HPLC SEPARATION AND QUANTITATIVE DETERMINATION OF GINSENOSIDES FROM *PANAX GINSENG, PANAX QUINQUEFOLIUM* AND FROM GINSENG DRUG PREPARATIONS

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Abstract

A new HPLC-method for separation and quantitative determination of ginsenosides in *Panax ginseng*, *Panax quinquefolium* and in pharmaceutical drug preparations is elaborated. A reversed-phase-system with μBondapak C_{18} column (3.9 mm I.D. × 30 cm) using acetonitrile-water (30:70) 2 ml/min and acetonitrile-water (18:82) 4 ml/min is suitable for the base-line separation of Rb_1, Rb_2, Rc, Rd, Rf, Rg_2, respectively Re, Rg_1 in 30 minutes. The ginsenosides are directly detected at 203 nm (without derivatization) with the LC-55 or LC-75 spectrophotometer (Perkin-Elmer) at 100 % transmission. Detection limit is 300 ng at a signal-to-noise ratio of 10:1. The ginsenosides-peak identification is carried out with HPTLC (high performance thin layer chromatography), with MIR-IR (multiple internal reflection-IR-spectroscopy) and with FD-MS (field desorption mass spectrometry). The calibration curve of each ginsenoside has a correlation coefficient very near to 1. Relative standard deviation for quantitative determinations depends upon the amount of ginsenosides and is approximately 1% for ginsenoside contents of 1%. This method is adaptable for routine analysis in quality control laboratories.

Introduction

Ten years after its discovery, high performance liquid chromatography (HPLC) has become an indispensable tool in research and quality control laboratories. Owing to the relative simplicity of this method and because of the high precision of the results it supplies, HPLC has already been introduced in 1975 as an official analytical method in the U.S. Pharmacopeia (1).

As continuation to our first communication (2) we present here a new HPLC-method for quantitative determination of the ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 in Panax species, ginseng extracts and pharmaceutical drug preparations.

Methods for the quantitative analysis of ginsenosides (see (2) et lit. cit.) such as colorimetry and gas-chromatography no longer comply with the requirements which are now demanded in an analytical laboratory. Although colorimetric methods enable a fast comparison of different products, they are not specific and supply higher results (3, 4). Also the gas-chromatographic determination of hydrolysis products of ginsenosides (panaxadiol and panaxatriol) is insufficient and supplies wrong results (5, 6).

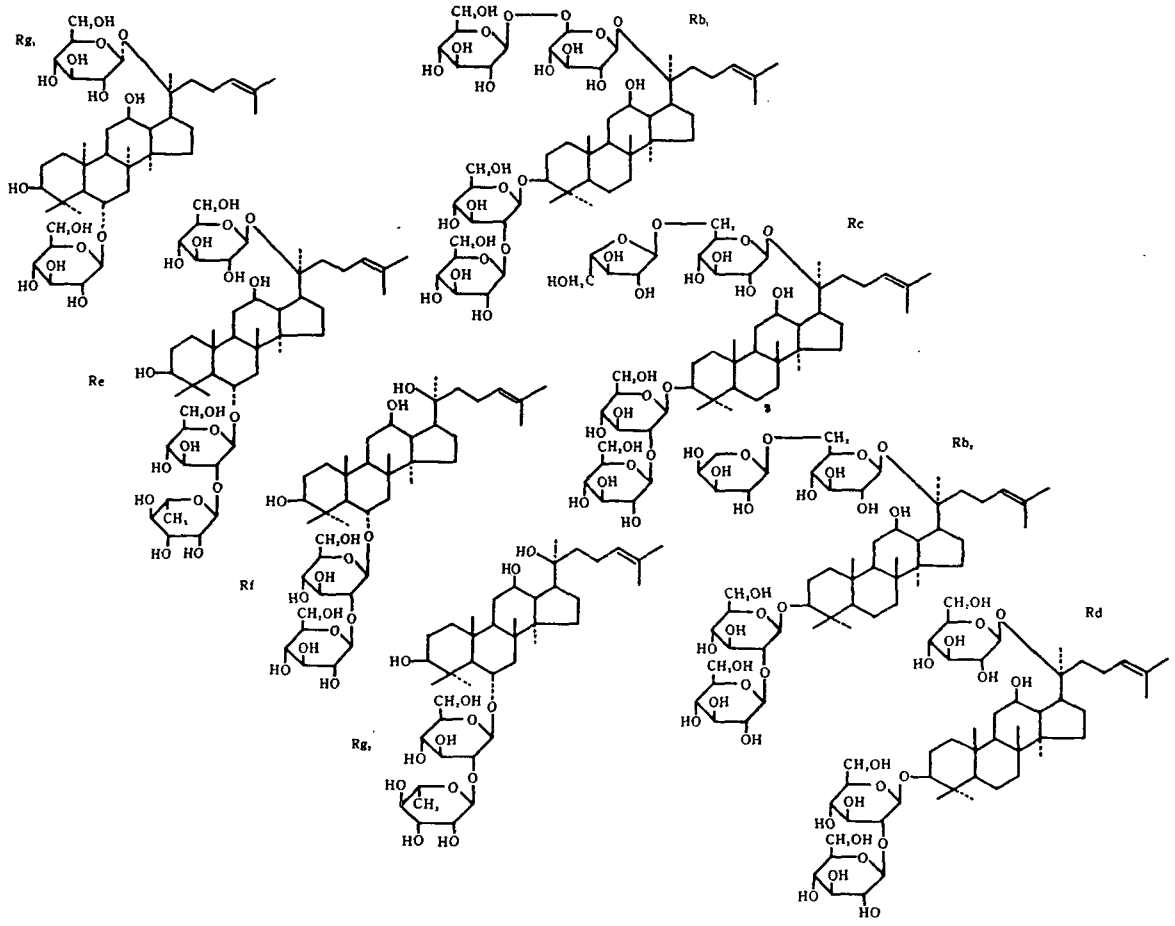

Fig. 1

More reliable values are obtained with the thin-layer chromatography-fluorimetric method. Unfortunately the ginsenosides Rb_1/Rb_2, Re/Rf, Rg_1/Rg_2 are not separated from each other, but are determined as a total (3, 5). Recently a HPLC-method for the determination of benzoylated ginsenosides has been published (7). The advantages and disadvantages of a UV derivatization have already been mentioned in our previous communication (2).

Experimental

Equipment

Pump, Waters model M-6000 A (Waters Assoc., Milford, Mass., USA); injector, Waters model U 6K; column, Waters μBondapak C_{18} (P/N 27324), 3.9 mm I.D. × 30 cm; detectors, Perkin-Elmer LC-55, Perkin-Elmer LC-75 (Coleman, Maywood, ILL, USA); recorder, W + W Recorder Series 1100 (W + W Electronic, Basel Switzerland); integrator, Autolab Minigrator (Spectra-Physics, Santa Clara, CA, USA).

Chemicals

Methanol UVASOL, n-butanol UVASOL, trichloroethylene p.a., Extrelut® were bought from Merck (Darmstadt, FRG) and acetonitrile HPLC-grade S was bought from Rathburn Chemicals(Walkerburn, Scotland). Freshly distilled water must always be used. The ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 have been isolated in our laboratories and identified with authentic substances (28–30).

Sample preparation

The basic principle of extraction is the same

for dry extracts, fluid extracts, roots, stems, leaves, tablets, hard-and soft gelatin capsules, teas, etc.

One can work either by means of the classical extraction with water saturated n-butanol (2, 7) or with Extrelut®-columns.

Dry extracts: Dissolve 1.0 g in 20.0 ml of water in a tared flask. Place onto an Extrelut®-column 4.0 ml of this solution and 6.0 ml of water. After 15 minutes, elute with 80.0 ml of water saturated n-butanol. Evaporate the eluate in a rotavapor and dissolve the residue in 2.0 ml of methanol. Inject 10–20 µl of this solution into the HPLC-system and carry out the chromatography.

Fluid extracts: Evaporate 5.0ml in a rotavapor. Dissolve the residue in 20.0 ml of water in a tared flask and treat further as described for dry extracts.

Roots, stems, leaves, teas: Extract 5.0 g (pulverized) with 3 × 50 ml of methanol 80% at 50° C each for 30 minutes. Evaporate the entire methanol extractions in a rotavapor. Dissolve the residue in a tared flask in 20.0 ml of water and treat further as described for dry extracts.

Tablats, hard gelatin capsules: These products usually contain pulverized roots and accessory agents. Extract in the same manner as described for roots from 10.0 g of pulverized tablets, or 5.0 g of hard capsule content.

Soft gelatin capsules: These also usually contain dry extracts and accessory agents. Introduce 4.0 g of soft gelatin capsule contents into 10 ml trichlorethylene and extract with 3 × 5 ml of water. Unite the aqueous phases, introduce them into a tared flask and fill up to 20.0 ml with water. Treat further as described for dry extracts.

Remarks: The plastic material of new Extrelut®-columns must be washed with methanol prior to its first use (dipping into methanol for 1 hour). For further use, washing with normal detergents is sufficient. The refilling material must be washed with methanol prior to use (for instance 6 bags of of refilling material with 1 litre of methanol for 6 hours in a Soxhlet, then dried at 80° C). The column requires 11.3 g of refilling material. For *further information see* (22).

HPLC-procedure

Column µBondapak C_{18}; eluent for Rb_1, Rb_2, Rc, Rd, Rf, Rg_2, acetonitrile-water (30:70) 2 ml/min.; eluent for Re, Rg_1, acetonitrile-water (18:82) 4 ml/min. Detection UV 203 nm with LC-55 or LC-75, absorbance 0.05. After each chromatography of test material wash the column with 30 ml of methanol 4 ml/min. Work at room temperature (22° C).

Quantification

For quantification use the external standard method. The Calibration curves are established with the ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 in concentrations of 1–40 µg/10 µl methanol. As parameter the peak height area can be used.

Results and discussion

Choice of the separation-system

From our experiences with the HPLC-quantitative determination of substances present in medicinal plants, we can establish without any doubt that reversed-phase-systems (RP-HPLC) are more simple than normal-phase-systems for analyses (2, 8–14). On µPorasil columns with the eluent n-heptane-n-butanol-acetonitrile-water we could determine the ginsenosides Re, Rf, Rg_1, Rg_2 (2). With the reversed-phase-system acetonitrile-water on µBondapak C_{18} columns we now can separate the major ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rg_1, and the minor ginsenosides Rf, Rg_2, and can estimate each quantitatively. As can be seen from Fig. 2 and 3 the protopanaxatriol-derivatives are eluted before the protopanaxadiol-derivatives.

The ginsenosides Rg_1 and Re cannot be separated with the eluent acetonitrile-water (30:70) 2 ml/min, because they are too polar and therefore cannot be retained. They can be well separated with the eluent acetonitrile-water (18:82) 4 ml/min (Fig. 3). Owing to bad reproducibility of retention times and because of the baseline drift

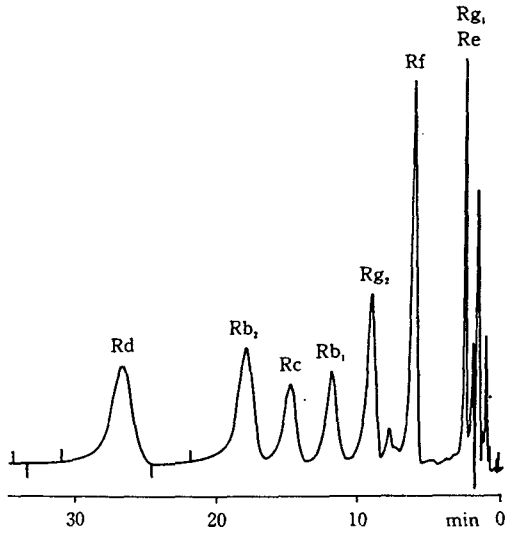

Fig. 2. HPLC-chromatogram of a mixture of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2. Column μBondapak C_{18} 3, 9 mm I.D. × 30 cm; eluent acetonitrile-water (30: 70); flow-rate 2 ml/min; detection UV 203 nm.

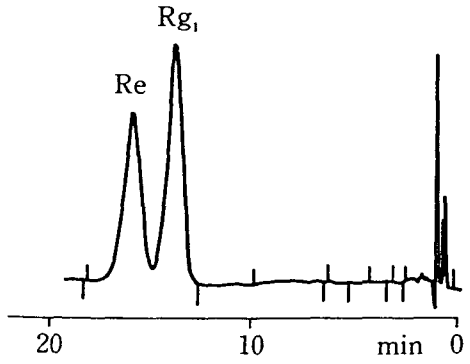

Fig. 3. HPLC-chromatogram of a mixture of ginsenosides Re, Rg_1. Column μBondapak C_{18} 3,9 mm I.D. × 30 cm; eluent acetonitrile-water (18: 82); flow-rate 4 ml/min; detection UV 203 nm.

Table I. Capacity factor k^1, relative retention α, resolution R (calculated for a new column). Conditions for Rb_1, Rb_2, Rc, Rd, Rf, Rg_2 see Fig. 2, conditions for Re, Rg_1 see Fig. 3.

Ginsenoside	k^1	α	R
Rg_1	29.16	1.16	1.37
Re	33.73		
Rf	5.26	1.59	3.33
Rg_2	8.37	1.30	2.36
Rb_1	10.88	1.27	1.87
Rc	13.78	1.25	1.73
Rb_2	17.25	1.50	3.58
Rd	25.90		

Table II. Retention time t_R, relative standard deviation of the retention time s_{rel}, number of measurements n (conditions see Table I)

Ginsenoside	t_R(sec)	s_{rel}	n
Rg_1	905	1.18%	28
Re	1042	2.96%	34
Rf	376	0.57%	36
Rg_2	562	1.57%	38
Rb_1	713	2.83%	36
Rc	887	2.90%	18
Rb_2	1095	2.08%	18
Rd	1614	1.62%	18

the use of a gradient elution is not recommended.

In Table I the most important chromatographic parameters of this separation are indicated. A resolution of 1.5 means a baseline separation (15). The high reproducibility of the separation is also shown by the relative standard deviations of the retention times in Table II.

Calibration curves and detection limit

In order to check the linear relationship between UV absorption intensity and amount of ginsenosides, calibration curves are established for Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 (Fig. 4 and 5).

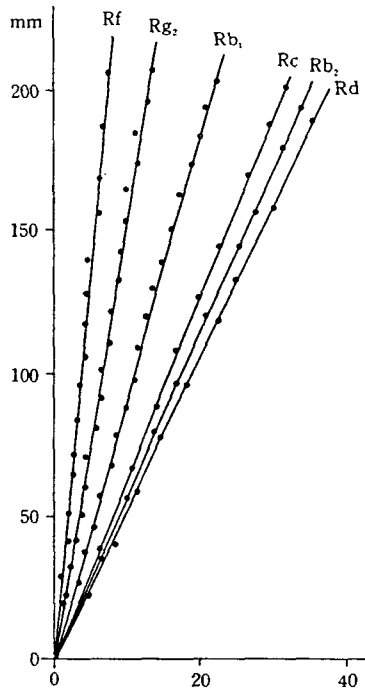

Fig. 4. Calibration curves of the ginsenosides Rb_1, Rb_2, Rc, Rd, Rf, Rg_2 (conditions see fig. 2.).

Table III. Equations of the least-squares regression — Function $y = C \cdot x + N$

	Rg_1	Re	Rf	Rg_2	Rb_1	Rc	Rb_2	Rd
C	5.6406	4.2659	25.5706	15.4561	9.1197	6.3025	5.6552	5.2672
N	0.3187	0.9191	5.3250	4.0965	1.6233	−0.4213	−0.2615	−0.6034
KK	0.9996	0.9998	0.9994	0.9996	0.9997	0.9999	0.9999	0.9999
F	(N1 = 1, N2 = 12)	(N1 = 1, N2 = 15)	(N1 = 1, N2 = 14)	(N1 = 1, N2 = 17)	(N1 = 1, N2 = 16)	(N1 = 1, N2 = 7)	(N1 = 1, N2 = 7)	(N1 = 1, N2 = 7)
	14832	36771	12897	24480	23828	148263	72725	39944
V	1.3971	0.8987	2.0758	1.1792	1.3004	0.2944	0.4936	1.0050
P	0.0463	0.0222	0.2251	0.0980	0.0591	0.0164	0.0210	0.0263
S	0.7887	0.4559	1.0886	0.6069	0.7453	0.2310	0.3737	0.6363

Symbols: C = slope, N = intercept, KK = correlation coefficient, F = F-value, V = standard deviation y-value to x-value, P = standard deviation of the correlation coefficient, S = standard deviation of the intercept.

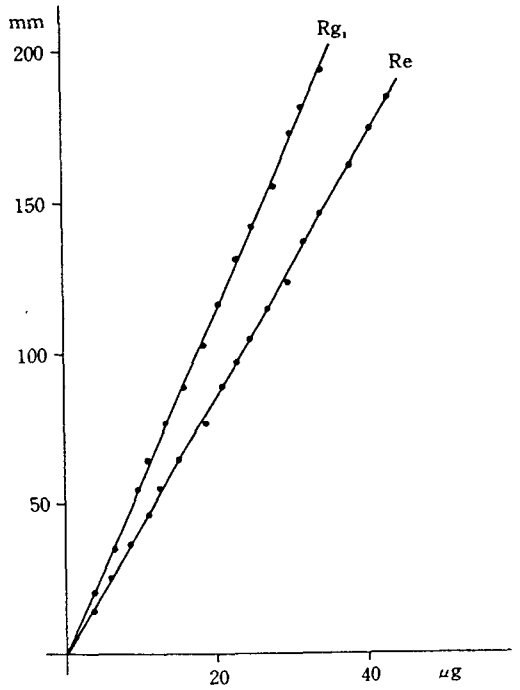

Fig. 5. Calibration curves of the ginsenosides Re, Rg_1 (conditions see fig. 3.)

Calibration curves can be established from the height of the peak as well as from the peak area. In Table III the equations of the least-squares regression are indicated: y signifies the height of the peak and x the injected quantity of ginsenoside in > g. For all close to the value 1.

The ginsenosides are detected directly at 203 nm (absorption maxima conditioned by the double bond at C(24) = C(25) with the UV spectrophotometers LC-55 or LC-75 (Perkin-Elmer) at 100% transmission. The detection limit is 300 ng at a signal-to-noise ratio of 10:1.

Extrelut® a new method of extraction

For the quantitative determination of roots, extracts and pharmaceutical specialities, the ginsenosides must be extracted. The classical extraction with methanol followed by extraction with water-saturated n-butanol (2, 7) gives good results, but demands a great deal of working time because of the phase separations. The Extrelut®-columns offer a much more elegant method.

The aqueous sample is brought onto a column filled with granulated carrier material and spreads as a stationary phase on the porous matrix. The column is then eluted with n-butanol saturated with water. During this process the ginsenosides are extracted from the aqueous phase into the n-butanol and eluted quantitatively. The disturbing accompanying materials (16–18) (mainly mono-, di- and oligo-saccharides) remain on the column. The eluate is free of emulsions. After evaporation of the n-butanol the residue is dissolved in methanol and injected directly into the HPLC-system. The recovery rate is 100%. With this new extraction method, one single person can prepare up to 30 extracts daily for HPLC-analysis.

HPTLC, MIR-IR and FD-MS to identify the ginsenosides (19)

An important aspect, which often is not considered in HPLC-separations, is the identification of the eluted substances. The examinations of purity of a peak is particularly important if there are substances to be determined in com-

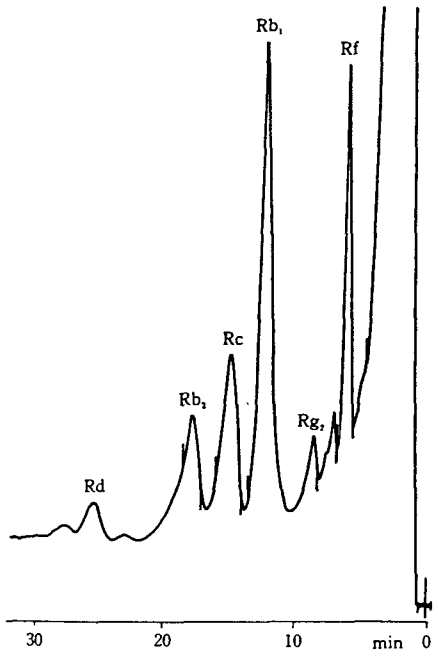

Fig. 6. HPLC-chromatogram of the ginsenosides Rb_1, Rb_2, Rc, Rd, Rf, Rg_2 contained in GINSANA ® soft gelatin capsules[1] (conditions see fig. 2.)

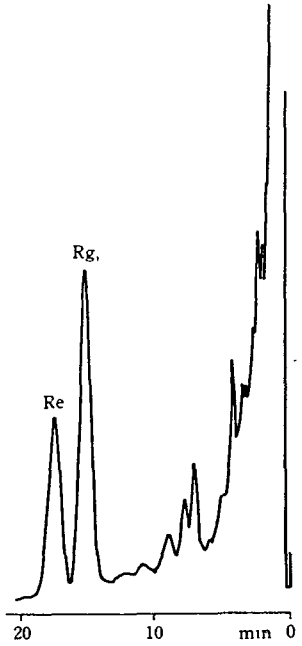

Fig. 7. HPLC-chromatogram of the ginsenosides Re, Rg_1 contained in GINSANA® soft gelatin capsules[1] (conditions see fig. 3.).

plex mixtures, such as body fluids or plant extracts. By means of HPLC-determination of the ginsenosides we report here about the possibilities and limits of the peak identification with on-line methods, like HPTLC, MIR-IR (multiple internal reflection-IR-spectroscopy) and FD-MS (field desorption mass spectrometry).

The steps of the possible off-line methods for peak-identification are shown in Fig. 8. The fractions corresponding to peaks Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Rg_2 are collected from the chromatograph in semi-micro flasks with conical bottom (step 1). In step 2 the solvent (acetonitrile-water) is evaporated. The residue is then dissolved in some microliters of methanol and drawn off with a syringe (step 3).

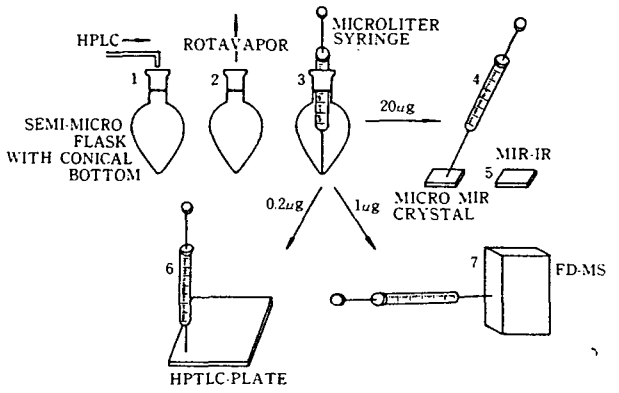

Fig. 8. Off-line methods to identify substances separated by HPLC.

HPTLC-Chromatograph with high performance thin layer plates (step 6) shows considerable advantages compared with the classical TLC: higher resolution, shorter analysis time, higher detection limit, higher reproducibility.

The results obtained with the HPTLC-spray-on technique as shown in Fig. 9. The ginsenosides can be separated on a running distance of 6.5 cm with the eluent A within 60 min. To carry out an accurate peak-identification, it is also possible to chromatograph with other eluents (for instance B). This technique allows a detection limit of 0.2 μg ginsenoside.

MIR-IR-A useful application for the multiple internal reflection-IR-spectroscopy is the identification of peaks separated by HPLC (steps 4 and 5).

[1] Manufacturer: GPL, Ginseng Products Ltd., Lugano-Switzerland

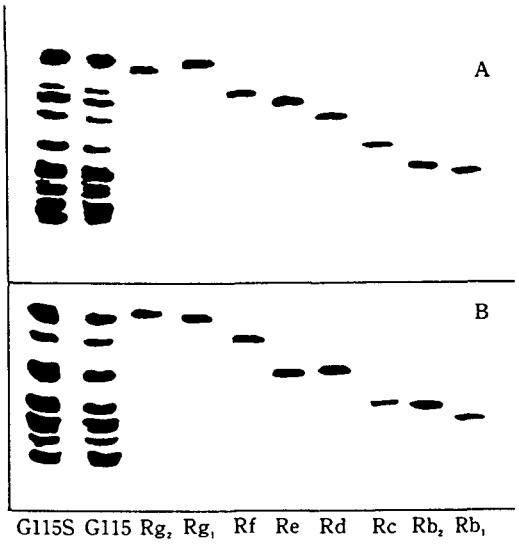

Fig. 9. HPTLC-chromatograms of G 115 ® (1 mg), G 115 S® (1 mg) and of the peaks Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2 (each 10 μg) collected from the HPLC.

This technique allows the recording of IR-spectra with very small amounts of substance. The spectra which we obtained with 20 μg each of the peak fraction, corresponding to Rg_1 (A), Re (B), are illustrated in Fig. 10. At these spectra the absorption bands at 1'640 cm^{-1} (C = C) and at 3'380 cm^{-1} (OH) are characteristic also for the other ginsenosides. As the finger print region does not differ substantially from one ginsenoside to another, peak-identification by this method is not suitable.

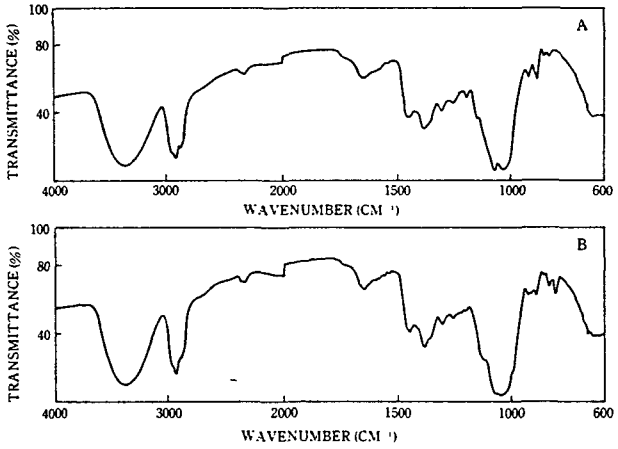

Fig. 10. MIR-IR spectra of the peaks Rg_1 (20 μg) (A), and Re (20 μg) (B), collected from the HPLC.

FD-MS-Electron impact-mass spectrometry (EI-MS) has been shown to be a very useful method for identification, determination of purity and structural determination of ginsenosides. However, for mass spectrometric investigation volatile derivatives (peracetates or permethyl ethers) have to be produced. Moreover the ginsenosides do not give molecular ions even derivatized and even when the spectrum is taken at low ionizing potential (20 eV). Fig. 11 gives the EI-MS of the ginsenoside Rg_2-TMSi-ether at 20 eV; the relative fragmentation scheme is given in Fig 12 (20).

FD-MS (Field desorption-mass spectrometry)

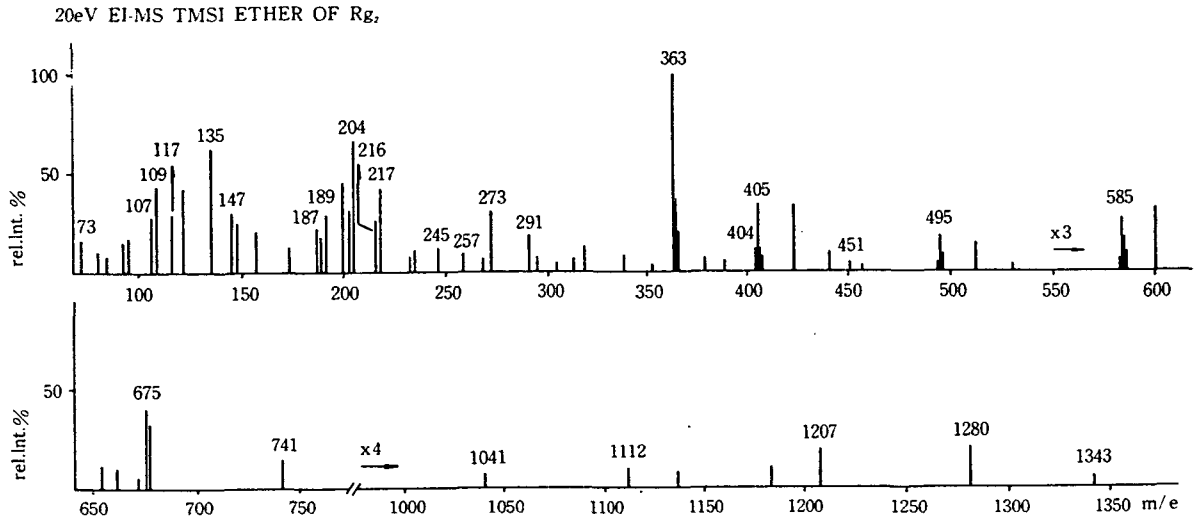

Fig. 11. Electron impact-mass spectrum of TMSi ether of Rg_2 at 20 eV.

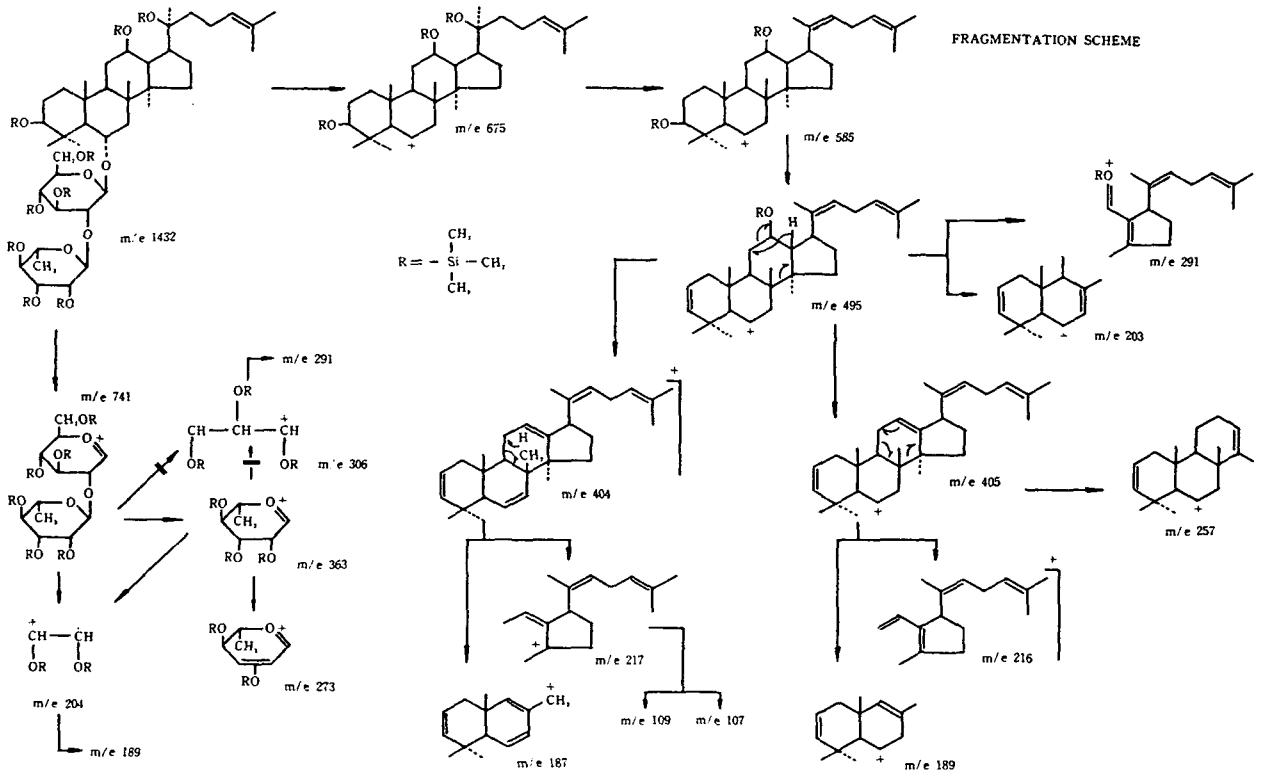

Fig. 12. Fragmentation scheme of EI-MS of TMSi ether of Rg_2.

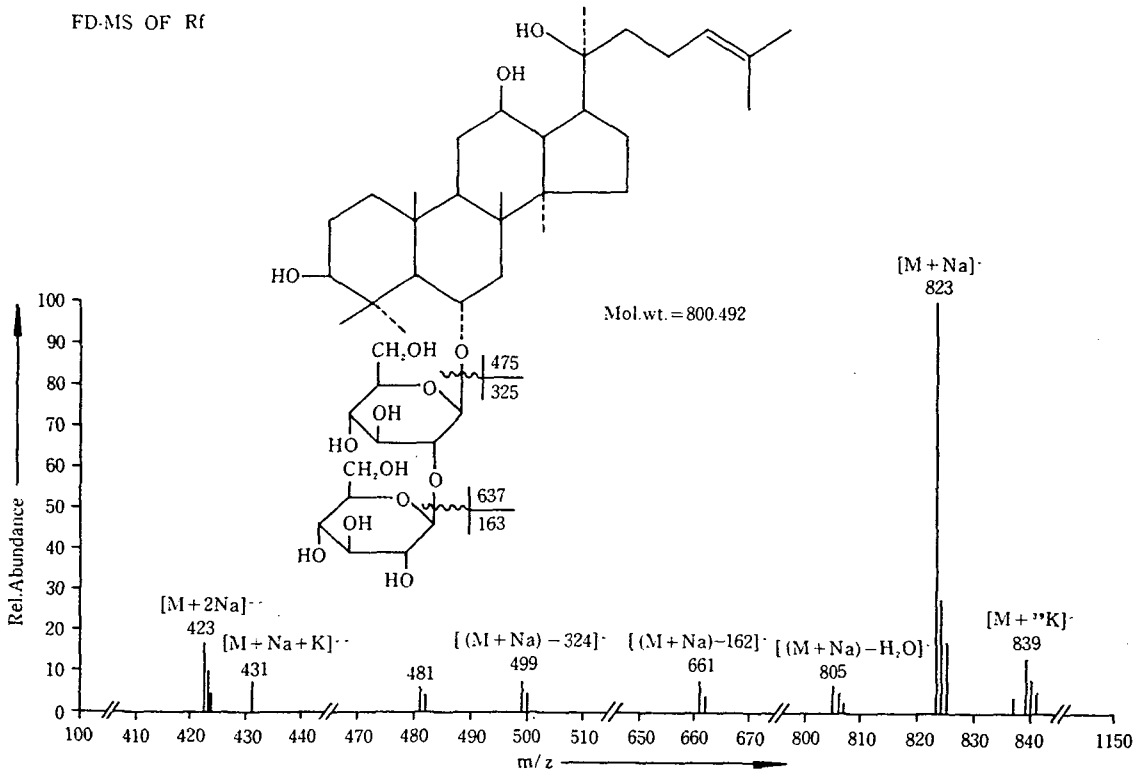

Fig. 13. Field desorption-mass spectrum of the peak Rf collected from the HPLC (0.1 μg).

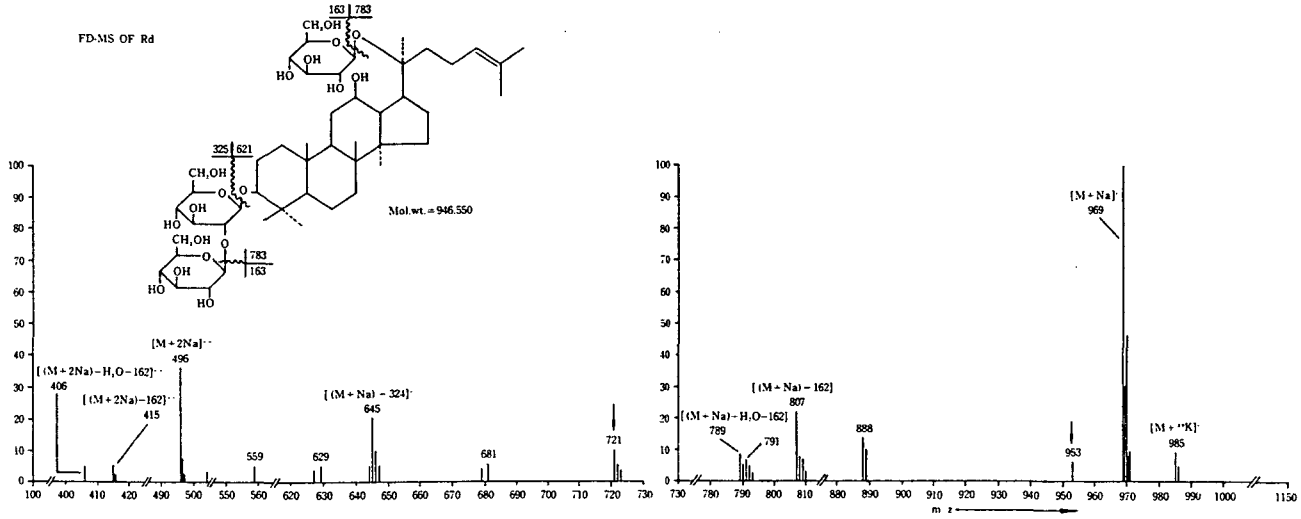

Fig. 14. Field desorption-mass spectrum of the peak Rd collected from the HPLC (0.1 μg).

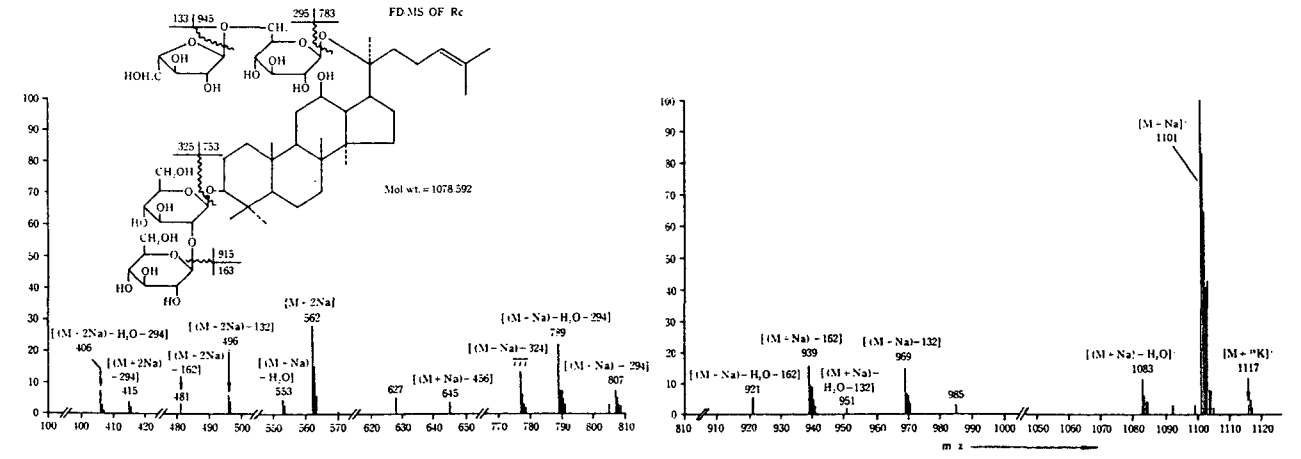

Fig. 15. Field desorption-mass spectrum of the peak Rc collected from the HPLC (0.1 μg).

(step 7) enables not only the molecular weight determination of underivatized glycosides, but yields important information about the sequence of the sugar moities in the molecule. Thus the method shows useful results for structural analysis. The characteristically high relative abundance of molecular or quasimolecular ions and the minimal fragmentation of compounds observed in field desorption-mass spectrometry provide good conditions for the application of FD-MS to identify substances separated by HPLC as the ginsenosides. For example in Fig. 13–15 the FD-MS spectra

Table IV. Distribution of ginsenosides in *Panax ginseng* C.A. Meyer

	% content								
	Rg_1	Re	Rf	Rg_2	Rb_1	Rc	Rb_2	Rd	Total
Leaves	1.078	1.524	—	—	0.184	0.736	0.553	1.113	6.188
Leafstalks	0.327	0.141	—	—	—	0.190	—	0.107	0.765
Stem	0.292	0.070	—	—	--	—	0.397	—	0.759
Main root	0.379	0.153	0.092	0.023	0.342	0.190	0.131	0.038	1.348
Lateral roots	0.406	0.668	0.203	0.090	0.850	0.738	0.434	0.143	3.532
Root hairs	0.376	1.512	0.150	0.249	1.351	1.349	0.780	0.381	6.148

of each 0.1 μg of the ginsenosides Rf, Rd and Rc are shown.

Distribution of ginsenosides in the ginseng plant

With this new HPLC-method one can trace exactly where and how the ginsenosides are located in a plant. Table IV demonstrates the average values which we have found for 20 Panax ginseng C.A. Meyer plants (4 years of age, from the region Kae Song, Korea).

The individual ginsenosides appear in different quantities in the different parts of the plant. The leaves contain mainly ginsenosides Rg_1, Re, Rd, the root hairs mainly ginsenosides Re, Rb_1, Rc. These results confirm and complete the results which have been found by the working team of Tanaka et al. (7, 21).

Chairman: Now the time is open to discussion.

Questioner (from Korea): In your HPLC of ginsenoside, have you ever used any internal standard?

Soldati: I didn't use any standard because I didn't place any standard in the chromatogram.

Questioner (from Korea): I think that volume of injection and the condition of instrument might be subject to change in the past time. How do you control that?

Soldati: We made the standard deviation of their attenuation time of each ginsenoside and we didn't use any interanl standard.

Questioner (from switzerland): On your last slide, we could see that the leaves of the Panax ginseng have a very high quantity of ginsenoside. This question is not to you but to our friends of Korea, Why are the leaves not used?

Someone: In leaves there are only a type of ginsenoside of Re, Rg_1, but there are not Rb ginsenoside.

Questioner (from switzerland): And another question is you can now separate about 8 to 10 ginsenoside and each was estimated quantitatively. What do you consider that we must now standardize which ginsenosides are in the ginseng roots?

Soldati: I think professor Tanaka made also publication about chromatography and I think the standardization of six ginsenoside Rb_1, Rb_2, Rc, Rd, Re, Rg_1 is important. The minor ginsenosides they are very small amount. It is also possible in the chromatogram to see that it is very probably that there are other minor ginsenoside in the roots.

Questioner: I am very appreciated your doctrine. And now I ask one thing. According to your explanation, ginseng leaves and roots have more precious field of saponins. I have question about it. Total 6.0 means all in two kinds of leaves and roots have. It's right?

Soldati: Yes, theoretically it's possible, but you must consider the light of the leaves and the roots have. You have only small light of leaves and the roots have and to produce ginseng produce is not so convenient.

References

1. The United States Pharmacopeia XIX, USP Convention, Inc., Rockville, Md., USA.
2. Sticher, O. and F. Soldati: Planta med. 36, 30 (1979)

3. Wagner, H. and A. Wurmböck: Deutsch. Apoth Ztg. 118, 1209 (1978)
4. Soldati, F.: GPL Symposium, September 28, 1979, Miami, USA.
5. Wagner, H.: Apoth. Journal 11, 38 (1979).
6. Betz, V., J. Deumig, H. Hess, C. Köhler and H. Schönmann: Deutsch. Apoth. Ztg. 119, 845 (1979)
7. Besso, H., Y. Saruwatari, K. Futamura, K. Kunihiro, T. Fuwa and O. Tanaka: Planta med. 37, 226 (1979)
8. Meier, B. and O. Sticher: J. Chromatogr. 138, 453 (1977)
9. Sticher, O. and B. Meier: Planta med. 33, 295 (1978)
10. Sticher, O. and B. Meier: Pharm. Acta Helv. 53, 40 (1978)
11. Sticher, O. and F. Soldati: Pharm. Acta Helv. 53, 46 (1978)
12. Sticher, O., F. Soldati and R.K. Joshi: J. Chromatogr. 166, 221 (1978)
13. Sticher, O., F. Soldati and D. Lehmann: Planta med. 35, 253 (1979)
14. Soldati, F.: Thesis ETH Zürich, Nr. 6347, 1979
15. Engelhardt, H.: Hochdruck-Flüssigkeits-Chromatographie. Springer-Verlag, Berlin, Heidelberg, New York, 1977
16. Takiura, K. and I. Nakagawa: J. Pharm. Soc. Japan 83, 298 (1963)
17. Takiura, K. and I. Nakagawa: J. Pharm. Soc. Japan 83, 301 (1963)
18. Takiura K. and J. Nakagawa: J. Pharm. Soc. Japan **83**, 305 (1963)
19. Soldati F. and H. -R. Schulten: J. Pharm. Sci., submitted.
20. Sticher O., F. Soldati, R. K. Joshi and D. Lehmann: Fitoterapia, **49**, 147 (1978)
21. Yahara S., K. Kaji and O. Tanaka: Chem. Pharm. Bull. **27**, 88 (1979)
22. Firm E. Merck, Darmstadt BRD: Leaflet 16/22/10/177 R.