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ORIGINAL ARTICLE

Metabolite profiling and fingerprinting of commercial cultivars of *Humulus lupulus* L. (hop): a comparison of MS and NMR methods in metabolomics

Mohamed A. Farag · Andrea Porzel · Jürgen Schmidt · Ludger A. Wessjohann

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Abstract Hop (*Humulus lupulus* L. Cannabaceae) is an economically important crop. In addition to its role in beer brewing, its pharmaceutical applications have been of increasing importance in recent years. Bitter acids (prenylated polyketides), prenylflavonoids and essential oils, are the primary phytochemical components that account for hop medicinal value. An integrated approach utilizing nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques was used for the first large-scale metabolite profiling in *Humulus lupulus.* Resins and extracts prepared from 13 hop cultivars were analysed using NMR, liquid chromatography (LC)-MS and fourier transform ion cyclotron resonance (FTICR)-MS in parallel and subjected to principal component analysis (PCA). A one pot extraction method, compatible with both MS and NMR measurement was developed to help rule out effects due to differences in extraction protocols. Under optimised conditions, we were able to simultaneously quantify and identify 46 metabolites including 18 bitter acids, 12 flavonoids, 3 terpenes, 3 fatty acids and 2 sugars. Cultivars segregation in PCA plots generated from both LC-MS and NMR data were found comparable and mostly influenced by differences in bitter acids composition among cultivars. FTICR-MS showed inconsistent PCA loading plot results which are likely due to preferential ionisation and also point to the presence of novel isoprenylated metabolites in hop. This comparative metabolomic approach provided new insights for the complementariness and coincidence for these different technology platform applications in hop and similar plant metabolomics projects.

Keywords *Humulus lupulus* L. · Hop · ^{1}H NMR-based metabolomics · ESI-FTICR MS · LC-MS · Humulones · Lupulones · Principal component analysis · Flavonoids · Isoprenoids · Plant secondary metabolites

Abbreviations

ESI	Electrospray ionisation.
FTICR	Fourier transform ion cyclotron resonance.
GC	Gas chromatography.
LC	Liquid chromatography.
MS	Mass spectrometry
MS^n	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
PDA	Photodiode array detection
PCA	Principal component analysis

1 Introduction

The hop plant (*Humulus lupulus* L., Cannabaceae) is an economically important crop cultivated in most temperate zones of the world for its female inflorescences, commonly referred to as "hop cones" or "hops". The bitter, resinous substance produced in the glandular hairs of the strobiles (lupulin glands) is used in brewing, baking and as cattle feed for its bacteriostatic action and preservative qualities, but in beer also for taste (Zhang et al. 2004). In addition, it

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M. A. Farag · A. Porzel · J. Schmidt · L. A. Wessjohann (✉)
Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany
e-mail: wessjohann@ipb-halle.de

Present Address:
M. A. Farag
Pharmacognosy Department, College of Pharmacy, Cairo University, Kasr El Aini st., P.B. 11562, Cairo, Egypt

is used in pharmaceutical applications. The resin is used as a mild sedative in European phytotherapy, and hop has been investigated for its estrogenic and, more recently, potential cancer chemopreventive activities (Chadwick et al. 2004; Lamy et al. 2007). Many investigators contributed to the identification of bioactive ingredients from hop and to elucidate the underlying mechanisms by which they exert their activities. Three major classes of secondary metabolites are synthesized and accumulate in hop lupulin glands; essential oils, bitter acids, and prenylflavonoids (Fig. 1). Much of attention has gone to hop prenylflavonoids, and specific compounds, such as xanthohumol (prenylated chalcone) and 8-prenylnaringenin (flavanone), have been identified as multipotent bioactive compounds (Stevens and Page 2004; Wessjohann et al. 2005; Gerhauser 2005; Chadwick et al. 2006; Wilhelm and Wessjohann 2006; Biendl and Pinzl 2008).

Moreover, increasing evidence reveals that the so-called hop bitter acids, which represent up to 30% of the total lupulin content of hop, exhibit interesting effects on human health (Van Cleemput et al. 2009). The hop bitter acids are resinous alicyclic phenolic acids and usually classified as α-acids and β-acids. They are present in hop as a complex mixture of varying composition and concentrations. The main α-acids are humulone, cohumulone, and adhumulone; the corresponding β-acids are lupulone, colupulone, and adlupulone (see Fig. 1). The β-acids differ structurally from the α-acids by having one extra isoprenyl group. Besides the two series of normal-, co-, and ad-homologs, there are some minor hop acids in the plant, including posthumulone/postlupulone, prehumulone/prelupulone and adprehumulone (Wang et al. 2008). Essential oils are the principal aroma components of hop with myrcene (30–50%), α-humulene (15–25%), and β-caryophyllene as major monoterpene and sesquiterpene components. Furthermore, hop leaves contain a wide range of phenolic acids, condensed tannins, and flavonoid glycosides (Arraez-Roman et al. 2006; Li and Deinzer 2006).

The composition of essential oils is characteristic of the hop genotype and, together with that of bitter acids and flavonoids, has been used for distinguishing hop products (Wang et al. 2008; van der Kooy et al. 2009). There are least 200 different hop varieties grown and cultivated worldwide and it is of interest in developing accurate methods for hop characterization that could be used to prevent adulteration and to classify hop from different geographical origins or countries. For reliable differentiation of hop cultivars, a systematic method including this myriad of metabolites (metabolite profiling) is desirable. With the recent developments in plant metabolomics

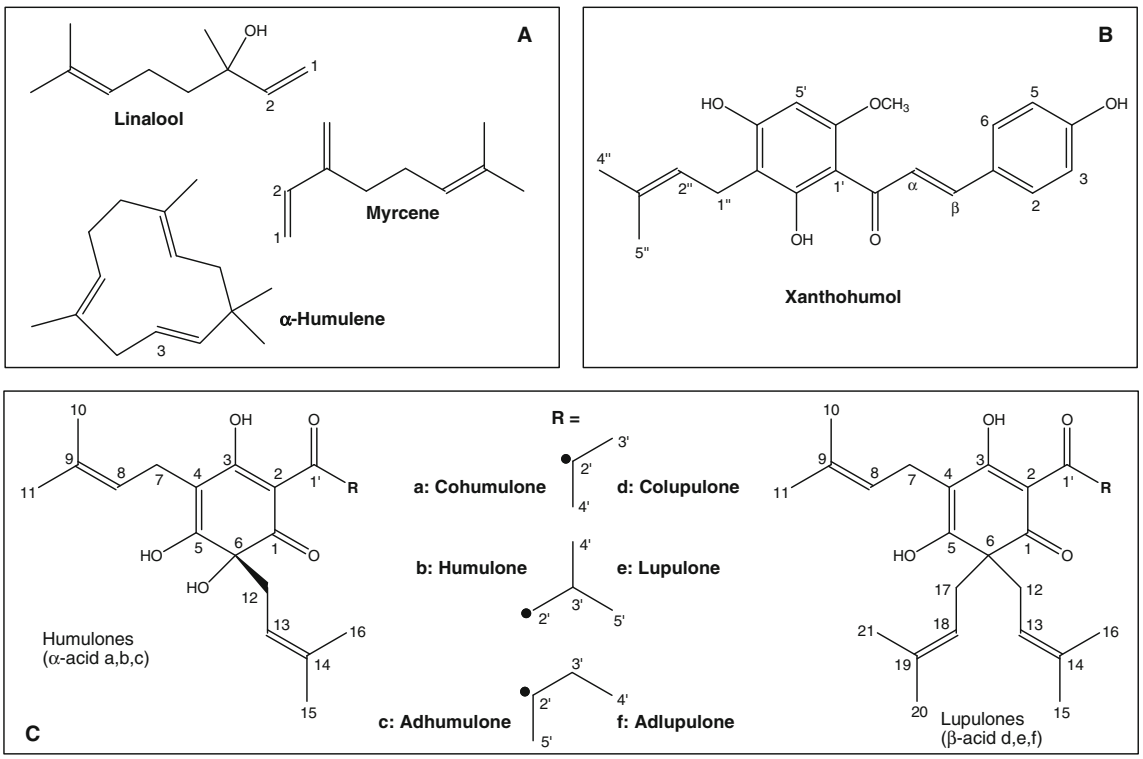

Fig. 1 Major classes of natural products; terpenes (**A**), flavonoids (**B**) and bitter acids (**C**) detected in hop with selected compound(s) discussed in the manuscript. Note: the carbon numbering system for each compound is used throughout the manuscript for NMR assignment, and thus is based on analogy rather than IUPAC rules

techniques, it is now possible to detect several hundred metabolites simultaneously and to compare samples reliably to identify differences and similarities in an untargeted manner. Several techniques have been developed for the nontargeted profiling of metabolites in plants. These include ^{1}H-NMR (Le Gall et al. 2003), HPLC-MS (Tolstikov and Fiehn 2002; von Roepenack-Lahaye et al. 2004), GC-MS (Fiehn et al. 2000), and direct injection FTICR-MS (Aharoni et al. 2002; Leon et al. 2009; Takahashi et al. 2008).

Growing evidence points to that no single method is adequate and that combining both MS and NMR, offers a powerful methodology for metabolomics projects. Separation based mass spectrometry (MS) approaches, such as liquid chromatography LC-MS, are relatively inexpensive, highly sensitive and provide excellent identifying capacity. Unlike MS-based analyses, nuclear magnetic resonance (NMR) spectroscopy is universal (i.e. independent of ionisation propensities) and provides a large amount of information regarding molecular structure, and absolute quantification of compounds within composite samples; NMR has a lower sensitivity compared to MS (Moco et al. 2007). Because of the complementary analytical features of NMR and MS, opportunities for leveraging both methods are being considered which will create more comprehensive metabolic profiling. While only few studies to date have combined both analytical techniques with multivariate analysis (Moco et al. 2008), this approach has exciting potential for the field of plant metabolomics. Such an approach was successfully used recently to investigate standardized *Ginkgo biloba* pharmaceutical preparations (Agnolet et al. 2010) and to demonstrate the metabolic similarity between transgenic and wild type potato (Catchpole et al. 2005).

This study aims to provide the first hop metabolic fingerprint and chemical profile using multiple approaches: LC-MS, ESI-FTICR-MS and NMR. For hop only one metabolomic study was reported on primary metabolites in response to nodulation using ^{1}H-NMR (Fortes et al. 2008). However, hop probably is the economically most important crop on earth grown exclusively for its secondary metabolites. In the present study, we present the first secondary metabolites profile of hop resins and cone extracts of 13 different cultivars, most of them of agricultural importance. The semi-polar metabolite content of the female cone was captured by using acetone as an extraction solvent. These extracts were successively analysed using the aforementioned technology platforms, and processed data were further subjected to multivariate analysis using principal component analysis (PCA) to reveal for compositional differences among cultivars. To the best of our knowledge, this comparative approach provides the first comprehensive study on the differences, complementariness and coincidence of these different technology platforms applied in plant metabolomics.

2 Materials and methods

2.1 Plant material

The 13 different hop cones and resins included in this study were provided from Hopsteiner (Mainburg, Germany). All information on collected samples and their origin is recorded in Table 1. Resins were obtained by standard extraction with ethanol. A detailed description of the resin preparation is given by (Biendl and Pinzl 2008).

2.2 Chemicals and reagents

Methanol-d_4 (99.80% d), acetone-d_6 (99.80% d) and hexamethyldisiloxan (HMDS) were provided from Deutero GmbH (Kastellaun, Germany). For NMR quantification and calibration of chemical shift, HMDS was added to a final concentration of 0.94 mM. Acetonitrile and acetic acid (LCMS grade) were obtained from J. T. Baker (The Netherlands), milliQ water was used for LC analysis. Standard for α-acids mixture (30.06% cohumulone and 69.93% humulone + adhumulone), β-acids mixtures (47.95% colupulone and 52.05% lupulone + adlupulone) and xanthohumol were provided by Hopsteiner (Mainburg, Germany). Chromoband C18 (500 mg, 3 ml) cartridge was purchased from Macherey & Nagel (Düren, Germany). Astragalin, isoquercetrin and xanthohumol were provided from Chromadex (Wesel, Germany). 8-Prenyl naringenin, desmethylxanthohumol and isoxanthohumol were provided

Table 1 Abbreviations and origins of hop cultivars harvested in 2009 included in this study

Cultivar	Country	Abbreviation
Hallertau Perle	Germany	HPE
Hallertau Hallertauer Tradition	Germany	HHT
Hallertau Hersbrucker	Germany	HHE
Hallertau Herkules	Germany	HHS
Hallertau Hallertauer Magnum	Germany	HHM
Hallertau Spalter Select	Germany	HSE
Hallertau Hallertauer Taurus	Germany	HTU
Elbe-Saale Magnum	Germany	EHM
Elbe-Saale Northern Brewer	Germany	ENB
Tettnang Perle	Germany	TPE
Mühlviertel Magnum	Austria	ATHM
Mühlviertel Perle	Austria	ATPE
Saaz Agnus	Czech Republic	CZAG

by Orgentis Chemicals GmbH, Gatersleben, Germany. All other chemicals and standards were provided from Sigma Aldrich (St. Louis, MO, USA).

2.3 Extraction procedure and sample preparation for NMR and LC-MS analyses

Dried hop cones were ground with a pestle in a mortar using liquid nitrogen. The powder (60 mg) was then homogenized with 5 ml acetone containing 8 µg/ml umbelliferone (an internal standard for relative quantification using LCMS) using a Turrax mixer (11000 RPM) for five 20 s periods. To prevent heating, a period of 1 min separated each mixing period. Extracts were then vortexed vigorously and centrifuged at 3000*g* for 30 min to remove plant debris. For NMR analysis, 4 ml were aliquoted using a syringe and the solvent was evaporated under a stream of nitrogen till dryness. Dried extracts were resuspended with 800 µl 100% acetone-d_6 containing HMDS (0.94 mM), which served as an internal chemical shift NMR standard. After centrifugation (13000*g* for 1 min), the supernatant was transferred to a 5 mm NMR tube. Acetone-d_6 was added only prior to NMR acquisition to prevent possible reaction with the sample. For FTICR- and LC-MS analyses, 500 µl were aliquoted and placed on a (500 mg) C18 cartridge preconditioned with acetone and water. Samples were then eluted using 6 ml acetone, the eluent was evaporated under a nitrogen stream and the obtained dry residue was resuspended in 1 ml methanol. Two µl were used for LCMS analysis, whereas for FTMS, samples were diluted at a ratio of 1:5 prior to analysis.

Extraction of hop resin for analysis was based on that developed by (Magalhaes et al. 2007) for hop resin analysis by LCMS with minor modifications. For NMR analysis, an aliquot (20 mg) of the resin was suspended in 1 ml of methanol-d_4 containing 0.94 mM HMDS. After sonication for 2 min, the samples were centrifuged at 20,000*g* for 1 min to remove insoluble material and 700 µl of the supernatant was transferred to a 5 mm NMR tube. All ^{1}H-NMR spectra for multivariate data analysis were acquired consecutively within a 48 h time-interval with samples prepared immediately before data acquisition similar to the cone extracts. Repeated control experiments after 48 h showed no additional variation. For LCMS and ESI-FTICR-MS analysis, 1 mg of hop resin was extracted with 1 ml 100% MeOH containing umbelliferone (IS) at a concentration of 8 µg/ml. After sonication for 2 min, an aliquot of 200 µl extract was loaded on a (500 mg) C18 cartridge preconditioned with methanol and water. Samples were then eluted using 5 ml 100% MeOH, eluent evaporated under nitrogen stream and the obtained dry residue was resuspended in 1.5 ml methanol. Two µl were used for LCMS analysis, whereas for FTMS, samples were diluted at a ratio of one to five prior to analysis.

2.4 LC-MS analysis

The LC-ESI and ESI-MS^n mass spectra were obtained from a LCQ Deca XP MAX system (ThermoElectron, San Jose, USA) equipped with a ESI source (electrospray voltage 4.0 kV, sheath gas: nitrogen; capillary temperature: 275°C). The Ion Trap MS system is coupled with a Surveyor micro-HPLC and equipped with a HYPERSIL GOLD RP18-column (5 mm, 150 × 1 mm, ThermoScientific). For HPLC a gradient system was used starting from H_2O:CH_3CN = 90:10 (each of them containing 0.2% HOAc) to 100% CH_3CN within 30 min, then isocratically for further 10 min; flow rate 70 µl/min. All mass spectra were averaged and background substracted. The Xcalibur 2.0 software was used for the data evaluation.

The MS^n spectra were recorded during the HPLC run by using the following conditions: isolation width ± 2 amu; normalized collision energy 35%; activation Q 0.25; activation time 30 ms.

2.5 High resolution UPLC-MS analysis

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 µm; Waters) applying the following binary gradient at a flow rate of 150 µl min^{-1}: 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B. The injection volume was 3.1 µl (full loop injection). Eluted compounds were detected from *m/z* 100 to 1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive and negative ion modes using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 l min^{-1}, 190°C; capillary, −5500 V (+4000 V); end plate offset, −500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; prepulse storage, 5 µs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 µl 10 mM lithium formiate in isopropanol/water, 1/1 (v/v), at a gradient time of 18 min using a diverter valve.

2.6 ESI-FTICR-MS analysis

The positive and negative ion high resolution ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FTICR) mass

spectrometer (Bruker Daltonics, Billerica, USA) equipped with an Infinity cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an APOLLO electrospray ion source (Agilent, off axis spray). Nitrogen was used as drying gas at 150°C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 2 ml/min. All data were acquired with a 512 k data points and zero filled to 2048 k by averaging 32 scans. The XMASS 6.1.2 and the Data Analysis Software (DA 4.0), respectively, were used for the calculation of the elemental composition.

2.7 Fatty acids derivatization and GC-MS analysis

One mg of dried hop resin (HHT) was resuspended in 0.5 ml chloroform and (trans-) esterified by adding 1 ml 1.25 M HCl in MeOH and incubating for 4 h at 65°C. Following the transesterification, HCl and solvent were evaporated under nitrogen. The sample was resuspended in 500 µl pyridine and transferred to a 200 µl glass insert, and analysed using a Thermo Trace GC coupled to a Voyager MS equipment (ThermoElectron, San Jose, USA) operating in EI mode at 70 eV and scanning from *m/z* 50–650. Samples were injected at a 1:1 split ratio, and the inlet and transfer line were held at 280°C. Separation was achieved with a temperature program of 80°C for 2 min, then ramped at 5°C min^{-1} to 315°C and held for 12 min, a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 µm film thickness) and a constant flow of 1.0 ml min^{-1}. MS interface and ion source were set at 280°C and 180°C, respectively.

2.8 NMR analysis

All spectra were recorded on a Varian VNMRS 600 NMR spectrometer operating at a proton NMR frequency of 599.83 MHz using a 5 mm inverse detection cryoprobe. ^{1}H NMR spectra were recorded with the following parameters: digital resolution 0.367 Hz/point (32 K complex data points); pulse width (pw) = 3 µs (45°); relaxation delay = 23.7 s; acquisition time = 2.7 s; number of transients = 160. Zero filling up to 128 K and an exponential window function with lb = 0.4 was used prior to Fourier transformation. 2D NMR spectra were recorded using standard CHEMPACK 4.1 pulse sequences (gDQCOSY, gHSQCAD, gHMBCAD) implemented in Varian VNMRJ 2.2C spectrometer software. The HSQC experiment was optimized for $^{1}J_{CH}$ = 146 Hz with DEPT-like editing and ^{13}C-decoupling during acquisition time. The HMBC experiment was optimized for a long-range coupling of 8 Hz; a 2-step $^{1}J_{CH}$ filter was used (130–165 Hz).

2.9 MS Data processing and PCA analysis

Relative quantification of hop metabolites profiles after LC/MS was performed using XCMS data analysis software, which can be downloaded freely as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) (Smith et al. 2006). This software approach employs peak alignment, matching and comparison (Smith et al. 2006). Native LCMS files from Xcalibur 1.4 (Thermo Fisher Scientific, Inc., Waltham, MA) were firstly converted into netCDF files using the File Converter tool. Files were arranged in one folder that was set as the file source. Peaks were subsequently extracted using XCMS under R 2.9.2 environment with signal-to-noise ratio set at 4. After peak extraction and grouping, nonlinear retention time correction of peaks was accomplished in two iterative cycles with descending bandwidth (bw). This was accomplished by manually decreasing the bw parameter (from 30 to 10 s). The resulting peak list was further processed using the Microsoft Excel software (Microsoft, Redmond, WA), where the ion features were normalized to the total integrated area (1,000) per sample and imported into the R 2.9.2 software package for principal component analysis (PCA). Absolute peak area values were autoscaled (the mean area value of each feature throughout all samples was subtracted from each individual feature area and the result divided by the standard deviation) prior to principal component analysis. This provides similar weights for all the variables, as described elsewhere (van den Berg et al. 2006). PCA was then performed on the MS-scaled data to visualize general clustering, trends, and outliers among all samples on the scores plot.

A similar approach was used for FTICR-MS data processing, except that files were converted to mzdata format in the R 2.9.2 software package and peak picking was made using the MSW method with a signal to noise threshold set = 3. Alignment was made based on high resolution mass with a mass window of 5 ppm.

2.10 NMR data processing and PCA analysis

The ^{1}H-NMR spectra were automatically Fourier transformed to ESP files using ACD/NMR Manager lab version 10.0 software (Toronto, Canada). The spectra were referenced to hexamethyl disiloxane (HMDS) at 0.062 ppm for ^{1}H-NMR and for CD_3OD signals at 49 ppm for ^{13}C-NMR, respectively. Spectral intensities were reduced to integrated regions, referred to as buckets, of equal width (0.04 ppm) within the region of δ 11.4 to −0.4 ppm. The regions between δ 5.0–4.7, δ 3.4–3.25 and δ 2.08–2.00 corresponding to residual water, methanol and acetone signals, respectively, were removed prior to multivariate analyses. Principal component analysis was performed with R

package (2.9.2) using custom-written procedures after scaling to HMDS signal and exclusion of solvent regions.

For the quantification of total α- and β-bitter acids, xanthohumol, α-humulene and linalool the peak area of selected ^{1}H NMR signals belonging to the target compounds, and the peak area of the IS (HMDS) were integrated manually for all the samples (details see Electronic Supplementary file1).

3 Results and discussion

3.1 Development of a single pot extraction method for MS and NMR analyses

We selected a study group of 13 hop cultivars and their derived resins, 10 of which originated from Germany and three others from Austria and the Czech Republic (see Table 1). To allow for a comparative analysis of the metabolite data derived from different technology platforms, a one pot extraction method was developed for extraction and was found compatible for both NMR- and MS-based metabolomics. Several solvents were initially tested including acetone, ethanol, methanol and 80% methanol. Solvent selection for sample preparation was evaluated with respect to reproducibility, quality and recovery of hop secondary metabolites as revealed by ^{1}H-NMR. The eventual decision for acetone was based on our interest in profiling hop secondary metabolites with an emphasis on those most abundant in hop resins (Vanhoenacker et al. 2001) and more likely to account for differences among hop cultivars (Wang et al. 2008). Compared with MS, NMR can detect metabolites universally without any derivatization step, thus providing an unbiased picture of differences among extraction methods. Compared with methanol and ethanol, acetone shows high recovery for bitter acids and xanthohumol signals as is revealed by resonances at aliphatic (δ 0.9–1.8 ppm) and olefinic/aromatic region (δ 5.5–8 ppm). As expected, methanol and to a lesser extent, ethanol extracts show high density signals for sugars in the region from δ 3–5 ppm (Supplementary Fig. 1). Most notably, resonance signals for methylene protons in bitter acids (δ 2.4–3.0 ppm) are better resolved in acetone versus the other two solvents (see inserts in supplementary Fig. 1). It should be noted that modifying solvent polarity by addition of water at a concentration of 20% did not enhance the recovery of flavonoid monoglycosides and aglycones but increased that of diglycosides (i.e. rutin) from hop cones as revealed by LCMS detection (data not shown). Acetone was therefore chosen for preparing the bulk extract from cones, further aliquoted for both MS and NMR sample preparation. Previously, acetone had been shown to be the most efficient and reproducible extraction solvent for anthocyanins in strawberries (Garcia-Viguera et al. 1998). The use of deuterated solvent, commonly used for NMR sample extraction was ruled out in our case due to possible deuterium exchange with sample components affecting further MS acquisition and metabolites assignment.

Two types of variance exist in metabolomics datasets: technical (or analytical) variance created by the experimental procedure, including sample preparation and instrumental analysis, and inherent biological variance existing within a population. To reveal for differences due to different detection methods as utilized in this study, it is crucial that the biological variance is minimized. Commercial hop resin, piloted from a large population of cone material, provides a uniform and consistent material for initial profiling of the different hop lines. To assess for the biological variance across each line, cone materials were later extracted in three biological replicates for all spectroscopic measurements.

3.2 NMR assignments

The use of NMR for metabolite profiling of hop has not been reported before, although HPLC-NMR has been used to characterize hop bitter acid content (Holtzel et al. 1996).

We chose the HHT cultivar to positively demonstrate the NMR spectroscopic characterisation of the metabolites investigated in this study. NMR signals were assigned using a combination of 2D-NMR experiments (^{1}H,^{1}H COSY, HSQC, and HMBC) in comparison with spectra of reference standards. Chemical shifts of compounds that were identified are listed in Table 2. Figure 2A shows example of representative ^{1}H-NMR spectra. The ^{1}H-NMR spectrum was characterized by three main regions: a low-field region between 5.5 and 8.0 ppm with signals principally due to aromatic protons of xanthohumol and olefinic protons of α-humulene and linalool; a mid-low field region between 5.5 and 4.5 ppm with signals due to anomeric protons of sugar units and olefinic protons of unsaturated fatty acids, and a high-field region between 3.2 and 0.8 ppm with high density signals due to methyl, methylene, and methine signals of α- and β-bitter acids, indicating that the resin contains a large amount of bitter acids. Some aglycon resonances were readily assigned due to their chemical shifts, together with their splitting and couplings. In particular, signals at δ 5.61 and 5.96 ppm were attributed to H-3 of α-humulene and H-2 of linalool, respectively (Figs. 1A, 2C). Other aromatic signals (Fig. 1B) at 6.04, 6.83, 7.51, 7.69 and 7.79 ppm (Fig. 2C) were assigned to xanthohumol by comparison with the ^{1}H-NMR spectrum of the standard compound, and with data reported in the literature (Chadwick et al. 2004). Bitter acid constituents of the humulone (α-acids, H) and lupulone

Table 2 Resonance assignments with chemical shifts of constituents identified in 600 MHz ^{1}H and ^{1}H-^{13}C 2D-NMR spectra of hop HHT resin extract (methanol-*d*4)

Molecule(s)	Assignment	^{1}H shift (δ)	^{13}C shift (δ)
Adlupulone	CH3-4′	0.89	12.3
	CH_3-5′	0.90	14.8
Adhumulone	CH_3-4′	0.93	12.1
	CH_3-5′	1.06 (d)	16.8
Humulone & lupulone	CH_3-4′& 5′,	0.95 (d)	23.6
	CH_2-2′	2.83 (m)	50.1
Cohumulone & colupulone[a]	CH_3-3′& 4′	1.08 (d), 1.09 (d)	19.6
H & L	CH_2-7a, CH_2-7b	3.01 (dd), 3.09 (m)	21.9
	CH_3-10	1.74 (s)	18.0
	CH_3-11	1.66 (s)	26.1
Humulone	CH_2-2′a	2.74 (dd)	48.4
Cohumulone	CH-2′	3.80 (m)	36.9
Colupulone	CH-2′	3.97 (m)	37.2
α-Humulene	CH-3	5.61 (dt, 16 & 7 Hz)	128.8
H	CH_3-16	1.51 (s)	18.0
	CH_3-15	1.60 (s)	26.1
	CH_2-12	2.51(m)	42.5
	CH-13	4.94 (m)	117.9
	CH-8	5.13 (t)	123.3
L	CH_3-15 & 20	1.55 (s)	26.1
	CH_3-16 & 21	1.57 (s)	18.2
	CH-13 & 18	4.77 (m)	119.5
	CH-8	5.03 (t)	123.3
Xanthohumol	CH_3-5″& 4″	1.71 (s)	25.9
	CH_2-1″	3.22 (d)	54.8
	OCH_3	3.90 (s)	56.3
	CH-5′	6.00 (s)	91.8
	CH-3 & CH-5	6.83 (d, 8.4 Hz)	116.9
	CH-2 & CH-6	7.51 (d, 8.4 Hz)	132.0
	CH-α	7.79 (d, 15.4 Hz)	126.6
	CH-β	7.69 (d, 15.4 Hz)	144.2
β-Glucose	CH-1	4.47 (d, 7.7 Hz)	98.0
Linalool	CH-2	5.96 (dd, 17 &10 Hz)	147.9
Myrcene[b]	CH-2	6.37 (dd, 17 &11 Hz)	140.3
Fatty acids	$\underline{CH_3}(CH_2)_n$–,	0.89	12.1
	$(CH_2)_n$	1.28	30.6

H (α-acid) series = adhumulone, cohumulone, humulone

L (β-acid) series = lupulone, adlupulone, colupulone

[a] Signal overlap but coupling could be distinguished

[b] Signal overlap in case of HHT resin but distinct in other cultivars

(β-acids, L) series (Fig. 1C) are characterized by the presence of a quinoid structure with isoprenyl side chains, that is, vinyl (4.7–5.0 ppm), allylic methyl (1.4–1.7 ppm), and allylic methylene protons (2.5–3.1 ppm) (see Fig. 2B). Except for adhumulone and adlupulone, resolved unique signals for other homologues could be identified in the resin proton NMR spectra. Terminal methyl protons at 4′ and 5′ positions were not well resolved in case of adhumulone and adlupulone, due to an overlap with other major proton signals at 0.95 ppm; the signal for methyl protons at 5′ in adhumulone (1.06 ppm) could be resolved as a doublet in HHT (Fig. 2), but not in most other cultivars. However, in HSQC experiments, it was easy to assign cross peaks unique for adhumulone and adlupulone, since the ^{13}C chemical shifts of C-5' appeared different for adhumulone and adlupulone at 16.2 and 14.8 ppm, respectively (Supp. Fig. 2). Using HSQC and HMBC experiments, all aliphatic signals of the humulone and lupulone series were also identified. It was demonstrated that among bitter acid signals, those of the characteristic region (0.89–1.3 ppm) were due to methyl and methylene proton resonances of the acyl side chain at C-2 in both H and L series (Fig. 2B). Signals due to fatty acids at δ 0.90 (terminal methyl groups) and 1.28 ppm (inner-chain CH_2 groups) could not be assigned to individual fatty acids. The assignments of HSQC correlation peaks in hop resin are shown in Supp. Table 1 and Supp. Fig. 3. All assignments were verified by HSQC and HMBC spectra of reference compounds. In case of α- and β-acids, only mixtures of cohumulone/humulone/adhumulone and colupulone/lupulone/adlupulone, respectively, were available. Due to very similar properties, the regioisomeric pairs humulone/adhumulone as well as lupulone/adlupulone could not be separated by chromatographic methods. However, the distinct differences in their HMBC correlation signals patterns allowed an unequivocal assignment of the corresponding ^{1}H and ^{13}C NMR signals.

3.3 Multivariate PCA analysis of NMR data

Similar metabolite patterns were observed by simple visual inspection of the ^{1}H-NMR spectra of hop resins. Therefore, we attempted to analyze the NMR spectra in a more holistic way using PCA to explore the relative variability within the different cultivars. PCA is an unsupervised clustering method requiring no knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance within (Goodacre et al. 2000). Duplicate NMR measurements from the same biological sample were found to be highly reproducible, as the scores of replicate measurements were virtually superimposed. Hence, one replicates is displayed in order to get a clearer display. Indeed, until now NMR technology provides the highest reproducibility of >98% among metabolomic measuring platforms (Dumas et al. 2006). The main principal component (PC) to differentiate hop, i.e. PC1, accounts for 61% of hop variance. The PC1/PC2 scores plot (Fig. 3A) shows that 3 major distinct clusters

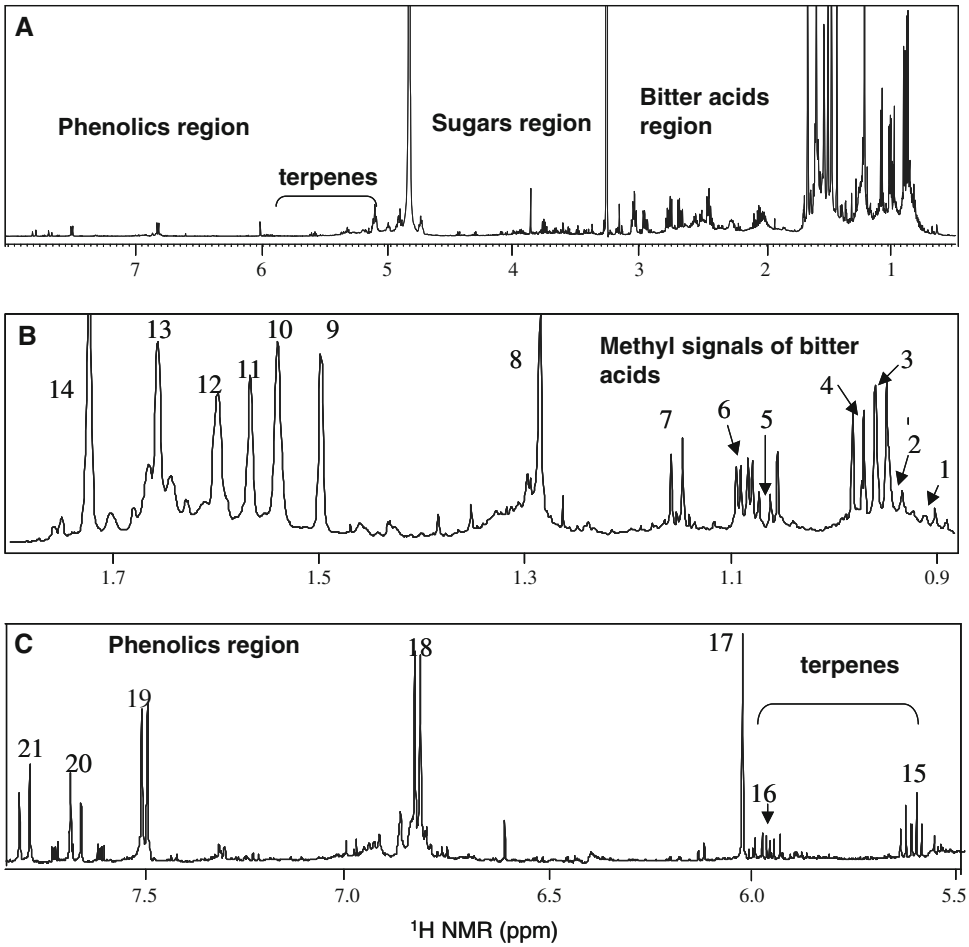

Fig. 2 Details of the ^{1}H-NMR spectrum of hop resin (HHT) demonstrating both signal richness and dynamic range of extract NMR spectra (**A**). Expanded spectral region from 0.90 to 1.75 ppm for bitter acids (**B**), and phenolics from 5.5 to 7.5 ppm (**C**) with assigned peaks: 1, adlupulone; 2, adhumulone; 3, humulone and lupulone; 4, humulone; 5, adhumulone; 6, colupulone and cohumulone; 7, cohumulone; 8, fatty acids; 9, humulones series; 10, lupulones series; 11, lupulones series; 12, humulones; 13, humulones and lupulones series; 14, humulones and lupulones series; 15, α-humulene; 16, linalool; 17, xanthohumol; 18, xanthohumol; 19, xanthohumol; 20, xanthohumol; 21, xanthohumol

are formed corresponding to the 13 different varieties studied. On the right side of the plot, hop cultivars HHE, HSE and HHT are positioned (positive PC1 values), whereas on the far left side, HHS, HTU, ATHM, and HHM are located (negative PC1 values). The other 6 cultivars ATPE, CZAG, ENB, EHM, TPE and HPE are spread in between. This group can still be separated along PC2. The separation observed in PCA can be explained in terms of the identified compounds, using the loading plots for PC1 exposing the most discriminatory signals. Two major groups stand out in this plot. The first corresponds to the chemical shifts of lupulones (L) and fatty acids, i.e. δ 1.56 and δ 1.28, contributing positively to PC1. The second, in the bitter acids region from methyl signals at δ 1.5, 1.6 and 2.5 ppm have a negative effect on PC1 and was assigned for the humulones (H) series (Fig. 3B).

These results suggest that hop segregation in PCA is mostly influenced by the ratio of α- versus β-bitter acids among examined hop cultivars. It should be noted that clear separation between hop based on geographical regions could not be observed from PCA as CZAG, ATPE and ATHM lines from Austria and Czech Republic clustered close to other cultivars originated from Germany. To confirm that discrimination between cultivars is related to bitter acid levels, quantifications were attempted for total α- and β-bitter acids in resins using ^{1}H-NMR methyl signals; (Me-15, Me-16 for α-acids) and (Me-15, Me-16, Me-20, Me-21 for β-acids). These signals were well separated for α- and β-acids as previously reported by (Hoek et al. 2001). For the quantification to be optimal, however, full relaxation of the protons of the bitter acids signals and the internal standard has to be achieved. For this purpose a rather large sum of relaxation delay and acquisition time of 25 s was used, as the longest relaxation times were 4.5 s for the HMDS protons. Results are represented in Fig. 4. In agreement with PCA results, hop cultivars HHS, HTU & HHM were found to be enriched in α-acids, higher levels of β-acids were determined in cultivar HHE, and to less extent in HSE. PCA results recognizing HHT, ENB and EHM cultivars as β-acids enriched could not be revealed from our absolute quantification. Albeit, lower levels of α-acids could be found in these lines compared with other cultivars as suggested from PCA. Quantifications of fatty acids have not been attempted in cultivars and could have accounted for this discrepancy. Myrcene, α-humulene and linalool, major terpenes in hop, appear at concentration

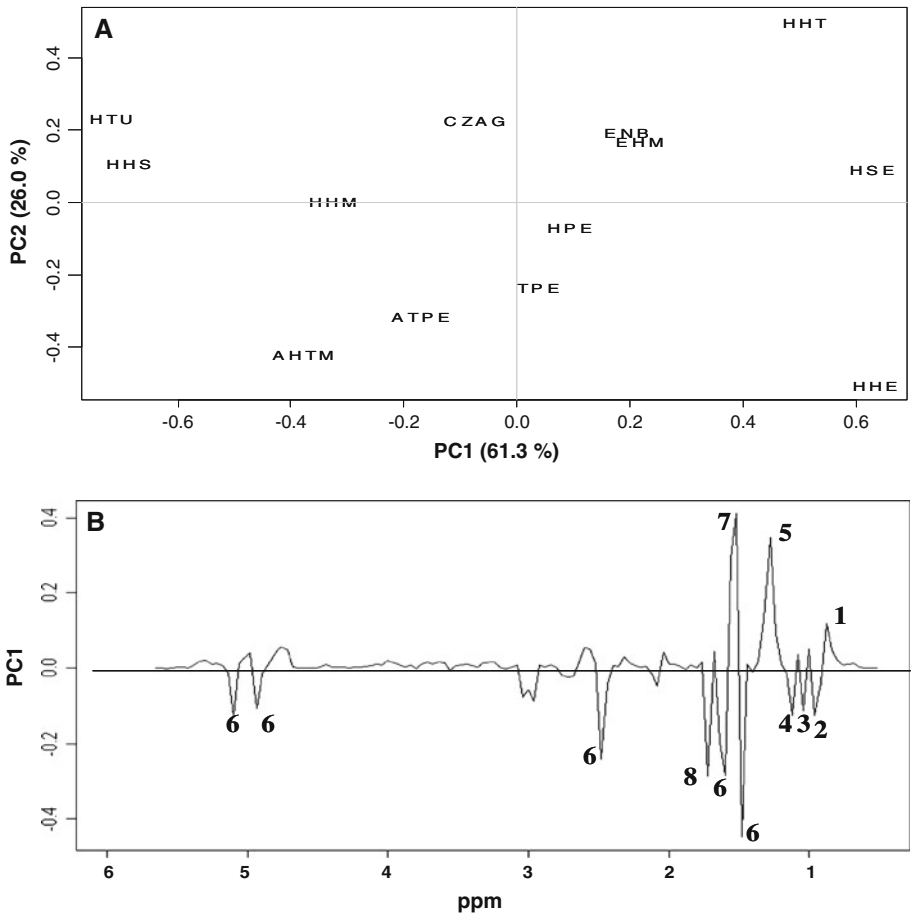

Fig. 3 ^{1}H-NMR (δ 0.4–11.0) peak based principal component analyses of hop resins of different cultivars. **A** Score Plot of PC1 and PC2 scores. **B** loading plot for PC1 components: 1, adlupulone and fatty acids; 2, lupulone and humulone; 3, adhumulone; 4, cohumulone; 5, fatty acids; 6, humlones; 7, lupulones; 8, humulones and lupulones. Group discrimination in samples is related to qualitative and quantitative differences in the bitter acids pattern. [δ 5.0–4.7, δ 3.4–3.25 regions excluded, 0.04 ppm buckets]

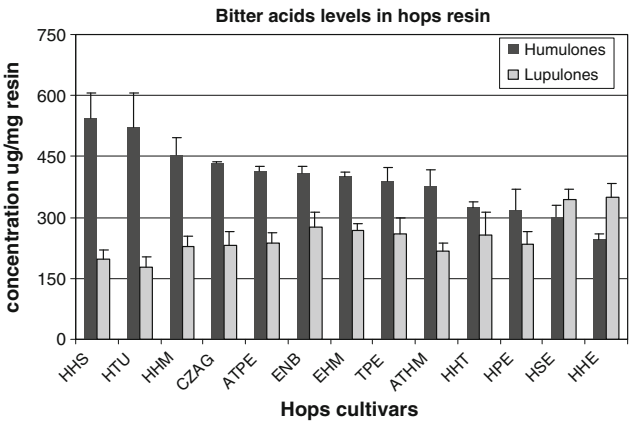

Fig. 4 Quantification of humulones (α-acids) and lupulones (β-acids) in hop resins using ^{1}H-NMR spectra. Note that these are averages from two different chemical shifts within the same sample. NMR signals (Me-15, δ 1.6) & (Me-16, δ 1.51) for α-acids; (Me-15 & Me-20, δ 1.55) and (Me-16 & Me-21, δ 1.57) for β-acids were used for quantification

levels in the range of 32–53, 41–61 and 72–107 μg g^{-1} resin, respectively. Xanthohumol, the major prenylated flavonoid in hop, was detected at an average level of about 30 μg g^{-1} resin. It is worth mentioning that the percentage of total bitter acids in most resins reached high values ranging from 59 to 74% among all cultivars. The current method in use for determining α- and β-acids is high-performance liquid chromatography (HPLC), which involves a more laborious, error prone and time-consuming procedure of sample preparation than NMR, but uses cheaper equipment (Culik et al. 2009). We could show that ^{1}H-NMR is an appropriate tool for bitter acids profiling without any chromatography step, especially as these metabolites are the main constituents in the hop resin and extract samples, similar to the case of using NMR for sugar analysis in fruit juices (Biais et al. 2009).

3.4 LC-MS peak identification

Simultaneously acquired HPLC-PDA and HPLC-MS total ion chromatograms of hop resin are presented in Fig. 5. The identities, retention times, UV characteristics, and observed molecular and fragment ions for individual components are presented in Table 3. A total of 39 metabolites were detected, and 30 were tentatively identified. Metabolite assignments were made by comparing retention time, UV/Vis spectra and MS data (accurate mass, isotopic distribution and fragmentation pattern in both positive and negative ion modes) of the compounds

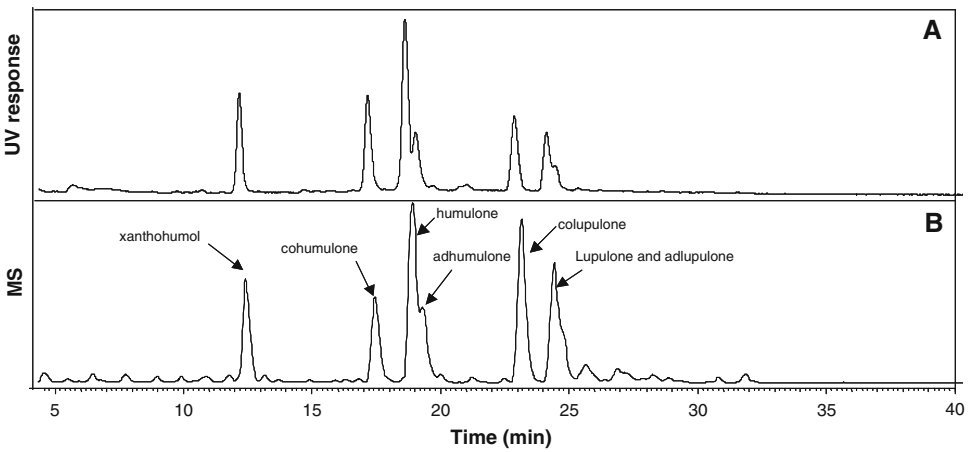

Fig. 5 LC-PDA (**A**) and LC-ESI-MS (**B**) total ion current chromatograms of hop resin HHT. Chromatographic conditions are described under Materials and Methods. The identities, R_t values, and basic UV and MS data of all peaks are listed in Table 3

detected with hop compounds reported in the literature and searching in the existing on-line public databases. Identifications were confirmed with standard compounds whenever available in-house. Identified metabolites belonged to various classes including sugar, chalcones, flavanones, flavonols and α/β-bitter acids, with the later accounting for the highest abundance in resin similar to results derived from ^{1}H-NMR spectra (see Fig. 2A). Xanthohumol was identified as the major principal prenylflavonoid in agreement with literature (Arraez-Roman et al. 2006). As expected, no peaks for xanthogalenol, 4′-*O*-methylxanthohumol, and 4′,6′-di-*O*-methylchalconaringenin, chemotaxonomic markers for North American hop, were found in the studied European hop (Stevens et al. 2000). Humulinone, a major oxidation product of the α-bitter acid humulone was tentatively identified, albeit at trace levels in all resins suggesting that bitter acids were not subject to any major chemical degradation (Arraez-Roman et al. 2006). The predominant loss of 69 amu ($-C_5H_9$, prenyl group) in the MS^n spectrum is diagnostic for the presence of the isoprenyl group in bitter acids and flavonoids; a total of 27 peaks show this pattern and the number of isoprenyl groups was predicted from the sequential loss of 69 amu from metabolites. A detailed description of MS fragmentation for prenylated bitter acids and flavonoids was previously described by (Zhang et al. 2004; Simons et al. 2009). The analysis of hop cones from which resins were derived was also made to determine whether qualitative and or quantitative differences exist between both materials and to check for biological variance within the cultivar. Most of the metabolites identified in resins were also detected in cones. In addition, several glycosidic conjugates of quercetin, and kaempferol aglycones (peaks 2, 3, 4, 5 & 7) were detected in cones, but not in resin (Table 2). These glycosides exhibit the predominant losses of 146 u (deoxyhexose), 162 u (hexose) or 86 (malic acid), in the MS^n spectrum diagnostic for flavonoid *O*-glycosides, and these compounds are, therefore, assigned as non-prenylated flavonoids. Absence of these glycosides from resin is likely due to their polar nature which precludes its presence within the resin lipophilic matrix during extraction. A quantitative analysis for different classes of compounds identified in hop cones is presented in (Supp. Table 2), which includes the relative quantification of each metabolite to the recovered amount of spiked umbelliferone (IS) to allow for comparison across cultivars.

3.5 Multivariate PCA analysis of LC-MS data

Resin extracts of cultivars were analysed in both positive and negative-ion electrospray ionization HPLC-(ESI)MS modes as changes in ESI polarity can often circumvent or significantly alter competitive ionization and suppression effects revealing otherwise suppressed metabolite signals (de Rijke et al. 2003). From the 13 resins, 772 and 610 mass signals were extracted by XCMS from the LC-MS data set acquired in negative and positive ionisation mode, respectively. Similar PCA results were observed in positive and negative mode and thus only the results derived from negative mode are presented in the following. PCA loading and score plots derived from picked MS peaks in negative mode were in general agreement with NMR derived data concerning the differences in α- and β-bitter acids composition among hop cultivars and accounting for its segregation. The PCA model (Fig. 6A) explains 66% of the total variance in the first component, PC1, whereas the second principal component, PC2, explains, 14% of the variance. The metabolome cluster of β-acids enriched cultivars HSE and HHE was found again at the far right side along PC1 (positive side); clusters of HTU and HHS lines enriched in α-acids were also separated at the left side along PC1 with negative scores values, as previously shown in the NMR-data derived score plot. Albeit, three cultivars (ENB, HHT and ATHM) were located at slightly different points in the two-dimensional score plot (positive side along PC1) compared with that plotted from NMR

Table 3 Compounds tentatively assigned in *H. lupulus* L. HHT resin and cone methanol extract; (+) and (−) indicate presence and absence of a metabolite

No.	rt (min)	UV max	Identification	$[M-H]^-$ (*m/z*)	error (ppm)	El. composition	$[M-H]^-$ (*m/z*)	MS^n product ions	Resin	Cone
1	2.05	nd	Maltose			$C_{12}H_{29}O_{11}^-$	341$[M-H]^-$	341, 239	−	+
2	8.18	265, 350	Rutin	609.1412	6.15	$C_{27}H_{29}O_{16}^-$	609 $[M-H]^-$	301, 271, 255, 179	−	+
3	8.76	265, 350	Isoquercitrin	463.0847	5.53	$C_{21}H_{19}O_{12}^-$	463 $[M-H]^-$	301, 271, 255, 179	−	+
4	9.44	285, 350	Isoquercitrin malonate	549.1546		$C_{24}H_{21}O_{15}^-$	549 $[M-H]^-$	505, 462, 301	−	+
5	10.01	265; 285shd, 350	Astragalin	447.910	3.00	$C_{21}H_{19}O_{11}^-$	447 $[M-H]^-$	285, 255	−	+
6	10.55	nd	Unknown glucoside	357.1164	4.75	$C_{16}H_{21}O_9^-$	357 [M-H]-	195	−	+
7	10.76	265, 350	Astragalin malonate	533.0926	0.19	$C_{24}H_{21}O_{14}^-$	533 $[M-H]^-$	489, 285	−	+
8	14.27	nd	Isoxanthohumol	353.1346	10.8	$C_{21}H_{21}O_5^-$	353 $[M-H]^-$		+	−
9	17.35	nd	8-Prenylnaringenin				339 $[M-H]^-$		−	+
10	18.23	nd	Cohulupone	317.1761	4.41	$C_{19}H_{25}O_4^-$	317 $[M-H]^-$	248, 220, 205, 180	+	+
11	18.9	nd	Humulinone	377.195	1.96	$C_{21}H_{29}O_6^-$	377$[M-H]^-$	263, 220, 180	+	−
12	19.75	nd	Unknown	331.1915	3.62	$C_{20}H_{27}O_4^-$	331$[M-H]^-$	262, 234, 194	+	+
13	20.69	290, 365	Desmethylxanthohumol	339.1243	4.36	$C_{20}H_{19}O_5^-$	339$[M-H]^-$	220, 175, 151,133	+	+
14	21.92	nd	Unknown	447.2192	6.75	$C_{21}H_{35}O_{10}^-$	447$[M-H]^-$	383, 314, 271	+	+
15	22.34	235, 370	Xanthohumol	353.1401	4.75	$C_{21}H_{21}O_5^-$	353$[M-H]^-$	233, 218,189,165	+	+
16	24.53	235, 360	Lupulone E	415.248	0.90	$C_{25}H_{35}O_5^-$	415$[M-H]^-$	371,302, 259	+	+
17	24.85	nd	Posthumulone				333$[M-H]^-$	264, 221,209	+	+
18	25.19	nd	Diprenyl naringenin	407.1846	1.53	$C_{25}H_{27}O_5^-$	407$[M-H]^-$	287, 243, 218	+	+
19	25.36	nd	Unknown	361.1998	2.82	$C_{21}H_{29}O_5^-$	361$[M-H]^-$	303, 292, 289	−	+
20	25.9	nd	Unknown				415$[M-H]^-$	346, 330, 287	+	−
21	26.32	285, 323	Cohumulone	347.1875	6.57	$C_{20}H_{27}O_5^-$	347$[M-H]^-$	278, 235, 223	+	+
22	27.2	290, 335	4-Deoxycohumulone	331.1921	5.46	$C_{20}H_{27}O_4^-$	331$[M-H]^-$	287, 219	+	+
23	27.6	285, 325	Humulone	361.201	0.49	$C_{21}H_{29}O_5^-$	361$[M-H]^-$	361, 292	+	+
24	28.1	285, 325	Adhumulone	361.202	3.26	$C_{21}H_{29}O_5^-$	361$[M-H]^-$	345, 292, 249,237	+	+
25	28.36	290, 335shd	4-Deoxyhumulone	345.2090	8.98	$C_{21}H_{29}O_4^-$	345$[M-H]^-$	301	+	+
26	29.09	290, 330	Adprehumulone	361.202	3.26	$C_{21}H_{29}O_5^-$	361$[M-H]^-$	292, 249, 237	+	−
27	29.29	280, 335	Postlupulone	385.2401	7.78	$C_{24}H_{33}O_4^-$	385$[M-H]^-$	316, 273, 248	+	+
28	29.83	285, 325	Prehumulone	375.2184	5.27	$C_{22}H_{31}O_5^-$	375$[M-H]^-$	306, 263, 251	+	+
29	30.48	nd	Unknown				683$[M-H]^-$	655, 639, 557, 385	+	+
30	30.51	nd	Unknown	447.2547	4.45	$C_{29}H_{35}O_4^-$	447$[M-H]^-$	378; 335, 310	+	+
31	30.81	280, 333	Colupulone	399.2495	8.03	$C_{25}H_{35}O_4^-$	399$[M-H]^-$	330, 287, 275, 262	+	+
32	31.99	280, 335	Lupulone	413.2714	7.49	$C_{26}H_{37}O_4^-$	413$[M-H]^-$	344, 301, 276	+	+
33	32.21	280, 335	Adlupulone	413.2712	7.00	$C_{26}H_{37}O_4^-$	413$[M-H]^-$	344, 301, 289, 276	+	+
34	32.95	nd	Adprelupulone	413.2709	6.28	$C_{26}H_{37}O_4^-$	413$[M-H]^-$	360, 291	+	+
35	33.35	nd	Prelupulone	427.2853	3.26	$C_{27}H_{39}O_4^-$	427$[M-H]^-$	358, 315, 290	+	+
36	36.15	nd	Adlupone				481$[M-H]^-$	412, 385	+	+
37	36.38	nd	Unknown				495$[M-H]^-$	399, 381,355	+	+
38	38.72	nd	Unknown				549$[M-H]^-$	411, 342	+	+
39	39.55	nd	Unknown				519$[M-H]^-$	450, 407, 381	−	+

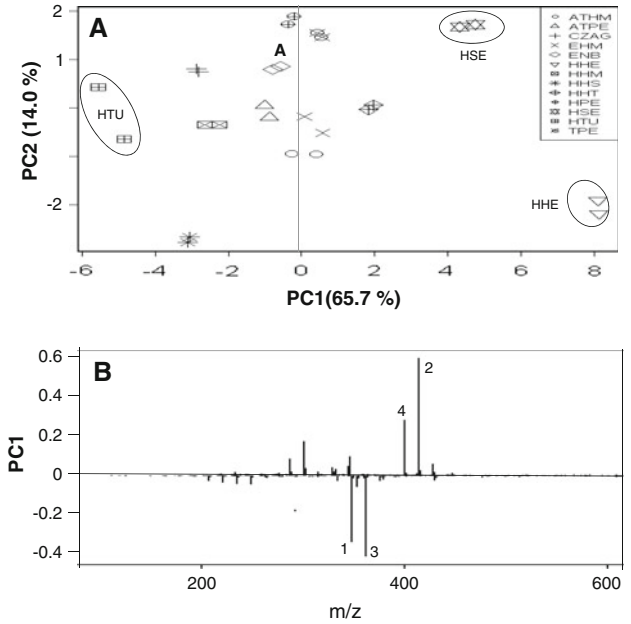

Fig. 6 LC-MS (*m/z* 100–1000) principal component analyses of different hop resins in negative mode. **A** Score Plot of PC1 and PC2 scores. **B** Loading plot for PC1 contributing mass peaks and their assignments: 1, humulone & adhumulone; 2, lupulone and adlupulone; 3, cohumulone; 4, colupulone. Group discrimination in samples is related to qualitative and quantitative differences in bitter acids pattern. It should be noted that ellipses do not denote statistical significance; the formula used for quantification is presented in Supplementary file 1. ATHM (○), ATPE (Δ), CZAG (+), EHM (x), ENB (◇), HHE (▽), HHM (⊠), HHS (✳), HHT (⊕), HPE (⊕), HSE (✡), HTU (⊞), TPE (⊗)

data (Fig. 6A). These differences could be ascribed to absence of mass signals for fatty acids in the MS loading plots (Fig. 6B), which were found to be discriminating for hop cultivar segregation in NMR plots (Fig. 3B). To help identify the nature of the fatty acids found in hop resin, additional GC-MS analysis was applied to the resin after methylation. Palmitic, linoleic and linolenic acid were identified as major fatty acids in hop resin HHT at a relative percentage of 25, 28 and 47%, respectively (See supp. Fig. 4). It has yet to be determined whether differences in fatty acid composition exist among hop cultivars; profiling of different resins after methylation using GC-MS could help clarify this issue. However, at least quantitative differences must exist to explain the differences of cultivars seen in PCA of NMR vs. LC-MS data.

3.6 Comparative PCA analysis of hop cones analysed by NMR and LCMS

To clarify and confirm the discrimination reasons observed between resins from different cultivars, hop cones from which resin was derived were extracted using the same extraction method for both LC-MS and NMR and further subjected to PCA. All hop cone material except for ATPE were analysed and this allowed also for measuring biological variance within each cultivar. PCA results from both NMR and LCMS datasets show a similar segregation (Fig. 7A, C) as in case of resin, with loading plots suggesting that the β-acids, α-acids and fatty acids dominantly contribute to hop cone extract discrimination (Fig. 7B, D). It should be noted that signals for sugars detected by NMR (Supp. Fig. 1) in cone extracts do not contribute for segregation in PCA loading plots along PC1 in the NMR dataset, suggestive that sugars are present at comparable levels in hop cultivars or at least their extracts.

3.7 Multivariate PCA analysis of ESI-FTICR-MS data

With its high mass accuracy and exceptional detection sensitivity, ESI-FTICR-MS is another important tool for metabolic fingerprinting of hop resins (Aharoni et al. 2002; Takahashi et al. 2008). Separation of the metabolites is achieved solely by ultra-high mass resolution; and identification of the putative metabolites or classes of metabolites was determined from the metabolites elemental composition based upon the accurate mass determination and isotope pattern. PCA plots from ESI-FTICR-MS show positive scores for cultivars HHE and HSE (far right), and negative scores for cultivars HHS and HTU (far left) along PC1 in agreement with both NMR and LCMS results (Fig. 8A). Nevertheless, for most cultivars, replicates derived from instrument measurements were dispersed, especially in the centre suggestive of relatively high technical variability for the FTMS measurements compared with LCMS. In positive mode, replicates were clustered closer along the centre axis compared with negative mode (Fig. 8B). Nevertheless, the CZAG cultivar (originated from Czech Republic) showed a clear separation from other cultivars along PC1 not previously observed from NMR and LCMS data sets (Fig. 8B). Examination of the loading plot for data derived from positive ionization mode revealed the presence of two strong mass signals found exclusively in CZAG, not detected in negative mode (Fig. 8C, D). Both signals showed an even mass weight of 574.41064 and 588.42699 amu with predicted molecular formulas of $C_{34}H_{56}O_6N^+$ and $C_{36}H_{58}O_6N^+$, respectively, with an error of 1.5 and 1.9 ppm. The presence of nitrogen containing compounds in hop is unlikely, with no previous reports of alkaloids or nitrogen containing secondary metabolites. After closer inspection, the masses were assigned by high resolution mass as tetramethyl ammonium adducts of the following chemical formulas $C_{30}H_{44}O_6$ and $C_{31}H_{46}O_6$. With the increased sensitivity for detection of nitrogen containing compounds in positive mode these masses appeared as strong signals in PCA results. Interestingly, analysing CZAG female cones in positive mode FTICR-MS revealed the presence of the same

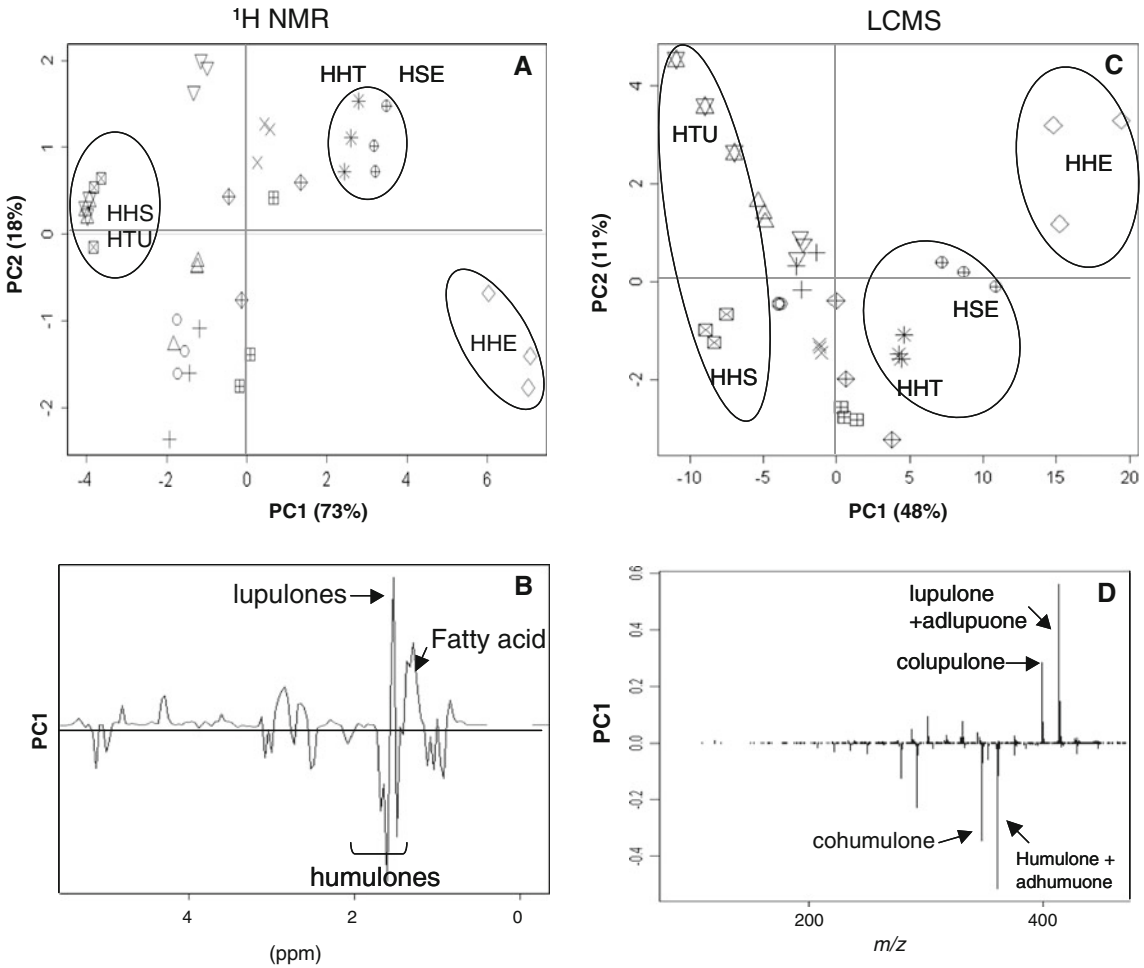

Fig. 7 Comparable scores and loading plots of 12 hop cone extracts based on ^{1}H-NMR (**A**, scores; **B**, loading) and LC-MS (**c**, scores; **D**, loading) datasets. Three biological replicates were measured. Group discrimination in both datasets is related to qualitative and quantitative differences in bitter acid patterns. It should be noted that ellipses do not denote statistical significance. ATHM (○), CZAG (Δ), EHM (+), ENB (x), HHE (◇), HHM (▽), HHS (⊠), HHT (✳), HPE (⊕), HSE (⊕), HTU (✡), TPE (⊞)

formulas, but in that case they appear as potassium adducts at *m/z* 539.27726 ($C_{30}H_{44}O_6K^+$, 0.6 ppm) and *m/z* 553.29233 ($C_{31}H_{46}O_6K^+$, 0.2 ppm), respectively. Careful inspection of the negative ionisation mode also revealed the presence of these metabolites, albeit at much lower intensities. We hypothize that these masses are from isoprenylated and oxidized derivatives, likely of the lupulone series with an additional isoprenyl moiety. Note the extra five carbon atoms in the predicted formula $C_{30}H_{44}O_6$ compared with that of colupulone $C_{25}H_{36}O_4$, and for $C_{31}H_{46}O_6$ compared with n-lupulone $C_{26}H_{38}O_4$. A mass difference of 14 amu between $C_{30}H_{44}O_6$ and $C_{31}H_{46}O_6$ is similar to that observed within the lupulone series (i.e. colupulone and lupulone). These data highlight the importance of acquiring data in both positive and negative mode in case of FTICR-MS and suggests for the presence of novel isoprenylated constituents in hop, preferentially ionized and detected in positive ion mode. The fact that these constituents evaded detection in NMR is likely due to their presence in trace amounts. NMR detects only the 3 major bitter acids from the humulone (H) and lupulone (L) series. Nevertheless, we cannot fully account for its absence in LCMS dataset from positive mode for both resin and cones. One possible explanation is the nature of the chosen mobile phase or elution gradient in our analysis, which might not be appropriate for the elution or ionisation and further detection of these special constituents. The identity of these new higher molecular weight prenylated structures is still under investigation.

Another key finding observed from loading plots in FITCR-MS was the appearance of colupulone at higher levels in the α-acid enriched cultivars HTU and HHS, contrary to results derived from both NMR and LCMS loading plots (Fig. 8C). This effect is likely due to mass discrimination effects caused by other components in the mixture competing for ionization (Sterner et al. 2000). Indeed, one can note a difference in abundance of chemical species in negative versus positive ion FTICR-MS for CZAG (Fig. 8E, F). Although, lupulone signals appear

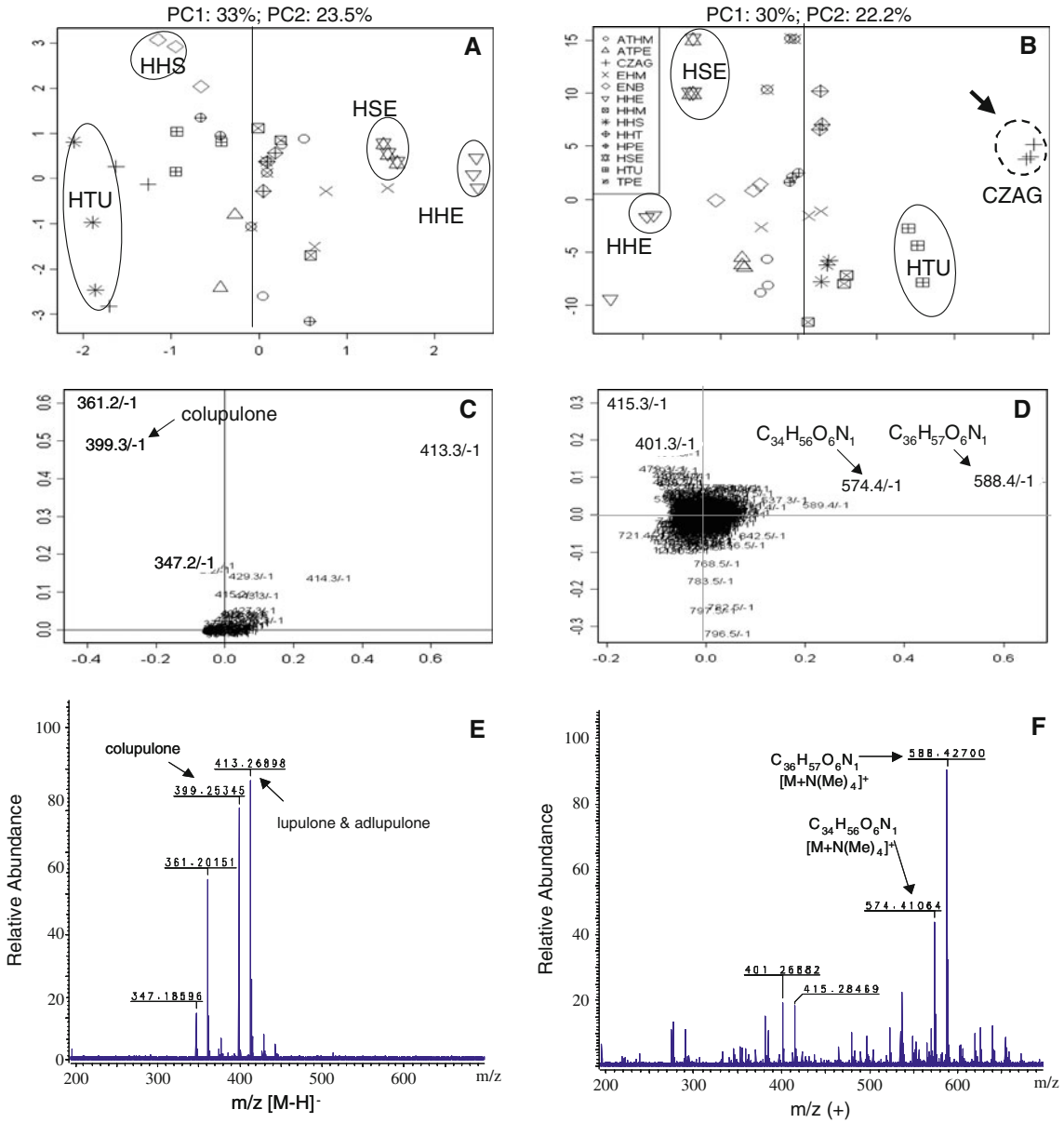

Fig. 8 ESI-FTICR-MS principal component analysis of different hop resin data in negative and positive ionisation mode. Score plot of PC1 and PC2 in negative (**A**) and positive mode (**B**). Loading plot for PC1 in negative (**C**) and positive mode (**D**). Cultivar CZAG segregation in positive mode is highlighted by an arrow in (**B**) and responsible masses are pointed out in plot (**D**). ESI-FTICR-MS fingerprint of CZAG resin extract shows differences in relative abundances of chemical species in negative (**E**) versus positive ionisation mode (**f**). ATHM (○), ATPE (Δ), CZAG (+), EHM (x), ENB (◇), HHE (▽), HHM (⊠), HHS (✳), HHT (⟡), HPE (⊕), HSE (✡), HTU (⊞), TPE (⊗)

more intense in the FTICR-MS spectra for the CZAG cultivar relative to humulones, the opposite trend was observed from both LCMS and NMR spectra (see Suppl. Fig. 5). The difference in FTICR-MS response is likely due to matrix effects altering the quantitative performance by limiting the sensitivity of metabolites detection and the precision of signal responses (Junot et al. 2010). These results warrant the added advantage of complementing different spectroscopic techniques to obtain a more comprehensive and quantitative metabolite profile.

4 Concluding remarks

To the best of our knowledge, this study provides the first comprehensive approach to reveal compositional differences between hop cultivars. NMR and MS techniques coupled with multivariate data analyses were used to obtain the experimental results, and interesting and meaningful differences between the various samples and or detection methods were identified. Principal component analysis consistently groups and separates the hop resins and plant

material studied. The results are generally independent of whether NMR chemical shifts, retention time/mass signal pairs (LCMS), or high resolution mass (FTICR-MS) was used as data basis.

Despite the advantages of FTICR-MS in unravelling the novel isoprenylated compounds in hop, this technique showed inconsistent loading results in PCA compared with NMR and LCMS derived datasets. One limitation in FTMS relates to the lack of quantification and the necessity of acquiring spectra in both positive and negative ion mode in order to obtain a comprehensive fingerprints of the samples; FTICR-MS also fails to distinguish between constitutional isomers as humulone and adhumulone pair. We do admit that our selection of cultivation sources does not cover all worldwide hop variations, but our approach is certainly feasible for analysing hop samples from such further sources. The same workflow of sample preparation, measurement and processing can be easily transferred for other plant metabolome studies of (commercially relevant) secondary metabolite producing plants. The comparative metabolomics approach developed in this work was found suitable for hop analysis in different cultivars which can be further applied for investigating the effect of other factors such as storage, harvesting time and/or seasonal variation on secondary metabolites composition.

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