2. Further validation should be performed when interference compounds appear in the samples.

**Reference**

Dong, Y., Yan, K., Ma, Y., Wang, S., He, G., Deng, J. and Yang, Z. 2015. A sensitive dilute-and-shoot approach for the simultaneous screening of 71 stimulants and 7 metabolites in human urine by LC–MS-MS with dynamic MRM. J. Chromatogr. Sci. 53: 1528-1535.

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## Reference chromatogram

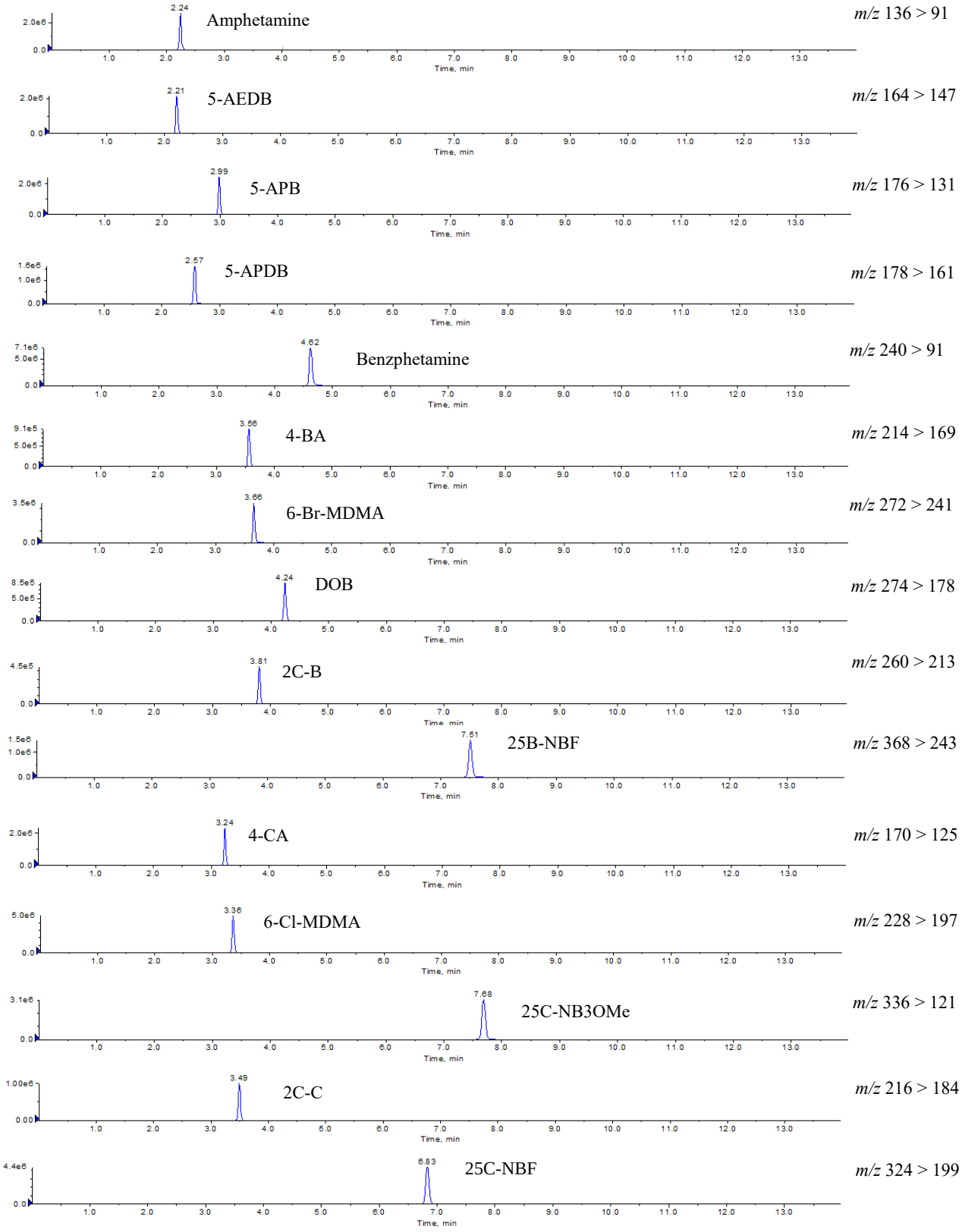


Figure. The MRM chromatograms of 74 phenethylamines and 10 isotope-labeled internal standards analyzed by LC-MS/MS.

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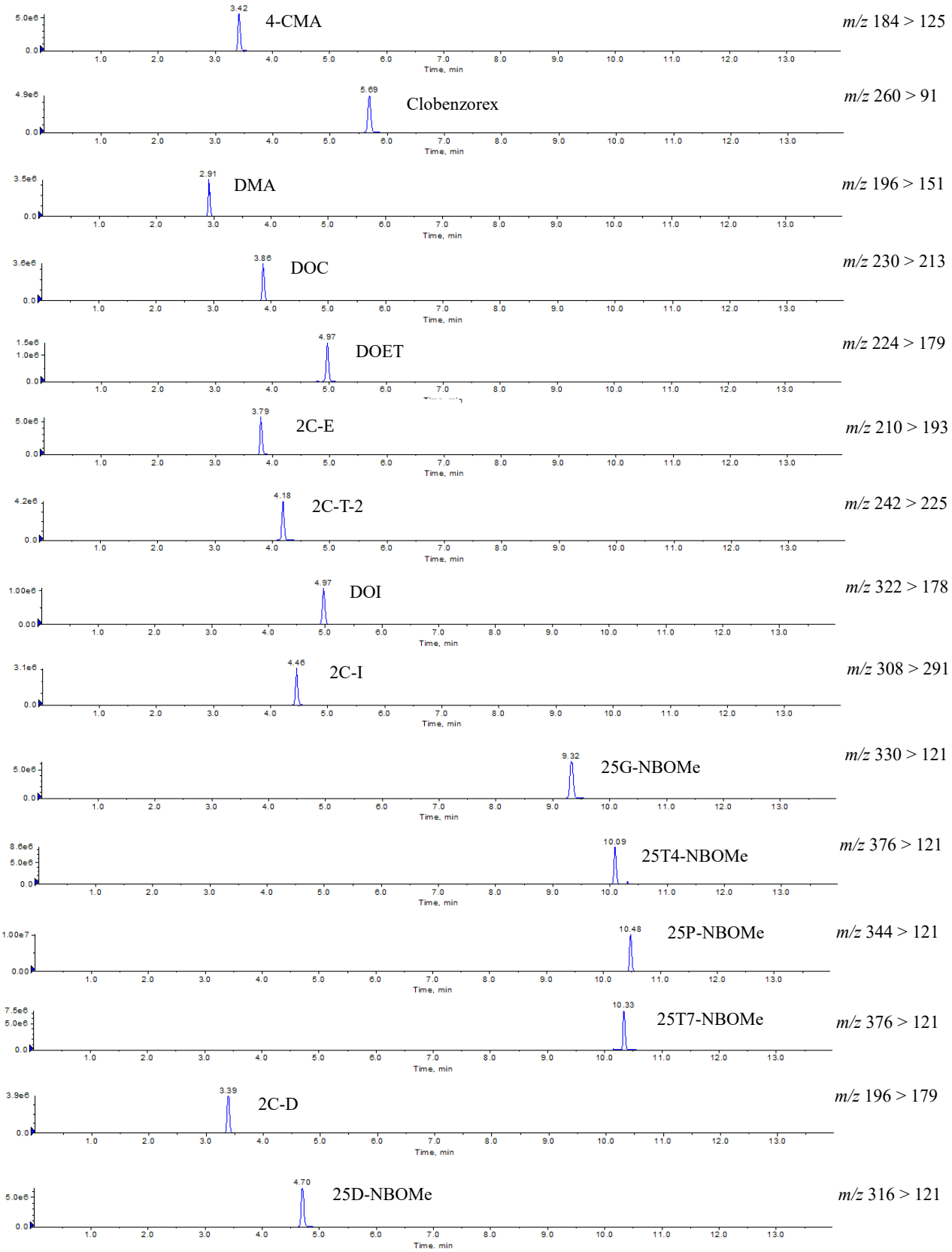


Figure. The MRM chromatograms of 74 phenethylamines and 10 isotope-labeled internal standards analyzed by LC-MS/MS (continued).

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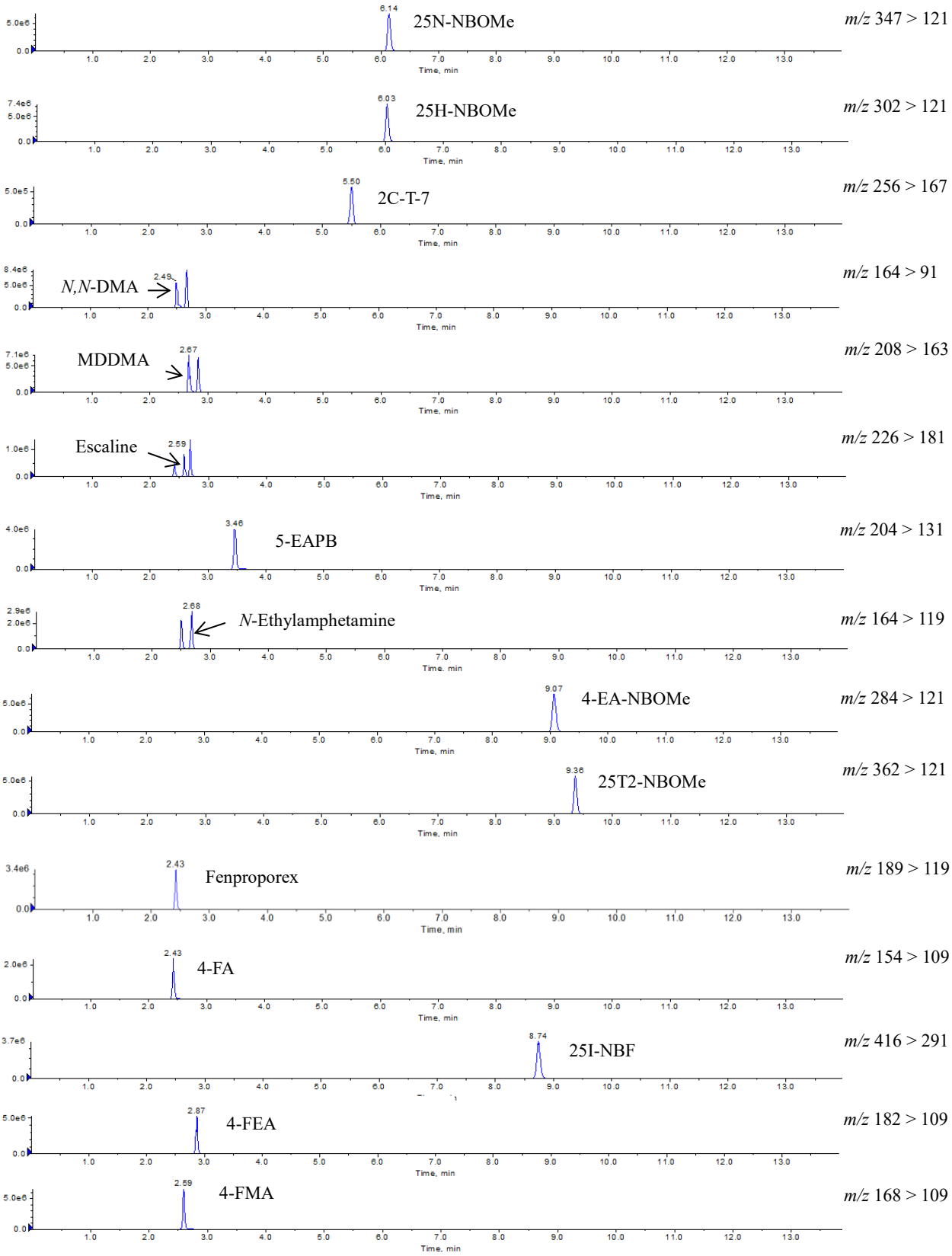


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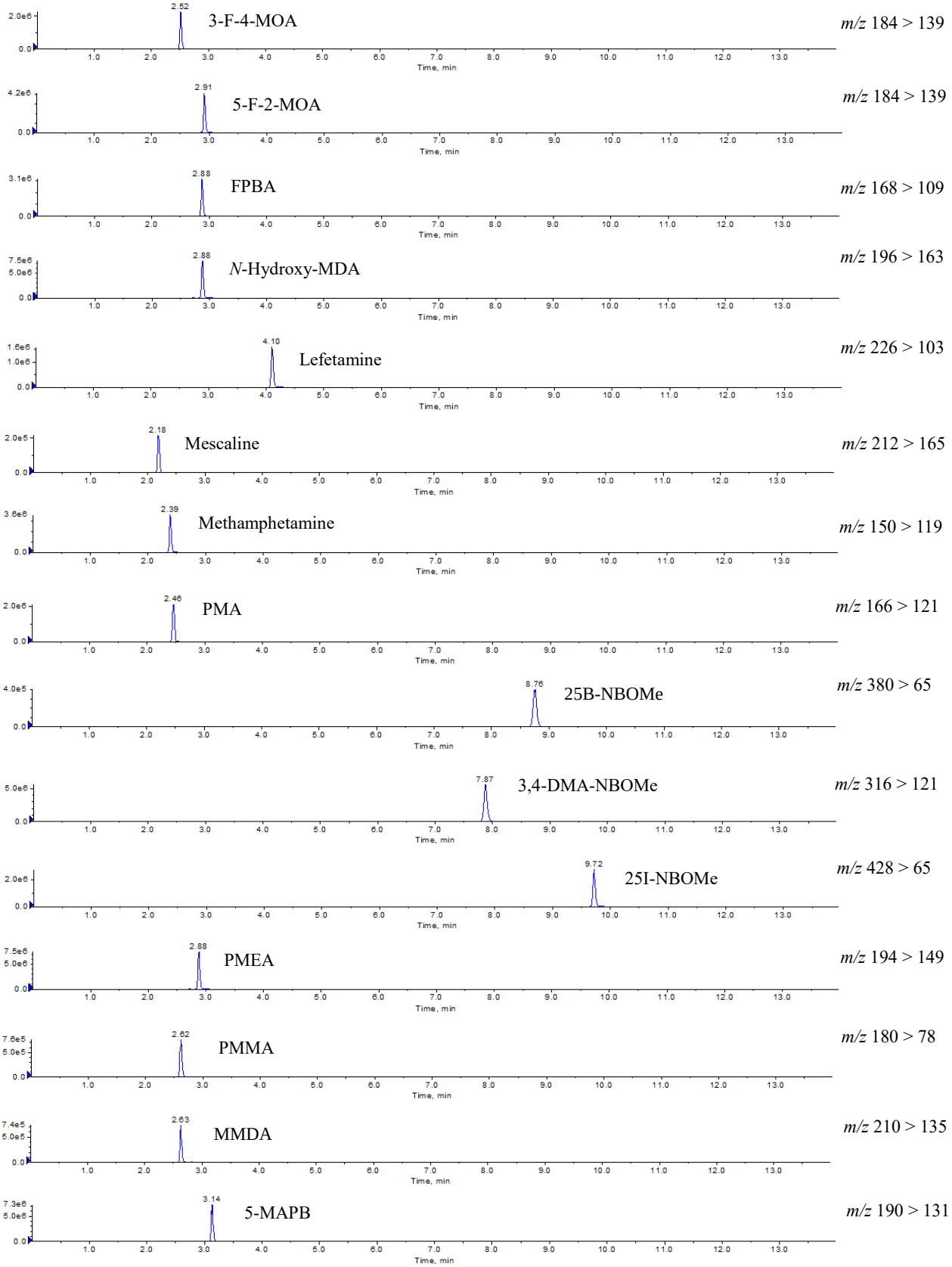


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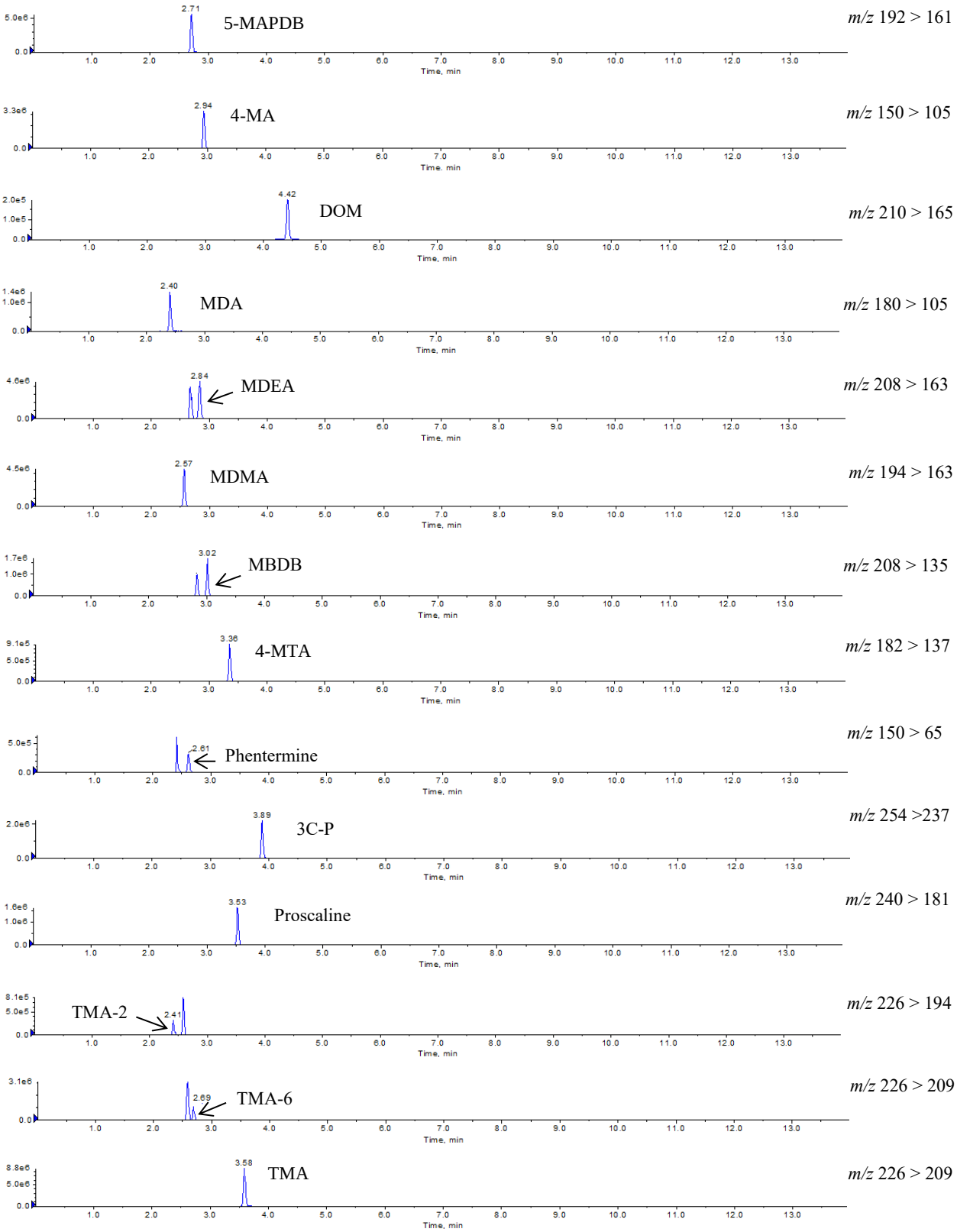


Figure. The MRM chromatograms of 74 phenethylamines and 10 isotope-labeled internal standards analyzed by LC-MS/MS (continued).

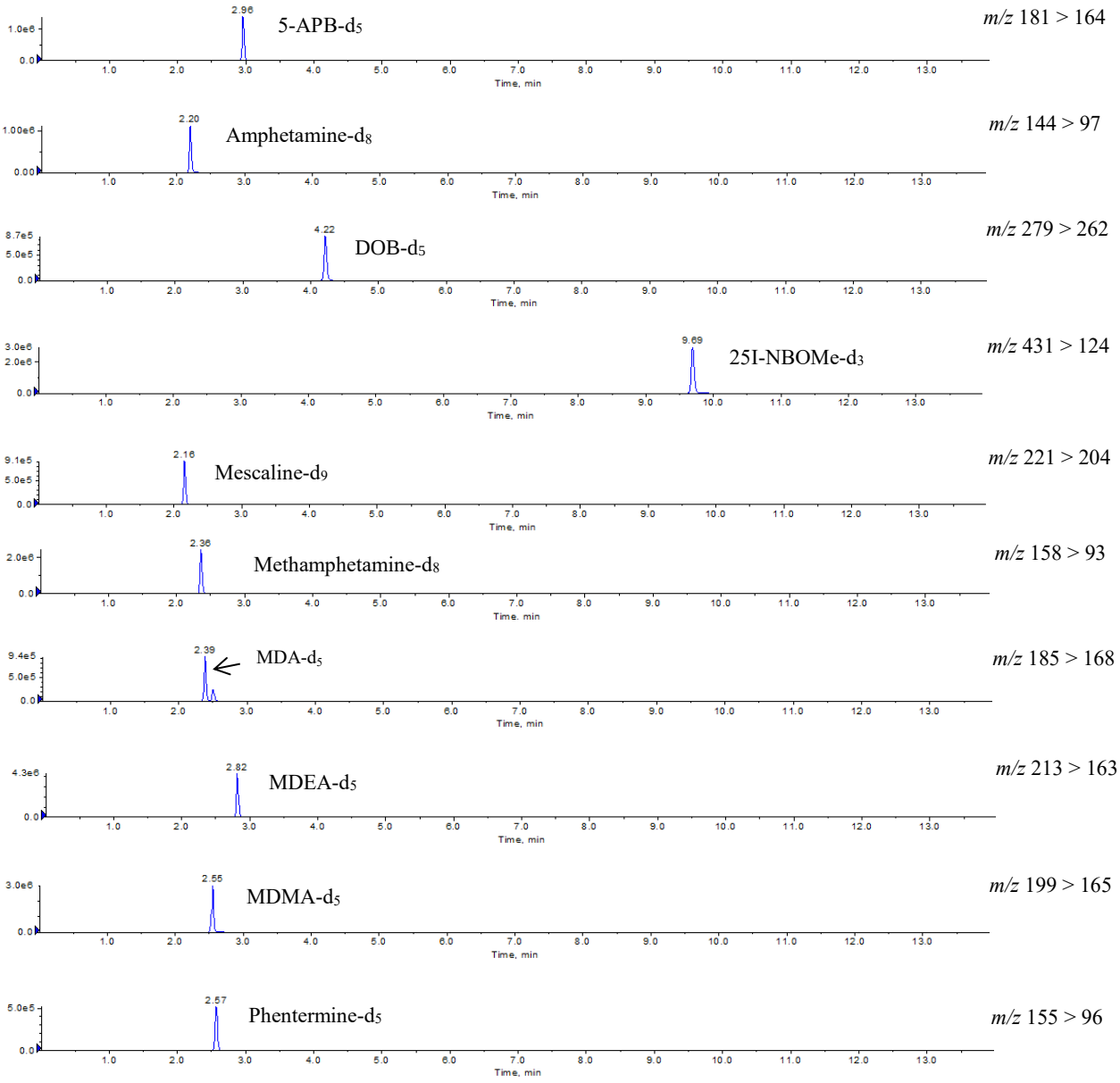


Figure. The MRM chromatograms of 74 phenethylamines and 10 isotope-labeled internal standards analyzed by LC-MS/MS (continued).

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Table. MRM parameters of 74 phenethylamines and 10 isotope-labeled internal standards

| Analyte | Ion pair: Precursor ion(*m/z*) >product ion (*m/z*) | Declustering potential (V) | Collision energy (eV) | Internal standard |
|---|---|---|---|---|
| 2-(2,5-Dimethoxy-4-nitrophenyl)-*N*-(2-methoxybenzyl)ethanamine (25N-NBOMe) | 347 > 121* | 30 | 23 | DOB-$d_5$ |
| | 347 > 91 | 30 | 59 | |
| 2-(2,5-Dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine (25H-NBOMe) | 302 > 121* | 33 | 23 | DOB-$d_5$ |
| | 302 > 91 | 33 | 51 | |
| 2,5-Dimethoxy-4-n-propylthiophenethylamine (2C-T-7) | 256 > 167* | 67 | 35 | DOB-$d_5$ |
| | 256 > 224 | 67 | 24 | |
| *N,N*-Dimethylamphetamine (*N,N*-DMA) | 164 > 91* | 57 | 30 | MDA-$d_5$ |
| | 164 > 119 | 57 | 18 | |
| *N,N*-Dimethyl-3,4-methylenedioxyamphetamine (MDDMA) | 208 > 163* | 21 | 19 | MDMA-$d_5$ |
| | 208 > 135 | 21 | 29 | |
| Escaline | 226 > 181* | 60 | 22 | Mescaline-$d_9$ |
| | 226 > 91 | 60 | 44 | |
| 5-(2-Ethylaminopropyl)benzofuran (5-EAPB) | 204 > 131* | 34 | 29 | 5-APB-$d_5$ |
| | 204 > 159 | 34 | 9 | |
| *N*-Ethylamphetamine | 164 > 119* | 68 | 16 | MDA-$d_5$ |
| | 164 > 65 | 68 | 56 | |
| 4-Ethyl-*N*-(o-methoxybenzyl)amphetamine (4-EA-NBOMe) | 284 > 121* | 44 | 24 | 25I-NBOMe-$d_3$ |
| | 284 > 91 | 44 | 59 | |
| 4-(Ethylthio)-2,5-dimethoxy-*N*-[(2-methoxy-phenyl)methyl]benzeneethanamine (25T2-NBOMe) | 362 > 121* | 23 | 27 | 25I-NBOMe-$d_3$ |
| | 362 > 91 | 23 | 65 | |
| Fenproporex | 189 > 119* | 40 | 15 | Amphetamine-$d_8$ |
| | 189 > 65 | 40 | 59 | |
| 4-Fluoroamphetamine (4-FA) | 154 > 109* | 40 | 28 | Amphetamine-$d_8$ |
| | 154 > 137 | 40 | 15 | |
| *N*-(2-Fluorobenzyl)-4-iodo-2,5-dimethoxyphenethylamine (25I-NBF) | 416 > 291* | 47 | 25 | 25I-NBOMe-$d_3$ |
| | 416 > 276 | 47 | 39 | |
| 4-Fluoroethamphetamine (4-FEA) | 182 > 109* | 65 | 31 | MDEA-$d_5$ |
| | 182 > 137 | 65 | 17 | |
| 4-Fluoromethamphetamine (4-FMA) | 168 > 109* | 50 | 30 | Amphetamine-$d_8$ |
| | 168 > 83 | 50 | 52 | |

* Quantitative ion pair.

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Table. MRM parameters of 74 phenethylamines and 10 isotope-labeled internal standards (continued)

| Analyte | Ion pair: Precursor ion(*m/z*) >product ion (*m/z*) | Declustering potential (V) | Collision energy (eV) | Internal standard |
|---|---|---|---|---|
| 3-Fluoro-4-methoxyamphetamine (3-F-4-MOA) | 184 > 139* | 36 | 24 | Amphetamine-$d_8$ |
| | 184 > 167 | 36 | 35 | |
| 5-Fluoro-2-methoxyamphetamine (5-F-2-MOA) | 184 > 139* | 40 | 25 | Amphetamine-$d_8$ |
| | 184 > 109 | 40 | 14 | |
| 1-(4-Fluorophenyl)butan-2-amine (FPBA) | 168 > 109* | 47 | 28 | 5-APB-$d_5$ |
| | 168 > 83 | 47 | 51 | |
| *N*-Hydroxy-3,4- methylenedioxyamphetamine (*N*-Hydroxy-MDA) | 196 > 163* | 34 | 15 | Amphetamine-$d_8$ |
| | 196 > 135 | 34 | 28 | |
| Lefetamine | 226 > 103* | 61 | 41 | DOB-$d_5$ |
| | 226 > 165 | 61 | 38 | |
| Mescaline | 212 > 165* | 40 | 30 | Mescaline-$d_9$ |
| | 212 > 180 | 40 | 24 | |
| Methamphetamine | 150 > 119* | 40 | 14.5 | Methamphetamine-$d_8$ |
| | 150 > 65 | 40 | 55.5 | |
| 4-Methoxyamphetamine (PMA) | 166 > 121* | 39 | 25 | Amphetamine-$d_8$ |
| | 166 > 91 | 39 | 42 | |
| *N*-(2-Methoxybenzyl)-4-bromo-2,5-dimethoxyphenethylamine (25B-NBOMe) | 380 > 65* | 72 | 105 | 25I-NBOMe-$d_3$ |
| | 380 > 93 | 72 | 44 | |
| *N*-(o-Methoxybenzyl)-3,4-dimethoxyamphetamine (3,4-DMA-NBOMe) | 316 > 121* | 17 | 24 | 25I-NBOMe-$d_3$ |
| | 316 > 91 | 17 | 55 | |
| *N*-(2-Methoxybenzyl)-4-iodo-2,5-dimethoxyphenethylamine (25I-NBOMe) | 428 > 65* | 117 | 113 | 25I-NBOMe-$d_3$ |
| | 428 > 272 | 117 | 23 | |
| Methoxyethylamphetamine (PMEA) | 194 > 149* | 42 | 18 | Methamphetamine-$d_8$ |
| | 194 > 121 | 42 | 27 | |
| Methoxymethamphetamine (PMMA) | 180 > 78* | 33 | 55 | Amphetamine-$d_8$ |
| | 180 > 65 | 33 | 62 | |
| 5-Methoxy-3,4-methylenedioxyamphetamine (MMDA) | 210 > 135* | 51 | 27 | Amphetamine-$d_8$ |
| | 210 > 165 | 51 | 26 | |
| 5-(2-Methylaminopropyl)benzofuran (5-MAPB) | 190 > 131* | 27 | 26 | 5-APB-$d_5$ |
| | 190 > 159 | 27 | 16 | |

* Quantitative ion pair.

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Table. MRM parameters of 74 phenethylamines and 10 isotope-labeled internal standards (continued)

| Analyte | Ion pair<br>Precursor ion(*m/z*)<br>>product ion (*m/z*) | Declustering potential (V) | Collision energy (eV) | Internal standard |
|---|---|---|---|---|
| 5-(2-Methylaminopropyl)-2,3-dihydrobenzofuran (5-MAPDB) | 192 > 161* | 15 | 19 | 5-APB-$d_5$ |
| | 192 > 133 | 15 | 33 | |
| 4-Methylamphetamine (4-MA) | 150 > 105* | 42 | 26 | Amphetamine-$d_8$ |
| | 150 > 133 | 42 | 12 | |
| 4-Methyl-2,5-dimethoxyamphetamine (DOM) | 210 > 165* | 63 | 24 | DOB-$d_5$ |
| | 210 > 178 | 63 | 25 | |
| 3,4-Methylenedioxyamphetamine (MDA) | 180 > 105* | 51 | 27 | MDA-$d_5$ |
| | 180 > 135 | 51 | 25 | |
| 3,4-Methylenedioxy-*N*-ethylamphetamine (MDEA) | 208 > 163* | 57 | 17 | MDEA-$d_5$ |
| | 208 > 135 | 57 | 30 | |
| 3,4-Methylenedioxymethamphetamine (MDMA) | 194 > 163* | 54 | 16 | MDMA-$d_5$ |
| | 194 > 105 | 54 | 36 | |
| *N*-Methyl-α-ethyl-3,4-methylenedioxyphenethylamine (MBDB) | 208 > 135* | 60 | 25 | 5-APB-$d_5$ |
| | 208 > 51 | 60 | 83 | |
| 4-Methylthioamphetamine (4-MTA) | 182 > 137* | 44 | 24 | DOB-$d_5$ |
| | 182 > 115 | 44 | 50 | |
| Phentermine | 150 > 65* | 38 | 52 | Phentermine-$d_5$ |
| | 150 > 133 | 38 | 14 | |
| 4-Propoxy-3,5-dimethoxyamphetamin (3C-P) | 254 > 237* | 54 | 12 | DOB-$d_5$ |
| | 254 > 107 | 54 | 38 | |
| Proscaline | 240 > 181* | 23 | 21 | DOB-$d_5$ |
| | 240 > 121 | 23 | 33 | |
| 2,4,5-Trimethoxyamphetamine (TMA-2) | 226 > 194* | 47 | 28 | 25I-NBOMe-$d_3$ |
| | 226 > 179 | 47 | 34 | |
| 2,4,6-Trimethoxyamphetamine (TMA-6) | 226 > 209* | 72 | 16 | 25I-NBOMe-$d_3$ |
| | 226 > 181 | 72 | 28 | |
| 3,4,5-Trimethoxyamphetamine (TMA) | 226 > 209* | 64 | 14 | 25I-NBOMe-$d_3$ |
| | 226 > 181 | 64 | 25 | |

* Quantitative ion pair.

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Table. MRM parameters of 74 phenethylamines and 10 isotope-labeled internal standards (continued)

| Analyte | Ion pair<br>Precursor ion(*m/z*)<br>>product ion (*m/z*) | Declustering potential (V) | Collision energy (eV) | Internal standard |
|---|---|---|---|---|
| 5-(2-Aminopropyl)benzofuran-$d_5$ (5-APB-$d_5$) (I.S.) | 181 > 164 | 27 | 12 | - |
| Amphetamine-$d_8$ (I.S.) | 144 > 97 | 50 | 24 | - |
| 4-Bromo-2,5-dimethoxyamphetamine-$d_5$ (DOB-$d_5$) (I.S.) | 279 > 262 | 67 | 16 | - |
| 4-Iodo-2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl] benzeneethanamine-$d_3$ (25I-NBOMe-$d_3$) (I.S.) | 431 > 124 | 26 | 26 | - |
| Mescaline-$d_9$ (I.S.) | 221 > 204 | 59 | 16 | - |
| Methamphetamine-$d_8$ (I.S.) | 158 > 93 | 36 | 33 | - |
| 3,4-Methylenedioxyamphetamine-$d_5$ (MDA-$d_5$) (I.S.) | 185 > 168 | 58 | 14 | - |
| 3,4-Methylenedioxy-*N*-ethylamphetamine-$d_5$ (MDEA-$d_5$) (I.S.) | 213 > 163 | 61 | 18 | - |
| 3,4-Methylenedioxymethamphetamine-$d_5$ (MDMA-$d_5$) (I.S.) | 199 > 165 | 22 | 19 | - |
| Phentermine-$d_5$ (I.S.) | 155 > 96 | 42 | 32 | - |