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Statistically designed two step response surface optimization of enzymatic prepress treatment to increase juice yield and lower turbidity of elderberry juice

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Abstract

This study examined the effects of different, statistically designed, enzymatic maceration treatments on juice yield, turbidity, and phenol yield (total phenols and total anthocyanins) in experimentally produced elderberry juice. Increased pectinolytic enzyme dose, longer maceration time, and elevated reaction temperature all had significantly positive effects on the juice yield. Increased enzyme dose and maceration temperature also increased the yields of anthocyanins in the elderberry juice, while none of the reaction parameters affected the juice turbidity. The juice yield was optimized further in a new experimental template, made by using the statistical steepest ascent optimization method. In the new response surface design template an optimal maceration treatment giving maximal juice yield and anthocyanin yields and low turbidity was identified. With the optimal treatment with a pectinolytic enzyme preparation, Pectinex BE 3L, produced by a cloned *Aspergillus* strain, a maximal juice yield of 77% w/w of the berry mash, an anthocyanin yield of 2380 mg/kg fresh berry mash, and a turbidity level of 128 formazin nephelometric units (FNU) were obtained. Enzymatic prepress treatment generally decreased turbidity levels by 30% as compared to pressing without prior enzymatic treatment. A comparison of the responses obtained after the optimal enzymatic treatment with five different pectinolytic enzyme preparations showed that the *Aspergillus niger* preparation Pectinex BE Color gave slightly better juice and phenol yields, and lower turbidity levels than the other enzyme preparations tested. In conclusion, the results demonstrated that juice yields and phenolic yields in elderberry juice could be improved with enzyme treatment and that the optimal reaction conditions for obtaining the best juice yield, highest phenolics, and lowest turbidity levels could be rationally identified via statistical factor level optimization.

Keywords: Pectinase treatment; Elderberry juice; Juice yield; Turbidity; Anthocyanins and phenols

Industrial relevance: This paper describes an optimization study on enzymatic pre-press treatment of elderberries for elderberry juice production. The aim is to increase juice yield, lower juice turbidity, and enhance the release of anthocyanins and other co-existing phenolics into the juice via enzyme catalyzed degradation of the cell wall polysaccharides in the elderberries. The work defines an optimal enzymatic treatment for increased elderberry juice yield, anthocyanin yield, and lowered juice turbidity, and also demonstrates how to shift a statistical design template to obtain improved results on selected parameters. Although the work is focused on elderberry juice the enzymatic as well as the statistical design strategy can easily be adapted for other fruit juice and food processes.

1. Introduction

Elderberries are mostly cultivated in northern and central Europe and have since antiquity been employed in herbal medicine for treating various illnesses ranging from asthma and colds to constipation and arthritis (Kilham, 2001). The industrial processing of elderberries mainly takes place in Northern Europe where the berries are processed for juice, juice concentrate, wine, and jelly manufacture (Jensen, Christensen, Hansen, Jørgensen, & Kaack, 2001). Recently, elderberries have received increased attention due to their high contents of anthocyanins, that are widely used as a color ingredients in various beverages and in home wine sets, and which may also provide nutritional benefits

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as antioxidant phytonutrients (Netzel et al., 2005). The anthocyanins of elderberries are all cyanidin-based with cyanidin-3-sambubioside (β-D-xylose-(1-2)-glucose) and cyanidin-3-glucoside, together accounting for ~85% of the anthocyanin content, being the most dominant (Goiffon, Mouly, & Gaydou, 1999). The anthocyanin glucosides in elderberries are excreted unmetabolized in human urine, albeit in low amounts, i.e.<0.1% of the ingested total, suggesting that at least a small fraction of the elderberry anthocyanin glucosides pass the intestine in an unchanged glucosidic form (Milbury, Cao, Prior, & Blumberg, 2002; Murkovic, Mülleder, Adam, & Pfannhauser, 2001; Netzel et al., 2005). Furthermore, an *in vivo* antioxidant effect of elderberry anthocyanins (and co-existing phenolics) in humans was recently indicated by the finding that both total phenolics and antioxidant capacity of plasma increased significantly in human subjects 1 h after ingestion of 400 ml of elderberry juice (Netzel et al., 2005).

In industrial berry juice production prepress addition of pectinases is prevalent as this enzyme addition increases the juice yield by breaking down the pectin in the plant cell walls and in the middle lamellae between the plant cells (Grassin & Fauquembergue, 1996). Apart from promoting juice extraction, our studies on black currant juice have demonstrated that enzymatic pre-press treatment with multicomponent pectinase preparations may be optimized to enhance the release of anthocyanins and other phenolics into the juice (Bagger-Jørgensen & Meyer, 2004; Landbo & Meyer, 2004). Although enzyme catalyzed break down of the plant cell wall matrix and middle lamella may first increase the immediate turbidity in the juice (Grassin & Fauquembergue, 1996) our studies with black currant juice have shown that forced enzymatic pre-press treatment, i.e. increased enzyme dosage and prolonged reaction time, may also improve the juice clarity (Landbo & Meyer, 2004). Immediate turbidity in fruit juices is generally assumed to be mainly due to the presence of pectin and other fractions of fruit cell wall material (Grassin & Fauquembergue, 1996). We therefore hypothesized that it might be possible to optimize the enzymatic pre-press treatment of elderberry juice to both target the turbidity and at the same time maximize the juice yield and the release of phenols and anthocyanins into the juice.

Since the enzyme catalyzed pre-press maceration reaction may be influenced by a number of factors, we chose to employ a statistically designed approach to the optimization in order to rationally examine the influence of different reaction parameters (i.e. factors) with a minimal number of experiments. Several methods have been developed to minimize the use of separate experiments to define the levels of experimental factors that have a significant effect on the selected experimental responses, and notably to define the distance between the factors so that a response maximum can be found. When the targeted response depends on two or more factors, the statistical state-of-the-art optimization methods include the steepest ascent method, simplex optimization and the simulated annealing method (Miller & Miller, 2000).

In this work we have optimized enzymatic prepress maceration of elderberries to obtain elevated juice yields, lowered juice turbidity and enhanced extraction of anthocyanins and other co-existing phenols into the juice. In the report we demonstrate the use of the statistical steepest ascent method to change the factor units in a quadratic central composite response surface frame to obtain an improved experimental design frame that encircle the optimal responses.

2. Materials and methods

2.1. Materials

A broadly representative mixture of mature elderberries (*Sambucus nigra* L) from several different cultivars harvested at the experimental field at the Danish Institute of Agricultural Sciences, Aarslev, Denmark. The berries had been stored at −20 °C from harvest until use.

2.2. Sample preparation and enzymes

Prior to juice processing, the berries were gently defrosted and crushed in a meat grinder (Jupiter, type 863, Germany). After the crushing aliquots of 220 g of crushed berries were vacuum packed, heated at 75 °C for 90 s, cooled rapidly, and frozen at −20 °C until further use. Five enzyme preparations, all intended for use in the juice and/or wine industry, were selected for evaluation in the prepress treatment of elderberry mash. Summaries of the available information regarding the enzyme preparations' activities are given in Table 1.

2.3. Experimental plan

The experimental plans were randomized, quadratic central composite response surface designs. The first template comprised 17 different combinations of the factors maceration time

Table 1
Enzymes employed in the experiments

Enzyme name	Pectinase activity [a]	Main activity [a]	pH optimum [a]	Temp. optimum [a]	Source [a]
Pectinex BE 3L[b]	1600 MOE/ml	Pectinesterase, pectin lyase and polygalacturonase	3.5	45–55 °C	*A. niger*
Pectinex BE Colour [b]	3600 MOE/ml	Pectinesterase, pectin lyase and polygalacturonase	3.5–4.0	45–55 °C	*A. niger*
Vinozyme G [b]	4000 FDU 20 °C/g	Pectin lyase, polygalacturonase, hemicellulase and cellulose	Not given	Not given	*A. niger*
Pectinex Smash XXL[b]	22,000 PG/ml	Pectinases and hemicellulases	3.5	45–55 °C	*A. aculeatus/oryzae*
Rapidase EX Color [c]	Not given	Pectinases	Not given	40–55 °C	*A. niger*

The table presents information concerning supplier, pectinase activity, main activity, pH optimum range, temperature optimum range and source.

[a] Activity given by the suppliers data sheets. MOE: (Most Einheit) measure of the reduction in the viscosity of apple juice; FDU: (Ferment Depectinization Units) measure of the depectinization of a natural substrate; PG: measure of the viscosity reduction in a solution of pectic acid for the polygalacturonase.

[b] Supplied by Novozymes A/S Bagsværd, DK.

[c] Supplied by DSM Gist-Brocades Delft, NL.

(10–50 min), maceration temperature (32–60 °C), and enzyme dose of Pectinex BE 3L (0.02–0.34% E/S) including six star points and three center points (Table 2). The star points refer to axial points going beyond the "cube" formed by the ranges of the chosen parameters. The % E/S addition level refers to dosage in milliliter enzyme preparation/100 g wet mash. As a control, the low enzyme dosage star point with an enzyme dose of zero was used. The second template comprised 11 different combinations of the two factors maceration temperature (46–74 °C) and enzyme dose; Pectinex BE 3L (0.18–1.0% E/S) with three center points. For each combination of enzyme reaction factors a control without enzyme added was included.

2.4. Maceration

Frozen elderberry pulp was gently defrosted in a thermostatic bath at 20 °C. Portions of 40 g of pulp were weighed out and the relevant enzyme preparation was added at the % E/S ratio required according to the experimental plan. Samples were mixed thoroughly and placed under agitation in a thermostatically controlled water bath in accordance with the reaction time and temperature given in the experimental design. After completion of the enzymatic maceration the pulp was pressed in a press (Schwanke Tinkturenpressen, type HP-2, Neuss, Germany) using customized nylon filter bags (Schwanke Tinkturenpressen, Neuss, Germany) and a pressure of 100 bar for 30 s. The juice was then pasteurized for 60 s at 90 °C, cooled, and purged with nitrogen in order to remove oxygen.

2.5. Enzyme dose response experiment with selected enzymes

A dose response experiment comparing the two enzyme preparations Pectinex BE 3L and Pectinex BE Colour (Table 1) with fixed maceration time (30 min) and temperature (63 °C) was done. The enzyme dose was varied: 0, 0.04, 0.08, 0.12, and 0.16% E/S.

2.6. Experimental statistical design and statistics

Linear regression and calculation of standard deviations were done with Excel (Microsoft). Significance of all the results was established at $P \leq 0.05$. The design of the experimental templates as well as the evaluation of the individual and interactive effects by multiple linear regression of the different maceration conditions were done with the aid of the computer program JMP 5.1.2 software (SAS Institute Inc., Cary, NC, USA). To define the factor ranges of the second experimental template the method of steepest ascent was applied on the juice yield response data obtained in the first template. The method of steepest ascent is based on defining the direction of the actual ascent, and in turn the new factor ranges, including the factor step size, from the slopes of the multiple linear regression lines, i.e. the coefficients, obtained in a preceeding experimental design (Miller & Miller, 2000). In the second experimental template, the maceration time was chosen to be kept constant at 30 min, and the responses juice yield, juice turbidity and anthocyanin levels were selected to have highest priority.

2.7. Analyses of total phenols, total anthocyanins, °Brix and juice turbidity

Total phenols in the juices were determined by the Folin–Ciocalteu procedure with total phenols expressed as milligram per liter gallic acid equivalents (GAE) (Singleton & Rossi, 1965). Total anthocyanins were determined by the pH differential method and anthocyanin concentrations in elderberry juice were calculated as cyanidin-3-glucoside equivalents (Wrolstad, 1976). Turbidity in Formazin Nephelometric Units (FNU) was measured by nephelometry at 90° light scattering, 860 nm, with a Nephla reader (Dr. Lange, Düsseldorf, Germany) calibrated against hexamethylene tetramine formazine. Prior to the FNU measurements all samples were diluted with the same dilution factor (50 times) to obtain a °Brix value of 0.3 °Brix.

Table 2
Influences of maceration time, maceration temperature and enzyme dose on juice yield, turbidity, water soluble carbohydrates, total anthocyanins and total phenols in the first experimental template

Exp. number	Time [min]	Temperature [°C]	Enzyme dose [%E/S g/g]	Juice yield [%]	Turbidity [FNU]	Water soluble carbohydrates [mg glucose/ml]	Anthocyanins [mg/g]	Total phenols [mg/g]
1	30	46	0.36	76.4	165	12.1	2.1	5.36
2	53	46	0.18	75.1	105	12.0	2.0	5.0
3	50	32	0.02	71.4	139	12.1	1.4	4.0
4	50	32	0.34	73.0	116	12.4	1.6	4.2
5	10	60	0.34	73.0	111	10.7	2.2	5.4
6	7	46	0.18	72.7	133	11.7	1.8	4.8
7	10	60	0.02	73.2	153	11.5	2.2	5.3
8	50	60	0.02	75.7	130	11.9	2.2	5.6
9	30	62	0.18	76.1	103	12.0	2.4	5.8
10	30	46	0	72.6	150	11.7	1.8	4.6
11	30	30	0.18	73.2	115	11.7	1.6	4.3
12	10	32	0.02	72.9	155	12.0	1.6	4.3
13	10	32	0.34	73.1	113	11.8	1.6	4.1
14	50	60	0.34	77.0	154	11.3	2.4	6.0
Center points[a]	30	46	0.18	74.6±0.6	118±11	12.4±0.2	1.9±0.1	5.0±0.2
Response ranges				71.4 – 77.0	103– 165	10.7 – 12.4	1.4 – 2.4	3.0 – 6.0

[a] Center point average of triplicates±SD.

Table 3
Multiple linear regression results for the parameters and the parameters and the significant interactions on the juice yield, total anthocyanins and the total phenols

Parameters and interactions (×)	Juice yield [%]		Total anthocyanins [mg/g]		Total phenols [mg/g]	
	Coeff.	P [a]	Coeff.	P [a]	Coeff.	P [a]
Maceration time	0.74	0.0093	0.03	No effect	0.09	No effect
Maceration temperature	1.11	0.0005	0.35	<0.0001	0.72	<0.0001
Enzyme dose	2.84	0.0127	0.07	0.0154	0.12	No effect
Time × temperature	1.01	0.0027	–	No effect	0.14	<0.0001
Time × dose	–	No effect	–	No effect	–	No effect
Temperature × dose	–	No effect	–	No effect	–	No effect
Constant	74.56	<0.0001	1.92	<0.0001	4.92	<0.0001

[a] *$P = 0.05$ indicates significance at 95% level.*

The °Brix value was determined by measuring the refractive index with a hand held refractometer (Carl Zeiss Gmbh, Vienna, Austria).

2.8. Extent of plant cell wall degradation

The extent of cell wall hydrolysis of mash was assessed by measuring total water-soluble carbohydrates liberated from the sample (Slominski, Guenter, & Campbell, 1993).

2.9. HPLC

Phenols and vitamin C were profiled by means of the HPLC procedure described by Lamuela-Raventòs and Waterhouse (1994) using a Hewlett Packard 1100 system equipped with a diode array detector, a Nova-Pak C18 column (3.9 × 150 mm, Waters), and controlled by a personal computer with HPChem station software. The major phenolic compounds, i.e. the two primary anthocyanins; cyanidin-3-sambubioside and cyanidin-3-glucoside, the flavonol; rutin, the hydroxycinnamic acids; neochlorogenic and chlorogenic acid, were identified against authentic standards by spectral and retention time analysis and quantified from linear regression curves of authentic standards, the only exception being neochlorogenic acid which was quantified as chlorogenic acid. Vitamin C was measured at 210 nm and quantified with L(+)-ascorbic acid.

3. Results and discussion

3.1. First response surface template

Based on results obtained previously with optimization of enzymatic prepress maceration of black currants (Landbo & Meyer, 2004) the multicomponent, pectinolytic enzyme preparation Pectinex BE 3L, produced by a cloned *Aspergillus* strain (Table 1), was chosen as the first enzyme product to be employed in the elderberry juice prepress optimization experiments.

Significant variations in juice yields, juice turbidity levels, water soluble carbohydrates, total anthocyanin and total phenol contents were found in response to the first round of different enzymatic maceration treatments (Table 2). The obtained juice yields ranged from 71.4–77.0% w/w based on the weight of macerated berries, and the turbidity levels of the juices in the differently macerated samples varied from 103–165 FNU (Table 2). Total phenols yields ranged from ~3.0 to 6.0 mg GAE/g wet weight equivalent to phenolic levels in the juices of 5895–8215 mg GAE/l. The extraction yields of anthocyanins ranged from 1.4 to 2.4 mg/g wet weight elderberry mash equivalent to a span of concentrations of anthocyanins in the juices of 2123 to 3273 mg/l. The content of water soluble carbohydrates in the samples varied from 10.7 to 12.4 mg/g mash indicating that the obtained extent of enzyme catalyzed cell wall degradation varied with the different prepress maceration treatments. In the non-enzyme treated control that had been incubated at 46 °C for 30 min, (Exp. no. 10, Table 2), the content of water soluble carbohydrates was 11.7 mg/ml. pH was measured to 3.9 in all the samples. The juice yield and the turbidity of the control sample were low (72.6% w/w) and high (150 FNU) respectively (Exp. no. 10, Table 2). Since high levels of immediate turbidity were recognized at both low levels of enzyme dosing (0.02% E/S) and at the highest enzyme dose level (0.36% E/S) the data indicated a complex effect of enzymatic maceration on the turbidity. The juice yield data were however consistent with the interpretation that increased juice extraction is a result of enzyme catalyzed degradation of the pectin in the plant cell wall matrix and in the middle lamella that acts as putty between the cells and binds water. The enzymes present in Pectinex BE 3L are pectin esterase (EC 3.1.1.11), pectin lyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15) (Table 1). The combined action of these enzymes should be able

Table 4
Steepest ascent calculations to determine the factor range for the second experimental plan based on juice yield data from the first experimental frame

Factors	1. Experiment					2. Experiment					
	Factor range			Step size [a]	Parameter estimates [b]	Factor range			Factor range		
	Genuine units					Indexed units [c]			Genuine units [d]		
	Low	Center	High			Low	Center	High	Low	Center	High
Maceration temperature	32 °C	46 °C	60 °C	14 °C	1.11	0	1	2	46 °C	60 °C	74 °C
Enzyme dose	0.02%E/S	0.18%E/S	0.34%E/S	0.16%E/S	2.84	0	2.56	5.13	0.18%E/S	0.59%E/S	1.00%E/S

[a] The distance between the low, the center, and the high factor values.
[b] Equals the coefficients of the multiple linear regression coefficients obtained for the effects of the different factors on juice yield in experiment 1 (see Table 3).
[c] Represent the calculated indexed center points and step sizes as calculated from the parameter estimates: (1.11/1.11)=1; (2.84/1.11)=2.56.
[d] The lowest values of each factor were decided to be equal to the center points of experiment 1. From there, the factor ranges for each factor in experiment 2 were calculated by adding the indexed unit multiplied by the step size: (46+14*1)=60; (0.18+0.16*2.56)=0.59.

Table 5
Influences of maceration temperature and enzyme dose on juice yield, turbidity, water soluble carbohydrates, total anthocyanins and total phenols in the second experimental plan

Exp. number	Temperature [°C]	Enzyme dose [% E/S g/g]	Juice yield [%]		Turbidity [FNU]		Water soluble carbohydrates [mg glucose/ml]		Total anthocyanins [mg/g]		Total phenols [mg/g]	
			+E	−E	+E	−E	+E	−E	+E	−E	+E	−E
1	74	1.00	77.5	73.8	157	201	10.4	9.7	2.4	2.4	6.0	5.6
2	76	0.59	76.5	74.4	161	192	9.7	9.3	2.2	2.3	5.7	5.6
3	60	0.12	76.7	71.3	125	208	9.4	9.7	2.3	2.2	5.5	5.0
4	46	0.18	76.7	73.7	120	210	9.3	9.8	2.1	1.9	5.0	4.6
5	74	0.18	75.7	73.2	150	191	9.3	9.5	2.3	2.3	5.9	5.5
6	46	1.00	77.5	71.6	146	211	9.8	9.3	2.0	1.8	5.1	4.5
7	44	0.59	75.6	72.6	135	206	9.1	9.5	1.9	1.8	4.8	4.5
8	60	1.06	76.3	69.4	147	212	9.8	8.8	2.3	2.0	5.7	5.1
CPs[a]	60	0.59	78.1±0.3	72.3±1.4	135±3	204±7	9.7±0.3	9.3±0.2	2.4±0.1	2.2±0.1	5.8±0.1	5.2±0.1
Response ranges			75.6–77.5	69.4 – 74.4	120–161	191–212	9.1–10.4	8.8–9.8	1.9–2.4	1.8–2.4	4.8–6.0	4.5–5.6
95% confidence intervals			75.3–77.8	67.9 – 75.9	117–164	183–220	8.8–10.8	8.5–10.1	1.8–2.5	1.7–2.5	4.7–6.1	4.4–5.8

[a] Center point average of three±SD.

to almost completely degrade the smooth regions of the pectic substances. Polygalacturonase acts on pectin with a low degree of esterification and hydrolyses the α-(1-4)-glycosidic bond between non-methylated galacturonosyl residues (Grassin & Fauquembergue, 1996). Pectin methyl esterase catalyzes the hydrolysis of the ester bond of the methylester of the galacturonosyl residues and this demethylation of pectin enhances the effect of polygalacturonase and therefore a synergistic effect of the combined use of these two enzyme activities is possible. If the demethylation is too fast or too extensive gelation may occur via interaction with calcium, which is amply present in fruit materials. Pectin lyase is an endo-enzyme attacking the α-(1,4)-glycosidic bond between two methylated galacturonosyl residues. The yield levels of total phenols and total anthocyanins in the non-enzyme treated control, Exp. no. 10, Table 2, were 4.6 and 1.8 mg/g mash respectively and thereby in the lower end of the obtained range for the whole template. The obtained values for juice yields, anthocyanins levels and turbidities are in accordance with previously obtained data on elderberry juice (Kaack, 1989). Multiple linear regression analyses of the data showed that an increase in the three factors maceration time, maceration temperature and enzyme dose all significantly increased the juice yield within the factor levels tested (Table 3). In addition, maceration time and maceration temperature exerted a significantly positive interaction effect on the juice yield. Both the anthocyanins and total phenols yields increased with increased maceration temperature, but increased enzyme dose only affected the yields of anthocyanins positively, while no effect of increased maceration time on neither total phenols or anthocyanins was found (Table 3). This pattern of response in relation to the experimental factors agreed with data obtained during the optimization of maceration for black currant juice where eight out of ten enzyme preparations did not increase the anthocyanin content in the juice with increasing maceration time (Landbo & Meyer, 2004). Although increased maceration temperature was the only factor which increased the levels of total phenols, an interaction between maceration time and maceration temperature had a significantly positive effect on the level of total phenols. With respect to turbidity and water soluble carbohydrates no significant effects of any of the three factors were found (data not shown).

3.2. Second response surface template

In order to examine if a further increase in juice yield was obtainable, and to assess if the immediate juice turbidity could be decreased by optimizing the prepress maceration conditions, the factor ranges for maceration temperature and enzyme dose in the surface response template were shifted. This was done by use of the method of steepest ascent (Miller & Miller, 2000) on the juice yield response data of the first template, while the maceration time

Table 6
Multiple linear regression results for the parameters and the parameters and the significant interactions on the juice yield, turbidity, total anthocyanins and the total phenols and water soluble carbohydrates

Parameters and interactions (×)	Juice yield [%]		Turbidity [FNU]		Total anthocyanins [mg/g]		Total phenols [mg/g]		W. slb.carbohydrates [mg glucose/ml]	
	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P
Temperature	0.01	No effect	10.7	<0.0001	0.16	0.0004	0.43	<0.0001	0.21	No effect
Dose	0.32	No effect	8.8	<0.0001	0.004	No effect	0.06	No effect	0.32	No effect
Temperature × dose	–	No effect	−5.0	0.0029	–	No effect	–	No effect	–	No effect
Temp × temp	–	No effect	8.6	0.0002	−0.22	0.0005	−0.29	0.0034	9.62	No effect
Constant/intercept	77.0	<0.0001	135.5	<0.0001	2.39	<0.0001	5.73	<0.0001	–	<0.0001

Table 7
Test of the optimal maceration treatment with Pectinex BE 3L and four new enzyme preparations

	Turbidity [FNU]	Juice yield [%]	Total anthocyanins [mg/g]	Total phenols [mg/g]
Pectinex BE 3L	136	76.6	2.34	5.58
Pectinex BE Colour	119	77.5	2.45	5.75
Pectinex Smash XXL	158	75.6	2.43	5.64
Rapidase EX Color	167	76.5	2.24	5.56
Vinozyme G	174	74.7	2.30	5.53

The average SD for turbidity was ±5 FNU, for juice yield ±0.5%, for total anthocyanins ±0.05 mg/g, for total phenols ±0.15 mg/g.

was kept constant at 30 min. The new factor range values were first estimated in indexed values from the multiple linear regression coefficients obtained for the juice yield data in the first template (Table 3). From there, the indexed numbers were converted to genuine units by multiplying the indexed numbers with the step size from the first template (Table 4). The test area of the second template (Table 4) was not far from the area of the first template because the responses obtained in the first template were presumed to already be close to an optimum prepress maceration reaction. For this reason it was decided that the second template included the center point values of the first experimental template (46 °C, 0.18% E/S) as the lowest factor limits. The new factor ranges thus became: maceration temperature 46–74 °C and enzyme dose 0.18–1.0% E/S (Table 4). With enzyme addition, the obtained range for the juice yields in the new template was 75.6–77.5% w/w while the juice yield range for the juices produced without enzyme gave a considerably lower range of 69.4–74.4% w/w (Table 5). However, despite the higher yield range with enzyme treatment, the differences in yields with and without enzyme addition respectively, did not reach full statistical significance as a little overlap between the 95% level confidence intervals was recognized: 75.3–77.8% w/w (with enzyme) versus 67.9–75.9% w/w (without enzyme) (Tables 5 and 6). A maximum elderberry juice yield was also found by others to be ~78% by weight of incoming elderberries (Kaack, 1989). The activities present in the enzyme preparation were apparently also able to catalyze degradation of the turbidity causing polysaccharides. This could be observed very clearly from the turbidity data that ranged from 120–161 FNU with enzyme addition, and thus on average were 30% lower, than those of samples produced without enzyme addition, that had turbidity levels ranging between 191–212 FNU (Table 5). Even though addition of the pectinolytic enzyme preparation during prepress treatment *per se* resulted in lowered juice turbidities, increased maceration temperature and enzyme dosage increased the juice turbidity when evaluated as single factors (Table 6). Together, however, the maceration temperature and enzyme dosage exhibited a strongly significant turbidity decreasing interaction effect (Table 6). Since the turbidity in the juices may be due to pectin and other plant cell wall substances released during the enzymatic prepress maceration, it seems logic that elevated turbidities may transiently result during enzyme catalyzed cell wall degradation — which can partly explain the positive effect coefficient of the enzyme dosage on the turbidity (Table 6). Subsequently, the turbidity causing pectinaceous cloud may be degraded by further enzymatic action (Grassin & Fauquembergue, 1996). However, since the detailed composition and mechanism of formation of the extensive,

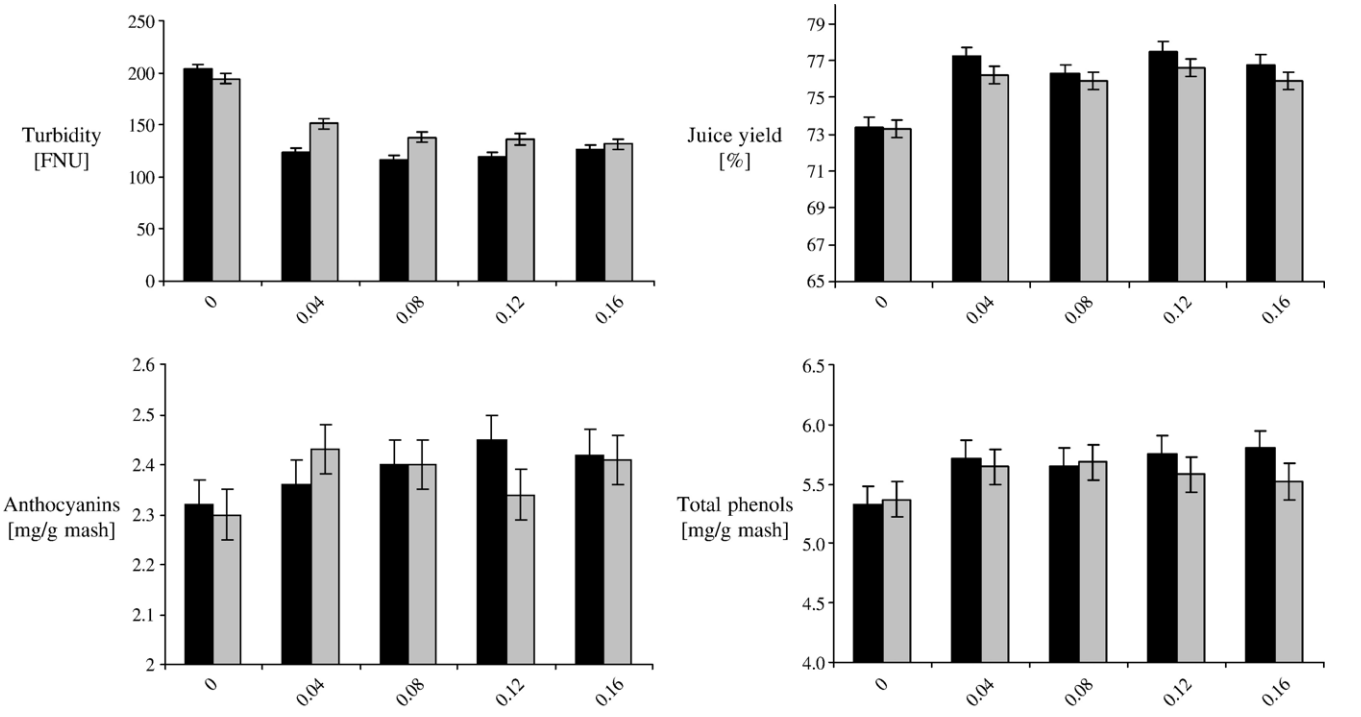

Fig. 1. Juice turbidity (a), juice yield (b), total anthocyanins (c) and total phenols (d) were measured in juices made with different enzyme preparations. Pectinex BE Colour (■) and Pectinex BE 3L (■).

immediate turbidity in elderberry juice is currently uncertain, additional research is needed to reach a firm interpretation of the effects. Any data on the composition of the turbidity causing substances, and on possible molecular interactions among different elderberry constituents in relation to turbidity, would provide a significantly improved foundation for tailoring enzymatic treatments to decrease elderberry juice turbidity.

Regarding anthocyanin and phenolics in the juices, multiple linear regression analyses of the data showed that the maceration temperature had a significantly positive effect on both the anthocyanin levels and on the levels of total phenols (Table 6). These data agreed well with what was seen in the first template. The ranges for water soluble carbohydrates, and total anthocyanin and total phenol levels were however independent of the added enzyme dosage (Table 6). Previously, we have demonstrated that aggressive enzyme catalyzed degradation of cell wall polysaccharides in black currants and black currant skins can significantly enhance the extraction of antioxidant phenolics into black currant juice, and notably that the extent of plant cell wall degradation correlated linearly to the release of phenolics (Bagger-Jørgensen & Meyer, 2004; Landbo & Meyer, 2001). Although a slightly elevated extent of water soluble carbohydrates could be discerned in the set of samples subjected to enzymatic maceration versus those macerated without enzyme addition, the increase was not statistically significant. Since the difference in carbohydrate degradation was thus not pronounced with enzyme addition, the release of anthocyanins and total phenols cannot be expected to increase as a result of the enzymatic treatment either. Although the experimental set-up was designed carefully, the highest reaction temperatures were above the optimal temperature range of the enzymes (Table 1), and this may explain these latter results. The independence of enzyme dose and even of addition of enzyme on anthocyanin levels and levels of total phenols was however also observed in black currant juice experiments, in temperature ranges of ~30–60 °C, after different maceration treatments with varying maceration factor levels and addition of different enzymes (Landbo & Meyer, 2004).

The distribution of the phenolics in the differently treated juice samples was investigated by HPLC analysis, but no significant differences in the profiles were obtained with the different maceration treatments (data not shown).

3.3. Identification of the optimal enzymatic prepress maceration treatment

An optimal prepress treatment was found via response surface modeling of the data, but had to be a compromise between the optimum ranges for the three responses; juice turbidity, total anthocyanins and juice yield. As the juice yield reached an optimum yield within the tested factor range focus was on the two other factors. For the turbidity the lowest levels were found when the enzyme dose ranged between 0.12 and 0.29% E/S and when the temperature ranged between 44 and 59 °C. For a maximum yield of total anthocyanins the ranges for maceration temperature and enzyme dose should be 63–69 °C and 0.12–1.06% E/S respectively. As the total anthocyanins were independent of the enzyme dose the low enzyme dose at 0.12% E/S was selected for the optimal treatment. With respect to the temperature 63 °C was chosen as this temperature gave a high level of total anthocyanins and a low level of turbidity (although the turbidity was not the lowest but still within the standard deviation of the turbidity measurements). The time was already chosen to be 30 min. The optimal process for elderberry maceration with Pectinex BE 3L was then 30 min, 63 °C and 0.12% E/S.

3.4. New enzymes

For this optimal maceration treatment the performance of four different enzyme preparations (Pectinex BE Colour, Pectinex Smash XXL, Vinozyme G and Rapidase EX Color (Table 1)) plus Pectinex BE 3L was tested. These four additional enzyme preparations were chosen based on their pectinolytic activity and on the fact that they had been produced with red berry juice production in mind. Especially their low pH optimum and high temperature tolerance was very important for these experiments. The enzyme preparation Pectinex BE Colour consistently penetrated as the best enzyme preparation for the five responses (Table 7). A reasonable explanation for this could be that this enzyme preparation should be at least twice as strong pectinolytic per unit volume as Pectinex BE 3L according to the producer (Table 1). The distribution of the phenolics in the five juices made with 30 min, 0.12% (E/S), 63 °C was again investigated by HPLC. In brief, the percentage of cyanidin-3-glucoside of the total anthocyanin content varied between 34% and 35% and between 62% and 63% for the cyanidin-3-sambubioside. Of the total content of flavonols, rutin constituted 67–68% and for the total content of hydroxycinnamic acids, chlorogenic and neochlorogenic constituted between 8–9% and 74–75% respectively. Hence, no significant differences in the profiles were obtained with the different enzyme preparations used for these macerations. No ascorbic acid was found in any of the juice samples. A dose-response experiment made to compare the results obtained with Pectinex BE 3L and Pectinex BE Colour emphasized that Pectinex BE Colour was the best enzyme of these two enzyme preparations for elderberry juice production (Fig. 1). Thus, for the turbidity response the enzyme Pectinex BE Colour gave a significantly lower turbidity in three out of four samples than juices made with the preparation Pectinex BE 3L (Fig. 1). The juice yields were also higher for juices made with Pectinex BE Colour than when juices were made with Pectinex BE 3L. For the total anthocyanins and total phenols yields the enzyme preparations were almost equally good. We ascribe the better performance of the Pectinex BE Colour to the stronger overall pectinolytic activity of this enzyme preparation (Table 1). From the data in Fig. 1 it seems possible that the enzyme concentration could be lower than 0.12% maybe even as low as 0.04% but according to the optimization made here we recommend a dosage of 0.12% (E/S).

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