Effect of Processing of Black Currant Press-Residue on Polyphenol Composition and Cell Proliferation

Linda Holtung,[*,†,‡] Stine Grimmer,[†] and Kjersti Aaby[†]

[†]Nofima Mat AS, Osloveien 1, N-1430 Aas, Norway

[‡]Department of Nutrition, Faculty of Medicine, University of Oslo, Postbox 1046 Blindern, N-0316 Oslo, Norway

ABSTRACT: The press-residue of black currants provides a good source of phenolic antioxidants. The purpose of this study was to optimize the extraction of phenolic compounds from the press-residue by use of extraction conditions compatible with food use. The effects of temperature, extraction duration, and use of ultrasound-assisted extraction on the juice yield, total phenolics (TP), and anthocyanin content of aqueous extracts were studied. Within the variables and response factors tested, the optimal conditions were a 15 min extraction at 90 °C. No significant effect from ultrasound-assisted extraction was found. The composition of anthocyanins and polyphenols was highly dependent on the extraction temperature. The percentage contribution of delphinidin- and cyanidin-3-rutinoside to TP had a negative linear correlation with temperature, while delphinidin- and cyanidin-3-glucoside had a positive linear correlation with temperature, with a maximum amount obtained at 80 °C and 55 °C, respectively. Furthermore, extracts obtained at higher temperatures showed a stronger inhibition of proliferation of Caco-2, HT-29, and HCT 116 cells than extracts obtained at lower temperatures. This may be due to the decomposition of complex polyphenols at higher temperatures, making them more accessible to the cells.

KEYWORDS: black currants, *Ribes nigrum* L., juice yield, polyphenols, anthocyanins, extraction, temperatures, HPLC, antiproliferation, Caco-2, HT-29, HCT 116

■ INTRODUCTION

Several epidemiological studies have shown an inverse association between the intake of fruits and vegetables and the risk of cancer, cardiovascular disease, and diabetes.[1−4] Furthermore, extracts rich in polyphenols from various fruits and berries have been shown to inhibit growth and induce apoptosis in a diverse range of human cancer cell lines.[5,6] One of the reasons for this protective effect is related to the polyphenols found in fruits and vegetables.[7,8] The bioavailability of the polyphenols differs greatly depending on their chemical structure. Smaller and less complex polyphenols such as gallic acid and isoflavones are more easily absorbed than larger and more complex polyphenols such as proanthocyanidins and anthocyanins.[9] Thus, the most abundant polyphenols in the human diet may not necessarily be those leading to the highest concentrations of active metabolites in the body. During food processing, complex polyphenols may be hydrolyzed[10] leading to smaller and less complex compounds that possibly are more bioavailable.

Black currant (*Ribes nigrum* L.) contains high levels of polyphenols, particularly anthocyanins, hydroxycinnamic acids, and flavonols.[11,12] Black currant is extensively used in juice manufacturing, where the peels and seeds of the black currant end up in press-residue. Since peels and seeds in fruits and berries are enriched with phenolic compounds, a large amount of the valuable polyphenols end up in press-residue instead of in the juice.[13,14] Thus, the press-residue is a promising source of polyphenols that can be extracted and added to the original juice in order to increase its concentration of polyphenols.

The yield of total phenols and anthocyanins are dependent on the extraction condition used. Typical extraction conditions that may influence the yield include the use of different enzymes, particle size of the sample, extraction temperature, extraction

solvent, number of extraction steps, solvent to solid ratio, and duration of extraction.[15−18] The most common organic solvents used in the extraction of polyphenols from berries are aqueous mixtures of ethanol, methanol, or acetone.[18] However, water or ethanol is preferred in the food industry because of the risk of methanol and acetone toxicity. The use of ultrasound-assisted extraction (UAE) can reduce the extraction time, decrease the extraction temperature, and increase the extraction yields.[19] Previously, it has been demonstrated that the use of pectinolytic enzymes and diminution of press-residue particles significantly enhanced the amount of polyphenols extracted from black currant press-residue.[17] Temperature, extraction time, and solvent to solid ratio have been shown to have a positive effect on the yield of total phenols in the extract from apple pomace.[20] However, the extraction time and temperature must be followed closely, as it has been shown that the extraction of polyphenols from black currant press-residue decreased with extended extraction time[21] and increasing the temperature resulted in reduced yield of anthocyanins from black currants.[16]

The aims of the current study were to (1) enhance the extractability of phenolic compounds from black currant press-residue using extraction conditions compatible with food use, (2) determine the antiproliferative effects of the extracts on human colon cancer cells in vitro, and (3) relate the antiproliferative effects to the phenolic composition of the extracts.

ACS Publications   © 2011 American Chemical Society

3632

dx.doi.org/10.1021/jf104427r | *J. Agric. Food Chem.* 2011, 59, 3632–3640

Table 1. Total Phenolics (TP) and Total Monomeric Anthocyanins (TMA) in Different Stages of Juice Processing of Black Currant

sample	TP (mg GAE/100 g of sample)	TMA (mg/100 g of sample)
black currant	625 ± 1	236 ± 2
mash	634 ± 79	194 ± 17
enzymatic treated mash	743 ± 77	267 ± 4
press-residue	2004 ± 17	686 ± 12
raw juice	478 ± 2	167 ± 18
clarified juice	575 ± 4	144 ± 6
filtered juice	449 ± 1	124 ± 11

■ MATERIALS AND METHODS

Chemicals and Enzymes. Gallic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Cyanidin- and delphinidin-3-O-β-glucopyranosides (glucosides) were purchased from Polyphenols Laboratories AS (Sandnes, Norway). Folin—Ciocalteu's phenol reagent, Triton X-100, and quercetin-3-rhamnosyl glucoside (rutin) were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid and chlorogenic acid were obtained from Fluka (Sigma-Aldrich, Buchs, Switzerland). Sodium carbonate, sodium acetate, potassium chloride, 2-propanol, acetone, methanol, acetontrile, and acetic acid were provided from Merck KGAa (Darmstadt, Germany). All solvents were of HPLC grade and water was of Milli-Q-quality (Millipore Corp., Bedford, MA). Dulbecco's Modified Eagle Medium (DMEM), McCoy's 5A Medium, fetal calf serum (FCS), nonessential amino acids, penicillin/streptomycin, and EDTA were purchased from Gibco (Invitrogen, Carlsbad, CA). MTT solution, the apoptose kit Cell Death Detection ELISA[PLUS], and Complete EDTA-free Protease Inhibitor Cocktail Tablets were obtained from Roche (Diagnostics, Mannheim, Germany). BCA protein assay was provided from Thermo Fisher Scientific Inc. (Waltham, MA), while hydrogen chloride (HCl) was obtained from Arcus Kjemi AS (Vestby, Norway). Rohapect 10L, rohapect DA6L, gamylozym AFL, and cellulase were purchased from AB Enzymes (GmbH, Darmstadt, Germany). Disodium hydrogen phosphate (Na_2HPO_4) was obtained from Chemi-Teknik AS (Oslo, Norway).

Plant Material. Black currants (*Ribus nigrum* L), a mix of the cultivars Ben Tron and Ben Nare, grown in Lier, Norway (59° 75′ N), were subjected to industrial juice processing at the berry processing company Findus (Lier, Norway). The berries were macerated, prior to a pectinolytic mash treatment at 40—50 °C using 0.02% rohapect 10L. The mash was pressed (HP 2500 and HP 3000, Bucher-Guyer), and the raw juice was clarified using 0.01% rohapect DA6L, 0.002% gamylozym AFL, and 0.005% rohapect 10L and then filtered. Samples were taken during black currant juice production (black currants, mash, enzymatic treated mash, press-residue, raw juice, clarified juice, and filtered juice) and frozen at −40 °C until further use. Frozen black currant press-residue (2 L) was homogenized (Combimax 700, Braun, Germany) before the sample was divided in portions (60 g), vacuum packed, and stored at −40 °C until used as raw material in further extraction trials.

The dry weight of black currants and press-residue was measured by the vacuum method[22] and was expressed as grams of dry matter per 100 grams of sample (g/100 g).

Extraction of Phenolic Compounds. To determine total content of phenolic compounds, the samples were extracted by organic solvents. Acetone and methanol were tested as the extraction solvents. More than 2-fold total phenolics were extracted from the black currant press-residue when acetone was used as the extraction solvent. Acetone was thus used for extraction. Frozen black currants, mash, enzymatic

treated mash, and black currant press-residue (2.5 g) pulverized in liquid nitrogen by an IKA-Universalmühle M20 (IKA Labortechnik, Staufen, Germany) was extracted with acetone (10 mL) by sonication (VWR Ultrasonic cleaner, Leuven, Malaysia, 45kHz) for 10 min. After centrifugation (4 °C, 1300g, 10 min, Heraus Multifuge 4 KR, Kendro Laboratory Products GmbH, Hanau, Germany), the supernatant was collected and the insoluble plant material re-extracted three times with 70% acetone (10 mL). Acetone was removed from pooled extracts by a nitrogen flow at room temperature (Sample concentrator, Techne, Stone, Staffordshire, U.K.) The volume of the extract was made up to 25 mL by water. The samples were extracted in duplicate.

Further extraction of polyphenols from black currant press-residue was performed under conditions compatible with food use, i.e., with water as extraction medium. In screening experiments several extractions treatments, i.e., enzymes (0.1% rohapect 10L or 0.015 or 0.15% cellulase), different temperatures (20, 55, 90 °C), sonication or water bath, and different extraction times (4, 10, 30, and 120 min) were tried. The extraction procedure was as follows: frozen black currant press-residue (5 g) was homogenized in water (15 mL) using a Polytron, PT 3100 homogenizer (Kineatica AG, Littaue, Switzerland), at speed 22000 rpm for 20 s before a selected treatment. After centrifugation (4 °C, 1300g, 10 min), the supernatant (juice 1) was collected and the insoluble plant material together with water (15 mL) was exposed to a second treatment prior to centrifugation and collection of the supernatant (juice 2). The volume of the extract was made up to 25 mL by water. The samples were extracted in duplicate.

On the basis of the results from the screening extraction experiments, different temperatures, use of ultrasound-assisted extraction, and different extraction times were combined in an experimental design. Frozen black currant press-residue (5 g) was homogenized (Polytron) in water (15 mL) for 20 s. The samples were extracted either for 4, 15, or 30 min at 20 °C, 40 °C, 55 °C, 80 °C, or 90 °C in a water bath (W) or an ultrasound bath (U) (see Table 1) before centrifugation (4 °C, 1300g, 10 min). Owing to the fact that ultrasound bath has a maximum temperature of 80 °C, 90 °C in combination with sonication was not tested. The yield of the juice was measured by weighing before the volume of the extract was made up to 25 mL by water. The samples were extracted in duplicate.

All the extracts were frozen (−80 °C) before analysis. The samples were filtered through a Millex HA 0.45 μm filter (Millipore Corp., Cork, Ireland) prior to HPLC analyses.

Analysis of Total Monomeric Anthocyanins and Total Phenolics. The pH differential absorbance method was used to determine content of total monomeric anthocyanins (TMA).[23] The absorbance of the samples was determined after 30 min reaction time and measured at both 520 and 700 nm (Agilent 8453 spectrophotometer, Agilent Technologies, Waldbronn Germany). The anthocyanin concentration in the samples was calculated as cyanidin-3-glucoside equivalents (mg/100 g of sample). To measure the total phenolic (TP) content the Folin—Ciocalteau method was used.[24] The absorption was measured at 765 nm after 60 min incubation at room temperature. TP content was expressed as gallic acid equivalents (GAE) in mg per 100 g of sample (mg of GAE/100 g of sample) or μg of GAE/mL extracts.

HPLC—DAD—ESI—MS^n Analyses of Anthocyanins. The analyses were carried out on an Agilent 1100 Series HPLC system (Agilent Technologies) equipped with an autosampler cooled to 6 °C, a DAD (190—600 nm), and an MSD XCT ion trap mass spectrometer fitted with an ESI interface. The anthocyanins were separated on a Betasil C18-column (250 mm × 2.1 mm i.d, 5 μm particles) equipped with a 5 μm C18 guard column (4.0 mm × 2.1 mm i.d.), both from Thermo Hypersil-Keystone (Bellefonte, PA). The separation was executed with mobile phases consisting of A; formic acid/water (5/95, v/v) and B; formic acid/acetonitrile (5/95, v/v) with the following gradient elution: 0—2 min 10% B, 2—17 min 10—20% B, 17—21 min 20—60% B, 21—25 min 60%B, 25—27 min 60—10% B. The column was allowed to

3633

dx.doi.org/10.1021/jf104427r |*J. Agric. Food Chem.* 2011, 59, 3632–3640

equilibrate for 5 min between injections (5 μL). The column temperature was held at 40 °C and the solvent flow rate was 0.25 mL/min.

After UV−vis detection the effluent was introduced directly, without splitting, to the ESI interface where ionization in positive mode was performed. The nebulizer pressure was 40 psi; dry gas flow, 10 L/min; dry temperature 350 °C; and capillary voltage 3.5 kV. Ions with m/z 100 to 2000 were measured, with a scan speed of 27000 amu/s. Fragmentation (MS^{2-3}) was carried out in the automatic mode; that is, the two most abundant ions in MS^{1-2} were fragmented. The fragmentation was performed with 50% energy (0.85 V) with helium as the collision gas.

The anthocyanins in the samples were identified based on their UV−vis spectra (190−600 nm), mass spectra, and retention times relative to external standards, and comparison with literature reports on anthocyanins in black currant.[11,15,25−27]

Cyanidin and delphinidin glycosides in the samples were quantified by external standards of cyanidin-3-glucoside and delpinidin-3-glucoside, respectively, with absorbance measured at 520 nm.

HPLC−DAD−ESI−MS[n] Analyses of Phenolic Compounds Other Than Anthocyanins.

The HPLC instrument and chromatographic column was the same as used for analysis of anthocyanins. The separation of the phenolic compounds was executed with mobile phases consisting of A, acetic acid/water (2/98, v/v), and B, acetic acid/acetonitrile (2/98, v/v), with the following gradient: 0−5 min 0% B, 5−14 min 0−12% B, 14−40 min 12−20% B, 40−45 min 20−80% B, 45−50 min 80% B, 50−52 min 80−0% B. The column was allowed to equilibrate for 7 min between injections (20 μL). The column temperature was held at 30 °C, and the solvent flow rate was 0.25 mL/min.

The HPLC effluent was directed to the ESI interface, where the phenolic compounds were analyzed in both negative and positive mode. The ionization and fragmentation parameters were as described for the analyses of anthocyanins. The phenolic compounds were identified and classified based on their UV−vis spectra (190−600 nm), mass spectra, and retention times relative to external standards, and comparison with literature reports.[11,13,25] The phenolic compounds were classified and quantified by external standards. The hydroxycinnamic acids were quantified as chlorogenic acid (at 320 nm) and the flavonols as rutin (at 360 nm).

Measurement of Cell Proliferation.

The Caco-2 and HT-29 cell lines were a generous gift from Professor Tor Lea, Norwegian University of Life Sciences. The HCT 116 cell line was a generous gift from Dr. Gunhild Mælandsmo, Dept. of Tumor Biology, the Norwegian Radium Hospital. All three cell lines originate from colorectal adenocarcinomas and were originally obtained from the American Type Tissue Collection (Rockville, MD). Caco-2 cells were grown in DMEM, containing 20% FCS, 1% of nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. The HT-29 was grown in DMEM, containing 10% FCS, 1% of nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. HCT 116 cells were cultured in McCoy's 5A medium containing 10% serum and 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37 °C and 5% CO_2 atmosphere in a humidified incubator.

The MTT assay was used to measure cell proliferation. Cells in growth media were seeded out in transparent 96-well cell culture plates. After 24 or 48 h of incubation at 37 °C in a 5% CO_2 atmosphere, the growth medium was replaced with 100 μL of medium containing 0, 20, 75, 100, or 125 μg GAE/mL black currant press-residue extracts obtained from extraction at 40 °C, 55 °C, 80 °C, and 90 °C for 30 min. Triplets of each concentration were added to the plates. At the end of the incubation (24 h), the cells were treated with MTT solution (15 μL) followed by incubation at 37 °C for 2 h. The cell proliferation rate was determined by the ability of the metabolic active cells to cleave tetrazolium sodium salt to purple formazan crystals.[28] After removal of the medium, the purple precipitate in each cell was dissolved in 100 μL of 0.04 M HCl in 2-propanol, and the absorbance was measured at

562 nm by a Titertek Multiscan plus MK II plate reader (Labsystems, Finland). The MTT-experiments were repeated three independent times.

The HT-29 cell line was analyzed for apoptosis using the Cell Death Detection ELISA[PLUS] assay. This assay is a photometric enzyme-immunoassay that measures cytoplasmic histone-associated DNA fragments that occur during apoptosis. The assay is a quantitative sandwich-enzyme-immunoassay using monoclonal mouse antibodies directed against DNA and histones that specifically detect mono- and oligo-nucleosomes.[29] Black currant press-residue extracts obtained from extraction at 40 and 90 °C for 30 min were mixed with growth medium to the concentrations of 20, 75, or 100 μg GAE/mL, added in parallel to the plate, and incubated for 24 h. The procedure was performed in accordance with the manufacturer's instructions.[29] Absorbance at 405 nm was measured by the Titertek Multiscan plus MK II plate reader.

The BCA protein assay was performed in parallel with the apoptosis experiment in order to correlate the protein quantity, and thereby the amount of cells, with signals generated in the Cell Death Detection ELISA[PLUS] assay. The same types of extracts and concentrations were used as in the apoptosis experiment. The samples were analyzed in parallel. After incubation for 24 h, the cells were first washed with ice-cold PBS and lysed for 10 min on ice with 100 μL of lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, 1% Triton X-100, pH 7.4; Complete EDTA-free Protease Inhibitor Cocktail were added to the lysis buffer before use, according to the manufacturer's instructions). The lysate from each well was transferred to microtubes and centrifuged at 14 000 rpm and 4 °C for 5 min. The supernatant was diluted three times with dH_2O and used further in the BCA protein assay. The procedure of the BCA protein assay was performed in accordance to the manufacture's instructions.[30] The Titertec Multiscan plus MK II plate reader was used to measure the absorbance.

Statistics.

The data from the screening experiments and experimental design were analyzed with one-way ANOVA, using the statistical program MINITAB 15 (Minitab Inc., State College, PA). In addition, in the experimental design, juice yield, TP, and TMA concentrations in response to different extraction conditions (temperature, extraction time, and sonication) were evaluated by a multiple regression model using MINITAB 15. Significance of all the results was established at $p \leq 0.05$.

■ RESULTS AND DISCUSSION

Phenolic Content and Mass Balance during Black Currant Juice Processing.

TP and TMA were measured in all the stages of black currant juice processing in order to get an overview for where the phenolic compounds ended up. As shown in Table 1, the amount of TP and TMA in press-residue was approximately 3 fold higher than in the berries, and approximately 4 fold higher than in the raw juice. The press-residue consisted of peels, seeds, branches, and some remaining flesh of the berries and had a higher concentration of dry matter (41.4%) than the berries (21.9%). If the results were calculated on a dry weight basis, the concentration of polyphenols (TP) in press-residue was still 1.7 fold higher than in the berries, demonstrating that the peels and seeds of black currant were especially rich in polyphenolic compounds.

The yield of raw juice was 75%, which meant that about 57% of the phenolic compounds originally present in the berries ended up in the raw juice. The remaining 43% was mainly found in press-residue and thus lost during the present manufacturing process.

Extraction of Phenolic Compounds from Black Currant Press-Residue.

One of the aims of the current study was to optimize the extraction of phenolic compounds, measured as TP

3634

dx.doi.org/10.1021/jf104427r |*J. Agric. Food Chem.* 2011, 59, 3632–3640

Table 2. Combinations of Temperature, Extraction Time, Water Bath (W)/Ultrasound (U) Bath in the Experimental Design

expt no.	temp (°C)	time (min)	water bath (W)/ultrasound bath (U)
1	20	4	W
2	20	4	U
3	20	15	W
4	20	15	U
5	20	30	W
6	20	30	U
7	40	4	W
8	40	4	U
9	40	15	W
10	40	15	U
11	40	30	W
12	40	30	U
13	55	4	W
14	55	4	U
15	55	15	W
16	55	15	U
17	55	30	W
18	55	30	U
19	80	4	W
20	80	4	U
21	80	15	W
22	80	15	U
23	80	30	W
24	80	30	U
25	90	4	W
26	90	15	W
27	90	30	W

and TMA, from the black currant press-residue. In a preliminary study the influence of different extraction conditions, i.e., temperature, duration of extraction, number of extraction steps, use of pectinase or cellulase, and sonication, was evaluated. The screening showed that two consecutive extraction steps, high temperatures, and longer extraction time increased the amout of TMA, while the use of enzymes gave no difference in the yields of TMA (data not shown). During the processing of black currant juice, the press-residue was subjected to an enzymatic treatment. This may explain why the enzymes used in this screening had no effect on the extraction of phenolic compounds from the black currant press-residue, in contrast to the findings of Landbo and Meyer.[17] The effect of sonication to increase the yield of TMA and TP was ambiguous and had to be further investigated.

On the basis of results from the screening experiments, the effect of different temperatures, extraction times, and use of sonication on juice yield and the amount of TP and TMA in the extracts from black currant press-residue were tested in an experimental design (Table 2). The juice yield, TP, and TMA concentrations varied in response to the different treatments in the experimental design. Juice yields ranged from 33.9% to 48.2% (Figure 1A). The juice yield would have probably been higher with a higher solvent to solid ratio.[16,20] However, if the extracts shall be used in foods (or dietary supplements), there is an advantage to have a lower solvent to solid ratio, due to the process required to remove water to obtain higher concentrations of polyphenols in the extracts. TP in the extracts were in the range
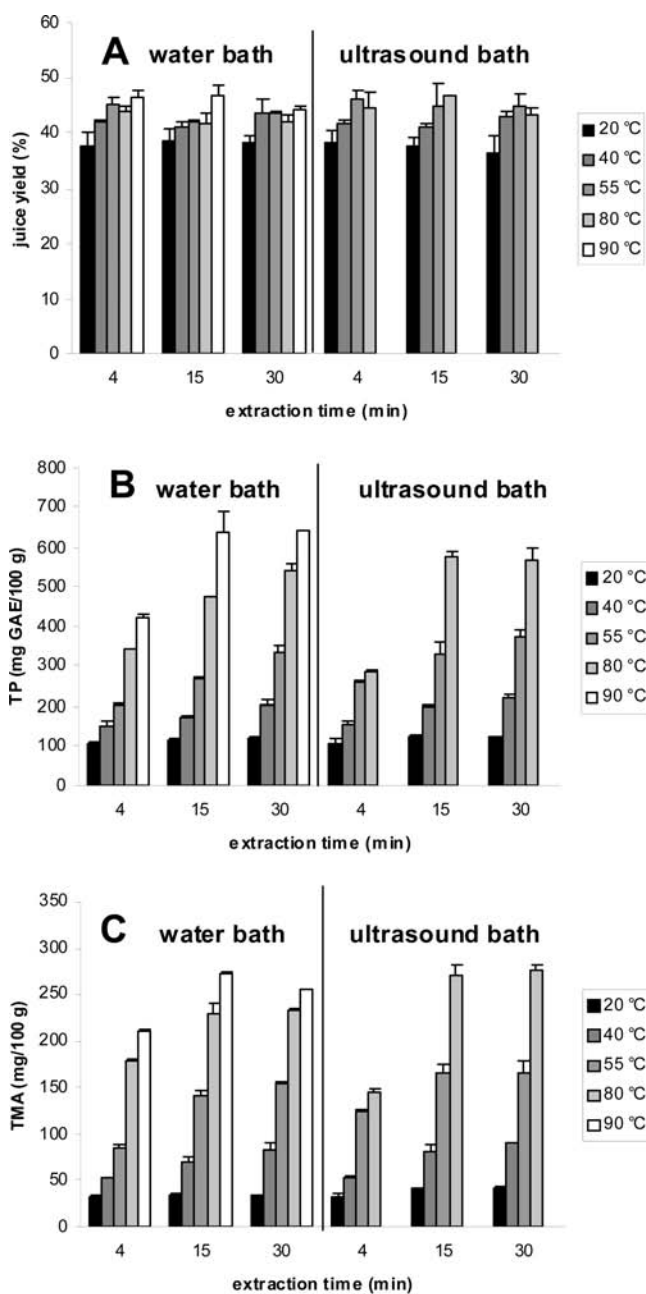

Figure 1. Juice yield (%) (A), total phenolics (TP) (mg GAE/100 g of press-residue) (B), and total monomeric anthocyanins (TMA) (mg/100 g of press-residue) (C) in aqueous extracts of black currant press-residue obtained after extraction for 4, 15, and 30 min at 20 °C, 40 °C, 55 °C, 80 °C, and 90 °C in either a water bath (left) or an ultrasound bath (right). The data are based on the average of two parallel analyses.

101−673 mg GAE/100 g of fresh press-residue (Figure 1B), which corresponded to 218−1295 mg GAE/L extract, while the TMA in the extracts were 31−295 mg/100 g of fresh press-residue (Figure 1C), which correspond to 67−567 mg/L extract. The extracts contained 5−34% of the TP originally present in press-residue (2004 mg GAE/100 g of fresh press-residue, obtained by extraction with acetone), while 5−43% of TMA originally present in press-residue (686 mg/100 g of fresh press-residue) were extracted.

Table 3. Effects of Extraction Parameters and Their Interactions on Total Monomeric Anthocyanins(TMA), Total Phenolics (TP), and Juice Yield of Aqueous Extracts of Black Currant Press-Residue Evaluated by a Multiple Regression Model

parameters and interactions	TMA (mg/100 g of press-residue)		TP (mg GAE/100 g of press-residue)		juice yield (%)	
	coeff	p-value	coeff	p-value	coeff	p-value
temperature	1.926	0.009	−0.284	0.824	0.394	<0.0001
extraction time	4.684	0.001	6.67	0.011	−0.02	0.883
temperature × extraction time	0.037	0.001	0.132	<0.0001	−0.001	0.246
temperature × temperature	0.007	0.277	0.042	<0.0001	−0.003	<0.0001
extraction time × extraction time	−0.145	<0.0001	−0.269	<0.0001	0.002	0.659
constant/intercept	−49.88	0.019	44.63	0.237	30.87	<0.0001
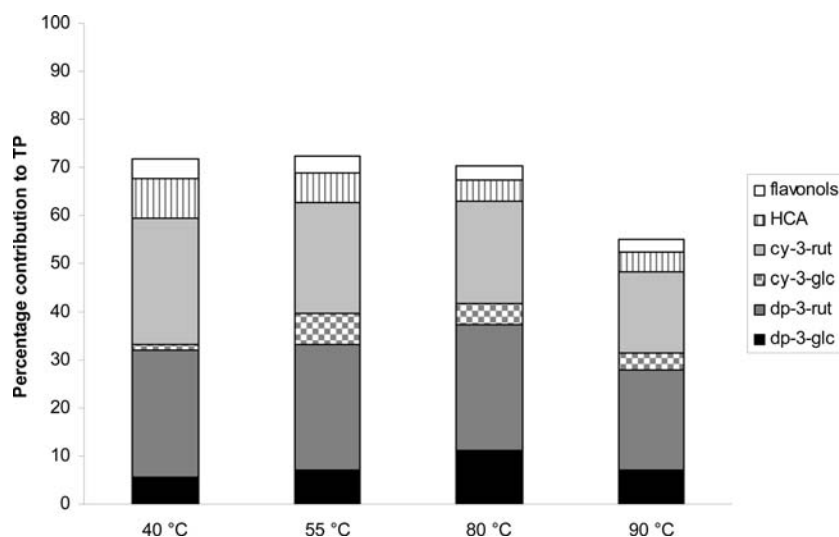
Figure 2. Percentage contribution to total phenolics (TP) of flavonols, hydroxycinnamic acids (HCA), cyanidin 3-rutinoside (cy-3-rut), cyanidin 3-glucoside (cy-3-glc), delphinidin 3-rutinoside (dp-3-rut), and delphinidin 3-glucoside (dp-3-glc) in aqueous extracts of black currant press-residue obtained after extraction for 30 min at 40 °C, 55 °C, 80 °C, and 90 °C. The phenolic compounds were identified and quantified by HPLC−DAD−MS.

In order to understand and show the significant effects of the extraction parameters, juice yield and TP and TMA concentrations in response to the different extraction conditions (temperature, extraction time, and sonication) were evaluated by a multiple regression model. A first-order multiple regression model was chosen so that estimates of selected second-order terms or combinations of them could be isolated. With respect to juice yield and TP and TMA concentrations, no significant effect of the ultrasound bath versus the water bath was found (data not shown). Thus, the parameters in the multiple linear regression with interactions were temperature, extraction time, temperature × extraction time, temperature × temperature, and extraction time × extraction time. Multiple linear regression analysis of the data showed that an increase in the temperature significantly increased the juice yield within the factor levels tested (Table 3). In addition, juice yield was curvilinear related (temperature × temperature) to temperature, meaning that the juice yield increased with increased temperature up to a point before the yield decreased or leveled out. As seen in Figure 1A, this temperature was about 55 °C. Both the TP and TMA concentrations increased with longer extraction time, while increased temperature only affected the contents of TMA positively. Although increased extraction time was the only factor which significantly increased the levels of TP, an interaction between temperature and extraction time had a significant effect on the level of TP. This

interaction also had an effect on the TMA levels. This means that the effect of extraction time on concentration of TP and TMA were dependent on extraction temperature. When 20 °C was used as the extraction temperature, duration of extraction had no effect on the yield of TP and TMA (Figure 1B,C). However, when higher temperatures were used during extraction, longer extraction time had a positive effect on the concentration of TP and TMA. It was also a significant curvilinear relationship between extraction time (extraction time × extraction time) and TMA yield, meaning that TMA yield increased with increasing extraction time to a certain point. As illustrated in Figure 1C, this duration time was about 15 min. A previous study on water extraction of polyphenols from black currant press-residue also showed that concentration of total anthocyanins decreased with longer extraction time, indicating that black currant press-residue contains unstable components.[21] Significant curvilinear relationships between both extraction time (extraction time × extraction time) and temperature (temperature × temperature), and TP concentration were also found. The curvilinear relationships may indicate a decomposition of the complex polyphenols in extracts obtained by more harsh treatment, i.e., high temperatures and long extraction time. According to other literature, the temperature during extraction affects the stability of the compound because of thermal decomposition,[31] and it has previously been shown that extraction
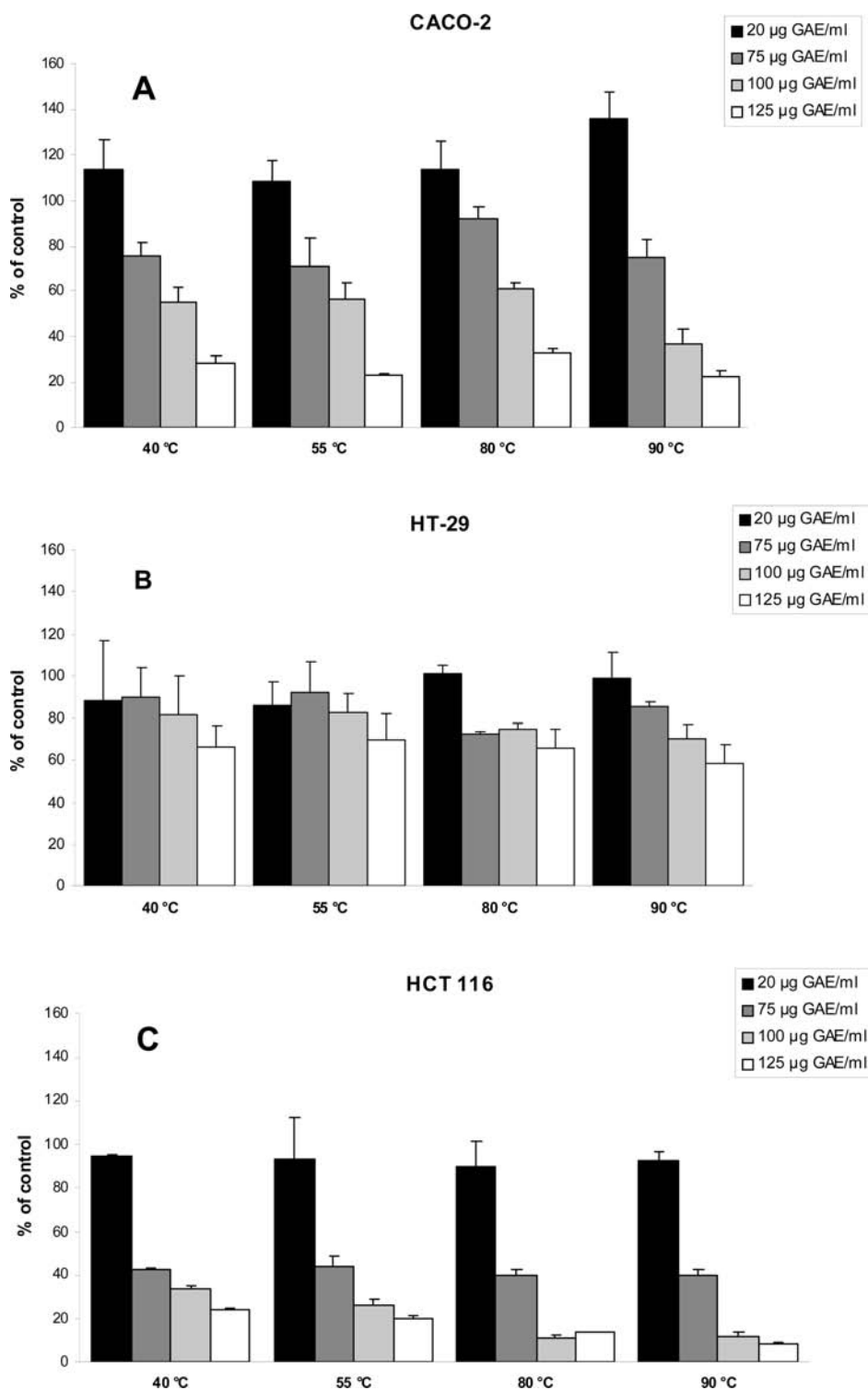
Figure 3. Effects on cell proliferation (MTT assay) of Caco-2 (A), HT-29 (B), and HCT 116 (C). The cells were incubated with different concentrations (20, 75, 100, and 125 μg GAE/mL) of aqueous black currant extracts obtained after extractions for 30 min at 40 °C, 55 °C, 80 °C, and 90 °C in cell culture medium for 24 h before cell proliferation was measured. Results are expressed as % absorbance of cells treated with black currant press-residue extracts compared to the control. The graphs represent the results of a typical experiment performed in triplicates.

temperatures beyond about 35 °C gave degradation of anthocyanins in black currants.[16] Within the limits of the factors tested, the highest juice yield and concentrations of TP and TMA were obtained with 90 °C extraction temperature and 15 min extraction time (Figure 1). However, there was no significant difference

in the concentrations of TP and TMA in extracts obtained at 90 °C for 15 and 30 min. Furthermore, although the highest yield of TP and TMA was obtained when the extraction temperature was 90 °C and extraction duration 15 min, the other extracts obtained at 40 °C, 55 °C, and 80 °C had the highest yield of TP
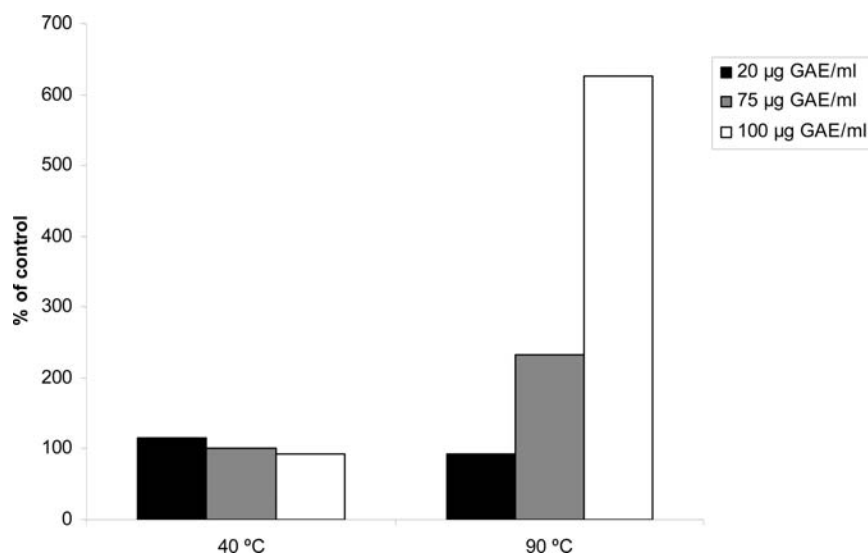
Figure 4. Effect on apoptosis from extracts of black currant press-residue obtained at 40 °C and 90 °C for 30 min. HT-29 cells were incubated with different concentrations (20, 75, and 100 μg GAE/mL) in cell culture medium for 24 h before measuring protein content and cytoplasmic histone-associated DNA fragments after induced cell death. The graph presents data from the Cell Death Detection ELISA[PLUS] assay related to the data from the protein content. The graph represents the results of a typical experiment performed in triplicates.

and TMA when 30 min was used as extraction duration. Thus, extracts obtained after 30 min extraction were used in the further studies.

Profile of Anthocyanins and Other Polyphenols. The four main anthocyanins in the black currants used for juice processing were delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside, contributing 11.8%, 38.8%, 7.6%, and 39.5% to the total anthocyanin content, respectively. This is in agreement with previous measurements of the anthocyanin distribution in black currant berries.[15,26]

In the extracts obtained from the experimental design, percentage contribution to the total anthocyanin content of delphinidin-3-glucoside varied between 2.7% and 20.1%, delphinidin-3-rutinoside varied between 36.6% and 47.8%, cyanidin-3-glucoside varied between 3.9% and 7.5%, and cyanidin-3-rutinoside varied between 34.1% and 46.3%. The values are in the range previously found in extracts from black currant press residue,[32] however with slightly higher contents of cyanidin-3-rutinoside in the present study. Flavonols and hydroxycinnamic acids (HCA) were the most abundant among other polyphenols. Percentage contribution to TP of flavonols varied between 2.6 and 4.6%, while HCA varied between 3.9 and 10.5%. Figure 2 shows the percentage contribution to TP of flavonols, HCA, cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside, and delphinidin-3-glucoside in extracts from black currant press-residue obtained by extraction for 30 min at 40 °C, 55 °C, 80 °C, or 90 °C. In extracts obtained at 40–80 °C, the percentage contribution to TP of all the above-mentioned polyphenols was about 70%. In extracts obtained at 90 °C, however, the contribution of these compounds had decreased to about 55%, which means that new unidentified compounds occurred in these extracts and made a considerable contribution to TP. In extracts obtained after 30 min, the percentage of delphinidin-3-rutinoside had a negative linear correlation with temperature, while delphinidin-3-glucoside had a positive correlation with temperature, disregarding the extract obtained at 90 °C. The same trend was shown in the percentage contribution to TP of cyanidin-3-rutinoside and cyanidin-3-glucoside. However, the maximum amount of cyanidin-3-glucoside in

the extracts was obtained at 55 °C. The results may indicate that the disaccharides (rutinosides) were decomposed to monosaccharides (glucosides) at higher extraction temperatures. Percentage contributions of both flavonols and HCA to TP had a negative correlation with the extraction temperatures.

Antiproliferative Effects of the Extracts on Cancer Cells. It has previously been demonstrated that black currant press-residue from juice production contains high concentrations of phenolic compounds,[13,17,21,32] and that the extracts from black currants inhibit the proliferation of different cancer cell lines.[5] However, the antiproliferative effects of black currant press-residue extracts obtained under different conditions, and how the effects are related to the phenolic composition of the extracts, have not been studied. Thus, black currant press residue extracts obtained after 30 min extraction time at 40 °C, 55 °C, 80 °C, and 90 °C were tested for their ability to inhibit the proliferation of the human colon cancer cell lines Caco-2, HT-29, and HCT 116 (Figure 3). All the extracts showed a dose-dependent inhibition of the cell proliferation in all three cell lines. This is in agreement with previous findings showing that black currant extract is able to inhibit cancer cell proliferation.[5,33] Interestingly, the black currant press-residue extracts inhibited the cell proliferation of the three cell lines in various degrees. The cell proliferation of HT-29 cells was on average inhibited to a lower extent than the cell proliferation of Caco-2 and HCT 116 cells. Furthermore, press-residue extracts obtained at 90 °C generally gave a stronger inhibition of the cell proliferation at the highest concentrations compared to extracts obtained at lower temperatures. In Caco-2 cells, 100 μg GAE/mL extract obtained at 90 °C inhibited the cell proliferation 1.6–1.9 fold more than extracts obtained at 40 °C, 55 °C, and 80 °C. This trend was also observed in HT-29 (1.1–1.5 fold) and HCT 116 cells (1.0–2.8 fold). The increased effect of extracts obtained at 90 °C on cell proliferation may be due to the decomposition of the complex polyphenols originally present, making them more accessible to the cells. Previously, it has been shown that glycosylation of phenolic compounds gave restrictive cellular antioxidant activity and more nonpolar compounds had a higher permeability coefficient.[34,35]

3638

dx.doi.org/10.1021/jf104427r |*J. Agric. Food Chem.* 2011, 59, 3632–3640

Extracts obtained at 40 °C and 90 °C were analyzed for induction of apoptosis using HT-29 cells. The extract obtained at 90 °C showed an increased induction of apoptosis compared to the extract obtained at 40 °C, indicating that the reduced cell proliferation is due to apoptosis in the HT-29 cells (Figure 4). The extract obtained at 40 °C did not show any effect on apoptosis with increasing concentrations, probably due to an induction below detection level.

The present study suggests an effective and environmentally friendly extraction procedure that can be transferred to industrial scale, giving extracts that can be used, for example, in food products enriched with natural phenolic compounds. By simple water extraction at 90 °C, 34% of the polyphenols in press-residue can be extracted. In addition, extracts obtained at 90 °C gave a stronger inhibition of the cell proliferation of three different cancer cell lines compared to extracts obtained at lower temperatures.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: + 47 64970468. Fax: + 47 64970333. E-mail: linda.holtung@nofima.no.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; BCA, bicinchoninic acid; DAD, diode array absorbance detector; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FCS, fetal calf serum; GAE, gallic acid equivalents; HCA, hydroxycinnamic acid; HCl, hydrogen chloride; HPLC, high performance liquid chromatography; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TMA, total monomeric anthocyanins; TP, total phenolic; UV—vis, ultraviolet—visible light.

■ REFERENCES

(1) WHO 2003. *Diet, nutrition and the prevention of chronic diseases*; WHO: Geneva, 2003.

(2) Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *134*, 1106–1114.

(3) Riboli, E.; Norat, T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am. J. Clin. Nutr.* **2003**, *78*, 559S–569S.

(4) Bazzano, L. A.; He, J.; Ogden, L. G.; Loria, C. M.; Vupputuri, S.; Myers, L.; Whelton, P. K. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first national health and nutrition examination survey epidemiologic follow-up study. *Am. J. Clin. Nutr.* **2002**, *76*, 93–99.

(5) Olsson, M. E.; Gustavsson, K. E.; Andersson, S.; Nilsson, A.; Duan, R. D. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. *J. Agric. Food Chem.* **2004**, *52*, 7264–7271.

(6) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.

(7) Hertog, M. G. L.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S.; Pekkarinen, M.; Simic, B. S.; Toshima, H.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B. Flavonoid intake and long-term risk of coronary-heart-disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381–386.

(8) Arts, I. C.; Hollman, P. C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81*, 317S–325.

(9) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242.

(10) Zafrilla, P.; Ferreres, F.; Tomas-Barberan, F. A. Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. *J. Agric. Food Chem.* **2001**, *49*, 3651–3655.

(11) Määttä, K. R.; Kamal-Eldin, A.; Törrönen, A. R. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes species*. *J. Agric. Food Chem.* **2003**, *51*, 6736–6744.

(12) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; Gonzalez-Paramas, A. M.; Törrönen, A. R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477–4486.

(13) Sandell, M.; Laaksonen, O.; Järvinen, R.; Rostiala, N.; Pohjanheimo, T.; Tiitinen, K.; Kallio, H. Orosensory profiles and chemical composition of black currant (*Ribes nigrum*) juice and fractions of press residue. *J. Agric. Food Chem.* **2009**, *57*, 3718–3728.

(14) Schieber, A.; Stintzing, F. C.; Carle, R., By-products of plant food processing as a source of functional compounds - recent developments. *Trends Food Sci. Technol.* **2001**, *12*, 401.

(15) Buchert, J.; Koponen, J. M.; Suutarinen, M.; Mustranta, A.; Lille, M.; Törrönen, R.; Poutanen, K. Effect of enzyme-aided pressing on anthocyanin yield and profiles in bilberry and blackcurrant juices. *J. Sci. Food Agric.* **2005**, *85*, 2548–2556.

(16) Cacace, J.; Mazza, G. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *J. Food Sci.* **2003**, *68*, 240–248.

(17) Landbo, A. K.; Meyer, A. S. Enzyme-assisted extraction of antioxidative phenols from black current juice press residues (*Ribes nigrum*). *J. Agric. Food Chem.* **2001**, *49*, 3169–3177.

(18) Escribano-Bailón, M. T.; Santos-Buelga, C., Polyphenol extraction from foods. In *Methods in Polyphenol Analysis*; Santos-Buelga, C.; Williamson, G., Eds. The Royal Society of Chemistry: Cambridge, 2003; pp 1—16.

(19) Ma, Y.-Q.; Ye, X.-Q.; Fang, Z.-X.; Chen, J.-C.; Xu, G.-H.; Liu, D.-H. Phenolic compounds and antioxidant activity of extracts from ultrasonic treatment of satsuma mandarin (*Citrus unshiu* Marc.) peels. *J. Agric. Food Chem.* **2008**, *56*, 5682–5690.

(20) Çam, M.; Aaby, K. Optimization of extraction of apple pomace phenolics with water by response surface methodology. *J. Agric. Food Chem.* **2010**, *58*, 9103–9111.

(21) Lapornik, B.; Prosek, M.; Golc Wondra, A. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food. Eng.* **2005**, *71*, 214–222.

(22) Bøgh-Sørensen, L. Dry matter in foodstuffs. The vacuum method. *Nordic Committee on Food Analysis*; Espoo, Finland, 2002; Vol. 169

(23) Giusti, M. M.; Wrolstad, R. E., Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons, Inc.: New York, 2001; Unit F1.2.1—F1.2.13.

(24) Aaby, K.; Skrede, G.; Wrolstad, R. E. Phenolic composition and antioxidant activities in flesh and achenes of strawberries (*Fragaria ananassa*). *J. Agric. Food Chem.* **2005**, *53*, 4032–4040.

(25) Anttonen, M. J.; Karjalainen, R. O. High-performance liquid chromatography analysis of black currant (*Ribes nigrum* L.) fruit phenolics grown either conventionally or organically. *J. Agric. Food Chem.* **2006**, *54*, 7530–7538.

(26) Slimestad, R.; Solheim, H. Anthocyanins from black currants (*Ribes nigrum* L.). *J. Agric. Food Chem.* **2002**, *50*, 3228–3231.

(27) Wu, X. L.; Gu, L. W.; Prior, R. L.; McKay, S. Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, *Aronia*, and *Sambucus* and their antioxidant capacity. *J. Agric. Food Chem.* **2004**, *52*, 7846–7856.

(28) Vistica, D. T.; Skehan, P.; Scudiero, D.; Monks, A.; Pittman, A.; Boyd, M. R. Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Res.* **1991**, *51*, 2515–2520.

(29) Roche, Cell Death Detection ELISA[PLUS], 2006.

(30) ThermoScientific, Pierce BCA Protein Assay Kit, 2007.

(31) Moure, A.; Cruz, J. M.; Franco, D.; Domínguez, J. M.; Sineiro, J.; Domínguez, H.; José Núñez, M.; Parajó, J. C. Natural antioxidants from residual sources. *Food Chem.* **2001**, *72*, 145–171.

(32) Kapasakalidis, P. G.; Rastall, R. A.; Gordon, M. H. Extraction of polyphenols from processed black currant (*Ribes nigrum* L.) residues. *J. Agric. Food Chem.* **2006**, *54*, 4016–4021.

(33) McDougall, G. J.; Ross, H. A.; Ikeji, M.; Stewart, D. Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown in vitro. *J. Agric. Food Chem.* **2008**, *56*, 3016–3023.

(34) Tian, X.-J.; Yang, X.-W.; Yang, X.; Wang, K. Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model. *Int. J. Pharm.* **2009**, *367*, 58–64.

(35) Wolfe, K. L.; Kang, X. M.; He, X. J.; Dong, M.; Zhang, Q. Y.; Liu, R. H. Cellular antioxidant activity of common fruits. *J. Agric. Food Chem.* **2008**, *56*, 8418–8426.

3640

dx.doi.org/10.1021/jf104427r |*J. Agric. Food Chem.* 2011, 59, 3632–3640