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Ontogenetic and Seasonal Variation in the Flavonoid Composition of *Sophora japonica* Cultivated in Bulgaria

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Abstract

Quantitative changes in the accumulation of the flavonol glycosides rutin and kaempferol 3-rutinoside and the isoflavone glycosides genistin and sophorabioside have been investigated in flowers and fruits of cultivated in Bulgaria *Sophora japonica* during ontogenetic and seasonal development. A method for extraction of flavonoids with good recovery was developed (98% for rutin and 97% for kaempferol 3-rutinoside). The subsequent high-performance liquid chromatography separation of flavonoid glycosides was achieved using Hypersil ODS C₁₈ column and UV detection. The mobile phase comprised methanol and phosphate buffer, pH 3.23, and linear gradient elution mode within 25 min was applied. Accumulation of rutin started with initiation of the flower buds, having the maximal content up to 287 mg/g (dry weight) in mid-July during the earliest stages of flower development and tended to have high levels until mid-August. A further decrease in the amount of rutin up to 44 mg/g was found proportionally with the decline of flavonoids throughout and after the flowering period. Rutin was the predominant glycoside and can account for up to 90% of flavonoid glycoside mixture in young buds, whereas sophorabioside (64.7 mg/g, calculated as rutin equivalent) and kaempferol 3-rutinoside (46.8 mg/g) dominated at later development stages of the fruits. No significant differences in flavonoid quantity between the analysis periods of two successive years were observed. This indicated clearly the ontogenetic dependence of flavonoid formation.

Keywords: Flavonoid glycosides, genistin, high-performance liquid chromatography, kaempferol 3-rutinoside, rutin, sophorabioside, *Sophora japonica*.

Introduction

Sophora japonica is widely cultivated in Bulgaria (Kuzmanov, 1976). The buds and fruits of this plant have been used in traditional Eastern medicine for their hemostatic properties, and flavonoids were discovered as hemostatic constituents (Ishida et al., 1989; Tang et al., 2001). Recent studies indicated that *Sophora japonica* contains a number of flavonol, flavone, and isoflavone glycosides in the buds, pericarps, and seeds, and they were demonstrated to be the major bioactive constituents (Tang et al., 2001, 2002; Wang et al., 2003). *Sophora* flavonoids have attracted attention because of some biological effects, for example, activity against lipid oxidation; multidrug-resistance modulation (Hendrich et al., 2002); IL-5 inhibitors (Min et al., 1999). Natural product manufacturers have recognized the flower buds of *Sophora japonica* as a rich source of rutin. The semi-synthesized Troxerutin is also used.

A number of methods have been reported for the analysis of rutin and other flavonoids in *Sophora japonica* by spectrophotometry (Genkina & Shakirov, 1973; Lin & Li, 1988), colorimetry (Balbaa et al., 1974), coulometric titration (Xu et al., 1989) and capillary electrophoresis with electrochemical detection (Chen et al., 2000). No HPLC method has been reported for separation of flavonoids in the species.

Although several reports on intraspecific variation in rutin pattern have been carried out, there are few reports addressing seasonal and developmental changes of flavonoids in *Sophora japonica* (Bandyukova, 1957; Genkina & Shakorov, 1973; Balbaa et al., 1974). In order to ensure reliable supplies of appropriate plant material, manufacturers require an efficient method for assessing the quality of the samples. Consequently, the development of an HPLC method for the quantification of the

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main flavonoid glycosides during the flower and fruit cycle in *Sophora japonica*, in which the qualitative and quantitative flavonoid composition appears very variable, would be desirable.

Materials and Methods

Plant material

Plant samples were collected from one tree cultivated in Sofia at an altitude of 620 m under natural garden conditions. Starting in mid-July, buds, flowers, and fruits were harvested until September for 2 years (2002–2003). Flower and fruit development was divided into different stages by morphologic features as follows: stage 1 (at the beginning of budding, flower buds from 2.5 to 4 mm in length); stage 2 (flower buds 4–5.5 mm in length); stage 3 (unexpanded flowers with closed corolla, length 6–7 mm); stage 4 (unexpanded flowers with protruding but still closed corolla, length 8 mm); stage 5 (beginning of flowering); stage 6 (fully expanded flowers); stage 7 (at the end of flowering, immature fruits from 4 to 11 mm); stage 8 (green mature but unripe fruits, 5–10 mm in diameter and 8–10 cm long having succulent pericarp and soft green seeds); stage 9 (mature fruits, pericarp, and seeds were separated from each other, the pericarp was leathery, and the seeds were hard and black). The whole inflorescences (terminal clusters) from the same tree, appearing in mid-July, were harvested every week up to mid-September. Mature leaves were collected in July. In all cases, the samples were dried to a constant weight at a room temperature.

Sample extraction

Dried plant materials of *Sophora japonica* were powdered, and an accurately weighed amount of the powder (0.2 g) was refluxed with methanol:water (70:30, v/v) at 90° (3 × 25 mL) for 15 min each time with constant stirring. The extracts were combined and made up to 100 mL into a measuring flask. An aliquot (1 mL) of extract was filtered through an 0.45 µm filter disk (Polypure II, Alltech, Lokeren, Belgium), and a 20 µL sample was subjected to HPLC analysis.

Chemicals and reagents

The standards of rutin (quercetin 3-*O*-rutinoside) and kaempferol 3-*O*-rutinoside were purchased from Extrasynthese (Genay, France). The standards of genistin (genistein 7-*O*-glucoside) and sophorabioside (genistein 4'-*O*-neohesperidoside) were kindly supplied by Prof. N. Nakov (Faculty of Pharmacy, Sofia, Bulgaria). HPLC-grade solvents and analytical-grade chemicals were provided by Merck (Darmstadt, Germany). The

water was double distilled. Solvents were filtered through an 0.45-µm filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath before use. Stock solutions were prepared in methanol and stored in a refrigerator. The working standard solutions of appropriate concentration were prepared daily by diluting the stock standard solutions with methanol.

HPLC analysis

The chromatographic analyses were performed on a Varian (Walnut Creek, CA, USA) chromatographic system equipped with a tertiary pump (model 9012), rheodyne injector with 20 µL sample loop, UV-Vis detector model 9050 set at 254 and 360 nm according to the UV absorption maxima of the compounds determined. A Varian Star Chromatography workstation and computer software (version 4.5) for controlling the system and collecting the data were used. A reversed-phase Hypersil ODS RP18, 5 µm, 250 × 4.6 mm i.d., Shandon (Runcom, England) column equipped with a precolumn 30 × 4.6 mm (Varian) filled with the same stationary phase was used. The mobile phase consisted of (A) methanol and (B) 20 mM potassium dihydrogen phosphate adjusted to pH 3.23 by phosphoric acid. The gradient program commenced at 30% A followed by a linear gradient to 45% A for 25 min, an isocratic step (45% A) for 5 min, and then changed to the initial condition in 5 min. The flow rate was 1 mL/min. The oven temperature was set at 27°C.

Quantitative analysis

The analysis of the flavonoids was carried out using the external standard method. Because of poor availability and expense, only rutin and kaempferol 3-rutinoside were used as standards. The responses of genistin and sophorabioside were related to rutin at 254 nm; the concentrations were estimated using the rutin calibration curve. The amounts of isoflavones are relative and not absolute. Working solutions containing 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 mg/mL rutin were prepared from a stock solution of 1 mg/mL in methanol. Solutions containing 0.1, 0.05, 0.02, and 0.01 mg/mL of kaempferol 3-rutinoside were prepared from a stock solution of 0.2 mg/mL.

TriPLICATE analyses were performed for each concentration, and the peak area was detected at 254 nm. Calibration curves were constructed from peak areas versus analyte concentrations. Slope, intercept, and other statistics of calibration lines were calculated with linear regression program using Statistikprogramm STL (Analytik-software, Leer, Germany). For rutin, good linearity of the response with a correlation coefficient of 0.9997 was obtained within the range 0.01–1 mg/mL and the regression equation was $y = 1.56 \times 10^7 x - 1.06 \times 10^4$. From calibration experiments, kaempferol 3-rutinoside

gave a correlation coefficient of 0.9988 within the range 0.01–0.2 mg/mL, and the regression equation was $y = 1.14 \times 10^7 x + 0.59 \times 10^4$.

For each sample, the complete assay procedure was carried out in triplicate and the standard deviation was calculated.

Results and Discussion

Most of the extraction methods published so far used water or aqueous-alcoholic mixtures as solvent for the extraction of rutin and other flavonoids from buds, fruits, or seeds of *Sophora japonica* (Genkina & Shakirov, 1973; Balbaa et al., 1974; Paniwnyk et al., 2001; Tang et al., 2001; Wang et al., 2003). The extraction of rutin was improved by ultrasound (Paniwnyk et al., 2001). In this study, the efficiency of the extraction procedure was tested using different techniques sequentially varying the composition of the aqueous methanol or the aqueous ethanol solvents, and the results are presented in Figure 1. The highest efficiency for rutin extraction was achieved using refluxing with 70% methanol. It was found that the amount of rutin extracted did not change significantly after 15 min of stirring. Hence, the plant materials were exhaustively extracted (3×25 mL solvent) for 15 min each time.

Using this extraction procedure, analysis of flower buds revealed levels of rutin as high as 28.7% (on a dry weight basis) in contrast with 19.5–20.5% reported by Genkina and Shakirov (1973) and 23.5% reported by Balbaa et al. (1974).

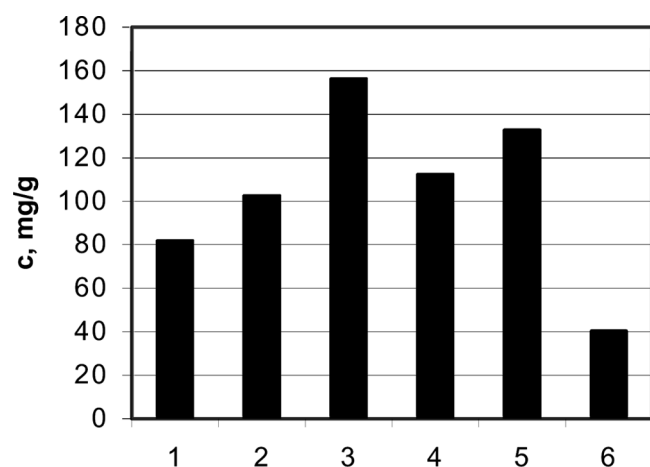


Figure 1. Amount of rutin (mg/g) extracted from the whole inflorescences with different extraction methods: 1–3, extraction by refluxing with constant stirring at 90° (3×15 min); 4–5, ultrasonically assisted extraction at ambient temperature for 20 min; 6, extraction by shaking at ambient temperature for 12 h. Extraction solvents: 1, methanol; 2, 4, and 6, 70% ethanol; 3 and 5, 70% methanol. Rutin was quantified by HPLC. See text for chromatographic conditions.

The subsequent high-performance liquid chromatography (HPLC) separation of flavonol glycosides rutin and kaempferol 3-rutinoside and isoflavone glycosides genistin and sophorabioside was achieved by a linear gradient of mobile phase methanol:phosphate buffer, pH 3.23, starting from 30% methanol and progressing to 45% in 25 min. rutin, sophorabioside, genistin, and kaempferol 3-rutinoside were identified by their retention time (Rt) and by coinjection with standards. The chromatograms of each sample were recorded at 254 and 360 nm to ensure reliable identification of assayed flavonoids. Data were collected at 254 nm because it gave the best signal-to-noise response for all flavonoids simultaneously as well as assisted in distinguishing between flavonol and isoflavone glycosides. In contrast with the flavonols, detection at 360 nm showed no absorption for the isoflavones.

Chromatograms at 254 and 360 nm of a typical flavonoid extract from *Sophora* fruit are presented in Fig. 2 and show the separation achieved for the main flavonoid glycosides. The retention times were as follows: genistin 15.06 ± 0.11 min; sophorabioside 16.51 ± 0.10 min; rutin 19.47 ± 0.08 min; kaempferol 3-rutinoside 24.57 ± 0.07 min. For triplicate analysis of both standards and plant samples, RSDs of the retention times were $\leq 0.7\%$ for flavonoids determined ($n = 6$).

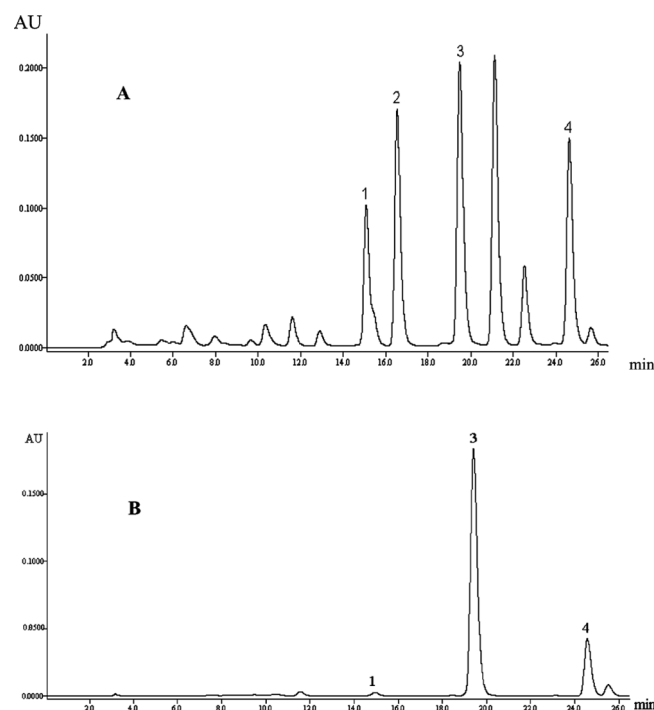


Figure 2. HPLC chromatograms (A, detected at 254 nm; B, detected at 360 nm) of a flavonoid extract from *Sophora japonica* fruits (stage 8). 1, genistin; 2, sophorabioside; 3, rutin; 4, kaempferol 3-rutinoside. See text for chromatographic conditions.

Table 1. Content of the flavonoids (mg/g) in *Sophora japonica* during the different stages for 2 years (2002–2003).

Stage	Rutin		Kaempferol 3-rutinoside		Genistin ^a		Sophorabioside ^a	
	2002	2003	2002	2003	2002	2003	2002	2003
1	287.4 ± 12.1	266.9 ± 11.2	4.6 ± 0.3	4.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	3.1 ± 0.3	2.3 ± 0.1
2	266.5 ± 6.0	234.9 ± 4.9	4.2 ± 0.2	4.1 ± 0.1	1.9 ± 0.1	1.8 ± 0.2	4.6 ± 0.6	4.1 ± 0.6
3	238.4 ± 12.9	234.3 ± 1.3	4.1 ± 0.1	4.1 ± 0.1	2.4 ± 0.2	2.5 ± 0.1	4.2 ± 0.4	4.2 ± 0.2
4	205.3 ± 34.7	234.6 ± 1.2	4.0 ± 0.8	4.8 ± 0.6	3.4 ± 0.8	4.2 ± 0.5	3.3 ± 0.1	3.8 ± 0.5
5	204.7 ± 15.1	211.2 ± 3.1	4.0 ± 0.3	4.0 ± 0.2	4.2 ± 0.8	4.4 ± 0.7	3.7 ± 0.3	3.7 ± 0.1
6	191.9 ± 2.0	199.5 ± 1.8	4.4 ± 0.1	4.2 ± 0.2	3.6 ± 0.1	3.5 ± 0.2	2.3 ± 0.03	2.4 ± 0.2
7	146.2 ± 4.3	145.9 ± 11.1	22.1 ± 1.6	19.2 ± 4.2	1.7 ± 0.3	1.4 ± 0.4	0.7 ± 0.05	0.4 ± 0.1
8	90.8 ± 9.6	81.6 ± 4.0	61.6 ± 3.6	64.8 ± 2.7	23.9 ± 3.8	22.5 ± 5.0	47.8 ± 9.2	50.8 ± 11.6
9	46.6 ± 2.1	44.5 ± 1.5	46.9 ± 3.3	44.1 ± 3.9	24.7 ± 2.2	23.2 ± 2.4	64.7 ± 5.4	60.6 ± 6.9

^aRelative amounts determined using calibration curve for rutin.

The instrument precision was composed of repeatability and reproducibility studies of rutin and kaempferol 3-rutinoside standard preparations. The repeatability was established by injecting the standard solutions of rutin and kaempferol 3-rutinoside (0.01 mg/mL) 10-times. The reproducibility was determined over 10 days by three injections per day of the same solutions. The relative standard deviations (RSDs) of the repeatability and the reproducibility were estimated to be $\leq 1\%$ and $\leq 1.64\%$, respectively.

Limit of detection (LODs) and limit of quantification (LOQ) were calculated according to ICH recommendation (International Conference on Harmonisation, 1995). The detection limits (LODs) of rutin and kaempferol 3-rutinoside were 0.5 and 0.4 $\mu\text{g/mL}$, respectively, and quantification limits (LOQs) were 1.5 and 1.2 $\mu\text{g/mL}$, respectively. The sensitivity obtained for rutin with this method was higher than that achieved by capillary electrophoresis—20 $\mu\text{g/mL}$ (Seitz et al., 1992).

The recovery of the method was checked by addition of the standard solutions of rutin (0.5 mg/mL) and kaempferol 3-rutinoside (0.2 mg/mL) to untreated samples of flower buds. Blank samples from the same plant material, without fortification, were treated and analyzed at the same time with spiked plant matrix under the conditions described in the “Materials and Methods” section. The complete assay procedure was carried out in triplicate.

The recovery of rutin and kaempferol 3-rutinoside from the plant matrix was 98 and 97%, respectively. The average precision of the analytical method, expressed by the RSDs, was estimated by measuring the within-day repeatability, being 3% for rutin and 2.5% for kaempferol 3-rutinoside.

In our view, the current method is suitable for quantitative analysis and quality control of extracts and commercial samples of *Sophora* flower buds and fruits.

The method developed was used for monitoring the dynamics of formation of the main flavonoids in *Sophora japonica*. Rutin was the predominant glycoside during

the earlier phenological stages (from 1 to 4) of flower development (Table 1; Fig. 3). The amount of rutin reached the maximum 287.4 ± 12.1 and 266.9 ± 11.2 mg/g in 2 successive years in youngest buds around mid-July when the flower itself was completely enclosed within a bud (stage 1). Significant differences between phenological stages 2, 3, and 4 with respect to rutin levels were not apparent. In 2002, there was a slight decrease in the amount of this compound detected from the start of the experiment until stage 4 in the unexpanded flowers. In this year, the average amount of rutin in stages 1–4 was 249.4 ± 35.6 mg/g. No difference in rutin quantity between the same phenological stages was observed in 2003 (average amount 242.7 ± 16.2 mg/g).

A further decrease in the amount of rutin up to 146.2 ± 4.3 mg/g (2002) and 145.9 ± 11.1 mg/g (2003) was found proportionally with the decline of flavonoids, throughout the flowering period (stages 5–7). The average amount of rutin in this period was 180.9 ± 30.7 mg/g (2002) and 185.5 ± 34.8 mg/g (2003). After this, a sharp decrease was observed during the latest phenological

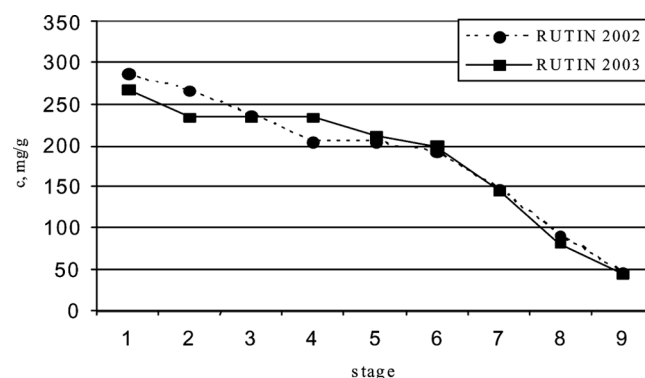


Figure 3. Accumulation of rutin (mg/g, mean values) during flower and fruit development in 2 years 2002 and 2003 (stages as described in “Plant material” section). Rutin was quantified by HPLC. See text for chromatographic conditions.

Table 2. Content of rutin (mg/g) in whole inflorescences over the monitoring period (July 17, 2002 to September 19, 2002).

Data	Rutin
17.07	166.9 ± 9.4
23.07	131.5 ± 6.2
28.07	132.3 ± 3.0
2.08	147.9 ± 3.3
8.08	126.3 ± 3.3
13.08	132.2 ± 5.5
28.08	63.1 ± 7.2
7.09	48.0 ± 2.9
19.09	28.8 ± 2.2

stages 8 and 9 up to 44.5 ± 1.5 mg/g in the period of fruit maturation. No significant differences in rutin quantity between the analysis periods of 2 successive years were observed; this clearly indicated the ontogenetic dependence of rutin formation.

In the whole inflorescences of *Sophora japonica*, rutin followed marked seasonal dependence. Accumulation of rutin started with initiation of the buds, having the highest content in mid-July (166.9 ± 9.4 mg/g), and tended to have high level until mid-August (132.2 ± 5.5 mg/g) (Table 2; Fig. 4). There was no significant difference from the end of July until mid-August. The average amount of rutin in this period was 134.7 ± 9.3 mg/g. After this, a sharp diminution was found. The amount of rutin decreased dramatically within 2 weeks, and further reduction was found after the flowering period—the quantity of rutin was five-fold smaller in mid-September compared with July.

Other main constituents of the flavonoid mixture were sophorabioside, genistin, and kaempferol 3-rutinoside. A clear pattern of these compounds was found with respect to the phenological stages and the sampling time (Table 1; Figs. 5 and 6).

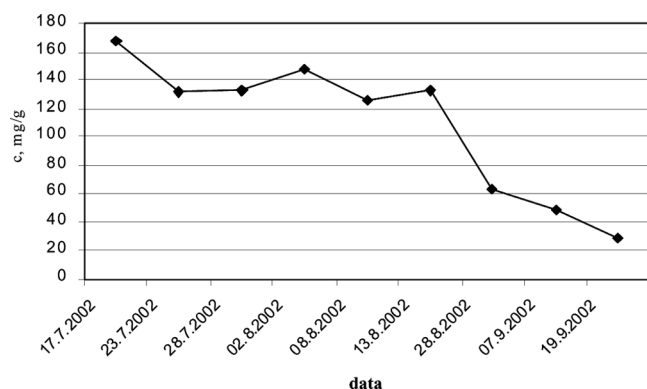


Figure 4. Accumulation of rutin in the whole inflorescences (mg/g, mean values) during seasonal development. Rutin was quantified by HPLC. See text for chromatographic conditions.

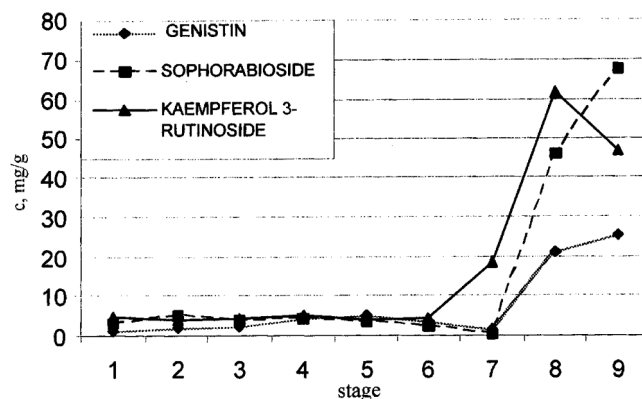


Figure 5. Accumulation of sophorabioside, genistin, and kaempferol 3-rutinoside (mg/g, mean values) during flower and fruit development for 2002 (stages as described in "Plant material" section). Flavonoid glycosides were quantified by HPLC. See text for chromatographic conditions.

In contrast with rutin, sophorabioside, genistin, and kaempferol 3-rutinoside accumulated extensively at the fruiting period but rose steadily until fruits approached maturity. The levels of the isoflavone glycosides were calculated as rutin equivalents. The content ranged from 1.2 ± 0.1 mg/g (genistin) and 4.6 ± 0.3 mg/g (kaempferol 3-rutinoside) in stage 1 (Fig. 6A) to 1.7 ± 0.1 mg/g (genistin) and 22.1 ± 1.6 mg/g (kaempferol 3-rutinoside) in stage 7 (Fig. 6B). Obvious changes in the earlier stages of flower development (between stage 2 and stage 5) included a decrease of sophorabioside and preferred accumulation of genistin and kaempferol 3-rutinoside. sophorabioside, already present in the youngest buds in small amounts, was the only determined flavonoid displaying a marked decrease throughout flower development and was the minor compound at the end of the flowering period (stage 7) (Table 1). However, it was at 64.7 ± 5.4 mg/g the predominant glycoside in fruits (stage 9) apart from rutin followed by kaempferol 3-rutinoside (46.9 ± 3.3 mg/g) and genistin (24.7 ± 2.2 mg/g) (Fig. 6C).

For one compound, kaempferol 3-rutinoside, there was no significant effect of sampling time during bud and flower development. A single maximum (61.6 ± 3.6 mg/g) at the beginning of fruit development was apparent for this compound.

Leaves from *Sophora japonica* contained a lower content of flavonoids than flowers and fruits in the 2 successive years. Rutin was the main compound in the flavonoid mixture of the leaves (29 mg/g), and other flavonoids determined were from 0.05 mg/g (genistin) to 0.7 mg/g (sophorabioside).

Previous studies on the accumulation of rutin in *Sophora japonica* during the ontogenetic development limited analysis to two or three stages of flower development (Bandyukova, 1957; Genkina & Shakirov, 1973;

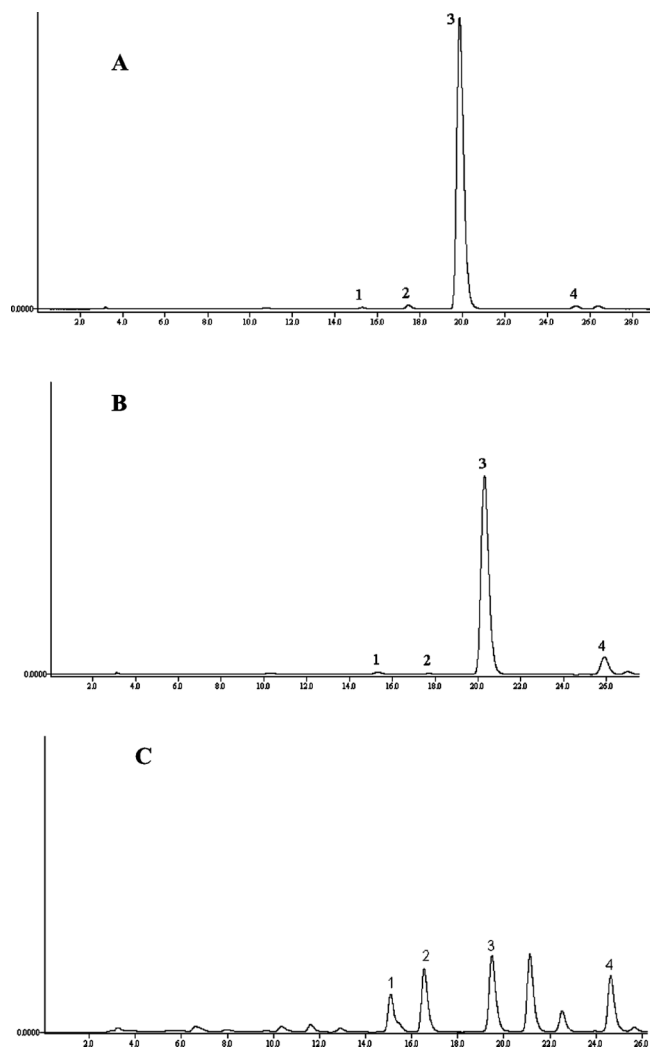


Figure 6. HPLC chromatograms (detected at 254 nm) of flavonoid extracts from *Sophora japonica*: (A) flower buds, stage 1; (B) flowers, stage 7; (C) fruits, stage 9 (for flavonoid numbering, see Fig. 2). Stages are as described in the "Plant material" section. See text for chromatographic conditions.

Balbua et al., 1974). These studies reported a very high concentration of rutin in the unopened flower buds ranging between 13% and 23.50% and decrease in the quantity of rutin as the buds opened. These trends are broadly similar to our results, but more detailed comparisons are not possible as the previous studies used different sampling methodology to those employed here.

Couch et al. (1952) reported that rutin disappeared in the green fruits. In contrast, Karyone et al. (1953) claimed that the green fruits of *Sophora japonica* contained a higher percentage of rutin (44.4%). Contradicting this statement, the studies of Genkina and Shakirov (1973) and Balbua et al. (1974) revealed 3% and 8.70%, respectively, in the green fruits, and a decrease in the amounts of rutin as the fruits approached maturity. The results obtained in our report correlate

with those of Balbua et al. (1974), and the determined content of rutin could be related to the range of rutin in his study.

With regard to the nature of the other flavonoids in the fruits, Szabo et al. (1967) mentioned that a kaempferol glycoside appeared in the fruits at the end of the flowering stage of the plant, followed successively by formation of sophoraflavonolloside, sophoricoside, and sophorabioside. Recent studies confirmed the presence of these compounds and revealed a large number of flavonoids in the pericarps and the seeds of *Sophora japonica* (Tang et al., 2001; Wang et al., 2003). Both sophorabioside and genistin were not detected in buds and flowers by Szabo et al. (1967) and Balbua et al. (1974). Our results revealed the presence of these isoflavonoids in the earlier phenological stages and indicated that sophorabioside and genistin exhibited significant differences with respect to the flower development, whereas kaempferol 3-rutinoside showed slight variation based on the factor considered. In recent years, *Sophora* isoflavonoids have attracted increased attention because of their ability to interact with lipid bilayers, and this fact could broaden the applicability of the plant (Hendrich et al., 2002).

Results obtained supported the ontogenetic dependence of flavonoid glycosides accumulation in *Sophora japonica*. The quantification of these compounds is essential in order to select those plant materials showing the highest flavonoid pattern. The total content of main flavonoids and rutin is maximal in the flower buds in mid-July. Although green unripe fruits contain the highest amounts of determined flavonoids, with regard to the isoflavonoids, mid-September is the best time for harvesting the mature fruits.

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