

Discovery of nitrogenous derivatives of dihydromyricetin in cell culture medium

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Abstract

Dihydromyricetin (DMY) is a health-beneficial flavanone in the family of flavonoids, extracted from *Ampelopsis grossedentata* (Hand.-Mazz.) W. Wang. When investigating the stability of DMY in Dulbecco's modified Eagle's medium (DMEM) solution at 37°C by LC-MS/MS, four nitrogenous derivatives of DMY were found except for the previously known dimers, oxidized products, and ring-cleavage products of DMY. Compound 1 (N-DMY) was identified to be 4'-amino (NH₂)-DMY based on MS and NMR data. The formation mechanism of N-DMY was thought to include two steps. First, the pyrogallol moiety on the ring B of DMY was prone to be oxidized to quinone. Second, quinone might further participate in the Strecker degradation of amino acids in DMEM to form N-DMY and corresponding aldehydes. Correspondingly, compounds 2–4 might be formed by the Strecker degradation reaction of amino acids in DMEM with quinones from the oxidation of DMY dimers and DMY ring-cleavage products. Consistent with the formation mechanism, it was observed that flavonoids with a catechol or pyrogallol moiety readily form their nitrogenous derivatives during the incubation in DMEM at 37°C, which might be because catechol or pyrogallol are easily oxidized to quinones. In addition, the nitrogenous derivatives of flavonoids may have a potential to participate in the metabolism of flavonoids during in vitro and in vivo experiments. Therefore, flavonoid nitrogenous derivatives may play a significant role in the actual effects of flavonoids, which need to be further investigated in the future.

KEYWORDS

amino acid, dihydromyricetin, DMEM, nitrogenous derivative, quinone, strecker degradation

1 | INTRODUCTION

As secondary metabolites of plants, flavonoids are a kind of important polyphenols, which widely exist in natural plants, mainly in the form of glycosides, and some in free form. Flavonoids have been gaining considerable attention for possessing various health-benefit effects, including antioxidant activity, anti-inflammatory activity, anticancer activity, antidiabetic activity, and anti-mutagenic

activity (Higbee et al., 2022; Maheshwari et al., 2022; H. Zhang et al., 2021).

However, the practical use of flavonoids is often limited due to the chemical instability of flavonoids caused by many factors, such as pH, temperature, light, oxygen availability, metal ions, and so on (Deng et al., 2018). A previous study found that flavonoids were rapidly degraded in cell culture medium, while they are relatively more stable in human fluid, plasma (Xiao & Högger, 2015). Therefore, the flavonoids might have

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been rapidly and dramatically degraded into other compounds before interacting with cells during the in vitro cell culture. These degradation products of flavonoids may have considerable effects on cells and cause artificial results for the cell experiments of flavonoids. DMEM is a commonly used cell culture medium during in vitro experiment, which contains numerous amino acids, glucose, vitamins, and so on. We have been focusing on investigating the stability of flavonoids in DMEM to avoid the underlying artifacts during the in vitro cell experiments. Previous studies indicated that many flavonoids were instable in DMEM due to the complex components of DMEM (Xiao & Högger, 2015; Xiao, 2018). Flavonoids, such as myricetin (Cao et al., 2020), quercetin (Cao et al., 2022), epigallocatechin gallate (EGCG) (Wang et al., 2022), and dihydromyricetin (DMY) (H. L. Zhang et al., 2022) easily degraded and were converted into dimers, oxidized products and ring-cleavage products in DMEM solution at 37°C.

Interestingly, during our previous study about the degradation products of some flavonoids in DMEM solution at 37°C, it was found that a class of flavonoid nitrogenous derivatives might commonly exist. However, the structure and formation mechanism of flavonoid nitrogenous derivatives were not clear.

Some past studies reported some nitrogenous derivatives of polyphenols, which were usually produced based on two steps. First, polyphenols were converted into quinones. Then, quinones with redox potential quickly further reacted with other amines to form nitrogenous derivatives of polyphenols (Haider et al., 1965; Rizzi, 2006). A study found that green tea extract could exhibit deodorizing effect on amines by eliminating amines to generate nitrogenous derivatives of tea catechins. For example, nitrogenous reaction products of (-)-epigallocatechin (EGC) with ammonia, methylamine, and ethylamine were detected, which were formed by replacing the hydroxyl group (OH) with an amino group (NH₂) at the 4' position of EGC (Kida et al., 2002). Another study reported some nitrogenous products formed by the reaction of condensed tannins and related polyphenols with ammonia (NH₃) water. The results indicated that regioselective amination occurred when the compounds containing pyrogallol-type groups were treated with NH₃ water in the presence of oxygen. For example, with NH₃ water treatment, with a pyrogallol-type group, pyrogallol, gallic acid, and epigallocatechin were transformed into 2-aminoresorcinol, 4-amino-3,5-dihydroxybenzoic acid and 4'-C-amino-epigallocatechin, respectively. While, for polyphenols with catechol-type groups, such as catechol, protocatechuic acid, and epicatechin, similar amination reaction will not occur. Besides, the possible amination mechanism was proposed as that the catechol nucleus was easy to be oxidized into *ortho*-quinone intermediate under alkaline conditions (NH₃ water treatment), and further generated nitrogenous derivatives (Braghiroli et al., 2013; Hashida et al., 2009).

In addition, another significant formation mechanism of nitrogenous derivatives of polyphenols was reported that nitrogenous derivatives of polyphenols were products of Strecker degradation of α -amino acids by polyphenol-derived quinones. The reaction term "Strecker degradation" was first proposed in 1948 for describing the degradation of α -amino acids by carbonyl compounds, to aldehydes and ketones containing one carbon atom less (Schönberg et al., 1948). Actually, as a carbonyl compound, quinones were also involved in the Strecker degradation, which often were ignored. Quinones are instable intermediates formed by oxidation of polyphenols and could react with nucleophilic reagents. Quinones could form covalent adducts with amino acids, peptides, and proteins (Rizzi, 2006; Schieber, 2018). Generally, high temperature could promote Strecker degradation during food processing (Fujimaki et al., 1968). However, at a lower temperature (22°C), Strecker degradation could also occur in nonenzymic models containing catechol-derived quinones and amino acids to form volatile aldehydes (Rizzi, 2006). In addition, Strecker degradation plays a significant role during the black tea fermentation, because volatile aldehydes could also be formed by coupled oxidation of catechins and amino acids (Chen et al., 2021).

Besides, a previous study showed that amination of myricetin could occur in vivo in myricetin-treated mice. A nitrogenous metabolite was characterized as 4'-NH₂-myricetin ($m/z = 316.046$) in the mouse fecal samples by UPLC-MS/MS under negative ion mode. The synthesis mechanism of 4'-NH₂-myricetin was proposed as that with a pyrogallol group on the B ring, myricetin could be first transformed into myricetin quinone, and further react with ammonia to form 4'-NH₂-myricetin (Zhang, Wang, et al., 2019). In another study, amination of EGCG was observed in mice and in humans. An aminated EGCG metabolite in mice, 4'-NH₂-EGCG, was thought to be formed by oxidation of EGCG quinone, and then quick reaction with ammonia. Especially, gut microbiota was found to play an important role in the formation of the aminated metabolite of EGCG. In addition, 4'-NH₂-EGCG showed similar antitumor effect on human colon cancer cells and relative anti-inflammatory effect on LPS-activated macrophages with that of EGCG (Zhang, Zhao, et al., 2019).

Our study focuses on a flavanone, DMY, extracted from *Ampelopsis grossedentata* (Hand.-Mazz.) W.T. Wang (H. Zhang et al., 2021). DMY was significantly instable in DMEM at 37°C due to its polyhydroxy structure. Especially, the pyrogallol-type structure on ring B of DMY is readily oxidized into high reactive *ortho*-quinone. Based on the previous results, DMY degraded rapidly and formed many degradation products via dimerization, further oxidation and ring-cleavage in DMEM at 37°C (H. L. Zhang et al., 2022). Interestingly, except for the above common degradation products of DMY, some unknown degradation products

of DMY in DMEM were identified as nitrogenous derivatives of DMY according to the exact mass and the nitrogen rule. More importantly, except for cell culture medium, nitrogenous derivatives of some flavonoids were also observed in intracellular extract during our *in vitro* cell experiments of flavonoids. Hence, flavonoid nitrogenous derivatives produced in cell culture medium might have considerable effects on the cells, which probably led to artifacts when evaluating the bioactivities of original flavonoids during the *in vitro* cell experiments. However, the formation and underlying mechanism of flavonoids nitrogenous derivatives are unclear. Therefore, further exploring the formation and underlying mechanism of DMY nitrogenous derivatives in DMEM are very meaningful.

In this study, we thoroughly investigated the structure and formation mechanism of DMY nitrogenous derivatives in DMEM solution at 37°C through LC-MS/MS and NMR.

2 | METHODS AND MATERIALS

2.1 | Chemicals and reagents

DMY (>97.0%) was provided by Tokyo Chemical Industry Co., Ltd. Dimethyl sulfoxide (DMSO) was acquired from Alfa Aesar Chemical Co., Ltd. DMEM (high glucose) and Phosphate Buffered Saline (1X) (PBS) were obtained from GE Healthcare Life Sciences HyClone Laboratories. Fetal Bovine Serum (FBS) was provided by Gibco. LCMS-grade acetonitrile and methanol were purchased from RCI Labscan LTD. Ultrapure water was prepared with a Milli-Q Integral water purification system (Millipore Corp.). Formic acid (>99.9%) was purchased from fisher scientific. All amino acids, including glycine (99%), L-arginine (99%), L-cystine (99%), L-glutamine (99%), L-histidine (98%), L-isoleucine (99%), L-leucine (99%), L-lysine (99%), L-methionine (99%), L-phenylalanine (99%), L-serine (99%), L-threonine (99%), L-tryptophan (99%), L-tyrosine (99%), and L-valine (99%), were purchased from J&K Scientific Ltd. The 2,4-dinitrophenylhydrazine (2,4-DNPH) and phenylacetaldehyde were obtained from Aladdin Industrial Corporation.

2.2 | UHPLC separation, MS detection and data processing

All samples were analyzed on a Thermo UltiMate 3000 UHPLC system and a Thermo Scientific LTQ Orbitrap XL hybrid FT Mass Spectrometer. Liquid phase system conditions were as follows: Chromatographic separation was performed on a Waters ACQUITY UPLC HSS T3 1.8 μm , 2.1 $\times$ 150 mm column (Waters Technology). The mobile phase was composed of 0.1% formic acid in

Milli-Q water (A) and acetonitrile (B). The temperature of column oven was at room temperature (23°C). The autosampler was set at 37°C. The flow rate was set at 0.3 mL/min. The injection volume was 10 μL . The total run time was 60 min. The samples were eluted with the following gradient: −10 to 0 min, 10% B; 0–10 min, 10% B; 10–35 min, 10%–35% B; 35–40 min, 35%–45% B; 40–45 min, 45%–40% B; 45–50 min, 40%–10% B. The conditions for mass spectrometry are as follows: Detection was using electrospray ionization (ESI) under positive (+) or negative (−) mode. Data were acquired in the full MS scans mode with a range of mass to charge ratio (m/z) = 50–1500 and a mass resolution of 30,000. And, the MS/MS data were obtained in the data dependent scan mode. Capillary temperature was 380°C. Sheath gas (N_2) flow rate was 48 arbitrary units (arb). Auxiliary gas flow rate was 15 arb. Ion spray voltage was 4.5 kV. The acquisition time was 50 min. Xcalibur software (Thermo Fisher Scientific) was used for data collection and analysis.

2.3 | Preparation of DMY stock solution and DMEM solution

DMY stock solutions: DMY was dissolved in DMSO to acquire stock solutions of 5×10^{-3} mol/L. Then, stock solution was stored at −20°C. DMEM solution: DMEM (high glucose) supplemented with 10% FBS were prepared in advance and stored at 0–4°C. After addition of FBS, the pH of DMEM still remained stable within the range of 7–8.

2.4 | In situ analysis exploring the changes of DMY in DMEM solution at 37°C via UPLC-MS/MS

In our previous study, *in situ* analysis had been used for investigating the stability of some flavonoids in DMEM solution based on UPLC-MS/MS (Cao et al., 2020, 2022; Wang et al., 2022; H. L. Zhang et al., 2022). The real-time and nondestructive features made *in situ* analysis perform well in studying the complex changes of flavonoids in DMEM solution. Therefore, in the present study, *in situ* analysis was applied to explore new products of DMY in DMEM solution at 37°C, especially DMY nitrogenous derivatives. In an autosampler vial, 100 μL of DMY standard solution (5×10^{-3} mol/L) was mixed with 900 μL of DMEM solution (high glucose DMEM supplemented with 10%FBS). Then, the vial was immediately put into the 37°C autosampler. Every 60 min, 10.0 μL of sample solution was automatically injected into the UPLC-MS/MS system. Under the same condition, DMEM and DMY stock solutions (5×10^{-3} mol/L) were used as blank control and standard control, respectively.

2.5 | Structural elucidation procedure

The raw LC-MS/MS data were collected using Xcalibur software (Thermo Fisher Scientific), including MS¹ and MS² data, retention times, quasi-molecular ions and fragment ions information. The structure characterization of new products peaks was based on a systematic procedure mentioned in our previous research (H. L. Zhang et al., 2022). At first, according to the measured exact mass in MS, molecular formulas of new product peaks were predicted. Then, combining with the characteristic fragment ions of DMY, new degradation products of DMY were preliminarily screened. Significantly, retro Diels–Alder (RDA) reaction on the C ring of flavonoids was a valuable fragmentation mechanism in MS, and could provide diagnostic product ions for various flavonoids to screen their products (de Rijke et al., 2006; Pinheiro & Justino, 2012). Finally, possible structures of new products were inferred based on fragment ions information provided by MS.

2.6 | Structural elucidation of nitrogenous derivatives of DMY by LC-MS/MS

Meanwhile, nitrogen rule was considered for the structure identification of DMY nitrogenous derivatives. Some peaks of new products with even m/z were predicted to contain odd (probably one) nitrogen atom and other peaks of new products with odd m/z also might contain even (probably two) nitrogen atoms. The structural elucidation of nitrogenous derivatives of DMY was based on the same methods as above in Section 2.5.

2.7 | Structure verification of compounds

Mass frontier 8.1 software (Thermo Fisher Scientific) was applied to evaluate the rationality of the predicted compound structures to a certain extent. Compare the actual fragments ions with top 15 intensity with theoretical fragments ions (obtained from Mass frontier 8.1 software) to verify whether the proposed structures are rational. In addition, Mass frontier 8.1 software could help predict the main fragmentation pathways of the compounds based on fragment ions and neutral loss. Finally, four DMY nitrogenous derivatives, compounds 1–4, were identified and their possible structures are shown in Figure 2. And compound 1 was named as N-DMY.

2.8 | The preparation of compound 1 (N-DMY)

To determine the structure more precisely, compounds need to be further identified by NMR. However, due to the complexity of cell culture medium, DMY

nitrogenous derivatives were difficult to be separated and purified for NMR detection. Combining the prediction results from MS, the structure of compound 1 (N-DMY) was predicted to contain one nitrogen atom. A previous study reported that a nitrogenous derivative, 4'-NH₂-myricetin, could be prepared by reaction of ammonia and the pyrogallol moiety on the ring B myricetin. Possessing a similar pyrogallol moiety on the ring B, N-DMY was attempted to be prepared by DMY and ammonia referring to the preparation method of 4'-NH₂-myricetin (Zhang, Wang, et al., 2019). The detailed procedures were as below. First, 10 mg of DMY powder was weighed and 2 ml of ammonia was taken. Then, mixed them by vortex for 1 min, immediately froze with the liquid nitrogen, then put them in a vacuum freeze drier for 5–6 h. Repeated the process several times, combined all obtained new products as the final product and stored it at –20°C for further research.

2.9 | Structural characterization of compound 1 (N-DMY)

The structure of prepared N-DMY was characterized by MS and NMR methods. MS detection was performed on a Thermo UHPLC-LTQ Orbitrap XL hybrid FT Mass Spectrometer. At first, the retention times, mass-to-charge ratio, and fragment ions of the final prepared N-DMY product were compared with those of the compound 1 produced in DMEM to verify whether the prepared N-DMY product is consistent with compound 1.

Then, we tried to separate the pure N-DMY from the prepared mixture by semi-preparative HPLC. However, the obtained N-DMY even contained more impurities, which might be because N-DMY was instable and easily degraded into many other products during the separation process. Therefore, we prepared N-DMY mixture referring to a previous method to prepare 4'-NH₂-myricetin (Zhang, Wang, et al., 2019), which was finally used for ¹H and ¹³C nuclear magnetic resonance (NMR) determination to characterize the structure of compound 1 (N-DMY). After dissolving the prepared N-DMY mixture in DMSO-*d*₆, the ¹H and ¹³C NMR spectra of the prepared N-DMY were recorded with a Bruker AVANCE III 600 M spectrometer (600 MHz) (Bruker, Germany). Data were processed with the MestReNova 11.0.3 software (Mestrelab Research S.L.).

2.10 | Investigation of the N-DMY formation mechanism

DMY was speculated to participate in the Strecker degradation of amino acids in DMEM to produce N-DMY and corresponding aldehydes. To verify this proposed formation mechanism of N-DMY, DMY was incubated in amino acid solutions in PBS to determine

TABLE 1 Chemical formulas and theoretical m/z comparison of individual dihydromyricetin (DMY), DMY dimers, ring-cleavage products of DMY dimers and their nitrogenous derivatives (compounds 1–4) in DMEM at 37°C at positive ion mode

DMY and its dimers, ring-cleavage products of dimers				Nitrogenous derivatives		
Name	Chemical formula	Theoretical m/z (H ⁺)	Changes of atoms	Name	Chemical formula	Theoretical m/z (H ⁺)
DMY	C ₁₅ H ₁₂ O	321.061	–O + NH	Compound 1	C ₁₅ H ₁₃ O ₇ N	320.076
DMY dimers	C ₃₀ H ₂₂ O ₁₆	639.098	–O + NH	Compound 2	C ₃₀ H ₂₃ O ₁₅ N	638.114
			–2O + 2NH	Compound 3	C ₃₀ H ₂₄ O ₁₄ N ₂	637.130
Ring-cleavage products of DMY dimers	C ₂₉ H ₂₀ O ₁₅	609.088	–O + NH	Compound 4	C ₂₉ H ₂₁ O ₁₄ N	608.104

whether the reaction products of Strecker degradation, N-DMY and corresponding aldehydes, could be formed.

Since aldehydes are prone to react with 2,4-DNPH to form the corresponding hydrazones, it is a common way to determine the aldehydes by LC-MS/MS after derivatization with 2,4-DNPH (Kuwata et al., 1983). In this study, N-DMY was detected by LC-MS/MS, while aldehydes were determined by LC-MS/MS after derivatization with 2,4-DNPH according to some modifications of previous method. The LC-MS/MS analysis was performed using the ultra-performance liquid chromatography system coupled to the time-of-flight mass spectrometry (Waters XEVO G2-XS QToF). The detailed conditions were present in Table 1.

2.11 | Incubation of DMY and amino acids in PBS at 37°C

Fifteen amino acids standard solutions were prepared by dissolving standards in PBS according to their concentration in DMEM, respectively. The final concentration of 15 amino acid solutions in PBS were as follows: glycine (0.4 mM), L-arginine (0.4 mM), L-cystine (0.2 mM), L-glutamine (4 mM), L-histidine (0.2 mM), L-isoleucine (0.8 mM), L-leucine (0.8 mM), L-lysine (0.8 mM), L-methionine (0.2 mM), L-phenylalanine (0.4 mM), L-serine (0.4 mM), L-threonine (0.8 mM), L-tryptophan (0.08 mM), L-tyrosine (0.4 mM), and L-valine (0.8 mM).

In 2-ml vials, 100 µl of DMY standard solution (5×10^{-3} mol/L) was mixed with 900 µl of every individual amino acid solution, respectively. Then these vials were incubated in water bath at 37°C. After 1 h, 100 µl of sample solution was taken for detection of N-DMY (Section 2.12.), and another 100 µl of sample solution was taken for detection of aldehydes (Section 2.13.).

2.12 | Determination of N-DMY

After incubating for 1 h, 100 µl of sample solution was taken, and mixed with 400 µl of methanol, then filtered with a 0.22 µm-pore-size filter for LC-MS/MS analysis.

The elution gradient was as below: 0–2 min, 15% B; 2–10 min, 15%–45% B; 10–12 min, 45%–15% B; 12–15 min, 15% B. Negative electrospray ionization mode was applied. In addition, DMSO was incubated in corresponding amino acid solutions in PBS as control.

2.13 | The determination of aldehydes by derivatization with 2,4-DNPH

After incubating for 1 h, 100 µl of sample solution was taken to be derived with 2,4-DNPH for the determination of aldehydes. In this study, only phenylacetaldehyde produced in the incubation model of DMY in phenylalanine in PBS was determined to verify the mechanism of N-DMY. While other corresponding aldehydes that may be produced by Strecker degradation of other amino acids by DMY-derived quinone have not been identified due to the lack of aldehyde derivatives. The steps were as follows. (1) Preparation of DNPH solution. Fifty milligram of 2,4-DNPH was dissolved in 20 ml of HCl aqueous solution (HCl: H₂O = 1: 1), and then diluted with H₂O to 50 ml total volume. (2) Preparation of the derivatives of phenylacetaldehyde with 2,4-DNPH (phenylacetaldehyde-DNPH). First, excess phenylacetaldehyde was added to DNPH solution. Then, the mixed solutions were incubated at 60°C for 20 min, and precipitation formed. Discard the supernatant and redissolve the precipitation with acetonitrile. Finally, the sample were identified as phenylacetaldehyde-DNPH by LC-MS/MS, which showed that the preparation was successful. (3) Determination of the aldehydes produced in sample solutions. The 100 µl of sample solution was mixed with 20 µL of DNPH solution. After the mixed solution was incubated at 60°C for 20 min, 100 µl of acetonitrile was added to mix the solution by vortex for 1 min. Then, sample solution was filtered with a 0.22 µm-pore-size filter for UPLC-MS/MS analysis. The elution gradient was as below: 0–2 min, 40%–80% B; 2–5 min, 80% B; 5–6.5 min, 80%–40% B; 6.5–10 min, 40% B. Negative electrospray ionization mode was applied.

2.14 | Statistical analysis

The 3D waterfall plots of stability data were analyzed and plotted by OriginPro 2019b software (OriginLab, Northampton). The experiment was performed in triplicate.

3 | RESULTS AND DISCUSSION

3.1 | Discovery of nitrogenous derivatives of DMY (compounds 1–4) in DMEM at 37°C

The formation and potential mechanism of nitrogenous derivatives of DMY were investigated in detail in the present study. Referring to the previous study based on LC-MS/MS, DMY ($m/z = 321.061$) degraded quickly in DMEM solution at 37°C. According to the characteristic fragment ions of DMY including $m/z = 303.050$, $m/z = 275.055$, $m/z = 195.029$, and $m/z = 153.018$ at positive ESI mode, DMY dimers ($m/z = 639.094$), their oxidation products ($m/z = 637.079$), and their ring-cleavage products ($m/z = 609.083$) were screened and identified (H. L. Zhang et al., 2022).

Except for the above compounds, four nitrogenous derivatives of DMY in DMEM solution at 37°C were identified according to the exact mass, nitrogen rule and

MS information from LC-MS/MS. These four compounds 1–4 were with quasi-molecular ion peaks $[M+H]^+$ at $m/z = 320.076$ (1), $m/z = 638.114$ (2), $m/z = 637.130$ (3), $m/z = 608.104$ (4). Meanwhile, these compounds also could be detected at negative ESI mode (data not shown). Figure 1 shows the extracted ion chromatograms (EIC) of DMY in DMEM solution at 37°C between 0 and 13 h, and the EIC was at $m/z = 319.045$, $m/z = 320.076$, $m/z = 321.061$, $m/z = 608.104$, $m/z = 609.088$, $m/z = 635.062$, $m/z = 637.082$, $m/z = 637.130$, $m/z = 638.114$, and $m/z = 639.098$. As shown in Figure 1, the content of new products was in order: $m/z = 321.061 > m/z = 320.076$; $m/z = 639.098 > m/z = 638.114 > m/z = 637.130$; $m/z = 609.088 > m/z = 608.104$.

Figure 2 is diagram of transformation of DMY in DMEM solution at 37°C. All new products are included, and their m/z , chemical formulas and predicted structures are also represented, especially the nitrogenous derivatives of DMY (compounds 1–4). Since C2, C3 of DMY are chiral carbon atoms, stereoisomerism existed in the new products of DMY in DMEM. However, through LC-MS/MS, stereoisomers of flavonoids were difficult to differentiate. Therefore, stereoisomerism was not considered.

In addition, results of Figures 3A, 4A, 5A indicated that EICs of every compound (1–4) mentioned in this

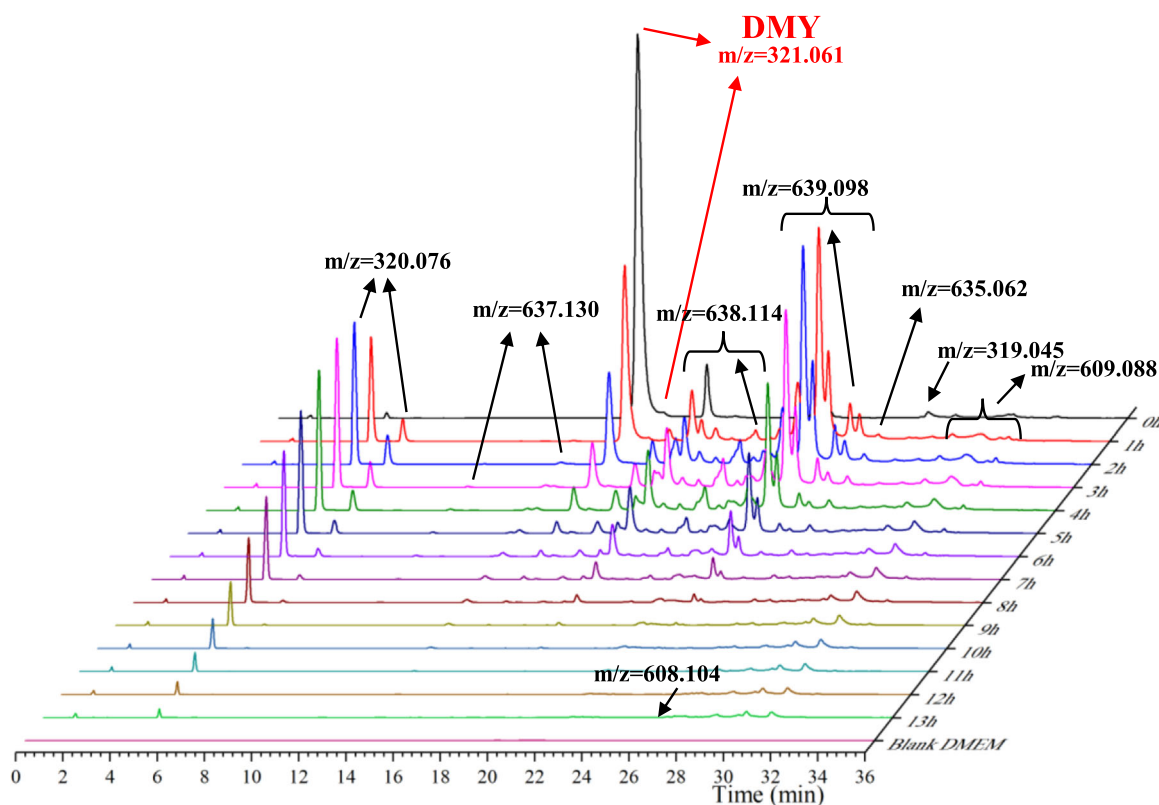


FIGURE 1 Extracted ion chromatograms (EICs) of dihydromyricetin (DMY) in Dulbecco's modified Eagle's medium (DMEM) solution at 37°C during the incubation of 13 h. The selected ions are at $m/z = 319.045$, $m/z = 320.076$, $m/z = 321.061$, $m/z = 608.104$, $m/z = 609.088$, $m/z = 635.062$, $m/z = 637.082$, $m/z = 637.130$, $m/z = 638.114$ and $m/z = 639.098$.

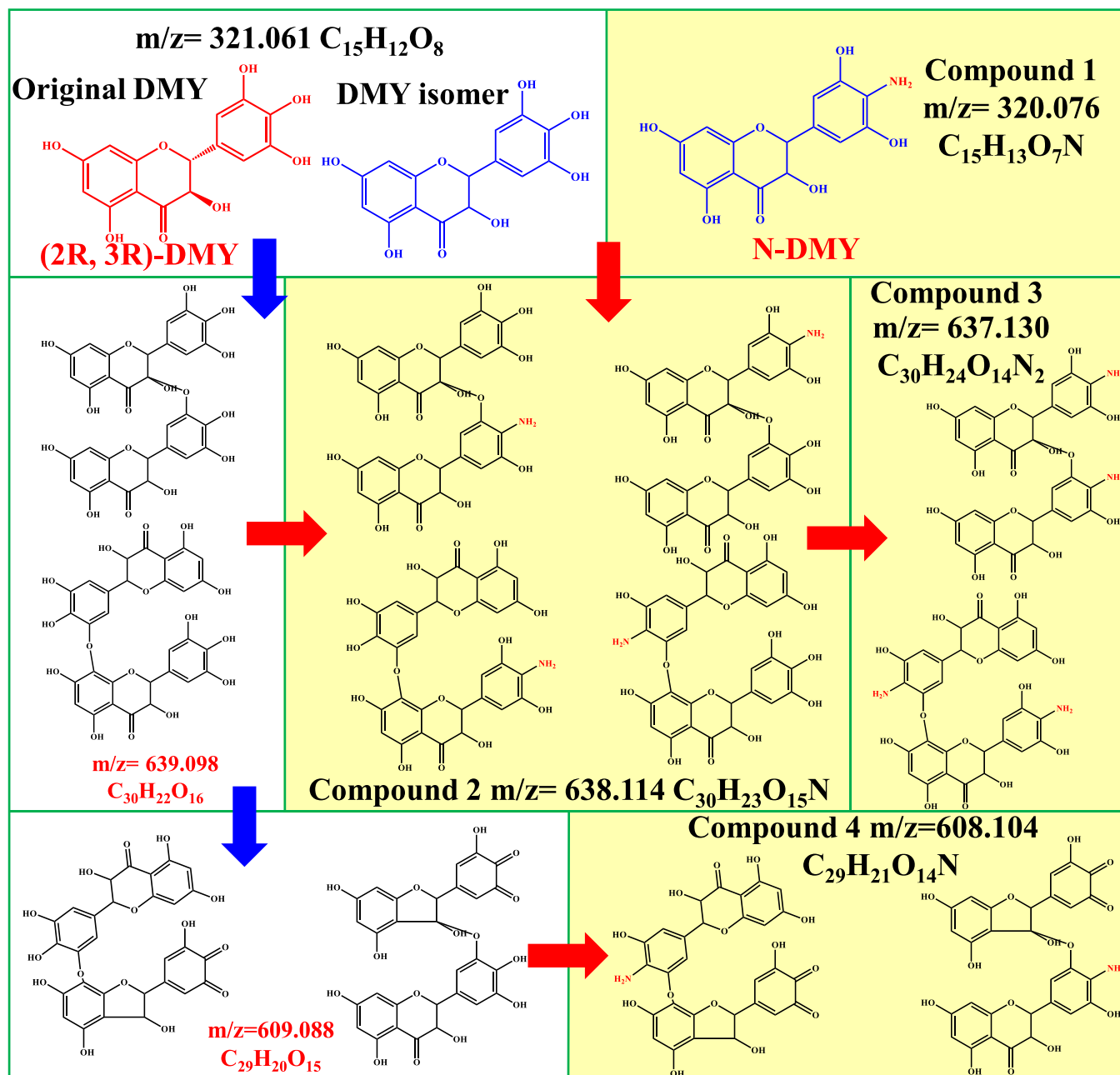


FIGURE 2 Diagram of transformation of dihydromyricetin (DMY) in Dulbecco's modified Eagle's medium (DMEM) solution at 37°C. The mass to charge ratio and chemical formulas of new products as well as their predicted structures are represented. Stereoisomers are not considered.

article actually all included several isomers with different retention times, respectively.

3.2 | Structural characterization of compounds 1–4 using LC-MS/MS

Structures of compounds 1–4 were characterized by LC-MS/MS according to the procedure in the Section 2.5. Particularly, compound 1 (N-DMY) was prepared according to the methods in Section 2.8, and its

structure was identified by MS and NMR as described in Section 2.9. Moreover, some previously identified compounds were listed in Table 1 for comparison, which possess similar chemical formular and similar *m/z* with compounds 1–4, but do not have nitrogen atom. The EICs and MS² of these compounds were also collected to help infer the reasonable structures of compounds 1–4. For every compound, there were many isomers, but only peak with highest intensity was chosen for fragment ions acquisition and structural characterization.

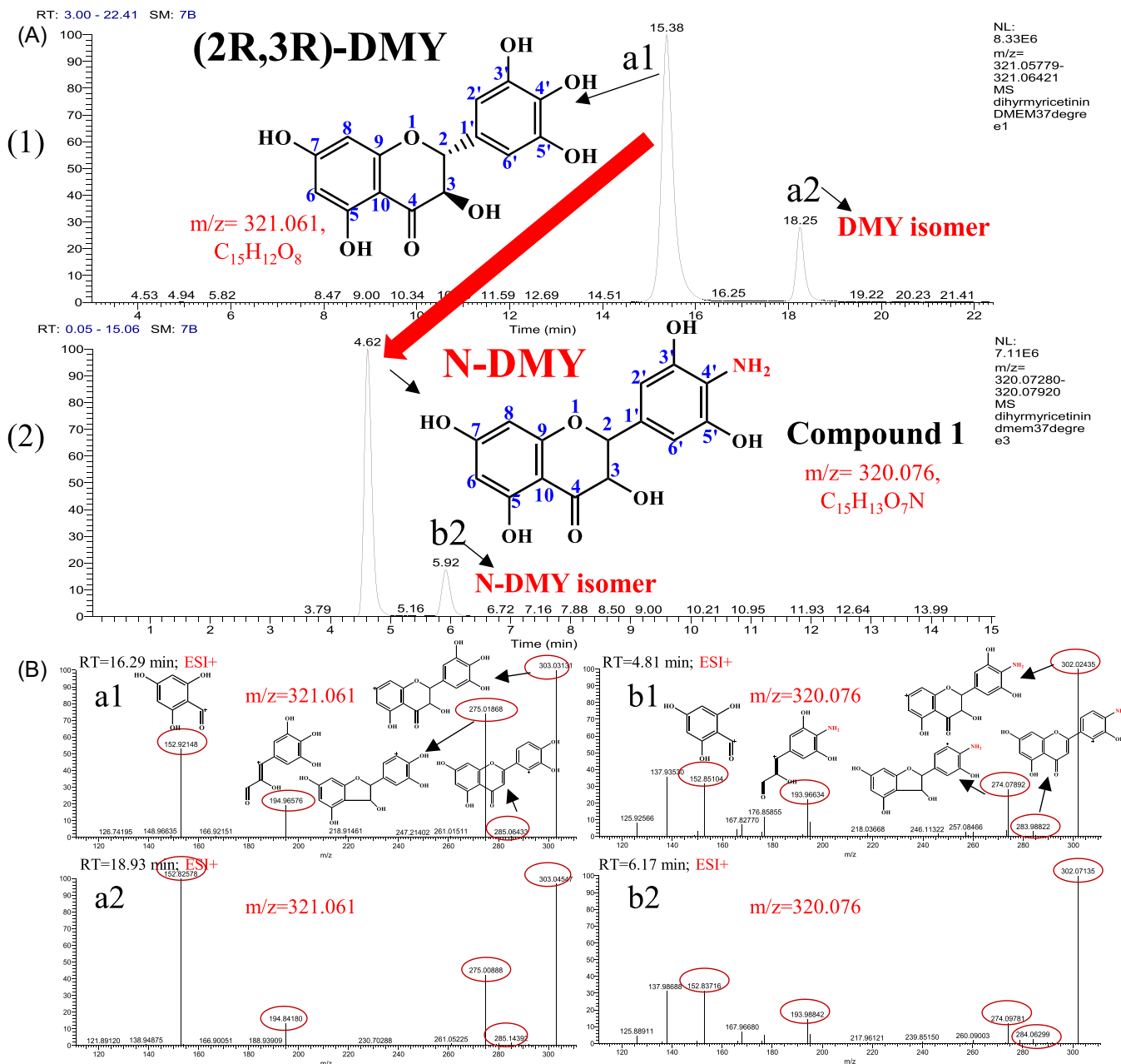


FIGURE 3 Extracted ion chromatograms (EICs), structures, MS² of dihydromyricetin (DMY) (at $m/z = 321.061$) and compound 1 (N-DMY at $m/z = 320.076$) at positive ion mode. (A) (1) EIC of (2R, 3R)-DMY (a1, RT = 15.38 min) and its isomer (a2, RT = 18.25 min); (2) EIC of N-DMY (b1, RT = 4.62 min) and its isomer (b2, RT = 5.92 min). (B) The MS² of DMY and N-DMY, as well as predicted fragment ion structures of a1 and b1.

3.3 | Structural characterization of compound 1 (N-DMY)

The compound 1 was named as N-DMY in this article. As shown in EICs of DMY and compound 1 in Figure 3A, there were both two isomers for DMY (a1, b2) and compound 1 (b1, b2). A previous study separated (2R, 3R)-DMY and its epimer (2R, 3S)-DMY, at a peak area ratio of 5:3 from the cultured media of human intestinal bacteria containing DMY (Y. Zhang et al., 2007). Similarly, in the present study,

a2 was is an isomer of (2R, 3R)-DMY (a1) found in cell culture medium with weaker peak intensity than DMY, which might be a stereoisomer of a1. Accordingly, b2 might be a corresponding stereoisomer of b1. The dominant b1 was used for identification of compound 1, and EICs and MS² of b1 are shown in Figure 3.

According to the $m/z = 320.076$, compound 1 was predicted to have a chemical formula of $C_{15}H_{13}O_7N$. Since the chemical formula of DMY is $C_{15}H_{12}O_8$, compound 1 was assumed to be formed by replacing

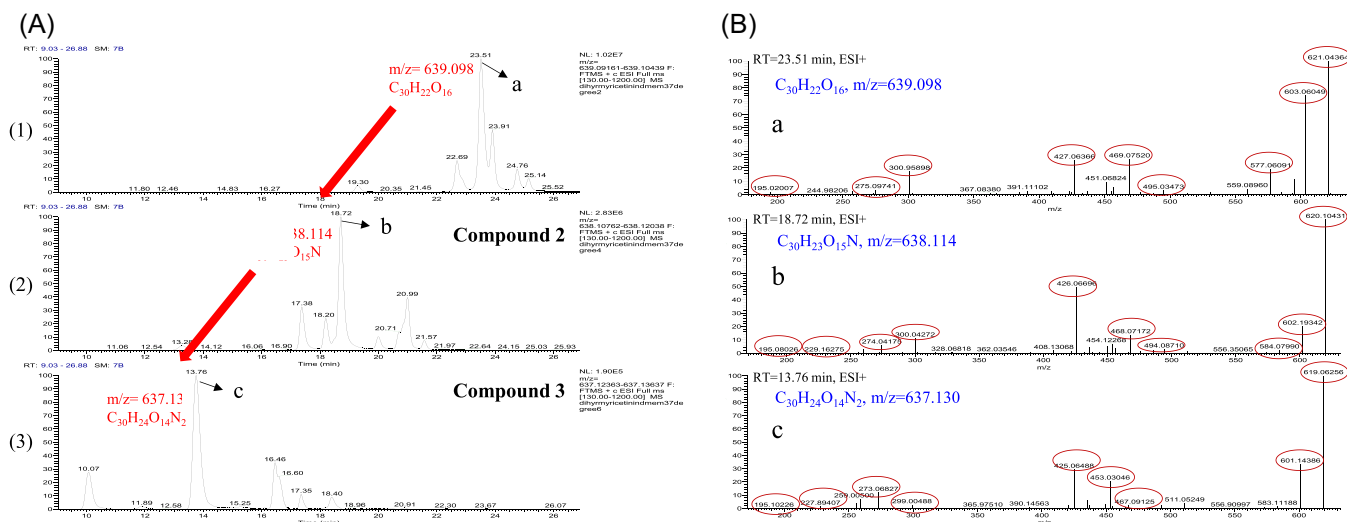


FIGURE 4 Extracted ion chromatograms (EICs) and MS² of dihydromyricetin (DMY) dimers (at m/z = 639.098) and their nitrogenous derivatives compound 2 (at m/z = 638.114) as well as compound 3 (at m/z = 637.130) at positive ion mode. (A) 1: EIC at m/z = 639.098; 2, 3: EICs of compound 2 (m/z = 638.114) and compound 3 (m/z = 637.130). The a–c represent the MS² of peaks with the highest intensity in the 1–3 of subfigure A, respectively. (B) The MS² of a–c.

one oxygen atom of DMY with one nitrogen atom and one hydrogen atom.

In addition, compound 1 (N-DMY) was prepared and further identified by NMR applying the preparation procedure in Sections 2.8 and 2.9. The final prepared N-DMY product was greyish-green powder. As shown in Figure 6A, the MS and UV results both indicated that the final product was a mixture of N-DMY and DMY. The results of MS showed that the N-DMY in the prepared N-DMY mixture was consistent with compound 1. Using the same LC and MS conditions, the N-DMY in the prepared N-DMY mixture showed identical retention time, exact mass and consistent fragment ions with compound 1 (data not shown). Therefore, the prepared N-DMY mixture was used as compound 1 produced in DMEM for NMR identification. Simultaneously, DMY was also detected by NMR as a comparison.

At 600 Hz, the ¹H and ¹³C NMR spectra of prepared mixture (including N-DMY and DMY) were obtained by using DMSO-*d*₆ as a solvent. Chemical shifts (in ppm) of ¹H and ¹³C NMR were measured. The peaks of DMY standard and prepared mixture (N-DMY and DMY) in ¹H and ¹³C NMR spectra as well as their assignments are shown in Figure 6B,C. The results showed that there were both two groups of peaks with similar chemical shifts and peak shape in ¹H NMR as well as ¹³C NMR spectra of the prepared N-DMY mixture. Combining the above MS results and the NMR spectra of DMY standard, it was thought that one group of peaks in the prepared N-DMY mixture spectra was attributed to DMY, and the other group of peaks was assigned to N-DMY. The ¹H NMR result of the prepared N-DMY mixture is shown in Figure 6B, two groups of peaks were very similar, which indicated that N-DMY had a similar structure to DMY. In

addition, integration results of the circled signals (2'H, 6'H) in ¹H NMR indicated a content ratio of N-DMY and DMY at about 1.2: 1 (Figure 6B). Significantly, the result of ¹³C NMR data of the prepared N-DMY mixture suggested that N-DMY might have a structure with just a replacement of the OH by one NH₂ on the 4'-C position of the DMY ring B (Figure 6C). Compared to DMY, the ¹³C NMR spectra of the prepared N-DMY mixture showed two significant different signals at δ 124.58 ppm and 124.31 ppm assigned to the C-1' and C-4'. Especially, C-4' in the prepared N-DMY was about 9 ppm shifted upfield comparing the δ 133.43 ppm for C-4' of DMY. This suggested that C-4' on the ring B was the position that the OH of DMY was replaced by NH₂ of the N-DMY, and the structure of N-DMY was shown in Figure 6. Besides, in previous studies, similar chemical shift changes were also observed in the NMR data of 4'-NH₂-myricetin (Zhang, Wang, et al., 2019) and 4'-NH₂-pyrogallol nucleus compounds, such as tannins (Hashida et al., 2009).

Besides, the above structure of compound 1 was verified again by Mass frontier 8.1 software according to the process in Section 2.7. As shown in Figures 3a,b1 (m/z = 320.076, RT = 4.62 min) with the larger peak area was chosen to acquire the top 15 high intensity actual fragment ions, and all 15 predicted fragment ions of N-DMY matched with that (data listed in Table 2). The possible fragment ions of b1 were deduced and shown in Figure 3B.

3.4 | Compounds 2 and 3

According to the m/z = 638.114, m/z = 637.130, the chemical formula of compounds 2, 3 were predicted to

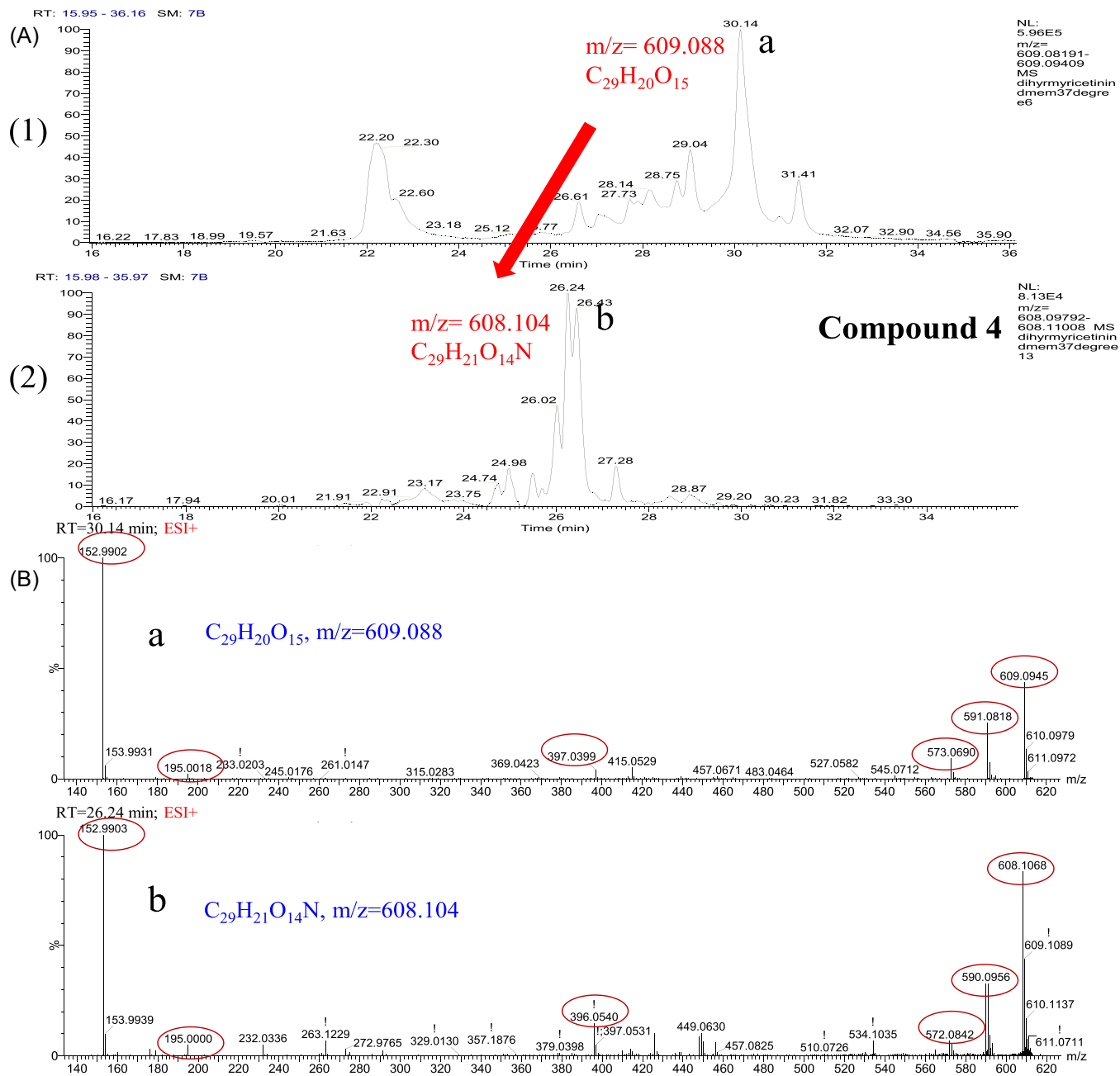


FIGURE 5 Extracted ion chromatograms (EICs) and MS^2 of ring-cleavage products of dihydromyricetin (DMY) dimers ($m/z = 609.088$) and their nitrogenous derivative (compound 4, $m/z = 608.104$) at positive ion mode. (A) 1: EIC chromatogram of $m/z = 609.088$; 2: EIC chromatogram of compound 4 ($m/z = 608.104$). The a and b represent peaks with the highest intensity in the subfigure 1 and 2, respectively. (B) The MS^2 comparison of a ($m/z = 609.088$) and b ($m/z = 608.104$).

be $C_{30}H_{23}O_{15}N$ and $C_{30}H_{24}O_{14}N_2$, respectively. Through a comparison with the MS information of DMY dimer ($m/z = 639.094$, $C_{30}H_{22}O_{16}$), compound 2 ($m/z = 638.114$) was inferred to be formed by linking one molecule of DMY and one molecule of N-DMY or converting the OH on the position of C-4' of $m/z = 639.094$ into NH_2 . While, compound 3 ($m/z = 637.130$) was predicted to be formed by linking two molecules of N-DMY or transforming two OH on the position of C-4' of $m/z = 639.094$ into NH_2 or converting one OH on the

position of C-4' of compound 2 ($m/z = 638.114$). Compounds 2 and 3 might be nitrogenous derivatives of DMY dimers. Four possible structures of compound 2 and two possible structures of compound 3 were shown in Figure 2. EICs and MS^2 of compounds 2 and 3 are shown in Figure 4A,B. Stereoisomerism was not considered.

Chose the isomer with the largest peak area (b at $m/z = 638.114$, RT = 18.72 min; c at $m/z = 637.130$, RT = 13.76 min in Figure 4A) to obtain the top 15 high

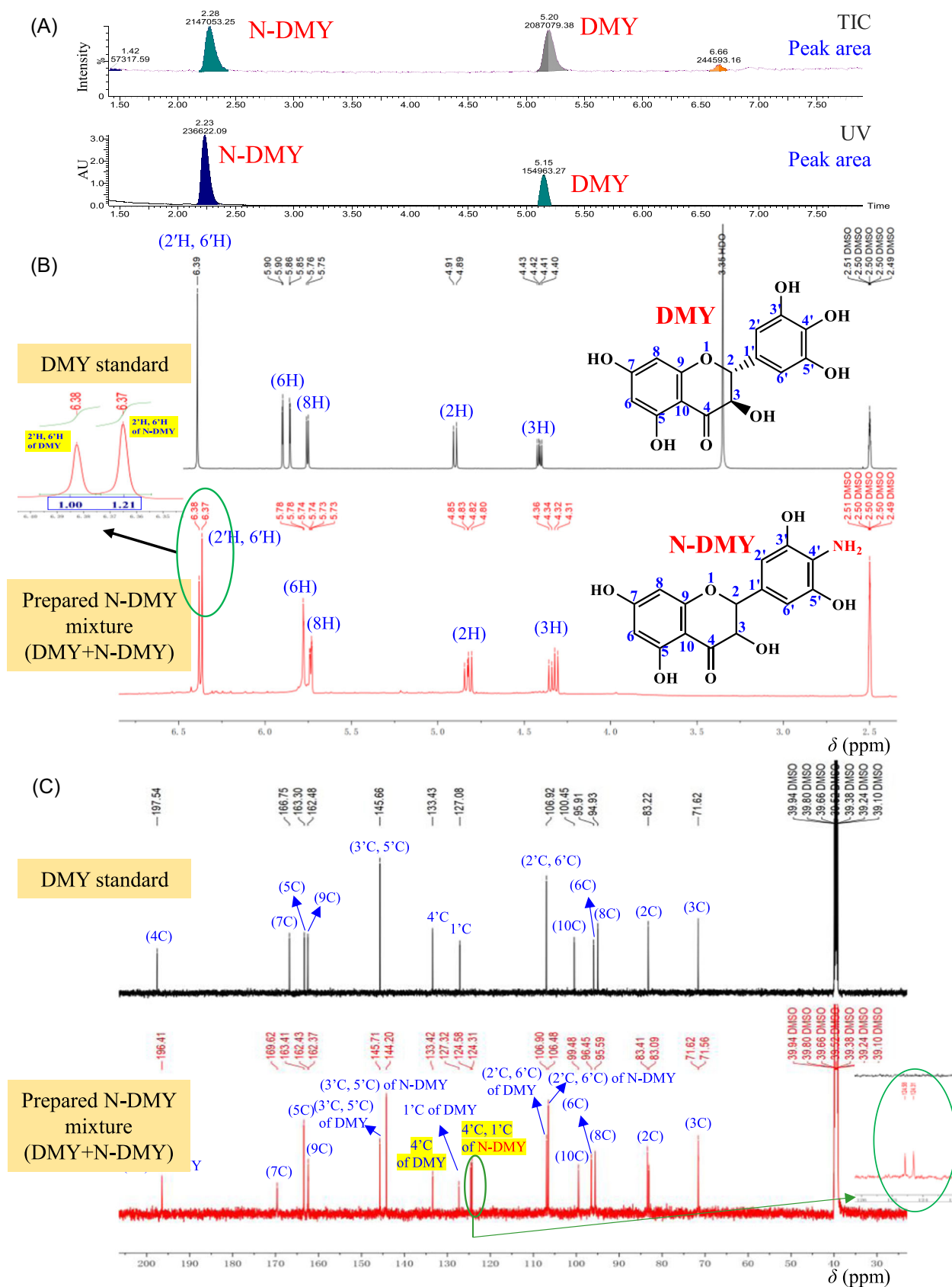


FIGURE 6 MS, UV, and nuclear magnetic resonance (NMR) results of the prepared N-DMY mixture. (A) TIC and UV of the prepared N-DMY mixture. (B) ^1H NMR spectra of dihydromyricetin (DMY) standard and the prepared N-DMY mixture. (C) ^{13}C NMR spectra of DMY standard and the prepared N-DMY mixture (δ in ppm, 600 MHz, $\text{DMSO}-d_6$ as a solvent).

TABLE 2 Structure verification of nitrogenous derivatives of dihydromyricetin (DMY) (compounds 1–4) by Mass frontier 8.1 software, including actual MS² and software predicted MS²

No.	Compound 1 (<i>m/z</i> = 320.076)		Compound 2 (<i>m/z</i> = 638.114)				Compound 3 (<i>m/z</i> = 637.130)				Compound 4 (<i>m/z</i> = 608.104)			
	Actual MS ²	Predicted MS ²	Actual MS ²		Predicted MS ²		Actual MS ²		Predicted MS ²		Actual MS ²		Predicted MS ²	
			1	2	1	2	1	2	1	2	1	2	1	2
1	302.0244	302.0659	620.1043	620.1035	620.1035	620.1035	619.0626	619.1195	619.1195	619.1195	152.9903	153.0182	153.0182	153.0182
2	137.935	138.0311	426.067	426.0820	426.0820	426.0820	601.1439	601.1089	601.1089	601.1089	608.1068	608.1035	608.1035	608.1035
3	152.8510	153.0182	602.1934	602.0929	602.0929	602.0929	425.0649	425.0979	425.0979	425.0979	590.0956	590.0929	590.0929	590.0929
4	274.0789	274.0710	468.0717	468.0925	468.0925	468.0925	453.0305	453.0816	453.0816	453.0816	396.054	396.0476	396.0476	396.0476
5	193.9663	194.0448	300.0427	300.0503	300.0503	300.0503	273.0683	273.0632	273.0632	273.0632	449.063	449.0503	449.0503	449.0503
6	176.8586	177.0420	454.1227	454.0894	454.0769	454.0769	259.005	259.0601	259.0601	259.0601	426.0725	426.0820	426.0820	426.0820
7	195.0122	195.0526	274.0418	274.0710	274.0710	274.0710	435.1696	435.1187	No	No	448.0573	448.0663	448.0663	448.0663
8	125.9257	126.0550	449.9701	450.0820	450.0820	450.0820	256.0812	256.0604	256.0604	256.0604	263.1229	263.0788	263.0788	263.0788
9	167.8277	168.0417	436.1719	436.1027	436.1027	436.0663	511.0525	511.0983	511.0983	511.0983	534.1035	534.1031	534.1031	534.1031
10	165.8549	166.0499	456.0491	456.0925	456.0925	456.0925	449.0815	449.0979	No	No	572.0842	572.0824	572.0824	572.0824
11	273.3488	273.0632	494.0871	494.0718	494.0718	494.0718	492.9708	493.0878	493.0878	493.0878	450.0414	450.0820	450.0820	450.0820
12	149.9873	150.0550	260.0642	260.0554	260.0679	260.0679	437.0007	437.0979	437.0979	437.0979	456.0852	456.0925	456.0925	456.0925
13	283.9882	284.0553	584.0799	584.0824	584.0824	584.0824	421.0009	421.1030	No	No	232.0336	232.0366	232.0366	232.0366
14	257.0847	257.0444	486.1835	486.1031	486.1031	486.1031	299.0049	619.1195	619.1195	619.1195	195.0000	195.0288	195.0288	195.0288
15	259.9608	260.0553	408.1307	408.0714	408.1077	408.0714	467.0913	601.1089	601.1089	601.1089	414.0682	414.0820	414.0820	414.0820

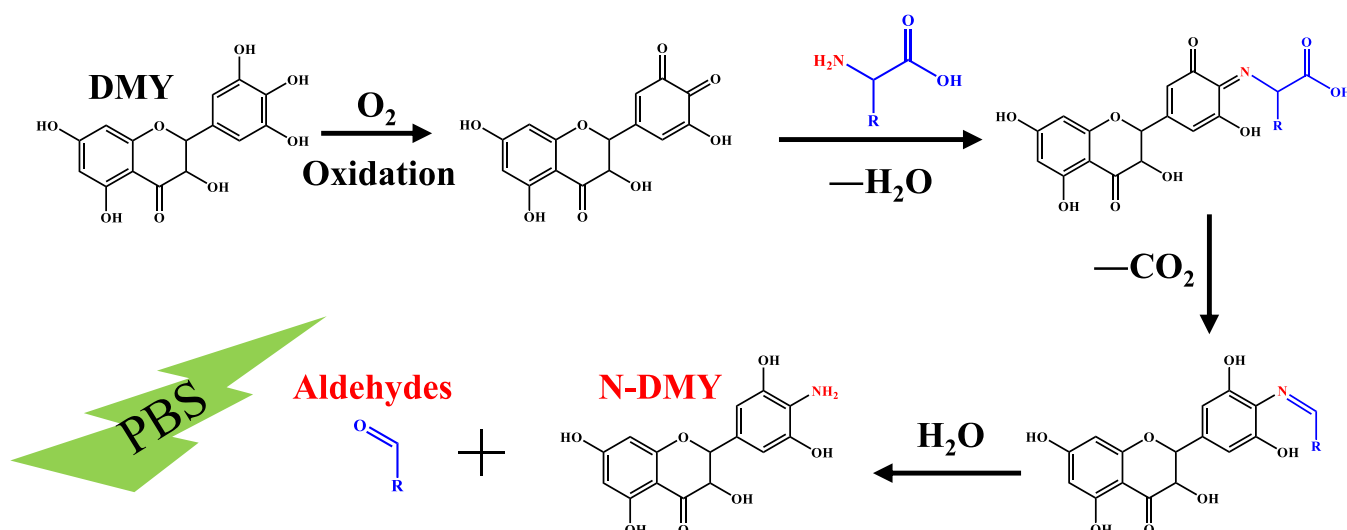


FIGURE 7 The proposed formation mechanism of N-DMY: dihydromyricetin (DMY)-derived quinone participated in the Strecker degradation of amino acids in Dulbecco's modified Eagle's medium (DMEM), and phosphate buffered saline (PBS) play an important role in the reaction.

intensity actual fragment ions. According to the procedure described in Section 2.7, for the four predicted structures of b, all 15 fragment ions matched. For the two predicted structures of c, 15, 12 fragment ions matched, respectively (data listed in Table 2). The results suggested that the predicted structures of compounds 2 and 3 were reasonable to some extent.

3.5 | Compound 4

Based on the $m/z = 608.104$, compound 4 was predicted to possess a chemical formula of $C_{29}H_{21}O_{14}N$. Combining the MS information of DMY dimers ring-cleavage products ($m/z = 609.088$, $C_{29}H_{20}O_{15}$), compound 4 possibly were nitrogenous derivatives of the ring-cleavage product $m/z = 609.088$ by replacing the OH on the position of C-4' with NH_2 . Two possible structures of compound 4 were shown in Figure 2 without considering stereoisomerism. EICs and MS^2 of compound 4 are shown in Figure 5.

Then, following the procedure described in Section 2.7, for the two predicted structures of $m/z = 608.104$ (RT = 26.24 min), both 15 fragment ions matched (data shown in Table 2), which suggested that the predicted structures were reasonable to some extent.

3.6 | Formation mechanism of N-DMY in DMEM

To understand the underlying mechanism of N-DMY formation in DMEM, all nitrogenous ingredients of DMEM were considered. The nitrogenous components in DMEM consist of inorganic salts (ferric nitrate nonahydrate), amino acids and some vitamins. Since no

ammonia was present in DMEM, it was unlikely to form N-DMY by regioselective amination on the position of C-4' of DMY, as described in some previous studies about other polyphenols possessing pyrogallol moieties (Zhang, Wang, et al., 2019; Zhang, Zhao, et al., 2019). Strecker degradation might be a more reasonable mechanism for the formation of N-DMY. According to the literature and our preliminary results, amino acids were thought to be the donor of nitrogen atoms for the DMY nitrogenous derivatives. Previous studies reported that polyphenols with special structures, such as a pyrogallol moiety, were prone to be oxidized to quinones. Then quinone could further participate in the Strecker degradation of amino acids to form volatile aldehyde and aminated polyphenols (Chen et al., 2021; Rizzi, 2006; Schieber, 2018). For possessing a pyrogallol moiety on the ring B of DMY, DMY are prone to be oxidized into quinone ($m/z = 319.045$ in Figure 1). We speculated that N-DMY might be a reaction product of DMY-derived quinone participating in Strecker degradation of amino acids in DMEM. While, compounds 2–4 might be formed by that first DMY dimers and DMY ring-cleavage products were oxidized to quinones, and then quinones participated the Strecker degradation reaction of amino acids in DMEM. The formation mechanism of N-DMY is proposed in Figure 7.

Therefore, according to the procedure in Section 2.11, we prepared 15 kinds of individual amino acid standard solutions dissolved in PBS according to their concentration in DMEM, respectively. Then, DMY standard solution was incubated with different amino acid standard solutions at $37^\circ C$. After incubating for 1 h, N-DMY and aldehydes were determined based on the methods mentioned in Sections 2.12 and 2.13. The results showed that N-DMY was found in all 15 kinds of mixed solutions of DMY and amino acids in PBS (Figure 8A).

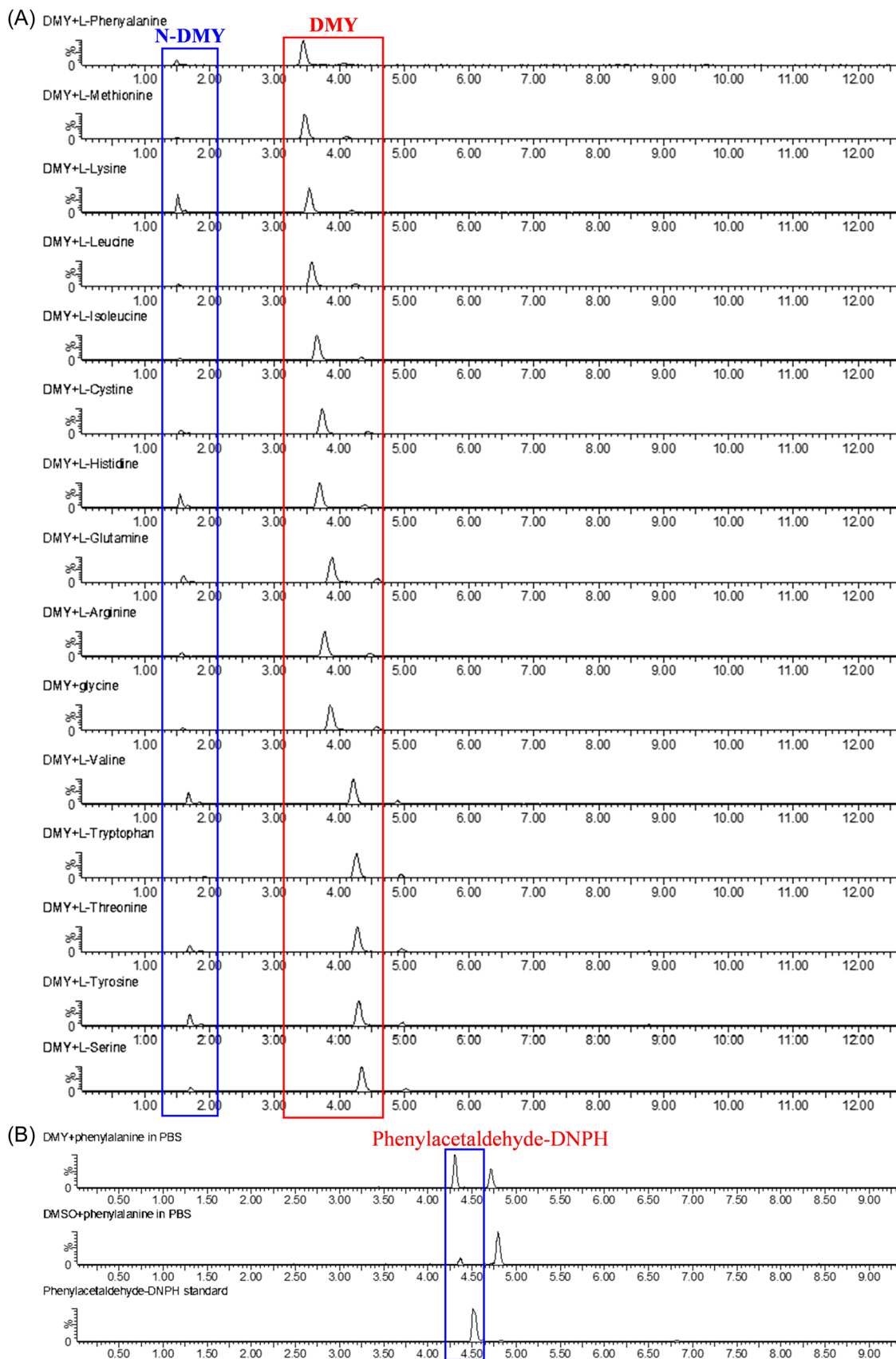


FIGURE 8 Incubation of dihydromyricetin (DMY) and amino acid solutions in PBS at 37°C. (A) Extracted ion chromatograms (EICs) of N-DMY ($m/z = 318.062$) and DMY ($m/z = 319.045$) under negative ion mode when DMY was incubated with individual amino acid solutions in PBS at 37°C. (B) Determination results of phenylacetaldehydes by derivatization with 2,4-DNPH.

While, the other possible reaction product, aldehydes, were determined by derivatization with 2,4-DNPH. Since the production of phenylacetaldehyde in the control (DMSO in phenylalanine in PBS) was very small and the control compound phenylacetaldehyde-DNPH was easy to prepare, we chose to detect phenylacetaldehyde for validation of mechanism. The phenylacetaldehyde-DNPH in samples was determined as described in Section 2.13. At first, phenylacetaldehyde-DNPH was prepared as control compound. After incubating for 1 h, phenylacetaldehyde-DNPH was significantly detected in the incubation model of DMY and phenylalanine in PBS than control solution (DMSO and phenylalanine in PBS). The data are shown in Figure 8B. This further confirmed the formation mechanism of N-DMY proposed in Figure 7.

Interestingly, when DMY standard solution was incubated in amino acid solutions in H₂O at 37°C, there was no N-DMY detected during 3-h incubation. Therefore, with main components of Na₂HPO₄, KH₂PO₄, NaCl, and KCl, phosphate buffered saline (PBS) might play an important role in the formation of N-DMY, which might provide an important environment for the reaction.

3.7 | Nitrogenous derivatives of flavonoids widely existed when flavonoids were incubated in DMEM solution at 37°C

Except for DMY, in our preliminary experiment, we also investigated the new products of many other flavonoids when they were incubated in DMEM solution at 37°C. Analyzing new products of these flavonoids by LC-MS/MS, and their chemical formulas were obtained based on the exact *m/z*. Interestingly, it was found that many flavonoids also formed corresponding nitrogenous derivatives in DMEM solution at 37°C, such as quercetin, luteolin, baicalein, fisetin, and so on (data not shown). The common characteristic of these flavonoids might be that they all have a catechol or pyrogallol moiety, which could be oxidized to form quinone structure, and further participate in the Strecker degradation of amino acids.

More importantly, these nitrogenous derivatives of flavonoids might be involved in the metabolism and bioactivities of DMY during in vitro and in vivo experiments. Take quercetin as an example. When we incubated quercetin (*m/z* = 303.048) in DMEM at 37°C, a nitrogenous derivative of quercetin (*m/z* = 302.064) was detected by LC-MS/MS under the positive (ESI) mode (Cao et al., 2022). Simultaneously, some nitrogenous metabolites of quercetin were also found during cell culture with quercetin in DMEM, including methylate (*m/z* = 316.082), glucuronide (*m/z* = 478.098), methylated glucuronide of quercetin nitrogenous derivative (*m/z* = 492.11) (unpublished). In addition, we investigated

the degradation of DMY in plasma of normal rat in vitro at 37°C in our preliminary experiment. DMY was dramatically more stable in plasma than in DMEM, but the degradation products of DMY produced in plasma in vitro were similar to that of DMY in DMEM, including DMY isomer, dimers, oxidized products and ring-cleavage products as well as their corresponding nitrogenous derivatives (unpublished). It indicated that the nitrogenous derivatives of DMY are possibly also present during in vivo experiments about DMY.

Hence, it is probably a common phenomenon that flavonoids with a catechol or pyrogallol moiety form their nitrogenous derivatives during the incubation in DMEM at 37°C. These nitrogenous derivatives of flavonoids may be involved in the metabolism of flavonoids and exhibit some effects on the bioactivities of flavonoids during in vitro and in vivo experiments.

4 | CONCLUSION

In this study, we thoroughly investigated the formation and potential mechanism of DMY nitrogenous derivatives produced in DMEM solution at 37°C through LC-MS/MS. Four nitrogenous derivatives (compounds 1–4) of DMY were found to be produced in DMEM solution at 37°C by using LC-MS/MS. Compound 1 (N-DMY) was prepared by the reaction of ammonia and DMY, and was identified as 4'-NH₂-DMY using MS and NMR. The formation mechanism of N-DMY in DMEM was proposed as that DMY first was oxidized to quinone, and then quinone participated in the Strecker degradation of amino acids in DMEM to form N-DMY as well as corresponding aldehydes. Correspondingly, compounds 2–4 might be formed by the Strecker degradation reaction of amino acids in DMEM with quinones from the oxidation of DMY dimers and DMY ring-cleavage products. In addition, flavonoids with a catechol or pyrogallol moiety may readily form their nitrogenous derivatives during the incubation in DMEM at 37°C, which may be involved in the metabolism and bioactivities of flavonoids during in vitro and in vivo experiments. Therefore, more work is needed in the future to investigate the in vitro and in vivo bioactivities of nitrogenous derivatives of flavonoids.

AUTHOR CONTRIBUTIONS

Haolin Zhang: Investigation; writing – original draft. **Minglong Wang:** Data curation; methodology. **Yuanwei Liang:** Validation. **Xiaomei Lv:** Investigation. **Ruiwei Xie:** Investigation. **Xiaojia Chen:** Supervision. **Jianbo Xiao:** Conceptualization; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

None declared.

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