DETERMINATION OF PHENOLIC COMPONENTS IN ETHANOLIC EXTRACTS FROM WOOD OF Maackia amurensis

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The phenolic components in an ethanolic extract from the wood of Amur maackia were determined quantitatively by the HPLC method in the isocratic regime using a column with a C_{19} reversed-phase sorbent and β-naphthol acetate as internal standard. Before injection into the chromatographic column, the extract was purified on a column of Florisil that had been treated with 30% acetic acid. The relative error in the determination of each component at a confidence level of 0.95 does not exceed 7%.

It has been established previously that the hardwood of Amur maackia, Maackia amurensis Rupr. et Maxim, family Fabaceae, subfamily Lotoideae, contains isoflavones: genistein (M_2), formononetin (M_1), and retusin (M_4); stilbenes: resveratrole (M_3) and 3,3',4',5-tetrahydroxystilbene (M_5); and also the isoflavonostilbene maackiasin (M_6), which was isolated for the first time [1, 2]. In the present communication we propose a method for the quantitative determination of these phenolic components in ethanolic extracts from Amur maackia based on the use of high-performance liquid chromatography, HPLC [3-6].

The mixture to be analyzed was separated on a column with a C_{18} reversed phase at room temperature in the isocratic regime. Preliminary experiments have shown that the pairs of compounds M_5-M_3 and M_3-M_4 were critical for the separation. In view of this, we tried three eluent systems, each of which contained acetic acid. Mobile phases containing no acid have been described for the separation of mixtures of isoflavonoids [3-5], and this, of course, led to a more prolonged retention of the separating capacity of the sorbent. However, for the mixture of phenolic components from the Amur maackia the presence of acetic acid in the systems proved to be necessary. It considerably decreases the spreading of the chromatographic bands that is particularly characteristic for compounds M_4 and M_6, improves the symmetry of the peaks, and raises the efficiency of separation.

Mixtures of solvents containing the minimum amount of acetic acid were selected experimentally. These were the following systems: 1) ethanol–2% acetic acid (20:50); 2) methanol–2% acetic acid (50:50); and 3) acetonitrile–2% acetic acid (20:50). The use of any of them (Table 1) permits the complete separation of the mixture to be analyzed, including the pairs of compounds M_5-M_3 and M_3-M_4. The separation criterion (R) [7] for these pairs of compounds amounted in these cases to not less than 1.5. Usually, such separation is regarded as adequate for subsequent analysis.

System 3 has the highest separating capacity, permitting work at comparatively low pressures, which has a favorable influence on the working life of the column. System 2 is the

TABLE 1. Chromatographic Characteristics of the Eluent Systems

Eluent system	Pressure, MPa	Time of analysis, min	R		Retention time of the standard, min	T_{rel} of the components of the mixture					
			M_5–M_3	M_3–M_4		M_5	M_3	M_4	M_2	M_1	M_6
1	22,5	60	2,3	2,4	37,28	0,06	0,12	0,19	0,34	0,70	1,59
2	16,0	15	1,5	3,3	11,28	0,13	0,19	0,35	0,48	0,85	1,34
3	10,0	40	4,5	2,9	32,44	0,09	0,12	0,17	0,27	0,46	1,23

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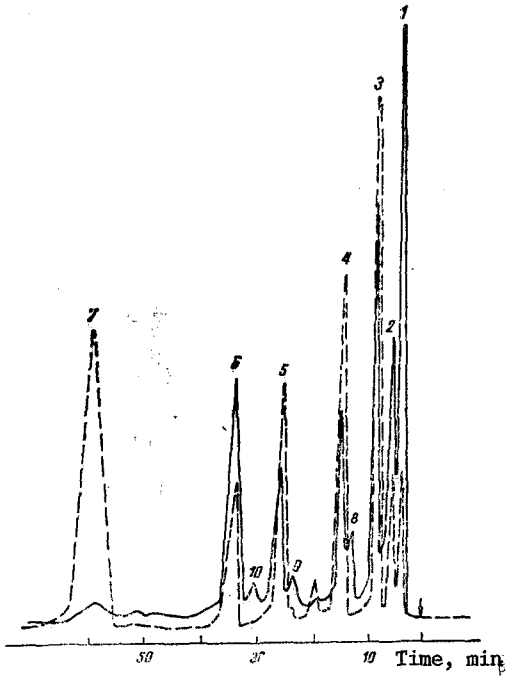

Fig. 1. Chromatograms of the separation of an artificial mixture of compounds M_1-M_6 (---) and of a purified ethanolic extract from the wood of the Amur maackia (——) in the presence of β-naphthol acetate in the eluting system ethanol–2% acetic acid (20:50) (1 ml/min) on a Separon SGX C_{18} column; detection at 254 nm; where the peaks represent the compounds indicated: 1) M_5; 2) M_3; 3) M_4; 4) M_2; 5) M_1; 6) β-naphthol acetate; 7) M_6; 8-10) unidentified components.

"fastest," and system 1, the "slowest;" however, it is the less toxic, which is not an unimportant factor in routine analyses.

The relative retention times T_{rel} showed (Table 1) that the order of elution of components M_1-M_6 was the same for all three systems.

Figure 1 shows a chromatogram with an artificial mixture of compounds (M_1-M_6) in which the peaks are separated from one another by valleys reaching practically to the zero line.

Quantitative calculations of the amounts of each component in the mixture were made relative to the internal standard – β-naphthol acetate – which issued as an individual peak between M_1 and M_6. The calibration coefficients K were determined by analyzing artificial mixtures of the individual compounds. The numerical values of K on the injection of from 1 to 5 μg of each compound into the column remained constant, i.e., the detector signal was linear within these concentration limits.

To determine the given phenolic components, Amur maackia wood sawdust was first treated with hexane. As shown in [1], the substances extracted in this way are nonphenolic components of the wood and amount to 0.8-1.0% of the weight of the sawdust. After elimination of the hexane, the phenolic compounds were extracted with 96% ethanol both at the boiling point and at room temperature for various intervals of time. Extraction at room temperature (3 h) and also boiling with ethanol for 0.5, 2, 4, and 6 h led to the revelation of the same amounts of the compounds M_1-M_6. This indicated that no transformations with the components to be determined, M_1-M_6, took place on hot extraction, and permitted the optimum extraction conditions to be selected – 30-min boiling in ethanol or 3 h steeping with ethanol at room temperature and with constant stirring.

On boiling, 9% of the extractive substances were dissolved out, and at room temperature 7%; the phenols detectable by HPLC in the ethanolic extract amounted to only 30-35%, the remainder consisting of complex polyphenolic compounds. The presence in the ethanolic extract of such large amounts of polyphenols excluded the possibility of its direct injection into the HPLC column and made preliminary purification necessary. This was performed on sorbents available to us: type KSK silica gel or Florisil, both impregnated with acetic acid. Com-

pounds M_1-M_6 were eluted with the benzene–acetone system rapidly, while the remaining extractive substances were strongly retained by the sorbents and were eluted only by methanol.

It is known that irreversible sorption on the sorbent that we used is characteristic for highly polar compounds. We therefore performed a number of experiments by passing artificial mixtures of known composition through a column containing Florisil, with the subsequent determination of each compound, and, on this basis, we calculated the "recovery" factors K_r. The values of K_r were affected by the degree of impregnation of the Florisil with acetic acid. Thus, for example, if the sorbent was impregnated with 15% acetic acid in a ratio of 90:10, respectively, the recovery of retusin was 49.99% (K_r = 2.39). The deposition on Florisil of 30% acetic acid in a ratio of 70:30 increased the recovery of this compound to 86.76% (K_r = 1.15).

We subsequently used Florisil (60-100 mesh) impregnated with 30% acetic acid in a ratio of 70:30. In this case, the values of the "recovery" factors for compounds M_1-M_6 were close to unity. This showed that under the purification conditions selected the irreversible sorption of the compounds to be determined is insignificant.

A typical separation of a natural mixture of phenolic compounds from Amur maackia is shown in Fig. 1. In the sample of wood investigated, the amounts of M_1, M_2, M_3, M_4, M_5, and M_6 amounted to 0.14, 0.13, 0.88, 0.14, 1.30, and 0.019%, respectively, on the absolutely dry mass. The procedure developed permits each component to be determined at a confidence level of 0.95 with a relative error of 7% and can be used for the qualitative and quantitative analysis of the phenolic components in extracts from the wood of Amur maackia.

EXPERIMENTAL

HPLC was performed on a GSP-100 instrument (Czechoslovakia) without a gradient. Glass columns with dimensions of 3.3 × 150 mm filled with the reversed-phase sorbent Separon SGX C_{18} (7 μm) were used. The space velocity of the eluent systems amounted to 1 ml/min. The components were detected by means of a UV detector at a fixed wavelength (254 nm). The areas of the peaks and the retention times were measured with the aid of a CI 100 integrator (Czechoslovakia).

Preparation of the Extract. Air-dry sawdust (≤1 mm) from Amur maackia wood (felled in late autumn) was covered in n-hexane (100 ml) and the mixture was stirred with a magnetic stirrer for 1 h. The hexane was poured off and the sawdust was dried in the air and was then transferred to a round-bottomed flask and, after the addition of 100 ml of ethanol, it was weighed and its contents were boiled under reflux for definite intervals of time. Then the flask was cooled and the weight was brought up to the initial level with ethanol.

Purification of the Extract. An aliquot of the extract (10 ml) was evaporated, and in the form of a "dry charge" on the Florisil (1.5 g; 60-100 mesh, impregnated with 30% acetic acid in a ratio of 70:30) was transferred to a column containing 1 g of the same sorbent equilibrated with benzene, and 30 ml of the benzene–acetone (2:1) system was passed through. A 10-ml portion of the eluate was evaporated to dryness and a known amount of β-naphthol acetate (0.5-0.6 mg) was added, the whole was dissolved in 0.5 ml of ethanol, and a 5- to 10-μl aliquot was injected into the column of the chromatograph.

CONCLUSIONS

A procedure is described for the qualitative and quantitative determination of phenolic compounds and ethanolic extracts from the hardwood of Amur maackia which is based on the use of reversed-phase HPLC. The relative error of the determination does not exceed 7%.

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