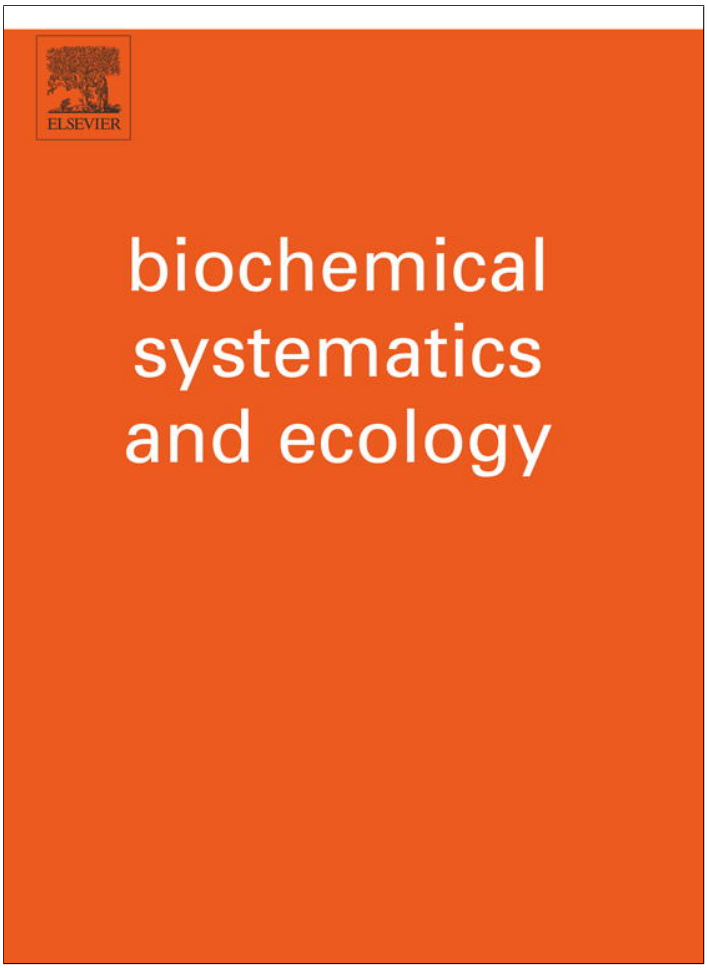

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Phenolic derivatives in raspberry (*Rubus* L.) germplasm collection in Bulgaria

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ABSTRACT

Leaves of 22 accessions of the Bulgarian raspberry (*Rubus* L.) germplasm collection – five Bulgarian and seven foreign cultivars, eight elite clones and two wild species, *Rubus occidentalis* L. and *Rubus odoratus* L., were analysed for phenolic constituents. The quantitative determination of caffeic (**1**), *p*-coumaric (**2**) and ferulic (**3**) acids, hyperoside (**4**), tiliroside (**5**), and isoquercitrin (**6**) was performed by RP-HPLC using linear gradient elution and UV detection at 254 and 310 nm. The detection limits ranged from 0.23 μg/ml (**4**) to 0.55 μg/ml (**2**). Caffeic acid was the dominant phenolic acid in the majority of the samples being present in amounts between 0.05 ± 0.01 mg/g dry weight in *R. occidentalis* and 1.43 ± 0.06 mg/g in the cultivars. The highest content of flavonols was found in the Bulgarian raspberry elite clones: 1.70 ± 0.002 mg/g (**6**), 0.60 ± 0.004 mg/g (**5**) and 0.97 ± 0.01 mg/g (**4**). Data were analyzed by hierarchical clustering (HC) and principal component analysis (PCA). The HC and PCA scoring plot showed that the samples could be classified into three clusters. Cluster C1 comprised cultivars characterized by high content of phenolic acids (**1–3**). Seven cultivars and the wild species *R. occidentalis* formed the cluster C2 presenting low content of phenolic compounds. Cluster C3 grouped the Bulgarian raspberry elite clones and *R. odoratus* sharing the highest content of flavonols (**5** and **6**). The Bulgarian elite clone E23617 displayed the highest content of the studied phenolic derivatives. The PCA loading plot showed that **1** can be used to distinguish between different raspberry varieties.

1. Introduction

The genus *Rubus* L. (e.g. raspberry, blackberry, and cloudberry) comprises around 700 species, naturally occurring in temperate climates (Alice and Campbell, 1999). *Rubus idaeus* L. leaves contain a notable amount of tannins, flavonoids, represented by derivatives of kaempferol and quercetin, phenolic acids, triterpenes as well as vitamin C (Gudej and Rychlinska, 1996; Gudej, 2003; Gudej and Tomczyk, 2004). The flavonoids and phenolic acids in *Rubus* berries and leaves have antioxidant, antimutagenic, anticarcinogenic, cytotoxic, and antimicrobial properties (Thiem, 2003; Badjakov et al., 2008; Kahkonen et al., 2012; Durgo et al., 2012). The phenolic compounds have been evaluated for free radical scavenging activity (Park et al., 2006; Michels et al., 2006; Kim et al., 2012). Other studies have reported that caffeic acid has antitumour

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and anti-metastatic effects (Chung et al., 2004); antiproliferative activity of isoquercitrin has been observed as well (Amado et al., 2009). In addition, tiliroside and *p*-coumaric acid exhibited anticoagulant and antiplateled activities (Han et al., 2012).

Previous reports showed that flavonol glycosides including quercetin 3-glucoside (isoquercitrin), quercetin 3-galactoside (hyperoside), kaempferol 3-galactoside, kaempferol 3-arabinoside, kaempferol 3-β-D (6"-*p*-coumaroyl)-glucoside (tiliroside), kaempferol 3-glucoside and quercetin 3-rutinoside (rutin) have been identified in *R. idaeus* and *Rubus chingii* leaves (Gudej, 2003; Han et al., 2012; Giao et al., 2012). In addition, salicylic and chlorogenic acids were identified (Han et al., 2012; Giao et al., 2012). Phenolic compounds in berries and leaves of *Rubus* species were determined by spectrophotometric, HPLC-UV, HPLC-DAD and HPLC-ESI/MS methods (Hakkinen et al., 1999a, 1999b; Määttä-Riihinen et al., 2004; Gudej and Tomczyk, 2004; Han et al., 2012). However, no quantitative data are available in the literature about the content of flavonol glycosides and hydroxycinnamic acids in raspberry leaves.

In the present study, the phenolic compounds caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), hyperoside (**4**), tiliroside (**5**) and isoquercitrin (**6**) were analysed quantitatively in 22 accessions of the Bulgarian raspberry (*Rubus* L.) germplasm collection. The aim of this study was to identify and quantify simultaneously phenolic acids (**1–3**) and flavonol glycosides (**4–6**) in the leaves of five Bulgarian and seven foreign cultivars, eight Bulgarian elite clones and two wild species, *Rubus occidentalis* L. and *Rubus odoratus*. Based on the content of phenolic derivates, the studied accessions were clustered and the dominant compound responsible for the clustering was identified. The phytochemical analysis of Bulgarian cultivars in comparison with foreign raspberry cultivars provides information about health promoting phenolic compounds and facilitates the accurate selection of genotypes with valuable characters in breeding programmes.

2. Materials and methods

2.1. Plant material

Twenty two accessions from the Bulgarian raspberry germplasm collection of the Institute of Agriculture (Kiustendil, Bulgaria) were selected for the assay. They included 5 Bulgarian and 7 foreign cultivars, 8 Bulgarian elite clones, and two wild raspberry species (Table 1). Raspberry leaves were collected in 2010 from experimental area in Kostinbrod region, Bulgaria (42°49′6.29″N, 23°13′29.91″E), 540 m above sea level.

2.2. Sample extraction

Dried leaves (0.50 g of each sample) were extracted with methanol:water (80:20, v/v) at room temperature (3 × 25 ml) for 24 h. The combined extracts were evaporated and concentrated mixture was extracted with dichloromethane to eliminate lipophilic compounds. The combined aqueous layers were concentrated and obtained mixture was extracted (×3) with ethyl acetate. The ethyl acetate extracts were evaporated *in vacuo*. The dry extract was dissolved in 1 ml methanol and filtered through a 0.45 μm syringe filter disc (Polypure II, Alltech, Lokeren, Belgium). Samples of 20 μl aliquots were injected into the chromatographic system.

Table 1
Description of accessions.

Accession name	Pedigree	Developed in:
R. odoratus		
R. occidentalis		
Shopska alena	[(Preussen × Lloyd George) × Newburgh]	Bulgaria, 1970
Samodiva	(Balgarian rubin × Shopska alena)	Bulgaria, 1985
Iskra	(Lloyd George × Preussen)	Bulgaria, 1974
Lyulin	[(Newburgh × Balg. rubin) × Heritigae]	Bulgaria, 1984
Balg. rubin	(Preussen × Lloyd George)	Bulgaria, 1957
E23617	(Iskra × Novost Kuzmina)	Bulgaria
E21878	(Balgarian rubin × Teodor Reimers)	Bulgaria
E26664	(Samodiva × Gradina)	Bulgaria
E27337	(Samodiva × 7667)	Bulgaria
E25927	(Samodiva × Podgorina)	Bulgaria
E23501	(Iskra × Novost Kuzmina)	Bulgaria
E27562	(Samodiva × Veten)	Bulgaria
E21840	(Shopska alena × Willamette)	Bulgaria
Meeker	(Willamette × Cuthbert)	US, 1967
Schoenemann	(Lloyd George × Preussen)	Germany, 1950
Willamette	(Newburgh × Lloyd George)	US, 1943
Autumn bliss	Complex parentage ncludes Lloyd George, *Rubus arcticu*, *R. occidentalis*, *R. idaeus* strigosus	UK, 1977
Zeva	(Rote Wadenswiler × Willamette)	Switzerland, 1963
Tulameen	(Nootka × Glen Prosen)	Canada, 2001
Latham	*(R. strigosus* × *R. ursinus)*	US

2.3. Chemicals and reagents

The standards of caffeic (**1**), *p*-coumaric (**2**) and ferulic (**3**) acids, and hyperoside (**4**), tiliroside (**5**), and isoquercitrin (**6**) were purchased from Extrasynthese (Genay, France). HPLC-grade solvents and analytical-grade chemicals were provided by Merck (Darmstadt, Germany). The stock standard solutions of these compounds were prepared in methanol and were stored at 4 °C in the dark. The working standard solutions of appropriate concentration were prepared by diluting the stock standard solutions with methanol.

2.4. Chromatographic equipment and conditions

The chromatographic analyses were performed on a Varian (Walnut Creek, CA, USA) chromatographic system, which consisted of a tertiary pump model 9012, a Rheodyne injector with a 20 μL sample loop and a UV–VIS detector model 9050. The chromatograms of each sample were recorded at 254 and 310 nm to ensure reliable identification of the phenolic acids. Data were collected at 254 nm because it gave the best signal-to-noise response for all phenolic acids and flavonol glycosides simultaneously. A Varian Star Chromatography workstation running version 4.5 software was used to control the HPLC system and to collect the data. The separation was carried out with a Varian Microsorb MV 100-5 column (150 × 4.6 mm i.d.; 5 μm: Varian USA), fitted with a Varian pre-column (30 × 4.6 mm i.d.) dry packed with Perisorb RP-18 (30–40 μm: Merck) and periodically changed: both columns were maintained at room temperature. The mobile phase was composed of methanol (A) and 20 mM potassium dihydrogen phosphate buffer (B) (adjusted to pH 2.92 with ortho-phosphoric acid). The high-performance liquid chromatography (HPLC) separation of phenolic acids and flavonoid glycosides was achieved by a linear gradient starting from 15% A and progressing to 42% A in 25 min followed by a linear gradient to 70% A for 10 min and then return to the initial condition in 5min. The solvents were filtered through Millipore (Watford, Ireland) 0.45 μm filters and degassed in an ultrasonic bath prior to use. The flow rate was 1 ml/min. The oven temperature was set at 30 °C.

2.5. Quantitative analysis and analytical performance

The analysis of phenolic acids (**1–3**) and flavonol glycosides (**4–6**) was carried out using the external standard method. Because of the similar molecular structure, the responses of isoquercitrin (**6**) were related to hyperoside (**4**), assuming the responses at 254 nm to be equal. External standard calibrations were established on five data points covering the concentration range of each analyte according to the level expected in the plant samples. Working solutions containing 0.5, 0.2, 0.1, 0.05, 0.005 mg/ml of **1–3** were prepared from stock solution of 1.0 mg/ml. The employed concentrations of **4** and **5** were 1.0, 0.5, 0.2, 0.1, 0.01 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 *vs* analyte concentrations. Slope, intercept, and other statistics of calibration lines were calculated with linear regression program using the Analytik-Software (Leer, Germany) STL statistics programme. The regression equations were, respectively, for compounds **1–5**: $y = 1.38 \times 10^7 x - 5.03 \times 10^4$ ($r^2 = 0.9998$); $y = 7.91 \times 10^6 x - 2.33 \times 10^4$ ($r^2 = 0.9999$); $y = 1.28 \times 10^7 x - 4964.70$ ($r^2 = 0.9999$); $y = 2.07 \times 10^7 x - 1.01 \times 10^4$ ($r^2 = 0.9999$) and $y = 1.36 \times 10^7 x + 9.38 \times 10^4$ ($r^2 = 0.9998$).

The examined compounds were assigned in the HPLC chromatograms by comparing individual peak retention times with these of authentic references standards, as well as by spiking techniques. For each sample, the complete assay procedure was carried out in triplicate and standard deviation was calculated.

The repeatability was established by injecting the standard solution (0.01 mg/ml) six times. The reproducibility was determined over 10 days by three injections per day of the same solution.

The limits of detection (LODs) were calculated according to the expression $3.3\sigma/S$, were σ was the standard deviation of the response and S the slope of the calibration curve (International Conference on Harmonisation, 1995). Limits of quantitation (LOQs) were established from the expression $10\sigma/S$. In order to check the percentage recovery, known quantities of standards of assayed phenolic acids and flavonoids were added to known amounts of *Rubus* leaves (*R. idaeus* Iskra). Recoveries of the analytes at following levels were examined: 0.55 mg/ml (caffeic acid), 0.25 mg/ml (*p*-coumaric acid), 0.45 mg/ml (ferulic acid), 0.30 mg/ml (hyperoside), 0.27 mg/ml (tilliriside) and 0.25 mg/ml (isoquercitrin). The fortified samples were than extracted and analyzed with the proposed sample preparation procedure and HPLC method. The percentage recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that had been prepared with the added standards, dividing by the amount added and multiplying by 100.

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Student's *t*-test was used to evaluate the differences between experimental and control groups. *P* values less than 0.05 were considered as significant.

2.6.1. Hierarchical clustering (HC)

Clustering is a process of dividing set of entities into subsets in which the members of each subset are similar to each other but different from members of other subsets (Barnard and Downs, 1992). In the present study, a hierarchical clustering using

the agglomerative algorithm (Barnard and Downs, 1992) was applied. According to this algorithm, the clusters are built from the bottom up, first by merging individual items into clusters, and then by merging clusters into superclusters, until the final merge brings all items into a single cluster.

The HC was applied as implemented in MDL QSAR v. 2.2 package (2004). The distance between clusters was calculated by average-linkage method, i.e. the average distance between the items in both clusters is taken into account.

2.6.2. Principal component analysis (PCA)

PCA is a multivariate data analysis designed to represent large, multidimensional data sets in a limited, but visually interpretable, number of dimensions, usually two to five, referred to as principal components (PC), such that an overview of the data is obtained. This overview may reveal groups of observations, trends, and outliers. It also uncovers the relationships between observations and variables and between the variables themselves (Eriksson et al., 2001). The results from PCA can be visualized on different plots: scores and loadings. Score plots (observation projects on PC) visualize groupings of the accessions based on their composition. Accessions with similar composition are clustered together. Loading plots (variable projections on PC) visualize the contributions of the phenolic compounds in the clustering of accessions. PCA was used as implemented in SIMCA-P 13.0 (2012).

3. Results

3.1. Quantitative determination of phenolic compounds

Based on the traditional approach, extraction of the raspberry leaves with hydroalcoholic solvent and further fractionation by liquid–liquid extraction (LLE) with ethyl acetate was applied in this study (de Rijke et al., 2006). In published extraction procedures ethyl acetate has been suggested as a more suitable solvent (Robbins and Bean, 2004). It has been shown that the recoveries of caffeic, syringic, ferulic, chlorogenic, *p*-hydroxybenzoic acids are higher in ethyl acetate than in diethyl ether (Fernandez de Simon et al., 1990). Most of the studies of flavonoid glycosides present in medicinal plants have been carried out by LLE using ethyl acetate (de Rijke et al., 2006).

To assess the natural variation of caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), hyperoside (**4**), tiliroside (**5**) and isoquercitrin (**6**) in the leaves of 22 raspberry accessions, a validated analytical RP-HPLC method was developed.

In the literature, the single most comment wavelength used for detection of phenolic acids and flavonoids is 254 nm since it gives the best signal to noise response for assayed compounds simultaneously as well as assists in distinguishing between flavonols and hydroxycinnamic acids (Robbins and Bean, 2004; de Rijke et al., 2006). Flavonols have their λ_{max} in the ranges 240–280 nm and 350–385 nm; cinnamate derivatives show an additional broad absorbance band from 270 to 360 nm. Therefore, to enhance selectivity, the longer λ_{max} was chosen for detection of the hydroxycinnamic acids and data were also collected at 310 nm. They can easily distinguish at different wavelength (310 *versus* 254 nm). Moreover, the relatively small structural features of the phenolic acids make a difference in retention on the C18 stationary phase as illustrated by caffeic and ferulic acids while no difference in UV absorbance maxima is observed (Robbins and Bean, 2004).

In respect to the analytical performance, for triplicate analysis of both standards and plant samples, RSDs of the retention times were $\leq$0.7% for the determined compounds ($n = 6$). The proposed method was found to be linear in the range 1.0–0.005 mg/ml for compounds **1–3** and in the range 1.0–0.01 mg/ml for compounds **4** and **5**. The instrument precision was composed of repeatability and reproducibility studies of the assayed compounds. The RSDs of the repeatability and the reproducibility were estimated to be $\leq$3.0% and $\leq$5.6% respectively. The detection limits (LODs) of **1–5** were 0.25 μg/ml, 0.55 μg/ml, 0.51 μg/ml, 0.23 μg/ml and 0.49 μg/ml, respectively and quantification limits (LOQs) were 0.76 μg/ml, 1.67 μg/ml, 1.55 μg/ml, 0.70 μg/ml and 1.49 μg/ml, respectively. As regards the recovery of the method, the responses from samples and fortified samples were compared and the following recovery data obtained 103.6 ± 3.4%, 101.3 ± 5.0%, 70.9 ± 11.9%, 105.2 ± 4.0%, 103.8 ± 3.9% and 91.4 ± 3.2% (RSD<16.9%) for compounds **1–6**, respectively. The average absolute recoveries were 91.9 and 100.1% for the phenolic acids and flavonoids, respectively. It was found that the percentage ratio is within the acceptance range of 90–110% (Huber, 1998). Only in the case of ferulic acid no satisfactory recovery was obtained. In the calculation of final results, the recoveries were not taken into account.

Typical HPLC chromatograms of standard mixture and raspberry samples are presented in Figs. 1 and 2, respectively.

The content of phenolic acids and flavonoids in the assayed samples is shown in Table 2. Mean values of individual phenolic compounds and their total content in each accession were compared by *t*-test: significant differences between *Rubus* varieties were found ($p \leq 0.05$). In the present study, caffeic acid (**1**) was the dominant phenolic acid in the majority of the samples being present in amounts up to 0.71 ± 0.02 mg/g dry weight in the wild species and up to 1.43 ± 0.06 mg/g in the cultivars. The content of caffeic acid was from 7% (*R. idaeus* Latham) (Fig. 2A) to 61% (*R. idaeus* Zeva) of the total amount of assayed compounds. Ferulic acid (**3**) had a low percentage (below 2%) in accessions E18878, E26664, E27337 and E21840. However, in some cultivars (*R. idaeus* Bulgarian rubin, Samodiva) it occurred in relatively higher concentration. This makes it suitable as marker compound of the chemical profile.

With respect to the flavonol glycosides, isoquercitrin (**6**) was present in the highest amounts, while content of **4** and **5** was considerably lower for both Bulgarian and foreign cultivars (Fig. 2A and B). The content of hyperoside (**4**) was substantially lower and ranged between 8.7% (Shopska alena) and 47.1% (E26664) of total in Bulgarian cultivars, and from 2.1% (Zeva) to 42.9% (Latham) (Fig. 2A) in foreign cultivars. Significant differences with respect to **5** in foreign varieties were no evident.

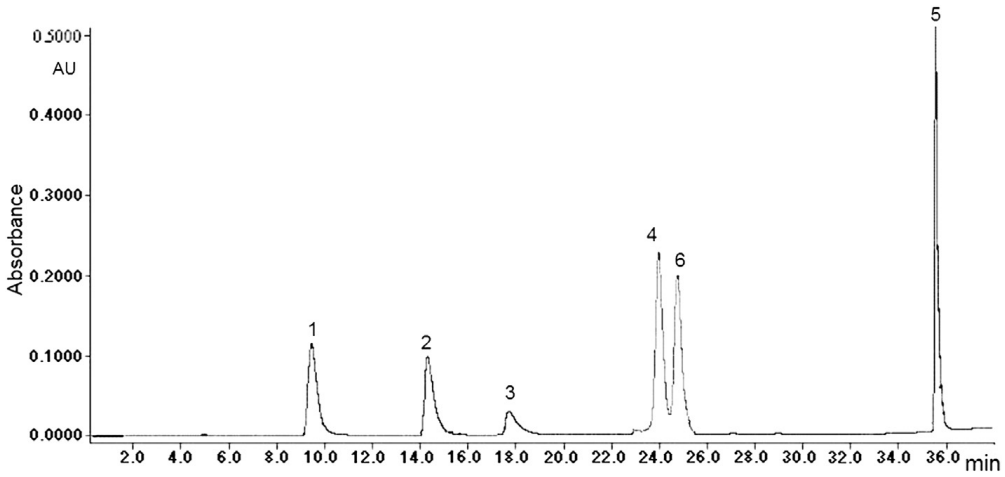

Fig. 1. HPLC chromatogram of standard solution of phenolic acids and flavonoids detected at 254 nm. Key to peaks identities: **1** – Caffeic acid; **2** – *p*-Coumaric acid; **3** – Ferulic acid; **4** – Hyperoside; **5** – Tiliroside; **6** – Isoquercitrin (For chromatographic protocol see 1.4.).

The *P* values were 0.1174 (Meeker *vs* Schoenemann), 0.1483 (Meeker *vs* Latham), 0.8084 (Schoenemann *vs* Latham), 0.7879 (Autumn Bliss *vs* Schoenemann) and 0.9868 (Autumn Bliss *vs* Latham).

The levels of (**6**) were calculated as hyperoside equivalents and achieved up to 1.70 ± 0.002 mg/g in Bulgarian elite clones (Table 2). Isoquercitrin represented from 16.2% (Bulgarian Rubin) to 71% (E21840) of the total amount of assayed compounds in the Bulgarian accessions, and from 18.4% (Tulameen) to 41.7% (Schoenemann) in the foreign varieties (Table 2).

The Bulgarian elite clone *R. idaeus* E23617 (4.23 mg/g) and Bulgarian variety *R. idaeus* Samodiva (3.14 mg/g) were the richest cultivars in total phenolic compounds due to the presence of high content of flavonol glycosides and phenolic acids, respectively. The variety Autumn Bliss with content of 0.12 mg/g was the poorest cultivar in total studied compounds. The main finding of this study is that the Bulgarian elite clones have a higher total content of assayed compounds and higher combined levels of **4** and **6** compared to the foreign cultivars (Table 2). In contrast to cultivars, the wild species *R. odoratus* and *R. occidentalis* were characterized with significantly lower amounts of phenolic compounds; **2** and **4** were not present in *R. odoratus* (Fig. 2C).

The RSD of the content of the studied phenolic acids was in the range 84.7% (**1**)–135.4% (**3**), while the RSD of the content of flavonoids **4**, **5** and **6** was 85.7%, 76.8% and 79.6%, respectively. Comparing to the variability in the content of phenolic acids, the variability of the flavonoids in the assayed accessions is moderate. Data clearly show that the content of assayed compounds among *Rubus* varieties is quite different, and this fact allows us to use them as a differentiating tool. These results were confirmed by a hierarchical clustering (HC) and a principal component analysis (PCA).

3.2. Hierarchical clustering on phenolic compounds

The hierarchical clustering of 22 raspberry accessions on the basis of their content of phenolic compounds is given in Fig. 3. Three well-defined clusters are distinguished which suggests three patterns of chemical composition. The first cluster C1, placed on the upper side of the dendrogram, consists of the following accessions: Zeva, Tulameen, E23501, Bulgarian rubin and Samodiva. The compounds most associated with this cluster were phenolic acids **1–3** being present up to 72.7% of the sum of studied compounds (*R. idaeus* Zeva) (Table 2). The highest content of caffeic acid (**1**) and *p*-coumaric acid (**2**) was found in the Bulgarian cultivar *R. idaeus* Samodiva; the highest amount of ferulic acid (**3**) was established in *R. idaeus* Bulgarian rubin. In cluster C1, a correlation in the occurrence of **1–3** was found, and this relationship could be explained by *p*-coumaric acid, a common precursor of phenolic acids in the biosynthetic pathway. In addition, the content of isoquercitrin (**6**) was very similar. No significant difference was observed in the total amounts of phenolic compounds assayed for Samodiva and his parent Bulgarian rubin ($p \leq 0.05$) (Table 2).

The second cluster C2 included the wild species *R. occidentalis,* all American varieties (Willamette, Meeker and Latham), the European accession Autumn Bliss and three Bulgarian varieties (Ljulin, Iskra and Shopska alena) (Fig. 3). The accessions were grouped by their low concentration in total compounds studied. Isoquercitrin (**6**) only appeared in representative concentrations (>25% of the sum of assayed compounds) in this cluster and was particularly important for the cluster differentiation.

The third cluster C3 grouped the wild species *R. odoratus* with almost all Bulgarian elite clones, E26664, E27562, E21840, E25927, E21878, and E27337; there was one exception – the European variety Schoenemann (Table 1). The cluster was characterized by the highest flavonol glycosides tiliroside (**5**), isoquercitrin (**6**), similar amounts of *p*-coumaric acid (**2**) and ferulic acid (**3**), and a significantly higher total amount of all analyzed compounds. No significant difference was observed in caffeic acid (**1**) content for E26664 *vs* E25927, and E27337 *vs* E27562 ($p \leq 0.05$) (Table 2).

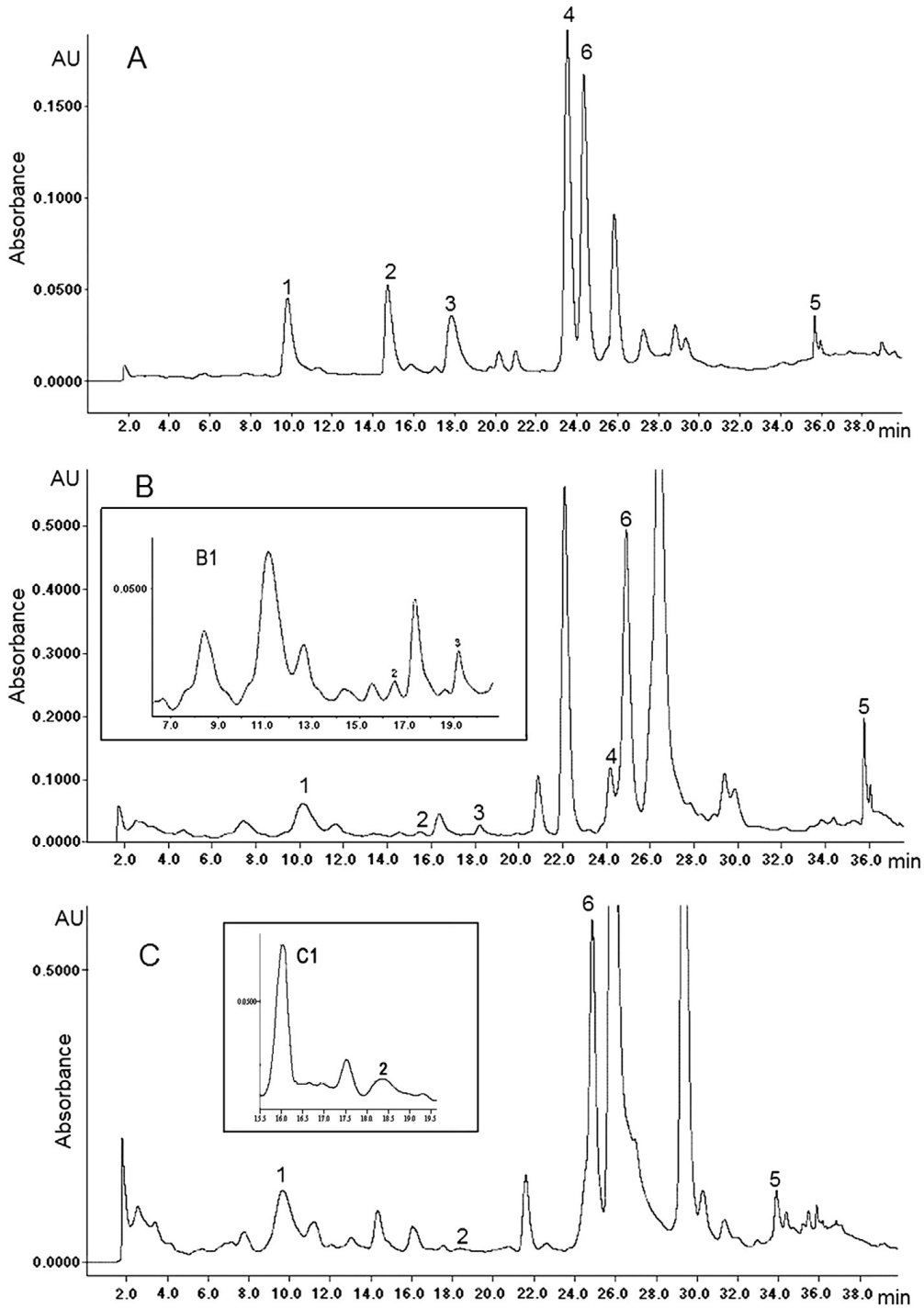

Fig. 2. HPLC chromatograms of: (A) cultivar Latham (*Rubus idaeus*); (B) Bulgarian elite clone 25927 (*Rubus idaeus*) (B1 – the same chromatogram from 7 to 19 min) and (C) wild species *Rubus odoratus* (C1 – the same chromatogram from 15 to 19 min). Chromatographic conditions and peak assignments as in Fig. 1.

The accession E23617 was an outlier. It was clearly separated from the considered raspberry accessions by the highest content of **1, 5, 6**, and total amount of phenolic compounds (Fig. 3, Table 2).

3.3. Principal component analysis on phenolic compounds

The PCA score plot is given in Fig. 4A. The first PC explains 43% of the variance, while the second adds 22%. The clusters here were more diffused than those generated by HC but in a good agreement with them. E23617 also was an outlier here. Bulgarian rubin and Samodiva were noticeably separated from the other accessions. The loading plot (Fig. 4B) pointed the caffeic acid (**1**) as the compound with the highest contribution in PC1. Isoquercitrin (**6**) and tiliroside (**5**) had positive contribution in PC2, while ferulic (**3**) and *p*-coumaric (**2**) acids were with negative contributions.

Table 2
Content of phenolic acids and flavonol glycosides in leaves of *Rubus* species (mg/g dry weight).

Accessions	Caffeic acid (**1**)	*p*-coumaric acid (**2**)	Ferulic acid (**3**)	Hyperoside (**4**)	Tiliroside (**5**)	Isoquercitrin (**6**)	Total
R. odoratus	0.71 ± 0.02[a,b]	–	0.08 ± 0.006a	–	0.10 ± 0.005a	0.98 ± 0.006	1.87 ± 0.01a
R. occidentalis	0.05 ± 0.01a	0.004 ± 0.0005a	0.006 ± 0.0006bc	0.07 ± 0.005a	0.33 ± 0.02bc	0.05 ± 0.003	0.51 ± 0.03b
R. idaeus							
Shopska alena	0.14 ± 0.005	0.03 ± 0.006b	0.008 ± 0.0006bg	0.04 ± 0.0007b	0.12 ± 0.004d	0.12 ± 0.004a	0.46 ± 0.01b
Samodiva	1.36 ± 0.12b	0.27 ± 0.01	0.15 ± 0.006	0.59 ± 0.006	0.19 ± 0.004	0.58 ± 0.004	3.14 ± 0.11cd
Iskra	0.23 ± 0.01c	0.09 ± 0.006c	0.005 ± 0.0006c	0.21 ± 0.002c	0.12 ± 0.005d	0.43 ± 0.008b	1.09 ± 0.02
Lyulin	0.29 ± 0.01d	0.03 ± 0.006d	0.03 ± 0.006de	0.09 ± 0.004	0.14 ± 0.001e	0.25 ± 0.003c	0.83 ± 0.02e
Balgarian rubin	1.00 ± 0.01	0.15 ± 0.006	0.29 ± 0.006	0.64 ± 0.005	0.14 ± 0.003e	0.43 ± 0.003b	2.65 ± 0.01c
E23617	1.43 ± 0.06	0.07 ± 0.006cf	0.05 ± 0.006eh	0.38 ± 0.002e	0.60 ± 0.004	1.70 ± 0.002	4.23 ± 0.01
E21878	0.59 ± 0.006	0.07 ± 0.006 fg	0.04 ± 0.006fhi	0.29 ± 0.004	0.30 ± 0.003b	1.60 ± 0.004	2.89 ± 0.02d
E26664	0.29 ± 0.02d	0.02 ± 0.001b	0.03 ± 0.006df	0.97 ± 0.01	0.20 ± 0.004	0.55 ± 0.005	2.06 ± 0.03
E27337	0.41 ± 0.01e	0.06 ± 0.006g	0.03 ± 0.006df	0.22 ± 0.005c	0.05 ± 0.004f	1.01 ± 0.002	1.78 ± 0.01a
E25927	0.32 ± 0.03d	0.09 ± 0.006c	0.06 ± 0.006ai	0.25 ± 0.005f	0.24 ± 0.003	1.31 ± 0.007	2.27 ± 0.02
E23501	0.79 ± 0.01	0.24 ± 0.02e	0.03 ± 0.006df	0.33 ± 0.004	0.43 ± 0.02	0.61 ± 0.006	2.43 ± 0.04g
E27562	0.41 ± 0.02e	0.04 ± 0.01d	0.07 ± 0.006a	0.75 ± 0.002	0.36 ± 0.009c	0.88 ± 0.002	2.51 ± 0.02g
E21840	0.36 ± 0.02	0.04 ± 0.006d	0.03 ± 0.006df	–	0.11 ± 0.01d	1.37 ± 0.02	1.91 ± 0.02f
Meeker	0.08 ± 0.01	0.03 ± 0.01d	0.02 ± 0.006df	0.08 ± 0.005ad	0.04 ± 0.004f	0.12 ± 0.0003a	0.37 ± 0.01
Schoenemann	0.39 ± 0.03e	0.08 ± 0.001cf	0.02 ± 0.006df	0.50 ± 0.005	0.06 ± 0.005f	0.75 ± 0.004	1.80 ± 0.04a
Willamette	0.21 ± 0.02c	0.13 ± 0.01	0.01 ± 0.0006g	0.13 ± 0.005	0.15 ± 0.003g	0.25 ± 0.002c	0.88 ± 0.02e
Autumn bliss	0.02 ± 0.003	0.005 ± 0.0006a	0.001 ± 0.0001	0.008 ± 0.0005	0.05 ± 0.002f	0.04 ± 0.001	0.12 ± 0.01
Zeva	1.19 ± 0.02b	0.20 ± 0.02e	0.02 ± 0.0006df	0.04 ± 0.004b	0.11 ± 0.005d	0.38 ± 0.005	1.94 ± 0.02f
Tulameen	0.84 ± 0.02	0.04 ± 0.006d	0.06 ± 0.006ah	0.36 ± 0.005e	0.16 ± 0.009g	0.33 ± 0.004	1.79 ± 0.02a
Latham	0.04 ± 0.006a	Below LOQ	0.01 ± 0.006df	0.24 ± 0.003f	0.05 ± 0.004f	0.22 ± 0.004	0.56 ± 0.01

[a] Values represent the mean of triplicate analyses ± SD (standard deviation).
[b] Means followed by the same letter within a column are not significantly different ($P < 0.05$).

4. Discussion

In the present study, three phenolic acids and three flavonol glycosides were analysed quantitatively in 22 accessions of the Bulgarian raspberry (*Rubus* L.) germplasm collection. Based on the content of phenolic derivates, the studied accessions were clustered and the dominant compound responsible for the clustering was identified. The phytochemical analysis showed that the Bulgarian elite clone E23617 displayed the highest content of the studied phenolic derivatives. The PCA loading plot pointed the caffeic acid (**1**) as the compound with the highest contribution in raspberry varieties differentiation.

The flavonoid content in the leaves of *Rubus* (raspberry and blackberry) from wild and cultivated varieties has been analysed after acid hydrolysis using spectrophotometric and HPLC methods (Gudej and Tomczyk, 2004; Han et al., 2012).

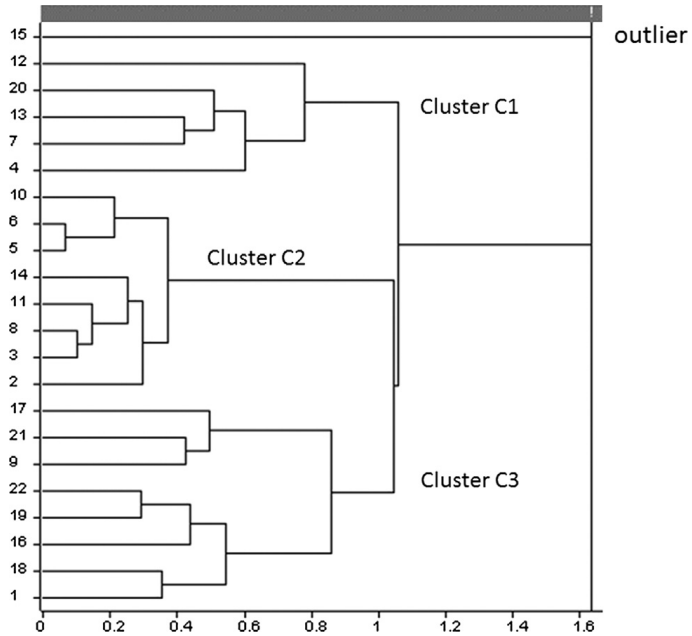

Fig. 3. Hierarchical clustering of 22 raspberry accessions on the basis of the phenolic compounds assayed. The content of phenolic compounds is reported in Table 1, Key to accessions identities: **1**-*R. odoratus*; **2**-*R. occidentalis*; **3**-Shopska alena; **4**-Samodiva; **5**-Iskra, **6**-Ljulin, **7**-Bulgarian rubin; **8**-Meeker; **9**- Schoenemann; **10**-Willamette; **11**-Autumn Bliss; **12**-Zeva, **13**-Tulameen; **14**-Latham; **15**-E23617, **16**-E21878; **17**-E26664; **18**-E27337; **19**-E25927; **20**-E23501; **21**-E27562; **22**-E21840.

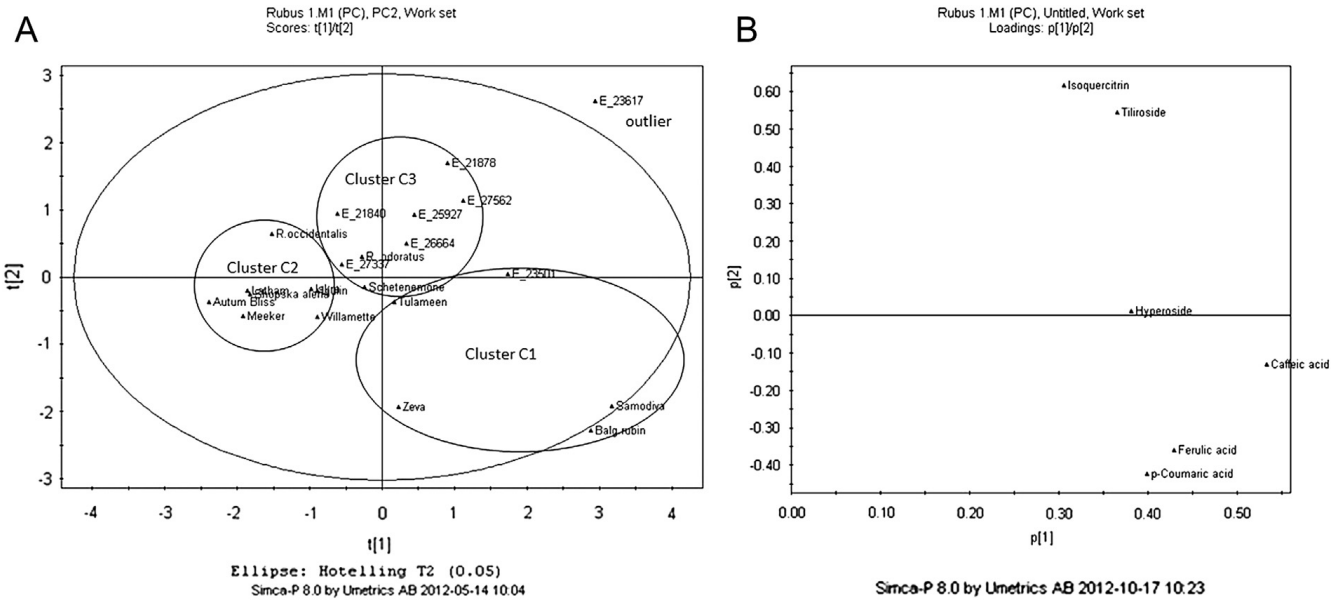

Fig. 4. A principal component analysis (PCA) on the phenolic compounds studied in Bulgarian raspberry collection: A – PCA score plot; B – PCA loading plot.

Gudej and Tomczyk (2004) reported 0.49%, 0. 38% and 0.32% flavonoids (as a sum of the flavonol aglycons quecetin and kaempferol) in the cultivars with polish provenance *R. idaeus* Tulameen, Willamette and Autumn Bliss, respectively, whereas our HPLC analysis revealed the presence of lower amounts of individual flavonoids **4** (hyperoside), **5** and **6** (Table 2). The flavonoid concentrations obtained by spectrophotometry in polish wild and cultivated *Rubus* L. leaves were generally higher (0.50–0.83% of dry weight, calculated as hyperoside) than values in the present study. This is due to the use of acid hydrolysis of the plant material before the analysis (Gudej and Tomczyk, 2004). We chose mild extraction conditions in our experimental setup to minimise the risk of hydrolyse of flavonol glycosides. More detailed comparisons are not possible as the previous studies used different sampling methodology from those employed here. In this study, the content of genuine free phenolic acids and flavonol glycosides in varieties of *Rubus* leaves is reported for the first time.

The data obtained for hydroxycinnamic acids and flavonols in the leaves of assayed raspberry varieties indicated higher concentrations compared to those reported in berries. Hydroxycinnamic acids and flavonols are minor phenolic classes in berries of *Rubus* species, being presented in the range 9–19 and 1–8 mg/kg fresh weight, respectively (Hakkinen et al., 1999a; Määttä-Riihinen et al., 2004). In this respect, the total amount of studied phenolic acids in the leaves of Bulgarian elite clones attained up to 1.78 mg/g dry weight, and the total amount of flavonols – 2.68 mg/g (Table 2). Our results suggest that the *Rubus* leaves could be of equal value as the medicinal plants *Agrimonia eupatoria*, *Salvia* sp., *Satureja montana*, as it has been described in previous study (Giao et al., 2012).

Since the plant material used in this study was collected at the same environmental conditions and agriculture practice, the differences in phenolic profiles could only be due to raspberry varieties. The statistical analysis showed that the distribution of the assayed phenolic acids and flavonols depends directly on the nature of cultivar or wild species.

Recently, the genetic profiling of Bulgarian raspberry germplasm collection has been undertaken using the Simple Sequence Repeat (SSRs) markers (Badjakov et al., 2005). Different levels of mean heterozigosity have been observed for the parental lines of the hybrid cv. Samodiva (25% for Bulgarian rubin and 75% for Shopska alena). There are differences in dendrograms generated from the SSR data and phenolics content, but partial agreement is also shown. Dendrogram generated by SSR analysis showed that the accessions *R. idaeus* Samodiva and one of its parents (Shopska alena) are separated in one of the subclusters. In our study there was no significant difference between the total content of assayed compound ($p \leq 0.05$) in the accessions Samodiva and the second parent, Bulgarian rubin. They were grouped in cluster C1 and showed high amounts of phenolic acids (**1–3**), hyperoside (**4**) and isoquercitrin (**6**) (Fig. 3, Table 2). The accessions Ljulin, Iskra, Shopska alena, Willamette and *R. occidentalis* have been gathered into one cluster by SSR data (Badjakov et al., 2005). In our study, tiliroside (**5**) and the total amounts of assayed compounds in these varieties were similar as well (cluster C2) (Fig. 3, Table 2).

In conclusion, HC and PCA clustering on phenolic compounds **1–6** in the leaves from Bulgarian raspberry collection tended to cluster the studied 22 accessions into three main groups. The profiles obtained demonstrate that the leaves of cultivars are characterized by greater amounts of phenolic derivatives than the leaves collected from wild species. Generally, Bulgarian elite clones showed a tendency of higher total content of assayed compounds and higher combined levels of hyperoside (**4**) and isoquercitrine (**6**) in comparison to the foreign varieties. The Bulgarian cultivar Samodiva and the elite clones E23617 and E21878 are the genotypes of highest potential for selection in terms of high phenolic content. *R. idaeus* E23617 revealed a unique chemical composition being with the highest content of caffeic acid (**1**), tiliroside (**5**), isoquercitrin (**6**) and total

amount of phenolic derivatives. A good agreement between the content of phenolic constituents and genetic profiling data would be of great benefit for the development of breeding strategies for plant improvement.

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