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Characterization of Phytoecdysteroid Glycosides in Meadowfoam (*Limnanthes alba*) Seed Meal by Positive and Negative Ion LC-MS/MS

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Meadowfoam (*Limnanthes alba*) is an oilseed crop grown in western Oregon. The seed meal has potential value as a biopesticide due to glucosinolate degradation products and phytoecdysteroids, a group of polyhydroxylated triterpenoids with potent activities as arthropod molting hormones. Liquid chromatography in combination with tandem mass spectrometry operated in the precursor ion mode revealed the presence of four ecdysteroid glycosides in meadowfoam seed meal. The carbohydrate sequence and the identity of the ecdysteroid aglycones, ponasterone A and 20-hydroxyecdysone, were determined by product ion scanning. Ecdysteroids were detected in the negative ion mode as $[M + \text{formate}]^-$ ions, which yielded $[M - H]^-$ and α-cleavage fragments with retention of hydroxyl groups in MS/MS experiments (not seen in the positive ion mode), allowing the determination of the number of hydroxyl groups in the side chain and in the steroid ring system. MS/MS of glycoside ions ($[MH]^+$ or $[M + \text{formate}]^-$) provided carbohydrate sequence information.

KEYWORDS: Meadowfoam; seed meal; *Limnanthes alba*; ecdysteroid glycoside; tandem mass spectrometry; electrospray ionization; atmospheric pressure chemical ionization (APCI)

INTRODUCTION

Ecdysteroids form a group of extensively hydroxylated steroids with variable side-chain lengths, derived from cholesterol (C_{27} steroid), ergosterol (C_{28} steroid), or sitosterol (C_{29} steroid). Characteristic for ecdysteroids is the presence of a 14α-hydroxy-7-en-6-one system (*1, 2*). These steroids function as molting hormones in insects. In plants, ecdysteroids are found at concentrations ranging from 0.001 to 2% (*1–3*). In the plant kingdom, phytoecdysteroids are frequently found in ferns (Polypodiaceae) and gymnosperms (Taxaceae and Podocarpaceae), whereas phytoecdysteroids show a scattered distribution in angiosperms (e.g., Ranunculaceae, Amaranthaceae, Verbenaceae, Lamiaceae, and Asteraceae) (*2, 4*). The accumulation of phytoecdysteroids in plants is generally considered to be the result of the coevolutionary arms race between plants and their herbivore pests. In insects, phytoecdysteroids cause disruption of the molting process, leading to death (*2*). There are at least 386 ecdysteroids and 34 ecdysteroid glycosides reported in the literature (*4, 5*).

The white meadowfoam (*Limnanthes alba* Hartw. ex Benth., Limnanthaceae) is native to southern Oregon and northern California (*6, 7*). The species received its name because fields of the plant in bloom (May−June) resemble sea foam. It received attention in the mid 1960s as a potential new oilseed crop (*8*). In the 1970s and 1980s, the species was subjected to a breeding program at Oregon State University with the aim to develop cultivars with improved agronomical properties and seed oil content (*9, 10*). Meadowfoam is now an established oil seed crop in western Oregon. The seed oil, which is rich in 20:1 and 22:1 fatty acids not commonly found in other seed oils (*11*), is mainly used by the cosmetic industry as an ingredient of skin care products. The spent seed material (seed meal) has potential value as a biopesticide due to the glucosinolate, glucolimnanthin (2−4% w/w), and its degradation products, that is, 3-methoxyphenylacetonitrile and 3-methoxybenzylisothiocyanate (*12*), and ecdysteroids (*4*). Glycosides of the ecdysteroids, ponasterone A (**1**) and 20-hydroxyecdysone (**2**), named "limnantheosides A−C" were reported from *L. alba* and *L. douglasii* by Dinan and co-workers (*13–15*). These authors used HPLC in combination with a radio-immunoassay method to detect ecdysteroids in HPLC fractions. Ecdysteroids have been reported from other species of *Limnanthes*, that is, *L. bakeri*, *L. floccosa*, *L. gracilis*, and *L. montana* (*15*).

The analysis of phytoecdysteroid glycosides in crude plant extracts presents a challenge. Our preliminary studies demonstrated that classical methods for analysis of glycosides, acid hydrolysis in particular, cannot be applied to phytoecdysteroid glycosides due to the instability (dehydration) of the aglycones. Our studies also indicated that glycosidases present in meadowfoam seeds show little or no hydrolytic activity for conversion

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of phytoecdysteroid glycosides into their aglycones, which would allow identification of the aglycones by chromatographic comparison with phytoecdysteroid standards. The analytical approach that we have employed in this study is based on cleavage of glycosides in the gas phase by collision-induced dissociation (CID) and fragmentation of the resulting aglycones to produce characteristic fragment ions for identification of the aglycones.

This investigation was designed to characterize the ecdysteroid glycosides by electrospray (ESI) and atmospheric pressure chemical ionization (APCI) LC-MS/MS on a hybrid linear ion trap triple-quadrupole mass spectrometer. Most previous studies reporting on the LC-MS/MS analysis of phytoecdysteroids were conducted in the positive ion mode, which yields mass spectra showing prominent loss of H_2O molecules from polyhydroxylated molecular ions, that is, $[MX - (H_2O)_n]^+$ where X = H, Na, or K (*16–20*). Because loss of H_2O molecules is generally less pronounced from polyhydroxylated molecular anions, we also explored the utility of LC-MS/MS in the negative ion mode for the structural characterization of ecdysteroids and their glycosides in meadowfoam seed meal. Few research groups have examined ecdysteroids by negative ion mass spectrometry. Examples include the analysis of metastable ions originating from $[M - H]^-$ by fast-atom bombardment (FAB) MS (*21*) and the analysis of pseudomolecular anions [M + acetate]$^-$ using a quadrupole mass analyzer with electrospray ionization (*17*). Ikeda and co-workers (*22*) studied the charge-remote fragmentation of $[M - H]^-$ ions of ecdysteroid monophosphates found in the silkworm, *Bombyx mori*. In Ikeda's study, charge-remote fragmentation of C−C bonds produced ions with retention of charge in the side chain and in the ring system fragments (*22*). We obtained $[M - H]^-$ and diagnostic C−C cleavage fragments by collision-induced dissociation of [M + formate]$^-$ ions of ecdysteroids and their glycosides.

MATERIALS AND METHODS

Chemicals. HPLC water was produced from reversed-osmosis water by a Milli-Q water purification system. HPLC-grade acetonitrile, hexane, and methanol were purchased from EMD Chemicals (San Diego, CA). Analytical grade EtOAc was from EMD Chemicals (San Diego, CA), and formic acid was from Fluka (Buchs, Switzerland). Ponasterone A (**1**), 20-hydroxyecdysone (**2**), ecdysone (**3**), and muristerone A (**4**) were purchased from A.G. Scientific (San Diego, CA). 20-Hydroxyecdysone (**2**) was also purchased from ChromaDex (Santa Ana, CA). Stock solutions of ecdysteroids (1 mM) were prepared in methanol and diluted to 100 or 10 μM with MeCN/H_2O (1:1, v/v).

Extraction of Ecdysteroids from Meadowfoam Seed Meal. Seed meal (6.0 g), from seeds grown in the Willamette Valley of Oregon, was stirred in MeOH (30 mL) for 60 min at room temperature using a magnetic stirrer. The suspension was allowed to settle, and the supernatant was centrifuged for 10 min. A 20 mL aliquot of the supernatant after centrifugation was mixed with 10 mL of brine. The resulting white precipitate was removed by centrifugation, and the clear liquid was extracted with hexane (1 × 30 mL) and then with EtOAc (1 × 30 mL, 2 × 15 mL). The combined EtOAc layers were washed with brine (30 mL), dried with anhydrous Na_2SO_4, and evaporated using a rotary evaporator. The residue was taken up in 2.0 mL of MeOH. A 100 μL aliquot of this solution was diluted with 900 μL of MeCN/H_2O (1:1, v/v). Five microliters of the diluted extract was injected onto the HPLC column. Alternatively, seed meal (1.0 g) was extracted with 5.0 mL of MeOH/H_2O (1:1, v/v) by sonication for 10 min and standing overnight at room temperature. The mixtures were centrifuged and the supernatants analyzed by LC-MS/MS after dilution as described above. The injection volume of these samples was also 5 μL.

LC-MS/MS Instrumentation and Conditions. Chromatographic separations were achieved on a 150 mm × 2.1 mm i.d., 4 μm, Cogent Bidentate C8 column (MicroSolv Technologies, Eatontown, NJ). The HPLC solvents were 0.01% aqueous formic acid (solvent A) and MeCN (solvent B). A linear solvent gradient was used starting from 5% solvent B in solvent A to 100% B over 15 min, followed by elution with 100% B for 5 min at a flow rate of 0.2 mL/min. After returning to the starting conditions in 1 min, the column was equilibrated for 10 min before the next injection. The HPLC system consisted of two Shimadzu Prominence LC-20AD pumps, a Shimadzu SIL-HTC autoinjector and system controller, and a switching valve (Shimadzu, Columbia, MD). The column effluent was directed to the mass spectrometer between 2 and 20 min of the chromatographic run and to a waste container during the remainder of the LC run.

The LC-MS/MS instrument consisted of a 4000 QTrap hybrid linear ion trap triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray (Turbo V) ion source operated at 400 °C (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Nitrogen (curtain and collision gas) and zero-air (heating and nebulizing gas) were provided by a gas generator. The spray needle was kept at +5.2 kV in the positive mode and at −4.5 kV in the negative mode. APCI spectra were recorded in the negative ion mode using the Turbo V source with a corona discharge needle current of −2 μA and a probe temp of 400 °C. Q1 mass spectra were recorded by scanning in the range of 200−1000 at a cycle time of 2 s with a step size of 0.2 u. MS/MS experiments (product ion and precursor ion scanning) were conducted at unit resolution for both Q1 and Q3 with the collision gas set at "medium". Enhanced product ion (EPI) scanning was performed by trapping fragment ions (produced in Q2 by CID) in Q3 (linear ion trap mode) with a dynamic fill time, followed by scanning of Q3 (quadrupole mass analyzer mode). Unless otherwise stated, the collision energy was 25 eV in the positive mode and 50 eV in the negative mode, with a collision energy spread of 10 eV. EPI spectra recorded in this manner show fragment ions (trapped in Q3 before scanning) arising from collisions at 15, 25, and 35 eV in the positive mode and 40, 50, and 60 eV in the negative ion mode.

Accurate mass measurements were performed on a Waters Micromass LCT Classic ToF instrument (Waters, Milford, MA) equipped with an electrospray ion source, interfaced to a Waters CapLC HPLC solvent delivery system. Sample solutions were introduced into the mass spectrometer by injecting 20 μL of analyte solution at a flow rate of 40 μL/min, using MeCN/H_2O (1:1 v/v containing 0.1% formic acid). The instrument was calibrated with polypropylene glycol (PPG) immediately before analysis. In-source fragmentation was achieved by setting the cone voltage at 45 V. The ecdysone (**3**) fragment ion, *m/z* 331.2278, deviated 1.5 ppm from the calculated mass of $C_{21}H_{31}O_3^+$ (331.2273). The 20-hydroxyecdysone (**2**) fragment ions, *m/z* 371.2225, 303.1959, and 301.1801, deviated 0.8, −0.3, and 1.0 ppm from the calculated masses of $C_{23}H_{31}O_4^+$ (371.2222), $C_{19}H_{27}O_3^+$ (303.1960), and $C_{19}H_{25}O_3^+$ (301.1798), respectively.

RESULTS AND DISCUSSION

Characterization of Ecdysteroid Aglycones by Positive Enhanced Product Ion MS/MS (+EPI). The four ecdysteroids examined (**1**−**4**; **Figure 1**) were resolved by reverse-phase HPLC and showed $[MH - nH_2O]^+$ ions in their +Q1 mass spectra (n = 0−3 at a declustering potential of 20 V). The in-source release of H_2O molecules from these polyhydroxylated aglycones was previously observed by Li and co-workers (*16*), who used a three-dimensional ion trap mass spectrometer for the characterization of ecdysteroids. Our +Q1 mass spectra provided little structural information for the distinction of the isobaric steroids, **1** and **3**, which were distinguishable by MS/MS using enhanced product ion (+EPI) scanning (**Figure 2**). Collision-induced fragmentation of $[MH]^+$ ions yielded fragment ions resulting from cleavage of carbon−carbon bonds in the side chain at C17, in addition to loss of H_2O neutrals from the molecular ions. For instance, the +EPI spectrum of **3** showed a prominent fragment ion with *m/z* 331 (**Figure 2C**) resulting from α-cleavage of the secondary alcohol at position 22 and

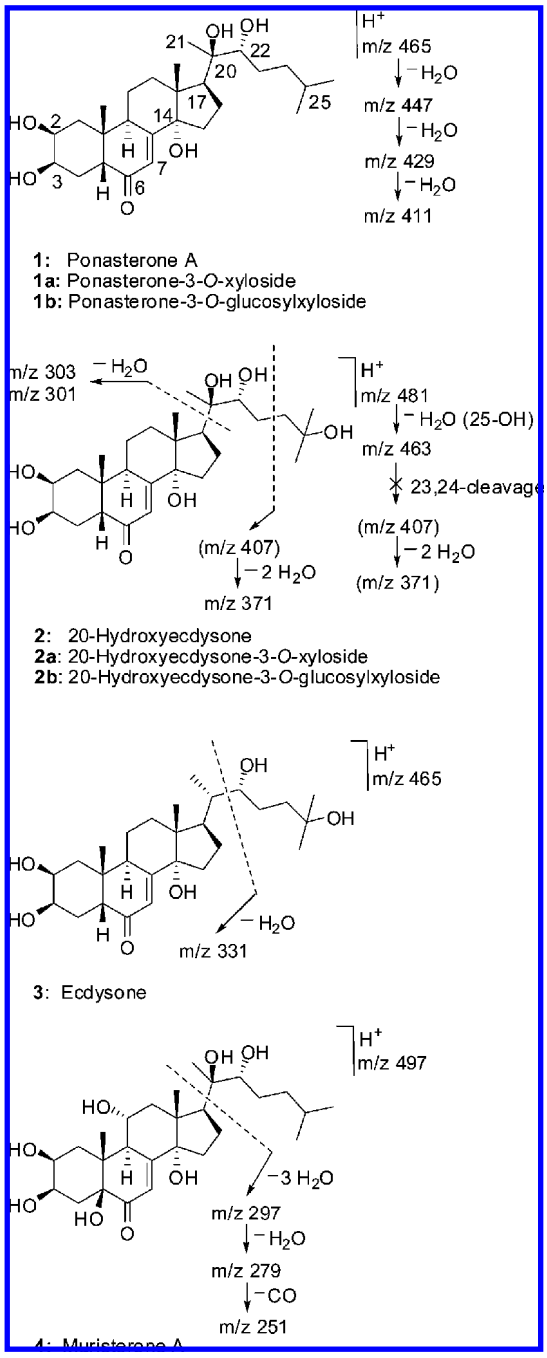

Figure 1. Structures of ecdysteroids **1**−**4** and their proposed MS/MS fragmentation in the positive ion mode.

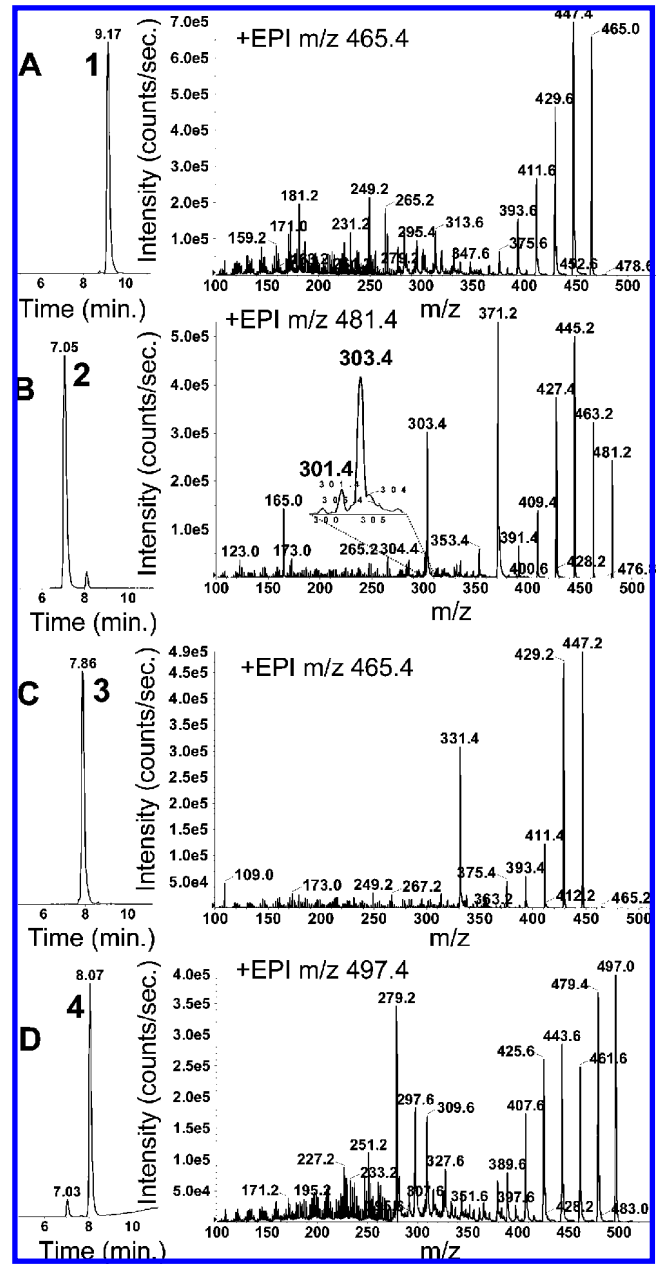

Figure 2. LC-ESI-MS/MS of ecdysteroid standards using enhanced product ion (EPI) scanning of the $[MH]^+$ ions of ponasterone A (**1**, *m/z* 465), 20-hydroxyecdysone (**2**, *m/z* 481), ecdysone (**3**, *m/z* 465), and muristerone A (**4**, *m/z* 497).

loss of H_2O. We propose that this H_2O molecule originates from the OH group at position 14, because the 2,3-diol functionality, the alternative source of H_2O molecules, should be resistant to loss of H_2O due to bridging of the proton between the vicinal diol oxygens (*16, 23*).

The elemental composition of the *m/z* 331 ion observed in the +EPI spectrum of **3**, determined by ESI-ToF MS analysis, was consistent with the fragmentation shown in **Figure 1**. The corresponding α-cleavage fragment with *m/z* 347 (331 + O) was observed for **1** at much lower intensity, presumably due to the charge stabilizing (proton-bridging) effect of the vicinal 20,22-diol functionality of **1** (*23*). Together with the lack of a hydroxyl group at C25 in **1**, this proton-bridging effect may explain why **1** is more resistant to loss of an H_2O molecule than **3** (*16*), which is evident from the marked differences between the intensities of the *m/z* 465 $[MH]^+$ and *m/z* 447 $[MH - H_2O]^+$ ions in the EPI spectra of **3** and **1** (**Figure 2**).

Of the steroids with a 20,22-diol functionality (**1**, **2**, and **4**), **2** yielded the lowest relative abundance of the molecular ion, which suggests that the OH at C25 is preferentially released first upon collisional activation of its $[MH]^+$ ion because **1** and **4** lack this hydroxyl group. Furthermore, protonation of C25-OH may initiate the cleavage between C23 and C24, giving rise to a fragment ion with *m/z* 371 (**Figure 1B**). This proposed fragmentation is consistent with the observation that CID of the *m/z* 463 $[MH - H_2O]^+$ ion of **2** generates the *m/z* 371 ion in poor yield (<10% relative intensity) and thus may be taken as additional evidence for the preferred release of the OH at C25. We hypothesize that the *m/z* 371 ion does not originate from the *m/z* 463 ion produced by loss of the 25-OH group (**Figure 1**) but that the low abundance of the *m/z* 371 ion observed in the EPI spectrum of *m/z* 463 results from CID of

a different *m/z* 463 precursor bearing OH groups at carbons 22 and 25. α-Cleavage of the C20-alcohol of **2** results in the formation of fragment ions with *m/z* 301 and 303 (**Figures 1** and **2B**). The proposed fragment ions with *m/z* 371 ($C_{23}H_{31}O_4^+$), *m/z* 303 ($C_{19}H_{27}O_3^+$), and *m/z* 301 ($C_{19}H_{25}O_3^+$) (**Figure 1**) are in agreement with accurate mass measurements performed on a ToF instrument. α-Cleavage of the C20-alcohol in **4** is accompanied by loss of multiple H_2O molecules, resulting in fragment ions with *m/z* 297 and 279 (**Figures 1** and **2D**).

The simultaneous loss of multiple H_2O molecules from protonated ecdysteroid species and α-cleavage of an alcohol moiety in the side chain at C17 presents a difficulty to determine how many hydroxyl groups are located in the steroid nucleus and how many in the side chain. The +EPI spectra of ecdysteroids can be used as fingerprints for the identification of ecdysteroids in extracts of plants by LC-MS/MS comparison, but seem to offer limited information for structure determination of unknown ecdysteroids. Because loss of H_2O molecules is generally less pronounced from molecular anions than from protonated molecules, we explored the use of negative ion MS/MS with the aim to produce carbon−carbon cleavage fragments with retention of hydroxyl groups.

Characterization of Ecdysteroid Glycosides in Meadowfoam Seed Meal by Positive Ion MS/MS. 20-Hydroxyecdysone-3-*O*-xyloside (**2a**), 20-hydroxyecdysone-3-*O*-glucosylxyloside (**2b**), and ponasterone-3-*O*-xyloside (**1a**) were previously identified in *L. douglasii* and *L. alba* (*13, 15*). To detect these ecdysteroid glycosides and possibly related compounds, a methanolic extract of meadowfoam seed meal was examined by LC-MS/MS using precursor ion scanning of the $[MH]^+$ ions of **1** and **3** (*m/z* 465), **2** (*m/z* 481), and **4** (*m/z* 497). The TIC of the precursors of *m/z* 465 showed six major peaks (Supporting Information Figure 1S). The precursor ions had the following *m/z* values: 465 (**1**), 465 and 483 (**2**), 597 (**1a** and **2a**), and 759 (**1b** and **2b**). The ions with *m/z* 465, 597, and 759 were subjected to +EPI scanning (**Figure 3**). The EPI spectrum and the chromatographic retention time (9.18 min) of **1** were identical with those of ponasterone A (cf. **Figure 2A**). Compound **1a** was identified as the xyloside of ponasterone A on the basis of the loss of 132 Da from its molecular ion to yield a fragment ion with *m/z* 465 and the similarity of its fingerprint region (100−465 Th) to the +EPI spectrum of **1**. The precursor ions of compounds **2**, **2a**, and **2b** that yielded *m/z* 465 by CID were subsequently identified as $[(^{13}C_2) - MH - H_2O]^+$ ions of **2** and its glycosides in EPI experiments, because the fragment ions appeared as a series of isotopomeric peaks.

Collision-induced dissociation of the *m/z* 465 ion of **2** revealed loss of multiple H_2O molecules and an isotopomeric series of fragment ions with *m/z* 301, 302, 303, 304, and 305 (**Figure 3C**). Ions with *m/z* 301 and 303 were also observed in the +EPI spectrum of 20-hydroxyecdysone, and therefore the *m/z* 465 ion of **2** was assigned to the $[(^{13}C_2) - MH - H_2O]^+$ ion of 20-hydroxyecdysone and not to the $[MH]^+$ ion of **1** or **3**. Likewise, the *m/z* 465 ion of **2** produced a series of isotopomeric fragment ions with *m/z* 371, 372, and 373 of low abundance (**Figure 3C**), consistent with the low yield of the *m/z* 371 fragment ion that resulted from CID of the *m/z* 463 $[MH - H_2O]^+$ ion of 20-hydroxyecdysone (not shown). Compound **2** was also identical to 20-hydroxyecdysone regarding chromatographic retention time (7.04 min) and by CID of its *m/z* 481 $[MH]^+$ ion.

Collision-induced dissociation of the *m/z* 759 ion of **1b** produced fragment ions consistent with sequential loss of glucose (162 Da) and xylose (132 Da), whereas the fingerprint region was characteristic of ponasterone A (100−465 Th, **Figure 3D**). The *m/z* 465 ion of **1b** appeared as a monoisotopic peak,

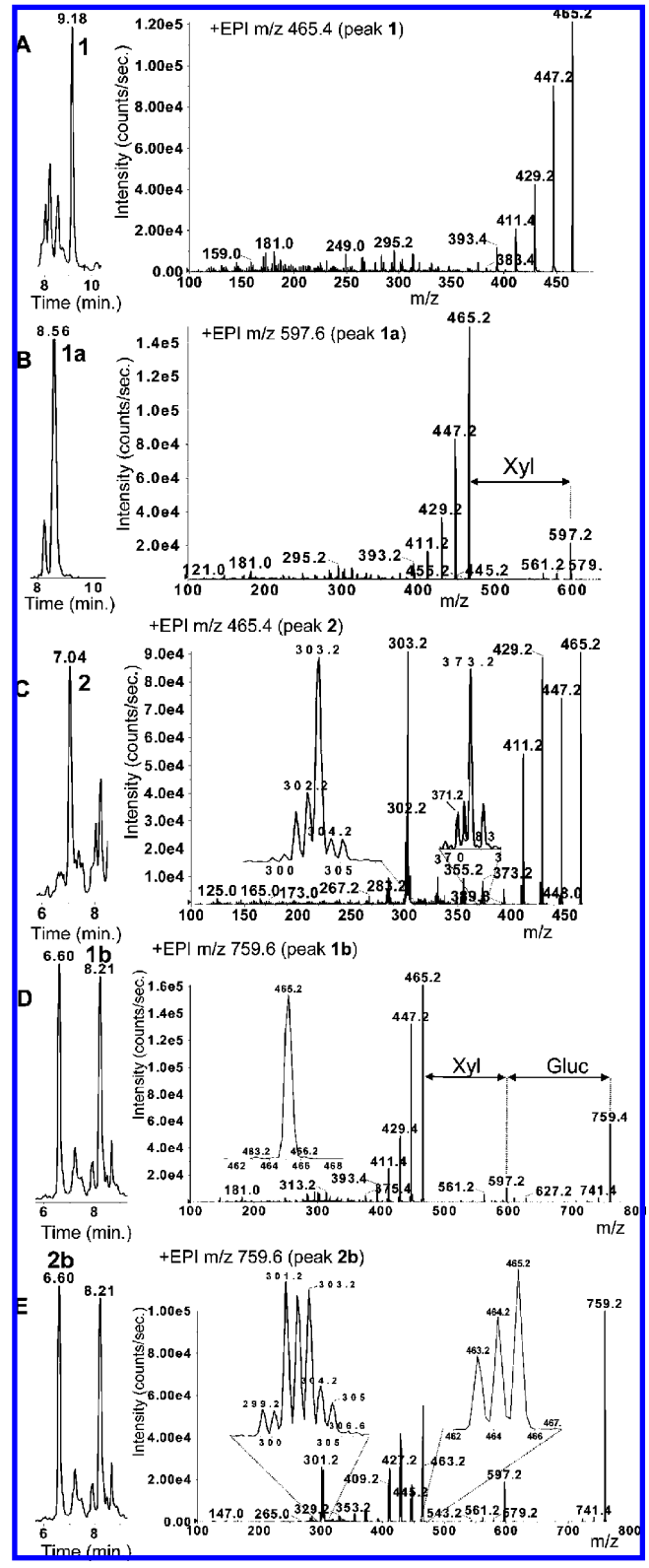

Figure 3. LC-ESI-MS/MS analysis of a meadowfoam seed meal extract showing +EPI spectra of ecdysteroids **1** (*m/z* 465 $[MH]^+$), **1a** (*m/z* 597 $[MH]^+$), **2** (*m/z* 465 $[(^{13}C_2) - MH - H_2O]^+$), **1b** (*m/z* 759 $[MH]^+$), and **2b** (*m/z* 759 $[(^{13}C_2) - MH - H_2O]^+$).

indicating that the precursor ion, *m/z* 759, represents the monoisotopic mass, [ponasterone A − *O*-xylose − *O*-glucose]H^+. CID of the *m/z* 759 ion of **2b** yielded a series of isotopomeric fragment ions with *m/z* 463, 464, and 465 that were assigned to $[2 - H_2O]H^+$ containing 0, 1, and 2 ^{13}C atoms, respectively (**Figure 3E**). Because the *m/z* 759 ion represents

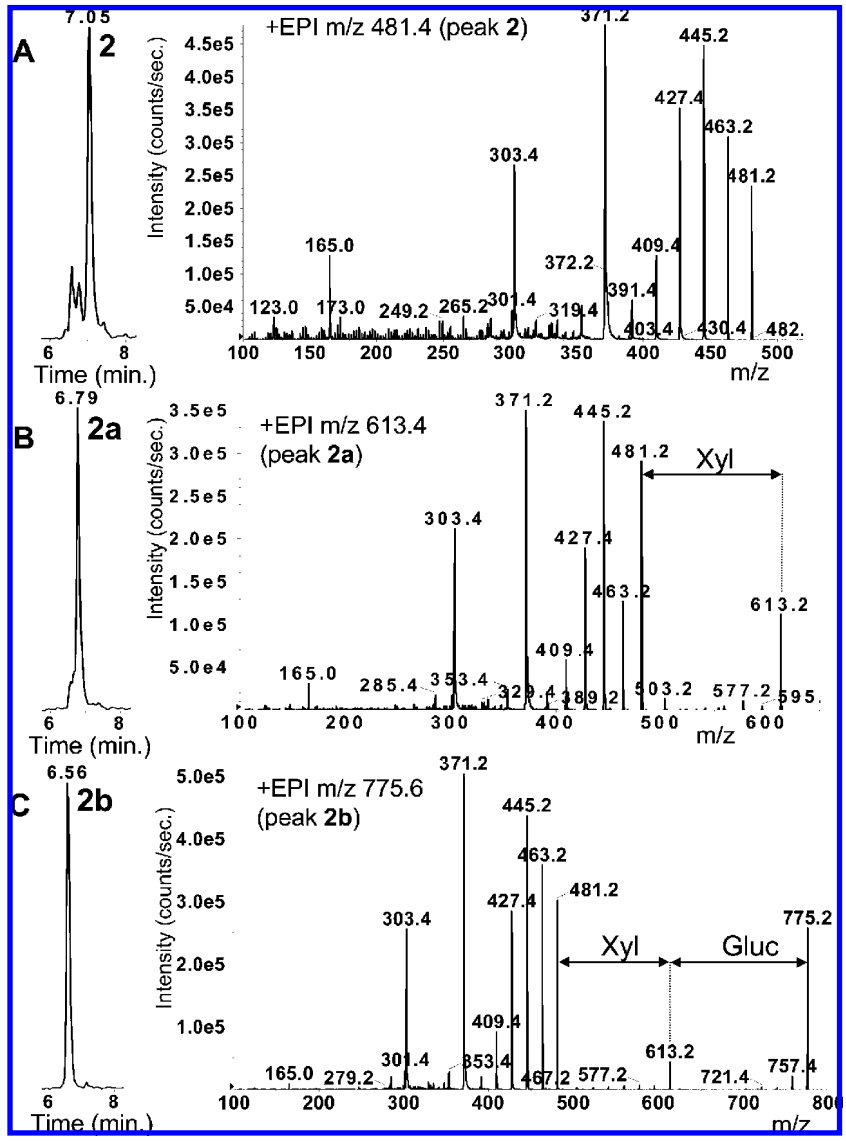

Figure 4. LC-ESI-MS/MS analysis of a meadowfoam seed meal extract showing +EPI spectra of ecdysteroids **2** (*m/z* 481 $[MH]^+$), **2a** (*m/z* 613 $[MH]^+$), and **2b** (*m/z* 775 $[MH]^+$).

$[(^{13}C_2)\mathbf{2} - H_2O]H^+$, the *m/z* 301 and 303 fragment ions of **2** (**Figures 1** and **2B**) appeared as a series of isotopomeric ions with *m/z* 301, 302, 303, 304, and 305 for **2b** (**Figure 3E**).

The relative intensity of the *m/z* 303 ion originating from *m/z* 465 in **2** (**Figure 3C**) was found too large by comparison with **2b** (**Figure 3E**) and with the +EPI spectrum of the *m/z* 465 ion $[(^{13}C_2) - MH - H_2O]^+$ of authentic 20-hydroxyecdysone (not shown). This discrepancy was further investigated as follows. The precursor ion spectrum of the *m/z* 465 ion of peak **2** showed a peak at *m/z* 611 in addition to peaks at *m/z* 465 and 483. CID of the *m/z* 611 ion yielded fragment ions with *m/z* 465 (loss of 146 Da, indicative of a deoxyhexosyl residue) and *m/z* 303 (loss of 162 Da from *m/z* 465, indicative of a hexosyl residue). This led us to investigate whether the coeluting compound was identical with quercetin-3-*O*-rhamnosylglucoside (rutin), a common flavonol glycoside found in many plants, including *L. douglasii* (*24*). An authentic sample of rutin indeed coeluted with **2** and produced the expected fragment ions, *m/z* 465 and 303, by CID of its molecular ion with *m/z* 611. Therefore, the large intensity of the *m/z* 303 ion in the +EPI *m/z* 465 spectrum of **2** (**Figure 3C**) was attributed to Q2 fragmentation of rutin's in-source fragment ion with *m/z* 465.

Q1 scanning with Q3 set at *m/z* 481 revealed major precursor ions with *m/z* 481 (residual intact ions, compound **2**), *m/z* 613 (**2a**), and *m/z* 775 (**2b**) (Supporting Information Figure 1S, panel B). The +EPI *m/z* 481 spectrum and the retention time of **2** were identical with those of 20-hydroxyecdysone (cf. **Figures 4A** and **2B**). Compound **2a** was characterized as the xyloside of 20-hydroxyecdysone on the basis of the loss of 132 Da from *m/z* 613 and the fingerprint region of the aglycone (100−481 Th). Similarly, the +EPI *m/z* 775 spectrum extracted at a retention time of 6.6 min is consistent with the identification of **2b** as the glucosylxyloside of **2** (**Figure 4C**).

Our LC-ESI-MS/MS examination of the meadowfoam seed meal extract and comparison of the data with literature reports (*13, 15*) indicate that peak 1 is ponasterone A, peak 1a is ponasterone A−3-*O*-xyloside (limnantheoside B), peak 1b is ponasterone A−3-*O*-glucosylxyloside (not previously reported in the literature), peak 2 is 20-hydroxyecdysone, peak 2a is 20-hydroxyecdysone-3-*O*-xyloside (limnantheoside A), and peak 2b is identical with 20-hydroxyecdysone-3-*O*-glucosylxyloside (limnantheoside C). Precursor ion scanning of *m/z* 497 did not indicate the presence of muristerone A glycosides, nor did +EPI scanning of *m/z* 497 show muristerone A in the seed meal extract. We also explored the use of LC-APCI-MS/MS in the

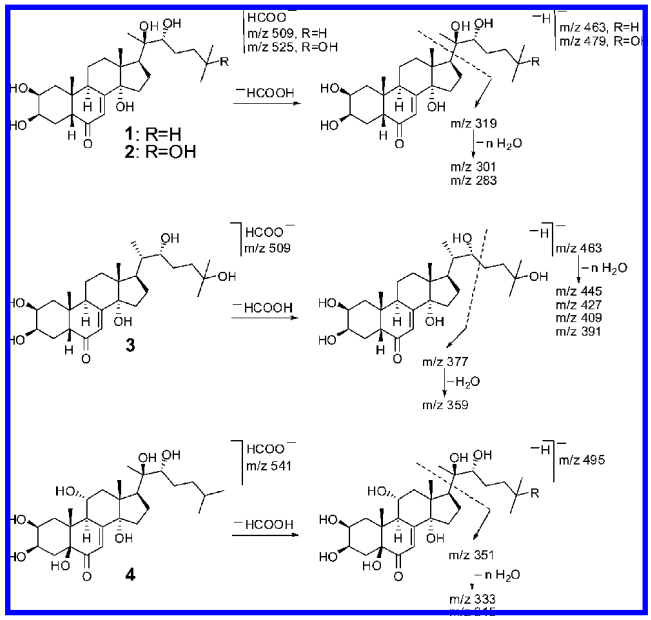

Figure 5. Proposed fragmentation pathway for ecdysteroids **1**−**4** by collisional activation of their formate adducts in the negative ion mode.

positive ion mode for analysis of ecdysteroids. APCI spectra recorded in the positive ion mode showed more prominent loss of H_2O molecules from $[MH]^+$ and fragment ions than +ESI spectra.

Characterization of Ecdysteroid Aglycones by Negative Enhanced Product Ion MS/MS (−EPI). The four ecdysteroid standards **1**−**4** produced negative ions by forming adducts with formate, that is, $[M + HCOO]^-$ ions, by LC-ESI-MS using Q1 scanning in the negative ion mode (the aqueous HPLC solvent contained 0.01% HCOOH). It was possible to dissociate these ions into molecular anions and formic acid by −EPI scanning. However, the intensity of the molecular ions was low compared to +EPI, presumably due to dissociation of the formate adducts into steroid neutrals and the formate ion. We observed slightly better sensitivity by using the APCI source compared to ESI in the negative ion mode due to a lower background signal. The study of negative ions was therefore continued with the APCI source. **Figure 5** shows the proposed fragmentation pathways for formate adducts of ecdysteroids.

Unlike MS/MS of ecdysteroid glycosides in the positive ion mode, CID of the formate−steroid adducts produced molecular anions and fragment ions resulting from α-cleavage of side-chain alcohols with retention of hydroxyl groups in the steroid ring system. These "intact" ring system ions were observed as peaks at *m/z* 319 (α-cleavage of the C20 alcohol in **1** and **2**), *m/z* 377 (α-cleavage of the C22 alcohol in **3**), and *m/z* 351 (α-cleavage of the C20 alcohol in **4**). These "ring system" ions and the molecular ions showed subsequent loss of H_2O molecules (**Figures 5** and **6**). Thus, the advantage of CID of ecdysteroid anions is that recognition of the "intact ring system" fragment ion allows the determination of the number of OH groups in the ring system and in the side chain. Although α-cleavage was observed in the +EPI spectra, it seemed to occur less predictably in the positive mode than in the negative mode. For example, **2** forms an α-cleavage ion with *m/z* 303, but not **1**, which has the same ring structure and 20,22-diol moiety.

Characterization of Ecdysteroid Glycosides in Meadowfoam Seed Meal by Negative Ion APCI-MS/MS. Due to loss of HCOOH neutrals upon CID of the steroid−formate adducts in Q2, we observed better signal by precursor ion scanning of the molecular anions $[M - H]^-$ compared to scanning with Q3

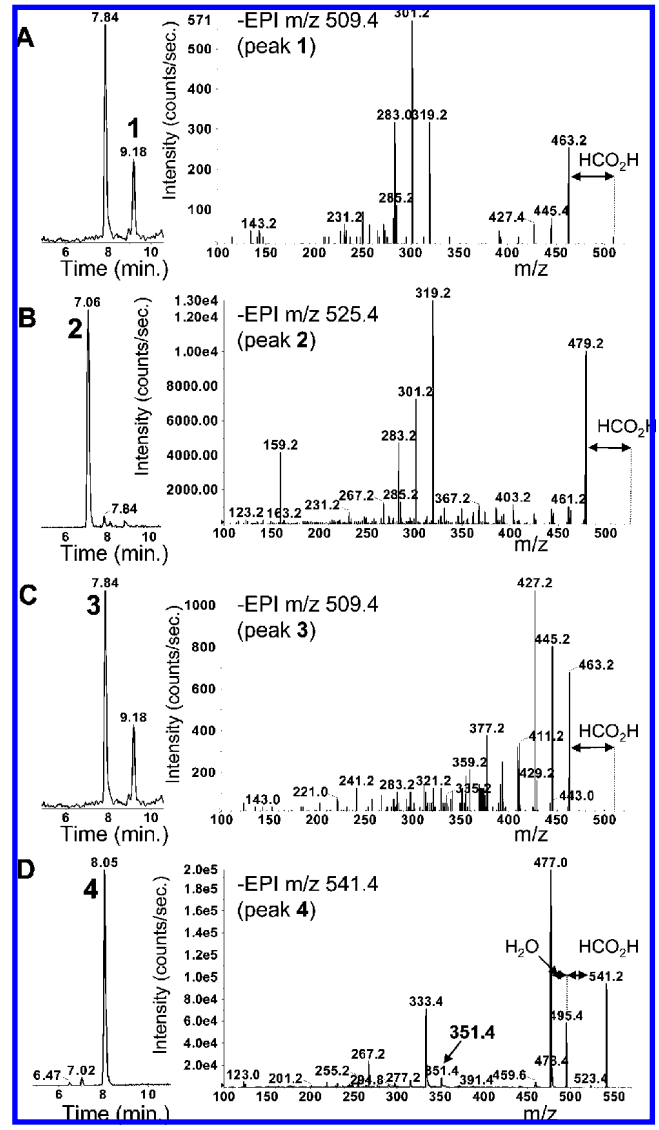

Figure 6. LC-APCI-MS/MS analysis of ecdysteroid standards using enhanced product ion (EPI) scanning of the [M + formate]$^-$ ions of ponasterone A (**1**, *m/z* 509), 20-hydroxyecdysone (**2**, *m/z* 525), ecdysone (**3**, *m/z* 509), and muristerone A (**4**, *m/z* 541). The collision energy was 50 eV for **1**, **2**, and **3** and 30 eV for **4**.

set at *m/z* values of $[M + HCOO]^-$ ions. As expected, the precursor ions of *m/z* 479 were found at *m/z* 525 [**2** + HCOO]$^-$, *m/z* 657 [**2a** + HCOO]$^-$, and *m/z* 819 [**2b** + HCOO]$^-$ (Supporting Information Figure 2S, panel A). These ions were subjected to CID in −EPI experiments (**Figure 7**). The −EPI spectra of compounds **2**, **2a**, and **2b** showed their molecular anions after loss of HCOOH. Glycosidic cleavage yielded fragment ions with *m/z* 479 [**2** − H]$^-$ for **2a** and fragment ions with *m/z* 611 and 479 for **2b**, consistent with the consecutive loss of a glucosyl and a xylosyl residue. Compounds **2**, **2a**, and **2b** all showed the intact ring system ion with *m/z* 319 originating from *m/z* 479. The −EPI spectra shown in **Figure 7** are thus in agreement with their proposed structures, that is, 20-hydroxyecdysone (**2**), 20-hydroxyecdysone-3-*O*-xyloside (**2a**), and 20-hydroxyecdysone-3-*O*-glucosylxyloside (**2b**), respectively.

Precursor ion scanning of the *m/z* 463 $[M - H]^-$ ion yielded $[M + HCOO]^-$ ions for compound **1a** (*m/z* 641) and compound **1b** (*m/z* 803) but not the expected *m/z* 509 ion for **1** (Supporting Information Figure 2S, panel B). −EPI scanning of [M + HCOO]$^-$ ions with *m/z* 641 and 803 yielded glycoside cleavage

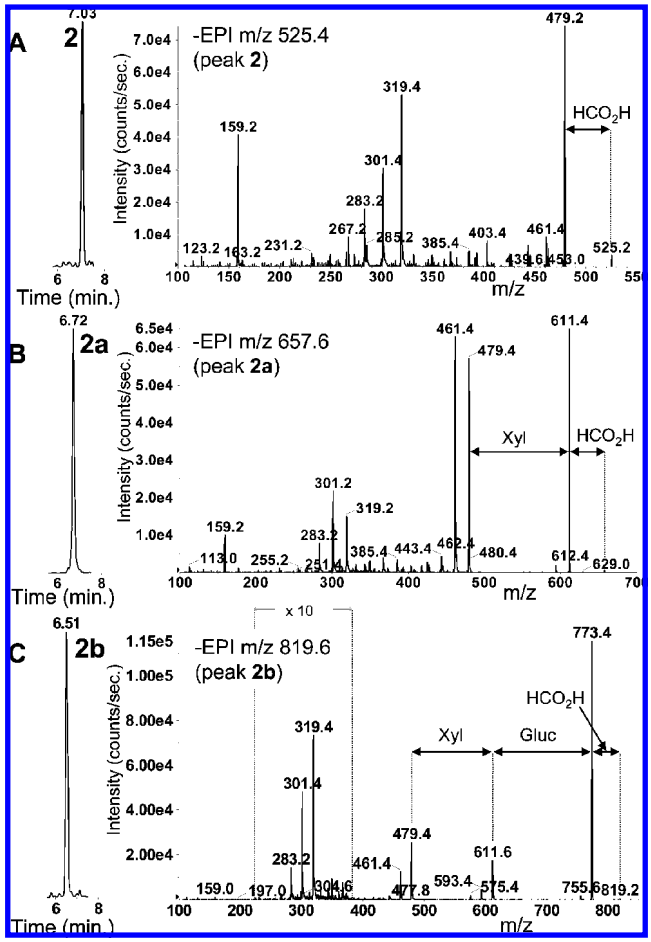

Figure 7. LC-APCI-MS/MS analysis of a meadowfoam seed meal extract showing −EPI spectra of peak **2** (*m/z* 525 [M + formate]$^-$), **2a** (*m/z* 657 [M + formate]$^-$), and **2b** (*m/z* 819 [M + formate]$^-$).

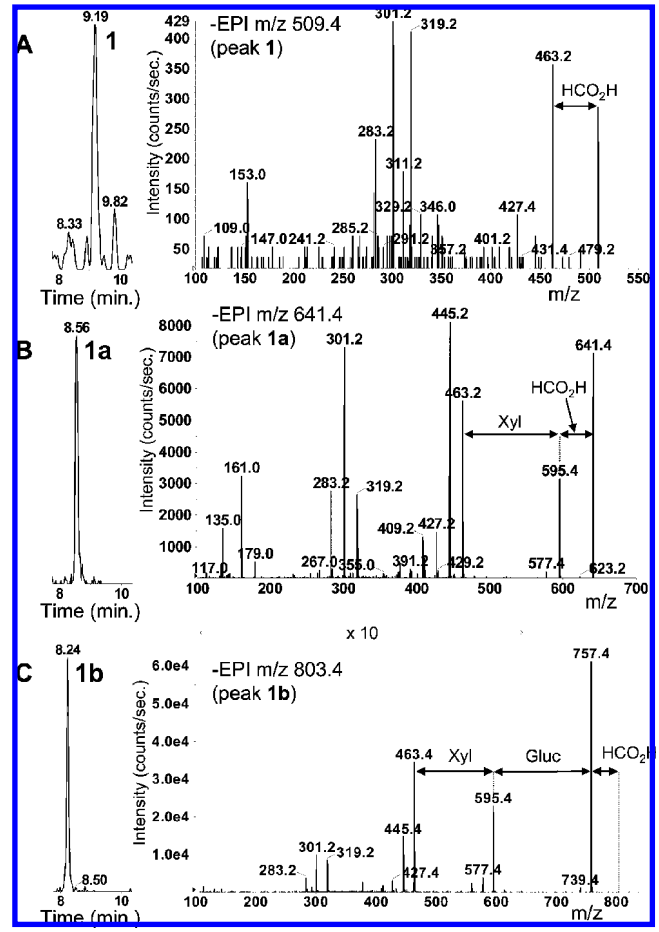

Figure 8. LC-APCI-MS/MS analysis of a meadowfoam seed meal extract showing −EPI spectra of peak **1** (*m/z* 509 [M + formate]$^-$), peak **1a** (*m/z* 641 [M + formate]$^-$), and **1b** (*m/z* [803 M + formate]$^-$).

fragments and an intact ring system ion with *m/z* 319, consistent with the characterization of peak **1a** as ponasterone A−3-*O*-xyloside and peak **1b** as ponasterone A−3-*O*-glucosylxyloside (**Figure 8**). Whereas **1** was not observed by precursor ion scanning of *m/z* 463, −EPI scanning of *m/z* 509 did reveal the presence of **1**, which showed a retention time of 9.2 min and a negative EPI spectrum similar to that of ponasterone A (cf. **Figures 8A** and **6A**). The precursors of the *m/z* 463 fragment ions of compounds **2**, **2a**, and **2b** were assigned to $^{13}C_2$ isotopomers of 20-hydroxyecdysone and its glycosides, which were not further investigated by −EPI scanning.

The strategy presented in this study allows the characterization of ecdysteroid glycosides by LC-MS/MS using a combination of precursor ion and product ion scanning in the positive and negative modes on a triple-quadrupole mass spectrometer. In our approach, the presence of ecdysteroid glycosides is revealed by precursor ion scanning, whereas the carbohydrate sequence and the identity of the ecdysteroid aglyonce are determined by product ion scanning. We used EPI scanning on a hybrid linear ion trap triple-quadrupole mass spectrometer because it is more sensitive than product ion scanning without Q3 trapping and because EPI allows Q3 trapping of fragment ions produced at different collision energies in Q2, resulting in EPI spectra with a greater diversity of fragment ion peaks. EPI experiments performed in the positive ion mode yield primarily [MH − nH$_2$O]$^+$ ions of ecdysteroids and, in some cases, fragments from α-cleavage in the side chain at C17. However, the simultaneous occurrence of both fragmentation routes precludes assignment of OH groups to the steroid nucleus or to the side chain. In contrast, EPI scanning in the negative mode yields α-cleavage fragments that retain OH groups, allowing assignment of OH groups to the steroid nucleus or to the side chain. Negative ion ESI-MS of ecdysteroids offers less sensitivity but higher selectivity (more diagnostic fragment ions) compared to positive ion MS. The sensitivity in the negative ion mode can be improved by using an APCI source and by using nonscanning MS/MS experiments such as selected reaction monitoring for quantitation purposes.

ABBREVIATIONS USED

LC-MS/MS, liquid chromatography−tandem mass spectrometry; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; EPI, enhanced product ion; TIC, total ion current; ToF, time-of-flight; Q, quadrupole.

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Supporting Information Available: Positive and negative precursor ion LC-ESI-MS/MS chromatograms showing the detection of glycosides of ecdysteroids **1** and **2** in meadowfoam seed meal. This material is free of charge via the Internet at http://pubs.acs.org.

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