Microdetermination of Diosgenin from Fenugreek (*Trigonella foenum-graecum*) Seeds

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Sulfuric acid hydrolysis of steroidal glycosides of Amber fenugreek was studied by capillary gas chromatographic analysis of diosgenin [(25*R*)-spirost-5-en-3-ol] and isomeric spirostadiene artifacts from 100 mg samples of seed material. Following extraction with 80% ethanol, highest recoveries of diosgenin occurred when hydrolyses were conducted in sulfuric acid, prepared at 1 molar (M) concentration in water containing 60−80% 2-propanol. Compared to a previous method with aqueous hydrochloric acid, the selected conditions of hydrolysis at 100 °C for 2 h with sulfuric acid in 70% 2-propanol reduced diene formation but did not completely eliminate these artifacts. Extraction of steroidal saponins with various alcohol/water mixtures prior to sulfuric acid hydrolysis gave similar recoveries of diosgenin. Application of the quantitative method to experimental samples of Amber, Quatro, and ZT-5 fenugreek, using 10 mg subsamples of crushed seed that had been defatted with petroleum ether and dried at 60 °C, gave diosgenin levels of 0.55, 0.42, and 0.75%, respectively. Levels of smilagenin and sarsasapogenin were very low in hydrolyzed seed extracts from ZT-5, a Canadian breeder line of fenugreek.

Keywords: *Steroidal sapogenins; fenugreek; sulfuric acid; diosgenin; quantitation; seed*

INTRODUCTION

Determination of diosgenin [(25*R*)-spirost-5-en-3-ol, Figure 1] in seeds of fenugreek (*Trigonella foenum-graecum* L.), an annual legume traditionally grown in India and Mediterranean countries, has been of interest for many years, primarily because diosgenin has been a valuable precursor for the synthesis of pharmaceutical steroids. Indeed, extracts from fenugreek seeds have been analyzed for diosgenin by IR (Hardman and Jefferies, 1972; Jefferies and Hardman, 1976), UV (Sanchez et al., 1972; Sharma and Kamal, 1982), TLC (Blunden et al., 1967; Provorov et al., 1996), GC (Bohannon et al., 1974; Brenac and Sauvaire, 1996; Taylor et al., 1997), and HPLC techniques (Ortuno et al., 1998).

In recent years, this crop has also been grown to some extent in western Canada, and levels of diosgenin in seed and foliage from commercially grown Amber fenugreek have been reported (Taylor et al., 1997). In these experiments, the mixture of steroidal saponins was extracted with aqueous ethanol before hydrolysis with hydrochloric acid to give diosgenin, the major steroidal sapogenin, as well as yamogenin [(25*S*)-spirost-5-en-3-ol] and other minor sapogenins. The above procedure was adopted in part from Miles et al. (1992) in their study on steroidal sapogenins from plants of Australia and New Zealand.

In our previous study with hydrochloric acid, acid-catalyzed dehydration reactions gave four spirostadiene side products in the final extracts, all of 396 molecular mass by GC-MS (Taylor et al., 1997). These artifacts presumably result from dehydration of the C-3 hydroxyl group of diosgenin and yamogenin to give a major pair of 25*R* and 25*S* spirosta-3,5-dienes (Bedour et al., 1964; Fazli and Hardman, 1971) and another minor pair of isomeric dienes of unknown structure.

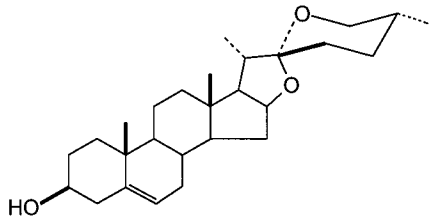

Figure 1. Structure of diosgenin.

Sauvaire and Baccou (1978a) have studied in detail the simultaneous extraction and hydrolysis of steroidal saponins from fenugreek seeds using sulfuric acid. With 0.5−2 M sulfuric acid in ethanol, propanol, or 2-propanol, they found by TLC with photodensitometric detection that the formation of dienes was suppressed and the levels of diosgenin (including yamogenin) were enhanced compared to previous methods with aqueous hydrochloric acid. Using several grams of defatted, powdered seed material contained in a round-bottom flask, a procedure with 1 M sulfuric acid in 70% 2-propanol heated with stirring for 12 h was suggested. A slight modification of that procedure (8 h reflux) was used by Petit et al. (1995) in their GC assessment of steroidal sapogenins from fenugreek seeds.

Similar techniques with sulfuric acid in 2-propanol have also been reported for assessing levels of diosgenin in various organs of fenugreek (Merkli et al., 1997; Ortuno et al., 1998) as well as in cell cultures (Drapeau et al., 1986; Savikin-Fodulovic et al., 1998) and tubers (Sauvaire and Baccou, 1978a) of *Dioscorea*, the traditional source of diosgenin for the synthesis of steroid drugs.

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In the present work, we have studied separately the conditions for extraction of steroidal saponins with various alcohols and the conditions for subsequent hydrolysis of the isolates with sulfuric acid mixtures, using defatted seed material from Amber fenugreek and an internal standard of 6-methyldiosgenin (Taylor et al., 1997). Diosgenin levels of Amber were determined by capillary GC and compared to another cultivar (Quatro) and to a Canadian breeder line (ZT-5) using 10 mg of seed material, approximately the equivalent of one seed.

MATERIALS AND METHODS

Chemicals. Diosgenin (~98%) was purchased from Sigma Chemical Co. (St. Louis, MO). 6-Methyldiosgenin was obtained from Steraloids Inc. (Wilton, NH). All organic solvents were of OmniSolv glass distilled grade (Merck, Darmstadt, Germany) and used as received. The labeled water contents of OmniSolv methanol, ethanol, and 2-propanol were 0.003, 0.03, and 0.042%, respectively. Aqueous mixtures of these alcohols were prepared by dilution with water (v/v). Sulfuric acid (ACS reagent grade) was purchased from Fisher Scientific (Nepean, ON, Canada) and freshly diluted with water or 2-propanol/water before use. Water was purified in the laboratory with a Millipore Super-Q system (Bedford, MA).

Gas Chromatography. GC was performed with a Hewlett-Packard (HP) 6890 instrument (Avondale, PA) equipped with an HP 6890 series autoinjector, a flame ionization detector (FID) (operated at 300 °C), an electronic gas control, and a fused silica capillary column of HP-5 (0.25 μm film thickness, 30 m × 0.32 mm i.d.). The split/splitless injection port, operated at 250 °C, was equipped with a silanized glass liner (HP part 5181-3316). The samples were injected directly (30 s) at an initial oven temperature of 200 °C. After 1 min, the temperature was raised at 10 °C/min to 270 °C and then at 1 °C/min to 290 °C. The carrier gas was helium with a 2 mL/min constant flow under electronic pressure control. Retention times and peak area counts were obtained with HP GC ChemStation software (ver. A.05.04).

Confirmation of peak identity was obtained by mass spectrometry on selected extracts, using an HP 5989A GC-MS as reported previously (Taylor et al., 1997).

Seed Material. Mature seeds from Amber and Quatro fenugreek, both released Canadian cultivars, and a Canadian experimental line of fenugreek (ZT-5) were ground with a Wiley mill (0.8 mm mesh sieve) and dried for 2.5 h with a convection oven set at 60 °C. The samples were defatted in a Soxhlet apparatus for 6 h with petroleum ether (bp 37.8−57.2 °C) as solvent and double-thickness cellulose extraction thimbles (Whatman). The material in the thimble was air-dried, ground with a mortar and pestle, and then oven-dried at 60 °C for 2 h. Samples were stored in a desiccator at room temperature before analysis for diosgenin content.

Optimization Experiments (Method A). These experiments were performed with Amber seed material (100 mg) using previously described procedures (Taylor et al., 1997), without the preparation of standard curves and with the following modifications. Aqueous hydrochloric acid was replaced with aqueous sulfuric acid (see Figure 3a), with sulfuric acid in 70% 2-propanol (see Figure 3b), and with sulfuric acid in other 2-propanol/water mixtures (see Figure 4). Extraction experiments with (aqueous) methanol, ethanol, and 2-propanol (see Figure 5) were conducted in duplicate trials with 5 mL of solvent, heated at 80 °C for 3 h in a capped test tube, followed by two room temperature extractions of the solid that remained. The combined extracts were evaporated to dryness with a centrifugal evaporator and hydrolyzed for 2 h at 100 °C with 1 M sulfuric acid in 70% 2-propanol. Unless otherwise stated, the solvent for saponin extraction was 80% ethanol.

Microscale Experiments (Method B). Subsamples (10 mg) of seed material from Amber, Quatro, and ZT-5 fenugreek were transferred to a test tube (100 × 13 mm) equipped with a Teflon-lined screw cap. After addition of 80% ethanol (5 mL),

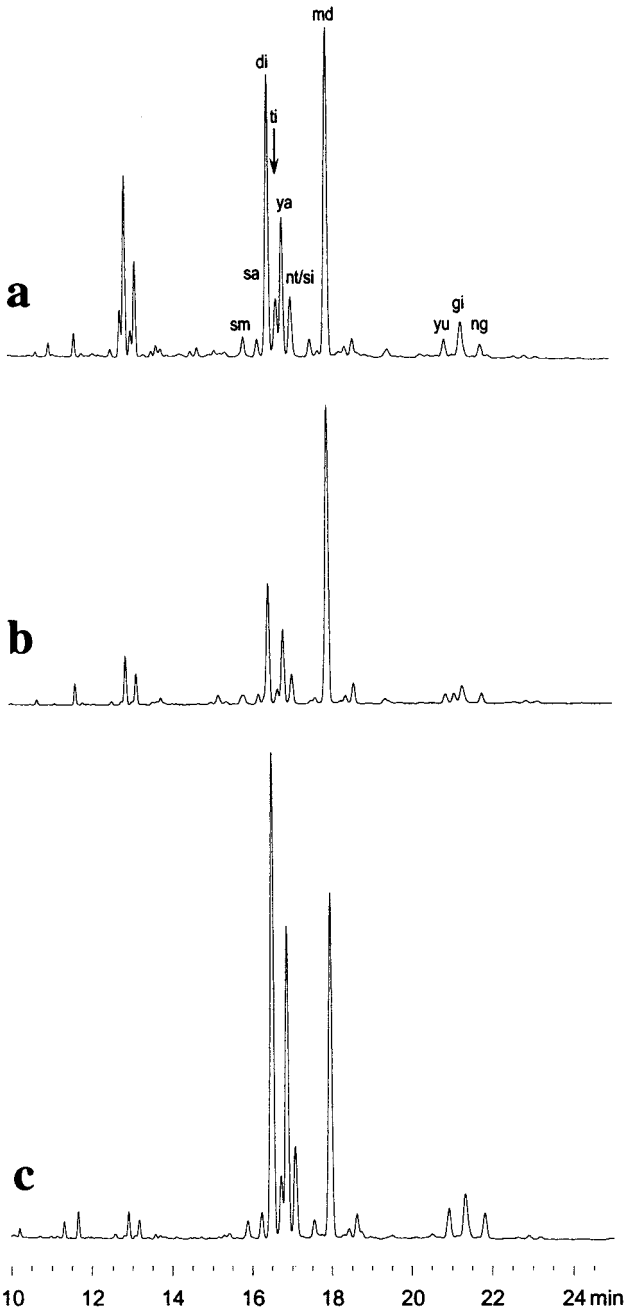

Figure 2. Chromatograms from GC analysis (HP-5 column) of Amber fenugreek seed extracts using method A with hydrolysis for 1 h at 100 °C with (a) 1 M aqueous hydrochloric acid, (b) 1 M aqueous sulfuric acid, and (c) 1 M sulfuric acid in 70% 2-propanol/30% water. Labels: sm, smilagenin; sa, sarsasapogenin; di, diosgenin; ti, tigogenin; ya, yamogenin; nt/si, mixture of neotigogenin and β-sitosterol; md, 6-methyldiosgenin; yu, yuccagenin; gi, gitogenin; ng, neogitogenin. Yamogenin, neotigogenin, yuccagenin, gitogenin, and neogitogenin were tentatively identified. Dienes eluted at 12.8−13.2 min.

the mixture was heated (80 °C) for 3 h with magnetic stirring in a metal block (Pierce Reacti-Therm III module). On cooling, the mixture was spun for 5 min at 700*g* (Eppendorf model 5403 centrifuge) and the solvent transferred with a glass pipet to another test tube. The solvent was evaporated at 43 °C with a Savant SpeedVac Plus (model SC 110A), and the pellet that remained in the test tube was dissolved with vortex mixing in 2 mL of 70% 2-propanol containing 1 M sulfuric acid. Each extract was hydrolyzed by heating the mixture at 100 °C for 2 h (metal block). After cooling, water (3 mL) was added. The internal standard of 6-methyldiosgenin (50 μg) was also added (with vortex mixing). The mixture was extracted three times

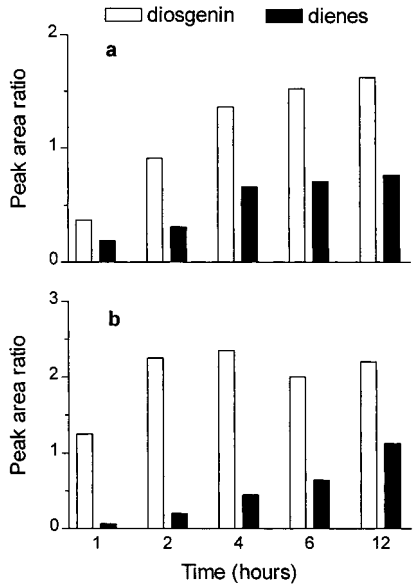

Figure 3. Influence of the time of hydrolysis at 100 °C with (a) 1 M aqueous sulfuric acid and (b) 1 M sulfuric acid in 70% 2-propanol on the recovery of diosgenin and dienes from Amber fenugreek using method A. Peak area ratio refers to the area of the peak for diosgenin (and the sum of areas of the integrated peaks for dienes) divided by the area of the peak for the internal standard. The data are average values (N = 2) from separate experiments.

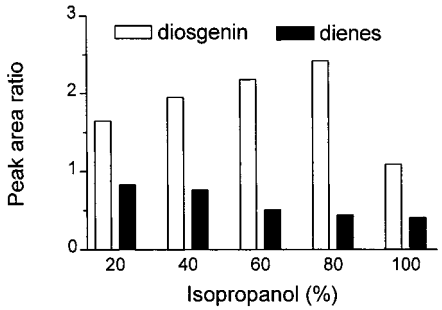

Figure 4. Influence of 1 M sulfuric acid in 20−100% 2-propanol at 100 °C for 2 h on the recovery of diosgenin and dienes from Amber fenugreek using method A. Other conditions were as described in Figure 3.

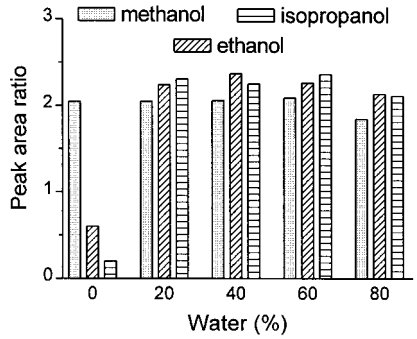

Figure 5. Influence of methanol, ethanol, and 2-propanol and solvent−water composition on the recovery of diosgenin from Amber fenugreek using method A with 1 M sulfuric acid in 70% 2-propanol at 100 °C for 2 h. Other conditions were as described in Figure 3.

with methyl *tert*-butyl ether (MBE; 2 mL), using a reciprocal shaker (Ames aliquot mixer) and centrifuge (2 min at 300*g*). The combined MBE extracts were washed twice with 1 M NaOH solution (1 mL) and once with water (1 mL). The MBE extracts were dried over anhydrous $MgSO_4$, briefly centrifuged, filtered through a small plug of cotton wool in a disposable pipet, and evaporated (Savant). The residue was dissolved in toluene (1 mL), and 2 μL was used for analysis by GC.

Method B was modified in one experiment by replacing sulfuric acid with 2 mL of aqueous hydrochloric acid (1 M) followed by heating at 100 °C for 1 h.

Quantitation and Statistics. Stock solutions of diosgenin were prepared in ethanol at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL. Portions (100 μL) of these solutions, delivering 0, 5, 10, 25, 50, 75, and 100 μg of diosgenin, were added to test tubes containing water (5 mL). The internal standard of 6-methyldiosgenin (50 μg, obtained from 100 μL of an ethanol stock solution prepared at 0.5 mg/mL) was added, and the samples were mixed by vortexing and then extracted with MBE, washed, dried, filtered, evaporated, and dissolved in toluene (1 mL) as described under method B with sulfuric acid. These calibration curve samples were analyzed by GC, along with the experimental seed extracts. Equations describing the calibration curves were obtained by linear regression analysis of the integrated peak area ratio of diosgenin to 6-methyldiosgenin versus the amount of diosgenin added. The amount of diosgenin found in the experimental seed samples 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 10 mg samples of defatted seed dried at 60 °C.

Statistical comparisons of diosgenin levels (see Table 1) were performed by one-way analysis of variance. A least significant difference test was used to evaluate differences among means.

Table 1. Microdetermination by Method B of Diosgenin in Extracts from Experimental Samples of Canadian Fenugreek Seed

	% diosgenin (N)		
analysis	Amber	Quatro	ZT-5
1	0.59 (5)	0.49 (2)	0.76 (2)
2	0.51 (2)	0.38 (5)	0.79 (2)
3	0.48 (2)	0.49 (2)	0.75 (5)
mean ± SD[a]	0.55 ± 0.06[a]	0.42 ± 0.06[b]	0.76 ± 0.07[c]

[a] Mean values (N = 9) with different superscripts are significantly different at the 1% level.

RESULTS AND DISCUSSION

Although 1 M aqueous hydrochloric acid effectively hydrolyzed the steroidal saponins extracted with 80% aqueous ethanol from fenugreek seeds, the quantitative determination of diosgenin by capillary GC of hydrolyzed samples had certain limitations, particularly with regard to control over artifact (diene) formation (Taylor et al., 1997). We initiated the present study in a new laboratory using similar seed material (Amber fenugreek), columns (HP-5), and instrumentation as before. Acid hydrolysis experiments were compared by examination of GC-FID traces of the separated mixtures of steroidal sapogenins and by calculating the peak area ratios of diosgenin and dienes to an internal standard.

GC traces obtained after hydrolysis for 1 h with 1 M solutions of hydrochloric acid in water, sulfuric acid in water, and sulfuric acid in 70% 2-propanol are shown in Figure 2. Peaks corresponding to the previously (or tentatively) identified steroidal sapogenins of Amber fenugreek, labeled in Figure 2a, were observed in all experiments but considerably reduced in the trial with aqueous sulfuric acid. The ratio of diosgenin to internal standard, indicative of the recovery of diosgenin, was highest in the experiment with sulfuric acid in 70% 2-propanol.

Peaks representing the dienes were relatively high in extracts from hydrochloric acid treatment and low in both treatments with sulfuric acid. Because the

recovery of diosgenin (and dienes) appeared to be low following hydrolysis for 1 h with aqueous sulfuric acid, an experiment was performed with longer hydrolysis times (Figure 3a). Although recoveries of diosgenin and yamogenin increased with time (until 6 h), there was also a corresponding increase in the amount of the four dienes produced. The use of aqueous sulfuric acid was therefore abandoned.

A similar time study was performed using sulfuric acid in 70% 2-propanol (Figure 3b). This experiment indicated that 2 h of heating was optimal. Dienes increased gradually with time, and they were particularly high in the 12 h samples. Subsequent hydrolysis experiments with sulfuric acid were performed for 2 h.

We also studied the influence of water in 2-propanol for sulfuric acid hydrolysis. The recovery of diosgenin increased gradually as the amount of water in the 2-propanol decreased (Figure 4). Dienes tended to be higher in treatments with 20 and 40% 2-propanol. With pure (>99%) 2-propanol, however, diosgenin levels fell considerably and were about the same as in trials with pure water. The optimum composition appeared to be 60−80% 2-propanol, and a 70% 2-propanol mixture was selected for subsequent experiments.

Although short-chain alcohols and aqueous alcohol mixtures are commonly used in the extraction of saponins from fenugreek (Sauvaire and Baccou, 1978b; Sauvaire et al., 1996; Benichou et al., 1999), it was of interest to compare the relative efficiencies of such solvents in the extraction of diosgenin glycosides from seed material followed by standard conditions of sulfuric acid hydrolysis and GC analysis. Extractions with 20−80% water in ethanol, methanol, and 2-propanol were performed, and the relative recoveries of diosgenin, evaluated by peak area ratios of diosgenin to internal standard, were very similar, with a trend toward slightly lower recoveries in treatments with methanol and with 80% water in the alcohols (Figure 5). The ratio of dienes to internal standard was also similar (0.3−0.4) in these treatments. In experiments without addition of water, ethanol and 2-propanol resulted in sharply reduced recoveries of diosgenin and dienes, whereas pure methanol showed the same recovery as in treatments with methanol/water mixtures.

The ratios of diosgenin to internal standard were compared in extractions of Amber seed material with 80% ethanol, 80% methanol, and 80% 2-propanol. Using four seed samples with each solvent, the relative recoveries of diosgenin were not significantly different.

Further modifications were studied with the objective of performing quantitative experiments with sulfuric acid using a small quantity of seed material (10 mg).

Compared to chromatograms from 100 mg of seed material hydrolyzed for 2 h with 1 M sulfuric acid in 70% 2-propanol, the 10-fold reduction in sample size did not change the peak area distribution of the nine steroidal sapogenins detected in Amber fenugreek. Reduced amounts of seed sample also meant that reduced amounts of internal standard were needed.

When 10 mg of seed material was extracted once with hot 80% ethanol and the extract hydrolyzed for 1 h with 1 M hydrochloric acid, the chromatograms showed a 2−3-fold reduction in peak areas for diosgenin and yamogenin as well as a concomitant increase, as expected, in areas of the four diene peaks. Compared to sulfuric acid, peaks for yuccagenin, gitogenin, and neogitogenin were also reduced in 10 mg experiments with hydrochloric acid, not only with Amber but also with other seed material (discussed below). This reduction in the last eluting components was not apparent in 100 mg experiments with Amber fenugreek (see Figure 2).

Three experimental seed samples were selected for determination of diosgenin levels by method B, using a design in which the defatted samples were analyzed on three different occasions, once in quintuplicate and twice in duplicate (Table 1). Quantitation was performed by reference to standard curves, prepared by adding to water various quantities of diosgenin and a fixed quantity of 6-methyldiosgenin and processing the calibration samples without the 80% ethanol extraction and acid hydrolysis step. Analysis by GC gave correlation coefficients (r^2) and slopes of 0.996−0.998 and 0.033−0.035, respectively.

Overall mean and standard deviation values (Table 1) showed that the precision (relative standard deviation) using method B was 9−14%. Analysis of variance indicated that diosgenin levels among the three fenugreek samples were significantly different, ZT-5 (0.76% diosgenin) giving nearly twice the levels of the Quatro seed material. These differences were also detectable with a smaller number of replicated samples. Diosgenin levels in Amber (0.55%) were nearly the same as previously reported levels (0.54%) with hydrochloric acid (Taylor et al., 1997).

On the basis of GC peak areas from representative chromatograms, the distributions of steroidal sapogenins from Amber and Quatro fenugreek were similar but the ratio of diosgenin to yamogenin was lower in Amber (2.0) than in Quatro (2.7). In ZT-5, this ratio was 2.3. Relative levels of tigogenin increased in the order ZT-5 > Quatro > Amber. Smilagenin and sarsasapogenin were easily detected in extracts from Amber and Quatro, whereas these sapogenins were at trace concentrations in extracts from ZT-5. It appears that seeds of ZT-5 accumulate higher levels of the glycosides of diosgenin, and possibly glycosides of yamogenin and tigogenin as well, at the expense of glycosides of smilagenin and sarsasapogenin. Areas of the four diene peaks represented 6−9% of the total peak areas of the mixtures.

This microprocedure with sulfuric acid in 2-propanol (method B) is useful for the determination of diosgenin from fenugreek seeds, including plant breeding or other research projects for which only small quantities of seeds are available. Excluding grinding, defatting, and drying steps, a skilled technician can process and analyze ~20 experimental and standard curve samples per week. The method described here appears to be time competitive with combined extraction and hydrolysis methods, allowing good flexibility in the choice of extraction solvent.

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