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Investigation of hop and beer bitter acids by coupling of high-performance liquid chromatography to nuclear magnetic resonance spectroscopy

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Abstract

HPLC–NMR coupling is becoming used for various applications, including the analysis of natural products. Its great potential is demonstrated by the analysis of hop bitter acids, such as humulones, isohumulones, dihydroisohumulones and tetrahydroisohumulones, using on-line and stopped-flow techniques. ^{1}H-NMR and two-dimensional NMR spectra recorded for all hop bitter acids allowed unambiguous identification. It is shown, that hyphenation of HPLC and NMR spectroscopy offers unique opportunities for analysis and quality control of hops and beer. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Nuclear magnetic resonance spectroscopy; Hop bitter acids; Humulones; Isohumulones; Dihydroisohumulones; Tetrahydroisohumulones

1. Introduction

Hop (*Humulus lupulus*) is a climbing herbaceous plant belonging to the family of the Cannabaceae in the order of Urticales. Because hops provides taste and flavor to beer analysis of the composition of varieties of hops and products derived therefrom is a major issue in the brewing industry [1]. The lupulin glands in the female cones produce humulones and lupulones as a mixture of so-called *n*-, co- and ad-compounds (Fig. 1).

During brewing, hops are boiled in the kettle with wort. This process leads to isomerisation of the humulones to *cis*/*trans* pairs of isohumulones (Fig. 1), which impart their bitter taste to beer and stabilize the beer foam.

In modern brewing practice, whole hops are frequently substituted by various hop products in order to better standardize the qualities of beer. Hop products, based on isohumulones, can be added directly to the beer for adjustment of bitterness. Hydrogenated or reduced isohumulones (Fig. 1) exhibit even more interesting properties, such as resistance to light.

The instability and the similarity in structure of the hop bitter acids create problems in their analysis. In this respect, application of hyphenated techniques, such as the coupling of high-performance liquid chromatography to mass spectrometry (HPLC–MS),

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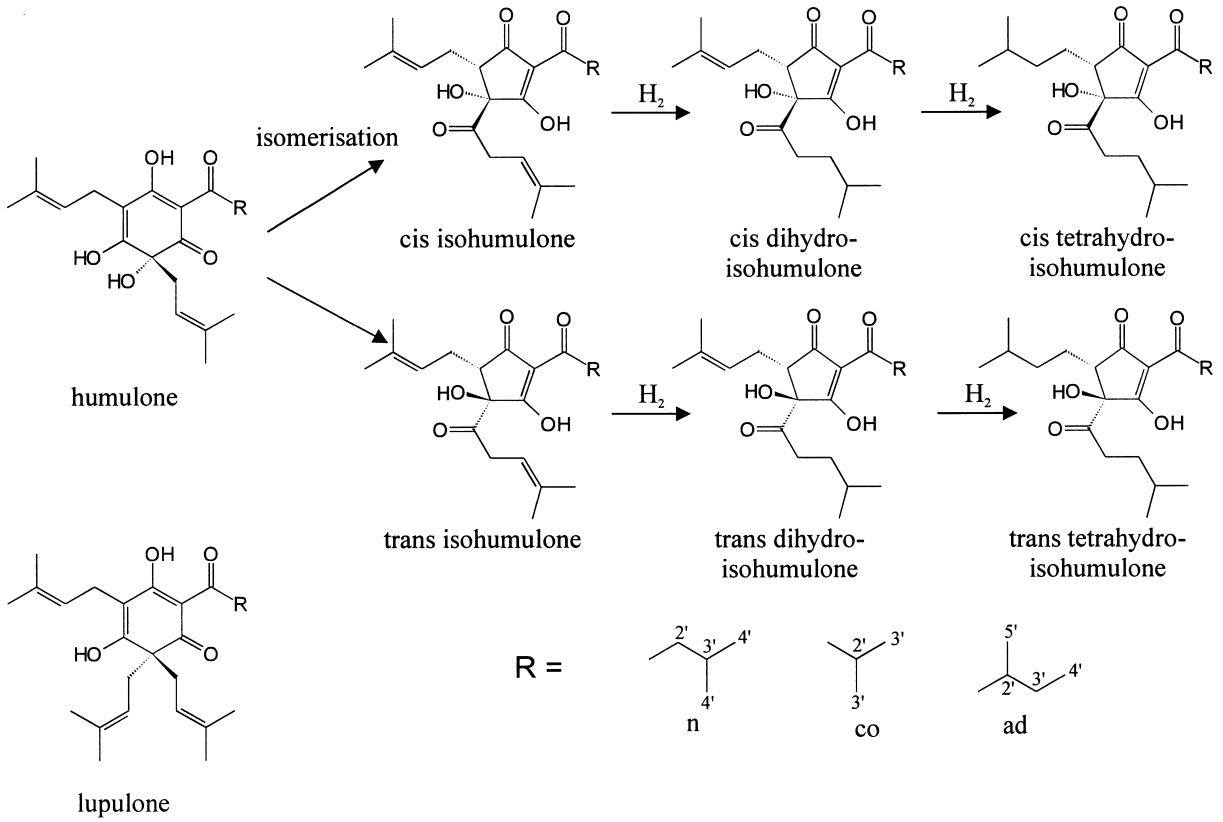

Fig. 1. Structures of the hop bitter acids.

should be very attractive. Unambiguous differentiation of stereoisomers is however in general elusive.

Nuclear magnetic resonance (NMR) spectroscopy is eminently suited to the structural elucidation of stereoisomeres. First experiments for an on-line coupling of HPLC with NMR were reported in the late 1970s [2,3], but broader application was limited by the low sensitivity of the NMR technique and the use of protonated solvents. Subsequent developments led to a dramatic increase in the sensitivity. Furthermore, the availability of NMR spectrometers with an increased dynamic receiver range and the improvement of the solvent suppression techniques allowed acquisition of signals of samples diluted in protonated solvents. Today, HPLC–NMR coupling can be routinely used for a wide range of applications [4–9]. The acquisition of stopped flow ^{1}H NMR spectra of constituents of a hop extract proved to be extremely suitable for hop analysis [10]. Futhermore, ^{1}H NMR spectroscopy was very helpful for the quantification of the components of hop standards [11].

In the present study, on-line and stopped-flow HPLC–NMR measurements afforded full structural information on hop bitter acids constituents of various hop products.

2. Experimental

2.1. Materials

Acetonitrile (NMR Chromasolve) was purchased from Riedel-de Haën (Seelze, Germany). Deuterium oxide (99.9%) was obtained from Deutero (Herresbach, Germany) and orthophosphoric acid (85%, analytical-reagent grade) was from Merck (Darmstadt, Germany). All hop extracts were provided by Hopstabil (Munich, Germany).

2.2. Instrumentation

A Merck–Hitachi (Darmstadt, Germany) L-6200A intelligent pump, a Merck–Hitachi L4000A UV detector and a Bruker (Rheinstetten, Germany) peak sampling unit BPSU-12 were used. The HPLC system was controlled by Bruker LC-Chromstar software and the Bruker LC244M interface. The NMR spectrometer was a Bruker AMX 600 equipped with an inverse 120-μL flow probe with a *z*-gradient system, as well as a Bruker ARX 400 with an inverse 120 μL flow probe. Bruker XWIN-NMR software was used for acquisition and processing of the NMR data. For a more detailed description of the used HPLC–NMR system see Refs. [5,8].

2.3. Samples

CO_2 hop extracts and isohumulones were a gift of Hopstabil (Munich, Germany). They were used without further sample preparation. Hydrogenation of the isohumulones was performed by liquid phase polymer-based catalysis [12]. Typical conditions were: 10% aqueous solution of the isohumulones (4 ml); 1 mg paladium–PVP_{k25} in aqueous solution, 25°C, 1 bar H_2 pressure.

2.4. HPLC conditions

Compounds were separated on a GromSil ODS-0 AB column (250 mm×4 mm I.D., 5 μm particle size) (Grom, Herrenberg, Germany). Under isocratic conditions solutions A: ACN (0.05% H_3PO_4) and B: D_2O (0.05% H_3PO_4) were used in the following proportions: 60% A and 40% B for the CO_2 hop extract (Fig. 2A) and the dihydroisohumulones (Fig. 4A); 55% A and 45% B for the isohumulones (Fig. 2B); 63% A and 37% B for the tetrahydro-isohumulones (Fig. 4B). The flow-rate was 0.3 ml/min. For stopped-flow measurements (Fig. 6) the

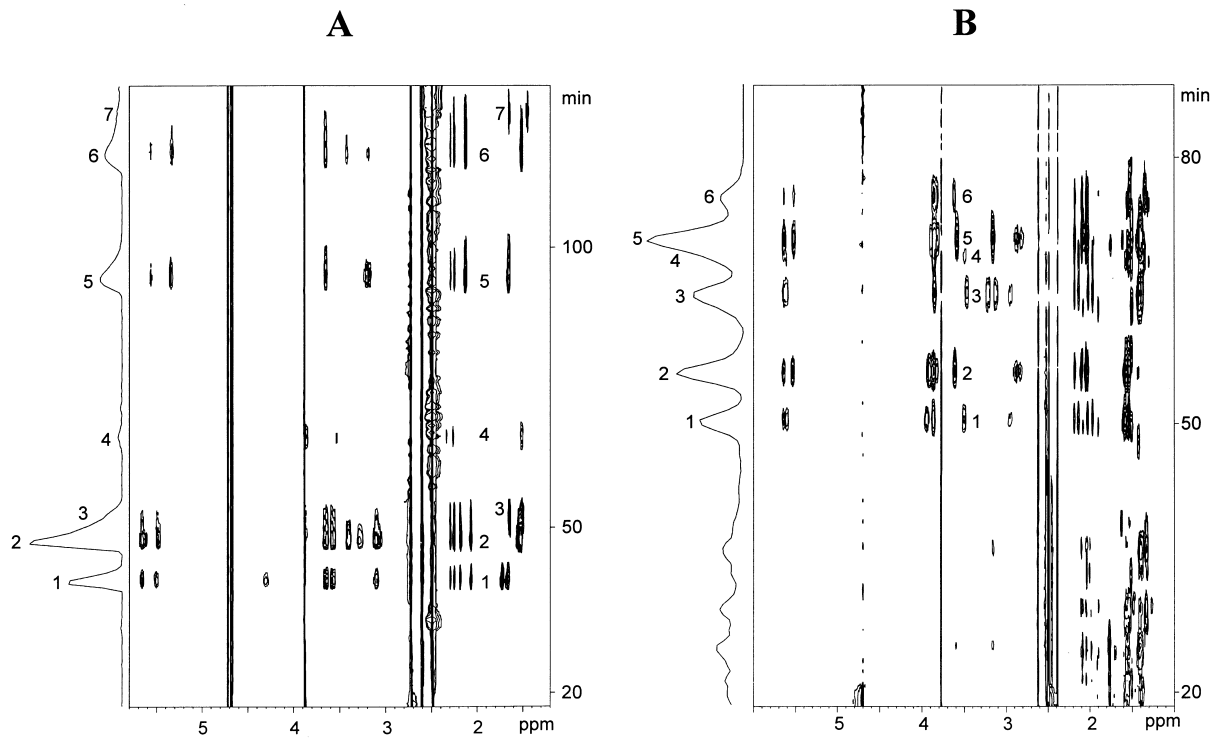

Fig. 2. NMR contour plots with NMR projection (left side) of (A) CO_2 hop extract: 1=co-humulone, 2=humulone, 3=ad-humulone, 4=unknown, 5=colupulone, 6=lupulone, 7=adlupulone; (B) isohumulones: 1=*trans*-iso-co-humulone, 2=*cis*-iso-co-humulone, 3=*trans*-isohumulone, 4=*trans*-iso-ad-humulone, 5=*cis*-isohumulone, 6=*cis*-iso-ad-humulone.

following gradient was used: 70% A and 30% B to 30% A and 70% B in 30 min. The flow-rate was 1.0 ml/min. Due to different absorption maxima the detector wavelength was 314 nm for the hop extract and 274 nm for the isohumulones and hydrogenated isohumulones. Samples were injected as solutions in acetonitrile (200 mg/ml, 15 μL).

2.5. NMR parameters

2.5.1. On-line NMR experiments

For continuous flow experiments, 32 transients with 4k complex data points and a spectral width of 6024 Hz were recorded using the Bruker AMX 600 spectrometer. A relaxation delay of 0.8 s and an acquisition time of 0.34 s/transient were used. During the separation, 128–256 experiments with an acquisition time of 36.4 s/FID were recorded. The WATERGATE technique was used for solvent suppression. Data were handled as a two-dimensional (2D) matrix. Fourier transformation was performed with a line broadening of 1 Hz in the t_2 direction only.

2.6.1. Stopped flow 2D experiments

The COSY experiments were performed with the Bruker AMX 600 spectrometer. Presaturation was used for solvent suppression. A total of 512 t_1 increments with 16 transients and 2 K complex data points were acquired with a spectral width of 9615 Hz in both dimensions. The total acquisition time was 7 h 36 min. The Bruker ARX 400 spectrometer

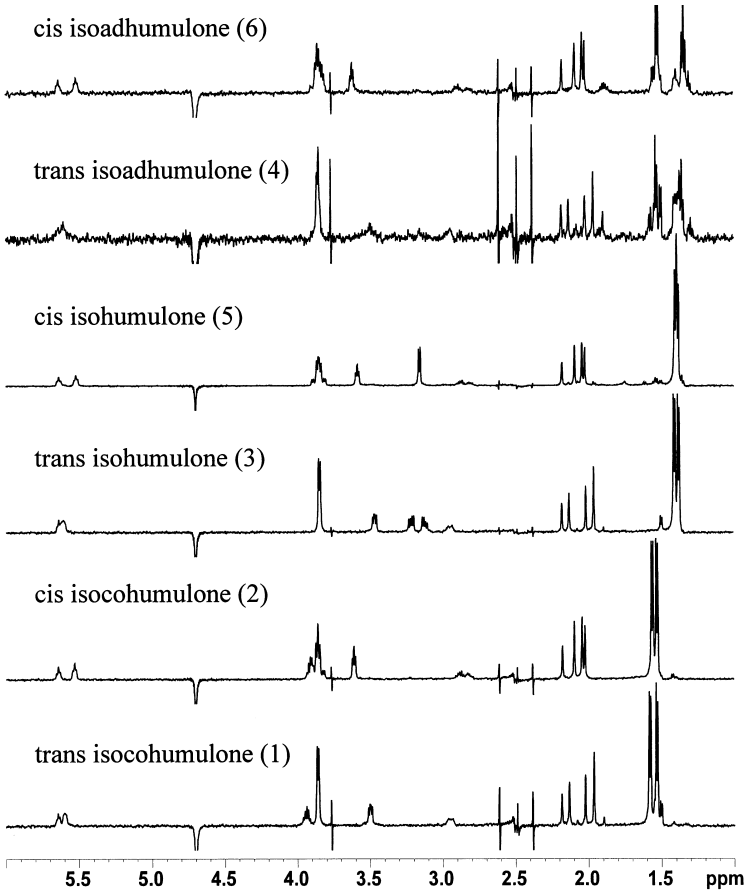

Fig. 3. ^{1}H NMR spectra of the isohumulones. All spectra are extracted from from the continuous-flow experiment. The numbers 1–6 are the corresponding peak numbers in Fig. 2B.

was used for the TOCSY experiments. Presaturation was again used for solvent suppression. A total of 320 t_1 increments with 32 transients and 4k complex data were acquired with a spectral width of 5617 Hz in both dimensions. A mixing time of 60 ms was used.

3. Results and discussion

3.1. CO_2 hop extract

The result of a HPLC–NMR separation of a CO_2 hop extract is shown in Fig. 2A which is viewed as a contour plot with the ^{1}H NMR chemical shift on the vertical axis and the HPLC retention time on the horizontal axis. The projection of the NMR signals on the left side illustrates the separation of the components.

Characteristic resonances of hop bitter acids are those of the double bonds (δ=5.4–5.7 ppm) and the methyl groups of the unsaturated side chains (δ= 2.0–2.4 ppm). The signals of the acyl side chains were used to differentiate between *n*-, co- and ad-humulones and isohumulones. Spectra of co-compounds showed a typical signal at 4.3 ppm resulting from the 2′-proton, while *n*-compounds were identified by the resonance of the methylene-group at 3.2 ppm and the ad-isomers by the doublet of the methyl-group of the 2-methylbutanoyl side chain at 1.6 ppm.

Unequivocal identification of individual constituents was possible based on these signals of the acyl side chain. Under the isocratic conditions and in view of the high loading of the column necessary for the NMR detection, the ad-isomers were partly coeluted with the *n*-isomers. However, the signal at 1.6 ppm (48–55 min) indicated this coelution of the ad-compound. In addition, an unknown substance (4) (about 2%) was detected (54–69 min). The spectra

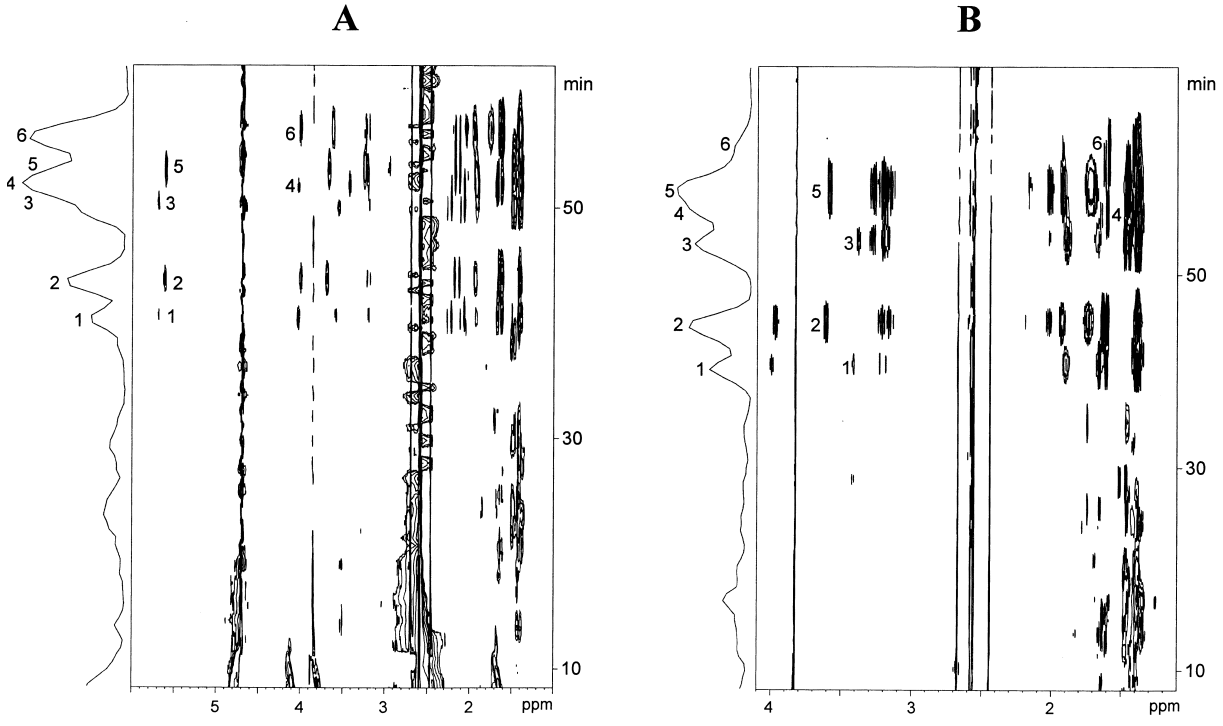

Fig. 4. NMR contour plots with NMR projection (left side) of (A) dihydroisohumulones: 1=*trans*-dihydroiso-co-humulone, 2=*cis*-dihydroiso-co-humulone, 3=*trans*-dihydroisohumulone, 4=*trans*-tetrahydroiso-co-humulone, 5=*cis*-dihydroisohumulone, 6=*trans*-tetrahydroiso-co-humulone; (B) tetrahydroisohumulones: 1=*trans*-tetrahydroiso-co-humulone, 2=*cis*-tetrahydroiso-co-humulone, 3=*trans*-tetrahydroisohumulone, 4=*trans*-tetrahydroiso-ad-humulone, 5=*cis*-tetrahydroisohumulone, 6=*cis*-tetrahydroiso-ad-humulone.

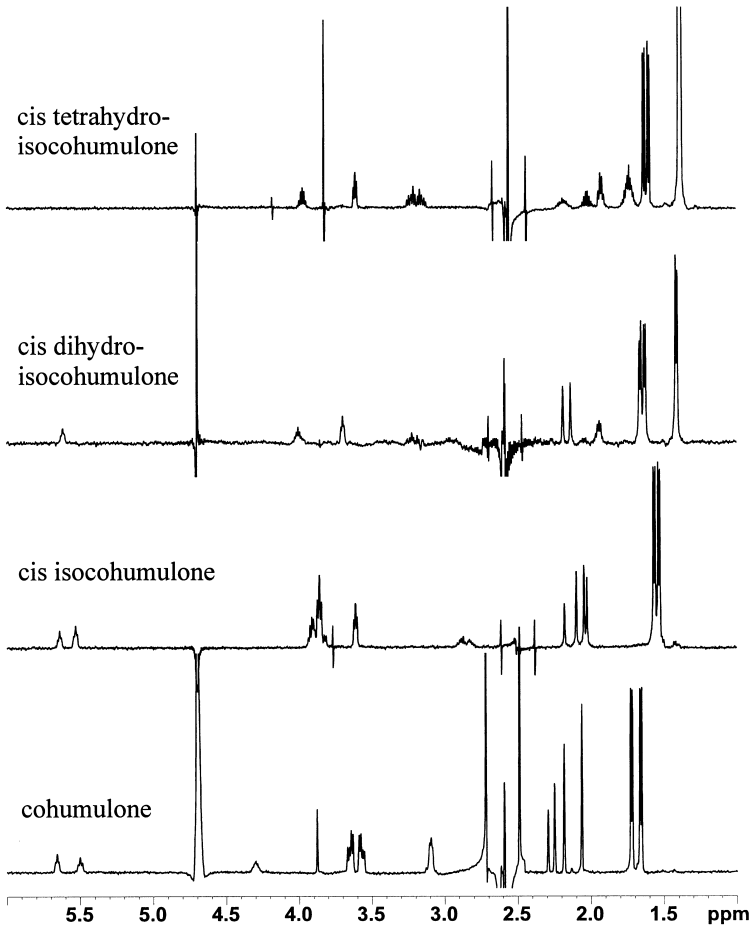

Fig. 5. Comparison of the ^{1}H NMR spectra of hydrogenated and not hydrogenated hop bitter acids. All spectra are extracted from from the continuous-flow experiments.

show a signal of the acyl side chain at 1.5 ppm, two signals of methyl groups of the unsaturated side chains at 2.3 and 2.4 ppm, and one olefinic proton. This pointed to the loss of one of the unsaturated side chains. Therefore, the compound (4) seems to be a decomposition product.

3.2. Isohumulones

Investigation of the isohumulones (Fig. 2B) demonstrated the superior features of HPLC–NMR with respect to HPLC–MS. Differentiation of all ishumulones by their mass spectra is not possible since *cis* and *trans* isomers as well as *n*- and ad-isomers have identical molecular masses and similar fragmentation patterns.

In contrast, all isohumulones displayed distinguishable ^{1}H NMR spectra (Fig. 3). The *n*-, co-, and ad-isohumulones were identified by the typical signals of the acyl side chains. The spectra of the *cis*-isohumulones significantly differed from those of the corresponding *trans*-isomers, e.g., the signals of the olefinic protons had a smaller chemical shift difference compared to the *trans*-isomer. Also, the signal of the ring proton was at 3.5 ppm for the *trans*-compounds and at 3.6 ppm for the *cis*-compounds. Under isocratic conditions it was not possible to separate *trans*-ad-isohumulone ($<$2%) from *cis*-*n*-isohumulone ($>$30%). Nonetheless on-line NMR experiments enabled detection and identification of this minor component. Fig. 6A shows a stopped-flow COSY spectrum of *cis*-iso-co-

humulone. An exact structural analysis was made by means of the 2D NMR connectivities.

3.3. Hydrogenated isohumulones

HPLC–NMR allowed direct identification of dihydro- and tetrahydroisohumulones (Fig. 4A and B). The contour plot (Fig. 4A) shows the separation of a sample containing dihydroisohumulones, which displayed only one olefinic proton (Fig. 5). Additional signals at 1.95 ppm and 2.05 ppm as well as the shift of the methyl signals from 2.05–2.10 ppm to 1.4 ppm confirmed that one of the side chains of the isohumulones had been hydrogenated, while 2D NMR experiments proved that selective hydrogenation of the pentenoyl side chain had occurred.

From the ^{1}H NMR data shown in Fig. 4B, the tetrahydroisohumulones were identified since olefinic protons are lacking. The 2D TOCSY NMR spectra confirmed the hydrogenation of the double bonds in the isohumulones (Fig. 6B). The sample did not contain any over-reduced products, nor was there evidence for the presence of ρ-isohumulones.

4. Conclusions

The unique applicability of the HPLC–NMR coupling in the investigation of hop bitter acids has been highlighted. From the on-line experiments detailed structural information was obtained, which allowed identification of all constituents, including

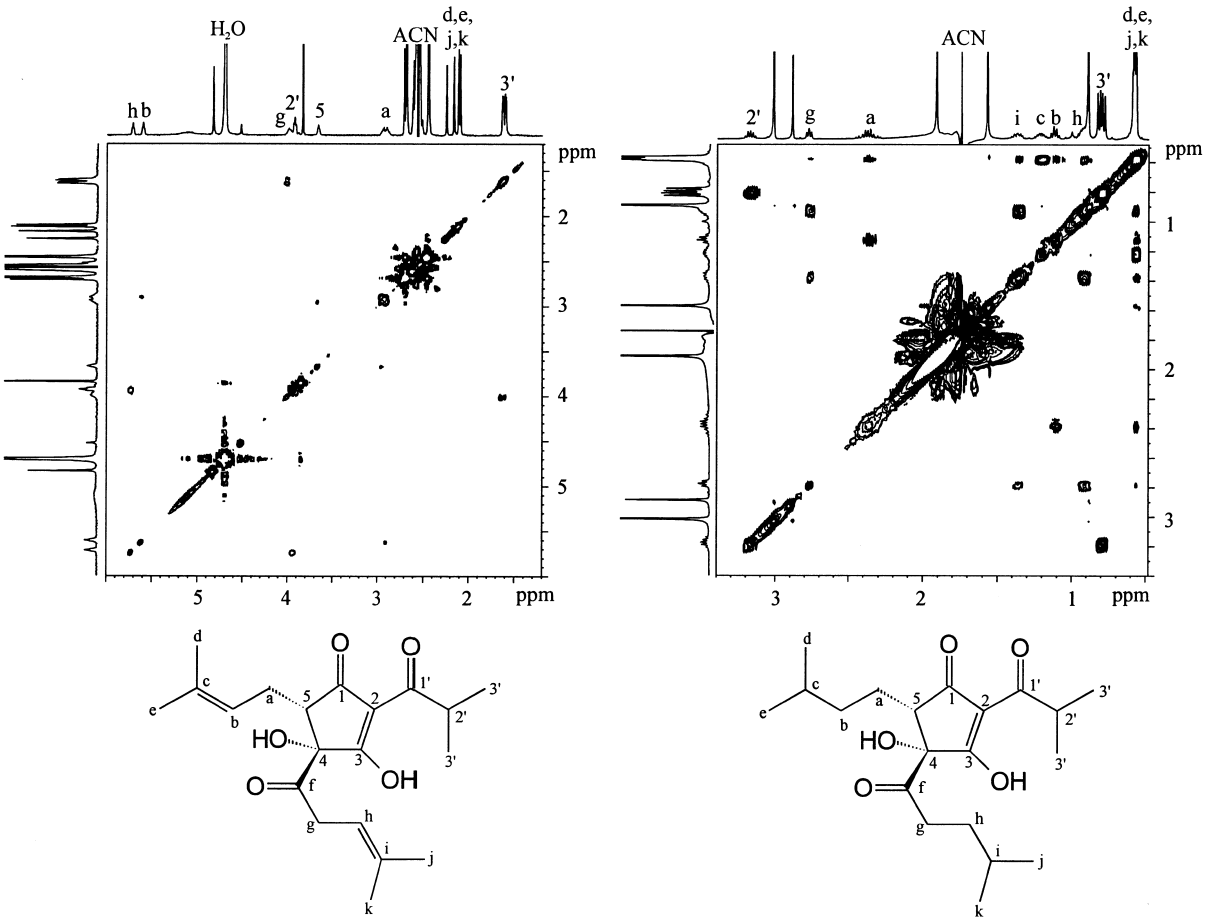

Fig. 6. 2D NMR stopped flow spectra of (A) H,H COSY spectrum of *cis*-iso-co-humulone; (B) TOCSY spectrum of *cis*-tetrahydroiso-co-humulone.

stereoisomers. The analysis times of 80–120 min is significantly shorter than that of conventional off-line NMR experiments. Furthermore, 2D NMR spectra enabled detailed structural analysis and assignment of the NMR signals.

This study represents, the basis for a wider approach to a number of interesting issues in analysis of reaction mixtures resulting from treatment of hop bitter acids, e.g. irradiation or oxidation.

Quantitative analysis of the hop bitter acids by HPLC–NMR should be of great interest both for monitoring the fate of hop constituents in the brewing process and in assessing the quality of hop-derived bitter components in beer.

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