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Identification of the major metabolites of resveratrol in rat urine by HPLC-MS/MS

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Abstract

To identify the major metabolites of resveratrol in rat, rat urine samples were pretreated by using solid-phase extraction technique (SPE) with polyamide cartridges. And a LC–MS/MS method with electrospray ionisation (ESI), negative ion mode and collision induced dissociation (CID), was used to elucidate the structures of the major metabolites of resveratrol. According to the results of our experiment, we found that the main metabolites of resveratrol were resveratrol monoglucuronide (M1), dihydroresveratrol monosulfate (M2), resveratrol monosulfate (M3) and dihydroresveratrol (M4).

Keywords: *Trans*-resveratrol; Metabolism; LC–MS/MS; Polyamide SPE

1. Introduction

Resveratrol (3,5,4′-trihidroxistilbene) is a phytochemical occurring naturally in various spermatophytes and present in peanuts [1], grapes [2], wine [3], and Polygonum cuspidatum [4]. Its levels are often increased in plants during pathogen attack, UV irradiation, or exposure to ozone [5–7]. When epidemiological studies indicated an inverse correlation between red wine consumption and the incidence of cardiovascular disease [8], the interest in compounds present in wine increased. Resveratrol is suggested to be one of the agents responsible for this protective effect [7].

Over recent years, the health protecting properties of resveratrol have been well described, such as an antioxidant [9], modulator of lipoprotein metabolism [7,10], inhibitor of platelet aggregation [11,12] and vasorelaxing agent [13,14]. The most important beneficial effect of resveratrol is its cancer chemopreventive activity as it is involved in the inhibition of tumor initiation, promotion and progression [15].

Although many studies have implicated a role of resveratrol in disease prevention, only a few studies have addressed the bioavailability and metabolism of resveratrol. In those papers reported, chromatography-mass spectrometry (LC–MS) and liquid chromatography/tandem mass spectrometry (LC–MS/MS) [16,18] methods were used for the analysis of metabolites of resveratrol in rat urine. Howerver, we found that the urine sample preparation methods in those experiments had some disadvantages such as time consuming, large sample volumes and severe interference. Here we use polyamide cartridges to pretreat the urine sample, and this method was proved to be successful.

2. Experimental

2.1. Chemicals and reagents

Trans-resveratrol (>99%) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were purchased from Merk KGaA (Darmstadt, Germany). Water was of deionized distilled water. The polyamide cartridge (180 mg, particle size 40 μm) was prepared in our laboratory.

2.2. Animals

Five male Sprague–Dawley rats (BW 200 ± 30 g) were housed in metabolic cages with water and a solid diet freely

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available, and maintained at 22 ± 3 °C with 40–70% relative humidity. Each rat was orally given a dose of 20 mg/kg resveratrol. Urine samples were collected from rat at −12 to 0 h predose and at 12 h postdose, and stored at approximately −20 °C before the sample preparation using polyamide solid phase extraction. The research using rats and mouse adhered to the "Principles of Laboratory Animal Care" (NIH publication #85–23, revised in 1985).

2.3. Investigation of the polyamide solid phase extraction (SPE)

2.3.1. Extraction of rat urine samples

Rat urine samples were thawed in a water bath at room temperature and then centrifuged at approximately 2000 × *g* for 5 min. polyamide SPE cartridges (180 mg) were conditioned with 5 ml of methanol and 10 ml of water. Aliquots of 1 ml urine samples were loaded onto the cartridges, which were washed with 10 ml of water and eluted with 4 ml of methanol. The organic layer was evaporated to dryness under nitrogen at 40 °C. The residues were dissolved in 200 μl of 80% acetonitrile and 20 μl was injected for HPLC analysis.

2.3.2. HPLC analysis

The analyses were carried out in Agilent 1100 Series (USA) liquid chromatograph equipped with two pumps (model G1312A), an autosampler (model G1313A) and a VWD detector (model G1314A). A Nucleosil 100 C_{18} reverse-phase column (150 mm × 4.6 mm; particle size, 5 μm; Knauer, Berlin, Germany) protected by a precolumn was used.

For determination of the metabolites of resveratrol, we used H_2O as solvent A, and acetonitrile/water (60/40, v/v) as solvent B, at a flow rate of 1.0 ml min^{-1} with the following gradient: 0–15% B linear (0–10 min), 15–45% B linear (10–16 min), 45–100% B linear (18–25 min), 100% B (25–28 min). This was followed by a 10 min equilibrium period with initial conditions prior to injection of the next sample. samples were filtered (0.45 μm, Millipore) and 20 μl was directly injected. Chromatograms were monitored at 290 nm using the UV detector.

2.4. LC–MS/MS analysis

LC–MS/MS analyses were performed using a system consisting of a Finnigan autosampler (Thermo Electron Corperation,

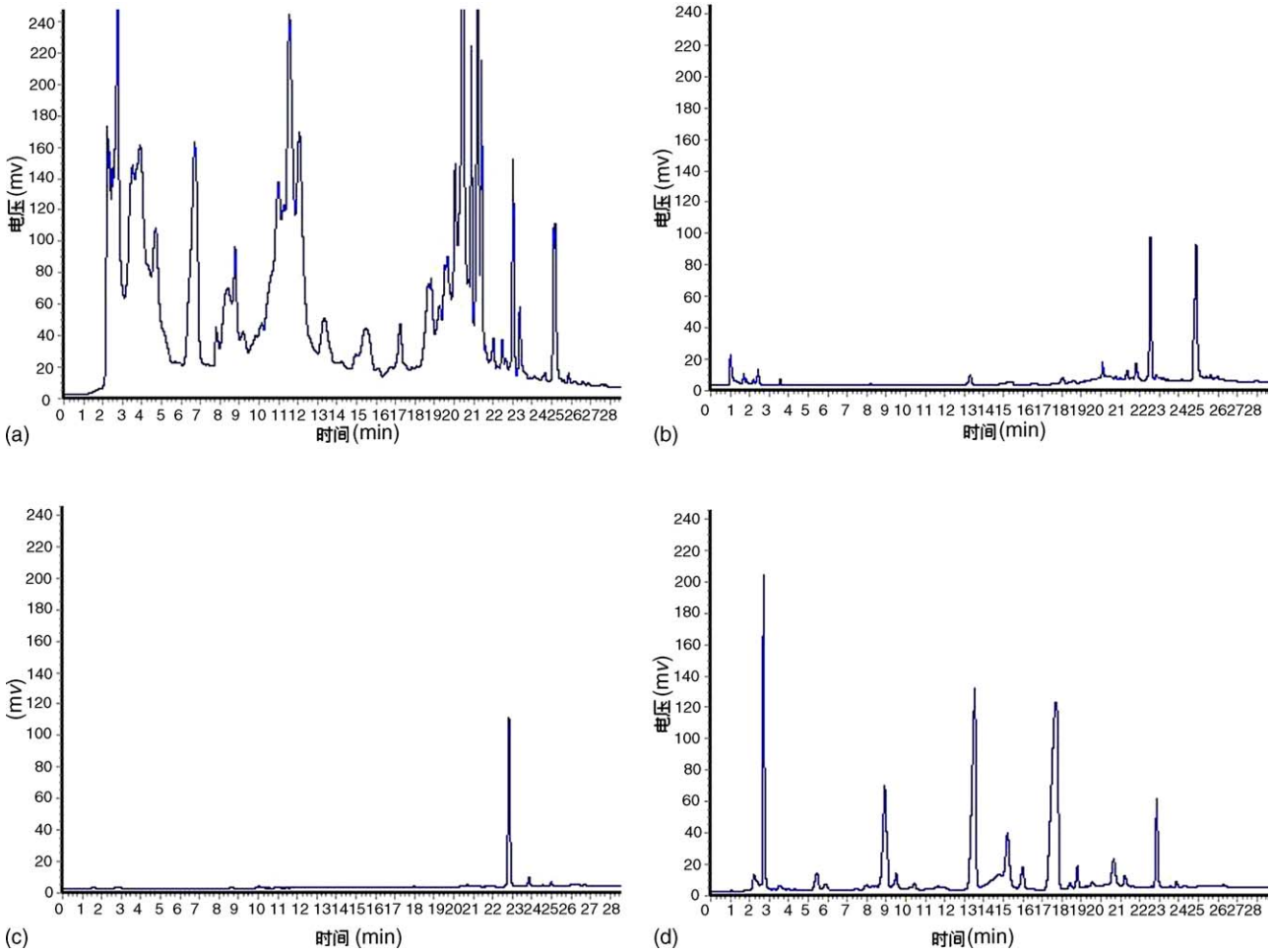

Fig. 1. HPLC chromatograms of resveratrol and its metabolites in rat urine, after oral adminstration of resveratrol. (a) Blank rat urine without SPE; (b) blank rat urine treated with SPE; (c) resveratrol standard; (d) urine sample treated with SPE after oral adminstration ofresveratrol.

USA), a Finnigan LC pump, a Finnigan TSQ Quantum Ultra equipped with an electrospray ion source and operated by XCalibur software.

The separation was carried out by using a C_{18} reverse-phase column (100 mm × 3 mm; particle size, 5 μm; Shimadzu, Japan) protected by a precolumn. we used water as solvent A, and methanol/water (80/20, v/v) as solvent B, at a flow rate of 1.0 ml min^{-1} with the following gradient: 0–15% B linear (0–10 min), 15–45% B linear (10–16 min), 45–100% B linear (18–25 min), 100% B (25–28 min). This was followed by a 10 min equilibrium period with initial conditions prior to injection of the next sample. samples were filtered (0.45 μm, Millipore) and 10 μl was directly injected.

The mass spectral analysis was performed in a negative electrospray ionization mode.The capillary and orifice voltages were set at −3.7 kV and −65 V, respectively. The nebulizer gas was set at 50 psi. The nitrogen auxiliary was ajusted to a constant flow rate of 2 l/min. The turbo-ionspray temperature was set at 400 °C. Collision induced dissociation (CID) studies were performed using collision energy of 30 eV. The ionspray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution.

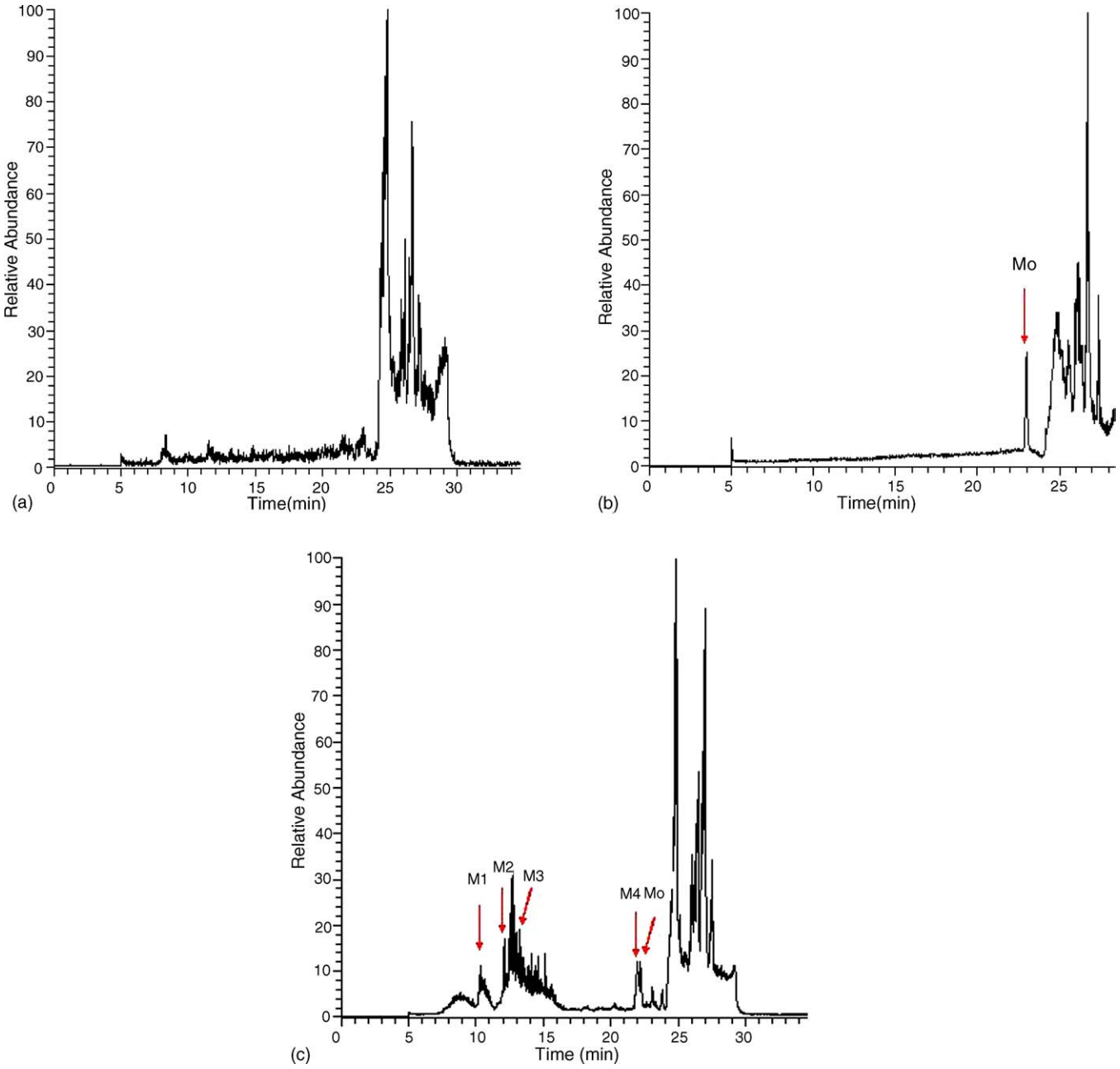

Fig. 2. TIC chromatograms of SPE extract of (a) blank rat urine; (b) resveratrol standard; (c) urine sample. M0: resveratrol; M1: metabolite 1; M2: metabolite 2; M3: metabolite 3; M4: metabolite 4.

3. Results and discussion

3.1. Chromatograms of rat urine sample before and after SPE treatment

In order to eliminate the endogenesis interferer, rat urine samples were cleaned up by using solid-phase extraction (SPE) with polyamide cartridges. The chromatograms of these samples were illustrated in Fig. 1. It is clear that the chromatograms of sample treated with SPE were excellent than that of untreated.

3.2. LC–MS/MS identification of the metabolites of resveratrol

3.2.1. Total ion current chromatograms of rat urine samples treated with SPE

The structures of metabolites were elucidated by electrospray LC–MS/MS using a combination of full and product ion scanning techniques. Under the HPLC condition described above, TIC chromatograms of blank urine, resveratrol standard and urine sample were listed below in Fig. 2. Compared chromatograms of urine sample treated with SPE after oral administration of resveratrol with that of the blank, we can find the peaks of M0, M1, M2, M3 and M4 that represent resveratrol (M0) and its metabolites (M1–M4), respectively.

3.2.2. Resveratrol (M0)

As illustrated in Fig. 3, the negative electrospray mass spectrum of resveratrol (M0, Tr = 22.1 min) showed a $[M-H]^-$ ion at *m*/*z* 227. The CID spectrum of *m*/*z* 227 generated a series of fragment ions at 185, 143, 117 and 119. The fragment ion at *m*/*z* 185 was generated from the loss of 42(C_2H_2O) from the ion at *m*/*z* 227. The *m*/*z* 185 further fragmented to *m*/*z* 143 after loss of 42(C_2H_2O). The *m*/*z* 119 and 117 were formed after the losses of 108($C_6H_4O_2$) and 110($C_6H_6O_2$), respectively. This result is consistent with that of the report [18]. The fragmentation pathway may be explained according to Fig. 4.

3.2.3. Metabolite M1

The mass spectra of M1 were listed in Fig. 5. Full mass of M1 which was detected at 10.1 min gave a $[M-H]^-$ ion at *m*/*z* 403, 176 Da higher than that of resveratrol. The CID spectrum of M1 showed a series of fragment ions at *m*/*z* 227, 185, 175, 117, and

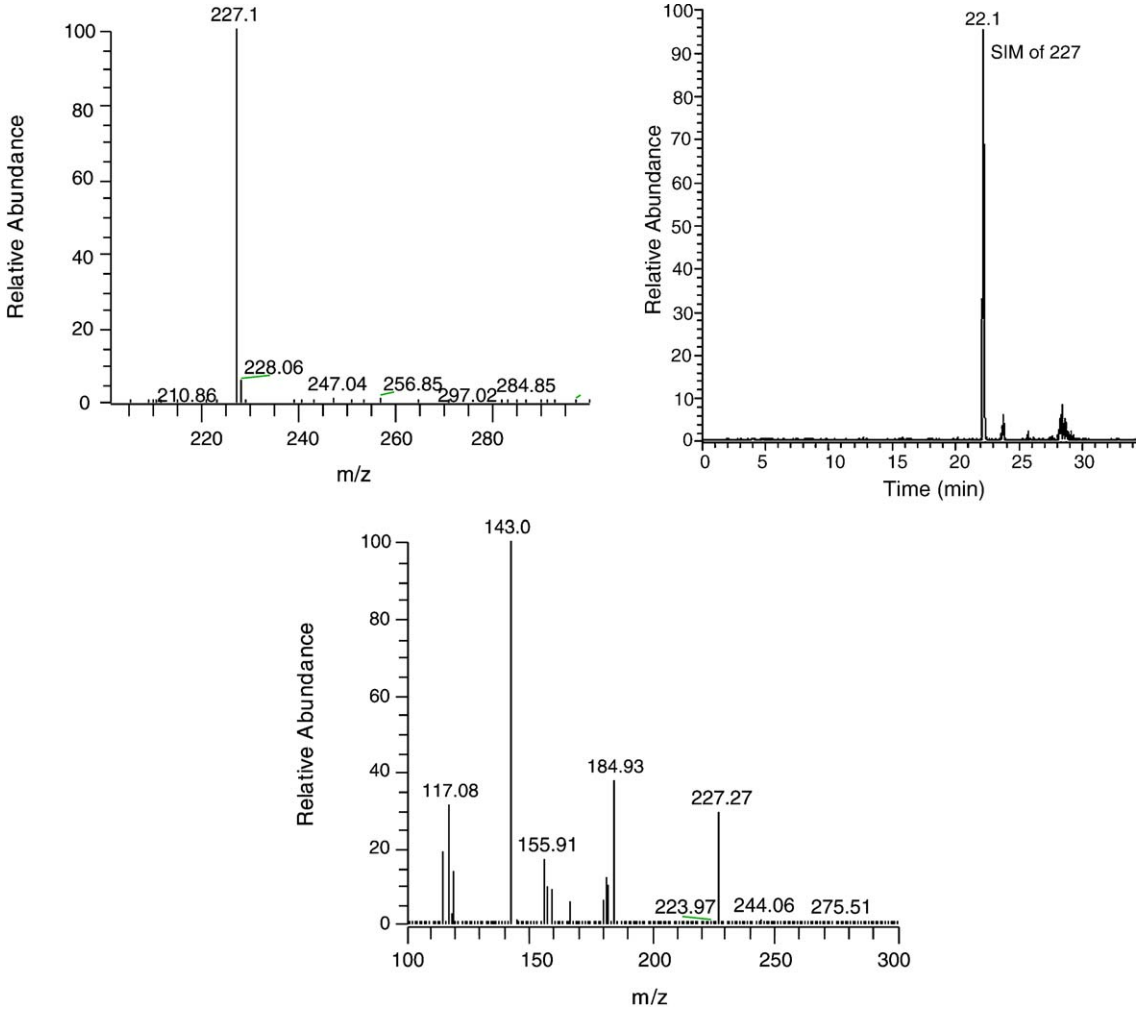

Fig. 3. LC–MS/MS spectra of resveratrol (M0, *m*/*z* 227) obtained using negative ion electrospray and CID.

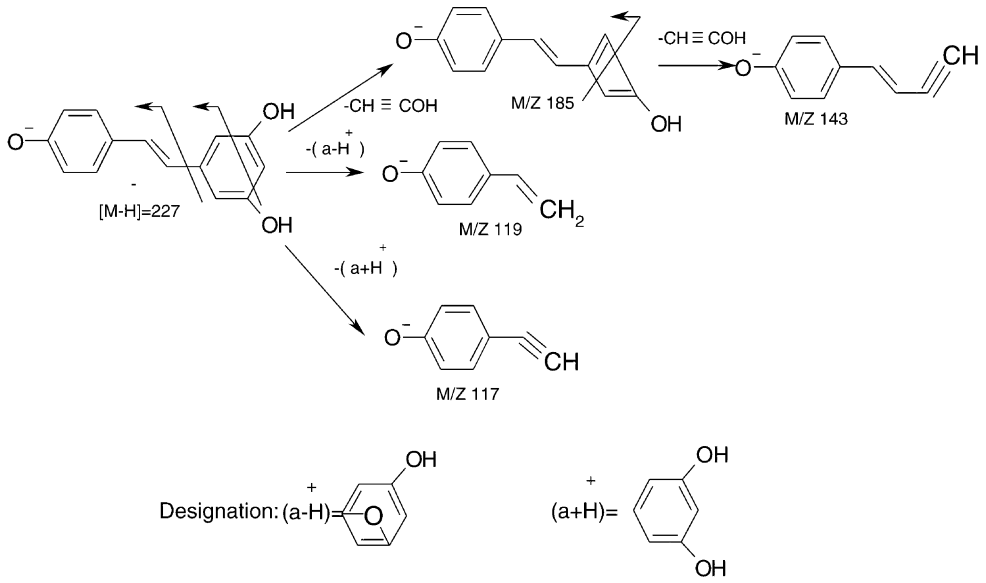

Fig. 4. Proposed mechanism for the decomposition of the *m/z* 227 $[M-H]^-$ ion of resveratrol.

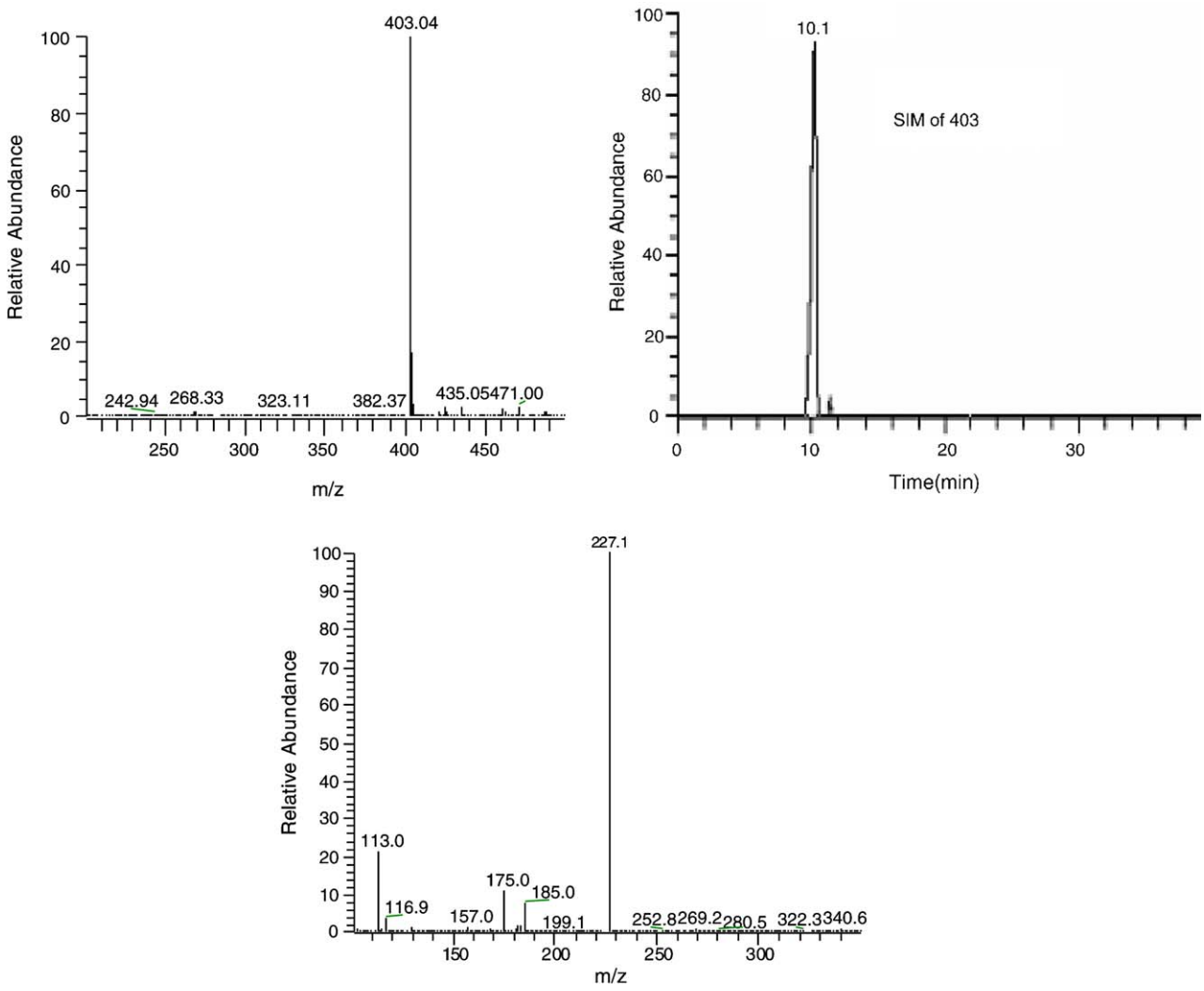

Fig. 5. LC–MS/MS spectra of M1 (*m/z* 403) obtained using negative ion electrospray and CID.

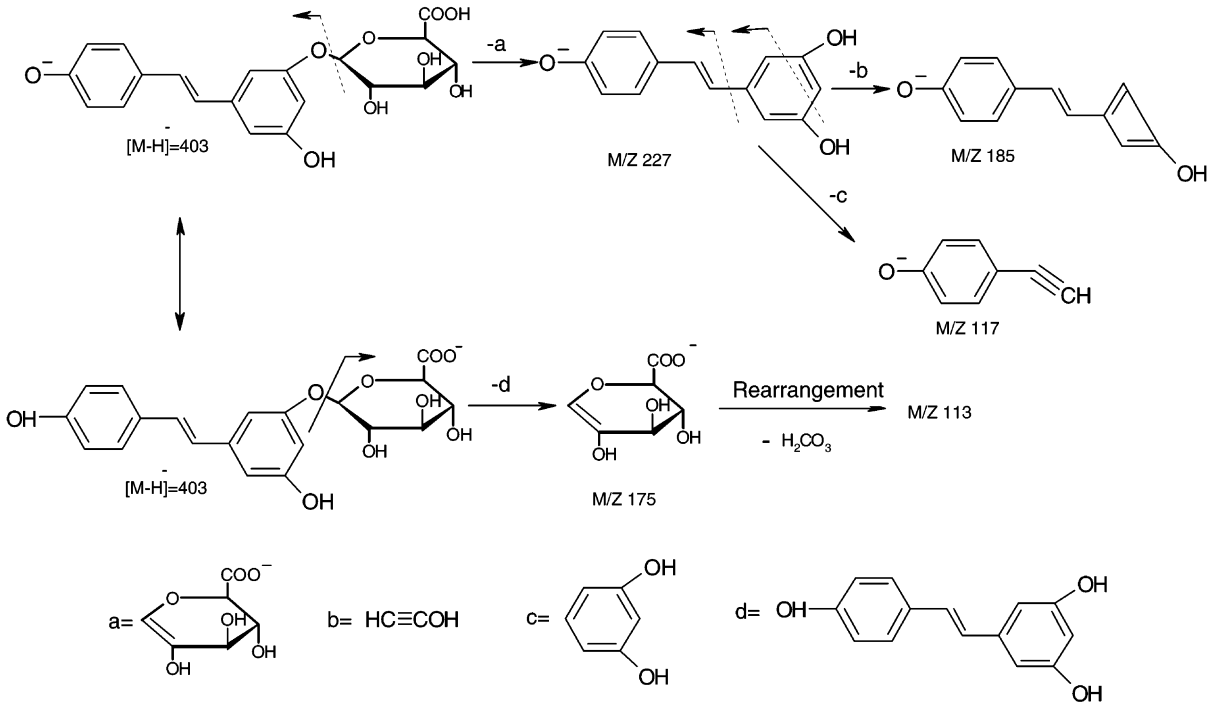

Fig. 6. Proposed mechanism for the decomposition of the *m*/*z* 403 $[M-H]^-$ ion of metabolite.

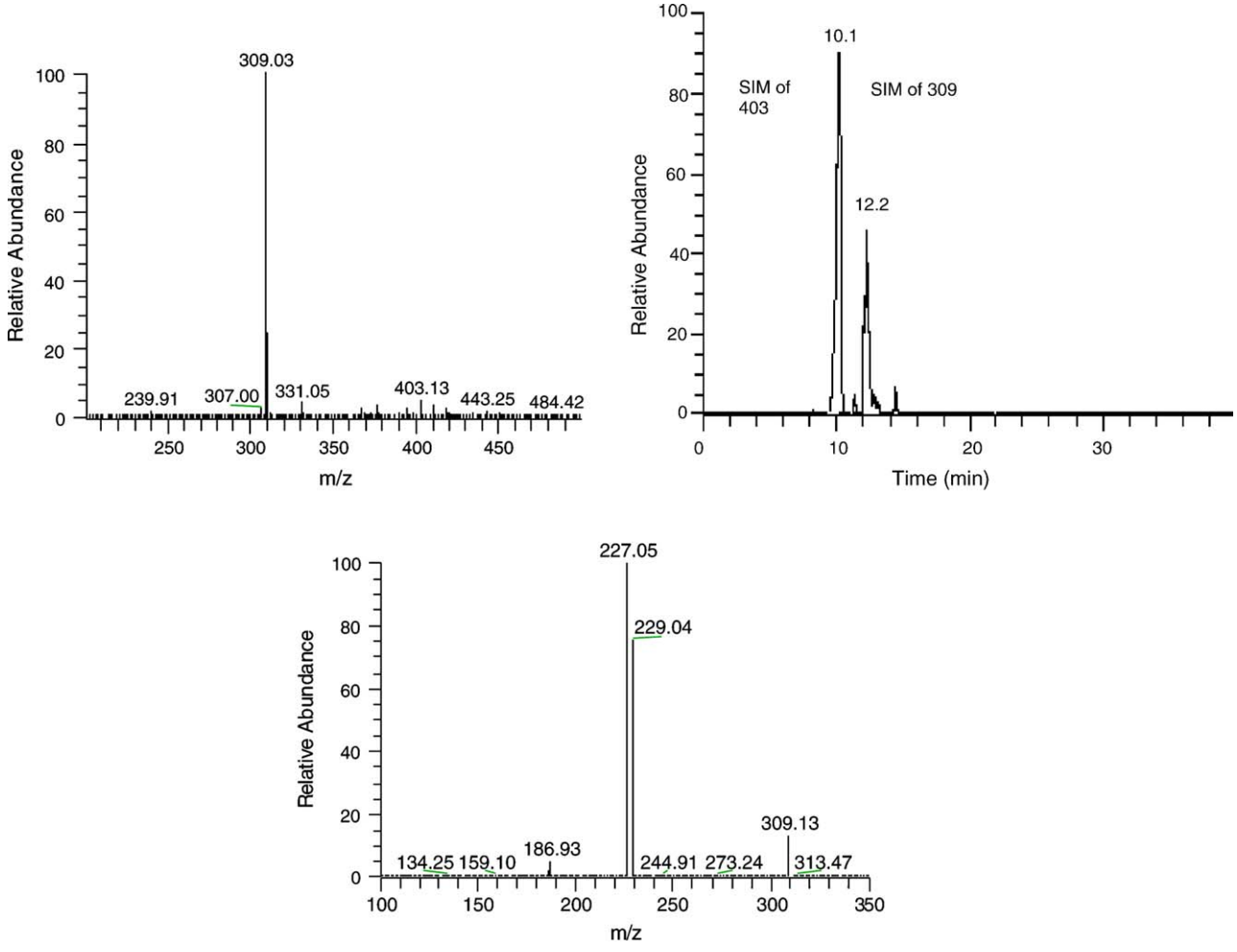

Fig. 7. LC–MS/MS spectra of M2 (*m*/*z* 309) obtained using negative ion electrospray and CID.

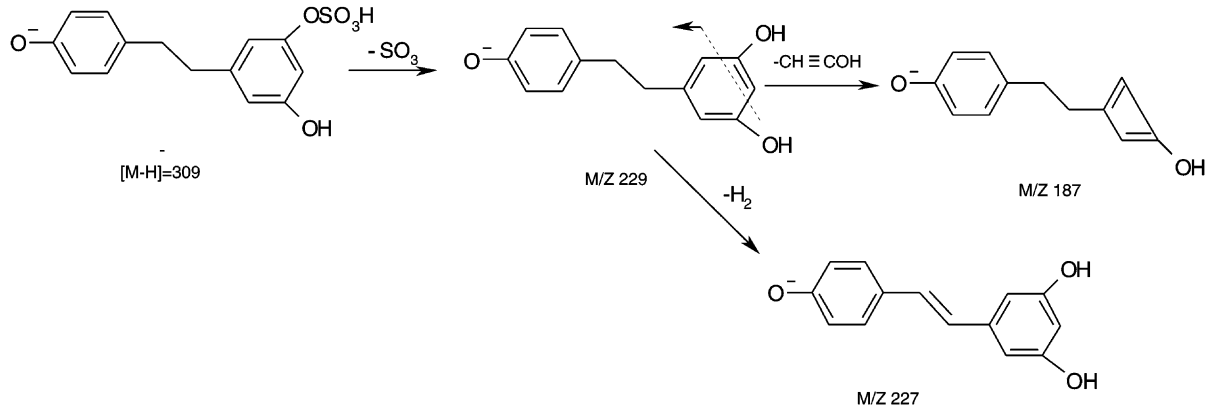

Fig. 8. Proposed mechanism for the decomposition of the *m/z* 309 $[M-H]^-$ ion of metabolite.

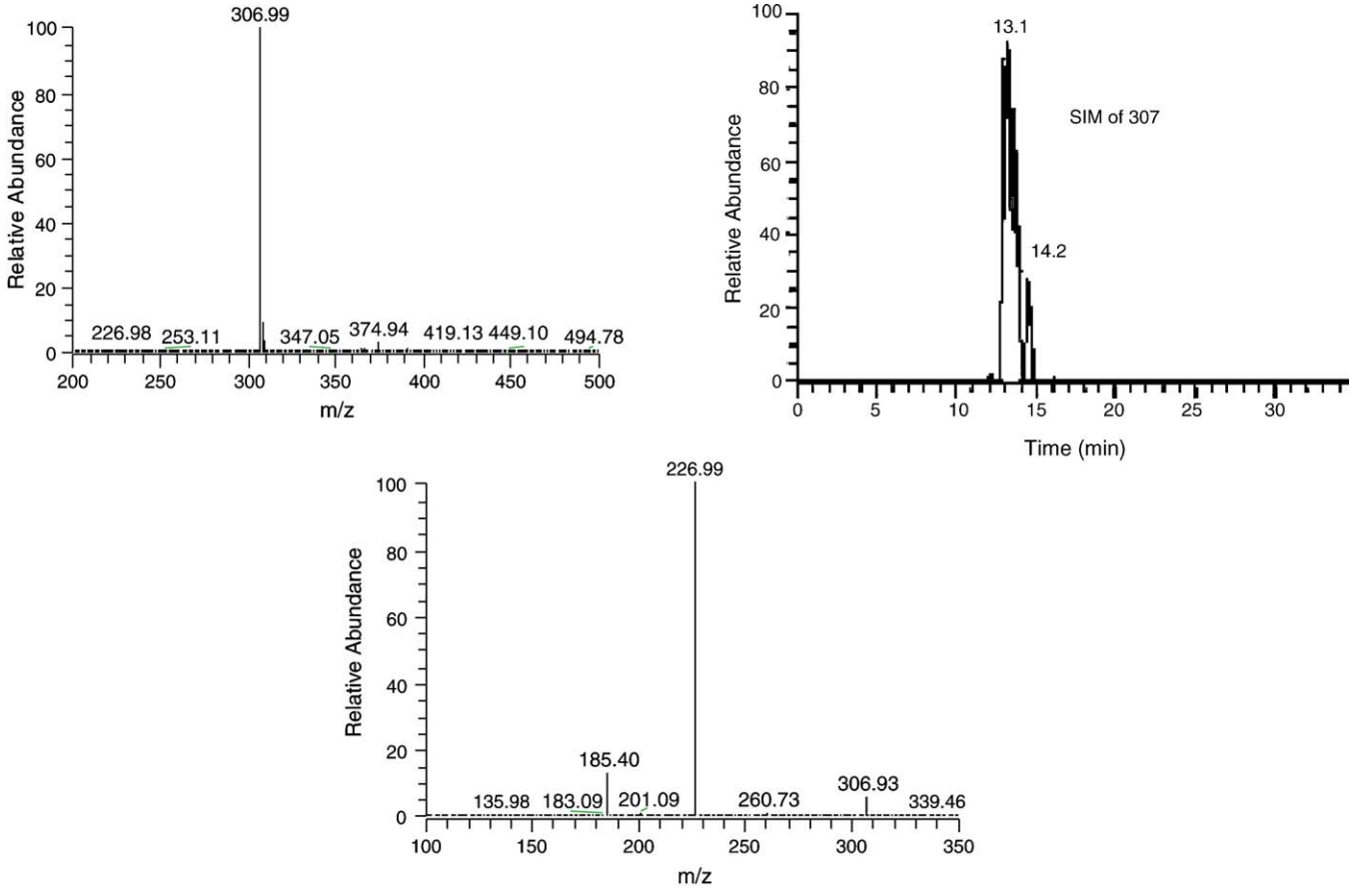

Fig. 9. LC–MS/MS spectra of M3 (*m/z* 307) obtained using negative ion electrospray and CID.

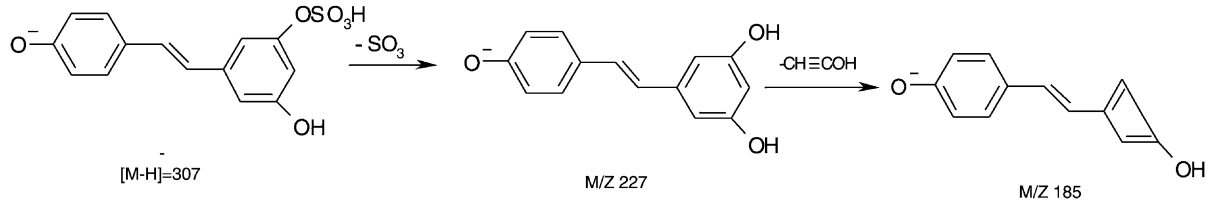

Fig. 10. Proposed mechanism for the decomposition of the *m/z* 307 $[M-H]^-$ ion of metabolite.

113 (Fig. 5). *m/z* 227, 185, 117 corresponded to the characteristic fragment ions of resveratrol. *m/z* 175 and 113 can be considered characteristic of the presence of a glucuronic acid moiety [19–21]. The ion at *m/z* 113 was formed by further dissociation of the fragment ion of *m/z* 175 and is common in the negative ion mass spectra of glucuronide metabolites. Therefore, the peak at 18.1 min was identified as resveratrol monoglucuronide. The fragmentation pathway may be explained in terms of Fig. 6.

3.2.4. Metabolite M2

The mass spectra of M2 were listed in Fig. 7. The $[M-H]^-$ ion of M2 (Tr = 12.1 min) was at *m/z* 309. The *m/z* 309 was 2 Da higher than that of resveratrol monosulfate (see M3), and was consistant with that of dihydroresveratrol monosulfate. The CID spectrum of M2 yielded fragment ions at *m/z* 229, 227 and 187. The *m/z* 229 was 2 Da higher than that of resveratrol (*m/z* 227), and was consistant with dihydroresveratrol. The *m/z* 229 was formed from *m/z* 309 after loss of 80(SO_3). The further loss of 42(C_2H_2O) from the *m/z* 229 produced the ion at 187. The loss of 2(H_2) from the *m/z* 229 produced the ion at 227. Therefore, M2 can be identified as dihydroresveratrol monosulfate. The fragmentation pathway may be explained according to Fig. 8.

3.2.5. Metabolite M3

The $[M-H]^-$ ion of *m/z* 307 of M3 (Tr = 13.1) fragmented to form a product ion of *m/z* 227 that corresponded to resveratrol itself after the loss of sulfate. The *m/z* 227 further fragmented to *m/z* 185. Therefore, the peak at 13.1 min was identified as resveratrol monosulfate (MW = 307) (see Fig. 9). Only one resveratrol monosulfate and no disulfate metabolites were detected. The fragmentation pathway may be explained according to (Fig. 10).

3.2.6. Metabolite M4

The $[M-H]^-$ ion of M4 (Tr = 22.0 min) was at *m/z* 229, 2 Da higher than that of resveratrol. The CID spectrum of M4 yielded fragment ions at *m/z* 187, 145 and 123. The fragment ions at 187 was formed by the loss of 42(C_2H_2O). The further loss of 42(C_2H_2O) from the *m/z* 187 produced the ion at 145. The fragment ion at *m/z* 123 was generated from the loss of 106(C_7H_6O). Peaks at *m/z* 459 and 481 may be of $[2M-H]^-$ and $[2M-2H+Na]^-$ (see Fig. 11), respectively. The LC–MS/MS data indicated that M4 was dihydroresveratrol. The fragmentation pathway may be explained according to Fig. 12.

3.3. The biotransformation pathway of resveratrol

According to the result of our experiment above, the main metabolites of resveratrol (M0) were resveratrol monoglucuronide (M1), dihydroresveratrolmonosulfate (M2), resveratrol monosulfate (M3) and dihydroresveratrol (M4). Therefore, the possible metabolic pathway of resveratrol in vivo were proposed as shown in Fig. 13.

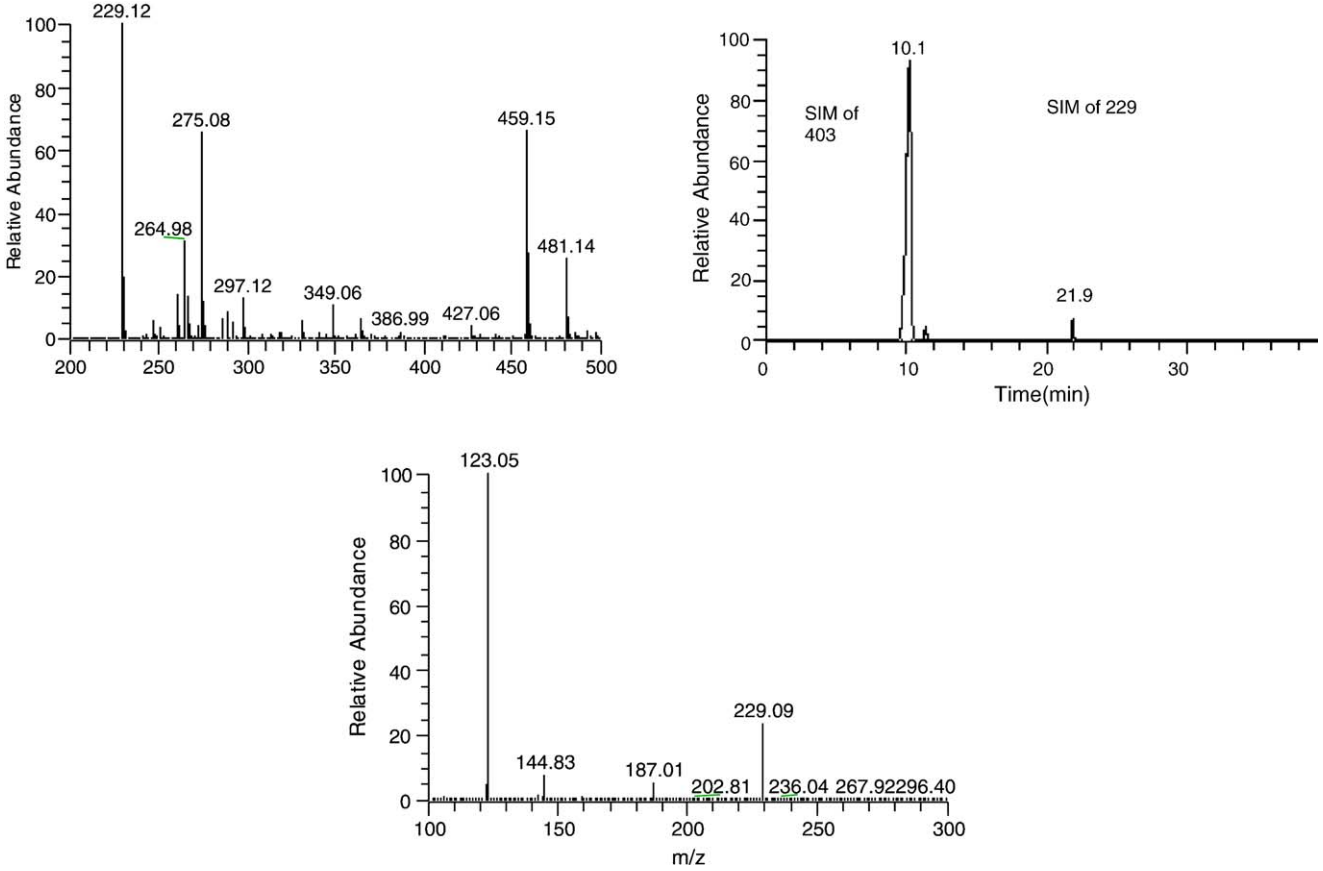

Fig. 11. LC–MS/MS spectra of M4 (*m/z* 403) obtained using negative ion electrospray and CID.

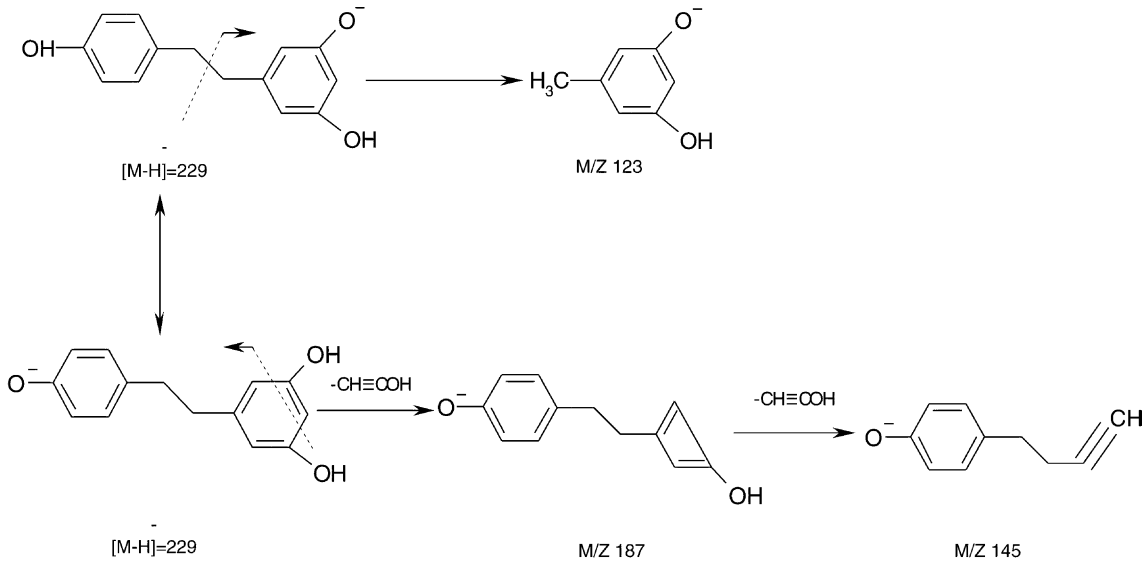

Fig. 12. Proposed mechanism for the decomposition of the *m/z* 229 $[M-H]^-$ ion of metabolite.

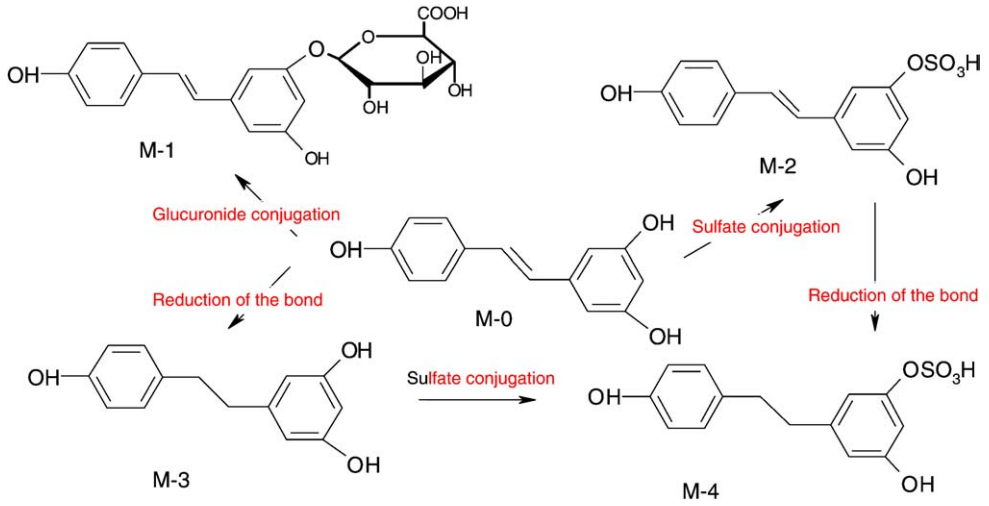

Fig. 13. The proposed metabolic pathway of resveratrol in vivo by rat.

4. Conclusions

The oldest and most basic sample preparation method is extraction, in which the analyst aims to separate the analyte of interest from a sample matrix using a solvent with an optimum yield and selectivity, so that as few potential interfering species as possible are carried through to the analytical separation stage. Solid-phase extraction technique (SPE) is one of the methods that usually used, and is often applied in the extraction of drugs and their metabolites from body fluids. In our experiment, we fond that polyamide cartridges, mainly used for the seperation of compound containning hydroxyl or carbonyl group, was best suitable for the pretreatment of urine sample of rat after ig resveratrol.

The superb sensitivity of ESI in combination with tandem mass spectrometry provides information for comprehensive structural characterization of the metabolites of resveratrol. Our LC–MS/MS data for the experiments indicated that resveratrol monoglucuronide (M1), 7,8-dihydroresveratrol (M2), resveratrol monosulfate (M3) and 7,8-dihydroresveratrol monosulfate (M4) were the main urinary metabolites, and these metabolites were confirmed by literatures, respectively [16,18,22].

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