Analytical Methods

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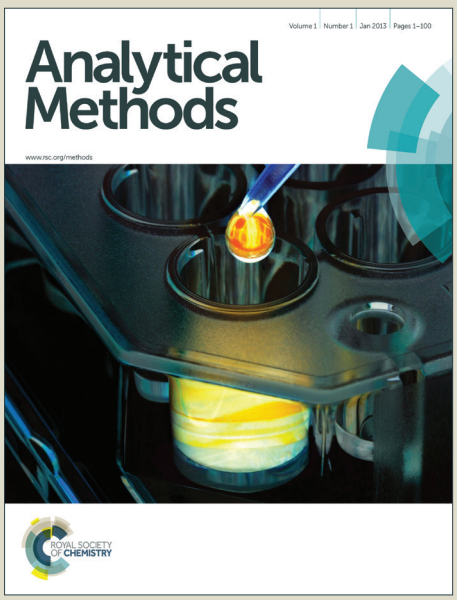

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Identification of metabolites of AHTN in rat urine and feces by liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry

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Abstract

6-Acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) is a kind of widely used polycyclic musk with the raising environmental and health concerns during the last decade, but the metabolites of AHTN in mode animal such as rat are unknown. In this study, major metabolites of AHTN in rat urine and feces were identified by ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC/ESI-QTOF-MS). Three hydroxylation metabolites and three carboxyl metabolites were found in urine and feces.

Keywords

AHTN; UPLC-QTOF-MS; metabolites

1. Introduction

6-Acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) is widely used as a fragrant ingredient in commercial household products such as perfumes, cosmetics, household and laundry cleaning products and air fresheners at a level of several percent.[1-3] This compound has been found ubiquitously in the environment[4-11] and can accumulate in animal and human tissues[12-21] because of their lipophilic nature. Due to the accumulation effect of AHTN, more and more concerns were paid on its toxicity.[22-24] Early in 1999, Seinen et al. demonstrated that AHTN has weak estrogenic activity and promiscuity of estrogen receptor because it induced a slight but dose-dependent stimulation of transcriptional activity in the transiently ERα transfected HEK293 cells.[25] Additionally, Api et al. observed that a statistically significant decrease in body weight and statistically significant increase in serum ALP and ALT levels over control group although there was no significant adverse effects and hormonal effects, in experiment of the oral subchronic toxicity of AHTN in the rat.[3] Although much of the toxicity of AHTN from gene to cell and *in vitro* had been reported, to the best of our knowledge, there were scarce in any care for metabolism research of AHTN *in vivo*, whereas, the metabolic fate of compound is commonly related to its bioaccumulation and toxicity, and some metabolites might be more toxic than the pro-drug. Therefore, it is necessary to perform *in vivo* study to investigate the major metabolic profiles and identify the possible metabolites for toxicological assessment of AHTN.

Currently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has

become the prevalent choice for drug metabolite identification because of its sensitivity and ability to analyze complex mixtures.[26] In recent years, with the resolution improvement in chromatography and mass spectrometer, such as the using ultra-high-performance LC coupled with time-of-flight MS, metabolite peaks can be resolved and identified from complex background matrix ions by accurate mass data and excellent chromatographic separation.[27] New data-processing techniques, such as mass defect filtering (MDF), can be used to remove the majority of matrix-related background ions and reduce the number of false-positives, providing a more confident result.[28] Additionally, the use of MS^E acquisition technique can provide intact and fragmental ion information of pro-drugs and metabolites in a single sample injection,[28] which enhance the identifying capability of untargeted metabolites.

In this paper, we describe the use of MS^E acquisition coupled with mass defect filtering technique to identify major metabolites of AHTN in rat urine and feces after UPLC chromatographic separation. This is also the first study on the metabolites of AHTN in rat. The results will be helpful for further assessment of the comprehensive toxic effects of AHTN on ecological and human health.

2. Experimental

2.1 Chemicals and reagents

HPLC grade of acetonitrile used for sample preparation was supplied by Dikma (Lake Forest, USA). Acetonitrile and water containing 0.1% formic acid used for mobile phases were LC-MS grade, purchased from Fluka (LC-MS CHROMASOLV®,

Sigma-Aldrich, Co., USA). Water was purified with a Milli-Q deionization system (>18.2MV) (Millipore simplicity, Millipore, France). AHTN was kindly donated by Dr. Berset (Water and Soil Protection Laboratory, Switzerland).

2.2 Drug administration

Male SD rats at 6-7 week age, 200-250 g body weight (bw) were purchased from experimental animal center of Academy of Military Medical Sciences, China. Before use for experiment, the rats were housed for 7 days in barrier facility for laboratory animal under the environmentally room (temperature 25±2ºC, humidity 60±5%, 12/12h light/dark cycle) and allow free access to food (soy free custom diet) and water. Then the rats (*n*=3) were orally administered a single dose of AHTN (300 mg/kg·bw with corn oil as Vehicle). Control group (*n*=3) were administrated a similar volume of corn oil. All procedures were approved by the animal care committee of Beijing Center for Disease Control and Prevention. After administration, the rats were placed in metabolic pans and the urine was collected each day for consecutive four days.

2.3 Sample preparation

After administration, the urine and feces were collected each day for consecutive four days. The feces were ground after freeze drying. Aliquots 0.1 g grounded feces were extracted with 1.0 ml acetonitrile by vortex for 1 min. The mixture was then ultra-sonicated for 15 min in ice bath and centrifuged at 10,000 rpm for 10 min at 4ºC. Urine was centrifuged at 10,000 rpm for 10 min at 4ºC, directly. The supernatant was

collected for LC-QTOF-MS analysis.

2.4 UPLC-QTOF-MS conditions

Liquid chromatographic separations were carried out using a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) with a BEH C18 column (2.1 mm ×100 mm, 1.7 μm) from Waters Co. (Milford, MA, USA). The column oven temperature was set at 40ºC, and the injection volume was 2 μL, and the flow was 0.3 ml/min. The mobile phase were A (water containing 0.1% formic acid) and B (acetonitrile). The gradient elution was began with 3% B, held for 2 min, then raised to 100% B at 14 min, and kept for 1 min, finally back to 3% B at 15.5 min for equivalent.

The MS^E analysis was performed on a Waters Synapt™ Q-TOF Mass spectrometer (Waters Corp., Milford, MA, USA) under positive electrospray ionization condition in V-mode. The capillary voltage, sampling cone and extraction cone voltage were set at 3.1 kV, 35 V and 4 V, respectively. The source and desolvation temperatures were held at 110ºC and 400ºC, respectively. The cone gas and desolvation gas using nitrogen were set at 50 L/h and 800 L/h, respectively. Two functions were run for data acquisition in centroid mode with *m*/*z* 50-1000. For MS scan, the trap collision energy and transfer collision energy were set 6.0 V and 4.0 V, respectively. In the MS^E scan of function 2, the trap collision energy was 10-35 V with energy ramp. External reference solution containing 1 mg/L of leucine enkephalin was used for mass lock.

2.5 Data analysis

The MS^E data were processed in Metabolynx (Waters Incor. Milford, MA, USA) using MDF and dealkylation tool to identify possible metabolite. The processing parameters were as follows: MS traces condition, retention time range from 0 to 12 min; expected metabolites chromatograms, mass values were calculated from parent compound and expected metabolites; unexpected metabolites chromatograms were created from mass range chromatograms with full acquisition range from 50 to 600 Da. False positive condition, the isotope entries with mass window 5 Da, was not reported; mass window was 1 Da and retention time (RT) window was 0.1 min matching with control sample, and the false positives could be not reported in the browser.

In this study, the software of Metabolynx was used to find the metabolites of AHTN in urine and feces. If a chemical formula of parent drug was been given, the chemical formula of all possible metabolites could be calculated according to all known possible pathways by Metabolynx software. And then, the software could automatically match these calculated formula to all mass information detected in sample by Tof-MS. If the detected molecular weight was same or similar as calculated molecular weight, the software will regard as a possible metabolite and record it. In this study, the chemical formula of AHTN as the parent drug was added into Metabolynx software, and all possible metabolic pathways were chosen. All matched records were listed. After cancelling all unreasonable results and results with big error, six metabolites (M1-M6) were picked out from the background matrix of urine and feces.

2.6 UPLC-MS/MS conditions

For UPLC-MS/MS Analysis, the condition of liquid chromatographic was the same to UPLC-QTOF analysis. Mass spectrometry was performed on a Micromass-Quattro Premier XE Mass spectrometer using the multiple reaction monitoring (MRM) and positive electrospray ionization modes. The capillary voltage, extractor voltage, RF lens voltage, and multiplier voltage were set at 3.5 kV, 3.0 V, 2.0 V, and 650 V, respectively. The source and desolvation temperatures were held at 100 and 350°C, respectively. The desolvation gas and cone gas were set at the flow rates of 650 and 50 L/h, respectively, and the ultrahigh purity argon collision gas was held at 0.13 mL/min.

3. Results and discussion

3.1 Separation and metabolites profiling

The total ion chromatorgraphy of urine and feces samples of control group and administrated group were acquired by UPLC-QTOF-MS. And then, according to the Mtabolnxy result several possible metabolites of AHTN were checked. But only six possible metabolites with protonated molecular ion m/z 275 (RT at 2.9 min, 4.2 min and 5.2 min, respectively) and m/z 289 (RT at 4.1 min, 4.6 min and 4.9 min, respectively) were finally identified in consideration of rationalities of metabolism pathway, detection error and the mass spectrometric cleavage regular. The TIC spectrum of feces sample of administrated group was shown in Fig.1(a). The

extraction spectrums of m/z 275 (M1~M3) and m/z 289 (M4~M6) were Fig. 1(b) and Fig.1(c), respectively. . The possible metabolites with protonated molecular ion at m/z 275 were detected in all urine and feces samples, and metabolites with protonated molecular ion at m/z 289 were only detected in feces samples. The retention time, elemental compositions, measured and calculated masses, as well as errors of six metabolites are summarized in Table 1.

3.2 Identification of metabolites

Firstly, the standard of AHTN was done a LC-MS/MS analysis to analyze the fragmentation rule of AHTN. The fragmentation analysis of AHTN was firstly performed to provide useful information on the identification and elucidation of the structures of unknown AHTN metabolites. As shown MS^E mass spectrum of AHTN in Figure 2, the protonated molecular ion at *m*/*z* 259.2087 caused loss of C_6H_{12} to result the product ion at *m*/*z* 175.1152 by alpha-cleavage of side six-member ring. But it is possible that alpha-cleavage was occurred at 9-10 bond (pathway A) or 5-6 bond (pathway B) because there were no obvious difference between the energy of 9-10 bond and 5-6 bond. Subsequently, the intermediate product *m*/*z* 175.1152 was further dissociated to *m*/*z* 147.0821 by loss of C_2H_4. The possible fragmentation pathways of AHTN were proposed in Figure 3.

As shown in Figure 4, the suggested compounds have the similar fragmentation ions such as m/z 175 to those of AHTN, which means they have the same mother nucleus. Metabolites M1, M2 and M3 were identical protonated molecular ions at *m*/*z*

275, which was 16 Da higher than their pro-drug with protonated molecular ion at *m*/*z* 259. This indicated that these metabolites were the oxidation products by hydroxylation. As shown in Figure 4, M1 and M2 have the same product ions at *m*/*z* 245, 215, 201, 175 and 159, which suggest they give similar fragment pathways, these product ions correspond to $[M+H-CH_2O]^+$, $[M+H-C_3H_8O]^+$, $[M+H-C_4H_{10}O]^+$, $[M+H-C_6H_{12}O]^+$, $[M+H-C_7H_{16}O]^+$, $[M+H-C_8H_{16}O]^+$ and $[M+H-C_9H_{16}O_2]^+$. Firstly, both M1 and M2 were dissociated to *m*/*z* 245 by loss of CH_2O. It was suggested that the OH group was not at ring but at side chain for both M1 and M2. Therefore, the location of hydroxylation for M1 and M2 maybe were at 14-methyl or 18-methyl. Further, M1 and M2 were identified as 14-OH-AHTN and 18-OH-AHTN, respectively, since the polarity of 18-OH-AHTN was lower than 14-OH-AHTN due to the effect of hydrogen bond between 18-OH and carbonyl. As shown in Figure 4, product ions at *m*/*z* 257, 215, 201, 187 and 159 were found for M3. M3 has no fragment ion at *m*/*z* 245, but has fragment ion at *m*/*z* 257. The fragment ion at *m*/*z* 257 was obtained by loss of H_2O from M3. It was suggested that the OH group was not at side chain but at ring for M3. Therefore, the possible structure of M3 was 8-OH-AHTN. The product ions at *m*/*z* 215, 201, 187 and 159 correspond to $[M+H-H_2O-C_3H_6]^+$, $[M+H-H_2O-C_4H_8]^+$, $[M+H-H_2O-C_5H_{10}]^+$, $[M+H-H_2O-C_7H_{14}]^+$ and $[M+H-H_2O-C_8H_{16}]^+$. According to the presumed fragment pathway of M3, the transition states from product ion at *m*/*z* 215 to product ion at *m*/*z* 159 were uncommon upper state tetra-atomic ring ions at *m*/*z* 187 and 173. But, it was possible because a stable three-ring product was obtained via an uncommon upper state. The

possible fragment pathway of M1, M2 and M3 was shown in Figure 5.

M4, M5 and M6 were gave the same protonated $[M+H]^+$ ion at *m*/*z* 289, which may be hydroxylation+ethylcarbonylation or carboxylation products. M4 produced the fragments ions at *m*/*z* 271 $[M+H-H_2O]^+$, 229 $[M+H-H_2O-C_3H_6]^+$, 201 $[M+H-H_2O-C_3H_6-CO]^+$ and 175 $[M+H-H_2O-C_3H_6-CO-C_2H_2]^+$. Thus, the structure of this metabolite may be 14-OH-8-carbonly-AHTN. M5 and M6 have the same fragment ions at *m*/*z* 243, 201, 159, and 145. The fragment ion of at *m*/*z* 243 was a decarboxylated product $[M+H-COOH]^+$. The latter lost an isopropyl (C3H6) resulted product ion at *m*/*z* 201, and then formed *m*/*z* 159 by loss of another isopropyl. For M6，the existence of *m*/*z* 187 was the same as the product ion of M3. Therefore, M5 and M6 may be 14-COOH-AHTN and 16-COOH-AHTN, respectively. The possible fragment pathways of M4, M5 and M6 were shown in Figure 6.

3.3 UPLC-MS/MS analysis of rat feces samples

According to the result of LC-QTOF-MS analysis, the possible metabolites M1 – M3 were found both in rat urine and feces, but M4 – M6 were only found in rat feces. It was possible induced that the contents of M4 – M6 were lower than M1 – M3. Therefore, a high sensitive method of UPLC-MS/MS (MRM mode) was performed for urine and feces samples. We used an orally dose (300 mg/kg) feces sample as reference sample to optimize the parameters of mass spectrum for 6 metabolites, since the standard was not available. All optimized parameters of AHTN as well as the metabolites for MRM acquisition were presented in Table 2. As shown in Figure 7, all

possible metabolites (M1– M6) were found in feces samples. Similarly to the results upon LC-QTOF-MS, M4, M5 and M6 were also not found in urine sample.

Semi-quantification test was performed by assuming that six metabolites pose the same molar response as AHTN upon LC-MS/MS. This might suffer from some inaccuracy due to the lack of authentic standards. As a result, the concentrations of AHTN and M1 – M6 in dried feces were 74157 ng/g, 98.7 ng/g, 2333.0 ng/g, 2654.1 ng/g, 98.1 ng/g, 7.2 ng/g and 105.4 ng/g, respectively, 24 hours later after 300mg/kg orally administration. While, AHTN and M1 – M3 in urine were only 1173.7 ng/g, 70.0 ng/g, 119.9 ng/g and 48.1 ng/g, respectively. It suggested that AHTN and metabolites are mainly excreted by feces.

4. Conclusions

With the use of LC-QTOF-MS, six metabolites of AHTN in rat have been structurally characterized based on the MS^E fragmentation and Metabolynx software. The six metabolites are namely 14-OH-AHTN, 18-OH-AHTN, 8-OH-AHTN, 14-OH-8-carbonly-AHTN, 14-COOH-AHTN and 16-COOH-AHTN.

Acknowledgement

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Table Captions

Table 1. Predicated elemental compositions, measured masses, calculated masses, and mass errors between measured and predicated masses of 6 metabolites

Table 2. LC-MS/MS acquisition paramters for 6 metabolites of AHTN.

Table 1. Predicated elemental compositions, measured masses, calculated masses, and mass errors between measured and predicated masses of 6 metabolites

Metabolite	RT time (min)	Elemental composition	Measured mass (Da)	Calculated mass (Da)	Error (mDa)	Error (ppm)
M1	2.87	C18H26O2	275.2038	275.1933	2.7	9.9
M2	4.20	C18H26O2	275.2028	275.1933	1.1	4.1
M3	5.25	C18H26O2	275.2039	275.1933	2.8	10.3
M4	4.08	C18H24O3	289.1807	289.1725	0.4	1.2
M5	4.63	C18H24O3	289.1822	289.1725	1.9	6.4
M6	4.97	C18H24O3	289.1814	289.1725	1.1	3.7

Table 2. LC-MS/MS acquisition paramters for AHTN and 6 metabolites of AHTN.

Metabolite	RT time (min)	Parent ion (m/z)	Daughter ion (m/z)	Collision energy (eV)	Cone voltage (V)
AHTN	8.	259.2	259.2>175.1	25	15
M1	2.97	275.1	275.1>245.0	30	25
M2	4.26	275.2	275.2>175.2	25	25
M3	5.32	275.2	275.2>159.2	20	25
M4	4.16	289.4	289.18>175.2	20	25
M5	4.68	289.4	289.18>159.1	25	25
M6	5.03	289.4	289.18>159.1	25	25

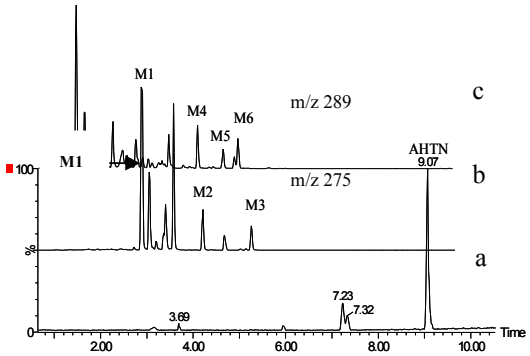

Figure 1. LC-QTOF-MS chromatogram of feces of administrated group. a, TIC spectrum; b, the extraction spectrum of *m/z* 275; c, the extraction spectrum of *m/z* 289.

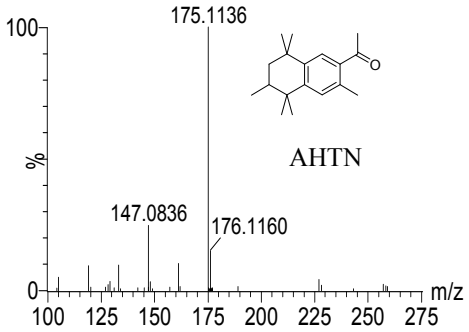

Figure 2. Mass spectra of AHTN

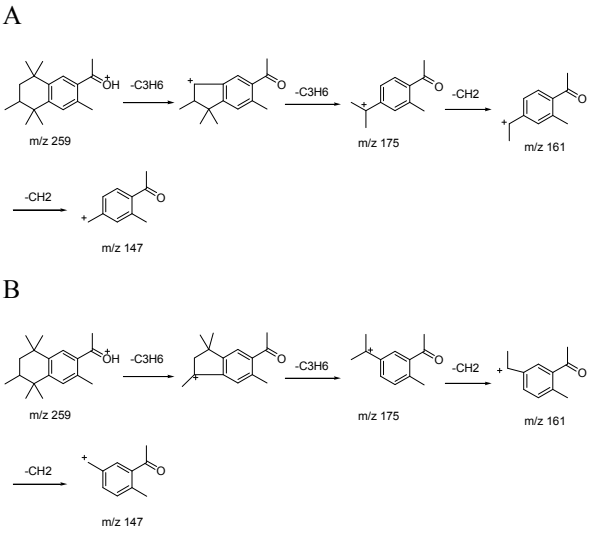

Figure 3. The hypothesis fragmentation pathway of AHTN.

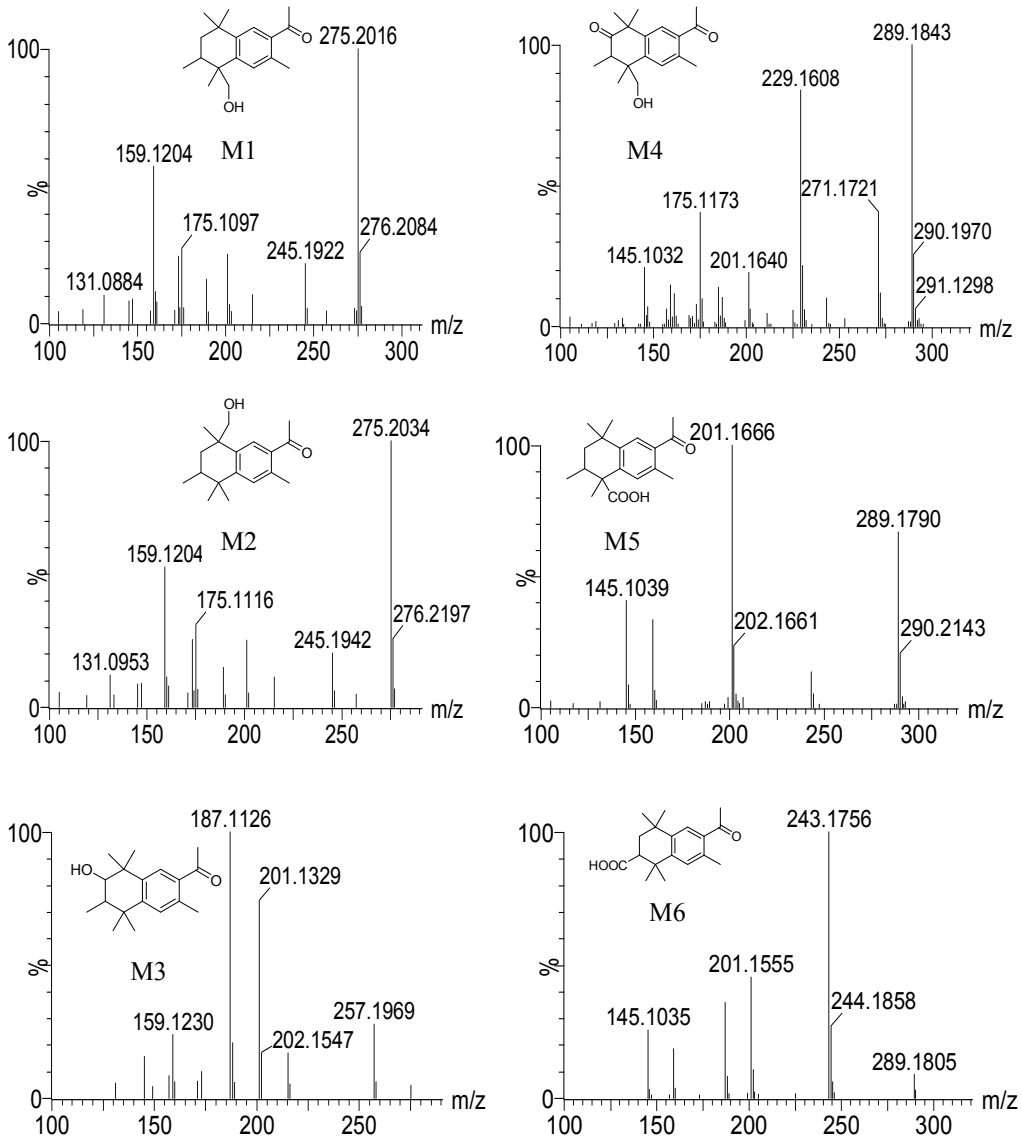

Figure 4. Mass spectra of the 6 metabolites (M1-M6).

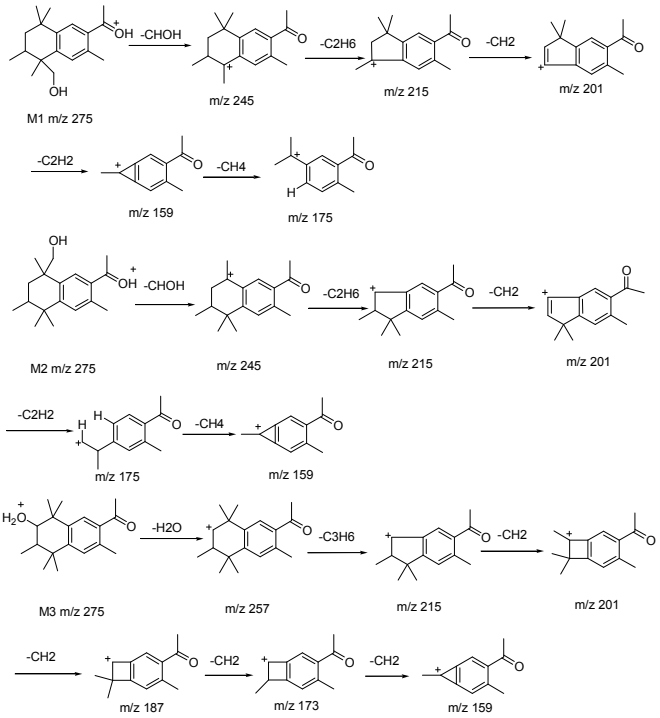

Figure 5. The hypothesis fragmentation pathways of M1, M2, and M3.

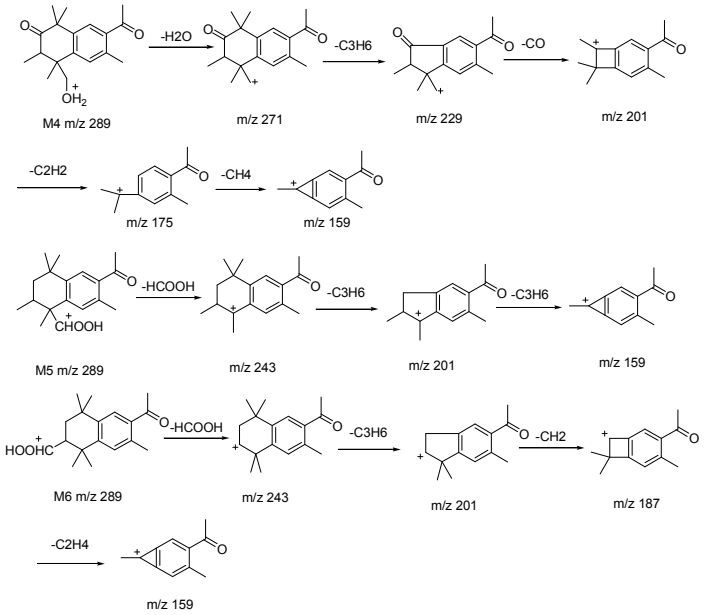

Figure 6. The hypothesis fragmentation pathways of M4, M5, and M6.

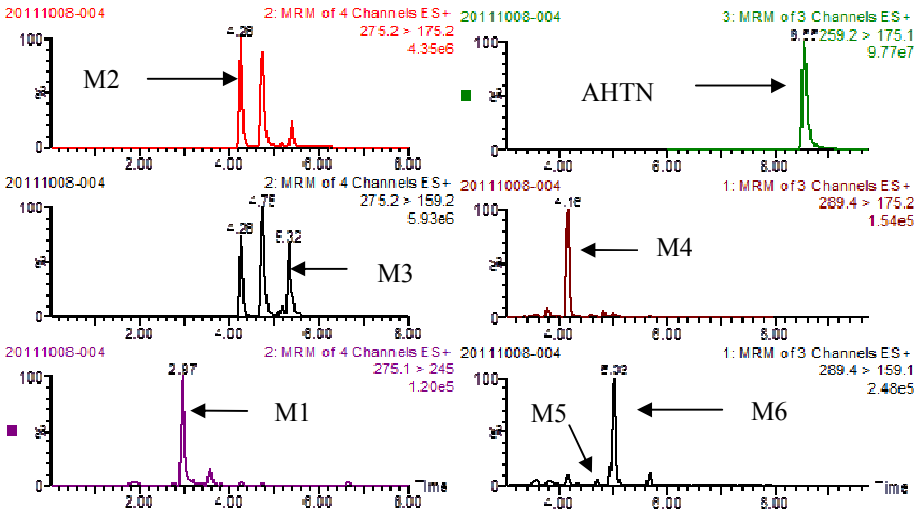

Figure 7. UPLC-MS/MS chromatograms of M1 to M6 in feces sample (300mg/kg dose).