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Characterization of prenylflavonoids and hop bitter acids in various classes of Czech beers and hop extracts using high-performance liquid chromatography–mass spectrometry

Lenka Česlová, Michal Holčapek*, Martin Fidler, Jitka Drštičková, Miroslav Lísa

Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 53210 Pardubice, Czech Republic

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ABSTRACT

Hops contain a wide range of polyphenolic compounds with antioxidant properties divided in various chemical classes. These compounds are detected in hop extracts and also in beer as its main product. Based on the careful optimization of column type, column packing, mobile phase composition and gradient steepness, two high-performance liquid chromatography–mass spectrometry (HPLC/MS) methods have been developed. The first method using Purospher Star RP-8e column and the gradient of aqueous acetonitrile containing 0.3% formic acid is optimized for the separation of low polar polyphenolic compounds, while the second one with Zorbax SB-CN column is used for more polar hops and beer components. In this work, more compounds are detected in comparison to previous reports. In total, 49 low polar and 37 polar compounds are detected in studied samples and their molecular weights are determined based on atmospheric pressure chemical ionization (APCI) mass spectra. Some compounds are identified based on the interpretation of their full scan and tandem APCI mass spectra, retention behavior and UV spectra, while the full structure elucidation of other species still requires further research. The quantitation of xanthohumol related prenylflavonoids and bitter acids is done with two detection techniques (APCI-MS and UV detection) providing comparable results. Both compound classes (i.e., prenylflavonoids and bitter acids) are separated and quantitated in a single HPLC run, where numerous other polyphenolics are detected as well.

1. Introduction

Hop plant (*Humulus lupulus* L.) is used in the beer production especially to add bitterness. The female inflorescences of the hop plant (hops) contain mainly hop resins, polyphenolic compounds, essential oils and other related compounds [1]. The main hop resins are bitter acids as a source of bitterness of beer. They are divided into two groups, humulones (α-acids) and lupulones (β-acids). Both types of these bitter acids have several homologs, such as normal-, co-, and ad-homologs (Fig. 1). Minor hop acids including post-, pre- and adpre-homologues [2] can be found in hops in addition to main types of bitter acids. During the brewing process, humulones are transformed into isohumulones (iso-α-acids) [3], which are responsible for the specific bitter taste and the stability of beer foam. The foam is one of the first qualitative signs of beer quality recognized by consumers. The foam stability can be affected by other compounds, such as proteins, metal ions, lipids and amino acids [4].

The hops contains many polyphenolic compounds in addition to bitter acids. The most important hop flavonoids (Fig. 2) are xanthohumol (XN) and related prenylflavonoids as isoxanthohumol (IXN), desmethylxanthohumol (DMX), 6-prenylnaringenin (6-PN), 8-prenylnaringenin (8-PN) and 6-geranylnaringenin (6-GN) [1,5,6]. These hop prenylflavonoids have a positive effect on the human health due to antioxidant, anticancer, antimicrobial and anti-inflammatory properties [1]. They also reduce the cholesterol level, protect against the cardiovascular diseases and inhibit many enzymes, e.g., 8-prenylnaringenin is known as a potent phytoestrogen [1]. The hops is used in the beer production, therefore one of the main dietary source of xanthohumol and related prenylflavonoids for people is beer. Nowadays, the hop extracts, prepared either by the extraction with organic solvents (methanol, ethanol, hexane or isooctane) [7] or by the supercritical fluid extraction with carbon dioxide [8], are used in the beer production. The content of prenylflavonoids in hops depends on the variety, stress factors and storage conditions. There is a possibility to distinguish the technology of beer production and find out the addition of hops

* Corresponding author. Tel.: +420 466 037 087; fax: +420 466 037 068.
E-mail address: Michal.Holcapek@upce.cz (M. Holčapek).

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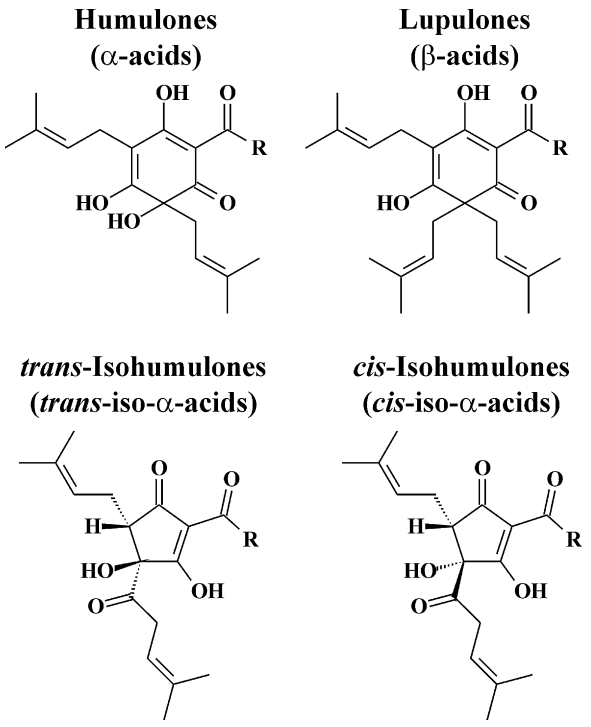

Fig. 1. Structures of individual classes of bitter acids: humulones (α-acids), lupulones (β-acids) and *cis*- and *trans*-isohumulones (iso-α-acids). R can correspond the following homologs: $-CH_2CH(CH_3)_2$ for humulone (H) and lupulone (L), $-CH(CH_3)_2$ for cohumulone (coH) and colupulone (coL), $-CH(CH_3)CH_2CH_3$ for adhumulone (adH) and adlupulone (adL), $-CH_2CH_2CH(CH_3)_2$ for prehumulone (preH) and prelupulone (preL), $-CH_2CH_3$ for posthumulone (postH) and postlupulone (postL), $-(CH_2)_4CH_3$ for adprehumulone (adpreH) and adprelupulone (adpreL). The same notation of R is used for *cis*- and *trans*-isohumulones.

after brewing process according to the amount of prenylflavonoids and bitter acids in beer. Therefore, the analysis of bitter acids and prenylflavonoids is important for the quality control of beer.

Beer samples can be analyzed without any pretreatment [3] or after the purification and preconcentration steps on solid phase extraction (SPE) columns packed with octadecylsilica [9] or octylsilica [10]. The most common separation technique to analyze flavonoids in food, drinks and other biological samples is reversed-phase high-performance liquid chromatography (HPLC) [11] with UV [9,12], electrochemical [13–15] or evaporate light-scattering [16] detection. Furthermore, gas chromatography [11] and electromigration techniques, such as capillary zone electrophoresis [11,17] or capillary electrochromatography [11], can be employed as well. To analyze hops and beer samples, the coupling of HPLC with mass spectrometry (MS) using electrospray ionization (ESI) [18–21] or atmospheric pressure chemical ionization (APCI) [2,3,6] in both polarity modes is often reported.

The qualitative and quantitative analysis of hop extracts of typical Czech varieties and Czech beer samples is presented in this paper. Two chromatographic methods are optimized for the separation of compounds with a lower polarity in beer and hops and compounds with the higher polarity in beer. To identify unknown prenylflavonoids, the APCI-MS in both polarity modes and tandem mass spectrometry (MS/MS) is used. The main prenylflavonoids, XN, IXN and 8-PN together with bitter acids (α, β and iso-α-acids) are quantified by HPLC with UV and MS detection in hop extracts and beer samples. The change in the flavonoid composition is monitored during the beer aging experiment.

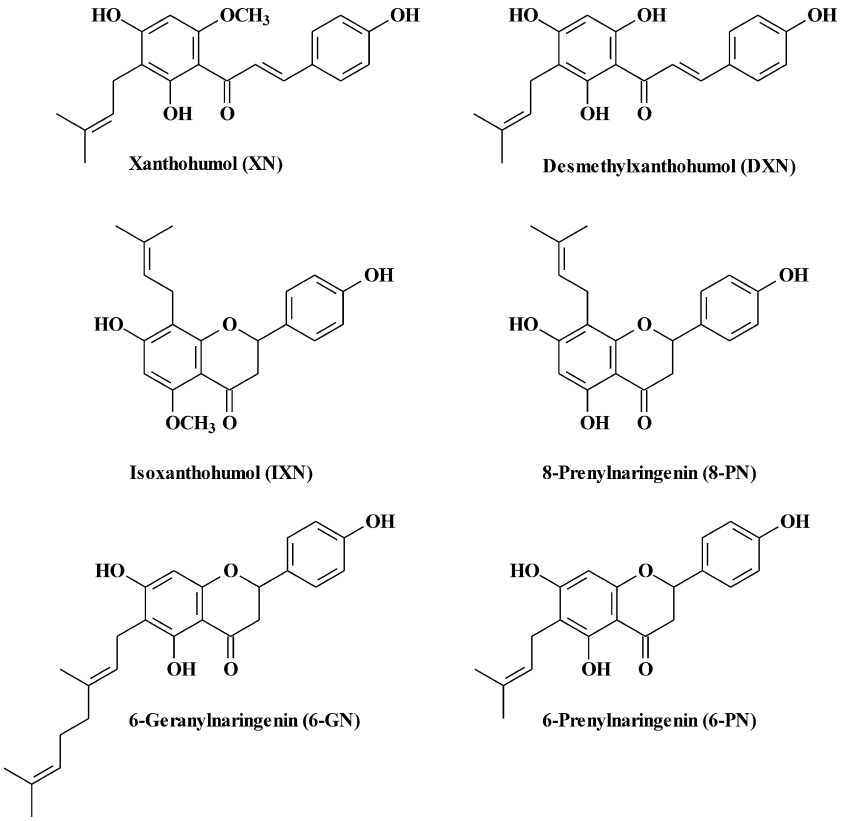

Fig. 2. Structures of common prenylflavonoids detected in hop and beer samples.

2. Experimental

2.1. Materials

Acetonitrile and methanol (both HPLC gradient grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). De-ionized water was prepared with Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic) and by Ultra CLEAR UV apparatus (SG, Hamburg, Germany). Formic acid was purchased from Lachema (Neratovice, Czech Republic). Standards of XN, IXN and 8-PN were provided by Hop research institute (Žatec, Czech Republic). Bitter acids were purchased from Labor Veritas (Zurich, Switzerland). Seven samples of hop extracts (E1–E7) were studied. Extracts E1, E2 and E3 were prepared by supercritical CO_2 extraction, E4 and E5 by alcohol extraction, extract E6 was prepared by supercritical CO_2 extraction with ethanol as a modifier from the rest after the first CO_2 extraction used for the beer production. Extract E7 was obtained by alkaline isomerization of supercritical CO_2 extract. Hop extracts E1–E4 were kindly provided by Hop research institute and extracts E5–E7 by Favea (Kopřivnice, Czech Republic). Different kinds of beer samples were bought in the local market. For our study, 13 beer samples were chosen: B1 – Pilsner Urquell (lager), B2 – Budweiser Budvar (lager), B3 – Budweiser Budvar (nonalcoholic beer), B4 – Krušovice (dark beer), B5 – Krušovice Mušketýr (ale), B6 – Radegast Premium (lager), B7 – Staropramen (lager), B8 – Stela Artois (lager), B9 – Zlatopramen (lager), B10 – Klasik (ale), B11 – Budweiser Superstrong (special beer), B12 – Staropramen (dark beer), B13 – Radegast Birell (nonalcoholic beer).

2.2. Chromatographic and mass spectrometric instrumentation

The chromatographic apparatus consisted of a Model 616 pump with a quaternary gradient system, a Model 996 diode-array UV detector, a Model 717+ autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). The outlet from the column was connected directly to Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) with APCI in both positive-ion and negative-ion modes.

2.3. Procedures

Solvents were filtered through a 0.45-μm Millipore filter and degassed by continuous stripping with helium. Standards were dissolved in acetonitrile–water mixture (90:10, v/v) and calibration solutions were prepared by sequential dilution. The concentration range of calibration solutions was 10 μg/L to 100 mg/L for XN, IXN and 8-PN and 100 μg/L to 1000 mg/L for α + β-bitter acids and dicyclohexylamin iso-α-bitter acids (DCHA-Iso). DCHA-Iso standard contains 64.3% of iso-α-bitter acids (IAA). 10 μL of each calibration solution was injected for the HPLC/MS analysis. The hop extracts were dissolved in acetonitrile:water mixture (90:10, v/v) at the concentration 2 g/L and 10 μL of this solution was injected for the HPLC/MS analysis. The beer foam was removed by ultrasonication and 100 μL of beer was directly injected without other pretreatment. All samples were filtered through a 0.45-μm filter prior the analysis. The change in the flavonoid composition was monitored during the beer aging experiment. Four samples of beer were analyzed immediately after opening the bottle, then after 6 h, 1 day, 3 days and 1 week. 120 mL of beer was kept at constant temperature 20 °C in a beaker. The loss of beer volume was measured before the analysis and concentrations of monitored flavonoids were corrected.

2.4. HPLC conditions

The optimization of HPLC conditions of phenolic compounds in hop extracts and beer samples was performed with the following columns: Purospher Star RP-18e, 250 mm × 4 mm, 5 μm, Purospher Star RP-8e, 250 mm × 4 mm, 5 μm (both from Merck, Darmstadt, Germany), Ascentis RP-Amide, 250 mm × 4.6 mm, 5 μm (Sigma–Aldrich, St. Louis, MO, USA), Zorbax Eclipse C18, 250 mm × 4.6 mm, 5 μm, Zorbax SB-CN, 250 mm × 4.6 mm, 5 μm (both from Waters, Milford, MA, USA), Phenomenex Luna C18, 250 mm × 4.6 mm, 5 μm, Phenomenex Ultracarb ODS, 150 mm × 3.2 mm, 5 μm (both from Phenomenex, Torrence, USA). Finally, two HPLC methods were optimized for the analysis of low polar and polar phenolic compounds. The mobile phase was composed from acetonitrile and water with 0.3% formic acid in both solvents. Method I (for low polar phenolic compounds) – the column Purospher Star C8-e, gradient elution: 0 min-40% acetonitrile in water, 40 min-100% acetonitrile. Method II (for polar phenolic compounds in beer) – the column Zorbax SB-CN, gradient elution: 0–5 min-5% acetonitrile in water, 35 min-20% acetonitrile in water, 55 min-40% acetonitrile in water, 60 min-100% acetonitrile. In all experiments, the flow rate and column temperature were kept constant at 1 mL/min and 40 °C, respectively. UV spectra of all chromatographic peaks were recorded in the range of 200–500 nm. The wavelength 330 nm was selected for monitoring of all flavonoids.

2.5. MS conditions

APCI mass spectra were recorded in the mass range of m/z 50–800 using both polarity modes and the following setting of tuning parameters:

(A) Direct infusion for the measurement of the fragmentation behavior of standards – the sample flow rate was 100 mL/min, pressure of the nebulizing gas was 15 psi, the flow rate of drying gas was 4 L/min, temperatures of drying gas and APCI heater were 300 and 350 °C, respectively. The mass spectrometer was tuned to give a maximum response for m/z 400 according to the molecular weights (MW) of measured compounds and the tuning parameter "compound stability" was set at 20%.
(B) HPLC/MS – the flow rate of mobile phase was 1 mL/min, pressure of the nebulizing gas was 70 psi, the flow rate of drying gas was 5 L/min, temperatures of drying gas and APCI heater were 350 and 450 °C, respectively. The mass spectrometer was tuned to give a maximum response for m/z 400 and the tuning parameter "compound stability" was set at 20%.

2.6. Fragmentation behavior

Isoxanthohumol (IXN), MW = 354. Positive-ion APCI-MS spectra: m/z 371 $[M+NH_4]^+$; m/z 355 $[M+H]^+$; m/z 299 $[M+H-C_4H_8]^+$, 100%. MS/MS of m/z 355: m/z 299 $[M+H-C_4H_8]^+$, 100%; m/z 235 $[M+H-C_8H_8O]^+$; m/z 179 $[M+H-C_4H_8-C_8H_8O]^+$. Negative-ion APCI-MS spectra: m/z 353 $[M-H]^-$, 100%. MS/MS of m/z 353: m/z 233 $[M-H-C_8H_8O]^-$.

Xanthohumol (XN), MW = 354. Positive-ion APCI-MS spectra: m/z 371 $[M+NH_4]^+$; m/z 355 $[M+H]^+$; m/z 299 $[M+H-C_4H_8]^+$, 100%. MS/MS of m/z 355: m/z 299 $[M+H-C_4H_8]^+$, 100%; m/z 235 $[M+H-C_8H_8O]^+$; m/z 179 $[M+H-C_4H_8-C_8H_8O]^+$. Negative-ion APCI-MS spectra: m/z 353 $[M-H]^-$, 100%. MS/MS of m/z 353: m/z 233 $[M-H-C_8H_8O]^-$; m/z 119 $[M-H-C_8H_8O-C_8H_2O]^-$.

8-Prenylnaringenin (8-PN), MW = 340. Positive-ion APCI-MS spectra: m/z 341 $[M+H]^+$, 100%; m/z 285 $[M+H-C_4H_8]^+$. MS/MS of m/z 341: m/z 285 $[M+H-C_4H_8]^+$, 100%. MS/MS of m/z 285: m/z 165 $[M+H-C_4H_8-C_8H_8O]^+$. Negative-ion APCI-MS spectra: m/z 339 $[M-H]^-$, 100%. MS/MS of m/z 339: m/z 219 $[M-H-C_8H_8O]^-$.

Desmethylxanthohumol (DXN), MW = 340. Positive-ion APCI-MS spectra: *m/z* 341 $[M+H]^+$; *m/z* 285 $[M+H-C_4H_8]^+$, 100%. MS/MS of *m/z* 341: *m/z* 285 $[M+H-C_4H_8]^+$, 100%; *m/z* 221 $[M+H-C_8H_8O]^+$; *m/z* 165 $[M+H-C_4H_8-C_8H_8O]^+$. MS/MS of *m/z* 285: *m/z* 165 $[M+H-C_4H_8-C_8H_8O]^+$, 100%. Negative-ion APCI-MS spectra: *m/z* 339 $[M-H]^-$, 100%. MS/MS of *m/z* 339: *m/z* 295 $[M-H-C_2H_3O]$; *m/z* 219 $[M-H-C_8H_8O]^-$, 100%.

Cohumulon (coH), MW = 348. Positive-ion APCI-MS spectra: *m/z* 349 $[M+H]^+$; *m/z* 293 $[M+H-C_4H_8]^+$; *m/z* 281 $[M+H-C_5H_8]^+$; *m/z* 223 $[M+H-C_4H_8-C_3H_6CO]^+$, 100%. MS/MS of *m/z* 223: *m/z* 167 $[M+H-2C_4H_8-C_3H_6CO]^+$, 100%. Negative-ion APCI-MS spectra: *m/z* 347 $[M-H]^-$, 100%; *m/z* 278 $[M-H-C_5H_9]^-$. MS/MS of *m/z* 347: *m/z* 278 $[M-H-C_5H_9]^-$, 100%. MS/MS of *m/z* 278: *m/z* 260 $[M-H-C_5H_9-H_2O]^-$; *m/z* 235 $[M-H-C_5H_9-C_3H_7]^-$, 100%.

n-Humulon (H) and adhumulon (adH), MW = 362. Positive-ion APCI-MS spectra: *m/z* 363 $[M+H]^+$; *m/z* 307 $[M+H-C_4H_8]^+$; *m/z* 295 $[M+H-C_5H_8]^+$; *m/z* 239 $[M+H-C_5H_8-C_4H_8]^+$, 100%. MS/MS of *m/z* 239: *m/z* 221 $[M+H-C_5H_8-C_4H_8-H_2O]^+$,100%. Negative-ion APCI-MS spectra: *m/z* 361 $[M-H]^-$, 100%; *m/z* 292 $[M-H-C_5H_9]^-$. MS/MS of *m/z* 361: *m/z* 343 $[M-H-H_2O]^-$; *m/z* 292 $[M-H-C_5H_9]^-$, 100%. MS/MS of *m/z* 292: *m/z* 274 $[M-H-C_5H_9-H_2O]^-$; *m/z* 249 $[M-H-C_5H_9-C_3H_7]^-$, 100%.

Colupulon (coL), MW = 400. Positive-ion APCI-MS spectra: *m/z* 401 $[M+H]^+$, 100%; *m/z* 345 $[M+H-C_4H_8]^+$. Negative-ion APCI-MS spectra: *m/z* 399 $[M-H]^-$, 100%. MS/MS of *m/z* 399: *m/z* 355 $[M-H-C_2H_3O]^-$; *m/z* 330 $[M-H-C_5H_9]^-$; *m/z* 287 $[M-H-C_5H_9-C_3H_7]^-$, 100%.

n-Lupulon (L) and adlupulon (adL), MW = 414. Positive-ion APCI-MS spectra: *m/z* 415 $[M+H]^+$; *m/z* 359 $[M+H-C_4H_8]^+$; *m/z* 291 $[M+H-C_4H_8-C_5H_8]^+$, 100%. MS/MS of *m/z* 291: *m/z* 235 $[M+H-2C_4H_8-C_5H_8]^+$, 100%. Negative-ion APCI-MS spectra: *m/z* 413 $[M-H]^-$, 100%. MS/MS of *m/z* 413: *m/z* 369 $[M-H-C_2H_3O]^-$; *m/z* 344 $[M-H-C_5H_9]^-$; *m/z* 301 $[M-H-C_5H_9-C_3H_7]^-$, 100%.

Iso-cohumulon (iso-coH), MW = 348. Positive-ion APCI-MS spectra: *m/z* 349 $[M+H]^+$, 100%; *m/z* 331 $[M+H-H_2O]^+$; *m/z* 313 $[M+H-2H_2O]^+$; *m/z* 281 $[M+H-C_5H_8]^+$. MS/MS of *m/z* 349: *m/z* 331 $[M+H-H_2O]^+$, 100%; *m/z* 313 $[M+H-2H_2O]^+$; *m/z* 281 $[M+H-C_5H_8]^+$. MS/MS of *m/z* 331: *m/z* 313 $[M+H-2H_2O]^+$, 100%; *m/z* 295 $[M+H-3H_2O]^+$; *m/z* 275 $[M+H-H_2O-C_4H_8]^+$. MS/MS of *m/z* 313: *m/z* 295 $[M+H-3H_2O]^+$, 100%; *m/z* 257 $[M+H-2H_2O-C_4H_8]^+$. Negative-ion APCI-MS spectra: *m/z* 347 $[M-H]^-$, 100%; *m/z* 251 $[M-H-C_6H_8O]^-$. MS/MS of *m/z* 347: *m/z* 329 $[M-H-H_2O]^-$; *m/z* 278 $[M-H-C_5H_9]^-$; *m/z* 251 $[M-H-C_6H_8O]^-$, 100%. MS/MS of *m/z* 251: *m/z* 233 $[M-H-C_6H_8O-H_2O]^-$; *m/z* 207 $[M-H-C_6H_8O-C_2H_3O]^-$.

Iso-n-humulon (iso-H) and iso-adhumulon (iso-adH), MW = 362. Positive-ion APCI-MS spectra: *m/z* 363 $[M+H]^+$, 100%; *m/z* 345 $[M+H-H_2O]^+$; *m/z* 327 $[M+H-2H_2O]^+$; *m/z* 295 $[M+H-C_5H_8]^+$. MS/MS of *m/z* 363: *m/z* 345 $[M+H-H_2O]^+$; *m/z* 327 $[M+H-2H_2O]^+$, 100%; *m/z* 307 $[M+H-C_4H_8]^+$; *m/z* 295 $[M+H-C_5H_8]^+$. MS/MS of *m/z* 345: *m/z* 327 $[M+H-2H_2O]^+$, 100%; *m/z* 289 $[M+H-H_2O-C_4H_8]^+$; *m/z* 277 $[M+H-H_2O-C_5H_8]^+$. MS/MS of *m/z* 327: *m/z* 309 $[M+H-3H_2O]^+$, 100%. Negative-ion APCI-MS spectra: *m/z* 361 $[M-H]^-$, 100%; *m/z* 265 $[M-H-C_6H_8O]^-$. MS/MS of *m/z* 361: *m/z* 343 $[M-H-H_2O]^-$; *m/z* 317 $[M-H-C_2H_3O]^-$; *m/z* 265 $[M-H-C_6H_8O]^-$, 100%. MS/MS of *m/z* 265: *m/z* 247 $[M-H-C_6H_8O-H_2O]^-$.

3. Results and discussion

3.1. Optimization of the separation

First, the composition of mobile phase is optimized with respect to the best separation of studied polyphenolic compounds. The successful separation requires the addition of acidic additives. Volatile organic acids (formic and acetic acids) have been considered because of the compatibility with the MS detection. Formic acid has a stronger effect on the peak shape and resolution, therefore acetonitrile:water and methanol:water mobile phases with the addition of formic acid have been tested. Mobile phases containing acetonitrile provide a higher resolution and shorter analysis time. Further, the steepness of the gradient elution and the amount of formic acid in the mobile phase have been optimized. Higher amount of formic acid improves the separation, especially for bitter acids (Figs. 3(A) and (B)). Finally, different stationary phases (see Section 2) are compared to obtain the best resolution of analyzed compounds [22]. The final method of low polar phenolic compounds is performed on Purospher Star C8-e column (Fig. 3(C)), where the full separation of all six homologs of α- and β-acids is achieved. Similar optimization procedure has been used for the analysis of polar phenolic compounds, where the Zorbax SB-CN column provides the best results in aqueous acetonitrile mobile phase containing 0.3% formic acid.

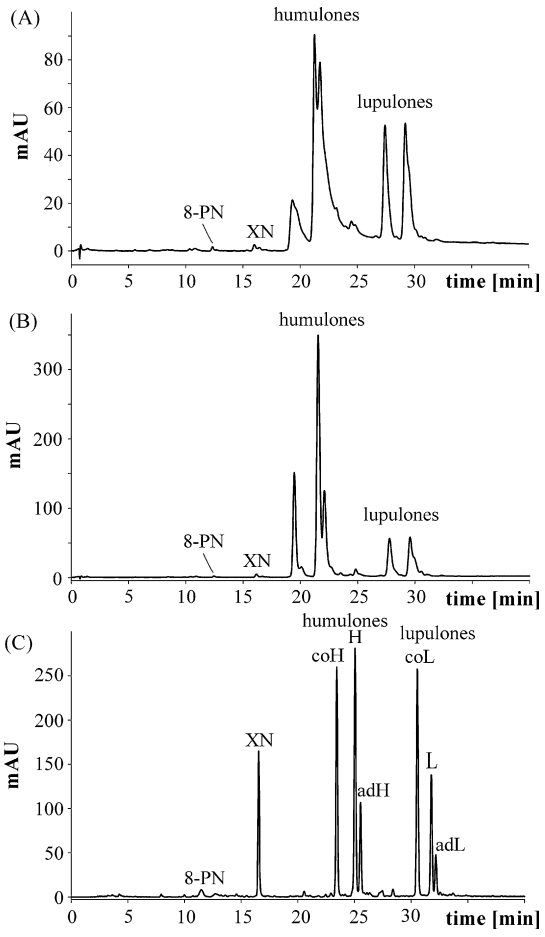

Fig. 3. Optimization of HPLC separation of hop extracts. Gradient elution: 0 min-40% acetonitrile in water, 40 min-100% acetonitrile, UV detection at 330 nm. (A) CO_2 extract, Ultracarb ODS column, addition of 0.1% HCOOH to the mobile phase, (B) CO_2 extract, Ultracarb ODS column, addition of 0.3% HCOOH to the mobile phase, (C) ethanol extract, Purospher Star RP-8e column, addition of 0.3% HCOOH to the mobile phase.

3.2. Identification of polyphenolic compounds

Optimized methods are used for the identification of polyphenolic compounds in hop extracts and beer samples. The complementary information from both positive-ion (i.e., $[M+H]^+$ ion) and negative-ion (i.e., $[M-H]^-$ ion) APCI mass spectra is used for the unambiguous determination of MWs of polyphenolic com-

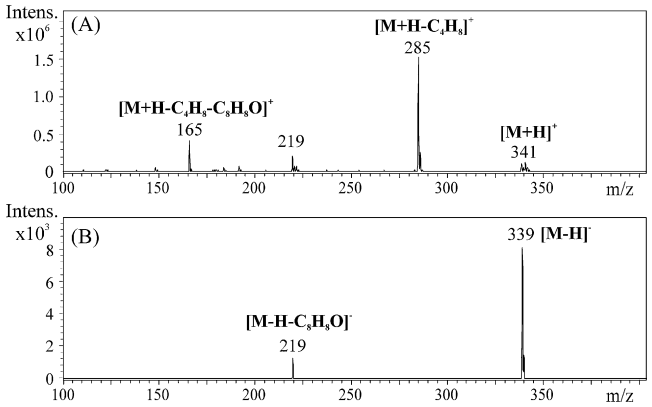

Fig. 4. Tandem mass spectra of 8-prenylnaringenin with MW = 340, (A) MS/MS spectrum of $[M+H]^+$ ion at $m/z = 341$, (B) MS/MS spectrum of $[M-H]^-$ ion at $m/z = 339$.

pounds. Additional structural information is obtained from tandem mass spectra, where the characteristic neutral losses are observed (see Section 2). To obtain information about the fragmentation behavior of prenylflavonoids and bitter acids, tandem mass spectra of their standards are measured using the direct infusion. Examples of tandem mass spectra for XN related compounds and bitter acids are shown in Figs. 4 and 5. The full scan mass spectra are not shown (see Section 2 for the list of observed ions), because they mostly exhibit as base peaks (or at least abundant peaks) protonated molecules $[M+H]^+$ in the positive-ion APCI mode and deprotonated molecules $[M-H]^-$ in the negative-ion APCI mode, which enables an unambiguous determination of MWs of individual compounds. ESI mass spectra (not shown) are similar, but the relative abundances of fragment ions are even lower in the full scan mass spectra and the sensitivity of HPLC/MS method is worse for ESI detection. Positive-ion (Fig. 4(A)) and negative-ion (Fig. 4(B)) tandem mass spectra of (de)protonated molecules illustrate the fragmentation behavior of XN related compounds on the example of 8-PN, while Fig. 5 depicts similar spectra for coH as a representative example of bitter acids. APCI mass spectra of studied polyphenolics have some common features applicable for the identification of particular functional groups in unknown analogs, for example the loss of prenyl fragment (C_4H_8, $\Delta m/z$ 56) is a diagnostic neutral loss for the confirmation of the presence of one or two prenyl groups in the molecule. Other important neutral loss is C_8H_8O ($\Delta m/z$ 120) corresponding to the ethylenephenol loss. Bitter acids often exhibit one to three consecutive losses of water, which does not have a high diagnostic value due to low specificity of water losses.

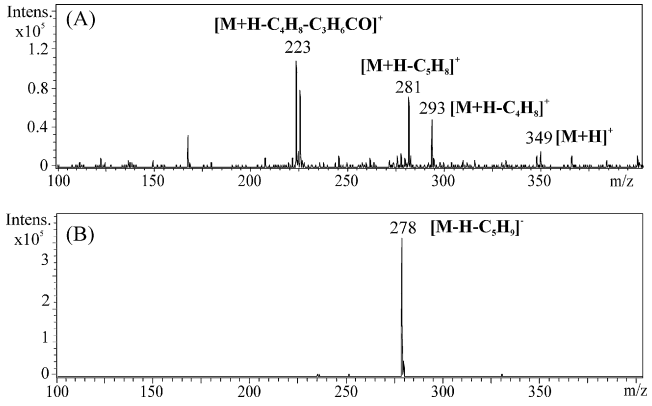

Fig. 5. Tandem mass spectra of cohumulone with MW = 348, (A) MS/MS spectrum of $[M+H]^+$ ion at $m/z = 349$, (B) MS/MS spectrum of $[M-H]^-$ ion at $m/z = 347$.

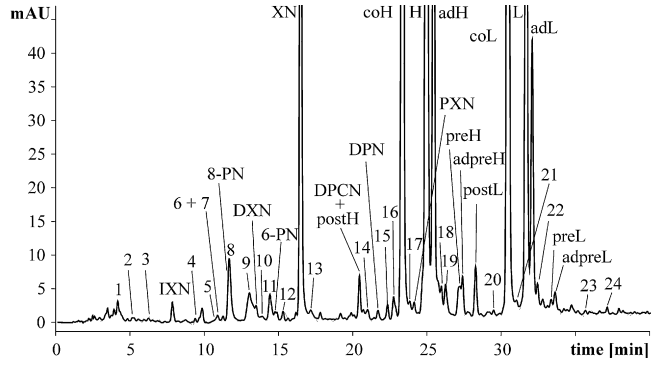

Fig. 6. HPLC chromatogram of hop ethanol extract K4 using UV detection at 330 nm. Purospher Star RP-8e column, gradient elution: 0 min-40% acetonitrile in water, 40 min-100% acetonitrile, addition of 0.3% HCOOH to the mobile phase.

The hop extract E4 is selected as a representative sample of hop extract containing a wide range of polyphenolic compounds (Fig. 6). MWs are determined for all 44 detected peaks including trace compounds (Table 1). In case of strongly coeluting peaks and/or trace peaks, the reconstructed ion currents for observed m/z values are monitored to confirm that the retention maximum for expected masses really corresponds to the assigned peak, which is useful tool for distinguishing trace peaks from the background noise. In many cases, important fragment ions are observed already in the full scan spectra, but it does not complicate MW determination. Twenty detected peaks are identified based on known MWs, the fragmentation behavior (characteristic neutral losses), UV spectra (characteristic maxima for chalcones and flavanones) and the retention behavior. In addition to main bitter acids (H, coH, adH, L, coL and adL), other minor bitter acids including post-, pre- and adpre-homologs of humulones and lupulones (postH, preH, adpreH, postL, preL and adpreL) are detected as well. Furthermore, eight phenolic compounds of the XN type are found. The identification of major prenylflavonoids (XN, 8-PN, IXN and DXN) is confirmed using identical standards. Other four prenyflavonoids (6-PN, 3′,5′-diprenylchalconaringenin – DPCN, 6,8-diprenylnaringenin – DPN and 5′-prenylxanthohumol – PXN) are identified on the basis of the fragmentation and retention behavior and UV spectra. Suggested structures are in agreement with compounds described previously in the literature [1,2,20,23]. The unknown phenolic compound No. 6 with MW = 370 probably corresponds to xanthohumol B or xanthohumol D. Both compounds have the same MW, the fragmentation and retention behavior. The full structural elucidation of other compounds would require the

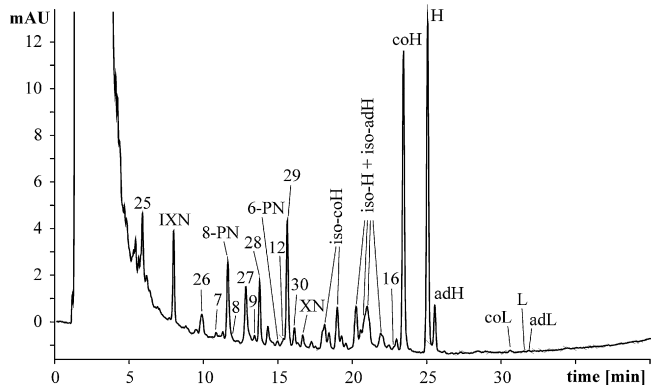

Fig. 7. HPLC chromatogram of low polar compounds in nonalcoholic beer Radegast Birell using UV detection at 330 nm. Purospher Star RP-8e column, gradient elution: 0 min-40% acetonitrile in water, 40 min-100% acetonitrile, addition of 0.3% HCOOH to the mobile phase.

Table 1
Low polar compounds detected in the hop ethanol extract E4 (Nos. 1–24) and in the nonalcoholic beer sample Radegast Birell (25–30).

Compound	t_R (min)	MW	Fragment ions in positive-ion APCI mode (m/z)	Fragment ions in negative-ion APCI mode (m/z)
1	4.2	380	363, 345, 327, 305, 275	361, 335, 317, 291, 265, 249, 223, 205, 165
2	5.2	394	377, 359, 275, 257, 233	375, 349, 331, 291, 263, 247, 205, 165
3	6.2	370	353, 325, 297, 269, 209	Low intensity
IXN	7.9	354	299, 235, 179	233, 165, 119
4	9.4	352	297, 233	Low intensity
5	10.6	363	346, 328, 320, 302, 246	344, 317, 293, 248
6	10.9	370	353, 299, 267, 251, 233, 179, 147	Low intensity
7	10.9	364	347, 329, 303, 277, 259, 221	249, 209, 141
8-PN	11.6	340	285, 221, 191, 165, 147	219
8	11.7	318	301, 275, 251, 233, 195	248, 205, 180
9	13.3	347	330, 312	328, 277, 250, 232, 180
DXN	13.5	340	285, 221, 165	Low intensity
10	13.8	306	251, 221, 181, 139	193
11	14.4	317	250, 232, 194, 177	247, 204, 179, 136
6-PN	14.8	340	285, 165	245, 219
12	15.3	378	Low intensity	308, 263
XN	16.5	354	299, 179	233, 119
13	17.2	364	Low intensity	319, 275, 251, 233, 206
DPCN	20.5	408	353, 297, 233	313, 301, 287, 243, 119
postH	20.5	334	Low intensity	315, 264
14	20.9	416	399, 381, 361, 349, 293, 275, 219	397, 371, 346, 329, 259, 179
DPN	21.0	408	353, 297, 233	313, 301, 287, 243, 119
15	22.4	416	399, 361, 343, 331, 273, 217	371, 346, 330, 303, 287, 263, 219
16	22.8	416	361, 349, 329, 293, 273, 235	371, 346, 303, 287, 219
coH	23.4	348	331, 313, 293, 281, 223, 167	278
17	23.9	430	413, 375, 357, 345, 291, 273, 217	385, 344, 317, 301, 289, 277, 233, 208
PXN	24.2	422	367, 311, 247, 191	406, 363, 338, 301, 286, 243
H	25.0	362	345, 327, 307, 295, 239, 223, 167	292
adH	25.5	362	345, 327, 307, 295, 239, 223, 167	292
18	26.0	444	427, 389, 377, 321, 303, 275, 219	425, 371, 329, 302, 259
19	26.3	346	291	327, 301, 261, 233, 208, 191
preH	27.2	376	Low intensity	306
adpreH	27.4	376	359, 321, 309, 273, 253, 223	306
postL	28.3	386	331, 319, 275, 263, 219	341, 316, 273, 248, 205
20	29.5	500	483, 465, 447, 415	481, 403, 385, 355, 249
coL	30.5	400	345, 333, 275, 277, 219	355, 330, 287, 262, 219, 194
21	31.1	514	497, 479, 461, 429	495, 417, 399, 387, 369, 263
L	31.7	414	359, 347, 291, 275, 219	369, 344, 301, 233
adL	32.1	414	359, 347, 291, 275, 219	369, 344, 301, 233, 208
22	32.5	426	371, 359, 303, 277	356, 313, 245
preL	33.4	428	373, 361, 305, 275, 219, 135	383, 358, 315, 290, 247, 222
adpreL	33.6	428	373, 361, 305, 275, 219, 135	383, 358, 315, 290, 247, 222
23	35.7	482	465, 447, 415	463, 385, 369, 341
24	37.2	552	535, 517, 467, 399, 343, 277	482, 413, 329
25	5.9	330	315, 287, 270, 245, 187	314, 299, 281, 263
26	9.9	380	Low intensity	361, 308, 283, 265, 253, 207
27	12.8	378	361, 343, 317, 277, 259, 221, 203, 175	359, 331, 281, 263, 235, 223, 207, 165, 141
28	13.8	366	Low intensity	347, 296
29	15.6	380	Low intensity	361, 310
30	16.1	380	Low intensity	361, 310, 251

support of other spectral data (e.g., nuclear magnetic resonance), but trace concentrations of these peaks and the complexity of samples do not enable preconcentration or isolation steps.

The final separation of low polar phenolic compounds in beer sample B13 is shown in Fig. 7. During the brewing process, all hop α-acids are transformed into the corresponding epimeric mixture of iso-α-acids (*cis* and *trans* isomers). Consequently, six iso-α-acids originate from three main α-acids. The beer sample B13 is nonalcoholic beer, which is prepared by different technology than the rest of studied beer samples. The second addition of hops is done at the final stage of beer production, hence this beer contains higher amount of bitter acids, which are not isomerized during the brewing process (Fig. 7). Similarly as for other beers, nearly all β-acids are transformed and therefore only trace concentration is detected in beer samples. Prenylflavonoids are also isomerized during the brewing process, e.g., XN to IXN and DMXN to 8-PN. Due to the high concentration of highly polar to ionic species in beer samples (see Fig. 7), the initial 5 min of the analysis have to be directed to the waste to avoid the contamination of mass spectrometer.

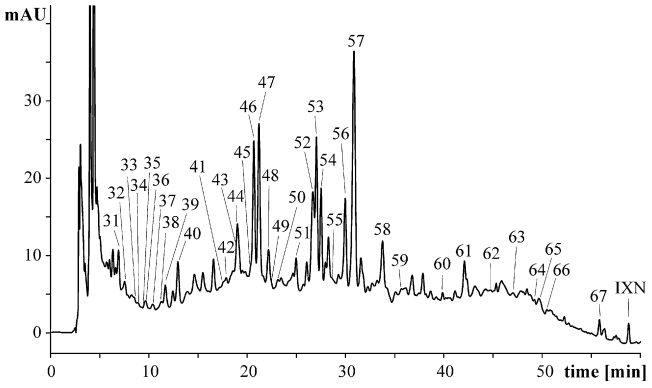

Fig. 8. HPLC chromatogram of polar compounds in nonalcoholic beer Radegast Birell using UV detection at 330 nm. Zorbax SB-CN column, gradient elution: 0 min-5% acetonitrile in water, 5 min-5% acetonitrile in water, 35 min-20% acetonitrile in water, 55 min-40% acetonitrile in water, 60 min-100% acetonitrile, addition of 0.3% HCOOH to the mobile phase.

Table 2
Polar compounds detected in the nonalcoholic beer sample Radegast Birell (31–67).

Compound	t_R (min)	MW	Fragment ions in positive-ion APCI mode (m/z)	Fragment ions in negative-ion APCI mode (m/z)
31	6.9	286	259, 241, 229, 211, 118	255, 241, 225, 211, 195
32	7.5	294	278, 166, 120	275, 257, 128
33	8.9	370	355, 326, 199, 147, 130	351, 325, 252, 223, 179, 145
34	9.1	261	244, 216, 205, 188, 159, 132, 116, 86	229, 216, 203, 169, 131
35	9.5	325	308, 209, 181, 118	147, 116
36	9.6	299	283, 225, 197, 171, 132	254, 236, 223
37	10.6	242	225, 197, 132	197
38	11.3	242	225, 197, 132, 86	197
39	11.7	292	275, 257, 247, 202, 182, 153, 136	247, 185, 163, 141, 127, 109
40	13.0	292	275, 257, 233, 216, 147	171, 119
41	17.5	276	259, 231, 186, 166, 120	231, 213, 186, 147, 127, 109
42	17.9	359	342, 325, 243, 231, 225, 197	340, 328, 298, 241, 223, 116
43	18.8	290	273, 207, 165, 151, 139, 123	245, 231, 205, 179, 161, 137
44	19.0	276	260, 217, 205, 147, 114	Low intensity
45	20.1	376	359, 243, 200, 173, 117	Low intensity
46	20.7	341	Low intensity	Low intensity
47	21.2	341	324, 296, 183, 132	296, 209, 197, 165, 130
48	22.1	306	290, 247, 181, 177, 114	Low intensity
49	22.9	290	273, 165, 151, 139, 123	271, 245, 205, 179, 125, 109
50	23.1	373	209, 181, 166, 120	328, 164, 147
51	25.0	315	298, 280, 270, 205, 188, 130	270, 197, 185, 141, 127, 109
52	26.7	358	Low intensity	195
53	27.1	–	–	–
54	27.5	–	–	–
55	28.9	355	338, 310, 225, 197, 132	310, 248, 223, 180, 130
56	29.9	432	Low intensity	Low intensity
57	30.9	–	–	–
58	33.6	372	Low intensity	209
59	35.9	372	Low intensity	209
60	39.9	497	Low intensity	482, 451, 346, 331, 248, 218, 175, 150
61	42.1	448	287	327, 299, 285, 255
62	44.9	490	Low intensity	327, 285, 255, 227, 203
63	47.1	492	Low intensity	473, 329, 311, 293, 193
64	49.2	328	Low intensity	309, 291, 229, 211, 171
65	50.1	364	Low intensity	345, 267, 248, 209, 179
66	50.8	318	Low intensity	289, 249, 180
67	55.8	330	Low intensity	311, 293, 275, 229, 211, 183, 171

Fig. 8 shows the final method for the separation of polar compounds in beer Radegast Birell. These compounds have initially coeluted in a huge peak eluted within first 5 min using conditions for low polar polyphenolics (see Fig. 7). The use of medium polar nitrile column and decreased organic content in the mobile phase has significantly improved the separation of these polar compounds, but even in this case there is excessive number of coeluting compounds at very low concentrations, which seriously complicates their identification. Some compounds have high responses with UV detection at 330 nm, but they do not provide any meaningful signal with APCI or ESI detection techniques. MWs and retention times of detected peaks are summarized in Table 2. There is an evidence that several compounds (Nos. 35, 52, 58, 59 and 61) are glycosides based on the characteristic neutral loss ($\Delta m/z$ 162) of glycoside moiety. Peaks 43 and 49 correspond to catechin and epicatechin, respectively. Compounds are identified based on determined MWs and the comparison of retention and fragmentation data with standards. The full structural assignment of other detected peaks would require further research probably including the isolation steps and the use of other spectral techniques.

3.3. Quantitation

APCI-MS and UV detection techniques are used for the quantification of XN, IXN, 8-PN, IAA and α- and β-acids. Parameters of the calibration equation for all standards are shown in Table 3. Correlation coefficients for all calibration curves with both MS and

Table 3
Parameters of calibration curves obtained with MS and UV detection.[a]

Compound	Detection	Slope	Intercept	R^2
Xanthohumol (XN)	MS	2.389 ± 0.010	−0.0665 ± 0.0794	0.9999
	UV	0.1498 ± 0.0009	−0.0585 ± 0.0418	0.9999
Isoxanthohumol (IXN)	MS	0.6111 ± 0.0035	−0.1300 ± 0.0403	0.9999
	UV	0.0453 ± 0.0003	−0.0392 ± 0.0120	0.9998
8-Prenylnaringenin (8-PN)	MS	6.920 ± 0.009	−0.1089 ± 0.3088	1.0000
	UV	0.0319 ± 0.0002	−0.0055 ± 0.0088	0.9997
Dicyclohexylamine iso-α-acids (DCHA-IAA)	MS	5.741 ± 0.048	−2.817 ± 0.928	0.9997
	UV	0.0351 ± 0.0002	0.0088 ± 0.0055	0.9999
α- and β-acids (α + β)	MS	7.493 ± 0.021	0.1881 ± 0.1670	0.9996
	UV	0.5995 ± 0.0028	0.1101 ± 0.1086	0.9996

[a] Three measurements have been performed for each calibration point.

Table 4
Limits of detection (LoD) and limits of quantification (LoQ) for MS and UV detection.

Compound	LoD		LoQ	
	MS (mg/L)	UV (mg/L)	MS (mg/L)	UV (mg/L)
XN	0.006	0.02	0.02	0.06
IXN	0.010	0.02	0.03	0.06
8-PN	0.006	0.03	0.02	0.10
IAA	0.006	0.06	0.02	0.20
α + β	0.010	0.10	0.03	0.30

UV detection are better than 0.9995. Limits of detection (LoD) and limits of quantitation (LoQ) are determined as 3 and 10 times signal-to-noise ratios, respectively (Table 4). The MS detection is more sensitive by 2–10 times depending on the compound. Quantitative results for XN, IXN, 8-PN, IAA and α- and β-acids in hop extract samples E1–E7 (see Section 2 for the description of extracts) are listed in Table 5 and for beer samples B1–B6 (see Section 2 for the description of beer types) in Table 6. Quantitative results obtained by both APCI-MS and UV detection techniques are in a good mutual agreement. The standard of α- and β-acids represents the sum of all α- and β-acids, therefore only the sum of both α- and β-acids is listed in all tables in accordance with the established practice in the beer analysis. Concentrations of determined polyphenolic compounds in hop samples depend on the type of extraction and hop variety. In extracts prepared using the supercritical CO_2 extraction (E1, E2 and E3), the concentration of XN is very low and the amount of IXN and 8-PN is lower than the LoD. The concentration of XN in alcohol extracts (E4 and E5) is significantly higher. Moreover, IXN and 8-PN are also found at detectable concentrations. The highest amount of XN is found in the extract E6 prepared by the supercritical CO_2 extraction with ethanol used as a modifier, which significantly improves the extraction recovery of XN. It is interesting to note that this extraction procedure is used for the rest after the CO_2 extraction used for the beer production, which is actually the industrial waste containing almost whole initial amount of XN and related compounds, because the CO_2 extraction without the polar modifier is not efficient for this group of polyphenolics. On the other hand, only low amounts of bitter acids are determined for the extract E6. The extract E7 is prepared by the alkaline isomerization of CO_2 extract, which caused remarkably altered composition of polyphenolics unlike to all other extracts. IAA has appeared only in this hop extract, the concentration of IXN is significantly enhanced and the concentration of bitter acids is almost three orders of magnitude lower in comparison to extracts E1–E5. Beer samples are analyzed without any pretreatment to avoid losses of labile polyphenolic compounds. The quantitative results are shown for six representative beer samples B1–B6 (Table 6). High amounts of IXN and IAA are observed in beer samples on contrary to hop extracts, because XN and bitter acids are transformed to IXN and IAA during the brewing process. Concerning the differences among different types of beer, the lowest amount of polyphenolics is found in nonalcoholic beers, while the highest contents are determined for premium Czech lager beers with the highest bitterness (e.g., Pilsner Urquell and Radegast), so the analytical results are in agreement with the beer taste. It is interesting that two different types of beers from one producer (B2 and B3) have comparable profiles of polyphenolics.

3.4. Beer aging

The change of flavonoid content during the beer aging is studied in beer samples B1–B4. The first measurement is performed

Table 5
Concentrations (mg/L) of monitored compounds in hop extract samples E1–E7 determined with MS and UV detection.

Compound	Detection	E1	E2	E3	E4	E5	E6	E7
XN	MS	0.78 ± 0.03	0.37 ± 0.01	1.61 ± 0.04	47.9 ± 1.5	53.6 ± 1.6	125 ± 3	23.4 ± 0.7
	UV	1.02 ± 0.02	0.59 ± 0.01	1.75 ± 0.03	45.1 ± 0.9	49.4 ± 1.1	107 ± 2	25.8 ± 0.5
IXN	MS	n.d.[a]	n.d.[a]	n.d.[a]	3.44 ± 0.09	3.50 ± 0.08	4.19 ± 0.11	20.0 ± 0.6
	UV	n.d.[a]	n.d.[a]	n.d.[a]	3.22 ± 0.06	3.70 ± 0.07	4.85 ± 0.09	17.3 ± 0.3
8-PN	MS	n.d.[a]	n.d.[a]	n.d.[a]	0.09 ± 0.00	0.11 ± 0.01	0.25 ± 0.01	0.16 ± 0.01
	UV	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
IAA	MS	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	2.69 ± 0.08
	UV	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	2.81 ± 0.07
α + β	MS	1040 ± 11	665 ± 6	966 ± 12	931 ± 10	1370 ± 16	40.8 ± 0.6	1.43 ± 0.02
	UV	1050 ± 10	695 ± 7	977 ± 10	948 ± 9	1390 ± 15	34.9 ± 0.5	1.69 ± 0.04

[a] n.d., not detected.

Table 6
Concentrations (mg/L) of monitored compounds in beer samples B1–B6 determined with MS and UV detection.

Compound	Detection	B1	B2	B3	B4	B5	B6
XN	MS	0.04 ± 0.00	n.d.[a]	n.d.[a]	0.09 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
	UV	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
IXN	MS	1.28 ± 0.04	0.64 ± 0.02	0.81 ± 0.03	0.67 ± 0.02	0.48 ± 0.01	0.90 ± 0.04
	UV	1.79 ± 0.03	1.18 ± 0.02	n.d.[a]	1.12 ± 0.02	0.98 ± 0.02	1.35 ± 0.03
8-PN	MS	0.02 ± 0.00	n.d.[a]	n.d.[a]	0.02 ± 0.00	n.d.[a]	0.02 ± 0.00
	UV	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
IAA	MS	31.4 ± 0.9	15.2 ± 0.5	13.4 ± 0.4	18.9 ± 0.06	25.2 ± 0.08	37.2 ± 1.1
	UV	30.6 ± 0.7	14.1 ± 0.3	13.6 ± 0.2	20.5 ± 0.4	25.8 ± 0.5	36.9 ± 0.7
α + β	MS	4.07 ± 0.13	0.76 ± 0.03	0.49 ± 0.02	1.74 ± 0.05	1.68 ± 0.05	3.13 ± 0.09
	UV	4.15 ± 0.09	0.91 ± 0.02	0.65 ± 0.01	1.94 ± 0.03	2.25 ± 0.04	3.57 ± 0.07

[a] n.d., not detected.

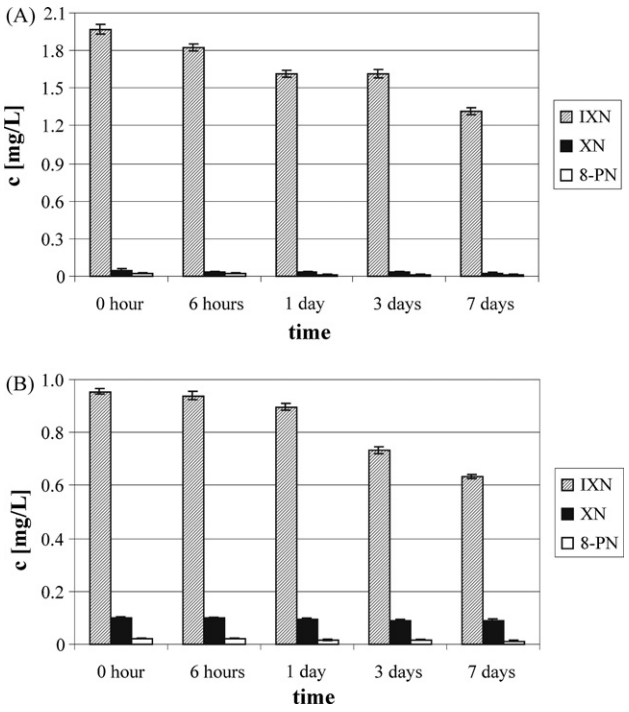

Fig. 9. Concentrations of xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8-PN) in beer during the beer aging experiment. (A) Pilsner Urquell lager, (B) Krušovice dark beer.

immediately after the bottle opening, further measurements are repeated after 6 h, 1 day, 3 days and 1 week after the bottle opening. The results for representative beer samples B1 (lager) and B4 (dark beer) are shown in Fig. 9. The measurement of beer volume is necessary during the whole experiment to compensate the evaporation at ambient conditions, which is almost 30% of the initial volume within 1 week. Nonalcoholic beer (B3) has mildewed during 3 days, so the experiment could not be finished. Probable explanation is that the presence of alcohol increases the resistance of beer against mildewing unlike to nonalcoholic beers. All beers become musty after 1 week. The content of IXN, XN and 8-PN slowly decreases during the aging experiment in all cases. In Pilsner Urquell lager (B1 – Fig. 9(A)), concentrations of IXN, XN and 8-PN after 1 day are 82, 80 and 83% related to the initial value defined as 100%, after 1 week 67, 65 and 50%, respectively. In Krušovice dark beer (B4 – Fig. 9(B)), the decrease as the relative concentration of polyphenolics is slightly slower, concentrations of IXN, XN and 8-PN after 1 day are 94, 94 and 90%, and after 1 week 66, 88 and 65%, respectively.

4. Conclusions

In this work, two HPLC/MS methods have been developed with the goal of superior separation of relatively low polar polyphenolic compounds found in hops and beer and highly polar polyphenolics in beer. Based on the combination of well optimized chromatographic separation and the interpretation of full scan and tandem APCI mass spectra, the presence of all previously described XN type prenylflavonoids and polyphenolic bitter acids is confirmed and MWs of numerous other compounds are determined. The quantitative analysis of main prenylflavonoids of XN type and bitter acids is correlated with various types of hops extraction. Finally, the beer aging experiment has shown that the absolute amount of polyphenolics is decreasing during time probably due to the oxidation reactions occurring at ambient conditions.

Acknowledgments

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