# High Performance Liquid Chromatographic Analysis of Beer Bitter Acids

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## ABSTRACT

A quantitative high performance liquid chromatography (HPLC) analysis for beer bitter acids is presented. The method uses 10-μm silica gel modified wih octadecyl groups and isocratic elution with tetrabutylammonium hydroxide in acidic methanol-water as ion pair former. Isocohumulones, isohumulones, and isoadhumulones were separated and eluted in that order. β-Phenylchalkone is proposed as an internal standard. Analysis time was less than 15 min.


Key words: *Beer bitter acids, HPLC, Iso-α-acids*

During the brewing process, the three major hops α-acids (humulone, cohumulone, and adhumulone) are isomerized into beer iso-α-acids, each α-acid yielding a *cis-trans* pair of stereoisomers. Beer therefore contains six major bitter iso-α-acids, ie, *cis*-isohumulone and *trans*-isohumulone, *cis*-isocohumulone and *trans*-isocohumulone, and *cis*-isoadhumulone and *trans*-isoadhumulone. Quantitation of these compounds in isomerized hop extracts, worts, and beers is important for the brewing industry.

Until now this analysis usually has been conducted on iso-α-acids as a group by direct photometry of a simple isooctane extract (1,5). This extraction does not achieve separation of the iso-α-acids from eventual by-products, contaminants, and other isooctane-extractable compounds. These limitations are well recognized, but the method is still rapid and quite useful.

An improved procedure achieving better separation of the iso-α-acids by ion-exchange chromatography (IEC) on Sephadex-diethylaminoethyl was described in 1972 (6). Modifications of this procedure have been and are still being evaluated by the European Brewery Convention and by the American Society of Brewing Chemists. We believed, however, that better group separation, or even individual compound separation, should be strived for and that this could be conveniently and rapidly achieved by modern high performance liquid chromatography (HPLC). The possibilities of various stationary phases were therefore studied and the results published recently (4). We did not succeed in separating the six iso-α-acids completely in the short time allowable for routine analysis. Instead of aiming for complete separation, we fell back on HPLC conditions whereby the *cis* and *trans* forms of each iso-α-acid elute together.

Whitt and Cuzner (9) have developed a gradient HPLC method with naphthalene as internal standard for the same purpose but did not achieve the separation of the isoadhumulones from the isohumulones. We wanted complete separation by an isocratic procedure. This is beneficial for ease of operation and precision of results. We also preferred an internal standard that was chemically closer to the bitter acids than naphthalene was. Our choice was β-phenylchalkone. A chromatogram under these conditions is shown in Fig. 1. Separation was sufficiently fast for routine analysis. The rest of this paper gives full details for the proposed analysis.

## EXPERIMENTAL

### Chromatography

*Instruments and Chemicals.* The HPLC instrument was a Varian 5020 LC with 10-μl Valco 7000 injector, Varichrom detector, and CDS-111 Varian integrator. The analysis column (25 × 0.46 cm) was filled with 10 μm of RSil-$C_{18}$-HL-D from RSL[1] and octadecylsilica gel that must be purified by boiling out three times for 4 hr with 2*N* HCl-$CH_3OH$ with magnetic stirring and thorough washing with distilled water as previously described (4,8). β-Phenylchalkone can be synthesized according to Clemo et al (3) or is available from RSL.[1] Water, methanol, and isooctane were of spectrograde quality. Phosphoric acid (85%), 3*N* HCl, and tetrabutylammonium hydroxide (40% Aldrich) were also used. The eluting solvent had the following composition: 72.5 ml of $CH_3OH$, 27.5 ml of $H_2O$, 1 ml of $H_3PO_4$ (85%), and 1.3 g of tetrabutylammonium hydroxide (40%).

[1] R. S. L., Begoniastraat, 6, B-9731 EKE, Belgium.

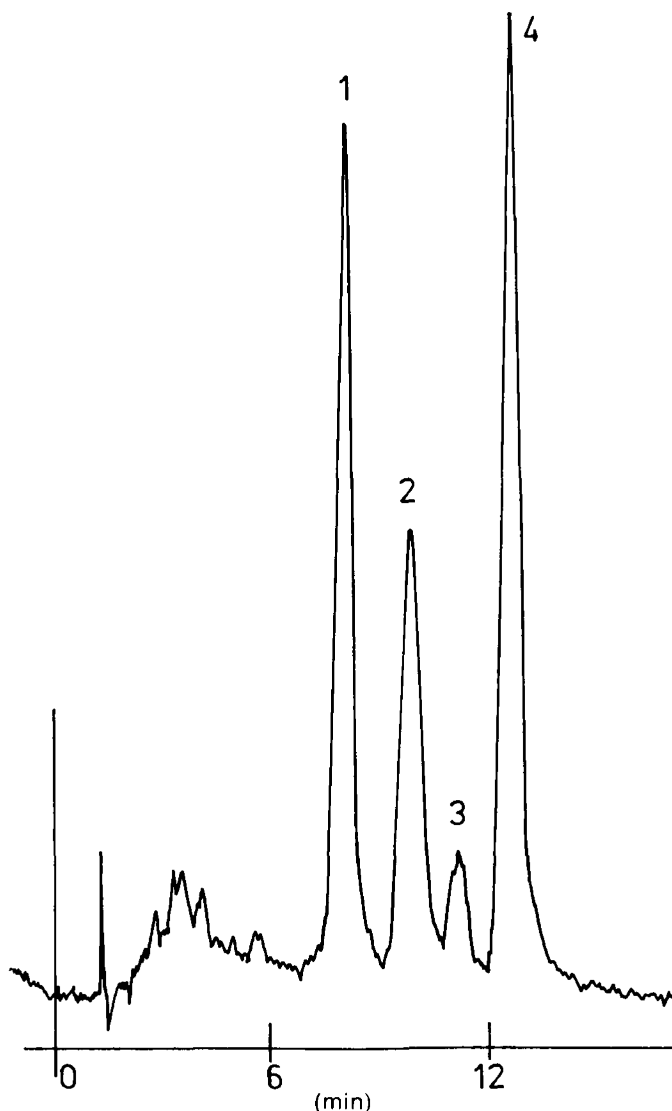


**Fig. 1.** Typical liquid chromatogram of an isomerized extract. EBC-ASBC reference sample: iso-α-I-x-1978-79; 120 ml/hr at 250 kg/cm$^2$. Peaks in order of appearance: **1**, *cis*-isocohumulone and *trans*-isocohumulone, **2**, *cis*-isohumulone and *trans*-isohumulone, **3**, *cis*-isoadhumulone and *trans*-isoadhumulone, **4**, β-phenylchalkone.

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Eluting speed was set at 2 ml/min, corresponding to a pressure of 200–250 kg/cm$^2$.

The Varichrom detector was used at 1 mV, with absorbance range 0.1 front cell position, a normal time constant, and a band width of 8 $\mu$m. The integrator was operated as follows: ID = , S/N = 2 N. IPW = 15 sec, TAN % = 3.20, AREA = 5,000 A, and STOP = 0.00M. The 1 mV full scale Varian A25 recorder ran at 30 cm/hr.

*Internal Standard and Correlation Coefficients.* The extinction coefficients of the different iso-$\alpha$-acids are not the same at all wavelengths. Based on previous work done in this laboratory (2), 270 nm was chosen as a compromise wavelength where these differences were minimal. *Trans*-isohumulone was recrystallized three times just before establishing the relationship between weight and area ratios against the internal standard. The relationship was linear over a wide range and extrapolated practically through the origin. The equation relating the ratio of surface areas (y) to the ratio of weights (x) of *trans*-isohumulone and the internal standard is then: $y = 0.792x - 0.028$. The similarity of the extinction coefficients of the various iso-$\alpha$-acids at 270 nm and their identical chemical stability allow this *trans*-isohumulone equation to be used for total iso-$\alpha$-acids content.

The quantitative recovery of *trans*-isohumulone added to beer was checked by direct addition of the same amount to the isooctane extracts. The standard deviation of the method was established for total iso-$\alpha$-acids content (0.76%) and for the individual stereoisomer pairs (1.05% for isocohumulones, 1.31% for isohumulones, and 5.68% for isoadhumulones). The figures were obtained from the data in Table I.

**TABLE I**
**Precision Data of the High Performance Liquid Chromatographic Method for Beer Iso-$\alpha$-Acids**

| Beer Analysis | Iso-cohumulones (%) | Iso-humulones (%) | Iso-adhumulones (%) | Iso-$\alpha$-Acids (mg/L) |
|---|---|---|---|---|
| 1 | 35.5 | 49.6 | 14.9 | 24.62 |
| 2 | 34.8 | 50.9 | 13.4 | 24.76 |
| 3 | 35.4 | 51.3 | 13.3 | 24.66 |
| 4 | 35.8 | 51.0 | 13.1 | 24.74 |
| 5 | 35.2 | 50.4 | 14.4 | 25.11 |
| $\bar{n}$[a] | 35.34 | 50.64 | 13.82 | 24.78 |
| s[b] | 0.37 | 0.66 | 0.78 | 0.19 |
| s %[c] | 1.05 | 1.31 | 5.68 | 0.76 |

[a] Mean values.
[b] Standard deviation.
[c] $S\,\% = s/\bar{n} \times 100$.

**TABLE II**
***Comparison of the HPLC[a] Method with Two Other Methods for Beer: Iso-$\alpha$-Acids, in mg/L, and the % Distribution Achieved by HPLC Analysis***

| Beer | HPLC | IEC[b] | Direct[c] |
|---|---|---|---|
| 1 | 24.6<br>36.7% isocohumulone<br>50.1% isohumulone<br>13.2% isoadhumulone | 25.4 | 23.2 |
| 2 | 24.9<br>32.6% isocohumulone<br>53.6% isohumulone<br>13.7% isoadhumulone | 26.8 | 22.2 |
| 3 | 27.3<br>47.8% isocohumulone<br>40.2% isohumulone<br>11.9% isoadhumulone | 27.1 | 21.4 |

[a] Liquid chromatography.
[b] Ion-exchange chromatography.
[c] Direct photometry.

**TABLE III**
**Comparison of the High Performance Liquid Chromatographic (HPLC) and Ion-Exchange Chromatographic (IEC) Methods for Isomerized Extracts**

| Beer | Sample | HPLC (+ allo) (%) | IEC (%) | Percent Iso-$\alpha$ Claimed |
|---|---|---|---|---|
| 1 | EBC-ASBC I-X 1978–79 | 15.0 | 16.9 | |
| | | 14.5 | 17.0 | |
| 2 | EBC 8-B 1976 | 18.9 (23.8) | 27.4 | |
| | | 18.5 (22.3) | 28.1 | |
| 3 | Commercial | 9.1 (10.05) | 17.0 | 20 |
| | | 8.6 (±10) | 17.1 | |
| 4 | Isofix 1979 EBC sample | 12.4 | 12.3 | 15 |

## Sample Preparation

*Beer.* Beer (±20 g), weighed into a beaker to ±10 mg, was transferred with minimal foaming into a 100-ml separation funnel, acidified with 2 ml of 3*N* HCl, and hand extracted for 5 min with 50 ml of isooctane. Twenty-five milliliters of the isooctane layer was completely evaporated on a rotavapor. The residue was dissolved in 1 ml of $CH_3OH$ containing the internal standard (~ 12 mg/100 ml). This final solution is kept in a brown colored container or is protected from light with aluminum foil. This solution contains about 0.25 mg of iso-$\alpha$-acids; the 10 $\mu$l used for the analysis then contains about 2.5 $\mu$g of the mixture.

*Isomerized Extract.* The homogenous extract was dissolved in methanol (100–150 mg of a 15–20% formulation in 100 ml of $CH_3OH$).

## Calculations

Isomerized hop extract (27.14 mg) was dissolved in 20 ml of methanol containing the internal standard. Concentrations were 135.7 mg of extract per 100 ml. Internal standard was 12.42 mg/100 ml.

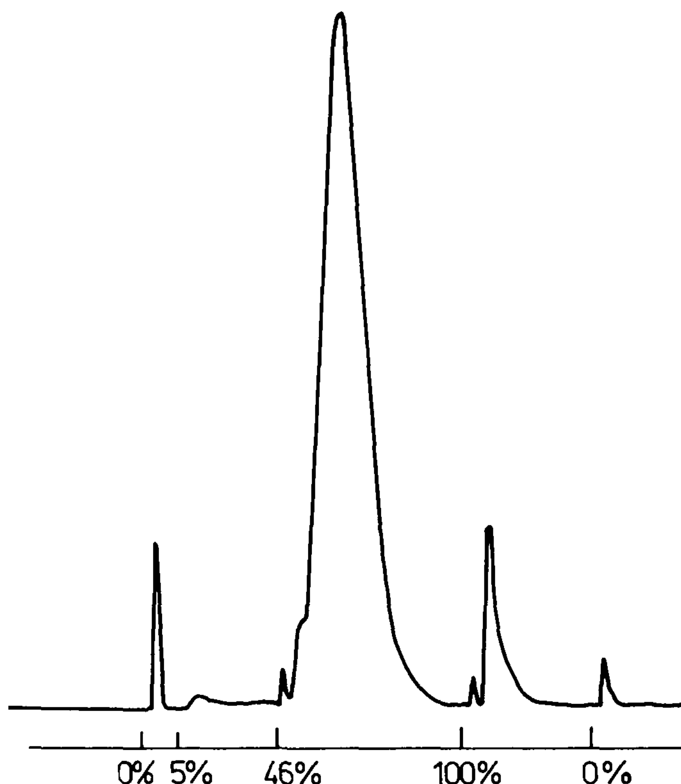


**Fig. 2.** Ion-exchange step gradient elution of iso-$\alpha$-acids with acetic acid in methanol/water. EBC-ASBC reference sample: iso-$\alpha$-I-x-1978-79.

The electronic integration gave a percentage to the peak areas or these were deduced by other means from the chromatogram. These percentages were converted into weights per 100 ml with the equation:

$$\text{isoacid, mg} = \left[\left(\frac{\text{area of isoacid peak, \%}}{\text{area of internal standard, \%}} + 0.028\right)\frac{1}{0.792}\right]12.42$$

If, as in a typical analysis, the percent peak area for isocohumulone is 13.55, for isohumulone 33.31, for isoadhumulone 8.98, and for the internal standard 28.28, then the total amount of iso-$\alpha$-acid is 7.95 + 18.90 + 5.42 = 32.27 mg of iso-$\alpha$-acid per 100 ml. The percentage iso-$\alpha$-acids in the extract is 32.27/135.7 × 100 = 23.78%.

**Comparative Methods for Iso-$\alpha$-Acids Analysis**

For comparison purposes, the direct photometry and a variation of IEC method were conducted in parallel on a number of samples.

*Direct Photometry.* Acidified beer was extracted with 2.5 times its volume of isooctane. The photometric value at 276 nm was used in the following equation:

$$\text{iso-}\alpha\text{-acids, mg/L} = \left(\frac{28.6 \times \text{volume of isooctane} \times E}{\text{volume of beer}}\right) - 5.8$$

where E is extinction (ie, absorbance).

*Ion-Exchange Analysis.* A modification of the Otter procedure (6) proposed by us to the EBC analysis committee[2] was followed. A glass column, 5 × 1 cm with a glass frit, was filled with Sephadex QAE 10–120 $\mu$m. About 1 mg of mixture containing iso-$\alpha$-acids in $CH_3OH$ was applied. The column was conditioned with methanol/water (80:20) and eluted with a step gradient of acetic acid in the methanol/water. $\beta$-Acids first and then $\alpha$-acids eluted at 5% HAC, unknown impurities at 15%, iso-$\alpha$-acids and hulupones at 46%, and all remaining material at 100%. After the 100% HAC flush, the column was reconditioned with methanol/water, 80:20. If only an iso-$\alpha$-acids determination was wanted, the 5% HAC flush was omitted.

The solvents, especially the acetic acid, were kept very pure.

The elutant rate was set at 200 ml/hr; the pressure was then about 2 $kg/cm^2$. Detection occurred at 275 nm, and the specific extinction of the iso-$\alpha$-acids under these conditions was 285.

Quantitiation of iso-$\alpha$-acids eluted at 46% HAC was based on photometry of the collected band brought to 50 ml in a measuring flask. A recorder trace of such an analysis for beer is shown in Fig. 2.

**Amounts Used**

Beer (250 ml) was acidified with 25 ml of 3*N* HCl and extracted with 625 ml of isooctane. Of the isooctane extract, 25 ml was used for HPLC analysis, 400 ml for step gradient ion-exchange analyses, and 5 ml for direct photometry. For ion exchange, the 400 ml was evaporated, the residue then dissolved in 15 ml methanol, and 5 ml of this was transferred to the column.

[2] M. Verzele, Communication to the Analysis Committee of EBC, March 1975.

## RESULTS AND DISCUSSION

Results for three major Western European lager beers are shown in Table II.

In the last years we have done many comparative analyses on beers and isomerized hop extracts. General conclusions are difficult to draw from the results; there seems to be least agreement between HPLC and direct photometry. Still, the results in Table II agree fairly well, except for the direct photometric value for Beer 3.

For isomerized extracts, the results differ much more, as is shown in Table III.

The very large differences for Samples 2 and 3 are explained partially by the appearance of small peaks just before and after isocohumulones. According to cochromatography experiments, this is most probably caused by allo-isomers (7).

To evaluate the bittering potential, the allo-isomer peaks must be included in the calculations by using the figures in parentheses in Table III.

Large differences still remain between the HPLC and IEC methods. For isomerized extracts 2 and 3 of Table III, the complex peak pattern in the beginning of the chromatogram is relatively more important. Perhaps the large deviations of Table III have to be correlated with the great variability observed for the utilization percentage of isomerized extracts. To us, it is obvious that only HPLC or, in other words, better analytical methods will bring an answer to this and to similar questions.


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