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Protease-Assisted Clarification of Black Currant Juice: Synergy with Other Clarifying Agents and Effects on the Phenol Content

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Conventional clarification with gelatin and silica sol removes a considerable amount of antioxidant phenolics from berry juices. This study examined the clarification and haze-diminishing effects of alternative clarification strategies on black currant juice including centrifugation and addition of acidic protease and pectinolytic enzyme preparations and gallic acid. Centrifugation of freshly pressed juice (10000*g* for 15 min) resulted in a ∼95% reduction of immediate turbidity and had a decreasing effect on haze development in the juice during cold storage without significantly compromising the total phenols levels. The extent of clarification and haze diminishment varied after individual treatments with five different acidic proteases, but one of the protease preparations, Enzeco, derived from *Aspergillus niger*, consistently tended to perform best. The individual and interactive effects on juice turbidity, total phenols, and total anthocyanin contents of clarification treatments involving the use of two selected acid proteases (Enzeco and Novozyme 89L), a pectinase (Pectinex BE 3-L), and gallic acid were evaluated in a full factorial 2^4 experimental design. Haze development during cold storage decreased when gallic acid or any of the enzyme preparations were employed individually, but negative interaction effects resulted when the pectinase was employed in combination with any of the proteases. After 28 storage days at 2 °C, the lowest levels of haze formation were achieved when the Enzeco protease preparation, added at 0.025 g/L, was added with 0.050 g/L of gallic acid and allowed to react in the juice for 90 min at 50 °C. The corresponding anthocyanin reduction was ∼12% (compared to ∼30% with gelatin silica sol treatment). The data support the hypothesis that phenol−protein interactions are involved in juice turbidity development during cold storage of berry juices and demonstrate that precentrifugation and protease-assisted clarification show promise as an alternative, phenolics-retaining clarification strategy in black currant juice processing.

KEYWORDS: Clarification; protease; pectinase; black currant juice; turbidity; haze; gelatin; silica sol

INTRODUCTION

Black currants are commonly grown in northern Europe, where the estimated annual harvest is around 500 000−600 000 tons. These berries are primarily processed for pure juice, juice concentrates, and jam production (*1*, *2*). As in other clear juice manufacturing processes, clarification is a fundamental step in the black currant juice production process. The purpose of the clarification step is to remove turbidity in the freshly pressed juice and to prevent the eventual development of haze during storage or after reconstitution of the juice concentrate. In general, the presence of cell fragments and small insoluble pectin species has been found to be responsible for the immediate turbidity in freshly pressed fruit juices, whereas the development of haze may result from interactions between polysaccharides, sugars, metal ions, and proteins or be due to oxidative polymerization of polyphenols, with protein−polyphenol interactions being considered as the most frequent cause of haze formation in beer, wine, and clear fruit juices (*3*−*6*). The protein−phenol haze forms via hydrogen and/or hydrophobic bonding between so-called haze-active, proline-containing proteins and phenolics (*5*, *7*). The hydrogen bonds occur between the hydroxyl groups of polyphenols and the carbonyl oxygen in the peptide backbone, whereas the hydrophobic interactions are generated via attraction between the aromatic structure of polyphenols and the nonpolar proline moiety in proteins (**Figure 1**) (*7*).

The use of gelatin and silica sol is one of the most widespread procedures employed to remove immediate turbidity and haze. Gelatin removes polyphenols that could combine with haze-active proteins or polymerize to give turbidity and haze, whereas the silica sol works by reacting with haze-active proteins and also removes surplus gelatin (*8*, *9*). When added in solution, the gelatin forms insoluble gel lumps, which are allowed to sediment slowly, and both the gelation and the sedimentation

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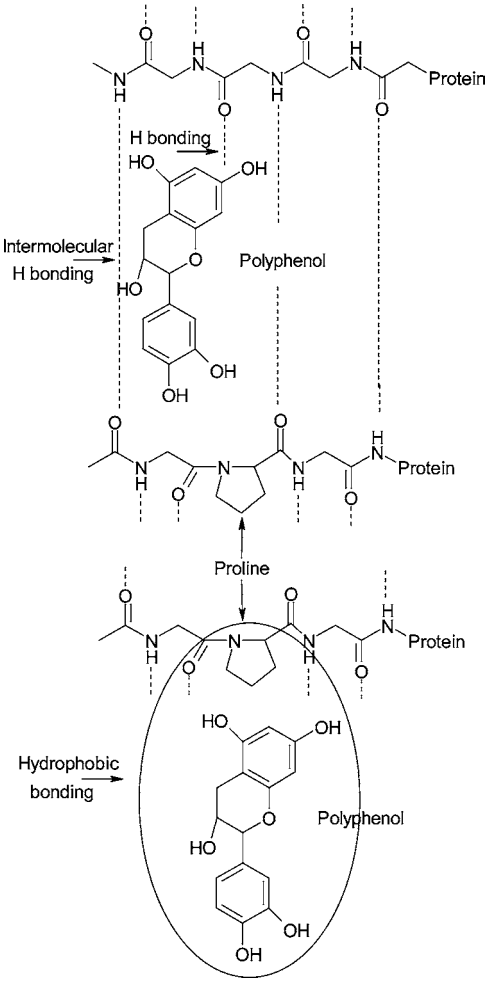

Figure 1. Hypothesized chemistry of haze-causing protein–polyphenol hydrogen and hydrophobic bonding, respectively, in beer, wines, and fruit juices (adapted from ref *7*).

process furthermore capture additional insoluble and particulate material to further reduce the immediate turbidity (*8*). The drawbacks derived from the use of this clarification technique relate to the long sedimentation times, which usually last 6−18 h, as well as the required use of vigorous downstream filtration procedures such as high-vacuum rotary filtration with diatomaceous earth to recover some of the juice captured in the colloid slurry (*8*, *9*). Not surprisingly, this clarification process causes a considerable reduction of the phenolic compounds in the juice (*10*).

Enzymatic depectinization of juice is widely used in conjunction with gelatin and silica sol for industrial juice clarification. The enzyme-catalyzed depectinization generally clarifies the juice both by hydrolysis of insoluble pectin fragments and by subsequent destabilization of the pectin cloud. The destabilization is a result of partial pectin hydrolysis that brings about aggregation and flocculation of oppositely charged pectin particles (*8*). Apart from their use in this enzymatic depectinization, microbial pectinases are also used in prepress fruit maceration and have recently been shown to be efficient in both enhancing black currant juice yields and favoring the release of some potentially beneficial health compounds such as phenolic acids and flavonoids during the berry maceration (*11*, *12*).

The use of proteases for clarification is based on the proteolysis of the haze-active proteins, thus preventing the binding with haze-active polyphenols and favoring the decrease of haze levels without removal of the phenolics. The principle of using proteases to prevent chill-haze in beer was already patented in 1978 (*13*), and the workability of enzymatically targeting the proline-rich proteins was very recently proven using proline-specific, microbial endoproteases to prevent chill-haze in beer (*14*). Protease-assisted clarification has also been studied for wine production (*15*, *16*), whereas only scarce literature can be found about the application of proteases in juice clarification (*17*, *18*).

In an attempt to minimize the loss of phenolics caused by the gelatin silica sol treatment, this study was focused on exploring, in laboratory-scale experiments, the use of centrifugation, protease or pectinase−protease treatments, and/or gallic acid addition as alternative strategies for black currant juice clarification. We report the individual and interactive effects of different pectinase, protease, and pectinase−protease treatments, as well as of gallic acid addition, on immediate juice turbidity, phenol and anthocyanin levels, and haze formation during cold storage of black currant juice. The data obtained are compared to those obtained by use of conventional gelatin silica sol clarification.

MATERIALS AND METHODS

Materials. The black currant berries (Ben Lomond, *Ribes nigrum*) and the industrially produced juice were provided by Vallø Saft A/S (Køge, Denmark). The latter had been subjected to pectinase-assisted maceration, pressing, and pasteurization, but had not been clarified. This juice and the berries were kept frozen (−20 °C) until use. Gallic acid and the reagents for the total phenols assay were purchased from Sigma-Aldrich (St. Louis, MO), whereas those used for measuring total anthocyanins content were from Merck (Darmstadt, Germany). Gelatin was purchased from SKW Biosystems (Boulogne Billancourt Cedex, France), and the silica sol (klar-Sol Super) was from Erbslöh Getränke-Technology (Geisenheim, Germany). The Grindamyl LB Pectinase for juice production was obtained from Danisco A/S (Brabrand, Denmark). The pectinase preparation and the five acid proteases used in the clarification experiments were from different enzyme manufacturing companies (**Table 1**). The pectinase preparations were selected because of their already widespread use in the fruit juice and wine industry, whereas the proteases were selected because of their acid tolerance and broad specificity.

Sample Preparation. Frozen black currant berries were initially defrosted and then gently crushed in a meat grinder (Jupiter; type 863, Germany). The berry mash was subsequently packed under vacuum in airtight polyethylene bags, heated at 80 °C for 2 min for inactivation of endogenous enzymes and pasteurization, and then stored at −20 °C until use. Once heated in a water bath at 20 °C, the berry mash was mixed with the Grindamyl LB Pectinase enzyme preparation at a 0.05% E/S addition level (E/S is enzyme/substrate ratio expressed as milliliters of enzyme preparation per 100 g of substrate). Then the samples were placed in a 50 °C water bath and incubated for 2 h with punctual, manual mixing using a glass spatula every 15 min. After this, the mash was pressed in a 5 L Tincture press (Essen, Germany) using nylon filter bags at a pressure of 100 bar. The juice was then pasteurized at 90 °C for 60 s, cooled, bottled, and then purged with nitrogen before the flasks were capped. As a final step and if required (following the requirements of each experiment), the juice was centrifuged at 10000*g* for 2 min. The centrifugation time was chosen to be only 2 min because longer centrifugation times at 10000*g* would be difficult to achieve industrially. After centrifugation, the juice was aliquoted into capped flasks (45 mL), purged with nitrogen, and frozen at −20 °C until use.

Centrifugation. Considering the turbidity reduction effect of centrifugation reported for cherry juice in our previous study (*18*), black currant juice samples were defrosted and centrifuged at 10000*g* for 15 min to study the influence of centrifugation on clarification.

Clarification with Gelatin, Silica Sol. The gelatin and silica sol clarification was made on industrially produced juice both with and without precentrifugation. A gelatin solution was prepared (12.5 g/L)

Table 1. Enzyme Preparations Employed in the Experiments (Data from Suppliers' Datasheets)

enzyme name	activity[a]	unit definition	pH optimum	temperature optimum (°C)	production strain
Amano Acid Protease A[b]	35000 U	production of 100 μg of tyrosin equiv during 60 min at pH 3.0 and 55 °C	3	55	*Aspergillus niger* strain
Denapsin 2P[c]	20000 APUN	liberation, per minute, of the digestion product not being precipitated with TCA, and which gives the same Folin color as 1 μg of tyrosine (10 min, pH 3.0, 30 °C)	3	50	*Aspergillus niger* strain
Pepsin 389P[d]	3000–3500 NF	must digest not less than 3000 and not more than 3500 times its weight of denatured egg albumen	3	48–50	extracted from the lining of hog stomach
Enzeco Fungal Acid Protease[e]	200000 APU	acid protease units (1100 milk clot units)	2.5–3.5	40–55	*Aspergillus niger* strain
Sumizyme AP[f]	200000 U	decomposing activity of hemoglobin (pH 4.7)	3	60	*Aspergillus niger* strain
Novozyme 89L[g]	0.18 AU	denatured hemoglobin, 0.02 M Ca^{2+} buffer at pH 5.5 and 37 °C	4.5–5.5	45–55	*Mucor miehei* strain
Pectinex BE 3-L	1600 MOE/mL	"Most Einheit" reduction in the viscosity of apple juice	2.5–3.5	50	*Aspergillus niger* strain

[a] Activities given by the suppliers' datasheets. [b] Supplied by Amano Pharmaceutical Co. Ltd., Nagoya, Japan. [c] Supplied by Nagase Biochemical Ltd., Tokyo, Japan. [d] Supplied by Biocatalysts Ltd., Pontypridd, U.K. [e] Supplied by Enzyme Development Corp., New York. [f] Supplied by Shin Nihon Chemical Co., Tokyo, Japan. [g] Supplied by Novozymes Ltd., Bagsværd, Denmark.

according to the manufacturers' datasheet, and the solution was added to the juice samples (0.5 mL of gelatin solution/100 mL of juice), which were then shaken for 5 min in a 50 °C water bath (thermostatically controlled Julabo SW-20C, Seelbach, Germany). Then, the silica sol solution (1.188 g/mL, addition level: 55 μL/100 mL of juice) was added with stirring, and the juice samples were shaken for another 5 min at 50 °C. In these experiments 6 h was enough time to allow complete spontaneous sedimentation of the formed flocs at 50 °C, and a sedimentation time of 6 h was therefore chosen as the benchmark treatment. Afterward, the juice samples were centrifuged at 10000*g* for 10 min to further separate the sedimenting flocs from the supernatant juice. The supernatant was then filtered through a Whatman filter no. 1, and the clarified juice was transferred into dark plastic bottles and purged with nitrogen. Turbidity, that is, haze development, and phenolics content of these traditionally clarified juices were subsequently compared with those of protease-clarified samples after 35 days of storage at 2 °C, which was chosen as a benchmark to compare the chill-haze turbidity values of the juices subjected to the different treatments.

Screening of Proteases. The clarifying effects of Amano Acid Protease A, Denapsin 2P, Pepsin 389P, Enzeco Fungal Acid Protease, and Sumizyme AP (**Table 1**) were tested in a two-factor, time and temperature, two-level design. Addition levels of the proteases were 0.5% E/S w/w, the haze-active protein concentration in the industrial juice (that is S) being estimated to be 0.5% w/w (the addition levels were thus equivalent to adding 0.025 g of protease preparation/L). No enzymatic treatment was applied to the controls, which underwent only thermal and treatment time variations. Two controls corresponding to uncentrifuged and centrifuged juice were included. After clarification, the samples were experimentally filtered under vacuum (Whatman filter no. 1), purged with nitrogen, and stored in dark plastic bottles at 2 °C for 35 days.

Clarification with Pectinase and Protease. Two acid proteases, Enzeco Fungal Acid Protease and Novozyme 89L, the latter had shown promising results in the clarification of centrifuged cherry juice (*18*), were individually tested in two identical two-factor Box−Behnken response surface design templates (*19*). Each template comprised 16 different combinations of reaction time (30−90 min), protease dosage (0−0.5% E/S, with E being the weight of the enzyme preparation and S being an estimated protein concentration of 0.5% w/w of the juice), Pectinex BE 3-L (0−0.5% v/v), and gallic acid (0−0.050 g/L) with three center points. Chemical features of gallic acid, that is, the presence of three ortho-positioned hydroxyl groups, were expected to have a positive effect on turbidity reduction. The temperature was held constant at 50 °C. After each treatment, the individual juice samples were vacuum filtered through a Whatman filter no. 1 and prepared for cold storage as described below.

Cold Storage for Haze Formation Monitoring. Experimentally clarified juice samples (i.e., nonindustrial juice samples) were aliquoted (5 mL) into sample vials, and potassium sorbate and sodium benzoate were added immediately after clarification to a final concentration of 0.5 ppm of each to prevent spoilage of the juice samples during storage. Before sealing, the sample vials were purged with nitrogen. The samples were stored in tightly capped vials at 2 °C in the dark and analyzed at day 0 (immediately after clarification) and after 7, 14, and 28 storage days. Samples from the protease screening experiments were analyzed after 35 days only. The rate of haze development during cold storage was calculated as the ratio between the average changes of FNU values (see below) during the storage time expressed in days (ΔFNU/day).

Determination of Turbidity. Turbidity in formazin nephelometric units (FNU) was measured by nephelometry at 90° light scattering and 860 nm, with a Nephla reader (Dr. Lange, Düsseldorf, Germany) calibrated against hexamethylene tetramine formazine. Prior to measurement, all samples were diluted with distilled water: the juice from Vallø Saft A/S and the juice samples made in the laboratory were diluted at 1:3 and 1:5, respectively, to cool the samples and to obtain 3 °Brix as required for the turbidity measurements. The °Brix value is proportional to the amount of sugar in the juice, and °Brix was determined by measuring the refractive index with a manual refractometer (Carl Zeiss GmbH, Vienna, Austria).

Determination of Total Phenols and Anthocyanins. Total phenols in the juices were determined according to the Folin−Ciocalteu procedure and expressed as milligrams per liter gallic acid equivalents (GAE) (*20*). Total anthocyanins were determined by the pH differential method and expressed as cyanidin-3-rutinoside equivalents (*21*).

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 (MLR). The responses in the MLR were level of immediate turbidity, rate of haze development, and phenol and anthocyanin contents. 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).

RESULTS AND DISCUSSION

Centrifugation. Centrifugation of the industrially unclarified black currant juice at 10000*g* for 15 min was found to be very effective in clarifying the juice as the centrifugation reduced significantly the immediate turbidity from 200 to 10 FNU (~95% reduction). Slightly decreased levels, equivalent to an

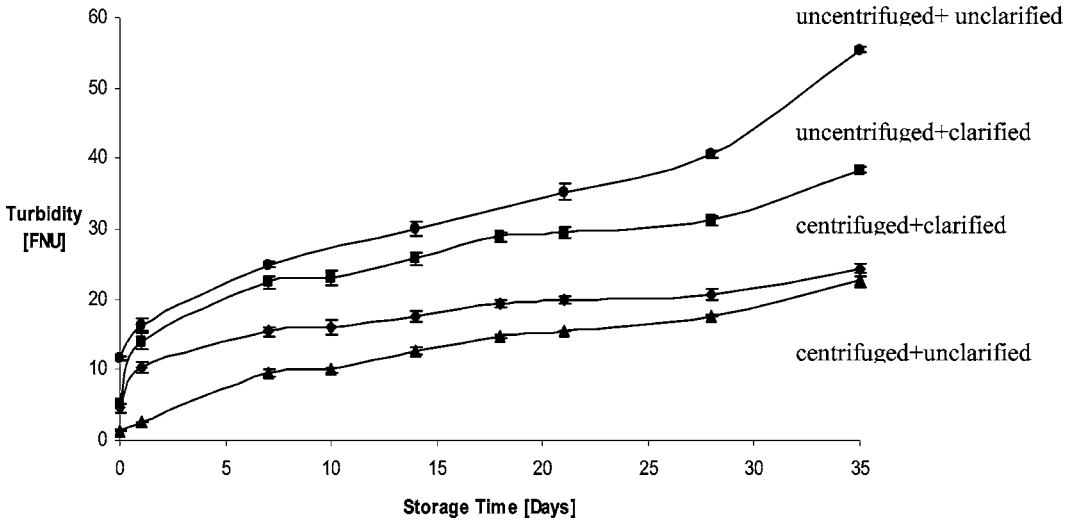

Figure 2. Evolution of turbidity, that is, FNU values, in industrially produced black currant juice during storage at 2 °C. Samples were subjected to different prestorage, clarification treatments: uncentrifuged plus unclarified (no centrifugation or chemical clarification treatment, but held at 50 °C as clarified samples); uncentrifuged plus clarified (no centrifugation, clarified with gelatin silica sol); centrifuged plus clarified (centrifuged at 10000*g* for 15 min and clarified with gelatin silica sol); centrifuged plus unclarified (centrifuged at 10000*g* for 15 min, no chemical clarification treatment, but held at 50 °C as clarified samples).

8% decrease in content of total phenols and anthocyanins, were detected in the centrifuged sample, with only the loss in anthocyanins being statistically significant. The slight decrease in these compounds during centrifugation could be ascribable to their capture by the pectin and insoluble plant cell wall fragments during their removal in the centrifugation process. With unclarified cherry juice (*18*) a similar decrease, that is, >98% reduction in turbidity, was obtained with high-speed centrifugation, in that case, 20000*g* for 20 min, signifying the efficiency of a centrifugation step for fast turbidity reduction before other clarification treatments (*18*).

Introduction of centrifugation prior to conventional clarification with gelatin and silica sol significantly decreased, but did not inhibit, haze development during cold storage at 2 °C for 35 days (**Figure 2**). Hence, centrifuged samples had lower turbidity levels than uncentrifuged samples irrespective of whether the juice samples had been subsequently clarified with gelatin silica sol or not (**Figure 2**). After 7 storage days, all samples exceeded the critical reference FNU value of 5, indicating the need to explore more efficient clarification methods. Surprisingly, centrifugation alone gave lower haze development in the juice samples during the cold storage than if the centrifuged juice had been subsequently subjected to the gelatin silica sol treatment (**Figure 2**). This signified that the clarification had a negative influence on the turbidity of the centrifuged samples. A possible explanation for this phenomenon could be an inadequate removal of gelatin. In fact, increased turbidity, that is, "overfining", can occur when addition of excess gelatin leads to the formation of positively charged gelatin−tannin colloid complexes, which will never flocculate. Also, as previously observed in apple juice and wine production (*22*), residual gelatin can remain in solution and cause further precipitation, leading to elevated turdibity. Nevertheless, precentrifugation prior to the addition of gelatin silica sol consistently resulted in lower turbidity during and at the end of the cold storage of the black currant juice (**Figure 2**). This renders precentrifugation recommendable, but the addition levels of gelatin should be adjusted to avoid overfining. On the basis of these results, all juice samples to be used for experimental enzymatic clarification studies were subjected to a previous centrifugation step. Particularly for the uncentrifuged

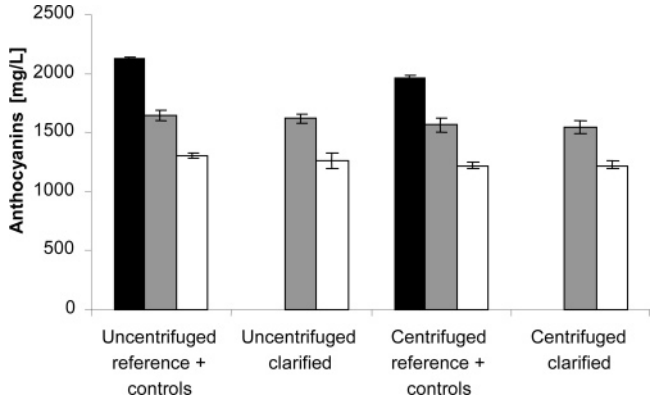

Figure 3. Anthocyanin content of different juices samples: industrially produced juices uncentrifuged and centrifuged at 10000*g* for 15 min, respectively (black bars); unclarified or clarified with gelatin and silica sol, respectively, at day 0 (gray bars); unclarified or clarified with gelatin and silica sol, respectively, at day 35 (white bars). The data are averages of five determinations.

samples, the rate of haze formation appeared to increase slightly at the very end of the storage period (between 28 and 35 storage days, **Figure 2**); unfortunately, our understanding of the mechanisms of haze formation in black currant juice during long-term storage is too limited to propose a plausible cause for this apparent increase at the end of the cold storage period.

Compared to the anthocyanin levels in unclarified, industrially manufactured juices (black bars, **Figure 3**), conventional clarification with gelatin silica sol brought about reductions in total anthocyanin levels of ∼20−25% immediately after clarification (gray bars) and ∼35% after 35 storage days (white bars) (**Figure 3**). A comparison of the anthocyanin levels between the gelatin silica sol clarified black currant juice samples (indicated in **Figure 3** as "uncentrifuged clarified" and "centrifuged clarified", respectively) with their relevant references (which had undergone only the heating associated with gelatin clarification and appear in **Figure 3** as "controls") demonstrated that both the thermal treatment and the gelatin silica sol treatment could be responsible for the anthocyanin losses. Presumably, the anthocyanin loss was a result of a combination of the gelatin−phenols reactions and the holding for (minimum)

Table 2. Immediate Turbidity Levels and Haze Development Rates of Industrial Black Currant Juices[a] Subjected to Protease Clarification at Different Temperature and Time Conditions.

		enzymatic treatment											
		Amano A		Denapsin 2P		Pepsin 389P		Enzeco		Sumizyme AP		control	
time (h)	temperature (°C)	level[b]	rate[c]	level	rate	level	rate	level	rate	level	rate	level	rate
1	20	17.9	1.3	3.4	1.4	10.8	1.8	17.4	1.5	9.4	1.2	3.3	1.3
4	20	11.8	1.3	3.3	1.3	8.9	1.7	14.7	1.5	7.5	1.1	2.2	1.3
1	50	8.1	0.8	2.9	1.2	6.5	1.9	5.8	0.7	6.6	0.9	2.9	1.5
4	50	4.9	0.7	2.2	1.4	6.4	1.5	3.4	0.7	4.4	0.9	3.2	1.3
2.5	35	14.9	0.9	2.9	1.3	8.4	1.8	9.7	1.0	5.2	0.9	3.1	1.5

[a] All samples were previously centrifuged at 10000*g* for 15 min. [b] FNU and [c] ΔFNU/day calculated for the data between 0 and 35 storage days. The average coefficient of variation on the FNU measurements was <6%. All measurements were done in duplicate.

Table 3. Multiple Linear Regression Coefficients Describing the Influence of Amano and Enzeco Protease Treatments on FNU Values for Immediate Turbidity and Haze Development Rate during 35 Cold-Storage Days of Industrial Black Currant Juice

	time					
	day 0		day 35		haze rate from day 0 to day 35	
	coeff	*P*[a]	coeff	*P*	coeff	*P*
Amano A						
constant[b]	12.083	0.023	47.617	1.66×10^{-4}	35.533	2.66×10^{-3}
time	–2.325	0.416	**–3.725**	**0.038**	–1.4	0.597
temperature	–4.175	0.209	**–13.975**	$\mathbf{2.88 \times 10^{-3}}$	**–9.8**	**0.049**
Enzeco						
constant	10.1	5.00×10^{-4}	47.717	1.61×10^{-3}	37.617	2.05×10^{-3}
time	**–1.275**	**0.044**	–1.55	0.577	–0.275	0.907
temperature	**–5.725**	$\mathbf{2.33 \times 10^{-3}}$	**–19.45**	**0.014**	**–13.725**	**0.022**
control						
constant	2.967	6.94×10^{-4}	49.533	1.23×10^{-3}	46.567	1.27×10^{-3}
time	–0.2	0.172	–3.425	0.248	–3.225	0.253
temperature	0.15	0.258	3.775	0.218	3.625	0.216

[a] $P < 0.05$ indicates significance at 95% level. Significant coefficients given in bold. [b] Linear regression coefficient constant.

6 h at 50 °C required for the gelation and settling of the gelatin flocs. This interpretation agrees well with previous data on the thermal stability of anthocyanins in black currant juice, which showed that holding of juice samples for >3 h at 50 °C tended to initiate losses in anthocyanin levels (*12*).

Protease Clarification. The effects of the treatments of black currant juice samples with each of the five different acidic protease preparations, Amano, Denapsin 2P, Pepsin 389P, Enzeco, and Sumizyme AP, on the immediate turbidity and on haze development during cold storage are shown in **Table 2**. Immediate turbidity levels varied from 2 to 18 FNU across the different treatments. The immediate turbidity levels obtained after treatments of the juice samples with the Denapsin 2P preparation were in the low end of the range (2−3 FNU). Turbidity levels obtained after treatments with either Denapsin 2P, Pepsin 389P, or Sumizyme AP indicated that neither the treatment time nor the treatment temperature used with these proteases affected the immediate turbidity levels of the juices, because the values did not differ significantly against time or temperature variations. In turn, this indicated that the variations of the initial FNU values obtained after these enzyme treatments were not necessarily ascribable to the enzymatic action of the enzyme preparations. Nevertheless, the most efficient enzymatic clarification was found after 4 h at 50 °C for all enzymes employed (**Table 2**). Both the Amano and the Enzeco treatments responded significantly to changes in the experimental treatment conditions. Hence, with the Enzeco treatment, both a prolongation of the treatment time (from 1 to 4 h) and an elevation of the temperature (from 20 to 50 °C) resulted in significantly improved turbidity levels immediately after treatment (day 0, **Table 3**). The rate of haze development during cold storage of the juice samples after protease treatment during the 35 day storage period varied from 0.7 to 1.8 after the different protease treatments, whereas the corresponding haze development rate in the controls ranged from 1.3 to 1.5 ΔFNU/day (**Table 2**). The recorded haze rates were particularly high in juice samples having been subjected to Pepsin 389P treatments, whereas the haze development rates of the Amano, Enzeco, and Sumizyme AP treated juices, respectively, were low and, furthermore, appeared to vary in response to the treatment conditions (**Table 2**). With the Sumizyme AP treated juices, no effect of the reaction parameters on turbidity or haze rate was observed (MLR results not shown). Because of the relatively constant values obtained with the control samples (**Tables 2** and **3**), it can be concluded that any eventual significant effect observed in the protease treatments was ascribable to the presence and putative action of the proteases. For both the Amano and the Enzeco treatments, temperature elevation during treatment resulted in a significantly lowered turbidity after the 35 days of cold storage of the juices and a matching, significantly lowered, rate of haze development (**Table 3**). The FNU values of the Amano and Enzeco treated juices (4 h, 50 °C) after 35 days of cold storage were 24.5 and 27.9, respectively, whereas turbidity in the corresponding control, with no enzymatic treatment, reached 40 FNU (data not shown). The positive effects of the higher treatment temperature may be a result of the proteases having maximum activity near 50 °C (**Table 1**). At this elevated temperature, the rate of the enzyme-catalyzed proteolysis of haze-active proteins might have been particularly high as a result of the general increase in reaction rates with increased temperature (up to a certain limit). In addition, a gradual denaturation

Table 4. Immediate and Reduction Rate of Anthocyanin Levels during 35 Days at 2 °C of Industrial Black Currant Juices[a] Subjected to Protease Clarification at Different Temperature and Time Conditions

		Amano A		Denapsin 2P		Pepsin 389P		Enzeco		Sumizyme AP		control	
time (h)	temperature (°C)	level[b]	rate[c]	level	rate	level	rate	level	rate	level	rate	level	rate
1	20	1823	6.8	1814	9.5	1786	8.0	1792	6.5	1783	7.7	1754	7.3
4	20	1737	6.6	1791	9.1	1702	6.0	1796	9.4	1749	7.9	1815	9.8
1	50	1732	9.1	1681	7.5	1643	4.9	1726	9.7	1672	7.7	1653	7.9
4	50	1423	4.9	1507	11	1472	8.9	1439	8.6	1380	7.6	1491	8.7
2.5	35	1713	6.8	1725	8.5	1651	6.3	1742	9.3	1730	9.6	1719	7.8

[a] Samples subjected to cold storage at 2 °C. The average coefficient of variation on the anthocyanin measurements was <3%. All measurements were done in duplicate. [b] mg/L. [c] Δmg/L per day.

Table 5. Multiple Linear Regression Coefficients Describing the Influence of Amano A and Enzeco Protease Treatments on Anthocyanin Content during 35 Cold-Storage Days of Industrial Black Currant Juice

	day 0		day 35	
	coeff[a]	*P*[b]	coeff	*P*
Amano A				
constant[a]	1690	4.85×10^{-5}	1439.66	2.74×10^{-4}
time	**−101.25**	**0.02**	−75.75	0.122
temperature	**−98.75**	**0.021**	−38.75	0.315
Enzeco				
constant	1706.1	1.14×10^{-4}	1396.5	4.93×10^{-5}
time	**−105.75**	**0.042**	**−122.75**	**0.009**
temperature	−70.75	0.087	**−90.25**	**0.017**
control				
constant	1691.83	6.45×10^{-5}	1405	2.55×10^{-4}
time	**−106.25**	**0.024**	−93.25	0.077
temperature	−25.25	0.268	−57.75	0.171

[a] Linear regression coefficient constant. [b] $P \leq 0.05$ indicates significance at the 95% level. Significant coefficients given in bold.

of the juice proteins to expose their haze-active sites may also have taken place at the elevated temperature (*7*).

Effect of Protease Clarification on Anthocyanin Levels. Compared to the initial, centrifuged juice (1958 mg of anthocyanins/L, **Figure 3**), considerable amounts of anthocyanins, that is, up to 24−30% of the initial level, were lost during protease clarification, particularly after the prolonged treatments done at 50 °C (**Table 4**). However, when the anthocyanins data of protease-clarified samples are compared with those of the corresponding control (which had been subjected to a thermal treatment), a direct influence of the protease clarification on the anthocyanin decrease could not be deduced. Significant effects of temperature and time on anthocyanins with the Amano and Enzeco treatments are shown in **Table 5**. The data obtained thus indicated that especially the long holding times of the clarification had a negative impact on the anthocyanins—despite flushing of the samples with N_2. The negative effect of the Enzeco preparation on the anthocyanin levels after 35 days of cold storage might indicate that this enzyme preparation harbored some side activities that were detrimental to the anthocyanin-glucosides in black currant juice. Such activities may include polyphenol oxidase or β-glucosidase activites, which are often produced by *Aspergillus niger* strains and may be present in enzyme preparations from these (*23*). Further studies are, however, needed to substantiate this hypothesis.

Despite the significant influence of the treatment conditions on the anthocyanin losses with Enzeco treatments, the total anthocyanin levels were not lower for Enzeco treated samples than for Amano and Sumizyme samples (**Table 4**). Hence, because of the higher effect of the Enzeco preparation on the turbidity data, this enzyme preparation was selected for further investigation of coupled pectinase−protease clarification.

Pectinase−Protease Clarification. Inclusion of the pectinase preparation Pectinex BE 3-L in clarification treatments resulted in significant increases in the immediate turbidity both when the Pectinex BE 3-L was used alone and in combination with either of the proteases Enzeco and Novozyme 89L (**Table 6**). Moreover, clarification treatment with the Novozyme 89L preparation alone resulted in significantly increased turbidity levels immediately after treatment, that is, at day 0 (**Table 7**). However, as discussed below, this effect reverted to a positive haze-preventing effect later during storage. The Novozyme 89L preparation was included as an additional protease in the evaluation because of the positive data previously obtained with this enzyme preparation on cherry juice clarification (*18*). Conversely, the use of the pectinase caused a significant decrease in haze development rate during storage of the black currant juices for 28 days at 2 °C, a decrease that was found to be statistically significant by MLR analysis of the data (**Table 7**). In addition, a surprising turbidity-enhancing interaction effect was identified between both of the proteases and the Pectinex BE 3-L, an effect that resulted in a slight increase of the haze rate during the cold storage of juices (with a low coefficient of haze rate: 0.134 and 0.186 for Enzeco and Novozyme 89L, respectively) (**Table 7**). Taken together, these data indicated that the pectinase action initially promoted the formation of the immediate turbidity after treatment, which could result as a consequence of the rupture of pectin polysaccharide fragments within the juice bulk. Afterward, an interaction or inactivation effect of the digested pectin fragments on non-protease-treated haze-active proteins apparently took place, resulting in a decrease of the haze development during cold storage; however, once a protease treatment had been included with the Pectinex BE 3-L treatment, the presumed proteolytic action of the proteases on the haze-active proteins perturbed the positive effect of the pectinase treatment on the haze development during cold storage. The present experimental setup did not, however, allow a detailed unveiling of the molecular mechanisms behind these effects, and further experimental evidence is clearly warranted to understand in more detail how enzymatically treated berry pectin and haze-active proteins interact to either retard or promote turbidity development during cold storage of fruit juices. Analyses of the effects of the different treatments with the Enzeco protease and the Novozyme 89L preparation, respectively, within this experimental framework (**Table 6**), with the treatment temperature kept constant at 50 °C and the treatment time shortened to last from 30 to 90 min, showed that treatments with either of these enzyme preparations both gave significantly decreased haze rates and turbidity levels of the juices after 28 days of cold storage (**Tables 6** and **7**). In addition, inclusion of extra gallic acid also had a positive effect

Table 6. Immediate Turbidity Levels and Haze Development Rates of Nonindustrial Black Currant Juices Subjected to Protease plus Pectinase Clarification[a] at Different Temperature and Time Conditions

				turbidity (FNU)							
				day 0[b]		day 7[b]		day 28[b]		rate[c]	
protease[a] (E/S %)	Pectinex BE 3 (E/S %)	time (min)	gallic acid (mg/L)	Enzeco	Novozyme 89L	Enzeco	Novozyme 89L	Enzeco	Novozyme 89L	Enzeco	Novozyme 89L
0	0	30	0	4	5	45	49	68	80	2	2.4
0.5	0	30	0	3	5	30	32	45	58	1.3	1.8
0	0.5	30	0	13	15	21	21	36	36	0.8	0.8
0.5	0.5	30	0	14	19	22	27	38	38	0.8	0.7
0	0	90	0	4	4	40	44	61	68	1.8	2
0.5	0	90	0	3	5	28	27	41	48	1.2	1.4
0	0.5	90	0	7	8	14	17	26	28	0.7	0.7
0.5	0.5	90	0	11	16	18	24	29	35	0.6	0.6
0	0	30	50	5	5	35	49	57	73	1.7	2.1
0.5	0	30	50	3	6	7	6	38	37	1.3	0.9
0	0.5	30	50	7	15	18	24	31	32	0.8	0.5
0.5	0.5	30	50	13	19	20	24	29	37	0.5	0.6
0	0	90	50	5	5	9	43	35	61	1.1	1.7
0.5	0	90	50	4	5	7	24	17	39	0.5	1
0	0.5	90	50	8	7	16	16	21	26	0.4	0.7
0.5	0.5	90	50	10	16	15	21	23	38	0.5	0.5
0.25	0.25	60	25	8	14	11	24	24	32	0.6	0.6

[a] Addition of either Enzeco or Novozyme 89L. [b] FNU. [c] ΔFNU/day. [d] Average of the three center points. The average coefficient of variation on the FNU measurements was <4%. All measurements were done in duplicate.

Table 7. Multiple Linear Regression Coefficients of the Effect of Protease plus Pectinase Treatment on FNU Values of Immediate Turbidity and Haze Development Rate during 35 Cold-Storage Days of Nonindustrial Black Currant Juice

	day 0		day 7		day 28		haze rate: 0 to 35 days	
	coeff[a]	*P*[b]	coeff	*P*	coeff	*P*	coeff	*P*
Enzeco								
constant	7.207	1.584×10^{-11}	19.777	2.865×10^{-8}	35.008	4.476×10^{-11}	0.942	3.36×10^{-10}
Enzeco	0.482	0.165	–3.288	0.079	**–4.766**	**0.028**	**–0.164**	**0.019**
Pectinex	**3.271**	$\mathbf{3.480 \times 10^{-7}}$	–3.392	0.071	**–7.984**	$\mathbf{1.181 \times 10^{-3}}$	**–0.365**	$\mathbf{4.59 \times 10^{-5}}$
time	**–0.738**	**0.043**	–3.159	0.090	**–5.503**	**0.014**	**–0.16**	**0.021**
gallic acid	–0.231	0.492	–5.685	0.006	**–5.853**	**0.010**	**–0.158**	**0.023**
Enzeco × Pectinex	**1.149**	$\mathbf{4.211 \times 10^{-3}}$	3.973	0.039	**5.416**	**0.015**	**0.134**	**0.047**
Novozyme 89L								
constant	10.439	5.040×10^{-10}	28.57	3.778×10^{-15}	43.203	1.388×10^{-12}	1.056	1.573×10^{-9}
Novozyme	**1.774**	**0.035**	**–3.448**	$\mathbf{5.445 \times 10^{-4}}$	**–4.991**	**0.017**	**–0.203**	**0.021**
Pectinex	**4.806**	$\mathbf{1.822 \times 10^{-5}}$	**–7.577**	$\mathbf{1.800 \times 10^{-7}}$	**–12.391**	$\mathbf{1.205 \times 10^{-5}}$	**–0.514**	$\mathbf{1.640 \times 10^{-5}}$
time	–1.498	0.068	**–2.302**	**0.010**	–3.509	0.075	–0.076	0.347
gallic acid	–0.018	0.981	–0.603	0.441	–3.316	0.091	–0.140	0.095
Novozyme 89L × Pectinex	no effect	no effect	**5.646**	$\mathbf{4.828 \times 10^{-6}}$	**7.466**	$\mathbf{1.217 \times 10^{-3}}$	**0.186**	**0.032**

[a] Linear regression coefficient constant. [b] $P \leq 0.05$ indicates significance at the 95% level. Significant coefficients given in bold.

on diminishing the haze rate, with the experimental run with the Enzeco preparation resulting in a particular strongly significant effect, whereas the effect of gallic acid in the Novozyme 89L run reached only ~90% significance (**Table 7**). After 28 days, the lowest turbidity value (17 FNU) was found when Enzeco (0.5 E/S %) and 50 mg/L of gallic acid were allowed to react in the juice for 90 min (**Table 6**). The values of turbidity after 0 and 7 storage days as well as the haze formation rate were also among the lowest of all after these treatment conditions (**Table 6**). These results implied that the protease-assisted clarification with the Enzeco preparation in the presence of gallic acid was at least as efficient as the conventional gelatin silica sol procedure, by which the turbidity of precentrifuged, gelatin silica sol clarified juice was 20 FNU after 28 storage days (**Figure 2**).

The fact that Novozyme 89L as well as Enzeco treatment produced a retarding effect on haze formation with precentrifugation provides support for the hypothesis that protein−phenol interactions are involved in black currant juice haze development during cold storage. As mentioned, the use of proteases—mainly broadly acting papain, obtained from the latex of papaya fruits—is well established for chill-haze proofing in beer brewing (*13*, *24*). However, protease treatment to prevent haze has been found to be less effective in wines. Bakalinsky and Boulton (*15*) used an immobilized protease for experimental clarification of different California wines, obtaining successful results only in one of four wines assayed. Likewise, in experimental Australian wines only one of five selected, commercial proteases diminished haze after an extended proteolytic reaction for 7 days at 25 °C, despite the fact that three of these five proteases were able to reduce the measured protein levels in the wines (*16*). In contrast, treatment of kiwifruit juice with a commercial, acid-stable fungal protease significantly improved the visual clarity of freshly processed kiwifruit juice and cured haze formation in such juice during storage (*17*). Seen in the light of these results, the clarification and haze prevention obtained with particularly the Enzeco and Novozyme 89L preparations in this study on black currant juice favor further studies on the principal use of proteases for clarification and haze retardation in berry and other fruit juice processes.

Table 8. Reduction Rates of Phenol and Anthocyanin Contents of Nonindustrial Black Currant Juices[a] Subjected to Protease plus Pectinase Clarification Treatments at Different Temperature and Time Conditions

				rate (mg/L per day for 28 days)			
				total phenols		anthocyanins	
protease[a] (E/S %)	Pectinex BE 3 (E/S %)	time (min)	gallic acid (mg/L)	Enzeco	Novozyme 89L	Enzeco	Novozyme 89L
0	0	30	0	–8.9	–0.7	–11.7	–4.8
0.5	0	30	0	–9.2	–2.32	–12.2	–9.2
0	0.5	30	0	–11.6	+5.2	–13.3	–4.7
0.5	0.5	30	0	–3.6	–1.6	–10.4	–8.9
0	0	90	0	–9.6	–0.5	–14.7	–7.6
0.5	0	90	0	–13.9	–0.4	–9.2	–6.9
0	0.5	90	0	–4.8	+6.1	–1.5	–8.7
0.5	0.5	90	0	–6.8	–5.2	–10.4	–5.3
0	0	30	50	–15.4	–6.8	–8.4	–6.1
0.5	0	30	50	–5.5	+4.1	–8.6	–4.4
0	0.5	30	50	–20.0	–1.8	–12.6	–8.1
0.5	0.5	30	50	–6.6	–0.2	–8.8	–3.6
0	0	90	50	–15.2	–3.2	–10.5	–8.6
0.5	0	90	50	–18.8	–5.2	–7.4	–9.4
0	0.5	90	50	–3.6	–6.6	–7.3	–6.9
0.5	0.5	90	50	–12.3	–3.4	–10.3	–6.5
0.25	0.25	60	25	–12.9[b]	–3.4[b]	–13.1[b]	–7.1[b]

[a] Addition of either Enzeco or Novozyme 89L. [b] Average of the three center points. The average coefficient of variation on the anthocyanin measurements was <3%. Measurements were done in duplicate on each juice sample.

Table 9. Multiple Linear Regression Coefficients Describing the Influence of Protease plus Pectinase Treatment on Total Phenolic and Anthocyanin Contents during 35 Cold-Storage Days of Nonindustrially Produced Black Currant Juice

	day 0		day 7		day 28		rate from 0 to 28	
	coeff[a]	*P*[b]	coeff	*P*	coeff	*P*	coeff	*P*
total phenols Enzeco								
constant	6209.74	1.91×10^{-28}	5757.37	9.81×10^{-27}	5908.26	6.518×10^{-29}	–10.768	6.03×10^{-9}
Enzeco	–19.063	0.383	–15.000	0.573	2.625	0.850	0.773	0.360
Pectinex	**–67.188**	**0.007**	**–60.000**	**0.037**	–19.500	0.176	1.704	0.058
time	–34.063	0.13	–23.750	0.377	**–41.375**	**0.010**	–0.259	0.755
gallic acid	42.187	0.066	**60.000**	**0.037**	–8.250	0.556	**–1.801**	**0.047**
Enzeco × time	no effect	no effect	no effect	no effect	**–51.375**	$\mathbf{2.348 \times 10^{-3}}$	**–3.096**	$\mathbf{2.494 \times 10^{-3}}$
total phenols Novozyme 89L								
constant	5913.86	1.412×10^{-25}	5790.53	3.71×10^{-26}	5865.53	1.473×10^{-27}	–1.72	0.041
Novozyme	49.688	0.065	–26.25	0.12	**39.688**	**0.007**	–0.357	0.672
Pectinex	–11.562	0.647	–3.75	0.815	1.563	0.900	0.469	0.58
time	**–65.937**	**0.019**	**–69.375**	$\mathbf{8.25 \times 10^{-4}}$	**–90.937**	$\mathbf{7.277 \times 10^{-6}}$	–0.893	0.299
gallic acid	33.438	0.198	**–59.375**	$\mathbf{2.58 \times 10^{-3}}$	–7.812	0.532	–1.473	0.098
Novozyme × gallic acid	**–59.063**	**0.032**	no effect	no effect	no effect	no effect	**2.076**	**0.026**
time × gallic acid	no effect	no effect	**–48.125**	**0.01**	**–69.062**	$\mathbf{1.003 \times 10^{-4}}$	no effect	no effect
Novozyme × time	no effect	no effect	no effect	no effect	**–36.562**	**0.011**	no effect	no effect
anthocyanins Enzeco								
constant	2407.32	2.79×10^{-24}	2263.43	1.12×10^{-25}	2105.30	1.71×10^{-23}	–10.344	1.704×10^{-9}
Enzeco	–6.625	0.608	–7.164	0.453	–1.334	0.918	0.172	0.837
Pectinex	–19.375	0.149	–5.498	0.563	–1.000	0.938	0.508	0.547
time	**–53.75**	$\mathbf{9.32 \times 10^{-4}}$	**–38.157**	$\mathbf{1.209 \times 10^{-3}}$	–25.329	0.067	0.924	0.281
gallic acid	–12.125	0.354	–14.497	0.142	9.665	0.460	0.601	0.478
anthocyanins Novozyme 89L								
constant	2295.987	1.441×10^{-25}	2281.96	1.212×10^{-28}	2116.10	2.548×10^{-25}	–6.90	1.646×10^{-10}
Novozyme	–13.331	0.188	4.999	0.518	–5.166	0.585	0.080	0.864
Pectinex	–8.999	0.365	0.333	0.965	2.498	0.791	0.268	0.570
time	**–44.654**	$\mathbf{4.466 \times 10^{-4}}$	**–52.319**	$\mathbf{6.828 \times 10^{-6}}$	**–63.817**	$\mathbf{1.060 \times 10^{-5}}$	–0.646	0.183
gallic acid	–11.664	0.245	–11.331	0.155	–8.831	0.356	0.163	0.728

[a] Linear regression coefficient constant. [b] $P \leq 0.05$ indicates significance at the 95% level. Significant coefficients given in bold.

The effects obtained with gallic acid addition agree well with previous findings that have indicated an ability of gallic acid to retard the formation of multimolecule protein−polyphenol cross binding—presumbaly via chemical blockage of the haze-active protein-binding sites, thus delaying the formation of both immediate turbidity and haze development (*5*, *6*, *18*). In accordance with the hypothesis that haze formation may take place via hydrogen bonding between the hydroxyl groups in the phenols and exposed carboxyl oxygens in proteins (**Figure 1**), the reactivity of the phenols with proteins will increase with the number of hydroxyl groups, being even higher when these groups are placed adjacently in the phenolic ring (*6*). Gallic acid possesses three ortho-positioned hydroxyl groups, so this feature may explain why the addition of even relatively low levels of gallic acid (50 mg/L equivalent to 0.3 mM) to a juice already abundant—with total phenols of ∼4000 mg/L—in mainly ortho-dihydroxylic phenols could induce reduced turbidity and haze formation (*5*, *18*).

Effects of Pectinase−Protease Clarification on the Levels of Anthocyanin Pigments. A reduction of ∼12% in the content

of total anthocyanins was found to occur when protease treatment was done at the best conditions to reduce turbidity: 0.5 E/S % Enzeco, 50 mg/L of gallic acid, and 90 min (row 16, **Table 8**). This loss should be compared to an estimated loss of anthocyanins of ~30% after gelatin silica sol treatment (and after similar cold storage) (**Figure 3**). The data indicate that protease treatment holds promise as an alternative clarification strategy.

In the statistically designed experimental framework made to evaluate the combined effects of pectinase−protease and gallic acid (**Table 8**), a negative effect of the treatment time on the total phenolic content at 0 and 28 storage days was observed—the only exception being with the total phenols in the Enzeco treatment framework, where the (negative) effect of the treatment time did not reach statistical significance (**Tables 8** and **9**). Because longer contact times favored decreasing turbidity, a compromise between time allowing acceptable levels of turbidity and the maintenance of the phenolic levels should be attained. Apart from time, only the presence of the pectinase in the Enzeco experiments and the interaction of Novozyme 89L and gallic acid had a significantly negative influence on the "immediate" phenolic content of juices (**Table 9**).

Other isolated influences, such as the negative interaction effect between both protease preparations and time on phenolic content after 28 days, were observed; this effect may be due to the significant dominance of the treatment time as a main effect. Gallic acid addition somehow induced a significantly stabilizing effect on the total phenols levels 7 days into storage in the Enzeco experiments and a corresponding opposite effect in the Novozyme 89L data (**Table 9**). Our present analyses do, however, not allow any firm conclusions to be drawn regarding these effects of gallic acid—which were not at all manifest in the anthocyanins levels. With respect to the anthocyanin content, the negative effect of prolonged treatment time was also apparent on the anthocyanins as time gave significantly negative coefficients on anthocyanin levels at day 0 and after storage for 7 and 28 days (except at day 28 in the Enzeco runs, when the statistical significance of the negative coefficient was only 93.3%) (**Table 9**). In spite of these results, neither the treatment time nor any of the other factors—protease, pectinase, or gallic acid—induced a statistically significant effect in the rate of anthocyanins loss during the cold storage period, as the coefficients of the haze rate from days 0 to 28 were nonsignificant (**Table 9**).

In light of the results shown in this study, it can be concluded that the introduction of a centrifugation step prior to clarification significantly decreases the immediate turbidity values as well as the haze development rate in black currant juices in postclarification cold storage. The protease treatment for clarification was found to be a promising alternative to the traditional gelatin silica sol treatment, minimizing the turbidity values of juice as well as retaining the anthocyanin and phenolic contents within acceptable levels. The effective role of proteases found in this study bears out the responsibility of haze-active proteins for the formation and development of turbidity in juices, particularly during cold storage. The use of the protease Enzeco coupled with gallic acid provided the best results, whereas the addition of pectinase in combination with protease had a surprising negative influence by increasing the turbidity values. It is well-known that phenolic compounds have the ability to prevent oxidative reactions taking place in the human body, which are responsible for the progression of a number of diseases. Changes in the levels and profiles of phenolic compounds of fruit and berry juices as a result of different clarification techniques may affect the antioxidant capacity and human-health beneficial properties of the resulting juices. The consequences of the changes in the phenols taking place with different clarification strategies with respect to antioxidant activity on in vitro oxidation of human low-density lipoproteins are being reported separately.

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