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Effect of Clarification Techniques and Rat Intestinal Extract Incubation on Phenolic Composition and Antioxidant Activity of Black Currant Juice

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This study examined the phenolic composition and the antioxidant potencies of black currant juices that had been experimentally clarified with acidic proteases and pectinases to retain the phenolics and which had been subjected to rat intestinal mucosa extract incubation to mimic gut cell mediated biotransformation of phenolics. When compared at equimolar levels of 2.5 μM gallic acid equivalents, the black currant juice samples prolonged the induction time of human low-density lipoprotein oxidation in vitro by 2.6−3.6 times, and the order of antioxidant potency of differently clarified black currant juices was centrifuged juice > gelatin silica sol clarified juice > enzymatically clarified juice ≈ raw juice. No immediate relationship between the, almost similar, phenolic profiles of the juice samples and their relative antioxidant activities could be established. Incubation of juices with a rat small intestine cell extract for 19 h promoted significant decreases in the contents of the anthocyanin 3-*O*-β-glucosides (cyanidin 3-*O*-β-glucoside and delphinidin 3-*O*-β-glucoside), but did not affect the anthocyanin 3-*O*-β-rutinosides (cyanidin 3-*O*-β-rutinoside and delphinidin 3-*O*-β-rutinoside) of the black currant juice. Black currant juice samples subjected to such intestinal cell extract incubation had ~30% decreased antioxidant capacity. Incubation of juices with the rat small intestine cell extracts at neutral pH appeared to decrease the levels of delphinidin glucosides more than the levels of cyanidin glucosides. The results provide an explanation for the predominant detection of anthocyanin rutinosides, and not anthocyanin glucosides, in plasma and urine in in vivo studies and provide important clues to better understand the complex mechanisms affecting dietary phenols in the gut.

KEYWORDS: Clarification; rat small intestine incubation; black currant juice; anthocyanins; in vitro LDL oxidation; antioxidant capacity

INTRODUCTION

Consumption of plant-based foods and beverages that are naturally rich in polyphenolic substances is known to correlate inversely to coronary heart disease mortality (*1*, *2*). The cardioprotective action of plant food consumption may be at least partly attributable to ascorbic acid, flavonoids, and other antioxidant phytochemicals in the products (*2*, *3*). The phytochemicals may act as antioxidants to inhibit oxidation of low-density lipoproteins (LDL). Because oxidation of LDL is considered to be a crucial step in the development of cardiovascular disease (*4*), the inhibition of LDL oxidation by dietary antioxidants may retard atherogenesis and cardiovascular disease development (*5*). From a health-protective point of view, ascorbic acid, phenolic acids, and flavonoids, particularly the anthocyanins, are some of the most important constituents of black currants. The anthocyanins in black currants are mainly found in the skins, and the total content is at least 2000 mg/kg on a fresh mass basis of skins (*6*). Although the chemical composition of black currants depends on the variety and agroclimatic conditions of their cultivation, the profile of the four major anthocyanins in black currants (**Figure 1**) has consistently been found to be cyanidin 3-*O*-β-rutinoside (33−38%), delphinidin 3-*O*-β-rutinoside (27−34%), cyanidin 3-*O*-β-glucoside (8−13%), and delphinidin 3-*O*-β-glucoside (8−13%) (*7*, *8*). Amounts of 115 mg/kg of total flavonols such as quercetin and myricetin aglycones have also been found in *Ribes nigrum* Öjebyn black currant samples (*9*).

Freshly pressed black currant juice is very rich in anthocyanins and phenolics and can contain 1340−3220 mg of anthocyanins/L of juice and 4550−7400 mg of gallic acid equivalents of phenols/L depending on the prepress treatment (*10*). The choice of cultivar has previously been shown to strongly affect the total phenols and the antiradical scavenging activity of black currant juices [as measured by the Trolox equivalent antioxidant capacity (TEAC) assay] (*11*), but also the different processing

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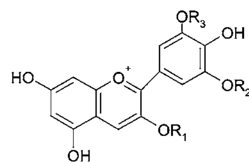

R_1	R_2	R_3	Common Name
β-D-glucopyranosyl	H	OH	Cyanidin 3-glucoside
6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl	H	OH	Cyanidin 3-rutinoside
β-D-glucopyranosyl	OH	OH	Delphinidin 3-glucoside
6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl	OH	OH	Delphinidin 3-rutinoside

Figure 1. Chemical structures of the four dominant anthocyanin glucosides present in black currants and black currant juice.

steps employed during the processing of black currants to juice and juice concentrates have been reported to negatively affect the amount of phenols and the antiradical scavenging activity of the resulting juices (*11*). Clarification, intended to remove cloud and haze-active substances from the juice, is one of the critical processing steps in this regard. Hence, industrial clarification with gelatin silica sol treatment has been shown to promote anthocyanin losses of 19−29% in the case of black currant juice (*12*). Furthermore, gelatin fining has been shown to induce a 10% reduction in the antiradical scavenging activity of black currant juices (*11*). Such data provide a strong incentive to develop alternative, gentler clarification strategies that can better retain the putatively beneficial antioxidant phenols in the juice. In a separate study (*13*) we have examined the clarification efficiencies on black currant juice turbidity and haze development of centrifugation, protease and pectinase addition, and other alternative clarification procedures. However, very little is known about the fate of phenolic compounds and antioxidant activity of juices subjected to alternative clarification strategies.

Obviously, the biological effects of polyphenols are related to their absorption and availability at the target organs and at the eventual molecular target sites (*14*−*16*). Apart from changes in the phenolics associated with the juice processing, phenolics are known to also undergo structural changes in the human body after intake (*14*−*16*). However, data about the detailed transformation mechanisms of phenolics occurring during gastrointestinal passage are still scarce. In rat and human gut systems anthocyanin glucosides have been shown to undergo enzymatic deglycosidation by the action of microbial or cytosolic β-glucosidases and to be degraded and/or further metabolized to simpler phenolics (*14*, *15*). Furthermore, a number of studies have demonstrated that the rate of absorption and the bioavailability of flavonoids are particularly related to the presence, type, and position of the attached sugar (*15*−*18*). A majority of studies thus indicate that flavonoid glucosides, including black currant anthocyanin glucosides, occur in an intact form in human plasma and urine, albeit in very low relative amounts, that is, ≤0.1%, of the dose ingested (*17*−*23*). In relation to black currant anthocyanins, administered orally either as black currant juice or as a mixture of the four main anthocyanins (cyanidin and delphinidin 3-*O*-β-rutinosides and cyanidin and delphinidin 3-*O*-β-glucosides), at least two independent studies have certified that a larger proportion of the 3-*O*-β-rutinosides than the 3-*O*-β-glucosides is absorbed and, furthermore, that the post-intake decrease in plasma is faster for the glucosides than for the rutinosides (*21*, *23*). Meanwhile, no consistent influence of the anthocyanin aglycone structure has been observed between cyanidins and delphinidins (*21*−*23*).

Data from in vitro radical scavenging assays indicate that antioxidant capacity and thus potentially health-beneficial properties of phenols are also largely affected by the phenolic structure and glycosidation state. Hence, an increase in the number of hydroxyl groups usually increases antioxidant activity (*24*), whereas the number of sugar units attached to the aglycone apparently decreases the antioxidant activity of anthocyanins (*25*).

As a continuation of our work on alternative clarification of black currant juice (*13*) the purpose of this work was to understand, at the proof-of-principle stage, the influence of different gentle clarification treatments on the phenolic profiles and antioxidant activities of black currant juice. In an attempt to mimic the biological transformations taking place in the gut, different black currant juice samples were incubated with rat intestine enterocyte extracts, and the anthocyanin profiles and antioxidant potencies of the juices in inhibiting in vitro human LDL oxidation were determined. As a consequence, an additional purpose of the work became to assess the influence of the rat intestinal extract contact, including the reaction pH, on the anthocyanins.

MATERIALS AND METHODS

Materials. Black currant berries (Ben Lomond, *Ribes nigrum*) were provided by Vallø Saft A/S (Køge, Denmark) and kept frozen (−20 °C) until use. Gallic acid and other reagents for total phenols determination, as well as the *p*-nitrophenyl-β-glucopyranoside assay substrate, were purchased from Sigma-Aldrich (St. Louis, MO). Anthocyanin standards were supplied by Polyphenols Laboratories AS (Sandnes, Norway). Gelatin was obtained from SKW Biosystems (Boulogne Billancourt, France), and silica sol was from Erbslöh Getränke-Technology (Geisenheim, Germany). The Grindamyl LB Pectinase used for juice production was obtained from Danisco A/S (Brabrand, Denmark). The pectinase and protease preparations employed for clarification were obtained from different enzyme manufacturing companies and were selected on the basis of our previous results with enzyme-assisted black currant juice clarification (*13*). Pectinex BE 3-L and Enzeco Fungal Acid Protease were both produced by *Aspergillus niger* strains and had pH optima between 2.5 and 3.5 and temperature optima of 50 and 40−55 °C, respectively.

Sample Preparation. Black currant berries were defrosted and then gently crushed in a meat grinder (Jupiter; type 863, Germany). The pulp was subsequently packed under vacuum in airtight polyethylene bags, heated at 80 °C for 2 min, and stored at −20 °C until use. Once defrosted in a water bath at 20 °C, the pulp was mixed with the Grindamyl LB pectinase preparation at 0.05% E/S ratio (expressed as milliliters of Enzyme preparation/100 g of wet berry mash Substrate). Then the samples were placed in a 50 °C water bath and incubated for 2 h with punctual manual mixing every 15 min. After this, the pulp was pressed in a hydraulic 5 L Tincture press (Essen, Germany) using nylon filter bags at a pressure of 100 bar. The juice samples were then pasteurized at 90 °C for 60 s, cooled, and poured into sample bottles; immediately after bottling, the samples were purged with nitrogen and the flasks closed.

Clarification Treatments. *Centrifugation.* On the basis of previous data for cherry juice (*26*), centrifugation was performed at 10000*g* for 15 min. Juice samples subjected to enzymatic clarification were also precentrifuged at 10000*g*, but for only 2 min. After centrifugation, the juice samples were aliquoted into capped flasks (45 mL), purged with nitrogen, and frozen at −20 °C until use.

Gelatin Silica Sol. Both gelatin and silica sol solutions were added to the juice samples at the same levels and following the same procedure as used in industrial berry juice processing (*26*). A gelatin solution (12.5 g/L) was added to the juice samples (0.5 mL of gelatin solution/100 mL of juice), which were then shaken for 5 min in a 50 °C water bath. Then, the silica sol solution (1.188 g/mL, addition level: 55 μL/100 mL of juice) was added with stirring, and the juice samples were shaken for another 5 min at 50 °C. To allow spontaneous sedimentation

of the formed flocs, the samples were maintained at 50 °C for 6 h. Afterward, the juice samples were centrifuged at 10000*g* for 10 min to further separate the sedimenting flocs from the supernatant juice. The supernatant was then filtered through a Whatman filter no. 1 and the clarified juice transferred into plastic bottles and purged with nitrogen.

Pectinase and Protease Treatment. Enzyme preparations were added at a percentage of 0.5 v/v % E/S (enzyme/substrate) and the temperature held constant at 50 °C during clarification (90 min). After clarification, the juice samples were vacuum filtered through a Whatman filter no. 1 and stored at 2 °C for 28 days as follows: clarified juice samples were aliquoted (5 mL) into sample vials and mixed with potassium sorbate and sodium benzoate to a final concentration of 0.5 ppm of each to prevent spoilage. Before sealing, the vials were purged with nitrogen.

Intestinal Enterocytes Incubation. After clarification, black currant juice samples were incubated in vitro with extracts from small intestine enterocytes and quantitative transformations of anthocyanin concentrations after incubation were followed by HPLC analysis (HPLC analysis described below). The incubation method with rat intestinal extracts was inspired by the assay used by Day et al. (*27*): In this assay, the flavonoid glycosides are mixed with intestinal extract. Day et al. (*27*) used human intestinal extracts, whereas we used rat intestinal extracts (see below) and phosphate buffer, with controls of heat-inactivated intestinal extracts run in parallel. The reaction was stopped by the addition of methanol followed by centrifugation.

Intestinal Extract Preparation. Male Wistar rats (270−300 g) were acclimated for a minimum of 7 days at controlled temperature (21 ± 2 °C). The rats were fed ad libitum on a commercial pelleted diet with free access to tap water. The rats were killed by decapitation, and the small intestine was excised. Immediately thereafter, the enterocytes were isolated according to the calcium chelation method (*28*). The isolated enterocytes were then suspended in ice-cold phosphate buffer to reach enterocyte concentrations of 0.1 g/mL. One milliliter of cell suspension was aliquoted into Eppendorf tubes and kept on ice. The cell suspensions were then disrupted by sonication for 30 s using a probe sonicator at an amplitude of 5 μ (MSE Ltd., Crawley, Surrey, U.K.) and centrifuged at 13600*g* for 10 min at 4 °C. The supernatant was retained and stored on ice prior to use.

Incubation Procedure. A 0.1 mL diluted juice sample was incubated with 0.4 mL of intestinal extract at 37 °C for up to 19 h. Controls were run in parallel with standard or juice samples incubated with heat-inactivated intestinal preparations (105 °C, 5 min). The reaction was stopped by the addition of methanol (0.5 mL), followed by centrifugation at 13600*g* for 10 min at 4 °C. The supernatant was then filtered through a 0.45 μm syringe-tip nylon filter and analyzed by HPLC.

Determination of β-Glucosidase Activity of Rat Intestinal Extracts. β-Glucosidase activity was assayed using 5 mM *p*-nitrophenyl-β-glucopyranoside as substrate in 50 mM citrate buffer principally as described in ref *29*, but with the reaction mixture adjusted to different pH values (pH 2.5, 4.0, 5.5, 6.0, 6.5, 7.0, or 7.5) to assess the effect of pH on β-glucosidase activity. In each run 1.0 mL of substrate solution and 0.1 mL of intestinal extract sample were incubated at 37 °C for 10 min. The reaction was stopped by adding 1 M Na_2CO_3, and the absorbance was read at 400 nm. Activity, defined as conversion of 1 μM substrate per minute under the reaction conditions, was calculated versus a standard curve of *p*-nitrophenol (*29*).

Quantification of Phenol Compounds. Total phenols in the juices were determined according to the Folin−Ciocalteu procedure and expressed as milligrams per liter of gallic acid equivalents (GAE) (*30*).

HPLC Analysis. HPLC analysis was carried out according to the procedure described by Lamuela-Raventós and Waterhouse (*31*) using a Hewlett-Packard (Houston, TX) 1100 system equipped with a diode array detector and a Nova-Pak C18 column (150 mm × 3.9 mm, Waters, Milford, MA) and controlled by a personal computer with HPChem station software. The phenolic compounds were identified by spectral and retention time analysis. The quantities of the different phenolic compounds were assessed from peak areas and calculated as equivalents of their representative standard compounds (linear regression curves of authentic standards) as follows: at 280 nm, catechin, epicatechin (flavan-3-ols), and gallic acid, respectively; at 316 nm (hydroxycinnamates), neochlorogenic as chlorogenic acid equivalents, *p*-coumaroylquinic and *p*-coumaric acid as *p*-coumaric acid equivalents, and caffeic acid; at 520 nm (anthocyanins), cyanidin 3-glucoside, delphinidin 3-glucoside, delphinidin 3-rutinoside, and cyanidin 3-rutinoside, respectively. Flavonols were quantified as rutin equivalents at 365 nm using rutin as the standard.

Inhibition of Human LDL Oxidation. The antioxidant power of juice samples after clarification and incubation to inhibit the copper-catalyzed oxidation of human LDL (37 °C, 5 mM $CuSO_4$) was assayed by diluting the samples to equimolar concentrations of total phenols and monitoring the formation of conjugated diene hydroperoxides spectrophotometrically (234 nm) for up to 240 min (*32*). At the same time, oxidation of human LDL was monitored without the addition of any juice sample or another antioxidant agent (control). The resulting absorbance−time curve could be divided into three phases: a lag phase (no or slow oxidation), a propagation phase (fast oxidation), and a decomposition phase. Antioxidant activities were then assessed by two different calculation methods: lag time and 50-factor (*33*). Lag time was calculated as the net prolongation of the induction time of the control, and the 50-factor was calculated as the time to attain 50% of maximum absorbance (T 50%) in samples versus controls. We expressed the antioxidant 50-factor from (T 50% of sample)/(T 50% of control). If the factor was <1, the tested sample was interpreted as having antioxidant activity to retard lipid peroxidation of LDL.

Statistical Analysis. Significance of the results was established at $P \leq 0.05$. Differences in responses were determined by one-way analysis of variance; 95% confidence intervals were calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

RESULTS AND DISCUSSION

Influence of Clarification on Phenol Content of Juice Samples. In general, all clarification treatments had a significant influence on the content of the different phenolic species in the black currant juice samples (**Table 1**). Clarification by gelatin silica sol treatment and centrifugation of the raw juice promoted the most remarkable changes, reducing approximately 42 and 32% the overall phenols content, respectively. The anthocyanins were apparently particularly sensitive to these clarification treatments that resulted in decreases in anthocyanins of 44 and 32%, respectively, relative to the initial values in the raw juice (**Table 1**). The observed gradual increases in caffeic and *p*-coumaric acid taking place simultaneously with decreases in the recoveries of neochlorogenic and *p*-coumaroylquinic acid (**Table 1**) may be a result of the (enzymatic) degradation of the quinic acid conjugation during the clarification treatment. The presence of depsidase activity able to hydrolyze quinic acid conjugates, that is, chlorogenic acids, to caffeic acid during the processing of black currant musts and wines has previously been reported to be present in a pectinolytic enzyme preparation (*34*). The presence of such enzymatic side activity in enzyme preparations used for clarification may not only change the phenolic profile but also affect the antioxidant activity of the juice products. The reduction of phenolic compounds during centrifugation may be ascribable to capture of phenols by the pectin and insoluble fragments of plant cell wall material during the forced sedimentation taking place during the centrifugation process. Although centrifugation thus induced losses of considerable amounts of phenols, the procedure was found to be extremely efficient for immediate turbidity removal and prevention of haze development in freshly pressed berry juices (*13*, *26*) and was therefore proposed as a prestep to other clarification procedures in berry juice processing (*26*). For this reason, juice samples subjected to gelatin silica sol treatment and to enzymatic clarification treatments in this study had all been centrifuged prior to the specific clarification treatment. Even when this was taken into account, the gelatin silica sol treatment was still poorest with respect to retaining phenolics and ascorbic acid in

Table 1. Contents of Ascorbic Acid, Selected Anthocyanins, Phenolic Acids, and Flavonols in Raw Black Currant Juice and in Juices Subjected to Different Clarification Methods[a]

	raw juice	raw centrifuged juice	gelatin silica sol	Enzeco Protease	Pectinex BE 3-L Pectinase	Enzeco Protease + Pectinex BE 3-L Pectinase
ascorbic acid	603	510 (84%)[b]	354 (59%)	368 (61%)	450 (75%)	599 (99%)
delphinidin-3-glucoside	537	357 (66%)	287 (53%)	370 (69%)	383 (71%)	375 (70%)
delphindin-3-rutinoside	1692	1149 (68%)	948 (56%)	1162 (69%)	1155 (68%)	1142 (67%)
cyanidin-3-glucoside	154	104 (68%)	72 (47%)	109 (75%)	115 (75%)	112 (73%)
cyanidin-3-rutinoside	1091	755 (69%)	634 (58%)	773 (71%)	758 (69%)	751 (69%)
total anthocyanins	3474	2365 (68%)	1941 (56%)	2414 (69%)	2411 (69%)	2380 (69%)
gallic acid	nd	nd	2	5	6	5
neochlorogenic acid	130	89 (68%)	93 (72%)	92 (71%)	67 (52%)	65 (50%)
caffeic acid	nd	nd	nd	0	9	7
p-coumaroyl quinic acid	46	32 (70%)	33 (72%)	26 (57%)	17 (37%)	17 (37%)
p-coumaric acid	1	1 (100%)	2 (200%)	3 (300%)	12 (1200%)	9 (900%)
total phenolic acids	177	122 (69%)	130 (73%)	126 (71%)	111 (63%)	103 (58%)
total flavonols	270	172 (64%)	206 (76%)	216 (79%)	214 (79%)	208 (77%)
total	3921	2659 (68%)	2277 (58%)	2756 (70%)	2736 (70%)	2691 (69%)

[a] All values are given in mg equiv standard/L. Data presented are the average of two measurements. The average coefficients of variation on the anthocyanin, phenolic acids, and flavonols data were <3, 4, and 8%, respectively. [b] Percent recovery of each compound versus in raw juice appears in parentheses. Standards used to quantify each phenolic species are given under Materials and Methods.

the black currant juice (**Table 1**). In fact, enzymatic clarification did not decrease either the total anthocyanin or the the total phenols content of the black currant juice beyond that induced by centrifugation (**Table 1**).

The maintenance of a high ascorbic acid recovery (**Table 1**) is an indication of the gentle clarification treatments employed, which were all accomplished under N_2 blanketing. Along the same line, the changes in anthocyanins and other phenols may thus not be due to oxidation during clarification treatments and sample handling, but mainly the result of other chemical, enzymatic, or physicochemical changes occurring during the treatments. Apart from having been documented to have a negative effect on the phenolic levels in berry (*12*) and apple juice processing (*35*), adsorption and consequent removal of anthocyanins by gelatin silica sol treatment has also been previously demonstrated with red wines, where the anthocyanin removal was found to have a negative effect on the wine color stability (*36*). Clearly, these results create an incentive for further development of alternative clarification strategies in the fruit juice industry. Despite the potential influence of several factors (e.g., cultivar, soil nutrients, temperature, and maturity stage) on the phenol content of black currant fruits and juices, the data obtained (**Table 1**) agree very well with the available reported contents of different phenolics in black currants (*37*, *38*).

Influence of Clarification on Antioxidant Activity of Black Currant Juice Samples. When the antioxidant potencies against in vitro oxidation of human LDL of the different black currant juice samples were compared at equimolar levels of 2.5 μM total phenols, significant differences in the antioxidant effects of the samples were recorded (**Table 2**). The control runs with no juice added had an average induction time of ~50 min, whereas all of the black currant juices prolonged the induction time of human LDL oxidation in vitro by 2.6−3.6 times. The order of antioxidant potency of differently clarified black currant juices was centrifuged juice > gelatin silica sol clarified juice > enzymatically clarified juice ≈ raw juice. No significant differences were found in the lag time and 50-factor values among samples corresponding to the different enzymatic clarification treatments, corroborating that the different enzymes used for clarification did not affect the overall phenolic profile of the black currant juice samples (**Table 1**). Because the juice samples were diluted to the same micromolar concentration of total phenols, any differences in the antioxidant activities have to be due to differences in the phenolic composition of the samples. However, no relationship between the relative antioxidant activities and the levels of individual phenols could be established by linear regression analysis. Previously, the antioxidant potencies of various berry extracts, red wines, and grape juices on human LDL oxidation have been shown to correlate to the relative abundance of specific phenols or phenolic classes in the samples being tested (*39*−*41*). The antioxidant potenciesof phenolic extracts from differently stored cherries were, for instance, somewhat surprisingly, found to correlate positively to the levels of *p*-coumaroylquinic acid but negatively to the cyanidin 3-rutinoside levels (*41*). Hence, even though the raw black currant juice had a considerably higher amount of anthocyanins, phenolic acids, and flavonols than the other juice samples (**Table 1**), the *relative* contents of the different phenolic species (and ascorbic acid) did apparently not vary sufficiently between the differently treated juice samples to establish correlations between antioxidant potency and contents of particular phenols. Compared to the other samples, the raw, centrifuged juice did, however, contain relatively lower levels of flavonols than the other samples. Although the levels of flavonols (rutin equivalents) in berry extracts were previously shown to correlate negatively to antioxidant potency toward LDL oxidation in vitro (*42*), the available data material is too limited to draw any firm conclusions in this regard for the black currant juice samples. Finally, the possibility that the slightly different potencies of the juice samples could be a result of a

Table 2. Antioxidant Activity as Inhibition of Human LDL Oxidation of Black Currant Raw Juices and of Juices Subjected to Different Clarification Treatments[a]

	lag time (±SD) (min)	50-factor (±SD)
control	49.7d (±0.8)	1.00 (±0.00)
raw juice	134.9c (±2.8)	2.39 (±0.05)
raw centrifuged juice	181.9a (±3.0)	3.14 (±0.05)
gelatin silica sol	154.9b (±4.4)	2.69 (±0.06)
Enzeco Protease	137.5c (±8.9)	2.42 (±0.14)
Pectinex BE 3-L Pectinase	132.5c (±5.3)	2.35 (±0.07)
Enzeco Protease + Pectinex BE 3-L Pectinase	139.3c (±5.1)	2.46 (±0.07)

[a] Samples tested at equimolar levels of 2.5 μM. Data presented are the average ± SD ($n = 3$). Different letters (a–d) indicate significant differences, $p < 0.05$, in lag time; ANOVA based on the pooled standard deviations of all determinations.

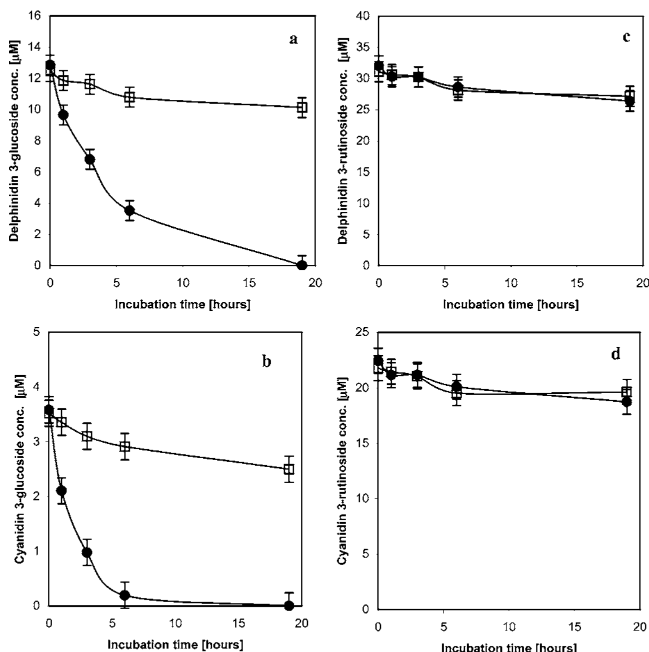

Figure 2. Concentrations of the four main anthocyanins contained in 10-fold-diluted black currant juice (phenol concentration = 704 μM) after incubation with rat intestinal mucosal cell extract and with heat-inactivated cell extract, final pH 5.2: (**a**) delphinidin 3-*O*-β-glucoside; (**b**) cyanidin 3-*O*-β-glucoside; (**c**) delphinidin 3-*O*-β-rutinoside; (**d**) cyanidin 3-*O*-β-rutinoside; (●) cell extract incubation; (□) heat-inactivated cell extract incubation.

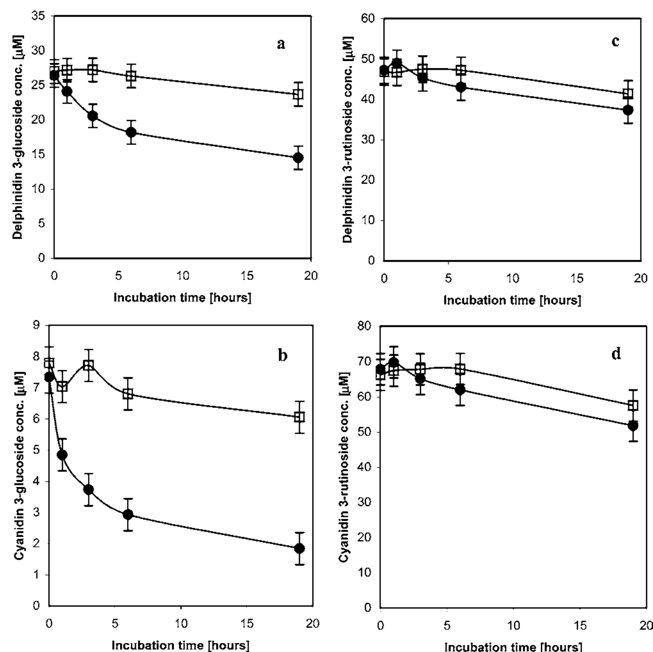

Figure 3. Concentrations of the four main anthocyanins contained in 5-fold-diluted black currant juice (phenol concentration = 1048 μM) after incubation in rat intestinal mucosal cell extract and in heat-inactivated cell extract, final pH 4.0: (**a**) delphinidin 3-*O*-β-glucoside; (**b**) cyanidin 3-*O*-β-glucoside; (**c**) delphinidin 3-*O*-β-rutinoside; (**d**) cyanidin 3-*O*-β-rutinoside; (●) cell extract incubation; (□) heat-inactivated cell extract incubation.

discrepancy between the Folin−Ciocalteu determination of total phenols and the true sum of phenolic antioxidants and ascorbic acid in the samples cannot be excluded. In any case, the data indicate that black currant juice exerts potential antioxidant activity on human LDL oxidation in vitro and that the antioxidant potency of the black currant juice constituents was distributed among several different phenolic compounds and ascorbic acid.

Influence of Intestinal Extract Contact in Vitro on Anthocyanins. *Evolution of Anthocyanin Contents.* When the effects on the anthocyanins of rat intestinal cell extract incubation at pH 5.2 of 10-fold-diluted juice, that is, final total phenol concentration of 704 μM, were assessed, dramatic decreases in the concentrations of delphinidin 3-*O*-β-glucoside and cyanidin 3-*O*-β-glucoside with time were observed, attaining complete disappearance of these glucosides after 19 h of incubation (**Figure 2a**,**b**). Meanwhile, the intestinal extract incubation did not affect the levels of the corresponding anthocyanin *O*-β-rutinosides (**Figure 2c**,**d**). When the concentration of black currant juice for intestinal extract incubation was doubled to a total phenol concentration of 1408 μM, the levels of the two anthocyanin *O*-β-rutinosides remained the same during the 19 h of incubation, whereas the levels of the 3-*O*-β-glucosides again fell significantly, albeit not as rapidly as in the previous case and not to complete exhaustion (**Figure 3**). The slower rate of degradation with the more concentrated phenolics sample could be due to the fact that the more concentrated juice also resulted in a lower pH; whereas the pH was 5.2 in the 10-fold-diluted juice incubations, it was 4.0 in the incubations with the 5-fold-diluted juice. The lower pH was induced by the acid pH of the black currant juice itself. When the β-glucosidase activity of the rat intestinal extract was measured on *p*-nitrophenyl-β-D-glucopyranoside in response to changes in pH, the maximum activity was found to be around pH 4−4.5, and activity was minimal at pH 6−7.5 and gradually decreased at pH values below 3.5 (**Figure 4**).

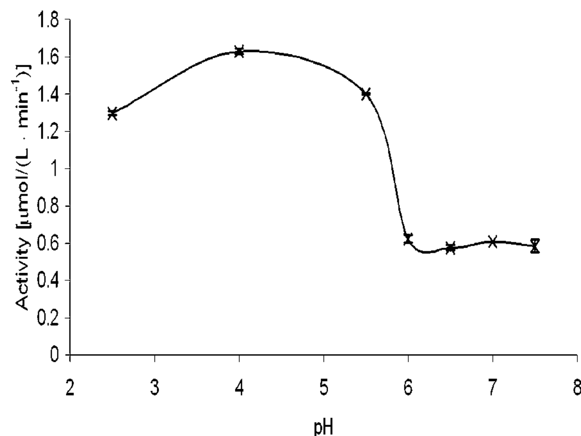

Figure 4. Response to changes in reaction pH of β-glucosidase activity in the rat intestinal mucosal cell extract at 37 °C as measured on *p*-nitrophenyl-β-D-glucopyranoside (5 mM). Data are averages of two assay measurements, and data points (×) are shown as averages ± SD.

The data obtained correspond extremely well with the available data on in vivo absorption of black currant anthocyanins in rats and humans, which have established that a larger proportion of anthocyanin rutinosides than glucosides are absorbed and that the structure of the anthocyanin aglycone does not significantly influence the absorption (or the urinary excretion) of glycosylated black currant anthocyanin species (*21*, *23*). The latter findings have been proposed to be due to cleavage of the anthocyanin glucosides, but not the rutinosides, by β-glucosidases present in the small intestine. Keppler et al. (*14*) have recently demonstrated deglycosylation of anthocyanin glucosides by intestinal microflora (using a microflora culture isolated from the cecum of freshly slaughtered pigs). They found

Table 3. Effects of Cell Extract Incubation on Four Main Occurring Anthocyanins Content in a 10-fold Black Currant Juice Adjusted to Neutral pH[a]

compound	incubation time (h)	cell extract concn[b] (μM)	cell extract recovery (%)	heat-inactivated cell extract concn[b] (μM)	heat-inactivated cell extract recovery (%)
black currant juice (683 μM) at pH 7.3					
Del-3-glu	3	0 ± 0	0	4.6 ± 0.1[#]	45
	19	0 ± 0	0	0 ± 0	0
Del-3-rut	3	0.6 ± 0.1	2	13.0 ± 0.2[#]	50
	19	0.1 ± 0.2	0	0.3 ± 0.3	1
Cy-3-glu	3	0.4 ± 0.0	14	1.6 ± 0.1[#]	54
	19	0 ± 0	0	0.5 ± 0.1	18
Cy-3-rut	3	6.9 ± 0.1	37	10.8 ± 0.1[#]	57
	19	1.0 ± 0.5	5	4.8 ± 0.4	25

[a] Data presented are the average ± SD ($n = 3$), except data with [#], which represent only one measurement. [b] Concentration assessed as the concentration remaining in the 0.5 mL mixture with the cell extract prior to addition of methanol.

that various anthocyanin glucosides, including both 3-β-D-*O*-glucosides, 3-β-D-*O*-rutinosides, and 3,5-β-D-*O*-diglucosides, were hydrolyzed by the pig cecum microflora and also that the rate of deglycosylation induced during the incubations depended on the sugar moiety (*14*). The ability of (human) cell-free intestinal extracts to deglycosylate various flavonoid glucosides have been documented for quercetin, kaempferol, naringenin, apigenin, and genistein glucosides previously (*27*); in that case 7-glucosides were rapidly deglycosylated during contact with cell-free intestinal extracts, whereas the 3,4′-diglucosides, 3-glucosides, 3-rhamnoglucosides, and 7-rhamnoglucosides remained unchanged (*27*). The available data thus suggest that different flavonoid glucosides are hydrolyzed differently but certainly support that intestinal β-glucosidase activity may have an important role in the uptake and metabolism of flavonoid glucosides, including anthocyanin glucosides, independent of the colonic microflora metabolism. Because the disappearance of the glucosides occurred directly during contact with the rat intestinal mucosal extract (**Figures 2** and **3**), our data strongly corroborate that significant changes in the anthocyanin glucosides most likely occur directly at the intestinal mucosal border and that important transformations may presumably be caused by mammalian enzyme systems in addition to the changes resulting from microbial metabolism in the intestinal canal.

Influence of pH in the Evolution of Anthocyanin Content. In an attempt to simulate the (presumed neutral) pH conditions of rat intestinal cells, a 10-fold-diluted black currant juice sample was adjusted to pH 7.3 and the anthocyanin concentration evolution was compared with that obtained with the 10-fold-diluted juice that had not been pH-adjusted (**Table 3**). With the pH adjusted to 7.3 significant reductions of all four black currant anthocyanin glucoside species were observed already after 3 h of incubation; the decrease in the level of delphinidins tended to be higher than the decrease in cyanidins, but the levels of both of the 3-*O*-β-glucosides reached 0 μM after 19 h of incubation (**Table 3**). Because the β-glucosidase activity of the intestinal rat extract was presumably minimal at pH 7.3 (**Figure 4**), the observed differences in stability at this neutral pH might be caused by differences in the phenolic structures rather than by differences in the glycosylation. To our knowledge there are no data available that support the idea that cyanidins would be more stable per se than delphinidins at neutral pH. Rather, the apparent higher loss of the delphinidin glucosides could be due to the putative higher tendency of delphinidin, carrying one more phenolic hydroxyl group than cyanidin, to bind to proteins in the cell extract. Reactivity of phenols with proteins has been

Table 4. Antioxidant Activity[a] of Black Currant Juices Subjected to Different Clarification Treatments after Cell Extract Incubation[a]

	lag time (±SD) (min)	50-factor (±SD)
control	49.7c (±0.8)	1.00 (±0.00)
raw juice	92.4b (±2.37)	1.73 (±0.03)
raw centrifuged juice	106.0a (±4.90)	1.95 (±0.07)
gelatin silica sol	90.8b (±2.02)	1.72 (±0.03)
Enzeco Protease	90.8b (±0.40)	1.72 (±0.01)
Pectinex BE 3-L Pectinase	99.4b (±1.39)	1.83 (±0.02)
Enzeco Protease + Pectinex BE 3-L Pectinase	94.7b (±1.74)	1.76 (±0.02)

[a] Samples tested correspond to a concentration of 2.5 M in the raw juice. The data presented are the average ± SD ($n = 3$). Different letters (a–c) indicate significant differences, $p < 0.05$, in lag time. The ANOVA was based on the pooled standard deviations of all determinations.

reported to increase with the number of hydroxyl groups at neutral pH, notably when the hydroxyl groups are adjacent in the phenolic ring (*43*). In case such phenol–protein binding might have occurred during the cell extract incubation, the resulting complexes would have been removed from the sample by precipitation during the centrifugation step, and this could thus explain the loss of anthocyanins. In the heat-treated controls, however, the deformation of proteins during heat inactivation would be expected to promote a lower binding ability, which in turn might result in a somewhat higher recovery of the anthocyanins (**Table 3**). At the neutral pH the levels of both delphinidins had disappared only after 19 h with the heat-inactivated cell extract (**Table 3**).

The reduction of anthocyanin levels in the samples incubated with the cell extract was, however, significantly higher than that obtained with the heat-inactivated controls; this finding confirmed the putative role of the action of β-glucosidases, responsible for breaking the sugar linkages resulting in the eventual subsequent degradation of the aglycone by spontaneous ring fission (*8*, *14*). Therefore, a comparison of the data of the non-pH-adjusted juice with those of the adjusted samples at pH 7.3 suggested a very strong effect of pH on the anthocyanin levels during incubation.

Influence of in Vitro Intestinal Extract Contact on Antioxidant Activity on Human LDL. Table 4 shows the antioxidant activity on in vitro human LDL oxidation after rat intestinal mucosa cell extract incubation of black currant juices subjected to the different clarification methods; the samples were diluted to an equal final phenol concentration of 2.5 μM in the LDL assay. Comparison of the results with those of the corresponding juice samples shown in **Table 2** revealed a decrease of about ∼33% in the antioxidant activity of the juice samples after cell extract incubation. This decrease is a little higher, but corresponds well, with the loss expected as a result of disappearance of the cyanidin and delphinidin 3-O-β-glucosides as these alone made up ∼20% of the total anthocyanins (**Table 1**).

Among the samples, the antioxidant potency of the centrifuged juice sample was still best, but the relatively good activity of the gelatin silica sol clarified sample, observed prior to cell extract incubation (**Table 2**), was no longer evident, as the antioxidant activity of this sample was now equal to that of the other clarified juice samples (**Table 3**). The data obtained thus suggest that the contact with the intestinal mucosal extract not only dampened the total antioxidant potency of the black currant juice samples but also somehow smoothed the subtle differences in antioxidant potency among the differently clarified juice samples. The decreases in antioxidant activity could be due to the instability of phenol aglycones as a consequence of the

deglycosidation (**Figures 2** and **3**), which in turn results in their degradation and in turn likely prevents any contribution to antioxidant activity. Although the original flavonol levels in the raw centrifuged juice were relatively lower than those of the other samples, the data do not offer an unequivocal explanation as to why the antioxidant potency of the centrifuged juice sample remained superior to the others. Clearly, the differences may be due to differences in the overall profiles of the phenolics in the juices, that is, the changes that appear to have occurred during the different clarification treatments (**Table 1**).

The results obtained regarding the deglycosylation of 3-*O*-glucosides and the lowered antioxidant activity of the juices after intestinal mucosal cell extract incubation can provide important clues about the fate of phenols after intake and about their bioavailability. Our results thus provide an explanation for the consistent finding of higher proportions of anthocyanin rutinosides than of glucosides in plasma and urine in in vivo studies involving berry juices (*21*−*23*). In addition, the observed effects of the mucosal cell extract incubations on in vitro antioxidant activities on human LDL may provide a new base for better in vitro evaluation of the potential cardioprotective effects of dietary phenolic compounds. Although still more studies on the absorption and the possible protective effects of naturally occurring fruit phenolics appear in the literature, limited research on the health effects of berries and berry juices has been conducted. Considering the high levels of phenolics and ascorbic acid in black currants and in black currant juice (**Table 1**), a targeted focus on their importance as a source of potentially health-protective phytochemicals seems to be warranted. In addition, the data also suggest that there are significant effects of the food-processing technology to consider in order to improve knowledge about the relationships between phenols intake and potential health-promoting effects of fruit juices.

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