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QUANTITATIVE HPLC ANALYSIS OF FLAVONOIDS AND CHLOROGENIC ACID IN THE LEAVES AND INFLORESCENCES OF *PRUNUS SEROTINA* EHRH.

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SUMMARY

Two quantitative reversed-phase high-performance liquid chromatographic (RP-HPLC) methods with UV detection have been developed for the separation and quantification of the flavonoids in *Prunus serotina* Ehrh. leaves and inflorescences. The first method entails hydrolysis of the flavonoid compounds and subsequent chromatographic assay of the aglycones obtained, quercetin and kaempferol, for rapid and simple quantification of the total flavonoid content (125 mm × 4 mm, 5-μm particle, ODS column; mobile phase 40–60% methanol gradient in a 0.5% aqueous solution of orthophosphoric acid; run time 7 min). The second method is a direct procedure for simultaneous quantification of six glycosidic compounds (rutin, hyperoside, reynoutrin, guajiverin, avicularin, and juglanin) and chlorogenic acid (250 mm × 4 mm, 5-μm particle, LiChrospher 100 column; mobile phase acetonitrile gradient in a 0.5% aqueous solution of orthophosphoric acid). The precision, accuracy, and reproducibility of the methods used were apparent from good linearity (R = 0.9805–0.9995), recovery of the internal standards (93.5–98.5%), and low *RSD* values (0.4–7.2% for biological plant material). The method for analysis of the aglycones, and its simple modifications can, moreover, be recommended for qualitative and quantitative analysis of three common flavonols – quercetin, kaempferol, and isorhamnetin. This was shown for hydrolysed extracts obtained from six popular medicinal plants (*Hypericum perforatum*, *Sambucus nigra*, *Calendula officinalis*, *Solidago virgaurea*, *Tilia sp.*, and *Gingko biloba*).

INTRODUCTION

Prunus serotina Ehrh. (American black cherry, bird cherry, Virginian prune, rum cherry), the largest of the native cherries in the subgenus

Padus of the family *Rosaceae*, is a very fast-growing tree indigenous to North America and planted, for timber and for ornamental purposes (handsome foliage), in areas with a continental climate, mainly in Europe, where it has become locally naturalised [1,2]. In Poland it occurs commonly, especially in lowlands on dry soils and in many types of forest cover [3].

American black cherry (in common with several other *Prunus* species) is known as a cyanogenic taxon, and until the 1990s its bark was used in the USA and British official pharmacy for treatment of irritating coughs, bronchitis, and asthma (mainly as the principal ingredient of *wild cherry syrup*, a popular vehicle for cough syrups), because of its hydrogen cyanide content [4,5]. Extracts of *P. serotina* have recently been revealed to have significant antioxidant activity also and are potentially useful for antioxidants in cosmetics and phytopharmaceutical production [6].

In previous work [7,8], the flavonoids have been recognised as the main chemical components of *P. serotina* leaves and inflorescences; this may be connected with the antioxidant activity of these plant materials. In the leaf flavonoid complex, fourteen compounds were characterised (isolated and structurally determined or co-chromatographically identified by use of authentic standards) as quercetin, kaempferol, and isorhamnetin monosides and biosides containing the D-galactopyranosyl, D-glucopyranosyl, L-arabinofuranosyl, L-arabinopyranosyl, L-rhamnopyranosyl, and/or D-xylopyranosyl units as the sugar residues.

Over the past decade evidence has accumulated that plant polyphenols and, especially, flavonoids are a most important class of defence antioxidants. With several endogenous antioxidants they play a role in optimum protection from oxidative stress caused by the increase in the level of reactive oxygen species (ROS) in the human organism. Under oxidative stress conditions, ROS (i.e. oxygen-centred free radicals, singlet oxygen, hydrogen peroxide) may be very damaging and play a causative role in aging and several degenerative diseases, for example heart disease, atherosclerosis, cataracts, cognitive dysfunction, hepatotoxicity, inflammation, tumour promotion, and cancer. This pathological process is caused by ROS attack on cell membrane lipids, proteins in tissues or enzymes, carbohydrates, and DNA. The documented mechanism of flavonoid antioxidant action includes suppressing ROS formation (by inhibition of enzymes or chelating trace elements involved in ROS generation), direct ROS scavenging and upregulating or protecting antioxidant defences. Finally, results from several epidemiological studies provide support for a protective effect against chronic diseases of dietary and supplementary intake of flavonoids [9,10].

This has led to more interest in searching for new rich plant sources of flavonoids and for simple and accurate methods of analysis of flavonoids.

The variety of flavonoids occurring in plant materials is usually large, the components of flavonoid fractions come from different classes of aglycone, mono and polyglycoside, or acylated compound, and differ from each other in polarity, molecular weight, and chromatographic and spectrophotometric properties. Every plant has an original and unique flavonoid profile, which makes quantification difficult. For this reason the methods frequently used to determine the total flavonoid content of herbal materials include hydrolysis of the glycosides to reduce the variety and number of analytes. The aglycones obtained can then be quantified by UV spectrophotometric determination as aluminium chelate complexes, as described in several pharmacopoeias [11,12]. For many herbal drugs, however, this method is not reproducible or not accurate, owing to disturbing proanthocyanidines or significantly different molar extinction coefficients of the aglycones analysed and the reference compound quercetin [13,14]. These obstacles can be overcome by combining hydrolysis with HPLC, and several HPLC methods have been used to separate and quantify the widespread flavonoid aglycones [13,14–27]. Several C_{18} (ODS) columns (Hypersil, LiChrosorb, Nucleosil, Vydac, Symmetry) of different length (100–250 mm), inner diameter (2–4.6 mm), and particle size (3 or 5 μm) have been used. The best results for the most critical separation, that of kaempferol and isorhamnetin, have been obtained by Hasler et al. on a 100 mm × 4.6 mm, 5-μm particle, Hypersil ODS column [13, 17]; this method, employing a 38–48.2% linear methanol gradient in 12 min at a flow rate of 2 mL min^{-1}, was adopted in the current work as the reference method. Although this reference method enables very successful separation of kaempferol and isorhamnetin with resolution, R_S, of 1.966 [13], it requires a relatively high flow rate, which can be dangerous for HPLC equipment during long-term, day-to-day routine analyses. Probably for this reason, when this method was recommended as an European pharmacopoeial procedure for standardisation of *Gingko biloba* leaves, the flow rate was reduced to 1 mL min^{-1} and the time of the methanol gradient (40–55%) was increased to 20 min on the 125 mm long column [12].

The objective of this work was, therefore, optimisation of the separation of three of the most widespread flavonol aglycones – quercetin, kaempferol and isorhamnetin – to obtain mild chromatographic conditions with a lower mobile-phase flow rate than the reference method discussed above and with a shorter analysis time than the pharmacopoeial method.

The optimum procedure was applied to *P. serotina* leaves and inflorescences, and simultaneous quantitative analysis of the six predominant flavonoid glycosides and chlorogenic acid was achieved. On the basis of the results, the seasonal dynamics of the plant material were studied.

The method and two simple modifications are also recommended as standard procedures for six other important medicinal plants.

EXPERIMENTAL

Reagents and Materials

Samples of flowers (racemes only or complete inflorescences, i.e. racemes with enclosed 2–3 leaves on short flowering tops) and leaves of *Prunus serotina* Ehrh., and *Gingko* leaves were collected in the Botanical Garden in Łódź and dried in air under normal conditions. Samples of other plant products (*Hyperici herba*, *Sambuci flos*, *Calendulae flos*, *Solidaginis virgaureae herba*, and *Tiliae inflorescentia*) were obtained commercially (Kawon, Gostyń, Polska). All samples were powdered and sieved (0.315 mm sieves, in accordance with Ref. [11]). Voucher specimens were deposited at the Department of Pharmacognosy.

The masses of the different plant materials (100–1000 mg) extracted were selected to obtain the maximum signal intensity, approximately 80–120 mAU.

Quercetin, kaempferol, isorhamnetin, rutin, and chlorogenic acid standards were HPLC-grade purity from Roth (Basel, Switzerland) whereas standards of the other flavonol glycosides (NMR spectroscopy grade purity) had been isolated previously – avicularin, reynoutrin (quercetin 3-*O*-β-D-xylopyranoside), guajiverin (quercetin 3-*O*-α-L-arabinopyranoside), and juglanin (quercetin and kaempferol 3-*O*-α-L-arabinofuranosides) from *Prunus spinosa* flowers [28,29] and hyperoside (quercetin 3-*O*-β-D-galactopyranoside) from *Prunus serotina* leaves [7].

Methanol (MeOH), acetonitrile (ACN), water, and orthophosphoric acid (Merck) were of HPLC-grade purity. Hydrochloric acid (POCh, Gliwice, Poland) was of analytical grade.

HPLC

RP-HPLC was performed with a Hewlett–Packard 1100 Series system (Perlan Technologies, USA) comprising a quaternary pump (HP 1311 A), a vacuum degasser (HP 1322 A), a UV–visible detector (HP 1314 A),

and a 20-µL sample injector (Rheodyne 7725). Compounds were separated on a 125 mm × 4 mm, 5-µm particle, Hypersil ODS column (HP) or a 250 mm × 4 mm, 5-µm particle, LiChrospher 100 column (Merck, Darmstadt, Germany). Chromatograms were acquired with an HP 3396 B reporting integrator, with a chart speed of 4 mm min^{-1} (methods A and B), 9 mm min^{-1} (method C), or 7 mm min^{-1} (method D).

Separated flavonoid peaks were initially identified by direct comparison of their retention times with those of standards. Standard solution was then added to the sample and peaks were identified by the observed increase in their intensity. This procedure was performed separately for each standard.

Optimisation of HPLC Procedures for Determination of Aglycones

Four RP-HPLC methods (A–D) were developed and compared with a widely known method [13] which was used in this work as a reference method (denoted method R). In methods A–C and R the mobile phase was prepared from a 0.5% aqueous solution of orthophosphoric acid (component A) and MeOH (component B). Detection was at 370 nm. In Method D the mobile phase was prepared from a 0.5% aqueous solution of orthophosphoric acid (component A) and ACN (component C) Detection was by UV absorption at 350 nm. Other conditions are listed in Table I.

Table I

Chromatographic conditions used for the methods tested

Method	Gradient profile	Flow rate (mL min^{-1})
A	0–1 min, 40% B; 1–13 min, 40–54% B; 13–14 min, 54% B; 14–15 min, 54–40% B; 15–15.5 min, 40% B (post time).	1.4
B	0–4 min, 40–55% B; 4–9 min, 55% B; 9–10.5 min, 55–40% B; 10.5–11 min, 40% B (post time)	1.2
C	0–0.5 min, 40% B; 0.5–2.0 min, 40–65% B; 2.0–4.0 min, 65% B; 4.0–6.0 min, 65–40% B; 6.0–6.5 min, 40% B; 6.5–7.0 min, 40% B (post time)	1.6
D	0.0–2.0 min, 5% C; 2.0–5.0 min, 5–10% C; 5.0–7.0 min, 10% C; 7.0–10.0 min, 10–15% C; 10.0–13.0 min, 15–20% C; 13.0–23.0 min, 20% C; 23.0–25.0 min, 20–30% C; 25.0–28.0 min, 30% C; 28.0–30.0 min, 30–50% C; 30.0–32.0 min, 50% C; 32.0–33.0 min, 50–5% C; 33.0–34.5 min, 5% C; 34.5–35.0 min, 5% C (post time)	1.0
R	0–12 min, 38–48.2% B	2.0

Quantification of Total Flavonoid Aglycone Content in *Prunus serotina* by HPLC (Method C)

Calibration

A standard stock solution of the aglycones (14.39 μg mL^{-1} for quercetin and 9.78 μg mL^{-1} for kaempferol) was prepared in MeOH and the linearity of the dependence of response on concentration was verified by regression analysis.

Sample Preparation

Plant material (100 mg) was first defatted by pre-extraction with chloroform. (After filtration, the chloroform extract was discarded.) The samples was then heated under reflux for 1 h with 6 mL 25% hydrochloric acid and 20 mL MeOH, and then twice with 20 mL MeOH, each time for 10 min. The hydrolysate was diluted with MeOH to 100 mL and filtered through a 13 mm, 2-μm pore, PTFE syringe filter (Whatman, UK). The filtrate was finally diluted with MeOH in the ratio 1:2 and 20 μL of this solution was injected for HPLC analysis. Analysis was performed after three separate extractions of each sample, and each extract was diluted and injected in triplicate.

Flavonoid Glycoside and Chlorogenic Acid Content (Method D)

Calibration

Standard stock solutions were prepared in MeOH (8.35 μg mL^{-1} for rutin, 25.30 μg mL^{-1} for hyperoside, 9.96 μg mL^{-1} for reynoutrin, 9.93 μg mL^{-1} for guajiverin, 20.80 μg mL^{-1} for avicularin, 7.93 μg mL^{-1} for juglanin, and 39.35 μg mL^{-1} for chlorogenic acid) and the linearity of the dependence of response on concentration was verified by regression analysis.

Sample Preparation

Plant material (100–150 mg leaves or 200 mg inflorescences) was first defatted by pre-extraction with chloroform. (After filtration, the chloroform extract was discarded.) The samples were then heated under reflux for 30 min with 30 mL MeOH and then twice with 20 mL MeOH, each time for 15 min. The extract obtained was diluted with MeOH to 100 mL, filtered through a 13 mm, 2-μm pore, PTFE syringe filter, and 20 μL of this solution was injected for HPLC analysis. Analysis was performed after three separate extractions of each sample, and each extract was diluted and injected in triplicate.

RESULTS AND DISCUSSION

In the course of optimisation of methods for separation and analysis of the common flavonol aglycones quercetin, kaempferol, and isorhamnetin, three methods, A, B, and C, were developed using a 125 mm × 4 mm i.d., 5-µm particle, Hypersil ODS column. The method was tested on six official herbal drugs containing the above mentioned aglycones – *Gingko bilobae folium* (Gingko leaf), *Hyperici herba* (St. John's wort), *Sambuci flos* (elder flower), *Calendulae flos* (calendula flower), *Solidaginis virgaureae herba* (European golden rod), *Tiliae inflorescentia* (lime flower) – and on *Prunus serotina* (bird cherry) leaves and inflorescences. The quality of the separations was described in terms of resolution, R_S, estimated on the basis of the height of the valley between the two adjacent bands kaempferol and isorhamnetin (Table II) [30].

Table II

Characteristic chromatographic data obtained by use of methods A–C and R

Method	Retention time $t_R \pm SD$ (min)[a]			R_S (K/I)[b]	
	Quercetin	Kaempferol	Isorhamnetin	1:1[c]	16:1[c]
A	7.360 ± 0.069	10.697 ± 0.087	11.623 ± 0.095	1.65	–
B	5.235 ± 0.012	6.781 ± 0.021	7.271 ± 0.025	1.46	>1.50
C	4.294 ± 0.015	5.153 ± 0.014	5.350 ± 0.018	1.24	>1.50
R	6.240 ± 0.025	9.670 ± 0.031	10.815 ± 0.052	1.98	–

[a]Mean values ± standard deviation, $n = 12$
[b]Resolution between kaempferol and isorhamnetin [30]
[c]Area ratios for kaempferol and isorhamnetin peaks

Method A, which included a 13-min linear methanol gradient (40–54%) followed by isocratic elution for 1 min, and a flow rate of 1.4 mL min^{-1}, enabled separation of critical bands in ca 11.70 min with satisfactory R_S (1.650), and was universal for all of the plant materials tested, especially *Gingko* (Fig. 1A) and *Tilia* (Fig. 1B), the hydrolysed extracts of which were the most complicated.

Method B incorporated a shortened elution procedure with a flow rate of 1.2 mL min^{-1} and a retention time for isorhamnetin of ca 7.30 min. It enabled still baseline separation ($R_S > 1.5$) of kaempferol and isorhamnetin when at least one of the compounds occurred at a lower concentration, for example in *Calendula*, *Sambucus* (Fig. 2A), *Solidago* (Fig. 2B), *Hype-*

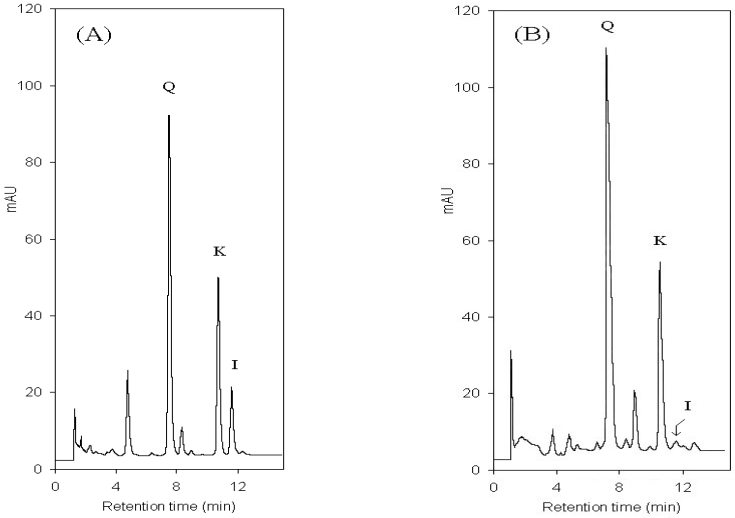

Fig. 1

Chromatograms obtained from hydrolysed extracts of (A) Gingko leaf (1000 mg) and (B) lime flower (500 mg) by use of method A. Detection was at 370 nm, chart speed was 0.4 cm min^{-1}, and full scale was 120 mAU. Peaks: Q, quercetin; K, kaempferol; I, isorhamnetin

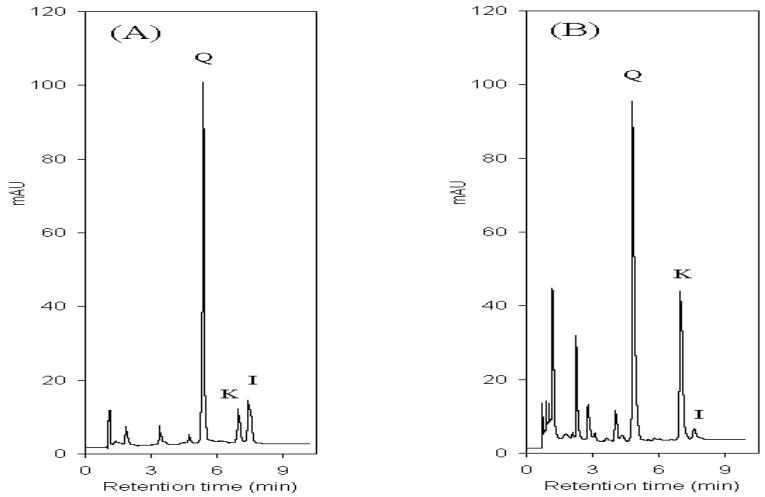

Fig. 2

Chromatograms obtained from hydrolysed extracts of (A) elder flower (200 mg) and (B) European golden rod (1200 mg) by use of method B. Detection was at 370 nm, chart speed was 0.4 cm min^{-1}, and full scale was 120 mAU. Peaks: Q, quercetin; K, kaempferol; I, isorhamnetin

ricum, and *Prunus serotina*), but gave unsatisfactory results for complicated extracts, for example that of *Gingko*.

Finally, method C was the shortest procedure, with elution of the last peak in ca 5.30 min and a flow rate of 1.6 mL min^{-1}. Although R_S was 1.250, this method enabled rapid analysis but only for plants furnishing hydrolysed extracts of simple composition (i.e. without many unidentified or interfering peaks) characterised by trace concentrations of at least one of the critical aglycones kaempferol or isorhamnetin (which could then be ignored). This characteristic was observed for *Hypericum* (Fig. 3A), *Calendula* (Fig. 3B), and *Prunus serotina*. For *Calendula* and *P. serotina* the area ratio of critical peaks was at least 16:1 causing an error in measurement of the area of the larger peak of 0.6–1.0% [30], which was acceptable.

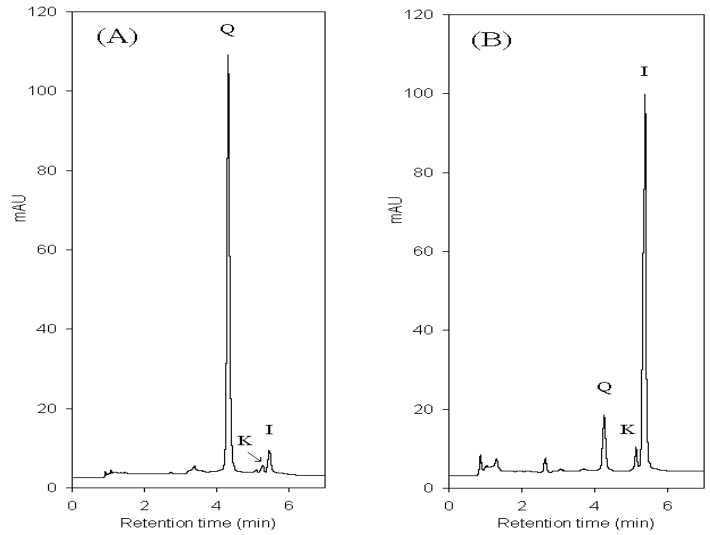

Fig. 3

Chromatograms obtained from hydrolysed extracts of (A) St. John's wort (150 mg) and (B) calendula flower (600 mg) by use of method C. Detection was at 370 nm, chart speed was 0.9 cm min^{-1}, and full scale was 120 mAU. Peaks: Q, quercetin; K, kaempferol; I, isorhamnetin

Method C was then used for systematic quantitative determination of the total flavonoid content of *Prunus serotina*. Two aglycones (quercetin and kaempferol) were determined and isorhamnetin was ignored because of its trace concentration (maximum peak area one-thirtieth that of quercetin) (Fig. 4).

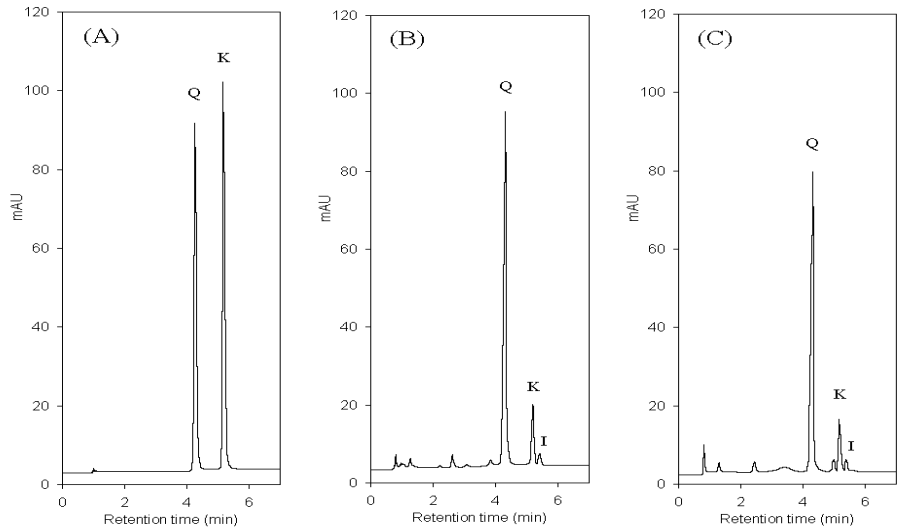

Fig. 4

Chromatograms obtained during determination of total aglycones in hydrolysed *Prunus serotina* extracts by method C. Detection was at 370 nm, the chart speed was 0.9 cm min^{-1}, and full scale was 120 mAU. (A) quercetin (Q, 14.39 μg mL^{-1}) and kaempferol (K, 9.78 μg mL^{-1}) standards, (B) extract of leaves (L 05. 05, 100 mg), (C) extract of inflorescence (F 05. 05, 200 mg)

In this study quercetin levels in bird cherry varied, depending on sample type and harvest time, from 0.87–2.11% in inflorescences to 1.90–3.32% in leaves (Table III). The kaempferol level was significantly lower (0.03–0.24%). The largest amounts of aglycones (3.36–3.56%) were found in young, spring leaves (before flowering). At flowering time the amounts of aglycones in the leaves decreased, stabilising at approximately 2.0%. In the inflorescences, aglycone concentration varied strongly at the bloom stage and was highest for buds (ca 2.0%); in full blossom it decreased by approximately 50% of the initial level. It must be pointed out that the total aglycone level was similar for inflorescences at the bud stage and leaves collected at the same time (ca 2.0% for both). The total aglycone concentration was, moreover, the same for separate racemes and for whole inflorescence shoots (racemes together with 2–3 small accompanying leaves). Similar seasonal dynamics in the flavonoid level have previously been observed for, among others, *Gingko biloba*, *Crataegus monogyna*, *Prunus spinosa*, and *Quercus robur* [14–16,31], so it is probably typical for trees and shrubs. In contrast, for annual and perennial plants amounts of secondary

metabolites are usually highest at flowering time and decrease rapidly after flowering. The total level of aglycones observed was significantly higher than that in other plants rich in flavonol drugs, for example *Sambuci flos*, *Solidaginis virgaureae herba*, *Betulae folium*, *Tiliae inflorescentia*, and *Prunus spinosa flos*, in which the maximum amounts of aglycones were 1.36%, 2.71%, 0.61%, 0.95%, and 2.45–2.64%, respectively [13,15,17]. In conclusion, *Prunus serotina* can be regarded as a valuable and rich source of plant flavonols with expected antioxidant action.

Table III

Total flavonoid content (%) of the leaves (L) and inflorescences (I) of *Prunus serotina* Ehrh.

Sample	Quercetin	Kaempferol	Total aglycones	Total glycosides
L 10. 2002	2.20 (0.9)	0.04 (7.2)	2.24	3.45
L 04. 2004	3.13 (1.0)	0.23 (1.7)	3.36	5.16
L 05. 2004	1.97 (3.3)	0.12 (3.3)	2.09	3.21
L 06. 2004	2.12 (0.4)	0.04 (4.0)	2.16	3.32
L 07. 2004	2.02 (2.9)	0.03 (4.3)	2.06	3.15
L 08. 2004	1.90 (1.0)	0.03 (5.0)	1.92	2.97
L 09. 2004	1.94 (3.2)	0.03 (4.9)	1.97	3.03
L 10. 2004	2.24 (3.7)	0.04 (5.0)	2.28	3.51
L 04. 2005	3.32 (1.2)	0.24 (1.3)	3.56	5.46
L 05. 2005	2.13 (1.0)	0.10 (4.0)	2.23	3.43
L 10. 2005	2.28 (0.6)	0.03 (4.6)	2.31	3.55
I 05. 2004				
Racemes only (floral buds)	1.98 (1.0)	0.13 (2.2)	2.11	3.24
Racemes only (full blossom stage)	0.87 (3.9)	0.11 (2.0)	0.98	1.50
Whole inflorescences (floral buds)	1.99 (0.8)	0.13 (1.5)	2.12	3.25
I 05. 2005				
Racemes only (floral buds)	2.11 (1.2)	0.10 (2.7)	2.21	3.39
Racemes only (full blossom stage)	1.08 (2.7)	0.11 (2.2)	1.19	1.82
Whole inflorescences (floral buds)	2.06 (1.0)	0.11 (1.8)	2.17	3.33

Values in parentheses are relative standard deviations, *RSD* (%) ($n = 3$). The conversion factors ($f_1 = 1.54$ for the aglycone quercetin (MW 302.20) and $f_2 = 1.46$ for kaempferol (MW 286.24)) were determined from the molecular weights of the glycosides hyperoside (quercetin 3-*O*-β-D-galactopyranoside, MW 464.38) and juglanin (kaempferol 3-*O*-α-L-arabinofuranoside, MW 418.20). Calculation: Total glycosides = (f_1 × total quercetin) + (f_2 × total kaempferol).

In the standardisation of phytomedicines, direct determination of the naturally occurring active constituents and the ratios of the predominant

components, at least, would be desirable. Six flavonoid glycosides (rutin, hyperoside, reynoutrin, guajiverin, avicularin, and juglanin) and chlorogenic acid in *P. serotina* were therefore quantified using method D after satisfactory baseline separation of methanol extracts (Table IV, Fig. 5). Seasonal

Table IV

Results from quantitative analysis of the flavonoid and chlorogenic acid content (%) of the leaves (L) and inflorescences (I) of *Prunus serotina* Ehrh.

Sample	Chlorogenic acid	Rutin	Hyperoside	Reynoutrin	Guajiverin	Avicularin	Juglanin	Total
L 10. 2002	1.29 (1.5)	0.14 (2.9)	1.37 (2.2)	0.29 (1.9)	0.08 (4.8)	1.20 (1.3)	0.05 (4.5)	3.13
L 04. 2004	2.30 (2.1)	0.35 (1.9)	2.11 (1.1)	0.40 (3.8)	0.20 (3.5)	1.75 (1.7)	0.20 (2.2)	5.01
L 05. 2004	1.98 (2.2)	0.21 (2.5)	1.24 (1.9)	0.26 (4.2)	0.11 (3.5)	1.08 (3.4)	0.13 (2.5)	3.03
L 06. 2004	1.81 (3.0)	0.13 (3.4)	1.27 (2.0)	0.29 (3.8)	0.08 (2.9)	1.18 (1.9)	0.07 (4.0)	3.09
L 07. 2004	1.54 (1.1)	0.11 (3.0)	1.20 (1.3)	0.28 (2.2)	0.07 (3.0)	1.10 (2.7)	0.05 (3.9)	2.99
L 08. 2004	1.08 (2.5)	0.11 (2.6)	1.28 (2.0)	0.27 (3.4)	0.07 (3.5)	0.98 (3.7)	0.04 (5.0)	2.75
L 09. 2004	1.14 (1.4)	0.10 (4.0)	1.30 (1.5)	0.29 (2.6)	0.07 (4.2)	0.96 (2.5)	0.05 (4.5)	2.77
L 10. 2004	1.18 (0.9)	0.16 (2.7)	1.58 (1.1)	0.31 (3.0)	0.08 (5.0)	1.16 (1.1)	0.05 (3.2)	3.34
L 04. 2005	2.25 (1.4)	0.32 (4.2)	2.23 (0.7)	0.44 (3.5)	0.22 (2.4)	1.82 (1.6)	0.22 (2.9)	5.25
L 05. 2005	2.05 (1.8)	0.16 (3.1)	1.37 (1.2)	0.35 (4.2)	0.12 (3.0)	1.15 (2.0)	0.11 (2.8)	3.26
L 10. 2005	1.23 (0.7)	0.18 (2.8)	1.50 (2.0)	0.32 (2.9)	0.10 (2.9)	1.22 (1.4)	0.04 (3.5)	3.36
I 05. 2004								
(a)	0.95 (1.6)	0.31 (2.0)	1.50 (2.1)	0.15 (4.8)	0.25 (2.5)	0.65 (1.8)	0.16 (2.0)	3.02
(b)	0.63 (2.6)	0.20 (1.8)	0.80 (1.2)	0.08 (5.2)	0.10 (2.7)	0.20 (2.6)	0.08 (1.9)	1.46
(c)	1.82 (1.5)	0.25 (3.4)	1.35 (1.7)	0.19 (3.7)	0.21 (2.5)	0.90 (1.1)	0.13 (3.2)	3.03
I 05. 2005								
(a)	0.96 (2.1)	0.29 (3.0)	1.59 (0.9)	0.17 (3.5)	0.28 (1.9)	0.69 (2.0)	0.15 (2.4)	3.17
(b)	0.71 (1.7)	0.17 (2.5)	0.86 (2.3)	0.10 (5.8)	0.11 (2.6)	0.39 (2.3)	0.08 (3.0)	1.71
(c)	1.90 (2.0)	0.22 (2.3)	1.41 (1.4)	0.21 (3.0)	0.18 (2.2)	0.95 (1.3)	0.13 (3.5)	3.10

Values in parentheses are relative standard deviations *RSD* (%) (n = 3)
(a), (b), (c) are samples of inflorescences as in Table III

dynamics in the flavonoid content was similar to that observed in the measurement of total flavonoids. The highest level was detected for hyperoside; the amount in the leaves was ca 2.1% in the spring (before flowering), 1.2–1.3% in the summer, and 1.5% in the autumn; in bud inflorescences it was approximately 1.5%. The amount of the second major glycoside – avicularin – in the leaves was 1.8% in the spring and 1.0–1.2% at other times; the amount in bud inflorescences was 0.7–0.9%. Levels of the other four flavonols were lower, approximately 0.1–0.4%, with the highest value (0.4%) observed for rutin in young spring leaves (before flowering) and in bud inflorescences, and for reynoutrin in young spring and autumn leaves. The lowest amounts were for juglanin in mature leaves (only 0.04–0.07%).

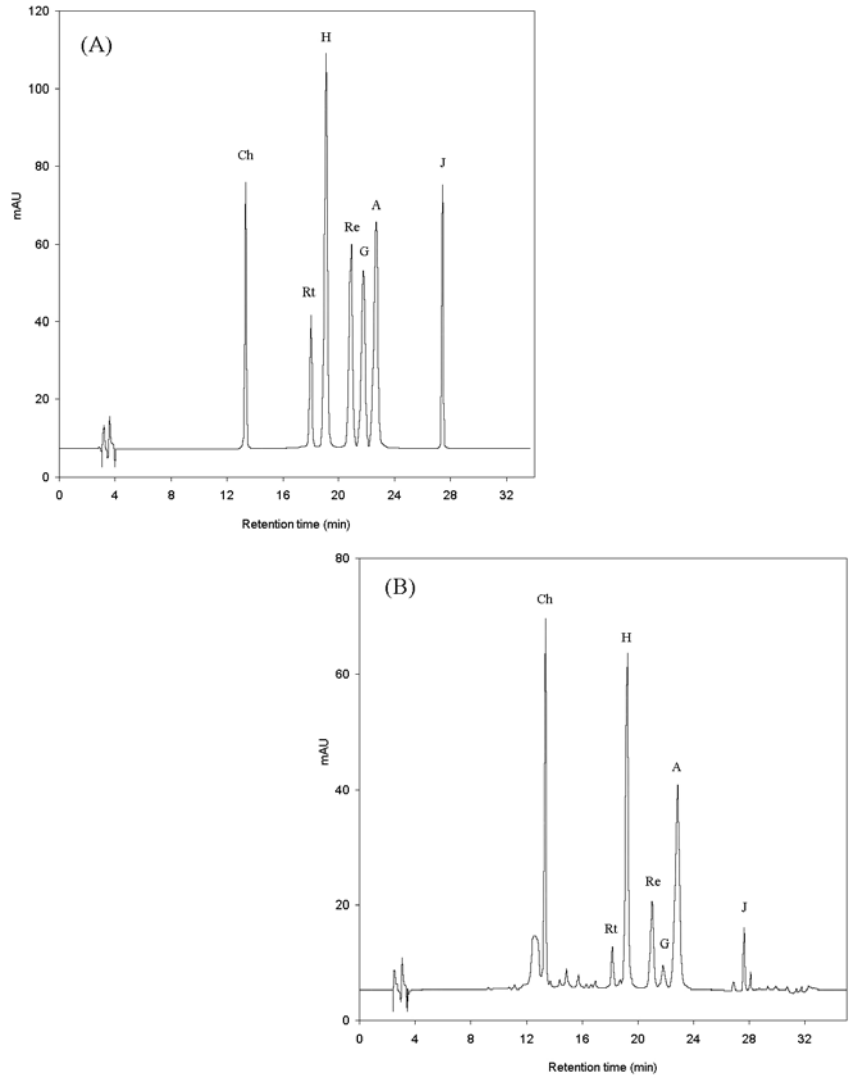

Fig. 5

Chromatograms obtained during determination of the major components of crude *Prunus serotina* methanol extracts by use of method D. Detection was at 350 nm, the chart speed was 0.7 cm min^{-1}, and full scale was 80–120 mAU. (A) Standard solution. Peaks: Ch, chlorogenic acid (39.35 µg mL^{-1}, t_R = 13.291 ± 0.087 min); Rt, rutin (8.35 µg mL^{-1}, t_R = 17.959 ± 0.087 min); H, hyperoside (25.3 µg mL^{-1}, t_R = 19.077 ± 0.113 min); Re, reynoutrin (9.96 µg mL^{-1}, t_R = 20.913 ± 0.178 min); G, guajiverin (9.93 µg mL^{-1}, t_R = 21.640 ± 0.151 min); A, avicularin (20.80 µg mL^{-1}, t_R = 22.780 ± 0.197 min); J, juglanin (7.93 µg mL^{-1}, t_R = 27.754 ± 0.061). t_R values are means ± *SD* from twelve measurements. (B): Leaf sample (L 05. 2005, 150 mg)

Apart from flavonoids, in the chromatograms obtained from the methanol extracts a significant band of another constituent was observed; this was identified as chlorogenic acid, a phenolic acid. The amounts present varied within the range 0.63–2.30%. The highest values (2.0–2.3%) were detected in young spring leaves (before and during flowering); during vegetation the level decreased steadily to 1.1–1.3%, so the seasonal dynamics of this analyte in the leaves was similar to those confirmed for the flavonoids. In contrast, the chlorogenic acid content of bud inflorescences (ca 1.0%) was approximately half that in whole inflorescence shoots (1.8–1.9%) and in leaves collected at the same time (ca 2.0%). In full blossom, the amount of chlorogenic acid decreased by approximately 50%, similarly to the flavonoid level, which indicated that harvesting at this time is not optimal.

According to recent systematic studies of the antioxidant activity of hyperoside [32] and chlorogenic acid [33] we can expect a direct correlation between high levels of these analytes and high biological activity of extracts which contain them, including *P. serotina* extracts.

The precision, accuracy, and reproducibility of the methods used were apparent from good linearity, recovery of the internal standards, and low

Table V

Linearity of calibration plots, and limits of detection and quantification for methods C and D

Compound	Linear range (μg mL^{-1})	$y = ax + b$ (linear model)[a]	Correlation coefficient, R[b]	LOD[c] (μg mL^{-1})	LOQ[c] (μg mL^{-1})
Method C					
Quercetin	2.40–14.39	$y = 655997x + 166863$	0.9978	0.076	0.253
Kaempferol	1.63–9.78	$y = 848327x + 122346$	0.9978	0.042	0.140
Method D					
Chlorogenic acid	4.72–39.35	$y = 131158x + 333533$	0.9888	0.378	1.259
Rutin	0.83–8.35	$y = 220759x + 93095$	0.9805	0.167	0.557
Hyperoside	4.86–25.30	$y = 288640x + 243053$	0.9884	0.243	0.810
Reynoutrin	1.33–9.96	$y = 394413x - 39725$	0.9988	0.266	0.885
Guajiverin	1.32–9.93	$y = 330477x + 120726$	0.9932	0.265	0.883
Avicularin	3.99–20.80	$y = 297886x + 175150$	0.9919	0.399	1.331
Juglanin	0.79–7.93	$y = 327258x + 64125$	0.9953	0.079	0.264

[a] y = peak area, x = concentration of standard (μg mL^{-1})
[b] For five points on the calibration curve, each concentration being injected three times – at the start, in the middle, and after the end of the assays
[c] *LOD* and *LOQ* are the limits of detection and quantitation, respectively

RSD values. The linearity of the dependence of response on concentration was verified by triplicate analysis of five solutions of each standard. The calibration plots for all the compounds were indicative of high correlation of peak area with flavonoid concentration; none of the regression coefficients was less than 0.98. The limits of detection and quantitation were determined by serial dilution, on the basis of a signal-to-noise ratio of 3:1 for the limit of detection and 10:1 for the limit of quantitation (Table V).

Complete analyses were performed in triplicate to calculate the average standard deviations as a measure of chromatographic precision and reproducibility. The results presented in Tables III and IV show the precision of the methods is relatively high – relative standard deviations (*RSD*) were less than 4% for all the samples except for low concentrations of kaempferol (4–7.2%) and the minor flavonol glycosides (occasionally 4–5.8% for reynoutrin, guajiverin, and juglanin).

Validation of the accuracy of the methods was achieved by six determinations of the recovery by the standard addition procedure. The average recoveries obtained were satisfactory, always higher than 93%, which confirmed the accuracy of the methods was good (Table VI).

Table VI

Recovery of the methods used for determination of the constituents of *Prunus serotina*

Compound	Initial mean content (%) of L 04. 2005	Initial conc. (μg mL^{-1})[a]	Amount added (μg) before extraction	Conc. after addition (μg mL^{-1})[a]		Recovery (%)[b]
				Expected	Measured	
Method C						
Quercetin	3.32	16.60	770	20.45	20.14	98.5 (0.9)
Kaempferol	0.24	1.20	804	5.22	5.10	97.7 (2.5)
Method D						
Chlorogenic acid	2.25	22.50	654	29.04	27.15	93.5 (0.8)
Rutin	0.32	3.20	403	7.23	6.89	95.3 (1.6)
Hyperoside	2.13	21.30	345	24.75	23.27	94.0 (1.3)
Reynoutrin	0.44	4.40	452	8.92	8.53	95.6 (2.8)
Guajiverin	0.22	2.20	530	7.50	7.10	94.7 (2.0)
Avicularin	1.80	18.00	414	22.14	21.37	96.5 (1.0)
Juglanin	0.22	2.20	425	6.45	6.31	97.9 (1.7)

[a]Mean concentration of the analyte in the final analytical solution
[b]Values in parentheses are relative standard deviations *RSD* (%) (n = 6)

CONCLUSIONS

Quantitative analysis of *Prunus serotina* flavonoids and chlorogenic acid was achieved for the first time in this work. The observed levels of the polyphenols confirm the importance of *P. serotina* leaves and inflorescences as excellent sources of plant antioxidants. The HPLC method proposed for analysis of the aglycones enables reproducible and accurate determination of three of the most common flavonols – quercetin, kaempferol, and isorhamnetin. This method and its two modifications can be recommended as simple and rapid standard procedures for six important medicinal plants (*Hypericum perforatum*, *Sambucus nigra*, *Calendula officinalis*, *Solidago virgaurea*, *Tilia sp.*, and *Gingko biloba*).

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