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Application of RP–HPLC for the Determination of Isoflavonoid Content in Soybean Roots as Effect of Chilling Stress*

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Soybean *(Glycine max.* [L] *Merr.)* is a subtropical legume and requires a temperature in range 25–30°C. Phenylalanine ammonia-lyase (PAL, EC.4.1.3.5) which catalyzes deamination of L-phenylalanine to *trans*-cinnamic acid is regarded as enzyme that leads to phenylpropanoids, such as flavonoids, which can protect plants againts biotic and abiotic stresses. Additionaly flavonoids are known as very effective anticancer protectants in herbal medicine. The very useful method for identification and quantitation of flavonoids is HPLC technique with the use of UV/VIS, DAD and MS detection. At a suboptimal root-zone temperature there is a decrease in soybean roots growth proportional to the decrease in temperature. In our experiment the PAL activity increases at 5° and 10°C but does not change at 15°C in comparison to the control. Preliminary RP–HPLC analyses of isoflavonoids in control roots letter transferred to the temperature 10°C showed that the composition of flavonoids were changed in the chilled roots. We suggest that PAL and its products can disturb: (1) root growth of seedlings, (2) nodule numbers and (3) symbiotic relationships between the soybean and *Bradyrrhizobium*.

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Soja *(Glycine max.* [L] *Merr.)* jest rośliną subtropikalną wymagającą do rozwoju temperatury w zakresie 25–30°C. Enzym amoniakoliaza L-fenyloalaniny (PAL, EC. 4.3.1.5) katalizuje deaminację L-fenyloalaniny do kwasu *trans*-cynamonowego i jest uważana za enzym metabolizujący fenylopropanoidy takie jak na przykład flawonoidy, pełniące rolę ochronną w przypadku stresu o charakterze biotycznym jak i abiotycznym. Dodatkowo flawonoidy są znane jako substancje o bardzo efektywnych właściwościach przeciwkancerogennych i stosowane są w tradycyjnym ziołolecznictwie. Bardzo efektywną metodą analizy ilościowej i jakościowej flawonoidów jest technika HPLC z zastosowaniem detektorów typu: UV/VIS, DAD oraz MS. W przedstawionych badaniach w suboptymalnej strefie temperaturowej następuje spadek wzrostu korzeni soi proporcjonalny do spadku temperatury. Aktywność PAL wzrasta w 5°C i 10°C lecz nie ulega zmianie w 15°C w porównaniu z próbką kontrolną. Przeprowadzona wstępna analiza zawartości izoflawonoidów, oznaczanych metodą RP–HPLC w kontrolnych próbkach korzeni przenoszonych do temperatury 10°C pokazuje, że zawartość flawonoidów zmienia się w schładzanych korzeniach. Sugeruje to, że PAL i jego produkty mogą przeszkadzać we: (1) wzroście brodawek korzeni, (2) liczbie narośli oraz (3) symbiotycznym reakcjom pomiędzy soją i *Bradyrrhizobium*.

Soybean (*Glycine max.* (L.) *Merr.*) is a subtropical legume, and for normal growth and production requires a temperature in the range of 25–30°C especially in the root zone. Suboptimal temperatures decrease nodulation and nodule function in these plants [1]. In the areas where the growing season is short, temperature is the main factor limiting growth and yield.

The phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) which catalyzes deamination of L-phenylalanine to *trans*-cinnamic acid is regarded as the primary enzyme that leads to phenylpropanoids, such as caffeic, ferulic, *p*-coumaric, sinapic acids, flavonoids, lignin and other biologically active substances. Some of phenylpropanoids can protect plants against various biotic (infections by viruses, fungi, insect attacks) and abiotic (UV radiation, wounding, nutrient deficiences, herbicide treatment, low temperatures) stresses [2]. Many of them *e.g.* flavonoids play an important role in the growth and development of plants but also induce *Rhizobium* nodulation genes that are required for the symbiotic association with legumes and they can also possess inhibitory activities. Isoflavonoids were also shown to induce resistance in *Bradyrrhizobium japonicum* and *Rhizobium fredii* to the soybean phytoalexin, glyceollin. Isoflavonoids which occur naturally in fruits and vegetables are an integral part of the human diet [3]. They were reported to exhibit a wide range of biological effects including: antiviral and antifungal action [4,5]. It has been sugested that the isoflavones may prevent chronic diseases including hormone-dependent cancers, coronary heart disease and antherosclerosis [6–8]. The isoflavonoids exert these effects as antioxidants, free radical scavengers and chelators of divalent cations [9–12]. One of the flavonoid compound, genistein, which is present in soybeans has been found to inhibit development of aberrant crypt foci, considered to be preneoplastic lesions in the colon of rats [13], as well as the growth of the human breast and prostate cancer cell lines [14, 15]. The plasma concentrations of isoflavone derivatives, including genistein, in Japanese men are known to be seven to 110 times higher than

those in Finnish men [16], suggesting that the large intake of soybean products may be related to the low mortality from prostate cancer in Japan [17].

The more effective and powerful techniqe used for determination and quantitation of flavonoids in plants and monitoring in human body fluids and human diet are HPLC [18–22] and GC [23–25] techniques.

This paper shows some of initial experiments to explain why penetration by a bradyrrizobium in soybean roots is temperature-dependent. What role is played by phenylpropanoids (for an example free phenolic acids) which are induced in roots by low temperature? The objectives of the present work were to investigate the effects of low temperature on isoflavonoid content in chilled soybean roots. The flavonoids were quantitatively determined by RP–HPLC technique with different methods of elution.

EXPERIMENTAL

Materials and methods

Preparation of biological materials. Seeds of the soybean (*Glycine max* (L.) Merr.) Polish cultivar Aldana were obtained from IHAR (Radzików). Seeds were surface-sterilized in tiuram and germinated in darkness (25°C). Some of the seedlings were kept at 25°C, these were the controls, some of them were transferred to different temperatures (5–15°C).

Phenylalanine ammonia-lyase (PAL) was extracted from the roots of 3-days-old soybean seedlings grown in the dark and transferred to low temperatures.

Flavonoids from the roots of 3-day-old soybean seedlings grown in the dark (25°C) and transferred to 10°C for 24 h were analyzed . Extracts were taken from the roots with 80% MeOH (v/v) (2g FW+10 ml MeOH) (J.T. Baker – USA)[26]. The supernatants were collected and further extracts from residues were obtained with 80% MeOH and then with absolute MeOH. After evaporation (Rotavapor, Unipan – Poland) the residues were obtained with ethyl acetate and the residues were redissolved in the 80% MeOH.

RP–HPLC analysis of flavonoids. The extracts in 80% MeOH were analyzed by RP-HPLC method (HP 1050 Hewlett–Packard – USA) on columns: Spherisorb ODS2 250 × 4 mm I.D., equipped with a precolumn Spherisorb ODS2 4 × 4 mm ID (Hewlett–Packard – USA); SynChropak SDC C_{18}, 250 × 4.6 mm I.D. (Alltech – USA); Econosil C_{18}, 250 × 4.6 mm I.D. (Alltech – USA); Ultrasphere C_{18}, 250 × 4.6 mm I.D. (Beckman – USA); LiChrosorb RP–18, 250 × 4.6 mm I.D. (Merck – Germany), Spherisorb S5 ODS2, 250 × 4.6 mm I.D. (Phase Sep – England), Nucleosil 5C_{18} (Machery–Nagel – Germany), NovaPak C_{18} (Waters – USA), Resolve C_{18} (Waters – USA) and μBondapak C_{18} (Waters – USA) (Table 1). Elution was performed with different mixtures of MeOH and H_2O, at a flow-rate 0.5 ml min^{-1}, and column temperature of 60°C, measuring the absorbance at 254 nm; volume of injection loop was 20.0 μl. Methanol and water for HPLC analysis were used from J.T. Baker (USA). Genistin and daidzein (Sigma – USA) in 80% MeOH were used as standards for quantitative determination of them in a biological material. The callibration lines were determined in mixture MeOH+H_2O (6+4) for range of 0.560–0.006 mg ml^{-1} (daidzein) and 0.830–0.013 mg ml^{-1} (genistin) respectively.

Table 1. Characterization of RP–HPLC C_{18} type columns from different producers used in experiments

No.	Support	Particle size (μm)	Pore size (Å)	Endcappin g (%C)	Surface m^2/g^{-1}	Run (cycles)
1.	Ultrasphere C_{18}	5	80	10	250	580
2.	SynChropak SDC C_{18}	5	100	14	50–100	625
3.	Econosil C_{18}	5	60	15	450	540
4.	Spherisorb S5 ODS2	5	80	12	220	630
5.	Spherisorb ODS2	5	80	12	200	555
6.	LiChrosorb RP–18	5	60	15	150	570
7.	Nucleosil $5C_{18}$	5	100	14	350	605
8.	Nova-Pak C_{18}	4	60	7	120	630
9.	Resolve C_{18}	5	90	10	200	595
10.	μBondapak C_{18}	10	125	10	300	610

1. Ultrasphere C_{18}, 250 × 4.6 mm I.D., (Beckman – USA).
2. SynChropak SDC C_{18}, 250 × 4.6 mm I.D., (Alltech – USA).
3. Econosil C_{18}, 250 × 4.6 mm I.D, (Alltech – USA).
4. Spherisorb S5 ODS2, 250 × 4.6 mm I.D., (Phase Sep. – England)
5. Spherisorb ODS2, 250 × 4.0 mm I.D., (Hewlett–Packard – USA).
6. LiChrosorb RP–18 250 × 4.6 mm I.D., (Merck – Germany).
7. Nucleosil $5C_{18}$, 250 × 4.0 mm I.D., (Machery–Nagel – Germany).
8. Nova–Pak C_{18}, 250 × 4.6 mm I.D., (Waters – USA).
9. Resolve C_{18}, 300 × 3.9 mm I.D., (Waters – USA).
10. μBondapak C_{18}, 250 × 4.6 mm I.D., (Waters – USA).

RESULTS AND DISCUSSION

The effect of low temperature on growth of soybean roots is given in Table 2. The PAL activity in roots cultivated in different temperatures is presented in Figure 1. The relation between relative retention times, logarithm of capacity factor and resolution of genistin and daidzein eluted by different mixtures of methanol and water are presented in Figures 2 and 3. The relation between retention time and selectivity of separation for diffrent C_{18} columns are given in Table 3. The chromatograms for different concentrations of genistin and daidzein separated by a RP–HPLC method on Spherisorb ODS2 with elution of MeOH+H_2O (6+4) are presented in Figures 4 and 5. The callibration lines for genistin and daidzein are presented in Figure 6. The typical separation of flavonoids from soybean roots by RP–HPLC method on column Spherisorb ODS2 are presented in Figures 7 and 8. The concentrations of determined flavonoids in biological material are given in Table 4.

Table 2. Effect of low temperatures on growth of soybean roots

Days in low temperature	Length of roots, cm			
	5°C	10°C	15°C	25°C
0 Day	3.7 +/- 0.61	3.7 +/- 0.61	3.7 +/- 0.61	3.7 +/- 0.61
1 Day	3.7 +/- 0.71	4.2 +/- 0.68	4.1 +/- 0.84	6.0 +/- 1.21
2 Days	4.2 +/- 0.59	4.3 +/- 0.72	6.0 +/- 1.20	10.6 +/- 3.30
3 Days	4.7 +/- 0.81	5.1 +/- 0.90	7.3 +/- 2.20	15.5 +/- 2.42
4 Days	5.2 +/- 0.76	5.4 +/- 0.54	8.6 +/- 1.87	18.2 +/- 1.81

Table 3. The relations between retention time, capacity factor, relative retention time and resolution equation for flavonoids standards separated by RP–HPLC method on different C_{18} columns

No.	Flavonoids	t_R, min	k`, log k`	α	R_S
Ultrasphere C_{18}					
1.	Genistin	5.315	0.940 (–0.027)	1.000	4.724
2.	Daidzein	8.237	2.007 (0.303)	2.134	
SynChropak SDC C_{18}					
3.	Genistin	4.629	0.918 (–0.037)	1.000	3.980
4.	Daidzein	6.987	1.896 (0.278)	2.464	
Econosil C_{18}					
5.	Genistin	4.539	0.864 (–0.063)	1.000	7.039
6.	Daidzein	7.485	2.074 (0.317)	2.400	
Spherisorb S5 ODS2					
7.	Genistin	4.705	1.035 (0.015)	1.000	4.964
8.	Daidzein	7.033	2.042 (0.310)	1.973	
Spherisorb ODS2					
9.	Genistin	4.712	0.867 (–0.062)	1.000	4.827
10.	Daidzein	7.024	1.783 (0.251)	2.057	
LiChrosorb RP–18					
11.	Genistin	2.975	0.872 (–0.059)	1.000	1.893
12.	Daidzein	4.378	1.755 (0.244)	2.012	
Nucleosil $5C_{18}$					
13.	Genistin	4.513	0.897 (–0.047)	1.000	3.934
14.	Daidzein	7.218	2.034 (0.308)	2.268	
Nova–Pak C_{18}					
15.	Genistin	2.453	0.123 (–0.911)	1.000	3.413
16.	Daidzein	4.180	0.913 (–0.039)	7.444	
Resolve C_{18}					
17.	Genistin	2.281	0.595 (–.0225)	1.000	2.445
18.	Daidzein	5.318	2.719 (0.434)	4.569	
μBondapak C_{18}					
19.	Genistin	4.517	0.820 (–0.086)	1.000	1.857
20.	Daidzein	6.253	1.520 (0.182)	3.859	

Table 4. Content of genistin and daidzein in soyben roots cultivated in 25°C and transferred to 10°C on 24h

Series	Content of flavonoids $\mu g\ g^{-1}FW$	
	Daidzein	Genistin
A_1	3.08 +/- 0.03	1.61 +/- 0.03
M_2	4.95 +/- 0.04	0

(A_1) – Soybean roots cultivated at 25°C.
(M_2) – Soybean roots cultivated at 25°C and transferred to 10°C on 24h.

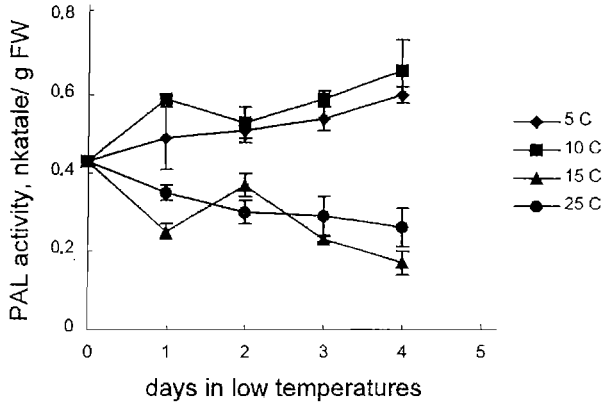

Figure 1. The PAL activity in soybean roots at low temperatures

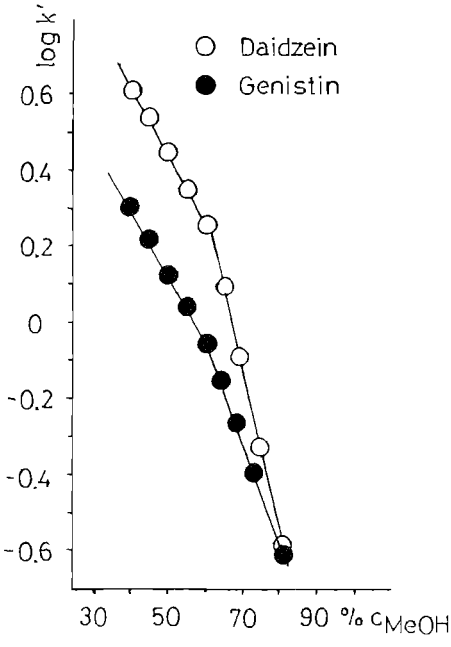

Figure 2. The relation between logarithm of capacity factors (log k') and concentration of methanol in eluent for genistin and daidzein flavonoids

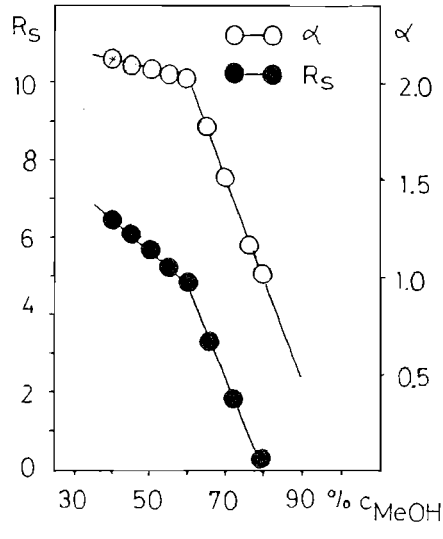

Figure 3. The relation between separation factor (α) and resolution (R_S) and concentration of methanol in eluent for genistin and daidzein flavonoids

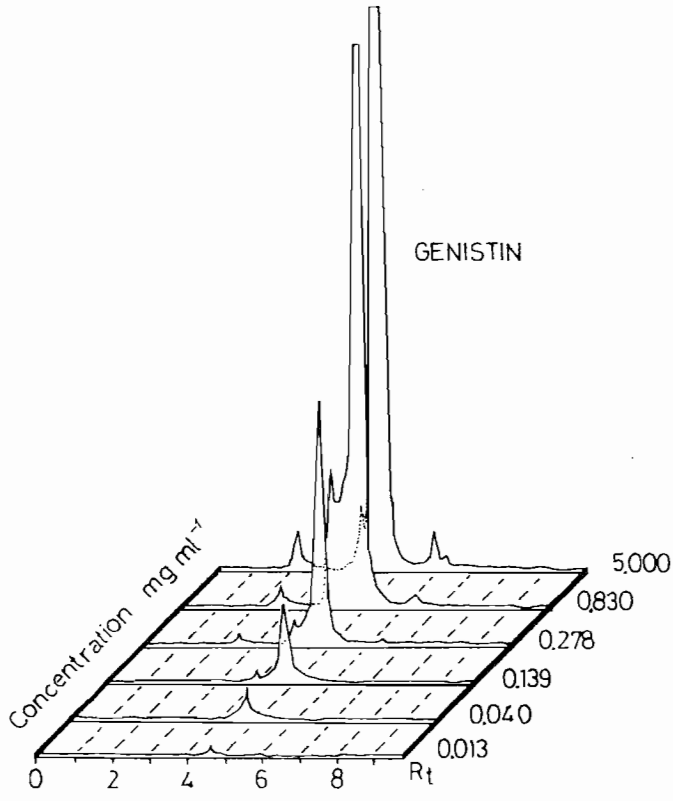

Figure 4. Separation of different concentrations of genistin standard by RP–HPLC method on Spherisorb ODS2 (column 250 × 4 mm I.D. with guard column 4 × 4 mm I.D., elution with MeOH:H_2O (6:4) a flow-rate 0.5 ml min^{-1} measuring the absorbance at 254 nm, column temperature 60°C)

The decrease of growth of soybean roots was a proportional to the decrease in temperature (Table 2). This means that, the primary effect of low temperature on soybean is the reduction of growth and metabolic processes. The isoflavonoids (daidzein and genistein and their glycosides as daidzin and genistin) are important compounds which accumulate in soybean roots. Flavonoid levels affect legume nodulation and N_2 fixation directly, it was reported that a hypernodulationg soybean mutant had a higher root concentration of isoflavone compounds, *e.g.* genistein, daidzein, coumestrol and *etc.* [1,25,27].

PAL activity in roots cultivated at 5°C and 10°C increased after 24 h to reach a maximum after 4 days in comparison to the control roots. The highest PAL activity was in the roots transferred to 10°C (near the biological minimum for soybean). When the seedlings were cultivated at 15°C PAL activity was similar to the control ones (Fig. 1). The enzyme activity, which is involved in the biosynthesis of flavonoids are changed at low temeperature. The extractable amount of PAL increases when the plants are acclimatized to low temperature conditions. PAL is turned over rapidly at normal temperatures, and it seems to be likely that the level of PAL increases at a low

temperature because the rate of synthesis is decreased less by cold than is the rate of degradation. The observed chilling stress had a very little effect on dry mass and soluble protein content over 4 days (data not show in figures).

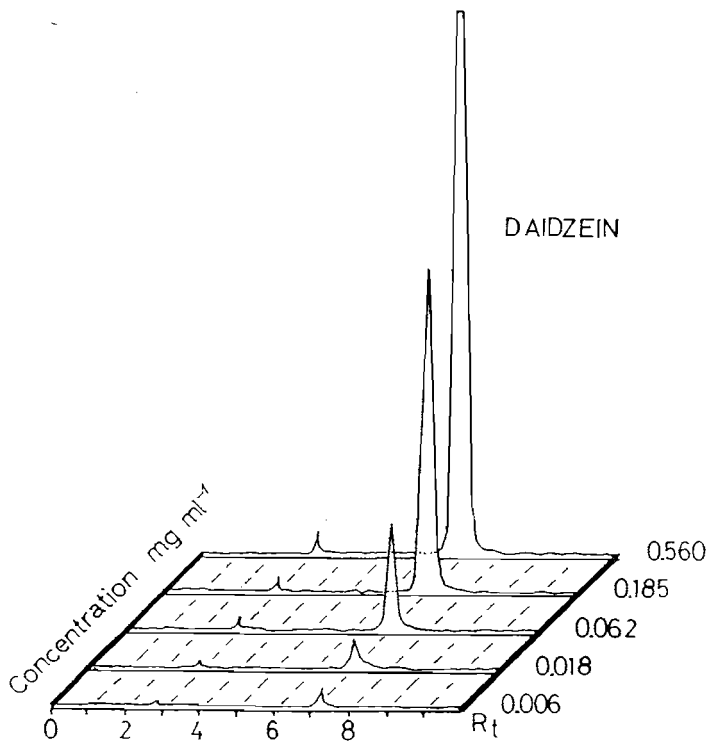

Figure 5. Separation of different concentrations of daidzein standard by RP–HPLC method on Spherisorb ODS2 (separation conditions as in Figure 4)

In the second part of experiment the standards of flavonoids were separated by RP–HPLC method on Spherisorb ODS2 column in different conditions and elution with mixture of MeOH and H_2O. Figures 2 and 3 show the changes in retention times (t_R), capacity factor (k'), logarithm of capacity factors ($\log k'$), separation factors (α) and resolutions (R_S) calculated from the chromatographic data according to equations (1)–(3):

$$k' = (t_R - t_O) / t_O \tag{1}$$

$$\alpha = (t_{R2} - t_O) / (t_{R1} - t_O) \tag{2}$$

$$R_S = 2\,(t_{R1} - t_{R2}) / (\omega_1 + \omega_2) = (\sqrt{N}/4)\,[(\alpha - 1) / \alpha][k_2'/(1 + k_2')] \tag{3}$$

The values of k' depend on the column (type of packing, age of column, *etc.*) and on the solvent composition, but therefore for each column best conditions for separation must be established. In this part of our experiment we tried to find the optimal conditions for RP–HPLC separation in mixture of MeOH and H_2O. Also we tested dif-

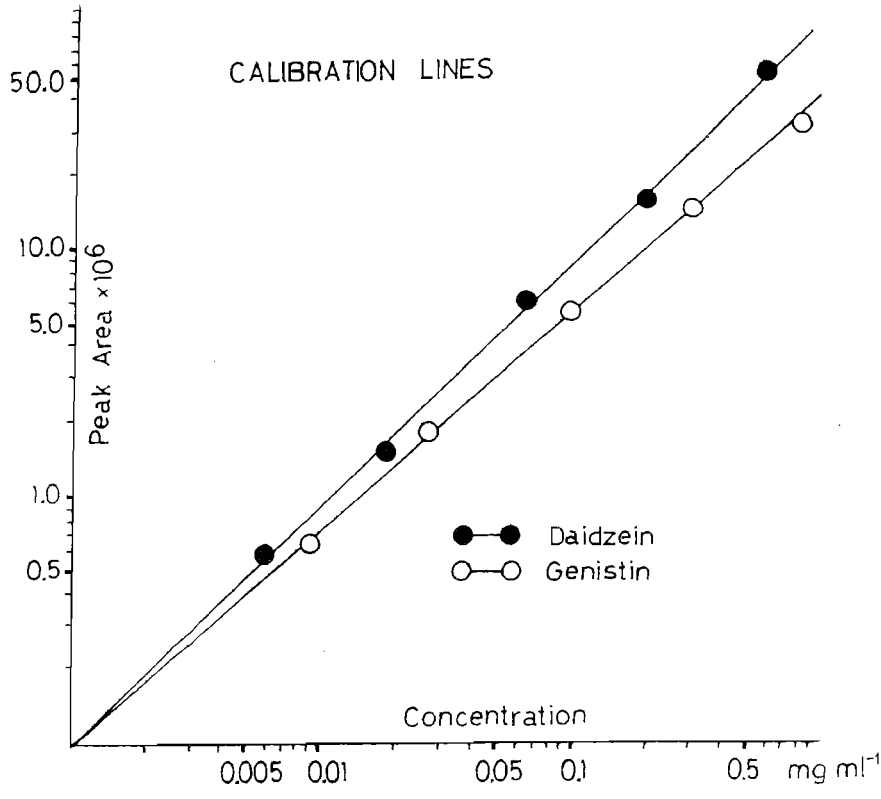

Figure 6. The calibration lines for daidzein and genistin standards

ferent types of C_{18} type columns with different concentration of carbon on surface, obtained from different manufacturers, but with the similar porosity (60–125 Å) and particle sizes (4–10 μm) (Tab. 1). The chromatographic parameters as: k', log k',α and R_S, calculated for different RP–HPLC columns (C_{18} type) with different porosity and carbon concentrations on the silica gel surface, were changed (Tab. 3). In according to changes of MeOH concentration the log k' values, α and R_S are changed (Fig. 2 and 3). We found two linear ranges of MeOH concentrations where k' changed linearly with composition of MeOH and H_2O mixture (Fig. 2 and 3). The observed changes in k' values can be caused by the changes in chemical structure of separated flavonoids or changes in the structure of bonded octadecyl (C_{18}) chains (hydrolysis, hydratation, effect of silanol groups, *etc.*,). For analytical practice the resolution (R_S) should be at least 1.0. The optimalization of chromatographic separation can be carried out by the trial and error method or by determination of relationship between retention and composition of eluent, which is described by the equation IV (Eq.IV)[28]:

$$\log k' = A - B\, c_{MeOH} \qquad (4)$$

where: A – the constant which is equal to log k' for the pure water, (100% H_2O in the eluent) and B – is the slope [29]. Combination of equations (3) and (4), proposed by Soczewiński, permits the precise determination of the eluent composition eq. (5):

$$c_{MeOH} = -\{\log 4R_S\alpha / [(\alpha - 1)(\sqrt{N} - 4R_S\alpha)] - A\} / B \quad (5)$$

Comparision of data presented at Figures 2 and 3 and equation (5) show that best results are obtained for mixture MeOH+H_2O (60+40, 60% MeOH) and this finding is similar to results obtained by Zhang and Smith [1]. The proposed method of elution is more effective (the mean recovery is about 93.6% for both tested flavonoids) and faster than the other elution methods using of buffers with different composition and acetonitrile [30–39]. The described method of determination of eluent composition is very effective and comfortable, and was used by the author for optimization of quantitative and qualititative analysis of iridoid glucosides [40–42].

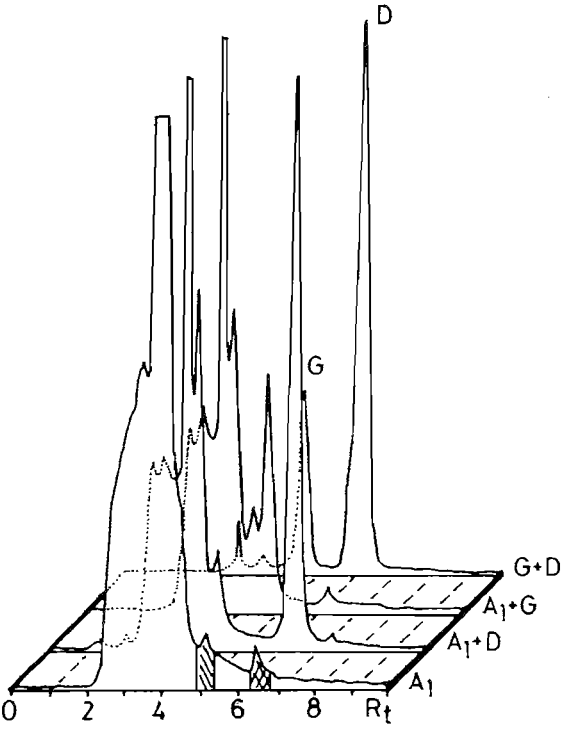

Figure 7. The separation by RP–HPLC method of flavonoids extract from soybean roots: (A_1) flavonoids from soybean roots cultivated in 25°C; (A_1+D) flavonoids with addition of daidzein standard; (A_1+G) flavonoids with addition of genistin standard; (G+D) separation of mixture of daidzein and denistin standards

The best separations were obtained with Spherisorb ODS2 from Hewlett–Packard and Spherisorb 5ODS2 from Phase Sep.Ltd. (Tab. 3). The concentration of C at silica gel support has clearly influence on retention time and other parameters of separation. All columns used in experiment had similar run about 540–630 cycles, but from

uncapped supports octadecyl groups linking faster as result of hydrolysis of siloxane bonds in alkilosiloxane chains. The changing of retention time are the effect of nonprotection of siloxane group and leanking of bonded phase [43–50].

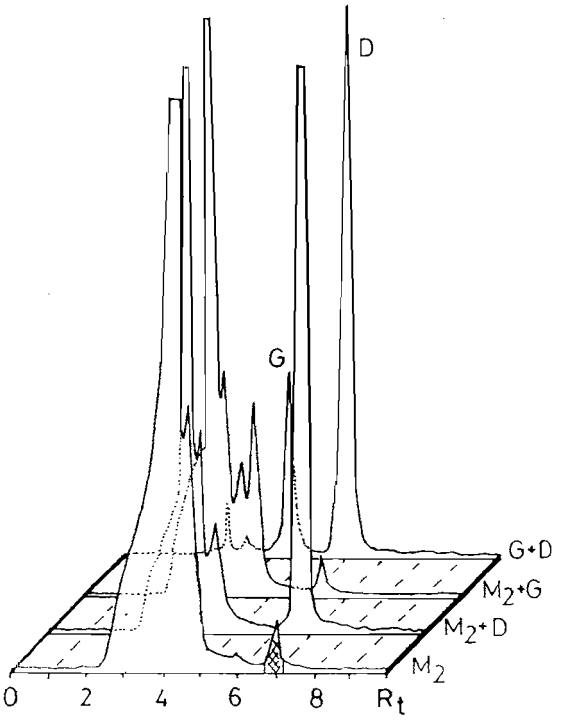

Figure 8. The separation by RP–HPLC method of flavonoids extract from soybean roots: (M_2) flavonoids from soybean roots cultivated in 25°C and transferred to 10°C on 24 h; (M_2+D) flavonoids with addition of Daidzein standard; (M_2+G) flavonoids with addition of Genistin standard; (G+D) separation of mixture of Daidzein and Genistin standards

The proposed conditions can be used for separation of flavonoids from soybean roots by RP–HPLC method. The tested method can be used for qualitative and quantitative determination of mixture of a few flavonoids, such as: daidzein and genistin. Extracted flavonoids from control roots and transferred to low temperature (10°C) were identified and quantified by the RP–HPLC method with the use of the commercially available genistin and daidzein standards. Preliminary tests showed that genistin (β-glycoside of genistein) disappeared but the content of daidzein increased in chilled soybean roots. Genistein concentration decreased, also in the soybean roots which were trasferred from the optimal temperature to lower temperature (13°–17°C) (Tab. 4, Fig. 7 and 8), what confirms the results obtained by Zhang and Smith [1].

CONCLUSIONS

We suggest that PAL and its products can play an important role in resistance to a low temperature and reduction in the genistein concentration at low temperatures and might in part disturb the symbiotic relationships between the soybean roots and bradyrhizobium and decrease nodule numbers.

The best conditions of separation of presented mixture of flavonoids were obtained for HPLC columns Spherisorb S5 ODS2 (Phase Sep.) and Sperisorb ODS2 (Hewlett–Packard) when using as an eluent the mixture MeOH:H_2O (6:4).

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