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ISOLATION AND GC-MS DETERMINATION OF FLAVONOIDS FROM *Glycyrrhiza glabra* ROOT

S. B. Denisova, E. G. Galkin, and Yu. I. Murinov

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Components of the ethylacetate extract of Glycyrrhiza glabra *root were separated based on their acid—base properties. The major components of the fraction soluble in aqueous NaOH were identified by GC-MS as glabridin, the principal component of the ethylacetate extract of licorice root (*Glycyrrhiza glabra *L.), 4-*O*-methylglabridin, and hispaglabridin B. The isoflavene glabrin was identified in addition to the isoflavan derivatives.*

Key words: *Glycyrrhiza glabra* L., Leguminosae, licorice, isoflavone, glabridin, hispaglabridin B, 4′-*O*-methylglabridin, GC-MS.

Crude extracts of licorice root (*Glycyrrhiza glabra* L., Leguminosae) contain many components, mainly a mixture of triterpenes, carbohydrates, and various classes of phenolic compounds. This is due to the fact that flavonoid (phenolic) compounds form a solubility series at one end of which lie those soluble in ether but insoluble in water such as nonglycosylated highly methylated derivatives and, at the other, glycosides that are insoluble in ether but soluble in water and contain up to three sugar units. In view of the great differences in the solubility of these compounds in water and organic solvents, there is no simple extraction method that is ideally suitable for separating them from the natural material [1]. According to the literature, methods used by researchers to isolate, identify, and analyze phenolic compounds are based mainly on their polarity, which is determined by both the number and steric features of the functional groups in the molecules. The difficulties that arise are due to the variety of isomeric structures involving the aromatic hydroxyls.

Chromatographic analysis of complex mixtures of phenolic compounds from plant extracts is also fraught with significant difficulties in separating glycosides, aglycons, and accompanying coumaric acids, esters, etc. with similar structures. Therefore, combined methods that use preliminary fractionation (extraction or chromatographic) followed by separation using one of the chromatographic methods were attempted.

A wide range of solvents, ether, benzene, methylenechloride, dichloroethane (DCE), $CHCl_3$, acetone, ethylacetate (EA), and ethanol, have been used to extract the total aglycons of flavonoids. We selected EA as the extractant because it is one of the most frequently used solvents that extracts from plant material (licorice root) not only nonglycosylated flavonoids but also coumaric and phenolic-carboxylic acids and coumarins [1-3]. By using the process conditions optimized by us, the yield of the EA extract of licoric root was 4% of the air-dried weight of the raw material.

We developed a scheme for preliminary separation of phenolic compounds of the EA extract as a function of their acid—base properties. We used the part of the EA extract that was soluble in DCE (about 60%) for the fractionation. The part of the extract that was soluble in DCE was extracted successively with aqueous solutions of Na_2CO_3 and NaOH to separate compounds with different acid—base properties. The so-called "acidic" compounds (phenolic-carboxylic and coumaric acids) that were extracted by aqueous Na_2CO_3 made up 14-15% (fractions 11 and 14) of the total. The amount of "weakly acidic" phenols or aglycons of flavonoids that were extracted by NaOH turned out to be slightly higher, 23% (fractions 8 and 9). "Neutral" compounds (nonpolar) were practically concentrated in fraction 6, >13%.

Institute of Organic Chemistry, Urals Scientific Center, Russian Academy of Sciences, 450054, Bashkortostan, Ufa, fax (3472) 35 60 66, e-mail: denisovas@anrb.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 236-240, May-June, 2006. Original article submitted September 15, 2005.

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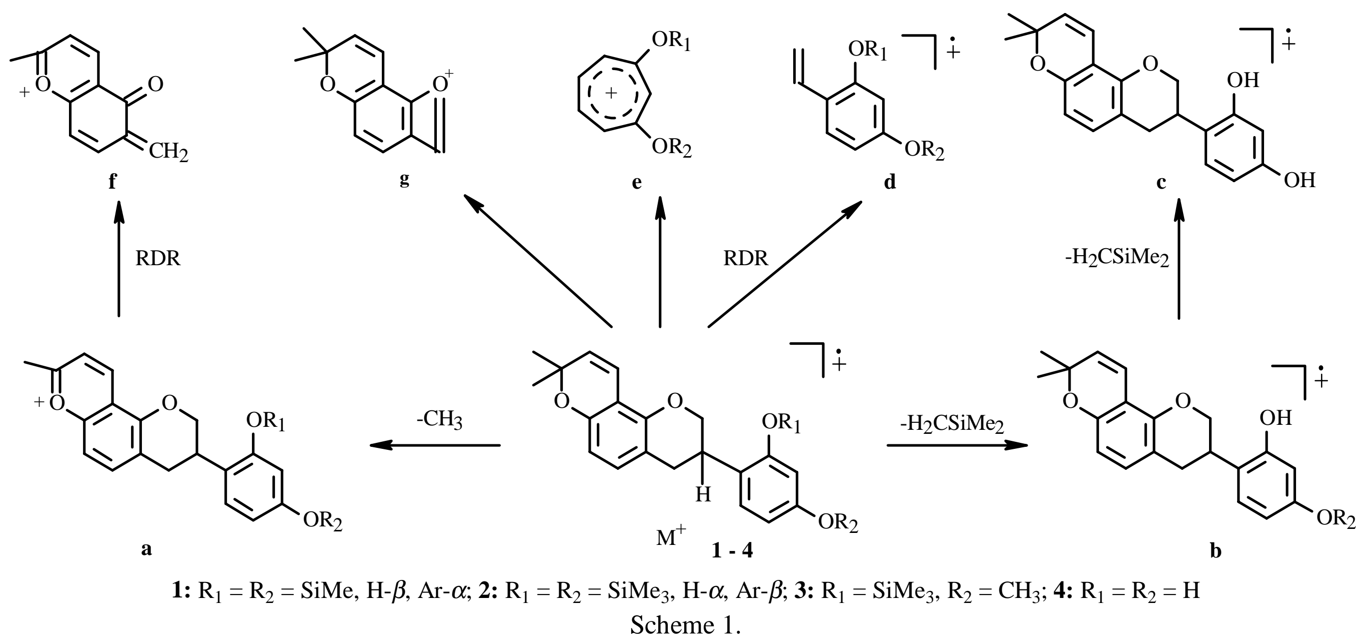
TABLE 1. *m/z* Values and Relative Intensities (%) of Diagnostic Fragments in Mass Spectra of Compounds **1-5**

Compound	Ion type									
	M^+	**a**	**b**	**c**	**d**	**e**	**f**	**j**	**h**	(**c**-CH_3)$^+$
1	468 (20.1)	453 (100)	395 (4.2)	323 (1.2)	280 (30.5)	267 (33.4)	187 (4.5)	173 (65.7)	73 (70.2)	307 (1.8)
2	468 (0.8)	453 (5.2)	395 (2.5)	322 (32.3)	280 (4.1)	267 (6.2)	187 (3.5)	173 (20.5)	73 (12.1)	307 (100)
										(**b**-CH_3)$^+$
3	410 (0.8)	395 (3.2)	338 (27)	-	222 (1.3)	209 (7.2)	187 (77.2)	173 (93.6)	73 (28.3)	323 (100)
4	324 (22.3)	309 (100)	-	-	136 (8.5)	123 (4.6)	187 (33.5)	173 (8.1)	-	-
5	462 (32.6)	447 (95)	-	-	274 (10.5)	261 (72.3)	187 (32.0)	173 (100)	73 (88)	-



1: $R_1 = R_2 =$ SiMe, H-β, Ar-α; **2:** $R_1 = R_2 = SiMe_3$, H-α, Ar-β; **3:** $R_1 = SiMe_3$, $R_2 = CH_3$; **4:** $R_1 = R_2 =$ H

Scheme 1.

Flavonoids were qualitatively identified using electron-impact mass spectrometry. Owing to the complex composition of the extracts, highly effective and sensitive gas chromatography-mass spectrometry (GC-MS) was more informative for resolving questions of structure and analysis. We prepared silyl derivatives [4] for the analysis by replacing active H atoms in the hydroxyls by trimethylsilyl (TMS). The presence of TMS substituents in the molecule should affect the decomposition pathway of the molecular ions (MI) and change the information value of the mass spectra.

A group of 7-10 peaks corresponding to the main part of the extract is clearly visible in the GC chromatogram of the TMS derivatives of fraction 8. Mass spectra of components for which the area of the chromatographic peaks was at least 1-2% of the total area (total content of these components was ~91%) were interpreted.

The MI of the flavonoids dissociate upon electron impact through a limited number of assumed pathways and, as a rule, the origin of diagnostically valuable fragments is explained by a retro-diene reaction (RDR) in the MI and fragment ions [5, 6]. Cleavage forms a set of fragments including the A- and B-rings. The combination of the mass values of the set (*m/z*) and their relative intensities are characteristic of each class of flavonoids [5-7].

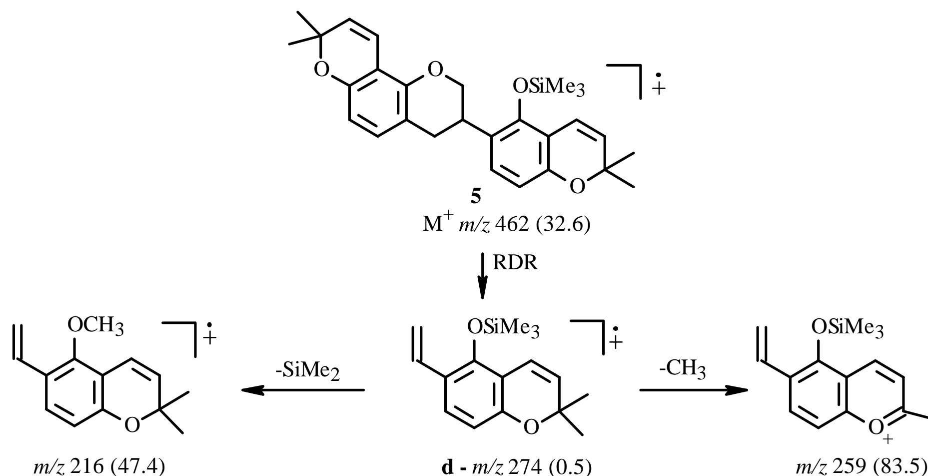
Mass spectra of compounds with TMS groups should give fragments with *m/z* 73, $[\cdot SiMe_3]^+$. The number of Si atoms in the molecule was determined reliably using known ratios for the distribution of ^{28}Si and ^{30}Si.

One peculiarity of the fragmentation of MI of silylated flavonoids **1-6** is the fact that it can occur through two competing pathways. The first is based on a RDR in the MI and in $[M - CH_3]^+$:

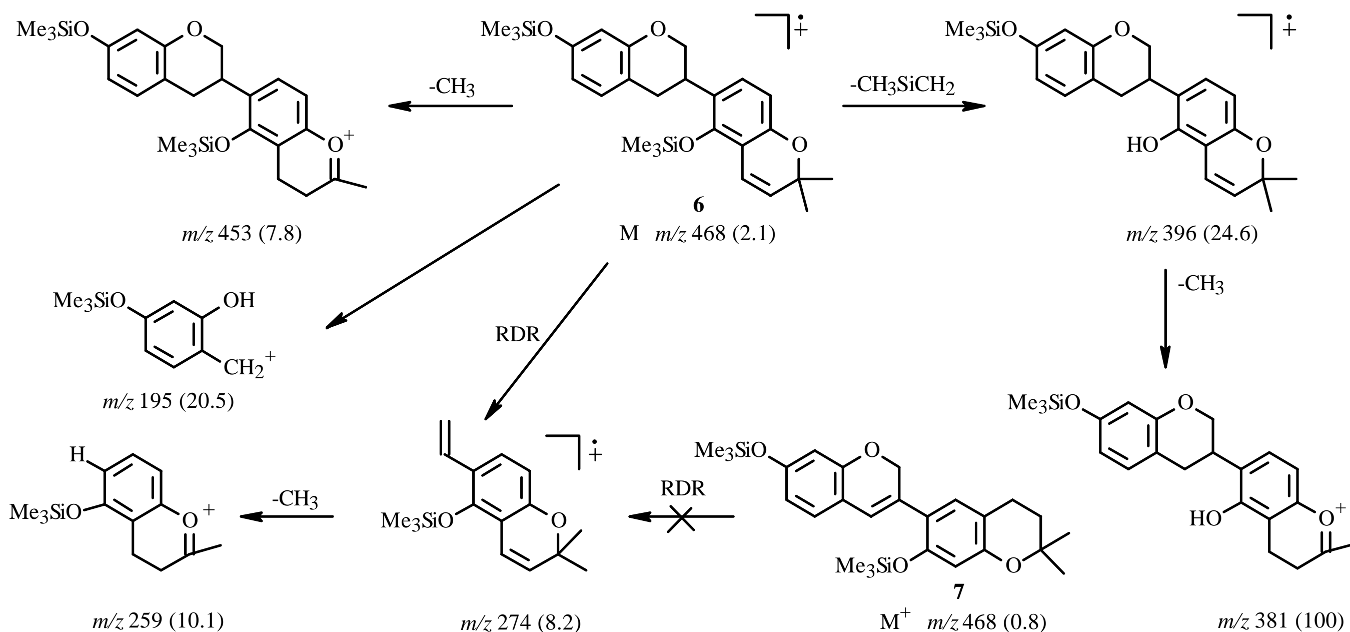
$$MI \xrightarrow{RDR} \textbf{d}\text{-type ions}; [M - CH_3]^+ \xrightarrow{RDR} \textbf{f}\text{-type ions}$$

and cleavage of the pyran ring at the third C atom to give peaks for **g**-type ions and tropylium (**e**).

The other pathway is due to successive cleavage of a silyl substituent as CH_2SiMe_3 or $\cdot SiMe_3$ (fragments **b** and **c**) and suppression of the RDR (Schemes 1-3, Table 1).



Scheme 2.



Scheme 3.

Schemes 1-3 illustrate well the formation pathways of the diagnostic fragments (**a**-**h**) in **1**-**7**.

Table 1 gives the set of diagnostically valuable fragments in the mass spectrum of the component with τ_R = 13.86 min (**1**). A comparison with the literature [7] showed that this component can be identified as the TMS derivative of the isoflavan glabridin.

Measurement of the ratios of peak intensities I$[M + 2]^+$/I$[M]^+$ and I$[M - CH_3 + 2]^+$/I$[M - CH_3]^+$ indicates that the molecule contains two Si atoms. The intense peaks for ions with *m/z* 73 (**h**) are, of course, due to $[\cdot SiMe_3]^+$. Fragments with *m/z* 280 (**d**) and 173 (**f**) arise from a RDR. Fragments of the **e**-type with *m/z* 267 confirm that the aromatic ring is bound to ring A and that ring B contains two TMS groups.

The decomposition of the TMS component with τ_R = 13.97 min (**2**) demonstrates the predominance of the second fragmentation pathway: **c**, *m/z* 322 (32.3), $[M - CH_3SiCH_2]$. Moreover, the spectrum of **2** exhibits the same set of characteristic fragments as that of **1**. Differences in the probability of forming fragment ions in the compounds with τ_R = 13.86 min and τ_R = 13.97 min indicate that they are most probably spectra of stereoisomers relative to the position of a H atom in the isoflavan

heterocycle [axial (Hβ and Arα) and equatorial (Hα and Arβ)]. It is known [8] that the size of the equatorial substituent is responsible for the stability of the MI ($W_M = I_{[M]^+}/\Sigma I \cdot 100\%$) of stereoisomeric pairs. The value of W_M of component **1** = 4.5% and is greater than W_M of **2** = 0.5%. Therefore, it can be assumed that the aryl group B is equatorial in the first instance (Arα and Hβ).

Several flavanoids have been isolated from licorice roots (*G. glabra*), including glabridin derivatives. The mass spectrum of the component with τ_R = 15.2 min (**3**) corresponds to the monotrimethylsilyl derivative of 4′-*O*-methylglabridin and demonstrates two competing pathways for decomposition of the MI, RDR into MI and $[M - CH_3]^+$:

$$\mathrm{MI} \xrightarrow{\mathrm{RDR}} \textbf{d}\text{-type ions, } m/z\ 188;\ [M - CH_3]^+ \xrightarrow{\mathrm{RDR}} \textbf{f}\text{-type ions, } m/z\ 173;$$

cleavage of the pyran ring gives **g**-ions, *m/z* 187, and tropylium (**e**), *m/z* 290, whereas competing cleavage of the TMS group with proton migration to the oxygen atom gives **b**, *m/z* 338, $[M - CH_3SiCH_2]^+$.

An unexpected result was the mass spectrum of the component with τ_R = 16.15 min (**4**), which we interpreted as the spectrum of unsilylated glabridin (*m/z* 324 $[M]^+$, 309, **a**; 187, **g**; 173, **f**; 136, **d**; 123, **e**]. Peaks for the aforementioned ions contribute 82% of the total ionic current. The spectrum of **4** lacks peaks for ions with *m/z* 73 $[SiMe_3]^+$. Measurements of the peak-intensity ratios I(*m/z* 326)/I(*m/z* 324) = 0.92% and I(*m/z* 311)/I(*m/z* 309) = 0.95% indicate that the molecule contains no Si atoms. Cleavage of the methyl and formation of the $[M - CH_3]^+$ fragment with *m/z* 309 (100) is a favorable process only for the 2,2-dimethylchromene ring [6, 7] whereas peaks of fragment ions with *m/z* 136 and 173 result from a RDR in the MI, $[M - CH_3]^+$. Finally, ring B with two hydroxyls gives rise to fragments **e** (*m/z* 123) and **g** (*m/z* 187).

The mass spectrum of the component with τ_R = 16.96 min (**5**) was identified with another derivative of glabridin, hispaglabridin B. The dissociation of the MI of the mono-TMS derivative occurs by the first type of fragmentation, *m/z* 462 $[M]^+$; 447, **a**; 274, **d**, 261, **e**, 187, **g**, 173, **f**. A RDR in the MI and $[M - CH_3]^+$ leads to peaks for ions with *m/z* 274 and 173; and cleavage of the pyran ring, with *m/z* 261 and 187.

The chromene ring conjugated to ring B gives rise to fragments with *m/z* 274, 261, and 259, 216. The formation of the last two is a feature of **5**, as shown in Scheme 2.

Isoflavenes are not basic structures in plant metabolism because they are unstable compounds. The isoflavene derivative glabrin was isolated from *G. glabra*. It is biogenetically related to the isoflavone glabrone. Another member of this group of compounds is pallidiflorin (*G. pallidiflora*). Mass spectra of the components with τ_R = 14.3 (**6**) and 15.9 min (**7**) are readily interpreted if **6** is considered to be an isoflavan derivative, the B ring of which is conjugated to a chromene ring; **7**, an isoflavene, the B ring of which is conjugated to a chromone ring. Each ring (A and B) contains a single TMS group. Decomposition of the MI of **6** combines both a RDR of $[M]^+$ to give a peak for ions with *m/z* 274 and elimination of a CH_3SiCH_2 group from $[M]^+$, *m/z* 396. In contrast with glabridin derivatives, the chromene ring is bound to ring B. Therefore, the observed peaks for ions with *m/z* 187 and 173 belong to fragments of ring B. Furthermore, *o*-hydroxybenzyl cations with *m/z* 195 (see Scheme 3) that are typical of flavans [11] are recorded. A RDR cannot occur in the MI of the isoflavene (**7**). Therefore, the spectrum of **7** does not exhibit peaks for ions with *m/z* 274 and 259.

Thus, the scheme developed by us for preliminary fractionation of phenolic compounds as a function of their acid—base properties enabled GC-MS to be used successively to analyze and identify several components of the EA extract of licorice root.

EXPERIMENTAL

Isolation of Flavonoids. Licorice roots (*G. glabra* L.) were collected in Turkmenia in the floodplain of the Amudar′ya river. Roots that were dried and ground in an industrial crusher and laboratory grinder underwent extraction. The raw material was freed of dust and large particles by sieving before use. The fractional composition of the ground raw material was determined by sieve analysis. Licorice root with an average particle size of 1.25-2.00 mm was used for the extraction.

Ground roots (1.5 kg) were treated three times at room temperature with hexane to remove lipids (extracted substance:extractant, 1:4). The pulp was filtered off. The solvent was removed from the extract in a rotary evaporator. The yield of lipids (licorice oil) was 7.2 g (0.49% of the dry weight). Then the pulp was extracted with EA (pulp:extractant, 1:4) with stirring at 40-50°C for 4 h. After the pulp was removed, the filtrate was evaporated in vacuo to dryness in a HP-1M2 rotary evaporator at temperatures less than 40-50°C to afford the dry EA extract of licorice root (yield 56.2 g, 3.75%).

The EA extract was fractionated as follows. EA extract (50 g) was dissolved in dichloroethane (DCE) (1 L). The solid (0) that was insoluble in DCE was filtered off and dried (20.10 g, 40.20%). The DCE solution of the EA extract (1) was treated with aqueous Na_2CO_3 (5%, 2 × 200 mL) to afford aqueous (3) and DCE (2) phases. The DCE phase (2) was treated with aqueous NaOH (5%, 3 × 300 mL) to afford DCE (4) and aqueous (5) phases. The DCE phase (4) was washed with distilled water (1 × 300 mL) to afford DCE (6) and aqueous (5) phases. The aqueous phase (5) was acidified with H_2SO_4 (10%) until the pH was 1 and extracted with DCE (3 × 1 L) to afford DCE (8) and aqueous (7) phases. The aqueous phase (7) was extracted with *n*-butanol (2 × 1 L) to afford butanol (9) and aqueous (10) phases. The aqueous phase (3) was acidified with H_2SO_4 (10%) until the pH was 1 and extracted with DCE (2 × 500 mL) to afford DCE (11) and aqueous (12) phases. The aqueous phase (12) was extracted with *n*-butanol (2 × 500 mL) to afford aqueous (13) and butnaol (14) phases. The aqueous phases (10) and (13) obtained by this scheme were evaporated to dryness. All organic phases were dried over $MgSO_4$. Solvent was removed in vacuo to dryness at 40-50°C to afford the corresponding extracts. Yield of DCE extracts: (8), 9.91 g (19.82% of EA extract); (6), 6.60 g (13.2%); (11), 2.18 g (4.93%); butanol: (9), 1.55 g (3.10%); (14), 4.83 g (9.65%).

Fractions of organic solvents were monitored using UV spectra recorded in the range 200-500 nm and TLC (Silufol plates). The eluent was hexane:acetone (6:4). Electronic absorption spectra (UV) were recorded on a Specord M 40 spectrophotometer in 0.1-cm quartz cuvettes.

Preparation of Trimethylsilyl (TMS) Derivatives. A weighed sample (fraction 8, 0.1 g) was dissolved in pyridine (10 mL), treated with trimethylchlorosilane (10 mL) and hexamethyldisilazane (5 mL), held for 2 h at 35°C on a water bath, and left for 12 h at room temperature. The precipitate was filtered off. The excess of reagents was distilled in vacuo at 100°C. The resulting sample was used for analysis.

GC-MS of TMS Derivatives of Flavonoids. The analysis was performed on an HP 5890 GC with a MSD HP 5972A-computer mass spectrometer (HP ChemStation data processing system). Components were separated in a HP-5MS 30 m × 0.25 quartz capillary column with a bound stationary phase consisting of 5% phenylmethylsilicone. The temperature was programmed in the range 40-250°C at 10°C/min. The energy of the ionizing electrons was 70 eV; the scanning rate, two spectra per second.

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