Enzyme-Assisted Extraction of Antioxidative Phenols from Black Currant Juice Press Residues (*Ribes nigrum*)

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Enzymatic release of phenolic compounds from pomace remaining from black currant (*Ribes nigrum*) juice production was examined. Treatment with each of the commercial pectinolytic enzyme preparations Grindamyl pectinase, Macer8 FJ, Macer8 R, and Pectinex BE, as well as treatment with Novozym 89 protease, significantly increased plant cell wall breakdown of the pomace. Each of the tested enzyme preparations except Grindamyl pectinase also significantly enhanced the amount of phenols extracted from the pomace. Macer8 FJ and Macer8 R decreased the extraction yields of anthocyanins, whereas Pectinex BE and Novozym 89 protease showed no effect. A decrease in pomace particle sizes from 500−1000 μm to <125 μm increased the phenol yields 1.6−5 times. Black currant pomace devoid of seeds gave significantly higher yields of phenols than pomace with seeds and seedless wine pomace. Four selected black currant pomace extracts all exerted a pronounced antioxidant activity against human LDL oxidation in vitro when tested at equimolar phenol concentrations of 7.5−10 μM.

Keywords: *Anthocyanins; black currant pomace; LDL oxidation; pectinase; phenols; plant cell wall degradation; protease*

INTRODUCTION

The oxidative modification of human low-density lipoprotein (LDL) is believed to play an important role in the initiation and development of atherosclerosis and coronary heart disease (*1*). Dietary antioxidants that protect LDL from oxidation and thereby from being atherogenic are therefore of great interest (*2*).

Epidemiological data have shown a consistent protective effect of fruit intake on cardiovascular diseases and coronary heart disease mortality (*3, 4*). Although there is still considerable uncertainty about the relationship between specific dietary components and cardiovascular disease risk, the current literature suggests that the cardioprotective action of fruit consumption is at least partly attributable to the antioxidant activity of ascorbic acid, β-carotene, tocopherols, and flavonoids present in fruits (*4*−*7*).

Much attention has been devoted to grapes and wine, especially red wine which contains a number of phenolic compounds that show antioxidative properties and have turned out as effective inhibitors of LDL oxidation in vitro (*8*−*10*). This ability of wine phenols to protect human LDL against oxidation has been proposed as a molecular mechanism behind the low incidence of coronary heart disease observed in certain regions of France where wine consumption is common (*8*). Antioxidant phenolics are also widely distributed in many other fruits and berries (*11*). Black currant are among those berries that contain a large array of phenolic compounds having antioxidant activity, including both phenolic acids and flavonoids, most notably anthocyanins (*12*−*14*). The anthocyanins contribute the dark red color and constitute the major type of phenolic compounds in ripe black currant (*12, 13*). The skins of black currant are particularly rich in anthocyanins and may contain at least 2 wt % on a fresh mass basis (*12*).

The purpose of this study was to examine enzyme-assisted extraction of phenolic antioxidants from black currant pomace by use of commercial plant cell wall degrading enzyme preparations. The extraction of phenols was optimized by varying different enzyme reaction parameters such as enzyme type, time of hydrolysis, substrate particle size, pomace seed content, and type of extraction solvent in statistically designed factorial experiments. The yields of selected samples were then compared to samples of wine pomace having undergone the same treatment. Finally, the antioxidant activities of selected black currant pomace extracts were investigated on human LDL oxidation in vitro.

MATERIALS AND METHODS

Pomace. Black currant pomace (*Ribes nigrum* var. Ben Lomond) with a percentage of skin and seeds of 51 and 49 wt %, respectively, was obtained from Vallø Saft A/S (Vallø, Denmark). Red grape pomace (*Vitis vinifera* var. Merlot) was obtained from Rutherford Brothers Winery, Napa, CA. The pomaces were freeze-dried, flushed with nitrogen, and kept frozen (−20 °C) until use.

Chemicals and Enzymes. Gallic acid, human LDL, and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, Folin-Ciocalteu phenol reagent, sodium potassium tartrate, copper sulfate, and sodium acetate-trihydrate were obtained from Merck (Darmstadt, Germany). Glucose was from AnalaR BKH Ltd. (Poole, U.K.). Grindamyl CA pectinase from *Aspergillus niger* was obtained from Danisco Ingredients (Brabrand, Denmark). The pectinases Macer8 FJ and Macer8 R from *Aspergillus* strains were from Biocatalysts Ltd. (Pontypridd, U.K.). Novozym 89 acid protease produced from *Mucor miehei* and the pectolytic enzyme Pectinex BE were from Novozymes A/S (Bagsværd, Den-

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10.1021/jf001443p CCC: $20.00
Published on Web 06/05/2001

mark). All the enzymes employed were liquid preparations and were selected for this study after preliminary trial tests.

Sample Preparation. The freeze-dried pomace was milled for 90 s under nitrogen in a water-cooled IKA-Universal mill model H20 (Jahnke & Kunkel, Staufen, Germany). The pomace was prepared in two ways before milling: with or without seeds. To obtain pomace samples of different particle sizes, a four-sieve sieving tower (Endecotts Filters Ltd., London, U.K.) with aperture sizes of 1000, 500, 250, and 125 μm, respectively, was used. The particle size of 375 μm was obtained by mixing substrates with particle sizes of 250 μm and 500 μm in the ratio 50:50. For each particle size interval it was assumed that the size of particles was evenly distributed.

Enzyme Hydrolysis. For enzymatic hydrolysis, samples (0.250 g) were incubated under nitrogen in doubly distilled water (1.0 mL). The selected hydrolysis conditions were based on evaluations of the enzyme suppliers' data sheets. Enzymes were dosed on the basis of enzyme/substrate ratio (E/S), that is, the total mass of the enzyme preparation relative to the dry mass of pomace. E/S ratios of 0 or 10% corresponding to enzyme concentrations of 0 or 1% (w/w) of the total reaction volume were used. All treatments were concluded with a heat treatment (100 °C, 10 min) prior to extraction of phenols.

Individual and interactive effects on plant cell wall degradation and phenol yield of the parameters were first screened in a factorial 2^5 design (*15*). The hydrolysis time was kept constant at 2 h while enzyme addition (Grindamyl pectinase and Macer8 FJ), hydrolysis temperature (20 °C versus 50 °C), particle size (250−500 μm or 500−1000 μm), and substrate type (with or without seeds) were varied according to the experimental plan. To evaluate in more detail the effect of particle size reduction on plant cell wall degradation, phenols, and anthocyanins yields, a separate experiment was carried out with Macer8 FJ treatment (50 °C, 8h) on differently sized pomace particles with and without seeds (<125, 125−250, 250−500, and 500−1000 μm). The blank was direct extraction after no hydrolysis and the control was treatment without enzymes at 50 °C for 8 h. Third, effects on plant cell wall degradation, total phenols, and anthocyanin yields of six factors were tested in a 2^6 factorial experimental plan composed of 32 different enzyme treatments with 3 center points (*15*). The six parameters tested were three different pectinases (Macer8 FJ, Macer8 R, Pectinex BE), an acid protease (Novozym 89 protease), hydrolysis time (0.5 versus 8 h), and substrate type (with or without seeds). The hydrolysis temperature (50 °C), E/S ratio (10%), and particle size (<250 μm) were kept constant throughout the investigation.

Extraction of Phenols. Every investigation was carried out in two parallel, identical systems: one was used for extraction with 60% methanol (*10*) and the other was used for extraction with doubly distilled water. The water extraction was included to evaluate the difference between methanolic and neat aqueous extraction, because pure water may be a more desirable solvent in large-scale operations. According to preliminary experiments, the optimal extraction time was found to be 10 min at 20 °C (data not shown). Measurements of total phenols, anthocyanins, and soluble carbohydrates were all done in duplicate on each sample. Before the analyses of the methanol extract, the methanol was evaporated and the extract was rediluted with doubly distilled water.

Determination of Total Phenols, Anthocyanins, and Total Water-Soluble Carbohydrates. Total phenols in pomace extracts were determined by the Folin-Ciocalteu procedure with total phenols expressed as mg/L gallic acid equivalents (GAE) (*16*). Anthocyanins were determined by the pH differential method, and anthocyanin concentrations in black currant and wine pomace extracts were calculated as cyanidin-3-rutinoside equivalents and malvidin-3-glucoside equivalents, respectively (*17*). The extent of cell wall hydrolysis of pomace was assessed by measuring total water-soluble carbohydrates liberated from the sample (*18*).

Inhibition of Human LDL Oxidation. The antioxidant activity of black currant pomace extracts to inhibit copper-catalyzed oxidation of human LDL (37 °C, 5 μM $CuSO_4$) was assayed by monitoring formation of conjugated diene hydroperoxides (234 nm) over 5 h (*19*). Immediately prior to assay, the extracts were diluted with doubly distilled water and tested at equimolar concentrations of 7.5 and 10 μM GAE. After triplicate analyses the antioxidant activity of each extract was evaluated on the basis of the lag time and compared to the lag time of the control sample which contained all the ingredients except the phenol extract.

Statistics. The computer program Modde (Umetri AB, Umeå, Sweden) was used to aid the statistical design of the factorial experiments and to fit and analyze the data by multiple linear regression. Significance of the results was established at $P \leq 0.05$. Differences in the responses were determined by either one- or two-way analysis of variance, where 95% confidence intervals were calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA). The statistical significance of linear correlations was tested by the dose−response *F*-test (*20*).

RESULTS

Influence of Parameters on Yields of Phenols and Plant Cell Wall Degradation. The yields of total phenols varied in response to the different treatments in the experimental design. Yields ranged from 62 to 383 mg/L GAE in the methanol extracts and between 58 and 247 mg/L in the water extracts (Table 1). In the methanol extracts the yield of phenols increased 1.2−1.8 times in seedless pomace extracts compared to extracts based on pomace with seeds (compare e.g., yields of experiments no. 3 and 4, Table 1). In the aqueous extracts an analogous increase in yields of 1.1−1.6 times was obtained for the substrate without seeds versus the substrate with seeds (Table 1). Reduction in particle sizes from 500−1000 μm to 250−500 μm increased the yield of phenols 2.4−3.5 times and 1.9−3.1 in the methanol and water extracts, respectively, compared to the yields obtained for the high particle size interval with each extraction solvent (calculations based on results from Table 1). The release of soluble carbohydrates ranged between 0.357 and 0.827 mg/mL (Table 1). The significance of responses to different parameters and interactions was similar for phenols extracted with water and those extracted with methanol (Table 2). Most pronounced were the significantly positive effects of using the small particle size (250−500 μm) and of extracting from seedless pomace (Table 2). No effect of the temperature was seen for either the methanolic or the aqueous extracts. Neither did the enzyme Grindamyl pectinase affect the extracted amounts of phenols (Table 2). Treatment with Macer8 FJ gave a significant increase in phenols if the extraction solvent was methanol, but no effect was obtained with water as extraction solvent (Table 2). For both types of extracts the same two interactions substrate type × particle size and particle size × Macer8 FJ were significant (Table 2). The positive coefficient of the substrate type × particle size interaction on phenols indicated that a significantly increased phenol extraction yield was obtained from the smaller seedless pomace particles (250−500 μm) as compared to that from the larger particles (500−1000 μm). The other interaction was negative, indicating that the enzyme treatment with Macer8 FJ mediated higher phenol yields on the small particles (250−500 μm) than those on the larger ones (500−1000 μm).

Both of the tested enzymes, Grindamyl pectinase and Macer8 FJ, had a significantly enhancing effect on the break down of the plant cell wall (Table 2). High temperature also had a significantly positive effect on

Table 1. Influences of Substrate Type, Particle Size, Enzyme Type, and Temperature on Release of Phenols and Plant Cell Wall Degradation

exp. no.	substrate type[a]	particle size (μm)[b]	Macer8 FJ (E/S)[c]%	Grindamyl pectinase (E/S)[c]%	Temperature (°C)	phenols methanol[d] (GAE[e] mg/L)	phenols water[f] (GAE[e] mg/L)	carbohydrates[g] (mg/mL)
1	0	250−500	0	0	50	276	201	0.384
2	1	250−500	0	0	20	206	154	0.383
3	0	500−1000	0	0	20	114	101	0.379
4	1	500−1000	0	0	50	62	70	0.357
5	0	250−500	10	0	20	358	233	0.624
6	1	250−500	10	0	50	276	148	0.811
7	0	500−1000	10	0	50	117	84	0.729
8	1	500−1000	10	0	20	78	58	0.496
9	0	250−500	0	10	20	326	215	0.553
10	1	250−500	0	10	50	237	133	0.627
11	0	500−1000	0	10	50	108	79	0.586
12	1	500−1000	0	10	20	80	69	0.483
13	0	250−500	10	10	50	383	247	0.776
14	1	250−500	10	10	20	237	171	0.690
15	0	500−1000	10	10	20	113	80	0.597
16	1	500−1000	10	10	50	92	71	0.827
17	0.5	375	5	5	35	188	113	0.632
18	0.5	375	5	5	35	198	132	0.594
19	0.5	375	5	5	35	206	133	0.657

[a] Substrate with (1) or with out seeds (0). [b] Black currant pomace particle size. [c] Enzyme/substrate ratio of enzyme dosage. [d] Extraction solvent 60% methanol. [e] GAE, gallic acid equivalents. [f] Extraction solvent pure water. [g] Yield of soluble carbohydrates, mg/mL glucose.

Table 2. Multiple Linear Regression Results for the Parameters and the Significant Interactions on Yield of Carbohydrates, Phenols, and Anthocyanins

	responses					
	carbohydrates[b]		total phenols methanol[d]		total phenols water[e]	
parameters and interactions (×)	regress. coef.	P[c]	regress. coef.	P[c]	regress. coef.	P[c]
substrate type[a]	no effect		−32.656	9.86×10^{-7}	−22.838	4.40×10^{-6}
particle size	no effect		−101.171	1.52×10^{-11}	−55.613	4.54×10^{-10}
Macer8 FJ	0.112	1.25×10^{-6}	15.494	3.66×10^{-4}	no effect	
Grindamyl pectinase	0.061	3.18×10^{-4}	no effect		no effect	
temperature	0.056	6.43×10^{-4}	no effect		no effect	
Macer8 FJ × Grindamyl pectinase	−0.032	0.02	no effect		no effect	
Macer8 FJ × temperature	0.036	0.011	no effect		no effect	
substrate type × particle size	no effect		15.281	4.03×10^{-4}	13.5	4.50×10^{-4}
particle size × Macer8 FJ	no effect		−10.969	3.54×10^{-3}	−7.725	0.017
constant[f]	0.589	1.05×10^{-14}	196.6	1.63×10^{-14}	131.095	1.58×10^{-14}

[a] Substrate with or with out seeds. [b] The limit of 95% confidence was ±0.026. [c] $P < 0.05$ indicates significance at 95% level. [d] The limit of 95% confidence was ±6. [e] The limit of 95% confidence was ±6. [f] Linear regression coefficient constant.

the cell wall breakdown. Neither the kind of substrate nor the particle sizes of the pomace influenced the plant cell wall degradation, however (Table 2). A positive significance was found for the interaction Macer8 FJ × temperature, indicating that the Macer8 FJ at an E/S ratio of 10% induced a higher degree of plant cell wall breakdown at the higher hydrolysis temperature (50 °C) than at the lower temperature (20 °C). A negative interactive effect between Grindamyl pectinase and Macer8 FJ was recorded. This may indicate some inhibitory effect between the enzyme preparations during catalytic hydrolysis of the pomace.

Effect of Reduced Particle Size. Reduction in particle size of pomace significantly increased the yield of total phenols and anthocyanins in both water and methanol extracts irrespective of whether seeds were present in the pomace substrate or not (Table 3). At all the tested particle size intervals yields of phenols and anthocyanins were significantly higher in extracts from seedless pomace samples than in extracts of pomace samples containing seeds (Table 3). For the phenols this result correlated well to the screenings experiment (Table 2). Significant linear correlations ($P < 0.05$) were found between yield of phenols and reduction in particle size with all combinations of substrate (with or without seeds) and extraction solvent. Thus, both pomace with and without seeds extracted with methanol gave $R^2 = 0.97$ with line estimates $y = -0.46x + 393.9$ and $y = -0.35x + 477.1$, respectively, for the yields of total phenols versus particle size. The negative slope thus signified a steep linear increase in phenols with reduced particle size. For the combinations of the two substrate types (±seeds) and water the analogous relations were $R^2 = 0.98$ with $y = -0.23x + 226.8$ and $R^2 = 0.91$ with $y = -0.17x + 290.6$, respectively (calculations based on results given in Table 3).

Except for the phenols extracted with methanol from pomace samples with seeds, the concentration of phenols did not differ in the blank vs the control signifying that there was no consistent effect of increased water contact time during enzyme hydrolysis (0−8 h) (Table 3). Irrespective of the presence or absence of seeds in the pomace, in both methanol and pure water extracts, the phenol yields of enzyme-treated samples (<125 μm) were higher than the yields of the blank and control of the same particle size (Table 3). Thus, the data demonstrate that there was a positively significant effect on phenol yields of treatment of black currant pomace with the enzyme Macer8 FJ prior to extraction (Table 3).

Table 3. Particle Size Experiment Results

conditions			carbohydrates[b] (mg/mL)		total phenols (mg/L[c] GAE)		anthocyanins (mg/L)	
treatment[a]	particle size	extraction solvent	+ seeds	− seeds	+ seeds	− seeds	+ seeds	− seeds
enzyme	<125 μm	methanol			386^{c}	471^{a}	9.7^{g}	15.5^{f}
enzyme	125−250 μm	methanol			302^{e}	410^{b}	7.2^{h}	11.1^{g}
enzyme	250−500 μm	methanol			193^{h}	320^{d}	3.8^{i}	6.7^{h}
enzyme	500−1000 μm	methanol			65^{k}	228^{g}	1.1^{j}	5.4^{h,i}
control	<125 μm	methanol			234^{f,g}	374^{c}	77.1^{c}	110^{b}
blank	<125 μm	methanol			316^{d,e}	383^{c}	110^{b}	117^{a}
enzyme	< 125 μm	water	0.740^{d}	0.833^{b}	212^{g}	286^{e}	5.7^{h,i}	9.8^{g}
enzyme	125−250 μm	water	0.783^{c}	0.842^{b}	193^{h}	266^{f}	4.8^{h,i}	8.0^{h}
enzyme	250−500 μm	water	0.957^{a}	0.804^{b,c}	130^{j}	202^{g,h}	2.9^{i}	5.0^{h,i}
enzyme	500−1000 μm	water	0.818^{b,c}	0.705^{d}	62^{k}	171^{i}	1.0^{j}	4.6^{i}
control	<125 μm	water	0.477^{e}	0.439^{e}	189^{i,h}	206^{g,h}	47.3^{d,e}	47^{e}
blank	<125 μm	water	0.341^{f}	0.358^{f}	160^{i}	212^{g,h}	48^{d,e}	51^{d}

[a] Enzyme, hydrolysis with Macer8 FJ at 50 °C for 8 h; control, hydrolysis without enzyme at 50 °C for 8 h; blank, no primary hydrolysis. [b] Yield of soluble carbohydrates, mg/mL glucose. [c] GAE, gallic acid equivalent. Results in couples of two columns under carbohydrates, total phenols, and anthocyanins, respectively, followed by different roman superscript letters $a-k$ are significantly different at $P < 0.05$.

Surprisingly, a statistically significant decrease in anthocyanins was seen when the samples were enzymatically treated with Macer8 FJ (Table 3). Moreover, the concentration of anthocyanins in the control and blank samples showed a significant decrease in yield with time except in the water-extracted seeded pomace samples, where no effect was evident (Table 3).

For the degradation of plant cell wall polysaccharides a significant effect of the enzymatic hydrolysis was observed with both substrate types. In all cases the yields of soluble carbohydrates were higher in enzyme-treated samples than in the untreated control and blank samples irrespective of the particle size (Table 3). Because the soluble carbohydrate levels were higher in the controls than in the blanks with both substrate types, there was an enhanced carbohydrate hydrolysis with increased holding time. Although a positive trend in carbohydrate yields of diminished particle size could be distinguished with the pomace samples devoid of seeds, the effect of reducing the particle size was not so significant on carbohydrates yield as it was for the yields of phenols and anthocyanins (Table 3). In the pomace devoid of seeds the largest marginal increase in soluble carbohydrates occurred when substrate particles were decreased from 500−1000 μm to 250−500 μm (Table 3). Further reduction of particle size resulted in smaller increases in soluble carbohydrate levels, and a leveling off of the plant cell wall breakdown in seedless pomace seemed to occur at a particle size around 250 μm. For pomace with seeds no general pattern was obvious (Table 3).

Effect of Other Enzyme Preparations on Yields of Phenols and Anthocyanins, and Plant Cell Wall Degradation. To compare additional plant-cell-wall-degrading enzyme preparations, the effects of the three commercial pectinases (Pectinex BE, Macer8 FJ, and Macer8 R) and one protease preparation (Novozyme 89) were determined on seedless black currant pomace versus pomace with seeds in a fractional two-level factorial 2^6 experimental design (*15*). The protease was included to attack specifically the protein network in the plant cell wall material.

The yields of phenols and anthocyanins, as well as the degree of carbohydrate breakdown, varied considerably in response to different treatments. The phenol and anthocyanins yields were higher in methanol extracts than those in water extracts (Table 4). In general, the yields obtained were higher than those obtained in the previous screenings experiment (Table 1). This finding

Table 4. Release of Total Phenols, Carbohydrates, and Anthocyanins in Response to the Variables Enzyme Type, Hydrolysis Time, and Substrate Type[a] in a 2^6 Factorial Design

response	range	average of center points
phenols (GAE[b] mg/L) methanol[c]	285−604	400
phenols (GAE[b] mg/L) water[d]	186−389	271
carbohydrates[e] (mg/mL)	0.351−1.084	0.770
anthocyanins (mg/L) methanol[c]	8−143	34
anthocyanins (mg/L) water[d]	5−87	23

[a] Enzymes: Pectinex BE, Macer8 FJ, Macer8 R, and Novozyme 89; time: 0.5−8 h; substrate type: with and without seeds. [b] GAE, gallic acid equivalents. [c] Extraction solvent 60% methanol. [d] Extraction solvent pure water. Enzyme dosage as in Table 1. The substrate particle size was <250 μm and the treatments were at 50 °C.

is most likely due to the smaller particle size of the pomace (<250 μm) employed in this second factorial experiment. From linear regression analysis of all the data in Table 4 the amounts of phenols obtained in both methanol and pure water extracts were found to be positively linearly correlated to the degree of plant cell wall breakdown with $r = 0.34$ ($P < 0.05$), $y = 188.2x + 281.8$ for the methanol extracts and $r = 0.36$ ($P < 0.05$), $y = 126.8x + 172.6$ for the water extracts. All the main parameters had a significant effect on the plant cell wall degradation (Table 5). Unexpectedly, the substrate type gave a positive regression coefficient for the carbohydrates, meaning that the carbohydrate breakdown was significantly higher in the pomace samples with seeds than in those without. The opposite effect of substrate with seeds was observed with phenols, as will be discussed below. Two interactions Pectinex BE × Macer8 FJ and Macer8 R × time showed negative and positive effects on the degree of carbohydrate breakdown, respectively. The negative interaction between the two enzymes Pectinex BE and Macer 8 FJ presumably indicated some inhibitory action between these two enzyme preparations during treatment. The positive interaction Macer8 R × time meant that a higher degree of plant cell wall degradation was obtained with the longer hydrolysis time (8 h) than with the shorter time (0.5 h) in the presence of Macer8 R. The significance of responses to the different parameters was almost similar for phenols extracted with water and methanol (Table 5) as it was also seen in the first screenings experiment (Table 2). Each one of the enzymes significantly improved the release of phenols in the methanol

Table 5. Multiple Linear Regression Results for the Parameters and the Significant Interactions on Yield of Carbohydrates and Phenols

parameters and interactions (×)	responses					
	carbohydrates[a]		phenols methanol[c]		phenols water[d]	
	regress. coef.	P^b	regress. coef.	P^b	regress. coef.	P^b
Pectinex BE	0.04	1.24×10^{-3}	11.903	0.03	10.63	1.15×10^{-3}
Macer8 FJ	0.069	1.35×10^{-6}	26.887	2.36×10^{-5}	no effect	
Macer8 R	0.101	1.55×10^{-9}	27.822	1.50×10^{-5}	20.736	1.66×10^{-7}
Novozym 89	0.031	0.009	18.084	1.78×10^{-3}	16.952	4.16×10^{-6}
time	0.06	1.23×10^{-5}	10.159	0.06	10.67	1.11×10^{-3}
substrate type[e]	0.025	0.032	−69.769	9.65×10^{-13}	−42.73	7.55×10^{-14}
Pectinex BE × Macer8 FJ	−0.025	0.034	no effect		no effect	
Macer8 R × time	0.023	0.044	no effect		13.814	$6.74\ 10^{-5}$
Pectinex BE × Macer8 R	no effect		−11.294	0.038	no effect	
Pectinex BE × Novozym 89	no effect		9.637	0.073	no effect	
Pectinex BE × time	no effect		11.131	0.041	no effect	
Novozym 89 × time	no effect		−12.75	0.021	no effect	
Pectinex BE × substrate type	no effect		no effect		−9.348	3.4610^{-3}
Macer8 R × Novozyme 89	no effect		no effect		5.964	0.05
constant[f]	0.733	5.83×10^{-31}	419.646	2.60×10^{-31}	268.156	1.01×10^{-33}

[a] The limit of 95% confidence was ±0.023. [b] $P < 0.05$ indicates significance at 95% level. [c] The limit of 95% confidence was ±11. [d] The limit of 95% confidence was ±6. [e] Substrate with or without seeds. [f] Linear regression coefficient constant.

Table 6. Multiple Linear Regression Results for the Parameters and the Significant Interactions on Yield of Anthocyanins

parameters and interactions (×)	responses			
	anthocyanins methanol[a]		anthocyanins water[c]	
	regress. coef.	P^b	regress. coef.	P^b
Pectinex BE	no effect		no effect	
Macer8 FJ	−18.827	1.59×10^{-6}	−10.868	7.81×10^{-5}
Macer8 R	−7.311	0.023	no effect	
Novozym 89	no effect		no effect	
time	−27.93	1.5×10^{-9}	−16.931	8.62×10^{-8}
substrate type	−14.538	6.00×10^{-5}	−7.324	4.13×10^{-3}
Macer8 FJ × time	−7.931	0.014	no effect	
constant[d]	68.529	1.14×10^{-18}	44.856	9.32×10^{-18}

[a] The limit of 95% confidence was ±6. [b] $P < 0.05$ indicates significance at 95% level. [c] The limit of 95% confidence was ±5. [d] Linear regression coefficient constant.

as they did in the water; the only exception was again Macer8 FJ (Table 5). The seedless substrate gave significantly enhanced phenol yields compared to those of the substrate containing seeds. A negative regression coefficient for substrate type signifies that the yields from seedless pomace (0) are higher than those from pomace with seeds (1) (Table 5). The time parameter was significant for the water extract and also pretty close for the methanol extract ($P = 0.06$). In contrast, there were no identical significant interactions for the methanol and water extracts among the total of eight significant interactions identified (Table 5). The interactions Pectinex BE × Novozyme 89 for the methanol extracts and Macer8 R × Novozyme 89 for the water extracts both had a positive coefficient indicating that a synergistic effect between the pectinases Pectinex BE and Macer8 R, respectively, and the protease Novozyme 89 occurred during treatment. For phenols extracted with methanol, there was a positive effect on yields of prolonged treatment with Pectinex BE, and a negative effect of prolonged treatment with Novozym 89 (Pectinex BE × time and Novozym 89 × time, Table 5). When phenols were extracted with pure water, elevated yields were obtained with prolonged Macer8 R treatment (Macer8 R × time, Table 5). Moreover, when phenols were extracted with pure water, an improved effect of seed removal penetrated with Pectinex BE treatment (Pectinex BE × substrate type, Table 5).

Neither Pectinex BE nor the acid protease Novozym 89 had an effect on anthocyanin yield; however, a significantly negative effect was obtained for both Macer8 FJ and for Macer8 R (Table 6). The parameters time and substrate type both had a significantly negative effect meaning that the long hydrolysis time contributed to the degradation of anthocyanins and that the seedless substrate gave higher anthocyanin yields, respectively (Table 6). The negative interaction Macer8 R × time recorded for the methanolic extract meant that a larger part of the anthocyanins degraded with longer hydrolysis time (8 h) than with the shorter time (0.5 h), in the presence of Macer8 R.

Antioxidant Activity toward LDL Oxidation. The antioxidant activities of methanol extracts from four different treatments in the 2^6 factorial experiment were compared toward human LDL oxidation in vitro at equimolar phenol concentrations of 7.5 and 10 μM GAE. The extracts, here named A, B, C, and D, were selected for their concentrations of phenols and anthocyanins: all extracts had high phenolic levels; extracts B and C had high anthocyanin levels, but extracts A and D had low levels of anthocyanins (Table 7). Moreover, extracts A and D were made from pomace with seeds that had been reacted with enzymes for 0.5 and 8 h respectively, whereas extracts B and C were from seedless pomace reacted with a similar enzyme composition for 0.5 and

Table 7. Antioxidant Activities of Black Currant Pomace Extracts on Human LDL

exp.	enzymes[a]	seeds ±	hydrolysis time	total phenols (TP)[b], anthocyanins (A)[c]	AO activity[d] (min) at 7.5 μM GAE[e]	AO activity[d] (min) at 10 μM GAE[e]
A	Pectinex BE, Macer8 FJ, Novozym 89	+	0.5 h	TP high, A low	102.4[b]	228.7[a,b]
B	Pectinex BE, Macer8 FJ, Macer8 R, Novozym 89	−	0.5 h	TP high, A high	122.7[a,b]	>280[a]
C	Pectinex BE, Macer8 FJ, Novozym 89	−	8 h	TP high, A high	146.0[a]	>280[a]
D	Pectinex BE, Macer8 FJ, Macer8 R, Novozym 89	+	8 h	TP high, A low	74.4[b,c]	>280[a]
	gallic acid[f]					180.7[c]

[a] Enzyme treatment prior to extraction. All particles sized <250 μm, $T = 50$ °C. [b] TP high, high level of total phenols. [c] A high, high level of anthocyanins; A low, low level of anthocyanins. [d] AO, antioxidant. AO activities are given as average net prolongation of induction time for conjugated diene hydroperoxide formation. Results in the same column followed by different roman superscript letters $a-c$ are significantly different at $P < 0.05$. [e] GAE, Gallic acid equivalents. [f] Antioxidant control compound.

Table 8. Phenol and Anthocyanin Yields in Wine and Black Currant Pomace Extracts

treatment	total phenols (GAE mg/L)		anthocyanins[a](mg/L)	
± seeds, extraction solvent	wine pomace	black currant pomace	wine pomace	black currant pomace
− seeds, methanol	342[c,y]	579[a,x]	13.0[a,y]	110.5[a,x]
− seeds, water	224[d,y]	330[c,x]	7.6[b,y]	70.3[b,x]
+ seeds, methanol	1393[a,x]	461[b,y]	4.9[c,x]	8.4[c,x]
+ seeds, water	839[b,x]	321[c,y]	2.5[d,x]	5.8[c,x]

[a] Amounts given as mg/L cyanidin-3-rutinoside or malvidin-3-glucoside equivalents for anthocyanins in black currant or wine pomace extracts, respectively. Results in individual columns followed by different roman superscript letters $a-d$ are significantly different at $P < 0.05$. Results in the same pair-wise rows followed by different roman superscript letters $x-y$ are significantly different at $P < 0.05$. All particles were sized <250 μm, $T = 50$ °C.

8 h, respectively (Table 7). Gallic acid was used as an antioxidant control compound.

At 7.5 μM GAE all extracts exerted a significant antioxidant effect compared to that of the control, but significant differences in antioxidant potency among the four extracts were found, with extract C exerting the highest antioxidant activity and extract D having the lowest (Table 7). At 10 μM GAE three out of four extracts (B, C, and D) totally inhibited the oxidation in the time interval measured (5 h), but the dose−response effect was less marked for extract A. At the same equimolar concentration of 10 μM GAE all the extracts were more effective antioxidants than pure gallic acid (Table 7).

Comparison with Extracts from Wine Pomace. The hydrolysis parameters used to produce the black currant pomace extracts B and D (Table 7) were applied on grape pomace from wine production as well, and the grape pomace and black currant pomace extracts were subsequently compared (Table 8). The phenol yields obtained for pomace samples without seeds were significantly higher for black currant pomace than they were for wine pomace. The opposite result was recognized for the treatments of pomace samples with seeds, where the amount of phenols was significantly higher for the wine pomace than for the black currant pomace in both methanol and water (Table 8). The same results for the two different kinds of substrates were observed for the anthocyanin yield results, with the exception of the substrates with seeds, where no significant effects were found (Table 8).

DISCUSSION

Although it is known that both lignin and phenolic compounds may inhibit plant cell wall degrading enzyme activities (*21*−*23*), the present data showed that increased extraction of phenols was positively correlated to enzymatic degradation of the black currant pomace polysaccharides. Furthermore, it was demonstrated that the yield of total phenols in both methanol and water extracts increased significantly after treatment with each of the pectinolytic enzyme preparations, Pectinex BE, Macer8 FJ, and Macer8 R, as well as with the protease Novozym 89, although not with Grindamyl pectinase (Tables 2 and 5). Macer8 FJ and Macer8 R had significantly negative effects on the yield of anthocyanins, whereas Pectinex BE and Novozym 89 did not (Tables 3 and 6). Enzyme-catalyzed anthocyanin pigment degradation is a recognized adverse effect of enzymes used in fruit juice processing (*24*). This degradation may be caused by polyphenol oxidase activity that induces coupled oxidative browning reactions, or it may be attributable to the presence of β-glucosidase, β-galactosidase, or α-L-arabinosidase side activities in multicomponent pectinolytic enzyme preparations that cause hydrolysis of the native glycosylated anthocyanins to produce unstable aglycons and in turn cause color loss (*24*). Pectinex BE is recommended for mash treatment and color extraction in berry juice processing and this particular preparation was previously shown to be superior to other commercial juice-processing pectinases in retaining the pigments cyanidin-3-galactoside and cyanidin-3-arabinoside responsible for cranberry juice color (*24*). In contrast, other pectinase preparations produced by the *Aspergillus niger* group have been shown to possess β-galactosidase, α-arabinosidase, and/or β-glucosidase activities that affect selected anthocyanin pigments in different fruit juices (cranberry, raspberry, grape) and thus affect juice color quality (*24*, *25*). In black currant the four major anthocyanins are the 3-*O*-rutinosides (α-L-rhamnosyl-D-glucoses) and 3-*O*-glucosides (β-D-glucopyranosides) of cyanidin and delphinidin (*13*). Both Macer8 FJ and Macer8 R appeared to possess glycosidase activities that destroyed the black currant anthocyanin pigments. According to the enzyme manufacturer's data sheet, the Macer8 R preparation has been developed for red wine processing with par-

ticular attention to the incorporation of β-glucosidase activity to enhance flavor development from the existing precursors. Our data signify that it is important that such β-glucosidase activity is very specific for this purpose so that undesirable color loss by degradation of anthocyanins is avoided. Although the enzyme concentrations employed in this study exceeded those employed in juice and wine making, the observed negative effect of the Macer enzymes on anthocyanins could have an adverse effect on color quality in their intended uses in fruit juice and wine processes. Better knowledge of the presence and specificity of different enzyme activities in multicomponent enzyme preparations is clearly needed to tailor new enzyme-catalyzed reactions in fruit processing.

Each of the four pectinases and the acid protease had significantly positive effects on the breakdown of the plant cell wall in black currant pomace (Tables 2 and 5). The finding that the Grindamyl pectinase efficiently degraded pomace polysaccharides, but as an individual factor did not necessarily improve total phenols yields, is in agreement with previous observations for the action of this enzyme preparation on grape pomace remaining from wine processing (*26*). The precise locations of phenolic compounds, their type of bonding, and possible physical entrapment in lignin and plant cell wall networks in heterogeneous fruit pomace substrates is largely unknown at present. Taken together, the data obtained strongly suggest that enzymatic degradation of plant cell wall polysaccharides increased pore formation, pore size, and overall substrate porosity, and thereby facilitated enhanced solvent penetration and extraction efficiency and that this − rather than direct enzyme-catalyzed release of phenols − improved the recovery of phenols from pomace.

It is widely accepted that a hydroxyproline-rich glycoprotein, extensin, forms a lattice that entangles the cellulose microfibrils in most primary plant cell walls. However, the nature of the cross-links between the extensin and the cell wall polymers has not been fully elucidated (*27*). The acid protease Novozym 89 was included in the hope that it would give a synergistic effect on the plant cell wall degradation with one or more of the pectinases. However, no interactions between the protease and the pectinases penetrated for the breakdown of plant cell wall carbohydrates, but the protease as an individual factor improved the degradation (Table 5). On the other hand, for the yields of phenols, synergistic interactions were recorded between the protease and the Pectinex BE and Macer8 R, respectively, and the protease alone also improved the recovery of phenols (Table 5). These results suggested that degradation of the protein network in itself, as well as concomitantly with enzymatic hydrolysis of the plant cell wall polysaccharides, enhanced overall substrate porosity and, with that, the extraction recovery of phenols.

The data obtained throughout this work showed that extracts made from seedless black currant pomace gave significantly higher yields of total phenols and anthocyanins than extracts made from pomace with seeds. Black currant pomace remaining after juice production mainly consists of skin and seeds. Hence, in agreement with the available knowledge that the anthocyanins are mainly localized in the skins of black currant (*12*), the highest amounts of phenols and anthocyanins were extracted from the skin fraction of the pomace and the presence of the crushed seed fraction in effect decreased phenol yields. This effect of seeds was opposite to what was previously observed for grape seeds (*10*). The negative effect of the presence of seeds on phenol yields from black currant pomace may be due to the seeds containing only a low concentration of phenols compared to the skin fraction, or may be due to the phenols in black currant seeds being anchored more strongly in the seed lignin network than in the skin plant cell wall networks.

The finding (Table 8) that the skin fraction in black currant pomace gave a significantly higher concentration of total phenols and anthocyanins than wine pomace skin may have root in the fact that the phenols in the skin fraction from red wine pomace for a large part have been extracted in the process of wine making. In the production of red wine the pomace is in close contact with the fermenting wine for several days (*28*) and the phenols are thus subjected to a mild, but prolonged, ethanolic extraction. In contrast, the contact time between skin, seeds, and juice after berry crushing in the production of black currant juice is comparatively very short, as the maceration step prior to pressing is usually accomplished in a few hours (*29*). Relatively little is known about the distribution of phenols in skin and seeds of black currant, and, as mentioned above, there is also a general lack of information about the types of bonding, possible physical entrapment, etc. of phenols in black currant berries as well as in other fruits. Further knowledge in this area is highly warranted as it would provide an improved foundation for developing targeted enzymatic hydrolysis that would be useful not only for utilization of juice and wine pomace, but also for enzymatic maceration in fruit juice and wine processing.

Another very significant parameter affecting phenol yields was the reduction in particle size diameter of the black currant substrate. Thus, significant correlations ($P < 0.05$) between reduction in particle size and yield of total phenols were found for all combinations of substrate and extraction solvent (Table 3). Also, for the anthocyanins a decreased particle size gave significantly higher yields (Table 3). Although the particle size did not penetrate as being of significant importance for the degradation of plant cell wall carbohydrates in the screenings experiment (Tables 1 and 2), it showed a significant trend in the enzymatic breakdown of the seedless substrate in the investigation of the particle size (Table 3). These results agreed well with results obtained for wine pomace (*26*). They were also in accordance with data obtained for enzymatic hydrolysis of other lignocellulosic byproducts from the food industry, such as sunflower and palm kernel meals, where the substrate particle size was found to be a major determinant in the enzymatic solubilization of nonstarch polysaccharides (*30*). Presumably, when the particle size is reduced the accessible surface for both enzyme attack and extraction may be increased. The present study was not aimed at elucidating the mechanisms for the particle size effect, but the data obtained confirmed the available knowledge that a decreased particle size improves the enzymatic hydrolysis of insoluble cellosic substrates and thus the enzymatic saccharifaction of complex plant cell wall structures (*23*, *30*). Furthermore, this study demonstrated that this enzymatic action resulted in better solvent penetration and improved recovery of phenolic compounds.

The results in Table 3 showed significantly different groupings within the yields of total phenols and anthocyanins. Important to notice was that if the seed containing pomace was milled fine enough (125−250 μm) water could be as good an extraction solvent as methanol on pomace with a larger particle size (250−500 μm), and that is an important issue in the food industry where use of methanol may be undesirable. In general, methanol is a more effective extraction solvent than water for a wide spectrum of phenols (*31*). Throughout this study the methanol extracts, on average, had 1.6 (± 0.2) times higher phenol concentrations than water extracts.

For all four selected extracts a significant antioxidant activity on human LDL oxidation in vitro was observed at 7.5 and 10 μM GAE. The two extracts with the highest antioxidative effects (B and C) had high concentrations of both phenols and anthocyanins and were produced from seedless substrate. All the extracts but one totally inhibited the oxidation at 10 μM GAE when measured for 5 h (Table 7). The extract unable to make the inhibition complete for 5 h was extract A, which also had the lowest total amount of phenols (356 mg GAE/L). Because the extracts were diluted to the same equimolar concentrations the differences in their antioxidant potencies is due to the differences in phenolic composition among the extracts, which in turn appears to depend on the sample pretreatment. This conclusion is in full agreement with what has been observed previously with grape pomace extracts (*26*).

In conclusion, the data obtained in the present work have demonstrated that black currant pomace left over from juice production provides a good source of phenolic antioxidants that may have nutritional benefits. Furthermore, the results of the present work demonstrate that separation of skins from seeds and diminution of pomace particle size, as well as enzymatic treatment with plant cell wall degrading enzymes, are techniques that deserve serious positive consideration in any eventual exploitation of black currant pomace for recovery of phenolic compounds for production of antioxidant concentrates.

ACKNOWLEDGMENT

Donations of enzyme samples from Biocatalysts Ltd., Danisco Ingredients, and Novo Nordisk A/S are gratefully acknowledged.

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Received for review November 30, 2000. Revised manuscript received April 3, 2001. Accepted April 9, 2001. This work was partially supported by the European Commission Research Directorates, Project QLRT-1999-00124.

JF001443P