Phenolic Fingerprints and Quality Assessment of Three Types of Beer

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German beer
Quality assessment
Phenolic fingerprint
High-performance thin-layer chromatography–2,2-diphenyl-1-picrylhydrazyl assay
Principal component analysis

Summary

The quality of three types of beer (dark, light and non-alcoholic) was assessed using high-performance thin-layer chromatography (HPTLC) combined with high-resolution mass spectrometry and chemometrics. An HPTLC separation of the polar beer components in the ethyl acetate extract was developed. The polar components were detected either by the *in situ* 2,2-diphenyl-1-picrylhydrazyl (DPPH*) assay or by derivatization with the Neu's reagent, followed by the PEG solution. This directly allowed the visual comparison and evaluation of the phenolic/flavonoid or radical scavenging (antioxidative) beer profile. Although the three types of beer showed a very similar chemical HPTLC pattern, the signal intensities were different. Detected by the Neu's reagent, the dark beer extracts contained a high amount of phenolic compounds, and the light beer extracts showed a moderate content, while the non-alcoholic beer extracts had the lowest phenolic content. The HPTLC–DPPH* assay confirmed the higher radical scavenging activity of dark beer extracts, if compared to light and non-alcoholic beer extracts. The most active bands with regard to the radical scavenging property were identified to be desdimethyl-octahydro-*iso*-cohumulone and *iso*-*n*/ad-humulone. The use of pattern recognition techniques showed a clear differentiation between dark and non-alcoholic beer extracts, while light beer extracts did overlap with both beer types. This HPTLC screening allowed the (1) direct comparison of beer samples/types *via* classification and pattern recognition, (2) the assessment of the beer quality with regard to its antioxidative potential, and (3) the reference to single components.

1 Introduction

Beer is a widely and frequently consumed, fermented alcoholic beverage in Europe, traditionally produced *via* water, malt, hop, and yeasts. Craft beer sorts caught attention due to novel brewing processes, new aromatic sorts of hops, higher alcohol contents, specific organoleptic properties (with regard to savor, flavor, aroma, *etc.*), and more pronounced haptic characteristics of the foam to mention a few. Changes in the brewing process do also change the chemical quality of the resulting beer. Not only the quality control during the processing is very important to achieve a constantly high beer quality, but also the quality assessment of the resulting beer product, especially under the perspective of varying (more flexible) brewing processes. Beer has a complex chemical composition, consisting of carbohydrates (*e.g.,* glucose, maltose, maltotriose, and dextrins), proteins and amino acids, and B-group vitamins as well as phenolic compounds to mention a few [1, 2]. On the one side, beer exhibits positive physiological properties such as antioxidative, antibacterial, anticancer, and antidiabetic activities, for example, due to the presence of phenolic compounds, such as prenylflavonoids, flavonoids, and phenolic acids. On the other side, there are health risks associated with the alcohol consumption for heavy drinkers, individuals with increased heart activity, teenagers, car drivers, pregnant, breast-feeding women, *etc.* [1–4].

The antioxidative activity related to the content of phenolics is an important aspect of the beer quality [3–6]. Spectroscopic and electroanalytical methods investigating the radical scavenging activity of food and natural products do not allow the assignment of individual antioxidative compounds and their contribution to the total activity. The time- and solvent-consuming conventional procedures for the isolation of active compounds can be avoided, if high-performance thin-layer chromatography (HPTLC) is directly combined with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*) assay. Directly after immersion of the HPTLC chromatogram in the DPPH* solution, radical scavenging compounds are detectable as yellow bands on a purple plate background [7–9]. In particular, HPTLC as a simple, low-cost, and high-throughput technique

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provides the analysis of more than 20 samples simultaneously under the same conditions. Other analytical techniques, such as high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC–HRMS) [2, 4], have been applied for the investigation of the phenolic profiles of beers and hops (*Humulus lupulus* L.) as well as Fourier transform infrared spectroscopy [5] and nuclear magnetic resonance [1] for the structure elucidation of target compounds. In our previous study, we applied diverse effect-directed assays *in situ* the chromatogram for identification of radical scavenging, antimicrobial and enzyme inhibiting compounds in 50 German beer extracts and classified them based on these beneficial effects for the first time. After online elution *via* an elution head-based interface, multipotent active zones such as isoxanthohumol were identified by high-resolution tandem mass spectrometry (HRMS/MS^2) [10]. However, it was evident that some polar compounds did remain at the start zone. In continuation of this research, the separation of the polar compounds was investigated in more detail. A simple HPTLC method for the control and assessment of the beer quality with regard to the phenolic content and, thus, health benefit was developed. HPTLC was combined with HRMS/MS^2 and chemometrics to figure out relevant compounds.

2 Experimental

2.1 Chemicals and Materials

Solvents used were of analytical grade. Acetic acid (100%), formic acid (96%), methanol, polyethylene glycol (PEG), 2-aminoethyl diphenylborinate (97%, Fluka), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*, 97%) were supplied by Sigma–Aldrich (Steinheim, Germany), while ethyl acetate and HPTLC plates silica gel 60 F_{254} (20 cm × 10 cm) were from Merck (Darmstadt, Germany).

2.2 Sample Preparation

Eleven dark beers, 25 light beers, and 10 non-alcoholic beers, plus 4 malt beers (No. 5, 36, 37 and 40) were purchased *via* a discounter in Giessen, Germany (Table S1 of Ref. [10]) and prepared as described in our previous study [10]. Briefly, 10 mL beer were extracted with ethyl acetate, concentrated to dryness, and taken up in 1.5 mL methanol.

2.3 HPTLC with Fluorescence Detection (HPLTC–FLD) and HPTLC–DPPH*

The beer extracts (15 µL each, or 6 µL for DPPH* assay [10]) were applied as 6 mm bands on the HPTLC plate silica gel 60 (20 cm × 10 cm) using the an automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland) with a dosage speed of 150 nL s^{-1}. The first sample was applied at a distance of 12 mm from the left side and 10 mm from the bottom side, with a track distance of 9.2 mm (20 samples per run). A mixture (10 mL) of ethyl acetate–formic acid–acetic acid–water 10:1.1:1.1:2.6 (*V*/*V*) was used as the mobile phase. After saturation with the mobile phase for 10 min in a twin-trough chamber (20 cm × 10 cm; CAMAG), the developing distance was 65 mm from the lower plate edge, followed by drying for 3 min with a hairdryer. By immersion using the TLC Immersion Device III (CAMAG; immersion time, 2 s; immersion speed, 4.5 cm s^{-1}), the chromatograms were derivatized with a (1) 0.5% methanolic solution of 2-aminoethyl diphenylborinate (natural product reagent or Neu's reagent), followed by immersion in a 5% methanolic polyethylene glycol (PEG) 400 solution for enhancement and stabilization of the fluorescent zones, or (2) 0.2% methanolic DPPH* solution. The obtained DPPH* chromatograms were placed in a vertical position to remove the excess of solution and dried at ambient temperature in the darkness for 30 s and then in a stream of warm air (hair dryer) for 5 min. The plate image was captured after 2 min [8, 10]. Documentation, also as JPEG files, was performed at white light illumination and UV 366 nm using the TLC Visualizer (CAMAG).

2.4 HPTLC–HRMS/MS^2 Analysis

A volume of 30 µL of sample 14 (dark beer) was applied and developed for MS analysis. Bands of interest were marked at UV 366 nm and eluted *via* the PlateExpress interface (Advion, Ithaca, NY, USA) or TLC–MS Interface 2 (CAMAG) to the HESI source of the Q Exactive Plus (ThermoFisher Scientific, Waltham, MA, USA). Experimental parameters were set as described [10].

2.5 Image Analysis and Multivariate Analysis

Using the PLS Toolbox software package (Eigenvector Research, Inc., Manson, WA, USA) for MATLAB (Version 7.12.0 R2011a), principal component analysis (PCA) was carried out as an exploratory data analysis by employing a singular value decomposition algorithm (SVD) and a 95% confidence level for Q and T^2 Hotelling limits for outliers.

3 Results and Discussion

3.1 Phenolic Profiles of German Beer Extracts by HPTLC–FLD

Some polar compounds remained at the start zone, when using the solvent mixture of methyl ethyl ketone, toluene, and formic acid (5:3:0.4), as described in our previous study [10]. Hence, a much more polar and more acidic mobile phase was developed. Finally, all beer samples were screened for these polar compounds with ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6). The flavonoid/phenolic compounds therein were derivatized with the Neu's reagent, followed by immersion in the PEG solution for zone enhancement/stabilization. The resulting chemical HPTLC fingerprints showed a similar phenolic pattern among the dark, light, and non-alcoholic beer extracts **(Figure 1).** It was also similar for the 4 malt beer samples, which were included in the study for comparison. Two pronounced blue fluorescent bands at hR_F 59 and 85 were observed at UV 366 nm for dark beers. In between these two blue zones, some brands of light and non-alcoholic beers (3, 4, 6, 7, 10, 15, 18, 41, 43 and 44) contained an orange (hR_F 64) and a yellowish olive fluorescent band (hR_F 70) at differing intensities. Altogether, up to 9 phenolic compound zones were observed at hR_F values 31, 36, 39, 46, 52, 59, 72, 85, and 95, exemplarily depicted for beer sample No. 14 **(Figure 2A).** Thus, in comparison to our previous study [10], two further

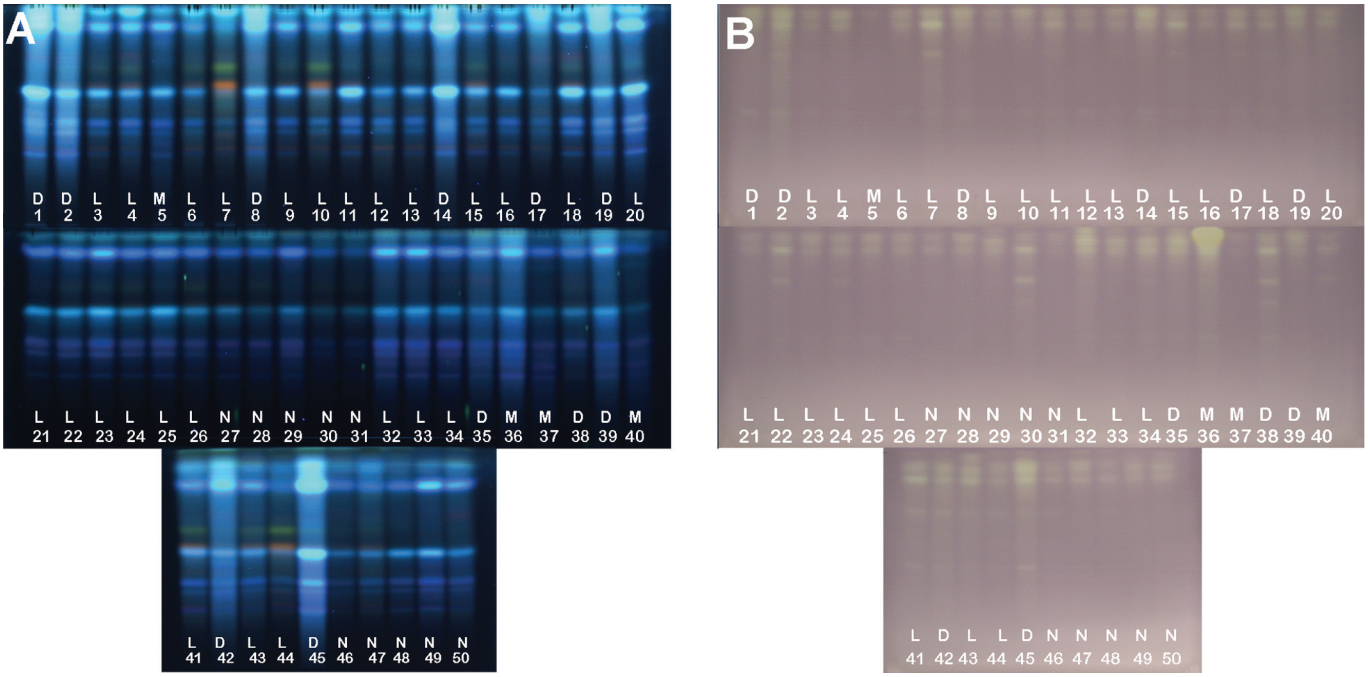

Figure 1

Phenolic fingerprints of German beer samples (light beer: L, dark beer: D, malt beer: M, non-alcoholic beer: N) *via* Neu's reagent/PEG solution (A) and DPPH* assay (B).

phenolic compound zones were observed. In agreement with our previous study and further literature [10–13, 15], dark beers contained a higher amount of phenols, while light and non-alcoholic beers had a lower amount of phenols. That could be explained by the different sorts of the kilned malt used, the different fermentation times, the different yeast strains employed in brewing alcohol-free beers, or losses brought about by the dealcoholisation process to mention a few [14].

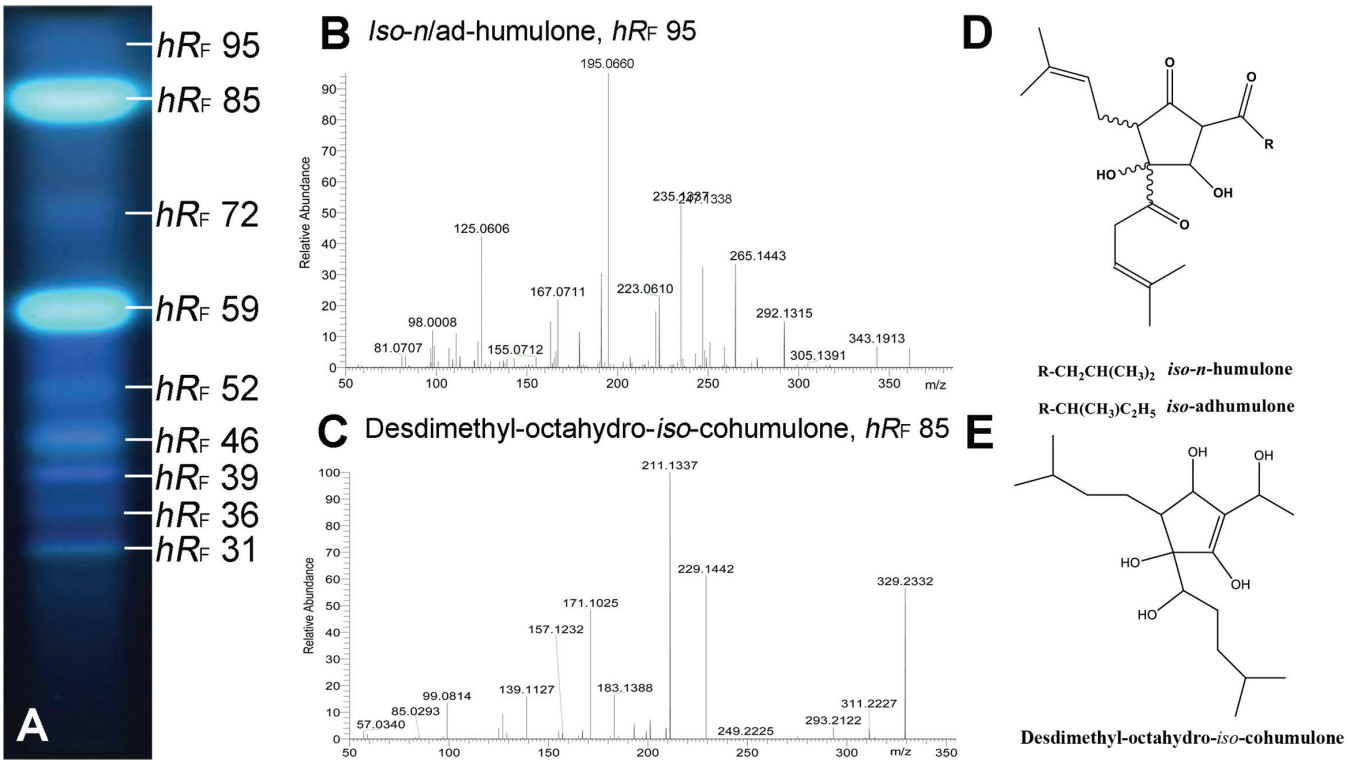

Figure 2

Phenolic fingerprint of dark beer sample No. 14 with nine separated polar compounds marked (A), HPTLC–HRMS/MS² spectra of the most pronounced zones *iso-n*/ad-humulone (B) and desdimethyl-octahydro-*iso*-cohumulone (C), and structures of *iso-n*/ad-humulone (D) and desdimethyl-octahydro-*iso*-cohumulone (E).

3.2 Radical Scavengers in German Beer Extracts by HPTLC–DPPH* and HPTLC–HRMS/MS²

Based on the obtained HPTLC–DPPH* image, bright bands observed on the violet background indicated compounds with radical scavenging effects. The dark beer extracts displayed the highest, and the light or non-alcoholic beer extracts exhibited a lower radical scavenging capacity. The active bands at hR_F 85 and 95 were recognized as the main antioxidants of the beer extracts (Figure 1). Based on HPTLC–HRMS/MS² measurements, the active zone at hR_F 95 showed a molecular ion at *m/z* 361.2014 and was assigned to be *iso*-*n*/ad-humulone, as MS² spectra showed several characteristic signals at *m/z* 343.1913, 265.1443, 247.1338, 223.0610, and 195.0660 [2, 10] **(Figure 2B).** The major active zone at hR_F 85 was confirmed to be desdimethyl-octahydro-*iso*-cohumulone with a molecular ion at *m/z* 329.2333 $[M - H]^-$, and two characteristic ions at *m/z* 229.1443 and *m/z* 211.1339 **(Figure 2C)** [2, 10]. These two compounds were identified as the main compounds in beer from Germany [10] and Spain [2]. Two further bands at hR_F 52 and 59 showed a lower radical scavenging activity against the purple background (Figure 1). Also, based on PCA models, desdimethyl-octahydro-*iso*-cohumulone was recognized as a marker for non-alcoholic and regular beers from Spain [2], which is in agreement with our study. Dark beer extracts displayed a strong antioxidative activity, while light and non-alcoholic beer extracts exhibited lower antioxidative activity, which is in agreement with our previous study [10]. Also, higher alcohol contents in beers (listed in Table S1 of [10]) correlated with a higher antioxidative capacity [12, 14, 15].

3.3 Multivariate Data Analysis Based on the Phenolic Pattern

PCA was applied to find a chemical pattern for differentiation of the 3 beer types and to identify the compounds responsible for discrimination between the samples [16]. The first principal component (PC1) accounted for 42.19% of the total variability, while PC3 accounted for 10.67%. Three types of German beer were grouped based on the chemical pattern: there is a clear difference between dark and non-alcoholic beer samples, while light beer samples did overlap with dark and non-alcoholic beers **(Figure 3A)**. The loading plot displayed relationships between variables and was used to identify variables that contributed to the positioning of the objects on the scores plot. The loading plots revealed that

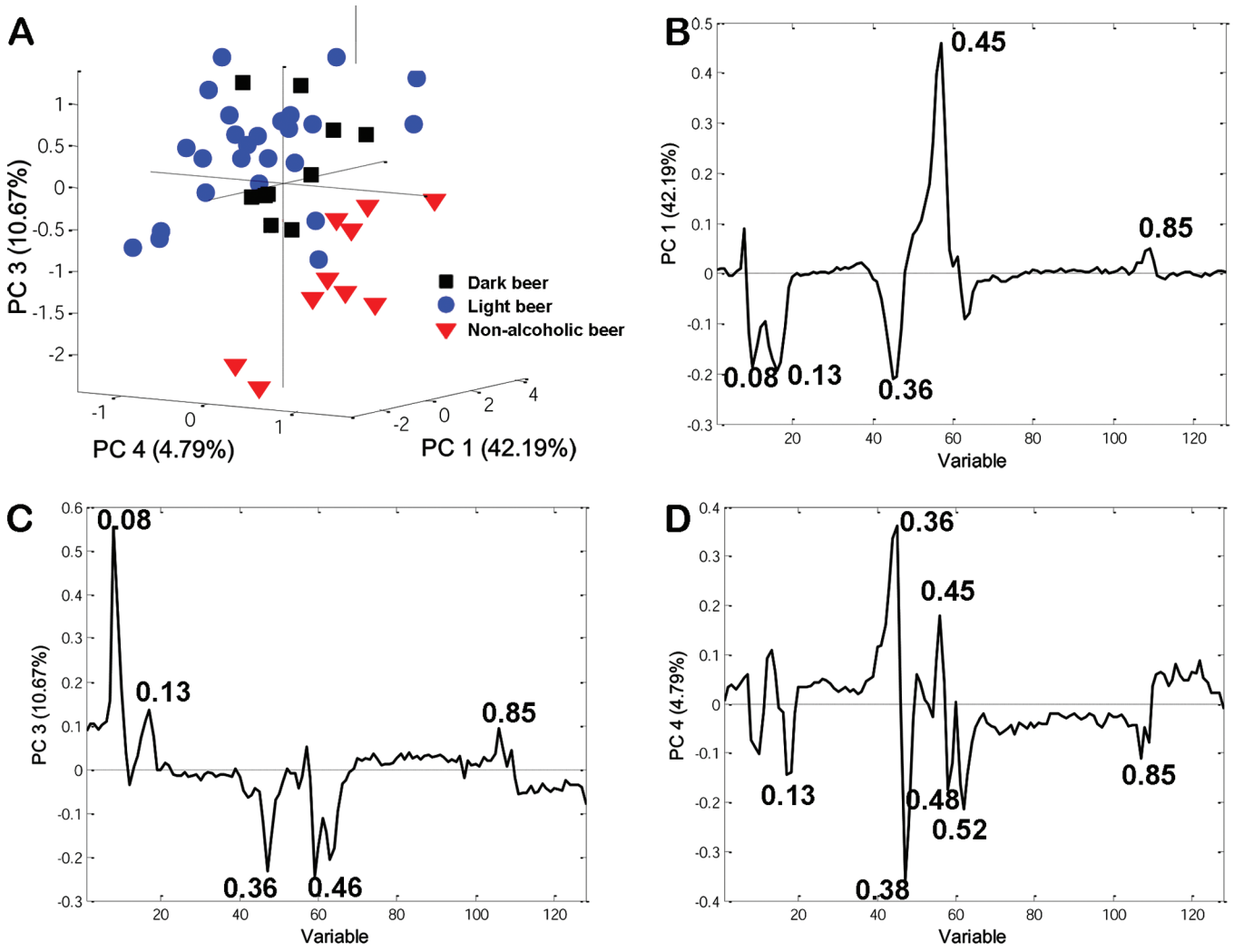

Figure 3

PCA of German beer samples after derivatization of the HPTLC chromatogram with Neu's reagent/PEG solution showing the PC score plots (A) and loading plots based on PC1 (B), PC3 (C), and PC4 (D).

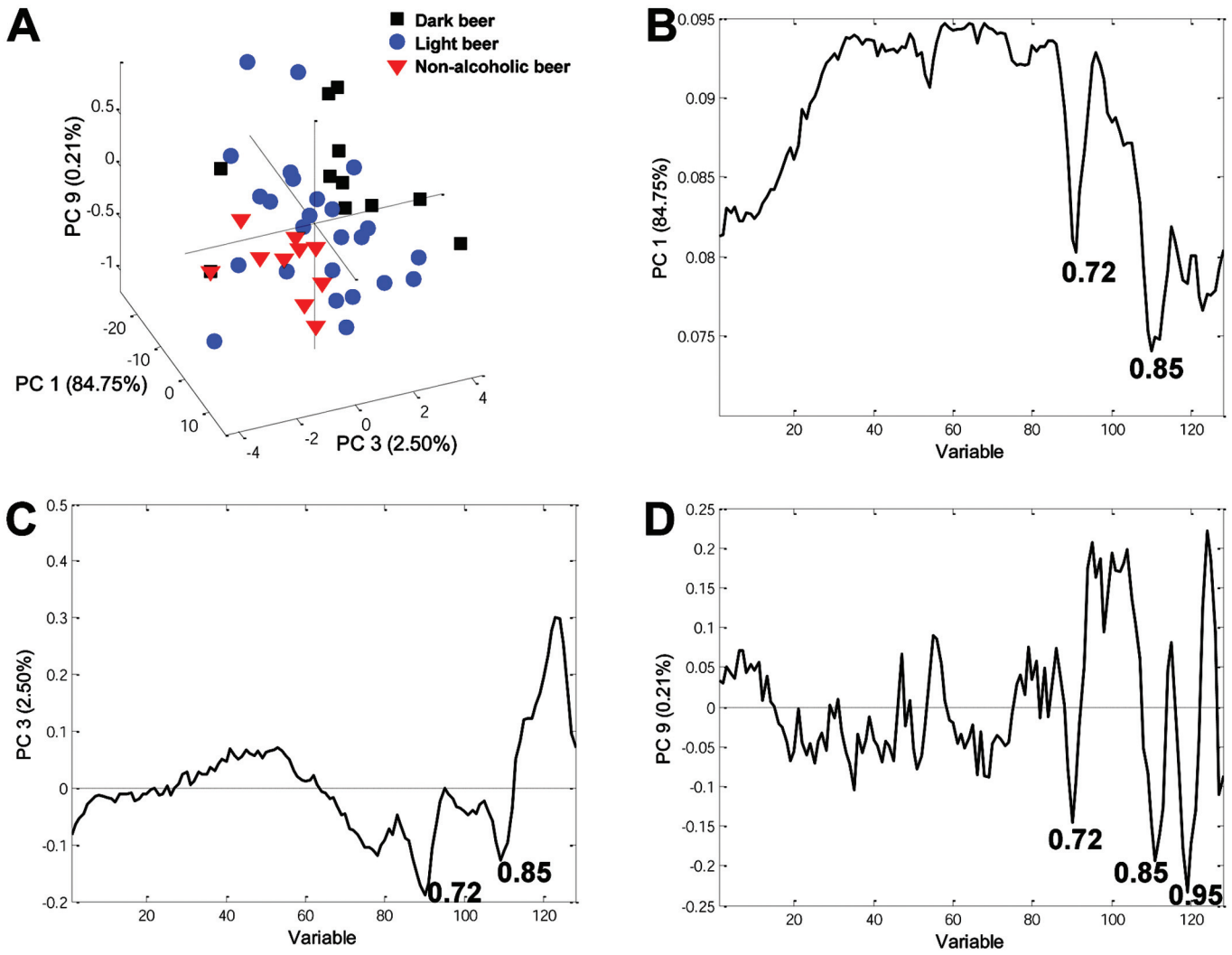

Figure 4

PCA of German beer samples after HPTLC-DPPH* assay showing the PC score plots (A) and loading plots based on PC1 (B), PC3 (C), and PC9 (D).

the bands at hR_F values of 8, 13, 36, 45, 52, and 85 (desdimethyl-octahydro-*iso*-cohumulone) had the most impact on the principal component (PC) direction and differentiation of the beer samples **(Figures 3B–D).** These zones were recognized as markers for discrimination between dark and non-alcoholic beers.

3.4 Multivariate Data Analysis Based on Radical Scavenging Potential

For the radical scavenging activity, PC1, PC3, and PC9 accounted for 84.75%, 2.50%, and 0.21% of the total variance, respectively. Non-alcoholic and dark beers formed two clearly separated groups of beer samples, while light beer samples were positioned between these two types of beer **(Figure 4A).** Dark beers showed a radical scavenging (antioxidative) pattern of higher intensity, while non-alcoholic beers contained a comparatively lower content of radical scavengers. The correlation optimized warping and autoscaling were applied to improve the multivariate model. The visual inspection of the eigenvectors confirms that phenols at hR_F 72, as well as hR_F 85 (desdimethyl-octahydro-*iso*-cohumulone) and hR_F 95 (*iso-n*/ad-humulone) were important variables in the differentiation of the 3 beer types with regard to the radical scavenging activity **(Figures 4B–D).**

4 Conclusion

The developed more polar and more acidic HPTLC separation, followed by derivatization with the Neu's reagent and PEG solution, provided a metabolite screening of the investigated beer extracts with regard to phenolics and flavonoids. Based on the HPTLC–DPPH* fingerprints, the dark beer extracts showed a higher amount of phenolic compounds and stronger antioxidative activity, compared to light and non-alcoholic beer extracts, which is in agreement with our previous research. Additionally, the DPPH* fingerprints provided the assignment of compounds with radical scavenging activity. The principal component analysis confirmed the classification and successful differentiation of dark and non-alcoholic beer samples, whereas light beers were positioned between the two groups. Beside other metabolites, desdimethyl-octahydro-*iso*-cohumulone and

iso-n/ad-humulone were identified as markers for the beer classification. Both HPTLC detection systems pointed out the good quality of dark beers with regard to the phenolic content and radical scavenging potential.

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