Analysis of Steroidal Sapogenins from Amber Fenugreek (*Trigonella foenum-graecum*) by Capillary Gas Chromatography and Combined Gas Chromatography/Mass Spectrometry†

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Mixtures of steroidal sapogenins isolated from the seed and combined aerial parts (foliage) of fenugreek (cultivar Amber) have been examined by capillary column gas chromatography with mass spectral and flame ionization detectors. Diosgenin [(25*R*)-spirost-5-en-3β-ol] was the major component in seed and foliage extracts hydrolyzed with hydrochloric acid. Yamogenin also appeared to be present. Tigogenin, neotigogenin, smilagenin, and sarsasapogenin were identified in the extracts. Dihydroxy steroidal sapogenins, tentatively identified as yuccagenin, gitogenin, and neogitogenin, were detected as minor components in hydrolyzed extracts from seed. A gas chromatographic method based on the use of an internal standard and a column of HP-5MS was developed to determine diosgenin in Amber fenugreek. Expressed on a dry weight basis, mean levels of diosgenin in seed were 0.54%. In field-grown foliage sampled during a season at 9, 15, and 19 weeks postseeding, diosgenin levels were 0.16, 0.07, and 0.07%, respectively.

Keywords: *Steroidal sapogenins; spirostanols; fenugreek; diosgenin; quantitation; seed; foliage*

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.), an annual legume traditionally cultivated in Europe, Africa, and Asia, represents a potentially new crop for Canada. A plant breeding program identified several new cultivars of fenugreek on the basis of seed production in the prairie provinces of western Canada. One new cultivar (Amber) is currently grown commercially. Besides the value of the seed as a condiment, there is also interest in developing this crop as a high-quality forage for cattle (Mir et al., 1993, 1997).

Diosgenin (**1**) and its C-25 epimer yamogenin (**2**) (Figure 1), the principal steroidal sapogenins obtained from fenugreek seed, are used as steroid intermediates in the pharmaceutical industry. Isolation and determination of **1** and **2** on an analytical scale are of interest, to help identify crops that are competitive economically with high-yielding tubers of the genus *Dioscorea*, the traditional source of **1** for chemical syntheses of steroid drugs (Morris et al., 1958; Liu et al., 1995). Extracts of fenugreek seed, which contain several steroidal saponin glycosides of the furostanol and spirostanol type (Mahato et al., 1982; Gupta et al., 1984, 1985), possess hypocholesterolemic (Sauvaire et al., 1991; Stark and Madar, 1993), hypoglycemic (Ribes et al., 1986), and oxytocic (Abdo and Al-Kafawi, 1969) properties. Saponins have been implicated in reduced animal growth and performance (Majak et al., 1980; Jenkins and Atwal, 1994). Complex mixtures of steroidal sapogenins have been obtained on acid hydrolysis of the saponins from fenugreek (Marker et al., 1947; Fazli and Hardman, 1971; Hardman and Jefferies, 1972; Petit et al., 1995).

Knight (1977) published a GC procedure for the qualitative analysis of steroidal sapogenins of fenugreek seed. After derivatization with trifluoroacetic anhydride, the trifluoroacetyl derivatives of diosgenin (**1**), yamogenin (**2**), yuccagenin (**3**), lilagenin (**4**), gitogenin (**5**), neogitogenin (**6**), tigogenin (**7**), and neotigogenin (**8**) were separated with a packed column. In studies on the identification of steroidal sapogenins from other plants, Miles et al. (1992, 1993) detected **1**, **2**, **7**, smilagenin (**9**), epismilagenin (**10**), sarsasapogenin (**11**), and episarsasapogenin (**12**) in underivatized samples using GC/MS with a capillary column of HP-1. After derivatization with acetic anhydride, Miles et al. (1994b) also studied mixtures of **1**, **2**, and **5**−**12** by GC/MS with HP-1. Petit et al. (1995) concluded from GC evidence with HP-1 that **1**−**3**, **5**−**9**, and **11** were present (as glycosides) in fenugreek seed harvested from an experimental field in France.

Diosgenin has been determined quantitatively in acid-hydrolyzed extracts from *Dioscorea* tubers by packed (Rozanski, 1972) and capillary (Liu et al., 1995) column GC with a flame ionization detector (FID) and an internal standard. Levels of **1** typically ranged from 1 to 8% on a dry weight basis (Rozanski, 1972). Using packed column GC-FID, Bohannon et al. (1974) estimated levels of **1** at 0.5−0.8% in acid-hydrolyzed extracts from fenugreek seeds and suggested that the peak for **1** probably included **2, 7**, and other components. Steroidal saponins (Varshney et al., 1977) and sapogenins (Fazli and Hardman, 1971) have been isolated from the leaves of fenugreek.

Levels of **1**, **2**, **7**, and **8** in some Australian plant species have been determined by GC/MS (Wilkins et al., 1994).

We report here the identification by GC/MS of steroidal sapogenins obtained from the seed and foliage of

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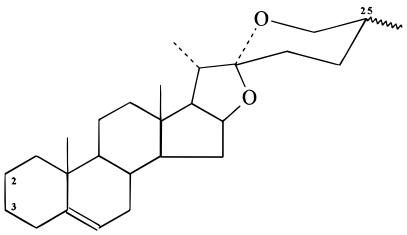
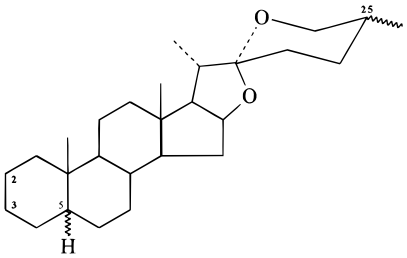


1 Diosgenin 25*R*, 3β-OH

2 Yamogenin 25*S*, 3β-OH

3 Yuccagenin 25*R*, 2α, 3β-$(OH)_2$

4 Lilagenin 25*S*, 2α, 3β-$(OH)_2$



5 Gitogenin 5α, 25*R*, 2α, 3β-$(OH)_2$

6 Neogitogenin 5α, 25*S*, 2α, 3β-$(OH)_2$

7 Tigogenin 5α, 25*R*, 3β-OH

8 Neotigogenin 5α, 25*S*, 3β-OH

9 Smilagenin 5β, 25*R*, 3β-OH

10 Epismilagenin 5β, 25*R*, 3α-OH

11 Sarsasapogenin 5β, 25*S*, 3β-OH

12 Episarsasapogenin 5β, 25*S*, 3α-OH

Figure 1. Structures of the sapogenins discussed in this work.

Amber fenugreek and the determination of diosgenin levels by GC-FID with a capillary column of HP-5MS.

EXPERIMENTAL PROCEDURES

Instrumentation. A Hewlett-Packard (Palo Alto, CA) 5830A gas chromatograph equipped with a FID (operated at 250 °C) and a fused silica capillary column of HP-5MS (0.25 μm film thickness, 30 m × 0.25 mm i.d.) was used. The split/splitless injection port, maintained at 225 °C, was equipped with a silanized glass liner (HP Part 18740-80200). The samples (1 μL) were injected direct (30 s) at an initial oven temperature of 200 °C. After 1 min, the temperature rose at 25 °C/min to 270 °C and then at 1 °C/min to 290 °C. The column was held at 290 °C for 10 min. Helium was used as the carrier gas with the inlet pressure adjusted to give linear flow velocities for hexane of 28−29 cm/s at 35 °C. Retention times and peak area counts were reported on a HP 18850A GC terminal.

Experiments with DB-1 (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA) were performed with a HP 5890A GC equipped with a FID under the conditions shown above for HP-5MS. A column of DB-1701 (30 m × 0.32 mm i.d.) was also used, with temperature programming from 200 to 260 °C at 10 °C/min and a hold at 260 °C for 40 min.

Mass spectra were obtained on a Hewlett-Packard 5989A GC/MS equipped with 30 m capillary columns of HP-5MS and DB-1701. The transfer line was at 280 °C (250 °C with DB-1701). For electron impact (70 eV) mass spectrometry (EI-MS), the temperature of the ion source was 275 °C. The ion source during chemical ionization mass spectrometry (CI-MS) was set at 250 °C (with isobutane as the reagent gas) or 275 °C (with methane). The samples were injected direct with helium as the carrier gas (under electronic pressure control). Column temperature programming was from 200 to 270 °C at 10 °C/min and then at 1 °C/min to 290 °C (HP-5MS) or from 200 to 260 °C at 10 °C/min (DB-1701).

NMR spectra were obtained in deuteriochloroform solution with a Bruker AM-400 spectrometer. Chemical shifts are reported relative to internal TMS (0 ppm).

Reference Chemicals. Samples of diosgenin, tigogenin, smilagenin, epismilagenin, sarsasapogenin, 6-methyldiosgenin, rockogenin [(5α,20α,22α,25*R*)-spirostan-3β,12β-diol)] and hecogenin [(5α,20α,22α,25*R*)-spirostan-3β-ol-12-one)] were obtained from Steraloids Inc. (Wilton, NH). Samples of β-sitosterol, stigmasterol, and cholesterol were also obtained from Steraloids.

A reference mixture of tigogenin and neotigogenin (90:10 peak area ratio by GC-FID with HP-5MS) was obtained by refluxing tigogenin in ethanol and hydrochloric acid according to the procedure of Wall et al. (1955). Diagnostic signals for each of the protons at C-26 can be found in the NMR spectra of 25*R* and 25*S* epimers of steroidal sapogenins (Puri et al., 1993; Miles et al., 1993). NMR analysis of the mixture indicated that the major component was tigogenin (a triplet for H-26α at 3.37 ppm and a doublet of doublets for H-26β at 3.47 ppm) and the minor component was neotigogenin (a doublet for H-26α at 3.29 and a doublet of doublets for H-26β at 3.95 ppm).

Using procedures from Blunden et al. (1979), oxidation of sarsasapogenin with chromium trioxide and pyridine gave sarsasapogenone, which was reduced with sodium borohydride to give an 85:15 mixture (DB-1701) of episarsasapogenin and sarsasapogenin. The NMR spectrum showed a multiplet at 3.63 ppm for H-3β of episarsasapogenin (the major component) and a broad singlet at 4.11 ppm for H-3α of sarsasapogenin (the minor component). Starting with tigogenin and using the same techniques (Blunden et al., 1979), a mixture of epitigogenin (the 3α hydroxy isomer of tigogenin) and tigogenin was obtained. The NMR spectrum showed multiplets at 4.05 ppm (*ca.* 10% of the mixture) and 3.59 ppm (*ca.* 90% of the mixture), which were assigned to H-3 of epitogogenin and tigogenin, respectively.

Plant Material. Certified seeds of Amber fenugreek were ground to pass through a 1 mm screen and oven-dried at 60 °C to constant weight to determine moisture content (Goering and Van Soest, 1970); 5 g samples were defatted with 250 mL of petroleum ether (bp 30−60 °C) in a Soxhlet apparatus. After 6 h, the solid in the thimble was air-dried and ground with a mortar and pestle; the moisture content was determined as described above. Loss of weight during defatting was calculated. A portion of defatted air-dried ground material (100 mg) was transferred to a test tube (100 × 13 mm) equipped with a Teflon-lined screw cap. Ethanol (4 mL) and water (1 mL) were added, and the mixture was heated (80 °C) for 3 h with magnetic stirring in a metal block (Pierce Reacti-Therm III module). After cooling, the mixture was centrifuged (5 min at 2000 rpm) and the solvent transferred with a disposable pipet to another test tube. The solid that remained was washed twice with 5 mL of ethanol−water (4:1 v/v). The combined extracts were split between two test tubes, and the volume in each tube was concentrated to approximately 1.5 mL with a Savant evaporator (Model SVC-100H). The concentrated extracts were combined in a test tube and 37% hydrochloric acid (0.3 mL) was added. Each extract was hydrolyzed by heating the mixture (1 M in HCl) at 100 °C for 1 h (metal block). After cooling, water (2 mL) was added. In quantitative experiments, the internal standard of 6-methyldiosgenin (500 μg) was also added (with vortex mixing). The mixture was extracted three times with methyl *tert*-butyl ether (MBE, 2 mL), using a reciprocal shaker (Ames aliquot mixer) and centrifuge (2 min at 1400 rpm). The combined MBE extracts were washed with 0.1 M NaOH solution (2 × 1 mL), dried ($MgSO_4$), filtered through a small plug of cotton wool in a disposable pipet, and evaporated under nitrogen with a Meyer N-EVAP apparatus (Organomation Associates, Berlin,

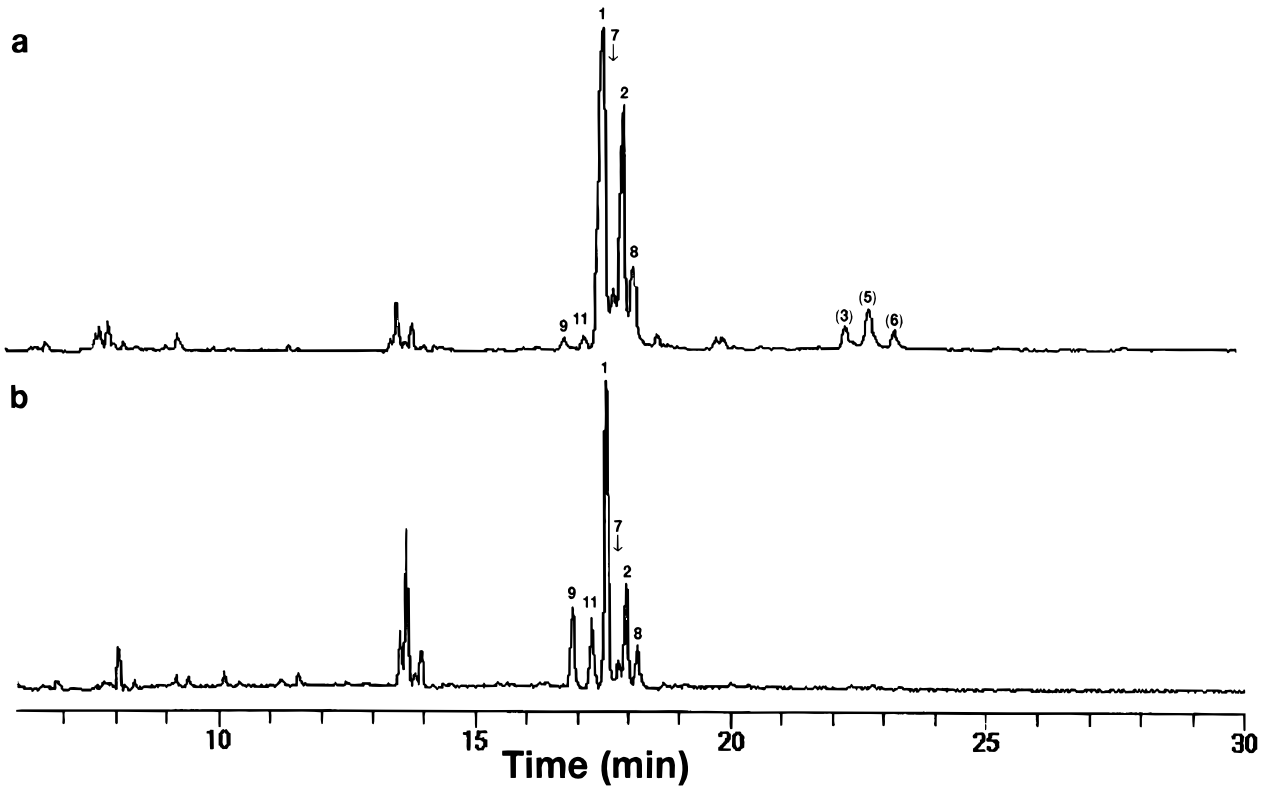


Figure 2. Total ion mass chromatograms from GC/MS analysis (70 eV) of acid-hydrolyzed extracts from (a) mature seed of Amber fenugreek and from (b) field-grown foliage of Amber fenugreek harvested at 15 weeks after seeding. For the identity (or tentative identity) of labeled peaks, see Figure 1. The spirostadienes eluted at 13.5−13.9 min. Other unlabeled components were not identified. The column was HP-5MS.

MA). The residue was dissolved in toluene (5 mL), and 1 μL was used for GC-FID and GC/MS analysis.

Foliage samples of Amber fenugreek, consisting of whole plants clipped 5 cm above ground level, were obtained at the Lethbridge Research Centre from an irrigated plot and from a greenhouse experiment. The plants were collected at 9, 15, and 19 weeks after seeding. At each collection, the plants were chopped, mixed, and freeze-dried. The freeze-dried material was ground and moisture content determined. Five-gram samples were defatted, moisture content was determined, and loss of weight during defatting was calculated. Defatted air-dried ground portions (100 mg) were analyzed (as described for seed). Rockogenin (500 μg) was added as an internal standard in experiments with foliage.

Quantitation and Statistics. Stock solutions of diosgenin were prepared in absolute ethanol at concentrations of 0, 0.5, 1, 2.5, 5, 7.5, and 10 mg/mL. Portions (100 μL) of these solutions, delivering 0, 50, 100, 250, 500, 750, and 1000 μg of diosgenin, were added to test tubes containing water (5 mL). The internal standards of 6-methyldiosgenin (500 μg, obtained from 100 μL of an ethanol stock solution prepared at 5 mg/mL) and rockogenin (500 μg) were added, the samples were vortexed, then extracted with MBE, washed, dried, filtered, evaporated, and dissolved in toluene (5 mL) as described for samples of plant material. These calibration curve samples were analyzed by GC-FID with HP-5MS. Equations describing the calibration curves were obtained by linear regression analysis of the integrated peak area ratio of diosgenin to 6-methyldiosgenin (or rockogenin) *vs* the amount of diosgenin added. The amount of diosgenin found in the experimental samples, expressed on a moisture-free basis, was obtained from the integrated peak area ratio of diosgenin to internal standard and by reference to the equation describing the appropriate calibration curve. Diosgenin levels were calculated as the amount found in the 100 mg samples of defatted dried plant material and corrected for the amount of weight lost during defatting.

Statistical comparisons of diosgenin levels (see Table 2) were performed with the general linear models procedure of SAS (SAS Institute Inc., 1989) by one-way analysis of variance. A least significant difference test was used to evaluate differences among means.

RESULTS AND DISCUSSION

Steroidal sapogenins have been obtained by acid hydrolysis of plant material, followed by extraction with an organic solvent such as chloroform or petroleum ether (Fazli and Hardman, 1968). Extraction of steroidal saponins with a water-miscible organic solvent has been done first, followed by acid hydrolysis (Bedour et al., 1964). In the present investigation, the steroidal saponins were isolated by extraction with 80% ethanol, and then the concentrated extracts were hydrolyzed with hydrochloric acid. This method was similar to a reported method (Miles et al., 1992) except that our experiments were carried out on a test tube scale and the crude saponin fraction was hydrolyzed without purification. On extraction of the hydrolysates with MBE, the MBE extracts were washed with dilute sodium hydroxide solution. Samples for GC/MS were obtained by dilution with toluene.

With the reported GC/MS conditions, a capillary column of HP-5MS was particularly useful for the separation of steroidal sapogenins of seed and foliage extracts (Figure 2).

The EI-MS of steroidal sapogenins obtained during GC/MS were characterized by the appearance of molecular ions (M^+) and an ion at m/z 139 (the base peak). Several other diagnostic ions were also observed. Fragmentation patterns of steroidal sapogenins have been described previously (Budzikiewicz et al., 1962; Faul and Djerassi, 1970; Blunden et al., 1980; Sauvaire et al., 1991).

Identification of diosgenin (**1**, M^+ 414 amu), tigogenin (**7**, M^+ 416 amu), smilagenin (**9**, M^+ 416 amu), and

Table 1. Gas Chromatographic Retention Times and Relative Peak Areas of Steroidal Sapogenins Found in Amber Fenugreek

	retention time[a] (min)			relative peak areas[b] (%)						
					field foliage			greenhouse foliage		
compound	HP-5MS	DB-1	DB-1701	seed	week 9	week 15	week 19	week 9	week 15	week 19
diosgenin (**1**)	23.27	17.95	42.29	38.9	27.1	25.0	24.3	22.5	26.2	40.7
yamogenin (**2**)[c]	23.83	18.37	44.22	20.0	7.1	7.7	8.3	12.8	10.0	17.2
yuccagenin (**3**)[c]	29.78	22.93		6.6						
gitogenin (**5**)[c]	30.39	23.45		7.6						
neogitogenin (**6**)[c]	31.18	24.02		3.3						
tigogenin (**7**)	23.59	18.35	43.19	6.6	3.5	5.0	5.2	4.0	5.1	5.6
neotigogenin (**8**)	24.17	18.55	44.96	5.7[d]	7.7[d]	7.2[d]	12.4[d]	15.7[d]	16.8[d]	11.8[d]
smilagenin (**9**)	22.36	17.40	38.76	1.8	10.5	9.6	8.6	9.3	9.8	6.0
epismilagenin (**10**)	22.36	17.40	39.63							
sarsasapogenin (**11**)	22.90	17.78	40.36	2.0	9.7	8.8	11.2	19.5	14.2	8.6
episarsasapogenin (**12**)	22.90	17.78	41.41							
spirostadienes[e]	17.50	13.61	22.99	1.6	6.9	7.4	4.4	2.6	3.0	1.5
	17.75	13.72	23.46	5.1	19.2	20.3	15.8	7.3	9.0	4.9
	18.00	13.94	23.85	1.0	2.9	3.0	3.1	2.3	2.7	1.4
	18.18	14.06	24.36	2.8	5.6	5.8	6.7	4.1	3.4	2.3

[a] With the GC-FID conditions described under Experimental Procedures. [b] Obtained from representative samples during GC-FID analysis with HP-5MS by (peak area of indicated compound/sum of peak areas × 100). [c] Tentative identification. [d] These values represent a mixture of neotigogenin and β-sitosterol. [e] Artifacts from diosgenin (first eluting pair) and probably from yamogenin (second eluting pair).

sarsasapogenin (**11**, M^+ 416 amu) in seed and foliage extracts was based on the comparison of retention times and mass spectra to those of authentic samples of these sapogenins. EI-MS and CI-MS on the component labeled as **2** in the extracts gave spectra that were nearly identical to the spectra of diosgenin, which supported an assignment to the epimeric yamogenin (**2**). Identification of neotigogenin (**8**), the 25*S* isomer of tigogenin, was accomplished by generating a mixture of **7** and **8** in the reversible iso reaction (Wall et al., 1955; Fieser and Fieser, 1959) and comparing the minor component in the mixture (**8**) to the component in the extracts with the same retention time.

Three minor components that eluted from 22.5 to 23.4 min in seed extracts were tentatively identified as yuccagenin (**3**, M^+ 430 amu), gitogenin (**5**, M^+ 432 amu) and neogitogenin (**6**, M^+ 432 amu) on the basis of molecular weight information from GC/MS and from previous reports of these same components occurring in fenugreek seed extracts in approximately the same ratio (Knight, 1977; Petit et al., 1995). The retention time of a reference sample of hecogenin (M^+ 430) was 23.6 min, longer than that of any component in the extracts.

The isobutane and methane CI-MS of these steroidal sapogenins were dominated by a quasimolecular ion (MH^+) and by loss of the elements of water from this ion. The $[MH^+ - H_2O]$ ion represented the base peak in all of the compounds examined. Ions of 10−30% abundance corresponding to $[MH^+ - CH_4]$ were also observed. The diols (**3**, **5**, **6**, and rockogenin) gave $[MH^+ - 2H_2O]$ ions as well. With methane, fragment ions corresponding to $[M - H]^+$ and $[M - H - H_2O]^+$ were also observed. These types of ions have been reported before in the CI-MS of steroidal alcohols related to cholesterol (Harrison, 1983).

Diosgenin and yamogenin have been shown to undergo dehydration reactions in the presence of hydrochloric acid to give 3,5-spirostadienes (M^+ 396 amu) as artifacts (Bedour et al., 1964; Fazli and Hardman, 1971). Four components of this molecular weight, with retention times of 13.5, 13.6, 13.8, and 13.9 min, were found in hydrolyzed extracts from seed and foliage (Figure 2). During CI-MS, these spirostadienes each gave a MH^+ ion of 100% abundance. With methane, the $[M - H]^+$ ion at *m/z* 395 was approximately half the abundance of the MH^+ ion.

It was interesting that a four-component mixture of spirostadienes was detected in the hydrolyzed extracts. Previous studies on the acid-catalyzed dehydration of 3-hydroxysteroids, including a detailed study with cholesterol (Patel and Peal, 1964), suggested that conjugated 3,5-spirostadienes should be formed. In separate spiking experiments with diosgenin, we found that hydrolysis with aqueous hydrochloric acid gave a major diene component (at 13.6 min in Figure 2) and a minor diene component (at 13.5 min). Yamogenin would be expected to behave similarly, thus rationalizing the peaks at 13.9 and 13.8 min in the extracts. The two minor components could possibly represent the 25*R* and 25*S* isomers of a rearranged 2,4-spirostadiene or an unconjugated 2,5-spirostadiene. However, the structure of all of these dienes needs to be confirmed. Hydrolysis of steroidal saponins of fenugreek seed with sulfuric acid in 2-propanol has been reported to suppress the formation of 3,5-spirostadienes (Sauvaire and Baccou, 1978; Petit et al., 1995), but that method was not investigated in the present study.

A capillary column of HP-5MS and a FID were used for the routine separation of steroidal sapogenins in extracts of Amber fenugreek. The appearance of chromatograms was similar to those from GC/MS (Figure 2) except that the retention times were longer (Table 1). DB-1 was also evaluated, but yamogenin and tigogenin could not be separated. On DB-1 and HP-5MS, it was impossible to separate smilagenin from epismilagenin or sarsasapogenin from episarsasapogenin, but mixtures of these α/β isomeric alcohols were easily resolved with a capillary column of DB-1701. Analysis of seed and foliage extracts by GC/MS with DB-1701 showed that only the β alcohols, smilagenin and sarsasapogenin, were present. However, a small peak (M^+ 414 amu) with the same retention time as episarsasapogenin (M^+ 416) was found in the extracts with DB-1701. This peak was identified as β-sitosterol, a compound known to occur in fenugreek (Fazli and Hardman, 1971). The α-hydroxy isomer of tigogenin (epitogogenin) was inseparable from tigogenin on all of the columns that were examined. It should be noted that these three pairs of epimeric alcohols have recently

been separated as their acetates with a capillary column of HP-1 (Wilkins et al., 1994).

The dihydroxy steroidal sapogenins did not elute from the column of DB-1701.

During GC-FID analyses with HP-5MS, the relative peak areas of steroidal sapogenins obtained by integration of chromatograms from representative seed and foliage samples were similar to those reported by Knight (1977) in a sample of fenugreek seed of unspecified origin. However, smilagenin and sarsasapogenin, each representing approximately 2% (seed) and 10% (foliage) of total sapogenins, were detected in extracts of Amber fenugreek. Recently, Petit et al. (1995) have also detected smilagenin and sarsasapogenin as minor components in extracts of fenugreek seed. Knight (1977) found lilagenin (0.6%) in seed extracts, but we were unable to confirm the presence of this sapogenin in our extracts. Yuccagenin (6.6%), gitogenin (7.6%), and neogitogenin (3.3%) were tentatively identified in seed extracts of Amber fenugreek. These diols were undetectable or at very low concentrations in foliage samples. Gitogenin has previously been reported to be present in seed but absent from the leaf, stem, and root of fenugreek grown in Asia (Fazli and Hardman, 1971). Relative peak areas of the six sapogenins were fairly constant in field foliage harvested at different intervals, but sarsasapogenin levels appeared to decrease and diosgenin levels appeared to increase as the plants matured in the greenhouse. Variations in the relative peak areas of neotigogenin could be related to different concentrations of β-sitosterol in the various samples because these compounds eluted with the same retention on HP-5MS.

To check on the distribution of unconjugated (free) sterols in fenugreek, seed and foliage samples were extracted for 3 h with 80% ethanol, omitting the defatting and hydrolysis steps. Examination of the toluene solutions by GC/MS indicated that a major component was β-sitosterol. Steroidal sapogenins were not detected in these extracts.

Although β-sitosterol appeared to be present in fenugreek in both free and conjugated forms, the other potentially interfering sterols, cholesterol (Fazli and Hardman, 1971) and stigmasterol (Marshall and Staba, 1976), could not be identified in any of the extracts.

In experiments with defatted seed samples extracted for 3 h with 80% ethanol, only trace levels of spirostadienes were detected when the extracts were hydrolyzed for 15 min with 1 M HCl or for 60 min with 0.5 M HCl. Under these conditions, area counts for diosgenin were low and the other sapogenins were barely detectable. Longer hydrolysis times were studied, and acceptable recoveries of sapogenins were judged to occur when the samples were hydrolyzed for 60 min with 1 M HCl. With these conditions (Miles et al., 1992), the spirostadienes accounted for 10−15% of the total peak areas of the sapogenins. With field foliage samples hydrolyzed for 60 min with 1 M HCl, spirostadiene formation was higher (30−40% of the total peak areas) compared to that shown in chromatograms from greenhouse foliage (10−20%). These differences could be related to a higher proportion of furostanol glycosides occurring in field samples because these glycosides have been reported to yield more spirostadienes on hydrochloric acid hydrolysis than those of the spirostanol type (Drapeau et al., 1986).

6-Methyldiosgenin, a synthetic derivative of diosgenin, seemed to represent an ideal internal standard because it eluted without interference on HP-5MS (at 19.3 min in Figure 2) and reference mixtures of 6-methyldiosgenin at a fixed concentration plus diosgenin of various concentrations gave a linear curve on plotting concentration *vs* peak area ratios. To avoid acid-catalyzed diene formation, 6-methyldiosgenin was added to the plant samples following dilution of the hydrolysates with water. Calibration samples, prepared by spiking water with appropriate amounts of diosgenin and 6-methyldiosgenin, were subsequently extracted with MBE in the same manner as the plant samples.

Although an internal standard should be added at the beginning of an analytical procedure, that approach would ideally require experimentation with pure samples of steroidal saponins or closely related glycosides, which were unavailable. Further, the formation of spirostadienes would still occur in calibration samples containing diosgenin if these samples were processed like the plant samples. This deficiency has been compensated for, in part, by posthydrolysis processing of the plant and calibration samples in the same manner. Rozanski (1972) added 5α-cholestan-3β-ol as an internal standard before hydrolysis to determine diosgenin levels of *Dioscorea* tubers, but the calibration samples were not hydrolyzed or extracted.

Experiments with foliage extracts spiked with 6-methyldiosgenin resulted in the partial degradation of this internal standard. The degradation product eluted with a long retention time (at 22.8 min in Figure 2), showed a molecular ion at m/z 442, and gave prominent fragment ions at m/z 139, 299, 313, and 370. This degradation product was not identified, but it might correspond to an aldehyde derivative of 6-methyldiosgenin, formed by allylic oxidation of the 6-methyl group. The degradation product was not found in seed samples. Apparently, a component in the foliage extracts catalyzed the degradation of the internal standard, similar to what could occur by allylic oxidation with selenium dioxide. On storage of toluene solutions of the extracts, GC-FID showed that the peak area of the degradation product gradually increased and, after 2 weeks, most of the 6-methyldiosgenin had disappeared. This conversion could not be completely suppressed by bubbling the toluene solutions with nitrogen gas.

Rockogenin was stable in foliage extracts, eluted without interference on HP-5MS (at 23.8 min in Figure 2), and represented a suitable replacement for 6-methyldiosgenin as an internal standard.

The seed sample of Amber fenugreek, analyzed on different days by the same techniques, gave a mean value of 0.54% diosgenin on a dry weight basis (Table 2). The overall precision (relative standard deviation) of these determinations was 13%. Other varieties of fenugreek have been shown to have similar levels, typically 0.5−0.9% for diosgenin plus yamogenin (Fazli and Hardman, 1971; Bohannon et al., 1974). Peak area counts for yamogenin represented approximately 50% of the counts for diosgenin. If the detector response factors were identical for these two sapogenins, which was a reasonable assumption (Rozanski, 1972), then the combined levels were close to 0.8%. Applying the same assumption to the other components, including the spirostadienes, led to an estimate of 1.3% total steroidal sapogenins in seed. That level falls in the range of the total sapogenin content (0.8−2.2%) reported for fenugreek seed grown in various countries and determined by an infrared method (Fazli and Hardman, 1968).

Foliage samples, taken at three stages of maturity and consisting of a mixture of leaves, stems, and pods,

Table 2. Gas Chromatographic Determination of Diosgenin from Amber Fenugreek Using Internal Standards of 6-Methyldiosgenin (Seed) and Rockogenin (Foliage)

	diosgenin[a] (%)			
		field foliage		
analysis day	seed	week 9	week 15	week 19
1	0.50, 0.51 0.59, 0.52 0.56			
2	0.49 0.55	0.17, 0.13 0.20, 0.15 0.13		
3	0.46 0.49		0.08, 0.06 0.05, 0.05 0.06	
4	0.57 0.66			0.06, 0.06 0.06, 0.06 0.06
5	0.37 0.53	0.14 0.17	0.09 0.12	0.07 0.08
6	0.61 0.61	0.11[b] 0.08[b]	0.09[b] 0.08[b]	0.19[b] 0.26[b]
mean ± SD (N)[c]	0.54 ± 0.07 (15)[d]	0.16 ± 0.02 (7)[e]	0.07 ± 0.02 (7)[f]	0.07 ± 0.02 (7)[f]

[a] Diosgenin levels were calculated on a moisture-free basis and corrected for the amount of weight lost during defatting. Percentage loss of weight during defatting ranged from 7 to 8 for seed and from 1 to 5 for foliage. [b] Greenhouse foliage. [c] Excluding data for greenhouse foliage. [d–f] Means with different superscripts are significantly different at the 1% level.

gave diosgenin levels of 0.05−0.26% on a moisture-free basis (Table 2). In samples collected from the field, the trend was toward higher levels in early (9 week) samples, whereas in greenhouse foliage, higher levels were found in the late samples obtained after 19 weeks of growth. These observations might reflect the relative maturities of fenugreek in the different environments, with peak levels of diosgenin expected at earlier stages (<9 weeks) in more mature plants from the field and at later stages (>19 weeks) in less mature plants from the greenhouse. Undoubtedly, levels of steroidal sapogenins will fluctuate in different years of growth.

The mixture of steroidal sapogenins found in the foliage of Amber fenugreek could be significant because pastures containing plants rich in these compounds have been implicated in hepatogenous photosensitization diseases of grazing ruminants, characterized by the presence of birefringent crystals in the bile (Holland et al., 1991; Munday et al., 1993; Flaoyen et al., 1993). Biliary crystals in sheep have recently been identified as insoluble salts of β-D-glucuronides of epismilagenin and episarsasapogenin (Miles et al., 1992, 1993, 1994a).

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