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Note

High-performance liquid chromatography with photodiode array detection of minor hop bitter acids in hops extracts and in beer

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Hops or rather the dried flowers of *Humulus lupulus* are only applied in brewing. They are boiled with wort, during which the hop bitter compounds are transferred to what will become beer, after fermentation and processing. The taste of beer is largely determined by its bitter flavour as derived from the hop bitter acids. Beer brewed without hops would be very different.

The hop bitter acids are present in hops as a rather complex mixture of varying composition and concentration. The main beer bitter principles are however not present in hops as such, but are transformation products formed during the brewing procedure. The chemical reactions involved are rather complex and this laboratory has played a large part in studying and elucidating them[1].

The main bitter acids of hop are the so-called α and β acids of which there are several homologues and analogues (I–III for the α and IV–VI for the β acids in Fig. 1). These compounds have only a faint bitter taste. The ring-contracted isomerization products of the α acids or the so-called iso-α acids, formed during the brewing process, are however very bitter and they are moreover well soluble in beer. There are two series of these iso-α acids: the *cis* series (VII–IX in Fig. 1) and the *trans* series (X–XII in Fig. 1). Considering that there are three major α acids, there are thus six major iso-α acids in beer. These are the main bitter compounds and their total concentration in beer varies between 15 and 80 ppm.

The analysis of the α acids in hops and of the iso-α acids in beers[2,3] is important to the brewery and is performed routinely on a very large scale, increasingly by high-performance liquid chromatography (HPLC).

All hops-derived bitter acids are very soluble in most solvents and the beer bitter acids can be extracted by all water-immiscible liquids. A solvent often used for this extraction is isooctane. From the discussion presented so far it could be deduced that such an isooctane extract of beer would produce six peaks on HPLC analysis. This is indeed the case, but there are also a rather large number of minor peaks most of which appear also to be derived from hop bitter acids[4]. Little has been done so far to isolate and identify these trace compounds in beer because the mixtures are very complex and cannot be resolved completely, not even by the highest efficiency HPLC. Furthermore, all bitter acids-derived compounds are very sensitive to oxidative degradation and their concentrations in beer are only in the ppb range. The problem is not without practical interest, since it is obvious to most brewing chemists

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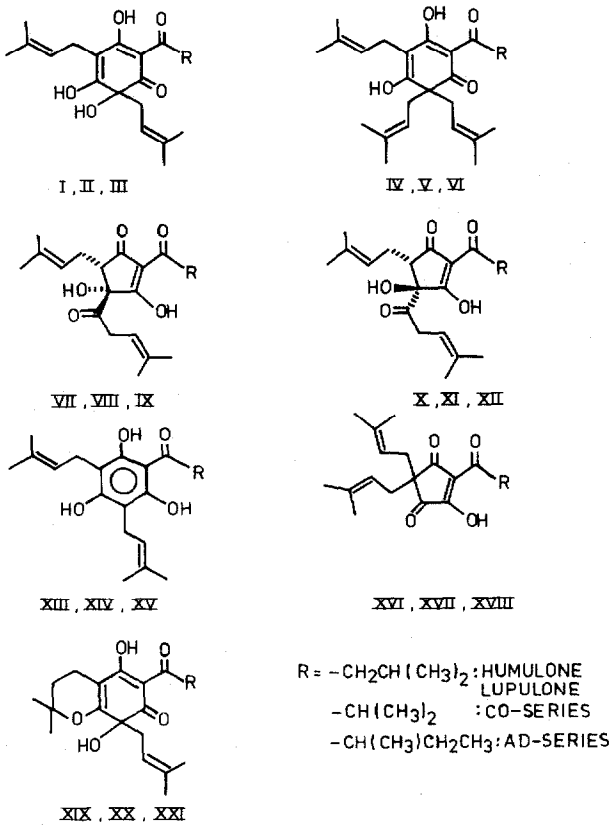


Fig. 1. Formulae of the hops-derived bitter acids as discussed in the text.

that these trace hops-derived compounds play a quality-determining rôle in beer.

Much chemical research —in which a wide variety of reduction, oxidation, acid and base treatments were applied— has been devoted to hop bitter acids[1]. Mostly this was carried out on the α acid humulone and on the β acid colupulone. This is a very complex field and literally hundreds of derivatives of the main bitter acids have been isolated from reaction mixtures of the individual α, iso-α or β acids. Many of these products have been studied thoroughly and their structures elucidated. Most of them, obtained, *e.g.*, by hydrogenation or chemical oxidation, cannot occur in beer as the chemistry involved is totally strange to the brewing conditions. Some reaction mixtures were however obtained under simulated brewing conditions and the reaction products could then of course be present in beer.

Chromatographic retention data are usually insufficient for identification purposes, especially with very complex mixtures. An on-line technique for indentification is therefore required. Photodiode array (PDA) detection is such a technique. Although the information obtained from an UV spectrum is not as rich as that from, *e.g.*, a mass or NMR spectrum, it can be used as evidence for peak identity. Moreover, the UV spectra of most of the hop bitter acids and their derived products have been recorded at one time or another, also in this laboratory, and are therefore well

known. Under these conditions it appears to be relatively easy to arrive at a positive identification with PDA detection. It is the aim of this paper to show this and to describe some results of the PDA identification of trace hop-derived compounds.

EXPERIMENTAL

Chromatography

An Hewlett-Packard (HP) 1090 liquid chromatograph provided with an autosampler injector and a photodiode array detector was employed. The data were recorded with an HP-35B computer, an HP-9121D flexible disc drive and an HP-Thinkjet printer. The columns were stainless-steel Lichroma tubes (25 cm × 0.46 cm) packed using a descending technique at 500 bar with a 20% acetone slurry of 5-μm ROSiL-C18-D (an octadecylated spherical and deactivated silica gel from RSL/Alltech-Europe). To remove trace metals from this material it was boiled with hydrochloric acid as described earlier[5]. This is essential for good HPLC of some of the hops-derived bitter acids which form strong complexes with trace metals. For some of the separations requiring very high efficiency, two columns were coupled with a 5 cm × 0.025 cm capillary tube. The anthracene bonded silica gel phase was synthesized by the reaction of 9-chloromethylanthracene with irregularly shaped 10-μm aminopropyl silica gel (10 μm RSiL-NH2, RSL/Alltech-Europe) as reported earlier[6].

Hops extraction

Hops extracts were either obtained as such from various suppliers, or made by hops extraction with isooctane and evaporation of the solvent. In the second method an apolar solvent extract was obtained analysing for 13.4% cohumulone, 30.5% humulone and adhumulone, 10.6% colupulone and 9.7% lupulone and adlupulone. The remaining 35.8% is unknown!

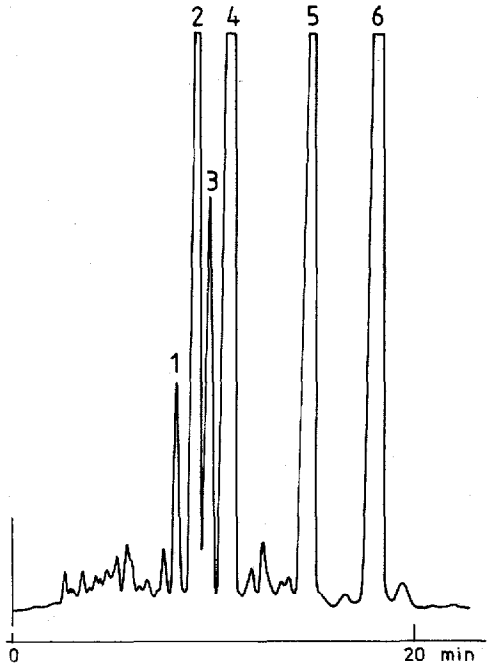
Beer extraction

A 250-ml volume of commercial lager beer was extracted with 60 ml isooctane for 30 min as described earlier[2,3]. The evaporated residue was redissolved in 200 μl methanol.

RESULTS AND DISCUSSION

Problems related to hops and hops extracts

Deoxy-α acids. As intimated above, a very large fraction of hops extracts is of unknown composition. All efforts to obtain information about this fraction have so far failed. We believe that the reason for this can only be the extreme complexity of this fraction in which no single compound occurs in sufficient concentration to allow its isolation. Still, when a hop extract is analysed with high sensitivity HPLC, with the main peaks going off-scale, two minor peaks with typical UV spectra are revealed. To observe these, the chromatographic column must have a rather high efficiency and the system must have low oxidative ability. Indeed the compounds appear to be the deoxy-α acids, XIII–XV in Fig. 1, which are extremely sensitive to oxidation and which are therefore often not noticed since they are removed from the mixture by



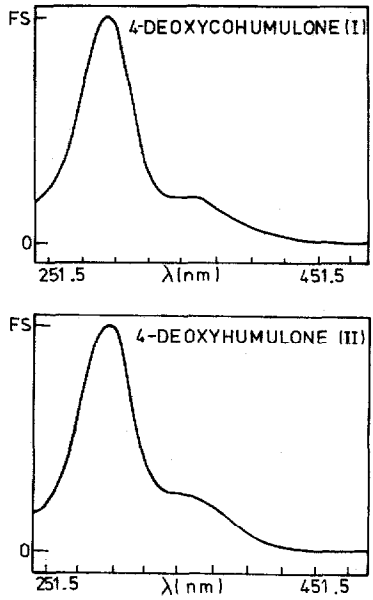


Fig. 2. Chromatogram at high sensitivity of an hop extract. Column: 25 cm × 0.46 cm packed with 5-μm ROSiL-C18-D. Mobile phase: methanol–water–phosphoric acid (85:17:0.5) at 1 ml/min. PDA detection, 314 nm for the chromatogram. Peaks: 1 and 3 = deoxy-α acids; 2 = cohumulone; 4 = humulone and adhumulone (co-eluted); 5 = colupulone; 6 = lupulone and adlupulone (co-eluted).

Fig. 3. UV spectra of the deoxy-α acids peaks 1 and 3 of Fig. 2 as obtained by PDA analysis. FS = Full scale.

such oxidation reactions. A chromatogram and the relevant PDA spectra are presented in Fig. 2 and 3. The practical identity of the two spectra shows that the peaks involved have the same basic structure and are therefore due to analogues or homologues. Furthermore the spectra are in complete agreement with those of the well known deoxy-α acids.

The oxidative ability of a chromatographic system is a rather vague notion. The sensitivity towards oxidation is often suppressed by an excess of other components in a mixture. However, once the sensitive compounds are separated from the protecting mixture their oxidation is often rapid. Very low concentration is also favourable for oxidation, so also is light. Furthermore we believe that trace metals in the stationary phases can also catalyse oxidation. This discussion of oxidation supports our claim about the variability of deoxy-α acids in the chromatograms produced from hops extracts.

Adlupulone. Strangely, adlupulone has never been isolated and its presence in hops has not been demonstrated unequivocally. In the usual HPLC analysis of α and β acids, the pairs humulone/adhumulone and lupulone/adlupulone are not separated and are determined together. This simplifies the analytical procedure. Separation of adhumulone from the two other major α acids is not easy, but is possible. The separation of adlupulone from the other two β acids is much more difficult. Furthermore,

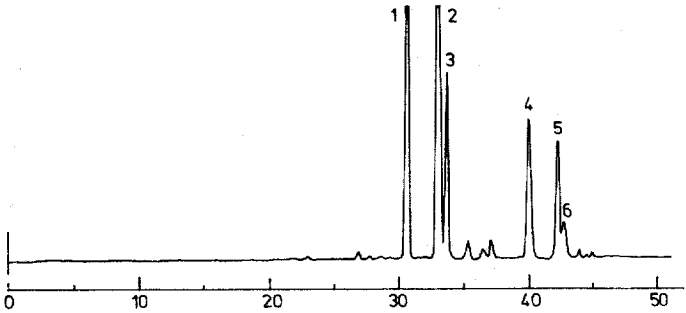


Fig. 4. chromatogram of an hop extract. Two 25 cm × 0.46 cm columns were coupled with a 5 cm × 0.025 cm capillary[11]. Column packing material: 5-μm ROSiL-C18-D. Gradient elution with acetonitrile–water–phosphoric acid from 50:50:0.5 to 100:0:0.5 in 60 min at 0.8 ml/min. PDA detection, 314 nm for the chromatogram. Peaks: 1 = cohumulone; 2 = humulone; 3 = adhumulone; 4 = colupulone; 5 = lupulone; 6 = adlupulone.

even when an additional peak is obtained by boosting the efficiency or changing the solvent system, proof is still needed that this peak is indeed adlupulone. We have now achieved this by coupling two highly efficient columns in series and by PDA analysis of a gradient experiment with an optimized solvent system (Fig. 4). The on-line PDA spectra shown in Fig. 5 speak for themselves; the extra peak, 6 in Fig. 4, is indeed a β acid, and considering its retention characteristics, it must be adlupulone.

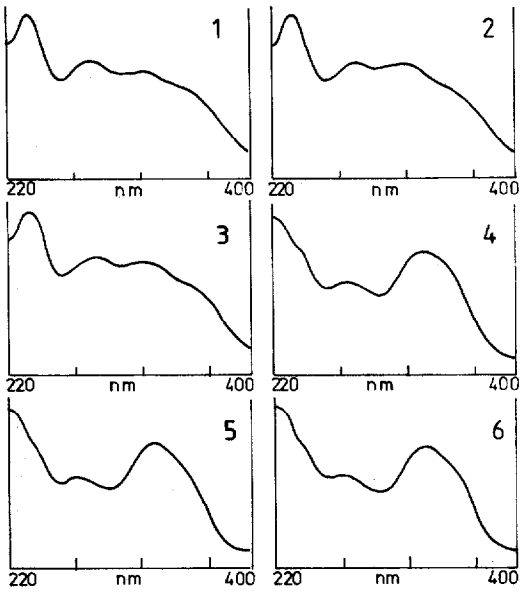


Fig. 5. UV spectra of the peaks of Fig. 4 as recorded by PDA analysis. Spectra: 1, 2 and 3, the three α acids; 4 and 5, colupulone and lupulone respectively; 6, a β acid (adlupulone).

Problems related to trace hop bitter acids in beer

As stated earlier, an isooctane extract of beer produces a most complex chromatographic pattern. Six major peaks, obviously due to the six major iso-α acids, stand out. Some of the minor components have been identified before. As long ago as 1958, Spetsig[7] described and identified the hulupones which are present in beer in ppm amounts (XVI–XVIII in Fig. 1). We have shown by purely chromatographic means that beer contains ppb amounts of the α and β acids[8] as well as traces of the so-called tricyclodehydroisohumulone[9]. The presence of peroxycolupulone is also very likely[10]. Still, these results only account for some of the minor constituents discussed in this paper.

A problem in applying PDA analysis to isooctane extracts of beer is the presence of the six iso-α acids in a concentration which is about 1000 times larger than that of the compounds we want to investigate. It is relatively easy to avoid this interference chromatographically, by detecting either at 280 nm (general wavelength) or at 310–350 nm (wavelength specific for six-membered hops derived compounds). Indeed, at the shorter wavelength all hop-derived bitter acids, five-membered as well as six-membered ring compounds, are detected. At the longer wavelength, only six-membered ring compounds are revealed, but their UV spectra cannot be recorded because of the interference at shorter wavelengths from the five-membered ring iso-α acids.

Group separation of the five- and six-membered ring compounds was achieved on a specially devised anthracene silica gel stationary phase[6]. While octadecylated phases cannot distinguish between compounds of different ring sizes, the anthracene phase does yield a group of early peaks which are the six-membered ring compounds and a more important group of later eluting peaks which are five-membered ring hop-derived bitter acids (mainly the iso-α acids). This special selectivity of the an-

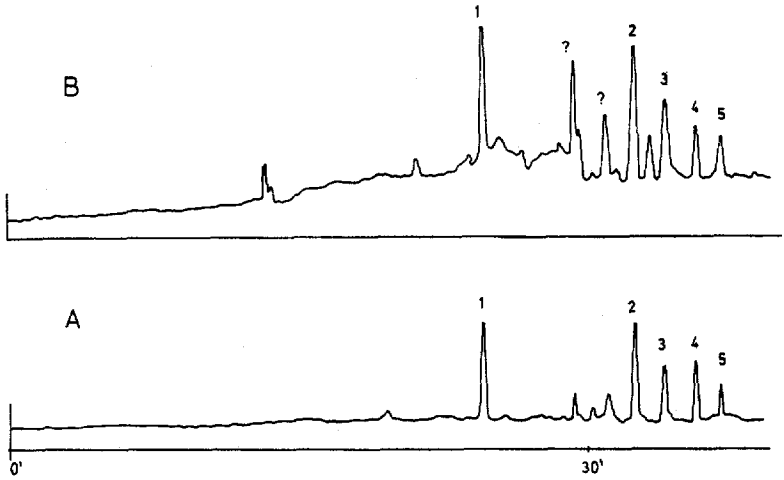


Fig. 6. Chromatogram of the six-membered ring hop-derived compounds extracted from beer and separated from five-membered ring interferences as explained in the text. Chromatographic conditions as in Fig. 4. Peaks: 1 = 9-methylanthracene-aminopropyl silane (by hydrolysis of the stationary phase during the gradient reversed-phase chromatography); 2 = dihydropyranylcohumulone; 3 = cohumulone; 4 = dihydropyranylhumulone; 5 = humulone. Peaks 2 and 4 are sometimes also called bicyclo isomerization products of the α acids. Detection: (A) at 336 nm; (B) at 280 nm.

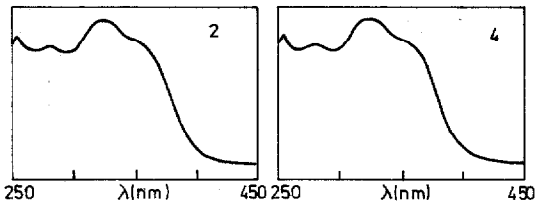


Fig. 7. UV spectra of peaks 2 and 4 of Fig. 6 obtained by PDA analysis. The maxima at 282, 320 nm and the shoulder at 345 nm are characteristic for the dihydropyranyl α acids.

thracene phase was established by chromatographing reference compounds such as the hop bitter acids humulone and *trans*-isohumulone. Five-membered hop-derived acids are much stronger than the six-membered hop acids. This is however not at the basis of the special selectivity of the anthracene silica gel. The aliphatic side chains of the bitter acids also play a rôle. For example, the special selectivity could not be demonstrated with other simple synthetic five- and six-membered vinylogous acids like dimedone or methylcyclopentanetrione.

The PDA analysis of the six-membered ring compounds separated by anthracene phase chromatography (extracted after acidification from the collected chromatographic peak) gives the trace in Fig. 6. Peaks 3 and 5 are the α acids cohumulone and the unseparated humulone and adhumulone respectively. This is revealed by cochromatography and by their PDA spectra. Peaks 2 and 4 are the bicyclic α acids formed by boiling the α acids in buffer or in wort[4]. This is revealed by cochromatography with authentic bicyclic or dihydropyranyl α acids (XIX and XX in Fig. 1), and by comparing the UV spectra of the compounds with those obtained on PDA analysis of peaks 2 and 4. Fig. 7 shows the spectra of the two fractions obtained.

In conclusion, the presence of the following minor hops-derived bitter acids has been shown in beer so far: the hulupones, α acids, β acids, tricyclodehydroisohumulone, peroxycolupulone, bicyclic derivatives of the α acids or dihydropyranyl derivatives of the α acids. Photodiode array detection was most helpful in obtaining this result. Considering the number of peaks in a chromatogram of an isooctane extract of beer, there must be still other so far unknown hop-derived bitter acids in beer. The ones mentioned are however the most important ones. Whether they contribute to beer taste has yet to be established.

ACKNOWLEDGEMENTS

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