HPLC Determination of the Composition and Stability of Blackcurrant Anthocyanins

Marina Rubinskiene[1], Ina Jasutiene[2], Petras Rimantas Venskutonis[3,*], and **Pranas Viskelis[1]**

[1]Laboratory of Biochemistry and Technology, Lithuanian Institute of Horticulture, Kauno g. 30, LT-54333 Babtai; [2]Food Institute of Kaunas University of Technology, Taikos pr. 92, LT-51001 Kaunas; and [3]Department of Food Technology, Kaunas University of Technology, Radvilenu pl. 19, LT-50015 Kaunas, Lithuania

Abstract

High-performance liquid chromatography with UV and mass spectrometry detectors are used to monitor the composition and stability of anthocyanins in blackcurrants harvested from different *Ribes nigrum* breeds at various ripeness phases. The highest amounts of pigments are found in overripe berries. The concentration of anthocyanins is higher in the berries of late blackcurrant breeds (Vakariai and Ben Alder). Delphinidin-3-rutinoside is the dominant component in the reddish color berries (onset of ripening), and cyanidin-3-rutinoside is a major pigment in the black ones (ripe berries). Studies of the effect of temperature and light on the stability of the main pigments in blackcurrants show that aqueous solution prepared from a dry colorant is more stable when compared with the liquid water and ethanol extracts of coloring substances. Cyanidin-3-rutinoside is found to be the most thermally stable anthocyanin.

Introduction

There is a worldwide interest in the development of natural food colorants as alternatives to synthetic ones (1–3) as a result of legislative actions and consumer concerns over the use of synthetic additives in the food. In general, consumers consider natural food ingredients to be more healthy compared with synthetic additives (4).

The interest in anthocyanins considerably increased when synthetic food colorants, particularly red ones, began to be questioned as additives for possessing adverse health effects. Antioxidant, radical scavenging, and antiviral activity of anthocyanins have also fostered their studies in different sources (5–9). However, the use of some natural colorants is limited because of their low stability.

The increasing importance of anthocyanins as well as their instability during processing has raised specific tasks for the methods of their determination. The development of combined extraction methods, chromatographic and spectrometric methods, had the biggest impact on the analysis of anthocyanins in different products. Thus, the use of size exclusion chromatography, high-performance liquid chromatography (HPLC), UV–vis spectroscopy, and electrospray mass spectrometry (MS) resulted in the characterization of 15 anthocyanin structures (10). A combination of column chromatography (CC) and reversed-phase (RP) HPLC enabled the preparative separation of anthocyanins without any reapplication of overlapping bands of the major and minor anthocyanins in a blackcurrant sample (11). Fractionation of anthocyanins by CC, thin-layer chromatography, and HPLC enabled the separation of fractions with potent antiviral activity against fluenza viruses A and B (7). Sequential extraction with ethyl acetate and methanol and an optimized RP-HPLC–diode array detection was also successfully applied for the analysis of anthocyanins (12). Other methods for the separation of blackcurrant anthocyanins, such as capillary zone electrophoresis (13) and gradient elution centrifugal partition chromatography (14), were also reported. Most recently, a rapid and sensitive HPLC method was validated, and quantitation of anthocyanins in 13 commercially available blackcurrant beverages was demonstrated (6). The method could be very useful when a rapid screening of anthocyanins in a great number of samples is required.

The objectives of this study were to characterize the seasonal variations in the composition of anthocyanin pigments in different blackcurrant cultivars using HPLC–UV–MS techniques and to assess the effect of temperature and light on the stability of the main pigments in the liquid colorant extracts and solution of spray-dried colorant.

Experimental

Preparation of blackcurrant pigments

Ribes nigrum L. fruits were harvested in the experimental fields of Lithuanian Institute of Horticulture (Babtai, Lithuania). The content of anthocyanins was determined in the berries collected at different harvest times (earliness) and ripeness phases in the

* Author to whom correspondence should be addressed: email rimas.venskutonis@ktu.lt.

following breeds of *R. nigrum*: (early breeds) Joniniai, Minaj Shmyriow, and Almiai; (late breed) Ben Alder; and (very late breed) Vakariai. The ripeness was assessed using the following color indicators: reddish (onset of ripening), dark brown (50% ripe), black (commercially ripe), intensive black (overripe). Freshly harvested berries were used for the preparation of coloring substances.

Fruit juices were pressed out in a conventional juicer and the cake was stored in a freezer until extraction. The coloring substances were isolated from the blackcurrant cakes with hot water acidified with 0.2% citric acid (grade reagent 99.8%, Lach-Ner, Neratovice, Czech Republic) for 3 h at 60°C. The extracts were spray-dried in a Büchi 190 Mini Spray Drier (Buchi, Flawil, Switzerland) using potato starch derivative Paselli MD (Avebe, Vendam, the Netherlands) as a carrier. The inlet temperature was 180–190°C, the outlet temperature was 80–90°C. Simultaneously, the cake was extracted with food-grade ethanol (AB Stumbras, Kaunas, Lithuania) and acidified with 0.1N HCl (grade reagent for analysis, 36.46M g/mol). The aqueous solutions of spray-dried colorant (0.1%, w/v), freshly prepared water, and ethanol extracts of coloring substances from blackcurrant cake were used for anthocyanin stability studies.

Total anthocyanin content

The pigments were extracted from 5 g of fresh berries with 95% (v/v) food-grade ethanol acidified with 0.1N HCl. The berries were ground with quartz sand and the extraction was continued with 20 mL portions of solvent until the sample became colorless. The extract was diluted with acidified ethanol at the ratio 1:9 (v/v); the absorption was measured on a spectrophotometer Genesys-5 (Thermo Spectronic, Rochester, NY) at 544 nm. The concentration of anthocyanins was determined from the calibration curve, which was constructed by measuring the absorption of Cyanidin-3-rutinosides (cy-3-rut) (M_w 595.2, ε = 28.800) reference solutions. Purified cy-3-rut was donated by the Danish Institute of Agricultural Sciences (Department of Fruit, Vegetable and Food Science, Tjele, Denmark). The concentration of anthocyanins was calculated using the following formula and expressed in mg of cy-3-rut in 100 g of berries:

$$C = \frac{c \times V \times k}{m \times 10} \qquad \text{Eq. 1}$$

where C is the concentration of anthocyanins in mg/L obtained from the calibration curve; *V* is the volume of the extract (mL); k is the dilution factor; and *m* is the amount of berries used for the extraction (g). Data were statistically assessed by one-way analysis of variance (vers. 3.43, 2002). Significance of difference was estimated at 5% level.

Determination of stability

Degradation reactions were carried out in a 1-mL quartz spectrophotometer cell. The samples were illuminated with a UV lamp (40 W) at a temperature of 40°C, which was placed at a distance of 15 cm from the sample. The absorption was measured every hour. To determine the effect of temperature on the stability of coloring substances, they were placed in closed test tubes and heated at 75°C, 85°C, and 95°C for 150 min in a water thermostat. The absorption was measured every 30 min. The composition of individual anthocyanins was monitored by HPLC with UV detector L-7400 LaChrom (Merck, Darmstadt, Germany) at 520 nm. Three replicate analyses were performed for each sample.

HPLC–UV–MS analysis of anthocyanins

Anthocyanin profiles of blackcurrant extracts were characterized by HPLC using a RP-C_{18} Lichrosher 100 RP 18e column (125- × 4-mm, 5 µm) (Merck). The eluents were 4% H_3PO_4 in water (A), and 100% HPLC-grade acetonitrile (ACN) (B) (Merck). Chromatographic conditions were as follows: 3% B in A at the time of injection (20 µL), 25% B in A (45 min), 30% B in A (46 min), initial conditions (47 min). The flow rate was 1 mL/min. Detection was performed with a UV detection system L-7400 LaChrom Merck Hitachi (Merck KGaA) at 520 nm and a Hewlett Packard 1100 series photodiode-array (PDA) detector (Agilent Technologies, Palo Alto, CA).

Mass spectra were registered by a Hewlett Packard 1100 MS (Agilent Technologies), operating in nitrogen flow at atmospheric pressure, applying electrical ionization. The voltage in the capillary was 4500 V; the voltage of fragmentation was 150 V. The flow rate of nitrogen was 13 L/min, and the temperature was 350°C. The scanning range was 100–1000 *m/z* with the interval of 0.1 *m/z*. Eluent A was water with 5% of acetic acid, 5% of ACN, and 0.5% of trifluoroacetyl; eluent B was 100% ACN.

Results and Discussion

Total concentration of anthocyanins

The changes in the total content of anthocyanins were monitored during blackcurrant fruit formation and ripening. The results show (Table I) that anthocyanins are synthesized during the whole period of ripening; however, the most remarkable increase in anthocyanin content was observed in the blackcurrants during the phase when their skins acquired a dark brown color. The concentration of red pigments in dark brown berries was 3.0–6.7 times higher when compared with the reddish ones. The concentration of pigments continuously increased during further steps of monitoring, and, consequently, the highest amounts of anthocyanins were determined in the overripe berries. The content of pigments in the overripe berries of different breeds was (on average) 11.8% higher when compared

Table I. Changes in Anthocyanin Content of Blackcurrants During Ripening (mg/100 g)

	Phase of maturity			
Cultivar	Reddish (onset of ripening)	Dark brown (50% ripe)	Black (commercial maturity)	Overripe fruits
Joniniai	42.6 ± 1.40	124.4 ± 2.65	249.7 ± 1.25	272.2 ± 3.86
Minaj Shmyriow	48.6 ± 1.04	235.3 ± 3.59	302.5 ± 3.09	356.9 ± 2.33
Almiai	35.6 ± 0.56	237.7 ± 2.87	329.4 ± 1.53	385.6 ± 3.52
Ben Alder	75.7 ± 1.66	284.6 ± 3.44	468.5 ± 1.45	494.0 ± 3.71
Vakariai	77.8 ± 0.81	312.9 ± 2.00	491.2 ± 3.49	512.2 ± 4.53
Mean	56.06	238.98	368.26	404.18

with the berries that were already suitable for processing. A dramatic increase in anthocyanin synthesis was also observed during the onset of ripening in a previously reported study (15). Comparing different *R. nigrum* breeds used in this study, it can be observed that higher amounts of pigments were found in the late berry breeds (Vakariai and Ben Alder), but very early breeds (Joniniai) accumulated the lowest amount of anthocyanins. Thus, the concentration of anthocyanins in the overripe berries of early breeds compared with commercially ripe berries increased from 9.0% (Joniniai) to 17.2% (Almiai), but the late breeds increased from 4.2% (Vakariai) to 5.2% (Ben Alder). The results obtained indicate that the concentration of anthocyanins in blackcurrant berries depends both on ripeness and genetic characteristics.

Individual anthocyanin profiles

First of all, ethanol extracts of blackcurrants were monitored using the PDA detector. Three-dimensional chromatograms enable differentiation of the spectra of color and noncolor substances. This can be clearly observed by comparing the chromatograms obtained by the PDA detector operating in the visible and UV wavelength range (Figure 1). Colorful substances, particularly anthocyanins, can be monitored in the range of visible light wavelengths, but the UV detection mode resulted in more than 70 peaks, representing a great number of various organic compounds present in natural extracts, such as phenols, acids, and other components absorbing the light in the UV wavelength range. So far as the composition of pigments was of the main interest in this study, LC–MS analysis was focused on the selected HPLC peaks detected in the range of visible light wavelengths.

Both positive and negative ionization was applied to obtain the mass spectra of the pigments; however, the cation spectrum was more informative because the main substances of interest acquire a positive charge. The intensity of fragmentation was based on the spectra of a reference anthocyanin, cy-3-rut, which was ionized at the intensities of 170, 150, 120, 70, and 30 V. The height of the mass spectral peaks change with the intensity of ionization; however, when the intensity of fragmentation was 150 V, the most representative peaks of cy-3-rut were the largest ones: 595 *m/z* corresponding to the molecular mass of cy-3-rut itself and 287 *m/z* corresponding to the molecular mass of aglycone, a cyanidin (Figure 2).

Two different methods were used to obtain the mass spectra; in the first one, acidified water and ethanol extracts of blackcurrant pigments were passed through the chromatographic column, and the separated components were transferred into the MS detector. However, in the second method, the extracts were directly injected into the MS detector. In general, fewer peaks in the mass spectra of water extract were obtained when compared with ethanol extract. Four main peaks were observed in the mass spectrogram of ethanol extract (Figure 3) corresponding to cy-3-rut (*m/z* 595), cyanidin (*m/z* 287), delphinidin-3-rutinosides (del-3-rut) (*m/z* 611), and delphinidin (*m/z* 303). The smaller peaks represent cyanidin-3-glucosides (cy-3-glc) (*m/z* 449), and delphinidin-3-glucosides (del-3-gcl) (*m/z* 465).

The fraction of ethanol extract mass spectra containing *m/z* ions of the main pigments of blackcurrants is shown in Figure 4. However, the peaks in the chromatogram (Figure 1) indicated that five more hydrophobic substances should be present in the extract; however, these peaks were not identified by MS. For this purpose, chromatographic separation conditions of anthocyanin derivatives should be further optimized or larger amounts of minor blackcurrant pigments should be collected by preparative chromatography (or both). The information on the substitutions

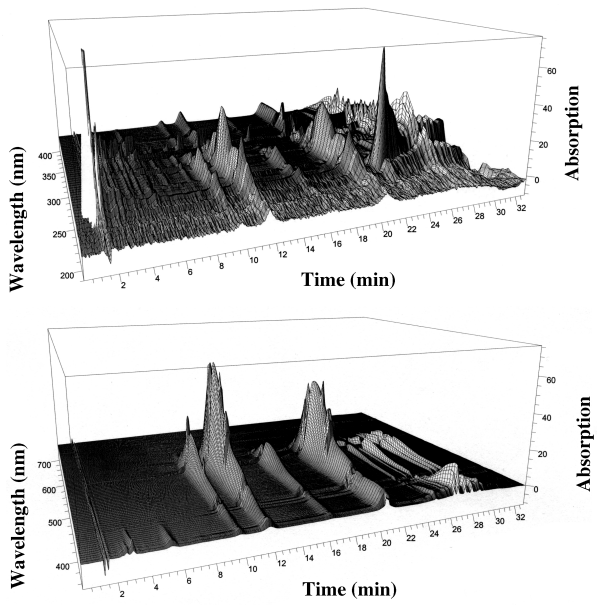

Figure 1. Typical chromatograms of blackcurrant anthocyanins obtained with PDA detector operating in UV (A) and visible (B) wavelength range; delphinidin-3-glucoside (3), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (4), and cyanidin-3-rutinoside (1).

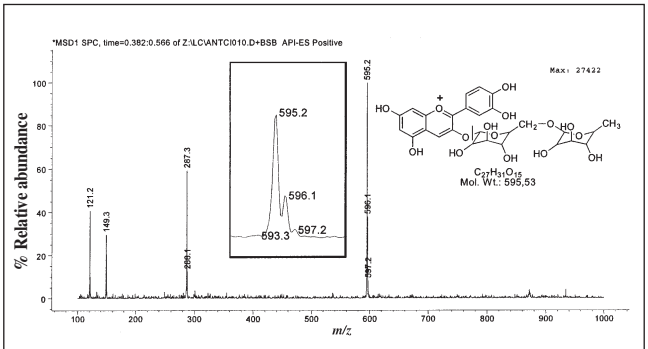

Figure 2. Mass spectra of cyanidin-3-rutinoside reference.

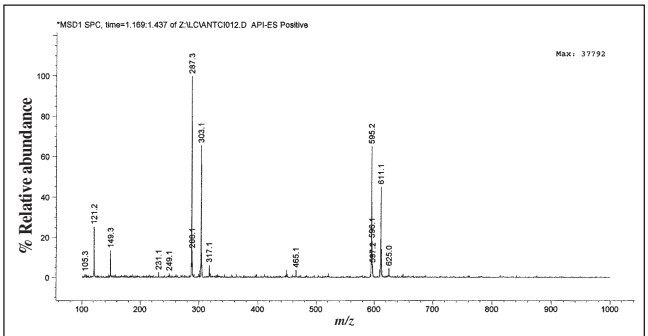

Figure 3. Mass spectra of blackcurrant ethanol extract.

in anthocyanin rings could be obtained by changing the level of fragmentation or using additional spectrometric techniques (or both), such as NMR. However, such work was beyond the scope of the present study.

For the identification of the pigments in blackcurrant extracts, UV spectra of all peaks were recorded during their elution. The main anthocyanins can be analyzed in isocratic regime; however, the gradient is needed for the separation of the rest of the pigments. It imparts serious difficulties in the analysis of UV spectra; when the composition of the mobile phase changes, it is rather difficult to compare the recorded UV absorption maximums with the characteristic maximums of absorption, which were obtained using a specified solvent and were provided in the previously published reports.

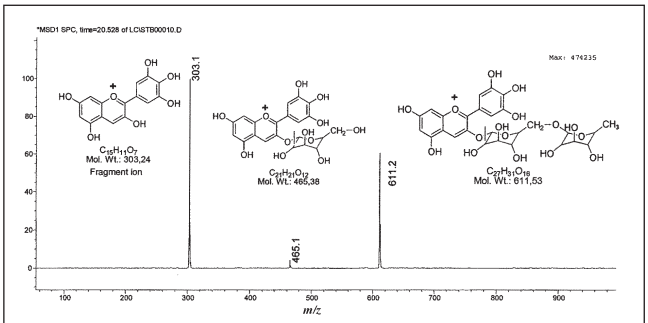

Figure 4. The fraction of mass spectra representing the main pigments in blackcurrant ethanol extracts.

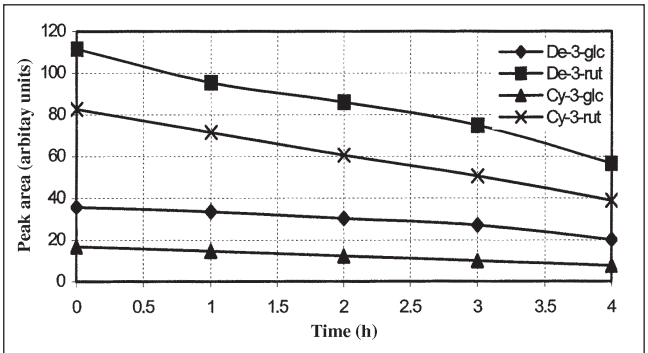

Figure 5. The influence of UV irradiation on the content of main black currant pigments in ethanol extract.

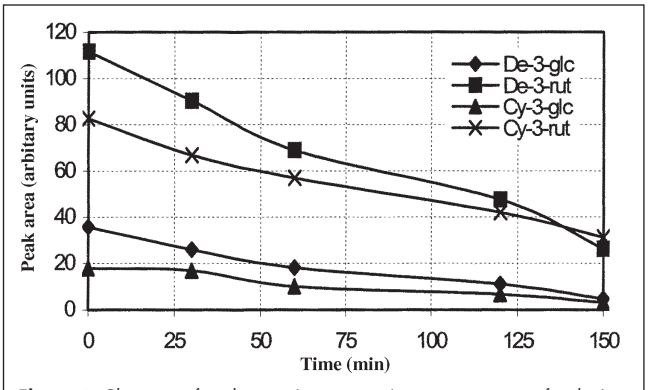

Figure 6. Changes of anthocyanin content in water extract of coloring substances during heating at 95°C.

Quantitative composition of anthocyanins in different *Ribes nigrum* breeds

It can be clearly observed that both the quantitative and qualitative composition of blackcurrant changes during ripening (Table II). Cyanidin and delphinidin rutinosides are dominating components in the fruits at different ripening phases. The concentration of cy-3-rut remarkably increases during ripening; this was the main pigment in the all berries from investigated *Ribes nigrum* breeds. Significantly higher percentages of cy-3-rut were found in Almiai and Joniniai breeds, 53.1% and 48.3%, respectively. The percentage content of anthocyanins in the berries of Lithuanian breeds (Almiai, Vakariai, and Joniniai) are in the following order: cy-3-rut (46.7%), de-3-rut (33.6%), de-3-glc (11.2%), and cy-3-glc (8.4%). The results are slightly different when compared with the percentage composition reported previously in some European breeds (16). For instance, de-3-glc was found to be a dominant glucoside in Lithuanian breeds of *R. nigrum*. The content of the dominant anthocyanin, cy-3-rut was higher by approximately 9% when compared with some European breeds. In addition, it has to be noted that the concentration of anthocyanins depends on the cultivation year; for instance the content of cyanidin and delphinidin rutinosides in Minaj Shmyriow breed fruits, as measured in this study, was on average by 20% higher when compared with earlier reported results (17). The content of anthocyanins in Ben Alder breed berries was in good agreement with earlier reported data (16).

Influence of UV light, temperature, and storage on the stability of anthocyanins

The stability of coloring substances in the water and ethanol extracts as well as in the aqueous solutions of a dry colorant was compared by exposing them to UV light at 40°C. The

Table II. Composition of Anthocyanins in Blackcurrants from Different Cultivars (%)*

Cultivar	Phase of maturity	Anthocyanin			
		cy-3-rut	cy-3-glc	de-3-rut	de-3-glc
Joniniai	r	39.06	3.86	48.31	8.77
	b	48.33	4.54	39.11	8.02
Almiai	r	36.40	6.06	44.31	13.23
	b	53.08	9.30	28.33	9.29
Minaj Shmyriow	r	30.36	4.02	52.36	13.25
	b	43.78	6.45	36.65	13.13
Vakariai	r	33.07	4.34	48.68	13.90
	b	38.76	11.36	33.46	16.42
Ben Alder	r	30.47	8.62	38.31	22.60
	b	36.63	11.9	31.14	20.33
Mean	r	33.87	5.38	46.39	14.35
	b	44.12	8.71	33.74	13.44
$LSD_{05(mean)}$	r	1.81	1.40	2.01	1.59
	b	3.00	1.92	1.97	1.64

* r = reddish berries (onset of ripening) and b = black berries (commercial ripeness).

changes of the main anthocyanins in blackcurrant preparations under the influence of UV light are presented in Figure 5; these changes were almost similar in all of the tested samples. The total content of anthocyanins after 4 h UV irradiation decreased on average by 45–55%. These results were also supported by HPLC data obtained at the beginning and at the end of irradiation by UV.

HPLC data show that cy-3-rut is the most thermally stable anthocyanin in blackcurrants (Figure 6). The content of cy-3-rut after heating at 95°C decreased by 35%, the content of cy-3-glc under the same conditions decreased by 53%, that of de-3-rut by 52%, and the content of de-3-glc by 63%. The same tendency was observed in the case of ethanol and water extracts of coloring substances obtained from fresh-pressed cake. The concentration of cy-3-rut in ethanol extract remained 96.2%, and the concentration of other anthocyanins amounted to 75.4–87.5% of the initial amount. It can be concluded that in terms of a thermal treatment cy-3-rut is the most stable anthocyanin in ethanol ($LSD_{0.5}$-8.33) and water ($LSD_{0.5}$-9.10) extracts.

In order to establish the influence of storage, the samples were stored 12 months at 8°C in hermetically closed glass vials, which were fully filled with ethanol extracts of pigments. The changes in the anthocyanin composition were determined by HPLC. It was found that the main pigments of blackcurrant (rutinosides of cyanidine and delphinidine) were quite stable; during 12 month of storage their content decreased on average by 25.8% and 28.1%, respectively. Glucosides were remarkably less stable; the content of de-3-glc and cy-3-glc decreased during the storage by one half.

Conclusion

The concentration of anthocyanins in *Ribes nigrum* fruits increases during the entire ripening period; the highest amounts of pigments were found in the overripe berries. Ben Alder and Vakariai accumulated higher amounts of anthocyanins of five investigated blackcurrant breeds. HPLC–UV–MS identified cy-3-rut, cy-3-glc, del-3-rut, and del-3-glc as four major blackcurrant pigments. The percentage of the identified anthocyanins depends on the breed and ripening period. It varied in the following range: cy-3-rut (36.6–53.0%), del-3-rut (28.3–39.1%), cy-3-glc (4.5–11.9%), and del-3-glc (8.0–20.3%).

Chromatographic analysis of coloring substance extracts demonstrated that the main four pigments of blackcurrants were equally sensitive to UV. Their concentration decreased on average by 45–55%. The most thermally stable component was cy-3-rut. Rutinosides were more stable than glucosides during 12 months of extract storage at 8°C.

Acknowledgments

The authors thank Dr. V. Luksa (SICOR Biotech UAB, Vilnius) for his assistance in MS and PDA analysis of anthocyanins. The project was supported by Lithuanian Foundation of Science and Studies (C03/2004).

References

1. F.J. Francis. Lesser-known food colorants. *Food Technol.* **41:** 62–68 (1987).
2. G.J. Lauro. A primer on natural colors. *Am. Assoc. Cereal Chemists* **36:** 949–53 (1991).
3. M.M. Giusti, L.E. Rodrigues-Saona, J.R. Baggett, G.L. Reed, R.W. Durst, and R.E. Wrolstad. Anthocyanin pigment composition of red radish cultivars as potential food colorants. *J. Food Sci.* **63:** 219–24 (1998).
4. D. Wiesenborn, J. Golz, J. Hanzel, D. Helgeson, N. Hettiarachehy, E. Holm, and J. Lindley. Red food colorant extract derived from purplehulled sunflower. *N.D. Farm. Res. Agric. Exp. Stn.* **49:** 19–21 (1991).
5. S. Benvenuti, F. Pellati, M. Melegari, and D. Bertelli. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of Rubus, Ribes and Aronia. *J. Food Sci.* **69:** 164–69 (2004).
6. I.L.F. Nielsen, G.R. Haren, E.L. Magnussen, L.O. Dragsted, and S.E. Rasmussen. Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. *J. Agric. Food Chem.* **51:** 5861–66 (2003).
7. Y.M. Knox, K. Hayashi, T. Suzutani, M. Ogasawara, I. Yoshida, R. Shiina, A. Tsukui, N. Terahara, and M. Azuma. Activity of anthocyanins from fruit extract of *Ribes nigrum* L. against influenza A and B viruses. *Acta Virol.* **45:** 209–15 (2001).
8. U. Viberg, G. Ekstrom, K. Fredlund, R.E. Oste, and I. Sjoholm. A study of some important vitamins and antioxidants in a blackcurrant jam with low sugar content and without additives. *J. Food Sci. Nutr.* **48:** 57–66 (1997).
9. L. Costantino, A. Albasini, G. Rastelli, and S. Benvenuti. Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine-oxidase. *Planta Med.* **58:** 342–44 (1992).
10. R. Slimestad and H. Solheim. Anthocyanins from blackcurrants (*Ribes nigrum* L.). *J. Agric. Food Chem.* **50:** 3228–31 (2002).
11. C. Froytlog, R. Slimestad, and O.M. Andersen. Combination of chromatographic techniques for the preparative isolation of anthocyanins applied on blackcurrant (Ribes nigrum) fruits. *J. Chromatogr. A* **825:** 89–95 (1998).
12. K. Maatta, A. Kamal-Eldin, and R. Torronen. Phenolic compounds in berries of black, red, green, and white currants (Ribes sp.). *Antiox. Redox Sign.* **3:** 981–93 (2001).
13. C.T. da Costa, B.C. Nelson, S.A. Margolis, and D. Horton. Separation of blackcurrant anthocyanins by capillary zone electrophoresis. *J. Chromatogr. A* **799:** 321–27 (1998).
14. J.-H. Renault, P. Thepenier, M. Zeches-Hanrot, L. Le Men-Olivier, A. Durand, A. Foucault, and R. Margraff. Preparative separation of anthocyanins by gradient elution centrifugal partition chromatography. *J. Chromatogr. A* **763:** 345–52 (1997).
15. T.B. Toldam-Andersen and P. Hansen. Growth and development in black currant (*Ribes nigrum*). 3. Seasonal changes in sugars, organic acids, chlorophyll and anthocyanins and their possible metabolic background. *J. Hort. Sci.* **72:** 155–69 (1997).
16. J. LeLous, B. Majoie, and J. Moriniere. Etude des flavonoides de *Ribes nigrum*. *Ann. Pharm. Fr.* **33:** 33–39 (1975).
17. I. Jasutiene and P. Viskelis. Pigments of black currant berries and their stability. *Food Chem. Technol.* **32:** 12–15 (1998).

Manuscript received February 3, 2005;
revision received August 2, 2005.