High-Performance Liquid Chromatographic Analysis of the Major Sweet Principle of Lo Han Kuo Fruits

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An analytical high-performance liquid chromatographic (HPLC) procedure has been developed for the triterpene glycoside mogroside V, the principal intensely sweet constituent of Lo Han Kuo fruits [*Thladiantha grosvenori* (Swingle) C. Jeffrey]. Concentration levels of this compound in whole, dried fruits, originating from the People's Republic of China, were in the range 0.81–1.29% w/w. Highest mogroside V levels occurred in the endocarp of the fruits rather than in the peel or in the seeds.

Thladiantha grosvenori (Swingle) C. Jeffrey (formerly *Momordica grosvenori* Swingle) is a vine which is cultivated by the Miao-tze people of Kwangsi Province in the People's Republic of China (Swingle, 1941; Jeffrey, 1979). The fruits of this plant, known to the Chinese as "Lo Han Kuo", are intensely sweet, and also find folklore use in the treatment of colds, sore throats, and stomach and intestinal troubles (Swingle, 1941). Lee (1975) purified a sweet constituent from *T. grosvenori* fruits and estimated its sweetness as being about 150 times that of sucrose. Recently, Takemoto et al. (1983a–c) have elucidated the structures of three sweet triterpene glycoside constituents of *T. grosvenori*, namely, mogroside V, the sweetest and most abundant such compound, and mogrosides IV and VI. Mogroside V has been assigned as the 3-*O*-[β-D-glucopyranosyl (1→6)-β-D-glucopyranoside]-24-*O*-[[β-D-glucopyranosyl (1→2)]-[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside] of the aglycone mogrol [10α-cucurbit-5-ene-3β,11α,24(*R*),25-tetraol] (Takemoto et al., 1983b,c).

We have studied mogroside V in the course of our research program on intensely sweet plant constituents with potential use as noncariogenic and noncaloric sucrose substitutes and wish to report here a method for the HPLC assay of this compound. Concentration levels of mogroside V in various parts [whole, peel (exocarp and mesocarp), endocarp, seeds] of Lo Han Kuo fruits have been determined. No previous analytical methods for any of the *T. grosvenori* fruit triterpene glycoside sweet constituents appear to have been published, although procedures have been reported for their isolation and purification (Takemoto et al., 1977, 1978; Nippon Chemical Research K.K., 1981a,b) and formulation in an aqueous sweetener mixture (Kamo et al., 1979).

EXPERIMENTAL PROCEDURES

Plant Material. Fruits of *Thladiantha grosvenori* (Swingle) C. Jeffrey (Cucurbitaceae), purchased in both Hong Kong and Chicago, were identified by D.D.S. Both samples were obtained from plants grown in the People's Republic of China.

Isolation of Reference Mogroside V. Gram quantities of mogroside V were generated in the following manner. Powdered *T. grosvenori* fruits (2.75 kg) were percolated with methanol–water (4:1, 20 L), and the percolate was evaporated under reduced pressure at 55 °C to yield a brown gum (800 g). A portion (400 g) was adsorbed to silica gel (Merck, Darmstadt, W. Germany) (1 kg, 0.063–0.2 mm), and loaded into a glass column containing an additional 2.5 kg of silica gel. Separation was carried out by elution with mixtures of chloroform–methanol–water of increasing polarity. Since chloroform has been listed as a carcinogen by the Environmental Protection Agency, chromatographic separations using this solvent were carried out in walk-in fume hood. Elution with chloroform–methanol–water (30:8:1) afforded a series of fractions containing mogroside V, that were combined and dried to produce 22 g of a residue. Aliquots (2 g × 5) of this residue were purified in batch-wise fashion, by dissolution in water (10 mL) and passage through an ion-exchange column (Amberlite IRA-410 C.P., strongly basic, 200 g) (Mallinckrodt Chemical Works, St. Louis, MO) by elution with 250 mL of water. On combining these eluates, partially purified mogroside V (5 g) was thereby obtained free from polar colored contaminants. Final purification of a portion of this material (3.6 g) was effected by isocratic elution over a gravity column containing silica gel (200 g) with chloroform–methanol–water (45:12:2). Altogether, 40 fractions (500 mL each) were collected, and when fractions 28 through 34 were pooled and dried, 2.3 g of pure mogroside V was obtained as a powder: mp 194–196 °C; $[\alpha]^{25}_D$ −16.3° (*c* 1.6, H_2O) [lit. mp 197–201 °C; $[\alpha]_D$ −9.4° (*c* 0.5, H_2O) (Takemoto et al., 1983a)]. Purity of the isolate was indicated by its appearance as a single zone after TLC on silica gel GHLF (Analtech, Inc., Newark, DE), using as solvents chloroform–methanol–water (10:10:1) (R_f 0.31), 1-butanol–acetic acid–water (4:1:1) (R_f 0.23), and 1-butanol–ethyl acetate–2-propanol–water (40:20:14:7) (R_f 0.15). Visualization was effected after TLC with 60% w/v sulfuric acid (110 °C, 10 min), whereupon mogroside V appeared as a purple spot in daylight. The molecular weight of this isolate was determined as 1286 dalton by fast-atom bombardment mass spectrometry, a value which is consistent with the elemental composition ($C_{60}H_{102}O_{29}$) of anhydrous mogroside V (Takemoto et al., 1983a). Confirmation of this identification was made by hydrolysis of 200 mg of mogroside V, dissolved in 200 mL of citric acid–disodium hydrogen phosphate buffer at pH 4, with 20 mL of commercial pectinase (Corning Biosystems, Corning, N.Y.) at 37 °C for 140 h. On extraction into chloroform, workup, and crystallization from methanol, 47 mg of mogrol was obtained: mp 120-122 °C; $[\alpha]^{25}_D$ +65.0° (*c* 0.76, $CHCl_3$). Anal. Found: C, 72.78; H, 10.48. $C_{30}H_{52}O_4 \cdot H_2O$ requires: C, 72.87; H, 10.93%. [Lit. mp 118–119 °C; $[\alpha]^{17}_D$ +70.0° (MeOH), $C_{30}H_{52}O_4$ (Takemoto et al., 1976)]. This hydrolyzate exhibited IR, ^1H NMR, and MS data that were closely comparable to literature data for mogrol (Takemoto et al., 1983b).

HPLC Analysis. HPLC analysis was conducted with a Model 324 gradient liquid chromatograph (Beckman Instruments, Berkeley, CA) equipped with a 421 micro-

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processor controller, Model 100A and 110A pumps, with an added stop-flow valve, and a Model 210 sample injector. A Model LC-85 variable wavelength UV spectrometer with LC-75 Autocontrol (Perkin-Elmer, Norwalk, CT) and an Altex C-R1A recorder (Shimadzu Seisakusho, Kyoto, Japan) was also employed. Solvents used in this study were of HPLC grade and were degassed before use by filtration through an Ultipor NR 0.2 μm filter (Rainin Instrument Co., Woburn, MA) by using a microfiltering apparatus (Sibata, Tokyo, Japan).

The following operating conditions were used for HPLC analysis: column, Zorbax NH_2, 25 cm × 4.6 mm i.d.; particle size, 7 μm (DuPont, Wilmington, DE); eluting solvent, acetonitrile–water (3:1, pH 5 with H_3PO_4); flow-rate, 2 mL/min; wavelength of UV detector, 210 nm; pressure, 1200 psi; sensitivity setting, 0.04 a.u.f.s.; recorder chart speed, 10 mm/min; temperature, ambient.

A Beer's law curve was obtained from triplicate injections of pure mogroside V, dissolved in acetonitrile–water (1:1), at levels of 2.0, 5.0, 10.0, 15.0, and 20.0 μg per injection. Peak height measurement was used and a linear regression equation was established. Mogroside V that eluted from the HPLC column was established as being pure after TLC analysis in the three solvent systems described previously.

Extraction of Plant Material for HPLC. Dried, powdered fruits of *T. grosvenori* (1.0 g) were suspended in water (20 mL), and heated at 45 °C for 4 h. The soluble portion was filtered, the marc washed with water (10 mL), and the filtrates (30 mL) were combined. Further extraction of the marc with water was shown by analytical TLC to provide no additional mogroside V. TLC was carried out according to previously mentioned protocols. When chloroform–methanol–water (10:10:1) was used as solvent, a detection limit of 0.25 μg of mogroside V was possible. The combined aqueous extract was washed with 3 × 30 mL chloroform, and the chloroform layers backwashed with water (30 mL). The total aqueous extraction (60 mL) was then evaporated under reduced pressure at 45 °C to a 10-mL volume. A 2-mL aliquot was then passed into a previously washed precolumn (7 cm × 5 mm, i.d.), packed with C_{18} Phase Bonded Hi-Flosil (Applied Science Laboratories, State College, PA), and polar impurities were removed by elution with 2 mL of water. An extract containing mogroside V was eluted from the precolumn with 3 mL of acetonitrile-water (1:1) and was filtered for HPLC injection.

Triplicate 10-μL injections of each sample extract obtained for *T. grosvenori* whole fruit, peel, endocarp (pulp), and seeds were made, and peak heights were measured. Data were compiled for two *T. grosvenori* samples that were available to this investigation.

Recovery Experiments. Standard mogroside V (7.0 mg) was added to 1.0 g of the exhausted marc from *T. grosvenori* fruits by dissolution in 5 mL of water and evaporation of solvent under reduced pressure at 45 °C. The spiked marc was taken through the entire extraction procedure, the triplicate 10-μL portions of the resulting extract were injected into the HPLC column to determine the percent recovery of mogroside V by the presently described method. The recovery experiment was performed a total of three times.

RESULTS AND DISCUSSION

In Figure 1, an HPLC chromatogram of underivatized mogroside V is shown. A retention time of 6.9 min was obtained for this compound under the chromatographic conditions used. A linear calibration curve ($y = 0.36 + 0.36x$, correlation coefficient $r = 0.99978$) for peak height

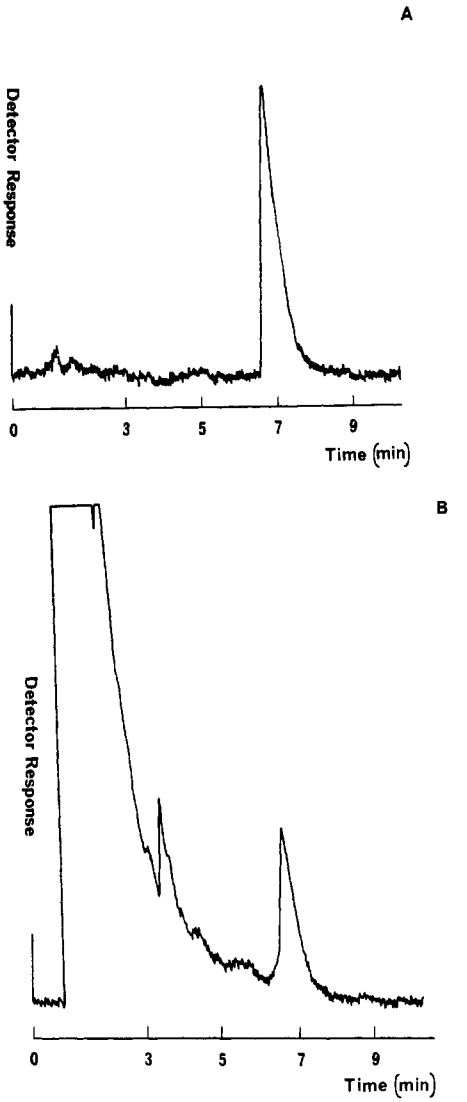

Figure 1. HPLC chromatograms of (A) pure mogroside V and (B) mogroside V as a constituent of an aqueous extract of *Thladiantha grosvenori* whole fruits, purchased in Hong Kong. For operating conditions, see text.

against quantity injected was obtained for mogroside V over the range 2–20 μg. The limit of detection at the 0.04 a.u.f.s. detector setting was 2 μg, as determined by injecting decreasing volumes of a standard 1 mg/mL solution of mogroside V, until the signal-to-noise ratio was about 3:1.

Although we found the isolation of mogroside V from *T. grosvenori* fruits in completely pure form to require considerable attention, it may be seen from Figure 1 that adequate resolution of this compound from other polar constituents was achieved by using the HPLC methodology described here, which involved comparatively little sample cleanup. After controlled experiments to determine the effectiveness of the extraction procedure, the recovery of a standard amount of mogroside V was determined as 82%. The concentration levels of mogroside V in two samples of authenticated *T. grosvenori* fruits available to us were sample 1 (purchased in Hong Kong), whole fruits, 1.29% w/w, peel, 1.26% w/w, endocarp, 1.56% w/w, seeds, 0.24% w/w and sample 2 (purchased in Chicago), whole fruits, 0.80% w/w, peel, 0.81% w/w, endocarp, 1.37% w/w, seeds, 0.51% w/w. Therefore, in both cases, the presence of this compound was observed at the highest concentration levels in the endocarp, when compared with other parts of the fruit. Also, we have been able to confirm the statement of Lee (1975) that the rind (peel) as well as the

pulp of Lo Han Kuo fruits contains a sweet principle.

Takemoto and co-workers (1983a) have reported the isolation of two other sweet triterpene glycoside constituents of *T. grosvenori* fruits in addition to mogroside V, namely, mogrosides IV and VI, which have molecular weights of 1124 and 1448 dalton, respectively. While mogroside VI was found to occur in very small quantities, mogroside IV was obtained in a yield almost as high as mogroside V (Takemoto et al., 1983a). During our work on *T. grosvenori* fruits, we have not observed by analytical TLC or HPLC either mogroside IV or any sweet triterpene glycoside less polar than mogroside V. However, since mogrosides IV and V are clearly separable by reversed-phase HPLC, in which a 25 cm × 4 mm Nucleocil C_{18} column was eluted with 42% ethanol (Takemoto et al., 1983a), it is not expected that significant amounts of mogroside IV in *T. grosvenori* samples will affect the validity of the present HPLC assay for mogroside V.

In other work performed in this laboratory on mogroside V, this compound has been shown to be nonmutagenic and to produce no mortality in acute toxicity experiments on mice at doses up to 2 g/kg body weight and to exhibit an equivalent molar sweetness intensity to the *ent*-kaurene glycoside, stevioside, when tested against a standard sucrose solution by a human taste panel (Kinghorn et al., 1985, unpublished results). These attributes, coupled with the high mogroside V levels in dried *T. grosvenori* fruits that are reported here, could serve to stimulate further study as to the suitability of extracts of the fruit of this plant and its constituents as alternative high-intensity sweeteners. It has been suggested already that *T. grosvenori* may be a suitable species for introduction into the United States (Swingle, 1941).

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Changes in the Polypeptide Composition of Peanut (*Arachis hypogaea* L.) Seed during Oil Roasting

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Free amino acids and sugars, released during roasting, are known to be major flavor precursors in roasted peanuts and they give rise to pyrazine compounds via Millard sugar–amine type reaction. In order to identify the protein/polypeptide source of these amino acids, peanut (*Arachis hypogaea L.*) seeds of Virginia, Runner, and Spanish market types were roasted in peanut oil for 0–12 min and protein was extracted and examined by gel filtration and gel electrophoresis. Gel filtration studies indicated that roasting caused a decrease in the methionine-rich proteins and aggregation of arachin proteins. Gel electrophoresis studies also showed a decrease in the methionine-rich protein and their polypeptides. In addition, a polypeptide with a molecular weight of 70 000 also gradually decreased during roasting. In contrast, the protein and polypeptide composition of arachin remained relatively unchanged during the 12-min roasting period. It is suggested that the polypeptide/s of methionine-rich protein may be involved in the formation of pyrazine compounds.

The sugar-amine nonenzymatic browning reaction has been shown to be involved in roasted peanut flavor formation (Pickett and Holley, 1952; Newell et al., 1967; Mason et al., 1969). Free amino acids and free sugars which are released during roasting are known to be major flavor precursors in roasted peanuts (Newell et al., 1967) and they give rise to pyrazine compounds via Millard sugar–amine type reaction (Mason et al., 1967; Johnson et al., 1971; Walradt et al., 1971). Koehler et al. (1969) investigated the pathway for the formation of alkylated

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