Identification and Quantification of Isoflavonoid and Triterpenoid Compliance Markers in a Licorice-Root Extract Powder

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Marker compounds are needed to determine dietary compliance in free-living human study populations participating in dietary intervention trials for cancer research. Two isoflavonoid and one triterpenoid marker compounds were detected and identified in a licorice-root extract powder. A convenient method that involves hydrolysis, solvent extraction, and liquid chromatographic analysis was developed to quantify the marker compounds in the licorice-root extract powder. The compounds and their concentrations are as follows: formononetin (1.92 mg/g), isoliquiritigenin (9.61 mg/g), and 18-β-glycyrrhetinic acid (43.9 mg/g).

INTRODUCTION

Southern Research Institute (SRI) undertook the identification and quantification of two isoflavonoid and one triterpenoid marker compounds present in an anticarcinogenic licorice-root extract powder on a contract with the National Cancer Institute. The function of marker compounds was discussed in the first paper in this series (Weinberg et al., 1993). Isoflavonoids previously isolated from licorice root include formononetin (Elgamal et al., 1972; Mitscher et al., 1978; Saitoh et al., 1978; Kobayashi et al., 1985), glabridin (Saitoh et al., 1976; Mitscher et al., 1978), glabrene (Saitoh et al., 1976; Mitscher et al., 1978), licoisoflavone A (Kinoshita et al., 1978; Hiraga et al., 1984), licoisoflavone B (Saitoh et al., 1978; Hiraga et al., 1984), licoisoflavone C (Hiraga et al., 1984), licoisoflavanone (Saitoh et al., 1978), glycyrrhisoflavanone (Hatano et al., 1988), glycyrrhisoflavone (Hatano et al., 1988), glabrone (Kinoshita et al., 1976), licoricone (Kaneda et al., 1973), glisoflavone (Hatano et al., 1988), and isoliquiritigenin (Hiraga et al., 1984).

Licorice root contains about 6–14% of glycyrrhizin, which is also known as glycyrrhizic acid, glycyrrhetinic acid glycoside, or glycyrrhizinic acid. It is a glycoside that contains the triterpenoid glycyrrhetinic acid, which is also known as glycyrrhetic acid, and an oligosaccharide (de Groot et al., 1988). The glycoside usually occurs in a combined calcium and potassium salt form (Hurst et al., 1983). Glycyrrhetinic acid can be liberated from glycyrrhizic acid by acid hydrolysis. Minor amounts of other triterpene sterols have also been isolated from licorice root. These include 18α-glycyrrhetic acid (Beaton et al., 1956), glabric acid, a hydroxylated 18α-glycyrrhetic acid (Beaton et al., 1956), and liquoric acid (Elgamal et al., 1965). Recently a number of new triterpene oligoglycosides have been isolated from licorice root (Kitagawa et al., 1988, 1989). However, it appears that all of these newly identified compounds would yield glycyrrhetinic acid upon hydrolysis.

MATERIALS, INSTRUMENTATION, AND METHODS

Materials. *Reagents.* High-purity acetone, methanol, methylene chloride, acetonitrile (UV grade), hexane (GC^2 grade), and pentane (GC^2 grade) were obtained from Burdick and Jackson (McGaw Park, IL). Acetone-d_6 (minimum isotopic purity 96.96 atom %) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Phenylthiourea (BDA microanalytical reagent) was obtained from British Drug Houses Ltd. (Poole, England). Ethyl acetate (Nanograde), pyridine (AR grade), concentrated hydrochloric acid (AR grade), formic acid (AR grade), and potassium carbonate (AR grade) were obtained from Mallinckrodt Inc. (St. Louis, MO). *N*-Methyl-*N*-(trimethylsilyl)fluoroacetamide (MSTFA), Tri-Sil, and pyridine (silylation grade) were obtained from Pierce Chemical Co. (Rockford, IL). Deionized water was prepared at the Institute using a Millipore Milli-Q water purification system.

Sample. A 500-g bag of a licorice-root extract powder was provided to SRI by the National Cancer Institute, which had obtained the product from MacAndrews and Forbes Co. (Camden, NJ) on a materials-transfer agreement. It was stored in a desiccator at ambient temperature.

Components. Formononetin was obtained from Indofine Chemical Co., Inc. (Somerville, NJ), and 18-β-glycyrrhetinic acid was obtained from Aldrich. The purity of each was listed as 99%.

Isoliquiritigenin was isolated from the licorice-root extract powder. A 1-L three-neck, round-bottom flask was equipped with a condenser, stirring motor and paddle, and stopper. Licorice-root extract powder (20.28 g) and 500 mL of 4 N aqueous hydrochloric acid were added. The suspension was stirred vigorously and heated to 60–74 °C over a period of 105 min. The suspension was extracted with three 300-mL portions of ethyl acetate, and the extracts were dried over anhydrous magnesium sulfate. The suspension was filtered and rotary evaporated to about 5 mL. The black tarry residue was diluted to 100 mL with ethyl acetate. A 90-mL portion was extracted with 500 mL of 2 N aqueous potassium carbonate. The phases were separated, and the aqueous phase was extracted with another 100 mL of ethyl acetate. The two ethyl acetate extracts were discarded. The basic aqueous phase was strongly acidified with 300 mL of 6 N aqueous hydrochloric acid and extracted with two 500-mL portions of ethyl acetate. The aqueous phase was discarded, and the ethyl acetate extract was dried over anhydrous magnesium sulfate, filtered, and rotary evaporated to dryness. The yield of black, viscous material was 2.22 g. The sample was diluted with ethyl acetate.

The solution (125 μL) was spotted on an Analtech, Inc., preparative TLC plate (Uniplate taper plate, silica gel GE) and developed using a solution containing 60% hexane–40% acetone. Five bands were detected. A deep yellow band at $R_f = 0.458$ was scrapped off, extracted with 10 mL of ethyl acetate, and centrifuged. The supernatant was removed and dried over anhydrous magnesium sulfate. An aliquot (4.0 mL) was evaporated to dryness, and a proton NMR spectrum was obtained. It clearly showed that only one component was present, a trihydroxychalcone that was identified as 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (2′,4,4′-trihydroxychalcone or isoliquiritigenin): ^1H NMR (Me_2SO-d_6) δ 13.62 (s, 1, O—*H*- - -O=C), 10.4 (br s, 2, *O*Hs), 8.15 (d, 1, aromatic H, J = 8.9 Hz), 7.74 (br d, 2 H, aromatic Hs, J = 8.6 Hz), 7.73 (s, 2

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H, H—C=C—H), 6.83 (br d, 2 H, aromatic Hs, J = 8.6 Hz), 6.37 (dd, 1, aromatic H, J = 2.1 Hz, J = 8.9 Hz), 6.24 (d, 1, aromatic H, J = 2.1 Hz). (Me_2SO-d_5 was used as an internal reference; δ = 2.50.)

A second aliquot (3.2 mL) was evaporated to dryness, yielding a significant quantity of a crystalline yellow solid, which was derivatized with 32 μL of Tri-Sil. The total-ion chromatogram showed two silylated components to be present: a high concentration of a disilylated component with an apparent molecular weight of 400 and a low concentration of a trisilylated component with an apparent molecular weight of 472. Silylation adds 72 mass units for each hydroxy group converted to a trimethylsilyl ether. The NMR spectrum discussed above showed clearly that only one component is present prior to silylation; presumably two silylated components were obtained because the hydroxy group ortho to the carbonyl group is hindered (Furlong et al., 1985) and is difficult to silylate. The mass spectrum of disilylated isoliquiritigenin is shown in Figure 4b in the supplementary material.

Isoliquiritigenin was isolated and identified after the LC/MS studies mentioned below were completed and, consequently, a mass spectrum of the underivatized isolated product was not obtained. However, the underivatized component (molecular weight m/z 256) can be detected among the components detected in a previous LC/MS run of extracted licorice-root extract powder. The preparative TLC procedure was repeated a number of times to obtain a larger quantity of the yellow crystalline compound.

Surrogate and Internal Standard Compounds. 4-Chloro-4-hydroxybenzophenone and 3-chloro-4-hydroxyphenylacetic acid were obtained from Aldrich. The purity was listed as 98 or 99%. Naphthalene-d_8 (98+ atom % D) was also obtained from Aldrich.

Instrumentation. A Waters (Division of Millipore, Milford, MA) Model 600 E multisolvent delivery system was connected to a Waters Model 484 variable-wavelength detector. Data from the variable-wavelength detector were sent to a Hewlett-Packard Co. (Atlanta, GA) Model 3396 recording integrator, which in turn was interfaced to a CompuAdd (Austin, TX) Model 286 personal computer. The communication between the recording integrator and personal computer was established using Chrom Perfect software distributed by Justice Innovations, Inc. (Palo Alto, CA). The liquid chromatographic/ultraviolet spectroscopic (LC/UV) data were collected as hardcopy on the recording integrator and stored on IBM-compatible disks in the personal computer.

A Hewlett-Packard Model 5890 gas chromatograph was coupled to a VG 70S high-resolution mass spectrometer by means of a direct inlet for capillary column gas chromatography. Alternatively, the liquid chromatograph was connected to an Extrel (Pittsburgh, PA) ThermaBeam particle-beam interface. The particle-beam interface in turn was connected to the VG Instruments, Inc. (Danvers, MA), Model 70S high-resolution mass spectrometer in place of the gas chromatograph. The effluent from the liquid chromatographic column passed through the UV cell in the liquid chromatograph before entering the particle-beam interface. Thus, both LC/UV and LC/MS data are generated in the same run. A Nicolet Co. (now General Electric NMR Instruments, Fremont, CA) NT 300B nuclear magnetic resonance spectrometer operated at 300.635 MHZ for observing protons. Data were stored on a magnetic disk, and hardcopy was produced using a Nicolet Zeta 8 digital printer. Analtech, Inc. (Newark, DE), supplied cylindrical (5 × 20 cm) and rectangular (20 × 20 cm) developing chambers, a portable UV lamp (8 W), a TLC plate desiccator chamber, tapered silica gel G preparative Uniplate-T (preadsorbent, channeled, scored) TLC plates, and analytical hard layer silica gel GHL Uniplates with inorganic binder TLC plates.

Methods. *Preparation of Sample for Identification of Components by LC/MS.* A 50-mL one-neck, round-bottom flask was equipped with a condenser. Licorice-root extract powder (4.962 g) and 15 mL of water were added to the flask, and the flask was occasionally swirled for 45 min. It was heated for 15 min at 82–85 °C. Then 5.0 mL of concentrated hydrochloric acid was added, and the suspension was heated at 82–85 °C for 30 min. The suspension was continuously swirled during this period. The reaction mixture was extracted with two 20-mL portions of methylene chloride, and the extracts were combined. The methylene chloride extract was extracted with two 20-mL portions of aqueous potassium carbonate (13.9 g/L). The aqueous potassium carbonate extracts were combined, acidified with 8 mL of concentrated hydrochloric acid, and extracted with two 20-mL portions of methylene chloride. The methylene chloride extracts were combined and washed with 40 mL of water. The methylene chloride extract was evaporated to dryness using a rotary evaporator with water aspirator vacuum and gentle heating. The residue was dissolved in 1 mL of methanol. Then 75 μL of the sample was combined with 25 μL of water and analyzed by LC/MS.

Typical Liquid Chromatographic/Mass Spectrometric Conditions. The sample was dissolved in 75% aqueous methanol. The liquid chromatographic column was a Keystone Scientific Co. (Bellefonte, PA) 250 × 3 mm, 5 μm, 300 Å, Deltabond ODS column. A solvent gradient and a flow rate of 0.3 mL/min were used during the analysis. The initial composition was 55% water, 40% methanol, and 5% formic acid. The composition was changed over 1 min to 95% methanol and 5% formic acid and held for 10 min. The particle-beam interface had a nebulizer temperature of 115 °C, a chamber temperature of 83 °C, and a helium flow rate of 1 L/min. The ion source of the mass spectrometer was maintained at 260 °C. The electron voltage was 70 eV. The magnet was scanned from mass 500 to 50 at 2 s/decade with a rest time of 0.5 s. The nominal resolution was 1000.

Gas Chromatographic/Mass Spectrometric Conditions. The gas chromatographic capillary column was a 10-m, 0.32-mm-i.d., HP-5 column coated with a 0.52-μm bonded-phase film of methyl silicone. The column was maintained at 35 °C for 5 min and then heated to 300 °C at 4 °C/min. The solvent delay was 3.45 min. Splitless injections were made. The injection port was maintained at 300 °C, and the flow of helium carrier gas was maintained by a head pressure of 4.2 psig. The mass spectrometer transfer line was operated at 300 °C and the ion source at 260 °C. The voltage was 70 eV. The magnet was scanned from mass 700 to 35 at 1 s/decade with a rest time of 0.5 s. The nominal resolution was 1000.

Liquid Chromatographic/Ultraviolet Spectroscopic Conditions. The liquid chromatographic column was a Keystone Scientific 250 × 3 mm, 5 μm, 300 Å, Deltabond ODS column. A solvent gradient and a flow rate of 0.65 mL/min were used during the analysis. The initial composition was 72% water, 23% acetonitrile, and 5% formic acid. The composition was changed over 0.5 min to 66% water, 29% acetonitrile, and 5% formic acid. The composition was maintained for 6.5 min and was then changed over 15 min to 20% water, 75% acetonitrile, and 5% formic acid. The wavelength was varied to increase the peak height produced by the various target compounds. The lamp was set to 280 nm for the first eluting compound of interest, 3-chloro-4-hydroxyphenylacetic acid. It was then reduced to 230 nm for formononetin and isoliquiritigenin. Then it was increased to 255 nm for 4-chloro-4′-hydroxybenzophenone, naphthalene-d_8, and 18-β-glycyrrhetinic acid.

Standards. A surrogate solution contained the following components in pyridine: 3-chloro-4-hydroxyphenylacetic acid (20.25 mg/mL) and 4-chloro-4′-hydroxybenzophenone (20.07 mg/mL). An internal standard solution contained naphthalene-d_8 (250 μg/mL) in acetonitrile. Instrument calibration standards contained the following components in 55% aqueous acetonitrile: formononetin (404 ng/mL), isoliquiritigenin (761 ng/mL), 18-β-glycyrrhetinic acid (7524 ng/mL), 3-chloro-4-hydroxyphenylacetic acid (4864 ng/mL), and 4-chloro-4′-hydroxybenzophenone (5200 ng/mL). By serial dilution, stock solutions were then prepared that contained $^1/_2$ and $^1/_4$ of these concentrations. Instrument calibration standards were prepared by adding 40 μL of an internal standard solution to each of these solutions. This yielded a concentration of 5000 ng/mL of naphthalene-d_8. Matrix spiking solutions containing appropriate concentrations of formononetin, isoliquiritigenin, and 18-β-glycyrrhetinic acid were also prepared.

Optimization of the Hydrolysis as a Function of Acid Concentration, Temperature, and Time. Licorice-root extract powder (0.25 g) was added to a culture vial, and 5 mL of 0.1, 1, 2, 4, or 6 N hydrochloric acid was added. The suspension was shaken and heated at 62, 74, or 80 °C for 15–120 min. The

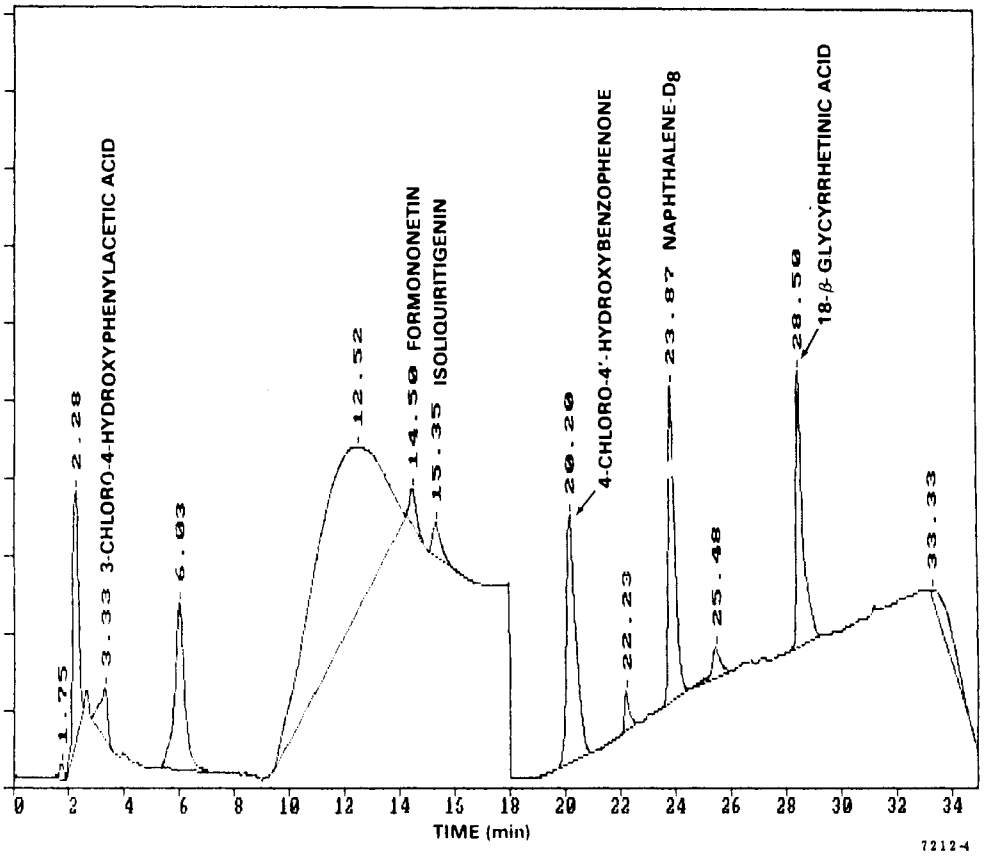

Figure 1. Liquid chromatogram of extract of hydrolyzed licorice-root extract powder containing surrogates and internal standard.

suspension was cooled in an ice bath, and 5 mL of ethyl acetate was added. The suspension was shaken vigorously and then centrifuged. One milliliter of the top ethyl acetate phase was removed, and 1 mL of MSTFA and 40 μL of an internal standard solution were added. The silylated sample was analyzed by GC/MS.

Optimization of the Hydrolysis as a Function of Time at Constant Temperature and Acid Concentration. Licorice-root extract powder (0.100 g) was added to a culture vial, and 5 mL of 4 N hydrochloric acid was added. A surrogate (250 μL) was added, and the suspension was mixed using a vibrator. The suspension was heated at 79 °C for 60, 85, 110, or 135 min. The suspension was cooled, and 10 mL of ethyl acetate was added. The suspension was shaken vigorously and centrifuged at 3000 rpm for 10 min. Then 20 μL of the top ethyl acetate was removed and blown to dryness using nitrogen. Forty microliters of an internal standard and 2 mL of 55% aqueous acetonitrile were added, and the solution was analyzed by LC/UV.

Quantification of Target Compounds in Licorice-Root Extract Powder. To 100 mg of licorice-root extract powder in a culture vial was added 125 μL of surrogate solution and 125 μL of pyridine. The surrogate solution contained 3-chloro-4-hydroxyphenylacetic acid (2531 μg) and 4-chloro-4'-hydroxybenzophenone (2509 μg). Then 5 mL of 4 N hydrochloric acid was added. The suspension was thoroughly mixed and heated for 1 h at 78 °C. (During the first 5 min, the caps of the culture vials were loosened to allow for expansion of air.) The sample was then cooled, 10 mL of ethyl acetate was added, and the suspension was vigorously shaken. The sample was then centrifuged at 3000 rpm for 10 min. Then 20 μL of the top ethyl acetate phase was removed and evaporated to dryness with mild heating. Two milliliters of 55% aqueous acetonitrile and 40 μL of internal standard solution were then added to the residue. The internal standard solution added contained naphthalene-d_8 (10 μg). An aliquot of the sample was then analyzed by LC/UV; a typical LC/UV chromatogram of the extract is shown in Figure 1.

RESULTS AND DISCUSSION

Identification of Components in a Licorice-Root Extract Powder. A sample of licorice-root extract powder was hydrolyzed in aqueous hydrochloric acid, the suspension was extracted with methylene chloride, and the extract was dried and evaporated to dryness. A solution containing an aliquot of the residue in 75% aqueous methanol was prepared, and a portion of the solution was analyzed by LC/MS. Formononetin and 18-β-glycyrrhetinic acid were tentatively identified on the basis of their mass spectra. A portion of a similarly prepared residue was silylated and analyzed by GC/MS. Formononetin was tentatively identified as its monosilylated derivative, isoliquiritigenein as its disilylated derivative (apparently the 2'-hydroxyl group does not silylate under the mild derivatizing conditions used), and glycyrrhetinic acid as its disilylated derivative.

For the identification of a compound, the mass spectrum of the tentatively identified compound must be essentially identical to that of the mass spectrum of the authentic compound. Although some extraneous peaks from the matrix may be present in the mass spectrum of the tentatively identified component, especially when the component is present at low concentration, peaks such as the parent peak, base peak, and key fragment peaks should have similar m/z values and relative intensities. Also, the LC/UV retention times of tentatively identified compounds and authentic compounds spiked into a sample containing the tentatively identified compounds should be the same with no peak broadening.

Pure samples of 18-β-glycyrrhetinic acid and formononetin were purchased. For the LC/MS runs, comparison of the mass spectrum of each tentatively identified compound (respectively, Figures 2a and 3a in the supplementary material) with the mass spectrum of the corresponding authentic compound (Figures 2b and 3b) shows that a good match is obtained. Also, for the GC/MS runs, comparison of the mass spectra of the silylated tentatively identified compounds (respectively, Figures

Table I. Liquid Chromatographic Relative Response Factors for Identified Components in Licorice-Root Extract Powder

component	linear regression slope	linear regression intercept	corr coeff
formononetin[a]	3.399	−0.0118	0.986
isoliquiritigenin[a]	0.649	−0.0136	0.987
18-β-glycyrrhetinic acid[a]	1.10	−0.0555	0.987
4-chloro-4′-hydroxybenzophenone[a,b]	1.97	−0.0360	0.996
3-chloro-4-hydroxyphenylacetic acid[a,b]	0.361	−0.00890	0.979

[a] Response factor relative to naphthalene-d_8. [b] Surrogates.

Table II. Optimization of Conditions for the Hydrolysis and Extraction of Licorice-Root Extract Powder[a]

run	time, min	temp, °C	hydrochloric acid concn, N	relative concn[b] formononetin	relative concn[b] 18-β-glycyrrhetinic acid
			GC/MS Analysis of Silylated Compounds		
			Data Set 1		
1	30	62	4.0	0.52	ND[c]
2	30	62	6.0	1.00	ND
3	30	80	2.0	Low	ND
4	30	80	4.0	0.90	ND
			Data Set 2		
5	30	74	0.1	0.028	0.092
6	30	74	1.0	0.11	0.23
7	30	74	4.0	1.00	1.00
8	30	74	6.0	0.28	1.22
			Data Set 3		
9	15	74	4.0	0.16	0.49
10	30	74	4.0	0.49	0.58
11	45	74	4.0	0.85	0.84
12	60	74	4.0	0.89	0.79
13	120	74	4.0	1.00	1.00
			LC/UV Analysis of Unsilylated Compounds		
			Data Set 4		
1	60	79	4.0	0.94	0.97
2	60	79	4.0	0.95	1.01
3	85	79	4.0	1.06	0.97
4	85	79	4.0	1.05	1.00
5	110	79	4.0	0.94	0.99
6	110	79	4.0	1.00	0.94
7	135	79	4.0	0.98	0.95
8	135	79	4.0	1.00	1.00

[a] Isoliquiritigenin is not included in this list because at the time this work was done its presence in the licorice-root extract powder had not been detected. [b] The relative concentration is calculated within each data set. [c] ND, Not detected. (A method for the quantification of 18-β-glycyrrhetinic acid was not available at the time these analyses were performed.)

2c and 3c) with the mass spectrum of the corresponding authentic compounds (Figures 2d and 3d) shows that a good match is obtained.

Isoliquiritigenin was isolated from licorice-root extract powder after the LC/MS studies were completed, and so LC/MS data were not obtained on the isolated product. However, as shown in Figure 4a in the supplementary material, the mass spectrum of a component observed in a hydrolysate of licorice-root extract powder generated a mass spectrum that shows the expected molecular ion at m/z 256 and key fragment ions at m/z 137 (molecular ion minus the 4-hydroxystyryl radical) and 120 (4-hydroxystyrene molecular ion). For the GC/MS runs, comparison of the mass spectra of the disilylated compound present in one of the hydrolysates (Figure 4b) matches the mass spectrum of the disilylated isolated compound (Figure 4c). However, the most compelling evidence for the identity of the isolated component is its NMR spectrum as previously discussed. Spiking the three authentic compounds into licorice-root extract powder caused no broadening of the peaks produced by the identified compounds in a LC/UV chromatogram.

Quantification of Components in a Licorice-Root Extract Powder. *Calibration of the Liquid Chromatograph.* A series of standards were prepared and employed to estimate the concentration of the targeted components in the licorice-root extract powder using the optimized hydrolysis, extraction, and analysis procedure. Surrogates were added to the sample prior to hydrolysis and extraction, and an internal standard was added to the isolated extract for quantification. 4-Chloro-4′-hydroxybenzophenone was selected as a surrogate for the isoflavonoids and 3-chloro-4-hydroxyphenylacetic acid as a surrogate for the triterpenoid. The surrogates do not occur in nature but contain structural features that are similar to those present in the target compounds, and they produce strong UV spectra. Naphthalene-d_8 was selected as the internal standard. It elutes in the vicinity of the target compounds and produces a strong UV spectrum.

After an initial analysis of the licorice-root extract powder, new standards were prepared that contained components whose concentrations bracketed the estimated concentrations of the components in the licorice-root extract powder. Then a three-point calibration curve for each component was generated. By linear regression analysis the typical slope and intercept values and correlation coefficients shown in Table I were obtained.

Hydrolysis, Extraction, and Analysis of the Licorice-Root Extract Powder. The goal was to generate a convenient but accurate method for determining the total concentration of formononetin, isoliquiritigenin, and 18-β-glycyrrhetinic acid in licorice-root extract powder. Because each of these compounds can exist as a glycoside, efforts were focused on developing a procedure that would liberate any aglycons from glycons present in the licorice-root extract powder.

Licorice-root extract powder was hydrolyzed in hydrochloric acid. The effect of the concentration of the hydrochloric acid, the temperature, and the time of hydrolysis on the concentration of formononetin and 18-β-glycyrrhetinic acid produced were investigated. (At the time that those studies were performed, a good method for the quantification of isoliquiritigenin was not available.) The results shown in Table II were obtained. Ultimately,

Table III. Measured Concentration of Identified Components in Hydrolyzed Licorice-Root Extract Powder

component	concentration, mg/g, or recovery of surrogates, %: run 1	2	3	4	5	6	av	% RSD[a]
formononetin	1.77	1.84	1.91	2.07	2.00	1.94	1.92	5.6
isoliquiritigenin	6.50	7.61	10.9	11.3	11.3	10.1	9.61	21.4
18-β-glycyrrhetinic acid	45.9	46.7	41.6	41.6	43.2	44.6	43.9	4.9
3-chloro-4-hydroxyphenylacetic acid[b]	133	131	111	109	114	112	118	9.2
4-chloro-4′-hydroxybenzophenone[b]	123	125	117	119	121	116	120	3.0

[a] % RSD, percent relative standard deviation. [b] Surrogates.

Table IV. Recovery of Components and Surrogates Spiked into Licorice-Root Extract Powder

component	sample concn, mg/g	run 1			run 2			run 3			run 4			run 5			run 6				
		spiking mix, mg/g	found concn, mg/g	recovery, %	spiking mix, mg/g	found concn, mg/g	recovery, %	spiking mix, mg/g	found concn, mg/g	recovery, %	spiking mix, mg/g	found concn, mg/g	recovery, %	spiking mix, mg/g	found concn, mg/g	recovery, %	spiking mix, mg/g	found concn, mg/g	recovery, %	av recovery, %	% RSD[c]
formononetin	1.92	1.84	3.87	106	1.92	3.45	80	1.81	4.07	118	1.81	4.42	139	1.87	4.16	120	1.86	3.89	106	111	18
isoliquiritigenin	9.61	0	8.12	NA[a]	0	6.90	NA	6.82	18.0	123	6.81	14.0	65	7.01	11.1	21	6.95	13.5	55	66	18
18-β-glycyrrhetinic acid	43.9	48.1	97.9	112	50.0	96.6	105	47.4	93.0	103	47.4	97.7	114	48.8	99.3	114	48.6	104	125	112	6.7
3-chloro-4-hydroxyphenylacetic acid[b]	0	48.6	67.7	139	50.6	68.8	141	48.0	54.1	113	47.9	55.9	117	49.3	58.1	118	49.1	56.8	116	124	10
4-chloro-4'-hydroxybenzophenone[b]	0	48.2	59.5	123	50.2	60.1	125	47.6	58.1	122	47.5	58.3	123	48.9	59.4	121	49.8	58.5	118	122	1.8

[a] NA, not applicable. [b] Surrogates. [c] % RSD, percent relative standard deviation.

LC/UV was used for all of the analyses because variable results were obtained during the attempted analysis of silylated 18-β-glycyrrhetinic acid by GC/MS. In data set 1, 18-β-glycyrrhetinic acid was not detected because, at the time the analysis was performed, a method for its quantification was not available. The results listed in all of the data sets clearly show that hydrolysis is needed to liberate both formononetin and 18-β-glycyrrhetinic acid and that a maximum yield of the products is obtained when the hydrolysis is conducted in 4 N hydrochloric acid at 79 °C for 1 h or more.

The average concentrations of formononetin, isoliquiritigenen, and 18-β-glycyrrhetinic acid are shown in Table III. The concentration of 18-β-glycyrrhetinic acid is close to the range 6–14% reported for its glucone glycyrrhizin in licorice root (de Groot et al., 1988). The average recoveries of the two surrogates are satisfactory. The recoveries of components and surrogates spiked into licorice-root extract powder are shown in Table IV. The values for isoliquiritigenin were somewhat erratic. (Isoliquiritigenin appears to be unstable in pyridine spiking solutions. We used pyridine because it is a good solvent for the components and dissolves in water. Moreover, it forms a hydrochloride salt when the solution is acidified with hydrochloric acid and is not subsequently extracted by ethyl acetate.)

Conclusion. Methodology to detect, identify, and quantify two isoflavonoids and one triterpenoid present in licorice-root extract powder after hydrolysis was developed. The concentrations of the components are relatively high, and the compounds found are as follows: formononetin (1.92 mg/g), isoliquiritigenin (9.61 mg/g), and 18-β-glycyrrhetinic acid (43.9 mg/g).

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Supplementary Material Available: Figures 2–4 showing mass spectra of compounds (3 pages). Ordering information is given on any current masthead page.

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