

ANALYSIS OF GINSENOSES AT THE NANOGRAM LEVEL BY CHROMATOGRAPHY AND MASS SPECTROMETRY

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To facilitate studies on the possible presence of ginseng products in serum, tissues and excretions, a procedure to optimize the analysis of ginseng specific products i.e. ginsenosides had to be worked out. Two genuine sapogenins, 20 (S)-protopanaxadiol and 20 (S)-protopanaxatriol can be released in a high yield from ginsenosides by using an optimized alkaline cleavage procedure. In contrast to acid hydrolysis, our alkaline conditions caused no epimerization, no hydroxylation, and no cyclization of the side chain. Furthermore, no unchanged ginsenosides were recovered.

The products of alkaline and acidic cleavage were separated and characterized by GC and GC-MS. The C-20-(S) epimers of the ginseng sapogenins could be distinguished from C-20-(R) epimers by difference in mass spectra and retention time. With the present method and data, the ginsenosides in some biological extracts and preparations were analyzed and identified at the nanogram level.

During the last decades, ginseng consumption has expanded to the level of the most commonly used drugs. Although several compounds in ginseng root extracts may exert biological effects(1) it is generally believed that the plant specific compounds i.e. ginsenosides are the compounds mainly responsible for the effects ascribed to this plant root extract(2). Due to lack of suitable analysis methods, it is incompletely known to which extent these compounds are absorbed from the intestine, distributed to the tissues and finally excreted. The acid conditions of the stomach and the alkaline medium in the intestine, indicate that the uptake, distribution and excretion of ginsenosides may occur after complete or partial hydrolysis(3). To simplify an analytical procedure for tissue or body fluids, a release of the sapogenin aglycones from more than a dozen parent ginsenosides and their metabolites seemed to be the most rational approach. In the present work, we have characterized(mass spectrometry and chromatography) the major compounds formed during acid and alkaline cleavage, in case they appear in biological extracts and some preparations.

Methods(4-6) for qualitative and quantitative analysis of sapogenins of ginsenosides in ginseng were based on determination of the two rearranged products, panaxadiol and panaxatriol, that were formed from the genuine sapogenins by epimerization and cyclization under acid hydrolysis of ginsenosides(6, 7). It would be more attractive to analyze the

genuine sapogenins, 20 (S)-protopanaxadiol and 20 (S)-protopanaxatriol. No entirely suitable method has, however, been described for cleavage of the glycosidic bond of ginsenosides that allows quantitative analysis of these compounds.

In this work, a number of the most important ginsenosides were analyzed. The products obtained after acid hydrolysis and alkaline cleavage were separated and identified by GC and GC-MS. The 20 (S)-protopanaxadiol and 20 (S)-protopanaxatriol can be released from ginsenosides in high yield by using optimized alkaline cleavage procedure without epimerization, cyclization or hydroxylation. Furthermore, no unchanged ginsenosides were recovered. The C-20-(S) epimers of ginseng sapogenins and related products can be distinguished from C-20-(R) epimers by difference in mass spectra and retention time.

With the present method, and with use of panaxatriol as internal standard, we have recently analyzed some commercial preparations of ginseng, human urine and feces after ginseng consumption. The results show that despite the alkaline procedure used some preparations contain side products which were otherwise only found as artifacts after acid hydrolysis of ginsenosides. The method was also used to demonstrate presence of small amounts of 20 (S)- and 20 (R)-protopanaxadiol ; 20 (S)- and 20 (R)-protopanaxatriol in human urine after consumption of ginseng.

MATERIALS AND METHODS

Materials

Butanol-1, methanol, sodium hydroxide, sodium methoxide were of analytical reagent grade(Merck, Darmstadt, Germany). Sep-Pak C₁₈ cartridges were from Waters(Milford, MA). Ginsenosides - Rg₁, - Rd, - Ro, - Rh₁, panaxadiol, panaxatriol and oleanolic acid were obtained from Chinese National Inst. for the Control of Pharm. & Biol. Products(Beijing, China). Ginsenosides Rb₁, - Rc - and - Re were obtained from Sigma (St. Louis, MO). N, O-bis(trimethylsilyl)-trifluoroacetamide(BSTFA) was obtained from Pierce(Rockford, IL), trimethylchlorosilane (TMCS) from Fluka(Buchs, Switzerland), trimethyl-silylimidazol(TMSI) from Macherey - Nagel(Düren, Germany).

Instrumentation

GC was performed with a HP 5890A gas chromatograph (Hewlett - Packard Co.) equipped with a flame ionization detector and a DB - 5 column (length 15m, i.d. 0.32mm, film thickness 0.25 μ m). Initial temperature was 220 $^{\circ}$ C for 1 min, it was then increased with 10 $^{\circ}$ C/min. to 300 $^{\circ}$ C, and finally kept constant for 11 min.; column (helium) flow rate 2.0ml/min., injector temperature 275 $^{\circ}$ C, detector temperature 290 $^{\circ}$ C. The split ratio was 1 : 15 and the peak areas were measured with an HP 3396A integrator.

GC - MS : GC - MS was performed with a HP 5890A gas chromatograph combined with a HP 5070B mass selective detector (Hewlett - Packard Co.) and a Hewlett - Packard 5995C GC - MS. Electron energy was 70eV. The column was a 13m, 0.18mm i.d., 0.4 μ m DB - 5. Initial temperature was 180 $^{\circ}$ C for 1.5min, it was then increased with 19 $^{\circ}$ C/min. to 270 $^{\circ}$ C and finally to 300 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min. with a hold for 8min. Injector temperature was 260 $^{\circ}$ C. Transfer Line temperature was 260 $^{\circ}$ C.

Sample preparation

To urine or aqueous extracts of ginseng preparations and feces, internal standard solution (panaxatriol) was added to give a concentration of 15ng/ml urine or 2 μ g/ml extract solution. The liquid (2 - 10ml) were applied to a Sep - Pak C₁₈ cartridge prewashed with 3ml of methanol and 5ml of distilled water. After washing the column with 10ml of water and 15ml of 30% methanol in water, the ginsenosides were eluted with 6ml of methanol. The solvent was evaporated to dryness at 60 $^{\circ}$ C under a stream of nitrogen.

Standard alkaline cleavage procedure

Procedure A : 0.1 - 100 μ g of the standard ginsenosides or total ginsenosides extract (cleaned up by Sep - Pak), 3ml of n - butanol and 0.2g of sodium hydroxide were added to a 10ml test tube with a teflon - lined cap. The reaction was carried out at 90 $^{\circ}$ C for 13h. The butanol phase was washed once with 1.2ml, and twice with 0.7ml of distilled water. The organic phase was evaporated to dryness at 70 $^{\circ}$ C under a stream of nitrogen.

Procedure B is identical to that described in procedure A, except that the sodium hydroxide was replaced with sodium methoxide (100mg) and a reaction time of only 5 h., since this was found to be equally efficient.

Effect of temperature, time course and base concentration during alkaline cleavage

Ginsenoside - Rg₁ was treated with the above procedure at various temperatures (60 - 95 $^{\circ}$ C) and then at a fixed temperature of 90 $^{\circ}$ C for varying time intervals. The reaction was also performed with several base concentrations with sodium hydroxide or sodium methoxide.

Derivatization and determination of the hydrolysis and cleavage products

Trimethylsilylation was performed by adding 50 - 100 μ l of a mixed reagent (BSTFA : TMSI : TMSCI = 3 : 3 : 2 v/v) to a tube containing hydrolysis or cleavage products. The tube was sealed with a teflon - lined cap and then heated for 20min at 70 $^{\circ}$ C. The resulting solution was transferred into a 100 μ l sample vial. An aliquot of the solution was analyzed by GC and GC - MS.

Recovery of extraction

A known amount (10 - 100 μ g) of standard ginsenosides (Rg₁, Rb₁ and Ro) with panaxatriol as internal standard was applied to a Sep Pak C₁₈ cartridge, followed by the sample preparation, the alkaline cleavage and derivatization procedures. The peak areas of TMSi - derivatives of 20 (S) - protopanaxadiol and 20 (S) - protopanaxatriol were compared to those obtained from which had not been purified Sep - Pak C₁₈. The recovery of the column purified reference compounds was between 95 - 99%

Standard solutions

In order to obtain standard curves for quantification of ginsenosides in samples, known amounts of ginsenoside - Rg₁ and - Rb₁ (10 - 100 μ g for GC ; 0.1 - 0.8 μ g for GC - MS) were transferred into a 10ml test tube containing panaxatriol (20 μ g or 0.15 μ g) as an internal standard. Such mixtures were then purified by Sep - Pak, cleaved and derivatized. The TMSi - derivatives of 20 (S) - protopanaxadiol and 20 (S) - protopanaxatriol were determined by GC or GC - MS.

RESULTS AND DISCUSSION

Products of acid hydrolysis of pure ginsenosides

No unchanged ginsenosides or prosapogenins were found after ordinary acid hydrolysis. The genuine sapogenins (20(S) - protopanaxadiol and 20(S) - protopanaxatriol) were always accompanied by ten side products obtained by cyclization, epimerization and hydroxylation of the side chain. Six products were formed from ginsenoside Rg₁ : panaxatriol, 20 (S) - protopanaxatriol, C - 25 - hydroxy - 20 (S) - protopanaxadiol and the corresponding C - 20 epimers. Panaxatriol was the main product. Another six products were obtained from ginsenoside Rb₁ : panaxadiol, 20 (S) - protopanaxadiol, C - 25 - hydroxy - 20 (S) - protopanaxatriol and the corresponding C - 20 epimers. Panaxadiol was the major product. At least thirteen products (Fig. 1) were obtained from the total ginsenosides. In addition to those mentioned above also oleanolic acid was formed. The amounts of C - 2(R) epimers were much higher than those of C - 20 - (S) epimers, possibly because the C - 20(R) epimers are more stable than the C - 20(S) epimers under strong acidic conditions (8).

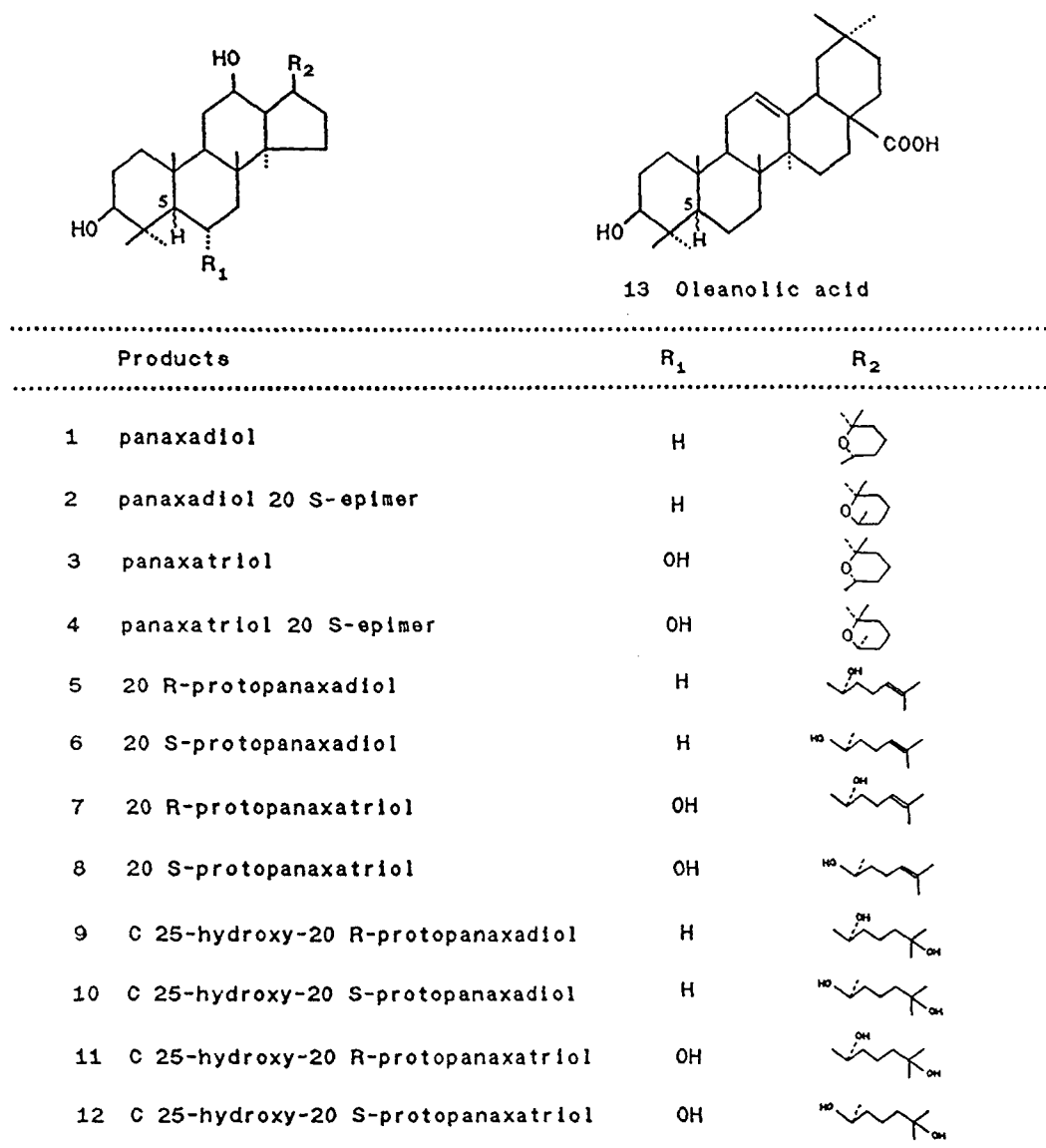


Fig. 1. Structures of cleavage products of ginsenosides. 13oleanol : c acid

Results of the alkaline cleavage of pure ginsenosides

The main products of ginsenosides were 20 (S) - proto-panaxatriol (Rg₁, Re and Rh₁) and 20 (S) - protopanaxadiol (Rb₁, Rc and Rd). No unchanged ginsenosides were found. In contrast to the acid hydrolysis no side products of epimerization, cyclization or hydroxylation were detected. The results suggest that two sapogenins are more stable under alkaline than acidic conditions.

As shown in figure 2, the best yield was obtained at a temperature of 90°C. The optimal duration for the cleavage was found to be 13h. with sodium hydroxide (Fig. 3). It was shown that 100mg of sodium methoxide gave essentially the same result as 200mg of sodium hydroxide under the conditions employed (Fig. 4).

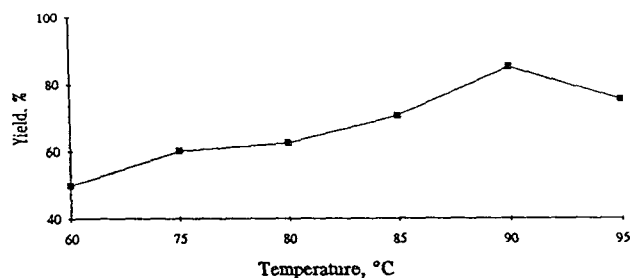


Fig. 2. Effect of temperature on the yield of 20 S - protopanaxatriol obtained from ginsenosides Rg₁ during alkaline cleavage. For details see Materials and Methods.

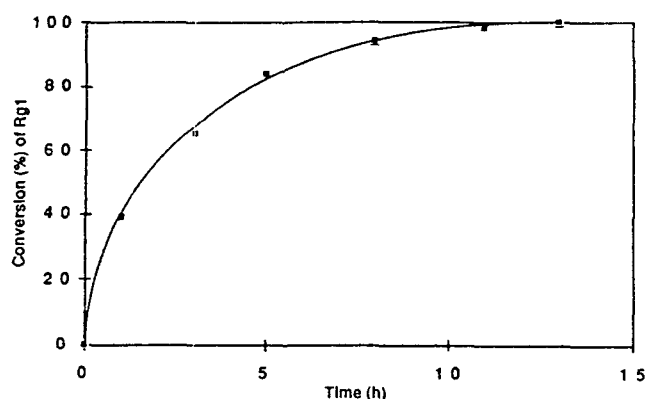


Fig. 3. Conversion of ginsenoside Rg₁, using alkaline cleavage procedure A.

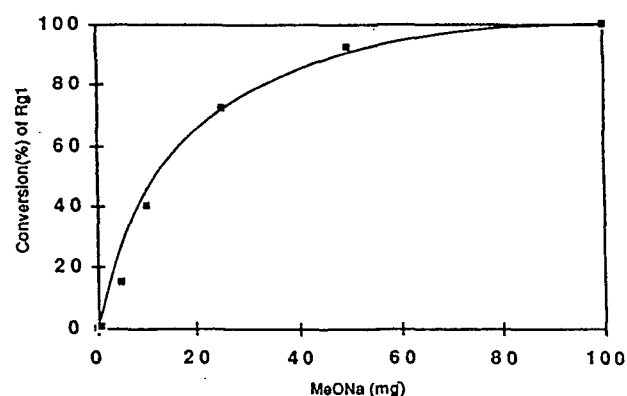


Fig. 4. Effect of MeONa conc. on cleavage of ginsenosides Rg₁ using alkaline cleavage B.

Identification of acid and base cleavage products

As shown previously C20 epimers of protopanaxadiols and triols afforded $M/Z=199$ as their base peaks. Since the ion at $m/z=593$ (or $m/z=681$) and ions related to this one (loss of one or more trimethylsilanol molecules) are more prominent in spectra of the 20 (S) - epimers as compared to 20 (R) - epimers, mass spectrometry allows a distinction between 20 (S) and 20 (R) compounds (Table. 1). In addition the retention times of 20 (S) compounds are somewhat longer than those of the corresponding 20 (R) epimers.

The results of quantitation of ginsenosides

Since the steroid moiety of ginsenosides is 20 (S) - protopanaxadiol or 20(S) - protopanaxatriol the specific detection and quantification of those epimers and the eventual presence of 20 (R) - epimers is important. Such quantifications were possible with the present method, using panaxatriol as internal standard.

Using the Sep - Pak C₁₈ purification procedure, commercial preparations were investigated. Typical results are displayed in figure 5 and 6. It is evident from the figure 5 that some commercial extracts of ginseng contained a mixture of

Table. 1 GC - MS Data of TMSi - derivatives of the Cleavage Products of Ginsenosides

Products	Rt(min)	M.W.	Characteristic ion(m/z)
1. panaxadiol	13.547	604	127(100) 183(4) 409(3) 341(1) 499(2) 589(3)
2. 20 (S) - panaxadiol	13.785	604	127(100) 341(1) 589(1)
3. panaxatriol	14.795	692	127(100) 183(5) 407(2) 497(1) 587(1) 677(1)
4. 20 (S) - panaxadtriol	15.152	692	127(100) 183(6) 497(1) 587(1)
5. 20 (R) - protopanaxadiol	14.935	676	199(100) 323(1) 406(1) 496(7) 593(2)
6. 20 (S) - protopanaxadiol	15.132	676	199(100) 323(7) 413(4) 496(7) 503(3) 593(16)
7. 20 (R) - protopanaxatriol	16.078	764	199(100) 494(1) 584(3) 681(2)
8. 20 (S) - protopanaxatriol	16.279	764	199(100) 321(3) 411(5) 501(4) 681(15)
9. C 25 - OH - 20 R - protopanaxadiol	17.878	766	131(16) 143(100) 199(28) 289(17) 593(6)
10. C 25 - OH - 20 S - protopanaxadiol	18.333	766	131(25) 143(100) 199(27) 289(14) 323(5) 413(4) 503(4) 593(36)
11. C 25 - OH - 20 R - protopanaxatriol	19.122	854	131(14) 143(100) 199(27) 289(21) 681(3)
12. C 25 - OH - 20 S - protopanaxatriol	19.792	854	131(22) 143(100) 199(37) 289(17) 321(5) 411(4) 501(4) 681(26)
13. oleanolic acid	15.446	600	189(44) 203(100) 306(10) 320(56) 482(40) 585(10) 600(11)

20(S) - and 20(R) epimers whereas less 20 (R) compounds was found in tablets or capsules containing pure root powder(Fig. 6). In some cases 20 - (R) -epimers and 25 - OH - products appeared. These products are normally only found after acid hydrolysis of ginsenosides.

The analytical procedure was also adopted to urine and feces from human individuals consuming ginseng. Significant amounts of protopanaxadiols and triols were detected in urine(Fig. 7) indicating that ginsenosides are absorbed and excreted in man. The occurrence of 20(S) - protopanaxadiol and 20(S) - protopanaxatriol in feces(Fig. 8) probably represents

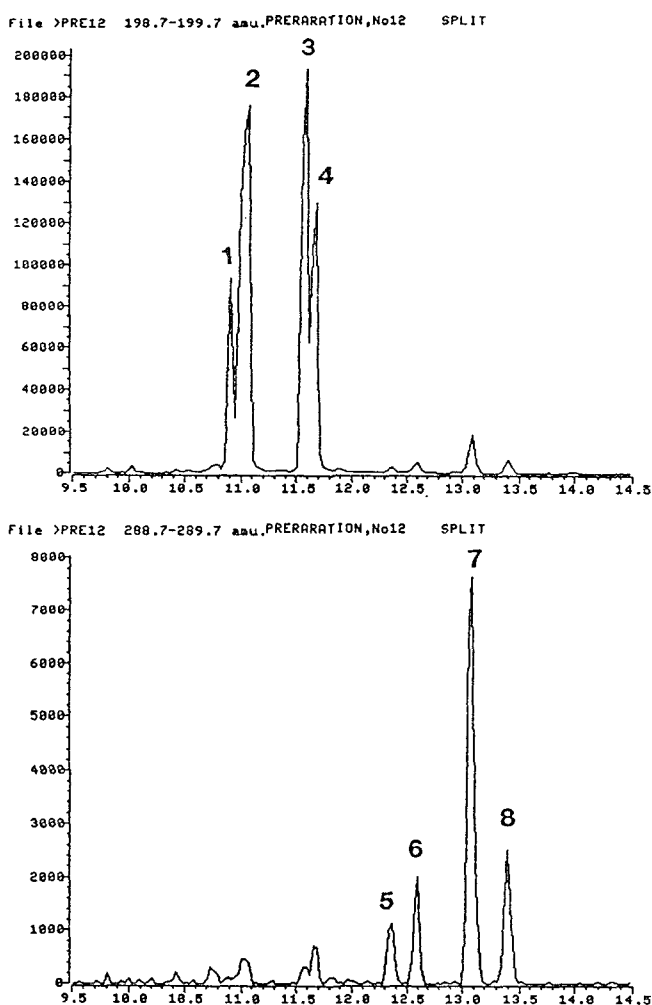


Fig. 5. GC - MS chromatogram of the TMSi - derivatives of products obtained from ginseng preparation. Peak identification : 1, 20R - protopanaxadiol ; 2, 20 S - protopanaxadiol ; 3, 20 R - protopanaxatriol ; 4, 20 S - protopanaxatriol ; 5, C - 25 - hydroxy - 20 R - protopanaxadiol ; 6, C - 25 - hydroxy - 20 S - protopanaxadiol ; 7, C - 25 - hydroxy - 20 R - proto-panaxatriol ; 8, C - 25 - hydroxy - 20 S - protopanaxatriol.

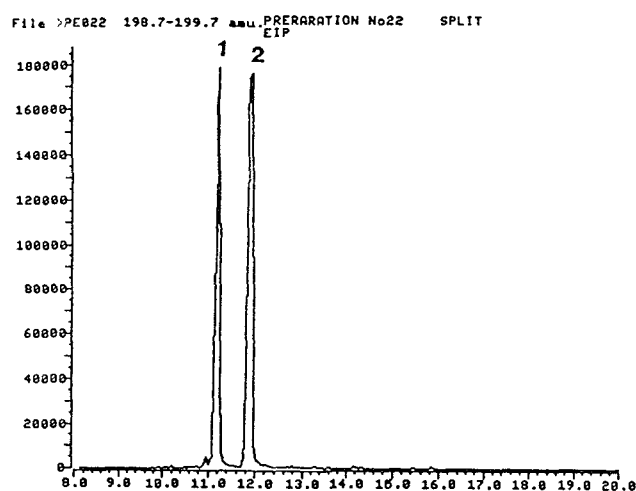


Fig. 6. GC - MS chromatogram of the TMSi - derivatives of products obtained from ginseng preparation. Peak Identification : 1, 20 S - protopanaxadiol ; 2, 20 S - protopanaxatriol

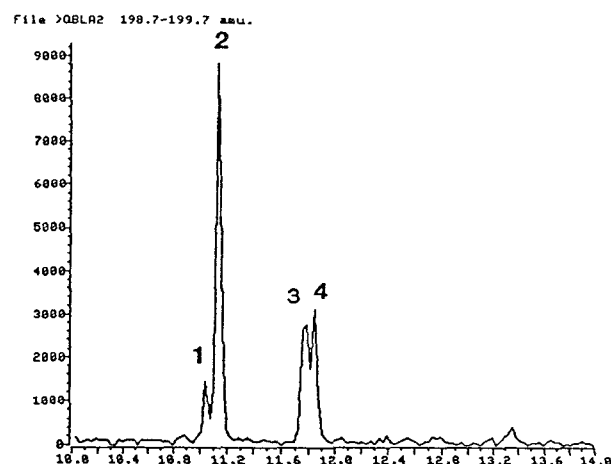


Fig. 7. GC - MS chromatogram of the TMSi - derivatives of products obtained from human urine after administration ginseng preparation Peak identification : 1.20R - protopanaxadiol ; 2, 20 S - protopanaxadiol ; 3.20 R - protopanaxatriol ; 4.20 S - protopanaxatriol.

unabsorbed ginsenosides or their gastrointestinal hydrolysis products(3). The amounts of 20(S) - protopanaxadiol and 20 (S) - protopanaxatriol found in the urine immediately after the administration was about 30ng/ml and 40ng/ml, respectively. The corresponding levels for feces were about 10µg/g feces and 10µg/g, respectively.

These preliminary results do not exclude the possibility that, after absorption, the protopanaxadiols and protopanaxa-

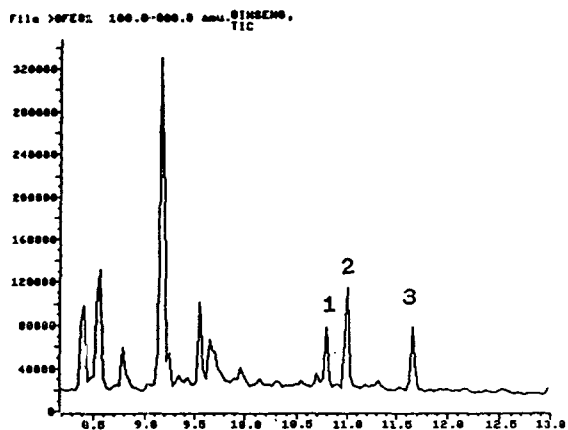


Fig. 8. GC-MS chromatogram of the TMSi-derivatives of products obtained from human feces after administration of ginseng preparation. Peak identification 1, panaxatriol(internal standard), 2, 20 S-protopanaxadiol ; 3, 20 S-protopanaxatriol.

triols are metabolized(as conjugated form) before excreted. Work on possible metabolism of ginsenosides is now performed in our laboratories.

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