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Fast Sampling, Analyses and Chemometrics for Plant Breeding: Bitter Acids, Xanthohumol and Terpenes in Lupulin Glands of Hops (*Humulus lupulus*)

Daniel P. Killeen,[a] Oliver C. Watkins,[a] Catherine E. Sansom,[b] David H. Andersen,[c] Keith C. Gordon[a,d] and Nigel B. Perry[a,b]*

ABSTRACT:

Introduction – The valuable secondary metabolites in hops (bitter acids, xanthohumol, volatile monoterpenes and sesquiterpenes) are sequestered in lupulin glands (extracellular trichomes) which can be collected and analysed with little or no sample preparation.

Objectives – To determine whether high throughput screening of lupulin glands composition, by fast analyses and chemometrics, could be used for breeder selection of hops with key flavour attributes.

Methods – Lupulin glands from 139 plants (39 cultivars/advanced selections) were analysed by Raman and ^{1}H NMR spectroscopy, and head-space solid-phase microextraction (HS-SPME) GC-FID. The digital X,Y-data were subjected to principal component analysis (PCA) and the results compared with conventional analyses of extracts of whole hops from the same plants. Quantitative ^{1}H NMR analyses were also done for the bitter acids.

Results – Raman spectroscopy rapidly identified hops cultivars with high xanthohumol concentrations and high α:β bitter acid ratios. ^{1}H NMR spectroscopy was slower, requiring a solvent extraction, but distinguished cultivars by cohumulone content as well as α:β acid ratios. HS-SPME-GC rapidly distinguished aroma hops with high myrcene and farnesene contents, and pinpointed a novel selection with unusual sesquiterpenes. The quantitative NMR analyses showed correlations between bitter acid concentrations related to biosynthetic pathways.

Conclusions – Analysis of lupulin glands gave reliable results for the main quality indicators used by hops breeders, potentially avoiding harvesting, drying and solvent extracting whole hops. PCA of digital X,Y-data rapidly discriminated different hops chemotypes, and highlighted plants with potential for new flavourcultivars. Copyright © 2016 John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: Hops; *Humulus lupulus*; lupulin glands; breeding; NMR; GC; HS-SPME; Raman

Introduction

Hops are the inflorescences (seed cones) of the female plants of *Humulus lupulus* L. (Cannabaceae). Their commercial value comes from the secondary metabolites in these cones (Neve, 1991). The most important of these metabolites are the phloroglucinol-derived bitter acids (Fig. 1) which give beer its characteristic bitterness (Zanoli and Zavatti, 2008; Schönberger and Kostelecky, 2011). The phloroglucinol-derived α-acids (Fig. 1) undergo thermal isomerisation in the brewing process to form the intensely bitter *iso*-α-acids (Fig. 1) (Hughes, 2000). Hops also produce β-acids (Fig. 1), which do not undergo thermal isomerisation in the brewing process and do not add substantial bitterness to finished beer (Hughes, 2000). However, β-acid derivatives have shown potential anti-Alzheimer's disease effects (Sasaoka *et al.*, 2014) and the bitter acids have a range of other bioactivities *in vitro* (Zanoli and Zavatti, 2008; Van Cleemput *et al.*, 2009) suggesting possible health benefits for hops extracts. The prenylated chalcone xanthohumol (Fig. 1), characteristic of hops, also has various bioactivities (Stevens and Page, 2004; Tan *et al.*, 2014; Miranda *et al.*, 2016). The pleasing aromas that hops add to finished beers are due to volatile monoterpenes and sesquiterpenes (Neve, 1991). The most abundant are usually myrcene, β-farnesene, caryophyllene and humulene (Fig. 1) (Inui *et al.*, 2013).

There is a worldwide effort to breed new hops cultivars with desirable combinations of these secondary metabolites (Beatson and Inglis, 1999; Koelling *et al.*, 2012; Mongelli *et al.*, 2016). For hops breeders and beer brewers the most important quality indicators are the humulene/caryophyllene ratio (H:C), α:β acid ratios and the cohumulone content (Kralj *et al.*, 1991; Field *et al.*, 1996;

* Correspondence to: Nigel B. Perry, The New Zealand Institute for Plant & Food Research Limited, Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand.
Email: nigel.perry@plantandfood.co.nz

[a] Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

[b] The New Zealand Institute for Plant & Food Research Limited, Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

[c] The New Zealand Institute for Plant & Food Research Limited, 55 Old Mill, RD 3, Motueka 7198, New Zealand

[d] Dodd-Walls Centre, University of Otago, Dunedin, New Zealand

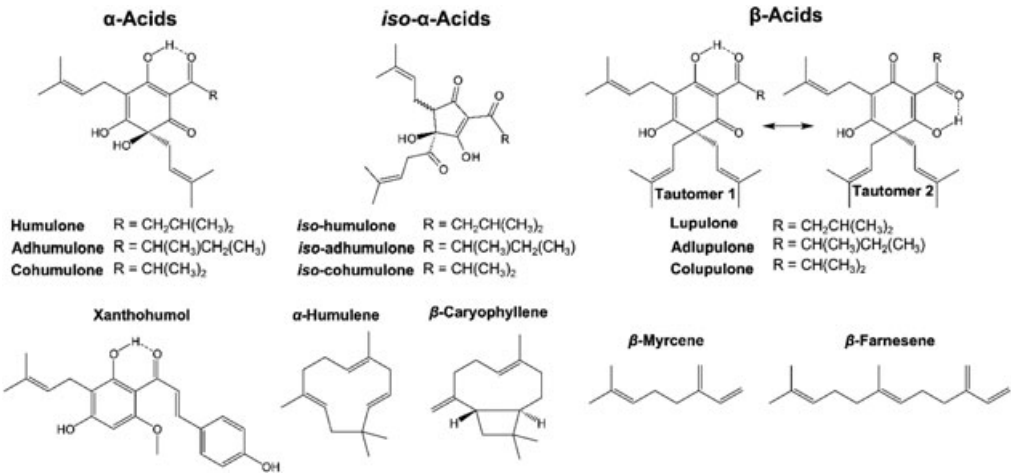

Figure 1. Chemical structures of the most abundant hops secondary metabolites and the *iso*-α-acids.

Beatson and Inglis, 1999; Intelmann *et al.*, 2009; Schönberger and Kostelecky, 2011). Draft hops genomes have been published (Natsume *et al.*, 2015; Hill *et al.*, 2016) but breeding programmes continue to require phenotyping of the metabolomes of new crosses and advanced selections. This involves the '"bottle-'neck" of labour-intensive hops harvesting, processing and chemical analyses.

Various analytical techniques have been used to quantitate the key hops metabolites. The bitter acids have been measured by UV spectrophotometry (Anon., 2010a), HPLC-UV (Anon., 2010b) and NMR spectroscopy (Hoek *et al.*, 2001; Farag *et al.*, 2014). HPLC-UV-ESI-MS/MS analysis has also been used to quantitate xanthohumol and isoxanthohumol (Magalhaes *et al.*, 2007). The standard industry analysis for the volatile monoterpenes and sesquiterpenes uses hydrodistillation then direct injection GC-FID (Anon., 2010c). More recently head-space solid phase microextraction (HS-SPME) has been combined with GC-FID, GC–MS and GC-Olfactometry for the analysis of hops volatiles (Field *et al.*, 1996; Gonçalves *et al.*, 2012; Van Opstaele *et al.*, 2013). Farag *et al.* (2012) combined NMR and LC–MS data to quantitate individual bitter acids, flavonoids and terpenes. Chemometric analysis of Raman, IR and near IR spectroscopy data has been used to rapidly analyse bitter acids (Killeen *et al.*, 2014).

All of these analyses have been carried out on hops cones after harvesting, drying and grinding, and almost all used solvent extraction. These steps are slow, severely hampering overall analytical throughput. However, all of the important secondary compounds are biosynthesised and stored in extracellular trichomes known as lupulin glands (Wang *et al.*, 2008; Clark *et al.*, 2013; Xu *et al.*, 2013), which can be physically separated from hops cones (Likens *et al.*, 1978; Nagel *et al.*, 2008) with potential for analysis directly or following a single solvent extraction.

For selection of new hops cultivars, hops breeders initially want qualitative analyses, to know whether new plant crosses fit on the spectrum of current commercial cultivars, or whether they stand out as potential new flavour and/or aroma chemotypes (Beatson *et al.*, 2003). Standard analytical approaches use peak-picking of known components prior to integration, but this can risk missing peaks of new components. An alternative, undirected approach to distinguishing cultivars is to apply principal component analysis (PCA) to digital X,Y-data direct from chromatograms or spectra. Farag *et al.* (2012) used PCA on NMR and LC–MS data from extracts of hops cones of 13 cultivars.

This present report describes rapid analyses of lupulin glands using Raman and ^{1}H NMR spectroscopy and HS-SPME-GC, with PCA-based discrimination of compositional variations. The results of the lupulin gland analyses are compared with conventional quantitative analyses of corresponding whole hops.

Experimental

Plant material

The hops sample set (n = 139) consisted of cones from one to nine individual plants of 39 cultivars. The different cultivars were advanced selections cultivated from the hops research plots at Plant & Food Research, near Motueka (41°5′48″S 172°58′24″E) in March 2013. Hops cones were kiln dried at 60 °C for times ranging from 18 to 36 h and stored at −20 °C. Lupulin glands (50–100 mg) were physically separated from the dried cones in January 2014 by rubbing through a 420 μm mesh sieve and stored at −20 °C prior to analysis. The weight of cones used to obtain lupulin glands varied between cultivars. The physical separation of lupulin glands from cones could not be performed exhaustively and therefore quantitation of lupulin glands in terms of cone weight was not possible. A larger pool of lupulin glands were collected from a single hops plant (variety '"Mount 'Hood"). This sample was analysed at regular intervals during the analytical data acquisitions as a check sample.

Reference compounds and identifications

Cohumulone, colupulone, a mixture of humulone and adhumulone, a mixture of lupulone and adlupulone, and xanthohumol were isolated as described previously (Killeen *et al.*, 2014). An aliquot of the International Calibration Extract 3 (ICE-3) containing certified concentrations of cohumulone, colupulone, mixtures of humulone/adhumulone and mixture of lupulone/adlupulone was purchased from the American Society of Brewing Chemists.

Myrcene, humulene and caryophyllene reference standards were purchased from Sigma-Aldrich. β-Farnesene, aromadendrene, α- and β-selinene and germacrene B were tentatively identified by their GC retention indices (Babushok *et al.*, 2011) and using their mass spectra, which were compared to a commercial MS library (Adams, 2007).

Whole hops analyses. The bitter acid and xanthohumol concentrations of whole hops samples were determined using the officially-described UV spectrophotometry and HPLC-UV analysis methods (Anon., 2010a, 2010b). The results of these analyses appear in a previous report (Killeen *et al.*, 2014). Volatile oils were hydrodistilled (4 h) from whole hops (100 g) and analysed by GC-FID using the officially-described method (Anon., 2010c)

with a Shimadzu GC2010, fitted with an AOC-20s autosampler and a SGE BP20 column (0.25 μm, 60 m × 0.25 mm i.d.). The helium carrier gas flow rate was 5.0 mL/min. The hop oil (0.1 μL in 1 mL of twice-distilled diethyl ether (LabServe)) was directly injected into the injection port operating at 180 °C, run in split mode 96:1 waste/column (injection load =1.04 nL). The column was heated from 60 to 138 °C at 6 °C/min, held for 5.5 min, then heated to 220 °C at 5 °C/min. The flame ionisation detector (FID) was at 245 °C and quantitative results were expressed as area % of total area of the GC trace.

Raman spectroscopy analyses of lupulin glands. Lupulin glands (~20 mg) were packed into aluminum divots for analysis by the method reported previously (Killeen *et al.*, 2014). Briefly, spectra were acquired using a FRA 106/5 Bruker Equinox FT-Raman spectrometer equipped with a Nd:YAG laser emitting at 1064 nm, Equinox 55 interferometer, and a liquid nitrogen-cooled germanium detector. The Raman Stokes shift was recorded from 200 to 3500 cm^{-1} with a spectral resolution of 4 cm^{-1}, laser spot size of 0.3 mm and power of 400 mW. Spectra were the average of 32 scans, and the sample acquisition time was ~1 min.

^{1}H NMR spectroscopy analyses of lupulin gland extracts. Lupulin glands (~10 mg) were accurately weighed and their contents were extracted into deuterochloroform containing 0.05 M dimethylformamide internal standard (800 μL), sonicated for 15 min and filtered (0.45 μm PTFE). An aliquot of the filtrate (500 μL) was diluted with deuterochloroform/0.05 M dimethylformamide (250 μL) and ^{1}H NMR spectra were measured at 293 K using a 400 MHz Varian Inova instrument: sweep width − 1 to 20 ppm; 32 transients; pulse width 90°. A delay (d1) of 20 s was used to ensure full proton relaxation between pulses, giving an acquisition time of ~10 min per sample. Calibration curves were generated for the individual bitter acids by integrating the appropriate signals in the hops ICE-3 analysed at 11 different concentrations ($r^2 > 0.99$ for each). The calibration standards were subjected to the same sample preparation steps described for the lupulin gland samples. The range of linearity of the calibration curves demonstrate that the components of interest in the lupulin glands are soluble in chloroform at analytically relevant concentrations.

Spectra were processed using MestReNova™ software, with phase correction, baseline correction (third order polynomial) and smoothing (Savitzky–Golay). The residual chloroform signal was used to reference spectra at 7.26 ppm.

Low-field (42.5 MHz) NMR spectra were acquired using a Magritek Spinsolve® benchtop NMR spectrometer using similar settings, but with 60 transients.

The α-acids were quantitated using the integrals of the proton signals at 18.87 (humulone), 19.00 (cohumulone) and 19.03 ppm (adhumulone) (Hoek *et al.*, 2001) and the β-acids were quantitated using the sum of integrals of the proton signals for the major and minor tautomers at 19.35 and 18.41 (adlupulone), 19.30 and 18.30 (colupulone) and 19.22 and 18.39 ppm (lupulone) (Borremans *et al.*, 1975; Tyrrell *et al.*, 2012).

GC-FID and GC–MS analyses of lupulin gland compositions. Lupulin glands (~10 mg) were accurately weighed into 20 mL silanised glass vials with a single silanised glass bead (~ 0.5 cm diameter) and sealed. Samples were equilibrated at 70 °C for 5 min with automated swirling at 30 rpm to puncture lupulin glands. This was followed by a 5 min adsorption onto a 100 μm polydimethylsiloxane SPME fibre (Agilent Technologies), also at 70 °C and delivered using a stainless steel injector sheath, which pierces and seals the glass vials. Desorption from the SPME fibre was performed over 1 min in an injector port held at 180 °C with concurrent splitless injection onto a SGE BP-20 column (0.25 μm, 25 m × 0.25 mm i.d.) using an Agilent 7890A gas chromatograph with a CTC Analytics ""PAL" autosampler and Agilent 5975C inlet XL MSD with triple axis detector. The carrier gas was hydrogen with a flow rate of 1.2 mL/min. The oven was heated from 40 to 240 °C at a rate of 10 °C/min. Simultaneous detection by both FID (at 250 °C) and 70 eV electron ionisation MS was facilitated using a two-way flow splitter (Agilent Technologies). The MS transfer line was held at 250 °C, the MS source was at 230 °C and the MS quadrupole mass analyser was held at 150 °C; the acquired mass range was *m/z* 35–300. Quantitative results were expressed as % of total area of the GC trace by FID (Supporting Information Table S1). The MS data was used for peak identification. The FID data was used to determine the area % results to allow direct comparison to GC-FID results from the whole hops cones without the need for response factors.

Chemometrics. Raman spectra were imported into the multivariate software '"The Unscrambler®"' (CAMO Software), reduced to the range 1710–1000 cm^{-1} (which contained the most important spectral data (Killeen *et al.*, 2014)) then subjected to a standard normal variate (SNV) transformation. PCA was performed using the non-iterative partial least squares (NIPALS) algorithm.

After phase correction, baseline correction (third order polynomial) and smoothing (Savitzky–Golay) the ^{1}H NMR spectra were imported into '"The 'Unscrambler" software. Pre-processing consisted of SNV transformation, baseline offset correction, deresolution and correlation-optimised warping (COW). The latter process was used to compensate for inter-sample variation in chemical shifts due to factors such as concentration, solvent acidity and water content. Attempts to use COW on the whole spectral region failed, therefore requiring the use of more targeted spectral regions. The final models were produced using the combined spectral ranges 14.60–14.67 ppm (xanthohumol) and 18.00–19.35 ppm (bitter acids).

Chromatograms from GC-FID analyses were imported into '"The 'Unscrambler" software (CAMO) and subjected to SNV transformation, baseline offset correction, deresolution and COW.

Results and discussion

Hops and lupulin glands samples

The hops samples used in this study included both well-known commercial cultivars and genetically novel selections chosen to encompass broad chemotype differences (Killeen *et al.*, 2014). The novel selections were designated with sequential numbering based on their associated breeding trials Alpha, Aroma, and Brew (Table S1).

Raman spectroscopy of lupulin glands

A summary of the PCA of Raman spectra of lupulin glands is shown in Fig. 2. The scores plot [Fig. 2(a)] showed that the check sample spectra clustered tightly and that the spectra of different hops cultivars generally clustered together (the number of cultivars plotted has been reduced for clarity, the ellipses are a visual aid with no formal statistical significance). The chemical basis for the separation of the hops cultivars and advanced selections was established from the loadings plots [Fig. 2(b) and 2(c)] and comparisons with Raman spectra of hops bitter acids and xanthohumol reported previously (Killeen *et al.*, 2014). The first principal component (PC1) was dominated by positive loading coefficients centred on characteristic xanthohumol Raman bands: 1606, 1550, 1261 and 1168 cm^{-1} [Fig. 2(b)]. The lupulin glands from selections '"Brew '6", '"Brew '5" and '"Alpha '8" had positive PC1 scores [Fig. 2(a)] and whole hops from these selections had the highest concentrations of xanthohumol as determined by HPLC-UV analyses (Killeen *et al.*, 2014). So Raman spectroscopy of lupulin glands could rapidly identify hops genotypes with high concentrations of this bioactive chalcone, which is also the precursor to numerous bioactive flavonoids (Stevens and Page, 2004; Tan *et al.*, 2014). This could potentially be useful to hops breeders seeking cultivars with higher concentrations of bioactive components for use in health products.

The separation by PC2 was predominantly derived from the vibrational bands around 1631 cm^{-1} and 1581 cm^{-1}, which were loaded inversely [Fig. 2(c)]. These vibrational bands are strongly associated with α-acids and β-acids, respectively

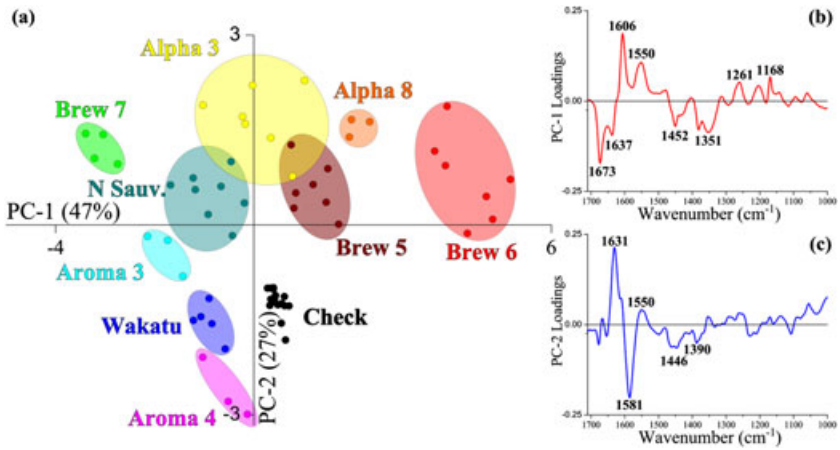

Figure 2. (a) Summary of principal component analysis (PCA) of Raman spectra of lupulin glands from nine hops cultivars and the check sample; (b) and (c) PC1 and PC2 loadings plots.

(Killeen *et al.*, 2014). The inverse loadings of these bands implied that PC2 was discriminating hops cultivars based on their α:β acid ratios and this was confirmed by inspection of the scores plot [Fig. 2(a)] and the results from the ^{1}H NMR analyses (Supporting Information Table S2). Whole hops of the selection '"Alpha '3" had high α:β acid ratios by HPLC-UV and UV spectrophotometry analyses, while whole hops of selection '"Aroma '4" and cultivar '"Wakatu" had low α:β acid ratios (Killeen *et al.*, 2014).

Therefore Raman spectroscopy of lupulin glands could be used as a rapid screening technique for hops genotypes with high α:β acid ratios, a key selection factor for breeding new cultivars of bittering hops (Hughes, 2000). New developments of miniature Raman spectrometers (Sorak *et al.*, 2012) would support putting this technique into the hands of hops breeders.

^{1}H NMR spectroscopy of lupulin gland extracts

Because Raman spectroscopy could not distinguish the individual α- and β-acids (Fig. 1) (Killeen *et al.*, 2014) ^{1}H NMR spectroscopy was explored as a tool for the analysis of lupulin gland extracts. It was found that the full NMR spectra could not be effectively interpreted chemometrically. The complexity and variety of signals in the low chemical shift (0.5–2.5 ppm) region of the spectra prohibited effective use of COW and the resultant PCA models did not produce varietal clustering. By contrast, Farag *et al.* (2014) were able to distinguish resins from different hops cultivars by signals in this region, and separated NMR spectra on their α:β acid ratios (Farag *et al.*, 2014). A higher field NMR instrument was used in that work (600 MHz versus 400 MHz for this work) on resins suspended in CD_3OD then centrifuged. This gave dominant bitter acid methyl signals (Farag *et al.*, 2014), whereas $CDCl_3$ solutions from this work also had strong terpene methyl signals.

Reproducible varietal clustering was observed in the scores plot [Fig. 3(d)] using only the very high chemical shift range 18.00–19.35 ppm, which covered the intramolecular hydrogen bonded proton signals of the bitter acids (Hoek *et al.*, 2001) (Fig. 4). Pre-processing, as shown for the spectral range 18.8–19.1 ppm in Fig. 3(a)–3(c), corrected misalignments of chemical shifts and different line widths between spectra, giving tightly clustered check sample spectra using PCA [Fig. 3(d)]. The ^{1}H NMR spectra of lupulin gland extracts from each hops variety generally clustered together [Fig. 3(d); the number of cultivars and selections plotted has been reduced for clarity; a complete PCA scores plot is provided in Supporting Information Fig. S1]. The loadings plot [Fig. 3(e)] showed spikes around 18.83, 18.97 and 19.00 ppm which corresponded to the signals of purified α-acids humulone, cohumulone and adhumulone respectively (Fig. 4). Close inspection of the loadings plot showed that the other spikes were in fact co-directional, overlapping pairs, e.g. 19.19 and 18.39 ppm with strong negative loading on PC1 [Fig. 3(e)]. The ^{1}H NMR spectra of the purified β-acids showed signals for two major tautomeric forms of each in $CDCl_3$ (Fig. 4) as noted by others (Molyneux and Wong, 1975). Each β-acid gave a pair of co-directional spikes in the loadings plot because these tautomer signals are inherently covariate.

Since the α-acid NMR signals were positively loaded on PC1 and the β-acid signals were negatively loaded [Fig. 3(e)], NMR spectra discriminated the hops by their α:β acid ratios. For example, as noted earlier, whole hops of the selection '"Alpha '3" had high α:β acid ratios by HPLC and UV analyses and had positive PC1 scores [Fig. 3(d)]. Whole hops of selection '"Aroma '4" and cultivar '"Wakatu" had low α:β acid ratios and had negative PC1 scores [Fig. 3(d)]. Whole hops of the cultivar '"Wai-'iti" had the lowest α:β acid ratios (Killeen *et al.*, 2014) and their NMR spectra had the most negative PC1 scores [Fig. 3(d)].

The 400 MHz NMR spectra gave resolved signals for the individual bitter acids (Fig. 4) and the PCA showed separated loadings for humulone and cohumulone on PC2 [Fig. 3(e)]. NMR spectra of lupulin gland extracts from the cultivar '"Green 'Bullet" were plotted in positive PC1 space and the whole hops had a relatively high α:β acid ratio (Killeen *et al.*, 2014). However, unlike the previously discussed high-α hops, '"Green 'Bullet" spectra were plotted in positive PC2 space [Fig. 3(d)]. This region of scores space was associated with cohumulone in the loadings plot [Fig. 3(e)]. This is consistent with the chemistry of whole '"Green 'Bullet" hops, which had some of the highest cohumulone contents in this sample set (Killeen *et al.*, 2014).

Therefore NMR spectroscopy of lupulin gland extracts could be used as a screening technique to distinguish hops by their α:β acid ratios and their cohumulone contents, both key selection factors for breeding (Kralj *et al.*, 1991; Field *et al.*, 1996; Beatson and Inglis, 1999; Hughes, 2000; Intelmann *et al.*, 2009; Schönberger and Kostelecky, 2011). However, another step of handling is required to prepare solution samples, and high-field NMR instruments are expensive and require liquid helium. This second drawback might

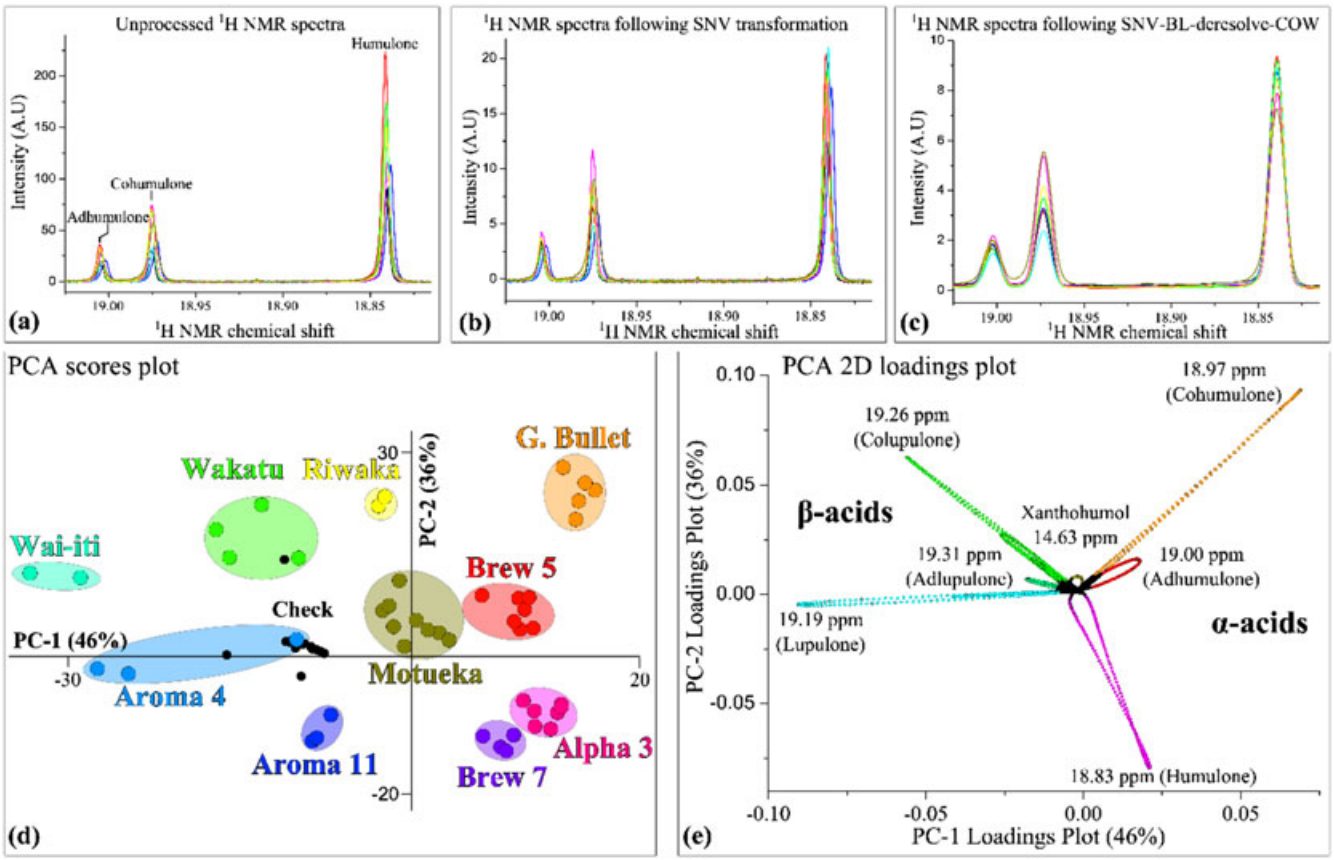

Figure 3. (a)–(c) Effects of pre-processing of ^{1}H NMR spectra of eight random lupulin glands extracts in the chemical shift range 18.8–19.1 ppm; (d) and(e) summary of PCA applied to pre-processed spectra of lupulin gland extracts from 10 hops cultivars and the check sample.

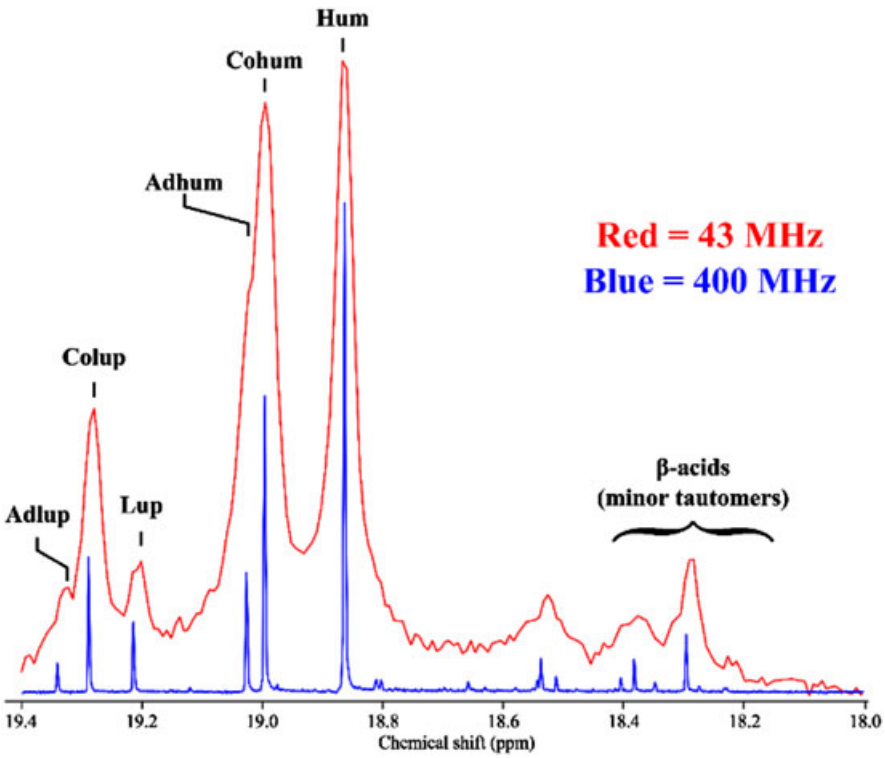

Figure 4. The 18.0–19.4 ppm range of ^{1}H NMR spectra of a lupulin glands extract in $CDCl_3$ acquired at 400 MHz and at 43 MHz.

be answered by the progress in miniaturisation of NMR systems (Zalesskiy *et al.*, 2014). The bitter acid profiles of lupulin glands were investigated using a low-field benchtop NMR spectrometer, aiming to take advantage of the uniquely high chemical shifts of the intramolecularly hydrogen-bonded signals in the bitter acids. A deuterochloroform extract of lupulin glands '("Brew '5") was analysed using a Magritek Spinsolve® 43 MHz NMR spectrometer, which has a permanent magnet, and compared to the 400 MHz ^{1}H NMR spectrum (Fig. 4). The signals of the α- and β-acids were resolved, so α:β acid ratios would be distinguished at 43 MHz. The cohumulone signal was not resolved from that of adhumulone (Fig. 4), but chemometrics might well give separations, as it did for the non-resolved Raman signals (Fig. 2). These preliminary results suggest that benchtop NMR spectrometers could be used by hops breeders at field stations for screening hops bitter acid profiles.

HS-SPME-GC of lupulin glands

Neither Raman nor NMR spectroscopy provided any information on the volatile monoterpenes and sesquiterpenes (Fig. 1) important for hops aroma. The non-conjugated terpenes were not excited by the Raman irradiation, and the terpene ^{1}H NMR signals mostly overlapped in the aliphatic region of the spectra (as discussed earlier) and were not well resolved in the olefin region. Furthermore, the 1,3-diene signals of myrcene and β-farnesene (Fig. 1) coincide. Therefore HS-SPME-GC, as previously used for analysis of whole hops volatiles (Field *et al.*, 1996; Gonçalves *et al.*, 2012; Van Opstaele *et al.*, 2013), was applied to lupulin gland analyses.

It was first shown that the sample set of hops from 39 cultivars and selections (139 plants) covered a wide range of volatile compositions, by using the standard method (Anon., 2010c), i.e. hydrodistilled essential oils from whole hops cones analysed by direct injection GC-FID. As expected (Neve, 1991) the main volatiles were myrcene (17–82%, uncorrected peak area as % of total peak area), caryophyllene (0–18%), humulene (0–42%) and β-farnesene (0–17%) (mean data for each cultivars and advanced selection are in Table S1).

Rapid HS-SPME-GC analyses were performed on lupulin glands from the same hops sample set and the digital X,Y-data from the GC traces were subjected to direct chemometric analysis after applying COW to compensate for retention time shifts between GC runs (Tomasi *et al.*, 2004). This allowed us to achieve greater overlap between chromatograms while retaining the peak resolution between humulene and β-farnesene, which eluted close together (Fig. 5). The effects of pre-processing are shown in Fig. 5(a)–5(c) giving reasonably clustered check sample GC traces [Fig. 5(d)]. PCA was performed on the full sample set, but for clarity the scores plot [Fig. 5(d)] shows a reduced number of samples (full PCA results are shown in Supporting Information Fig. S2). The scores plot separated the GC traces into genotype-specific clusters [Fig. 5(d)] and the chemical origins of the genotypic clustering were shown in the two-dimensional (2D) loadings plot [Fig. 5(e)].

Lupulin glands with high myrcene were separated from high humulene plus caryophyllene cultivars along PC1 [Fig. 5(d)]. The New Zealand aroma hops cultivars ""Motueka" and ""Riwaka" (> 75% myrcene in oils, Table S1) were plotted in

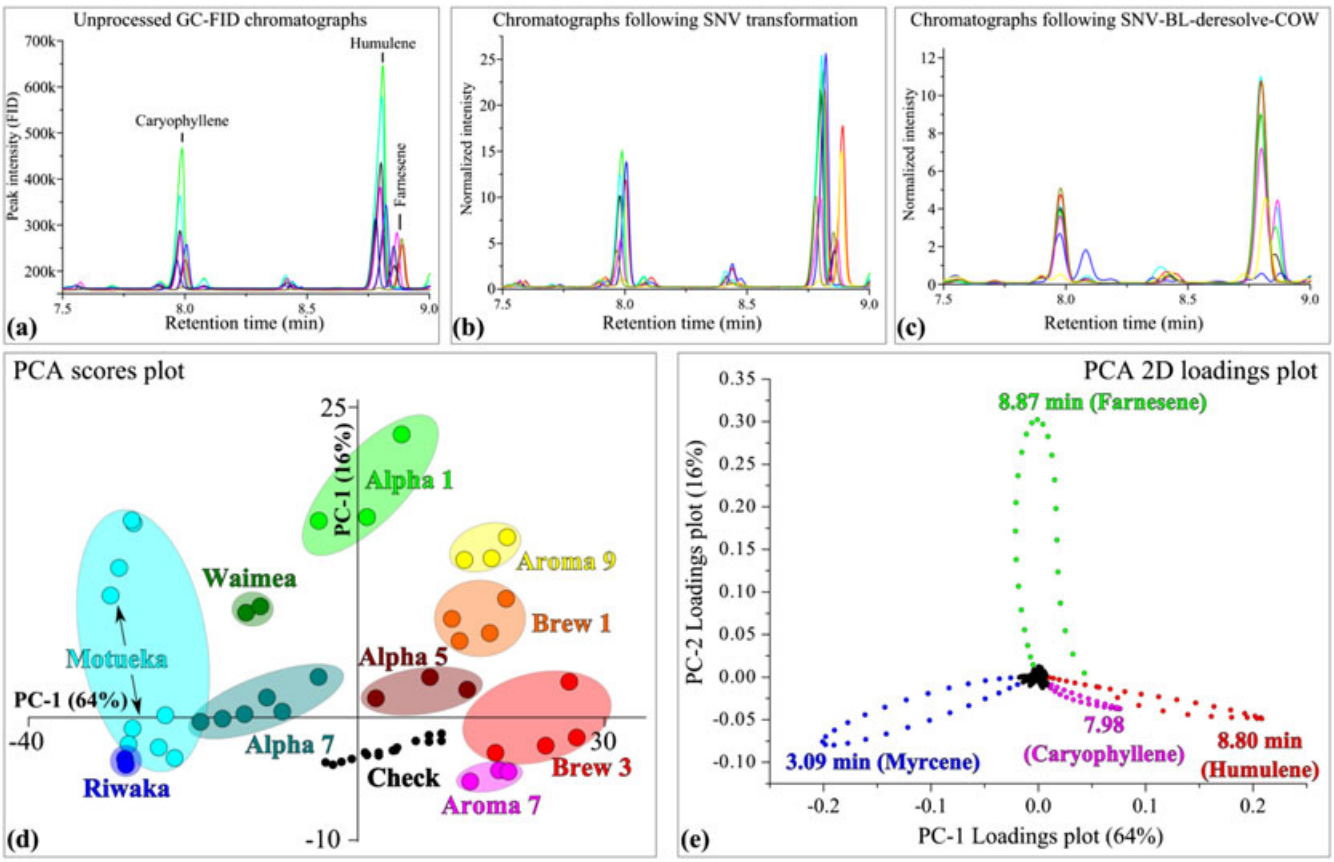

Figure 5. (a)–(c) Effects of pre-processing of selected HS-SPME-GC-FID chromatographs of eight random lupulin glands samples in the range 7.5–9.0 min; (d) and (e) summary of PCA applied to pre-processed chromatographs of lupulin glands from 10 hops cultivars and the check sample.

negative PC1 space [Fig. 2(d)], associated with the myrcene GC peak at 3.09 min in the loadings plot [Fig. 2(e)]. Conversely, lupulin glands from hops rich in humulene and caryophyllene (e.g. Brew 3) were plotted in positive PC1 space, associated with the humulene (8.80 min) and caryophyllene (7.98 min) GC peaks [Fig. 2(e)]. This rapid PCA did not bring out the H:C ratio of interest to hops breeders (Kralj *et al.*, 1991; Schönberger and Kostelecky, 2011) because the proportion of H to C was strongly correlated overall in this hops sample set (r^2 = 0.82 for the whole hops oils, r^2 = 0.73 for the lupulin gland HS). Therefore this selection parameter would have to be extracted by the traditional integration method: a strong correlation (r^2 = 0.95) was found between the H:C ratios measured on whole hops oils and lupulin glands HS.

PC2 of the lupulin gland HS data was associated with high proportions of β-farnesene (8.87 min) in the 2D loadings plot [Fig. 2(e)], so that hops selections such as '"Alpha '1" rich in this sesquiterpene (Table S1) were automatically distinguished. The '"Motueka" lupulin glands separated into two clusters on PC2 [Fig. 2(d)] and inspection of the oils analyses for the individual plants revealed that some gave hops with moderate concentrations of β-farnesene, whereas this compound was not detected in hops from other plants. Rapid detection of variability within established hops cultivars using lupulin glands HS-SPME-GC with chemometric methodology would also be useful to breeders.

The PCA (Fig. 5) showed that most of the variation in the lupulin glands HS-SPME-GC data set was accounted for by PC1 (64% of the variation) and PC2 (16%) covering variation of the four major terpenes expected from previous hops analyses (Neve, 1991; Inui *et al.*, 2013). However, PC3 (8%) had large loadings coefficients associated with other GC peaks (Fig. S2), which were identified by GC retention indices (Babushok *et al.*, 2011) and MS (Adams, 2007) as the following sesquiterpenes/aromadendrene (9.30 min), α- and β-selinene (9.36 and 9.94 min) and germacrene B (10.46 min). These compounds have previously been reported as major volatiles in a few hops cultivars (Tressl *et al.*, 1983) and have been associated with resistance to powdery mildew (Hartley and Fawcett, 1969). The PCA of the HS-SPME-GC data automatically highlighted the novel selection '"Aroma '6" on the PC3 scores plot. In this genotype the four usual '"major" volatile components accounted for only 33% of the total peak area for the hydrodistilled oils (n = 3, Table S1). Rapidly highlighting hops with unusual volatile profiles is very valuable to hops breeders seeking to produce cultivars with novel aroma and disease-resistance properties (Beatson *et al.*, 2003).

GC instruments are routinely used by hops research programmes, and the cost of adding automated HS-SPME sampling would be quickly offset by reduced time spent drying and distilling for primary screening of hops volatiles profiles.

Quantitative analyses of bitter acids in lupulin by ^{1}H NMR spectroscopy

The results discussed earlier demonstrate that qualitative analyses of lupulin glands by three fast analytical methodologies, combined with chemometric analyses, can rapidly pinpoint useful selections for hops breeders. The next step before cultivar development would be harvesting and drying whole hops, then conventional extractions and analyses; to confirm their interesting chemistry and to give quantitative measures of bitter acids and volatiles contents.

Quantitative analyses of hops bitter acids can also be carried out by ^{1}H NMR spectroscopy (Molyneux and Wong, 1975; Hoek *et al.*, 2001). A quantitative ^{1}H NMR method, calibrated for the six main hops bitter acids, was used to measure these compounds in lupulin gland extracts (Table S2). The average bitter acid concentration of lupulin gland extracts from 139 individual plants spanning 39 hops genotypes was 52% *w/w* dry weight [range 43–63%, standard deviation (SD) ± 9%, Table S2]. A recent study on lupulin glands chemistry from 14 hops cultivars (by HPLC-UV) found an average bitter acid content of 40.7% *w/w* fresh weight (27.6–47.3%) corresponding to 63.1% *w/w* dry weight (Patzak *et al.*, 2015). The only other expansive study of lupulin chemistry (by UV spectrophotometry) reported an average bitter acid concentration of 73.3% *w/w* dry weight (51–86%, SD ± 5.8%) in lupulin glands from 112 hops genotypes (Likens *et al.*, 1978). So the NMR results from the present hops lupulin glands sample set are somewhat lower than both of those two previous results, which used different analytical methods and different sample sets. It has previously been shown that UV spectrophotometry tends to overestimate bitter acid concentrations by about 10% compared to HPLC-UV analysis (Killeen *et al.*, 2014).

Results from this report are the first dataset (to our knowledge) with all six bitter acids (Fig. 1) quantified individually across a large number of lupulin glands or whole hops samples. This gave an opportunity to examine quantitative relationships and to rationalise these in terms of knowledge of bitter acid biosynthetic pathways. There were strong correlations between the relative amounts of the analogous α- and β-bitter acids expressed as percentages of their respective families. Humulone as a % of total α-acids was correlated with lupulone as a % of total β-acids (r^2 = 0.87), and similarly colupulone with cohumulone (r^2 = 0.89). This relationship did not apply to adhumulone with adlupulone (r^2 = 0.23), but these compounds only spanned a small range of concentrations (9–15%, Table S2) so other factors may have confounded biosynthetic correlations. The correlated pairs share common branched-chain acyl-CoA thioester precursors: isovaleryl-CoA for humulone and lupulone, and isobutyryl-CoA for cohumulone and colupulone (Clark *et al.*, 2013). These precursors are specifically biosynthesised in lupulin glands (Clark *et al.*, 2013), and it seems that their ratios affect the ratios of the bitter acids with the different acyl side chains.

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