Chemical Studies of Phytoestrogens and Related Compounds in Dietary Supplements: Flax and Chaparral (43824)

W. R. OBERMEYER,[1] S. M. MUSSER, J. M. BETZ, R. E. CASEY, A. E. POHLAND, AND S. W. PAGE

Divisions of Natural Products and General Scientific Support, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, District of Columbia 20204

Abstract. **High-performance liquid chromatographic (HPLC) and mass spectrometric (MS) procedures were developed to determine lignans in flaxseed (*Linum usitatissimum*) and chaparral (*Larrea tridentata*). Flaxseed contains high levels of phytoestrogens. Chaparral has been associated with acute nonviral toxic hepatitis and contains lignans that are structurally similar to known estrogenic compounds. Both flaxseed and chaparral products have been marketed as dietary supplements. A mild enzyme hydrolysis procedure to prevent the formation of artifacts in the isolation step was used in the determination of secoisolariciresinol in flaxseed products. HPLC with ultraviolet spectral (UV) or MS detection was used as the determinative steps. HPLC procedures with UV detection and mass spectrometry were developed to characterize the phenolic components, including lignans and flavonoids, of chaparral and to direct fractionation studies for the bioassays.** [P.S.E.B.M. 1995, Vol 208]

Epidemiological studies (1–3) indicate that foods containing phytoestrogens may reduce the risk of certain hormonally related cancers, particularly breast and prostate, and cardiovascular disease. Soybeans (*Glycine max*) are important components of Asian diets that have been, associated with a reduced risk of breast and prostate cancer (4). Soy contains more than ten isoflavones, with genistein and daidzein as the major phytoestrogens (4). Dietary supplementation is being proposed to increase consumption of phytoestrogens. Although the evidence for beneficial effects of food containing these components is mounting, the potential for toxicity from high levels of phytoestrogens has not been adequately investigated. The reported adverse effects of the synthetic antiestrogen, tamoxifen, used in clinical trials to prevent breast cancer, are examples of the potential for the toxic as well as the beneficial effects of hormonal therapies (5). Estrogenic substances can also have pronounced effects on fertility. The effects of phytoestrogens on livestock reproduction have been well documented (6). Setchell and co-workers (7) have postulated that the soy phytoestrogens were responsible for observed infertility and liver disease in captive cheetahs. Hormonal estrogens are known to be associated with hepatotoxicity (8).

This report describes preliminary work in the characterization of flax products, which contain high levels of lignan phytoestrogens, and chaparral, which has been associated with toxicity in humans and contains lignans which are structurally similar to known estrogenic substances. Lignans are structurally diverse and are characterized by the presence of two phenylpropanoid units. Their wide distribution in the plant kingdom may be related to lignin, the natural polymer and major constituent of plants. Many lignans have been reported to have antitumor, antimitotic, antioxidant, and weak estrogenic activities (9).

Flax (*Linum usitatissimum*) seed products are currently being marketed as dietary supplements and are being used as food constituents. Flaxseed is poten-

[1] To whom requests for reprints should be addressed at Divisions of Natural Products and General Scientific Support, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C Street, SW, Washington, DC 20204.

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tially the richest source of phytoestrogens (10). The primary lignan found in flaxseed is 2,3-bis(3-methoxy-4-hydroxybenzyl)butane-1,4-diol (secoisolariciresinol) 1. Secoisolariciresinol occurs naturally in flaxseed is as a glucoside. Upon ingestion, this glucoside is enzymatically hydrolyzed to the aglycone and transformed by intestinal microflora into enterodiol 2 and enterolactone 3 (11). The metabolites of enterodiol and enterolactone are found in bile and urine as glucuronide conjugates. Few nondegradative extraction procedures have been reported for the phytoestrogens in flaxseed. In order to prevent artifact formation and improve quantitation of the total flaxseed lignans, a mild enzymatic hydrolysis procedure to release the bound conjugates was developed. High-performance liquid chromatographic (HPLC) techniques were used as the determinative steps.

Chaparral (*Larrea tridentata*) is a dominant desert shrub in certain areas of the southwestern United States and Mexico. Infusions of chaparral have been used as a native American traditional medicine for treatment of a variety of diseases. Chaparral has also been used as a contraceptive agent in Mexico (12). Studies in the 1920s showed some uterine relaxation activity (13). Some of the components of chaparral have estrogenic activity and anti-implantation activities (14). Chaparral has been promoted as a "dietary supplement" in the treatment of cancer and as a "blood purifier." In 1990, Katz and Saibil (15) reported a case of acute nonviral hepatitis associated with the consumption of a chaparral product. This report included the reoccurrence of the disease on patient-elected rechallenge. In 1992, more than 10 cases of acute nonviral hepatitis associated with chaparral were reported to the U.S. Food and Drug Administration (FDA) and the Center for Disease Control (CDC) (16). Chemical and microbiological analyses of the chaparral products failed to show contamination (17). Microscopic examination (B. D. Tall, unpublished results) and chromatographic analyses described in this report confirmed the case-related material to be *Larrea tridentata*.

The major secondary chemical components of *Larrea* are waxes, volatile compounds, saponins, and phenolics (14, 18–20). The phenolic compounds account for most of the extractable dry weight (83%–91%). These include flavonoid aglycones and glycosides, and nordihydroguaiaretic acid (NDGA) 4 and related lignans (Fig. 1). NDGA is the most abundant lignan in the plant and is present in the leaves at approximately 5%–10% of the dry weight. NDGA was previously used commercially as an antioxidant. However, its approval as generally recognized as safe (GRAS) was withdrawn after animal studies revealed evidence of kidney toxicity (21).

HPLC procedures were developed to characterize

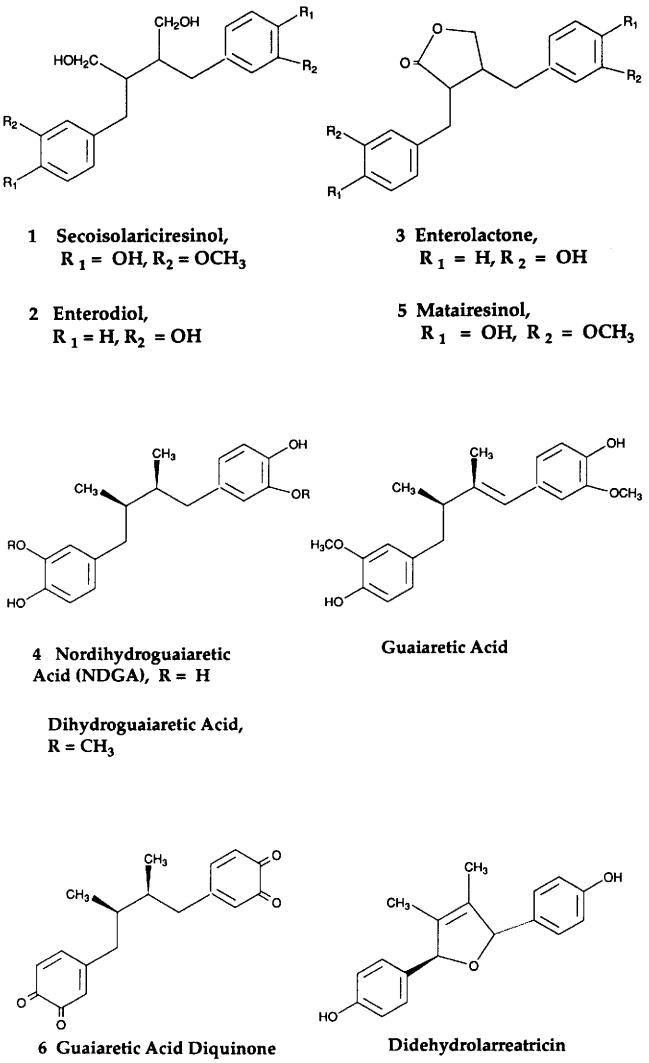

Figure 1. Representative lignans found in chaparral.

the phenolic and flavonoid components of chaparral for chemotaxonomic comparisons of the chaparral samples. These procedures were also used for the characterization of fractions for the bioassays.

Materials and Methods

The analytical reference standards for secoisolariciresinol, matairesinol, enterodiol, and enterolactone were purchased from Dr. T. A. Hase, University of Helsinki, Finland. The NDGA and β-glucuronidase were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were reagent grade. All solvents were HPLC grade and purchased from Baxter Healthcare Corp. (Burdick and Jackson Div., Muskegon, MI). The C18 solid-phase extraction (SPE) columns were purchased from Millipore-Waters (Milford, MA). Flaxseed products were obtained from the Food Industries Section, Arthur D. Little (Cambridge, MA). Authentic specimens of Mexican (Chihuahuan)

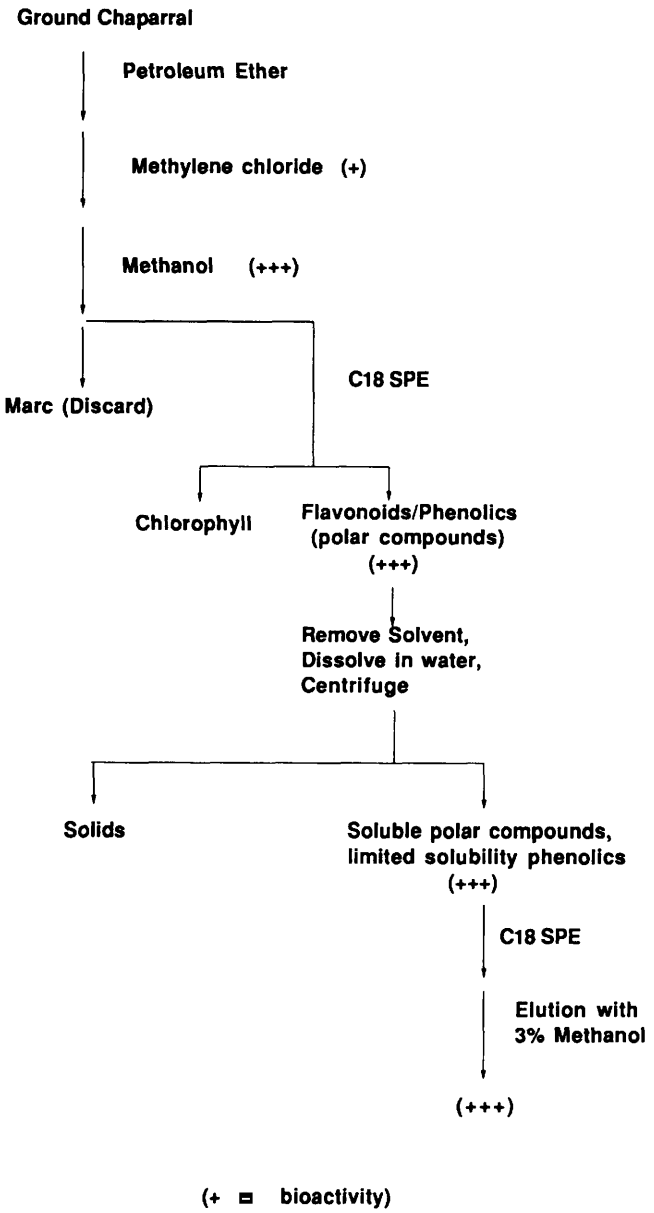

Figure 2. Chaparral fractionation scheme.

and U.S. (Sonoran) *Larrea tridentata* were kindly provided by Dr. Alvin B. Segelman, Nature's Sunshine Products (Spanish Fork, UT).

Hydrolysis, Extraction, and Concentration of Flaxseed Lignans. Flaxseed (0.1 g) was added to 4.0 ml of sodium acetate buffer (pH 5.0). This mixture was homogenized with a Tekmar Tissumizer. β-glucuronidase (200 μl) (107,000 units/ml glucuronidase and 470 units/ml sulfatase) was added, and the suspensions were placed in a shaking water bath (37°C, 80 rpm) for 24 hr. The tubes were then centrifuged for 15–20 min at 2000 *g*. The supernatant was decanted into a clean tube. The pellet was rinsed with 2.0 ml acetonitrile and centrifuged, and the supernatants were combined. The combined supernatants were loaded onto an activated C18 SPE column. The column was rinsed with 3.0 ml water, and this rinse was discarded. The lignans were than eluted with 5.0 ml methanol. The methanol was removed under nitrogen. The samples were then redissolved in the mobile phase for HPLC analyses.

HPLC/UV Determination of Flaxseed Lignans. Data were collected using a Waters Model 600E pump equipped with a BioRad Model AS100 refrigerated autosampler (200 μl loop) and a Waters 991 Photodiode Array Detection System set to monitor at 280 nm. Chromatographic separation was achieved with an Alltech C18 Econosil cartridge (5.0 μm; 250 × 4.6 mm i.d.) with a guard cartridge (30 × 4.6 mm i.d.). An isocratic mobile phase consisting of 70% aqueous 0.1% glacial acetic acid and 30% acetonitrile was used for the separation. The flow rate was 1.0 ml/min. The injected volume was 100 μl from 2.0 ml total volume. Coumarin was used as an internal standard.

HPLC/MS Determinations of Flaxseed Lignans. Data were collected using a Vestec Model 201 LC/MS system operated under thermospray ionization conditions. The block, tip, and controller temperatures were 300°, 207°, and 170°C, respectively. The lens conditions and temperature were optimized using the solvent ions. Chromatographic separation was achieved using a 250 × 4.6 mm i.d., 5 μm Alltech C18 Econosil cartridge. The mobile phases employed were 50 m*M* ammonium acetate in water (Solvent A) and acetonitrile (Solvent B). A gradient solvent program was used, with the initial solvent at 20% B with a linear increase to 30% B over 15 min, then held at 30% B for

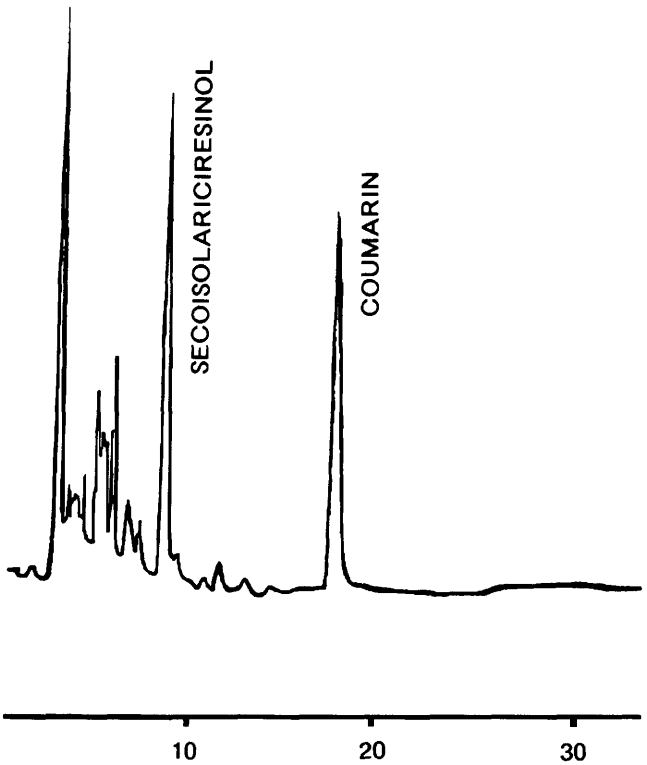

Figure 3. HPLC/UV chromatogram of flaxseed lignans.

Table I. Concentration of Lignans in Flaxseed and Flaxmeal

	Secoisolariciresinol (μg/g sample)	Matairesinol (μg/g sample)	Enterodiol (μg/g sample)	Enterolactone (μg/g sample)
Flaxseed	817	0	0	0
Flaxmeal	2260	0	0	0

20 min. This gradient was required for optimum separation of the lignans. The flow rate was 1.0 ml/min. The manual injector loop volume was 20 μl.

Extraction and Cleanup of Chaparral. Samples were homogenized to a powder (less than 20 mesh) using a Wiley mill. A 0.5-g portion was extracted with 10 ml of methanol in a shaker bath at room temperature for 16 hr. This mixture was centrifuged, and the supernatant collected. A 500-mg SPE cartridge was conditioned with 4 ml of methanol, 4 ml of water, and 4 ml of methanol under a vacuum of 10 mm Hg. When the last of the rinse solvent reached the surface of the packing material, the collection tube was removed, and the solvent discarded. A new collection tube was placed under the cartridge and 2 ml of the extract was added to the cartridge. All of the extract was allowed to exit the cartridge, the vacuum was turned off, and the tube with the eluent was removed. Prior to HPLC analysis, the eluent was filtered through a Centrex centrifugal microfilter for 10 min at 1500 rpm.

An extraction scheme (Fig. 2) for *in vitro* toxicologic evaluations was developed in order to determine which component or components might account for the observed hepatotoxicity.

HPLC/UV Characterization of Chaparrel. Data were collected using a Waters Model 600E pump equipped with a BioRad Model AS100 refrigerated autosampler (200 μl loop, maintained at 4°C) and a Waters 991 Photodiode Array Detection System set to monitor at 280 nm. Chromatographic separation was achieved with an Alltech C18 Econosil cartridge (5.0 μm; 250 × 4.6 mm i.d.) with a guard cartridge (30 × 4.6 mm i.d.). The injected volume was 50 μl. The mobile phase consisted of 70% of a 0.1% aqueous glacial acetic acid/30% acetonitrile (Solvent A) and 100% acetonitrile (Solvent B). A gradient solvent program was

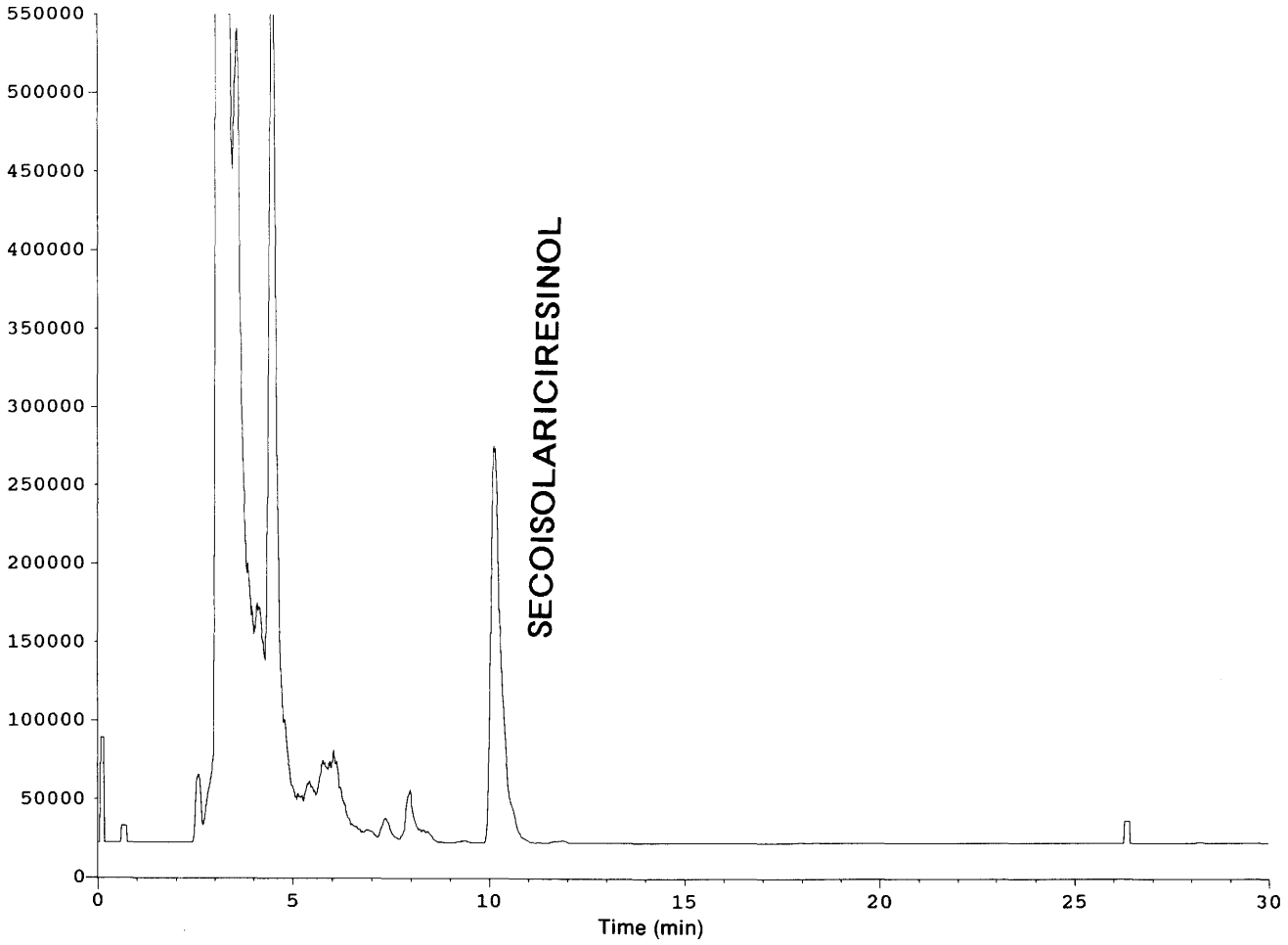

Figure 4. HPLC/MS chromatogram of flaxseed lignans.

used, with the initial solvent at 30% B with a linear increase to 80% B over 45 min at a flow of 0.8 ml/min. At 45 min, the percentage B was stepped to 10%, and the flow increased to 1.0 ml/min to flush the column. From 50 to 55 min, a linear gradient to 30% B at 0.8 ml/min was carried out. At 57 min, the system was equilibrated and ready for the next injection.

HPLC/MS Characterization of Chaparral Extract. Data were collected using a Vestec Model 201 Thermospray LC/MS system operated in the discharge mode. A Kratos Model 783 Spectroflow UV detector at 210 nm was used in line prior to the thermospray interface. Chromatographic separation was achieved on a 250 × 4.6 mm i.d., 5 μm YMC Inc. C18AQ column. The mobile phases employed were 90:10 water/acetonitrile with 0.1% trifluoroacetic acid (Solvent A) and 10:90 water/acetonitrile with 0.1% trifluoroacetic acid (Solvent B). The gradient was 25% B for 1 min, followed by a linear increase to 60% B over 50 min, then held at 60% B for 5 min. The flow rate was 0.9 ml/min.

Results

Flaxseed. Representative HPLC/UV and HPLC/MS chromatograms for the flaxseed lignans are shown in Figure 3 and 4, respectively. Table I shows results of the analyses of lignans in specific flaxseed and flaxmeal products. Matairesinol 5, which was previously reported as a flaxseed lignan, was not found in any of the specimens examined.

Chaparral. HPLC chromatograms of methanolic extracts of chaparral from the United States and from Mexico are shown in Figure 5 and 6, respectively. The HPLC chromatogram of a methanolic extract of chaparral is compared with a chromatogram of an infusion made from an identical quantity of the same chaparral in Figure 7.

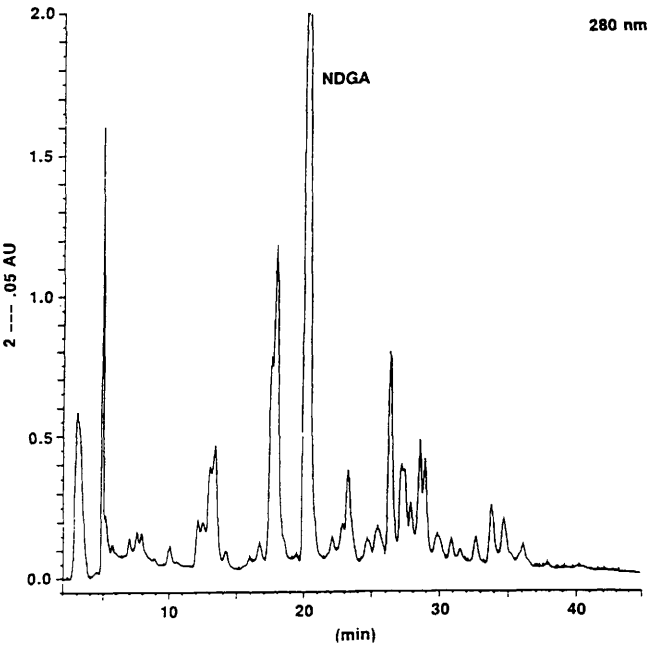

Figure 5. HPLC/UV chromatogram of methanolic extract of U.S. chaparral.

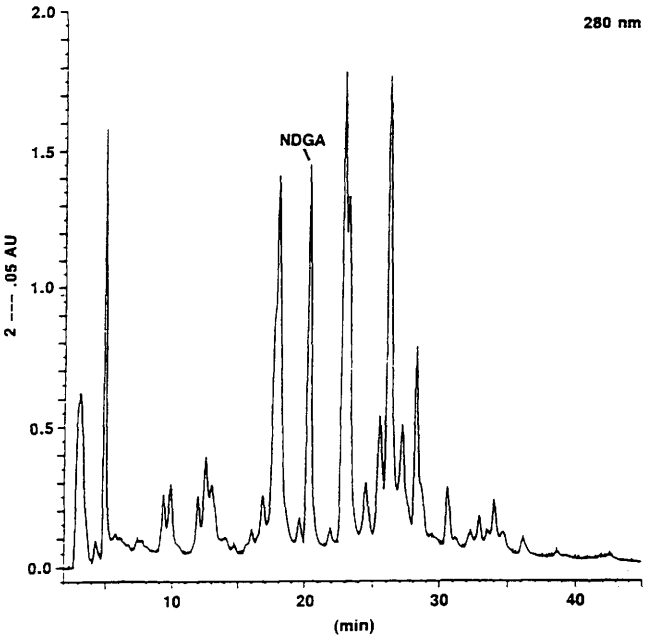

Figure 6. HPLC/UV chromatogram of methanolic extract of Mexican chaparral.

Discussion

Flaxseed. The enzymatic hydrolysis procedure released significantly larger amounts of the lignan from the seed matrix than simple solvent extraction. Other reported procedures using heat, acid, or base extraction were evaluated and found to produce significant amounts of related lignan compounds, including matairesinol. Since the mild hydrolysis conditions used in our studies did not produce these products, some of the previously reported lignan products may be artifacts of the work-up procedures (23). While no adverse effects have been reported from the consumption of flaxseed products, the long-term effects of human exposure to high levels of phytoestrogens from increased consumption of these products are not known.

Chaparral. Cases of hepatitis were associated with both Mexican and U.S. plant materials (17). In native American cultures, chaparral was traditionally used prepared as an infusion (20). Most of the chaparral dietary supplements associated with toxic hepatitis were in capsule or tablet forms. Comparisons of the lignan components in the infusion with those in the methanolic extracts indicate that exposure to many of the lignan components of chaparral would be much greater from the capsule or tablet forms than from a steeped tea. Preliminary studies with *Artemia salina* and cultured rat hepatocytes indicated that most of the toxicity was accounted for in the methanolic fraction

(24, 25). The component or components of chaparral which can cause acute toxic hepatitis in certain individuals have not yet been identified. Molecular modeling studies have indicated very close structural similarities among several of the lignan components of chaparral and enterolactone/enterodiol and diethylstilbestrol (F. S. Fry, Jr., and S. W. Page, unpublished results).

Reductive-Oxidative Activities. In addition to the estrogenic activity exhibited by some of the lignans and isoflavones, many of these compounds can act as antioxidants. The antioxidant activity of NDGA and related compounds is well known. Preliminary studies indicate that secoisolariciresinol, with soybean lipoxidase as the autoxidation initiator, is 90% as effective as butylated hydroxy anisole (BHA) (23). Antioxidants can provide protective effects by reacting with toxic reactive oxygen species. However, under certain conditions, they can become reactive oxygen species. If detoxification mechanisms are compromised, these compounds can also have toxic effects. These processes are outlined in Figure 8. Preliminary electron paramagnetic resonance and chemiluminescence studies have indicated the presence of a number of reactive free radical species in chaparral extracts (P. A. Yasei and G. C. Yang, unpublished results). The purported toxic metabolite from NDGA is the ortho-quinone 6 (Fig. 1) (21), which is also found in chaparral. Recent studies have shown that NDGA can act as a prooxidant *in vitro* (K. Ellwood, unpublished results). Phytoestrogens can be hypothesized to cause hepatotoxicity through mechanisms similar to those of hormonal estrogens. However, the potential for toxicity resulting from the formation of reactive oxygen species in reductive-oxidative processes cannot be ruled out.

Dietary exposures to phytoestrogens have traditionally been through food products. Substituting extracts of these compounds for the food itself not only raises questions regarding the safety of consuming such high concentrations of phytoestrogens, but may remove other beneficial components, such as fiber, from the diet as well.

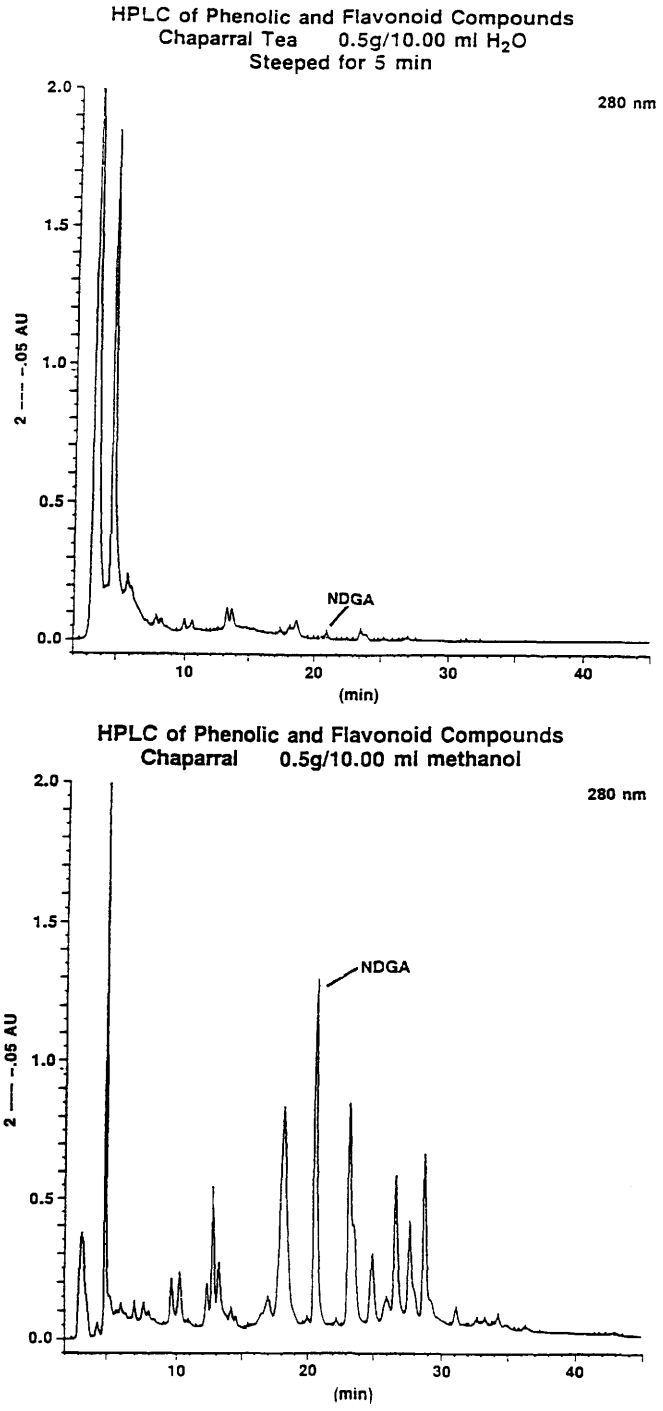

Figure 7. Comparison of HPLC/UV chromatograms of methanolic extract and infusion from chaparral.

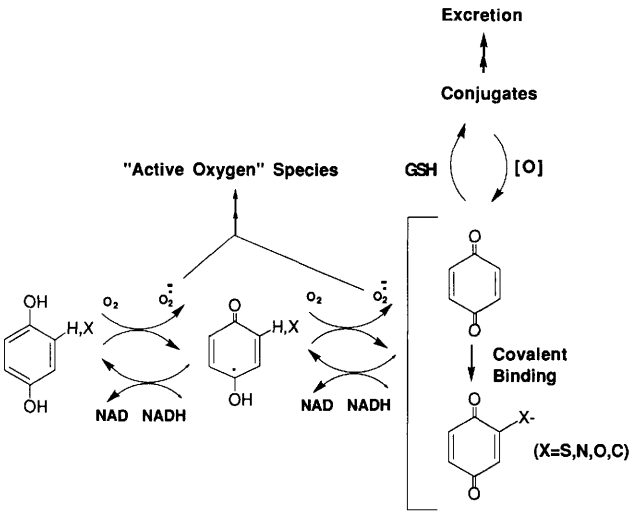

Figure 8. Biological reductive-oxidative cycling process schematic.

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