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GAS–LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF GINSENOSIDES IN *PANAX GINSENG*

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SUMMARY

The most important saponins contained in *Panax ginseng* were determined by gas–liquid chromatography after trimethylsilylation. Results are reported for the analysis of ginseng roots, leaves and flowers of different origin.

INTRODUCTION

Ginseng was in ancient times claimed to be a divine herb for retaining youth and prolonging life, and detailed statements have appeared in many oriental materia medica for at least 2000 years.

The pharmacological effect of ginseng roots has been tested in recent years by various techniques, and research being concentrated on the saponins, which are responsible for the anti-stress and anti-fatigue activity. In accordance with the literature, we have been able to verify that they induce an action at the adrenal cortex level, analogous to certain steroids but through a different mechanism.

The most abundant saponins contained in *Panax ginseng* roots are divided into two groups: ginsenoside Rd, Rc, Rb_1 and Rb_2, which possess 20 (*S*)-protopanaxadiol as aglycone, and ginsenoside Rg_1, Rg_2, Rf and Re, which, possess 20 (*S*)-protopanaxatriol as aglycone. Their structures (Fig. 1) have been determined by Shibata and co-workers[1–3]. In addition to these compounds other minor saponins have been isolated from *Panax ginseng*, such as ginsenoside Rh_1[4] (in a yield of 0.0015%) and ginsenoside Rb_3, possessing a protopanaxatriol unit. In addition, the ginsenoside Ro, containing an oleanolic acid as aglycone, has been identified[2]. From the leaves and flowers of *Panax ginseng*, in addition to the saponins Rd, Re, Rg_1, Rc, Rb_2 and Rb_1, three new saponins, F_1, F_2 and F_3, have been isolated[5].

As the saponins constitute one of the active principles of the drug, the first problem to be faced to day for the therapeutic utilization of ginseng extracts field is the establishment of the relative abundance of the constituents.

Recently, the qualitative and quantiative analysis of crude ginseng drugs has been investigated. Such methods are based on colorimetric procedures or UV absorption of derivatized panaxatriol and panaxadiol obtained on hydrolysis of the saponins[6]. In addition, some workers have reported the determination of panaxadiol

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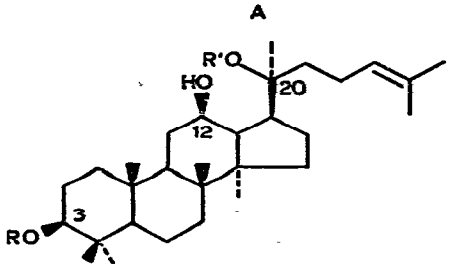
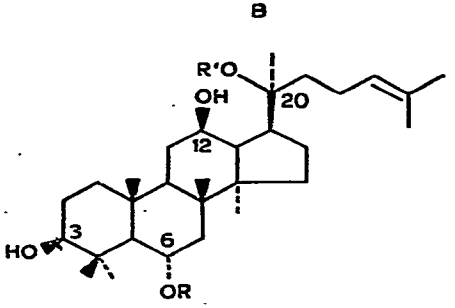


	R	R'
20(*S*)-Protopanaxadiol	H	H
Rc	glc^{2}-^{1}glc	glc^{6}-^{1}ara (fu)
Rd	glc^{2}-^{1}glc	glc
Rb_1	glc^{2}-^{1}glc	glc^{6}-^{1}glc
Rb_2	glc^{2}-^{1}glc	glc^{6}-^{1}ara (py)
Rb_3	glc^{2}-^{1}glc	glc^{6}-xyl (py)
F_2	glc	glc



	R	R'
20(*S*)-Protopanaxatriol	H	H
Rg_1	glc	glc
Rg_2	glc^{2}-^{1}rham	H
Rf	glc^{2}-^{1}glc	H
Re	glc^{2}-^{1}rham	glc
Rh_1	glc	H
F_1	H	glc
F_3	H	glc^{6}-^{1}ara (py)

Fig. 1. Ginseng saponins. Glc = β-D-glucopyranosyl; rham = α-L-rhamnopyranosyl; xyl = β-D-xylopyranosyl; ara (py) = α-L-arabinopyranosyl; ara (fu) = α-L-arabinofuranosyl.

and panaxatriol as trimethylsilyl ethers by gas–liquid chromatography (GLC)[7,8]. However, these procedures are not able to give the percentage of certain ginsenosides. More recently, a high-performance liquid chromatographic (HPLC) method was reported by Sticher and Soldati[9] for separating and determining ginsenosides Rg_1, Rg_2, Rf, Re and Rd in ginseng extracts.

In this paper, we report a GLC procedure for the determination of the most abundant saponins of ginseng. In addition to the assay of the ginsenosides in different drugs, the aim of this work was also to define the instrumental parameters and the analytical procedures necessary to avoid decomposition of the trimethylsilyl-saponins, which can occur on active surfaces at the high temperatures that are necessary to ensure reasonable elution times.

In previous work[10] we studied the qualitative behaviour of ginseng saponins after derivatization with the silylating reagents, and the components were identified by GLC–mass spectrometry (MS). The mass spectra of the saponins, as the TMS-ethers, have been reported elsewhere after GLC or by direct inlet system (DIS) introduction[10–12]. In a preliminary study[11] we suggested the determination of each saponin by GLC, but only the calibration graph for ginsenoside Rg_1 (for which the problems of decomposition are less critical) was reported and no quantitative evaluation of the high-molecular-weight saponins was considered.

Here, in addition to the ginsenoside contents of different ginseng components

and varieties, we report the strictly defined analytical conditions that are necessary to obtain reproducible results.

EXPERIMENTAL

Materials

All of the solvents were of analytical-reagent grade and were used as received. Pyridine and Sylon BTZ [consisting of trimethylchlorosilane, N,O-bis(trimethylsilyl)-acetamide and trimethylsilylimidazole (2:3:3)] were of silylation grade (Supelco, Bellefonte, PA, U.S.A.). Pure samples of ginsenosides Rg_1, Re, Rd, Rc, Rb_2, Rb_1 and F_1 were used as obtained in our laboratories. The stachiose internal standard and Oxisorb gas driers were supplied by Supelco.

Preparation of ginseng sample from plant materials

About 2 g, exactly weighed, of finely powdered roots were placed in a 22 × 80 mm cellulose thimble. The plant material was placed in a Soxhlet extraction apparatus fitted with a round-bottomed boiling flask containing 150 ml of methanol and extracted for 4 h. The methanol extract was concentrated under vacuum and the residue transferred into a 20-ml volumetric flask with a mixture of 5 ml of methanol and 2 ml of water and subsequently with aliquots of methanol. The solution was diluted to volume with methanol and filtered. An exactly measured volume of the resulting solution (from 0.20 to 1.00 ml according to the total ginsenosides content) was transferred into a tapered Reacti-vial, and 0.25 ml of stachiose internal standard solution (1.00 mg/ml in methanol–water, 4:1) was added. The solvent was evaporated under a stream of nitrogen at 60°C on a heating block. Trimethylsilylation was performed by adding 0.70 ml of anhydrous pyridine and 0.30 ml of Sylon BTZ. The vial, sealed with a PTFE-lined cap, was heated for 30 min at 60 °C. An 0.25-ml aliquot of hexamethyldisilazane (HMDS) was then added and the solution was heated for 10 min at 60°C (HMDS was added to prevent reactivation, *i.e.*, to keep glass surfaces and supports well deactivated.) After cooling, aliquots of 1 μl were injected.

Preparation of ginseng samples from dry extracts

About 500 mg of dry ginseng extract were transferred into a 20-ml volumetric flask and 2 ml of water were added. After stirring, 2 ml of methanol were added and the sample was heated on a water-bath at 60°C to dissolve as much as possible the product. After the addition of 10 ml of methanol, the solution was heated at 60°C with stirring, then cooled and diluted to volume with the same solvent. After filtration, 0.5 ml of the resulting solution was transferred into a Reacti-vial and the solvent evaporated under a stream of nitrogen at 60°C. The sample was treated and silanized as described above for the samples from plant materials.

Standard solutions

A ginsenosides stock solution containing the ginsenosides Rg_1, Re and Rb_1 at a concentration of 1.0 mg/ml in methanol–water (9:1) was prepared. Known amounts of each component (*e.g.*, 0.1, 0.2, 0.4 and 0.6 mg) were transferred into Reacti-vials by pipetting 0.10, 0.20, 0.40 and 0.60 ml of the solution. An aliquot of

stachiose internal standard solution (0.25 ml of a 1.00 mg/ml solution in methanol–water, 4:1) was added to each sample and the solvent evaporated under a stream of nitrogen at 60°C. The samples were treated with 0.7 ml of anhydrous pyridine and 0.3 ml of Sylon BTZ and the vials were sealed with PTFE-lined caps and heated for 30 min at 60°C. The solution was heated for 10 min at 60°C after adding 0.2 ml of HMDS. Aliquots of 1 μl were then injected.

Gas–liquid chromatography

GLC analysis was performed on a Hewlett-Packard Model 5830A gas chromatograph equipped with a 30 cm × 2 mm I.D. glass column, containing 0.5% OV-101 on Chromosorb W HP (100–120 mesh). The temperature programme was 250–350°C at 5°C/min with a hold at 350°C for 10 min; helium flow-rate, 25 ml/min; injector temperature, 350°C; and flame-ionization detector temperature 350°C. Peak areas were measured with an Hewlett-Packard System 18850A integrator. In order to prevent surface-catalysed thermal decomposition of the components on the column the following procedure for column preparation and deactivation was used. The glass column (Pyrex) was first washed with 20% hydrochloric acid solution and rinsed consecutively with distilled water, methanol and acetone. Then it was dried at 200°C under a nitrogen flow and filled with the stationary phase.

The column ends themselves, and not special connecting tubing (especially if made of stainless steel because of its high catalytic activity), must act as part of the injection system. In addition, the vaporisation zone of the column was filled only with the support, Chromosorb W HP (100–120 mesh).

The column was conditioned under a helium flow from 50 to 360°C at 1°C/min, with the detector temperature at 370°C and the injection port temperature kept off. After the maximal temperature has been reached, the injection port temperature was set at 370°C and the column was kept under these conditions overnight. The deactivation was executed by injecting 5-μl aliquots of HMDS at 180°C and during several programming runs carried out from 180 to 350°C. The injection port was maintained at 180°C and then its temperature increased gradually with column temperature during this phase. Subsequently it was set at 370°C for about 5 h. This deactivation procedure probably involves irreversible thermal dehydration of the interacting silanols after their silanization at low temperature (180°C).

To avoid contact of oxygen and traces of water with the products and to extend the column life, the carrier gas was passed through a molecular sieve trap, followed by an oxygen trap.

RESULTS AND DISCUSSION

To develop a successful GLC method for the separation and determination of the ginsenosides in different ginseng drugs and extracts we employed a short column with improved temperature stability and deactivation. Several factors of importance for the preparation of such columns are still poorly understood, but the procedure described gives a satisfactory column for the analysis of trimethylsilylated triterpenoidic glucosides containing up to four sugar units. To obtain the best reproducibility of the results, the vaporisation of the samples into the column must take place on the deactivated support, otherwise decomposition peaks may occur in the gas chromatogram.

Column deactivation by silanization with HMDS gave the best results. The effectiveness of HMDS was shown by Grob *et al.*[13], who used it to deactivate glass surfaces at high temperatures. Attempts to carry out deactivation with other silylating reagents, such as trimethylchlorosilane–hexamethyldisilazane (1:3), trimethylsilylimidazole orN, O-bis(trimethylsilyl)acetamide, or a mixture of them, proved unsuccessful. In addition, columns that are used for analysis over extended periods may show a gradually impaired deactivation. To restore such columns, HMDS is injected into the column during some temperature programming runs as for deactivation procedure. The column is tested by injection of a standard solution (prepared as described under Experimental) containing the ginsenosides Rg_1, Re and Rb_1. Symmetrical peaks should be obtained. In addition, every ginsenoside should give rise to only one peak in the gas chromatogram, as reported in Fig. 2.

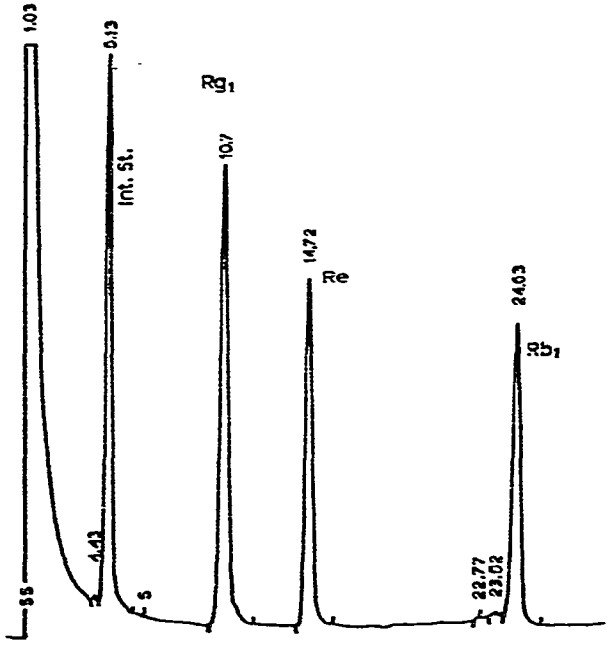


Fig. 2. Gas chromatogram of standard ginsenosides Rg_1, Re and Rb_1 as TMS derivatives.

The very small peaks at 22.77 and 23.52 min are impurities due to the ginsenosides Rc and Rb_2, respectively. If decomposition peaks are observed with an activated or re-activated column, reconditioning can be achieved by injecting aliquots of HMDS during some programming runs. Fig. 3 shows a typical gas chromatogram, obtained as described under Experimental, due to a *Panax ginseng* root extract. The peaks of ginsenosides Rg_1 and Rf are superimposed.

The identity of the components was confirmed by GLC–MS and from authentic sample retention times comparison as reported elsewhere[10].

Calibration graphs for the ginsenosides Rg_1, Re and Rb_1 are shown in Fig. 4. A linear detector response was found for all the standard data, which were fitted with a straight line by the least-squares technique.

On the basis of the very close identity of responses of ginsenosides Rd and Re

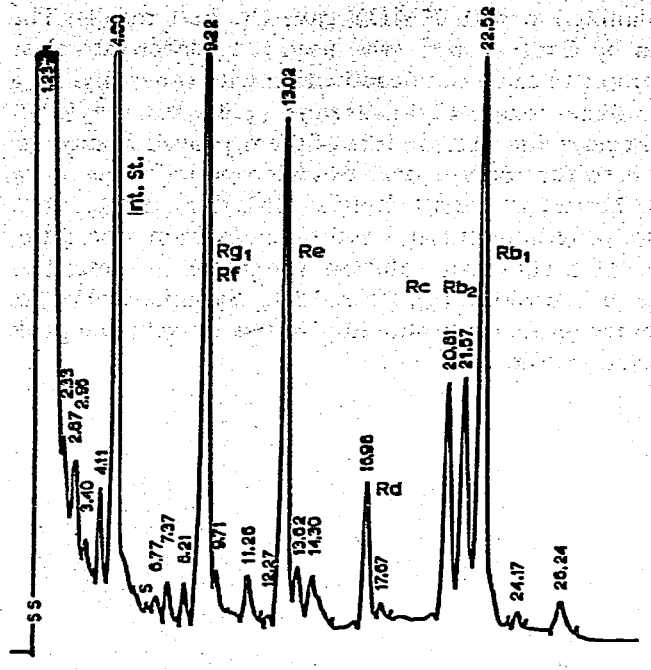


Fig. 3. Gas chromatogram of *Panax ginseng* extract after treatment with silylating reagents.

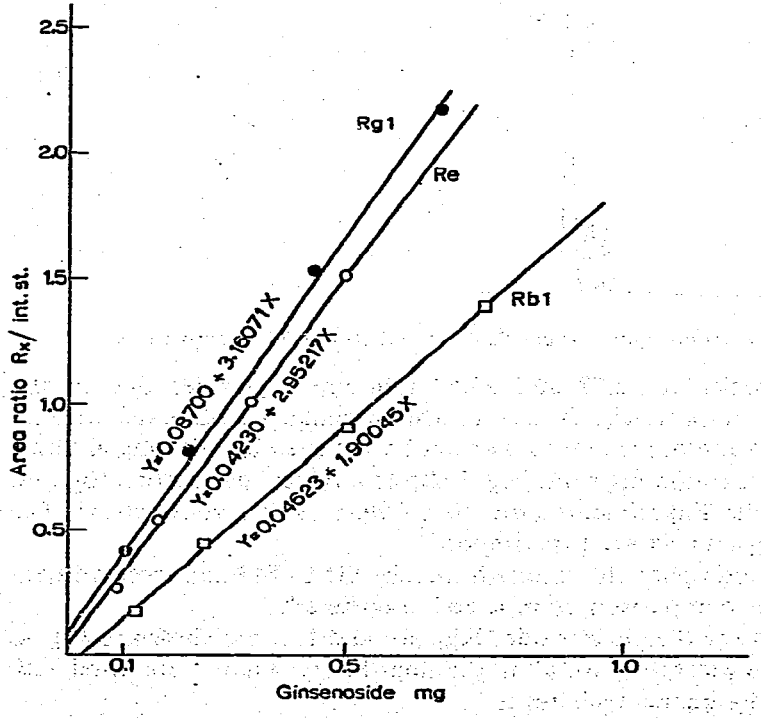


Fig. 4. Calibration graph for ginsenosides Rg_1, Re and Rb_1 (internal standard, stachiose).

TMS-ethers, and of ginsenosides Rc, Rb_2 and Rb_1 TMS-ethers, all of the most important components can be assayed using the calibration graphs shown above.

In order to determine the contents of the ginsenosides, different parts of the plant and materials of different origin were investigated. Gas chromatograms for some different drugs are reported in Fig. 5 and the results of quantitative analysis are given in Table I.

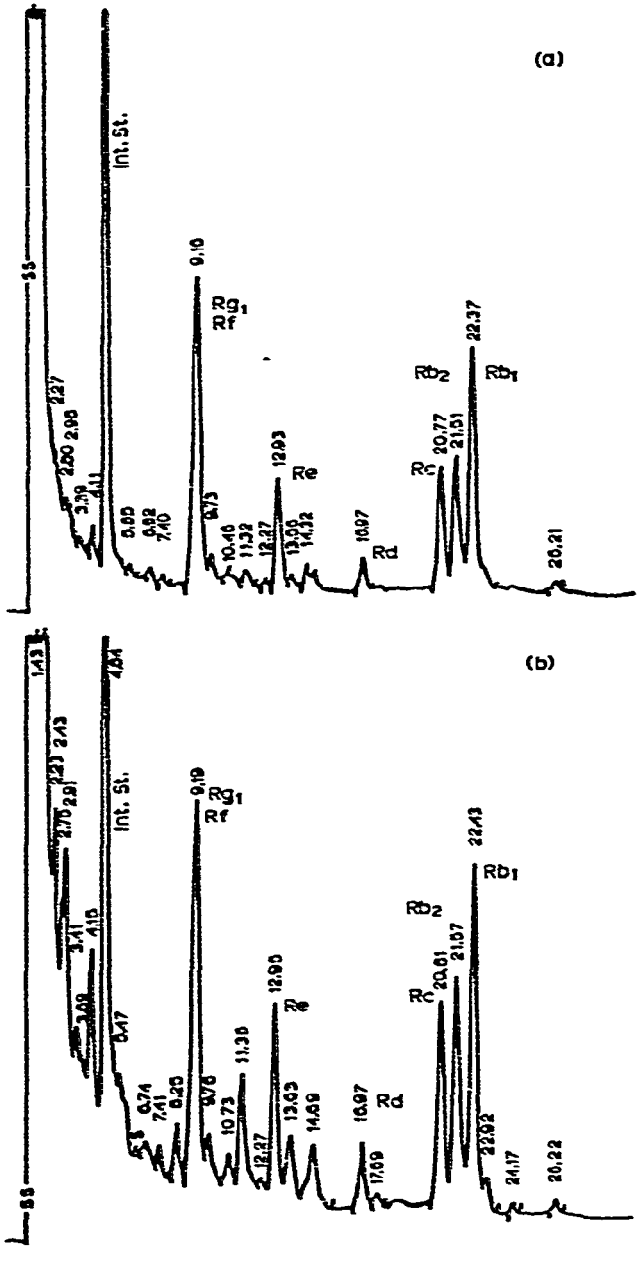


Fig. 5.

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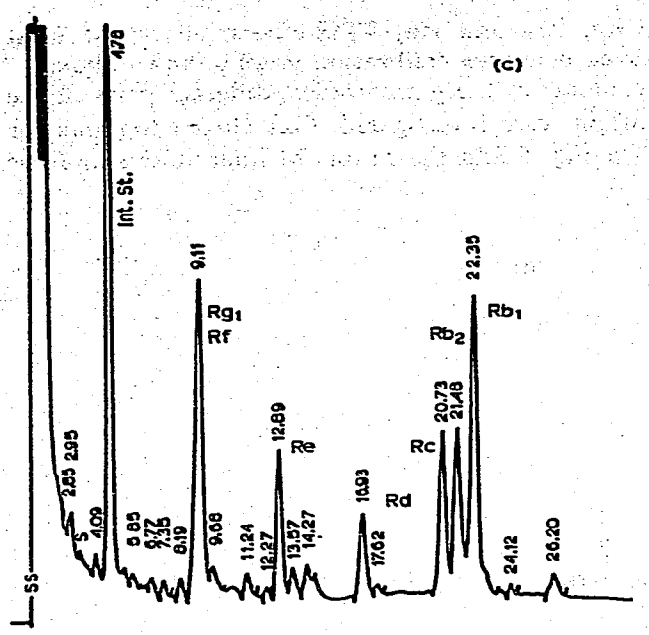
(c)
Int. St.
Rg1
Rf
Re
Rd
Rc
Rb2
Rb1
2.85
2.95
4.09
5.85
6.72
7.35
8.19
9.11
9.66
11.24
12.27
12.89
13.57
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24.12
26.20
SS

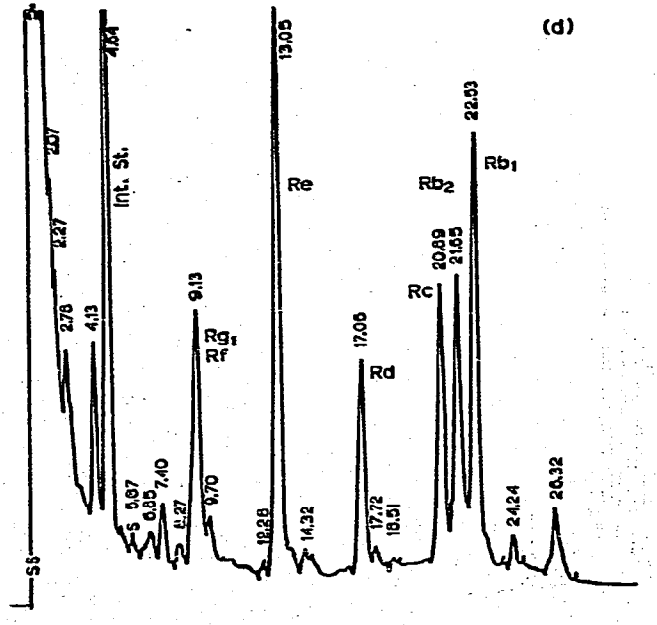
(d)
Int. St.
Rg1
Rf
Re
Rd
Rc
Rb2
Rb1
2.57
2.78
4.13
4.84
5.87
6.85
7.40
8.27
9.13
9.70
10.28
13.05
14.32
17.06
17.72
18.51
20.89
21.65
22.53
24.24
26.32
SS

Fig. 5.

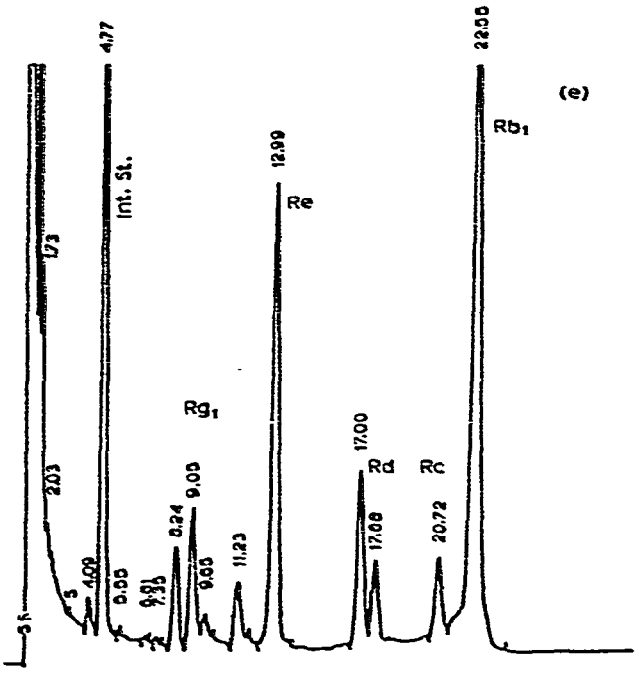

Fig. 5. Gas chromatograms of different ginseng extracts: (a) straight; (b) red; (c) curved; (d) fasern; (e) *Panax quinquefolium*.

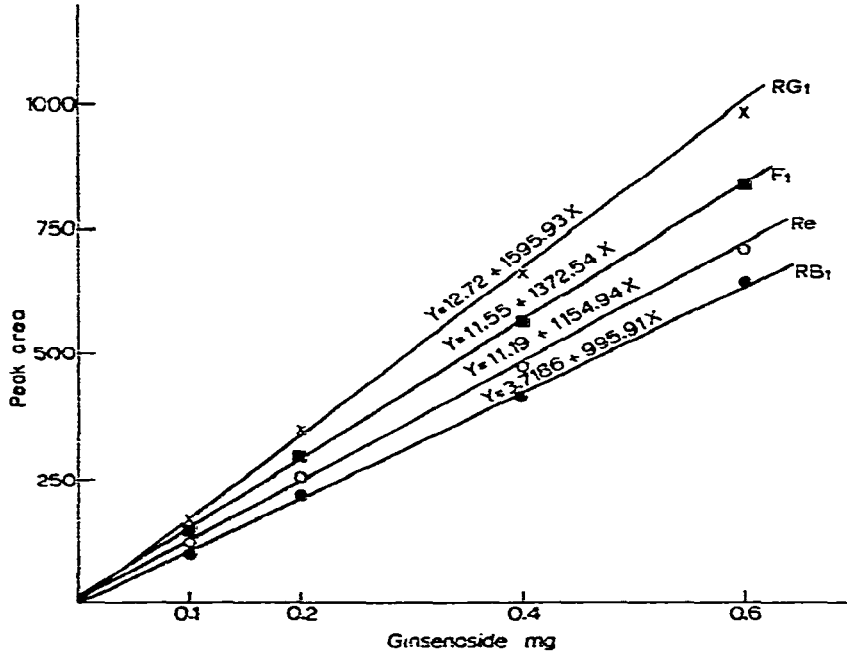

Fig. 6. Calibration graphs of ginsenosides Rg_1, Re, Rb_1 and F_1 (external standard).

TABLE I

GINSENOSIDE COMPOSITION OF SOME GINSENG ROOT SAMPLES

Species	*Origin*	*Remarks*	*Rg_1 + Rf* (% ± S.D.)	*Re* (% ± S.D.)	*Rd* (% ± S.D.)	*Rc* (% ± S.D.)	*Rb_2* (% ± S.D.)	*Rb_1* (% ± S.D.)
Korean ginseng (*Panax ginseng*)	Korea (Sam Geon Sam)	White	0.394 ± 0.020	0.317 ± 0.009	0.073 ± 0.003	0.275 ± 0.003	0.324 ± 0.013	0.713 ± 0.036
	Korea	Straight	0.184 ± 0.009	0.052 ± 0.002	0.002 ± 0.001	0.154 ± 0.005	0.187 ± 0.009	0.318 ± 0.016
	Korea	Red	0.180 ± 0.009	0.082 ± 0.003	0.017 ± 0.002	0.198 ± 0.007	0.246 ± 0.012	0.330 ± 0.017
	Korea	Curved	0.196 ± 0.010	0.076 ± 0.003	0.038 ± 0.002	0.207 ± 0.007	0.243 ± 0.013	0.400 ± 0.020
	Korea (Kiboshi)		0.280 ± 0.014	0.700 ± 0.006	0.030 ± 0.002	0.170 ± 0.006	0.181 ± 0.011	0.501 ± 0.025
	Korea	Sliced roots	0.260 ± 0.013	0.150 ± 0.010	0.010	0.190 ± 0.009	0.200 ± 0.015	0.460 ± 0.023
	Korea	Slender tails	0.520 ± 0.026	1.38 ± 0.042	0.421 ± 0.017	2.410 ± 0.072	2.42 ± 0.097	3.450 ± 0.172
	Korea	Fasern	0.126 ± 0.006	0.312 ± 0.009	0.096 ± 0.040	0.276 ± 0.012	0.322 ± 0.012	0.439 ± 0.025
American ginseng (*Panax quinquefolium*)	U.S.A.		0.200 ± 0.010	1.132 ± 0.034	0.381 ± 0.016	0.409 ± 0.015	–	3.888 ± 0.195

TABLE II

GINSENOSIDE COMPOSITIONS OF LEAVES AND FLOWERS OF *PANAX GINSENG*

Sample	*F_1* (%, w/w, ±S.D.)	*Rg_1* (%, w/w, ±S.D.)	*Re* (%, w/w, ±S.D.)	*Rd* (%, w/w, ±S.D.)	*Rc* (%, w/w, ±S.D.)	*Rb_2* (%, w/w, ±S.D.)	*Rb_1* (%, w/w, ±S.D.)
Leaves (dried)	1.17 ± 0.03	1.59 ± 0.04	2.59 ± 0.05	1.88 ± 0.04	0.52 ± 0.02	0.72 ± 0.02	0.10 ± 0.02
Flowers (dried)	0.47 ± 0.01	1.06 ± 0.03	4.29 ± 0.10	2.34 ± 0.05	0.47 ± 0.02	0.71 ± 0.03	0.55 ± 0.05

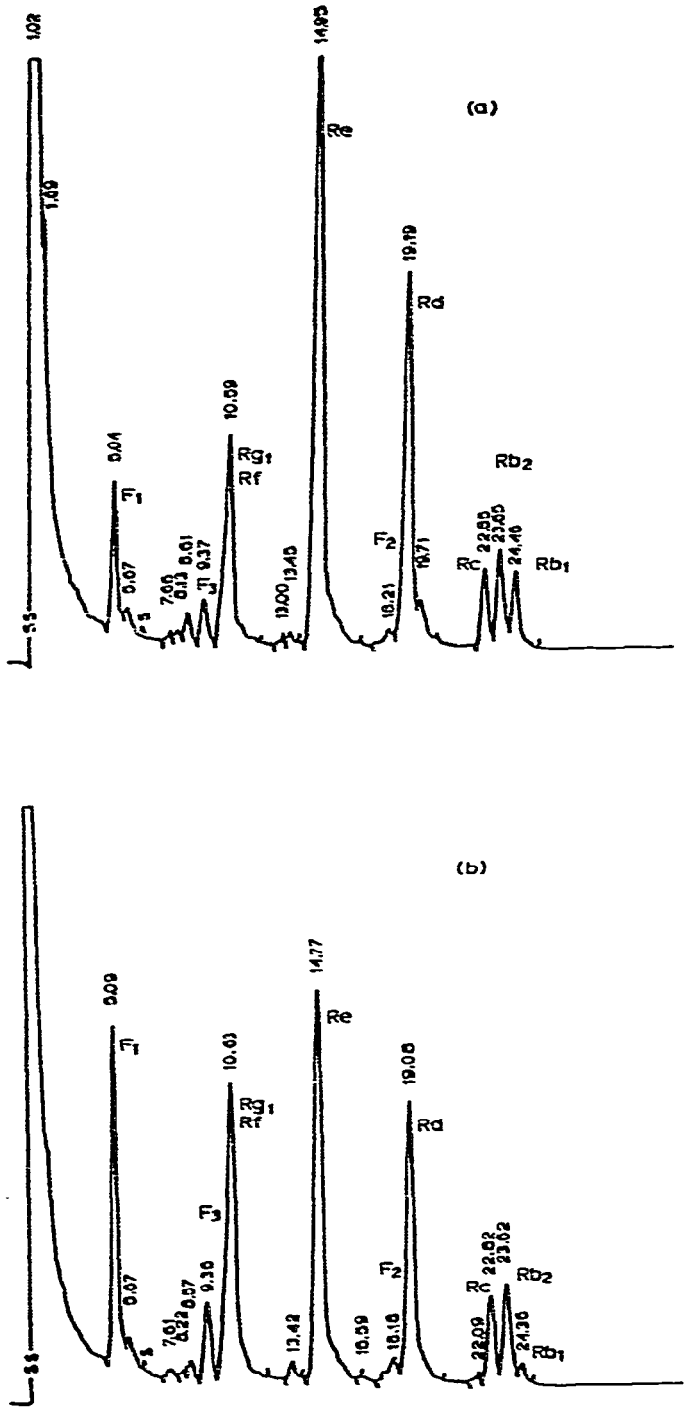

Fig. 7. Gas chromatograms of (a) flowers and (b) leaves of *Panax ginseng* after treatment with silylating reagents.

The mean calibration errors (Syx %) calculated with the aid of a computer[14] for the ginsenosides Rg_1, Re and Rb_1 were 1.70%, 1.13% and 2.89%, respectively. To control the reproducibility of the method, six samples of the same batch of the drug were analysed. The results obtained for the ginsenosides Rg_1, Re, Rd, Rc, Rb_2 and Rb_1 gave standard deviations of 4.0, 2.1, 4.1, 2.1, 2.8 and 3.6%, respectively. In spite of the variable total content of the ginsenosides in old *Panax ginseng* roots, the ratio of the amount of the ginsenoside Rb_1 to that of the ginsenoside Rg_1 seems constant with a value of about 2.

Very young roots (fasern) or slender tails show a lower content of the ginsenoside Rg_1. *Panax quinquefolium* (also called American ginseng) roots show a very different saponin composition and a characteristic absence of Rb_2 peak.

In order to evaluate the amounts of the ginsenosides in the aereal part of *Panax ginseng*, leaves and flowers were examined. The evaluation of the components was performed by using an external standard calibration graph (Fig. 6), due to the presence of an interfering peak (ginsenoside F_1) near that of the internal standard.

The gas chromatograms are reported in Fig. 7. Calibration graphs for ginsenosides F_1, Rg_1, Re and Rb_1 also give good straight lines. The mean calibration errors (Syx %) are 1.54, 2.56, 2.17 and 2.79, respectively. Results of the quantitative determinations are reported in Table II.

CONCLUSIONS

The results show that GLC can be used satisfactorily as a simple and rapid means for the determination of high-molecular-weight saponins. Further, the application of this method permits careful quantitative examination of the saponins in *Panax ginseng*, which are considered to be the active principles of the extract.

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