LANZHOU INSTITUTE OF HUSBANDRY AND PHARMACEUTICAL SCIENCES OF CAAS

In vitro and *in vivo* metabolism of aspirin eugenol ester in dogs by LC-MS

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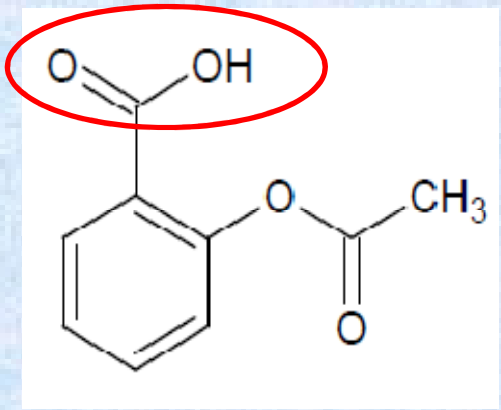
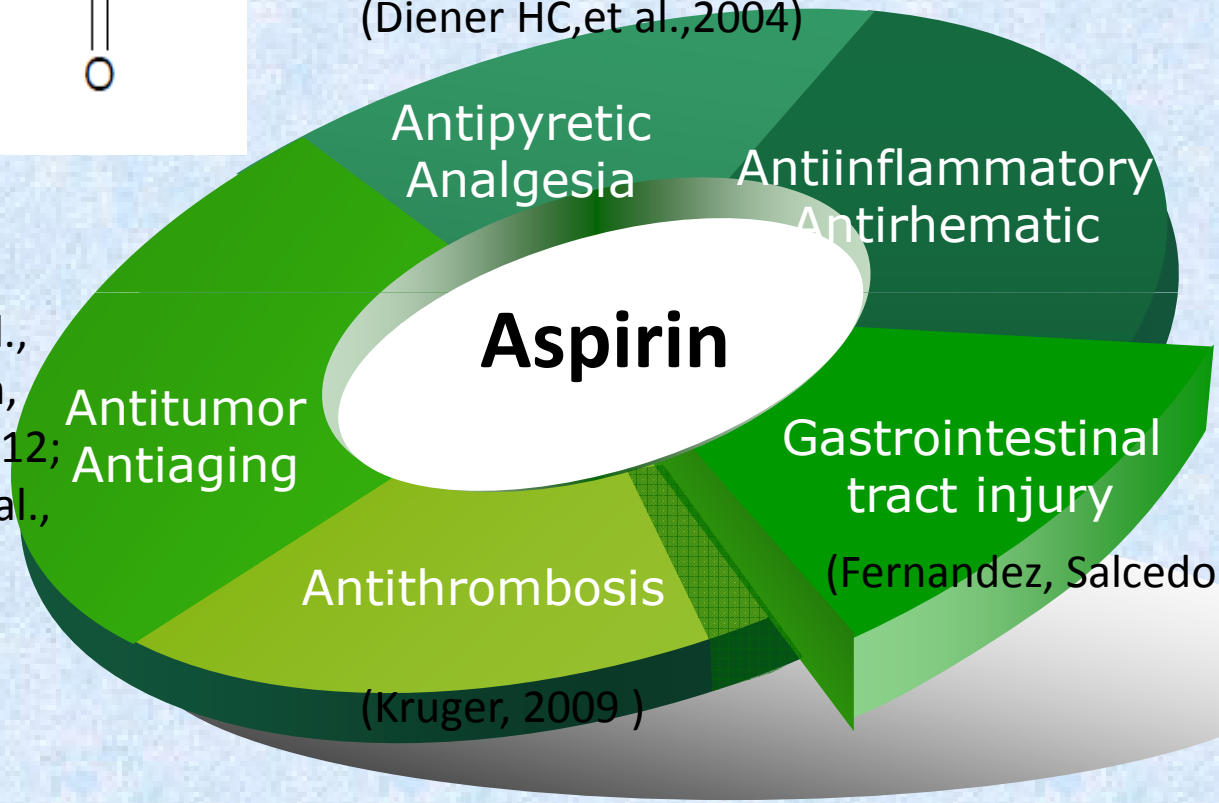
Outline

- Research background
- *In vitro* metabolism of AEE
- *In vivo* metabolism of AEE
- Conclusion
- Acknowledgement

Research backgroud



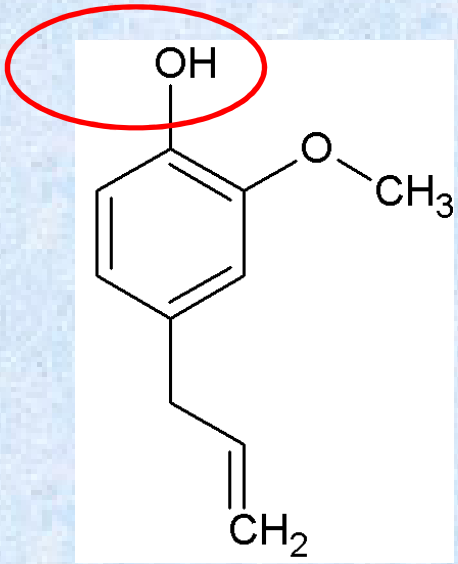
(Diener HC,et al.,2004)



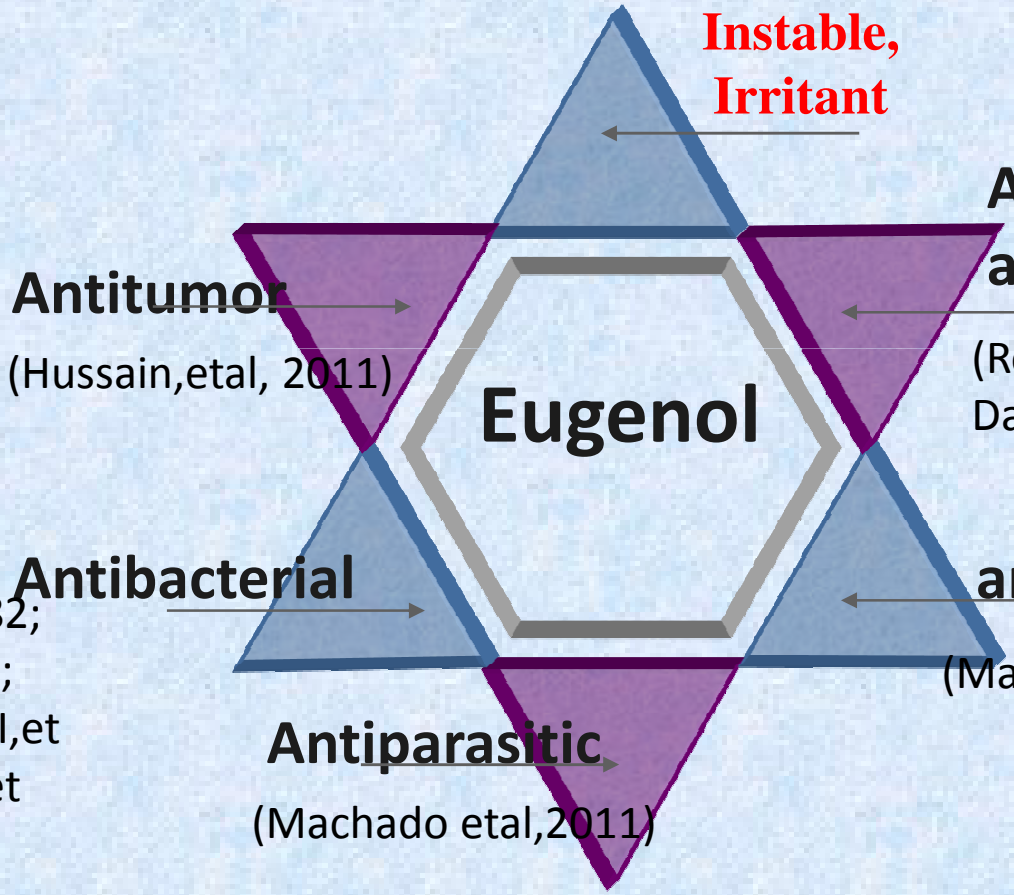
(Lim, Han et al., 2008; Hussain, Javeed et al., 2012; Wan, Zheng et al., 2013)

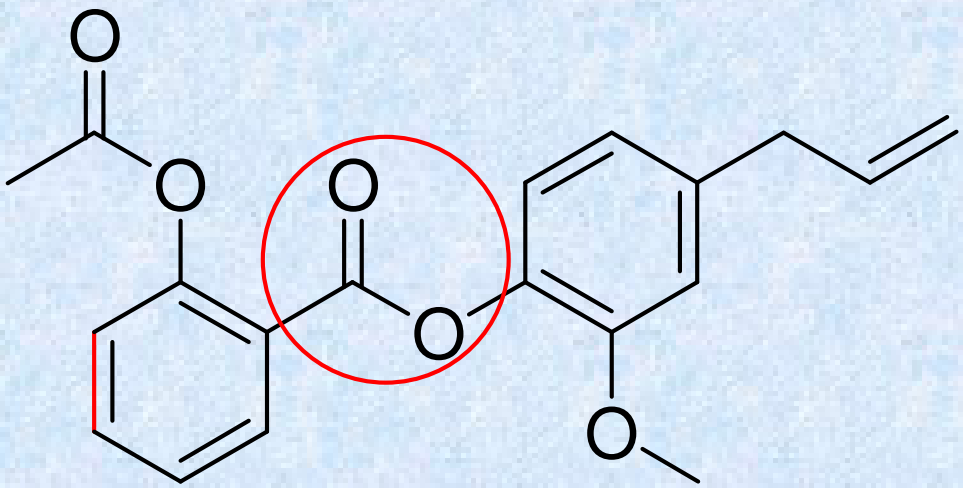
(Fernandez, Salcedo et al., 1995)

(Kruger, 2009)



(Azzouz ,et al.,1982; Bishop,et al.,1995; Konstantopoulou I,et al.,1992; Pandey,et al.,2000)





AEE(Aspirin Eugenol Ester)

The aim is to reduce the side effects（Gastrointestinal tract irritant, volatile, instable, pungent odor）and improve the synergistic therapeutic effect of aspirin and eugenol.

The result of AEE experiments indicated:

- Many pharmacodynamics such as analgesic, anti-inflammatory, antipyretic, bacteriostat and antioxidant activity, but lasted longer than aspirin or eugenol.
- Very low acute and sub-acute toxicity （LD_{50}=10.94 g/kg , NOAEL ≥50mg/kg）.
- No genotoxicity (teratogenicity and mutagenicity).

Li, *et al*., Medicinal Chemistry Research, 21(7), 2012, 995-999.
Li, *et al*., Food and Chemical Toxicology, 50(6), 2012, 1980-1985;
Li, *et al*., Food and Chemical Toxicology, 62, 2013, 805-809. Liu, Li *et al*., Acta Cryst. (2011). E67, o1621.
Li, *et al*., Journal of Animal and Veterinary Advances, 2012,11(23):4401-4405

In our design idea, AEE was speculated as a pro-drug and decomposed into aspirin and eugenol after absorption, and then showed the original biological activities of aspirin and eugenol. The objective of the present study was to investigate both *in vivo* and *in vitro* metabolism of AEE in dog, and next step to illustrate its action mechanism, establish its residual mark compound and to formulate its dosage.

In vitro metabolism of AEE

Method:

1. Dog liver microsomes were prepared by differential centrifugation.

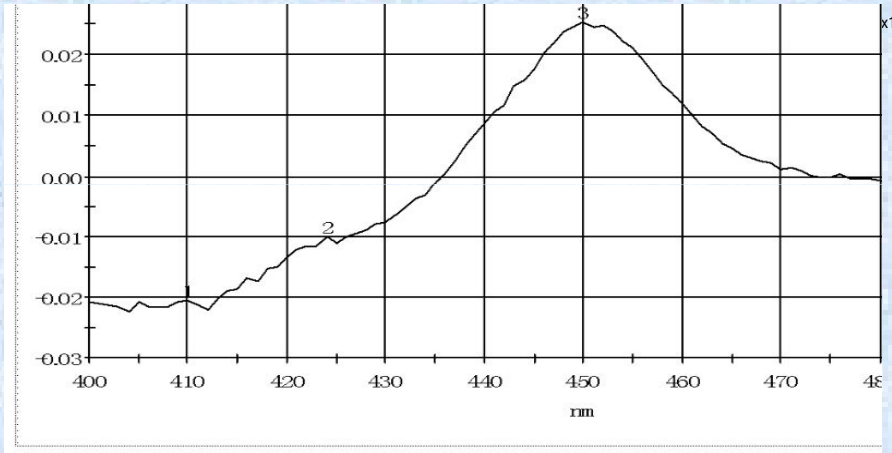
2. the protein concentrations of dog liver microsomes were measured using BSA method（9.5-10.1mg·mL^{-1}）.

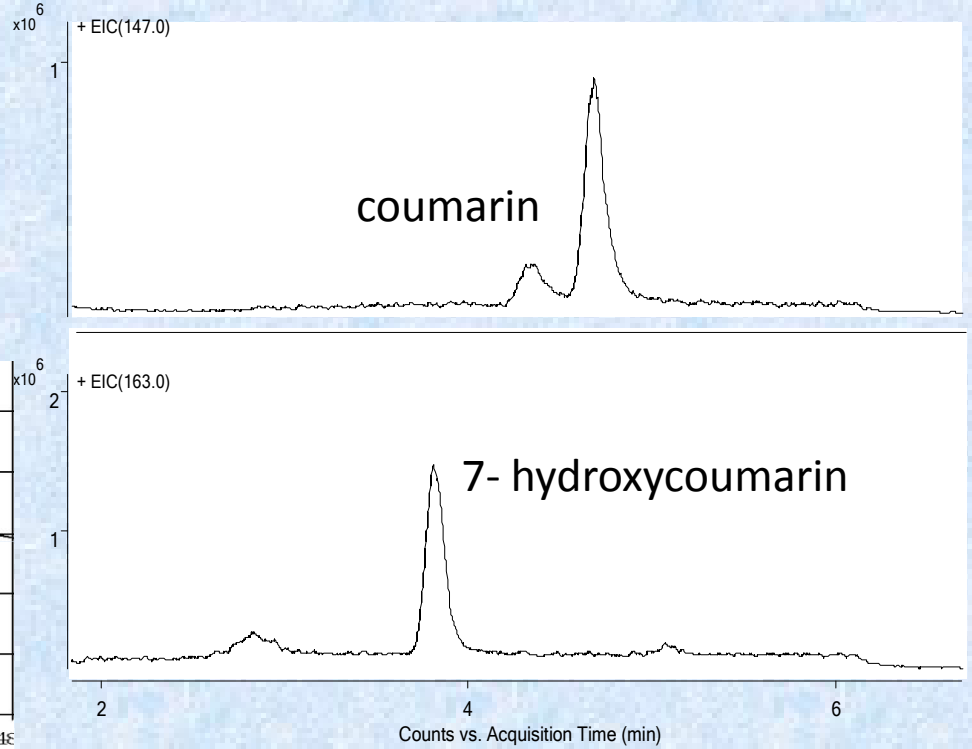
3. The content of CYP450 in liver microsome were measured with CO differential spectroscopy （0.18 nmol·mg^{-1}）.

4. liver microsome enzyme activity was measured by coumarin transforming into 7-hydroxycoumarin.

5. *In vitro* incubation system was established, including Tris-HCl 0.05mol·L^{-1}, liver microsome 0.6mg·mL^{-1} , $MgCl_2$ 5mmol·L^{-1} , 6-phosphate glucose 10 mmol·L^{-1} , NADP 2 mmol·L^{-1} , 6-phosphate glucose dehydrogenase 5 U·mL^{-1}， total volume 400 μL.

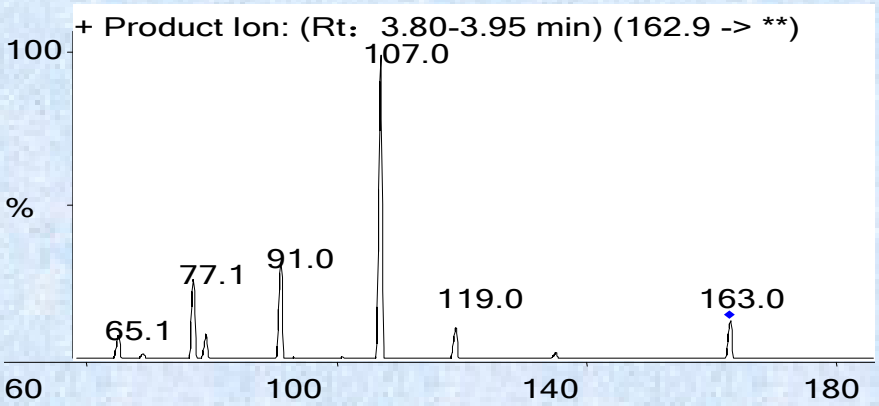
Liu, *et al*., 2009; Smith, *et al*., 1985





CYP has activity

coumarin transformed into 7-hydroxycoumarin



Sample preparation

Incubation mixture

↓ 37¡æ for 3min

4¦ÌL drug (100 mmol¡Ë$^{-1}$ in system)

↓ 37¡æ for 30min

400¦ÌL ice-cold acetonitrile (5% formic acid)

↓ vortexed for 1 min

↓ centrifuged at 16000g for 10min (4¡æ)

↓ filtered with a 0.22¦Ìm nylon membrane

supernatant

↓ 5¦ÌL aliquot

LC/MS/MS analysis

Chromatography condition

HPLC： Agilent 1200；

Column： Agilent Zorbax Eclipse plus C18 column (3.0 mm×100 mm, 1.8 μm)；

Flow rate： 0.4ml·min^{-1}；

Column temperature： 30℃.

Mobile phase：
A acetonitrile ；
B water（0.1% formic acid）

0-1min	90%B
1-3min	90-60%B
3-5min	60-50%B
5-10min	50-42%B
10-12min	42-38%B
12-14min	28-25%B
14-15min	25-5%B
15-20min	5%B

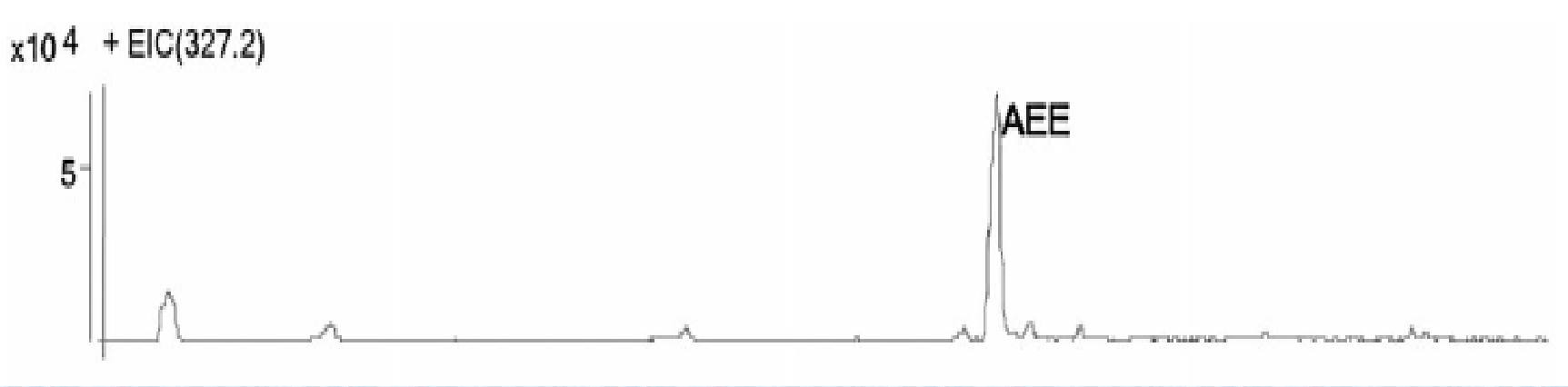
Mass spectrum condition

Mass spectrum apparatus： Agilent 6410A,

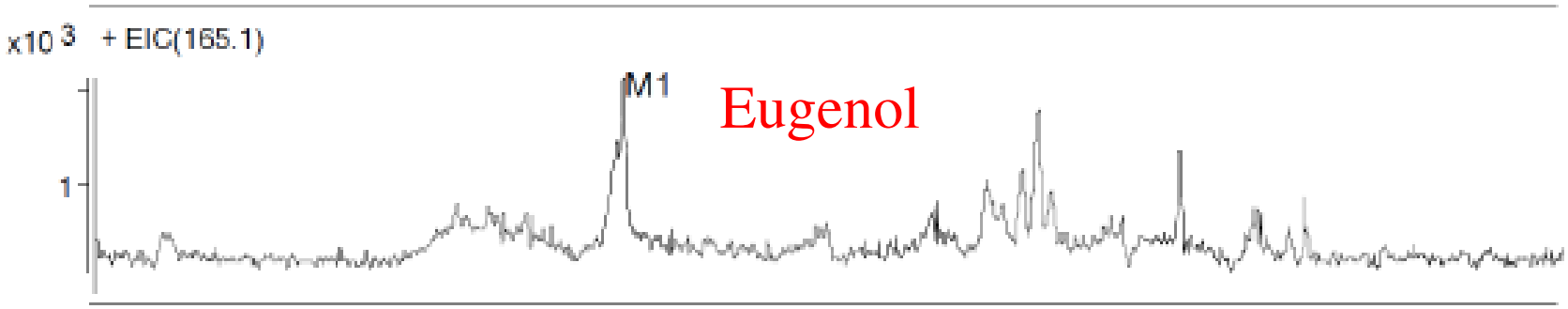
Detect mode: ESI, positive and negative modes,

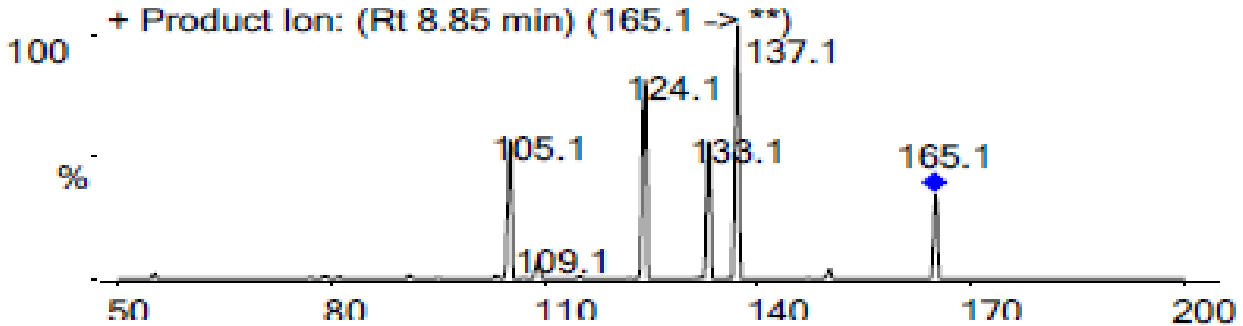
Nebulizer pressure： 30psi, Gas flow rate： 10L·min^{-1},
Gas temperature ： 300℃,

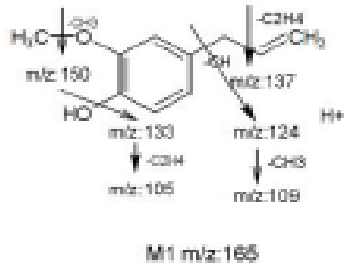
Capillary potential of MS： 4000V

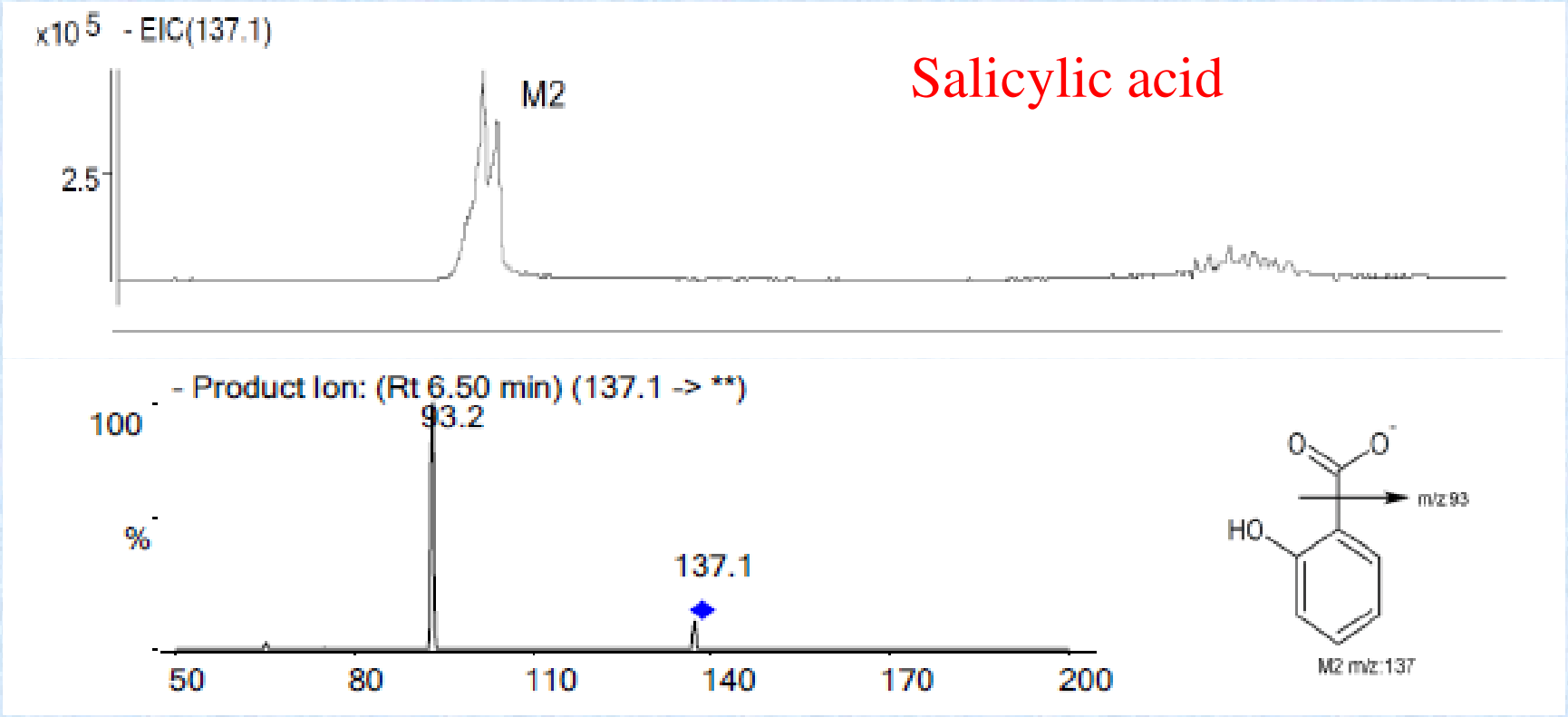


Detection results （EIC and fragment ion)

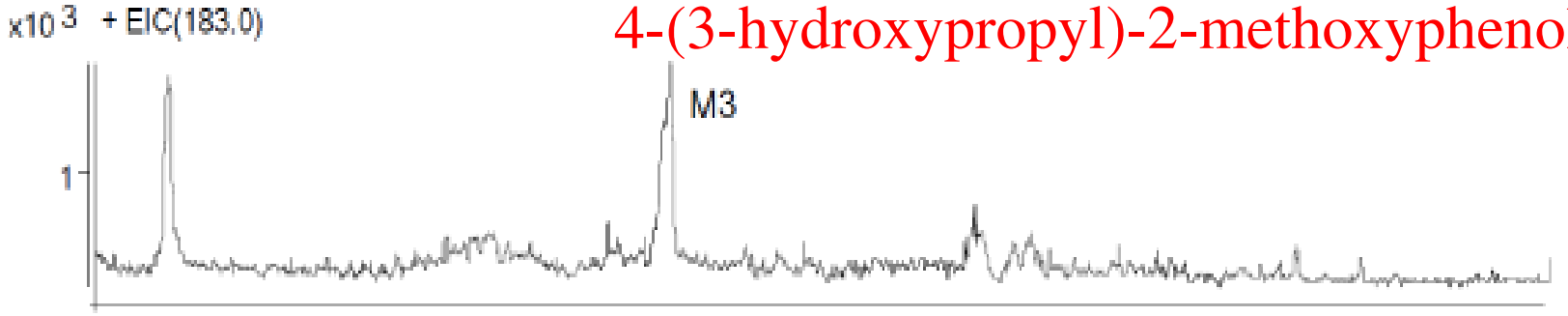


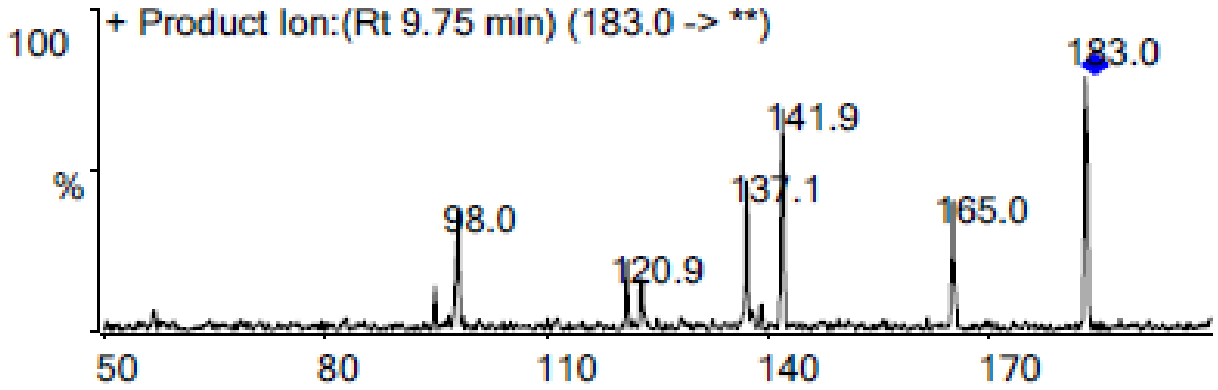


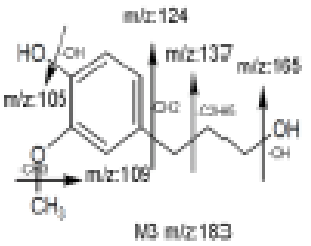


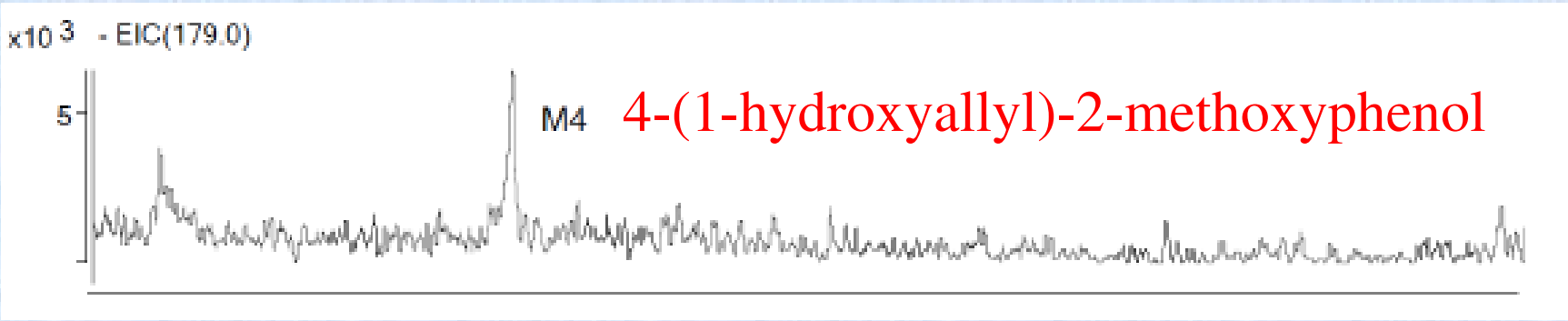

x10 5 - EIC(137.1)
M2
2.5
Salicylic acid
- Product Ion: (Rt 6.50 min) (137.1 -> **)
100
%
93.2
137.1
50
80
110
140
170
200
O
O
HO
m/z 93
M2 m/z:137

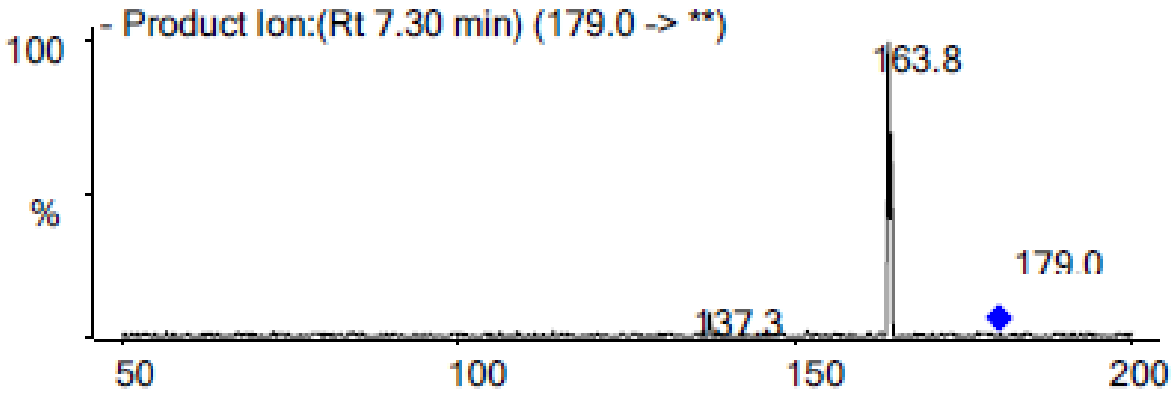
4-(3-hydroxypropyl)-2-methoxyphenol

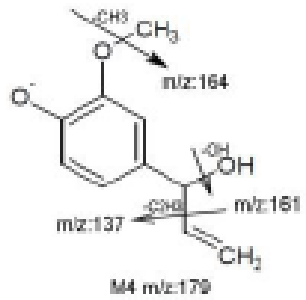




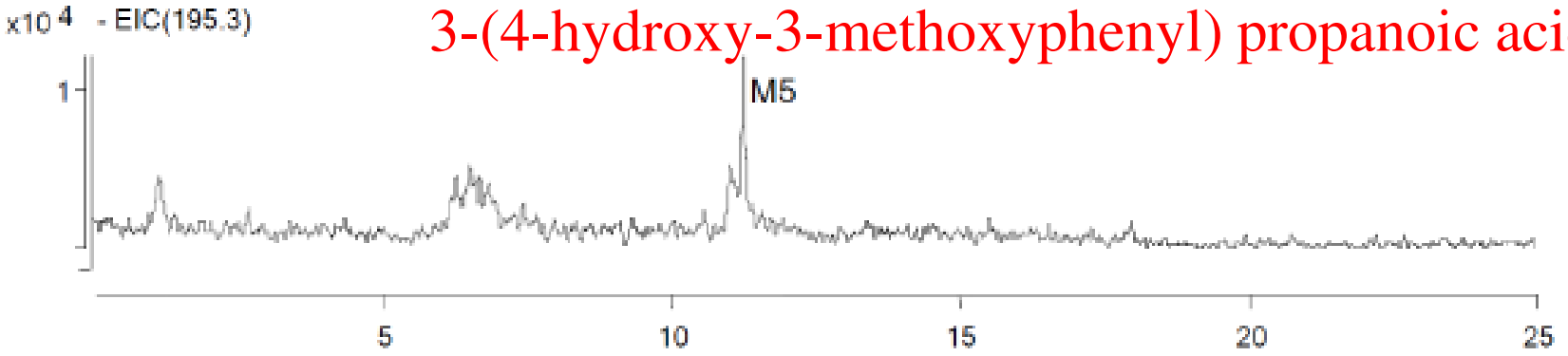


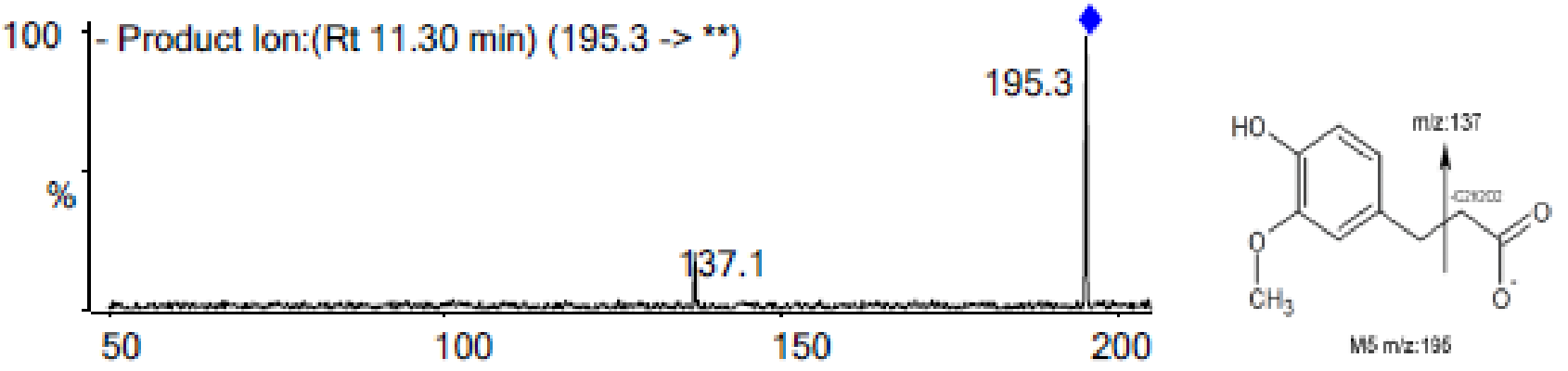

x10 3 - EIC(179.0)
5
M4
4-(1-hydroxyallyl)-2-methoxyphenol


- Product Ion:(Rt 7.30 min) (179.0 -> **)
100
%
163.8
179.0
137.3
50
100
150
200


CH3
CH3
O
m/z:164
O
OH
OH
CHO
m/z:161
m/z:137
CH2
M4 m/z:179

3-(4-hydroxy-3-methoxyphenyl) propanoic acid





The retention times (RT), mass ions, elemental compositions, and major fragment ions of AEE and its metabolites *in vitro*

	Compound	RT (min)	Mass ion	Elemental composition	Identification
	AEE	15.95	327.3	$C_{19}H_{19}O_5^+$	aspirin eugenol ester
	M1	8.85	165.1	$C_{10}H_{13}O_2^+$	eugenol
	hydrolysis				
In	M2	6.50	137.1	$C_7H_5O_3^-$	salicylic acid
vitro	M3	9.75	183.0	$C_{10}H_{15}O_3^+$	5-(2-hydroxypropyl)-2-methoxyphenol
	M4	7.30	179.0	$C_{10}H_{11}O_3^-$	4-(1-hydroxyprop-2-en-1-yl)-2-methoxyphenol
	M5	11.30	195.0	$C_{10}H_{11}O_4^-$	3-(3,4-dihydroxyphenyl)propanoic acid

In vivo metabolism of AEE

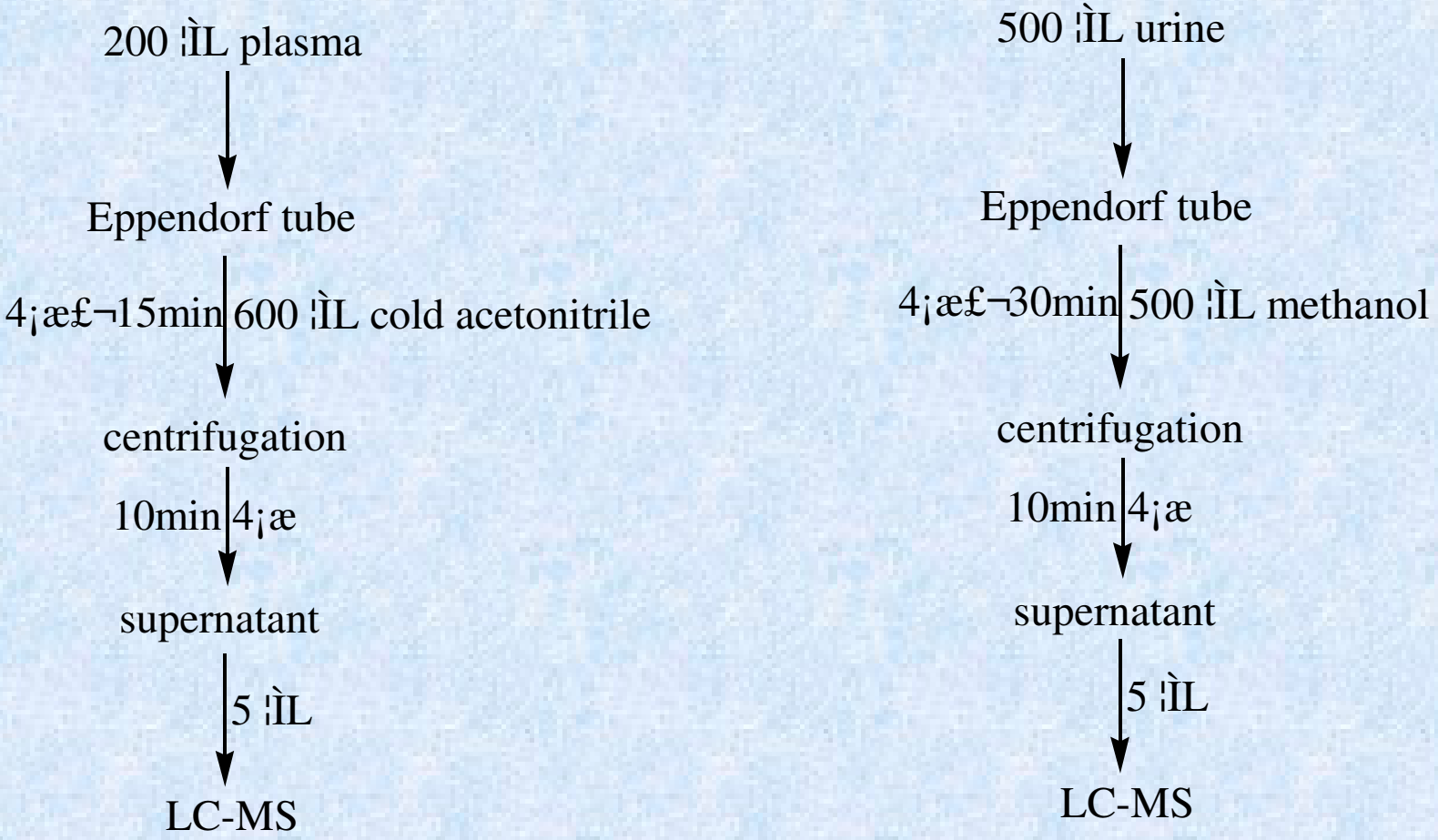
Materials

AEE tablet： each tablet included AEE 150mg.

Dosage : 20mg·kg^{-1} .

Animals： 6 beagle , half and half of male and female. After administrating AEE, bloods and urines at different time were collected.

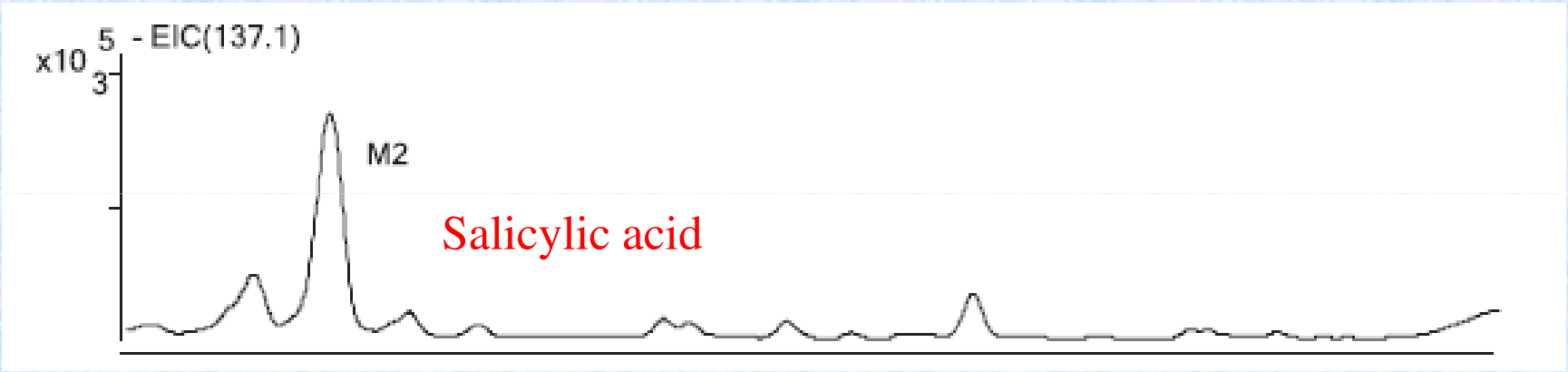
Sample processing method

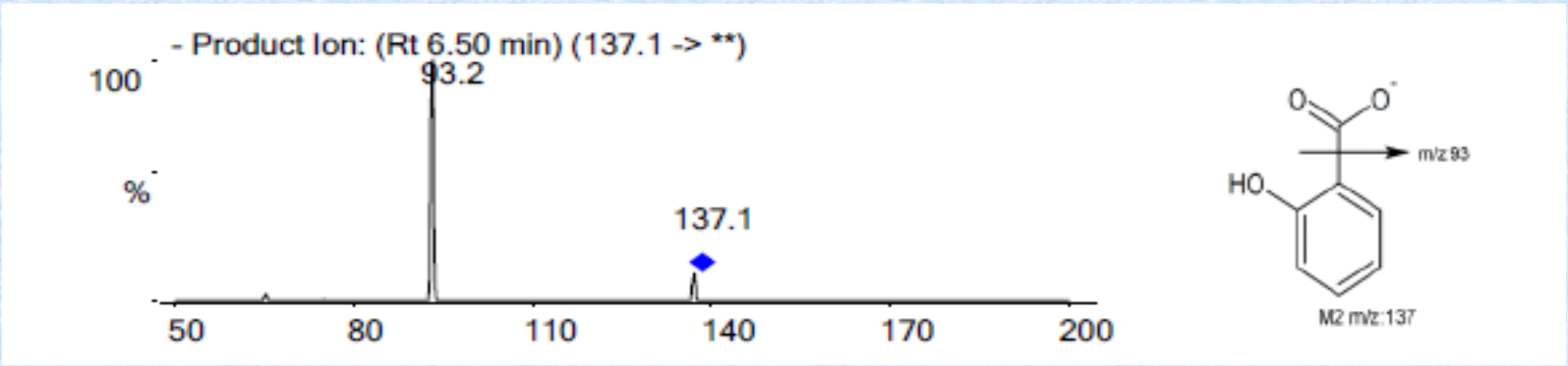


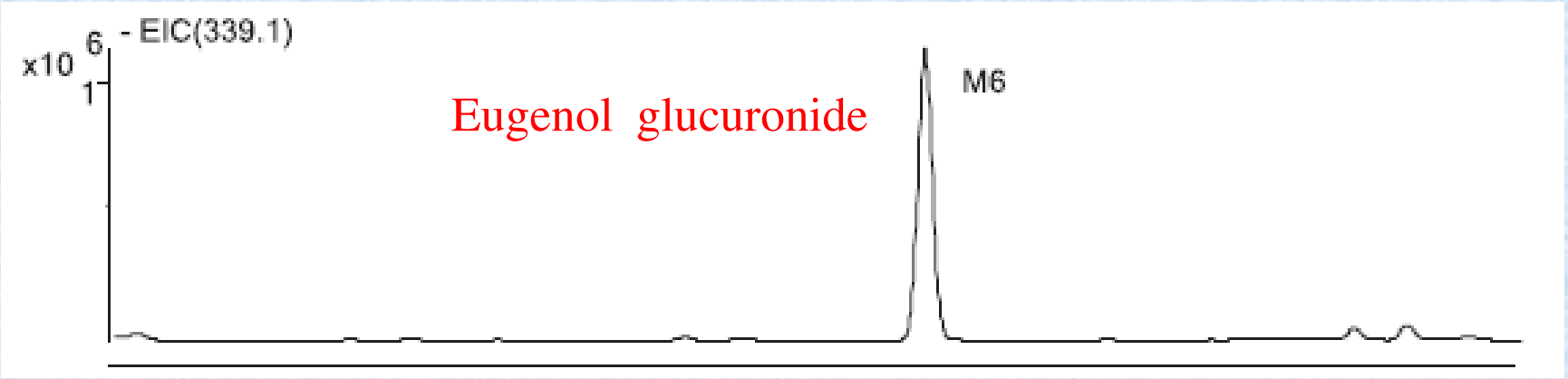
Chromatography condition and mass spectrum condition as the same with *in vitro* metabolism.

Result

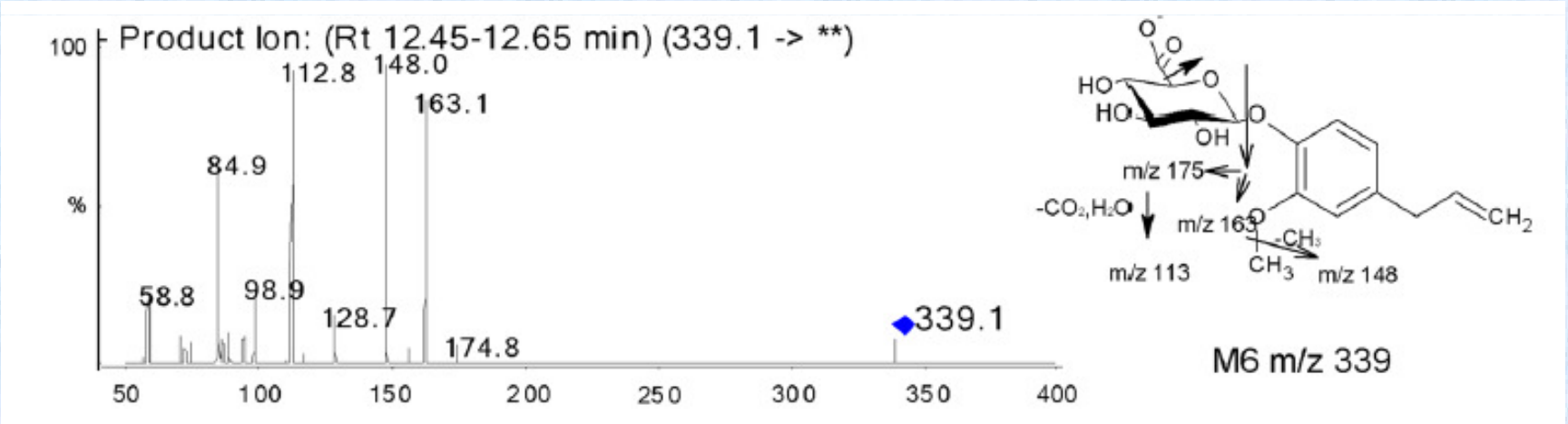
six metabolites (M2，M6，M7，M8，M9，M10) were found, and all of them existed in plasma and urine.



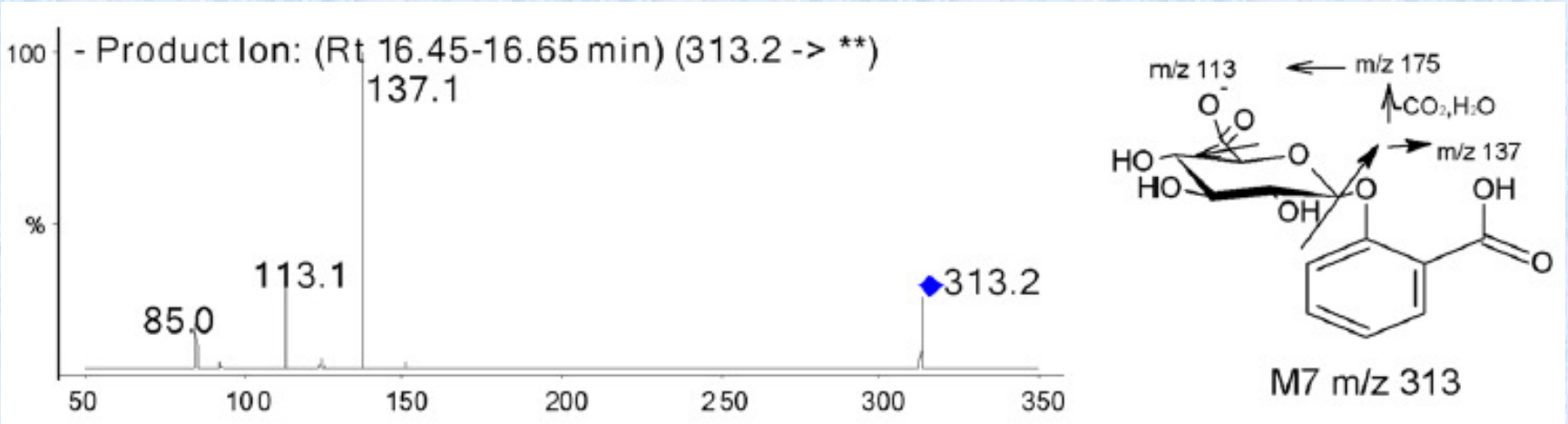


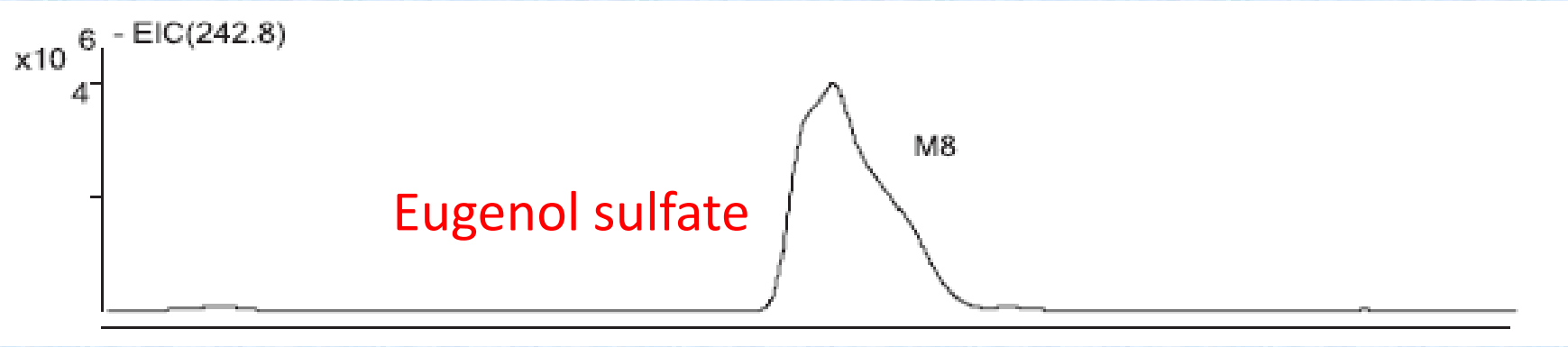


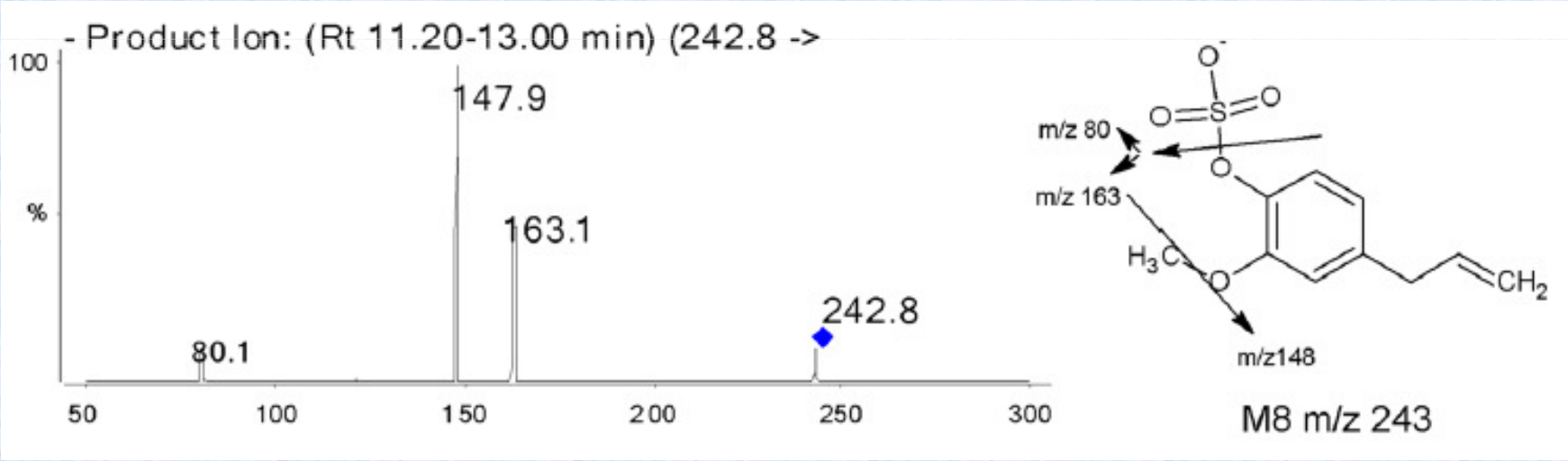
Eugenol glucuronide

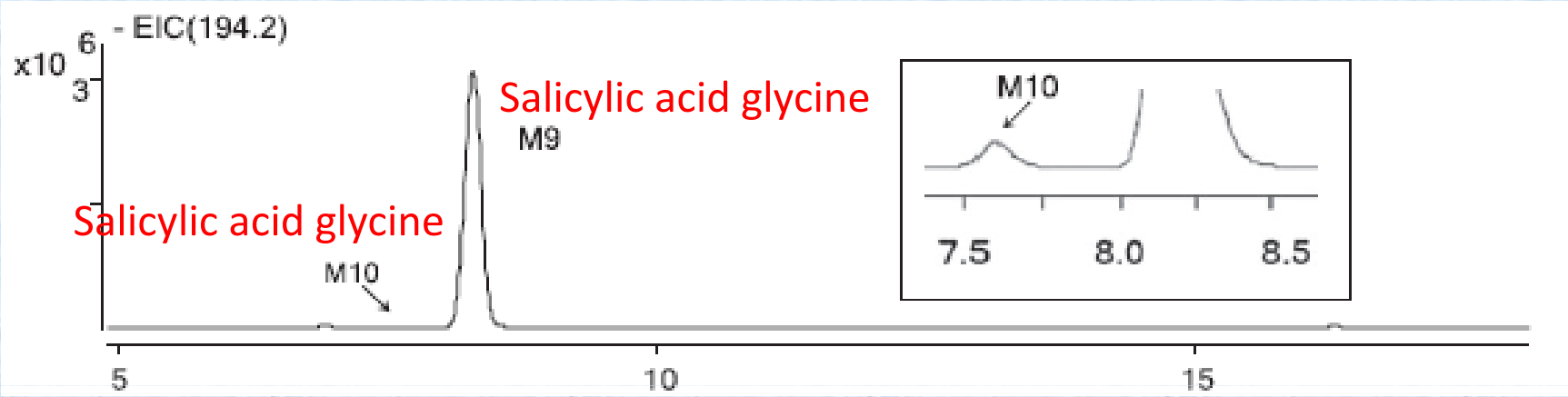


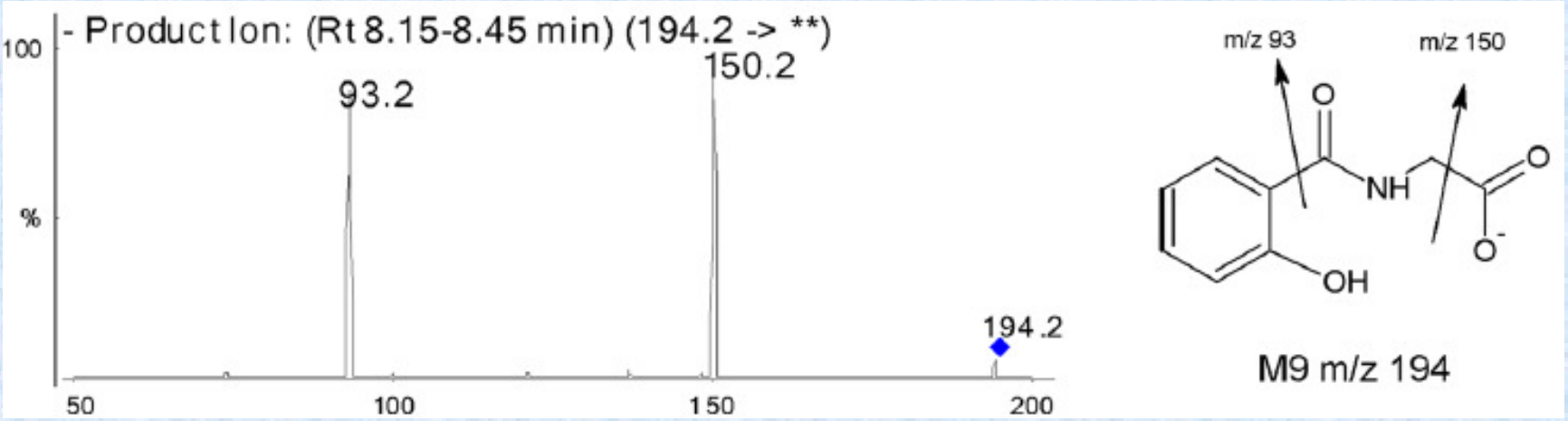

- EIC(313.3)
x10 6
2
M7
Salicylic acid glucuronide

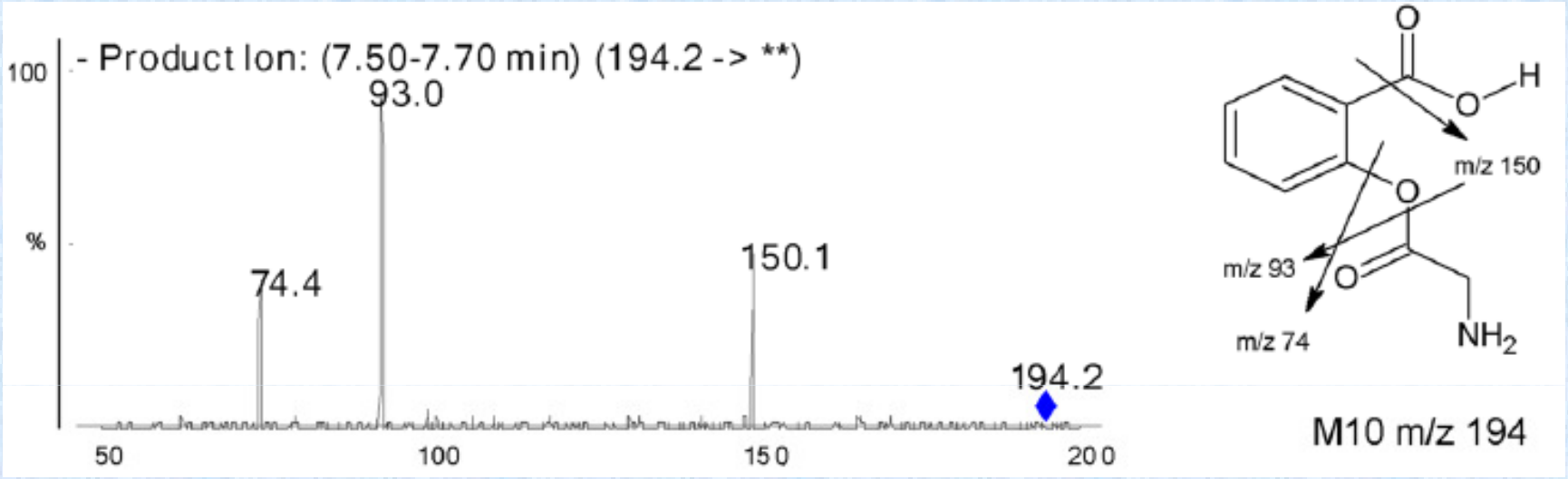

100
%
- Product Ion: (Rt 16.45-16.65 min) (313.2 -> **)
137.1
113.1
85.0
313.2
50
100
150
200
250
300
350
m/z 113
m/z 175
CO2,H2O
m/z 137
HO
HO
OH
OH
O
M7 m/z 313


x10 6
4
- EIC(242.8)
M8
Eugenol sulfate


- Product Ion: (Rt 11.20-13.00 min) (242.8 ->
100
%
147.9
163.1
242.8
80.1
50
100
150
200
250
300
m/z 80
m/z 163
m/z148
M8 m/z 243


- EIC(194.2)
x10 6
3
Salicylic acid glycine
M9
Salicylic acid glycine
M10
M10
7.5
8.0
8.5
5
10
15


- Product Ion: (Rt 8.15-8.45 min) (194.2 -> **)
100
%
93.2
150.2
194.2
50
100
150
200
m/z 93
m/z 150
O
NH
O
O-
OH
M9 m/z 194



In vivo metabolism, without M2 as salicyclic acid for decomposition reaction, all other metabolites were second phase metabolites, mainly including glucuronic acid product and other conjugation metabolites.

The retention times (RT), mass ions, elemental compositions, and major fragment ions of AEE and its metabolites *in vitro*

	Compound	RT (min)	Mass ion	Elemental composition	Identification
In vivo	M2	6.80	137.1	$C_7H_5O_3^-$	salicylic acid
	M6	12.55	339.1	$C_{16}H_{19}O_8^-$	eugenol glucuronide
	M7	16.55	313.3	$C_{13}H_{13}O_9^-$	salicylic acid glucuronide
	M8	11.50	242.8	$C_{10}H_{11}O_5S^-$	eugenol sulfate
	M9	8.30	194.2	$C_9H_9O_4N^-$	salicylic acid glycine
	M10	7.60	194.2	$C_9H_9O_4N^-$	salicylic acid glycine

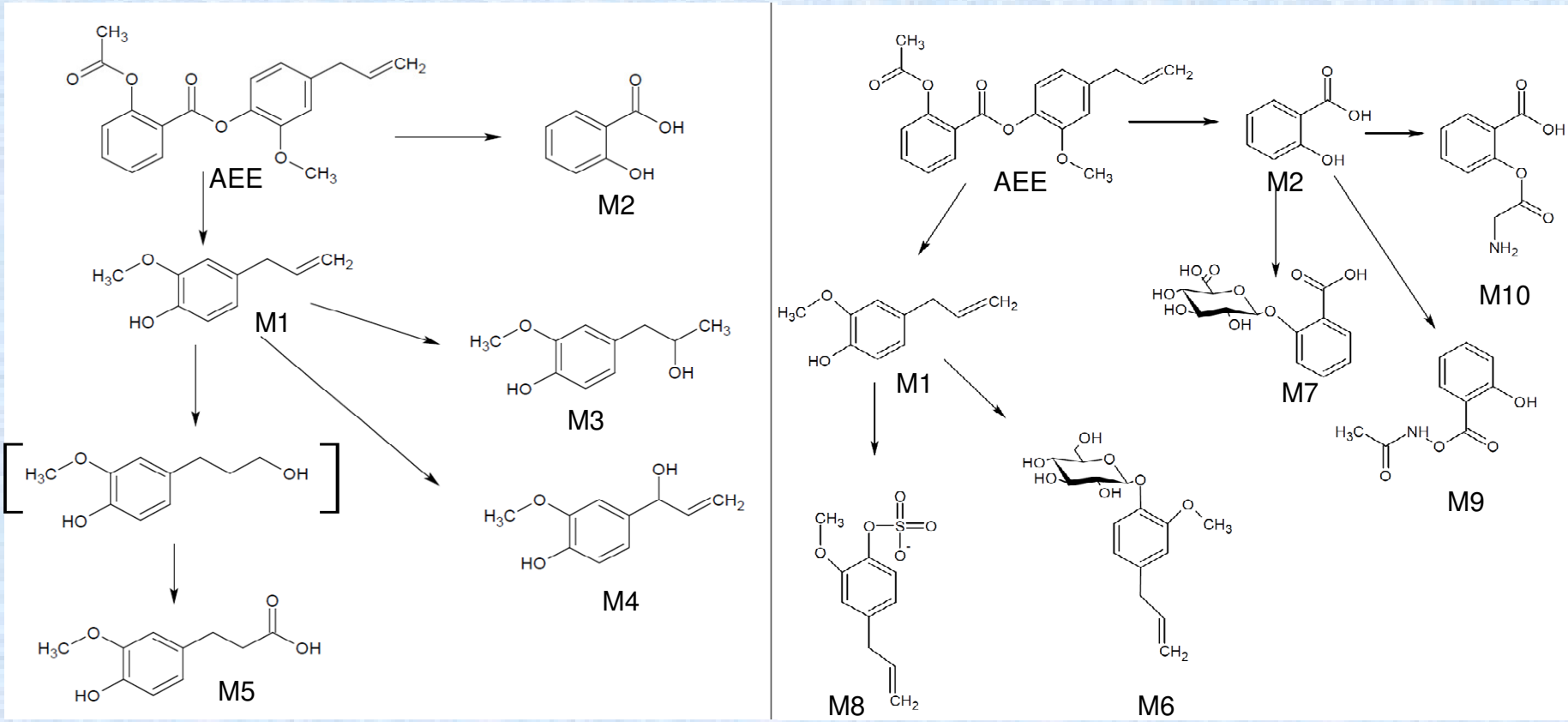
AEE metabolites *in vitro* and *in vivo*

Table 2. The retention times, mass ions, elemental compositions and major fragment ions of aspirin eugenol ester (AEE) and its metabolites

	Compound	Retention time (min)	Mass ion	Elemental composition	Major fragment ions	Identification
In vitro	AEE	15.50–16.30	327.3	$C_{19}H_{19}O_5^+$	162.9, 121.1	Aspirin eugenol ester
	M1	8.60–9.10	165.1	$C_{10}H_{13}O_2^+$	137.1, 133.1, 124.1, 109.1, 105.1	Eugenol
	M2	6.15–6.90	137.1	$C_7H_5O_3^-$	93.2, 65	Salicylic acid
	M3	9.60–9.85	183.0	$C_{10}H_{15}O_3^+$	165.0, 141.9, 137.1, 120.9, 98.0	5-(2-Hydroxypropyl)-2-methoxyphenol
	M4	7.15–7.40	179.0	$C_{10}H_{11}O_3^-$	163.8, 137.3	4-(1-Hydroxyprop-2-en-1-yl)-2-methoxyphenol
	M5	11.25–11.40	195.0	$C_{10}H_{11}O_4^-$	179, 137.1	3-(3,4-Dihydroxyphenyl) propanoic acid
In vivo	M2	6.75–6.95	137.1	$C_7H_5O_3^-$	93.2, 65	Salicylic acid
	M6	12.45–12.65	339.1	$C_{16}H_{19}O_8^-$	174.8, 163.1, 148.0, 128.7, 112.8, 84.9	Eugenol glucuronide
	M7	16.45–16.65	313.3	$C_{13}H_{13}O_9^-$	137.1, 113.1, 85	Salicylic acid glucuronide
	M8	11.20–13.00	242.8	$C_{10}H_{11}O_5S^-$	163.1, 147.9, 80.1	Eugenol sulfate
	M9	8.15–8.45	194.2	$C_9H_9O_4N^-$	150.1, 93.2	Salicylic acid glycine
	M10	7.50–7.70	194.2	$C_9H_9O_4N^-$	150.1, 93.0, 74.4	Salicylic acid glycine

Oxidization metabolism

conjugation metabolism



The proposed metabolic pathways of AEE *in vitro* (left) and *in vivo* (right) in dogs

Conclusion

1. *In vitro* metabolism, 5 metabolites (M1,M2,M3,M4,M5) were obtained from AEE hydrolysis and oxidization.

2. *In vivo* metabolism, 6 metabolites (M2,M6,M7,M8,M9,M10) were obtained from conjugation of glucuronic acid, glycine, sulfation, mainly including salicylic acid and eugenol conjugation products .

You Mingshen, Jianyong Li*, *et al*. *In vitro* and In *vivo* metabolism of aspirin eugenol ester in dog by liquid chromatography tandem mass spectrometry. Biomedicinal Chromotography, 2014 Jun 16. doi: 10.1002/bmc.3249.

Acknowledgement

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中国农业科学院兰州畜牧与兽药研究所

LANZHOU INSTITUTE OF HUSBANDRY AND PHARMACEUTICAL SCIENCES OF CAAS

My research groups

Key Lab of New Animal Drug Project of Gansu Province
Gansu Provincial Engineering Research Center for New Animal Drug
Key Lab of Veterinary Pharmaceutical Development, Ministry of Agriculture

Thank for your attention!