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Acylphloroglucinol Glucoside from Hops: Isolation, Identification and Haze-activity

Polyphenols can complex with proteins in the beer matrix, thereby forming undesirable temporary or permanent hazes. Conversely, polyphenols possess desirable qualities as they are antioxidants and have health benefits. Selectively removing haze-sensitive polyphenols can therefore be an important tool for optimising beer quality. In this study, one predominant compound was detected in PVPP washing solution as well as unfiltered beer and thus was suspected of being able to form haze. The compound was isolated from a hop tannin extract by using preparative HPLC and subsequently identified as 1-[(2-methylpropanoyl)-phloroglucinyl]-β-D-glucopyranoside (co-multifidol glucoside) by NMR (1H, 13C and 2D) coupled with MS. In separate trials using model solutions, its tendency to form haze was confirmed. As a complement, various phenolic compounds from the hop tannin extract and the co-multifidol glucoside were identified by using HPLC-DAD.

Descriptors: haze, chill-haze, PVPP, NMR, co-multifidol glucoside

1 Introduction

The conservation of the clarity of beer during the period of its indicated shelf-life is presently one of the major problems facing breweries. Haze can occur as chill-haze which emerges during cold storage of beers. It is reversible and can dissolve again at room temperature or warmer. Chill-haze is regarded as the precursor of the formation of irreversible permanent haze which in general presupposes oxidation reactions and, as a consequence, the formation of covalent bonds. Haze formation is predominantly caused by reactions between condensable polyphenols and proteins [2-5, 8, 9, 14-16, 39-40, 42-44, 46-48]. Although authors reported varying proportions of the single substances which are present in haze, it is accepted that proteins range from 14-77% followed by polyphenols ranging from 15-75% [2, 3]. In addition to polyphenols and proteins, polysaccharides and inorganic substances can be found in proportions of 1-14%.

Various investigations [10-13, 23, 26, 30-32] support the general consensus that simple polyphenols like (+)-catechin, or the (-)-epicatechin have little or no chill-haze forming capabilities until they have been oxidatively polymerized. These polymerization reactions can be promoted by oxidation or acid catalyzed, respectively, and thus are promoted by the influx of minimal amounts of oxygen during filtration and filling. In this connection, the dimers procyanidin B3 and prodelphinidin B3 have been identified as very haze-active proanthocyanidins the haze activity of which rises with increasing rate of polymerization and molecular size [4, 24, 27, 40].

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Siebert et al. [38] found that only those flavonoids bearing two hydroxyl groups on the phenyl group show haze-active behaviour. Vicinal hydroxyl groups appear to exert the strongest effect. In additional trials, Siebert et al. [38] demonstrated that proanthocyanidins bearing three hydroxyl groups (e.g. gallocatechin, prodelphinidin B3) are more reactive than those with only two hydroxyl groups (e.g. catechin, procyanidin B3).

Substances promoting oxidation reactions in beer such as iron in its ferrous state (Fe^{2+}) which can act as a catalyst in the Fenton reaction system contribute to polymerization reactions of polyphenols. Recent studies demonstrated that, at beer pH, the polyphenol-protein complexes can form complexes with the reaction products of the Fenton-/Haber-Weiss reaction system such as iron in its higher valence state (ferric iron, Fe^{3+}). This, in turn, is mainly formed after the consumption of the endogenous antioxidative potential thereby promoting the temperature dependent formation of chill-haze [17-19].

To face the haze challenge in bottled beer, the chemical-physiological stability of a beer or their resistance against haze formation, respectively, are improved by e.g. applying stabilizing agents such as polyvinylpolypyrrolidone (PVPP) and silica gels. In practice, breweries achieve the best results when using a combination of both PVPP and silicagel; however, their usage simultaneously yields a loss of physiologically relevant and antioxidative beer ingredients such as polyphenols. When working apart from the German purity law, a further approach consists in the application of certain proteolytic enzymes, e.g. papain, which provokes a hydrolysis of proteins and/or their precipitation. Some enzymes can also act selectively thereby breaking down proteins at specific binding sites, e.g. at the proline binding site [36]. Increasing the antioxidative potential of a beer by e.g. adding ascorbic acid or sulfur dioxide also helps slowing down beer staling mechanisms and, consequently, haze formation. However, adding too high concentrations of these preservatives negatively impacts the taste of beer [36]. Ultimately, there is an agreement in the present literature that preventing oxygen

influx during processing and reducing the amount of pro-oxidative transition metal ions are indispensable measures to delay the occurrence of beer haze during extended storage.

Unfortunately, there is no adequate model for predicting a beer's tendency to form haze which, in turn, often leads to over stabilizing of beers thus unnecessarily lowering their organoleptic quality and also raising the process costs. Studies proved that the intake of foods and beverages (including beer), which are rich in polyphenols, can reduce the risk factors for cardiovascular disease, certain types of cancer, inflammation and others [22, 34]. In addition, there is evidence that polyphenols are capable of slowing down or preventing beer staling reactions thereby having a significant effect as they can diminish oxidative damage [7, 20, 21, 29, 33]. Therefore, an excessive removal of polyphenols may be unwanted.

Isolating beer haze and analyzing its composition can provide an approximation of its individual fractions. Although these findings have a more general character and only support conclusions to a very limited extent. However, examining selected substances and substance groups can help to quantitatively assess the effects of the stabilizing steps. Moreover, it can provide useful information for a better understanding of mechanisms which are responsible for the formation of beer haze thus helping to improve stabilizing agents and applications.

There are various methods and approaches to analyze colloidal stability, raw materials, beer or beer haze, respectively. The most commonly used method for determining a beer's colloidal stability is to conduct a forcing test where warm and cold phases are alternated periodically during storage and the beer's turbidity is then measured after the particular cold phase at 0 °C [28]. This data can additionally be correlated with a beer's endogenous antioxidative potential as perceived from measurements using electron spin resonance spectroscopy [18]. *McMurrough* et al. [25] used a model system which shows the influence of certain flavonoids and sensitive proteins in regard to haze formation. Quantitatively measuring the content of polyphenols or anthocyanogens by spectrophotometry according to MEBAK is a rather imprecise method as related to determining haze-relevant polyphenols [28]. At this juncture, the determination of monomers, dimers and trimers of potential phenolic haze-precursors by using high performance liquid chromatography (HPLC) can provide more detailed information as it displays the rate of polymerization which, in turn, allows drawing conclusions about the beer's condition. When assessing selected substances, the composition of beer, the beer haze or raw materials, nuclear magnetic resonance (NMR) spectroscopy coupled with mass spectrometry (MS) is the method of choice. It offers qualitative and quantitative identification and characterization of a variety of components and allows correlating them with a variety of quality parameters.

In the present study, a hop tannin extract was fractionated and single phenolic compounds were characterized by HPLC-DAD. One dominant polyphenol 1-[(2-methylpropanoyl)phloroglucinyl]-β-D-glucopyranoside (co-multifidol glucoside) – a biologically active compound of the group of so-called "multifidol glucosides" – was detected in both unstabilized beer and PVPP regeneration solution. After isolating it by using preparative HPLC, it was identified by NMR coupled with MS and tested on its haze-activity in model systems.

2 Materials and methods

2.1 Reagents and materials

Catechin (±), epicatechin (-), protocatechuic, 4-hydroxybenzoic, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, cinnamic acid and gliadin (from wheat) were purchased from Sigma-Aldrich, Steinheim, Germany. Caffeic acid was obtained from Fluka, St. Gallen, Switzerland. Sodium acetate, acetic acid, iron(III)-chloride ($FeCl_3 \cdot 6H_2O$) and all organic solvents used were obtained from Merck, Darmstadt, Germany. All reagents were of analytical grade or higher.

Unstabilized beer samples and PVPP washing solution samples were obtained from a German brewery.

The hop tannin extract used in the study was the (poly-)phenolic fraction of an ethanolic hops extract and was supplied by Simon H. Steiner Hopfen GmbH, Mainburg, Germany.

The hop tannin extract is a by-product of the extraction of hops with ethanol. In this process, cone hops are blended with ethanol (90 %, v/v) and the cones are crushed in a wet mill and pumped into an extractor. The solvent ethanol, which is finally enriched with hop resins, tannins and aroma substances, will be evaporated and the resulting extract passes a centrifuge to separate the resins and oils from the tannin fraction, which was used for the tests. This tannin extract contains 40 to 50 % (v/v) water, a large amount of high molecular polyphenols but also low molecular substances like catechin and multifidol glycosides, quercetin and kaempherol.

2.2 Extraction of 1-[(2-methylpropanoyl) phloroglucinyl]-β-D-glucopyranoside (co-multifidol glucoside) from PVPP washing solution, unstabilized beer and hop tannin extract

Extraction and isolation of the (poly-)phenolic fraction from the hop tannin extract was carried out according to the method described by *Vancraenenbroeck* et al. [45] and *Anger* [1] with modifications. The adsorption apparatus was prepared by pouring a polyamide slurry (20 g of polyamide in deionized water, SC6, 0.05 - 0.16 mm, Macherey-Nagel, Germany) into the apparatus and removing the water. The hop tannin extract was mixed with deionized water at 33 g/L. Prior to adsorption, a 300 mL aliquot of the aqueous solution was filtered through a membrane filter (0.45 μm, Sartorius, Germany) and poured in the adsorption apparatus. The mixture was stirred for 1 hour to allow the adsorption of the phenolic fraction to the polyamide. After removing the liquid phase, the supernatant was washed and water-soluble substances were removed by adding 300 mL deionized water and stirring for 15 min before pumping out the liquid phase. The (poly-)phenols were then desorbed from the polyamide by adding an aliquot of 300 mL of an acetone-water mixture (3:1, vol./vol.) to the slurry and stirring for another hour. To prevent oxidation of phenolic material, a constant stream of nitrogen gas was introduced into the adsorption apparatus. The mixture was then transferred to a 500 mL round-bottom flask

and the solvent was evaporated under vacuum at a temperature of 25 °C. The remainder was partitioned three times with 20 mL nhexane using a separating funnel to remove residues of hop bitter acids and other unwanted compounds. Prior to further steps, the n-hexane was removed under vacuum at a temperature of 40 °C.

Further purification of this aqueous solution was required, so it was separated using Strata C18-E cartridges (2 g/12 mL, Phenomenex, Germany). First, the cartridge was conditioned with 10 mL of methanol followed by 10 mL of deionized water. Next, the cartridge was loaded with 20-40 mL of the sample and washed with 50 mL of a methanol-water mixture (30:70, vol./vol.). The majority of the polyphenols remained on the column until eluted with 50 mL of 100 % methanol. The obtained methanol-water and methanol fractions were subsequently analyzed by HPLC.

The same procedure was used for two other samples: unstabilized beer and PVPP washing solution from the PVPP regeneration. Prior to fractionating, the PVPP washing solution (300 mL) was acidified to pH 6.5 with hydrochloric acid and the unstabilized beer sample (300 mL) was degassed in an ultrasonic bath for 20 min.

2.3 Extraction and identification of catechins and phenolic acids from the hop tannin extract

The hop tannin extract (1 g) was dissolved in 20 mL of acidified deionized water (adjusted to pH 2 with hydrochloric acid) and was partitioned with ethyl ether (3x5 mL). Ether layers were combined and dried using a rotary evaporator at 25 °C. The residue was then dissolved in 0.5 mL of methanol and subsequently analyzed by HPLC.

Chromatographic analyses of polyphenols were carried out using an Agilent 1100 series equipped with a UV-DAD detector. The samples were filtered through 0.45 μm syringe filters (Sartorius, Germany) before injection. A 10-μL sample was injected onto a 5 μm Zorbax Eclipse XDB-C18 column (Agilent Technologies, 4.6×150 mm) and eluted at a constant flow rate of 0.3 mL/min at a temperature of 35 °C using two mobile phases. Mobile phase A was 0.01 % (v/v) phosphoric acid in water and mobile phase B was 100 % acetonitrile. The following gradient conditions were used: 0-15 % B from over 15 min; 1045 % B from 15-65 min; return to 0 % B over 3 min and reequalibration for 10 min. The absorbance was measured at 260, 280 and 325 nm. The phenolic peaks were identified by comparing retention times with that of the standards and also from the individual UV-spectra associated with each peak. A mixed standard solution of the co-multifidol glucoside, phenolic acids and catechins was prepared by dissolving polyphenol standards in methanol to yield concentrations of 100 ppm each.

2.4 Isolation and identification of 1-[(2-methylpropanoyl)phloroglucinyl]-β-D-glucopyranoside (w-multifidol glucoside)

Isolation of the co-multifidol glucoside was performed on an Agilent preparative HPLC 1200 series (Agilent Technologies, Böblingen, Germany) equipped with a UV-DAD detector. Prior to separation, the methanol-water fractions were evaporated under reduced pressure at 25 °C to dryness and the residue was dissolved in 2 mL of methanolwater (50:50, vol./vol.). Samples were eluted at a flow rate 4 mL/min at a constant temperature of 40 °C on a Nucleosil 100-C18 column (Masherey Nagel, 250×21 mm, 5 μm). Two mobile phases were used. Mobile phase A was 100 % water, and mobile phase B was 100 % acetonitrile. The following gradient conditions were used: 10-17.5 % B over 22.5 min; 17.5-25 % B from 22.5-45 min; 25 % B from 45-68 min isocratically; return to 10 % B over 0.5 min and reequilibration for 14.5 min. Absorbance was measured at 210, 250, 290, 310 and 360 nm. Fractions containing the desired compound were collected and combined for further analysis.

Following identification was carried out by spectral methods using ^{1}H and ^{13}C NMR and 2D-correlations (COSY, TOCSY, HMQC, HMBC) on a Bruker AM400 (400 MHz). The sample was dissolved in CD_3OD. Mass spectra (ESI) were recorded by using a HCT Ultra ETD II instrument.

2.5 Testing for haze activity

The following model systems were prepared to test the samples' tendency of forming haze:

Test condition 1: 200 ppm of the test sample was dissolved in a mixture of 5 % (v/v) ethanol in acetic acid buffer (0.025 M; pH 4.0) and stirred. Then, 400 μL of iron chloride solution (0.01 M) were added to the obtained mixture to achieve a final Fe^{3+} concentration of 22 ppm.

Test condition 2: 200 ppm of the test sample was dissolved in a mixture of 5 % (v/v) ethanol in acetic acid buffer (0.025 M; pH 4) and stirred. Then, gliadin at a concentration of 1000 ppm was added.

Test condition 3: Same preparation as for sample 2 but with the ferric iron salt added.

All treatments were incubated for 24 h at 0 °C test their tendency to form chill-haze. The samples' cloudiness was assessed visually at 0 °C.

3 Results and discussion

Fractionation and HPLC analysis of the phenolic fraction of unstabilized beer samples by using a modified method previously described by Vancraenenbroeck et al. [45] and Anger [1] showed a dominant peak (P1) eluting at a retention time of 23.7 minutes (Fig. 1, A). In subsequent trials examining the PVPP washing solution from PVPP regeneration, a peak eluting at the same retention time was detected (Fig. 1, B). Based on this data and findings from Siebert and *Lynn* [40] who demonstrated that predominantly haze-active polyphenols bind to PVPP, it was assumed that the detected compound P1 may play a notable role in reactions causing beer haze.

As a complement to the previous analysis, a hop tannin extract was fractionated by the method described above and analyzed by using HPLC. The resulting chromatogram of methanol-water fraction (Fig. 1, C) showed a similar response as in the previous

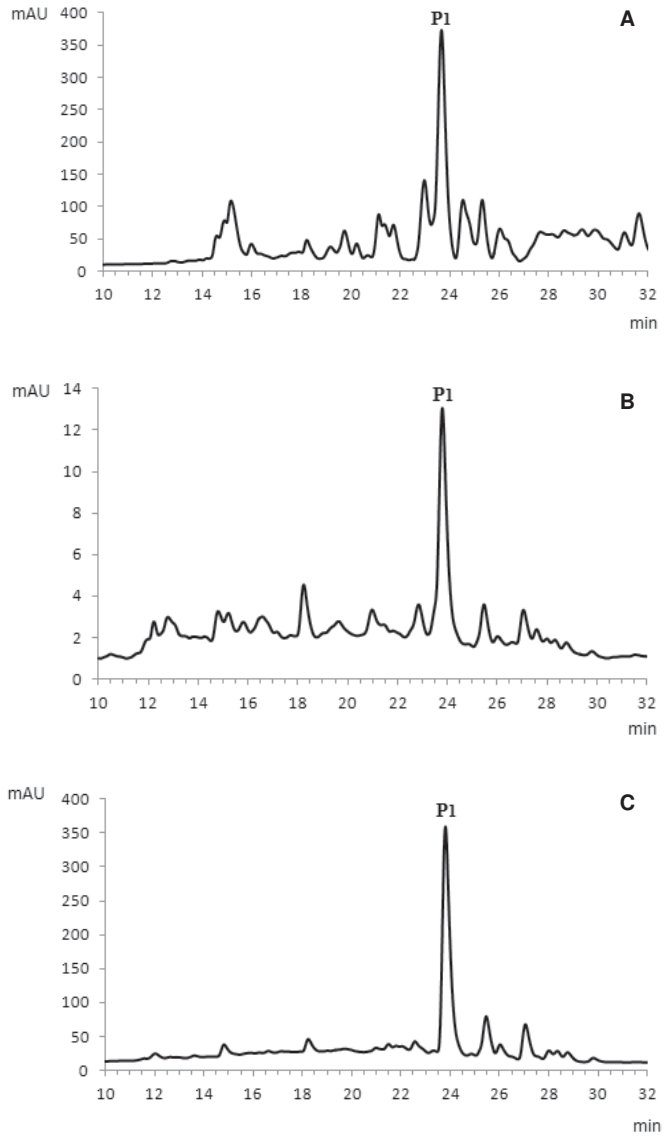

Fig. 1 HPLC-UV-DAD chromatograms of the methanol-water fraction of unstabilized beer (A), PVPP washing solution (B) and a hop tannin extract (C) after fractionation. The dominant peak is designated as P1

trials and the compound P1 with elution at a retention time of 23.7 minutes was reaffirmed.

Subsequently trials were carried out and the compound P1 was isolated by preparative HPLC and fractions containing the compound were collected. In a next step, the compound P1 was identified as 1-[(2-methylpropanoyl)phloroglucinyl]-β-D-glucopyranoside by using ^{1}H, ^{13}C NMR and 2D-correlations (COSY, TOCSY, HMQC, HMBC) and MS spectra. The compound's NMR data and structure is shown in table 1 and figure 4, respectively. The NMR data of the isolated compound was recently published by *Bohr* et al. [6] and belongs to the group of multifidol glucosides which were shown to have significant anti-inflammatory activity.

Cloudiness in beer is related to the formation of complexes between haze-active polyphenols and certain proteins and their concentrations thus directly influence the haze intensity [38, 41, 43]. Siebert and Lynn [42] reported that the haze intensity in a

Table 1 ^{1}H and ^{13}C NMR data of co-multifidol glucoside (in CD_3OD, 400 MHz)

Position	δ 1H, ppm (J, Hz)	δ 13C, ppm
1	–	161.6 (C)
2	–	106.2 (C)
3	–	167.5 (C)
4	5.95 d. (2.3) 1H	98.3 (CH)
5	–	165.6 (C)
6	6.17 d. (2.3) 1H	95.3 (CH)
1'	–	212.0 (C)
2'	3,98 sept. (6.3) 1H	40.5 (CH)
3'	1.14 d. (6.3) 3H	20.2 (CH3)
4'	1.13 d. (6.3) 3H	19.5 (CH3)
1"	5.04 d. (7.3)1H	101.5 (CH)
2"	3.50 dd. (9.0/7.3) 1H	74.8 (CH)
3"	3.44 m. 1H	78.7 (CH)
4"	3.38 t. 1H	71.2 (CH)
5"	3.44 m. 1H	78.4 (CH)
6" α	3.91 dd. (12.1/2.0) 1H	62.5 (CH2)
6" β	3.71 dd. (12.1/5.5) 1H	62.5 (CH2)

protein-polyphenol model system is also greatly influenced by its pH level and ethanol content. Trials where ethanol levels and pH were varied under model conditions demonstrated that the highest turbidity occurred at pH 3.8-4.3 and raised with increasing ethanol concentrations from 6 to 12% (v/v) ethanol. Further experiments showed that the haze intensity was noticeably greater with gliadintannic acid ratios of 2:1 and 5:1 (w/w) than with other mixtures [37]. At this juncture, iron in its ferric state also plays an important role as it is capable of forming complexes with oxidized and polymerized polyphenols thereby yielding particles with a higher molecular weight [18]. Moreover, the sample's temperature significantly impacts the cloudiness as low temperatures facilitate the formation of chill haze while permanent haze also occurs at room temperature [17]. Permanent haze is often age-related because it is related with oxidation reactions [35].

As compound P1 was suspicious of contributing to beer haze, its

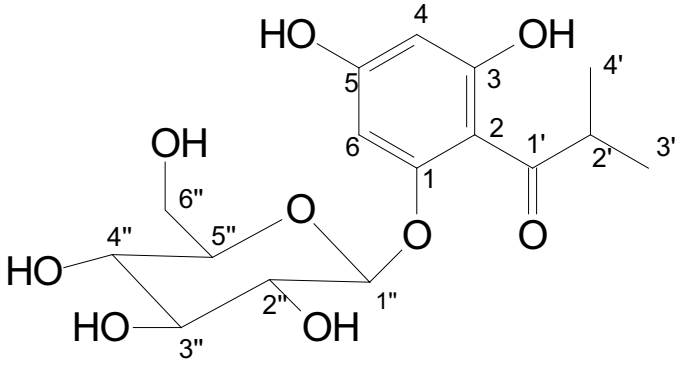

Fig. 2 The chemical structure of 1-[(2-methylpropanoyl) phloroglucinyl]-β-D-glucopyranoside (co-multifidol glucoside)

Table 2 Retention times and spectral characteristics of phenolic standards

Compound no.	Name	Retention time Rt, min	Spectral characteristics	
			λ_{max1}, nm	Λ_{max2}, nm
1	protocatechuic acid	15.20	260	294
2	4-hydroxybenzoic acid	20.18	256	–
3	(±)-catechin	22.98	280	–
4	vanillic acid	23.89	260	292
5	caffeic acid	24.68	324 (298 sh.[1])	236
6	syringic acid	25.82	274	–
7	(-)-epicatechin	27.33	278	–
8	p-coumaric acid	30.40	310	226 sh.[1]
9	ferulic acid	33.50	322 (294 sh.[1])	236
10	cinnamic acid	49.07	278	–
P1	co-multifidol glucoside	35.52	286	222 sh.[1]

[1] sh. = shoulder

tendency to form haze was tested in model solutions at pH 4.3 and at a gliadin-polyphenol ratio of 5 to 1 (w/w) with and without addition of ferric iron. These testing conditions were chosen because they were most suitable for simulating the beer matrix. The mixtures were incubated for 24 h at 0 °C to allow the formation of potential chill-haze.

Mixing compound P1 with ferric iron solely did not yield chill haze as assessed visually (Fig. 2, test condition 1). However, when gliadin was mixed with compound P1 under absence of ferric iron the appearance of chill haze could be detected (test condition 2). Using test condition 2 and adding Fe^{3+} also led to the formation of chill haze but with the appearance of a red-brownish colour. The change in the haze colour to the typical red-brownish colour appears through the enclosure of Fe^{3+} in the haze formation and indicates that ferric iron may play a role in the visible complexes being formed.

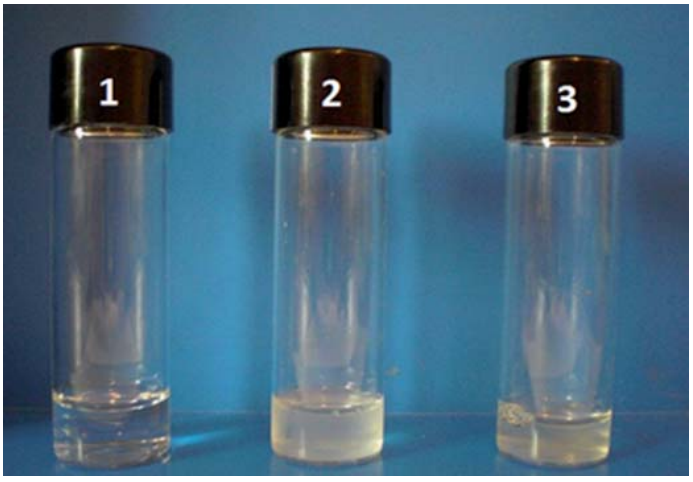

Fig. 3 Testing of co-multifidol glucoside (200 ppm) for chill-haze formation in acetic buffer (pH 4, 0.025 M). 1: with ferric iron (0.01 M) solely; 2: with gliadin (1000 ppm) and 3: with gliadin (1000 ppm) and ferric iron (0.01 M). All test samples were incubated at 0 °C for 24 h prior to assessing visually the formation of chill-haze

Taken together, these outcomes strongly suggest that the detected compound co-multifidol glucoside is a hop-derived polyphenol with the concomitant negative impact as being a promoter of beer-haze.

In a last experiment, the phenolic profile of the used hop tannin extract was examined by extraction with ethyl ether and measuring the phenolics and polyphenols by using HPLC-DAD after solvent evaporation and resolving in methanol. The resulting chromatogram was then compared to retention times from commercially available standards (Table 2) and the co-multifidol glucoside as obtained from the extraction and isolation procedures previously described. The resulting chromatogram is shown in figure 4. The majority of the polyphenols and phenolic acids from the hop tannin extract could be identified.

4 Conclusions

Outcomes of this study demonstrate that 1-[(2-methylpropanoyl)-phloroglucinyl]-β-D-glucopyranoside (co-multifidol glucoside) is a polyphenol deriving from the phenolic fraction of hops. The compound belongs to the group of "multifidol glucosides". Moreover, the co-multifidol glucoside was detected in unstabilized beer and washing solution from PVPP regeneration after beer filtration. Investigating its haze activity in model solutions confirmed the

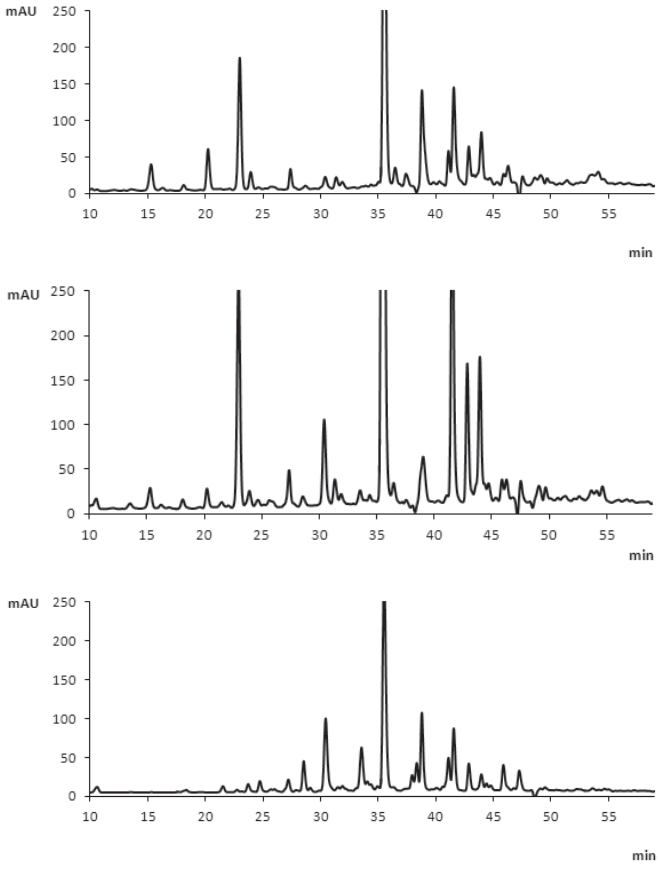

Fig. 4 HPLC-DAD chromatograms of a hop tannin extract (ethyl ether fraction) recorded at 260, 280 and 325 nm. 1: protocatechuic acid; 2: 4-hydroxybenzoic acid; 3: (±)-catechin; 4: vanillic acid; 5: caffeic acid; 6: syringic acid; 7: (-)-epicatechin; 8: p-coumaric acid; 9: t-ferulic acid; 10: cinnamic acid; P1: co-multifidol glucoside

compounds' tendency to form chill-haze with haze active proteins. Based on the results as received from this investigation, diminishing or removing multifidol glucoside from beer or raw materials, respectively, is suggested. This, in turn, would result in an increase of the beer's colloidal stability and to a reduction of costs for stabilizing agents, such as PVPP, which was shown to be markedly inactivated by binding to the identified compound. In additional trials, various phenolic compounds of the hop tannin extract were characterized by HPLC-DAD.

Abbrevations used

DPPH, 2,2-diphenyl-1-picryl-hydrazyl; 2-DR, 2-deoxyribose; EDTA, ethylenediaminetetraacetic acid; TBA, thiobarbituric acid.

Acknowledgement

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