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Note

Improved high-performance liquid chromatographic method for the analysis of ginsenosides in Panax Ginseng extracts and products

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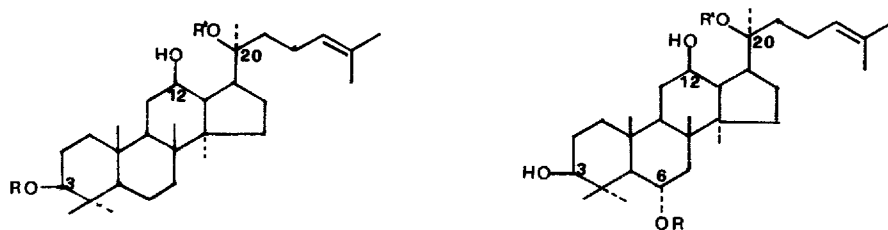
Ginseng, the roots of Panax Ginseng C. A. Meyer (Araliaceae), has long been used as a folk medicine and many studies suggest that its pharmacological effects such as an antistress or rehabilitative action[1–3] are due to ginsenosides (Fig. 1).

As part of our interest[4,5] in high-performance liquid chromatography (HPLC) of medicinal plant extracts, we have evaluated the HPLC procedures currently available for ginsenosides. It is evident that (a) the Extrelut method[6,7] of sample preparation is time-consuming and (b) the purification of ginsenosides from complex pharmaceutical products is unsatisfactory.



20-S-Protopanaxadiol(R=R'=H)

Ginsenoside

- R_{b1} R=D-Glc(β1-2)D-Glc; R'=D-Glc(β1-6)D-Glc
- R_{b2} R=D-Glc(β1-2)D-Glc; R'=L-Ara(pyr)(α1-6)D-Glc
- R_c R=D-Glc(β1-2)D-Glc; R'=L-Ara(fur)(α1-6)D-Glc
- R_d R=D-Glc(β1-2)D-Glc; R'=D-Glc

20-S-Protopanaxatriol(R=R'=H)

Ginsenoside

- R_e R=L-Rha(α1-2)D-Glc; R'=D-Glc
- R_f R=D-Glc(β1-2)D-Glc; R'=H
- R_{g1} R=D-Glc; R'=D-Glc
- R_{g2} R=L-Rha(α1-2)D-Glc; R'=H

Fig. 1. Structures of ginsenosides referred to in the text.

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The method described here involves a simple extraction and clean-up of ginsenosides in Panax Ginseng extracts and products using Sep-Pak C_{18} cartridges prior to an isocratic elution on a RP-18 Spheri 5 column.

EXPERIMENTAL

Materials

Acetonitrile and methanol were of HPLC grade (Chromasolv; Riedel-de Haën, Hannover, F.R.G.). Water was distilled in glass and then passed through a 0.45-μm membrane filter (Type HA, Millipore). Petroleum ether, b.p. 40–60°C (analytical reagent grade), was purchased from C. Erba (Milan, Italy).

The ginsenosides were kindly provided by Pharmaton S.A. (Lugano-Bioggio, Switzerland); the Ginseng extracts were obtained from different commercial sources. Sep-Pak (C_{18}) cartridges (Waters Assoc.) were used for HPLC sample preparation.

Sample preparation

Ginseng extracts. A 65-mg amount of dry extract was dissolved in 2 ml of water and applied to a Sep-Pak C_{18} cartridge pre-washed with methanol (2 ml) and water (5 ml). After washing the column with water (10 ml) and 30% methanol (15 ml), the ginsenosides were eluted with methanol (5 ml). The solvent was evaporated to dryness under vacuum and the residue was dissolved in 1 ml of the eluent.

Capsules. An accurately weighed amount from a capsule (Geriatric and Ginsala capsules*) corresponding to 50 mg of Ginseng extract was introduced in a centrifuge-tube and extracted with petroleum ether (3 × 5 ml). The resulting powder was suspended in water (2 ml), centrifuged at 5300 *g* for 5 min and applied to a Sep-Pak C_{18} cartridge. Then the sample was processed as described for Ginseng extracts.

Fluids. A 2-ml volume of fluid (Ginsana syrup, elixir, tonic*) was diluted to 5 ml in water and directly applied to a Sep-Pak C_{18} cartridge. Then the sample was treated as described for Ginseng extracts.

Calibration graphs

Several aliquots of standard stock solutions (1 mg/ml) of ginsenosides R_{b2}, R_{b1}, R_c and R_d were diluted in the eluent to obtain reference solutions containing 2, 4, 6, 8 and 10 μg per 10 μl. These solutions were injected and the corresponding peak areas were integrated against the masses of ginsenoside injected.

Recovery

Ginseng products prepared using a standardized Ginseng extract in the range of 25–50 mg/g for the capsules and 8–10 mg/ml for the fluids were subjected to the described procedure and the peak areas were compared to those obtained from the corresponding standardized extract processed analogously.

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* Geriatric and Ginsana capsules, Ginsana syrup, elixir and tonic are preparations of Pharmaton S.A. and G.P.L. (Lugano-Bioggio, Switzerland) containing Ginseng extract, accessory agents and, for Geriatric, dimethylaminoethanol, vitamins and mineral salts.

Chromatographic conditions

HPLC was performed on a Waters (Milford, MA, U.S.A.) liquid chromatograph equipped with a Model 590 pump, a Model 510 pump, a Model 680 automated gradient controller, a Model U6K universal injector, a Model Lambda-max 480 ultraviolet detector and a Model 730 data module. The columns were μBondapak C_{18} (30 cm × 3.9 mm I.D.) (Waters), and PRP-1 cartridge (100 mm × 4.6 mm, 10 μm) and RP-18 Spheri 5 cartridge (100 mm × 4.6 mm, 5 μm), both from Kontron (Milan, Italy). Suitable pre-columns (Waters Part No. 84550 and Kontron RP-18, 5 μm, OD-GU) were used to protect the column.

The separations were obtained isocratically with acetonitrile–water (28.5:71.5, v/v) or by gradient elution, using the eluents 15% acetonitrile (A) and 80% acetonitrile (B) according to the following profile: 0–12 min, 100% A; 12–30 min, 72% A, 28% B (curve 5); 30–40 min, 72A, 28% B (curve 6). The flow-rate was 3 ml/min and the peaks were monitored at 203 nm (0.05 a.u.f.s.).

RESULTS AND DISCUSSION

A typical chromatogram of ginsenosides reference compounds on RP-18 Spheri 5 (Fig. 2) shows symmetrical, well resolved peaks, whose retention times remain unchanged with an accurately identical eluent. In comparison, other C_{18}-bonded phases (μBondapak and PRP-1) gave broad, poorly resolved peaks.

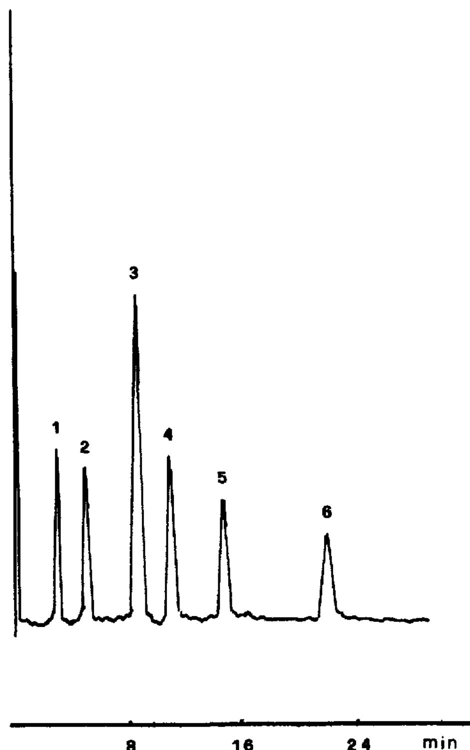

Fig. 2. Separation of a mixture of standard ginsenosides on a RP-18 Spheri 5 column. See text for operating conditions. Peaks: 1 = R_f; 2 = R_{g2}; 3 = R_{b1}; 4 = R_c; 5 = R_{b2}; 6 = R_d.

Figs. 3 and 4 show the chromatograms of samples obtained from Ginseng extracts and products according to the Extrelut method[6] and our procedure. The preliminary purification with the C_{18} cartridge strongly reduces the front also in samples arising from complex mixtures and provides a better baseline separation between the ginsenosides and the background. Moreover, the proposed clean-up method requires significantly less time in comparison to the Extrelut technique (2 *vs.* 12 h). It should be also noted that neither evaporation of large volume of organic solvent nor washing of the analytical column every three injections is required.

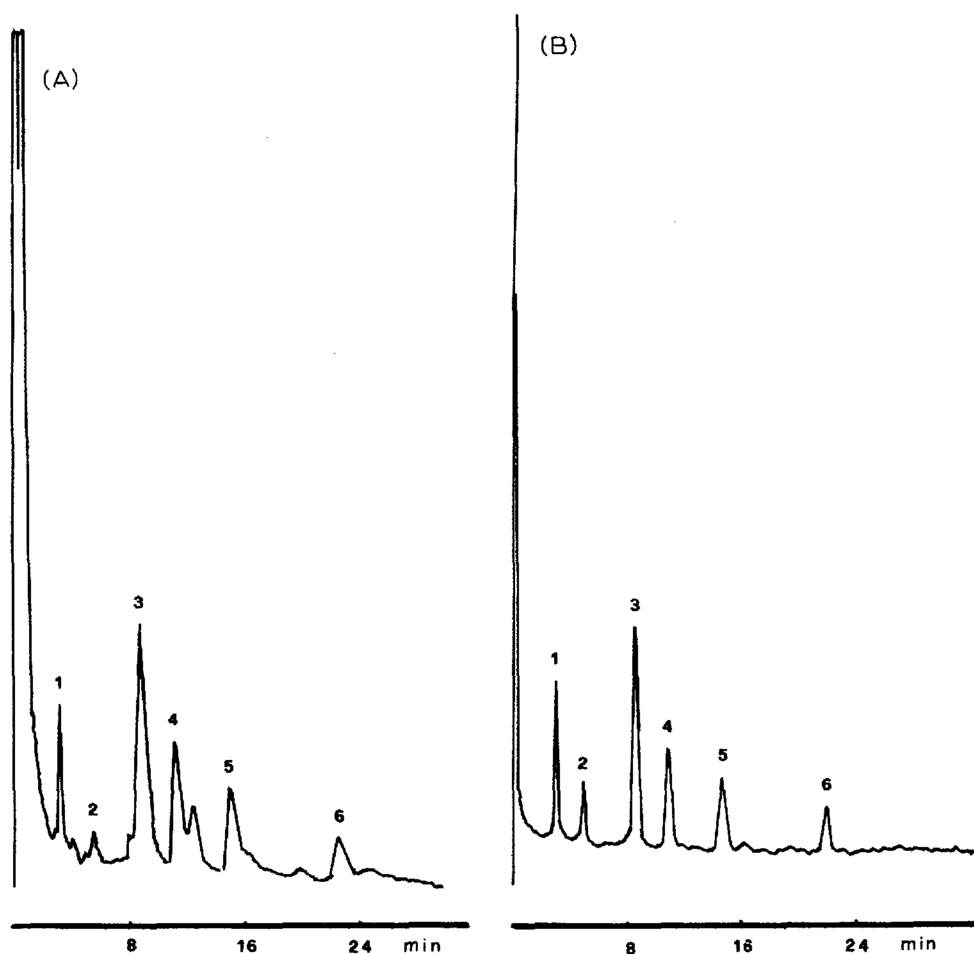

Fig. 3.

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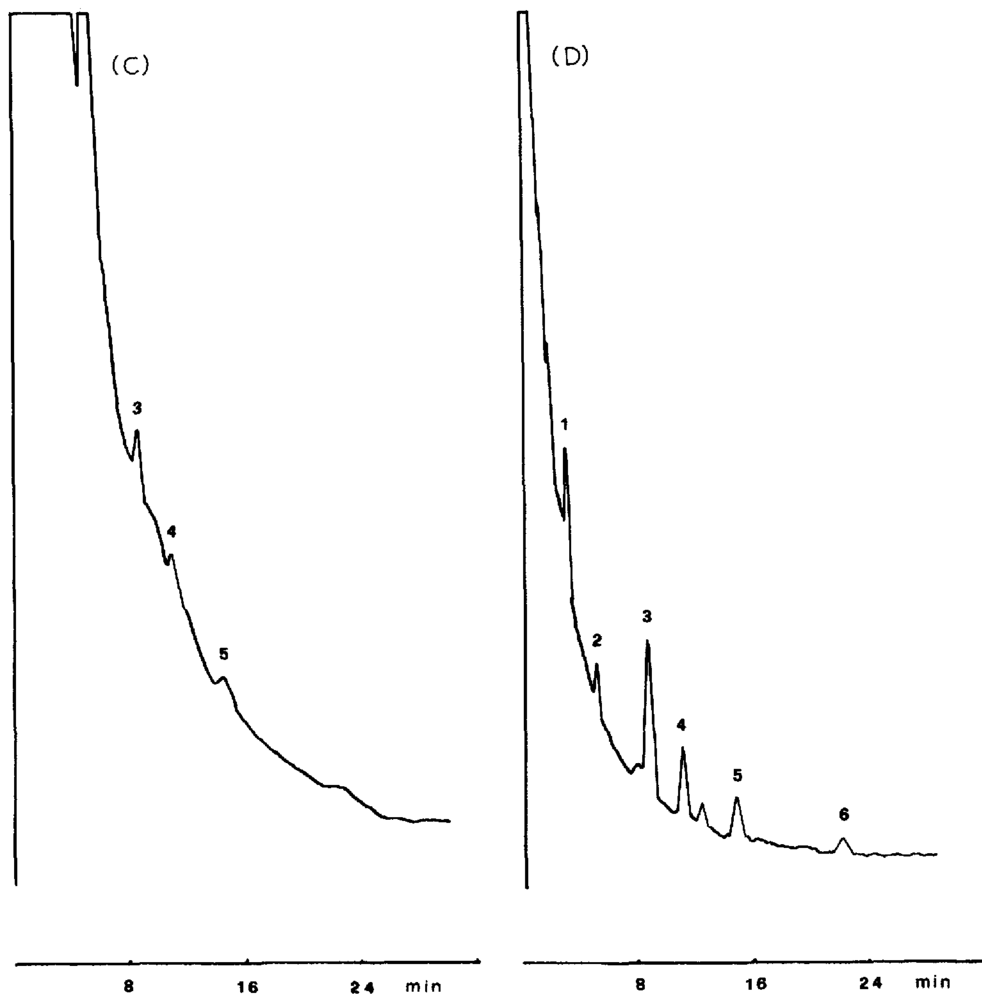

Fig. 3. HPLC of a Ginseng extract (A, Extrelut; B, Sep-Pak C_{18}) and Geriatric capsules (C, Extrelut; D, Sep-Pak C_{18}). Peaks as in Fig. 2.

Solvents with different proportions of acetonitrile and water were tried and a 28.5:71.5 mixture was found to give the best and most rapid separation. With this eluent, rectilinear responses between peak areas and injected amounts were obtained from three replicate injections of R_{b1}, R_c, R_{b2} and R_d standard solutions in the range of 2–10 μg, as indicated by the following equations

$y = 2.80\,x - 0.02 \quad r = 0.998\ (R_{b1})$
$y = 3.61\,x - 0.05 \quad r = 0.997\ (R_c)$
$y = 3.57\,x + 0.03 \quad r = 1.000\ (R_{b2})$
$y = 3.82\,x + 0.04 \quad r = 0.999\ (R_d)$

where y represents the peak area and x the amount injected (μg).

The mean recovery of known amounts of R_{b1}, R_c, R_{b2} and R_d in various Ginseng products was 100% (S.D. = 0.99%; C.V. 1.99%) in five different experiments and the limit of detection of each ginsenoside was 500 ng.

Owing to the suitability of the isocratic elution mode for a rapid quantification of the main ginsenosides, the gradient approach, although investigated as shown in Fig. 5, was not applied as a routine method.

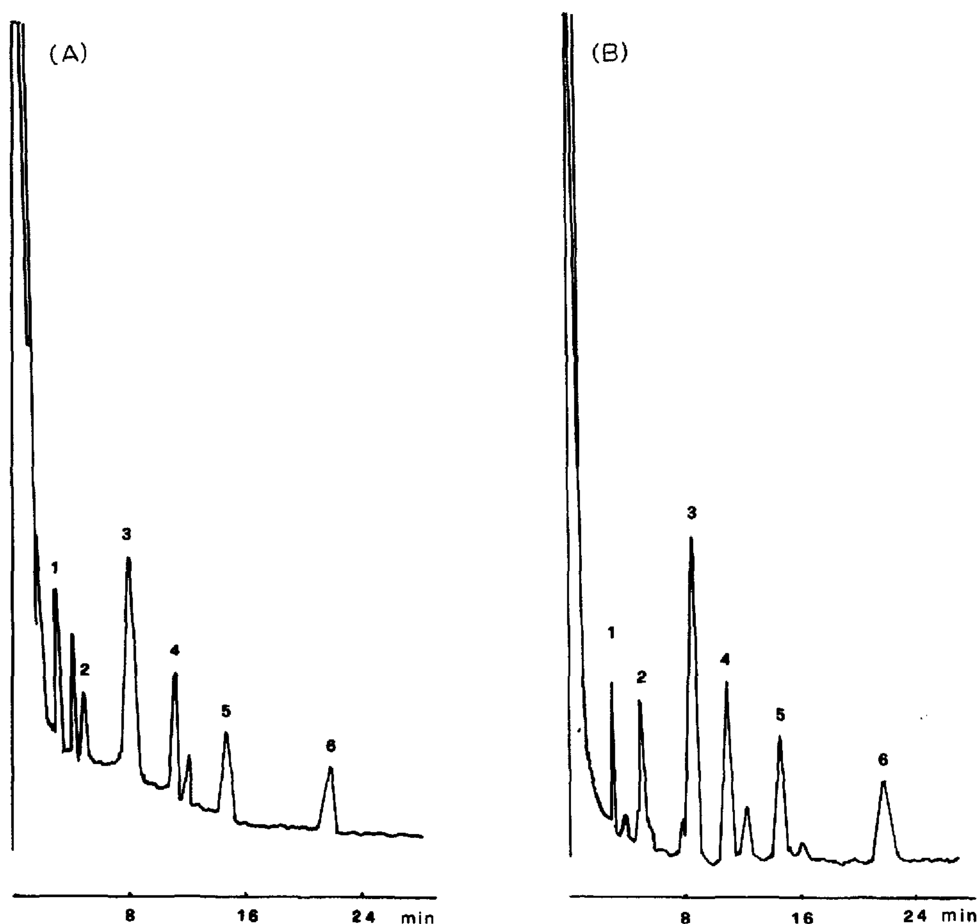

Fig. 4.

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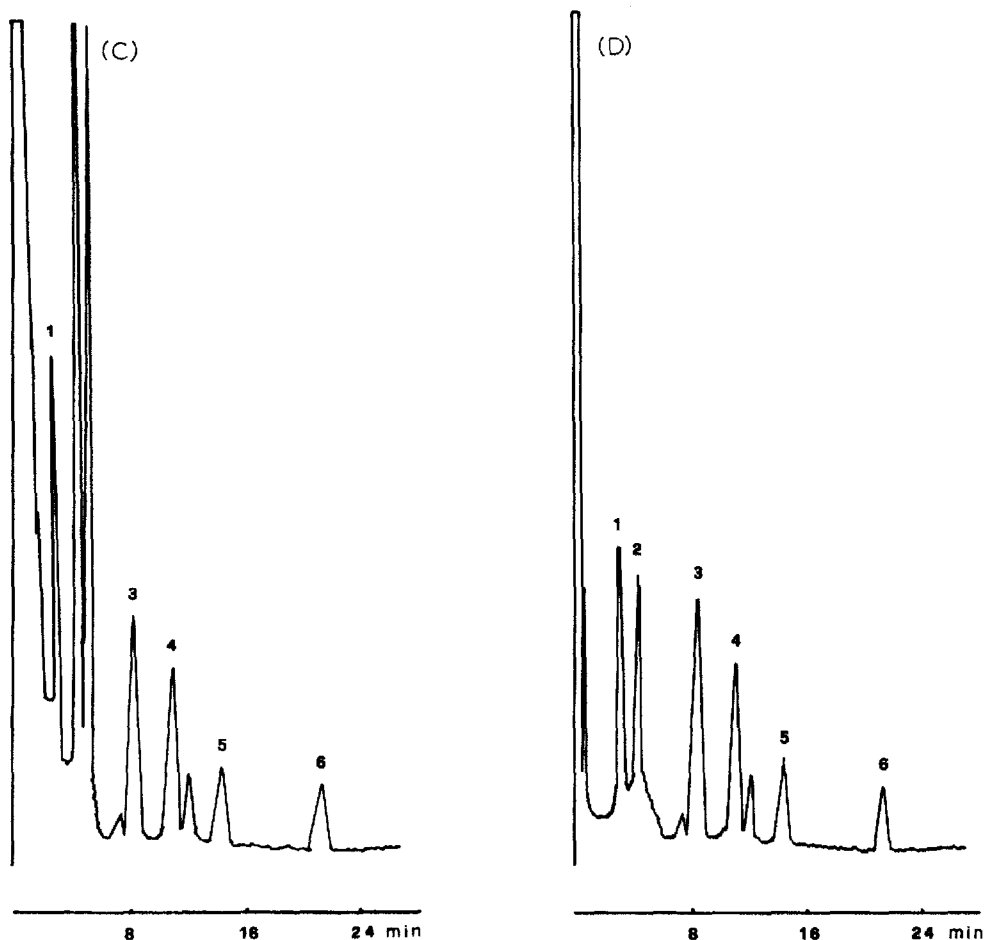

Fig. 4. HPLC of different Ginseng products purified with Sep-Pak C_{18}: (A) Ginsana capsules; (B) Ginsana syrup; (C) Ginsana tonic; (D) Ginsana elixir. Peaks as in Fig. 2.

In conclusion, the proposed extraction procedure coupled with isocratic RPLC on RP-18 Spheri 5 allows a rapid, clean and accurate analysis of Ginseng extracts and products without loss of column efficiency over long periods of use. This new method will be also useful for studying the stability of Ginseng extracts in complex matrices under different pH and temperature conditions[8].

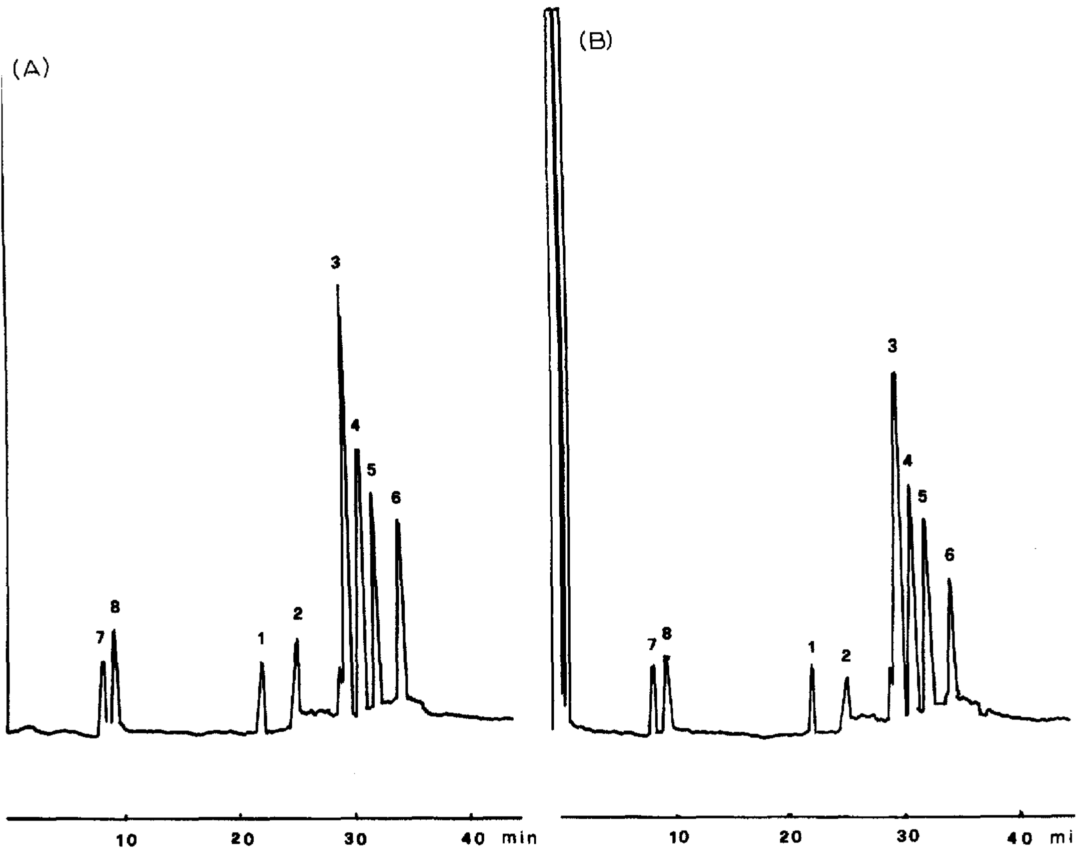

Fig. 5. HPLC with gradient elution of a Ginseng extract (A) and Geriatric capsules (B) on RP-18 Spheri 5. See text for eluents and profile. Peaks as in Fig. 2 except: 7 = R_{g1}; 8 = R_e.

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