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ORIGINAL PAPER

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INFLUENCE OF THE TIME OF BILBERRY (*VACCINIUM MYRTILLUS* L.) ADDITION ON THE PHENOLIC AND PROTEIN PROFILE OF BEER

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ABSTRACT

Background. In recent years, there has been an increased fruit addition to the brewing process, especially in craft breweries. Fruit addition leads to changes in the organoleptic characteristics and chemical composition of beers. Bilberries are rich in phenolic compounds and possess significant antioxidant capacity. The effects of bilberry addition and brewing process parameters on the changes in the phenolic and protein profile of beer have not been sufficiently studied. The aim of this research was to investigate the changes in the individual phenolic compounds and the protein fractions in beer when bilberries were added at different maturation stages.
Materials and methods. An infusion mashing method was applied for the purpose of obtaining wort with an original extract of 14°P after boiling. Pilsner malt, bitter and aromatic hops 60/40 (Perle and Cascade, respectively), dry yeast *Saccharomyces pastorianus* (*carlsbergensis*) Saflager W 34/70, and bilberries (*Vaccinium myrtillus* L.) were used. All processes were conducted in a Home Brew 50 all-in-one 50 dm^3 brewing system. The fermentation was carried out in a stainless steel cylindroconical fermenter at a temperature of 14°C. The "green beer" was transferred to small stainless-steel fermenters after 60% of the original wort extract had been fermented. The maturation continued for 14 days at 14°C, and the beer lagering for 5 days at 2°C. The bilberries were pasteurised in a water bath for 10 minutes at 70°C. After cooling, they were added to small fermenters at a concentration of 167 g/dm^3 at the beginning and on the seventh day of beer maturation. All variants were carried out in duplicate. After lagering, the beer was bottled using a "beer gun". The beer samples from the experiments were filtered on the day of bottling and frozen until analysis. HPLC/UV-VIS and electrophoresis were used to determine the phenolics and proteins, respectively. The total monomeric anthocyanins were determined by the pH differential method. The original wort extract and alcohol concentration were evaluated, and the sensory analysis was performed according to EBC standard methods.
Results. The changes in 10 phenolic acids, 7 flavonoids, and 10 protein fractions in beer with bilberries added at the beginning and on the seventh day of maturation were studied. The addition of bilberries led to an increase in the phenolic acid (3-fold) and flavonoid (6.2-fold) concentrations. The highest enrichment was observed in terms of rutin, chlorogenic, caffeic, and 3,4-dihydrobenzoic acids. Rosmarinic acid and monomeric anthocyanins were only detected in the bilberry beers. Chlorogenic and neochlorogenic acids, rutin, and catechin dominated in the bilberry beers. Neochlorogenic and gallic acids, epicatechin, and catechin dominated in the bilberry-free beers. The addition of bilberries reduced the protein content by 93 to 96%. The number of protein fractions decreased from 10 to 4. The influence of the bilberry addition time on the phenolics and proteins was different, and it affected the individual protein fractions in a different way. More phenolic acids and flavonoids were determined when bilberries were added at the beginning of maturation.

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The losses of some protein fractions were higher at the beginning of maturation and of others on the seventh day of maturation, whereas the addition time did not matter for some of the fractions.

Conclusion. This study provides new information related to the changes in the phenolic and protein profile of beer with bilberries depending on the time of bilberry addition during beer maturation. The protein concentration and number of protein fractions decreased dramatically. In spite of the significant protein losses, the bilberry addition improved the phenolic profile of the beer and its organoleptic characteristics. The presence of more phenolic compounds is related to the antioxidant capacity respective to the biological value of beer. Further research in this direction is needed.

Keywords: beer, bilberries, phenolic acids, flavonoids, proteins

INTRODUCTION

Fruit addition to beer has been practised for a long time. This trend has intensified in recent years, especially in craft breweries. Tips for beer reinforcement by the addition of a variety of fruits, including bilberries, can be found on home brewing forums and sites. It is recommended that the bilberries be added at the maturation stage (between 60 g/dm^3 and 360 g/dm^3), but information related to the changes that occur in beer composition when bilberries are added is scarce. Mileva et al. (2018) have reported a 1.3- to 1.4-fold increase in polyphenols and a 1.2- to 1.4-fold increase in flavonoids in beer with bilberries added during maturation at a concentration of 100 g/dm^3. According to these researchers, the polyphenol and flavonoid concentrations in beer are higher when bilberries are added during wort boiling. Nevertheless, no data are available on individual phenolic compounds in bilberry beer.

Beer contains different phenolic compounds, including phenolic acids, flavonoids, condensed tannins, and non-flavonoid polyphenols. They originate from malt, cereals, and hops. The variety of individual compounds is great due to differences in the raw materials and brewing process parameters.

There are numerous studies on the phenolic compounds in beer. Most of them describe ferulic and p-coumaric acids as the most abundant phenolic acids in beer. Vanillic, gallic, and sinapic acids have also been reported as being important for beer (Wannenmacher et al., 2018). The greater part of phenolic acids in beer is present in bound form as glycosides, esters, and bound complexes (Nardini and Ghiselli, 2004; Wannenmacher et al., 2018). The variety of flavonoid compounds in beer is great. Catechin, epicatechin, quercetin, rutin, kaempferol, myricetin, procyanidin B3, procyanidin B2, prodelphinidin B3, and other flavonoids in smaller quantities have been identified in different studies (Wannenmacher et al., 2018).

Phenolic compounds play various roles in beer. Most of them possess antioxidant properties. They are involved in beer stability and aging during storage. Some phenolic acids act as flavour precursors in beer, others influence bitterness perception.

Proteins in beer originated from barley, malt, and yeast and have an impact on beer quality. According to Leiper et al. there are three major groups of proteins in beer. The first consists of a group of proline rich fragments originating from hordein, ranging in size from 15–32 kDa, which are involved with haze formation. The second is LTP1-lipid transfer protein 1 (9.7 kDa in pure form), which is involved in foam stability, and the third is protein Z (40 kDa), which appears to have no direct function but may play a role in stabilizing foam once it has been formed (Steiner et al., 2011). Earlier classification of proteins by their molecular weight split them into three categories: high molecular weight fraction: >40 kDa, middle molecular weight fraction: 15–40 kDa and low molecular weight fraction: <15 kDa. Foam-positive proteins can be divided into high molecular weight proteins (35–50 kDa) and low molecular weight proteins (5–15 kDa; Steiner et al., 2011).

The phenolic compounds and proteins in beer are known as the main substances responsible for beer turbidity and sediment formation (Leiper and Miedl, 2009). The addition of fruits rich in phenolic compounds, such as bilberries, boosts protein precipitation. Bilberries are rich in procyanidins, which are

considered the main polyphenols in beer related to turbid appearance (Zhao and Sun-Waterhouse, 2018).

Bilberries are rich in phenolic compounds; they possess significant antioxidant capacity and present huge benefits for human health. The main phenolic compounds found in bilberries are anthocyanins. Georgieva et al. (2018) reported 12 individual anthocyanins in bilberries (*Vaccinium myrtillus* L.) from Bulgaria, including glycosides of delphinidin, cyanidin, malvidin, petunidin, and peonidin, where delphinidin glycosides were the predominant compounds. Similar anthocyanins were identified in Slovenian blueberries (*Vaccinium corymbosum* L.) and bilberries (*Vaccinium myrtillus* L.) (Može et al., 2011).

Bilberries also contain flavonoids and phenolic acids. Phenolic acids are significantly fewer, chlorogenic acid being the predominant one. Flavonoids like catechin, epicatechin, quercetin, myricetin rutin, and other flavonoid glycosides have been identified in blueberries (*Vaccinium corymbosum* L.) and bilberries (*Vaccinium myrtillus* L.) (Može et al., 2011; Diaconeasa et al., 2014).

To our knowledge, the changes in the phenolic and protein profiles of beer when bilberries are added have not been studied or there are insufficient data in the available literature. The effects of the brewing process parameters have not been sufficiently studied, either. The aim of this study was to investigate the changes in the individual phenolic compounds and the protein fractions in beer when bilberries were added at different maturation stages.

MATERIALS AND METHODS

Raw materials

Pilsner malt (Weyermann, Germany), Perle and Cascade hops (Bulhops, Bulgaria), and dry yeast *Saccharomyces pastorianus* Saflager W 34/70 (Fermentis, France) were used. The bilberries (*Vaccinium myrtillus* L.) were obtained in a frozen state from Bulfruct Ltd., Bulgaria, and kept in a freezer until use.

Chemicals

The standard compounds (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, catechin, epicatechin, rutin, myrecetin, quercetin, quercetin-3-glucoside and kaempherol); ACRYLAMIDE, suitable for electrophoresis ≥99%; N,N'-METHYLENE-BIS-ACRYLAMIDE, for electrophoresis ≥98%, powder; SODIUM DODECYL SULFATE for electrophoresis, ≥98.5% (GC); TRIZMA(R) BASE, Primary Standard and Buffer, ≥99.9% (titration), crystalline; ETHYLENEDIAMINETETRAACETIC ACID, FREE ACID SIGMA GR BioUltra, anhydrous, ≥99% (titration); TEMED MOLECULAR BIOLOGY REAGENT BioReagent, for molecular biology, ≥99% (GC); AMMONIUM PERSULFATE for electrophoresis, ≥98%; GLYCINE, for electrophoresis, ≥99%; BRILLIANT BLUE R; BROMPHENOL BLUE SULTONE; 2-MERCAPTOETHANOL, for molecular biology, for electrophoresis, bioreagent 99%; SIGMA MARKER (TM), Wide Range, mol. wt 6500–200 000 Da; BRILLIANT BLUE G; HPLC-grade acetonitrile; acetic acid were purchased from Sigma-Aldrich (Steinheim, Germany). HCl, 37% from Merck. All other chemicals used were of analytical grade and were purchased from local suppliers.

Beer production

Pilsen malt (15 kg) was milled using a Corona hand mill and mixed with 55 dm³ pre-heated water to obtain wort with an original extract of 14°P after boiling. An infusion mashing method was applied under the following conditions: 20 minutes at 60°C, 20 minutes at 65°C, 25 minutes at 72°C, and 1 minute at 78°C. After the mashing process, the sweet wort was lautered, the spent grains were sparged and the entire amount of wort was boiled. The bitter hops (20 g Perle – 9% α-bitter acids) were added 10 minutes after the boiling started, and the aromatic hops (17.14 g Cascade – 7% α-bitter acids) were added 7 minutes before the end of the boiling process. The total amount of α-bitter acids was 60 mg/dm³. After removal from the hot trub, the wort was cooled to 14°C. All processes were conducted in a Home Brew 50 all-in-one 50 dm³ brewing system (TM INOX, Bulgaria).

The wort was aerated and placed in a stainless steel cylindroconical fermenter (TM INOX, Bulgaria). The yeast was sprinkled into the wort, according to the manufacturer's instructions. The fermentation temperature was 14°C. The main fermentation was monitored hydrometrically. For maturation, the "green beer" was transferred to small stainless-steel fermenters when 60% of the original wort extract had been fermented.

Beer maturation continued for 14 days at 14°C, and beer lagering for 5 days at 2°C. Both were conducted under pressure.

The bilberries were pasteurised right before being added to a water bath for 10 minutes at 70°C and cooled to 14°C after that. They were added at a concentration of 167 g/dm^3, in an empty small fermenter which had previously been flushed with CO_2. At the beginning of maturation, the "green beer" was slowly transferred from the stainless steel cylindroconical fermenter to the small fermenter with bilberries using a hosepipe which reached the bottom of the small fermenter. After the transfer, a pressure of 0.5 bar was reached in the fermenter using CO_2. On the seventh day of maturation, the "green beer" was transferred under pressure from one small fermenter to other one with bilberries. The presence of CO_2 kept the bilberries and "green beer" away from oxidation.

All variants were carried out in duplicate. After lagering, the beer was bottled using a "beer gun" (Blichmann Engineering, USA).

Sample preparation

The beer samples for the experiments were filtered through Macherey-Nagel MN 619 ¼ Ø 320 filter paper on the day of bottling and frozen until analysis. The bilberries (10 g) were extracted with 100 cm^3 of acidified methanol (0.1% HCl, v/v) for 24 hours at 4°C in the dark. The liquid was separated through filtration, and the extract volume was filled up to 100 cm^3 with the extraction solvent. The extract was kept in a freezer until analysis.

HPLC analysis of phenolic compounds

HPLC analysis of the phenolic components was performed according to Denev et al. (2019) using an Agilent 1220 HPLC system (Agilent Technology, USA) equipped with a binary pump and a UV-Vis detector (Agilent Technology, USA). Separation was performed using an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25°C using a wavelength of 280 nm. The following mobile phases were used: 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 cm^3/min. The gradient elution started with 14% B; it increased linearly to 25% B between the 6th min and the 30th min, then to 50% B in the 40th min.

The individual compounds were identified by comparing the retention times of the standards used (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myrecetin, quercetin, naringenin, and kaempherol). The results were calculated using standard curves built on the basis of the peak areas of standard solutions of each individual compound and expressed as mg/dm^3 sample.

Total monomeric anthocyanins

The total monomeric anthocyanins were determined using the pH differential method (Giusti and Wrolstad, 2001). The results were calculated using a molar absorptivity of 26 900 dm^3/(mol·cm) and molecular weight of 449.2 g/mol, and expressed as equivalent of cyanidin 3-glucoside (CGE). The measurements were performed using a Helios Omega UV-Vis spectrophotometer equipped with VISIONlite software (Thermo Fisher Scientific, Madison, WI, USA).

Electrophoretic analysis of proteins

The proteins were isolated from the beer by precipitation using trichloroacetic acid (TCA) (Tattersall et al., 1997). An Eppendorf microcentrifuge was used. Electrophoretic analysis was performed using SDS-PAGE according to Laemmli at a concentration of 15% acrylamide in gel (Laemmli, 1970; Lyutskanov et al., 1994). The molecular weight of the protein was determined by markers, SigmaMarker™ 6,500–200,000 Da, and TotalLab specialised software, trial version, for image analysis of electrophoretic gels (TotalLab, n.d.). The quantity and percentage of each fraction in the total quantity of the determined protein fractions were calculated using TotalLab.

Quantitative analysis of proteins

The protein concentration was determined using the Bradford method (1976), with Stosheck's modification (cited in Owusu-Apenten, 2002). The results were expressed in mg/dm^3 bovine serum albumin (BSA).

Basic characteristics of beer

The original extract (method 8.2.1) and alcohol concentration (method 9.2.1) were measured according to EBC standard methods (Analytica, 2005).

Sensory analysis

Sensory evaluation of the beverages was performed by a trained 5-member tasting panel. The samples were evaluated using the descriptive method (method 13.10) and a ranking test (method 13.11; Analytica, 2005).

Statistics

The results reported in the present study are the mean values of at least three analytical determinations, and the coefficients of variation expressed as the percentage ratios between the standard deviations and the mean values were found to be <5% in all cases. The means were calculated with Microsoft Excel™ at a 95% confidence level. One-way ANOVA and Scheffe's multiple range test at $p < 0.05$ as described by Donchev et al. (2007) were used too.

RESULTS AND DISCUSSION

Beer characteristics

The basic characteristics of the bilberry-containing beers and the control beer (without bilberries) have been summarised in Tables 1 and 2. The alcohol content was in the range of 4.79 ±0.12% (w/w) and 5.45 ±0.19% (w/w). The real extract was between 4.36 ±0.12% (w/w) and 4.62 ±0.16% (w/w). These are normal values for beer produced from wort with an original extract of 14°P. The bilberry addition time did not affect the alcohol content of the beer. The beer with bilberries added on the seventh day of maturation was preferred according to the results of the sensory evaluation by ranking test (Table 2). The bilberry beers were intense ruby in colour. This was due to the presence of anthocyanins extracted from the bilberries. The addition of bilberries did not change the body of the beer or the foam stability. The other organoleptic characteristics of the beers have been summarised in Table 2.

Phenolic acids

The main phenolic acids detected in the bilberry-free beer were neochlorogenic (38.5%), gallic (28.2%), and chlorogenic (12.4%). They represented 72.2% of the total concentration of the phenolic acids determined. Except for p-coumaric and ferulic acids, the concentration

Table 1. Basic characteristics of beers

Sample	Alcohol, % (w/w)	Real extract, % (w/w)
Control beer	5.45 ±0.19[a]	4.62 ±0.16
Beer with bilberries added at the beginning of maturation	4.79 ±0.12[b]	4.36 ±0.12
Beer with bilberries added on the seventh day of maturation	5.13 ±0.41[a,b]	4.45 ±0.20

The same letter for a given sample means no significant differences (95% confidence level).

Table 2. Results of the ranking test and descriptive sensory analysis

Sample	Description	Ranking evaluation (1 – the best)
Control beer	Colour: goldish Aroma: hoppy/malty Taste: harmonious, typical of light lager beers with a slight bitterness; medium to full body	3
Beer with bilberries added at the beginning of maturation	Colour: intense ruby Aroma: fruity, a bit sweet Taste: no bitter taste; medium body	2
Beer with bilberries added on the seventh day of maturation	Colour: intense ruby Aroma: fruity, with good harmony and freshness Taste: no bitter taste; medium to full body	1

of phenolic acids in the bilberry-free beer (Table 3) was higher than the data reported by other researchers (Nardini and Garaguso, 2020; Wannenmacher et al., 2018). This can be explained by the differences in the raw materials and brewing process parameters.

Chlorogenic and vanillic acids were the predominant phenolic acids identified in bilberries, 62.5% and 19.5% respectively. The percentages of other phenolic acids were lower: rosmarinic (6.5%), ferulic (5.7%), caffeic (4.3%), p-coumaric, and cinnamic acids (under 1%). The quantities of neochlorogenic, gallic, and 3,4-dihydrobenzioc acids were not determined and not included in the total quantity of the identified phenolic acids because there were not enough separated peaks.

According to Može et al. (2011), Su et al. (2017), and Diaconeasa et al. (2014), chlorogenic acid is the

Table 3. Content of phenolic compounds in bilberries and beers

Compounds	Bilberries mg/100 g	Control beer sample mg/dm^3	Beer with bilberries added	
			at beginning of maturation mg/dm^3	on seventh day of maturation mg/dm^3
Phenolic acids				
Chlorogenic	57.39 ±1.73	13.51 ±0.38	116.86 ±0.54	90.19 ±1.97
Neochlorogenic	NSP	41.89 ±0.01	65.28 ±0.37	52.24 ±1.44
Vanillic	17.90 ±0.53	8.50 ±0.01	18.18 ±0.00	13.75 ±0.86
Caffeic	3.97 ±0.22	1.95 ±0.04	16.03 ±0.79	13.01 ±0.75
3,4-dihydrobenzoic	NSP	7.43 ±0.19	56.43 ±0.97	30.46 ±0.33
Ferulic	5.28 ±0.04	3.79 ±0.06	7.90 ±0.34	5.87 ±0.71
p-coumaric	0.83 ±0.09	0.59 ±0.00	3.15 ±0.19	2.33 ±0.30
Gallic	NSP	30.73 ±0.03	35.32 ±0.92	39.22 ±0.70
Cinnamic	0.53 ±0.01	0.45 ±0.04	1.24 ±0.04	0.79 ±0.05
Rosmarinic	5.96 ±0.17	ND	2.28 ±0.04	1.31 ±0.23
Flavonoids				
Quercetin	3.76 ±0.04	3.31 ±0.03	5.02 ±0.37	3.27 ±0.36
Quercetin-3-glucoside	13.41 ±0.61	3.71 ±0.45	5.95 ±0.47	3.58 ±0.21
Rutin	175.69 ±5.69	3.35 ±0.37	185.86 ±4.00	163.68 ±2.95
Myricetin	4.80 ±0.16	3.47 ±0.51	8.36 ±0.16	5.62 ±0.15
Kaempferol	0.46 ±0.01	1.05 ±0.06	2.36 ±0.24	1.27 ±0.18
Catechin	138.79 ±0.66	17.97 ±0.64	105.39 ±5.30	68.55 ±5.95
Epicatechin	47.82 ±0.86	23.17 ±0.40	36.52 ±3.08	21.55 ±0.30
Total monomeric anthocyanins				
	569.0 ±31.5	ND	140.3 ±21.2	136.0 ±22.6

Each value is the mean ±SD of triplicate determination.
NSP – peaks not separated well enough.
ND – not detected.

Fig. 1. Beer with bilberries added on the seventh day of maturation (on the left) and control beer (on the right)

main phenolic acid found in blueberries (*Vaccinium corymbosum* L.) and bilberries (*Vaccinium myrtillus* L.). Su et al. (2017) reported the presence of vanillic, ferulic, caffeic, gallic, and 3,4-dihydrobenzoic acids. Caffeic, ferulic, and p-coumaric acids were identified in bilberries analysed by Može et al. (2011).

The addition of bilberries resulted in up to a 3-fold increase in phenolic acids in beer, but the concentration of individual acids changed to different degrees. Chlorogenic, caffeic, and 3,4-dihydrobenzoic acids increased 8.6-fold, 8.2-fold, and 7.6-fold, respectively. Neochlorogenic, cinnamic, ferulic, and vanillic acid increased between 1.6- and 2.8-fold. The change in gallic acid was insignificant. Furthermore, the ratio between individual phenolic acids changed. In the control beer sample, neochlorogenic and gallic acids had the highest concentrations, followed by chlorogenic acid. In the bilberry beer samples, chlorogenic and neochlorogenic acids had the highest concentrations, followed by 3,4-dihydrobenzoic acid. Unlike the control beer sample, the bilberry beer samples contained rosmarinic acid, which most probably originated from the bilberries added.

The phenolic acid concentration in the beer samples with bilberries added on the seventh day of maturation was 23% lower than in the samples with bilberries added at the beginning of maturation. The highest decrease was observed in the concentration of 3,4-dihydrobenzoic acid (46%) and cinnamic acid (36%), and the lowest in the caffeic acid (19%) and neochlorogenic acid (20%) concentrations.

The presence of more phenolic acids in the beer samples with bilberries added at the beginning of maturation could be attributed to the longer contact time between the bilberries and the fermented medium improving the extraction process.

The reduction of the 3,4-dihydrobenzoic and cinnamic acids in the beer samples with bilberries added at the beginning of maturation was significantly lower than that of the other acids. It could be assumed that they were more difficult to extract from the bilberries. The reason for this may be the bound form they appear in and their abundance in bilberry skin; hence, the slower extraction process.

The calculation of the mass balance for all phenolic compounds demonstrated that the bilberry beer contained more chlorogenic, caffeic, and p-coumaric acids (expressed in mass) than the bilberries. These acids had not only been extracted from the bilberries; they were found in the control beer (without bilberries), too. Nevertheless, for each of the three acids, the sum of its concentration in the control beer and its quantity extracted from the bilberries (if 100% of the acid was transferred from the bilberries to the beer) was lower than the acid concentration in the bilberry-free beer. It can be hypothesised that a biotransformation or biosynthesis took place during beer maturation with bilberries.

Chrzanowski (2020) reviewed yeast metabolism for polyphenol biosynthesis, discussing the different pathways for biosynthesis of the aromatic ring in *S. cerevisiae* and the connection between aromatic amino acids and phenolic compounds, as well as the enzymes involved in the biosynthesis. According to the author, the overexpression of ARO1 (pentafunctional enzyme converting DAHP into 5-enolpyruvylshikimate-3-phosphate) and ARO2 (chorismate synthase) in *S. cerevisia* positively influenced the production of p-coumaric acid.

Flavonoid compounds

The main flavonoids in the bilberry-free beer sample were epicatechin and catechin. Their percentages in the total concentration of the identified flavonoids

were 41.4% and 32.1%, respectively. The quantity of kaempferol was the lowest (1.9%). The percentages of quercetin, rutin, myricetin, and quercetin 3-glucoside were between 5.9% and 6.6%. The concentrations of catechin, epicatechin, quercetin, rutin, kaempferol, and myricetin in the control beer sample were higher than those in other beers as reported by Wannenmacher et al. (2018), Kellner et al. (2007), Dvořáková et al. (2007), Jandera et al. (2005), and Socha et al. (2017).

As mentioned above, the reason for the higher flavonoid concentration can be found in the differences in the raw materials and brewing process parameters, as well as in the filtration processes.

Rutin (45.7%) and catechin (36.1%) constituted the largest part of the flavonols and flavanols determined in the bilberries used. Diaconeasa et al. (2014) also reported a high rutin content in blueberries. Slovenian bilberries contain more quercetin and myricetin, and the epicatechin concentration is higher than that of catechin (Može et al., 2011).

The bilberry addition led to a rise in the flavonoid concentration by up to 6.2-fold, rutin demonstrating the highest increase (56-fold). The increase in the other flavonoids was between 1.6- and 5.9-fold. The rutin and catechin concentrations in the bilberries were the highest (Table 3). Their percentages in the total flavonoid content were 45.7% and 36.1%, respectively. This affected the ratio between individual flavonoids in the bilberry beer samples. Unlike the control beer sample, the rutin content was the highest and reached 53%, followed by catechin (30%). The amount of the other determined flavonoids decreased significantly.

The concentration of the individual flavonoids in the beer samples with bilberries added on the seventh day of maturation was 33–46% lower than that in the samples with bilberries added at the beginning of maturation. The decrease in the rutin concentration was only 12%. This resulted in an additional increase in the rutin content and decrease in the overall content of the other flavonoids.

The total monomeric anthocyanins in the bilberries used were 568.99 μmol CGE/100 g. A similar concentration was reported by Georgieva et al. (2018) for Bulgarian bilberries. The control beer sample did not contain anthocyanins, but they were found in the bilberry beers. The bilberry addition time did not affect the anthocyanin concentration (Table 3).

The results presented indicate that the addition of bilberries enriched the beer with phenolic compounds by increasing their concentration, on the one hand, and by introducing new phenolic compounds, on the other hand. This definitely improved the biological value of the beer. Phenolic compounds including anthocyanins, flavonols, and phenolic acids have multiple biological effects such as antioxidant, antimutagenic, anti-inflammatory, and antimicrobial activity (Georgieva et al., 2018). Many epidemiological studies suggest a strong association between the consumption of food rich in phenolics and the prevention of many human diseases associated with oxidative stress (Socha et al., 2017). The phenolic acids that provide beneficial health effects are: chlorogenic acid, gallic acid, coumaric acid, and caffeic acid (Diaconeasa et al., 2014). The potential preventive effects of hydroxycinnamic acids on several chronic diseases have been widely reviewed in recent years (Bento-Silva et al., 2020).

Proteins

The protein concentration in the control beer sample was 83.7 mg/dm^3 BSA (Fig. 2). It was between 93 and 96% higher than that in the bilberry beer samples. The time of bilberry addition during maturation did not

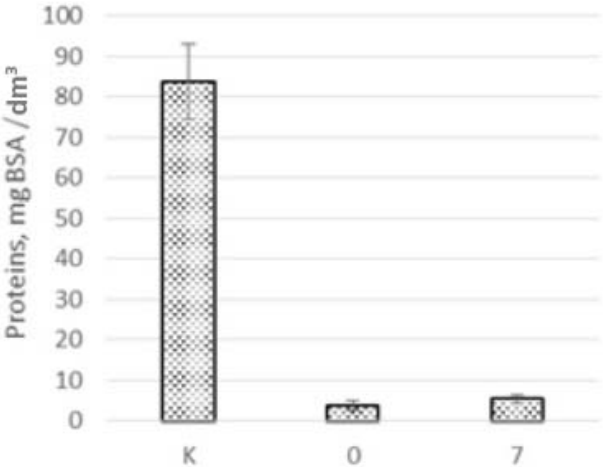
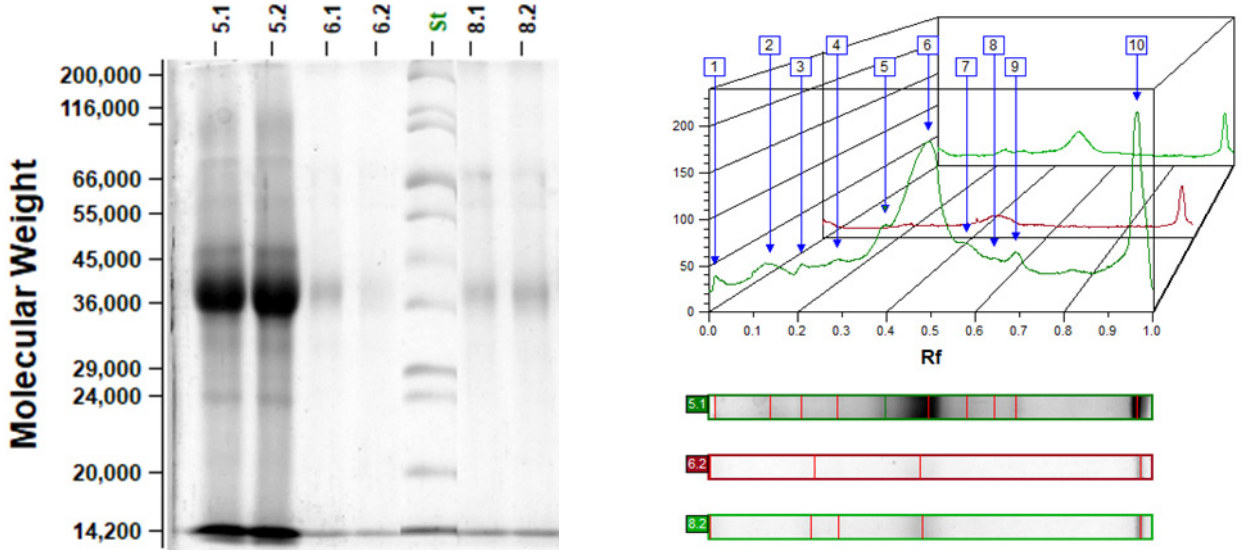


Fig. 2. Protein concentration in beers. The results are expressed as bovine serum albumin in mg per litre beer, means ±SD, n = 3: K – control beer sample, 0 and 7 – beers with bilberries added at the beginning and on the seventh day of maturation, respectively



Fraction number	1	2	3	4	5	6	7	8	9	10
Molecular weight, kDa	220	101	65	49	37	31	28	27	26	25
Part of total fractions, %										
Control beer sample	1.2	9.0	3.3	4.7	11.3	35.5	13.3	3.0	4.6	14.2
BB0	10.1	ND	12.9	ND	ND	52.0	ND	ND	ND	25.0
BB7	5.9	ND	15.0	8.5	ND	50.1	ND	ND	ND	20.5
Degree of protein reduction compared to the control sample, %										
BB0	0	100	58	100	100	80	83	100	100	80
BB7	21	100	30	72	100	78	100	100	100	78

Control beer sample – beer without bilberries (bands 5.1/5.2 in the electrophoresis).
BB0 – beer with bilberries added at the beginning of maturation (bands 6.1/6.2 in the electrophoresis).
BB7 – beer with bilberries added on the seventh day of maturation (bands 8.1/8.2 in the electrophoresis).
ND – not detected.

Fig. 3. Protein fractions in beers

affect the protein concentration significantly. The protein content in the samples with bilberries added at the beginning and on the seventh day of maturation was identical. In addition to the significant reduction in the protein concentration, there was a remarkable change in the protein fractions. The bilberry-free beer sample contained ten protein fractions, the molecular weight of which varied between 25 and 220 kDa (Fig. 3). The main fractions were those with molecular weights of 31 and 25kDa. Their share of all fractions was 35.5% and 14.2%, respectively. The percentages of the fractions with molecular weights of 27, 65, and 220 kDa were the lowest.

The protein fractions in the bilberry beer samples remained only four or five, which was a 50% reduction. The fractions with molecular weights of 26, 27, 37, and 101 kDa were the most affected ones; they were not detected in the bilberry beers. The fractions with molecular weights of 25, 31, 65, and 220 kDa were the most resistant to precipitation. They were present in the samples with bilberries and the reduction in their concentration was different.

It can be assumed that the protein fractions with molecular weights of 26, 27, 37, and 101 kDa are derived from malt hordein and contain considerable proline and glutamine residues. It has been proved

that proline and glutamatic acid rich hordeins with molecular weights of 10–30 kDa according to some researchers (Nadzeyka and Altenhofen, 1979), or 1–40 kDa according to others (Asano et al., 1982), are the main initiators of haze development in beer (Zhao and Sun-Waterhaus, 2018). The proline residues are involved in the site where the polyphenols are attached to the protein and adjacent proline and glutamine tend to enable strong binding to polyphenols (Siebert, 2009; Zhao and Sun-Waterhaus, 2018).

The time of bilberry addition affected the individual protein fractions in a different way. The longest contact time between the bilberries and the fermented beer led to the highest loss of protein fractions with molecular weights of 49 and 65 kDa. The opposite trend was observed with the fractions with molecular weights of 28 and 220 kDa. The 28 kDa fraction was present in the samples with bilberries added at the beginning of maturation but was not detected in the samples with bilberries added on the seventh day of maturation. The loss of the 220 kDa fraction was detected only when the bilberries were added on the seventh day of maturation. This could be attributed to reversible coagulation. It is known that the protein-polyphenol interaction is reversible at least initially (Zhao and Sun-Waterhaus, 2018). The degree of reduction for most protein fractions (25, 26, 27, 31, 37, and 101 kDa) was not affected by the bilberry addition time.

Despite the serious reduction in the amount of protein fractions with molecular weights of 25 and 31 kDa, their percentage in the bilberry beers remained the highest. The main protein fraction in the bilberry beers was the one with a molecular weight of 31 kDa. It constituted between 50 and 52% of all fractions. The share of the fraction with a molecular weight of 25 kDa was between 20 and 25%.

CONCLUSION

This study provides new information related to the changes in the phenolic and protein profiles of bilberry beer, as well as to the influence of the time of bilberry addition during the beer maturation. On the basis of the results, a conclusion can be drawn that, in spite of the significant protein losses, the bilberry addition improved the phenolic profile of the beer and its biological value, respectively. Rosmarinic acid and monomeric anthocyanins were only detected in the bilberry beer. The influence of the time of bilberry addition on the phenolics and proteins was different. The total protein concentration was not affected by the addition time, but more phenolic acids and flavonoids were obtained when the bilberries were added at the beginning of maturation. The new data obtained in this study can be useful for better management of the technological processes in the production of bilberry beer.

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