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Flavonols, Phenolic Acids and Antioxidant Activity of Some Red Fruits

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Summary

Red fruits (blueberry, blackberry, chokeberry, strawberry, red raspberry, sweet cherry, sour cherry, elderberry, black currant and red currant) were analyzed for flavonols (quercetin, myricetin, and kaempferol), hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric acid) and hydroxybenzoic acids (*p*-hydroxybenzoic, ellagic acid) by using HPLC method. Compounds were analyzed as aglycons after acid hydrolysis with 1.2 mol dm^{-3} HCl. Each fruit sample was analyzed for total polyphenols (TP) and total anthocyanins (TA). TP ranged from 1763 to 7194 mg kg^{-1} fresh weight (FW) in red raspberries and chokeberries, respectively. TA ranged from 169 to 4069 mg kg^{-1} FW in strawberries and blueberries, respectively. Flavonols varied from 4 mg kg^{-1} in red raspberries to 183 mg kg^{-1} in blueberries. Hydroxycinnamic acids were found in relatively high concentrations in blueberries (92 mg kg^{-1}) and in black currant (70 mg kg^{-1}) and represented significant portion in sour and sweet cherry phenolics. The amount of ellagic acid was high in blackberry (121 mg kg^{-1}), strawberry (41 mg kg^{-1}) and red raspberry (32 mg kg^{-1}). The strongest antioxidant activity determined by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays showed chokeberry, followed by blueberry, black currant and elderberry. Antioxidant activity correlated better with TP than with TA, total flavonols (TF), or total hydroxycinnamic acids. Additionally, linear relationship was observed between antioxidant activity of fruits and content of caffeic acid, quercetin and *p*-coumaric acid. Overall results showed that red fruits can serve as good source of bioactive polyphenols in human diet, but due to high concentrations of anthocyanins, flavonols, phenolic acids and strong antioxidant activity, chokeberry, blueberry, elderberry and black currant can be regarded as good candidates for nutritional supplement formulations.

Zusammenfassung

Die Konzentration der Flavonole (Quercetin, Myricetin, und Kaempferol), der Hydroxyzimtsäure (Kaffeesäure, Ferulasäure und *p*-Cumarsäure) und der Hydroxybenzoesäure (*p*-Hydroxybenzoesäure und Ellagsäure) aus roten Früchten (Süß-und Sauerkirschen), sowie aus Beerenfrüchten (Blaubeere, Brombeere, Aronia, Erdbeere, Himbeere, Holunderbeere, schwarze und rote Johannisbeere) wurde mittels HPLC-Methode bestimmt. Die Verbindungen wurden als Aglykon nach der Hydrolyse mit 1,2 mol dm^{-3} HCl analysiert. In jeder Frucht wurden auch die Gesamtpolyphenole (TP) und Gesamtanthocyane (TA) bestimmt. TP variierte von 1763 in der Himbeere bis 7194 mg kg^{-1} (FW) in der Aronia. TA variierte von 169 (Erdbeere) bis 4069 mg kg^{-1} FW (Blaubeere). Die Konzentration der Flavonole variierte von 4 mg kg^{-1} in der Himbeere bis 183 mg kg^{-1} in der Blaubeere. Die höchsten Konzentrationen an Hydroxyzimtsäure wurden in der Blaubeere (92 mg kg^{-1}) und in der schwarzen Johannisbeere (70 mg kg^{-1}) gefunden und stellten einen bedeutenden Anteil der Phenole in den Süß- und Sauerkirschen dar. Die Ellagsäure-Konzentration betrug in der Brombeere 121 mg kg^{-1}, in der Erdbeere 41 mg kg^{-1} und in der Himbeere 32 mg kg^{-1}. Die stärkste antioxidative Aktivität, die mittels $DPPH^{\bullet}$- und $ABTS^{\bullet+}$-Methoden bestimmt wurde, zeigten Aronia, schwarze Johannisbeere und Holunderbeere. Die antioxidative Aktivität korreliert besser mit TP als mit TA, mit den Gesamtflavonolen (TF) oder mit der Gesamthydroxyzimtsäure. Es wurde eine lineare Korrelation zwischen der antioxidativen Aktivität der Früchte und der Kaffeesäure-, Quercetin- und *p*-Cumarsäure-Konzentration festgestellt. Alle Ergebnisse weisen darauf hin, dass die Beerenfrüchte und Kirschen als eine reiche Quelle bioaktiver Polyphenole in der menschlichen Ernährung dienen können. Aronia, Blaubeere, Holunderbeere und schwarze Johannisbeere haben die höchste Anthocyan-, Flavonol-, und Phenolsäure-Konzentration. Sie zeigen auch die stärkste antioxidative Aktivität und können aus diesem Grunde als gute Rohstoff-Kandidaten in der Produktion von funktionellen Lebensmitteln eingesetzt werden.

Keywords: Red fruits, berries, flavonols, phenolic acids, antioxidant activity / rote Früchte, Beerenfrüchte, Flavonole, Phenolcarbonsäuren, antioxidative Aktivität

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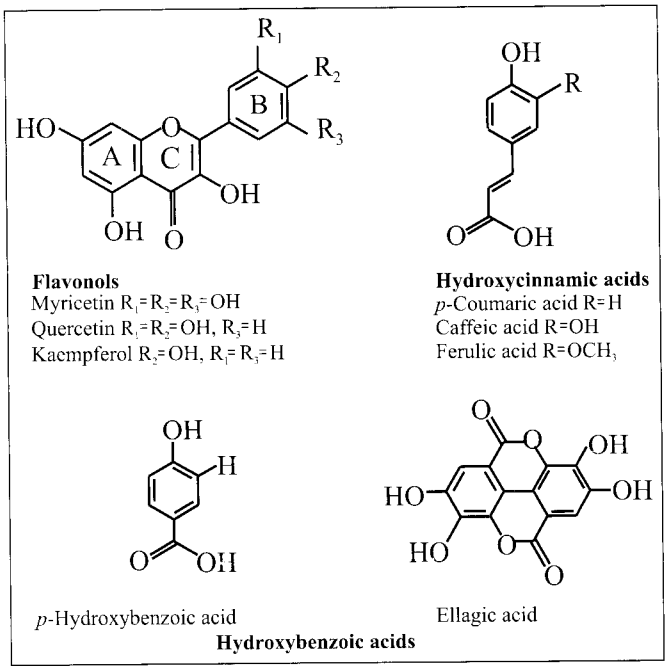

Fig 1 Structures of aglycons of flavonols and phenolic acids

Introduction

Flavonols (Fig. 1) belong to the large group of polyphenolic compounds. They contain a common molecular structure that consists of the tricyclic C_6-C_3-C_6 "flavon skeleton" and are widespread in plants where they occur usually as *O*-glycosides. Although over 200 flavonol aglycons have been identified in plants, only four of these, quercetin, kaempferol, myricetin and isorhamnetin, are common in fruits[1]. Phenolic acids (Fig. 1) (hydroxycinnamic and hydroxybenzoic acids) belong to the group of polyphenolic compounds as well, and occur in fruits as esters, glycosides and amides. The most common hydroxycinnamic acids are *p*-coumaric, caffeic and ferulic acid, while the corresponding hydroxybenzoic acids are *p*-hydroxybenzoic, gallic, ellagic, 3,4-dihydroxybenzoic, vanillic, and syringic acid[2–4].

In the last few decades flavonols and phenolic acids, are studied intensively because of their potent positive role in human health. Flavonols, phenolic acids and other polyphenols are potent antioxidants, free radical scavengers[5] and metal chelators; they exhibit various physiological activities including anti-inflammatory, anti-allergic, anti-carcinogenic, anti-arthritic activities[1,6–8]. They may have a positive role in decreasing the risk of some chronic diseases such as cancer or heart diseases[9–11]. Most of these biological effects of polyphenols are believed to come from their antioxidant properties[12]. One of the most important sources of polyphenolic compounds among dietary plants is small red fruits[13]. Strongly coloured berries contain high levels of polyphenols such as anthocyanins, flavonol glycosides and hydroxycinnamic acids[14–18]. Some berries like strawberry and red raspberry contain high levels of ellagic acid[16,18]. All these polyphenolic antioxidants, together with vitamins and carotenoids, are responsible for beneficial effects of diet rich in fruits and vegetables on human health[12,19].

Because of the positive effects of fruits polyphenols on human health, the interest in consuming fruits and their products is growing. In order to fully understand the roles of fruit's polyphenols, further studies are needed. As a first step it is important to determine the distribution of polyphenols in fruits rich in polyphenols, and to investigate antioxidant activity of those fruits. These data will enable evaluation of fruits as a source of polyphenols in diet. Furthermore, they can be the basis for further studies of effects of individual polyphenols on fruit's antioxidant activity. Epidemiological studies will help in understanding the relation between the intake of fruit's polyphenols and the risk of developing various diseases. With that kind of knowledge it will be possible to choose raw materials for preparation of functional foods with better biological activity.

The aim of this study was to determine the content of individual flavonols (quercetin, myricetin, kaempferol) and phenolic acids (*p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, ellagic acid) present in various red fruits (blueberry, blackberry, chokeberry, strawberry, red raspberry, sweet cherry, sour cherry, elderberry, black currant and red currant) by using high performance liquid chromatography (HPLC) equipped with a photodiode array detector (PDA) in order to examine the distribution of these polyphenols in red fruits. Additionally, a concentration of total polyphenols and total anthocyanins was measured. Antioxidant activity of red fruits was determined by using DPPH and ABTS assays. The existence of possible correlation between total or individual polyphenols and antioxidant activity of fruits was examined as well.

Materials and methods

Chemicals

4-hydroxybenzoic acid, ellagic acid, caffeic acid, ferulic acid, *p*-coumaric acid, myricetin, quercetin dihydrate, kaempferol, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from *Sigma-Aldrich* (St. Louis, MO, USA). Methanol (HPLC grade) was obtained from *Merck* (Darmstadt, Germany); *o*-phosphoric acid (85 %, HPLC grade) and ammonium peroxodisulfate were purchased from *Fluka* (Buchs, Switzerland). Hydrochloric acid (36.2 %), L-(+)-ascorbic acid, potassium chloride, sodium acetate trihydrate, sodium carbonate and Folin-Ciocalteau reagent were obtained from *Kemika* (Zagreb, Croatia).

Fruit samples

Fruits [black currant (*Ribes nigrum*), red currant (*Ribes sativum*), red raspberry (*Rubus idaeus*), blackberry (*Ru-*

bus fruticosus), sour cherry (*Prunus cerasus*), sweet cherry (*Prunus avium*), strawberry (*Fragaria anannassa*), chokeberry (*Aronia melanocarpa*), elderberry (*Sambucus nigra*), and blueberry (*Vaccinium myrtillus)*] were harvested in Slavonia (Croatia) at the commercial maturity stage. Immediately after harvesting, fruits were frozen and stored at –20°C until analysis.

Sample preparation for determination of total anthocyanins, total polyphenols and antioxidant activity

For determination of total anthocyanins, total polyphenols, and antioxidant activity, fruit extracts were prepared in three replicates according to the following procedure[20]. Approximately 100 g of fruits were mixed in a blender in order to obtain a homogenized fruit sample. 20 g of homogenized fruit was mixed with 20 ml of methanol/HCl 2 % (95:5, v/v) solution. After 60 min the solution was filtered under vacuum in a 50-ml volumetric flask. The residue was extracted again in the same way. The extracts were combined and the solution was diluted to volume with methanol/HCl 2 % (95:5, v/v) solution.

Determination of total polyphenols

Total polyphenols were determined by Folin-Ciocalteau micro method[21]. Blueberry and chokeberry extracts were diluted 1:4 (v/v) with methanol/HCl 2 % (95:5, v/v) solution prior to analysis. Other fruit extracts were analyzed without dilution. An aliquot (20 µl) of fruit extract was mixed with 1580 µl of distilled water and 100 µl of Folin-Ciocalteau reagent. 300 µl of sodium carbonate solution (200 g l^{-1}) was added to the mixture. After incubation in water bath at 40 °C for 30 min, absorbance of the mixture was read against the prepared blank at 765 nm with a UV-Vis spectrophotometer (UV 2005, Barcelona, Spain). Total polyphenols were expressed as mg of gallic acid equivalents (GAE) per kg of fruits. Data presented are mean ± standard deviation (SD).

Determination of total anthocyanins

Total anthocyanins were determined by a pH-differential method[22]. Two dilutions of each fruit extract were prepared, one with potassium chloride buffer (pH 1.0) (1.86 g KCl in 1 l of distilled water, pH value adjusted to 1.0 with concentrated HCl), and the other with sodium acetate buffer (pH 4.5) (54.43 g $CH_3CO_2Na{\cdot}3H_2O$ in 1 l of distilled water, pH value adjusted to 4.5 with concentrated HCl), diluting each by the previously determined dilution factor (strawberry, red raspberry, red currant 1:10 (v/v); blackberry, sour cherry 1:20 (v/v), sweet cherry 1:30 (v/v); black currant, elderberry, chokeberry and blueberry 1:100 (v/v)). Absorbance was measured simultaneously at 510 and 700 nm after 15 min incubation at room temperature. The content of total anthocyanins was expressed in mg of cyanidin-3-glucoside equivalents (CGE) per kg of fruits using a molar extinction coefficient (ε) of cyanidin-3-glucoside of 26 900 l mol^{-1} cm^{-1} and molar weight (MW) (449.2 g mol^{-1}). Data presented are mean ± standard deviation (SD).

Antioxidant activity

The antioxidant activity of fruit extracts was measured spectrophotometrically with a UV-Vis spectrophotometer (UV 2005, Barcelona, Spain) by using two assays, DPPH and ABTS assay. The ability of the fruit extract to act as free radical scavenger against DPPH• radical was tested by measuring the disappearance of the absorbance at 517 nm after the addition of fruit extract and by comparing it with the disappearance of the absorbance produced by the addition of a known amounts of Trolox, a water-soluble vitamin E analogue, under the same conditions. In the ABTS assay, the amount of $ABTS^{\bullet+}$ radical cation scavenged by fruit extract was measured by monitoring the decrease of absorbance of $ABTS^{\bullet+}$ radical cation at 734 nm, and by comparing the decrease of absorbance with the decrease of absorbance produced by the addition of a known amount of Trolox.

In the DPPH method[20], five dilutions of each fruit extract were analyzed. A 50 µl of diluted fruit extract was mixed with 300 µl of methanolic DPPH• solution (1 mmol dm^{-3}) and brought to 3 ml with methanol. The solution was kept in dark at room temperature for 15 minutes. The absorbance ($A_{extract}$) was read against the prepared blank (50 µl diluted fruit extract, 2950 µl methanol) at 517 nm. A DPPH• blank solution was prepared each day (300 µl of 1 mmol dm^{-3} DPPH•, 2.7 ml of methanol) and its absorbance (A_{DPPH}) was measured daily. Trolox standards of final concentration 0–2500 µmol dm^{-3} were prepared in methanol and assayed under the same condition. A 50 µl of Trolox was mixed with 300 µl of methanolic DPPH• solution (1 mmol dm^{-3}) and brought to 3 ml with methanol. After 15 min, the absorbance (A_{Trolox}) was read against the prepared blank at 517 nm. Calibration curve of Trolox was constructed by linear regression of the absorbance value (A_{Trolox}) versus concentration. Trolox calibration curve was used to calculate antioxidant activity of each diluted fruit extract and to express the antioxidant activity in µmol of Trolox equivalent (TE) per g of fruits. Moreover, the percent inhibition of DPPH• radical caused by each diluted fruit extract was calculated according to formula:

$$\% \text{ inhibition} = [(A_{DPPH} - A_{extract})/A_{DPPH})] \times 100$$

Percent of inhibition of DPPH• radical caused by each diluted fruit extract was plotted against antioxidant activity (µmol TE/g). Using the curve obtained, final results were expressed as µmol of TE per g of fruits needed to reduce DPPH• radical by 50 %.

In the ABTS method[23], five dilutions of each fruit extract were analyzed. $ABTS^{\bullet+}$ radical cation was chemically generated with ammonium peroxodisulfate solution by mixing 0.2 ml of ammonium peroxodisulfate (65 mmol dm^{-3}) with 50 ml of ABTS solution (1 mmol dm^{-3}, prepared in 0.1 mol

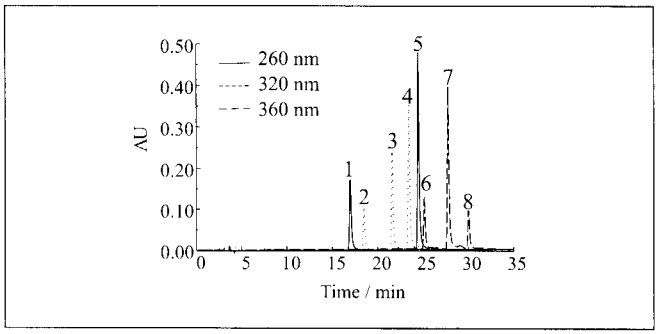

Fig. 2 Overlayed HPLC chromatograms of standards detected at 260, 320 and 360 nm. Compounds: **(1)** *p*-hydroxybenzoic acid, **(2)** caffeic acid, **(3)** *p*-coumaric acid, **(4)** ferulic acid, **(5)** ellagic acid, **(6)** myricetin, **(7)** quercetin, **(8)** kaempferol

dm^{-3} phosphate buffer pH = 7.4) and the mixture was left to stand overnight. A 0.5 ml of $ABTS^{\bullet+}$ radical cation stock solution was mixed with 2 ml of phosphate buffer (pH = 7.4) in cuvette and the absorbance (A_{ABTS}) was read at 734 nm. Subsequently, 0.1 ml of diluted fruit extract was added into the cuvette, the solution was mixed quickly, and the absorbance (A_{extr}) was read after 60 s at 734 nm. The decrease in absorbance ($\Delta A = A_{ABTS} - A_{extr}$) after 60 s was calculated for each diluted fruit extract. The decrease in absorbance caused by the addition of Trolox (0–400 µmol dm^{-3}) was measured according to the same procedure. A 0.5 ml of $ABTS^{\bullet+}$ stock solution and 2 ml of phosphate buffer (pH=7.4) were mixed in cuvette and the absorbance (A_{ABTS}) was read at 734 nm. A 0.1 ml of Trolox was added into the cuvette, the solution was mixed quickly, and the absorbance (A_{trolox}) was read after 60 s at 734 nm. The decrease in absorbance was calculated according to formula: ($\Delta A_{trolox} = A_{ABTS} - A_{trolox}$). Calibration curve of Trolox was constructed by linear regression of the decrease in absorbance (ΔA_{trolox}) versus concentration. Antioxidant activity for each diluted fruit extract was calculated on the basis of the Trolox calibration curve and expressed in µmol of Trolox equivalent (TE) per g of fruits. Additionally, the percent inhibition of $ABTS^{\bullet+}$ radical cation caused by addition of each diluted fruit extract was calculated according to formula:

$$\% \text{ inhibition} = [(A_{ABTS} - A_{Exstr})/A_{ABTS})] \times 100$$

Percent of inhibition of $ABTS^{\bullet+}$ radical caused by each diluted fruit extract was plotted against antioxidant activity (µmol TE/g). Using the curve obtained, final results were expressed as µmol of TE per g of fruits needed to reduce $ABTS^{\bullet+}$ radical by 50 %.

HPLC analysis of flavonols and phenolic acids

Flavonols and phenolic acids were analyzed as aglycons after acid hydrolysis with 1.2 mol dm^{-3} HCl. Two extracts of each fruit were prepared, one for analysis of flavonols and the other for analysis of phenolic acids. The procedure applied for extraction and hydrolysis of flavonols and phenolic acids was already described in ref. [24,25].

For flavonol analysis, fruits (~100 g) (random selection) were homogenised in blender. Ascorbic acid (80 mg) was dissolved in 5 ml of distilled water and 5 g of homogenized fruits sample, 25 ml of methanol and 10 ml of HCl (6 mol dm^{-3}) were added to ascorbic acid solution. Water was added to this mixture to obtain a final volume of 50 ml and final concentration of HCl 1.2 mol dm^{-3}. This solution was refluxed on water bath for 2 h at 85 °C. After cooling, extracts were filtered. A 20 ml portion of the filtrate was evaporated to dryness on a rotary evaporator using water bath and temperature 35 °C. The residue was dissolved in 2 ml of methanol and filtered through a 0.45 µm filter (*VariSep PTFE*, 0.45 µm, 25 mm-Varian) prior to injection into the HPLC.

For phenolic acids analysis, fruits (~100 g) (random selection) were homogenised in blender. Ascorbic acid (80 mg) was dissolved in 5 ml of distilled water and 5 g of homogenized fruit sample, 25 ml of methanol and 10 ml of HCl (6 mol dm^{-3}) were added to ascorbic acid solution. Water was added to this mixture to obtain a final volume of 50 ml and final concentration of HCl 1.2 mol dm^{-3}. This solution was refluxed on water bath for 16 h at 35 °C. After cooling, extracts were filtered. A 20 ml portion of the filtrate was evaporated to dryness on a rotary evaporator using water bath and temperature 35 °C. The residue was dissolved in 2 ml of methanol and filtered through a 0.45 µm filter (*VariSep PTFE*, 0.45 µm, 25 mm-Varian) prior to injection into the HPLC. All fruit extracts were analyzed on HPLC system immediately, the same day when prepared.

The analytical HPLC system employed consisted of a *Varian* LC system (USA) equipped with a ProStar 230 solvent delivery module, and ProStar 330 PDA Detector. Phenolic compounds separation was done in an OmniSpher C18 column (250 x 4.6 mm inner diameter, 5 µm, *Varian*, USA) protected with guard column (ChromSep 1 cm x 3 mm, *Varian*, USA). The data were collected and analysed on IBM computing system equipped with Star Chromatography Workstation software (version 5.52).

The same solvents and gradient elution program were used in determination of phenolic acids and flavonols. Solvent A was 0.1 % phosphoric acid and solvent B was 100 % HPLC grade methanol. The elution conditions were as follows: 0–30 min from 5 % B to 80 % B; 30–33 min 80 % B; 33–35 min from 80 % B to 5 % B; with flow rate = 0.8 ml min^{-1}. Operating conditions were as follows: column temperature 20 °C; injection volumes, 10 µl of the standards and samples. A 10-minute re-equilibration period was used between individual runs. UV-Vis spectra were recorded in wavelength range from 190-600 nm (detection wavelength was 260 nm for ellagic and *p*-hydroxybenzoic acid; 320 nm for caffeic, *p*-coumaric and ferulic acid; 360 nm for myricetin, quercetin and kaempferol). The overlayed HPLC chromatograms of separated standard compounds are shown in Figure 2. Calibration curves of the standards were made by diluting stock standards in methanol to yield 1–80 mg

l^{-1} (*p*-hydroxybenzoic acid, caffeic acid, myricetin and kaempferol); 1–250 mg l^{-1} (ellagic acid, ferulic acid and quercetin); 2–240 mg l^{-1} (*p*-coumaric acid).

Identification and peak assignment of flavonols and phenolic acids in all fruit extracts was based on comparison of their retention times and spectral data (190–600 nm) with those of authentic standards. Additional identification was carried out by spiking the fruit extracts with phenolic standards. Identified flavonols and phenolic acids were quantified using calibration curve of authentic standards. Data presented are mean ± standard deviation (SD). The concentrations of total flavonols, total hydroxycinnamic acids and total hydroxybenzoic acids were obtained by summing up the concentrations of individual compounds.

All phenolic compounds showed a linear response within range studied (r^2=0.9963 to 0.9999). Precision of method was evaluated by determining within-day variation of the HPLC analysis. To gain the data for studying precision of method, black currant and red raspberry extracts were analyzed six times within one day. Coefficients of variations (CV) of peak areas were 5.4 % for myricetin, 5.4 % for quercetin, 8.0 % for kaempferol, 0.6 % for ellagic acid, 0.6 % for caffeic acid, 1.0 % for *p*-coumaric acid, 1.4 % for ferulic acid in black currant; 4.8 % for quercetin, 1.5 % for *p*-hydroxybenzoic acid, 7.2 % for ellagic acid, 11.6 % for caffeic acid, 2.3 % for *p*-coumaric acid, 5.5 % for ferulic acid in red raspberry. Recoveries were measured by adding known amounts of standards (10–50 mg l^{-1}) to fruit extracts prior to HPLC analysis. The recoveries ranged from 88 to 103 % for quercetin, from 86 to 102 % for myricetin, from 85 to 99 % for *p*-hydroxybenzoic acid, from 93 to 101 % for ellagic acid, from 84 do 100 % for caffeic acid, from 93 to 100 % for *p*-coumaric acid, and from 85 do 103 % for ferulic acid. In the calculation of final results, no correction for recovery was applied to data. The following limits of detection were estimated using a signal-to-noise ratio of 3: 0.76 mg l^{-1} by *p*-hydroxybenzoic acid, 1.12 mg l^{-1} by ellagic acid, 0.32 mg l^{-1} by caffeic acid, 0.49 mg l^{-1} by *p*-coumaric acid, 0.44 mg l^{-1} by ferulic acid, 0.15 mg l^{-1} by miricetin, 0.07 mg l^{-1} by quercetin, 0.13 mg l^{-1} by kaempferol.

Statistical analysis

Correlation and regression analyses were performed using Statistica 7.1 (*Statsoft*, Tulsa, USA). Differences at $p \leq 0.05$ were considered significant.

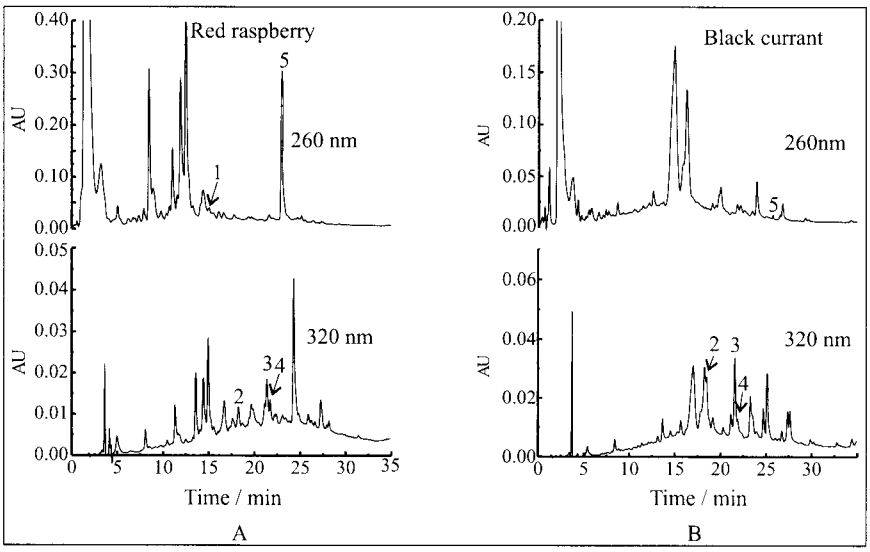

Fig. 3 HPLC chromatograms of **(A)** red raspberry and **(b)** black currant extracts detected at 260 and 320 nm. Compounds: **(1)** *p*-hydroxybenzoic acid, **(2)** caffeic acid, **(3)** *p*-coumaric, **(4)** ferulic acid, **(5)** ellagic acid

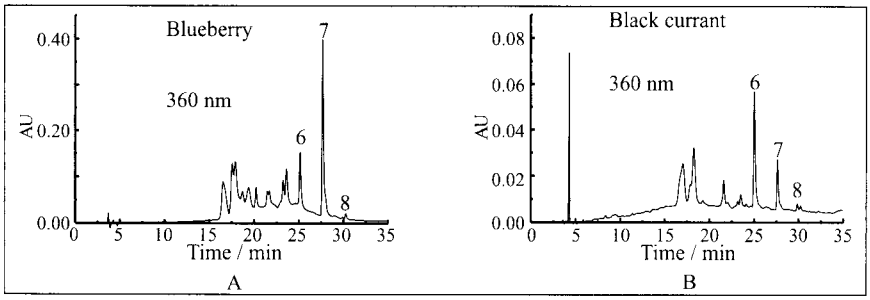

Fig. 4 HPLC chromatograms of **(A)** blueberry, **(B)** black currant extracts detected at 360 nm. Compounds: **(6)** myricetin, **(7)** quercetin, **(8)** kaempferol

Results and Discussion

Flavonols and phenolic acids were determined by using HPLC method in order to examine the distribution of these compounds in fruits. HPLC chromatograms of red raspberry and black currant samples representing separated phenolic acids detected at 260 and 320 nm are shown in Figure 3. Figure 4 shows HPLC chromatograms of separated flavonols in blueberry and black currant extracts recorded at 360 nm. The concentrations of individual flavonols and phenolic acids are reported in Table 1.

The highest concentrations of flavonols (183 mg kg^{-1}) and phenolic acids (92 mg kg^{-1}) were found in *blueberries*. The dominant flavonol in blueberries was quercetin (137 mg kg^{-1}), followed by myricetin (43 mg kg^{-1}) whereas kaempferol was found at considerably lower concentration (3 mg kg^{-1}). Data presented by other authors also confirmed that the major flavonols in blueberry (*Vaccinium myrtillus*) are quercetin and myricetin whereas kaempferol was present at significantly lower concentration[15]. Phenolic acids found in our sample of blueberry were *p*-coumaric as a dominant one (55 mg kg^{-1}), caffeic (27 mg kg^{-1}) and ferulic

Tab. 1 Concentrations of hydroxybenzoic acids, hydroxycinnamic acids, and flavonols in red fruits (mg kg^{-1} of fresh weight); determined by HPLC method: values are means ± SD (n=2)

Red fruits	Hydroxybenzoic acids [mg kg^{-1}]			Hydroxycinnamic acids [mg kg^{-1}]				Flavonols [mg kg^{-1}]			
	p-HBA	EA	Σ	CA	*p*-CouA	FA	Σ	M	Q	K	Σ
Ericaceae											
Blueberry				27.29±0.6	54.90±3.9	9.57±0.3	91.76	43.02±1.1	136.97±3.2	2.88±0.1	182.87
Rosaceae											
Blackberry		121.07±0.2	121.07	5.65±0.4	5.94±0.0	5.11±0.0	16.7		55.42±0.5	2.13±0.5	57.55
Chokeberry		3.97±0.1	3.97	38.39±0.5			38.39		92.15±0.5	6.86±0.3	99.01
Strawberry		41.40±0.2	41.40		16.86±0.3		16.86		6.24±0.2	7.71±0.3	13.95
Red raspberry	4.95±0.1	31.74±2.1	36.69	3.23±0.1	4.24±0.1	4.29±0.1	11.76		3.85±0.2		3.85
Sweet cherry				11.47±0.2	5.40±0.2		16.87	0.17±0.1	8.58±0.1	5.97±0.3	14.72
Sour cherry				9.05±0.5	19.12±0.9		28.17		5.05±0.3	2.38±0.1	7.43
Caprifoliaceae											
Elderberry				15.84±0.2	10.80±0.2		26.64				145.71
Saxifragaceae											
Black currant		3.15±0.1	3.15	21.31±0.1	31.69±0.3	17.48±0.1	70.48				73.19
Red currant	12.54±0.1		12.54	12.76±0.3	8.26±0.2		21.02	1.72±0.3	8.87±0.2	0.34±0.0	10.93

p-HBA: *p*-Hydroxybenzoic acid; EA: Ellagic acid; CA: Caffeic acid; *p*-CouA: *p*-Coumaric acid; FA: Ferulic acid; M: Myricetin; Q: Quercetin; K: Kaempferol

acid (10 mg kg^{-1}). These phenolic acids were identified in blueberries in previous studies as well[15,17,26]. *p*-Coumaric acid dominated over caffeic and ferulic acid[17] which agrees with our results. According to other investigation the concentration of caffeic acid in blueberry (*Vaccinium myrtillus*) can be higher than the concentrations of *p*-coumaric and ferulic acid[26].

The main characteristic of *blackberry* was high concentration of ellagic acid (121 mg kg^{-1}). Apart from ellagic acid, blackberry contains lower amounts of caffeic (6 mg kg^{-1}), *p*-coumaric (6 mg kg^{-1}) and ferulic acid (5 mg kg^{-1}). These phenolic acids were identified in blackberry in previous study as well[27]. In that study the predominant phenolic acid in blackberry was ellagic acid, and the amounts of caffeic, *p*-coumaric and ferulic acid were considerably lower[27] which agrees with the results of present study. Blackberry contains flavonol quercetin as a dominant one (55 mg kg^{-1}) and kaempferol (2 mg kg^{-1}). Previous study confirmed that the major flavonol in different varieties of blackberry fruits was quercetin[28].

Chokeberry contained high concentrations of flavonols (99 mg kg^{-1}). The main flavonol was quercetin (92 mg kg^{-1}), followed by kaempferol at a considerably lower concentration (7 mg kg^{-1}). According to previous studies quercetin was the predominant flavonol in chokeberry[15,18,29,30], whereas kaempferol was not identified[29,30] or its concentration was low[15,18] which agrees with our results. Phenolic acids found in our sample of chokeberry were caffeic (38 mg kg^{-1}) and ellagic acid (4 mg kg^{-1}). Previous studies confirmed that chokeberry contains caffeic acid or caffeic acid derivatives[15,18,29] and low concentration of ellagic acid[18]. Chokeberry contained the highest concentration of caffeic acid among the fruits studied.

In *strawberry*, the concentration of phenolic acid was higher than concentration of flavonols. The main phenolic acid found in strawberry was ellagic acid (41 mg kg^{-1}), followed by *p*-coumaric acid (17 mg kg^{-1}). According to previous study, the main representative of phenolic acids in strawberry was ellagic acid[18] or its derivatives[16], followed by *p*-coumaric acid[16,18] which is consistent with the results in this study. Flavonols found in low concentrations in strawberry were kaempferol (8 mg kg^{-1}) and quercetin (6 mg kg^{-1}). These results are in accordance with those reported by *Häkkinen* et al.[30], *Hertog* et al.[31], and *Määttä-Riihinen* et al[16]. Along with flavonols quercetin and kaempferol, some authors reported low level of myricetin[18].

Like strawberry, *red raspberry* was characterized by higher concentrations of phenolic acids in comparison to flavonols. Ellagic acid (32 mg kg^{-1}) was the dominant phenolic acid whereas caffeic (3 mg kg^{-1}), ferulic (4 mg kg^{-1}), *p*-coumaric (4 mg kg^{-1}) and *p*-hydroxybenzoic acid (5 mg kg^{-1}) were found at lower concentrations. These results are in accordance with those reported by *Häkkinen* et al.[18], *Määttä-Riihinen* et al.[16] and *Mattila* et al.[26]. The only flavonols found in red raspberry was quercetin (4 mg kg^{-1}) which agrees with previous study[30]. Apart from quercetin, *Häkkinen* et al.[18], identified also lower levels of myricetin and kaempferol.

Flavonols identified in *sweet cherry* were quercetin (9 mg kg^{-1}), kaempferol (6 mg kg^{-1}) and myricetin (0.17 mg kg^{-1}). Quercetin and kaempferol were identified in sweet cherry in previous study as well[32]. Among five investigated phenolic acids, only caffeic acid (12 mg kg^{-1}) and *p*-coumaric acid

(5 mg kg^{-1}) were identified in our sample of sweet cherry. According to the literature data, sweet cherry contains derivatives of caffeic and *p*-coumaric acids[32].

Sour cherry contains flavonols quercetin (5 mg kg^{-1}) and kaempferol (2 mg kg^{-1}). These results are in accordance with those reported by *Kim* et al.[32]. Phenolic acids identified in sour cherry were *p*-coumaric (19 mg kg^{-1}) and caffeic acid (9 mg kg^{-1}). The derivatives of these phenolic acids were found in sour cherry in previous study as well[32].

Elderberry contains high concentrations of flavonols (146 mg kg^{-1}). The dominant flavonol was quercetin (144 mg kg^{-1}), and its concentration in elderberry was the highest in comparison to other red fruits studied. The concentration of kaempferol was low (2 mg kg^{-1}). Previous study[15] confirmed that elderberry is characterized by high concentration of quercetin and low concentration of kaempferol. Phenolic acids identified in elderberry were caffeic (16 mg kg^{-1}) and *p*-coumaric acid (11 mg kg^{-1}). *Määttä-Riihinen* et al.[15] found *p*-coumaric and caffeic acid in elderberry as well along with ferulic acid which was not identified in our sample of elderberry.

Black currant contained high concentrations of flavonols (73 mg kg^{-1}) and phenolic acids (74 mg kg^{-1}). The main flavonol was myricetin (44 mg kg^{-1}), followed by quercetin (21 mg kg^{-1}) and kaempferol (8 mg kg^{-1}). Some studies[15,30] confirmed that myricetin was the dominant flavonol in black currant as it was found in our study, while others reported quercetin as the main flavonol[14,18]. Phenolic acids found in our sample of black currant were *p*-coumaric acid (32 mg kg^{-1}) as a main phenolic acid, caffeic acid (21 mg kg^{-1}), ferulic acid (17 mg kg^{-1}) and ellagic acid (3 mg kg^{-1}). These results are in accordance with those already published[14,15,18].

In *red currant*, the concentration of phenolic acids was higher than the concentration of flavonols. Phenolic acids found in our sample of red currant were caffeic (13 mg kg^{-1}), *p*-coumaric (8 mg kg^{-1}) and *p*-hydroxybenzoic acid (13 mg kg^{-1}). These phenolic acids (*p*-coumaric, caffeic, *p*-hydroxybenzoic acid)[18] or derivatives of *p*-coumaric and caffeic acids[14,15] were found in red currant in previous study as well, along with lower concentrations of ellagic and ferulic acid[18]. The dominant flavonol was quercetin (9 mg kg^{-1}), while myricetin (2 mg kg^{-1}) and kaempferol (0.34 mg kg^{-1}) were present at lower concentrations. According to some previous studies, flavonols present in red currant were quercetin, myricetin and kaempferol, among which quercetin was the dominant one[14,15,18] which is consistent with the results of present study. Some authors reported only the presence of quercetin whereas myricetin and kaempferol were not identified[30].

Tab. 2 Concentrations of total polyphenols (TP) [mg GAE kg^{-1}], total anthocyanins (TA) [mg CGE kg^{-1}], TA/TP ratio and antioxidant activity of red fruits; [a] values are means ± SD (n=3)

Red fruits	Σ Polyphenols	Σ Anthocyanins	TA/TP	DPPH	ABTS
	[mg kg^{-1}]			[μmol TE/g]	
Ericaceae					
Blueberry	6180.23±157	4069.03±129	0.66	125.52	53.28
Rosaceae					
Blackberry	3657.57±167	1055.70±31	0.29	41.89	23.94
Chokeberry	7194.40±78	3571.96±48	0.50	181.07	78.90
Strawberry	1999.24±88	169.17±2	0.08	6.11	12.08
Red raspberry	1763.19±71	231.72±1	0.13	18.61	12.10
Sweet cherry	2010.67±50	192.52±13	0.10	4.22	13.62
Sour cherry	2904.54±101	1145.89±37	0.39	29.49	23.74
Caprifoliaceae					
Elderberry	4415.33±124	3175.14±34	0.72	100.16	37.91
Saxifragaceae					
Black currant	5435.06±31	2189.26±20	0.40	109.89	44.67
Red currant	1947.94±23	197.76±2	0.10	13.73	13.15

The concentrations of flavonols and phenolic acids of red fruits examined in this study are comparable to the results of earlier studies[14–16,26–28,30–32]. Some differences in flavonol and phenolic acid content can be explained by differences in fruit cultivars, growing conditions, degree of ripeness, handling after storage, sample preparation treatments, etc.

Red fruits were analyzed using a pH-differential and Folin-Ciocalteau method in order to examine their total anthocyanin (TA) and total polyphenol (TP) content. The portion of anthocyanins in total polyphenol concentration was evaluated by calculating TA/TP ratio and the results are presented in Table 2. Polyphenols were found in the highest concentrations in chokeberry, blueberry, black currant and elderberry (7194 mg kg^{-1}, 6180 mg kg^{-1}, 5435 mg kg^{-1}, 4415 mg kg^{-1}, respectively). High concentrations of polyphenols were found in blackberry and sour cherry as well (3658 mg kg^{-1}, 2905 mg kg^{-1}, respectively), while sweet cherry, strawberry, red currant and red raspberry had relatively lower concentrations of polyphenols (2011 mg kg^{-1}, 1999 mg kg^{-1}, 1948 mg kg^{-1}, 1763 mg kg^{-1}, respectively). The concentrations of total polyphenols found in our study are in accordance with previous studies[20,27,32,33].

Anthocyanins were found in the highest concentrations in blueberry, chokeberry, elderberry, black currant, sour cherry and blackberry (4069 mg kg^{-1}, 3572 mg kg^{-1}, 3175 mg kg^{-1}, 2189 mg kg^{-1}, 1146 mg kg^{-1}, 1056 mg kg^{-1}, respectively) whereas the concentration of anthocyanins in red raspberry, red currant, sweet cherry and strawberry were considerably lower (232 mg kg^{-1}, 198 mg kg^{-1}, 193 mg kg^{-1}, 169 mg kg^{-1}, respectively). The concentrations of total anthocyanins are

Tab. 3 Correlation coefficients (r) between antioxidant activity of fruits (DPPH and ABTS) and concentrations of total polyphenols, total anthocyanins, total flavonols, total hydroxycinnamic acids, total hydroxybenzoic acids, quercetin, caffeic acid, and *p*-coumaric acid in red fruits

	r	
[Substance/mg kg^{-1} FW]	**DPPH [µmol TE/g]**	**ABTS [µmol TE/g]**
ΣPolyphenols	0.98***	0.98***
ΣAnthocyanins	0.93***	0.90***
ΣFlavonols	0.79**	0.73*
ΣHydroxycinnamic acids	0.66*	0.64*
Quercetin	0.74*	0.68*
Caffeic acid	0.91***	0.94***
***p*-Coumaric acid**	0.74*	0.82**

*,**,*** designate significance at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

in the range of, or similar to those reported in previous studies[20,27,32,33]. As it can be seen from TA/TP ratio, anthocyanins represented important part of polyphenols in investigated fruits. They are the predominant polyphenolic components in elderberry (72 %) and blueberry (66 %) which indicates a lower proportion of other polyphenols in these fruits. In chokeberries (50 %), black currant (40 %) and sour cherries (39 % %) anthocyanins represented significant proportion in total polyphenol concentration but the proportion of anthocyanins were considerably lower in blackberry (29 %), red raspberry (13 %), sweet cherry (10 %), red currant (10 %) and strawberry (8 %) indicating a higher proportion of other polyphenols in these fruits.

In order to evaluate antioxidant activity of red fruits studied, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays were applied and the results are presented in Table 2. The values of antioxidant activity of fruits obtained by DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assay differ due to differences in methods used, in free radical that was applied and in reaction time. By far, the strongest antioxidant activity against both radicals showed chokeberry (181 µmol TE/g, DPPH method; 79 µmol TE/g ABTS method), followed by blueberry, black currant and elderberry (126, 110, 100 µmol TE/g respectively, DPPH method), (53, 45, 38 µmol TE/g respectively ABTS method). The fruits with higher concentrations of polyphenols (Table 1 and 2) showed also the strongest antioxidant activity. Other examined fruits like blackberry, sour cherry, red raspberry, red currant, strawberry and sweet cherry posses relatively lower antioxidant activity (42, 30, 19, 14, 6, 4 µmol TE/g respectively; DPPH method), (24, 24, 12, 13, 12, 14 µmol TE/g respectively; ABTS method). There are already a number of reports on the antioxidant activity of fruit extracts determined by several methods such as oxygen radical absorbance capacity (ORAC), ABTS or DPPH method indicating that chokeberry, blueberry, black currant and elderberry possess strong antiradical activities[20,29,33–35] as it was found in this study. Chokeberry exhibited the highest antioxidant activity determined by ORAC procedure, followed by elderberry and black currant[34]. Chokeberry also showed higher antioxidant activity than fruits like lingonberry, blueberry and cranberry[29]. In the evaluation of commercial red fruit juice concentrates, the strongest antioxidant activity determined by DPPH method had chokeberry juice concentrate as in present study, and was followed by black currant and elderberry, while other fruit concentrates like red currant, strawberry, red raspberry and cherry concentrate showed lower antioxidant activity[35]. The strongest antioxidant activity evaluated by ABTS assay showed black currant, chokeberry and elderberry juice concentrate, whereas the antioxidant activity of red currant, strawberry, raspberry and cherry juice concentrate were significantly lower[35].

The correlation between antioxidant activity and total polyphenols, total anthocyanins, total hydroxycinnamic acids and total flavonols are presented in Table 3. Total polyphenols were found to correlate with the antioxidant activities of fruits (DPPH, r = 0.98; ABTS, r = 0.98). Total anthocyanins correlate with antioxidant activity as well (DPPH, r = 0.93; ABTS, r = 0.90) but the correlation coefficient was lower for total anthocyanins versus antioxidant activity than for the total polyphenols versus antioxidant activity. Total flavonols (DPPH, r = 0.79; ABTS, r = 0.73) and total hydroxycinnamic acids (DPPH, r = 0.66; ABTS, r = 0.64) correlate with antioxidant activities of fruits as well. According to the data presented by others, total polyphenols of various small fruits correlate better with antioxidant activity than total anthocyanins do[34], which agrees with the results of our study. Moreover, total polyphenols of fruits belonging to *Vaccinium*, *Rubus* and *Ribes* species correlate better with antioxidant activity than total anthocyanins[33,36]. Total flavonols of red fruit concentrates correlate with antioxidant activity[35] which is consistent with our findings. In the investigation of antioxidant activity of various blueberries, linear correlation was found between total polyphenols, total anthocyanins, total flavonols, total hydroxycinnamic acids and antioxidant activity[37]. The correlation between total flavonols and antioxidant activity was better than the correlation between total hydroxycinnamic acids and antioxidant activity[37] which is consistent with our results. Good linear correlations between mentioned polyphenol groups indicate possible influence of these polyphenols on antioxidant activity of fruits.

Moreover, linear relationship was observed between antioxidant activity of fruits and concentration of some individual phenolics (Tab. 3). Quercetin correlates with the antioxidant activity of red fruits (DPPH, r = 0.74; ABTS, r = 0.68). In previous studies[5] quercetin showed higher antioxidant activity against ABTS$^{\bullet+}$ radical than other flavonol aglycons like myricetin and kaempferol. Considering antioxidant activity of quercetin, the existence of linear relationship between antioxidant activity of fruits and the concentrations

of quercetin is possible. Among various hydroxycinnamic acids, the highest antioxidant activity determined by ORAC assay showed caffeic acid and was followed by *p*-coumaric > vanillic > chlorogenic acid[29]. Our results are showing that there is also a linear relationship between the concentration of caffeic acid and antioxidant activity of fruits (DPPH, 0.91; ABTS, r = 0.94) and between concentration of *p*-coumaric acid and antioxidant activity of fruits (DPPH, r = 0.74; ABTS, r = 0.82). Correlation coefficient was higher for caffeic acid versus antioxidant activity than for the *p*-coumaric acid versus antioxidant activity.

From the results of this study it can be seen that, among the red fruits studied, chokeberry, blueberry, elderberry and black currant stand out in high concentrations of flavonols, phenolic acids, and in strong antioxidant activity. Blueberry contains the highest concentrations of flavonols and phenolic acids, and is followed by elderberry which has high concentrations of flavonols, especially quercetin. Chokeberry showed by far the highest antioxidant activity. Furthermore, chokeberry has the highest amount of total polyphenols and is rich in flavonols as well. Black currant has high concentrations of flavonols and phenolic acids. Other red fruits have considerably lower concentrations of flavonols and phenolic acids, and lower antioxidant activity, but it should be emphasized that some fruits like blackberry, strawberry and red raspberry contain relatively high concentration of ellagic acid. The diversity in the polyphenolic profiles between different fruit species may relate to different biological availability and activity in human organism. Although all examined fruit species can serve as good source of bioactive polyphenolic compounds, chokeberry, blueberry, elderberry and black currant stand out in high concentrations of anthocyanins, flavonols and phenolic acids and in high antioxidant activity, and therefore could be used in nutritional supplement formulations. Moreover, quercetin, myricetin, and caffeic acid were reported to have strong antiradical activity among various flavonols and phenolic acids[5,29]. Therefore, chokeberries (which are abundant in quercetin and caffeic acid derivatives), elderberries (abundant in quercetin derivatives) and blueberries (abundant in quercetin and myricetin derivatives) can serve as good source of these individual phenolics.

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Zimt und Cumarine: bittere „Wahrheiten" – Beitrag zur Extrapolation von Risiken

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Zusammenfassung
Die aktuellen und hochgradig artifiziellen Debatten zur Sicherheit von Lebensmitteln deuten auf ein grundsätzliches Problem mit der Extrapolation von Risiken aus den Eigenschaften isoliert betrachteter sekundärer Pflanzeninhaltstoffe hin. Beispiele sind die Diskussion der Morphingehalte in Backwaren mit Mohnzusatz, die Übertragung der toxikologischen Daten hoher Konzentrationen von Estragol auf Gewürze wie Estragon, oder die Ableitung eines Leberrisikos von Cassia-Zimt, basierend auf den Eigenschaften eines isolierten Cumarins. Aktuell gipfelte dies im Vorschlag, Furanocumarine in Engelwurz zu limitieren, weil das Furanocumarin 8-Methoxypsoralen – selbst gar nicht in Engelwurz nachgewiesen – in hohen Dosen und unter Bestrahlung mit UV-Licht im Verdacht steht, Hautkrebs auslösen zu können. Bevor die Bodenhaftung in der Risikobewertung gänzlich verloren geht, ist es an der Zeit für eine kritische Betrachtung der derzeitigen Mechanismen einer „Risikobewertung". Die Zielsetzung der Riskobewertung ist der weitestmögliche Ausschluss aller auch nur denkbaren Risiken. Dies ist weder praktikabel noch realistisch. Zimt und Engelwurz sind typische Beispiele.

Summary
The recent, highly artificial debates on the safety of foodstuff point to a basic problem with the extrapolation of risks derived from the properties of singled out secondary plant metabolites. Examples are the discussion of morphine contents in poppy seed, the transfer of toxicological data from high concentrations of estragol to spices such as estragon, or the derivation of a liver risk of Chinese cinnamon from the properties of an isolated coumarin. Only recently the suggestion was made to limit furanocoumarins in Angelica roots, because the furanocoumarin 8-methoxypsoralen – which was not even detected in Angelica – is suspected to cause skin cancer when applied in high doses and combined with UV-irradiation. It is time to critically evaluate the current mechanisms of "risk assessment" before common sense is completely lost. The aim of risk assessment is to exclude any conceivable risk as far as possible. This is neither practicable nor realistic. Cinnamon and Anglica are typical examples.

Keywords: Zimt, *Angelica*, Cumarine, Furocoumarine, Toxizität / Cinnamon, *Angelica*, coumarins, furocoumarins, toxicity

Einleitung

„Zimt: eine bittere Wahrheit" – unter dieser Überschrift wurde noch vor wenigen Monaten von der als wissenschaftlich und fachlich fundiert anerkannten Zeitschrift für Phytotherapie vor den Folgen des Konsums von Zimtsternen gewarnt. Nach dieser Meldung, für die als Quelle die Verbraucherminister der Länder angegeben wurden, könnte wegen des Cumaringehaltes von Zimt (*Cinnamomum aromaticum* Nees, Lauraceae) für Kinder bis 15 kg Körperge-