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Effect-directed classification of biological, biochemical and chemical profiles of 50 German beers

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ABSTRACT

Biological and biochemical fingerprints were investigated for the first time for the feasibility of effect-directed classification, and thus, to allow the choice of a distinct beer with regard to beneficial health effects. A high-performance thin-layer chromatography method was newly developed and combined with *in situ* effect-directed analysis for profiling 50 German beers for multipotent active compounds, and thus, their health-related potential. Discovered multipotent active zones were online eluted and characterized by high resolution mass spectrometry. For example, isoxanthohumol, *iso- α -ad/n-humulone* or its isomers, desdimethyl-octahydro-*iso-cohumulone* and *ad/n-humulone* were proven as antimicrobial compounds, isoxanthohumol as an acetylcholinesterase inhibitor, and isoxanthohumol and *iso- α -ad/n-humulone* or its isomers as radical scavengers. Investigating multivariate data analysis of effect-directed fingerprints for the first time, the pattern recognition and classification results showed the power of clustering non-alcoholic beers from other types of beer, or it showed the differentiation of dark and non-alcoholic beers.

Keywords

Effect-directed clustering, Fingerprinting, Profiling, Direct bioautography, Multivariate data analysis, Healthy food

1. Introduction

A powerful bioanalytical technique is high-performance thin-layer chromatography (HPTLC) directly combined with *in situ* enzymatic, biological and chemical assays (effect-directed analysis, EDA) to screen complex samples with regard to effects or active components. For example using direct bioautography, antimicrobials were detected by changes in the microbial growth directly on the HPTLC chromatogram (Morlock, 2013). Due to its simplicity, speed and cost-efficiency, bioautographic methods were developed in academic and industrial laboratories to discover and characterize, *e.g.*, antimicrobial, antifungal, anti-tumour, anti-protozoa and oestrogen-effective compounds in complex food, botanicals and environmental samples (Morlock, 2013; Klingelhöfer & Morlock, 2015a; Jamshidi-Aidji & Morlock, 2016).

Beer is one of most popular and consumed alcoholic beverage in the world (Kondo, 2004). It contains carbohydrates, B-vitamins, peptides, amino acids, amines, minerals, such as potassium, magnesium and sodium, as well as phenolic compounds. Most phenolic compounds in beer originate from the added malt (70–80%) and hops (20–30%) (Quifer-Rada et al., 2015). They mainly consist of prenylflavonoids and phenolic acids (hydroxybenzoic, cinnamic and ferulic acids as well as their derivatives). On the one hand, beer is highly sensitive to oxidation. On the other hand, such prenylflavonoids and phenolic acids exhibit a strong antioxidant activity, and thus, increase the shelf-life of beer (Kondo, 2004; Stevens & Page, 2004; Zhao, Chen, Lu & Zhao, 2010; Kavalier, Figueroa, Kincaid, Matthews & Kennelly, 2014; Quifer-Rada et al., 2015). Diverse physiological effects and health benefits in cardiovascular disease, diabetes, specific cancer types, (post)menopausal hot flushes and osteoporosis have been associated with beer consumption (Kondo, 2004; Stevens et al., 2004; Zhao et al., 2010; Kavalier et al., 2014; Quifer-

Rada et al., 2015). These effects may be referred to the ability of prenylflavonoids to modulate cell-signaling.

To date, several analytical techniques have been used for determination of phenolic compounds in beers and hops, including high performance liquid chromatography (HPLC) coupled to UV or electrochemical detection (Kellner, Jurková, Čulík, Horák & Čejka, 2007; Zhao et al., 2010), high-resolution mass spectrometry (Quifer-Rada et al., 2015), UV/vis spectrophotometry, cyclic voltammetry, Fourier transform infrared spectroscopy and gas chromatography-mass spectrometry (Masek, Chrzescijanska, Kosmalska & Zaborski, 2014). Although planar chromatography offers specific advantages, HPTLC analysis of the phenolic compounds in beer has not been described so far.

Digital image evaluation via multivariate data analysis is an emerging field in HPTLC. Flatbed scanners or CCD cameras were used for image acquisition and commercial or open source software for image processing and evaluation (Olech, Komsta, Nowak, Cieśla & Waksmundzka-Hajnos, 2012; Fichou, Ristivojević & Morlock, 2016; Ristivojević & Morlock, 2016). For example, different software for image processing and videodensitometry was compared to evaluate antioxidants from varieties of *Medicago sativa* (Kowalska, Oniszczyk & Stochmal, 2013). To minimize influences like non-uniformity of the plate background on the subsequent chemometric evaluation of such images, several preprocessing steps such as baseline removal, denoising, warping and normalization were applied (Ristivojević et al, 2016). Such a processing for image analysis of chromatograms after the 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) assay was described using commercial and self-developed software (Olech et al., 2012).

Another increasing field that made substantial progress in recent years has been the effect-directed analysis of planar chromatograms. The improved efficiency, performance and

reproducibility of the effect-directed chromatograms or (bio)autograms triggered first bioquantifications (via densitometric measurement of the microorganism response), shown for oestrogen-effective compounds in surface and waste water or antibiotics in herbal extracts (Klingelhöfer et al., 2015b; Jamshidi-Aidji et al., 2016). Though effect-directed analysis of different botanicals and food products was already demonstrated using HPTLC combined with bioassays and high resolution mass spectrometry (HRMS) (Krüger, Hüsken, Fornasari, Scainelli, & Morlock, 2017; Krüger, Bergin, & Morlock, 2018), the current study is the first report with a novel concept: Until now, there has been no published procedure for the application of pattern recognition techniques on enzymatic or biological autograms, or in other words for the classification of samples according to different bioactivities. Such a new combination would support the effect-directed classification of samples according to their health-related potential. Hence in the current study, the effect-directed classification of 50 German beer extracts was performed for the first time using biological, biochemical and chemical fingerprints. As a planar chromatographic separation of phenolic compounds in beer did not exist, an HPTLC method was new. The resulting HPTLC chromatograms were subsequently subjected to assays such as *Bacillus subtilis*, *Aliivibrio fischeri*, acetylcholinesterase (AChE) and DPPH' assays. Discovered multipotent active zones were characterized by HRMS after online elution via an elution head-based interface. Pattern recognition and effect-directed classification of bioautograms and further effect-directed chromatograms followed via multivariate data analysis. The influence of data preprocessing and the selection of variables of principal component analysis (PCA) were investigated for effect-directed classifications.

2. Materials and methods

2.1. Chemicals

Fast Blue Salt B, polyethylene glycol (PEG) 400, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•], 97%), isoxanthohumol ($\geq 95.0\%$), AChE from *Electrophorus electricus* Linnæus, 2-aminoethyl diphenylborinate (97%), all inorganic substances and most solvents (at least of analytical grade), if not stated otherwise, were purchased from Fluka Sigma-Aldrich, Schnelldorf, Germany. Formic acid (96%), citric acid, toluene, ethyl acetate, HPTLC plates silica gel 60 F₂₅₄ and *B. subtilis* spore suspension (Deutsche Sammlung von Mikroorganismen DSM 618) were obtained by Merck, Darmstadt, Germany. Thiazolyl blue tetrazolium bromide (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Carl Roth, Karlsruhe, Germany, and α -naphthyl acetate was from Panreac, Barcelona, Spain. The *A. fischeri* strain (DSM 7151, NRRL-B11177) was delivered by the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Berlin, Germany. Bidistilled water was prepared with a Destamat Bi 18E, Heraeus, Hanau, Germany.

2.2. Sample preparation

50 commercially available beers were purchased on the German market (Table S1), *i.e.* 11 dark beers, 25 light beers (pale beer with ca. 5 vol% alcohol), 10 non-alcoholic beers (blond, <0.5 vol% alcohol) and 4 barley malt beers. The extraction of the phenolics from beer (Kavalier et al., 2014) was slightly modified. Briefly, beers (each 10 ml) were degassed in an ultrasonic bath for 15 min and transferred to 50 ml tubes. Ethyl acetate (5 ml) was added, followed by vortexing for 2 min, then methanol (0.4 ml) was added and the upper ethyl acetate phase was collected. Extraction of the aqueous beer phase was repeated and the united ethyl acetate phases were centrifuged at $3000 \times g$ for 10 min. Ethyl acetate was evaporated at the rotary evaporator

(140 mbar, 35 °C) and the residue was dissolved in methanol (1.5 ml) prior to storage at -20 °C. Thus, a 1.5 ml ethyl acetate extract was made out of 10 ml beer, though finally dissolved in methanol.

2.3. HPTLC analysis and image acquisition

The beer extracts (15 µl each) were sprayed as 6-mm bands using the Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland). The sample application volume was adjusted to the detectability of each assay to be 6 µl for the DPPH[•] assay, 5 µl for the *A. fischeri* bioassay and for the AChE assay as well as 2 µl for the *B. subtilis* bioassay. The track distance was at least 8.4 mm, the distance to the side edge minimum 10 mm and the distance from the lower plate edge 10 mm. After pre-equilibration for 10 min, development was performed in a Twin Trough Chamber (CAMAG) with methyl ethyl ketone – toluene – formic acid (5:3:0.4, V/V/V) up to 70 mm. For 3 s, chromatograms were immersed in a 0.5% methanolic 2-aminoethyl diphenylborinate solution (Neu's reagent) at an immersion speed of 4.5 cm/s using the Chromatogram Immersion Device (CAMAG). After 5 min of drying in the air, plates were immersed for 3 s in a 5% methanolic PEG 400 solution at an immersion speed of 4.5 cm/s for enhancement and stabilization of fluorescent zones. With the TLC Visualizer (CAMAG), image acquisition and documentation (JPEG format) was performed at UV 366 nm, and after EDA, under white light illumination in reflection mode, if not stated otherwise.

2.4. Effect-directed analyses of beer extracts

Before each assay application, the chromatogram was neutralized by immersion in phosphate/citrate buffer (pH 7.8, immersion speed 3.5 cm/s and immersion time 5 s) and documented at UV 366 nm to notice any potential slight shift of zones (Fig. 1C).

For the DPPH[•] assay, the neutralized chromatogram was immersed at an immersion speed of 3 cm/s for 5 s into a 0.02% methanolic DPPH[•] solution prepared according to (Krüger, Urmann, Morlock, 2013) and stored in a brown flask at 4 °C until use. The wet plate was set vertically on a filter paper to let remove any excessive solution, dried at ambient temperature (25 °C) in the dark for 30 s and heated for 30 s in a stream of warm air. Image acquisition was performed every 30 s for a period of 10 min. The image taken after 3 min was used for multivariate data analysis.

For the *B. subtilis* bioassay, the neutralized chromatogram was immersed in the prepared bacterial suspension (immersion speed 3.5 cm/s and immersion time 5 s), incubated (37 °C, 2 h), immersed in a 0.2% PBS-buffered MTT solution and incubated again (37 °C, 0.5 h) (Jamshidi-Aidji & Morlock, 2015). The bioautogram was dried on the TLC Plate Heater (CAMAG) at 50 °C for 5 min and documented.

For the luminescent *A. fischeri* bioassay, the nutrient medium (100 ml, prepared as in (DIN EN ISO 11348-1, 2009) was inoculated (200 µl of a cryostock: 10 ml cell suspension centrifuged at 2500 x g for 3 min, cells washed with nutrient medium, centrifuged and resuspended in 1 ml nutrient medium and glycerol, 4:1) and cultivated at room temperature on a shaker (85 rpm, SM-30, Edmund Bühler, Hechingen, Germany) for about 20 h. The neutralized chromatogram was immersed in the bacterial suspension (immersion speed 2.0 cm/s and immersion time 2 s). The instant bioluminescence of the bioautogram was captured and processed with the BioLuminizer 1.0.2.6107 (CAMAG) (Krüger, Urmann & Morlock, 2013). Ten images (50 s exposure time

each) were taken in time intervals of 1.5 min. The image taken after 6 min (fifth image) was used for multivariate data analysis.

For the AChE assay, the neutralized chromatogram was immersed (immersion speed 2.5 cm/s and immersion time 5 s) in the enzyme solution (AChE 666 units plus 100 mg BSA ad 100 ml 0.05 M TRIS buffer, pH 7.8), incubated at 37 °C for 25 min, immersed in a substrate solution (25 mg α -naphthyl acetate plus 50 mg Fast Blue salt B ad 90 ml ethanol-water, 1:2, V/V) and documented (Marston, Kissling & Hostettmann, 2002; Hage & Morlock, 2017).

2.5. Characterization of active zones via HPTLC-HRMS

The sample was applied threefold on a HPTLC plate, prewashed with methanol and water 4:1, V/V, and dried at 120 °C for 30 min. After chromatography, phenolic compounds were marked at UV 366 nm with a pencil and eluted via PlateExpress (Advion, Ithaca, NY, USA) to the HESI source of the Q Exactive Plus (ThermoFisher Scientific, Waltham, MA, USA). Full scan mass spectra were recorded in the positive and negative ion mode (m/z 100–750) with the following parameters: ESI voltage 3.3 kV (-3.3 kV), capillary voltage -0.6 V, source voltage 25 V (-25 V), source voltage dynamic 25 V, capillary temperature 320 °C.

2.6. Image evaluation via multivariate data analysis

Image analysis was carried out with ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA). PCA was performed via PLS Tool Box (version 6.2.1) for MATLAB (version 7.12.0, R2011a; Eigenvector Research, Wenatchee, WA). All data preprocessing techniques investigated were applied to improve the PCA models. PCA was carried out as an exploratory

data analysis by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T^2 Hotelling limits for outliers (Ristivojević et al., 2016).

3. Results and discussion

There are few investigations of the effects on the food intake side compared to the tonnage of food eaten. Due to the natural complexity, processing in industries and at home, matrix influences, differing storage conditions as well as individual resorption of food, such studies are limited in their meaning. However, information obtained by a fast and cost-efficient effect-directed fingerprinting, here exemplarily developed for beers, points out for which food further studies are severely needed unless proven as irrelevant. Hence, it is a good basis and source to start thinking.

50 German dark, light, non-alcoholic and malt beers were bought on the local market (Table S1). Three commonly consumed types of beer, *i.e.* dark, light and non-alcoholic beers, were selected for multivariate data analysis. Few malt samples were also analyzed to compare their effect-directed responses with the other three types of beer. From 10 ml beer, a 1.5 ml ethyl acetate extract was prepared (enrichment factor of 6.7). For example, if 15 µl/band of this extract were applied, the response equaled to 100 µl beer. Assuming a regular and a typical beer consumption of by a factor of 2000 higher dose (200 ml drink), any effect-directed response obtained by 100 µl beer does matter.

3.1. Method development and phenolic fingerprints of beer extracts

For the first time, a chemical profiling of phenolics in German beer extracts was developed, *i.e.* on HPTLC plates silica gel 60 with methyl ethyl ketone - toluene - formic acid 5:3:0.4, V/V/V. The chromatographic system was streamlined. The 70-mm separation took 30 min and provided

a fast analysis (1.5 min/sample). After derivatization with Neu's reagent/PEG 400 and documentation at UV 366 nm, the profiling of the phenolics (primarily with vicinal hydroxyl or hydroxyl/keto groups) in beers showed differences in their chemical pattern with regard to zone intensities and the pattern itself. Dark beers showed the strongest response, and thus, contained a high amount of phenolics, while light beers showed moderate and non-alcoholic beers low responses. For example, dark beer extracts (Fig. S1, no. 1, 2, 8, 14, 19, 35, 42 and 45) showed a higher intensity of phenolic zones 2, 3 and 5, if compared to non-alcoholic beer extracts (Fig. S1, no. 27, 28, 30, 31 and 46-50). In total, seven characteristic phenolic compound zones were observed at hR_F values 6, 10, 46, 54, 60, 66 and 77 (Fig. 1A-C), whereby zone 4 was assigned to isoxanthohumol (Fig. S1, co-chromatography of the standard). Additionally, two blue bands at hR_F 33 and 38 were found in few beer extracts. For example, beer extracts no. 5, 7, 37, 40 and 45 displayed a blue fluorescent band at hR_F 33. Several dark, light and malt beer extracts revealed a hardly visible blue fluorescent band at hR_F 77 (Fig. S1, no. 1-3, 9, 11, 15, 32-34, 37, 40 and 41). Few beer extracts showed a characteristic orange band at hR_F 6, while it was an intense blue fluorescent band in other beer extracts. To conclude, zone intensities and thus quantitative differences in the phenolic profiles were evident, whereby dark beers were leading in the response of phenolics, and thus considered as interesting source for beneficial health effects.

Literature data obtained by quite different analytical techniques, such as polarography (Gorjanović, Novaković, Potkonjak, Leskosek-Cukalović & Suznjević, 2010) and HPLC-MS (Ceslová, Holcapek, Fidler, Drsticková & Lísa, 2009), showed that dark beer contained the highest, light beer a moderate and non-alcoholic beer the lowest content of phenolics. The highest contents of xanthohumol, isoxanthohumol, 8-prenylnaringenin, *iso- α* -acids were found in

dark beer, if compared to non-alcoholic and light beers (Ceslová et al., 2009). Such data were in accordance with our findings and confirmed the outcome of this newly developed method.

The developed method for fingerprinting and profiling was also suited for routine control of the beer quality over time (Figs. 2, S1 and S2). Exemplarily, the same beer (Table S1, Köstritzer samples 2, 8, 14 and 19) was bought on different dates over 8 months. Three samples were found to give similar profiles, but sample no. 19 showed a quite weaker response in the phenolic fingerprints and in the subsequently discussed DPPH[•] scavenging fingerprints. Of course, this might be explained by natural variances of the raw materials or slight variances in the brewing process.

3.2. Effect-directed fingerprints of beer extracts

Beneficial health effects of prenylflavonoids have been ascribed to the ability of phenolic compounds to scavenge free radicals and to prevent rheumatoid arthritis, diabetes, Alzheimer's and Parkinson's diseases (Ceslová et al., 2009). Prenyl groups have a marked influence on the antioxidant activities of the different prenylflavonoids containing phenol B rings, as prenylation could endow hydroxylated flavonoids with enhanced antioxidant activity (Burda & Oleszek, 2009; Ceslová et al., 2009). A moderate antioxidative activity of isoxanthohumol, *e.g.*, the ability to inhibit *in vitro* oxidation of human low-density lipoprotein (LDL), was described (Burda et al., 2001). So far, almost all activities found in beers, *e.g.* DPPH[•] scavenging activity, AChE inhibition and activities against bacteria or fungi, have been determined using photometric or electrochemical assays (Miranda et al., 2001; Natarajan, Katta, Andrei, Babu Rao Ambati, Leonida & Haas, 2008; Nair & Hunter, 2004). As these sum parameter approaches do not attract attention to individual active compounds, the developed HPTLC method was hyphenated to four

quite different assays to demonstrate the fast discovery of single DPPH[•] scavengers, Gram-negative (*A. fischeri*) and Gram-positive (*B. subtilis*) antimicrobials as well as AChE inhibitors in beers. Additionally, a recent video demonstrated the detection of oestrogen-effective compounds in beers using such a hyphenated technique (Klingelhöfer & Morlock, 2015b).

Before the use of each assay for the beer extracts, a system suitability check with a positive control was performed. Also, each chromatogram was neutralized before the assay application.

3.2.1. Radical scavenging properties of beer via HPTLC-DPPH[•] fingerprints

The antioxidative compound pattern of beer extracts and the contribution of individual phenols to the total antioxidative activity were investigated by HPTLC *in situ* combined with the DPPH[•] assay. Substances exhibiting free radical scavenging properties (bright zones on a purple background) were located between hR_F 6 and 66 (Figs. 1D and S2). One of the most dominant zones in the HPTLC-DPPH[•] fingerprints was isoxanthohumol (zone 4). Few dark beer extracts showed bright bands at the position of the previously detected phenolic compound zone 1. Dark and light beer extracts as well as a malt beer extract (Fig. S2, no. 36) showed strong antioxidative activities, mainly owed to the previously detected phenolic compound zones 4-6, while most non-alcoholic beer extracts revealed only two weak bands against the purple background. An exception was observed for some dark beer extracts (Fig. S2, no. 19, 38 and 39) that showed a lower antioxidative response similar to non-alcoholic beers. Few beer extracts (Fig. S2, no. 7, 22, 30 and 38) displayed characteristic, though weaker antioxidative zones at hR_F 33 and 38. All in all, the DPPH[•] scavenging activity of dark beer extracts differed only slightly from light beer extracts. Especially dark beer extracts displayed a comparatively higher antioxidative potential

than non-alcoholic beers. The HPTLC-DPPH[•] fingerprints (Fig. S2) assorted well with the phenolic fingerprints (Fig. S1).

3.2.2. Gram-negative antimicrobials in beer via HPTLC-A. *fischeri* fingerprints

To the best of our knowledge, there was no study dealing with the investigation of the antimicrobial properties of beer, though individual compounds, such as humulone, lupulone and xanthohumol, were reported as antimicrobial agents of hops (*H. lupulus*) (Miranda et al., 2000). Hence, for studying the antimicrobial activity of beer, two different bioautographic assays were applied. First, the beer extracts were investigated on their impact on the bioluminescence of Gram-negative *A. fischeri* bacteria (Fig. S3, depicted as greyscale image), as one of the most studied sum parameter assays (DIN EN ISO 11348-1, 2009). In almost all beers, isoxanthohumol (zone 4) and compound zone 5 showed the most intense (darkest) bands against the bioluminescent background (Figs. 1E and S3). Almost all dark and light beer samples showed a minor band for compound zone 3 (Fig. S3). Almost no response was given for malt beer extracts (Fig. S3, no. 5, 37 and 40), except for malt beer extract no. 36. Most light beer extracts (Fig. S3, no. 3, 4, 6, 7, 10-13, 16, 18, 20, 41, 43 and 44) showed moderate activity against *A. fischeri* bacteria, while some (Fig. S3, no. 15, 21-25, 32-34 and 43) exhibited comparatively stronger antimicrobial activities. This was similar for five non-alcoholic beer extracts (Fig. S3, no. 27, 28, 30, 31 and 48) that showed a low response against *A. fischeri* bacteria, while the five others showed comparatively stronger antimicrobial activities. Obviously, the beer types did not correlate with this antimicrobial effect.

3.2.3. Gram-positive antimicrobials in beer via HPTLC-B. *subtilis* fingerprints

For the second antimicrobial assay, the HPTLC chromatograms were immersed in a Gram-positive *B. subtilis* bacteria suspension (Jamshidi-Aidji et al., 2015). Any antimicrobials in the beer extracts were indicated as bright bands against a purple background (Figs. 1F and S4). The beer extracts showed none or one characteristic zone at the position of compound zone 7 with a highly varying activity against Gram-positive *B. subtilis* bacteria. The strongest antimicrobial activity showed some light and non-alcoholic beer extracts (Fig. S4, no. 6, 10, 12, 13, 23, 25, 47 and 48). In contrast, no response was obtained for extracts of all four beer types (Fig. S4, no. 4, 5, 17, 29, 32-35, 38-43, 46, 49 and 50). Obviously, the beer types did not correlate with this antimicrobial effect.

3.2.4. AChE inhibiting compounds in beer via HPTLC-AChE fingerprints

Different neurological disorders, such as Alzheimer's disease, senile dementia, ataxia and myasthenia gravis, were related to AChE activity, involved in terminating synaptic transmissions in the central nervous system, erythrocytes and cholinergic brain synapses. Thus, AChE inhibitors are meant to enhance muscle contraction and strengthen the cholinergic neurotransmission (Natarajan et al., 2008; Nair et al., 2004). After the HPTLC-AChE assay, bright zones on a pink background showed AChE inhibiting compounds (Figs. 1G and S5). One characteristic AChE inhibiting band evident in almost all beer extracts at differing intensities (more intense zones in Fig. S5, no. 7, 22, 24, 30, 32-36 and 38) originated from isoxanthohumulol (zone 4). In few beer extracts, minor AChE inhibiting bands were observed for compound zones 5 and 6 (Fig. S5, no. 32-36). To conclude, a similar AChE inhibiting potential was found in the beer extracts, except for some beers with a higher AChE response (Fig. S5, no. 7, 30 and 36).

3.3. Assignment of the multipotent compounds 3-7 by HPTLC–HRMS

For structural elucidation of the active compounds found in the beer extracts, the light beer sample no. 15 (Table S1, Franken Bräu Pilsner) was considered as a representative sample with regard to the EDA results. HPTLC was coupled to a Q Exactive Plus Orbitrap mass spectrometer, zones of interest were online eluted and the respective mass spectra recorded within a minute each. The following tentative assignments were based on HRMS data, spectral and chromatographic properties as well as literature. All prenylflavonoids with a phenyl group attached in the flavonoid backbone, which are characteristic for hops and beers (Zhao et al., 2010), followed the same fragmentation pattern and showed ions corresponding to retro-Diels Alder fragmentation in the C-ring, involving 1,3 scission in MS² spectra, as described previously for other flavonoids like naringenin (Quifer-Rada et al., 2015). Only the multipotent compound zones 3-7 were selected for the subsequent characterization, as these zones showed more than one effect-directed response, and thus, were of special interest.

The blue fluorescent active compound zone 3 at hR_F 46 showed a molecular ion at m/z 329.2333 and was assigned to be the deprotonated molecule $[M-H]^-$ of desdimethyl-octahydro-*iso*-cohumulone. Characteristic fragment ions at m/z 229.1443 and 211.1339 were obtained (Table 1, Fig. 3). This result confirmed previous data for low-alcoholic and non-alcoholic beer using ultra-performance liquid chromatography–mass spectrometry (Stevens, Taylor, Clawson & Deinzer, 1999).

The hardly visible blue fluorescent active compound zone 4 at hR_F 54 with a molecular ion at m/z 353.1394 was tentatively assigned as isoxanthohumol. Isoxanthohumol was found in almost all German beer extracts and showed a response in each assay, except for the *B. subtilis* assay. Isoxanthohumol produced several fragments at m/z 119.0511, 165.0919, 218.0583 and 233.0816,

(Table 1, Fig. 3). Our result confirmed previous findings (Zhao et al., 2010; Andrés-Iglesias, Blanco, Blanco & Montero, 2014; Haseleu & Hofmann, 2009), also at a higher amount in German and Spanish beers, while in agreement with our study xanthohumol was not detected (Zhao et al., 2010; Česlová, Holčapek, Fidler, Drštičková & Lída, 2009).

The blue fluorescent active compound zones 5 at hR_F 60 and 6 at hR_F 66 were tentatively assigned as *iso- α -ad/n-humulone* and its geometric isomer. The molecular ion at m/z 361.2020 produced characteristic fragment ions at m/z 235.1337, 265.1444 and 343.1913 (Zhao et al., 2010; Quifer-Rada et al., 2015; Andrés-Iglesias, 2014). Here, compound zone 5 produced further fragment ions at 247.1338, 223.0611 and 195.0660, while compound zone 6 had characteristic ions at m/z 292.1314 (loss of C_5H_9), 221.1545 (loss of $C_9H_{16}O$) and 179.0713 (Table 1, Fig. 3) (Zhao et al., 2010; Andrés-Iglesias, 2014; Haseleu et al., 2009). The two types of bitter acids, *i.e.* α -acids (humulones) and β -acids (lupulones), play an important role in the beer quality, organoleptic properties, bitter flavour and foam stability. During boiling of the wort, humulones isomerize into the more soluble *iso*-acids having the same molecular mass. Most common hop acids are normal (*n*-), *co*- and *ad*-homologues (Klingelhöfer et al., 2015b; Stevens et al., 1999; Andrés-Iglesias et al., 2014).

The antimicrobial compound zone 7 was tentatively assigned as *ad*- or *n*-humulone. The molecular ion at m/z 361.2017 led to the same characteristic fragment ion at m/z 292.1316, but at a higher intensity (Table 1, Fig. 3) (Zhao et al., 2010; Andrés-Iglesias et al., 2014; Česlová et al., 2009). Further fragment ions were at m/z 249.0767 and 235.1338.

3.4. Image analysis and chemometrics

Precondition for chemometrics was a reliable effect-directed profiling. ImageJ is difficult to perform due to the high number of parameters that have to be properly optimized to obtain variables. Two procedures for HPTLC image evaluation were found to be of interest for the different (bio)autograms (Masek et al., 2014; Olech et al., 2012; Ristivojević et al., 2016). The first approach used pixels along the track as variables, as previously described in literature (Fichou et al., 2016; Ristivojević et al., 2017; L. Komsta, 2012). The effect-directed fingerprint is treated as unique pattern and active compounds could be observed as peak on the line profile consisting of a series of pixels. The main advantages of this approach are simplicity and possibility of multivariate image evaluation of samples with one active band in the bioautogram; it is possible to convert one band to a series of pixels (variables). The second approach used the calculated peak areas of active bands as variables. In this case, it was possible to combine several assays, if peak areas from different assays were obtained as variables prior multivariate analysis. Specialized software for fast calculation of peak areas can be used for that (Ristivojević, Trifković, Vovk & Milojković-Opsenica, 2014).

3.4.1. Multivariate data analysis based on line plot along the track

3.4.1.1. Preprocessing techniques used for effect-directed chromatograms

Several preprocessing procedures such as denoising using median filter (3 pixels), correlation optimized warping (COW), standard normal variate (SNV) as well as autoscaling were conducted to minimize any drawbacks in the HPTLC image after detection via bacteria suspension/MTT solution, enzyme/substrate solutions or DPPH[•] reagent. In the case of bioautography, there are several sources of noises: non-uniformity of adsorbent particles, derivatization by living organisms, slight damages of the stationary phase during neutralization

or drying, imperfection of the detection (*e.g.*, fluctuations during instrumental measurement and variation of parameters). An aggressive denoising could distort the analytical signal, whereas a minor denoising would remove only a small part of the noise (Fichou et al., 2016; Ristivojević et al., 2016).

In HPTLC, slight hR_F differences of bands may be caused, *e.g.*, by vapour phase fluctuations during development, local change of the stationary phase through vapour deposition, inefficient drying of application zones, inconsistent humidity, temperature variations and human errors. Hence, warping techniques were developed to mitigate such band shifts, of which COW is the most recommended. Normalization was applied to compensate for any interference by different sample volumes applied on the HPTLC plate. It is recommended to compare the results with and without normalization via, *e.g.*, the commonly applied SNV. Scaling is an important step that divides each variable by a scaling factor, which is different for each variable. After the autoscaling procedure, all variables become equally important (Fichou et al., 2016; Ristivojević et al., 2016; Komsta, 2012; Ristivojević et al., 2017). In case of (bio)autograms, we found mandatory the application of the baseline correction procedure (weighted least squares) to keep the random variance of the baseline smaller than that of the (bio)autograms (Komsta, 2012; Ristivojević et al., 2017). The mentioned procedures were combined to choose the best PCA model. In case of assays with one active band (*B. subtilis* bioassay and AChE assay), just one compound had influence on the model.

3.4.1.2. Principal components (PCs) identified for effect-directed chromatograms

PCA is a commonly used variable reduction method and reduces a multidimensional data set into the 2D or 3D space. The score plot is a map of the samples (objects) and shows their distribution

for the calculated PCs (Fichou et al., 2016; Ristivojević et al., 2016). The loading plot depicts the identification of important variables, and the three PCs indicate whether samples are similar, dissimilar, typical or represent an outlier. In the case of the fingerprints of the HPTLC-DPPH[•] assay, PC1 accounted for 85.35%, PC2 for 5.05%, PC3 for 2.73% and PC 4 for 2.14% of the total variance (Fig. 4A). Two preprocessing techniques such as COW and mean centering were chosen to improve the multivariate model. Non-alcoholic beer extracts formed one cluster on the right side of the PC score plot, while almost all dark beer extracts were positioned on the lower left side of the PC score plot, except for two dark beer extracts (Table S1, no. 38 and 39). Light beer extracts did overlap with dark beer extracts because of their similar DPPH[•] response. PC1 and PC2 were highly influenced by compound zone 1, desdimethyl-octahydro-*iso*-cohumulone and the compound zone at hR_F 38, whereas PC4 was positively linked to compound zone 1, desdimethyl-octahydro-*iso*-cohumulone and isoxanthohumol (Fig. S6).

In the case of the fingerprints of the *B. subtilis* bioassay, *n*-humulone was recognized as a characteristic marker with potential Gram-positive antimicrobial activity. After baseline correction, COW, SNV and autoscaling, PCA resulted in a five-component model that explained 86.23% of the total variance. The beer extracts were projected along three PC directions, *i.e.* PC1 plotted against PC2 vs. PC5 (Fig. 4). Via this PC1-PC2-PC5 scatter point plot, the separation of the dark and non-alcoholic beer extracts was obvious; while two dark beer extracts did overlap with non-alcoholic beer extracts. Light beer extracts were positioned between non-alcoholic and dark beer extracts. There is no clear separation between the beer types because of the similar bioautographic pattern (Fig. 4B).

In the case of the fingerprints of the *A. fischeri* bioassay, PCs 1-5 accounted for 33.49%, 22.96%, 13.32%, 9.40%, and 4.24% of the total variance, respectively. Several preprocessing techniques

such as COW, baseline correction (Weighted Least Squares), and autoscaling significantly enhanced the separation between three types of beer. In the score plot of PC1 against PC 2 vs. PC5, the Gram-negative *A. fischeri* bioassay showed a good separation between the dark and non-alcoholic beer extracts, however, light beer extracts misclassified with both types (Fig. 4C). Desdimethyl-octahydro-*iso*-cohumulone and/or isoxanthohumol had negative correlations with PC1, PC2 and PC5 (Fig. S7).

According to the Eigenvalues of the correlation matrix and AChE inhibiting compounds in the beer extracts, the first three PCs described 98.48%, 0.82% and 0.25% of the total variance, while PC4 did 0.12 % (Fig. 4D). In this case, 50 pixels were chosen for the PCA model, instead of 128 pixels for the previous assays. Autoscaling was used as preprocessing technique to improve the PCA model. All beer extracts seemed to be grouped into two patterns. Pattern 1 included non-alcoholic beer extracts, while light and dark beer extracts were not distinguishable (Fig. 4D). Isoxanthohumol was identified as important phenolic marker for the discrimination of beer extracts according to the AChE assay (high loading values along PC1 and not close to the origin).

To conclude, all PCA models based on single effect-directed fingerprints achieved a clustering and differentiation between dark and non-alcoholic beer extracts, while light beer extracts did overlap. The clustering was based on qualitative and especially quantitative differences in the profiles between the three types of beer.

3.4.2. Multivariate data analysis based on peak areas

In the second approach, the peak areas of active zones of effect-directed fingerprints were calculated using ImageJ and used as variables for the PCA modelling. In the case of the

combination of the fingerprints of all (bio)assays, a five-component model explained 95.25% of the total data variability. PC1 described 43.26% of the total variation, whereas PC2 and PC3 contributed to 23.40%, and 15.56%, as well as PC4 7.49% of the total variability, respectively. This is illustrated in a 3D-score value plot and the projection of loading vectors for the three PCs (Fig. 5A and B). Similar as in the above mentioned PCA model, the Gram-positive antimicrobial activity of *ad*- or *n*-humulone as well as the AChE inhibiting and antioxidative activity of isoxanthohumol did contribute to the separation between dark and non-alcoholic beer extracts. The main advantage of this approach was the possibility of combining all activities to identify and cluster the best health-related beers with the most bioactive compounds and thus beneficial impact. Also the DPPH[•] assay fingerprints were found to be appropriate as data set for the second approach because of four recognized compounds with antioxidative properties used as variables (Fig. 5C and D). Mutual projections of PC scores for PC 1 against PC2 vs. PC4 reached the best classification results. The percent cumulative contribution of the variance of the first four PCs was 100.00%, wherein PC1, PC2 and PC4 accounted for 56.22%, 28.29% and 5.21% of the variability, respectively. The 3D PC1-PC2-PC4 score plot showed a good differentiation between the dark and non-alcoholic beer extracts, while light beer extracts did overlap. Isoxanthohumol (zone 4) and *iso-α-ad/n*-humulone and its isomer (zones 5 and 6) showed the highest influence on the separation between dark and non-alcoholic beers. Non-alcoholic beer extracts were clustered best here, as they showed a lower response against DPPH[•] than dark and light beer extracts.

4. Conclusion

For the first time, effect-directed fingerprints were investigated for image processing and multivariate data analysis to evaluate their potential for clustering and classification of samples with regard to beneficial activities. As the developed method used the same sample preparation for all 50 beers and the same sample application volume, the resulting effect-directed fingerprints of the beer extracts could directly be compared not only visually, but also chemometrically. It allowed the assessment of beers with regard to potential health-promoting activities. Isoxanthohumol (zone 4) as a main phenolic beer component showed the widest spectrum of activities, *i.e.* DPPH[•] scavenging and AChE inhibiting activities as well activity against Gram-negative *A. fischeri* bacteria, while *ad*- or *n*-humulones (zone 7) were only active against Gram-positive *B. subtilis* bacteria and desdimethyl-octahydro-*iso*-cohumulone (zone 3) against Gram-negative *A. fischeri* bacteria. *Iso- α -*n*/*ad*-humulone (zones 5 and 6) and its isomer showed DPPH[•] scavenging activity and antimicrobial activity against Gram-negative *A. fischeri* bacteria. Such effect-directed fingerprints combined with multivariate data analysis succeeded in classification and identification of active marker compounds in a high number of samples. The two different approaches investigated were successful for classification and differentiating of beer types. The first approach was fast, simple and appropriate if the samples contained one active compound, while the second was appropriate for more than four active compounds. The clustering approach that combined all activities using multivariate data analysis based on peak areas also showed the potential to identify and cluster the beers with regard to health-related activities, and thus, to find beers with the most beneficial potential. Finally, the method was also suited for quality control of beer.*

Acknowledgment

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. List of multipotent active compound zones, along with their hR_F value, calculated mass, accurate mass, mass error, fragments, calculated sum formula as well as tentative assignment.

Zone	hR_F	Calculated mass [M-H] ⁻	Accurate mass [M-H] ⁻	Mass error (mDa)	Fragments	Sum formula	Tentative assignment	Reference
3	46	329.2328	329.2333	0.5	229.1443, 211.1339	C ₁₈ H ₃₃ O ₅	Desdimethyl-octahydro- <i>iso</i> -cohumulone	Stevens et al., 1999
4	54	353.1394	353.1394	0.0	233.0816, 218.0583, 165.0919, 119.0511	C ₂₁ H ₂₁ O ₅	Isoxanthohumol	Zhao et al., 2010 Andrés-Iglesias et al., 2014 Haseleu et al., 2009
5	60	361.2020	361.2022	0.2	343.1913, 265.1444, 247.1338, 235.1337, 223.0611, 195.0660	C ₂₁ H ₂₉ O ₅	<i>Iso-α-n</i> /ad-humulone	Zhao et al., 2010 Andrés-Iglesias et al., 2014 Haseleu et al., 2009
6	66	361.2020	361.2020	0.0	343.1914, 265.1444, 235.1337, 292.1314, 221.1545, 179.0713	C ₂₁ H ₂₉ O ₅	<i>Iso-α-n</i> /ad-humulone (or isomer)	Zhao et al., 2010 Andrés-Iglesias et al., 2014 Haseleu et al., 2009
7	77	361.2020	361.2017	0.3	292.1316, 249.0767, 235.1338	C ₂₁ H ₂₉ O ₅	<i>n</i> -humulone or ad-humulone (<i>n</i> -humulone isomer)	Zhao et al., 2010 Andrés-Iglesias et al., 2014

List of figures

Fig. 1. HPTLC fingerprints of four types of German beer extracts showing (A) the phenolic profile at UV 366 nm (15 μ l/band applied), (B) after derivatization with Neu's reagent/PEG solution, (C) after neutralization with phosphate/citrate buffer (proof of any zone shift) as well as (D) after DPPH[•] assay (6 μ l/band applied), (E) after *A. fischeri* bioassay (5 μ l/band applied), (F) after *B. subtilis* bioassay (2 μ l/band applied) and (G) after AChE assay (5 μ l/band applied).

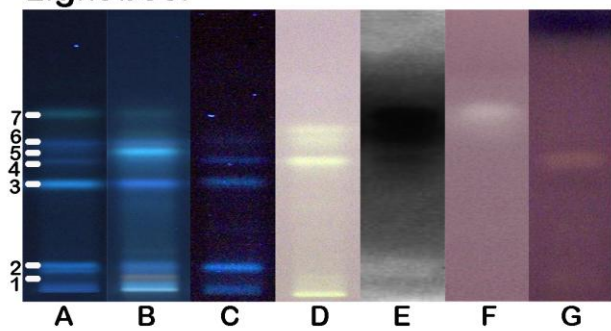
Fig. 2. HPTLC fingerprints of 50 German beer extracts showing (A) the phenolic profile at UV 366 nm (15 μ l/band applied), (B) after DPPH[•] assay (6 μ l/band applied), (C) after *A. fischeri* bioassay (5 μ l/band applied), (D) after *B. subtilis* bioassay (2 μ l/band applied) and (E) after AChE assay (5 μ l/band applied).

Fig. 3. HPTLC-HRMS mass signals of multipotent compounds 3-7 in light beer sample no. 15 (Table S1, Franken Bräu Pilsner, 14 μ l/band applied).

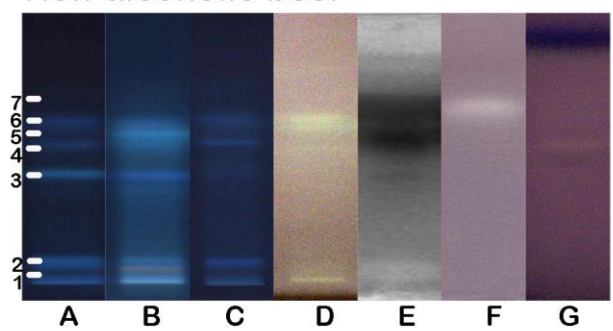
Fig. 4. 3D PCA score plots based on (A) DPPH[•] assay, (B) *B. subtilis* bioassay (C) *A. fischeri* bioassay and (D) AChE assay.

Fig. 5. 3D PCA score plots and loading plots based on (A, B) all bioassays and (C, D) DPPH[•] assay.

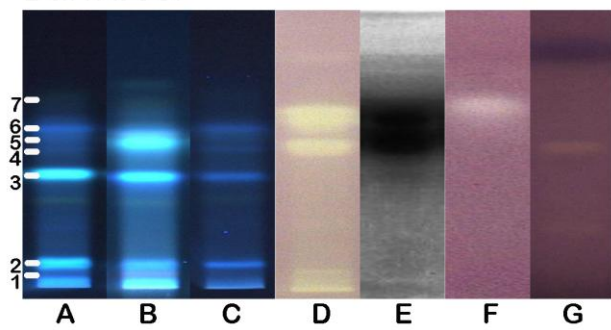
Light beer



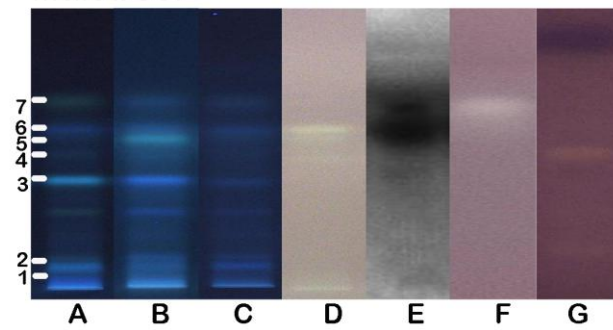
Non-alcoholic beer

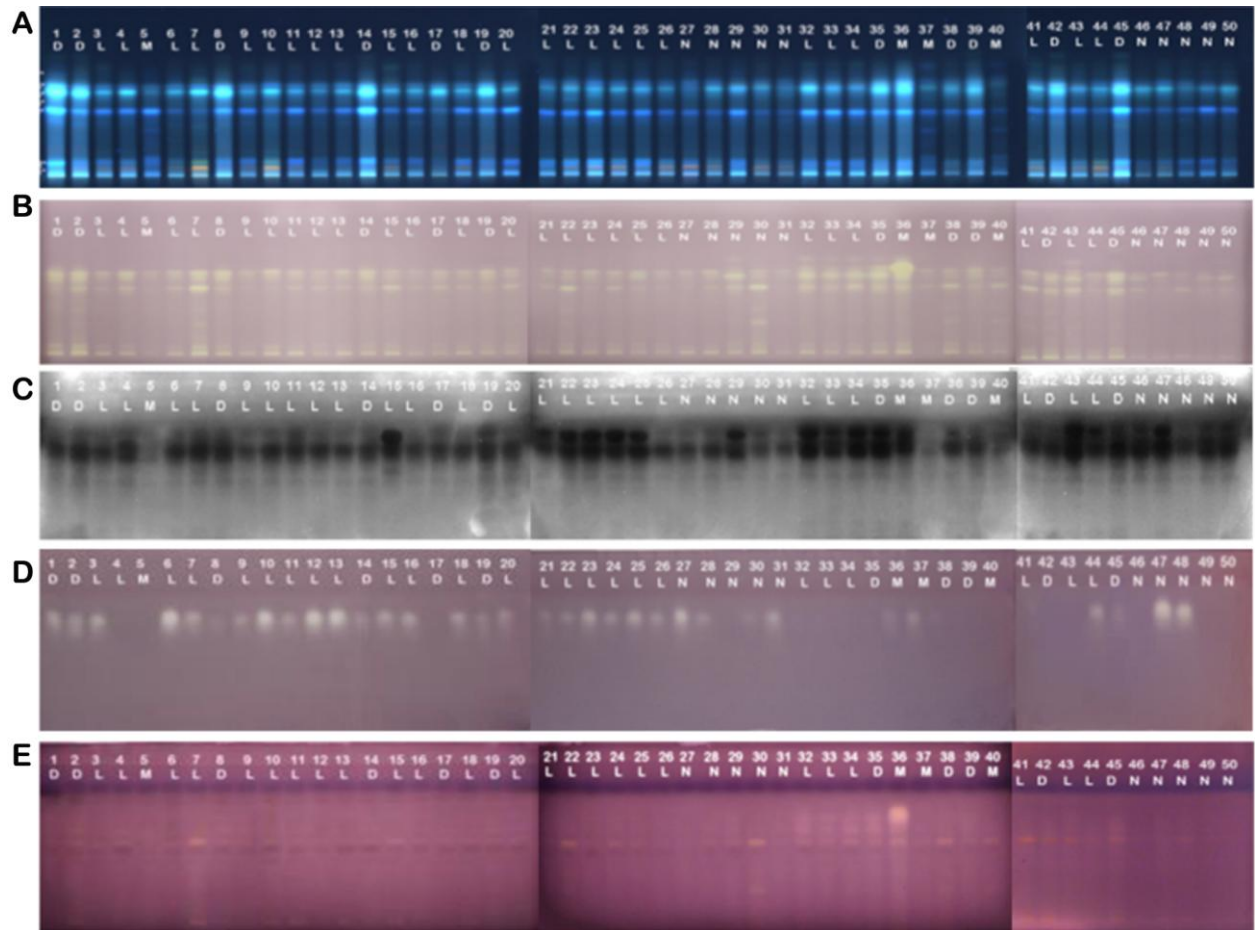


Dark beer

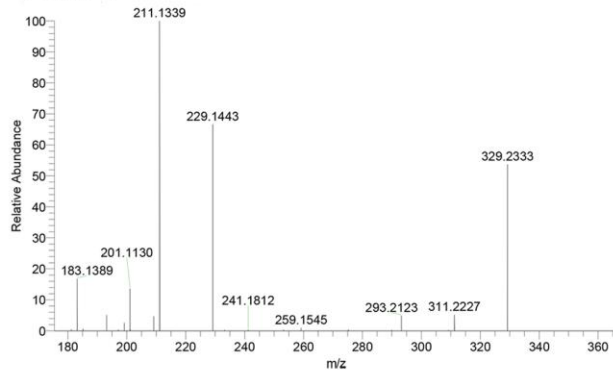


Malt beer

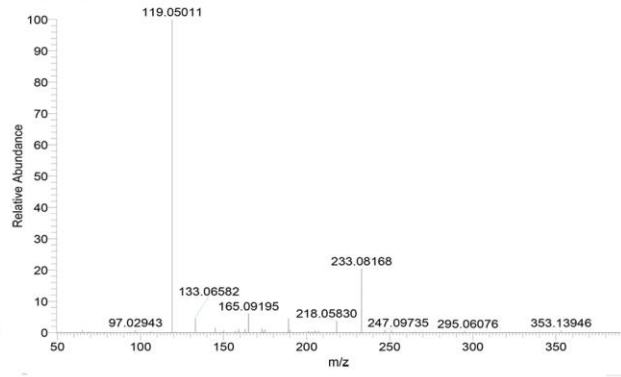




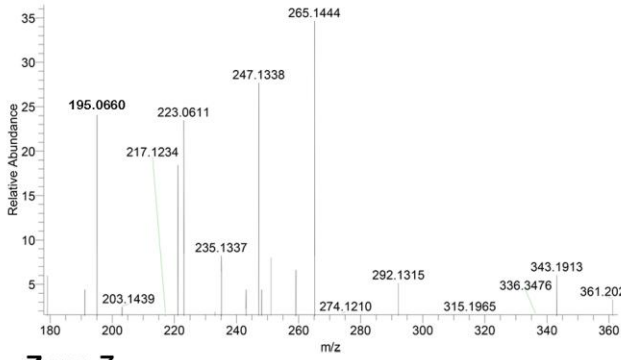
Zone 3



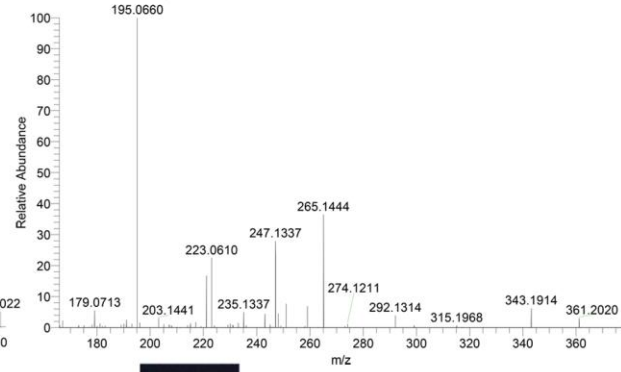
Zone 4



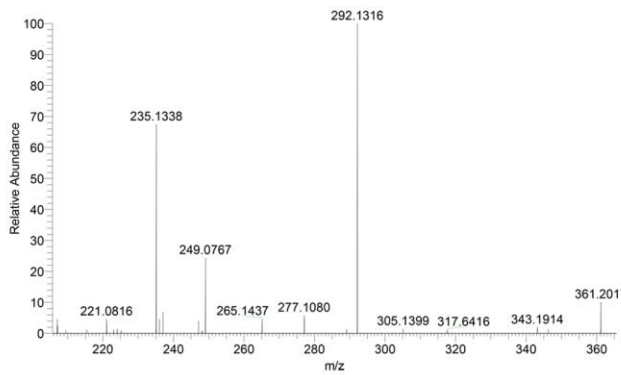
Zone 5



Zone 6



Zone 7

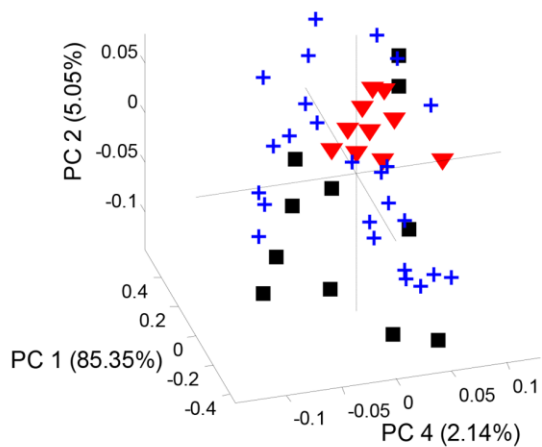


Multipotent compound zones 3-7:

- 7 *Ad-* or *n*-humulone
- 6 *Iso-α-ad/n*-humulone
- 5 and isomer
- 4 Isoxanthohumol
- 3 Desdimethyl-octahydro-*iso*-cohumulone

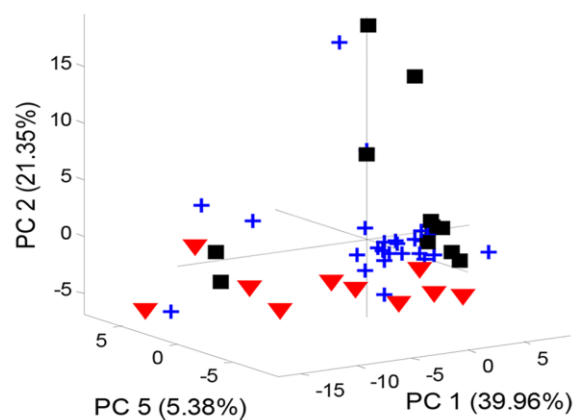
- 2
- 1

A HPTLC-DPPH[•] assay

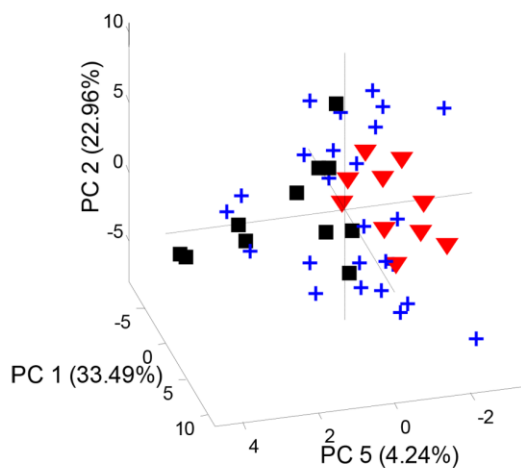


B HPTLC-*B. subtilis* bioassay

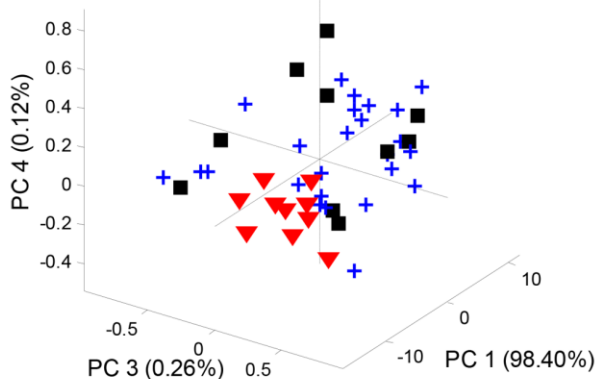
▼ Non-alcoholic beer
■ Dark beer
+ Light beer

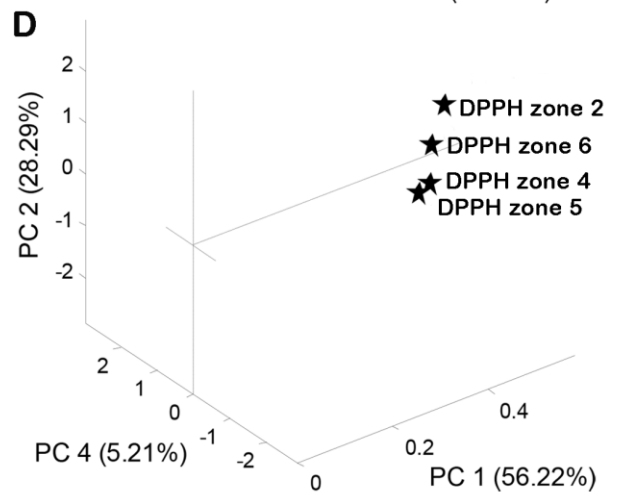
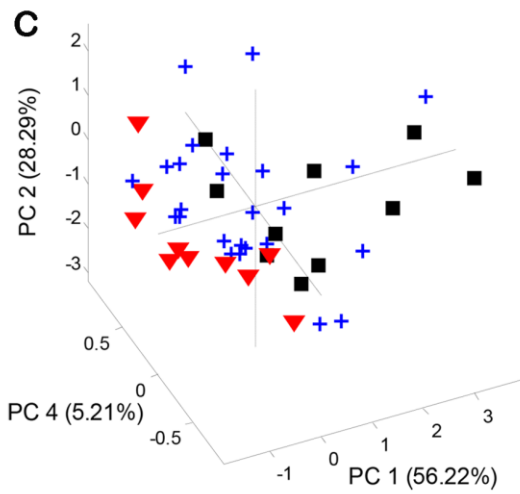
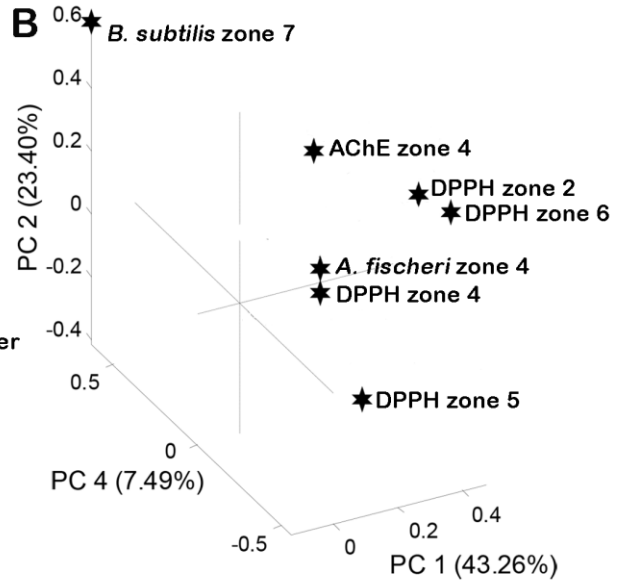
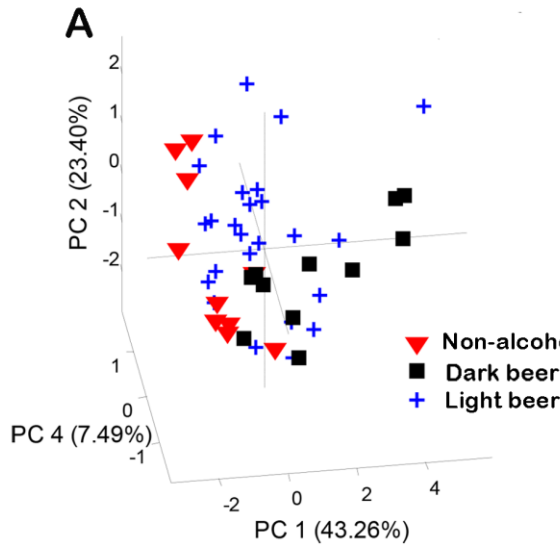


C HPTLC-*A. fischeri* bioassay



D HPTLC-AChE assay





Highlights

- Fast classification of food with regard to health-related potential is made possible.
- Profiling of 50 beers was combined with four effect-directed assays and HPTLC-HRMS/MS²
- For the first time effect-directed classification is based on biological and biochemical fingerprints.
- HPTLC method for analysis of phenolic compounds in beer is newly developed.
- For the first time reliable pattern recognition and clustering of bioautograms is performed.