Mass Spectrometry and Tandem Mass Spectrometry of Citrus Limonoids

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Methods for atmospheric pressure chemical ionization tandem mass spectrometry (APCI-MS/MS) of citrus limonoid aglycones and electrospray ionization tandem mass spectrometry (ESI-MS/MS) of limonoid glucosides are reported. The fragmentation patterns of four citrus limonoid aglycones (limonin, nomilin, obacunone, and deacetylnomilin) and six limonoid glucosides, that is, limonin 17-β-D-glucopyranoside (LG), nomilin 17-β-D-glucopyranoside (NG), nomilinic acid 17-β-D-glucopyranoside (NAG), deacetylnomilinic acid 17-β-D-glucopyranoside (DNAG), obacunone 17-β-D-glucopyranoside (OG), and obacunoic acid 17-β-D-glucopyranoside (OAG) were investigated using a quadrupole mass spectrometer in low-energy collisionally activated dissociation (CAD). The four limonoid aglycones and four limonoid glucosides (LG, OG, NAG, and DNAG) were purified from citrus seeds; the other two limonoid glucosides (NG and OAG) were tentatively identified in the crude extract of grapefruit seeds by ESI mass spectrometry in both positive and negative ion analysis. Ammonium hydroxide or acetic acid was added to the mobile phase to facilitate ionization. During positive ion APCI analysis of limonoid aglycones, protonated molecular ion, [M + H]+, or adduct ion, [M + NH3 + H]+, was formed as base peaks when ammonium hydroxide was added to the mobile phase. Molecular anions or adduct ions with acetic acid ([M + HOAc - H]− and [M + HOAc]) or a deprotonated molecular ion were produced during negative ion APCI analysis of limonoid aglycones, depending on the mobile-phase modifier used. Positive ion ESI-MS of limonoid glucosides produced adduct ions of [M + H + NH3]+, [M + Na]+, and [M + K]+ when ammonium hydroxide was added to the mobile phase. After collisionally activated dissociation (CAD) of the limonoid aglycone molecular ions in negative ion APCI analysis, fragment ions indicated structural information of the precursor ions, showing the presence of methyl, carboxyl, and oxygenated ring structure. CAD of the adduct ion [M + H + NH3]1+ of limonoid glucosides produced the aglycone moiety corresponding to each glucoside. The combination of mass spectrometry and tandem mass spectrometry provides a powerful technique for identification and characterization of citrus limonoids.

Citrus limonoids are a group of chemically related, highly oxygenated, tetracyclic triterpenoids present in Rutaceae and M. alaiceae families in aglycone and glucosidic forms (see limonoid structures in Figure 1). Citrus limonoids have been found to possess important anticancer activities in laboratory animals,1–4 to inhibit cancer cell proliferation, and induce apoptosis of human breast cancer cells in culture,5,6 and also to exhibit antifeedant activities against several kinds of insects.7,8

To date, more than 50 individual limonoid aglycones and limonoid glucosides have been isolated and structurally characterized from citrus and its related genera.9 High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) are the most commonly used methods for limonoid separation, isolation, and identification.10,11 Because of the limited availability of standards (currently only limonin and nomilin are commercially available) and the lack of characteristic UV/vis absorption spectra, nuclear magnetic resonance (NMR) was often employed for identification of citrus limonoids, requiring extensive and laborious purification to conduct experiments, which have often made positive characterization of limonoids a challenge to analytical chemists. Mass spectrometry has been one of the most sensitive and rapid methods in organic compound identification and characterization, but very few studies have been conducted in the citrus limonoid field because of the nonvolatile character of this class of compounds.12 The advent of soft ionization methods, such as atmospheric pressure chemical ionization (APCI) and electrospray

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ionization (ESI), now provide opportunities to rapidly characterize these nonvolatile naturally occurring compounds. Electron ionization mass spectrometry and APCI mass spectrometry were reported for the analysis of some limonoid aglycones, but no detailed fragmentation pathways were proposed. Electrospray ionization mass spectrometry has also recently been successfully applied for the characterization and quantification of limonoid glycosides. Tandem mass spectrometry is a method that can assist in identification and structural elucidation of organic compounds. In this paper, we report the first application of tandem mass spectrometry for characterization of limonoid aglycones and limonoid glucosides.

Figure 1. Chemical structures of limonoids (A) limonin (M 470), (B) obacunone (M 454), (C) nomilin (M 514), (D) deacetylnomilin (M 472), (E) limonin 17-β-D-glucopyranoside (M 650), (F) nomilin 17-β-D-glucopyranoside (M 694), (G) obacunone 17-β-D-glucopyranoside (M 634), (H) deacetyl nomilinic acid 17-β-D-glucopyranoside (M 670), (I) nomilinic acid 17-β-D-glucopyranoside (M 712), and (J) obacunoic acid 17-β-D-glucopyranoside (M 652).
EXPERIMENTAL SECTION

Reagents and Standard Limonoids. HPLC grade methanol and ACS reagent grade glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Reagent grade ammonium hydroxide was bought from Corco Chemical Co. (Fairless Hills, PA). Four limonoid aglycones (limonin, nomilin, obacunone, and deacetyl nomilin) and four limonoid glucosides, that is, limonin 17β-α-glucopyranoside (LG), nomilinic acid 17β-α-glucopyranoside (NAG), obacunone 17β-α-glucopyranoside (OG), deacetyl nomilinic acid 17β-α-glucopyranoside (DNAG), were purified and characterized from the seeds of Citrus tangerina (Tanaka) Tsen (Zhejiang Province, China) according to our previously published method, and the purity (>95%) and structure of the eight limonoids were further confirmed by analytical HPLC and NMR.15,17,18 In brief, citrus seeds were defatted by continuous extraction with boiling hexane for 24 h in a Soxhlet apparatus. The residue was reextracted for 24 h with acetone for limonoid aglycones. The acetone extract was evaporated under vacuum (<50 °C) with a rotavapor, the residue was dissolved in a minimum amount of dichloromethane, and two volumes of 2-propanol were added to crystallize the limonin. Limonin was repeatedly crystallized in dichloromethane and 2-propanol. The remaining solution was vacuum-dried and dissolved in a minimum amount of acetone for further purification of other limonoid aglycones by open column chromatography on silica gel and preparative TLC. Limonoid glucosides in the remaining seed residue was extracted with boiling methanol in a Soxhlet apparatus for 24 h. The methanol extract was evaporated under vacuum and redissolved in deionized water, and the water solution was repeatedly chromatographed using XAD-2 (Rohm & Haas, Philadelphia, PA) and WA-30 resins (Mitsubishi Chemicals, Chiyoda-Ku, Tokyo). Fractomatographed using XAD-2 (Rohm & Haas, Philadelphia, PA) and methanol extract was evaporated under vacuum and redissolved with boiling methanol in a Soxhlet apparatus for 24 h. The column chromatography on silica gel and preparative TLC was vacuum-dried and dissolved in a minimum amount of acetone for further purification of other limonoid aglycones by open column chromatography on silica gel and preparative TLC. Limonoid glucosides in the remaining seed residue was extracted with boiling methanol in a Soxhlet apparatus for 24 h. The methanol extract was evaporated under vacuum and redissolved in deionized water, and the water solution was repeatedly chromatographed using XAD-2 (Rohm & Haas, Philadelphia, PA) and WA-30 resins (Mitsubishi Chemicals, Chiyoda-Ku, Tokyo). Fractions of the methanol eluant containing limonoid glucosides (identified using TLC with Ehrlich’s reagent19) were evaporated to dryness under vacuum and further purified by preparative HPLC on a Waters C18 column (250 × 20 mm, 10 μm) for each individual limonoid glucoside. Standard solutions of limonoid aglycones (sonication for ~5 to 10 min) and glucosides with concentrations up to 100 μg/mL were prepared in methanol and filtered though 0.2 μm nylon filters (Waters Co., Milford, MA).

Plant Materials. Fresh Ruby Red grapefruits (Rio Grande Valley, TX) were purchased from a local supermarket. Three seeds were separated from one fruit and ground in a mortar and pestle after freezing with liquid nitrogen. Twenty milliliters of methanol−water (1:1, v/v) was used to extract the seed meals in a test tube and the solution was sonicated for 30 min. The extract was evaporated with a rotavapor (<50 °C) under vacuum to remove methanol. The aqueous solution was reextracted with methylene chloride (20 mL × 2) to remove the minor limonoid aglycones. After separation, the water fraction was loaded to a preactivated Sep-Pak C18 cartridge (Waters Corporation, Milford, MA). The cartridge was first washed with deionized water, and the limonoid glucosides were eluted with methanol.

Apparatus. All spectra were obtained using a quadrupole mass spectrometer (Quattro Ultima, Micromass UK limited, Manchester, U.K.). The mass spectrometer was interfaced to a Waters 2695 HPLC system equipped with an autoinjector and a photodiode-array detector (Milford, MA). Methanol containing 0.1% ammonium hydroxide or acetic acid at a flow rate of 0.8 mL/min was used as the carrying solvent during mass spectrometry and tandem mass spectrometry. Direct infusion was carried out by introducing the limonoid solutions with a Harvard model 11 single syringe pump (Holliston, MA) through a micro splitter valve (Upchurch Scientific, Oak Harbor, WA) at a flow rate of 0.01 mL/min to the carrying solvent and then to the interface of the mass spectrometer. The mass spectrometer was calibrated with sodium iodide and cesium iodide, and the data were processed by Masslynx V3.5 software.

Mass Spectrometry of Limonoids. APCI-MS analysis of limonoid aglycones was performed in both positive and negative ion modes. The extract cone voltage, corona current, vaporizer temperature, and drying gas temperature were optimized with regard to maximum signals of protonated ions, deprotonated ions, or adduct ions, depending on whether positive or negative ion analysis was performed. The optimum positive ion APCI conditions included an extract cone voltage of 100 V, a corona current of 4.70 μA, and a desolvation gas temperature of 350 °C at 4.3 L/min. In negative ion APCI, the extract cone voltage was maintained at ~85 V and corona current, at 5.6 μA, and other parameters were kept the same as in positive ion analysis.

Both positive and negative ion ESI-MS were also used for limonoid glucoside analysis and the mass spectrometer was optimized with regard to the maximum abundance of adduct ions, [M + NH3 + H+]+, [M + Na]−, and [M + K]−, or deprotonated molecular ions in positive and negative ion mode, respectively. Typical positive ion ESI conditions were as follows: capillary voltage, 3.35 kV; cone voltage, 63 V; source temperature, 120 °C; and desolvation temperature of 300 °C at a flow of 16 L/min. In negative ion ESI analysis, the capillary voltage was maintained at ~3.5 kV and cone voltage, at ~65 V. Other parameters were identical to positive ion ESI conditions.

Tandem Mass Spectrometry of Limonoids. Collisionally activated dissociation tandem mass spectrometry of limonoid aglycones was carried out in negative ion APCI analysis. Fragmentation of the precursor ion (M−) was enhanced by CAD using argon gas in the collision cell of the mass spectrometer. The collision energy was adjusted so that the abundance of the selected precursor ions was attenuated to 50% typically around 20 eV. For limonoid glucoside analysis, the collision energy was also set to attenuate the precursor ion [M + H + NH3]− to ~50% The CAD conditions were optimized for each analyte.

RESULTS AND DISCUSSION

Mass Spectrometry and Tandem Mass Spectrometry of Limonoid Aglycones. Initially, both ESI and APCI were used to analyze the four limonoid aglycones in positive and negative ion modes, respectively. Unlike limonoid glucosides,15 recognizable spectra of the four limonoid aglycones were not obtained with ESI in both positive and negative ion analysis. During APCI analysis, different mobile-phase modifiers were used to enhance the protonation or deprotonation of limonoid aglycones. Acidification of the mobile phase with acetic acid increased the abundance.

of the molecular anions of the four limonoid aglycones during negative ion APCI, whereas formic acid did not show the expected results (data not shown). When ammonium hydroxide was added to the carrying solvent, the abundance of both the protonated molecular ions and deprotonated molecular ions of the four limonoid aglycones was increased in positive ion and negative ion APCI analyses, respectively.

During positive ion APCI analysis, abundant protonated molecular ions, \([M+H]^+\), were observed as base peaks in the spectra of limonin, obacunone, and deacetylnomilin and as the second abundant peak in the spectrum of nomilin when ammonium hydroxide was added to the carrying solvent (Figure 2). In addition, one adduct ion, \([M+NH_3+H]^+\), was also observed in significant abundance in the spectra of the four limonoid aglycones. In the spectrum of nomilin, two fragment ions at \(m/z\) 472.41, 455.43 corresponding to the molecular ion of deacetylnomilin and the protonated molecular ion of obacunone were observed, which were produced by loss of acetyl or acetoxy radicals and accompanied by hydrogen rearrangement from the protonated molecular ion of nomilin, respectively (Figure 2C). The observation of these fragment ions indicated the structural relationship among the three limonoid aglycones (nomilin, obacunone, and deacetylnomilin).

During negative ion APCI-M S analysis, different adduct ions were observed, depending on the mobile-phase modifiers used (Figure 3). Adduct ions with acetic acid were formed for all the four limonoids when acetic acid was added to the carrying solvent. In the spectra of limonin and deacetylnomilin, \([M+HOAc−H]^−\) was produced as base peaks or in high abundance (Figure 3A, D). In addition, an abundant fragment ion at \(m/z\) 455.43 resulting from the loss of a methyl radical from the molecular anion was also observed in the spectrum of limonin. Unlike limonin and deacetylnomilin, \([M+HOAc]^−\) was formed for obacunone and nomilin. The \([M+HOAc]^−\) was observed in much lower abundance in the spectrum of nomilin, which was possibly due to the acetoxy group in the A-ring of nomilin inhibiting the adduct ion formation of nomilin with acetic acid (see structures in Figure 1). Additionally, a fragment ion at \(m/z\) 454.36 corresponding to the molecular ion of obacunone that was formed by loss of an acetoxy radical and one hydrogen from the molecular ion of nomilin (Figure 3C) was also observed in the spectrum of nomilin. Another fragment ion at \(m/z\) 546.40 in the spectrum of nomilin might have been produced by loss of carbon monoxide from the adduct ion \([M+HOAc]^−\) at \(m/z\) 574.43. Further loss of a hydroxyl radical from \(m/z\) 546.4 formed the fragment ion at \(m/z\) 529.42. The spectrum of deacetylnomilin also showed the molecular ion information of obacunone at \(m/z\) 454.48 and the nomilin molecular ion at \(m/z\) 514.46 (Figure 3D). These two ions were possibly produced by elimination of water from deacetylnomilin (\(m/z\) 472.47) and loss of hydroxyl radicals from the adduct ion \([M+HOAc−H]^−\) of deacetylnomilin, respectively (Figure 3D).

When ammonium hydroxide was added to the carrying solvent during negative ion APCI-M S analysis, an unusual ion of \([M+46−H]^−\), possibly a sodium adduct ion, \([M+2Na−H]^−\), was observed in the spectra of limonin, obacunone, and deacetylnomilin (Figure 3E, F, H) rather than the adduct ion with ammonia detected in positive ion analysis. In addition, the deprotonated molecular ions, \([M−H]^−\), were also observed as base peaks in the spectra of limonin and obacunone, although in low abundance in the spectra of nomilin and deacetylnomilin. But nomilin and deacetylnomilin formed abundant deprotonated molecular ion of obacunone as base peaks by loss of acetic acid from the deprotonated molecular ion of nomilin at \(m/z\) 513.38 and loss of one molecule of water from the deprotonated molecular ion of deacetylnomilin at \(m/z\) 471.34, respectively (Figure 3G, H). Additionally, another ion at \(m/z\) 499.37 was observed, which might have been produced by loss one molecule of water from the adduct ion \([M+46−H]^−\) of deacetylnomilin (Figure 3H).
Comparing the spectra of negative ion APCI-MS analysis of limonoid aglycones using different mobile-phase modifiers, we speculate that the A-ring of limonoids may affect the formation of adduct ions. For example, ions adducted with acetic acid were observed in high abundance in the spectra of limonin, obacunone, (C) nomilin, and (D) deacetylnomilin; and with 0.1% ammonium hydroxide as the mobile-phase modifier (E) limonin, (F) obacunone, (G) nomilin, and (H) deacetylnomilin.

Figure 3. Negative ion APCI-MS of limonoid aglycones with 0.1% acetic acid added to the mobile phase (A) limonin, (B) obacunone, (C) nomilin, and (D) deacetylnomilin; and with 0.1% ammonium hydroxide as the mobile-phase modifier (E) limonin, (F) obacunone, (G) nomilin, and (H) deacetylnomilin.

Comparing the spectra of negative ion APCI-MS analysis of limonoid aglycones using different mobile-phase modifiers, we speculate that the A-ring of limonoids may affect the formation of adduct ions. For example, ions adducted with acetic acid were observed in high abundance in the spectra of limonin, obacunone, and deacetylnomilin (Figure 3A, B, D), but in low abundance for nomilin (Figure 3C). The [M + 46 – H] of nomilin was not observed when ammonium hydroxide was added to the solvent under negative ion APCI (Figure 3G), whereas abundant [M + 46 – H] ions were produced for the other three limonoids (Figure 3E, F, H), indicating that the acetoxy group in nomilin might inhibit the formation of adduct ions with acetic acid or sodium.

Because the molecular anions (M⁻) were preferred over deprotonated ions or quasimolecular ions in mass spectrometry characterization of limonoids, MS/MS analysis of the four limonoid aglycones was carried out in negative ion mode by collisional dissociation of the molecular anions. Methanol containing 0.1% glacial acetic acid was used as carrying solvent during direct infusion experiments.
Limonin. The most abundant fragment ion in the CAD spectra of limonin was at m/z 454.91 (Figure 4A). This ion was formed by loss of a methyl radical from the deprotonated molecular ion $[M - H]^-$. Further loss of one molecule of carbon dioxide ($CO_2$) from m/z 454.91 produced m/z 410.9. The second abundant ion at m/z 109.81 possibly resulted from the cleavage of the C-ring after elimination of one methyl radical from the deprotonated molecular ion m/z 469.94. Another low-abundance fragment ion at m/z 366.76 might have been produced by elimination of another carbon dioxide molecule from m/z 410.9.

Obacunone. In the CAD spectra of obacunone, the deprotonated molecular ion at m/z 453.78 and fragment ion at m/z 438.75 produced by loss of a methyl radical from the deprotonated molecular ion were observed in low abundance (Figure 4B). A
fragment ion at m/z 394.98 [M − H − CH₃ − CO₂]⁻ indicated the further elimination of carbon dioxide from the m/z 438.75. The most abundant fragment ion, which was formed from the cleavage of C-ring following the same fragmentation pattern as limonin, was observed in the spectrum at m/z 190.87. In addition, an abundant fragment ion observed at m/z 341.05 was possibly produced by the fragmentation of the A-ring, as shown in Figure 4B.

Nomilin. As the precursor of all the limonoids in plant biosynthesis,²⁰ the CAD spectrum of nomilin produced more informative fragments in the high mass region (Figure 4C). The spectrum was also dominated by the most abundant fragment at m/z 190.85, which was formed by cleavage of C-ring of nomilin with the same fragmentation pattern as limonin and obacunone. The molecular anion (m/z 514.02) was observed in low abundance and fragmented into another low abundant ion at m/z 499.24 by elimination of one methyl radical. The fragment at m/z 454.23 corresponding to the molecular ion of obacunone was formed by loss of the acetoxy group and one hydrogen from the molecular anion of nomilin. Further loss of one molecule of carbon dioxide and one hydrogen formed the fragment ion at m/z 409.97. Although the structure of the fragment ion at m/z 382.13 was not determined, this fragment ion further differentiated nomilin from the other limonoid aglycones investigated.

Deacetylnomilin. Like the three limonoid aglycones discussed above, the CAD MS/MS spectra of deacetylnomilin was also dominated by the same major fragment at m/z 190.06 (Figure 4D). The primary difference among the spectra of the three structural similar limonoids (deacetylnomilin, nomilin, and obacunone) was that the deacetylnomilin formed the [M − 2H]⁻ ion instead of the molecular anion or deprotonated molecular ion observed in the other two limonoid aglycones, indicating that an extra hydrogen, possibly the hydrogen of the hydroxyl group in the A-ring of deacetylnomilin was easily eliminated. Another fragment ion at m/z 148.1 might have been formed following a pathway similar to that of the fragment ions at m/z 148 or 149 in the spectra of the other three limonoids.

Mass Spectrometry and Tandem Mass Spectrometry of Limonoid Glucosides. Similarly to our previous report,¹⁵ four limonoid glucosides (LG, OG, DNAG, and NAG) produced abundant deprotonated molecular ions with few fragmentations in negative ion ESI analysis when acetic acid was used as the mobile-phase modifier (data not shown). In positive ESI analysis, ammonium hydroxide was used to enhance protonation of limonoid glucosides instead of trifluoroacetic acid, which was used in our previous study.¹⁵ Interestingly, in addition to two typical adduct ions, [M + Na]⁺ and [M + K]⁺, observed in high intensity, an adduct ion with ammonia, [M + NH₃ + H]⁺, was also produced as the base peak for the four limonoid glucosides (Figure 5). Additionally, a significantly abundant molecular cation was also observed in the spectrum of NAG (Figure 5D).

To demonstrate the application of the ESI-MS method, Ruby Red grapefruit seed extract was analyzed using both positive and negative ion ESI mass spectrometry. Two major limonoid glucosides, nomilin 17-β-D-glucopyranoside (NG) and obacunioic acid 17-β-D-glucopyranoside (OAG), were found to be present in the grapefruit seed extract (Figure 6). Although it was difficult to differentiate the presence of NG and OAG on the basis of the positive ion spectrum because of the complexity of components in the crude extract (Figure 6A), negative ion ESI spectrum clearly showed that on the basis of the high abundant deprotonated molecule ions of NG at m/z 693.68 and OAG at m/z 651.64 (Figure 6B), only two major limonoid glucosides were present. It should be noted that the same adduct ions as in the analysis of the four purified limonoid glucosides were formed during positive ion ESI-

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Figure 6. Positive and negative ion ESI-MS analysis of grapefruit seed extract with ammonium hydroxide as the mobile-phase modifier in positive ion mode and acetic acid as mobile-phase modifier in negative ion mode: (A) positive ion ESI-MS spectrum and (B) negative ion ESI-MS spectrum.

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MS of the extract. The ions \([M + H + \text{NH}_3]^+\) of NG at \(m/z\ 712.78\) and OAG at \(m/z\ 670.74\); the sodium adduct ions, \([M + \text{Na}]^+\), of NG (\(m/z\ 717.82\)) and OAG (\(m/z\ 675.79\)); as well as the potassium adducts, \([M + \text{K}]^+\), of NG at \(m/z\ 733.66\) and OAG at \(m/z\ 691.76\) were all observed in high abundance.

Tandem mass spectrometry of limonoid glucosides was carried out by collisional activation of the adduct ion \([M + H + \text{NH}_3]^+\). Similarly to our investigation of limonoid aglycones, MS/MS spectrometry of limonoid glucosides was conducted in both positive and negative ion analysis during our initial study. By CAD of the deprotonated molecular ions of limonoid glucosides in negative ion ESI analysis, the spectra were found to be dominated by one fragment ion, which corresponded to the loss of one hydrogen from the deprotonated molecular ions. No additional informative fragments were observed in addition to this fragment (data not shown), whereas for collisional activation of the adduct ion \([M + H + \text{NH}_3]^+\) in positive ion ESI analysis, molecular ion information of the corresponding aglycone was obtained for each limonoid glucoside. Tandem mass spectrometry was also applied to the analysis of the fragmentation of NG and OAG identified in Figure 7.

**Figure 7.** Positive ion MS/MS mass spectra of limonoid glucosides (A) limonin 17-\(\beta\)-D-glucopyranoside, (B) nomilin 17-\(\beta\)-D-glucopyranoside, (C) obacunone 17-\(\beta\)-D-glucopyranoside, (D) deacetyl nomilinic acid 17-\(\beta\)-D-glucopyranoside, (E) nomilinic acid 17-\(\beta\)-D-glucopyranoside, and (F) obacunoic acid 17-\(\beta\)-D-glucopyranoside.
grapefruit seed extract by ESI-MS.

LG differentiates from the other five limonoid glucosides in structure by having intact A,A′-rings (see Figure 1). The CAD spectrum of LG was dominated by the corresponding aglycone molecular ion (limonin) at m/z 470.97 (Figure 7A). Another unusual fragment ion at m/z 425.03 was possibly formed by neutral elimination of one molecule of carbon dioxide and loss of one hydrogen from limonin. One unusual fragment ion at m/z 374.83 in the spectrum of LG might have been produced by the elimination of the furan ring in limonin (see Figure 8 for fragmentation pathways).

**Figure 8.** Proposed fragmentation pathways of limonin 17-β-D-glucopyranoside and nomilinic acid 17-β-D-glucopyranoside in MS/MS: (A) fragmentation of limonin 17-β-D-glucopyranoside and (B) fragmentation of nomilinic acid 17-β-D-glucopyranoside.
NG and OГ have similar structures with infused A-A rings but an intact A ring. In the CAD spectra of NG (Figure 7B) and OГ (Figure 7C), the major fragment ions corresponding to the protonated aglycone molecular ions (nomilin and obacunone) were formed as base peaks. A fragment at m/z 469.34 in the spectrum of NG was possibly formed by loss of CH2O2 from the protonated molecular ion of nomilin (m/z 515.1). An analogous fragment ion at m/z 408.68 in the CAD spectrum of OГ was also observed, but was accompanied by the loss of one extra hydrogen from the protonated molecular ion of obacunone. Like LG, low abundant fragment ions formed by loss of the furan ring from the corresponding aglycone molecular ion were observed in the spectra of both NG and OГ.

DNAG, NAG, and OAG are three acidic limonoid glucosides with infused A-A rings and an open A ring. In the spectrum of DNAG, the corresponding aglycone cation (deacetyl nomilinic acid) at m/z 490.85 was produced as the base peak (Figure 7D). In addition, a fragment ion at m/z 473.09 corresponding to the protonated aglycone of obacunolic acid was also formed by loss of one molecule of water and accompanied by a hydrogen migration from deacetyl nomilinic acid. Another structurally informative fragment m/z 454.96 corresponding to the molecular ion of obacunone might have been produced by further loss of one molecule of water from obacunolic acid. Additionally, a fragment ion of [aglycone – H2O – 95]+ was also observed at m/z 377.3, indicating a molecule of water was easily eliminated in deacetyl nomilinic acid. The corresponding aglycone molecular ions of NAG and OAG (nomilinic acid at m/z 532.92, obacunolic acid at m/z 472.84) were also observed in high abundance. In the CAD spectrum of NAG (Figure 7E), the second abundant ion at m/z 514.85 corresponding to the molecular ion of nomilin was produced by loss of one molecule of water and rearrangement of the A ring from nomilinic acid. Further loss of one molecule of acetic acid from nomilin resulted in obacunone at m/z 454.89 (see Figure 8 for fragmentation pathways). Another two fragments at m/z 436.88 and 418.99 were possibly formed by loss of the furopyridine cations from m/z 532.92 (nomilinic acid) and m/z 514.85 (nomilin), respectively. Although the corresponding aglycone molecular ion was observed in high abundance in the CAD spectrum of OAG (Figure 7F), another fragment ion at m/z 426.63 was produced as the base peak, which might have been formed by loss of CH2O2 from the opened A ring of obacunolic acid (m/z 472.84). The loss of furopyridine ring [aglycone – 95]+ was also observed at m/z 377.18. In addition, the protonated molecular ion of obacunone at m/z 455.02 was produced by loss of one molecule of water and rearrangement of the A ring, as in NAG. Although the structures of some abundant fragment ions in the CAD spectrum of OAG were not determined, OAG was found to produce more fragments in the CAD experiments, as compared to other limonoid glucosides investigated. Comparing the structure of OAG with the other limonoid glucosides, it was noted that the double bond in the open A ring of OAG might have induced extensive fragmentation.

CONCLUSIONS

The current study has demonstrated that both positive and negative ion APCI and ESI mass spectrometry are suitable for identification and characterization of limonoid aglycones and glucosides. The formation of adduct ions during mass spectrometry depended on the additives used in the mobile phase and was also affected by the structure of limonoids. Generally, acetic acid adducts were observed in negative ion APCI-MS of limonoid aglycones when acetic acid was used as the mobile-phase modifier, and ammonia adducts were observed in positive ion APCI-MS when ammonium hydroxide was added to the mobile phase. In addition, the mobile-phase modifiers also affected the formation of molecular anions or deprotonated molecular ions in negative ion APCI-MS of limonoid aglycones. The protonated molecular ions were normally observed in positive ion APCI-MS of limonoid aglycones. For electrospray ionization mass spectrometry of limonoid glucosides, adduct ions with ammonia, sodium, and potassium were produced in positive ion ESImS using ammonium hydroxide as the mobile-phase modifier, while deprotonated molecular ions were observed in negative ion ESI/MS when acetic acid was added to the mobile phase. The use of different mobile-phase modifiers not only enhances the protonation or deprotonation of limonoids but also assists in identifying the presence of limonoids. The employment of both positive and negative ion analyses provided complementary techniques for the positive identification of limonoids.

Negative ion APCI tandem mass spectrometry of limonoid aglycones and positive ion ESI-MS/MS of limonoid glucosides produced informative fragments, assisting in the identification of the limonoids. The CAD spectra of four limonoid aglycones produced characteristic fragmentation patterns that showed structural information of the precursor ions, indicating the presence of methyl, acetoxy, or oxygenated ring structure. In addition to the molecular anion or deprotonated molecular ions observed in the MS/MS spectra, the fragmentation pattern also showed the structural relationship between the limonoid aglycones, such as the predominant fragment ion at m/z 190 indicating the identical C, D, and E rings present in the structure of the four limonoid aglycones. The protonated aglycone molecular ion of the aglycone cation corresponding to each glucoside was obtained in high abundance after CAD of the limonoid glucoside adduct ion [M + NH3 + H]+. Structurally informative fragment ions, such as fragment ions related to loss of the furopyridine cation, water, or acetic acid in the CAD spectra, were also identified, which may be used to confirm the functional groups present in the structure. Furthermore, the MS/MS spectra reduced the background noise and eliminated ions from other constituents that may interfere with the interpretation of mass spectrometry data. In conclusion, mass spectrometry in combination with tandem mass spectrometry can be used as an important tool for identification and characterization of citrus limonoids and represents a powerful analytical technique for further studies in this field.

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