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Nutritional quality of berries and bioactive compounds in the leaves of black currant (*Ribes nigrum* L.) cultivars evaluated in Estonia

Piret Raudsepp[a,*], Hedi Kaldmäe[b], Ave Kikas[b], Asta-Virve Libek[b] and Tõnu Püssa[a]
[a]*Department of Food Hygiene, Estonian University of Life Sciences, Tartu, Estonia*
[b]*Polli Horticultural Research Centre, Viljandimaa, Estonia*

Abstract. Polli Horticultural Research Centre (58°7′N; 25°32′E) in Estonia has focused on selecting cultivars with high productivity and suitable to use in local climatic conditions since 1945. Besides important agronomic characteristics, more attention has been recently paid to fruit quality and content of various bioactive compounds.

The results of a biochemical analysis of 4 prospective black currant selections (10B, 2-96-51, 1-96-16, 4-96-1), 4 new cultivars ('Karri', 'Almo', 'Ats', 'Elo') from our own breeding program and 7 introduced cultivars ('Öjebyn', 'Zagadka', 'Ben Sarek', 'Intercontinental', 'Pamyati Vavilova', 'Titania' and 'Pilenai') are presented.

In addition to the analysis of main biochemical characteristics, the anthocyanin content of the berries was determined using high performance liquid chromatography (HPLC). The total anthocyanin content of the berries varied in a wide range. The highest anthocyanin content was found in the cultivar 'Almo' (212 ± 9 mg/100 g) and the lowest in 'Ben Sarek' (83 ± 24 mg/100 g). The ascorbic acid content varied from 98 mg/100 g with 'Ats' to 209 mg/100 g with elite selection 4-96-1.

The polyphenol composition of the black currant leaves was determined by HPLC, the compounds were identified using polyphenol commercial standards and/or compounds mass spectrometric (MS) characteristics.

Keywords: Black currant, seedlings, fruit quality, leaves, poly phenol composition

1. Introduction

Ascorbic acid and anthocyanins are the most highly valued health beneficial compounds in black currants contributing to the antioxidant capacity of the fruit [13]. Anthocyanins have also been associated with fruit colour and are, therefore, important contributors to the quality of the product, they appear to be very stable and remain active after a prolonged frozen storage, processing into juice, wine and jam [9, 13]. The change of focus to quality and nutritional factors in breeding programs in Europe stems from the retail price reduction due to the overproduction of black currants in Europe in 2003–2005 [1]. The focus on the nutritional aspects of the berries is an important sales argument for promoting black currants for fresh consumption. The processors' main requirements are high levels of ascorbic acid and antioxidant properties together with a low acidity and improved sensory characteristics [2]. The anthocyanin content of 4 prospective black currant selections (10B, 2-96-51, 1-96-16, 4-96-1), 4 new cultivars ('Karri', 'Almo', 'Ats', 'Elo') from our own breeding program and 7 introduced cultivars ('Öjebyn', 'Zagadka', 'Ben

*Corresponding author: P. Raudsepp, Department of Food Hygiene, Kreutzwaldi 58A, Tartu 51014, Estonia. Tel.: +372 7313432; Fax: +372 7313432; E-mail: raudsepp.piret@gmail.com.

Sarek', 'Intercontinental', 'Pamyati Vavilova', 'Titania' and 'Pilenai') were analysed in this study, additionally to the main biochemical characteristics.

The leaves of black currant have been used in folk medicine for their diaphoretic and diuretic properties as well as to relieve rheumatic pain [18]. The pharmacological effect other than anti-inflammatory effect of black currant leaves has not been scientifically proved [3, 4]. Their anti-inflammatory effect may be due to the polyphenol compounds that are originally synthesised by plants to protect themselves from pathogens [5]. Surveys have indicated that some of the polyphenol compounds may be exposed on the cuticle of leaves to give a quick response to the microbial attack [25]. Although black currant leaves are widely in use as tea material [17], there are not many scientific studies about the composition of the chemical compounds responsible for the health beneficial effect. We used a mixture of known polyphenol standard compounds, previously known to be present in the leaves of black currant [17, 18] and some additional standards that have been found to be present in other leaves of pharmacological value [6] to determine the composition of polyphenol compounds in the leaves.

2. Materials and methods

2.1. Materials

The anthocyanidin standards and other chemicals, used in sample preparation or chromatographic separation were all with HPLC purity grade: catechin, kaempherol, quercetin-3-glucoside, myricetin, quercetin-3-galactoside from Sigma-Aldrich Inc.; cyanidin-chloride from Carl Roth 4545.1, Germany; delphinidin-chloride from Carl Roth 4537.1, Germany; formic acid from Fluka, USA; quercitrin, Alfa-Aesar; acetonitril, ethanol 96%, hydrochloric acid 37%, NaOH and methanol were with analytical grade; water, ultra pure made prior to analysis using Milli Q by Millipore Corporation.

The chemicals used for biochemical analysis: potassium ferricyanide (III), sulfuric acid, lead subacetate, potassium iodate, NaOH, 2,6-dichloroindophenol, methylene blue and oxalic acid were of analytical grade.

2.2. Collecting of samples

The research was carried out in 2005–2007 and 2009. 500 g of berry samples per cultivar were collected in the middle of the harvesting season from the cultivar evaluation test plots established in 2000. Four prospective selections (10B, 2-96-51, 1-96-16, 4-96-1) and 4 new cultivars ('Karri', 'Almo', 'Ats', 'Elo') [11] from our own breeding program and 7 introduced cultivars ('Öjebyn', 'Zagadka', 'Ben Sarek', 'Intercontinental', 'Pamyati Vavilova', 'Titania' and 'Pilenai') were analysed in three consecutive years 2005, 2006 and 2007.

Fully developed leaves for the qualitative analysis of polyphenols were collected in August 2009 randomly from 6 different bushes of the same cultivar ('Karri') including equal proportions of leaves from different sides and inner and outer range of the bush.

2.3. Methods

The representative samples were prepared from the field samples, 200 g of berries were homogenized using a kitchen blender and analysed on the same day for sugar, soluble solids and ascorbic acid content. The soluble solids content in the homogenized samples were recorded in a refractometer (Abbe WYA-1S, *Optic Ivymen System*) at 20°C, organic acids were determined by titration with 0.1 NaOH. Ascorbic acid was determined using the modified Tillman's method. Ascorbic acid was titrated with 2,6-dichloroindophenol under acid conditions [16]. The ferricyanide method was used for the sugar content analysis [23]. The results are expressed in mg per 100 g of fresh berries. The average weight of berries was determined by weighing 20 berries per sample.

The samples for the anthocyanin content determination were collected at the same time and frozen at 40°C for further preservation.

The leaves were dried at 50°C for 24 h until air-dry and were ground prior to analysis.

2.4. Sample preparation for HPLC analysis of anthocyanins and leaf polyphenols

For the anthocyanidin analysis, 200 g of fresh frozen berries were homogenized with a Heidolph DIAX 900, 2 g of homogenizate was weighed with an *OHAUS* analytical standard scale into a 50 ml centrifuge tube, 3 parallel samples of each. The samples were extracted for 30 min at room temperature with 20 ml ethanol/water/HCl (70:30:1), after which the samples were shaken on a minishaker IKA MS 1 by *IKA-WORKS* for 30 s twice after 5 min. The tubes were then centrifuged with an Eppendorf centrifuge 5810 R at 3220 g at 20°C for 10 min, the supernatant was removed and the samples were extracted once more with 20 ml ethanol/water/HCl (70:30:1) using the same procedure, and for the third time with 5 ml ethanol/water/HCl (70:30:1). The supernatants were combined and 1 ml of the final solution was filtered through a Spartan 13 mm regenerated cellulose membrane filter with a pore size of 0.2 μm (modified [26] and [24]). For the acid hydrolysis of the samples, the final filtrate was taken to 1 M HCl concentration and heated in the Binder oven at 90°C for 60 min [25].

For the polyphenol analysis of the leaves: 0.1 g of dried leaves were weighed into a 15 ml centrifuge tube, 10 ml of 40% ethanol was added, and 3 parallel samples were made. The samples were extracted at room temperature for 24 h. The extracts were centrifuged at 3220 g at 20°C for 10 min. The supernatant was filtered through *Spartan* 13 mm regenerated cellulose membrane filter with a pore size 0.2 μm

2.5. Chromatographic conditions

For detection and quantification of anthocyanins, *Agilent* 1100 Series LC/MSD Trap-XCT with an ESI interface (m/z interval 50–1000 in positive ion mode) and UV-Vis diode array detector was used. HPLC reversed phase column Zorbax 300SB-C^{18} with dimensions of 2.1 × 150 mm with a particle size of 5 μm was used at 35°C, and the speed of the mobile phase was 0.3 ml min^{-1}. The elution was carried out with 1% formic acid in water (mobile phase A) and acetonitril (solvent B), a multistep mobile phase gradient was used as follows: 0 min 95:5 (A:B), linear gradient until 30 min 70:30 (A:B), by 40 min the ratio of A:B was 10:90 and maintained for 50 min, after what the concentration of mobile phase A was raised again to 95% while mobile phase B was dropped to 5% and the system was re-equilibrated 10 min. For the quantification of the anthocyanins, the peak area at 510 nm was used. The detection limit for anthocyanins was 0.1 μg/ml and the quantification limit 0.2 μg/ml. The calibration curve was constructed using a standard mixture of delphinidin and cyanidin.

The analysis of the polyphenol composition of the leaves was conducted on the same chromatographic system but in the negative ionisation mode. The elution gradient was set as follows: 0 min 90:10 [0.1% formic acid in water (A):acetonitril (B)], the linear gradient until 30 min 70:30 (A:B), by 40 min the ratio of A:B was 10:90 and maintained until 50 min and from 50.1 min the concentration of solvent A was raised again to 90% and the concentration of solvent B dropped to 10% and maintained until 60 min to re-equilibrate the system. The calibration curves were constructed using a standard mixture of catechin, chlorogenic acid, quercetin galactoside, quercetin glucoside, myricetin, quercetin rhamnoside also known as quercitrin, and kaempherol.

3. Results and discussion

3.1. Biochemical and morphological characteristics of fruit

The average fruit weight was 1.25 g. The cultivar 'Öjebyn' had the smallest fruits (0.9 g) and 'Karri' the largest ones 1.7 g.

The ascorbic acid content varied from 98 mg/100 g with 'Ats' to a very high level of 209 mg/100 g with elite selection 4-96-1 with average ascorbic acid content of 132.5 mg/100 g (Table 1). 130 mg has been set as the requirement by some producers to ensure the healthiness of the processed product [12].

The sugar content and sugar acid ratio, that are major factors affecting the sweetness of the taste of the berries, were highest with the cultivar 'Pilenai' (12.6% and 4.7, respectively) and lowest with 'Zagadka' (8.4% and 2.7 respectively). Above-the-average sugar acid ratio (3.7) was recorded for the cultivars 'Pamyati Vavilova' 'Titania' 'Intercontinental' and elite selections 4-96-1 and 2-96-51. These selections both have the cultivar 'Pamyati Vavilova' as

Table 1
The average biochemical and morphological characteristics of berries, measured in 2005 and 2007

Cultivar	Soluble solids in juice (%)	Water (%)	Titratable acids (%)	Sugars (%)	Sugar acid ratio	Ascorbic acid (mg/100g)	Average fruit weight (g)
10 B	17.3	79	2.6	9.8	3.5	138	1.2
1-96-16	17.2	76	2.7	11	4.1	134	1
2-96-51	15.6	83	2.7	10.4	3.9	141	1.2
4-96-1	16.3	79	2.6	10.7	4.2	209	1.2
Almo	17.8	79	2.8	9.5	3.4	112	1.2
Ats	16.9	78	2.4	8.8	3.7	98	1.4
Ben Sarek	16.3	80	3.6	8.7	2.4	115	1.5
Elo	17	79	2.6	9.2	3.6	117	1.2
Intercontinental	18.5	79	2.8	10.4	3.7	132	1.5
Karri	16.9	77	2.4	8.9	3.7	134	1.7
Pamyati Vavilova	16.4	79	2.7	11.9	4.4	164	1.2
Pilenai	16	81	2.7	12.6	4.7	110	1.3
Zagadka	16	78	3.1	8.4	2.7	107	1.2
Titania	16	77	3.1	12.6	4.1	141	1.1
Öjebyn	17.9		2.7	8.7	3.2	133	0.9

one of the crossing parents. Both selections have also high acorbic acid contents (209 mg and 141 mg/100 g) similar to their parental genotype 'Pamyati Vavilova'(ascorbic acid content 164 mg/100 g). Comparison with our earlier research [11] including the 4 new cultivars from our breeding program and cultivar 'Öjebyn'used as the standard cultivar, showed that the sugar content in the current study years was slightly higher than average and might have been influenced by the weather conditions and relatively modest yields (data not shown) in these years. The ascorbic acid levels obtained with the above listed cultivars are consistent with the average results obtained previously and show a similar variation between genotypes.

3.2. Analysis of the anthocyanin content of the berries

Our analysis revealed that anthocyanins are present in form of cyanidin and delphinidin glycosides in black currant berries. The prevailing glycosides are delphinidin glucoside (1), delphinidin 3-O-rutinoside (2), cyanidin-glucoside (3), cyaniding 3-O-rutinoside (4) (Fig. 1). Previous studies have revealed similar results [10, 14, 19]. Some authors have discovered a total of 15 different anthocyanin glycosides in black currant berries [21, 22]. After the acid hydrolysis of the samples, it was revealed that cyanidin was slightly dominant over delphinidin (Table 2) in most of the studied cultivars. It is interesting to remark that at the beginning of the berry ripening in the example of 'Pilenai', the cyanidin was largely prevailing over delphinidin 73:23, but in the fully ripened berries, the ratio 57:43 was closer to equal (Table 2). The highest content of anthocyanins was found in cultivars 'Titania', 'Almo', 'Öjebyn', 'Ats', 'Intercontinental' and 'Elo' from which 'Almo', 'Elo' and 'Ats' are from our own breeding programme. Seedling 1-96-16 from our breeding programme showed also a high content of anthocyanins in three consecutive years (Table 2).

It has to be mentioned that in the second year of the study (2006), the weather conditions were not favourable for the berry production, and the content of anthocyanins in the berries gathered approximately at the same time as in the two other years was lower than in the first (2005) and the third (2007) year. It may be the result of incomplete ripening by that time. Previous studies have revealed that anthocyanin content depends on the ripening stage of the berries, increasing during ripening and reaching the maximum when berries are fully ripened [7, 8, 15]. The anthocyanin content may also be influenced by weather conditions [12]. The overall crop yield of berries was likewise lower in the year 2006.

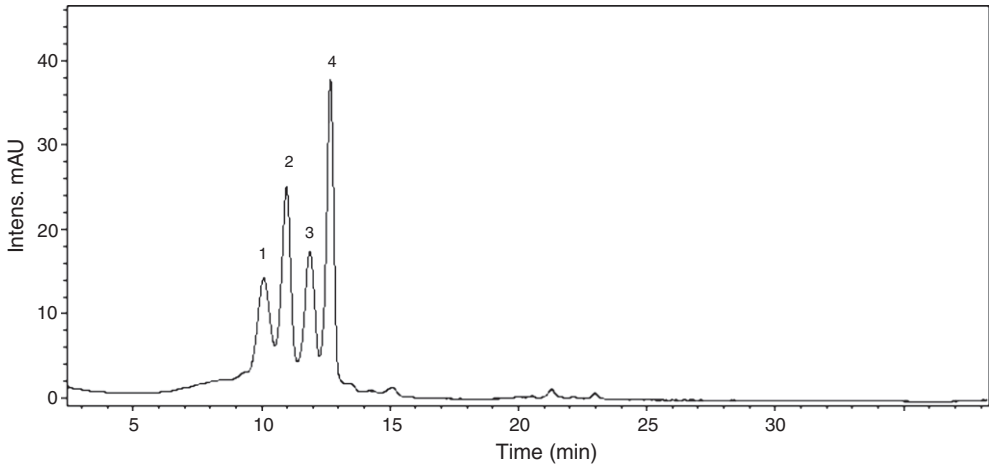

Fig. 1. UV-chromatogram of fruit anthocyanin analysis at 510 nm. 1) delphinidin glucoside, 2) delphinidin 3-O-rutinoside, 3) cyanidin-glucoside, 4) cyaniding 3-O-rutinoside.

Table 2
The average anthocyanin content of black currant berries in 3 consecutive years and the percentage of the main aglycones after acid hydrolysis

	Anthocyanin content of berries (ug/ml)	Delphinidin (%)	Cyanidin (%)
10B	158 ± 21	44	56
1-96-16	221 ± 29	55	45
1-96-51	150 ± 66	41	59
4-96-1	148 ± 33	42	58
Almo	213 ± 9	38	63
Ats	185 ± 65	39	61
Ben Sarek	84 ± 24	37	63
Elo	172 ± 21	40	60
Intercontinental	177 ± 6.5	44	56
Karri	154 ± 89	41	59
Pamjati Vavilova	143 ± 22	40	60
*Pilenai I	0	–	–
*Pilenai II	14 ± 4	27	73
*Pilenai III	111 ± 32	40	60
*Pilenai IV	177 ± 93	43	57
Zagadka	179 ± 43	52	48
Titania	216 ± 29	49	51
Öjebyn	194 ± 52	42	58

*The cultivar 'Pilenai' was measured in four different phases of ripening; stage I being green berries and IV stage the fully ripened stage, collected at the same time as the berries of the other cultivars and seedlings.

3.3. Polyphenol composition of leaves

The results of the HPLC analysis of the leaves are presented in Fig. 2. The analysis was conducted using polyphenol standards (2-catechin, 3-chlorogenic acid, a-quercetin galactoside, 6-quercetin glucoside, b-myricetin, 8-quercitrin,

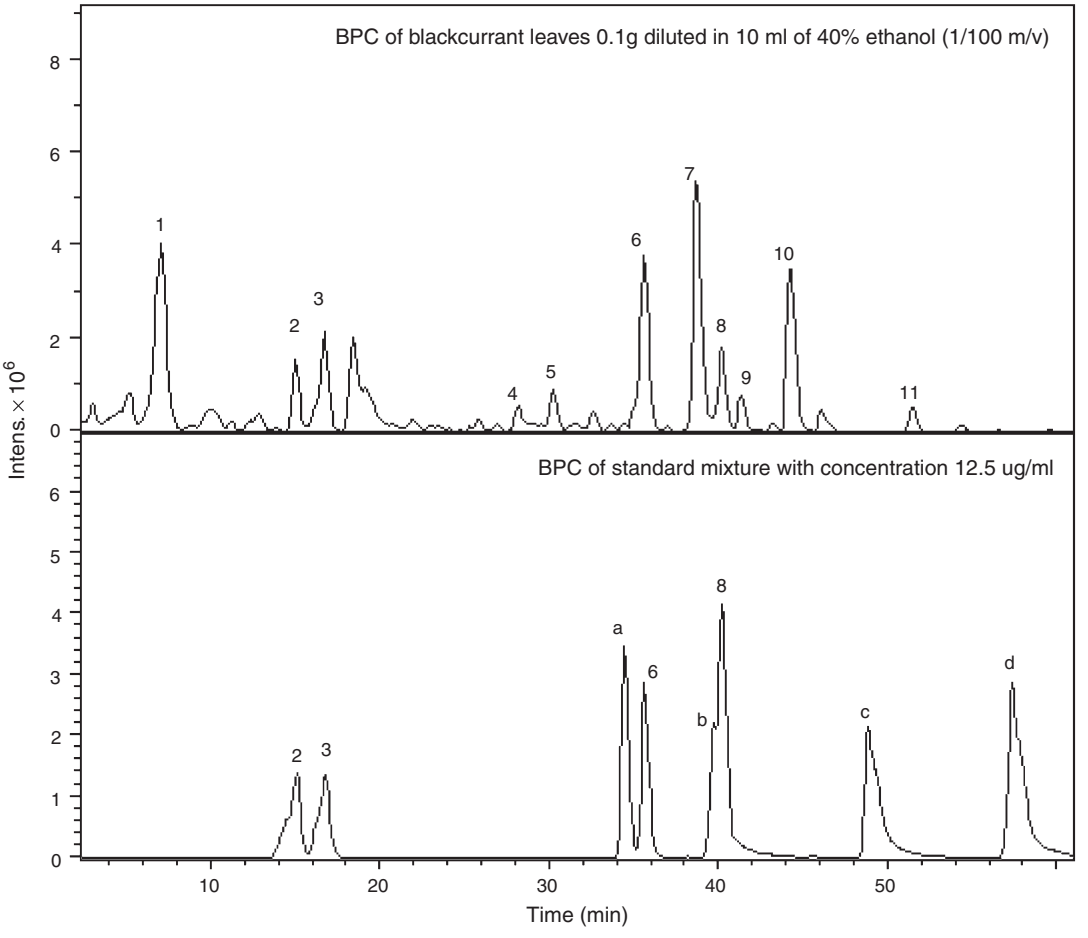

Fig. 2. The base peak chromatogram (BPC) of analysis of the leaves and standard mixture of the known leaf specific polyphenols. The polyphenols found in black currant leaves are marked with numbers repeating the peak numbers on leaf BPC and the standard compounds not found in black currant leaves are marked with letters. 1. gallocatechin; 2. epigallocatechin; 3. chlorogenic acid; 4. unidentified compound with $[M\text{-}H]^- = 383$; 5. myricetin glucoside; 6. quercetin glucoside; 7. quercetin malonylhexoside; 8. mixture of kaempferol glucoside, quercetin rutinoside and quercetin acetylglucoside; 9. mixture of isorhamnetin rutinoside and an unidentified glucoside with $[M\text{-}H]^- = 373$; 10. kaempferol malonylhexoside; 11. isorhamnetin glucoside.

c-quercetin, d-kaempherol) previously known as present in the black currant leaves [17, 18] or in the other leaves of pharmacological value [6]. Some of the standard compounds were present in the black currant leaves as well (peaks 2, 3, 6 and 8). The compounds that were not overlapping with any of the used standards were identified using their molecular weight and MS^2 collision fragments.

The quantitative analysis was performed using the peak area under MS extracted ion chromatograms. The analysis revealed that the concentration of catechin in the dried leaves was 786 mg/100 g; chlorogenic acid 1493 mg/100 g; quercetin glucoside 1947 mg/100 g and quercetin rutinoside 399 mg/100 g. The content of catechin was lower than found in the green tea by Rusak et al. (3330 mg/100 g), but the content of quercetin was significantly higher than found in green tea by Rusak et al. (28 mg/100 g) [20]. The health beneficial effect of black currant leaves compared to the green tea needs to be investigated further.

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