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Activity-Guided Identification of *In Vitro*-Antioxidants in Beer

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ABSTRACT

In order to locate the key antioxidants contributing to oxidative stability of beer, activity-guided fractionation in combination with the oxygen radical absorbance capacity (ORAC) assay, hydrogen peroxide scavenging (HPS) assay and linoleic acid (LA) assay was applied to a pilsner-type beer. LC-MS and 1D/2D NMR experiments led to the identification of a total of 31 antioxidants, amongst which 3-methoxy-4-hydroxyphenyl-*β*-D-glucopyranoside (tachioside), 4-(2-formylpyrrol-1-yl)butyric acid, 4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid, *n*-multifidol-3-*O*-*β*-D-glucoside, quercetin-3-*O*-(6‘‘-malonyl)-glucoside, 4-feruloylquinic acid, syringaresinol, saponarin, and hordatines A-C have been isolated from beer for the first time. On a molar comparison, the hordatines A-C, saponarin, and quercetin-3-*O*-*β*-D-(6’’-malonyl)glucoside were evaluated with the highest antioxidant activities of all identified beer constituents, reaching values 10 – 17.5 (ORAC), 2.0 – 4.1 (HPS), and 1.1 – 6.1 *μ*mol TE/*μ*mol (LA) for hordatines A-C.

Keywords:

Antioxidants, beer, hordatines, tachioside

INTRODUCTION

Freshly brewed beer has been attracting consumers for thousands of years due to its refreshing character, desirable aroma, and its typical bitter taste profile. Although microbiological spoilage and haze formation have been limiting factors determining the shelf life of beer, technological improvements in beer manufacturing and a better understanding of the chemical transformations occurring during the brewing process helped to keep these problems largely under control. However, the instability of the attractive aroma as well as the typical bitter taste of beer became the main unresolved limiting factor of beer quality.[1,2]

Strecker aldehydes like phenylacetaldehyde and 2-methylpropanal were identified as important staling compounds formed upon beer ageing,[3-6] while undesirable changes in taste are particularly caused by the degradation of the iso-*α*-acids, the main contributors to beer's bitterness which derived from hops.[7,8] Two types of degradation mechanisms have been assigned for the storage-induced iso-*α*-acids depletion, a *trans*-specific, oxygen-independent and proton-catalyzed cyclization reaction converting *trans*-iso-α-acids into the less bitter, but harsh and lingering bitter tricyclohumols and tricyclohumenes[9,10] and, by an autoxidative mechanism, leading to *cis*- and *trans*-configured hydroperoxy- and hydroxyl-allo-iso-α-acids.[11] While the proton-catalyzed cyclization of iso-α-acids can be controlled by keeping the beer's pH value high and storage temperatures low,[10] antioxidants are believed to counteract the autoxidative mechanisms and to increase the shelf life of beer.[12,13] Therefore, a series of studies has been performed to evaluate the antioxidant activity of beer components.[1] Most previous studies focused on the role of polyphenols as beer antioxidants by acting as metal ion chelators,[14] inhibiting fatty acid oxidation[15] and avoiding formation of unwanted staling aldehyde

precursors.[16,17] Among the polyphenols, hydroxybenzoic acids (**1−3**, Figure 1) and hydroxycinnamic acid derivatives (**4−7**) were considered as antioxidants.[18-24] Originating from both hops and malt,[25] they occur in both free and bound form.[21] Ferulic acid (**6**), the most predominant hydroxycinnamic acid in beer,[18,19,21] has been shown to slow down the degradation of iso-*α*-acids.[26] In addition, Maillard reaction products, primarily formed during the kilning process,[27] were proposed as antioxidants in beer.[21,28-31] Moreover, sulphur dioxide was found to act as an antioxidant[15] and scavenger for staling aldehydes in beer by forming monothioacetals.[32,33] Whereas all the previous studies focused on the antioxidant activity of literature-known compound classes in beer, any activity-guided strategy to locate and identify the key antioxidants in beer is still lacking.

It was therefore the objective of the present study to locate the key antioxidants in pilsner-type beer by means of an activity-guided fractionation approach using three *in vitro* antioxidant assays, namely the oxygen radical absorbance capacity (ORAC) assay, the hydrogen peroxide scavenging (HPS) assay, and the linoleic acid (LA) assay. The target antioxidants should then be isolated, purified and determined in their chemical structure as well as their antioxidative activity.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: 2,2′-azo-bis(2-methylpropinamidine) (AAPH), fluorescein sodium salt, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), acetic acid,

disodium hydrogen phosphate, ethylenediaminetetraacetic acid hemoglobine, hydrogen peroxide, iron(II)sulfate heptahydrate, peroxidase from horseradish, linoleic acid, 2-methoxyhydroquinone, phenylalanine, *p*-hydroxyphenyllactic acid, sodium tetraborate, syringic acid, trifluoroacetic acid (99 %), triton X-100, tryptophan, tween 20, tyrosol (Sigma-Aldrich, Steinheim, Germany); caffeic acid, formic acid (98−100 %), hydrochloric acid (32 %), *p*-hydroxybenzoic acid, potassium dihydrogen phosphate, potassium hydroxide, sodium hydroxide (Merck, Darmstadt, Germany); ferulic acid, fluorescein, 2-isopropylmalic acid, *p*-coumaric acid, sinapic acid, vanillic acid (Fluka, Neu-Ulm, Germany); D_2O, methanole-d_4 (Euriso-Top, Saarbrücken, Germany); sodium hydroxide (Riedel-de-Haen, Seelze, Germany); *cyclo* (Pro-Tyr) (Bachem, Weil am Rhein, Germany); benzoylleucomethylene blue (TCI Europe, Zwijndrecht, Belgium). Water for high-performance liquid chromatography (HPLC) separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). Solvents were of HPLC grade (J.T. Baker, Deventer, Netherlands) and ethyl acetate was purified by distillation in vacuum at 40 °C. Pilsner-type beer was purchased from a German brewery. A flavor hop polyphenol extract was obtained from Simon H. Steiner GmbH (Mainburg, Germany).

Solvent Extraction of Beer. Beer (500 mL) was adjusted to pH 2.5 with traces of formic acid and then extracted with ethyl acetate (3 × 500 mL). The combined organic extracts (I) and the aqueous layer (II) were separated from organic solvents in vacuum at 40 °C and freeze-dried for 48 h. The dried aqueous phase (38 g) was extracted with acetonitrile/water (70/30, v/v; 3 × 100 mL) for 10 min during ultrasonification. After centrifugation (4000 rpm, 5 min), the combined supernatants were separated from organic solvents in vacuum at 40 °C and

lyophilised for 48 h to obtain the acetonitrile/water soluble fraction IIA (yield: 40 %), which was kept at −20 °C until further used.

Separation of Fractions I and IIA by Medium Pressure Liquid Chromatography (MPLC). Fraction I (3.9 g) and fraction IIA (16 g), respectively, was dissolved in 20 mL or 80 mL methanol/water (30/70, v/v), and 20 mL were injected through a 6-way-injection valve on a Sepacore system (Büchi, Flawil, Switzerland) consisting of two C-605 pumps, a C-620 control unit, a C-660 fraction collector, and a C-635 UV detector. The separation was performed on a 460 × 16 mm glass column (Büchi, Flawil, Switzerland) filled with 25−40 *μ*m LiChroprep RP18 material (Merck KGaA, Darmstadt, Germany). Operating with a flow rate of 30 mL/min, the solvent system consisted of aqueous formic acid (0.1 %, A) and methanolic formic acid (0.1 %, B) and a gradient was used as follows: 0 min / 5 % B, 20 min / 25 % B, 23 min / 60 % B, 33 min / 68 % B, 40 min / 100 % B, and 55 min / 100 % B. Prior to the next injection, the column was flushed to 5 % B for 3 min, and kept for 10 min. The effluent was monitored at λ=254 nm, data recorded by using Sepacore Control Chromatography Software, version 1.0 (Büchi, Flawil, Switzerland), and a total of 9 MPLC fractions collected, namely I-1 to I-9 (**Figure 2**). Separation of fraction IIA was performed using the same conditions, but using another solvent gradient: 0 min / 5 % B, 20 min / 25 % B, 23 min / 60 % B, 33 min / 68 % B, 40 min / 100 % B, and 55 min / 100 % B. The column was flushed to 5 % B for 3 min, and kept for 10 min prior to next injection. Monitoring the effluent at 254 nm, another 9 MPLC fractions, namely IIA-1 up to IIA-9, were collected. The subfractions obtained were separated from solvent in vacuum at 40 °C and freeze-dried for 48 h, before storing at −20 °C until further used.

Identification of Key Antioxidants in MPLC Fraction I-7. Fraction I-7 (84.5 mg) was dissolved in acetonitrile/water (20/80, v/v; 5 mL) and, after membrane filtration, 1 mL was injected onto a 250 × 21.2 mm i.d., 5 *µ*m, HyperClone C18 column (Phenomenex, Aschaffenburg, Germany) using a 2 mL sample loop. Using a binary gradient of 0.1 % aqueous formic acid as solvent A and acetonitrile as solvent B, chromatography (flow rate: 21 mL/min) was performed with the following gradient: 0 min / 10 % B, 5 min / 15 % B, 20 min / 22 % B, 23 min / 100 % B, 27 min / 100 % B, 30 min / 10 % B, and 35 min / 10 % B. The effluent, monitored λ=228 and 288 nm, was separated into 10 subfractions, namely I-7-1 up to I-7-10, which were collected individually in several runs. The corresponding fractions were combined and separated from solvent in vacuum at 40 °C, followed by lyophilization. The subfractions were further purified by re-chromatography by means of semipreparative RP-HPLC using a 250 × 10.0 mm i.d., 5 *µ*m, Luna PFP column (Phenomenex, Aschaffenburg, Germany), operated with a flow rate of 5.5 mL/min and using 0.1 % aqueous formic acid in water as solvent A and methanol as solvent B. For separation of fraction I-7-9, the following solvent gradient was used: 0 min / 35 % B, 10 min / 48 % B, 20 min / 48 % B, 28 min / 55 % B, 33 min / 55 % B, 43 min / 80 % B, 44 min / 100 % B, 47 min / 100 % B, 48,5 min / 35 % B, and 55 min / 35 % B. The effluent, monitored at λ=280 nm, was collected into 27 subfractions, namely I-7-9-1 up to I-7-9-27. After re-chromatography and verifying the purity of each fraction by means of analytical HPLC, the target compounds were separated from solvent in vacuum, freeze-dried for 48 h and, then, analyzed by LC-TOF-MS and 1D/2D-NMR spectroscopy for structure determination. Spectroscopic data (UV/Vis, LC-TOF-MS, ^{1}H-NMR) and chromatographic data (retention time), followed by co-chromatography with commercially available reference compounds led to the identification of *p*-

hydroxybenzoic acid (**1**), vanillic acid (**2**), syringic acid (**3**), *p*-coumaric acid (**4**), caffeic acid (**5**), ferulic acid (**6**), sinapic acid (**7**), *p*-hydroxyphenyllactic acid (**8**), 2-isopropylmalic acid (**9**), *cyclo*(Pro-Tyr) (**10**), tyrosol (**11**), and cinnamic acid (**15**, **Table 1**). *Co*-, *n*- and *ad*-multifidolglucoside (**18a−c**),[34] quercetin-3-*O*-*β*-D-glucoside (**19**),[35] kaempferol-3-*O*-*β*-D-glucoside (**20**),[35] quercetin-3-*O*-*β*-D-(6-*O*-malonyl)glucoside (**21**),[36] and kaempferol-3-*O*-*β*-D-(6-*O*-malonyl)glucoside (**22**),[35] were assigned by comparison of spectroscopic data with literature and by co-chromatography with the corresponding reference material isolated from a hop polyphenol extract. In addition, comparison of spectroscopic data with literature led to the identification of 3-, 4- and 5-feruloylquinic acid (**12−14**),[37] *N*-feruloyltyramine (**16**),[38] and syringaresinol (**17**).[39]

Identification of Key Antioxidants in MPLC Fractions IIA-5, IIA-7, IIA-8 and IIA-9. *Identification of Antioxidants in Fraction IIA-5.* Fraction IIA-5 was separated on a 250 × 21.2 mm i.d., 5 *μ*m, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) with a flow rate of 21 mL/min. Using a binary gradient of 1 % formic acid in water (solvent A) and 1 % formic acid in acetonitrile (solvent B), chromatography was performed with the following gradient after dissolving fraction IIA-5 (9.4 mg) in 800 *μ*L starting condition solvents: 0 min / 10 % B, 5 min / 15 % B, 20 min / 22 % B, 23 min / 100 % B, 27 min / 100 % B, 30 min / 10 % B, 35 min / 10 % B. Monitoring the effluent at 280 nm, 10 subfractions (IIA-5-1 to IIA-5-10) were collected and their antioxidant activity evaluated by means of the ORAC-, HPS- and LA-assay, respectively. The highly active subfraction IIA-5-9 was purified by re-chromatography, separated from solvent in vacuum, lyophilised for 48 h and, then, used for LC-TOF-MS, LC-MS/MS as well as 1D/2D-NMR spectroscopy to identify phenylalanine (**23**) and tachioside (**24**) by comparison with the corresponding reference compound.

Identification of Antioxidants in Fraction IIA-7. Fraction IIA-7 (4.2 mg) was dissolved in acetonitrile/1 % formic acid (20/80, v/v; 500 µL) and, after membrane filtration, was injected onto a 250 × 21.2 mm i.d., 5 µm, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) with a flow rate of 21 mL/min. Using a binary gradient of 1 % formic acid in water as solvent A and 1 % formic acid in acetonitrile as solvent B, the following method was used for separation: 0 min / 0 % B, 5 min / 0 % B, 10 min / 5 % B, 25 min / 5 % B, 27 min / 100 % B, 29 min / 100 % B, 32 min / 0 % B, and 35 min / 0 % B. The effluent, monitored at λ=280 nm and by means of an ELSD, was separated into 11 subfractions, namely IIA-7-1 to IIA-7-11, and the solvent was removed in vacuum at 40 °C, followed by lyophilisation and analysis by means of the ORAC-, HPS- and LA-assay, respectively. Analysis of the most active subfraction IIA-7-9 by by LC-TOF-MS, LC-MS/MS and 1D/2D-NMR led to the identification of tryptophan (**25**).

Identification of Antioxidants in Fraction IIA-8. For further subfractionation, fraction IIA-8 (6.3 mg) was separated on a 250 × 21.2 mm i.d., 5 µm, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany). Operating with a flow rate of 21 mL/min, the solvent system consisted of aqueous formic acid (0.1 %, A) and acetonitrile (B) and a gradient was used as follows: 0 min / 5 % B, 25 min / 25 % B, 32 min / 30 % B, 34 min / 100 % B, 37 min / 100 % B, 40 min / 5 % B, 45 min / 5 % B. Monitoring the effluent at 280 nm, 15 subfractions were collected, namely IIA-8-1 to IIA-8-15. Hordatines (**29−31**) were purified by an alternative method using a 250 × 10.0 mm i.d., 5 µm, Luna PFP(2) column (Phenomenex, Aschaffenburg, Germany) with 0.1 % trifluoroacetic acid in water as solvent A and 0.1 % trifluoroacetic acid in methanol as solvent B. The following gradient was used for separation at a flow rate of 5.5 mL/min: 0 min / 15 % B, 10 min / 30 % B, 30 min / 48 % B, 32 min / 100 % B, 34 min / 100 % B, 36 min / 15 % B,

40 min / 15 % B. After separation from solvent in vacuum, the subfractions and purified compounds were freeze-dried and then analyzed by LC-TOF-MS, LC-MS/MS, 1D/2D-NMR spectroscopy as well as ORAC-, HPS- and LA-assay, respectively. In accordance with the spectroscopic data (UV/Vis, LC-TOF-MS, $^{1}H/^{13}C$-NMR), the structures of the previously reported 4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid (**28**)[40] and hordatine A (**29**)[41] was confirmed. The chemical structures of hordatine B (**30**) and hordatine C (**31**) have been determined by means of UV/vis, LC-TOF-MS, and 1D/2D-NMR experiments and, to the best of our knowledge, no detailed NMR-data have been earlier reported.

*Hordatine B, **30**, **Figure 1***. UV/vis (0.1 % aqueous formic acid/acetonitrile): λ_{max} = 228 / 300 / 320 nm; LC-TOF-MS $(ESI)^-$: *m/z* (%) 625.3089 (20; measured), 625.3098 (calculated for $[C_{29}H_{40}N_8O_5+HCOOH-H]^-$) 579.3038 (100; measured), 579.3043; LC-TOF-MS (calculated for $[C_{29}H_{40}N_8O_5-H]^-$); $(ESI)^+$: *m/z* (%) 581.3198 (5; measured), 581.3200 (calculated for $[C_{29}H_{40}N_8O_5+H]^+$), 291.1641 (100; measured), 291.1640 (calculated for $[C_{29}H_{40}N_8O_5+2H]^{2+}$); ^1H NMR (500 MHz, D_2O, COSY): δ (ppm) 1.29–1.60 [m, 8H, H_2-C(2‘‘), H_2-C(2‘‘‘), H_2-C(3‘‘), H_2-C(3‘‘‘)], 2.97–3.32 [m, 8H, H_2-C(1‘‘), H_2-C(1‘‘‘), H_2-C(4‘‘), H_2-C(4‘‘‘)], 3.89 [s, 3H, H_3-C(10)], 4.32 [d, 1H, *J* = 7.4 Hz, H-C(8‘)], 5.91 [d, 1H, *J* = 7.4 Hz, H-C(7‘)], 6.06 [d, 1H, *J* = 12.2 Hz, H-C(8)], 6.86 [d, 1H, *J* = 12.2 Hz, H-C(7)], 6.88 [s, 1H, H-C(2)], 6.94 [d, 2H, *J* = 8.4 Hz, H-C(3‘/5‘)], 7.07 [s, 1H, H-C(6)], 7.32 [d, 2H, *J* = 8.4 Hz, H-C(2‘/6‘)]; ^{13}C NMR (125 MHz, D_2O, HSQC, HMBC): δ (ppm) 26.1, 26.2, 26.3, 26.6 [C(2‘‘), C(2‘‘‘), C(3‘‘), C(3‘‘‘)], 39.7 [C(1‘‘‘)], 40.0 [C(1‘‘)], 41.7 [C(4‘‘), C(4‘‘‘)], 57.1 [C(10)], 58.1 [C(8‘)], 89.5 [C(7‘)], 114.5 [C(6)], 116.8 [C(3‘/5‘)], 118.1 [C(2)], 124.1 [C(8)], 127.7 [C(3)], 128.9 [C(2‘/6‘)], 131.1 [C(1)], 131.6 [C(1‘)], 137.2 [C(7)], 144.7 [C(5)], 148.4 [C(4)], 157.3 [C(4‘)], 157.7 [C(5‘‘), C(5‘‘‘)], 171.6 [C(9)], 173.9 [C(9‘)].

Hordatine C, ***31****,* ***Figure 1****.* UV/vis (0.1 % aqueous formic acid/acetonitrile): λ_{max} = 228 / 300 / 320 nm; LC-TOF-MS $(ESI)^-$: *m/z* (%) 655.3221 (20; measured), 655.3204 (calculated for $[C_{30}H_{42}N_8O_6+HCOOH-H]^-$), 609.3147 (100; measured), 609.3149 (calculated for $[C_{30}H_{42}N_8O_6-H]^-$); LC-TOF-MS $(ESI)^+$: *m/z* (%) 611.3289 (5; measured), 611.3306 (calculated for $[C_{30}H_{42}N_8O_6+H]^+$), 306.1691 (100; measured), 306.1691 (calculated for $[C_{30}H_{42}N_8O_6+2H]^{2+}$); ^{1}H NMR (500 MHz, D_2O, COSY): *δ* (ppm) 1.31–1.62 [m, 8H, H_2-C(2‘‘), H_2-C(2‘‘‘), H_2-C(3‘‘), H_2-C(3‘‘‘)], 2.98–3.32 [m, 8H, H_2-C(1‘‘), H_2-C(1‘‘‘), H_2-C(4‘‘), H_2-C(4‘‘‘)], 3.87 [s, 3H, H_3-C(10‘)], 3.92 [s, 3H, H_3-C(10)], 4.35 [d, 1H, *J* = 7.4 Hz, H-C(8‘)], 5.93 [d, 1H, *J* = 7.4 Hz, H-C(7‘)], 6.07 [d, 1H, *J* = 12.2 Hz, H-C(8)], 6.88 [d, 1H, *J* = 12.2 Hz, H-C(7)], 6.90 [s, 1H, H-C(2)], 6.96 [s, 1H, H-C(2‘)], 6.97 [d, 1H, *J* = 7.4 Hz, H-C(5‘)], 7.08 [d, 1H, *J* = 7.4 Hz, H-C(6‘)], 7.10 [s, 1H, H-C(6)]; ^{13}C NMR (125 MHz, D_2O, HSQC, HMBC): *δ* (ppm) 26.2, 26.3, 26.6 [C(2‘‘), C(2‘‘‘), C(3‘‘), C(3‘‘‘)], 39.8 [C(1‘‘‘)], 40.0 [C(1‘‘)], 41.7 [C(4‘‘), C(4‘‘‘)], 57.1 [C(10‘)], 57.1 [C(10)], 58.2 [C(8‘)], 89.4 [C(7‘)], 111.2 [C(6‘)], 114.6 [C(6)], 116.7 [C(5‘)], 118.0 [C(2)], 120.2 [C(2‘)], 124.0 [C(8)], 127.8 [C(3)], 131.3 [C(1)], 132.3 [C(1‘)], 137.1 [C(7)], 144.6 [C(5)], 146.7 [C(4‘)], 148.5 [C(4)], 148.7 [C(3‘)], 157.6 [C(5‘‘), C(5‘‘‘)], 171.6 [C(9)], 173.9 [C(9‘)].

Identification of Antioxidants in Fraction IIA-9. Fraction IIA-9 (11.3 mg) was dissolved in acetonitrile/1 % formic acid (40/60, v/v; 1 mL) and, after membrane filtration, injected onto a 250 × 21.2 mm i.d., 4 *μ*m, Synergi Hydro-RP column (Phenomenex, Aschaffenburg, Germany). Chromatography (flow rate: 21 mL/min) was performed using a binary gradient of 1 % formic acid in water (A) and 1 % formic acid in acetonitrile (B), the following chromatographic method was used: 0 min / 5 % B, 12 min / 12 % B, 32 min / 32 % B, 40 min / 50 % B, 42 min / 100 % B, 44.5 min / 100 % B, 47 min / 5 % B, and 50 min / 5 % B. Monitoring the effluent at λ=280 nm, 20 subfractions were collected, namely IIA-9-1

to IIA-9-20, solvent separated in vacuum, lyophilised for 48 h, and their antioxidant activity analysed by means of the ORAC-, HPS- and LA-assay, respectively. After re-chromatography, comparison of spectroscopic data (UV/Vis, LC-TOF-MS, $^{1}H/^{13}C$-NMR) with literature data revealed saponarin (**26**)[42] and 4-(2-formylpyrrol-1-yl)butyric acid (**27**)[40] as the key antioxidants.

Isolation of Antioxidants from Hop Polyphenol Extract. A commercial flavor hop polyphenol extract (20 g) was suspended with water (200 mL), the pH adjusted to 2.5 by adding traces of formic acid and, then, extracted with ethyl acetate (3 × 200 mL). The combined organic extracts were separated from organic solvents in vacuum at 40 °C, dissolved in methanol/water (70/30, v/v) and, after membrane filtration, injected onto a 250 × 21.2 mm i.d., 5 µm, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) using a 2 mL sample loop. Chromatography (flow rate: 21 mL/min) was performed using 0.1 % aqueous formic acid as solvent A and acetonitrile as solvent B, and the following gradient: 0 min / 20 % B, 20 min / 20 % B, 24 min / 25 % B, 26 min / 25 % B, 29 min / 100 % B, 32 min / 100 % B, 35 min / 20 % B and 40 min / 20 % B. Monitoring the effluent at λ=264 nm, a total of 7 subfractions was collected, namely III-1 to III-7, separated from solvent in vacuum at 40 °C, followed by lyophilization. Comparison of spectroscopic data (UV/vis, LC-TOF-MS, $^{1}H/^{13}C$-NMR) with literature confirmed the structure of *co*-, *n*- and *ad*-multifidolglucoside (**18a−c**),[34] quercetin-3-*O*-*β*-D-glucoside (**19**),[35] kaempferol-3-*O*-*β*-D-glucoside (**20**),[35] quercetin-3-*O*-*β*-D-(6''-*O*-malonylglucoside (**21**),[36] and kaempferol-3-*O*-*β*-D-(6''-*O*-malonylglucoside (**22**)[35] as key antioxidants.

Chemical Synthesis of Tachioside (24) and Isotachioside (24a). Following a literature protocol with some modifications,[43] 2-methoxyhydroquinone (4 mmol) was dissolved in acetone (10 mL), acetobromo-*α*-D-glucose (3 mmol) and

298 sodium hydroxide (2 mmol/L in water; 1.5 mL) were added, followed by stirring for
299 24 h at room temperature in the dark. After filtration, the solvent was removed in
300 vacuum, sodium hydroxide (1 mmol/L in methanol/water, 50/50, v/v; 10 mL) was
301 added and, after incubation for 5 min to achieve de-acetylation, the pH value was
302 adjusted to 5.5 with hydrochloric acid, followed by purification by means of MPLC
303 using a 150 × 40 mm cartridge (Büchi, Flawil, Switzerland) filled with 25−40 *µ*m
304 LiChroprep RP18 material (Merck KGaA, Darmstadt, Germany). Chromatography
305 (flow rate: 40 mL/min) was performed using a binary gradient of 0.1 % aqueous
306 formic acid (solvent A) and methanol (solvent B): 0 min / 0 % B, 5 min / 0 % B,
307 30 min / 30 % B, 37 min / 100 % B, 45 min / 100 % B, 50 min / 0 % B, and
308 60 min / 0 % B. After solvent separation, the target compounds were re-
309 chromatographed by HPLC on a 250 × 21.2 mm i.d., 5 *µ*m, Luna Phenyl-Hexyl
310 column (Phenomenex, Aschaffenburg, Germany) operated at a flow rate of
311 21 mL/min. Monitoring the effluent at 280 nm, the target molecules were eluted with
312 3 % acetonitrile in 0.1 % aqueous formic acid, separated from solvent in vacuum
313 and freeze-dried for 48 h to afford tachioside (**24**, 12 mg) und isotachioside (**24a**, 20
314 mg).

315 *3-Methoxy-4-hydroxyphenyl-β-D-glucopyranoside (tachioside),* ***24****,* ***Figure 1****.*
316 UV/vis (0.1 % aqueous formic acid/acetonitrile): λ_{max} = 224 / 284 nm; LC-TOF-MS
317 $(ESI)^-$: *m/z* (%) 301.0919 (100; measured), 301.0923 (calculated for $[C_{13}H_{18}O_8–H]^-$),
318 139.0399 (40; measured), 139.0401 (calculated for aglycone $[C_7H_8O_3–H]^-$); LC-
319 TOF-MS $(ESI)^+$: *m/z* (%) 325.0894 (100; measured), 325.0894 (calculated for
320 $[C_{13}H_{18}O_8+Na]^+$); ^1H NMR (500 MHz, D_2O, COSY): *δ* (ppm) 3.42–3.63 [m, 4H, H-
321 C(4‘), H-C(3‘), H-C(2‘), H-C(5‘)], 3.73 [dd, 1H, *J* = 5.9, 12.5 Hz, H_a-C(6‘)], 3.85 [s,
322 3H, H_3-C(1‘‘)], 3.92 [dd, H, *J* = 2.0, 12.5 Hz, H_b-C(6‘)], 4.99 [d, H, *J* = 7.6 Hz, H-
323 C(1‘)], 6.66 [dd, 1H, *J* = 2.7, 8.7 Hz, H-C(6)], 6.84 [d, 1H, *J* = 2.7 Hz, H-C(2)], 6.87

[d, 1H, *J* = 8.7 Hz, H-C(5)]; ^{13}C NMR (125 MHz, D_2O, HSQC, HMBC): *δ* (ppm) 56.9 [C(1‘‘)], 61.6 [C(6‘)], 70.1 [C(4‘)], 74.0 [C(2‘)], 76.6 [C(3‘)], 77.1 [C(5‘)], 102.3 [C(1‘)], 103.9 [C(2)], 109.8 [C(6)], 116.6 [C(5)], 141.6 [C(4)], 149.0 [C(3)], 151.8 [C(1)].

2-Methoxy-4-hydroxyphenyl-β-D-glucopyranoside (Isotachioside), ***24a****,* ***Figure 1****.* UV/vis (0.1 % aqueous formic acid/acetonitrile): λ_{max} = 224 / 284 nm; LC-TOF-MS $(ESI)^-$: *m/z* (%) 301.0922 (100; measured), 301.0923 (calculated for $[C_{13}H_{18}O_8–H]^-$), 139.0406 (45; measured), 139.0401 (calculated for aglycone $[C_7H_8O_3–H]^-$); LC-TOF-MS $(ESI)^+$: *m/z* (%) 325.0891 (100; measured), 325.0894 (calculated for $[C_{13}H_{18}O_8+Na]^+$); ^{1}H NMR (500 MHz, D_2O, COSY): *δ* (ppm) 3.40–3.57 [m, 4H, H-C(4‘), H-C(3‘), H-C(2‘), H-C(5‘)], 3.74 [dd, 1H, *J* = 4.6, 12.3 Hz, H_a-C(6‘)], 3.84 [s, 3H, H_3-C(1‘‘)], 3.88 [dd, H, *J* = 1.6, 12.3 Hz, H_b-C(6‘)], 4.85 [d, 1H, *J* = 7.5 Hz, H-C(1‘)], 6.41 [dd, 1H, *J* = 2.6, 8.7 Hz, H-C(5)], 6.57 [d, 1H, *J* = 2.6 Hz, H-C(3)], 7.05 [d, 1H, *J* = 8.7 Hz, H-C(6)]; ^{13}C NMR (125 MHz, D_2O, HSQC, HMBC): *δ* (ppm) 58.6 [C(1‘‘)], 63.7 [C(6‘)], 72.5 [C(4‘)], 76.2 [C(2‘)], 78.8 [C(3‘)], 79.3 [C(5‘)], 103.7 [C(6)], 105.1 [C(1‘)], 109.6 [C(5)], 121.3 [C(3)], 142.5 [C(1)], 153.1 [C(2)], 155.5 [C(4)].

Oxygen Radical Absorbance Capacity (ORAC) Assay. The antioxidant capability of extracts, fractions and purified compounds was measured by using the ORAC-Assay following a literature protocol.[44] Sample solutions were prepared using phosphate buffer (10 mmol/L, pH 7.4) and aliquots in four replicates (each 25 *µ*L) were pipetted into the wells of a black 96-well-plate (Greiner Bio-One, Frickenhausen, Germany). After addition of fluorescein (10 nmol/L in phosphate buffer, 150 *µ*L) to each well and incubation of the microplate (30 min, 37 °C) the decline of fluorescence was recorded every 90 s at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). After three cycles, AAPH

(240 mmol/L in phosphate buffer, 25 μL) was added to each well before performing another 57 cycles (90 min in total). For calibration, a serial dilution of trolox (200, 100, 50, 25, 12.5 μmol/L) from a stock solution (4 mmol/L in ethanol) was treated in the same way as well as a blank of phosphate buffer (25 μL) and a C1-well (50 μL of phosphate buffer instead of sample and AAPH). Calculation was carried out as described in literature[45] with slight modifications. After normalising to C1-well (C1-value = 1) and determination of the area under the fluorescence versus time curve (AUC) the AUC of the blank was subtracted. With the obtained netAUC the antioxidant capacity was expressed as trolox equivalents (TE) using the calibration curve of trolox standards.

Hydrogen Peroxide Scavenging (HPS) Assay. Following a literature protocol with slight modifications,[46] stock solutions of the samples in water were diluted stepwise (1+1, v+v; 8 dilution steps) using a phosphate buffer (100 mmol/L, pH 6.0) and afterwards, three replicates (each 20 μL) of each dilution step were pipetted into the wells of a clear 96-well-plate (Greiner Bio-One, Frickenhausen, Germany). Phosphate buffer (100 mmol/L, pH 6.0, 100 μL) was added to each well as well as aqueous solutions of hydrogen peroxide (250 μmol/L, 20 μL), horseradish peroxidase (150 U/ml, 40 μL) and ABTS (0.1 %, 40 μL). After incubation at 37 °C, the absorption at λ=414 nm was recorded four times within 15 min using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Considering the different dilutions of a sample, the EC_{50}-value of the gained absorption versus concentration curve was calculated on the base of a logistic dose-response-model with Origin 8G as software. The antioxidant activity was expressed as trolox equivalents (TE) dividing the EC_{50}-value of trolox by the EC_{50}-value of the sample.

Linoleic Acid (LA) Assay. Following a literature protocol,[47,48] the samples were dissolved and diluted stepwise (1+1, v+v, 8 dilution steps) using an

ethanol/water mixture (1+1, v+v). Aliquots of three replicates (each 25 µL) were pipetted into the wells of a clear 96-well-plate (Greiner Bio-One, Frickenhausen, Germany) before addition of oxygen-saturated phosphate buffer (200 mmol/L, pH 6.75, 60 µL), an aqueous hydrogen peroxide solution (16 mmol/L, 20 µL) and an aqueous iron(II)sulfate/EDTA solution (16 mmol/L/ 15 mmol/L, 20 µL) to each well. After mixing linoleic acid (25 µL), tween 20 (25 µL), sodium hydroxide (1 mol/L, 300 µL) and water (5 mL) and filling up to 10 mL with borate buffer (50 mmol/L, pH 9.0), this linoleic acid substrate (20 µL) was added to each well and the degradation of linoleic acid was performed for exactly 10 min at room temperature. To stop this process, 80 µL of a colorant solution, consisting of haemoglobin (28 mg), benzoylleucomethylene blue (25.5 mg) and triton X-100 (7 mL), which were dissolved in 250 mL phosphate buffer (400 mmol/L, pH 5.0), were added to each well. After a further incubation for 30 min at room temperature, the absorption at λ=666 nm was recorded using an infinite M200 spectrometer (Tecan, Männedorf, Switzerland) with i-control 1.10 as software. Out of the sigmoidal absorption versus concentration curve of the serial dilution, the EC_{50}-value of each sample was calculated on the base of a logistic dose-response-model with Origin 8G as software. The antioxidant activity was expressed as trolox equivalents (TE) dividing the EC_{50}-value of trolox by the EC_{50}-value of the sample.

High Performance Liquid Chromatography (HPLC). The HPLC system (Jasco, Groß-Umstadt, Germany) consisted of two PU-2087 Plus pumps, a DG-2080-53 degaser, respectively, and a MD-2010 Plus diode array detector monitoring the effluent in a range between 220 and 500 nm using Chrompass 1.8.6.1 (Jasco, Groß-Umstadt, Germany) as software. For sample injection, an AS-2055 Plus autosampler was used in analytical mode and a 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany) in preparative and semipreparative mode.

For detection with ELSD a Sedex LT-ELSD Model 80 (Sedere, Alfortville, Frankreich) was used and the split ratio was set to 1.0 mL/min for the ELSD.

UPLC/Time-of-Flight Mass Spectrometry (UPLC-TOF-MS). Aliquots (2 μL) of all antioxidants were injected into an Acquity UPLC core system (Waters, Manchester, UK), consisting of a binary solvent manager, a sample manager and a column oven. The chromatographic separation was performed on a 150 × 2 mm i.d., 1.7 μm, BEH C18 column (Waters, Manchester, UK) at a flow rate of 0.3 mL/min and a temperature of 40 °C. Aqueous formic acid (0.1 %, A) and acetonitrile (B) were used as solvents for the following gradient: 0 min / 5 % B, 3 min / 100 % B, and 4 min / 100 % B. High-resolution mass spectra were recorded on a Synapt G2-S HDMS (Waters, Manchester, UK) in negative and positive ESI resolution mode using −3.0 and +2.5 kV capillary voltage, respectively, 30 kV sampling cone, 4.0 kV extraction cone, 150 °C source temperature, 450 °C desolvation temperature, 30 L/h cone gas and 850 L/h desolvation gas. The instrument was calibrated (*m/z* 50−1200) using a solution of sodium formate (0.5 mmol/L) dissolved in 2-propanol/water (9/1, v/v). All data were lock mass corrected using leucine enkephaline as reference (*m/z* 554.2615, $[M-H]^-$ and *m/z* 556.2771, $[M+H]^+$). Data acquisition and interpretation were performed using MassLynx (version 4.1) and the tool “elemental composition” as software.

Nuclear Magnetic Resonance Spectroscopy (NMR). 1D- and 2D-NMR spectra were recorded on a 400 MHz ultrashield Avance III spectrometer with a Broadband Observe BBFOplus probehead and a 500 MHz ultrashield plus Avance III spectrometer with a Triple Resonance Cryo Probe TCI probehead (Bruker, Rheinstetten, Germany), respectively. Using methanol-d_4 and D_2O as solvents, the chemical shifts were quoted in parts per million relative to the solvent signal. The pulse sequences for recording 2D NMR experiments (i.e. COSY, HSQC, and

HMBC) were taken from the Bruker software library. Data processing was performed by using XWin-NMR version 3.5 (Bruker, Rheinstetten, Germany) and Mestre-Nova 8 (Mestrelab Research, Santiago de Compostela, Spain) as software.

RESULTS AND DISCUSSION

Using several tests as recommended for antioxidant screening,[49,50] a Pilsner-type beer was iteratively fractionated and each fraction analysed by three different *in vitro*-assays, namely the oxygen radical absorbance capacity (ORAC) assay, the hydrogen peroxide scavenging (HPS) assay, and the linoleic acid (LA) assay, respectively.

Antioxidant Assay-Guided Fractionation of Beer. First, beer was extracted with ethyl acetate to afford the ethyl acetate soluble fraction I and the aqueous fraction II (**Figure 2**). Without considering the yields of these fractions, fraction I showed with 210 (ORAC assay), 79 (HPS assay), and 38 *µ*mol TE/g (LA assay) by far high antioxidant activity when compared to the aqueous fraction II with 97 (ORAC assay), 23 (HPS assay), and 16 *µ*mol TE/g (LA assay), thus indicating hydrophobic antioxidants to be more active than the water-soluble compounds. However, considering the high yield of 90% of fraction II when compared to fraction I (10%), the aqueous fraction II seems to have a higher contribution to the antioxidant activity of beer, e.g. fraction II showed antioxidant activity with 3600 (ORAC), 840 (HPS), and 580 *µ*mol TE/L beer (LA), whereas fraction I showed lower activity with 800 (ORAC), 310 (HPS), and 150 *µ*mol TE/L beer (LA). As consideration of the natural ratios of the fractions is crucial to evaluate the

454 contribution to the antioxidant activity of the beer, the following activity
455 measurements were done by taking the different yields of fractions into account.

456 **Identification of Key Antioxidants in Fraction I.** To achieve the isolation of
457 individual antioxidants, fraction I was separated by means of RP18-MPLC to yield 9
458 fractions, namely I-1 to I-9 (**Figure 2**), which were then subjected to antioxidant
459 activity measurement. Recombination of aliquots of these fractions revealed an
460 antioxidant activity of the mixture with values of 670 (ORAC), 270 (HPS), and 130
461 *μ*mol TE/L (LA) which is comparable to the activity found for the entire fraction I,
462 thus confirming the stability of the key antioxidants during separation. Among the
463 individual fractions, fraction I-7 was found to exhibit by far the highest activity with
464 530 (ORAC), 220 (HPS), and 58 *μ*mol TE/L (LA), respectively, and was therefore
465 further sub-fractionated by means of preparative HPLC. A total of 10 subfractions (I-
466 7-1 to I-7-10) was collected and analysed using the three antioxidant assays.
467 Except for fraction I-7-10, all fractions showed some relevant antioxidant activity. In
468 particular, fraction I-7-2 with 93 (ORAC), 87 (HPS), and 7 *μ*mol TE/L (LA), and
469 fraction I-7-9 with 32 (ORAC), 36 (HPS), and 24 *μ*mol TE/L (LA) showed the highest
470 activities, followed by fractions I-7-1, I-7-4, and I-7-7, respectively.

471 UV/Vis, LC-TOF-MS, and NMR analysis and comparison with data obtained
472 from the corresponding reference substances revealed tyrosol (**11**) in fraction I-7-2,
473 *p*-hydroxybenzoic acid (**1**) in fraction I-7-3, vanillic acid (**2**), caffeic acid (**5**) and
474 syringic acid (**3**) in fraction I-7-4, *p*-coumaric acid (**4**) in fraction I-7-6, ferulic acid (**6**)
475 and sinapic acid (**7**) in fraction I-7-7, and cinnamic acid (**15**) in fraction I-7-8 as most
476 active antioxidants (**Table 1**), thus confirming literature data.[18-23,51-53] In addition, *p*-
477 hydroxyphenyllactic acid (**8**) and 2-isopropylmalic acid (**9**), both well-known
478 metabolites of *Saccharomyces cerevisiae*,[54,55] and the diketopiperazine *cyclo*(Pro-

Tyr) (**10**) were identified in fraction I-7-1 and, to the best of our knowledge, have not yet been reported in beer.

Next to 3-feruloylquinic acid (**14**), known from barley and beer,[56,57] also 4- and 5-feruloylquinic acid (**13**, **14**) could be unequivocally identified in fraction I-7-5. *N*-feruloyltyramine (**16**), recently reported to be present in hops and beer,[58] was found in fraction I-7-9-15, while fraction I-7-9-21 contained syringaresinol (**17**), which has been reported earlier in barley and brewer's spent grain,[59,60] but not yet in beer. Comparison of spectroscopic chromatographic data with those of reference substances isolated and purified from a hop polyphenol extract, followed by co-chromatography, led to the identification of *co*-multifidolglucoside (**18a**) and quercetin-3-*O*-*β*-D-glucoside (**19**) in fraction I-7-8, quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**21**) in fraction I-7-9-12, *ad*-multifidolglucoside in fraction I-7-9-17 (**18c**), *n*-multifidolglucoside (**18b**) in fraction I-7-9-19, and kaempferol-3-*O*-*β*-D-glucoside (**20**) and kaempferol-3-*O*-*β*-D-(6''-*O*-malonylglucoside (**22**) in fraction I-7-9-20. Apart from compounds **18a**, **18c**, **19**, **21** and **22**, which have already been reported as constituents in beer,[34,62] *n*-multifidolglucoside (**18b**) and quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**20**) were just known from hops.[58,61]

Identification of Key Antioxidants in the Fraction II. Due to its complex composition, the lyophilized fraction II was extracted with an acetonitrile/water (70/30, v/v) to separate less polar compounds from highly polar carbohydrates and peptides. As the non-extractable residue exhibited only a negligible antioxidant activity compared to the highly active extract fraction IIA with 3300 (ORAC), 770 (HPS), and 460 *μ*mol TE/L (LA), fraction IIA was further separated by means of RP18-MPLC to give subfractions IIA-1 to IIA-9, which were again assayed for antioxidant activity. The highest ORAC-activities were found for fraction IIA-9 (1700 *μ*mol TE/L), IIA-8 (1200 *μ*mol TE/L), followed by IIA-7 (800 *μ*mol TE/L). The

HPS-assay identified fractions IIA-9 (580 μmol TE/L), IIA-8 (470 μmol TE/L) and IIA-5 (340 μmol TE/L) as the most active ones, while fractions IIA-5 (300 μmol TE/L), IIA-8 (160 μmol TE/L) and IIA-9 (160 μmol TE/L) exhibited the highest impact in the LA-assay. Taking all these data together, fractions IIA-5, IIA-7, IIA-8, and IIA-9 were most active and, therefore, further separated by means of preparative HPLC to give the subfractions IIA-5-1 to IIA-5-10, IIA-7-1 to IIA-7-11, IIA-8-1 to IIA-8-15, and IIA-9-1 to IIA-9-15.

Within fraction IIA-5, subfraction IIA-5-9 showed by far the highest antioxidant activity (200 μmol TE/L in ORAC, 91 μmol TE/L in HPS, 140 μmol TE/L in LA; **Figure 2**). Subfraction IIA-7-9 (230 μmol TE/L in ORAC, 13 μmol TE/L in HPS, 13 μmol TE/L in LA) contained the most important antioxidants of fraction IIA-7. In comparison, the antioxidant activity in the subfractions of IIA-8 and IIA-9 was rather spread over the fractions.

UV/vis, LC-TOF-MS and 1D/2D-NMR analysis of the compounds isolated from fraction IIA-5-9 revealed, besides the amino acid L-phenylalanine (**23**), a phenolic compound exhibiting two UV-maxima at 224 and 284 nm. LC-TOF-MS showed *m/z* 301.0919 ($[C_{13}H_{18}O_8–H]^-$) as the pseudomolecular ion ($[M−H]^-$) and a cleavage of 162 Da, thus indicating the presence of a hexose moiety. ^{1}H NMR spectroscopy revealed proton signals at 6.66 (H-C(6)), 6.84 (H-C(2)), und 6.87 ppm (H-C(5)), thus indicating an three-fold substituted aromatic system. Moreover, a *β*-D-glucopyranoside moiety was assigned with the anomeric proton (H-C(1‘)) resonating at 4.99 ppm and showing a coupling constant of 7.6 Hz. Heteronuclear correlation spectra (HMBC) revealed the connection between H-C(1‘) and C(1) at 151.8 ppm and between H_3-C(1’’) at 3.85 ppm and C(3) at 149.0 ppm, thus indicating the presence of tachioside (**24**) or isotachioside (**24a**) as outlined in **Figure 3**. Independent chemical synthesis and comparison of chromatographic and

spectroscopic data, followed by co-chromatography, revealed tachioside (**24**; **Figure 1**) as the key antioxidant in fraction IIA-5.

In addition, tryptophan (**25**) was identified in fraction IIA-7-9 by comparison with the reference substance, and saponarin (**26**), previously reported in barley and the PVPP residue of beer,[63,64] was identified in fraction IIA-9-11 by means of LC-MS and NMR spectroscopic experiments. Moreover, LC-TOF-MS analysis of fraction IIA-9-11 revealed a major compound (**27**) with *m/z* 180.0668 as the $[M-H]^-$ ion, fitting well with the molecular formula of $C_9H_{11}NO_3$, and *m/z* 94.0300 as the fragment ion ($[C_5H_5NO-H]^-$), thus indicating a butyric acid side chain. The ^{1}H NMR spectrum exhibited an aldehyde proton (H-C(1)) at 9.46 ppm and the typical proton coupling pattern of a pyrrol system with three aromatic double doublets resonating at 6.26 (H-C(4)), 7.04 (H-C(3)), and 7.17 ppm (H-C(5)) and exhibiting characteristic coupling constants of 1.7, 2.5, and 4.1 Hz, respectively. The HMBC spectrum of **27** revealed connectivity of the signal of C(2) at 132.7 ppm with the protons at 4.37 ppm (H_2-C(4')) as well as the aldehyde proton (H-C(1)) and the aromatic proton signals H-C(3), H-C(4), and H-C(5), thus indicating the butyric acid side chain to be linked to the pyrrol via the nitrogen atom. Taking all spectroscopic data into consideration, 4-(2-formylpyrrol-1-yl)butyric acid (**27**) was identified as the key compound in fraction IIA-9-7.

LC-TOF-MS analysis of the major compound (**28**) in fraction IIA-8-10 showed a similar fragmentation pattern as observed for compound **27**, although a mass shift of 30 Da was observed, e.g. *m/z* 210.0770 was found as the pseudomolecular ion ($[C_{10}H_{13}NO_4-H]^-$) and *m/z* 124.0404 ($[C_6H_7NO_2-H]^-$) as the main fragment ion (**Figure 4**). In comparison to **27**, the ^{1}H NMR spectrum of compound **28** showed an additional signal of a hydroxymethyl group (H_2-C(6)) at 4.64 ppm, while the pyrrol proton at C(5) was lacking. Although isolated earlier as a Maillard reaction product

from carbohydrate/aminobutyric acid model reactions,[40,65] 4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid (**28**), isolated from fraction IIA-8-10, has not been previously been reported as a constituent of beer (**Figure 1**).

HPLC-UV/vis analysis of fractions IIA-8-6, IIA-8-8, and IIA-8-11, respectively, showed typical local absorption maxima at 228 nm, 300 nm and 320 nm as expected for hydroxycinnamic acid derivatives. LC-TOF-MS of the main compounds **29−31** revealed *m/z* 551.3091, 581.3194, and 611.3296 as pseudomolecular ions ($[M+H]^+$), fitting well with the molecular formula of $C_{28}H_{38}N_8O_4$, $C_{29}H_{40}N_8O_5$, and $C_{30}H_{42}N_8O_6$, respectively, and indicated the presence of hordatines (**29−31**), which has been reported earlier.[66] Full spectroscopic signal assignment has been achieved by 1D/2D-NMR experiments (Table 2). The ^3J proton coupling constant for the protons H-C(7) and H-C(8) of the double bond of 12.2 Hz indicated a *cis*-configuration, that seemed to be caused by a *trans* to *cis* isomerisation during the isolation procedure.[67] Moreover, the ^3J proton coupling constant of 7.4 Hz between the protons H-C(7') and H-C(8') demonstrated a *trans*-configuration of the amide side chain and the hydroxyphenyl side chain. HMBC spectroscopy confirmed the position of the methoxy groups by the ^3J coupling of H_3-C(10) at 3.89 ppm and C(5) at 144.7 ppm of hordatines B (**30**) and C (**31**) and the ^3J coupling of H_3-C(10') at 3.87 ppm and C(3') at 148.7 ppm of hordatine C (**31**). This is the first time, that detailed $^1H/^{13}C$ NMR data are available for hordatine B (**30**; **Figure 1**), although it has been suggested earlier as a natural product from barley.[68] Since its first suggestion in barley,[66] the chemical structure of hordatine C (**31**, **Figure 1**) has, to the best of our knowledge, not been confirmed earlier.

Antioxidant Activity of Identified Compounds. Prior to the analysis of their antioxidant activity by means of the ORAC-, HPS-, and LA-assay (**Table 3**), respectively, a purity of more than 98% was confirmed for each target compound by

583 HPLC-TOF-MS and ^{1}H NMR spectroscopy. The highest antioxidant activity of 10 –
584 17.5 (ORAC), 2.0 – 4.1 (HPS), and 1.1 – 6.1 μmol TE/μmol (LA) was found for the
585 hordatines (**29−31**). In the ORAC-assay, only saponarin (**26**) reached also a high
586 activity of 13 μmol TE/μmol, while the LA-assay indicated high antioxidant activity
587 for compounds exhibiting an aromatic *ortho*-dihydroxy moiety, such as, e.g. caffeic
588 acid (**5**; 2.8 μmol TE/μmol), quercetin-3-*O*-*β*-D-glucopyranoside (**19**;
589 1.8 μmol TE/μmol), and quercetin-3-*O*-*β*-D-(6-malonyl)glucoside (**21**;
590 3.0 μmol TE/μmol). While mono-phenolic compounds, such as, e.g. *p*-
591 hydroxybenzoic acid (**1**), *p*-coumaric acid (**4**), *p*-hydroxyphenyllactic acid (**8**), *cyclo*
592 (Pro-Tyr) (**10**), and tyrosol (**11**) showed almost negligible activities
593 (< 0.2 μmol TE/μmol) in the TE assay, *p*-coumaric acid (**4**) showed high antioxidant
594 activity of 8 μmol TE/μmol in the ORAC-assay and 1.0 μmol TE/μmol in the HPS-
595 assay, thus indicating the different specificities of the test systems used. In
596 comparison, 4-(2-formylpyrrol-1-yl)butyric acid (**27**) and 4-[2-formyl-5-
597 (hydroxymethyl)pyrrol-1-yl]butyric acid (**28**) showed only low activity of 0.13 and
598 0.12 μmol TE/μmol in the LA-assay, and 2-isopropylmalic acid (**9**), cinnamic acid
599 (**15**) and phenylalanine (**23**) did not show any significant antioxidant activity
600 (< 0.01 μmol TE/μmol).

601 For the first time, an activity-guided fractionation, followed by unequivocal
602 structure determination by means of LC-TOF-MS, LC-MS/MS, and NMR
603 spectroscopy led to the identification of 31 antioxidant compounds in beer. Whereas
604 previous studies focused on the antioxidant activity of literature-known compound
605 classes in beer, such as, e.g. chalcones, flavanones, procyanidins, hydroxybenzoic
606 acids, and hydroxycinnamic acids,[14,18-24] the activity-guided strategy applied in the
607 present study demonstrate for the first time hordatines (**29−31**), saponarin (**26**), and
608 quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**21**) to exhibit with the highest

antioxidant activities. To answer the question as to which of the compounds identified contribute most to the antioxidant activity of beer, quantitative and dose-activity studies are currently under investigation and will be published separately.

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SUPPORTING INFORMATION AVAILABLE

MPLC and HPLC chromatograms and antioxidant activity of the activity-guided fractionation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1: Assignment of the Isolated Antioxidants to the HPLC-Fractions Referring to Figure 2.

fraction	analyte (**no.**)
I-7-1	*p*-hydroxyphenyllactic acid (**8**) 2-isopropylmalic acid (**9**) *cyclo* (Pro-Tyr) (**10**)
I-7-2	tyrosol (**11**)
I-7-3	*p*-hydroxybenzoic acid (**1**)
I-7-4	vanillic acid (**2**) syringic acid (**3**) caffeic acid (**5**)
I-7-5	5-feruloylquinic acid (**12**) 4-feruloylquinic acid (**13**) 3-feruloylquinic acid (**14**)
I-7-6	*cis/trans*-*p*-coumaric acid (**4**)
I-7-7	*cis/trans*-ferulic acid (**6**) sinapic acid (**7**)
I-7-8	*co*-multifidolglucoside (**18a**) cinnamic acid (**15**) quercetin-3-*O*-*β*-D-glucoside (**19**)
I-7-9-12	quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**21**)
I-7-9-15	*N*-feruloyltyramine (**16**)
I-7-9-17	*ad*-multifidolglucoside (**18c**)
I-7-9-19	*n*-multifidolglucoside (**18b**)
I-7-9-20	kaempferol-3-*O*-*β*-D-glucoside (**20**) kaempferol-3-*O*-*β*-D-(6''-*O*-malonylglucoside (**22**)
I-7-9-21	syringaresinol (**17**)
IIA-5-9	phenylalanine (**23**) tachioside (**24**)
IIA-7-9	tryptophan (**25**)
IIA-8-5 to IIA-8-8	hordatine A (**29**)

IIA-8-6 to IIA-8-9	hordatine B (**30**)
IIA-8-10	4-[2-formyl-5-(hydroxymethyl) pyrrol-1-yl]butyric acid (**28**)
IIA-8-11 to IIA-8-12	hordatine C (**31**)
IIA-9-7	4-(2-formylpyrrol-1-yl)butyric acid (**27**)
IIA-9-11	saponarin (**26**)

Table 2. ^{1}H and ^{13}C NMR Data (500 / 125 MHz, D_2O plus 5 % MeOH-d_4) of Hordatine A (**29**), Hordatine B (**30**) and Hordatine C (**31**), Using the Numbering as Shown in Figure 1.

position	hordatine A (**29**)				hordatine B (**30**)				hordatine C (**31**)			
	δ_C [ppm][a]	HSQC[b]	δ_H [ppm][c]	M (J [Hz])[d]	δ_C [ppm][a]	HSQC[b]	δ_H [ppm][c]	M (J [Hz])[d]	δ_C [ppm][a]	HSQC[b]	δ_H [ppm][c]	M (J [Hz])[d]
C(1)	130.1	[C]			131.1	[C]			131.3	[C]		
C(2)	125.6	[CH]	7.25	s	118.1	[CH]	6.88	s	118.0	[CH]	6.90	s
C(3)	126.9	[C]			127.7	[C]			127.8	[C]		
C(4)	159.9	[C]			148.4	[C]			148.5	[C]		
C(5)	110.6	[CH]	6.93	d (8.3)	144.7	[CH]			144.6	[CH]		
C(6)	131.9	[CH]	7.32	d (8.3)	114.5	[CH]	7.07	s	114.6	[CH]	7.10	s
C(7)	137.2	[CH]	6.85	d (12.3)	137.2	[CH]	6.86	d (12.2)	137.1	[CH]	6.88	d (12.2)
C(8)	123.3	[CH]	6.01	d (12.3)	124.1	[CH]	6.06	d (12.2)	124.0	[CH]	6.07	d (12.2)
C(9)	171.6	[C]			171.6	[C]			171.6	[C]		
C(10)	-	-			57.1	[CH_3]	3.89		57.1	[CH_3]	3.92	s
C(1‘)	131.8	[C]			131.6	[C]			132.3	[C]		
C(2‘)	128.8	[CH]	7.31	d (8.3)	128.9	[CH]	7.32	d (8.4)	120.2	[CH]	6.96	s
C(3‘)	116.7	[CH]	6.93	d (8.3)	116.8	[CH]	6.94	d (8.4)	148.7	[CH]		
C(4‘)	157.2	[C]			157.3	[C]	-		146.7	[C]		
C(5‘)	116.7	[CH]	6.93	d (8.3)	116.8	[CH]	6.94	d (8.4)	116.7	[CH]	6.97	d (7.4)
C(6‘)	128.8	[CH]	7.31	d (8.3)	128.9	[CH]	7.32	d (8.4)	111.2	[CH]	7.08	d (7.4)

C(7‘)	88.8	[CH]	5.85	d (7.3)	89.5	[CH]	5.91	d (7.4)	89.4	[CH]	5.93	d (7.4)
C(8‘)	57.7	[CH]	4.28	d (7.3)	58.1	[CH]	4.32	d (7.4)	58.2	[CH]	4.35	d (7.4)
C(9‘)	174.1	[C]			173.9	[C]			173.9	[C]		
C(10‘)	-	-			-	-			57.1	[CH_3]	3.87	s
C(1‘‘)	40.0	[CH_2]	3.03−3.35	m	40.0	[CH_2]	2.97−3.32	m	40.0	[CH_2]	2.98−3.32	m
C(1‘‘‘)	39.7	[CH_2]	3.03−3.35	m	39.7	[CH_2]	2.97−3.32	m	39.7	[CH_2]	2.98−3.32	m
C(2‘‘), C(2‘‘‘), C(3‘‘), C(3‘‘‘)	26.1, 26.2, 26.3, 26.6	[CH_2]	1.34−1.61	m	26.1, 26.2, 26.3, 26.6	[CH_2]	1.29−1.60	m	26.2, 26.2, 26.3, 26.6	[CH_2]	1.31−1.62	m
C(4‘‘), C(4‘‘‘)	41.7	[CH_2]	3.03−3.35	m	41.7	[CH_2]	2.97−3.32	m	41.7	[CH_2]	2.98−3.32	m
C(5‘‘), C(5‘‘‘)	157.6	[C]			157.7	[C]			157.6	[C]		

[a] Chemical shift in the ^{13}C NMR spectrum in ppm; [b] C-H substitution degree deduced from the HSQC (heteronuclear single quantum coherence) spectrum; [c] Chemical shift in the ^{1}H NMR spectrum in ppm; [d] multiplicity of the signals ^{1}H NMR signals including distinguishable coupling constants in Hz.

Table 3. Antioxidant Activity of the Compounds Isolated from Beer.

Analyte (**no.**)[a]	antioxidant activity (μmol TE/μmol) in the		
	ORAC-assay[b]	HPS- assay[c]	LA-assay[c]
p-hydroxybenzoic acid (**1**)	4.92 ± 0.40	1.08 ± 0.08	< 0.01 ± 0.00
vanillic acid (**2**)	3.39 ± 0.12	1.26 ± 0.14	0.41 ± 0.03
syringic acid (**3**)	1.16 ± 0.18	1.49 ± 0.13	1.27 ± 0.13
p-coumaric acid (**4**)	3.81 ± 0.38	0.95 ± 0.09	0.15 ± 0.02
caffeic acid (**5**)	4.17 ± 0.12	1.17 ± 0.09	2.75 ± 0.59
ferulic acid (**6**)	5.48 ± 0.13	1.10 ± 0.05	0.86 ± 0.12
sinapic acid (**7**)	2.10 ± 0.22	1.34 ± 0.08	1.13 ± 0.18
p-hydroxyphenyllactic acid (**8**)	0.80 ± 0.08	1.79 ± 0.23	< 0.01 ± 0.00
2-isopropylmalic acid (**9**)	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00
cyclo (Pro-Tyr) (**10**)	1.12 ± 0.08	0.50 ± 0.04	< 0.01 ± 0.00
tyrosol (**11**)	1.33 ± 0.18	0.78 ± 0.05	0.04 ± 0.00
5-feruloylquinic acid (**12**)	1.63 ± 0.30	0.83 ± 0.13	1.37 ± 0.18
4-feruloylquinic acid (**13**)	1.69 ± 0.21	0.85 ± 0.11	1.08 ± 0.26
3-feruloylquinic acid (**14**)	1.87 ± 0.29	0.83 ± 0.14	1.13 ± 0.24
cinnamic acid (**15**)	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00
N-feruloyltyramine (**16**)	4.27 ± 0.45	0.68 ± 0.03	0.50 ± 0.02
syringaresinol (**17**)	2.28 ± 0.04	1.21 ± 0.10	0.80 ± 0.05
co-multifidolglucoside (**18a**)	2.16 ± 0.03	0.63 ± 0.08	0.55 ± 0.07
n-multifidolglucoside (**18b**)	2.23 ± 0.10	0.45 ± 0.08	0.83 ± 0.08
ad-multifidolglucoside (**18c**)	2.06 ± 0.11	0.54 ± 0.08	0.74 ± 0.07
quercetin-3-*β*-D-glucoside (**19**)	3.94 ± 0.20	2.09 ± 0.34	1.81 ± 0.26
kaempferol-3-*O*-*β*-D-glucoside (**20**)	4.75 ± 0.13	1.59 ± 0.21	0.51 ± 0.06
quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**21**)	6.61 ± 0.45	1.74 ± 0.29	2.97 ± 0.46
kaempferol-3-*O*-*β*-D-(6''-*O*-malonyl)glucoside (**22**)	4.45 ± 0.34	1.95 ± 0.31	0.91 ± 0.17
phenylalanine (**23**)	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00
tachioside (**24**)	2.62 ± 0.14	0.98 ± 0.16	1.77 ± 0.23
isotachioside (**24a**)	3.29 ± 0.17	0.72 ± 0.09	1.58 ± 0.38
tryptophan (**25**)	2.05 ± 0.36	0.65 ± 0.04	< 0.01 ± 0.00
saponarin (**26**)	12.92 ± 0.19	2.02 ± 0.25	1.17 ± 0.18
4-(2-formylpyrrol-1-yl)butyric acid	< 0.01 ± 0.00	< 0.01 ± 0.00	0.13 ± 0.01

(**27**)						
4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid (**28**)	< 0.01	± 0.00	< 0.01	± 0.00	0.12	± 0.01
hordatine A (**29**)	10.01	± 0.97	1.97	± 0.44	1.05	± 0.13
hordatine B (**30**)	12.58	± 0.98	3.02	± 0.53	3.19	± 0.32
hordatine C (**31**)	17.50	± 0.77	4.10	± 1.01	6.14	± 1.28

[a] Numbering of compounds refer to structures given in **Figure 1**; [b] Errors express standard deviation of four replicates; [c] Errors express the confidence interval (α = 5 %) of each three replicates.

Figure Captions

Figure 1. Chemical structures of antioxidants identified in beer: *p*-hydroxybenzoic acid (**1**), vanillic acid (**2**), syringic acid (**3**), *p*-coumaric acid (**4**), caffeic acid (**5**), ferulic acid (**6**), sinapic acid (**7**), *p*-hydroxyphenyllactic acid (**8**), 2-isopropylmalic acid (**9**), *cyclo* (Pro-Tyr) (**10**), tyrosol (**11**), 5-feruloylquinic acid (**12**), 4-feruloylquinic acid (**13**), 3-feruloylquinic acid (**14**), cinnamic acid (**15**), *N*-feruloyltyramine (**16**), syringaresinol (**17**), *co*-multifidolglucoside (**18a**), *n*-multifidolglucoside (**18b**), *ad*-multifidolglucoside (**18c**), quercetin-3-*O*-*β*-D-glucopyranoside (**19**), kaempferol-3-*O*-*β*-D-glucopyranoside (**20**), quercetin-3-*O*-*β*-D-(6''-*O*-malonyl)glucopyranoside (**21**), kaempferol-3-*O*-*β*-D-(6''-*O*-malonyl)glucopyranoside (**22**), phenylalanine (**23**), tachioside (**24**), isotachioside (**24a**), tryptophan (**25**), saponarin (**26**), 4-(2-formylpyrrol-1-yl)butyric acid (**27**), 4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid (**28**), hordatine A (**29**), hordatine B (**30**), and hordatine C (**31**).

Figure 2. Separation scheme used to locate antioxidants in beer, with the antioxidant activity of single fractions, investigated by ORAC-assay (blue), HPS-assay (red), and LA-assay (green), respectively. Each activity was normalized to the fraction with the highest activity of each separation step.

Figure 3. (**A**) HPLC chromatogram and antioxidant activity of fraction IIA-5 of the acetonitrile/water-extract (IIA) prepared from beer, investigated by ORAC-assay (blue), HPS-assay (red), and LA-assay (green), respectively, and (**B**) excerpt of the HMBC spectrum (500 / 125 MHz, D_2O) and structure of tachioside (**24**) isolated from subfraction IIA-5-9.

Figure 4: TOF-MS spectra of 4-(2-formylpyrrol-1-yl)butyric acid (**A**) and 4-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]butyric acid (**B**), and excerpt of the HMBC spectrum (500 / 125 MHz, MeOH-d_4) of 4-(2-formylpyrrol-1-yl)butyric acid (**C**).

Figure 1 (Spreng & Hofmann)

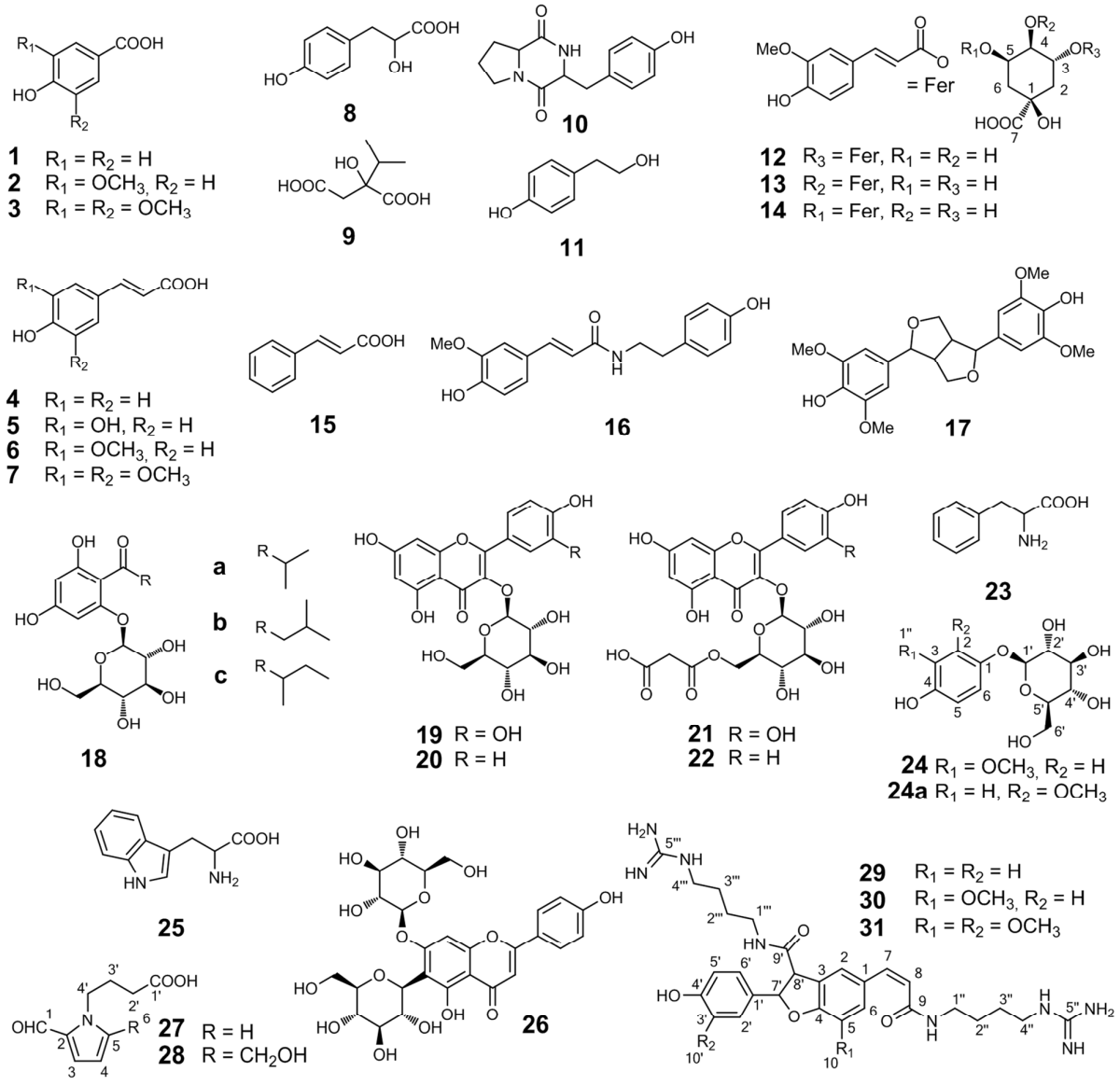


Figure 2 (Spreng & Hofmann)

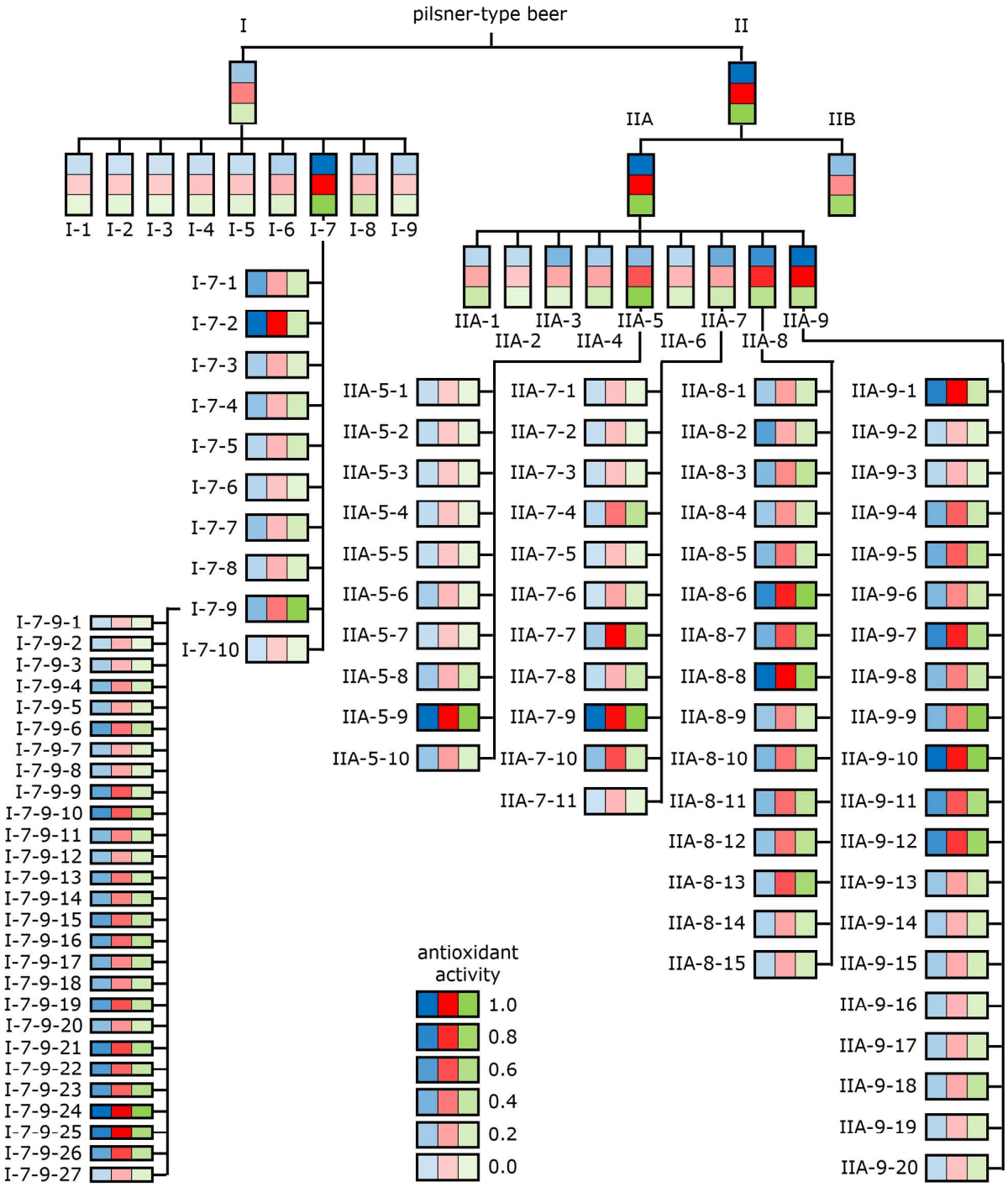


Figure 3 (Spreng & Hofmann)

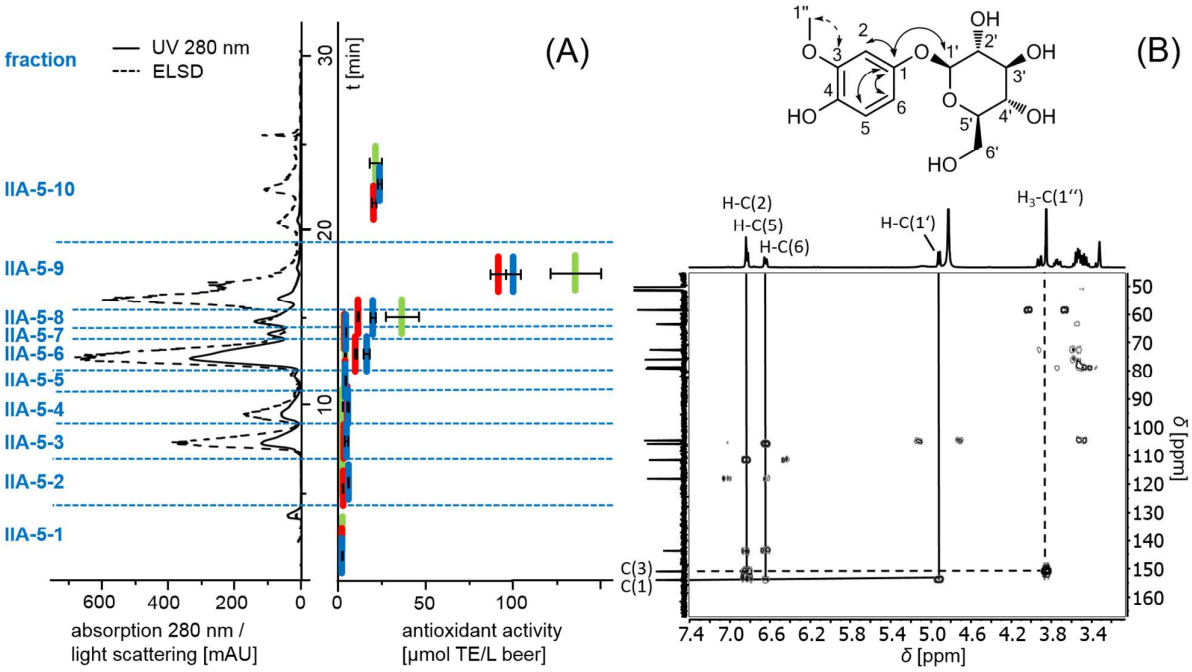
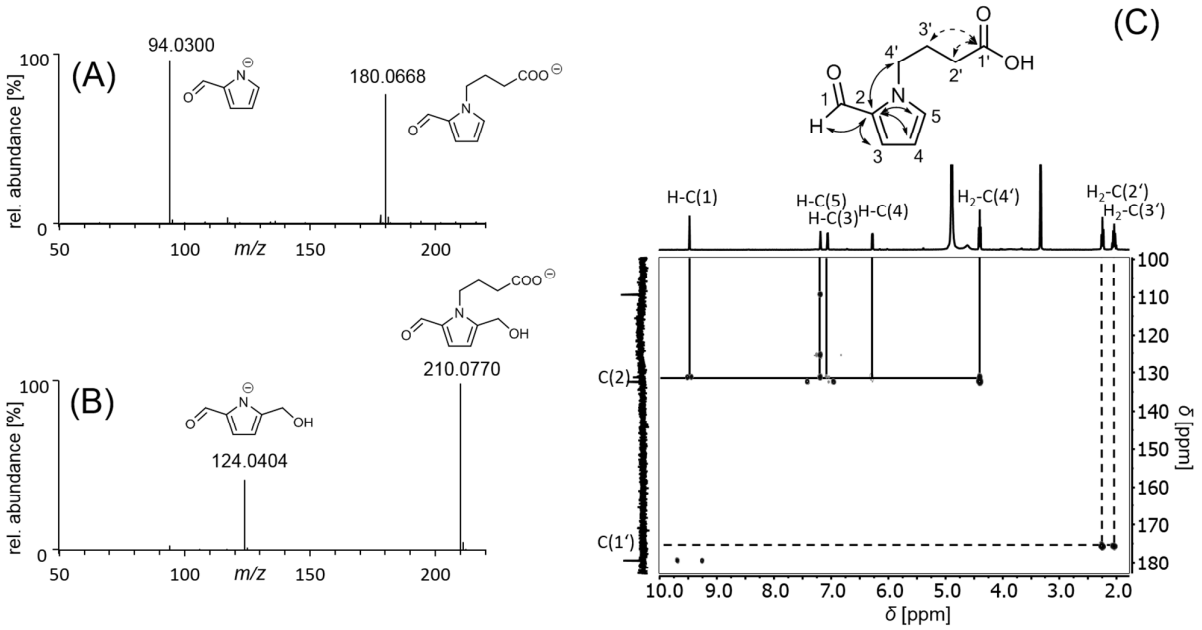
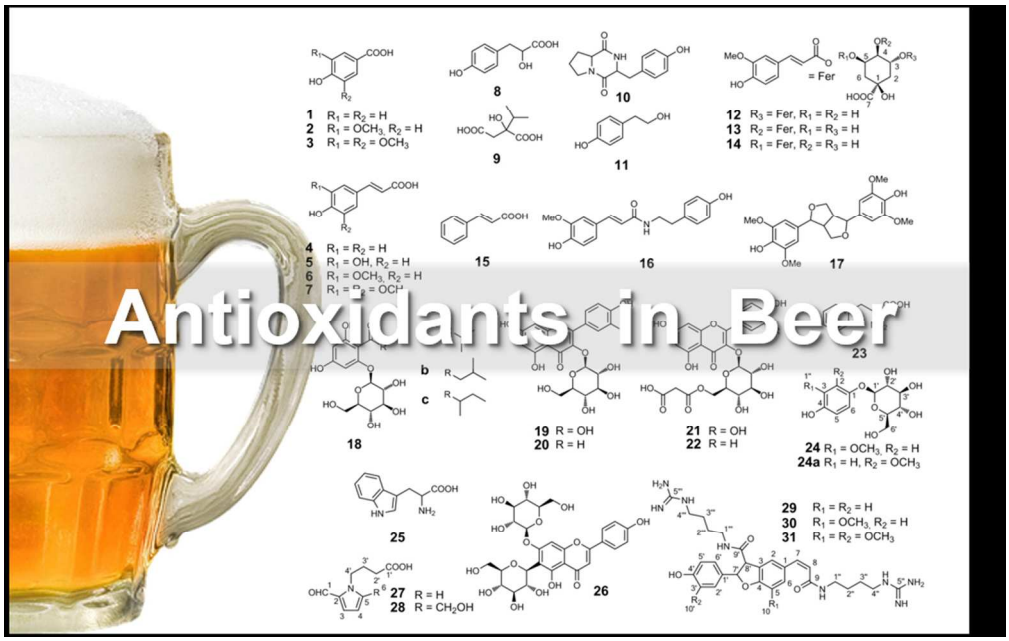


Figure 4 (Spreng & Hofmann)





TOC Graphic Spreng

183x115mm (150 x 150 DPI)