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ARTICLE

Effect of a Cellulase Treatment on Extraction of Antioxidant Phenols from Black Currant (*Ribes nigrum* L.) Pomace

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The effect of a commercial cellulase preparation on phenol liberation and extraction from black currant pomace was studied. The enzyme used, which was from *Trichoderma* spp., was an effective "cellulase−hemicellulase" blend with low β-glucosidase activity and various side activities. Enzyme treatment significantly increased plant cell wall polysaccharide degradation as well as increasing the availability of phenols for subsequent methanolic extraction. The release of anthocyanins and other phenols was dependent on reaction parameters, including enzyme dosage, temperature, and time. At 50 °C, anthocyanin yields following extraction increased by 44% after 3 h and by 60% after 1.5 h for the lower and higher enzyme/substrate ratio (E/S), respectively. Phenolic acids were more easily released in the hydrolytic mixture (supernatant) and, although a short hydrolysis time was adequate to release hydroxybenzoic acids (HBA), hydroxycinnamic acids (HCA) required longer times. The highest E/S value of 0.16 gave a significant increase of flavonol yields in all samples. The antioxidant capacity of extracts, assessed by scavenging of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation, the oxygen radical absorbance capacity, and the ferric reducing antioxidant potential depended on the concentration and composition of the phenols present.

KEYWORDS: Black currant; *Ribes nigrum* L.; pomace; cellulase; anthocyanins; flavonoids; phenolic acids; cell wall; antioxidant capacity; antioxidants; polyphenols

INTRODUCTION

The growing interest in the exploitation of bioactive constituents, especially flavonoids and phenolic acids, from processed plant residues has encouraged research on the application of cell wall hydrolyzing enzymes to various agricultural byproducts. Black currants and press residues from black currant juice manufacture are rich sources of anthocyanins and other antioxidants and, therefore, constitute useful sources of these compounds for their further application as functional food additives (*1*, *2*). In our recent published work (*2*) we identified, by RP-HPLC, the major phenol classes (predominantly anthocyanins, as well as flavonols and hydroxycinnamic acid derivatives) in black currant skins and related byproducts and demonstrated that black currant pomace had a significant portion of "tightly bound" antioxidant phenols which could not be easily extracted with the use of organic solvents. For this reason, we decided to follow a series of experiments using commercially available enzymes for enabling polyphenols, anthocyanins, and antioxidant activity release from black currant press residues.

In this study, we investigated the effect of a multiactivity cellulolytic preparation on antioxidant phenol and anthocyanin release from black currant pomace (the press residue produced during black currant juice processing, consisting of skins, pulp, seeds, and stalks) prior to extraction with acidified methanol, which was chosen as a good solvent for the extraction of anthocyanins and other groups of phenolics present in black currants. Our main objective was to enhance cell wall degradation from black currant pomace by including a "cellulase-assisted" hydrolysis step as an essential treatment for the production of polyphenol-rich extracts that could be further processed for the manufacture of dietary supplements or food additives. To optimize the enzyme-mediated extraction procedure, certain enzyme reaction parameters, such as enzyme concentration, hydrolysis time, and temperature, were investigated. Finally, the antioxidant activity of all hydrolysates (supernatants) and phenolic extracts was determined.

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MATERIALS AND METHODS

Plant Material and Sample Preparation. Pomace byproduct from black currant juice processing was supplied frozen by GlaxoSmithKline (Coleford, U.K.). The sample contained mainly skins, seeds, and stems. The pomace was freeze-dried, purged with nitrogen, and kept frozen (−18 °C). Freeze-dried black currant pomace was initially milled, in an APEX-Comminuting Mill (type 214, Apex Construction Ltd., London, U.K.), for 30 s and passed through a 610 μm aperture sieve. The seeds and bulky wooden particles were removed, and pomace was homogenized in a coffee grinder. A sample with a particle size range of 125 − 300 μm was obtained by using a two-stage sieving tower with aperture sizes of 125 and 300 μm. The sample was packaged under nitrogen and stored at −18 °C until needed.

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Chemicals and Reagents. 3,5-Dinitrosalicyclic acid (DNS), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri[2-pyridyl]-*s*-triazine (TPTZ), ferric chloride, potassium persulfate, 2,2′-azobis(2-amidopropane) dihydrochloride (AAPH), fluorescein disodium salt, ±6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), quercetin-3-rutinoside (rutin), caffeic acid, gallic acid, formic acid, bovine serum albumin (BSA), substrates, reagents, and sugar standards used for enzyme activity and protein determinations were purchased from Sigma-Aldrich (Poole, U.K.). Folin−Ciocalteu reagent and buffer salts were from BDH (Lutterworth, U.K.). Cyanidin-3-rutinoside was donated by Dr. Christina Garcia-Viguera (CEBAS-CSIC, Murcia, Spain). Cyanidin-3-glucoside, delphinidin-3-glucoside, and delphinidin-3-rutinoside (in the form of chloride salts) were purchased from Polyphenols Laboratories (Norway). Cellulase 13 L (C013L) liquid enzyme preparation from *Trichoderma reesei* was supplied by Biocatalysts (Pontypridd, U.K.). According to the supplier′s specification sheets, the working pH lies between 3.5 and 6.0 and the optimum temperature ranges from 40 to 70 °C. This enzyme preparation was selected for the study after preliminary testing of various commercial cellulases. All solvents were supplied by Rathburn Chemical Co. (Walkerburn, U.K.) and were of HPLC grade.

Enzyme Activity Assays and Basic Kinetic Parameters. The conditions of pH 4.6 (using 0.05 M phosphate/citrate buffer) and temperature of 40 °C for the various assays of enzyme activity were selected according to the manufacturer′s recommendation and because these conditions were selected for enzyme hydrolysis of black currant pomace prior to extraction. Protein content and reducing sugar levels of C013L enzyme preparation were determined according to the Lowry method (*3*) and the DNS assay (*4*), respectively. Endoglucanase (endocellulase), exoglucanase (cellobiohydrolase), cellobiase (β-glucosidase), total cellulase, and xylanase activity were assessed according to the methods of Ghose (*5*) and Wood and Bhat (*6*). Pectinase activity (*7*) and aryl-β-glucosidase and other *p*-Np-sugar activity determinations were determined according to the initial rate of reaction method as proposed by Price and Stevens (*8*). The kinetic parameters (K_m and V_{max}) of the endocellulase component (major activity of C013L preparation) on CMC were determined by conducting a substrate concentration dependence study following an initial rate approach (*8*).

Enzyme Hydrolysis Treatment and Extraction of Phenols. Enzyme reaction parameters were determined by varying the mass of the enzyme preparation relative to the mass of freeze-dried pomace (E/S) (E/S values of 0, 0.032, and 0.16 ww^{-1} were applied), temperature (40 and 50 °C), and time (0, 1.5, 3, and 6 h). Enzyme hydrolysis was performed (in duplicate) in 50 mL polyethylene centrifuge tubes with continuous stirring. Briefly, 0.250 g of black currant pomace was accurately weighed (±0.001 g) and suspended in phosphate/citrate buffer, pH 4.6, reaching 62.5% wv^{-1} substrate concentration in the final reaction mixture. After pre-equilibration at the desired temperature for 7 min, enzyme C013L was added, and the mixture was incubated for the desired time with continuous stirring in a thermostatically controlled water bath. Appropriate enzyme blanks (no pomace) and a buffer blank were also included. Reaction was terminated by immersing the tubes in a boiling water bath for 5 min. After rapid cooling, each sample was centrifuged (3800 rpm, 10 min), and the resulting supernatant was collected and filtered through a 0.45 μm filter. All supernatants were stored at −18 °C until analyzed. Formic acid in methanol (10 mL of 3%) was added to the remaining solid pomace residue (in the same 50 mL centrifuge tubes), and the mixture was vigorously vortexed for 1 min, followed by centrifugation (3800 rpm, 5 min). The extract was collected into a round-bottom evaporation flask and kept in the dark. The procedure was repeated with another 20 mL portion of methanolic formic acid solvent for complete extraction. Extracts were combined and evaporated to dryness using a rotary evaporator at 40 °C. The resulting phenolic concentrate was redissolved in 4 mL of water. The extracts were stored at −18 °C until analyzed.

Analytical Methods. Total phenol content (TPC) was determined according to the Folin−Ciocalteu procedure (*9*). Individual phenol groups, namely, anthocyanins (detection 520 nm), flavonols (detection 360 nm), hydroxycinnamic (detection 320 nm), and hydroxybenzoic acids (detection 280 nm), were analyzed by reversed phase HPLC (*2*). Antioxidant capacity was also determined by the $ABTS^{\bullet+}$ radical cation decolorization assay (*10*). All of the methods listed above, including certain modifications, were described recently (*2*). Reducing sugar content was determined according to the DNS method (*11*, *4*). The ORAC assay was performed as described by Ou and co-workers (*12*), using a fluorescence spectrophotometer (Perkin-Elmer, PE 3000) set at 493 nm excitation and 515 nm emission, connected to a thermostatically controlled water bath. The ORAC values were calculated as proposed by Cao and Prior (*13*). The FRAP assay was performed as described by Benzie and Strain (*14*).

Statistical Analysis. The statistical program GenStat (6th edition) was employed to analyze the numerical results by general linear regression. The significance of the experimental variables was determined by analysis of variance. Significant differences were considered at $P \leq 0.05$. Correlation coefficients were calculated using Minitab statistical software.

RESULTS

Enzyme Activities and Kinetic Properties. The CO13L enzyme preparation contained 193 ± 18.7 mg mL^{-1} protein, a high reducing sugar content, 19.8 ± 0.47 mg mL^{-1}, and various enzyme activities. The main activity detected was endocellulase (or endoglucanase) activity (8.4 units/mg of protein), followed by the hemicellulase (xylanase) activity (2.8 units/mg of protein) (**Table 1**). Total cellulase activity was high, whereas pectinase activity was almost absent. An array of "side activities" was also determined, and the results showed that β-glucosidase and β-cellobiosidase activities were relatively high, followed by β-D-galactopyranosidase, β-D-xylopyranosidase, and α-L-arabinopyranosidase activity, respectively. Some activities, including β-D-glucuronidase or α-D-xylopyranosidase, were not present. The K_m (substrate concentration when the reaction velocity is half-maximal) and V_{max} (maximum reaction rate) values, calculated according to the method fo Price and Stevens (*8*), are shown in **Table 2**. The highest values were those derived from the Lineweaver−Burk equation, although they were not significantly different ($P > 0.05$).

Polysaccharide Degradation. Cell wall polysaccharide hydrolysis was monitored through the measurement of reducing sugar level in the samples. As expected, reducing sugar concentration varied in response to the different reaction parameters and ranged from 3.86 to 6.13 mg mL^{-1} for the supernatants and from 0.37 to 2.04 mg mL^{-1} for the extracts (**Figure 1**). The interaction of enzyme to substrate ratio and time was very significant ($P < 0.001$) in determining the exponentially increasing rate of reducing sugar release in all supernatants after enzyme treatment as shown in **Figure 1a**. However, the interaction E/S × temperature was not significant. The interaction E/S × temperature × time was highly significant ($P < 0.001$) in determining the increasing rate of reducing sugar release in the extracts (**Figure 1b**).

Total Phenol Content. Enzyme addition, in the concentration range studied, had a highly significant effect ($P < 0.001$) on the release of total phenols in the samples (**Figure 2**). At 40 °C, there was a maximum release of total phenols in the supernatants after 3 h of incubation (**Figure 2a**). When the temperature was increased to 50 °C (**Figure 2b**), maximum phenol release was achieved more quickly (1.5 h) and followed a linear decrease after this time up to 6 h, suggesting thermal degradation of phenols (temperature; $P < 0.001$, temperature × time; $P < 0.05$). At the lower temperature

Table 1. Enzyme Activity Profile of C013L Enzyme Preparation

enzyme	activity (specific activity)	substrate
endocellulase[a]	1513 (7.84)	medium-viscosity CMC
endocellulase	1620 (8.39)	medium-viscosity CMC
cellobiohydrolase[a]	41 (0.21)	Avicel
cellobiase	13.4 (0.07)	cellobiose
β-D-glucopyranosidase	36.9 (0.19)	*p*-Np-β-D-glucopyranoside
total cellulase[a]	82 (0.42)	filter paper
xylanase	539 (2.79)	nirchwood xylan
pectinase	4.6 (0.02)	pectin from citrus
polygalacturonase	3.8 (0.02)	polygalacturonic acid
β-D-cellobiosidase	35.3 (0.18)	*p*-Np-β-D-cellobioside
α-L-arabinopyranosidase	8.8 (0.05)	*p*-Np-α-L-arabinopyranoside
β-D-galactopyranosidase	12.3 (0.06)	*p*-Np-β-D-galactopyranoside
β-D-xylopyranosidase	9.7 (0.05)	*p*-Np-β-D-xylopyranoside
α-D-xylopyranosidase	0	*p*-Np-α-D-xylopyranoside
β-D-glucuronidase	0	*p*-Np-β-D-glucuronide

[a] Activities are expressed as the corresponding IUPAC units, whereas the other enzyme activities were expressed as international units (IU = 1 μmol of reaction product min^{-1} mL^{-1}). All results are quoted as units/mL enzyme preparation; in parentheses, results are expressed as units/mg of protein. Each determination was performed in quadruplicate, and each standard deviation was <6% of the quoted value.

Table 2. Kinetic Data for the Endocellulase Activity of the C013L Preparation

equation type	V_{max} (mg mL^{-1} min^{-1})	K_m (mg mL^{-1})
Lineweaver−Burk	0.038	13.8
Eadie−Hofstee	0.036	13.4
Hanes	0.035	12.4
direct linear plot	0.035	12.2

of 40 °C, extracted phenols (following enzyme treatment) increased linearly (after 1.5 h) compared to the change of the total phenol levels of the control extract (E/S 0). When 6 h of incubation with enzyme was applied prior to extraction, E/S 0.16 and E/S 0.032 treatments produced 74 and 43% higher phenol yields compared to the control, respectively. The E/S 0.16 and E/S 0.032 treatments gave quite similar yields of phenols. These were more rapidly released, at 50 °C, and again the yields were significantly higher than the controls (up to ∼50% after 1.5 h of incubation).

Effect on the Individual Groups of Phenolic Compounds. *Total Monomeric Anthocyanins*. The cellulase treatment had a small but significant effect on increasing the total monomeric anthocyanin content of all samples. The concentration of anthocyanins reached a maximum in the supernatants at 1.5 h of incubation, reaching concentrations that were 8 and 9% higher than the control for E/S 0.032 and E/S 0.16, respectively, at 40 °C, whereas at 50 °C the corresponding values were 5 and 3% higher than those of the control. This was followed by a decrease in anthocyanin concentration over the time scale studied at both temperatures and particularly at 50 °C (**Figure 3**). Anthocyanin degradation in the supernatants increased mainly at the higher temperature and longer times, and statistical analysis showed that the only significant interaction was temperature × time ($P < 0.001$). Enzyme treatment had a very significant effect on the subsequent extractability of anthocyanins by formic acid in methanol (E/S × temperature × time; $P < 0.001$). After 6 h of hydrolysis at 40 °C, the relative anthocyanin concentration increased 25 and 32% for the 0.032 E/S and 0.16 E/S treatments, respectively, compared to the control. At 50 °C, the relative increase in subsequent extractability of anthocyanins was higher after enzyme treatment with the cellulase at 0.032 E/S ratio than at 40 °C (**Figure 3b**). The maximum

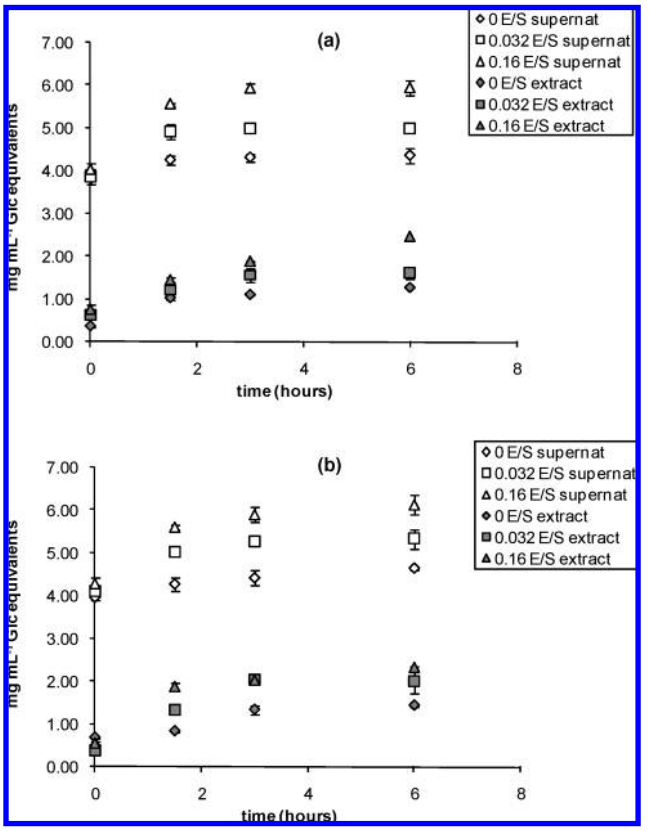

Figure 1. Reducing sugar concentration of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results are expressed as milligrams per milliliter of glucose (Glc) equivalents.

increase in anthocyanin yield was 44% relative to the control for the 0.032 E/S treatment and was obtained when hydrolysis time prior to extraction was 3 h at 50 °C, whereas the increase in yield was larger (60%) and was reached more rapidly (1.5 h) when a higher enzyme dosage (E/S 0.16) was applied at 50 °C.

Hydroxybenzoic Acid (HBA), Hydroxycinnamic Acids (HCA), and Flavonols. HBA concentration in the supernatant and in subsequent extracts was increased significantly by the cellulase treatment compared to the respective controls (**Figure 4**). Similar release of HBA in the supernatants was observed at both 40 and 50 °C (**Figure 4**), but the effect of changes in E/S ratio was highly significant ($P < 0.001$). The release of HBAs in the supernatants was highest at the higher enzyme dosage level (E/S 0.16) throughout the experiment (E/S × time; $P < 0.001$), and this effect was greater at 50 °C (**Figure 4b**) than at 40 °C (**Figure 4a**). After 1.5 h of hydrolysis, the HBA yield was 80% higher than for the control, whereas the maximum increase was 50% for the lower enzyme dosage. When extracts were analyzed, the HBA concentration again increased, in an enzyme dosage- and time-dependent manner. All reaction parameters affected HBA extraction, because the interaction E/S × time × temperature was significant ($P < 0.05$). The enzyme treatment generally improved HBA extraction, particularly at 50 °C (**Figure 4**). It is noteworthy that at the same temperature after 6 h of hydrolysis, the increases in HBA extraction yield compared to the control were 80 and 120% for the E/S ratios 0.032 and 0.16, respectively (**Figure 4b**). Cellulase treatment increased release of HCA in the supernatants, and the magnitude of the effect was dependent on all

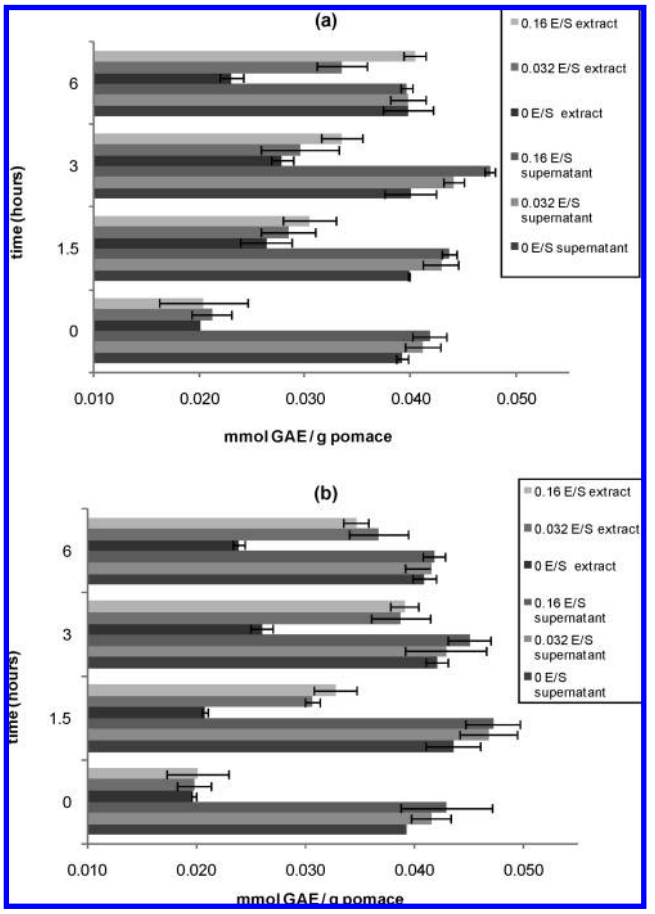

Figure 2. Total phenol content of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results are expressed as gallic acid equivalents (GAE): millimoles of gallic acid per gram of pomace.

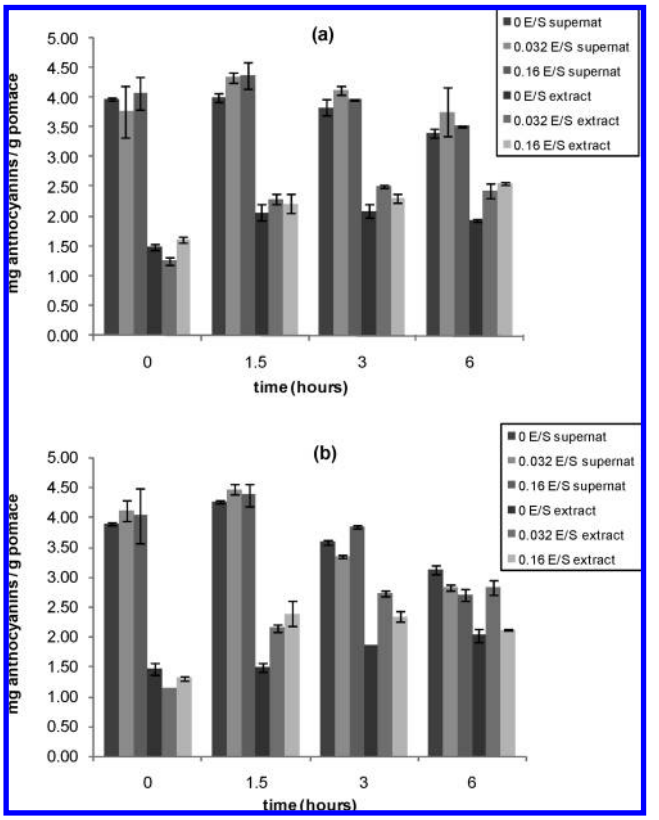

Figure 3. Total monomeric anthocyanin concentration of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results (mg of anthocyanins per g of pomace) were calculated as the sum of the concentration (expressed as mg of cyanidin-3-rutinoside per g of pomace) of all major individual anthocyanins (cyanidin-3-rutinoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and delphinidin-3-glucoside).

variables studied, with the interaction E/S × time × temperature being significant (**Figure 5**). After 6 of h hydrolysis at 50 °C, the relative increases in HCA content, for the supernatants, were 40 and 120% higher for the lower and upper enzyme levels tested, respectively, in comparison with E/S 0 (**Figure 5b**). After 3 h of hydrolysis, the corresponding values were 40 and 80% higher than the control, suggesting that the lower enzyme dosage (E/S 0.032) reached its maximum HCA release earlier. Enzyme concentration also had a significant impact ($P < 0.001$) on HCA levels of the extracts, the higher enzyme dosage enhancing extraction of HCA (**Figure 5**). The increase in extractability of HCA depended on hydrolysis time and enzyme dosage (E/S × time < 0.05), but there was no interaction between E/S ratio and temperature. Flavonol content of supernatants and extracts increased with cellulase treatment in a dose-dependent manner ($P < 0.001$) and ranged from 0.141 to 0.306 mg of rutin equiv/gram of pomace for both fractions analyzed (**Figure 6**). After 6 h of treatment with cellulase at the 0.16 E/S ratio, the flavonol concentrations in the supernatant were 60 and 70% higher than the control, at 40 and 50 °C, respectively. Extractability of flavonols from the residue after cellulase treatment (0.16 E/S) increased by 100% compared to the respective control after 6 h at 40 °C and after 1.5 h at 50 °C, respectively.

Antioxidant Capacity Evaluation. *ABTS$^{\bullet+}$ Scavenging Activity*. Cellulase treatment at 40 °C caused an increase (~16% for the 0.032 E/S ratio and ~9% for the 0.16 E/S ratio relative to the controls) in the ABTS$^{\bullet+}$ scavenging activity of the supernatant, which peaked at 1.5 h for E/S 0.032 and at 3 h for E/S 0.16 but decreased significantly for both treatments after 6 h of hydrolysis (**Figure 7a**). Similarly, at 50 °C (**Figure 7b**), ABTS$^{\bullet+}$ scavenging activity of the supernatant increased rapidly up to 1.5 h of enzyme hydrolysis, followed by a progressive decrease up to 6 h. E/S ratio, temperature, and time were all significant in determining the radical scavenging activity of the supernatant. The decrease in radical scavenging activity at longer hydrolysis times can be attributed to thermal degradation. Radical scavenging activity of extracts was higher at the higher E/S ratio over the time scale studied (except 6 h at 50 °C) (E/S × time; $P < 0.001$). The radical scavenging activity of extracts following hydrolysis increased with cellulase concentration and hydrolysis time at 40 °C (**Figure 7a**), but at 50 °C (**Figure 7b**) the scavenging activity of extracts reached a plateau for 1.5−6 h for the higher E/S ratio, whereas the lower E/S ratio showed a maximum at 3 h.

ORAC Assay. The supernatants and methanolic−formic acid extracts had similar ORAC values in the range 0.072−0.245 mmol of Trolox equiv/g of pomace for all samples analyzed. Enzyme-treated supernatants had higher ORAC values than the controls over the time scale studied (**Figure 8a**,**b**). At 40 °C, there was a rapid increase in ORAC values up to 1.5 h for 0.032 E/S (58% higher relative to the control), followed by a steady decrease of ORAC values until 6 h. The elevated enzyme dosage of 0.16 E/S favored higher ORAC values (47% higher than control) after 3 h of

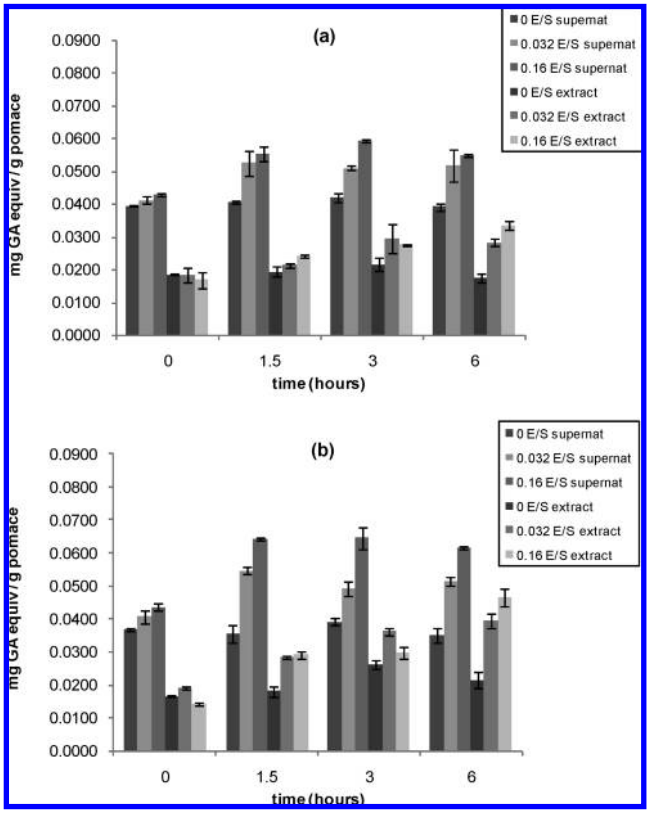

Figure 4. Hydroxybenzoic acid content of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results are expressed as gallic acid (GA) equivalents: milligrams of gallic acid per gram of pomace.

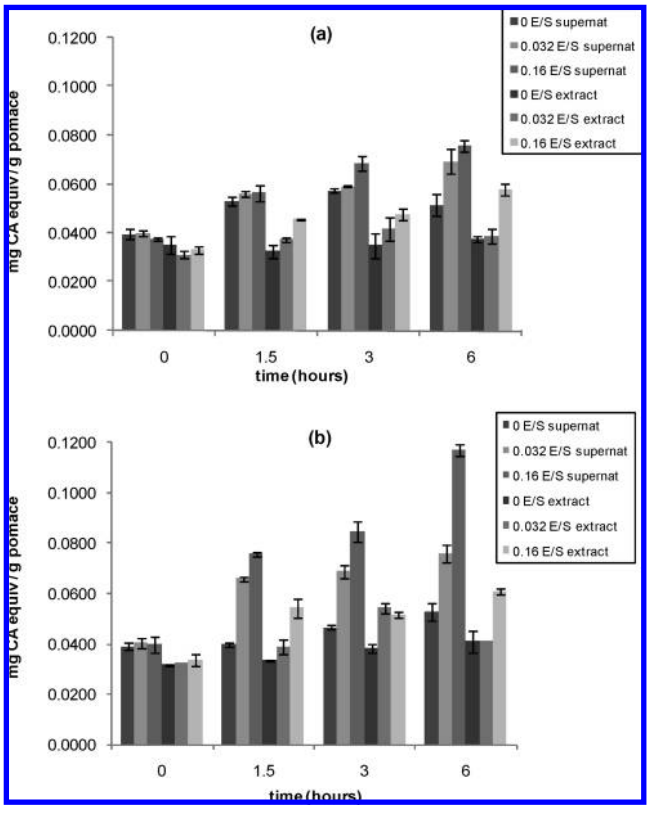

Figure 5. Hydroxycinnamic acid content of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results are expressed as caffeic acid (CA) equivalents: milligrams of caffeic acid per gram of pomace.

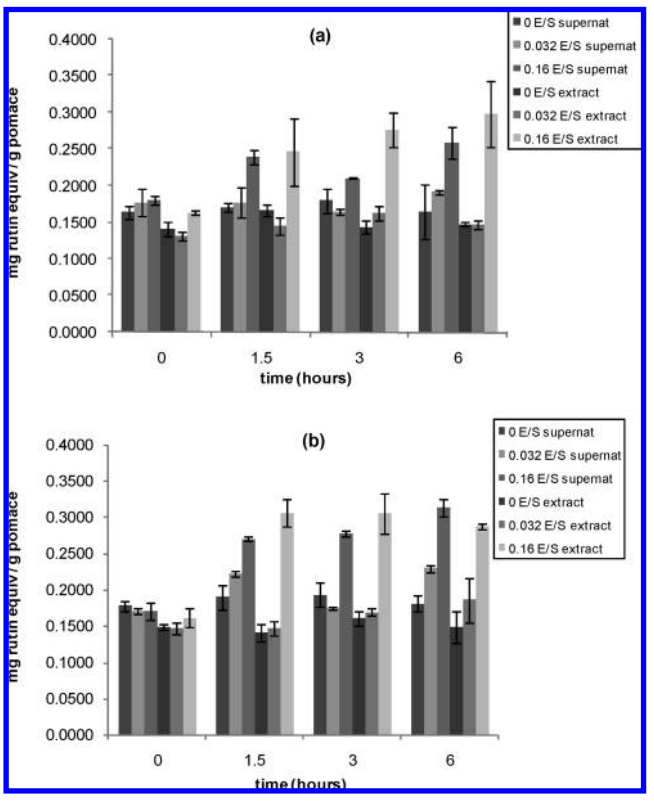

Figure 6. Flavonol content of supernatants and extracts from enzyme-treated black currant pomace treated at (a) 40 °C and (b) 50 °C. Results are expressed as rutin equivalents: milligrams of quercetin-3-rutinoside per gram of pomace.

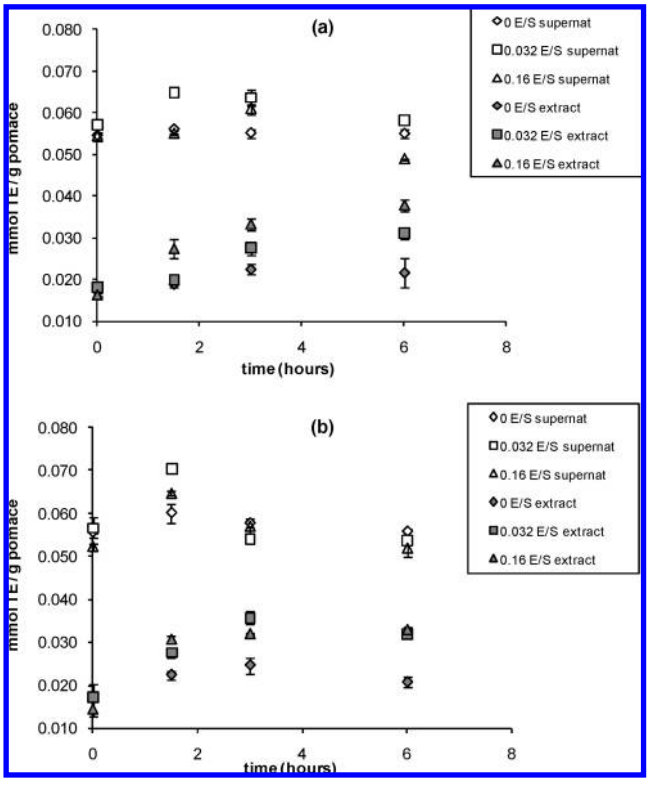

Figure 7. ABTS$^{\bullet+}$ scavenging activity of supernatants and extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results are expressed as millimoles of Trolox equivalents (TE) per gram of pomace.

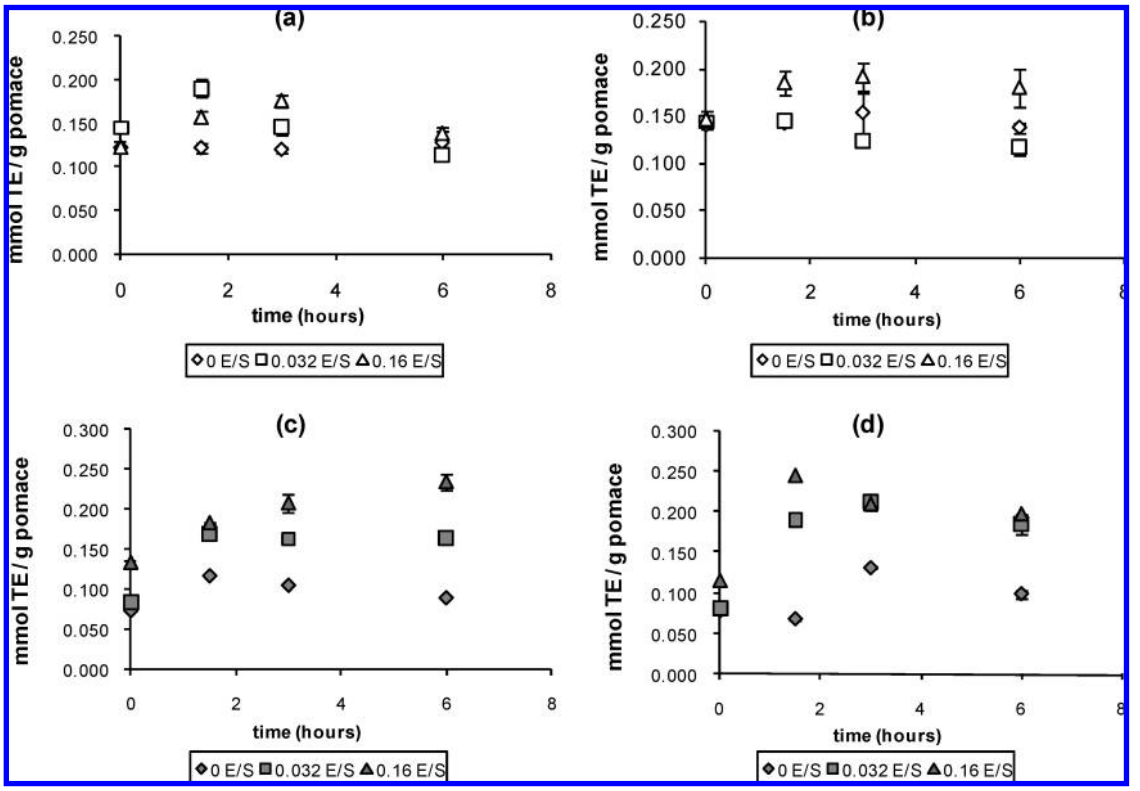

Figure 8. ORAC of supernatants (**a**, **b**) and extracts from enzyme-treated black currant pomace treated at (**a**, **c**) 40 °C and (**b**, **d**) 50 °C. Results are expressed as millimoles of Trolox equivalents (TE) per gram of pomace.

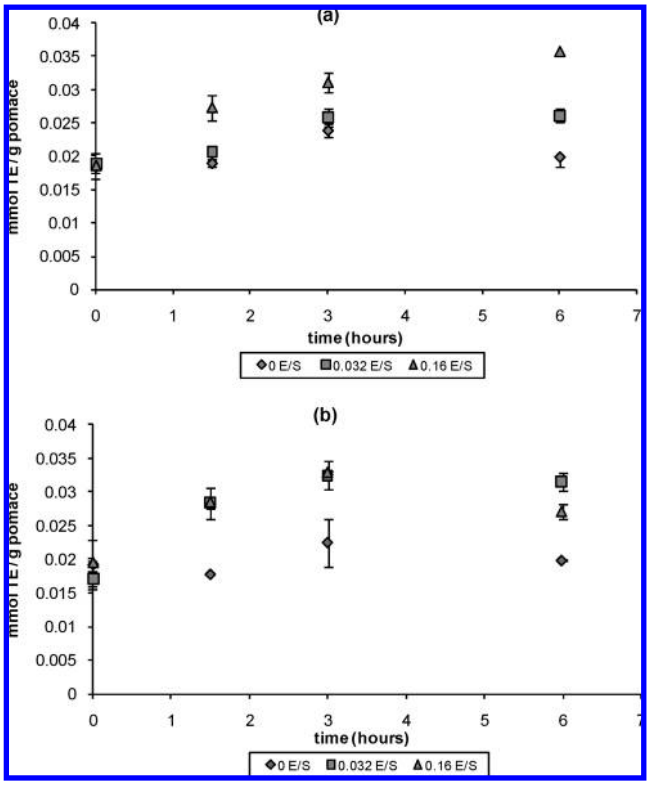

Figure 9. FRAP values of extracts from enzyme-treated black currant pomace treated at (**a**) 40 °C and (**b**) 50 °C. Results expressed as millimoles of Trolox equivalents (TE) per gram of pomace.

enzyme hydrolysis (**Figure 8a**). At 50 °C, the higher enzyme concentration gave constantly higher ORAC values compared to the controls (**Figure 8b**), reaching a maximum increase of 54% at 3 h of hydrolysis (E/S × time; $P < 0.001$). These values were significantly higher than for the identical E/S ratio treatment at 40 °C. In the case of the extracts (**Figure 8c**,**d**), enzyme hydrolysis, hydrolysis temperature, and time had a highly significant effect on the ORAC values ($P < 0.001$). At 40 °C, the 0.032 E/S ratio cellulase treatment caused an increase in the ORAC value, which remained steady, but that for the higher enzyme dose became greater over time. After 6 h, ORAC values increased from 0.088 (for the 0 E/S ratio) to 0.165 (90% increase) and 0.234 (160% increase) mmol of Trolox equiv/g of pomace for the 0.032 and 0.16 E/S ratios, respectively. Again at 50 °C, there was a regular increase of ORAC values for the enzyme-treated samples when compared to the controls (**Figure 8d**) and, in addition, higher ORAC values were obtained at earlier times (temperature × time; $P < 0.001$). After 1.5 h of hydrolysis at 50 °C, the relative increases of ORAC values of the extracts were 180 and 260% for the 0.032 and 0.16 E/S ratios, respectively.

FRAP Values. Ferric reducing antioxidant potential determination could only be applied to the extracts, because the buffer components of the supernatant interfered with the assay by causing haze formation with the FRAP reagent. FRAP values of the extracts increased with time and were dependent on E/S ratio and temperature (E/S × temperature × time; $P < 0.001$). At 40 °C (**Figure 9a**), higher FRAP values were obtained for the higher enzyme dose treatment, reaching a maximum at the lengthened hydrolysis time of 6 h (80 and 30% higher than the control for the 0.032 and 0.16 E/S ratios, respectively). When the hydrolysis temperature was 50 °C (**Figure 9b**), changes in FRAP values were largely parallel for the two enzyme levels tested. The highest FRAP value for both E/S ratios was achieved at a hydrolysis time of 3 h (~60% increase).

DISCUSSION

Enzyme activity determination results demonstrated the ability of the commercial cellulase to degrade various substrates other than carboxymethylcellulose (**Table 1**). The major activity of the C013L preparation was endocellulase (8.4 units/mg of protein), but cellobiohydrolase and β-glucosidase activities were also present, and these contributed to the overall cellulolytic activity (expressed as total cellulase) and possible degradation of highly ordered (crystalline) and amorphous cellulose. Xylanase activity was the main secondary activity (2.8 units/mg of protein). Pectinase and endopolygalacturonase activity were relatively low. These results are consistent with previous reports that the cellulase system in fungi includes three major hydrolytic enzymes: (a) an endocellulase that cleaves β-linkages at random; (b) a cellobiohydrolase, which releases cellobiose from either the reducing or the nonreducing end of cellulose; and (c) a β-glucosidase that releases glucose from cellobiose and short-chain cellooligosaccharides (*15*, *16*). The low level of β-glucosidase activity was consistent with previous reports that *Trichoderma reesei* cellulases exert low β-glucosidase activity (*17*, *18*)). It has also been reported (*19*) that *Trichoderma* cellulases additionally contain at least three endoxylanases, and these enzymes might contribute to the xylanase activity in the C013L preparation. According to Vinzant and co-workers (*20*), the *T. reesei* cellulase complex consists of many glycosyl hydrolases, of which five are β-1,4-endoglucanases (EGI-EGV), two β-1, 4-exoglucanases (cellobiohydrolase CBHI and CBHII), two xylanases (XYNI and XYNII), one each is a β-D-glucosidase, an α-L-arabinofuranosidase, an acetyl xylan esterase, a β-mannanase, and an α-glucuronidase. However, interpretation of results from enzymatic assays is limited because no substrate allowing specific determination of only cellobiohydrolase activity is available, and it is not possible to distinguish the five endoglucanases from *T. reesei* (*21*). Desphande and co-workers (*22*) used synthetic substrates including *p*-nitrophenol-β-D-cellobioside to measure cellobiohydrolase activity in the presence of endoglucanase activity; however, other enzymes such as β-glucosidases and some endoglucanases and endoxylanases may hydrolyze this substrate (*22*). This study has shown that the enzyme preparation C013L had a broad spectrum of activities, relating to black currant cell wall polysaccharide hydrolysis, and can be described as a "cellulase−hemicellulase" blend with various secondary activities, but lacking pectinase activity. The kinetic parameters (K_m and V_{max}) were calculated because they show the specificity of the endocellulase (main activity) for the particular substrate. The reducing sugar content of the enzyme preparation was quite high and confirms the findings of Nieves and co-workers (*23*) that certain sugars are present in commercial enzyme mixtures for stabilization purposes.

In a recent study (*2*), it was demonstrated that black currant pomace was a plant material with notably low "free" phenol content but with "cell wall-bound" phenols present at moderately high concentrations. Consequently, in this study, we decided to apply a suitable enzyme treatment that would enable release or facilitate extraction of antioxidant phenols from black currant pomace. Analysis of black currant pomace revealed reasonably high contents of lignin, hemicellulose, and cellulose (*24*), and consequently enzyme preparation C013L was used because of its cellulase and hemicellulase activity.

The cellulase treatment caused an increase in the reducing sugar concentration in all black currant pomace samples, together with an increase in total phenol contents in the extracts. Enzyme treatment that caused increases in soluble carbohydrate in the supernatants also resulted in improved extractability of total phenols by methanolic formic acid solvent from the residue, and this showed that direct enzyme-assisted liberation of total phenols did not occur. At the lower E/S ratio, the relative amount of each enzyme component present in the reaction mixture was 12.9 units of endoglucanase (or endocellulase), 4.3 units of xylanase, 0.66 unit of total cellulase, 0.33 unit of cellobiohydrolase, 0.29 unit of β-glucosidase, 0.28 unit of β-cellobiosidase, 0.098 unit of β-galactosidase, and 0.070 unit of α-arabinosidase. Although the mechanism of action of these enzymes in complex substrates is largely unknown, it is well established that the degradation of cellulose and hemicellulose is effected by a group of glycosidases which hydrolyze glycosidic bonds within the polymer. Each polymer is cleaved into large oligomers by endoglycanases (endoglucanases and endoxylanases) and then further degraded into smaller oligomers, disaccharides (cellobiose and xylobiose) and monosaccharides, by exoglycanases and disaccharide hydrolases (cellobiase, β-glucosidases, and β-xylosidases) (*25*). Because xylans and xyloglucanases, major components of hemicellulose, are also covalently substituted with acetyl moieties and other sugars, such as L-arabinose and D-galactose, other enzyme activities (arabinosidases, galactosidases, and xylan esterases) play an important role (*25*). The release of reducing sugars (**Figure 1**) can be mainly attributed to the endoglucanase and xylanase activities in the reaction mixture. It has been previously shown that effective degradation of cell wall embedded cellulose involves the combined action of endoglucanase and cellobiohydrolase (major components of the cellulolytic system) (*26*). In addition, it was proved that the xyloglucanase (hemicellulase) activity enhanced the accessibility of cellulose for the consortium of cellulolytic enzymes (*27*). This is possibly because xyloglucans are long enough to tether cellulose microfibrils via hydrogen bonding (*28*), hence rupturing of these cross-links probably assisted the degradation of the cellulose−xyloglucan network (*29*), resulting in easier access for the cellulolytic enzymes. As mentioned above, enzyme action and the resulting polysaccharide degradation had an effect on total phenol content in the extracts as shown in **Figure 2**. The better extraction of total phenols for the lower enzyme concentration (0.032 E/S), at the higher hydrolysis temperature of 50 °C, clearly suggests that the higher activity of the individual enzyme components of the preparation, or even the "activation" of some minor activities, had a significant effect on total phenols extracted. However, differences were not significant between the higher and lower enzyme dosages, even though effects were highly significant when compared to the controls. This may be due to nonproductive, competitive adsorption of the enzyme components on the polysaccharide substrate, when a higher enzyme dosage was used, because nonproductive adsorption may sterically block access of enzymes that can catalyze hydrolysis at these sites (*30*). The presence of lignin may also limit black currant pomace hydrolysis and subsequent extractability of phenols. Optimal extractability of total phenols required a longer enzyme treatment at 40 °C (6 h) than at 50 °C (3 h), which is consistent with enzyme-catalyzed hydrolysis improving the subsequent extractability of the phenols. There was a significant reduction of phenol concentration in the supernatants over time, particularly at 50 °C, which indicates that phenolic compounds were lost by thermal degradation.

Total monomeric anthocyanin concentration increased significantly in the enzyme-treated extracts over the time scale studied. The high correlation between total monomeric anthocyanins and reducing sugars ($r = 0.942$) is consistent with black currant pomace polysaccharide hydrolysis contributing to total anthocyanin recovery from the cellulase-treated plant material. Extracted anthocyanin concentration increased at higher temperature, which is also consistent with the importance of the enzyme activity. At 50 °C, extracted anthocyanins reached a maximum concentration after 1.5 h, but the concentration decreased at longer times when the higher enzyme dosage was used, whereas the concentration continued to increase during the experiment when the lower enzyme concentration was used (**Figure 3b**). This may be due to the β-glucosidase activity of the enzyme, which hydrolyzed anthocyanins to the less stable aglycone, leading to a reduced concentration of anthocyanins with longer hydrolysis time. At 50 °C, with the higher enzyme concentration, this effect became dominant, whereas at 40 °C, polysaccharide hydrolysis continued to lead to increased anthocyanin extractability. In a previous study (*31*), β-glucosidase also proved to have a detrimental effect on anthocyanin concentration. Total monomeric anthocyanins in the supernatants also decreased in concentration over time, particularly at 50 °C, but this decrease was not enzyme dependent, but was due to thermal degradation, because the only significant interaction ($P < 0.001$) was temperature × time. This finding is consistent with the literature, which recommends short time of extraction and processing treatments to avoid anthocyanin degradation (*32*). The findings of this study agree well with the findings of Buchert and co-workers (*33*), who reported an increase in total phenols and total anthocyanin content after application of a commercial cellulase to assist juice extraction from black currants.

The increase in phenolic acid (hydroxybenzoic and hydroxycinnamic acid) concentration, especially in the supernatants, demonstrates the importance of the enzyme activities present in the cellulase preparation for the direct release of these compounds from black currant pomace. However, cellulase treatment caused increases in flavonol concentration in both supernatants and extracts (**Figure 6**), which may indicate that both the cell wall and vacuolar membrane had to be enzymatically degraded to enable optimal recovery of these compounds, which would be consistent with the localization of flavonoid glycosides mainly in the cell vacuole (*34*). It is likely that the resulting cellulolytic and xylanolytic action of the cellulase and the xylanase caused release of the phenolic acids and flavonols from the complex cell wall structure, allowing release of the intracellular contents. In general, enzyme reaction parameters (E/S × time × temperature) had a major impact on HCA concentration, whereas the effect on HBA was less important, showing that the latter were more easily released. The conjugation of HCA through ester bonds with cell wall polymers or even with lignin may account for this result. Most phenolic compounds in plants are naturally present in conjugated forms; in dicotyledonous plants low molecular weight phenols occur as glycosides or esters with polysaccharides or related compounds. Although, there are no studies concerning conjugation of phenolic acids and localization of these compounds in black currants, it has been shown that in some plants HCA can be found in small quantities in various saccharide fractions of the cell wall (*35*). It has been proved that in several dicots some HCA (ferulic acid and *p*-coumaric acid) are esterified to either the C2 of α-L-arabinose or the C6 of β-D-galactose in the neutral side chains of pectins (*36*). Hydroxycinnamic acids also have the potential to link with the cell wall proteins via cysteine or tyrosine residues (*37*). The possible presence of such cross-links in black currants could largely explain the apparent increase in HCA concentration, because α-arabinosidase and β-galactosidase side activities present in the enzyme preparation (**Table 1**) could hydrolyze these bonds. In addition, according to Fry (*38*), α-L-arabinofuranosidase can hydrolyze other major wall polymers, mainly rhamnogalacturonan I (pectin polymer), xylans, and extensins (cell wall proteins), whereas β-D-galactopyranosidase can utilize rhamnogalacturonan I and xyloglucan as potential substrates, showing the significance of the specific secondary activities present in the cellulase preparation for plant cell wall degradation. In general, our results are in accordance with earlier studies (*39*, *40*), which observed release of HCA, HBA, and flavonols from the tested plant material by using commercial plant cell wall degrading enzyme preparations.

The increase in phenolic content of the supernatants and extracts from cellulase-treated black currant pomace was responsible for the high antioxidant capacity of the samples as determined by two radical scavenging activity methods ($ABTS^{\bullet+}$ scavenging activity and ORAC assay) and FRAP for the extracts. The total phenols of the extracts correlated well with $ABTS^{\bullet+}$ scavenging activity ($r = 0.856$), ORAC values ($r = 0.884$), and reducing sugar content ($r = 0.840$), suggesting that enzyme-mediated cell wall polysaccharide degradation caused the increase in total phenols extracted, which determined the increase in antioxidant capacity. Antioxidant activity increased in all cases for shorter enzyme treatments but under some conditions, especially with higher enzyme concentration and higher temperature, the activity declined for longer times of treatments. This is consistent with degradation of the antioxidants under these conditions. Both $ABTS^{\bullet+}$ scavenging activity and ORAC values correlated well with total monomeric anthocyanins and HBA ($0.869 < r < 0.931$) and quite well with HCA and flavonols ($0.388 < r < 0.740$) for all samples. The correlations for the extracts alone were even better for $ABTS^{\bullet+}$ scavenging activity and ORAC values ($r = 0.916$). In the case of the supernatants, the $ABTS^{\bullet+}$ scavenging activity correlated well with HBA ($r = 0.884$), HCA ($r = 0.863$), and flavonols ($r = 0.798$), whereas the correlation between ORAC values and phytochemical content was weaker with HBA ($r = 0.581$), anthocyanins ($r = 0.364$), and flavonols ($r = 0.369$). The relatively high reducing sugar concentration of the supernatants and the differences in the antioxidant activity of the different phenolic compounds present in these samples might account for these differences. The highest progressive increase in antioxidant capacity ($ABTS^{\bullet+}$ scavenging activity, ORAC, and FRAP) for all extracts at the lower temperature of 40 °C was achieved for the higher E/S ratio of 0.16, but when the temperature was increased to 50 °C, the effect of enzyme concentration was less pronounced, indicating that the higher reaction temperature and the corresponding increase in enzymatic degradation of the cell wall polysaccharides did not affect drastically the antioxidant capacity of the extracted samples. Overall, the antioxidant and enzyme activity data suggest that the enzyme preparation used contained enzymes that (a) directly, but mainly indirectly, promoted the selective release of antioxidant phenols and (b) possibly modified liberated phenols to more active compounds which, in the case of the higher enzyme concentration, caused loss of the measured activity. The influence of process parameters on composition and antioxidant activity, such as elevated temperature causing

loss of phenols, is clearly demonstrated in the reduction of $ABTS^{\bullet+}$ scavenging activity for the supernatants and the decrease in anthocyanin concentration for the same conditions.

The results obtained in this study clearly demonstrated that utilization of a multiactivity commercial cellulase enhanced cell wall polysaccharide degradation and assisted the liberation and extraction of antioxidant phenols, especially anthocyanins, from black currant pomace. Enzyme treatment enabled the production of extracts rich in anthocyanins and other phenols with increased antioxidant activity, but reaction parameters do affect the phenol content and consequently influence antioxidant capacity values.

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