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# PREPARATIVE LIQUID CHROMATOGRAPHY OF HOP AND BEER BITTER ACIDS

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## SUMMARY

Methods and liquid chromatographic (LC) systems for the separation on a preparative scale (Prep-LC) of hop alpha acids and of beer iso-alpha acids were developed. The optimization of the selectivity factor for the *cis–trans* pairs of the iso-alpha acids was particularly studied. The highest values were achieved on reversed-phase packing materials with acetonitrile–water mixtures containing a relatively high proportion of water, to which magnesium salts are added, as eluent. Up to 400 mg of isomerized hop extract could be treated in one run on a laboratory Prep-LC column of dimensions 25 × 2.2 cm I.D. This application illustrates some critical points of Prep-LC, *viz.*, the need for a high selectivity value, the problem of detection at high concentrations and the difficulties of obtaining the separated compounds undegraded from the collected Prep-LC fractions.

## INTRODUCTION

In the beer brewing process, the three major hop alpha acids (humulone, cohumulone and adhumulone) are converted into six major beer-bittering iso-alpha acids (three *cis* forms and three *trans* forms). The large-scale separation of these six intensely bitter compounds is of interest for the evaluation of their individual contributions to the bitter taste of beer. We have used counter current distribution to isolate large amounts of the most prominent iso-alpha acids, but this is a very lengthy and costly procedure. Today, it is obvious to turn to liquid chromatography (LC) on a preparative scale (Prep-LC) for this purpose. Several LC systems for separating the iso-alpha acids have been described[1], but they are useless for preparative purposes because the selectivities are too low. Analytical LC of the iso-alpha acids does not aim at the highest possible selectivity between the indiviual compounds, but rather tries to obtain them separated from the matrix, preferably all together in a single peak[2]. This simplifies quantification, which is the major aim of the analytical LC of iso-alpha acids of beer and of isomerized hop extracts. Most analytical LC systems for determining iso-alpha acids use a reversed-phase octadecylated silica gel with a water–

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acetonitrile or methanol–phosphoric acid mixture as solvent. Such systems do not separate the *cis* and *trans* forms of the iso-alpha acids, or insufficiently so. They also do not separate *cis*- and *trans*-isohumulone from *cis*- and *trans*-isoadhumulone. They do separate *cis*- and *trans*-isocohumulone (as a single peak) from the other four iso-alpha acids, but for preparative LC purposes this is not sufficient.

We have evaluated many LC systems for the larger scale separation of beer bitter acids, initially with little success. Buffered silica gel as used by Schwarzenbach[3] and ourselves[1] gave problems with the recovery of the compounds from the column. Silica gel, derivatized with a wide variety of silanes, did not markedly improve the selectivity obtainable on octadecylated silica gel reversed-phase column packing materials. Increasing the selectivity value of the intended separation is, however, of such great importance for Prep-LC[4] that the search was continued, but now directed at the composition of the solvent. This was rewarded with a decisive improvement, obtained by adding magnesium salts to the reversed-phase eluent and by using a relatively large proportion of water.

## EXPERIMENTAL

The chromatograph was a Varian 5000 Series instrument with a Varian UV-50 detector (Varian, Walnut Creek, CA, U.S.A.). The injector was a Valco 7000 p.s.i. sample loop injector (Vici, Houston, TX, U.S.A) fitted with external loops of the required volume (10 $\mu$l to 2 ml). The columns (RSL, Eke, Belgium) were packed with 5-$\mu$m RoSil-$C_{18}$ (RSL) for analytical LC (25 × 0.46 cm I.D.) and with the same material of 10-$\mu$m size for Prep-LC (25 × 2.2 cm I.D.). The solvents were of LC quality. The amounts indicated are in millilitres. The phosphoric acid used had a content of 85%.

### *Optimization of selectivity by eluent and pH adjustment*

The iso-alpha acids are known to give chelated salts with bivalent cations. The influence of magnesium cations on their chromatographic separation, as revealed in this work, is therefore not surprising. We also evaluated other divalent cations such as beryllium, calcium, strontium and barium, but without much success. The last three cations have a similar effect to magnesium, but not so pronounced. Beryllium salts have the curious effect of causing extreme broadening of the iso-alpha acid peaks while leaving the alpha acid peaks unaffected. This was verified with pure *trans*-isohumulone. The reason for this effect of beryllium is not clear. Magnesium bromide, which is readily soluble in the eluents in question, poses the problem that the stainless-steel parts of the LC instrument are sensitive to bromide, and these parts have to be washed carefully after each series of chromatographic runs in which this salt was used. Magnesium sulphate is better in this respect, but it needs at least 45% water in the eluent system because of solubility problems. Magnesium acetate is also not acceptable for solubility reasons.

Optimization of the chromatographic conditions was achieved on analytical columns with *trans*- and *cis*-isohumulone as the probe pair of diastereoisomers to be separated. These are readily available by isomerization of pure humulone in brewing conditions. Without adding magnesium salts, with methanol in the usual concentration (10–30%) in the reversed-phase eluent, the *cis* isomer elutes first ($\alpha$ = 1.03),

whereas the opposite occurs with acetonitrile ($\alpha$ = 1.07). Therefore, we use a mixture of methanol and acetonitrile in the procedure for the determination of iso-alpha acids[2]. In this way the isomers are compressed together in a single peak. Chromatograms for the separation of *cis*- and *trans*-isohumulone with methanol or acetonitrile as the organic component in the eluent mixture and in proportions that are usual for reversed-phase LC are shown in Fig. 1. The selectivity factor of these separations is insufficient for Prep-LC. It can be increased significantly with acetonitrile by using a large proportion of water in the eluent. With a 50:50 composition of acetonitrile and water the selectivity factor increases to 1.13 but the retention times then become very long. The latter can be shortened again, while the selectivity factor is further increased, by lowering the pH and by adding magnesium salts to the eluent. This is partly illustrated in Fig. 2.

The best results are obtained at about pH 2 with 50% water in the eluent and with 0.1 *M* magnesium sulphate added. The chromatograms further show that the retention time of humulone (some residual humulone is always present in the isomerization mixtures) does not change on adding magnesium salts to the eluent, whereas it does for the *cis* and *trans* isomers (decreasing with higher magnesium salt concentration). The selectivity factor attained can be as high as 1.3, which indicates good Prep-LC possibilities. A preparative separation is shown in Fig. 3. Although the detection wavelength was 320 nm or far from the absorption maximum, the chromatogram shows that a still longer wavelength is indicated for the larger samples that the system obviously can handle.

In the same way, a mixture of *cis*- and *trans*-isocohumulone can be separated. This mixture can be obtained by isomerization of pure cohumulone. While humulone

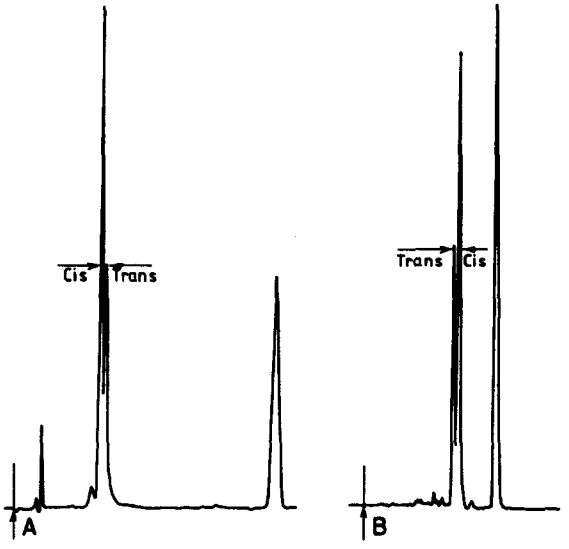


Fig. 1. LC of *cis*- and *trans*-isohumulone. Column, 25 × 0.46 cm I.D. packed with 5-$\mu$m RoSil-$C_{18}$. Reversed-phase eluent: (A) methanol–water (85:15)–0.25% phosphoric acid (selectivity factor, $\alpha$ = 1.03); (B) acetonitrile–water (70:30)–0.25% phosphoric acid ($\alpha$ = 1.07). Detection at 270 nm. Flow-rate, 1 ml/min. Note the reversal of elution order and the higher selectivity with the acetonitrile system. The third peak is unchanged humulone still present in the isomerization mixture.

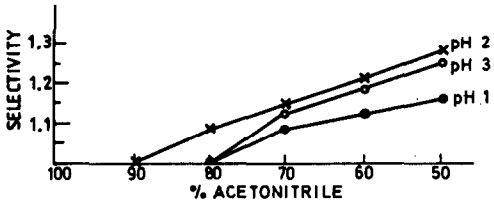


Fig. 2. Influence of pH and acetonitrile concentration on the selectivity factor for the *cis–trans*-isohumulone pair. Column, 25 × 0.46 cm I.D. packed with 5-μm RoSil-$C_{18}$. Detection at 270 nm. Flow-rate, 1 ml/min.

can be prepared by repeated crystallization of an alpha acid–*o*-phenylenediamine complex, pure cohumulone cannot be isolated in this way and is therefore much more difficult to obtain. A Prep-LC system for the separation of cohumulone from an alpha acids mixture was therefore first developed. Fig. 4 shows the separation of 400 mg of alpha acids on a 25 × 2.2 cm I.D. preparative LC column. This chromatography afforded 140 mg of cohumulone at each pass. This was repeated, sometimes more than ten times, until the desired amount of cohumulone was obtained. After isomerization, as explained above for humulone, the *cis*- and *trans*-isocohumulone were separated under identical conditions to those in Fig. 3. The retention times of these isocohumulones are shorter than those of the humulone-based analogues.

*Optimization of magnesium ion concentration*

A desirable possibility would be to separate the iso-alpha acids from a pre-isomerized hop extract, rather than to have to isolate pure humulone or pure cohumulone first. However, with the solvent system is Fig. 3 the peaks of *cis*-isocohumulone and *trans*-isohumulone coelute. This problem is solved by lowering the pH to 1

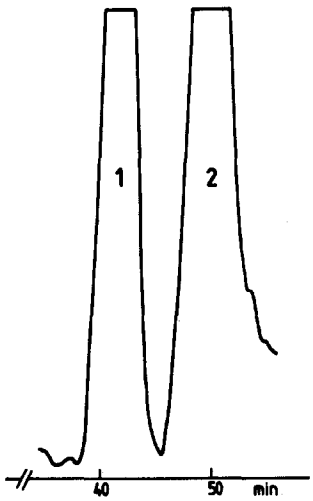


Fig. 3. Prep-LC of 40 mg of a mixture of *cis*- and *trans*-isohumulone. Column, 25 × 2.2 cm I.D. packed with 10-μm RoSil-$C_{18}$. Eluent: acetonitrile–water (50:50)-0.1 *M* $MgSO_4$ with phosphoric acid to pH 2. Flow-rate, 10 ml/min. Detection at 320 nm. Sample loop, 1 ml. With this acetonitrile-based solvent system, the first peak is *trans*-isohumulone and the second peak is *cis*-isohumulone.

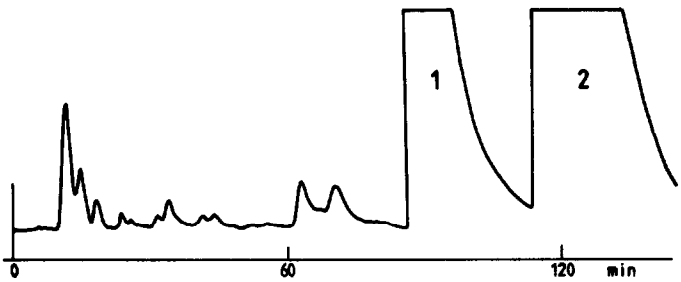


Fig. 4. Prep-LC of alpha acids for the isolation of cohumulone. Column, 25 × 2.2 cm I.D. packed with 10-μm RoSil-$C_{18}$. Eluent: acetonitrile–water–phosphoric acid (50:50:1). Flow-rate, 10 ml/min. Detection at 340 nm. Sample loop 1 ml. Sample size, 400 mg of alpha acids (hop extract) dissolved in the eluent. Elution sequence as in Fig. 3.

by adding more phosphoric acid. The selectivity for the *cis–trans* pairs is then lower, but the above-mentioned coelution is avoided. The phosphoric acid concentration must always be high, because at lower concentrations the peaks begin to tail and even disappear because of interaction with residual trace iron (iron traces are present in the column packing material and come from the whole LC system). The further problem of long retention times can be solved by adjusting the magnesium salt concentration. The retention times for the four most prominent iso-alpha acids as a function of the magnesium salt concentration are given in Table I.

The chromatograms in Table I are shown in Fig. 5. As a compromise, taking also the time element into consideration, the optimum concentration of magnesium salt was calculated to be 6 g/l. A chromatogram obtained under these conditions on a preparative column with an analytical sample size is shown in Fig. 6. For preparative purposes, a sample of 400 mg of isomerized hop extract, dissolved in 1 ml of eluent and containing about 70% iso-alpha acids, gave the chromatogram in Fig. 7. Fractions of 10 ml were collected and analysed on the analytical column in Fig. 5. From

TABLE I

RETENTION TIMES OF FOUR ISO-ALPHA ACIDS AS A FUNCTION OF MAGNESIUM SALT CONCENTRATION

Column, 15 × 0.46 cm I.D. packed with 5-μm RoSil-$C_{18}$. Eluent: acetonitrile- water-phosphoric acid (50:50:2). The magnesium salt, in this instance the sulphate, was added to the water; 12 g in 500 ml of water corresponds to 12 g/l in the LC eluent or to 0.1 *M*.

| *$MgSO_4$ (g)* | *Retention time (min)* | | | | *P (atm)* |
|---|---|---|---|---|---|
| | *trans-Isocohumulone* | *cis-Isocohumulone* | *trans-Isohumulone* | *cis-Isohumulone* | |
| 0 | 16.42 | 19.17 | 24.34 | 28.64 | 233 |
| 0.6 | 16.86 | 19.82 | 25.00 | 28.79 | 235 |
| 1.2 | 16.01 | 19.06 | 23.56 | 27.38 | 235 |
| 2.4 | 14.44 | 17.43 | 21.03 | 24.77 | 236 |
| 4.8 | 12.55 | 15.50 | 18.08 | 21.76 | 240 |
| 7.2 | 11.35 | 14.23 | 16.19 | 19.75 | 246 |
| 12 | 9.92 | 12.61 | 13.96 | 17.25 | 261 |

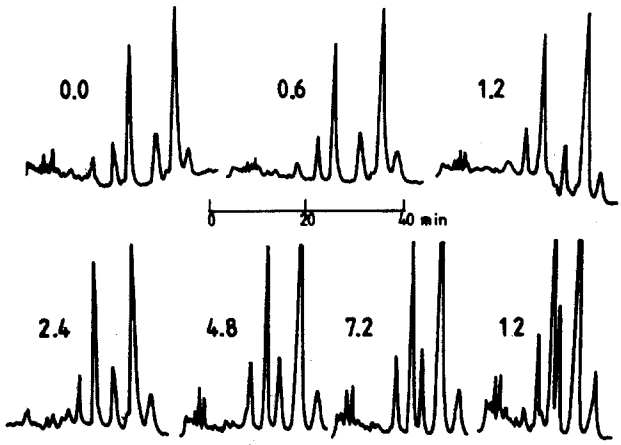


Fig. 5. Analytical separations of isomerized hop extract as a function of the amount magnesium sulphate (g/l) added. Column, 15 × 0.46 cm I.D. packed with 5-$\mu$m RoSil-$C_{18}$. Eluent: acetonitrile–water–phosphoric acid (50:50:2). Sequence of major peaks: *trans*-isocohumulone, *cis*-isocohumulone, *trans*-isohumulone, *cis*-isohumulone + *trans*-isoadhumulone, *cis*-isoadhumulone.

the marked parts in Fig. 7, iso-alpha acids with over 95% purity could be isolated. These results illustrate that the separation in Fig. 7 is better than the chromatographic trace indicates. UV detection is obviously not optimal for Prep-LC, as has been reported previously[5]. An insensitive linear detector seems to be desirable for Prep-LC. Results of the determination of the individual iso-alpha acids content of the sample and of the actual amounts obtained from Fig. 7 are given in Table II.

A problem with working with iso-alpha acids is their instability. They cannot be kept unchanged for more than a few days, even in a freezer. This is a most frustrating situation, as it implies that reference iso-alpha acids (*e.g.*, *trans*-isohumulone) have to be repurified every time they are needed.

One purpose of this work was to isolate pure iso-alpha acids for organoleptic studies. This was achieved for the *cis*- and *trans*-isohumulones and -isocohumulones. The concentration of *cis*- and *trans*-isoadhumulones in the mixtures was too low for successful isolation.

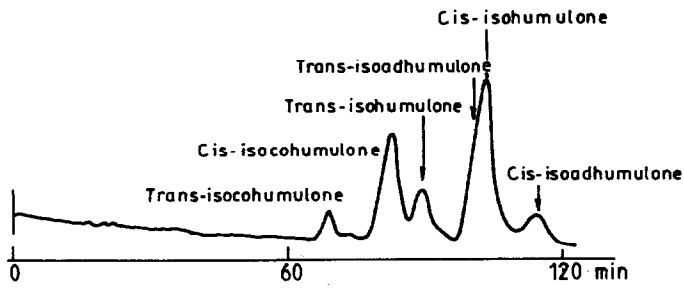


Fig. 6. Prep-LC of isomerized hop extract. Column, 25 × 2.2 cm I.D. Eluent: acetonitrile–water–phosphoric acid (50:50:2) + 6 g/l $MgSO_4$. Flow-rate, 10 ml/min. Sample size, 1 mg of isomerized extract in 10 $\mu$l of eluent. Detection at 270 nm. Elution sequence as in Fig. 5.

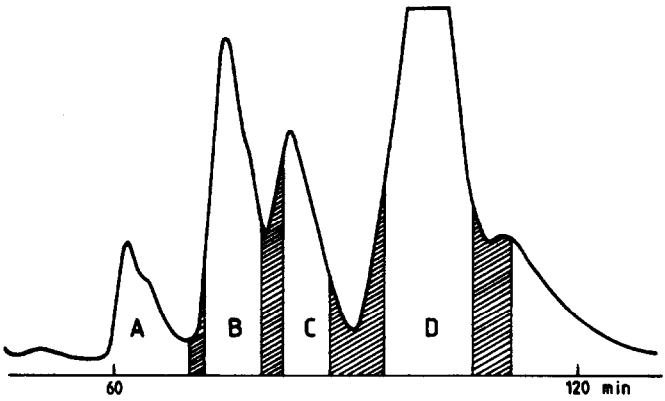


Fig. 7. Prep-LC of isomerized hop extract. Conditions as in Fig. 6 except the sample size, which was about 400 mg of isomerized hop extract in 1 ml of eluent. Detection at 320 nm. The areas marked A, B, C and D gave the amounts as indicated in Table II in each run.

Preliminary results indicate that the *cis* compounds are slightly more bitter than the *trans* compounds. There are also slight taste differences. It was very difficult to obtain the separated compounds unchanged and uncontaminated for a taste panel. Oxidation, degradation and contamination on working up collected Prep-LC fractions are major concerns. Fractions from several Prep-LC runs, collected over several days, are worked up together. Rechromatography at the end is always necessary. The finally collected solution, which is a reversed-phase solvent mixture containing salts and phosphoric acid, can obviously not be evaporated as such to obtain the desired iso-alpha acid. To remove the inorganic involatile chemicals, the final chromatographic solution has to be extracted with, *e.g.*, hexane. Removing this hexane completely without damaging the compounds is not easy. Work on these aspects and problems is continuing.

TABLE II

RECOVERY OF INDIVIDUAL ISO-ALPHA ACIDS OBTAINED FROM ONE PREP-LC SEPARATION IN FIG. 7

| *Iso-alpha acid* | *Amount injected (mg)* | *Amount obtained (mg)* |
|---|---|---|
| *trans*-Isocohumulone (A) | 21 | 12 |
| *cis*-Isocohumulone (B) | 75 | 33 |
| *trans*-Isohumulone (C) | 40 | 7 |
| *cis*-Isohumulone (D) | 120 | 33 |

## CONCLUSION

Optimized chromatographic systems have been described for the preparative separation of some hop and beer bitter acids on an octadecylated silica gel reversed-phase column. For hop alpha acids this packing material can handle about 6 mg of alpha acid mixture per gram. For beer iso-alpha acids, a relatively high water content and magnesium ions in the reversed-phase eluent lead to a selectivity factor that can be as high as 1.3 for *cis*- and *trans*-isohumulone. The column capacity is lower for mixtures of the iso-alpha acids (up to 4 mg/g).

Working up the collected fractions from a Prep-LC run without degrading the compounds is an underestimated aspect of Prep-LC.

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