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[1]Kaunas Botanical Garden, Vytautas Magnus University, Kaunas, Lithuania
[2]Food Institute of Kaunas University of Technology, Kaunas, Lithuania
[3]Botanic Garden, Faculty of Biology, University of Warsaw, Warsaw, Poland

Investigations of anthocyanins, organic acids, and sugars show great variability in nutritional and medicinal value of European cranberry (*Vaccinium oxycoccos*) fruit

Laima Česonienė[1*], Remigijus Daubaras[1], Ina Jasutienė[2], Inga Miliauskienė[2], Marcin Zych[3]

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Summary

The rising interest in nutraceutical-rich foods in relation to human health has resulted the increased use of cranberries in the modern diet. Berries of wild European cranberry clones and cultivars show great variation in yield, colour and quantities of biologically active compounds. In the present study, we estimated the production of different phytochemical components in 40 genotypes of *Vaccinium oxycoccos*. Our main goal was to identify genotypes that have superior biochemical properties, such as high levels of anthocyanins, organic acids and easily digestible sugars. As a result, wild clones of Lithuanian origin and certified cultivars were selected as the most valuable in terms of total amount of anthocyanins, organic acid, fructose and glucose concentrations.

Introduction

Native Europeans, especially those from Central and Northeast Europe, use the fruit of the European cranberry (*Vaccinium oxycoccos* L.) for food and medicine (Kardell, 1986). Cranberry products, and especially cranberry juice, have long been consumed for health reasons, primarily because of their therapeutic effect on urinary tract infections (Terris et al., 2001; Viškelis et al., 2009). However, other nutritional and therapeutic properties of cranberries are widely acknowledged in various studies (see, e.g., Borowska et al., 2009; Côté et al., 2010; Česonienė et al., 2011), and cranberry fruit is reported to possess a wide range of biological properties including anti-carcinogenic effects, and is useful in treating cardiovascular disorders. In general, the beneficial effect of cranberries, including *V. oxycoccos* and *V. macrocarpon* Aiton, on human health has been attributed in particular to phenolic compounds (Borowska et al., 2009; Viškelis et al., 2009; Côté et al., 2010) and organic acids (Povilaitytė et al., 1998; Zuo et al., 2002). Furthermore, the red colour of cranberry fruit is due to the presence of anthocyanins; compounds which have important therapeutic effects, including anti-tumor, anti-ulcer, antioxidant, and anti-inflammatory properties (Wang and Wang, 2009). The renewed interest in anthocyanins is not only due to their health-promoting activities, but also their use as natural colourants by the food industry (Lima et al., 2009). According to Bridle and Timberlake (1997), the specific anthocyanin content of foods and beverages is an important indicator in estimating their nutritional value. Similarly, the relative amounts of organic acids present can be used to distinguish between fresh and processed berries. For example, organic acids known to impart specific flavour are often used to control pH and can be used as an indicator of product quality. The use of natural organic acids is considered a good alternative to artificial compounds by the fruit-processing industry because of their preserving, antioxidant, flavouring and acidifying properties, as well as their low cost (Raybaudi-Massilia et al., 2009). For instance, investigations of antimicrobial activities revealed that citric acid strongly inhibited the growth of *Shigella dysenteriae*, resulting in a 5-log reduction (In et al., 2013), and a transmission electron microscopy study showed that malic acid caused cytoplasmic disruption in bacterial pathogens, seemingly without damaging the cell membrane (Raybaudi-Massilia et al., 2009). According to other studies, carboxylic acids, such as malic, citric and succinic acid etc., behave as antioxidants because they also have the ability to chelate metals. Therefore they are classified as preventive or synergistic compounds (Pero et al., 2008). The soluble carbohydrates present in the fruit, namely glucose and fructose, contribute to the sweetness of cranberries. These sugars are important indicators of metabolic processes during fruit development.

Vaccinium oxycoccos clones and cultivars express great morphological diversity (Gugnacka-Fiedor, 1986; Suda and Lysak, 2001; Česonienė et al., 2013), which seems to translate into differences in their production of medicinally useful phytochemicals (Česonienė et al., 2011). Promising results have been achieved by breeding of *V. oxycoccos* in Russia when new productive cultivars 'Dar Kostromy', 'Krasa Severa' and 'Sazonovskaja' cultivars were originated (Makeev et al., 2000). Our previous investigations corroborated big differences among *V. oxycoccos* clones according to berry size and productivity. Consequently, the most perspective clones were selected with an average berry weight from 1.1 g to 1.3 g, which could be equal to the average weight of *V. macocarpon* cultivars (Daubaras et al., 2004). Our aim was to assess this variation by quantifying the amounts of nutritionally and medicinally important compounds, namely fructose, glucose, anthocyanins, quinic, malic and citric acids, in 40 different genotypes of *V. oxycoccos*, and, thus, determine the most promising genotype in terms of potential nutritional value.

Material and methods

The species

European cranberry *Vaccinium oxycoccos* L. (syn. *Oxycoccus quadripetalus* Gilib., *Oxycoccus palustris* Pers.; Ericaceae Juss.), is a dwarf, woody, evergreen clonal shrub with slender, rooting stems, occasionally up to 0.8-1.0 m length, with short, usually erect, flowering shoots (Jacquemart, 1997). Its fruit is an over-wintering, edible berry (the cranberry), which is harvested from the wild or from cultivated varieties.

Plant material

For the present study, we selected 40 genotypes of *V. oxycoccos*: 13 certified cultivars and 27 clones collected from the wild. The cultivars 'Kuresoo', 'Soontagana', 'Virussaare', 'Nigula' and 'Maima' were of Estonian origin, 'Sazonovskaja', 'Krasa Severa' and 'Dar Kostromy' were selected from Russian stock, and the remaining 'Reda', 'Amalva', 'Zuvinta', 'Vaiva', 'Vita' and twenty seven wild clones were of Lithuanian origin. All genotypes, both wild clones and cultivars, have been cultivated in the collections of Kaunas

* Corresponding author

Botanical Garden of Vytautas Magnus University under uniform ecological conditions in acid peat beds, pH 3.5-5.0. This collection is located in the central part of Lithuania with typical growing season lasting 180-190 days, an average temperature -6 °C in January and +16 °C in July. An average annual rainfall is 600 mm. Studied cultivars were propagated using cuttings that were planted in acid peat (pH 4.0-5.0) beds.

These genotypes were selected as the most promising for further breeding purposes on the basis of a long-term monitoring programme that included phenological observations, resistance studies to fungal diseases and pests, as well as productivity studies (Daubaras et al., 2004; Česonienė et al., 2013). Berry samples for analysis were collected at the full maturity stage (brown seeds and typical berry coloration). Approximately 250-300 g berry samples were collected from a single genotype. Part of the sample was frozen and later used for benzoic acid analysis. Rest of berries were pressed in a conventional juicer and the compressed cakes and juices obtained were stored in a freezer (at -28 °C) prior to extraction.

Biochemical analyses

For analyses two biological replicates, i.e. two year yields, were used. Total anthocyanins (TAC), malic, quinic, citric acids and sugars were investigated in berry juice, meanwhile qualitative and quantitative analysis of anthocyanins was accomplished in berry cake.

The total anthocyanin content in cranberry was determined by a pH differential method. For TAC measurements two dilutions of the sample were prepared namely one with potassium chloride buffer (pH 1) and the other one with sodium acetate buffer (pH 4.5), diluting by the 10 dilution factor (1 ml of juice + 9 ml of buffer). Absorbance was measured using a Genesy 5 spectrophotometer (USA) at a wavelength of 510 nm and 700 nm in buffers of pH 1.0 and 4.5. The TAC was calculated as follows:

$$\frac{(A_{510pH1} - A_{700pH1}) - (A_{510pH4.5} - A_{700pH4.5})}{\varepsilon \cdot L} \times MW \times DF \times 10^3$$

MW – molecular weight, for cyanidin-3-galactoside 445.2 g/mol
DF – dilution factor
ε – a molar extinction coefficient, for cyanidin-3-galactoside 41700
L – pathlength in cm
A – absorbance

Results were expressed in terms of mg/kg of cyanidin-3-galactoside (a molar extinction coefficient 41700) (Wrolstad, 1976).

For qualitative analysis of anthocyanins, berry cakes were homogenized and 3 g of homogenate were extracted over a period of 1 h at room temperature with 10 ml of acidified ethanol (95% [v/v] food grade ethanol containing 0.1 M HCl). The extracts were analyzed by HPLC using a reversed phase C_{18} LiChrospher®100 RP 18e column (125 × 4 mm, 5mm) (Merck, Darmstadt, Germany). The eluents were (A) 4% aqueous H_3PO_4, and (B) 100% HPLC-grade acetonitrile (Merck) (Viškelis et al., 2009). Chromatographic conditions were as follows: 10% B in A at the time of injection, 14% B in A (4 min), 16% B in A (10 min), 30% B in A (25 min), initial conditions (26 min). Flow rate was 0.8 ml/min, 20 µL was injected. The samples were filtered through a 0.45-µm cellulose syringe filter prior to analysis. Detection was performed using a diode-array detection system Agilent 1200 (Agilent Technologies, USA) at 520 nm. ChemStation software was used.

Determination of benzoic acid was performed according to the ISO 22855 "Fruit and vegetable products – Determination of benzoic acid and sorbic acid concentrations – High-performance liquid chromatography method". Berries were homogenized and 5 g of homogenate were extracted for 30 min (periodically stirring) in a water bath at 70 °C with 75 ml of extraction solution. The extract was subsequently clarified with Carrez I and Carrez II solutions and made up to 100 ml with extraction solution. The samples were filtered through a paper filter and a 0.45-µm cellulose syringe filter before analysis. Chromatographic conditions were as follows: the eluent was a mixture of 50 parts by volume of ammonium acetate and 40 parts by volume methanol (pH adjusted to 4.5-4.6), flow rate was 0.8 ml/min, 20 µl was injected. The reversed phase ZORBAX Eclipse XDB-C18 (5mm) 150 × 4.6 mm (Agilent Technologies, USA) column was used. Detection was performed using a diode-array detection system Agilent 1200 (Agilent Technologies, USA) at 235 nm. ChemStation software was used. Benzoic acid content mg/kg was calculated as follows:

$$C = (V \times c_{st} \times 100)/(V_{st} \times m),$$

V – peak area of the sample
V_{st} – peak area of the standard solution
c_{st} – concentration of standard solution, 50 mg/l
m – amount of berry sample used for the extraction, g

For determination of malic, citric and quinic acids, 1 ml of juices was diluted with ~50 ml distilled/deionized water, clarified the solution with Carrez I and Carrez II solutions and made the solution up to 50 ml with distilled/deionized water. After 5 min, the samples were filtered through a paper filter and a 0.22 µm nylon syringe filter before analysis. A standard solution of organic acid mixture was prepared by dissolving 2 g of malic (Sigma-Aldrich, Germany), citric (Sigma-Aldrich, Germany) and quinic (Sigma-Aldrich, Germany) acids in 100 ml of water. Standard solutions of 0.1%, 0.5%, and 1% (w/v) organic acid mixture were prepared by appropriate dilution of the original standard solution. Chromatographic conditions were as follows: the eluent was 20 mM Na_2HPO_4 buffer solution (pH adjusted to 2.8 with acetic acid), flow rate was 1.0 ml/min, 20 µl was injected. The reversed phase Hydrosphere C18 (5 µm, 12 nm), 150 × 4.6 I.D. (YMC Co., Ltd., Japan) column was used. Column temperature was set at 30 °C. Detection was performed using a diode-aray detection system Shimadzu Prominence LC20AD (Shimadzu Corp., Japan) at 220 nm and Lab Solutions software. Quinic, citric, malic acid content were calculated from the calibration curves of each organic acid standard solutions (concentration 0.01, 0.05, 0.1 and 0.2 g/l) multiplied by the dilution factor (50).

For determination of sugars, 10 ml of juices were diluted with ~80 ml of water, clarified with Carrez I and Carrez II solutions, and made up to 100 ml with distilled/deionized water. After 5 min, the samples were filtered through a paper filter and a 0.22 µm nylon syringe filter before analysis. A standard solution of a sugars mixture was prepared by dissolving 0.4 g each of fructose (Sigma-Aldrich, Germany), glucose (Sigma-Aldrich, Germany) and sucrose (Sigma-Aldrich, Germany) in 100 ml of distilled/deionized water. A 2 mg/ml and a 0.4 mg/ml standard solution of carbohydrate mixture was prepared following dilution with distilled/deionized water. Chromatographic conditions were as follows: the eluent was a mixture of 75 parts by volume of acetonitrile and 25 parts by volume water, flow rate was 1.2 ml/min, 20 µl was injected. The YMC-Pack Polyamine II 250 × 4.6 mm, 5 µm (YMC Co., Ltd., Japan) column was used. Column temperature was set at 28 °C. Detection was performed using an Evaporative Light Scattering Detector ELSD-LTII (Shimadzu Corp., Japan). Sugar content g/kg was calculated as follows:

$$C = (V \times c_{st} \times 1000)/(V_{st} \times m),$$

V – peak area of the sample
V_{st} – peak area of the standard solution
c_{st} – concentration of standard solution (4 mg/ml)
m – amount of juice sample used for the analysis in g

Statistical analysis
Investigations were carried out in three replications for each genotype. Averages of the data and standard deviations were calculated using STATISTICA, version 7.

Results and discussion

Anthocyanin content
Our fruit samples clearly differed in their total anthocyanin content (TAC). Although the mean TAC was 59.4 mg/kg, the TAC values for juices of *V. oxycoccos,* expressed as cyanidin-3-galactoside, ranged from 12.2 mg/kg (‘Virussaare‘) to 227.8 mg/kg (98-C-17).
Most investigated genotypes were characteristic of moderate TAC values. However the wild clones 96-Z-02, 98-C-17, 96-K-19, and 99-Z-06, were distinguished for exceptionally high TAC values 100.2, 227.8, 126.2 and 103.0 mg/kg, respectively (Tab. 1). These even exceeded the range reported in earlier studies from 43.3 to 96.8 mg/kg (Povilaitytė et al., 1998; Borowska et al., 2008). Similar variation in anthocyanin content was also reported for fruit of different cultivars and clones of the large cranberry *V. macrocarpon* (Vvedenskaya and Vorsa, 2004; Viškelis et al., 2009). Accumulation of anthocyanins is influenced by numerous factors such as berry ripening, air temperature etc., and may also be genetically determined. Interestingly, in our study, berries of clones with highest TAC values had coloured pulp, although, in general, TAC is mainly concentrated in the vacuoles of fruit epidermal cells, whereas the pulp is practically devoid of anthocyanin.

Chromatographic analysis revealed the presence of six anthocyanins (Fig. 1). Overall, the average anthocyanin profile was as follows: cyanidin-3-galactoside (19.3±0.53%), cyanidin-3-glucoside (2.8±0.22%), cyanidin-3-arabinoside (20.2±0.17%), peonidin-3-galactoside (29.6±0.48%), peonidin-3-glucoside (8.1±0.31%), and peonidin-3-arabinoside (19.8±0.23%). Proportions of different compounds varied between the genotypes studied. For example, berries of 96-Z-13, 97-J-07, 99-Z-01, 99-Z-02, 99-Z-05, 99-Z-15 and ‘Soontagana’ did not contain cyanidin-3-glucoside, whereas 99-Z-08 and 98-C-15A contained 10% and 9% of cyanidin-3-glucoside, respectively. A similar anthocyanin profile was reported for fruit of the related species *V. macrocarpon* (Viškelis et al., 2009). Its cultivars, like *V. oxycoccos*, differ in terms of the anthocyanin mass fraction (Vvedenskaya and Vorsa, 2004; Borowska et al., 2009). The anthocyanin profile of the fruit provides important commercial information, since the proportion of individual anthocyanins may affect the colour stability of cranberry products, such as cranberry juice and cranberry sauce (Starr and Francis, 1968).

The mean benzoic acid content for our study group was 52.4 mg/kg (Tab. 1). Particularly high concentrations of benzoic acid were found in berry extracts of clones 98-C-15, 98-C-17, and ‘Maima’ (147.0±6 mg/kg, 115.2±11 mg/kg, 129.4±2 mg/kg). Our results fell well within the ranges previously reported by Park et al. (2008), i.e. 10.1-240 mg/kg.

The investigated genotypes varied with regard to organic acid content (Tab. 1). Quinic acid concentration ranged from 3.81 to 13.3 g/kg (on the average 7.65 g/kg), malic acid from 14.1 to 43.3 g/kg (on the average 26.5 g/kg), and citric acid from 10.8 to 54.3 g/kg (on the average 33.2 g/kg). The highest concentrations of quinic acid were found in berries of clone 96-Z-03 (13.3 g/kg), malic acid in clone 96-K-04 (43.3 g/kg) and citric acid in clone 96-K-19 (54.3 g/kg). Like *V. oxycoccos*, citric, malic, and quinic acids are the main organic acids present in the berries of *V. macrocarpon* (Miller et al., 2009). However, Viljakainen et al. (2002) reported that total acid content in *V. oxycoccos* juice is the highest, compared with the juices of wild berries, such as those of bilberry, lingonberry, cloudberry, red raspberry, and black crowberry.

In our study cranberry, fruit accumulated, on average, 42.1 g/kg fructose and 45.1 g/kg glucose (Tab. 1). The highest fructose content was detected in the Estonian cultivar ‘Soontagana’ (56.9±1.4 g/kg), followed by the Lithuanian clones 98-C-15 and 95-A-05, which contained 54.9±6.0 g/kg and 53.9±3.0 g/kg, respectively. Berries of the cultivar ‘Maima’ accumulated the largest amount of glucose (62.9±4.0 g/kg). The mean concentration of total sugar was 84.4 g/kg. Our results show that fruit of some clones may be superior in terms of sugar concentration to the berries of cultivars of *V. macrocarpon*, which, according to some authors, contain 45.7-80.4 g/kg of total sugars (Wang and Wang, 2009; Povilaitytė et al., 1998). However Viljokainen et al. (2002) found concentrations of fructose and glucose, respectively 20.69 g/l and 27.75 g/l. Such variation may be caused by geographical and climatic conditions.
The glucose and fructose contribute sweetness to cranberries. These sugars are known to be indicators of the metabolic processes that occur during ripening. For breeding purposes, it is important to select cranberry clones that accumulate significant amounts of sugars that can be rapidly absorbed by the human digestive system. Furthermore, breeders seek to develop cultivars with more natural sweetness because this reduces the amount of sugar that needs to be added during processing and may thus increase the market demand for fresh fruit (Trehane, 2004).

Conclusions

Our present study revealed great variation in TAC and organic acids and sugar content in fruit of both cultivars and wild clones of *V. oxycoccos*. The wild clones, 98-C-17, 96-K-19, 96-Z-02, 99-Z-06 were the most promising genotypes with exceptionally high amounts of TAC. Because of prevailing termostable galactoside (48.9%) and glucoside (10.9%) conjugates berries of *V. oxycoccos* are perspective for processing by high temperature. Of the genotypes studied so far, ‘Soontagana’, ‘Sazonovskaja’, ‘Vaiva’ and 99-Z-05 could be used to reduce supplement sugar intake by preparation of food products with cranberries. We suggest, therefore, that these genotypes should be investigated further as a source of valuable characters for future breeding programmes.

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Tab. 1: Total anthocyanin (TAC), benzoic acid, quinic acid, malic acid, citric acid, fructose and glucose content in berry juice of 40 genotypes of *Vaccinium oxycoccos*

Cultivar / Genotype	TAC [mg/kg]	Benzoic acid [mg/kg]	Quinic acid [g/kg]	Malic acid [g/kg]	Citric acid [g/kg]	Fructose [g/kg]	Glucose [g/kg]
'Amalva'	53.2	39.2	8.87	18.9	23.3	41.0	42.3
'Dar Kostromy'	47.2	67.3	11.43	36.8	43.9	25.0	29.8
'Krasa Severa'	24.2	12.2	7.62	29.1	50.3	40.7	47.2
'Kuressoo'	40.5	64.7	6.21	25.5	37.8	48.2	57.6
'Maima'	19.3	129.0	7.10	28.7	54.3	47.4	62.9
'Nigula'	12.9	57.7	5.42	27.9	37.1	36.2	52.4
'Reda'	35.2	27.0	10.00	18.5	19.1	28.9	32.4
'Sazonovskaja'	99.0	65.1	6.93	32.4	49.3	52.5	59.3
'Soontagana'	34.2	35.2	6.00	30.5	32.5	56.9	57.6
'Vaiva'	48.1	16.4	7.32	18.4	13.2	53.9	59.7
'Virussaare'	12.2	41.6	5.92	31.7	54.3	49.5	57.8
'Vita'	28.2	16.6	9.45	18.5	26.1	36.7	33.5
'Zuvinta'	32.5	39.1	11.83	25.2	43.6	33.9	52.4
95-A-03	53.5	39.5	5.96	21.0	10.8	36.4	36.1
95-A-04	43.3	64.0	6.12	22.2	12.1	35.9	37.8
95-A-07	66.5	37.2	6.01	18.0	16.9	35.2	35.1
96-K-04	98.1	64.6	10.50	43.3	41.4	50.1	49.5
96-K-19	126.2	69.3	7.36	20.6	54.3	39.5	51.3
96-Z-02	100.2	45.2	6.73	14.9	25.4	28.5	29.4
96-Z-03	77.1	28.4	13.30	16.9	27.1	43.6	39.5
96-Z-13	55.0	78.2	9.86	22.6	40.6	40.6	37.5
97-J-04	76.1	61.7	6.93	35.7	28.4	35.5	34.4
97-J-07	44.1	39.8	9.51	43.2	38.8	54.9	43.2
98-C-15	56.3	147.0	7.61	25.3	33.6	49.5	48.7
98-C-15 A	52.7	66.3	5.52	28.7	30.0	38.3	30.0
98-C-17	228.0	115.0	8.52	32.9	36.6	49.5	48.7
99-Z-01	41.8	67.4	6.67	25.8	25.9	46.1	52.5
99-Z-02	36.9	17.2	6.85	24.8	33.6	41.6	42.0
99-Z-04	66.4	30.5	8.71	28.0	35.3	42.5	53.0
99-Z-05	73.1	74.7	9.03	37.7	36.1	52.4	54.2
99-Z-06	103.0	14.3	6.22	22.8	42.2	36.3	47.5
99-Z-08	52.1	65.3	8.72	32.8	21.0	36.4	42.6
99-Z-11	31.4	83.6	8.40	32.7	33.7	48.3	47.0
99-Z-13	62.9	17.1	7.01	25.3	27.6	29	26.6
99-Z-14	78.1	27.4	6.53	17.9	27.4	43.2	47.1
99-Z-15	29.6	7.1	6.25	31.6	37.5	44.3	42.9
99-Z-16	74.7	36.7	6.23	27.8	30.5	39.3	44.4
99-Z-17	51.1	97.0	3.81	14.1	16.4	47.6	54.2
99-Z-18	64.3	37.8	8.25	24.2	42.3	43.4	38.8
Nr. 184	48.2	52.9	5.23	26.0	37.6	39.8	43.9
M	59.4	52.4	7.65	26.5	33.2	42.1	45.1
SD	37.62	31.62	1.99	7.21	11.4	7.76	9.55
$M_{min} – M_{max}$	12.2-227.8	7.1-147.0	3.81-13.3	14.1-43.3	10.8-54.3	25.0-56.9	26.6-62.9

M – mean value; SD – standard deviation; M_{min}-M_{max} – range of mean values

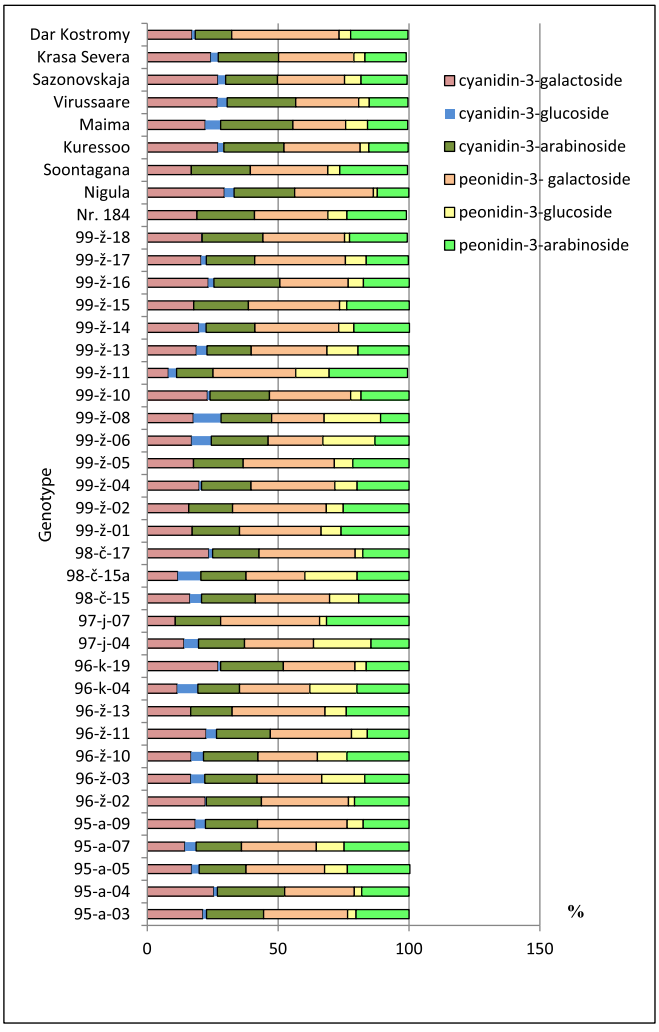

Fig. 1: Quantitative composition of anthocyanins in *V. oxycoccos* genotypes, %

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Address of the authors:
Laima Česonienė, PhD; Remigijus Daubaras, PhD, Kaunas Botanical Garden, Vytautas Magnus University, Žilibero 6, Kaunas, LT-46324, Lithuania
E-mail: laimac@hotmail.com
E-mail: remigijusd@hotmail.com
Ina Jasutienė, PhD; Inga Miliauskienė, Food Institute of Kaunas University of Technology, Taikos pr. 92, LT-51180, Kaunas, Lithuania
E-mail: ina.jasutiene@lmai.lt
E-mail: inga.miliauskiene@lmai.lt
Marcin Zych, PhD, Botanic Garden, Faculty of Biology, University of Warsaw, Aleje Ujazdowskie 4, 00-478 Warsaw, Poland
E-mail: mzych@biol.uw.edu.pl