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Note

High-performance liquid chromatography of hop bitter substances

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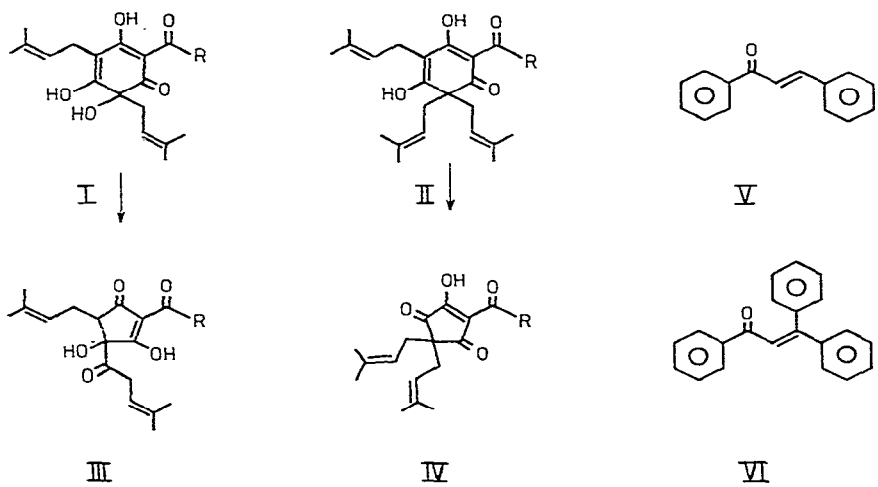
The bitter acids of hops mainly consist of α-acids (I), β-acids (II) and their oxidation products. In the brewing process, these bitter acids undergo complex chemical transformations. Some of the products which are formed remain in solution up to the final beer stage. The bitter taste of beer is mainly due to the iso-α-acids (III) and the hulupones (IV). Each α-acid homologue leads to two diastereoisomeric iso-α-acids. Therefore, the three major α-acids generate six major iso-α-acids (Fig. 1).



R = $CH_2CH(CH_3)_2$: humulone (I) and lupulone (II)
R = $CH(CH_3)_2$: cohumulone and colupulone
R = $CH(CH_3)CH_2CH_3$: adhumulone and adlupulone

Fig. 1. Structures of the compounds studied.

Analysis of these compounds as individuals or as a group is important for the brewery, the hop growing and the hop transforming industry. This importance is reflected by the following considerations. The determination of the α-acids content

is needed because it is the basis for commercial transactions. Knowledge of the relative amount of cohumulone is important since this can be correlated with the origin of the hops and with quality factors which are still largely unspecified. The iso-α-acids content of beer is variable and depends on a large number of factors. The utilization yield or transformation of the hop α-acids into iso-α-acids in the beer can vary from only 20 to 40% even with similar brewing techniques. Analysis of this conversion at the various stages of production is therefore important.

The determination of hop α-acids and iso-α-acids can be performed by conductimetry, spectrometry, counter-current distribution and several chromatographic techniques[1–13]. These methods are, however, not selective. Other compounds interfere and these analyses mostly give no indication of the content of the individual bitter acids. A difficulty is that there is no trustworthy reference method with which any method can be evaluated. At one time counter-current distribution was used as a reference, but it is now recognized that this method is inadequate.

With the development of high-performance liquid chromatography (HPLC) it is of interest to study the possibilities of this technique in the analysis of hop bitter acids. Such studies have been reported before by several workers, but the results were unsatisfactory and not up to the standards of excellence obtainable by optimized HPLC. This paper presents some of our results on HPLC of hop bitter acids, with special emphasis on the development of a possible routine liquid chromatographic method for the α-acids.

EXPERIMENTAL

All of the chromatographic runs were carried out on a Varian 8520 high-performance liquid chromatograph, equipped with a Valco injection device (10-μl loop) and a variable-wavelength Varichrom detector. Peak areas were measured with a Varian CDS 111 electronic integrator. Columns (25 cm × 0.46 cm I.D.) were of stainless-steel precision-bore Lichroma tubing. Connections to the injector and detector were stainless-steel reducing unions (1/16–1/4 in.) modified for zero dead-volume. Columns were filled by a modified method described by Webber and McKerrel[14]. Octadecyl silica gel was prepared according to Kirkland[15], and the ion exchangers according to Parr and Grohmahn[16] and Cox *et al.*[17].

RESULTS AND DISCUSSION

Chromatography of hop bitter substances on silica gel gives unusable results. Separations can be carried out on buffered silica gel but this method is rather difficult to reproduce.

An amino phase ($\equiv Si-CH_2-CH_2-CH_2-NH_2$), a cyano phase ($\equiv Si-CH_2-CH_2-CH_2-CN$), 12 different ion exchangers based on chloropropyl silica gel ($\equiv Si-CH_2-CH_2-CH_2-NR_3^+$) and on bromobenzyl silica gel ($\equiv Si-C_6H_4-CH_2-NR_3^+$) and an apolar octadecyl phase, chemically bonded to 10-μm microparticulate silica gel, were studied for the chromatography of the hop compounds. The amino phase and the cyano phase gave poor results. Some separations can be performed on the ion-exchange materials, but poor column efficiency and strong tailing peaks cause considerable loss of resolution. Octadecyl silica gel is the most useful stationary phase

for the analysis of hop bitter acids. In contradiction with literature data, where it is claimed that complete deactivation is necessary on a reversed-phase silica gel[18], some separations of the iso-α-acids could not be achieved on phases deactivated with trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS) and bis(trimethylsilyl)acetamide (BSA). The best results were obtained in a reproducible way on silica gel treated only with octadecyltrichlorosilane and containing *ca.* 17% bonded organic material.

Elution on a 5-μm SiO_2–C_{18} phase with buffered mobile phases in mixtures of methanol and water leads to complete separation of all the individual hop acids, as shown in Fig. 2 for the six-membered ring α-acids and in Fig. 3 for the five-membered ring iso-α-acids. The separations are only obtained under the optimized chromatographic conditions indicated. Slight differences in the composition of the solvent cause big shifts for some compounds, with residual loss of resolution. This fact explains the methanol:water ratio of 59:41 used for the analysis of the hop α-acids and the high buffer capacity needed for a good chromatogram of iso-α-acids.

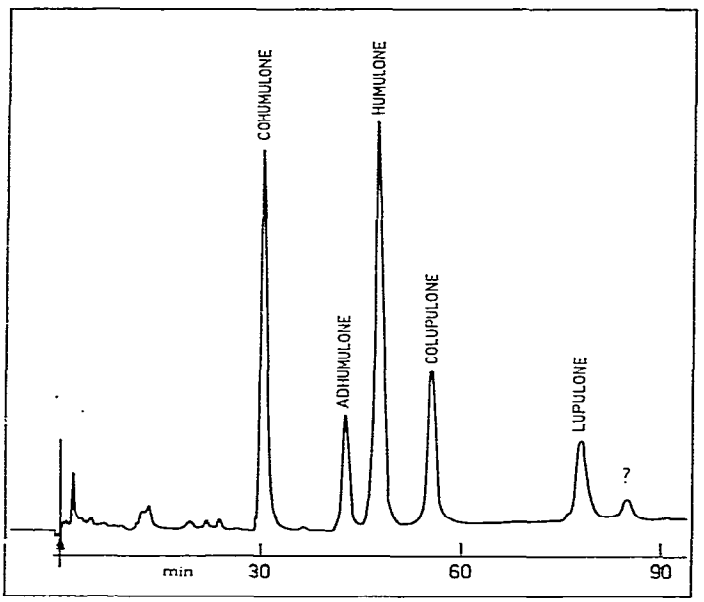


Fig. 2. Chromatogram of hop extract sample on 5-μm SiO_2–C_{18} (17%). Column (25 × 0.46 cm), Lichroma tubing. Detection at 334 nm. Sodium acetate–acetic acid buffer (pH 7.0), 0.2 *M* in methanol–water (59:41). Flow-rate, 60 ml/h at a pressure of 550 kg/cm.

The resolution between the three α-acid homologues on a 10-μm SiO_2–C_{18} column, as shown in Fig. 4, is sufficient to develop a quantitative analysis using chalkone (V) as internal standard. 10-μm SiO_2–C_{18} columns are preferred because they are easier to fill and because of the high back pressure of a 5-μm phase (see Fig. 2). Chalkone solutions should be kept in the dark to avoid light-induced isomerization of the *trans* to the *cis* form.

The detection of the compounds is performed at 334 nm with a variable-

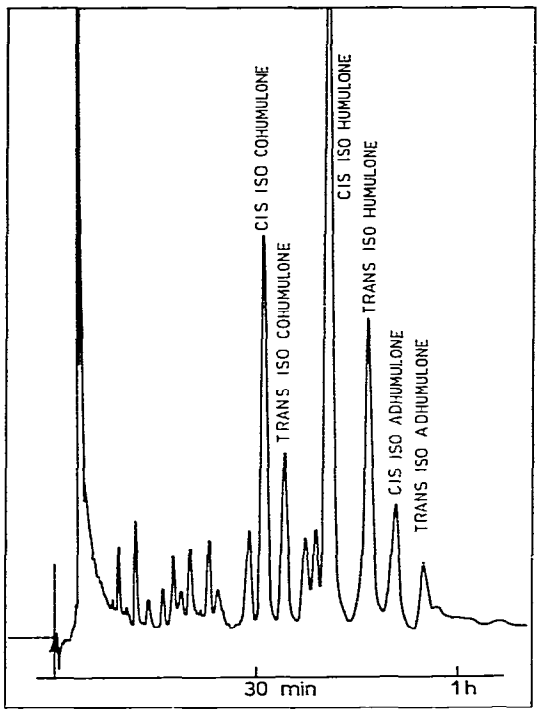


Fig. 3. Chromatogram of an isomerised hop extract sample on 5-μm SiO_2–C_{18} (17%). Column and flow-rate as in Fig. 2. Detection at 280 nm. Citrate buffer (pH 3.0), 1 *M* in methanol–water (60:40). Peaks as indicated on the chromatogram; others unknown.

wavelength detector. At this wavelength the three α-acids have maximum light absorption. Chalkone shows a maximum at 313 nm so that much attention must be given to the correct calibration of the detector monochromator*. The selectivity of detection is well increased at this wavelength, since iso-α-acids and most oxidation products of the bitter acids do not absorb at 334 nm.

A calibration curve determined for pure humulone ($y = 0.436\ x - 0.038$, $r = 0.9997$) is linear over a sufficient concentration range (0.02 mg/ml to 0.4 mg/ml). Cohumulone and adhumulone can be determined using the same calibration curve since their extinction coefficients in the solvent were found to be the same[19]. With the knowledge of the extinction values of pure colupulone and lupulone, the determination of the β-acids on the same chromatogram is made possible by the introduction of a correction factor in the calculation.

The analysis time needed for complete resolution (90 min) under the described conditions is too long for routine purposes. For a faster analysis, the solvent system of methanol–water–acetic acid (85:15:1) was suitable. Humulone and adhumulone are not separated under these conditions, but so far there seems to be less need for this analytical detail.

* This can be done by observing the emission line of the deuterium lamp at 486.1 nm, or by observing the maximum at 311 nm in the UV spectrum of naphthalene.

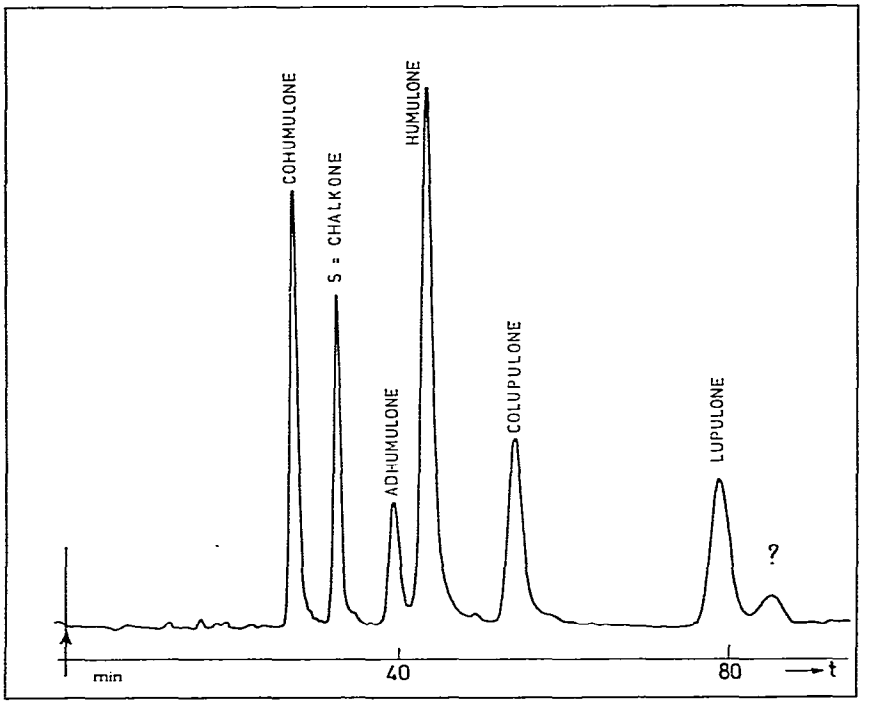


Fig. 4. Chromatogram of a hop extract obtained as in Fig. 2 but with 10-μm SiO_2–C_{18} (17%). Back pressure ~200 kg/cm^{-2}. Chalkone as internal standard.

Chalkone (V) is less suitable as an internal standard in this solvent system since it is eluted at the same retention volume as a small unknown compound present in all of the hop extracts examined so far. A better internal standard is β-phenylchalkone (VI) (2-benzoyl-1,1-diphenylethene) synthesised by addition of 2.5 equivalents of phenylmagnesium bromide, prepared from magnesium and bromobenzene, to 1 equivalent of dibenzoylmethane, followed by dehydration of the tertiary alcohol in an acid medium[20]. A chromatogram under these conditions is shown in Fig. 5. The detection wavelength is now 324 nm, the absorption maximum for the α-acids. β-Phenylchalkone (VI) shows a maximum at 318 nm. Since β-phenylchalkone cannot undergo isomerization reactions, the stability of the solutions is better than for chalkone. The calibration curve for pure humulone against this standard gives $y = 0.62418\ x - 0.01946$ and $r = 0.9986$. For the analysis of the total α-acids content, humulone and adhumulone are determined together; the extinction coefficients of both compounds are similar in the solvent used[19]. Due to a slight difference in extinction value, a correction factor (1.0594) must be introduced to calculate the cohumulone concentration. The β-acids can be determined from the same chromatographic run if their extinction coefficients are known. An advantage of the proposed method is the possibility for automation, with an automatic sampling device and an electronic integrator for calculation of the results.

The relative standard deviation and accuracy of the method described so far is 2–3%. This is on the high side for the type of analysis involved. One reason for

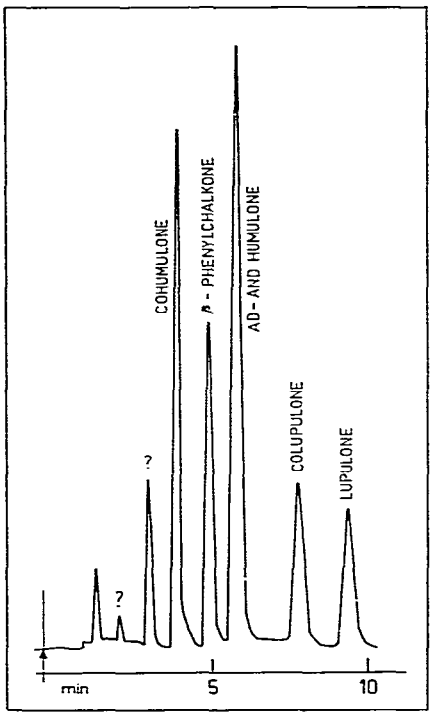


Fig. 5. Chromatogram of a hop extract sample on 10-μm SiO_2–C_{18} (17%). Column as in Fig. 2. Detection at 324 nm. Solvent system: methanol–water–acetic acid (85:15:1). β-Phenylchalkone as internal standard. Analysis time, 10 min.

this was found to be oxidation of the bitter acids during the chromatographic run. Addition of BHT (butylhydroxytoluene, a well established anti-oxidant) to the sample solution (1%) and to the eluting solvent (1%) improved the results. BHT does not interfere with the detection at 324 nm and the relative standard deviation is now of the order of 1%.

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