Molar Absorptivities and Reducing Capacity of Pyranoanthocyanins and Other Anthocyanins

Monica Jordheim,[†] Kjersti Aaby,[‡] Torgils Fossen,[†] Grete Skrede,[‡] and Øyvind M. Andersen*,[†]

Department of Chemistry, University of Bergen, Allégt. 41, N-5007, Bergen, Norway, and Matforsk AS, Norwegian Food Research Institute, Osloveien 1, N-1430 Aas, Norway

To improve accuracy in the determination of anthocyanin purity and succeeding antioxidant capacity, ^1H and ^{13}C nuclear magnetic resonance spectroscopy have been combined with high-performance liquid chromatography (HPLC) equipped with a diode array detector and UV–vis spectroscopy in the analysis of anthocyanidin 3-glycosides and 5-carboxypyranoanthocyanidin 3-glycosides. The molar absoptivity (ε) values were found to be relatively similar, in contrast to previously reported literature values, and the average ε values for both anthocyanidin 3-monoglycosides and 5-carboxypyranoanthocyanidin 3-glycosides were proposed to be 22 000 and 23 000 in acidified aqueous and methanolic solutions, respectively. To assess the influence of structure on the potential antioxidant capacity of anthocyanins, the 3-glucosides of pelargonidin (**1**), cyanidin (**2**), peonidin (**3**), delphinidin (**4**), petunidin (**5**), malvidin (**6**), 5-carboxypyranopelargonidin (**8**), 5-carboxypyranocyanidin (**9**), 5-carboxypyrano-delphinidin (**11**), 5-carboxypyranopetunidin (**12**), and 5-carboxypyranomalvidin (**13**) and the 3-galac-tosides of cyanidin (**7**) and 5-carboxypyranocyanidin (**14**) were examined by a ferric ion reducing antioxidant power (FRAP) assay. The reducing capacities of the individual anthocyanins were in the range of 0.9−5.2 μmol of Trolox equivalents/μmol. The two 5-carboxypyranoanthocyanins **11** and **9** and the four common anthocyanins **2**, **4**, **7**, and **14**, all possessing pyrogallol or catechol type of B rings, showed the highest antioxidant capacity measured by FRAP. However, the inclusion of the 5-hydroxyl in the D ring and just one oxygen substituent on the B ring in **8** diminished the reducing capacity considerably. Correspondingly, electrochemical behavior of 5-carboxypyranoanthocyanidin 3-glucosides and anthocyanidin 3-glucosides was derived using HPLC coupled to a coulometric array detector set from 100 to 800 mV in increments of 100 mV. The relative order of the reducing capacity of the various 5-carboxypyranoanthocyanidin 3-glucosides and anthocyanidin 3-glucosides were nearly alike, whether determined by coulometric array detection or FRAP.

KEYWORDS: Anthocyanins; pyranoanthocyanins; purity; molar absorptivity; reducing capacity; FRAP; coulometry; antioxidant capacity

INTRODUCTION

Increasing evidence that reactive oxygen species and oxidative damage are involved in degenerative diseases has resulted in increased interests in the efficacy of antioxidant activity of naturally occurring molecules including various vitamins and polyphenolic compounds in food and biological systems. However, some recent human intervention trials involving vitamins have presented negative results in this context, while other studies have shown that consumption of antioxidant-rich foods decreases levels of oxidative damage *in vivo* in humans (*1*). The antioxidants serve to keep the levels of free radicals low, permitting them to perform useful biological functions without too much damage. A variety of methods for the determination of antioxidant capacity of anthocyanin samples have been described (*2–7*), and one will find many terms used by different researchers to describe antioxidant capacity (*8, 9*). To obtain comparable results, the measurements of individual antho-cyanins have usually been compared to similar measurements of ascorbic acid or Trolox, often expressed as Trolox equivalents (*10*). After surveying the literature, it is obvious that these results are strongly dependent upon the antioxidant assays applied (*2–4, 10–23*), and most comparative studies conclude that each methodology gives different responses for the same compounds or samples (*24–27*). The relative antioxidant capacity order of various antho-cyanins has even been altered just by changing the concentration of the examined compounds (*3*).

Our opinion is that the relevance of much antioxidant anthocyanin literature is limited because of inadequate consid-

* To whom correspondence should be addressed. Telephone: +47-5558-3460. Fax: +47-5558-9490. E-mail: oyvind.andersen@kj.uib.no.
† University of Bergen.
‡ Matforsk AS, Norwegian Food Research Institute.

10592 *J. Agric. Food Chem.,* Vol. 55, No. 26, 2007

Jordheim et al.

Table 1. Variations of Molar Absorptivity (ε) Values in the Literature (ref *35* and References therein, *42* and *43*) Showing the Lowest and Highest Reported ε Values for Pg3glc, Cy3glc, Cy3gal, Pn3glc, Dp3glc, Pt3glc, and Mv3glc[a]

ε values in the literature	Pg3glc	Cy3glc	Cy3gal	Pn3glc	Dp3glc	Pt3glc	Mv3glc
lowest	14 300	16 520	30 200	11 300	13 000	12 900	13 900
highest	36 600	34 300	46 230	15 100	29 000	21 300	36 400

[a] Structures of individual anthocyanins are shown in **Figure 1**.

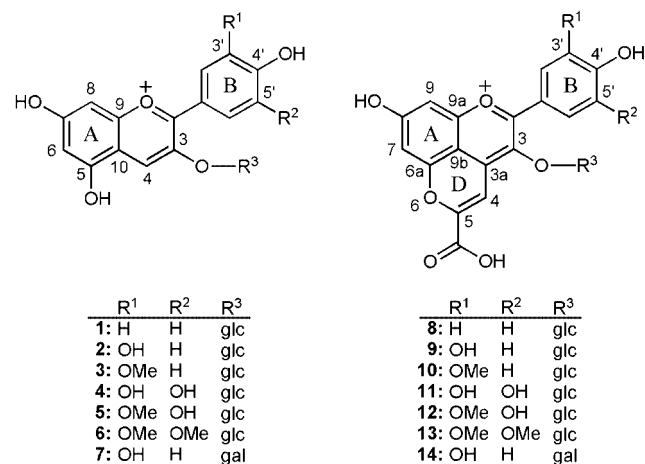|	R¹	R²	R³			R¹	R²	R³
1:	H	H	glc		**8:**	H	H	glc
2:	OH	H	glc		**9:**	OH	H	glc
3:	OMe	H	glc		**10:**	OMe	H	glc
4:	OH	OH	glc		**11:**	OH	OH	glc
5:	OMe	OH	glc		**12:**	OMe	OH	glc
6:	OMe	OMe	glc		**13:**	OMe	OMe	glc
7:	OH	H	gal		**14:**	OH	H	gal

Figure 1. Structures and nomenclature of anthocyanins (**1**–**7**) and carboxypyranoanthocyanins (**8**–**14**). **1** = pelargonidin 3-*O*-β-glucopyranoside (Pg3glc); **2** = cyanidin 3-*O*-β-glucopyranoside (Cy3glc); **3** = peonidin 3-*O*-β-glucopyranoside (Pn3glc); **4** = delphinidin 3-*O*-β-glucopyranoside (Dp3glc); **5** = petunidin 3-*O*-β-glucopyranoside (Pt3glc); **6** = malvidin 3-*O*-β-glucopyranoside (Mv3glc); **7** = cyanidin 3-*O*-β-galactopyranoside (Cy3gal); **8** = 5-carboxypyranopelargonidin 3-*O*-β-glucopyranoside (pPg3glc); **9** = 5-carboxypyranocyanidin 3-*O*-β-glucopyranoside (pCy3glc); **10** = 5-carboxypyranopeonidin 3-*O*-β-glucopyranoside (pPn3glc); **11** = 5-carboxypyranodelphinidin 3-*O*-β-glucopyranoside (pDp3glc); **12** = 5-carboxypyranopetunidin 3-*O*-β-glucopyranoside (pPt3glc); **13** = 5-carboxypyranomalvidin 3-*O*-β-glucopyranoside (pMv3glc); and **14** = 5-carboxypyranocyanidin 3-*O*-β-galactopyranoside (pCy3gal).

erations concerning the purity state of the examined anthocyanin samples. The majority of antioxidant tests subjected to single anthocyanins have addressed purity by the use of high-performance liquid chromatography (HPLC) equipped with a diode array detector (DAD), liquid chromatography−mass spectrometry (LC−MS), or both methods (*28–30*). When molar absorptivity values have been used, one has to bear in mind that existing literature presents substantial variation between the molar absorptivity values given for the same anthocyanin, even in the same solvent, as well as inconsistent values for structurally similar anthocyanins (**Table 1**).

The first part of the present paper illustrates limitation by the use of HPLC−DAD alone in the determination of anthocyanin purity. Thereafter, a new set of molar absorptivity values for anthocyanidin 3-monoglycosides and pyranoanthocyanidin 3-monoglycosides is presented. The second part of the paper describes the electrochemical behavior of a series of pure pyranoanthocyanidin 3-monoglycosides and corresponding anthocyanin 3-monoglycosides with the aim of linking variation in anthocyanin structure to reducing potentials obtained by both a ferric ion reducing antioxidant power (FRAP) assay and hydrodynamic voltammograms.

Table 2. Molar Absorptivity (ε) Values of Selected Anthocyanidin 3-Glycosides and 5-Carboxypyranoanthocyanidin 3-Glycosides in Acidified Aqueous and Methanolic Solvents (A and B)[a]

pigment	solvent	molar absorptivity (ε)	λvis-max (nm)	Mr[b]
pelargonidin 3-glc (**1**)	A	21 000	497	546.40
	B	24 000	502	546.40
cyanidin 3-glc (**2**)	A	20 000	510	484.83[c]
petunidin 3-glc (**5**)	A	21 000	515	514.86[c]
	B	23 000	527	592.43
malvidin 3-glc (**6**)	A	23 000	517	528.88[c]
	B	25 000	529	606.45
cyanidin 3-gal (**7**)	A	23 000	508	562.40
	B	22 000	519	562.40
5-carboxypyranopelargonidin 3-glc (**8**)	B	22 000	495	614.43
5-carboxypyranocyanidin 3-gal (**9**)	B	21 000	506	630.43

[a] A = aqueous buffer (pH 1.0) consisting of 0.2 M KCl/0.2 M HCl (25:67, v/v). B = concentrated HCl/MeOH (0.01:99.99, v/v). [b] Molecular mass including the mass of chloride counterion. See **Figure 1** for structures. [c] Molecular mass including the mass of trifluoroacetate counterion. See **Figure 1** for structures.

MATERIALS AND METHODS

Anthocyanins. Pg3glc (**1**) was isolated from strawberries (*Fragaria* x *ananassa*), while Cy3glc (**2**) and Pn3glc (**3**) were isolated from black rice (*Oryza sativa*) (**Figure 1**). Dp3glc (**4**), Pt3glc (**5**), and Mv3glc (**6**) were isolated from black beans (*Phaseolus vulgaris*), while Cy3gal (**7**) came from black chokeberry (*Aronia melanocarpa*). The acidified aqueous methanolic extracts containing the various anthocyanins were concentrated under reduced pressure, purified by partition with ethyl acetate and various type of column chromatography support, Amberlite XAD-7, Sephadex LH-20, and Toyopearl HW-40F (*31, 32*). In some cases, the same type of chromatography technique was applied twice at different stages in the isolation procedure. The 5-carboxypyranoanthocyanins (**8**–**14**) (**Figure 1**) were hemisynthesized from their corresponding anthocyanidin 3-glycosides (**1**−**7**) according to the published procedure (*32, 33*). The purity of individual anthocyanins was checked by integration of their HPLC−DAD chromatograms recorded at 520 ± 20 and 280 ± 10 nm, respectively, and confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy. Solid samples were achieved by evaporation to dryness under nitrogen followed by freeze-drying to stable mass.

HPLC with UV–Vis Detection. The HPLC system used with UV–vis detection (Agilent 1100 Series, Waldbronn, Germany) was equipped with a HP 1050 DAD, a 20 μL loop, and a 200 × 4.6 mm i.d., 5 μm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, A, water [0.5% trifluoroacetic acid (TFA)] and B, acetonitrile (0.5% TFA) were used for elution. The elution profile consisted of initial conditions with 90% A and 10% B, followed by gradient elution for 10 min (14% B), isocratic elution for 10–14 min, and the subsequent gradient conditions: 18 min (16% B), 22 min (18% B), 26 min (23% B), 31 min (28% B), and 32 min (40% B), isocratic elution for 32–40 min, gradient elution for 40–43 min (10% B), and final linear elution for 43–46 min (10% B). The flow rate was 1.0 mL/min, and aliquots of 15 μL were injected with a Micro Autosampler (Agilent 1100 Series). The UV–vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240–600 nm in steps of 2 nm.

HPLC with Coulometric Array Detection. The HPLC system (HP 1050 series, Waldbronn, Germany) used with coulometric array detection was interfaced to a coulometric array detector (ESA, Inc., Chelmsford, MA) with eight porous graphite working electrodes with associated palladium reference electrodes (*5*). The array detector was set from 100 to 800 mV in increments of 100 mV. The ESA CoulArray operating software was used to collect voltammetric data. Raw data were processed using Microsoft Excel, and the results were presented as peak areas at the electrodes expressed as microcoulombs per nanomole of antioxidant (μC/nmol) and as cumulative peak areas (μC/nmol). The cumulative peak area was the response across several electrodes; for example, the cumulative response at 300 mV was the sum of the peak areas at 100, 200, and
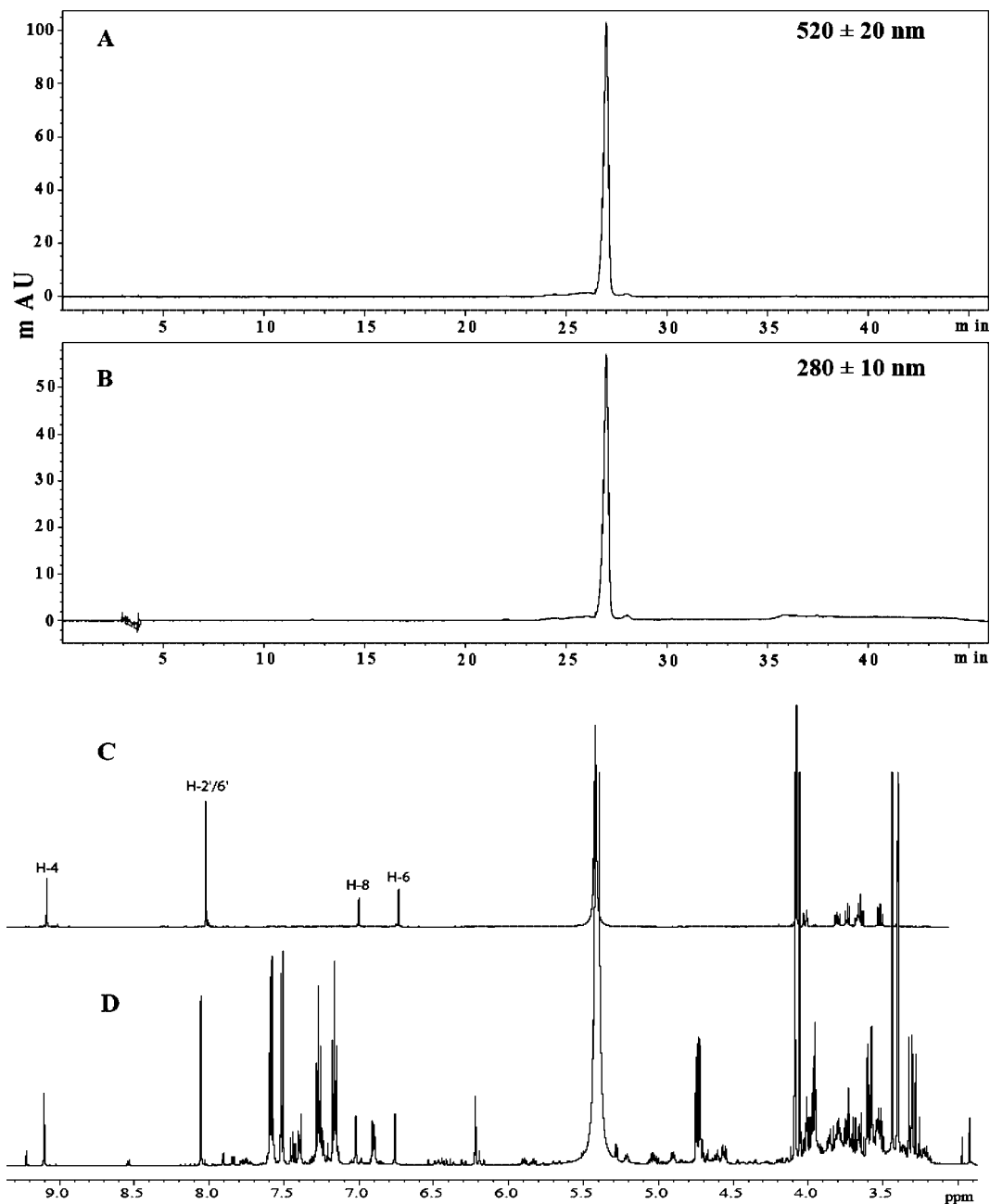
Molar Absorptivities and Reducing Capacity of Anthocyanins

J. Agric. Food Chem., Vol. 55, No. 26, 2007   **10593**

Figure 2. HPLC chromatograms [detected at 520 ± 20 nm (**A**) and 280 ± 10 nm (**B**), respectively] of Mv3glc (**6**) after purification by XAD-7, Sephadex LH-20, and Toyopearl HW-40F column chromatography. (**C**) ^{1}H NMR spectra (600.13 MHz) of pure **6** (concentrated 11 mM). (**D**) ^{1}H NMR spectra (600.13 MHz) of the same sample, **6**, as shown in the HPLC chromatograms (**A** and **B**). NMR samples dissolved in 5:95 CF$_3$CO$_2$D/CD$_3$OD (v/v) were recorded at 25 °C.

300 mV. Hydrodynamic voltammograms (HDVs) were achieved by plotting the cumulative response versus electrode potential. Chromatographic separation was performed on a Betasil C18 column (250 × 4.6 mm i.d., 5 μm particle size) equipped with a 5 μm C18 guard column (4.0 × 4.6 mm i.d.) both from Thermo Hypersil-Keystone (Bellefonte, PA). The mobile phase consisted of acetonitrile (A) and 0.2% phosphoric acid and 2% acetic acid (v/v) in water (B). The program followed a linear gradient from 5 to 20% A in 25 min and from 20 to 40% A in 5 min. The column temperature was 40 °C; the solvent flow rate was 1 mL/min; and the column was allowed to equilibrate for 10 min before each sample (15 μL) was injected. The samples were filtered through a Millex HA 0.45 μm filter (Millipore, Molsheim, France) before injection.

NMR Spectroscopy. One-dimensional ^{1}H and 1D ^{13}C compensated attached proton test experiment (CAPT), two-dimensional heteronuclear single-quantum coherence (^{1}H$-^{13}$C HSQC), heteronuclear multiple-

bond correlation (^{1}H$-^{13}$C HMBC), double-quantum-filtered correlation (^{1}H$-^1$H DQF$-$COSY), total correlated (^{1}H$-^1$H TOCSY), and nuclear Overhauser effect (^{1}H$-^1$H NOESY) spectroscopy were obtained at 600.13 and 150.90 MHz for ^{1}H and ^{13}C, respectively, on a Bruker Avance 600 instrument (Fällanden, Switzerland), equipped with a 600 MHz Ultrashield Plus magnet (Bruker Biospin AG) and a triple-resonance cryogenic probe (5 mm CPTCI ^{1}H$-^{13}$C/^{15}N/D Z-gradient coil). Sample temperatures were stabilized at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (5:95 CF$_3$CO$_2$D/CD$_3$OD, v/v) were used as secondary references [δ 49.0 and 3.40 from tetramethylsilane (TMS), respectively].

Determination of Molar Absorptivity (ε) Values. Weighed portions of purified anthocyanins were dissolved in aqueous buffer (pH 1.0) consisting of 0.2 M KCl/0.2 M HCl (25:67, v/v) (solvent A in **Table 2**) or concentrated HCl/MeOH (0.01:99.99, v/v) (solvent B in **Table 2**) to give accurate pigment concentrations around 1.0×10^{-4} M. The molar absorptivity values, δ (L cm^{-1} mol^{-1}), were calculated according

10594 *J. Agric. Food Chem.,* Vol. 55, No. 26, 2007

Jordheim et al.

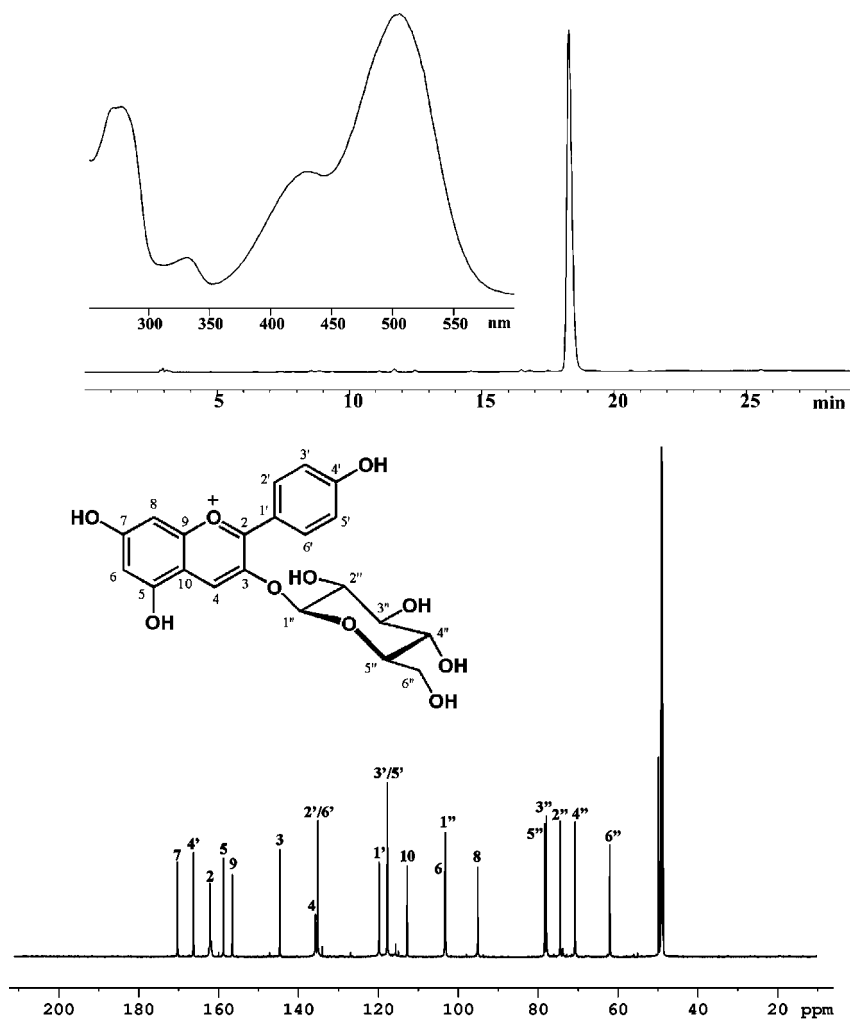**Figure 3.** Online UV–vis spectrum and HPLC chromatogram (detected at 280 ± 10 nm) of pure Pg3glc (**1**) and 1D ^{13}C NMR spectrum of pure **1**.
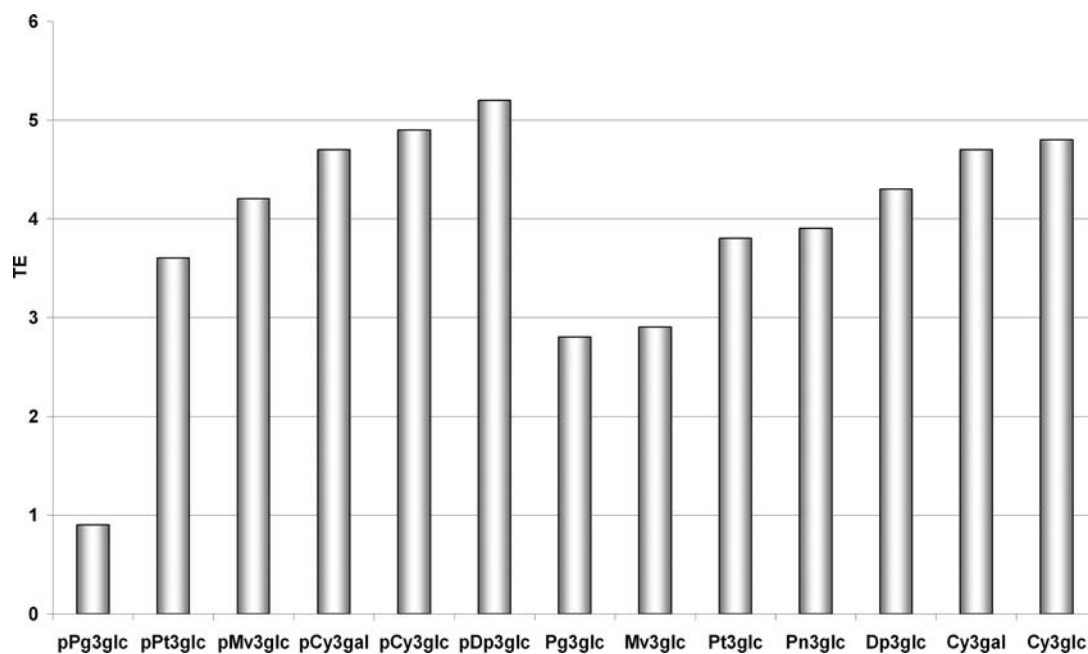
Figure 4. Antioxidant activity of anthocyanidin and pyranoanthocyanidin 3-glucosides (**1–9** and **11–14**) in the FRAP assay expressed as Trolox equivalents (TE). For pigment identification, see **Figure 1**.

to Lambert–Beer's law using the molecular mass, including the mass of the counterion of the individual anthocyanins. The absorbance measurements were made in triplicate.

FRAP Assay. The FRAP assay was performed according to the procedure described by Benzie and Strain (*34*), with modifications. The measurements were carried out on a FLUOstar OPTIMA plate reader
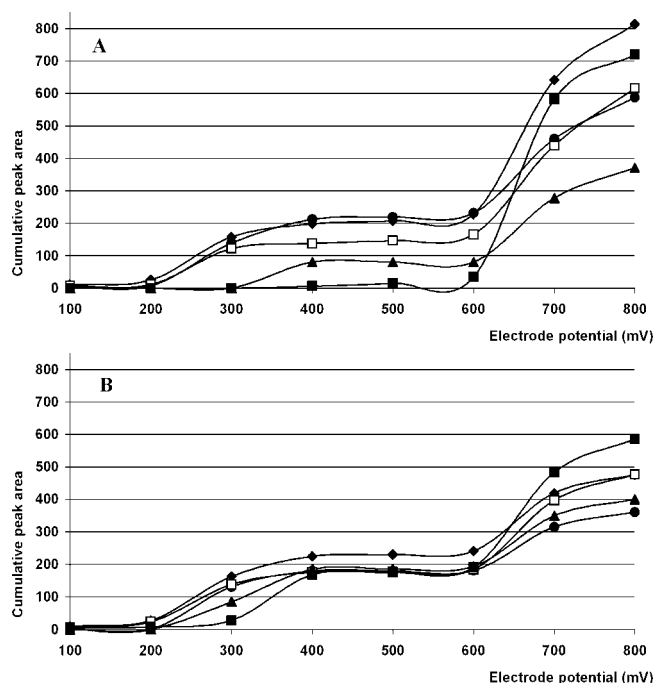
Molar Absorptivities and Reducing Capacity of Anthocyanins

J. Agric. Food Chem., Vol. 55, No. 26, 2007 **10595**

Figure 5. (A) HDVs showing cumulative peak areas (μC/nmol of anthocyanin) of pPg3glc (**8**) (■), pCy3glc (**9**) (◆), pPn3glc (**10**) (▲), pDp3glc (**11**) (●), and pPt3glc (**11**) (□). (B) HDVs showing cumulative peak areas (μC/nmol of anthocyanin) of Pg3glc (**1**) (■), Cy3glc (**2**) (◆), Pn3glc (**3**) (▲), Dp3glc (**4**) (●), and Pt3glc (**5**) (□). For pigment identification, see **Figure 1**.

(BMG Labtech GmbH, Offenburg, Germany) using the 595 nm absorbance filter. Anthocyanin solution (250 μM, 10 μL) was added manually to the plate and mixed with freshly prepared FRAP reagent (190 μL) added by the plate reader. The reaction was conducted at 27 °C, and absorbance was measured every 2 min for 60 min. Aqueous solutions of Fe^{II} (FeSO$_4$·6H$_2$O) in the concentration range of 125–1000 μmol/L were used for the calibration of the FRAP assay. FRAP values, derived as average values from triplicate analysis of three solutions of the anthocyanins, were expressed as micromoles of Trolox equivalents per micromole of anthocyanin (μmol of TE/μmol).

RESULTS AND DISCUSSION

Determination of Anthocyanin Purity. The merit of antioxidant capacity values for individual anthocyanins depends upon the precision in the determination of pigment purity or sample concentration. Whether anthocyanins are purchased from commercial sources or isolated by research groups, the purity of individual compounds has mainly been determined by HPLC−DAD, LC−MS, or both methods (*28–30*). However, as shown in **Figure 2**, anthocyanin purity values obtained by HPLC−DAD have their limitations. After purification by successive use of various types of column chromatography (Amberlite XAD-7, Sephadex LH-20, and Toyopearl HW-40F), the absence of additional peaks in the HPLC chromatograms recorded at 520 ± 20, 280 ± 10 (parts **A** and **B** of **Figure 2**), 320 ± 10, and 360 ± 10 nm indicated high sample purity. However, when the 1D ^{1}H NMR spectrum of the same sample (**Figure 2D**) was compared to a similar spectrum of pure Mv3glc (**6**) (**Figure 2C**), it was clear that **6** in the former sample was not even the major aromatic compound. Pure Mv3glc was achieved by subjecting the sample shown in **Figure 2D** a second time through a Toyopearl HW-40F column.

Purity analyses based on HPLC−DAD chromatograms have to reflect the following considerations: Chromatograms recorded in the visible area (typical between 500 and 550 nm) fail to detect aromatic compounds absorbing at shorter wavelengths.

When additional HPLC chromatograms recorded in the UV–visible region of the spectrum are included (typically around 280 nm), other aromatic compounds may be detected. Impurities lacking a UV-absorbing chromophore will still be invisible. These impurities might be perceived by the use of MS. However, eventual water and inorganic salt content will normally not be determined by these methods. Furthermore, compounds with different chromatographic properties to those of anthocyanins might not show up in the HPLC chromatograms, independently of the HPLC detector, because of the strong interaction with the stationary phase of the column. This latter case is most likely the reason for the discrepancy between the HPLC−DAD and ^{1}H NMR results obtained for the anthocyanin sample examined in **Figure 2**. The reliability of HPLC−DAD purity analysis of anthocyanin may thus be improved considerably by including complementary determination of purity by NMR. Purity control was, in this paper, performed by the integration of ^{1}H NMR signals. When the presence of the baseline separated signals other than those belonging to the examined compound and the solvent peaks were accumulated below 15% of those of the examined anthocyanin, the sample was defined as pure. A standardized method using NMR integration for the determination of impurity amounts has limitations caused by crowded spectral regions with significant signal overlap and baseline effects, which may artificially enhance the signal integrals of trace compounds. The purity control was further checked by observation of signals in the ^{13}C NMR spectrum.

Another routinely used approach employed to define or measure purity/concentration of anthocyanin samples includes the use of molar absorptivity (ε) values. Major difficulties here with respect to exact mass determinations are reflected by the huge variations among the reported ε values (**Table 1**). For instance, the ε value of Mv3glc (**6**) dissolved in 0.1% HCl in methanol has been reported separately to be both 13 900 and 29 500 L cm^{-1} mol^{-1} (reviewed by Giusti et al., *35*). In addition to substantial variation between ε values given for the same anthocyanin, even in the same solvent, there exist inconsistent differences between structurally very similar anthocyanins (**Table 1**). The presence of other impurities than anthocyanins (and other pigments) implies in most cases the calculation of too low of ε values, which according to Lambert–Beer's law ($A = \varepsilon$cl) give too high of anthocyanin concentrations. Consequently, impurities or too low ε values will imply that the measured antioxidant capacities are presented to be lower than reality. Additionally, some reported ε values and purity determinations are hampered by the lack of anthocyanin counterions in the calculations.

To improve the control procedure in estimations of anthocyanin purity, we have combined ^{1}H (**Figure 2C**) and ^{13}C NMR spectroscopy with HPLC−DAD and UV–vis spectroscopy in the purity analysis of various anthocyanins. As shown in the ^{13}C NMR spectrum of Pg3glc (**1**) (**Figure 3**), there exist no significant signals but those belonging to the examined pigment (**1**). Samples obtained using this NMR standardization have been applied for determination of ε values of various anthocyanidin 3-glycosides and 5-carboxypyranoanthocyanidin 3-glycosides (**Table 1**). Contrary to what has been proposed in the literature (reviewed by Giusti et al., *35*), the ε values of various anthocyanidin 3-monoglycosides have been found to be relatively similar for all components investigated (**Table 1**). Even the two analogous pyranoanthocyanins showed ε values in a similar range of those recorded for anthocyanidin 3-monoglycosides (**Table 1**). On the basis of these measurements, we suggest the average ε values for both anthocyanidin 3-monogly-

10596 *J. Agric. Food Chem.*, Vol. 55, No. 26, 2007

Jordheim et al.

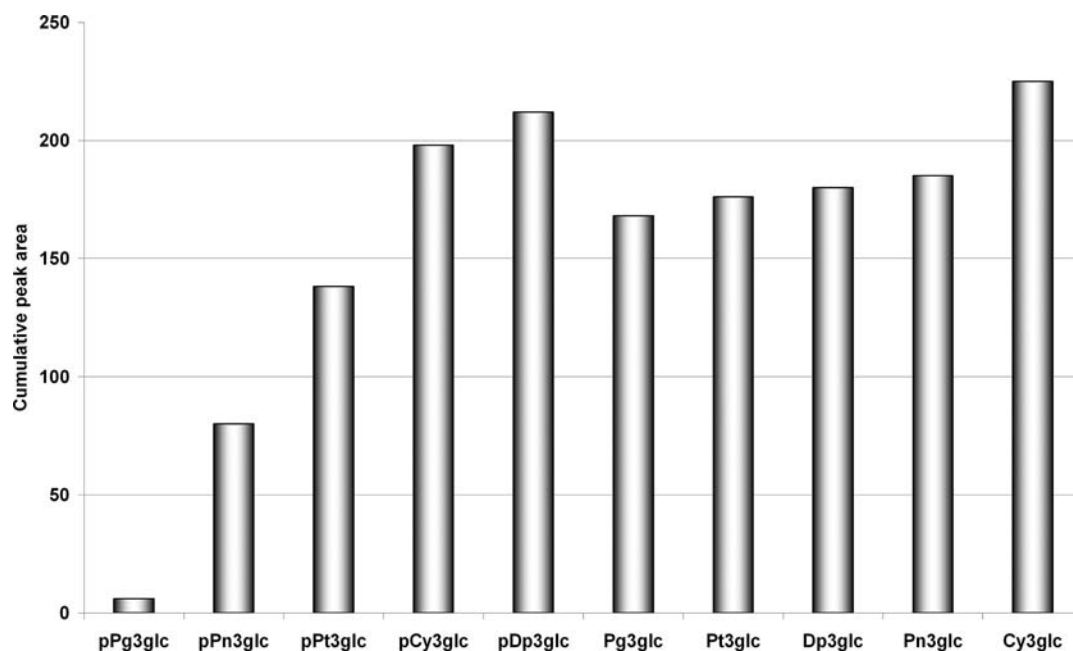**Figure 6.** Cumulative peak area (μC/nmol) recorded at 400 mV derived from the HDVs of anthocyanidin and pyranoanthocyanidin 3-glucosides (**1**–**5** and **8**–**12**). For pigment identification, see **Figure 1**.

cosides and 5-carboxypyranoanthocyanidin 3-monoglycosides to be 22 000 and 23 000 L cm^{-1} mol^{-1} in acidified aqueous and methanolic solutions, respectively.

Reducing Capacity of Anthocyanins Determined by the FRAP Assay. To assess the influence of structure on the potential antioxidant capacity of anthocyanins, the 3-glucosides of Pg (**1**), Cy (**2**), Pn (**3**), Dp (**4**), Pt (**5**), Mv (**6**), pPg (**8**), pCy (**9**), pDp (**11**), pPt (**12**), and pMv (**13**) and the 3-galactosides of Cy (**7**) and pCy (**14**) were examined by the FRAP method (**Figure 4**). The concentration of each anthocyanin dissolved in acidified methanolic solutions was first determined by absorption spectroscopy using the obtained molar absorptivity (ε) value of 23 000 L cm^{-1} mol^{-1} at the visible absorption maxima for the 13 anthocyanins. The reducing capacities of the individual anthocyanins were in the range of 0.9 to 5.2 μmol of TE/μmol. The two 5-carboxypyranoanthocyanins **11** and **9** showed the highest potential antioxidant capacity measured by FRAP subjected to anthocyanins (5.2 and 4.9 μmol of TE/μmol, respectively). However, nearly similar values were obtained for **2**, **4**, **7**, and **14** (**Figure 4**). These six pigments possess vicinal trihydroxyl (pyrogallol type) or *o*-dihydroxyl (catechol type) groups on their B rings. Compounds **8** and **1** gave the lowest reducing capacities (0.9 and 2.8 μmol of TE/μmol, respectively) among the examined anthocyanins. These pigments have only one hydroxyl group on their B rings. The large difference between the latter two values shows that the inclusion of the 5-hydroxyl in the D ring seem to have a significant negative effect on the reducing capacity, when there is just one oxygen substituent on the B ring.

When the reducing capacity of ferric ions is monitored, the FRAP method has previously been used to analyze the potential antioxidant capacity of anthocyanin-containing extracts and mixtures (*36*–*39*). In a few cases, this method has been used for the determination of the potential antioxidant capacity of pure anthocyanins (*5, 10*). García-Alonso et al. (*10*) have reported the reducing capacity of **4**–**6** and **11**–**13** to be between 1.6 and 2.5 expressed as ascorbic acid equivalents. The FRAP values for ascorbic acid and Trolox (water-soluble α-tocopherol analogue) are considered to be identical (*40*), and thus, the corresponding reducing capacities of **4**–**6** and **11**–**13** (2.9–5.2

TE) shown in **Figure 4** are considerably higher than those reported by Garcia-Alonso et al. (*10*). Their antioxidant capacities were measured after a relatively short reaction time (6 min) (*10*), when the reactions may have been incomplete (*5, 40*). Furthermore, their purity determinations seem to be based on HPLC chromatograms detected at 520 nm, which is only adequate for the detection of compounds absorbing in the visible spectral region. The major difference concerning the reducing capacities shown in **Figure 4** compared to literature values is that the 5-carboxypyranoanthocyanins in previous literature (*10*) were reported to have lower antioxidant capacities than the corresponding common anthocyanins.

Reducing Capacity of Anthocyanins Determined by Coulometric Array Detection. The reducing capacity of the 3-glucosides of Pg(**1**), Cy (**2**), Pn (**3**), Dp (**4**), Pt (**5**), pPg (**8**), pCy (**9**), pPn (**10**), pDp (**11**), and pPt (**12**) were derived from coulometric analyses using HPLC coupled to a coulometric array detector set from 100 to 800 mV in increments of 100 mV. HDVs for each of the 10 anthocyanins were achieved by plotting the cumulative responses versus electrode potential (**Figure 5**). Flavonoids present several waves of oxidation across the coulometric array, corresponding to several moieties capable of undergoing oxidation (*5, 41*). According to Aaby et al. (*5*), the cumulative responses at low to medium oxidation potentials (300–500 mV) were most relevant for addressing the potential antioxidant capacity of various phenolics. Hence, the relative cumulative peak area at 400 mV was used as a measure for the reducing capacity of the individual anthocyanins (**Figure 6**).

When the reducing capacity of the individual anthocyanins was examined, the most pronounced effect was observed for pPg3glc (**8**) (**Figure 5A**). This pigment remained without any significant cumulative responses even at electrode potentials as high as 600 mV. In full agreement with FRAP measurements (**Figure 4**), the reducing capacity of this compound with a D ring and only one hydroxyl group on the B ring was very low compared to the other examined anthocyanins. In fact, the relative order of the reducing capacity of the 5-carboxypyranoanthocyanidin 3-glucosides were alike whether determined by coulometric array detection (**Figure 6**) or FRAP (**Figure 4**). With the exemption of a slightly decreased value for Dp3glc

Molar Absorptivities and Reducing Capacity of Anthocyanins

J. Agric. Food Chem., Vol. 55, No. 26, 2007   **10597**

(**4**) measured by coulometric array detection, there was similar agreement between the relative reducing capacity of the examined anthocyanidin 3-glucosides.

LITERATURE CITED

(1) Halliwell, B. Polyphenols; antioxidant treats for healthy living or covert toxins. *J. Sci. Food Agric.* **2006**, *86*, 1992–1995.

(2) Satué-Gracia, M. T.; Heinonen, M.; Frankel, E. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin–liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362–3367.

(3) Kähkönen, M. P.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **2003**, *51*, 628–633.

(4) Rahman, M. M.; Ichiyanagi, T.; Komiyama, T.; Hatano, Y.; Konishi, T. Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure−activity relationship and their synergism. *Free Radical Res.* **2006**, *40*, 993–1002.

(5) Aaby, K.; Hvattum, E.; Skrede, G. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: Relationship to antioxidant activity. *J. Agric. Food Chem.* **2004**, *52*, 4595–4603.

(6) Tsuda, T.; Horio, F.; Osawa, T. Dietary cyanidin 3-*O*-β-D-glucoside increases ex vivo oxidation resistance of serum in rats. *Lipids* **1998**, *33*, 583–588.

(7) García-Alonso, M.; Rimbach, G.; Sasai, M.; Ankara, M.; Matsugo, S.; Uchida, Y.; Rivas-Gonzalo, J. C.; de Pascual-Teresa, S. Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. *Mol. Nutr. Food Res.* **2005**, *49*, 1112–1119.

(8) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(9) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

(10) García-Alonso, M.; Rimbach, G.; Rivas-Gonzalo, J. C.; de Pascual-Teresa, S. Antioxidant and cellular activities of anthocyanins and their corresponding vitisins A—Studies in platelets, monocytes, and human endothelial cells. *J. Agric. Food Chem.* **2004**, *52*, 3378–3384.

(11) Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S.-W.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-β-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407–2410.

(12) Miller, N. J.; Rice-Evans, C. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem.* **1997**, *60*, 331–337.

(13) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304–309.

(14) Pool-Zobel, B. L.; Bub, A.; Schröder, N.; Rechkemmer, G. Anthocyanins are potent antioxidants in model systems but do not reduce endogenous oxidative DNA damage in human colon cells. *Eur. J. Nutr.* **1999**, *38*, 227–234.

(15) Degenhardt, A.; Knapp, H.; Winterhalter, P. Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *J. Agric. Food Chem.* **2000**, *48*, 338–343.

(16) Stintzing, F. C.; Stintzing, A. S.; Carle, R.; Frei, B.; Wrolstad, R. E. Color and antioxidant properties of cyanidin-based anthocyanin pigments. *J. Agric. Food Chem.* **2002**, *50*, 6172–6181.

(17) Seeram, N. P.; Schutzki, R.; Chandra, A.; Nair, M. G. Characterization, quantification, and bioactivities of anthocyanins in *Cornus* species. *J. Agric. Food Chem.* **2002**, *50*, 2519–2523.

(18) Seeram, N. P.; Nair, M. G. Inhibition of lipid peroxidation and structure−activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *J. Agric. Food Chem.* **2002**, *50*, 5308–5312.

(19) Kim, M.-Y.; Iwai, K.; Onodera, A.; Matsue, H. Identification and antiradical properties of anthocyanins in fruits of *Viburnum dilatatum* Thunb. *J. Agric. Food Chem.* **2003**, *51*, 6173–6177.

(20) Chun, O. K.; Kim, D.-O.; Lee, C. Y. Superoxide radical scavenging activity of the major polyphenols in fresh plums. *J. Agric. Food Chem.* **2003**, *51*, 8067–8072.

(21) Kim, D.-O.; Lee, C. Y. Comprehensive study on vitamin C equivalent antioxidant capacity (VEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 253–273.

(22) Lapornik, B.; Wondra, A. G.; Prosek, M. Comparison of TLC and spectrophotometric methods for evaluation of the antioxidant activity of grape and berry anthocyanins. *J. Planar Chromatogr.— Mod. TLC* **2004**, *17*, 207–212.

(23) Awika, J. M.; Rooney, L. W.; Waniska, R. D. Properties of 3-deoxyanthocyanins from *Sorghum*. *J. Agric. Food Chem.* **2004**, *52*, 4388–4394.

(24) Arnao, M. B.; Cano, A.; Acosta, M. Methods to measure the antioxidant activity in plant material. A comparative discussion. *Free Radical Res.* **1999**, *31*, 89–96.

(25) Baderschneider, B.; Luthria, D.; Waterhouse, A. L.; Winterhalter, P. Antioxidants in white wine (cv. Riesling). Part 1. Comparison of different testing methods for antioxidant activity. *Vitis* **1999**, *38*, 127–131.

(26) Perez, D. D.; Leighton, F.; Aspee, A.; Aliaga, C.; Lissi, E. A comparison of methods employed to evaluate antioxidant capabilities. *Biol. Res.* **2000**, *33*, 71–77.

(27) Schwarz, K.; Bertelsen, G.; Nissen, L. R.; Gardner, P. T.; Heinonen, M. I.; Hopia, A.; Huynh-Ba, T.; Lambelet, P.; McPhail, D.; Skibsted, L. H.; Tijburg, L. Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *Eur. Food Res. Technol.* **2001**, *212*, 319–328.

(28) Santos-Buelga, C.; Williamson, G. *Methods in Polyphenol Analysis*; Royal Society of Chemistry: Cambridge, U.K., 2003; pp 383.

(29) Andersen, Ø. M.; Francis, G. W. Techniques of pigment identification. In *Plant Pigments and Their Manipulation*; Davies, K., Ed.; Blackwell Publishing: London, U.K., 2004; pp 293–341.

(30) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV−visible spectroscopy. In *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*; Hoboken, N. J., Ed.; John Wiley and Sons: New York, 2005; pp 19–31.

(31) Andersen, Ø. M.; Fossen, T.; Torskangerpoll, K.; Fossen, A.; Hauge, U. Anthocyanin from strawberry (*Fragaria ananassa*) with the novel aglycone, 5-carboxypyranopelargonidin. *Phytochemistry* **2004**, *65*, 405–410.

(32) Jordheim, M.; Fossen, T.; Andersen, Ø. M. Preparative isolation and NMR characterization of carboxypyranoanthocyanins. *J. Agric. Food Chem.* **2006**, *54*, 3572–3577.

(33) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401–1407.

(34) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

(35) Giusti, M. M.; Rodríguez-Saona, L. E.; Wrolstad, R. E. Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **1999**, *47*, 4631–4637.

(36) Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frei, B.; Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519–525.

Jordheim et al.

(37) Nilsen, I. L. F.; Haren, G. R.; Magnussen, E. L.; Dragsted, L. O.; Rasmussen, S. E. Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidant potency. *J. Agric. Food Chem.* **2003**, *51*, 5861–5866.

(38) Vangdal, E.; Slimestad, R. Methods to determine antioxidative capacity in fruit. *J. Fruit Ornamental Plant Res.* **2006**, *14*, 123–131.

(39) Walton, M. C.; Lentle, R. G.; Reynolds, G. W.; Kruger, M. C.; McGhie, T. K. Anthocyanin absorption and antioxidant status in pigs. *J. Agric. Food Chem.* **2006**, *54*, 7940–7946.

(40) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(41) Manach, C. The use of HPLC with coulometric array detection in the analysis of flavonoids in complex matrixes. In *Methods in Polyphenol Analysis*; Santos-Buelga, C., Williamson, G., Eds.; Royal Society of Chemistry: Cambridge, U.K., 2003; pp 63–91.

(42) Cabrita, L.; Fossen, T.; Andersen, Ø. M. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. *Food Chem.* **2000**, *68*, 101–107.

(43) Torskangerpoll, K.; Andersen, Ø. M. Colour stability of anthocyanins in aqueous solutions at various pH values. *Food Chem.* **2005**, *89*, 427–440.