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THE ISOLATION AND IDENTIFICATION OF STEROIDAL SAPOGENINS IN SWITCHGRASS

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ABSTRACT

Switchgrass (*Panicum virgatum* L.) has been reported to be hepatotoxic, causing photosensitization in lambs and horses. In this study we show the presence of steroidal saponins in two samples of switchgrass that has been implicated in the poisonings of sheep and horses. After hydrolysis of the saponins, diosgenin was determined to be the major sapogenin in both switchgrass samples. We also confirmed the presence of diosgenin in kleingrass after hydrolysis of saponins extracted from it.

INTRODUCTION

Many plants of the *Panicum* genus have been reported to cause hepatogenous photosensitization in animals throughout the world (Flaoyen, 2000). Some of the *Panicum* species that have been implicated in secondary photosensitization are *P. capillare* (Smith and O'Hara, 1978), *P. coloratum* (Steyn, 1928; Remington and Quin, 1934; Dollahite et al., 1977; Muchiri et al., 1980; Bailey, 1985; Bridges et al., 1987; Cornick et al., 1988; Patamalai et al., 1990; Regnault, 1990), *P. dichotomiflorum* (Smith and O'Hara, 1978; Holland et al., 1991; Miles et al., 1992), *P. decompositum*, *P. maximum* (Clare, 1955), *P. effusum* (Conner, 1951; Clare, 1955), *P. laevifolium* (Remington and Quin, 1934; Clare, 1955; Smith and O'Hara, 1978), *P. miliaceum* (Conner, 1951; Clare, 1952, 1955; Smith and O'Hara, 1978), *P. schinzii* (Button et al., 1987; Lancaster et al., 1991; Miles et al., 1992), and *P. virgatum* (Pouli et al., 1992).

The presence of glycosidic steroidal saponins has been established in some species of the *Panicum* genus, and these compounds have been suggested as one of the primary agents causing hepatogenous photosensitization in animals grazing these grasses (Patamalai et al., 1990; Holland et al., 1991; Miles et al., 1992). The hydrolysis of the saponins isolated from plant material yields sapogenins. The sapogenin diosgenin (**1**) has been intensified in *P. dichotomiflorum* and in

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P. schinzii (Holland et al., 1991; Miles et al., 1992), while diosgenin (**1**) and an isomer, yamogenin (**2**), have been isolated from *P. coloratum* (Patamalai et al., 1990).

In 1992, Puoli et al. (1992) reported hepatogenous photosensitization in lambs grazing switchgrass (*Panicum virgatum* L. cv. Cave-in-Rock). In the summer of 2000, poisoning of horses grazing a pasture of switchgrass (*Panicum virgatum* L.) in eastern Nebraska was reported to our laboratory. Five horses were housed in a 4.6-ha pasture that had been seeded to switchgrass several years previously. The botanical composition of the pasture was not determined at the time it was being used by the horses. After several weeks a two-year-old filly became ill, developed liver disease, and died. The other four horses had elevated serum enzymes suggestive of hepatic damage. A liver biopsy was taken on one of the living horses. The results of the liver biopsy were similar to that of the horse that had died. The horses were removed from the pasture, and over the subsequent three months they were monitored biochemically to determine if the hepatic disease was resolving. All four horses recovered and appear to have normal hepatic function. The purpose of this study was to determine if saponins and their hydrolysis products, sapogenins, were present in the Cave-in-Rock cultivar switchgrass that was reported to cause photosensitization in lambs and in a sample of switchgrass (*Panicum virgatum* L.) from the pasture where the horses were poisoned. We also analyzed a sample of Kleingrass 75 (*Panicum coloratum* L.), previously implicated in a photosensitization case, for sapogenins to compare with our findings from switchgrass.

MATERIALS AND METHODS

Materials

Switchgrass sample 1 (*Panicum virgatum* L. cv. Cave-in-Rock) was harvested at heading by Kenneth P. Vogel from research fields at the University of Nebraska - Agricultural Research and Development Center, located about 50 km west of Omaha. Switchgrass sample 2 was provided by Ted Forke, Lincoln, NE, and was harvested at heading from a pasture in Lancaster County, NE, where the liver problems with horses occurred. It was identified as *Panicum virgatum* L. by the Intermountain Herbarium (voucher #230944), Utah State University, Logan, UT. Kleingrass 75 (*Panicum coloratum* L.) was provided by Byron L. Burson (USDA, ARS, Southern Crops Research Laboratory, College Station, TX). Diosgenin was purchased from Steraloids, Inc. (Newport, RI). *N,O*-bis(Trimethylsilyl)trifluoroacetamide silylation reagent was purchased from Pierce Chemical Co. (Rockford, IL).

Saponin Isolation

All grass samples were dried, ground, and saponins extracted using the methods of Patamalai et al. (1990) and Wall et al. (1952). The ground grass sample (100 g) was extracted by refluxing (16 hrs) with 70% ethanol (1000 mL). The extract was filtered and the residue extracted (3 hrs) and filtered again (2X). The filtrates from the three extractions of the same plant material were combined and the solvent removed by rotary-evaporation to a volume of ~300 mL. The concentrated aqueous extracts were extracted with chloroform (300 mL) (4X). The aqueous phase was then extracted with water-saturated n-butanol (100 mL) (3X). Sodium chloride (1.5 g) was added to the initial n-butanol extraction to aid in separation of the water and n-butanol layers. The n-butanol extracts were combined and the solvent removed by rotary-evaporation to dryness. The dark-brown residue was dissolved in methanol (~20 mL) and introduced drop-wise into acetone (~900 mL) to produce a light-brown precipitate. The precipitate was collected, re-

dissolved in methanol, and precipitated in acetone three additional times. After the fourth and final time, the precipitate was allowed to stand in acetone for 16 hrs. The precipitate was collected by filtration and allowed to dry overnight at room temperature in a fume hood. Isolated saponin yields were 0.893 g (switchgrass #1), 0.223 g (switchgrass #2), and 1.217 g (kleingrass).

Hydrolysis of Saponins to Sapogenins

The hydrolysis of saponins to sapogenins was accomplished using methods described previously by Patamalai et al. (1990) and Wall et al. (1952). Saponin (446 mg from switchgrass #1, 112 mg from switchgrass #2, and 608 mg from kleingrass) isolated from the grass samples was dissolved in 9.0 mL ethanol, 30.8 mL distilled water, and 7.0 mL concentrated HCl and refluxed (3 hrs). The mixture was allowed to cool to room temperature, filtered, and the residue washed (50% ethanol). The residue was collected and then refluxed (1 hr) in a mixture consisting of 30 mL benzene, 10 mL methanol, and 2 g potassium hydroxide. The mixture was allowed to cool to room temperature, filtered, and the residue washed (hot 90:10 benzene:ethanol). The filtrate and washings were combined and partitioned between benzene (50 mL) and water (50 mL) by thorough mixing in a separatory funnel. The benzene phase was collected and the aqueous phase extracted two additional times with benzene (50 mL). The benzene extracts were combined, rotary-evaporated to dryness, and the residue collected for further analysis. Sapogenin yields were 70.2 mg (switchgrass #1), 77.3 mg (switchgrass #2), and 90.6 mg (kleingrass).

Flow Injection and High-Performance Liquid Chromatography Mass Spectrometry

Flow injection atmospheric pressure chemical ionization (APCI) mass spectral data were acquired on a Finnigan LCQ Mass Spectrometer from Finnigan Corporation (San Jose, CA). Samples were loop injected (20 μL) into the APCI source in a 50:50 methanol:20 mM ammonium acetate solution at a flow rate of 0.5 mL/min.

Ionization was achieved using an APCI source vaporizer temperature of 450°C and corona discharge current of 5 μamps. The inlet capillary temperature and voltage were 200°C and 16V, respectively. Full scan mass data were collected for a mass range of 100-2000 amu. MS^n product ion spectra were collected after isolation of the target ion (± 5 amu) and the relative collision energy manually adjusted to observe significant fragmentation of the selected ion.

High-performance liquid chromatography was performed on a reversed-phase Betasil C18 column (100 mm x 2 mm I.D., 5 μm particle size, 100 Å pore size) from Keystone Scientific, Inc. (Bellefonte, PA). A Uniguard guard column (3 mm x 2 mm) obtained from Keystone Scientific, Inc. (Bellefonte, PA) was placed inline before the chromatographic column. The mobile phase was 85:15 methanol:20 mM ammonium acetate. The flow rate was 0.5 mL/min and the injection volume was 20 μL. The detector was a Finnigan LCQ Mass Spectrometer utilizing atmospheric pressure chemical ionization.

Diosgenin: APCI-MS, m/z (%) 415 (76, $[M+H]^+$), 397 (100), 271 (72), 253 (77).

Gas Chromatography-Mass Spectrometry

Trimethylsilyl (TMS) derivatives of sapogenin residues were prepared by adding pyridine (200 μL) and *N,O*-bis(Trimethylsilyl)-trifluoroacetamide silylation reagent (100 μL) to a vial containing ~15 mg of the sapogenin residue. The vials were then sealed with a teflon lined cap and heated at 70°C for 30 min, allowed to cool to room temperature, and diluted with 0.5 mL of chloroform.

Gas chromatography-mass spectrometry was performed on a Finnigan GCQ

system (Finnigan, Austin, TX). The gas chromatography column was a J&W Scientific (Folsom, CA) DB-5MS Capillary Column (30 m, 0.25 mm I.D., 0.25 μm film). Manual injections of 2.0 μL were made. The GC temperature for analysis was 100°C for 1 min, then 40°C/min to 250°C followed by 10°C/min to 320°C, and held at 320°C for 5 min for a total analysis time of 16.75 min. Under these conditions the TMS derivative of a diosgenin (**1**) standard eluted at 13.02 min.

Diosgenin-TMS: EI-MS (70 eV), *m/z* (%) 486 (0.07, $[M]^+$), 414 (0.79), 372 (8.26), 343 (9.01), 282 (100), 267 (33.16), 253 (22.25), 187 (18.78).

RESULTS AND DISCUSSION

The precipitates obtained from the saponin isolation procedure of the two switchgrass samples and a single kleingrass sample all displayed saponin-like foaming properties in water. Saponin precipitates (8 mg) from all three grass samples were dissolved separately in water (2 mL) and shaken vigorously (5 min). All three samples produced a foam when shaken, which persisted for over 16 hrs.

The saponin precipitates were hydrolyzed to sapogenin residues. These sapogenin residues were initially analyzed by APCI-MS. A strong protonated molecular ion at m/z = 415 was observed in the mass spectrum from all three grass samples corresponding to the possible steroidal aglycones diosgenin (**1**) and yamogenin (**2**), previously isolated from kleingrass (Patamalai et al., 1990), and/or similar isomeric compounds (Fig. 1).

Reverse-phase HPLC, using APCI-MS as a detector, was performed on the sapongenin residues isolated from all three grass samples and a standard sample of diosgenin (**1**). While the diosgenin standard eluted as a single peak at Rt = 9.9 min, it was evident that the grass samples contained multiple components that were unresolved under the chromatographic conditions employed. The APCI mass spectra of the unresolved components were similar to the diosgenin standard producing a strong protonated molecular ion at m/z = 415 and major fragment ions at m/z = 397 (MH^+ - H_2O), 271 (-$C_8H_{16}O_2$, loss of rings e and f) and m/z = 253 (MH^+ - $H_2O/C_8H_{16}O_2$).

The isomeric sapogenins could not be resolved by HPLC; therefore, the sapogenin residues and the diosgenin standard were derivatized with a trimethylsilylating reagent and analyzed by the capillary gas chromatography/mass spectrometry (GC/MS) method described. Figure 2 shows the reconstructed ion chromatograms for the diosgenin standard, kleingrass sapogenin residue and switchgrass sapogenin residues. The kleingrass and switchgrass samples all contained several peaks that eluted in two regions (11.37 - 11.57 min and 12.99 - 13.66 min). The peaks eluting in these regions all had EI-MS fragmentation patterns essentially identical to the standard diosgenin (Materials and Methods). Based on the retention times, diosgenin was identified as the major sapogenin in both switchgrass samples. We speculate that the major peak in the kleingrass sample, eluting at 13.16 min, is yamogenin (**2**) based on the earlier report (Patamalai et al., 1990) that yamogenin and diosgenin were isolated from kleingrass. Diosgenin and yamogenin appear to be the major sapogenin components in both switchgrass and kleingrass. The chromatograms of the sapogenin residues indicate that all grass samples contain as many as four spirostadiene side products from acid-catalyzed dehydration reactions with a molecular mass of 396 that elute in the retention time of 11.39 - 11.57 min (Bedour et al., 1964; Fazli and Hardman, 1971).

The non-hydrolyzed saponin material was also analyzed by APCI mass spectrometry in order to gain further understanding of the attached glycosidic residues. A protonated molecular ion was observed at m/z = 1177

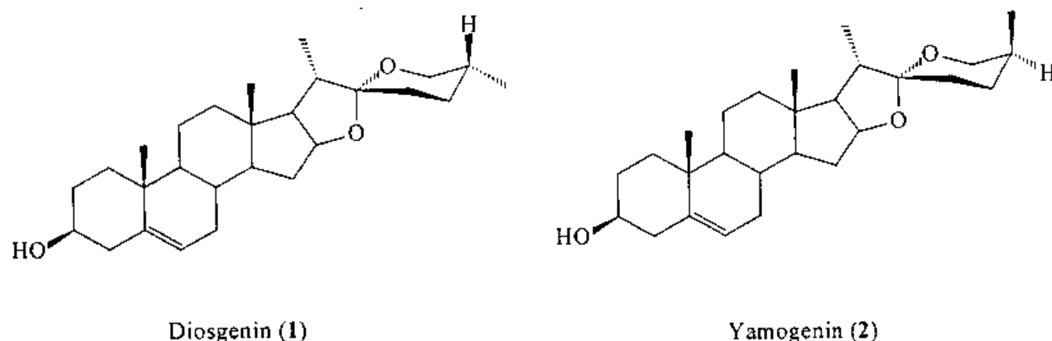

Figure 1. Chemical structures diosgenin (1) and yamogenin (2).

(Fig. 3A). The mass difference between the protonated molecular ion MH^+ (m/z = 1177) and the aglycone fragment (m/z = 415) is 762 amu and can only be accounted for by attachment of two hexose (e.g., glucose) and three deoxyhexose (e.g., rhamnose) units. The tandem mass product ion spectrum (MS^2) resulting from collision-induced dissociation of the protonated molecular ion resulted in a more detailed fragmentation pattern characterized by sequential losses of the glycosidic units (Fig. 3B). MS^2 fragment ions at m/z = 1031 and 1015 indicate losses of a terminal deoxyhexose (MH^+ - 142) and a terminal hexose (MH^+ - 162) unit. A hexose sugar (162 amu) must be added at the alpha position to the aglycone to account for the fragment ion at m/z = 577 (diosgenin-hexose). A second deoxyhexose unit is added at the beta position accounting for the ion at m/z = 723 (diosgenin-hexose-deoxyhexose). The glycosidic sequence either branches after this point or is a mixed sequence to account for the ions at m/z = 869 (diosgenin-hexose-deoxyhexose-deoxyhexose) and m/z = 885 (aglycone-hexose-deoxyhexose-hexose). The two remaining glycosidic moeities could then be added in an unknown sequence (Fig. 4). Although direct APCI-MS of the intact saponin material is useful for determining the molecular weight of the major saponin fraction and some useful structural information can be deduced from the fragmentation pattern, the APCI-MS data cannot differentiate between the possible anomeric glycoside residues or their exact linkage (Fang et al., 1999).

The kleingrass and both switchgrass samples have similar APCI mass spectra for the nonhydrolyzed saponin material. However, the differences between the kleingrass and switchgrass samples result from the relative ratios of the isomeric steriodal aglycone compounds and not necessarily in the arrangement of the hexose and deoxyhexose sugar moeities.

In this study we showed the presence of steroidal saponins in two samples of switchgrass that has been implicated in providing the hepatotoxins to sheep and horses. After hydrolysis of the glycosides, diosgenin (1) was determined to be the major sapogenin in both switchgrass samples. We confirmed the presence of diosgenin in a

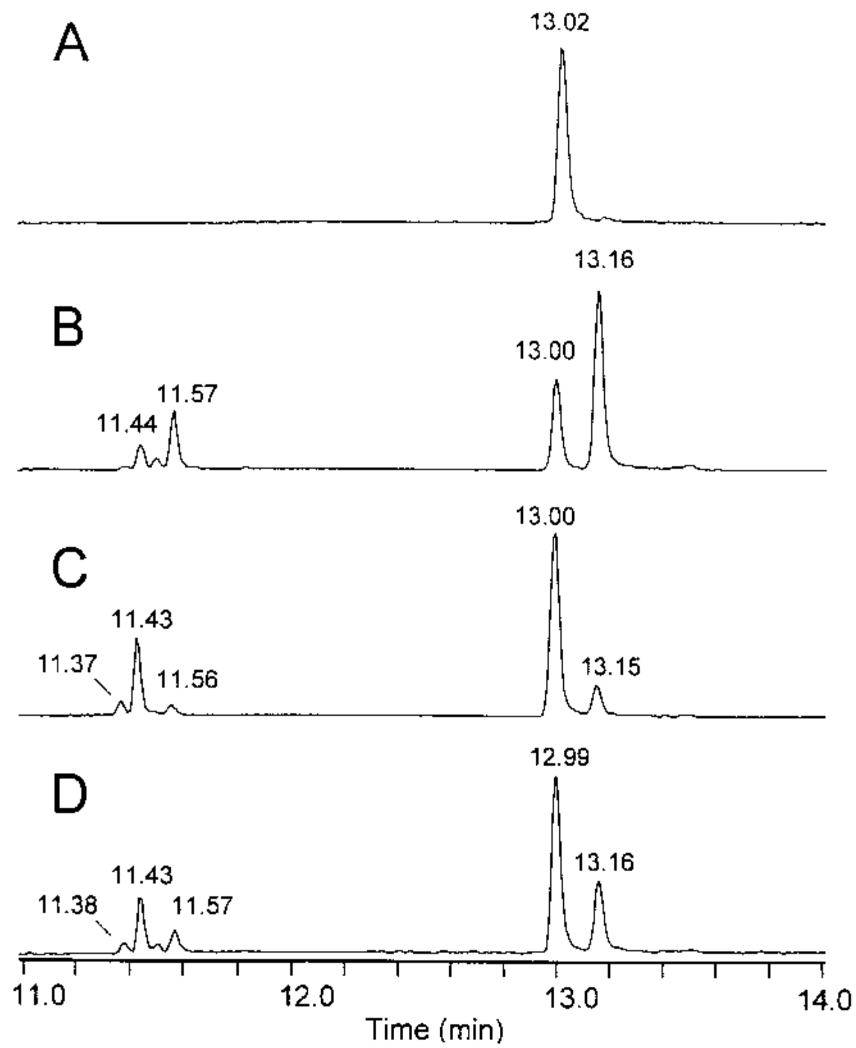

Figure 2. Reconstructed ion chromatograms (m/z = 282.3) for sapogenin samples. (**A**) Diosgenin standard. (**B**) Kleingrass 75 (P. coloratum L.). (**C**) Switchgrass sample 1 (P. virgatum L. cv. Cave-in-Rock). (**D**) Switchgrass sample 2 (P. virgatum L.).

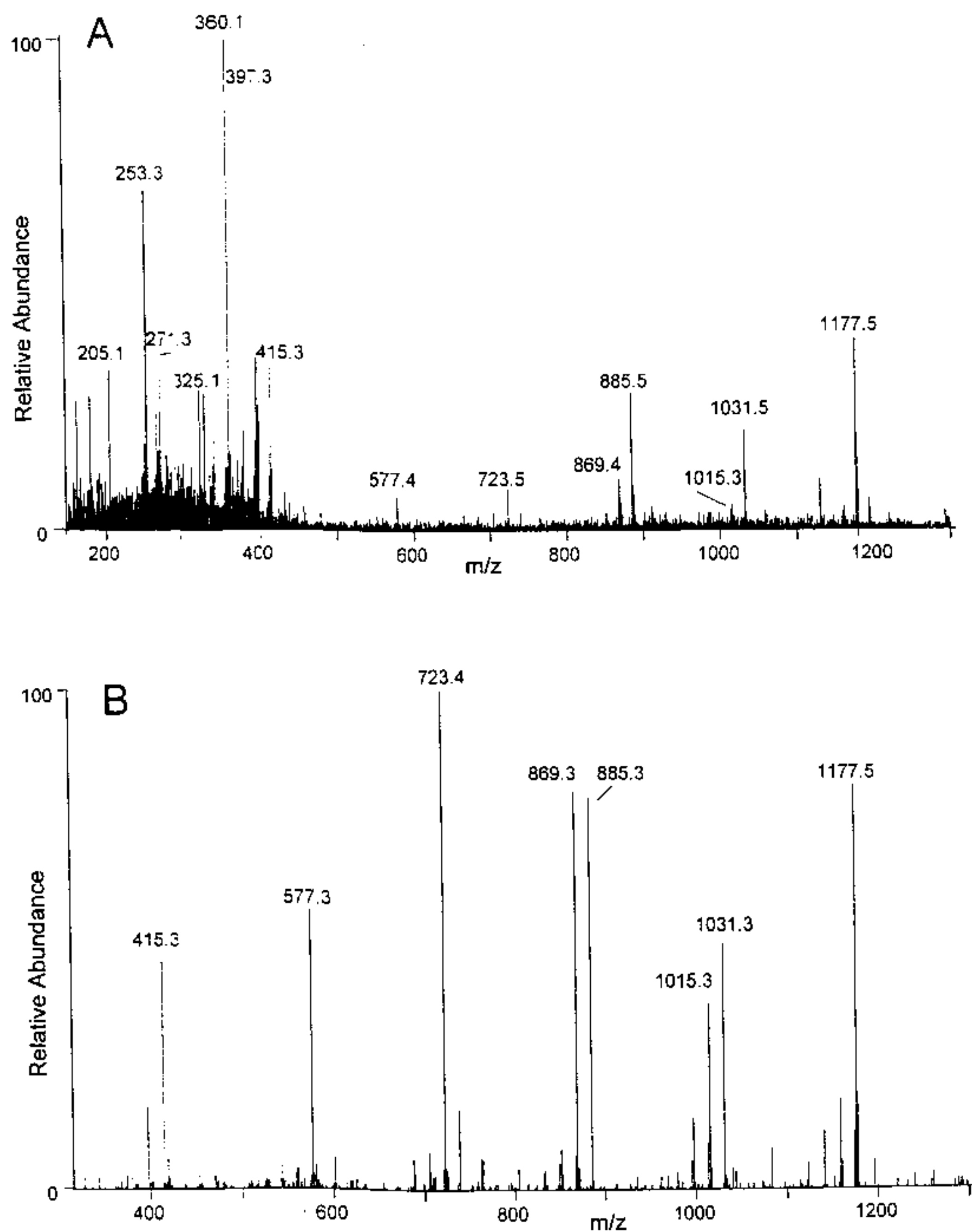

Figure 3. **(A)** APCI mass spectrum of isolated saponin material from switchgrass sample 2 (P. virgatum L.). **(B)** MS^2 product ion spectrum of MH^+ = 1177.

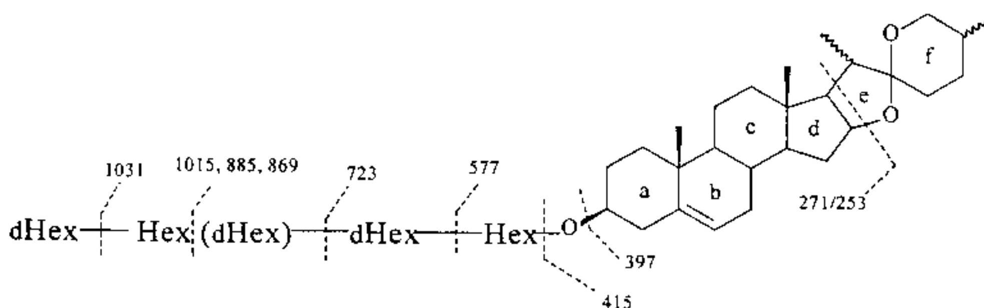

Figure 4. Proposed structure of major saponin from switchgrass and kleingrass plant material and plausible APCI ion fragments.

kleingrass sample after acid hydrolysis of the glycosides as well as an additional steroidal isomer, which we speculate is yamogenin (2). The nonhydrolyzed saponin components were shown to be glycosides of disogenin, yamogenin, and other isomeric steroidal aglycones with the glycoside portion being composed of two hexose and three deoxyhexose sugar units.

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