EVALUATION OF THE SAPONIN CONTENT IN GINSENG EXTRACTS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Ezio Bombardelli, A. Bonati, B. Gabetta and E. M. Martinelli
Inverni della Beffa S.P.A-Research Laboratories
Via Ripamonti Milano, ITALY

It is well known that *Panax ginseng* has been widely used all over the Oriental world for many centuries, due to its stimulating properties in a general sense. Our attention towards the components of the extract of this plant has been focused on the saponin constituents which are definitely responsible for the anti-stress and anti-fatigue activity attributed to this plant. In accordance with the literature we have been able to verify that they explicate an action at the adrenal cortex level analogous to certain steroids but through a different mechanism.

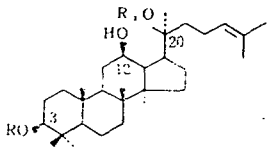
Since the saponins are surely at least one of the active principles of this drug, the first problem to be faced today for the utilization of ginseng extracts in the therapeutic field is the standardization and the analytical definition of these constituents, indispensable condition for a rational therapy and for the reproducibility of the results.

From a chemical point of view, a fundamental contribution to the study of the most important constituents of this plant has been given by Shibata *et al.*, who elucidated the structures of a number of saponins named ginsenosides Rb_1, Rb_2 etc. on the basis of the chromatographic behaviour. These components are derivatives of protopanaxadiol, protopanaxatriol and oleanolic acid and their structures are reported in Fig. 1. From an analytical view point, the gas-chromatography of the trimethylsilylethers of these ginsenosides revealed a valid means for their identification and determination in the extracts.

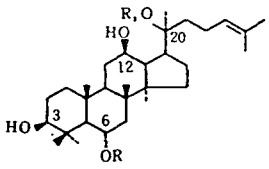
As an example Fig. 2 reports the gas-chromatogram of a ginseng total extract obtained from a six-year-old ginseng root of Korea origin which is the drug used in popular medicine.

In the preliminary phase of our research the experiments have been carried out by investigating the mass spectra and gas-chromatographic properties of the TMS derivatives of the pure compounds. In order to proceed to the analysis of mass spectra, each compound has been silylated, the reagent excess has been directly evaporated in the source and the various spectra have been registered at their respective evaporation temperatures, that is, 140° C for ginsenoside Rg_1, 150° C for ginsenoside Re, etc., at 70 eV.

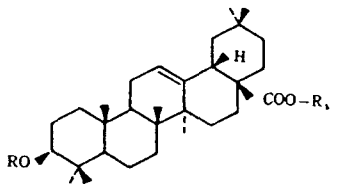
Since the gas-chromatographic analysis is performed at relatively high temperatures, it has been confirmed that the mass spectra obtained by direct introduction on the individual compounds are identical to the spectra obtained through the GLC-MS analysis which had been carried out to investigate possible thermic re-arrangements or demolitions. The compounds showed stability in the given operative conditions; it has therefore been possible to evaluate the homogeneity of the individual peaks and the possibliity of applying



20 S-Protopanaxadiol R = R' = H
Ginsenoside Rb_1 R = D-Glc-β(1 → 2)D-Glc-
R' = D-Glc-β(1 → 6)D-Glc-
Rb_2 R = D-Glc-β(1 → 2)D-Glc-
R' = L-Ara(pyranose)β(1 → 6)-D-Glc-
Rc R = D-Glc-β(1 → 2)D-Glc-
R' = L-Ara(furanose)β(1 → 6)-D-Glc-
Rd R = D-Glc-β(1 → 2)D-Glc-
R' = D-Glc-
Rf_2 R = R' = D-β-Glc-



20 S-Protopanaxatriol R = R' = H
Ginsenoside Re R = L-Rha(1 → 2)D-Glc-
R' = D-Glc-
Rf R = D-Glc-β(1 → 2)-D-Glc-
R' = H
Rg_1 R = D-Glc-
R' = D-Glc-
Rf_1 R' = D-β-Glc-
Rf_3 R' = L-α-Ara(pyranose)(1 → 6)D-β-Glc-
Rg_2 R' = H R = L-Rha(1 → 2)D-β-Glc-



Oleanolic Ac. R = R' = H
Ginsenoside Ro R = D-β-Glc(1 → 2)Glucuronyl
R' = D-β-Glc-

Fig. 1.

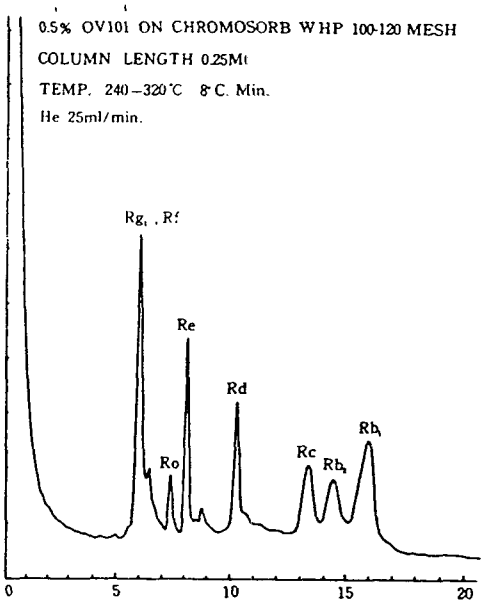


Fig. 2. Gas chromatogram of a crude extract of *Panax ginseng* roots after treatment with the silylating reagents.

this technique to the analysis of ginseng total extracts as they are, or in various pharmaceutical formulations. During the analysis, the peaks due to the ginsenosides have been identified by GLC-MS as well as through compârison with the retention times of standard products. As far as the gas-chromatographic technique is concerned, certain precautions must be taken so as to obtain satisfactory and reproducible separations, due to the low volatility of these substances, most of which have very high molecular weights. The glass columns must be very short and silanised, the carrier gas must be freed from any trace of oxygen present by means of purifiers and the gas chromatograph must have an injector that ensures evaporation of the compound directly in the column and the product must not get in contact with metal parts.

Regarding the mass spectra, the various ginsenosides may be identified on the basis of their characteristic fragmentation patterns. As examples we may take into consideration the mass spectra of one of the ginsenosides of protopanaxadiol, protopanaxatriol and oleanolic acid. In these compounds, the molecular ions are extremely small or absent and generally a high sample pressure has to be maintained in the ion source if the molecular

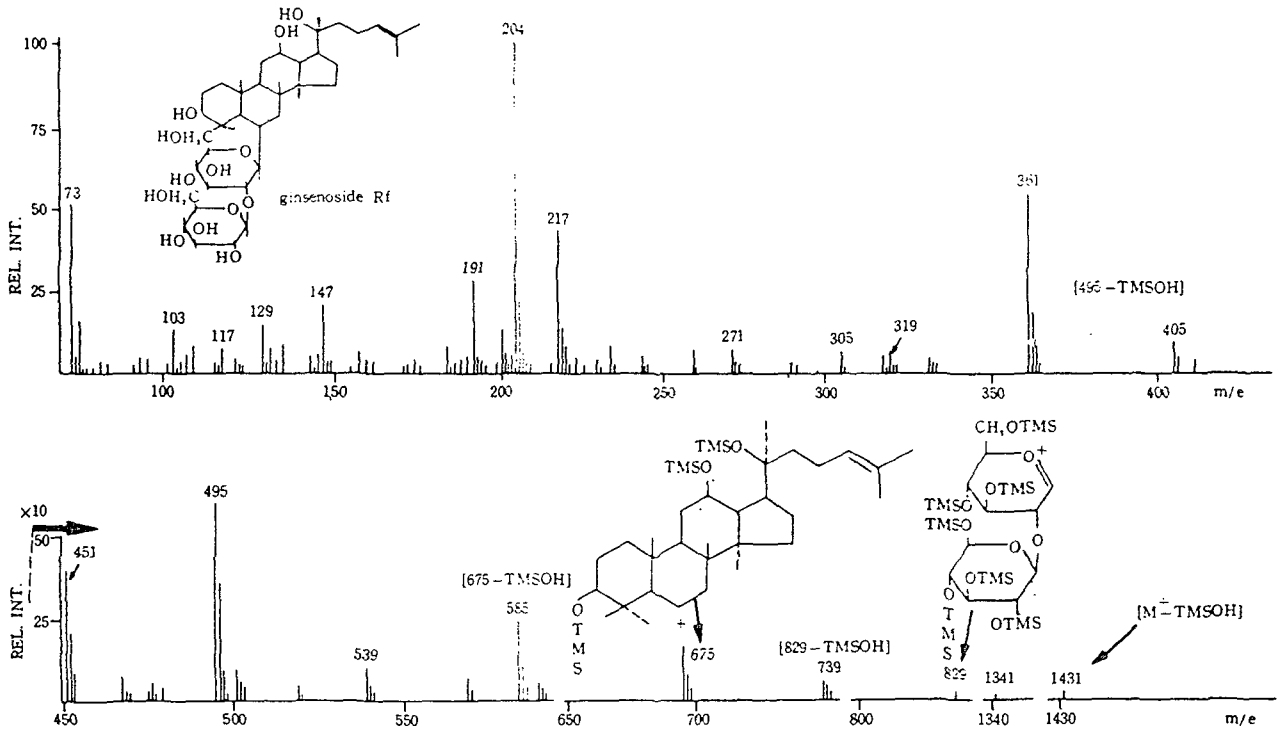


Fig. 3. Mass spectrum of ginsenoside Rf TMS-ether.

ion is to be seen. The important ions present in high mass range are due to the loss of the side chain at C-20. In the MS of the ginsenoside Rf (Fig. 3) the molecular ion is lacking, while the ion resulting from the loss of trimethylsilanol at *m/e* 1431 is present. Another significant peak, in the high region of the spectrum is the ion at *m/e* 829 due to the diglucoside unit. The ion at *m/e* 675 results from the loss of the saccharide unit in the molecular ion.

The ions at *m/e* 585, 495 and 405 result from the loss of trimethylsylanols from the ion at *m/e* 675.

Ions at *m/e* 451 and 361 are due to the trimethylsylilglucose unit, as well as the remaining ions occurring in the first part of the spectrum.

As regards the protopanaxadiol compounds, the ginsenoside Rd spectrum is quoted as example (Fig. 4). The molecular ion is lacking, while the ion at *m/e* 1343, resulting from the sugar loss at C-20, is present.

The ion at *m/e* 829 which is characteristic of the saccharidic unit at C–3 and the ion at *m/e* 497 resulting from the loss of both chains are also present.

The two further protopanaxadiol derivatives Rc and Rb_2, which differ from one another only in the pyranosidic and furanosidic form of the terminal arabinose of the chain at C-20, give rise to the two mass spectra reported in Fig. 5 and 6, respectively. Through the analysis of mass spectra of ions at *m/e* 204 and 217 it is possible to differentiate them. As it is well known, the contribution of the ion *m/e* 217 results from the occurence of the furanosidic unit whereas the contribution of *m/e* 204 is due to the pyranosidic one. As shown by the ratios between the ion at *m/e* 217 and 204 the ginsenoside Rc proves to be the one possessing the pyranosidic unit. As regards the general fragmentation scheme, the compounds behave like the ones above.

A saponin differing from the previous ones is that containing oleanolic acid as aglycone (Ro). After the injection into the gas-chromatography this saponin gives rise to a desglucosidation reaction involving the C–28 carboxyl group and, as

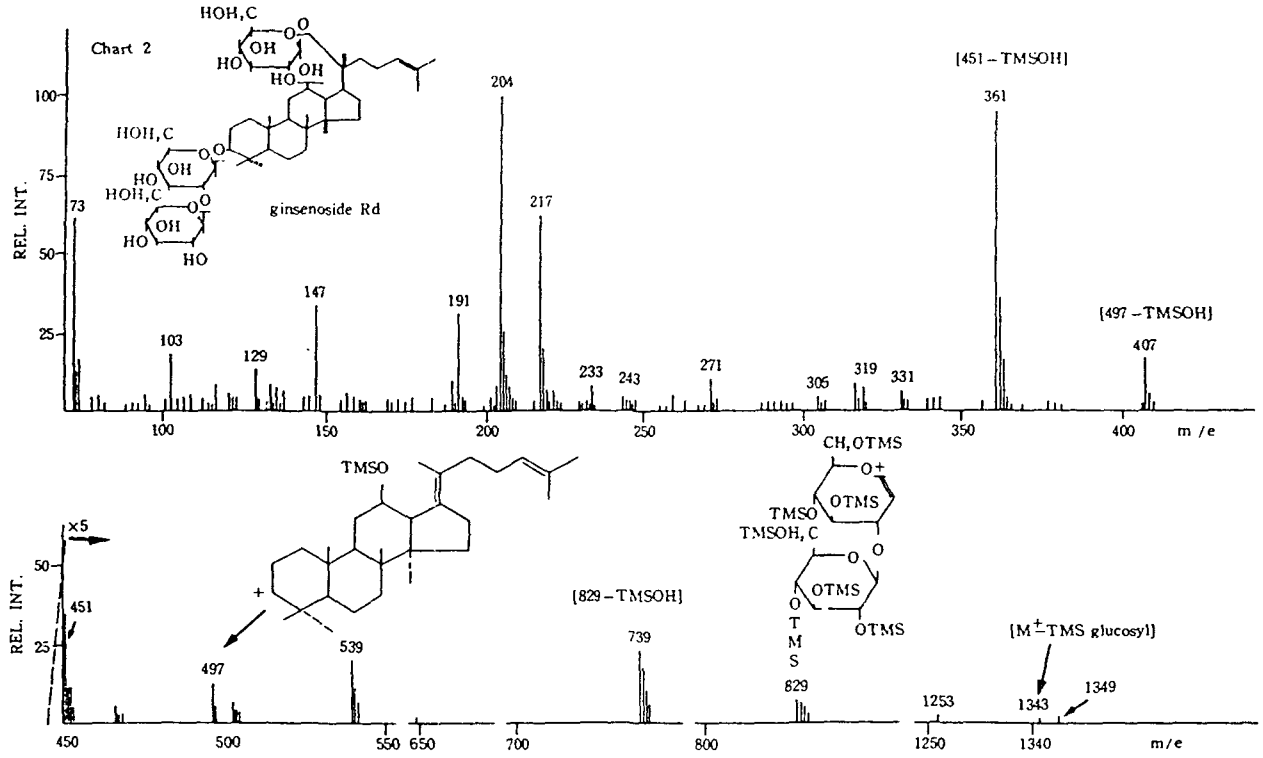


Fig. 4. Mass spectrum of ginsenoside Rd TMS-ether.

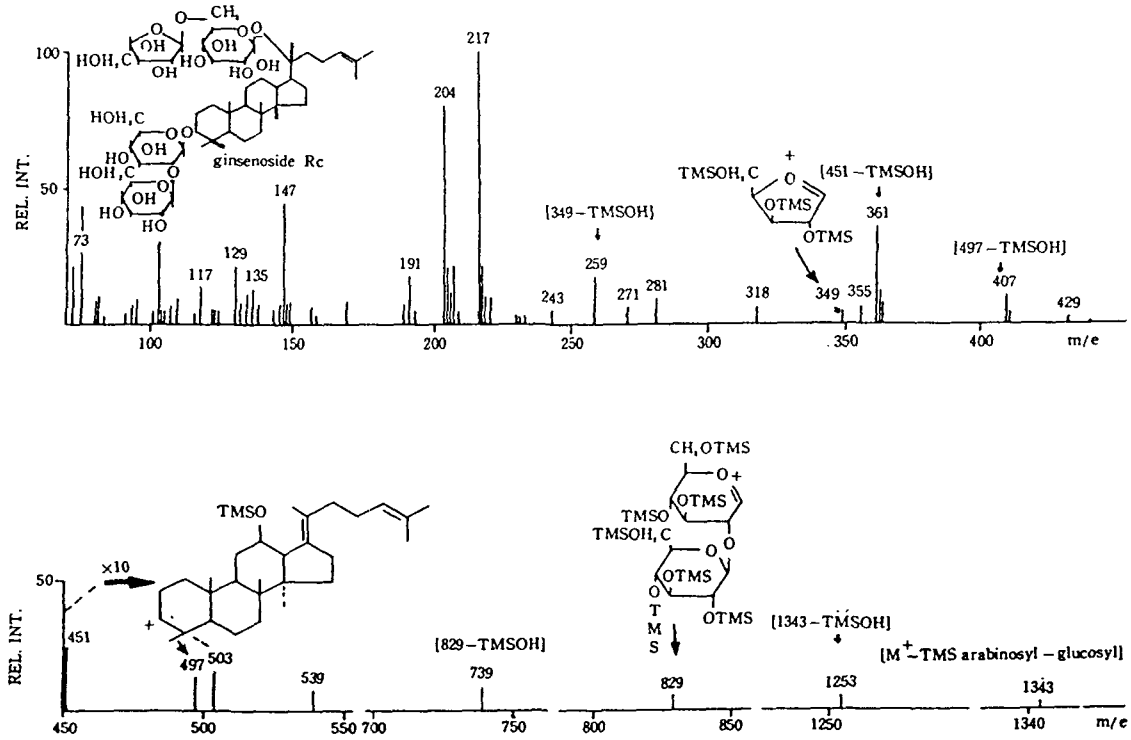


Fig. 5. Mass spectrum of ginsenoside Rc TMS-ether.

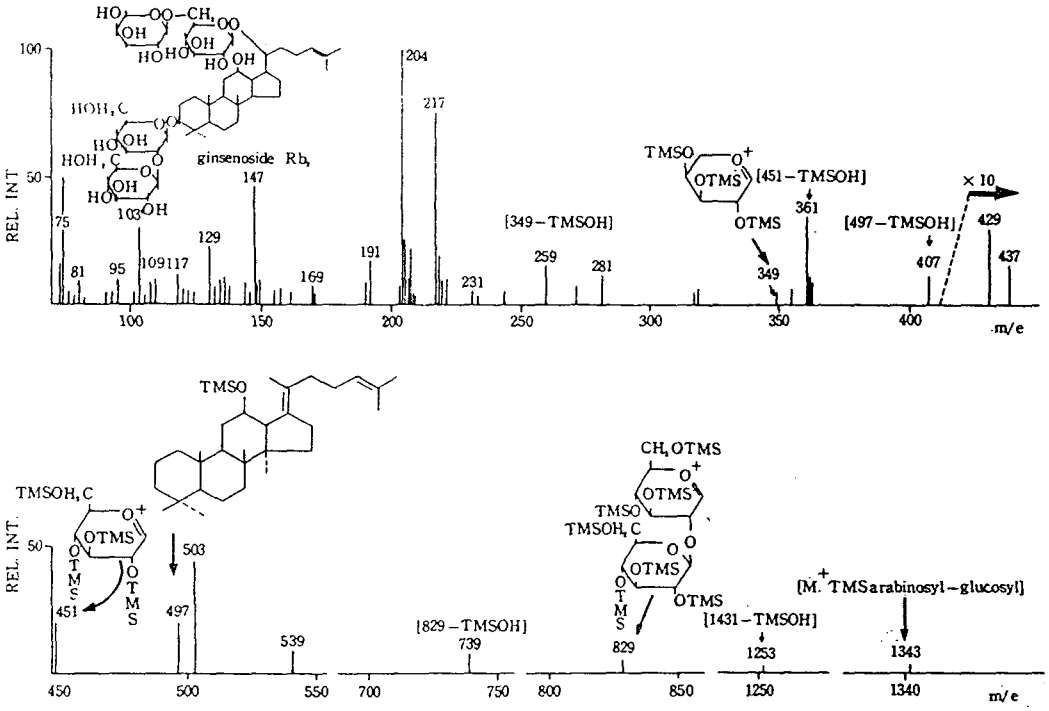


Fig. 6. Mass spectrum of ginsenoside Rb_2 TMS-ether.

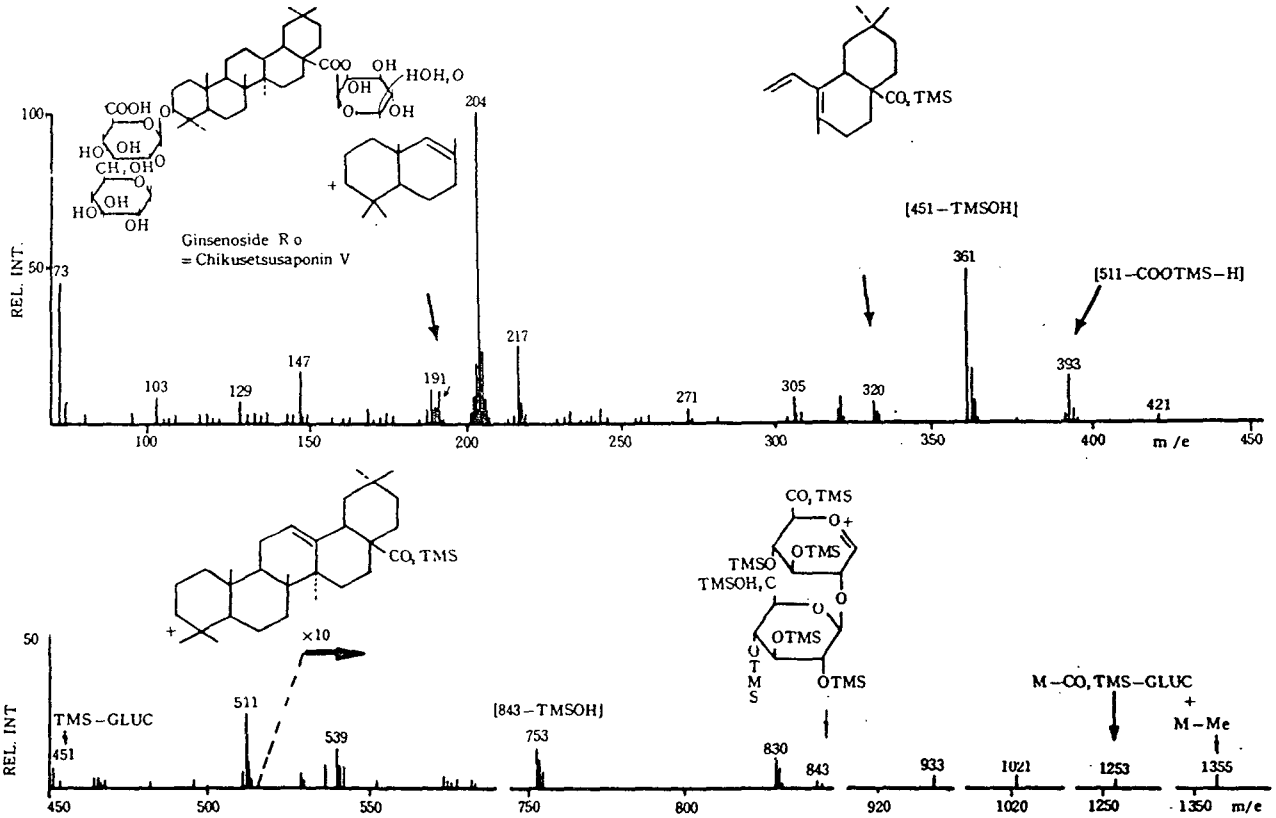


Fig. 7. Mass spectrum of 3-0-glucosylglucuronyl-oleanolic acid TMS-ester obtained after gas chromatography of ginsenoside Ro-TMS ether.

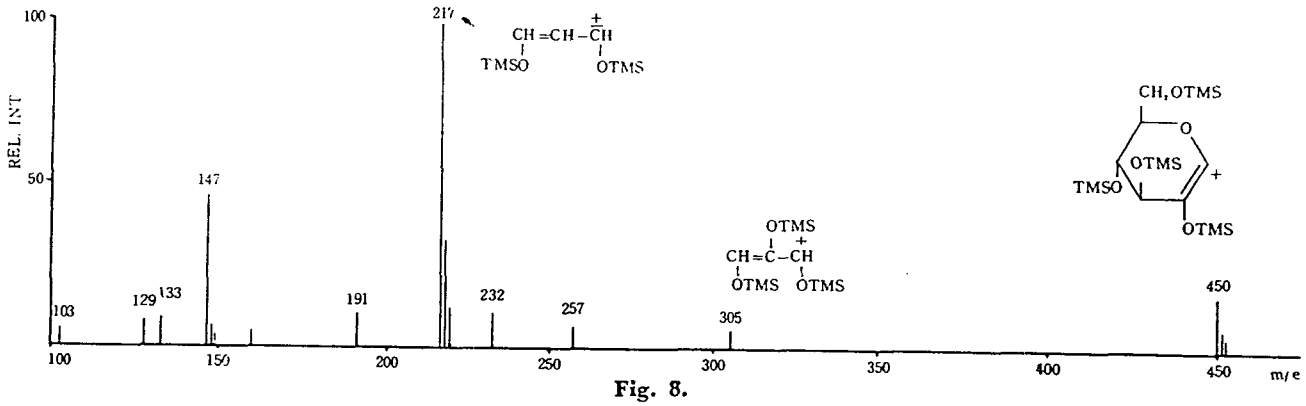


Fig. 8.

consequence, the mass spectrum reported in Fig. 7 is obtained.

The temperature of the injector port takes a very important role in this transformation: we have well documented that this behaviour is characteristic for all the esterosaponins having high molecular weights. In the case of the ginsenoside Ro the peak due to this desglucosidation reaction falls at *m/e* 511. The detached sugar unit can be characterized through an independent GLC-MS experiment and can be assigned the structure reported in Fig. 8 on the basis of its mass spectrum.

Coming back to the gas chromatogram reported in Fig. 1 it is evident that it is posisble to determine quantitatively each saponin by this technique. In fact, we analyse them by using stachiose as an internal standard and preparing a calibration curve for each constituent. The ratio of the area of each ginsenoside peak to the area of stachiose peak is calculated and the obtained value plotted versus the total amount of the relative ginsenoside contained in a standard sample.

The standard curve was computer-estimated by a least squares fit. The amount of ginsenosides in the unknowns is calculated from the extracted standard curve. The program calculates the averaged results, the mean calibration error and confidence limits using a 95 % confidence level.

Fig. 9 reports the calibration curve for ginsenoside Rg_1. Using this technique we noted that the drugs commonly on the market present, from a quantitative point of view, remarkable variations for both total saponins contents and their reciprocal ratios, as it appears evident from the gas chromatograms reported in Fig. 10. So far for the evaluation of the quali- and quantitative saponin content of the ginseng extracts it is advisable, being hard to use a perfect standardisation of each single component, to utilize a more simple way in addition to the GLC technique.

For this reason we suggest a technique which employs a colorimetric reaction, specific for this kind of saponins. The saponins are previously purified through a passage on a XAD4 resin column, which absorbs them selectively, and then eluted with methanol. A portion of the eluted methanol is treated with a solution of antimonium trichloride in acetic acid and perchloric acid. A red coloring develops, the intensity of which is proportional to the ginsenosides content. As reference substance for the calibration curve of this reaction ginsenoside Rg_1 can be used. Therefore the title of the extracts can be given in percentage of saponins expressed as Rg_1. The gas chromatography connected with this technique allows the selection of a drug having a relative composition in ginsenosides as much as possible standardised. Furthermore it also allows the control of possible sophistications of materials of different origin and the rapid individuation of other ginseng species.

As an example the gas chromatograms of the ginseng extracts prepared from the leaves, flowers and young roots of *Panax ginseng* and *Panax*

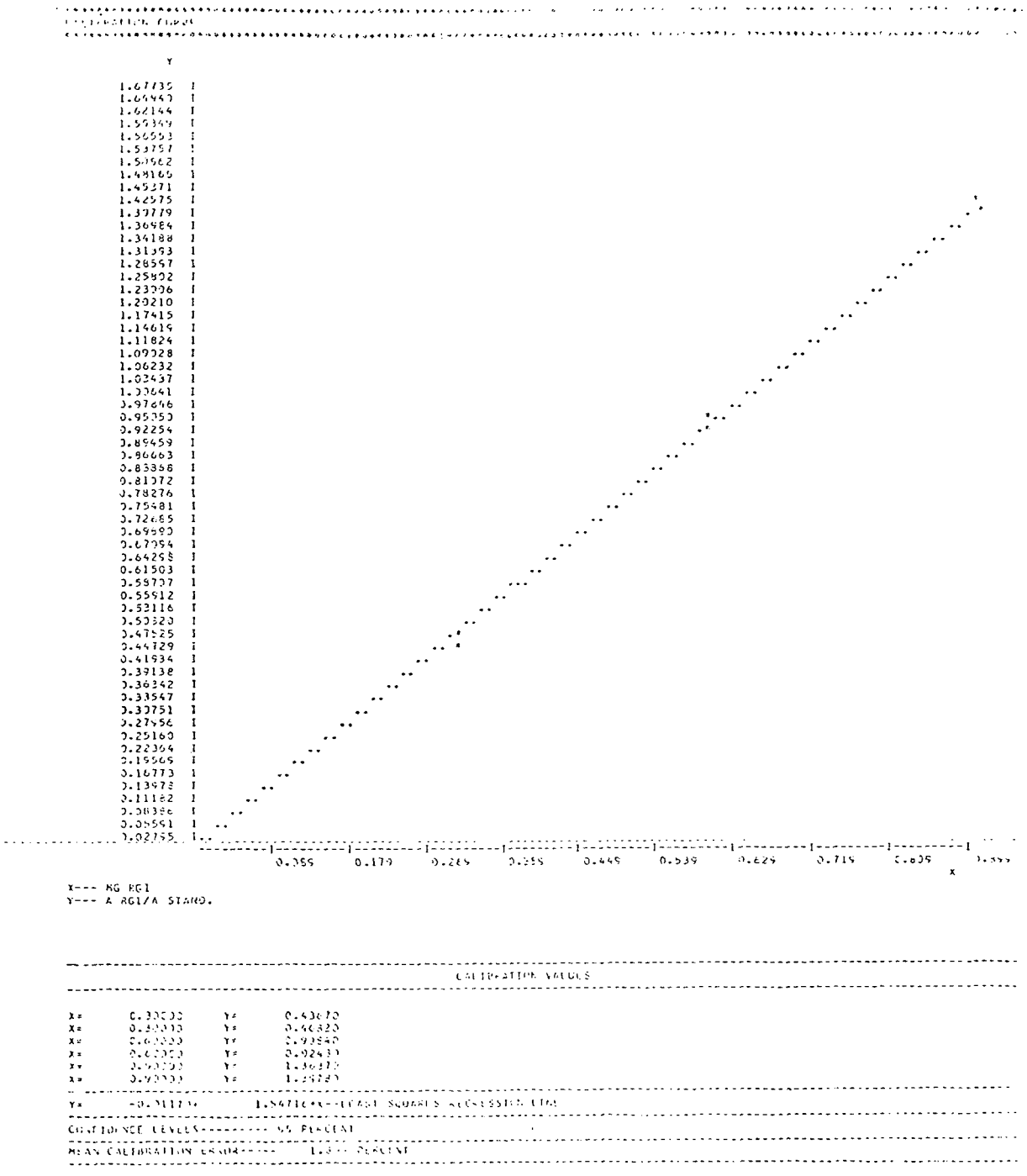


Fig. 9. Calibration curve of ginsenoside Rg_1.

quinquefolium roots are here reported. The gas chromatogram corresponding to the extract of ginseng leaves (Fig. 11) shows the presence of the same ginsenosides contained in the roots (Rg_1, R_1,Rd,Rc,Rb_1,Rb_2), which, however, are present in completely different ratio. It is important to note that ginsenosides Rg_1 and Re are present in quantities even superior to the total saponins content in the roots of best quality.

In addition this extract contains other ginsenosides (Rf_1,Rf_2 and Rf_3) absent in the roots. These ginsenosides already described by Shoji *et al.* have

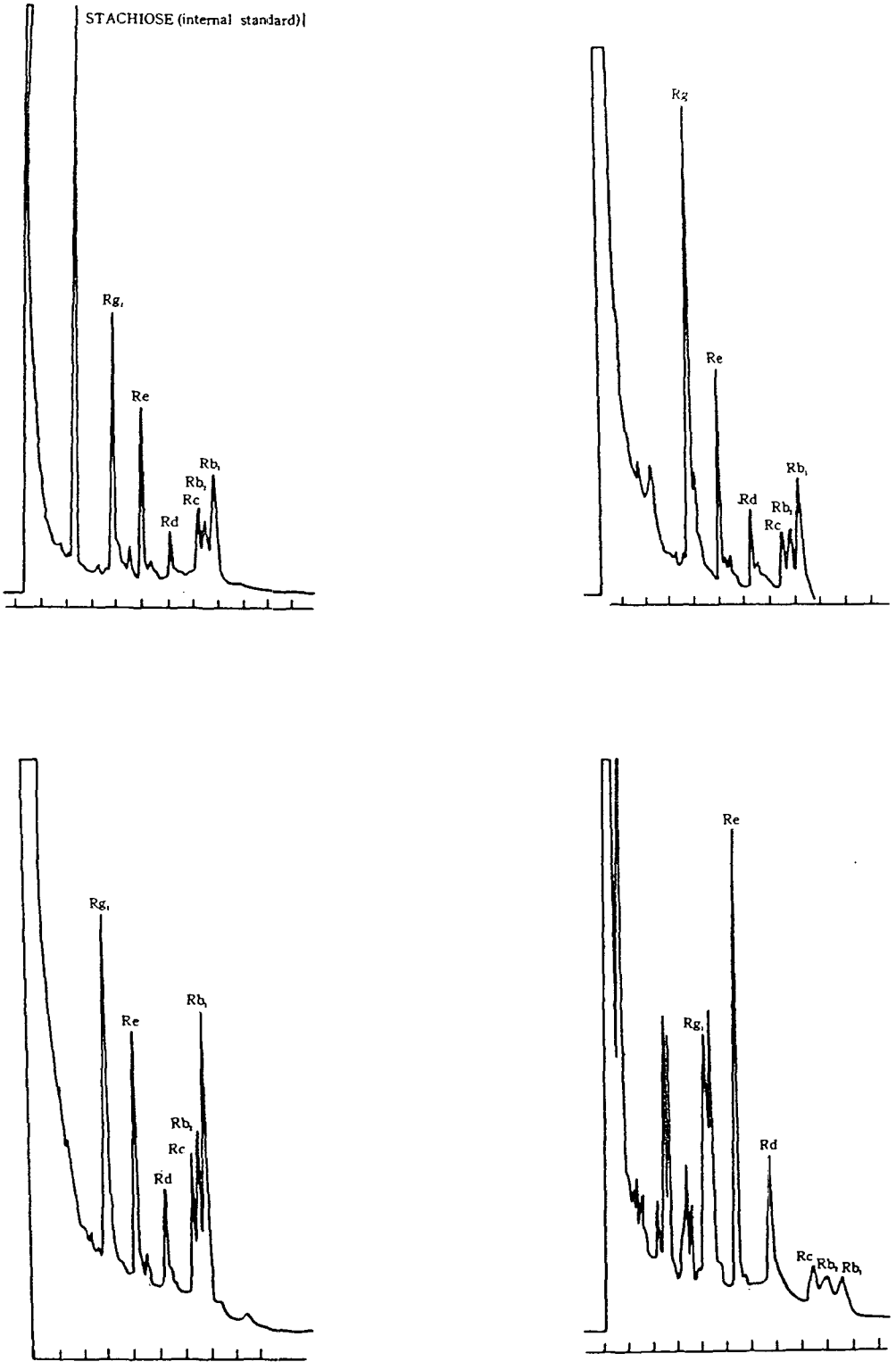

STACHIOSE (internal standard)
Rg1
Re
Rb1
Rb2
Rc
Rd
Rg1
Re
Rd
Rb1
Rb2
Rc
Rg1
Re
Rb1
Rb2
Rc
Rd
Re
Rg1
Rd
Rc
Rb2
Rb1

Fig. 10

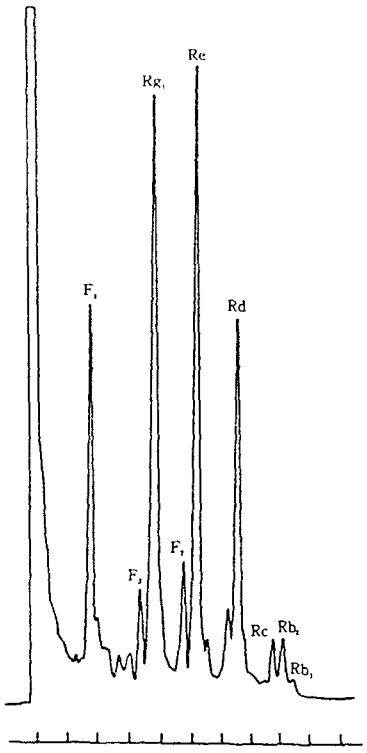


Fig. 11. Gas chromatogram of a crude extract of *Panax ginseng* leaves after treatment with silylating reagents.

been characterised by GLC-MS. Their MS spectra are reported in Fig. 12–14.

Similar situations can be verified in flowers (Fig. 15), where ginsenoside Re is the most abundant one. In several samples of flowers quantities of over 4% of ginsenoside Re have been detected. Ginsenosides Rf_1,Rf_2,Rf_3 and Rg_2 are present also in flowers.

The roots of *Panax quinquefolium* are characterised (Fig. 16) by the lack of the ginsenoside Rb_2 and by the insignificant quantity of Rg_1. The presence of other ginsenosides unknown in *Panax ginseng* is also evident.

In conclusion, these methods permit a rapid examination of an unknown sample from a quali- and quantitative point of view at research level. If the GLC qualitative analysis is carried out by weighing and injecting in the gas chromatograph a sample in a quantity equivalent to that of an extract having a known composition, it is possible also to obtain in few minutes semi-quantitative data about its constitution.

We think that such a method can be usefully employed in the control of cultivations, for the selection of future cultivations dedicated to the production of a single component showing a particular pharmacological activity.

In the pharmaceutical field, the gas chromatographic method permits a rapid assay of the stability of the preparations and a control of eventual sophistications which in the past, in Western countries sometimes had negatively influenced this drug so interesting above all in many syndromes of advanced age.

Experimental

Gas chromatographic analysis

The samples were prepared by dissolving 20 mg of crude ginseng extracts in 0.4 ml of anhydrous pyridine in a screw-capped vial with PTFE cap liners. The resulting solutions were treated with 0.1 ml of TBT (trimethylchlorosilane, N,O-bis-trimethylsilyl) acetamide, trimethylsililimidazole 2:3:3, purchased from Pierce, Rockford, Ill., U.S.A..

The samples were then heated at 60°C for 10 minutes.

A Varian 1,400 aerograph gas chromatograph, equipped with a hydrogen flame ionization detector (FID), was used. The column (0.25 m × 2 mm) of coiled glass tube contained 0.5% OV-101 liquid phase loaded on chromosorb WHP (100-120 mesh).

The temperature of the oven was programmed from 240°C up to 320°C at 8°C/min. The flow of the carrier gas (He) was 25 ml/min. Temperature of injection port: 320°C; temperature detector: 330°C. Injected volume: 2μl.

Mass spectrometry

The spectra were recorded by DIS on Varian MAT model CH7 mass spectrometer. The pure individual ginsenosides were trimethylsilylated as described for GLC analysis using 2 mg of pure ginsenosides. The solution (3μl) was placed in the crucible filled with silanized glass wool.

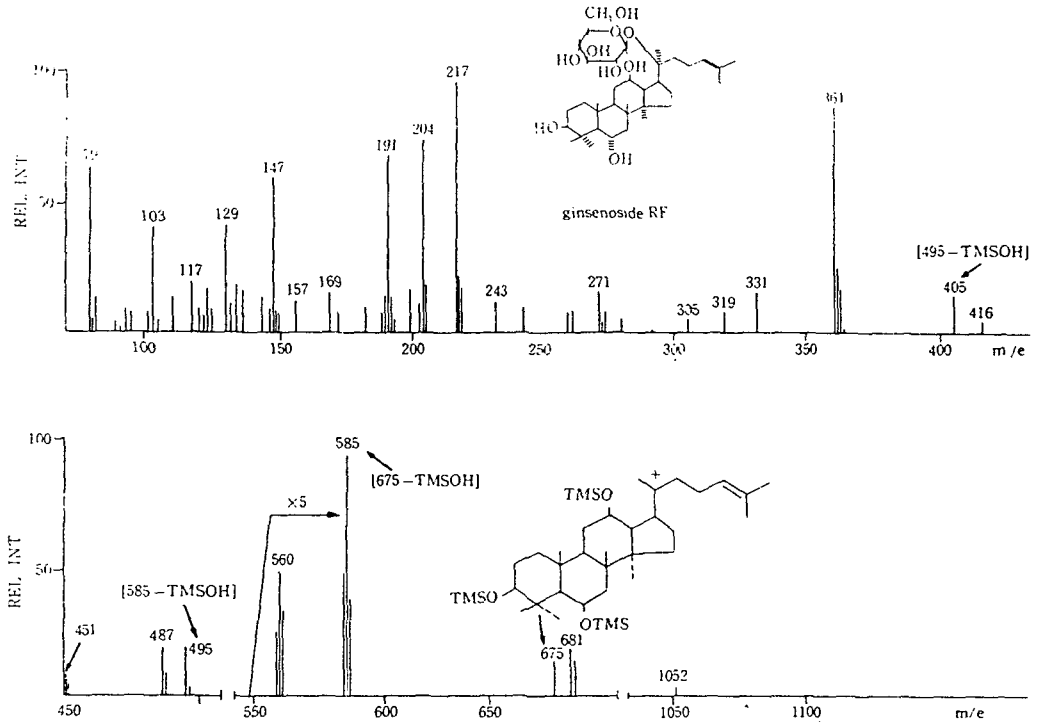


Fig. 12. Mass spectrum of ginsenoside F_1 TMS-ether.

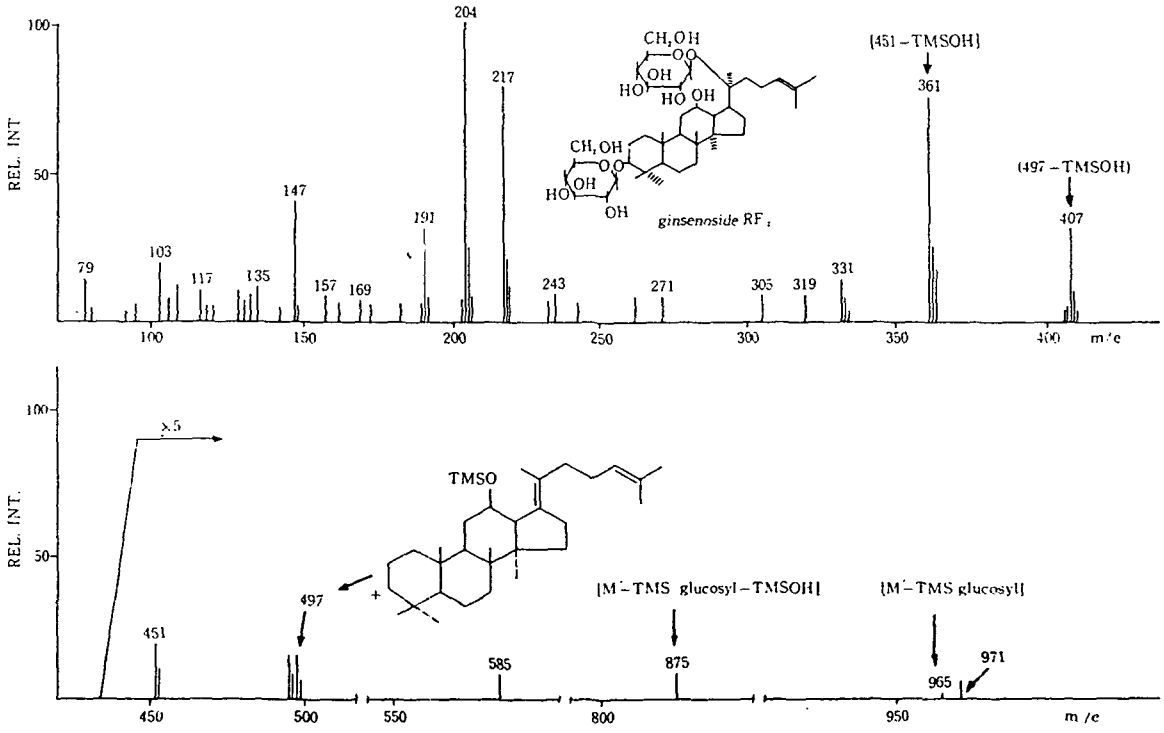


Fig. 13. Mass spectrum of ginsenoside F_2 TMS-ether.

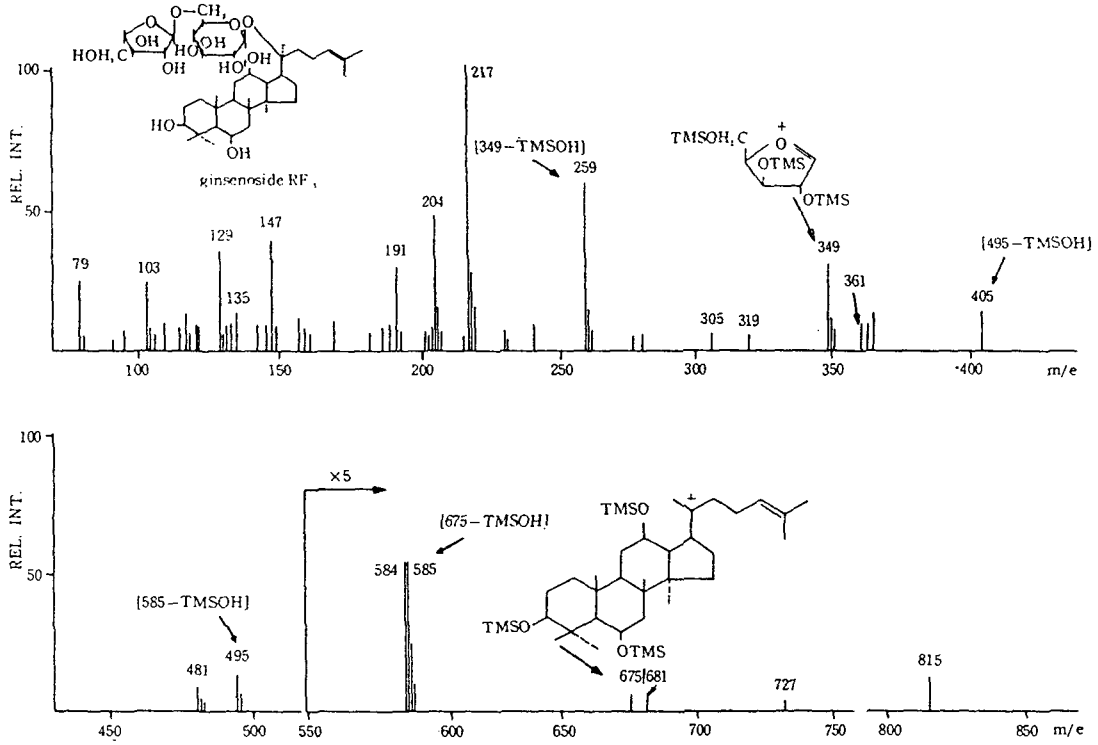


Fig. 14. Mass spectrum of ginsenoside F_3 TMS-ether.

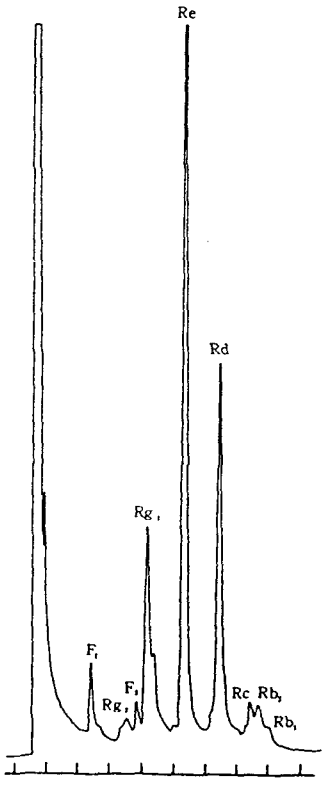


Fig. 15. Gas-chromatogram of a crude extract of *Panax ginseng* flowers after treatment with silylating reagents.

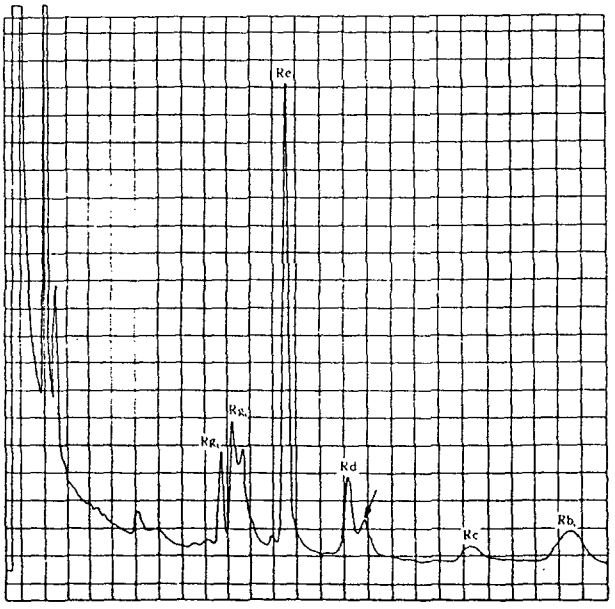


Fig. 16. Gas chromatogram of a crude extract of *Panax quinquefolium* after treatment with the silylating reagents.

The reaction excess reagent in the probe was evaporated at 40° C by the diffusion pump. All the spectra were measured at 70 eV. The temperature of the source was about 200° C. The ion trap current was 300 μA.

Gas-liquid chromatography — Mass spectrometry

The mass spectra were recorded on the Varian mass spectrometer combined with the Varian gas chromatograph.

The temperature of the two-stage jet separator was 360° C and the line off-side temperature 300° C. The values of m/e in the high mass region were determined using perfluorokerosene, tris-(pentadecafluoroheptyl)-S-triazine as internal standards.