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Development and validation of a high-performance liquid chromatography system for the analysis of hop bitter acids

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Abstract

An isocratic HPLC system is developed for the baseline separation of the six main hop bitter acids on a RP C_{18} column within 25 min. The eluent contains 0.05 *M* triethanolamine in methanol–water (65:35, v/v), the pH is set at 6.85 with phosphoric acid. The contribution of each of the components of the eluent on retention and resolution is investigated as well as the linearity for determination of the six main bitter acids.

1. Introduction

During the beer brewing process, hop (*Humulus lupulus* L.) extracts are added to the wort which is subsequently boiled. Part of the bitter acids in the extract are thus transformed into the iso-α-acids, which largely contribute to the specific bitter taste of beer. Consequently, the analysis of the bitter acids in hop extract is important for quality control. In our laboratory the biosynthesis of the bitter acids in hop is studied [1,2]. Hence a simple and reliable method for their analysis is required.

Hop extracts are prepared from dried hop cones, the fullgrown female inflorescence of hop. These cones contain a high concentration of the desired bitter acids. The acids are obtained either by extraction with organic solvents (ethanol, hexane, isooctane) or either by liquid or supercritical fluid extraction with carbon dioxide. The main compounds of hop extract are the bitter acids of which the analogous α- and β-acids are the most important. There are several homologues of both types (see Fig. 1). The composition of the hop extract depends strongly on the extraction method and conditions. The normal-, co- and ad-homologues are the six main bitter acids; of these humulone is in most extracts the compound with the highest concentration (around 25%) and adlupulone the compound with the lowest concentration (around 3%). The other acids are present as minor compounds.

The analysis of the bitter acids is difficult [3]. Due to their instability, gas chromatography has not yet been successful. By micellar electrokinetic chromatography [4,5] and micro column liquid chromatography [6] the main six bitter acids can be separated. With "classical" HPLC so far only with a gradient system separation of these acids could be achieved [7–9]. Best results were obtained by David *et al.* [9] who used octyltrimethylammonium hydroxide in the eluent; with this system the objective of

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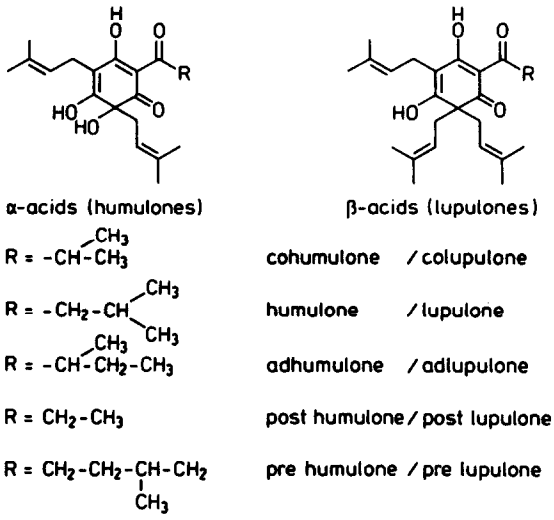

Fig. 1. Structural formulae of humulones and lupulones. In this paper the pre-fix "normal" is sometimes used for humulone and lupulone to distinguish these compounds from their homologues.

baseline separation has been met. The use of the above described methods either involves special equipment or the use of large amounts of HPLC eluent. Hann [10] described an isocratic HPLC system for the fast analysis of bitter acids using an ion-pairing reagent containing eluent and a column temperature of 40°C. In this system the β-acids are separated, but humulone and cohumulone co-elute. For routine analysis, HPLC systems consisting of methanol or acetonitrile, water and phosphoric acid, combined with an RP C_{18} column specially designed for bitter acid analysis, are mostly used [11]. In these fast, isocratic systems, however, the normal- and ad-homologues of the α- and β-acids co-elute. The resolution between these two pairs is the problem in most separation methods.

In this paper an HPLC system for baseline separation of the six main bitter acids is described. The system is isocratic, fast, stable, linear for quantitative analysis and requires only standard HPLC instrumentation and column materials. The influence of the components of the eluent on retention and resolution is described and linearity for quantitative analysis and stability are investigated. Moreover a comparison of the performance of six columns, comparing selectivity, resolution and retention times, is made.

2. Experimental

2.1. Chromatography

The chromatographic system used consisted of an LKB pump, type 2150 (Bromma, Sweden), an autosampler, type WISP 710B (Waters), a photodiode array detector type 990 (Waters) and a Waters 5200 printer plotter. Columns used were: Polymer PL RP-S 5 μm, pore type 100 Å, 150 × 4.6 mm (Polymer Labs., Zeist, Netherlands); Chromsep Microspher C_{18}, 3 μm, 100 × 4.6 mm (Chrompack, Bergen op Zoom, Netherlands); Shandon Hypersil ODS 5 μm, 250 × 4.6 mm (Life Sciences International, Zeist, Netherlands); Phenomenex Hypersil 5 C_{18}, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA); Macherey–Nagel Nucleosil 5 C_{18} Hop, 250 × 4 mm (Macherey–Nagel, Düren, Germany); and Phenomenex Ultracarb 5 ODS (30), 150 × 4.6 mm.

Mobile phases were filtered over a 0.45-μm nylon membrane filter (type NL 17, Schleicher & Schüll, Dassel, Germany) and degassed by vacuum.

2.2. Chemicals

Hexadecyltrimethylammonium bromide (CTAB, Merck–Schuchardt, Hohenbrunn near Munich, Germany, for synthesis) and triethanolamine (Sigma, St. Louis, MO, USA) were used for the preparation of the mobile phases. Organic solvents were distilled before use. Hop carbon dioxide extract was a kind gift of Mr. L.C. Verhagen (Heineken Brewery, Zoeterwoude, Netherlands). This extract contained 45.4% α-acids and 21.2% β-acids. Humulone, cohumulone and adhumulone, used as test and reference compounds, were isolated from hop extract by means of centrifugal partition chromatography, as described earlier [12]. The identity of these compounds was confirmed by NMR, UV and elution order in earlier published works [12]. The peaks for the β-acids were

identified using the UV spectra and the data from earlier published works, in relation to elution order and relative amounts present.

2.3. Sample preparation

Hop extract was dissolved in methanol, centrifuged for 2 min at 15 500 *g* (14 000 rpm) in an Eppendorf centrifuge and diluted with methanol to the desired concentration.

3. Results and discussion

In our attempts to develop an isocratic HPLC system, in which at least the six main bitter acids (Fig. 1, the co-, ad- and normal-homologues) could be analyzed, we took as a starting point the gradient system as described by David *et al.* [9]. This system consists of a Hypersil C_{18} column and a gradient of octyltrimethylammonium hydroxide and water in 0.5% H_3PO_4 in acetonitrile. We used the Chromsep Microspher C_{18} column and CTAB as the ion-pairing reagent in the eluent. Many modifications of the mobile phase composition, with respect to CTAB molarity and water–acetonitrile ratio did not yield a system in which our demands for a fast and isocratic analysis for all six bitter acids could be fulfilled.

Recently we described the preparative separation, isolation and purification of the three main α-acids, by means of centrifugal partition chromatography (CPC) [12]. In this liquid–liquid chromatography technique triethanolamine proved to be very efficient for the resolution of the three α-acids. Hence, we tested this compound as an ion-pairing reagent for HPLC eluents replacing CTAB. Two organic solvents (methanol and acetonitrile) in several concentrations in water at different pH values were assayed to obtain sufficient resolution and suitable retention. Using the Chromsep Microspher C_{18} column a complete separation for the α-acids was obtained with the eluent parameters 0.1 *M* triethanolamine in methanol–water (56:44, v/v), pH set at 6.5 by means of phosphoric acid. This set was used as a starting point to compare the performance of several columns for this particular separation.

3.1. Columns

We tested the six columns as listed in the experimental section. The Polymer PL RP-S, was tested because of its compatibility with basic eluents (to pH 13). From our CPC experiments it was known already that in liquid–liquid chromatography the β-acids require a rather high pH value of the mobile phase for transport. For the α-acids a pH of 8.4 of the CPC mobile phase proved to be optimal for resolution and retention, while this value for the β-acids was about 10 (unpublished results). Retention of the bitter acids on this polymeric column proved to be very strong. Acetonitrile was required as the organic modifier because of its higher eluting force. The retention times were unacceptably long using methanol. For this column, eluents varying in pH, water–acetonitrile ratio, triethanolamine molarity and sodium phosphate buffer concentration, were tested. The chosen eluent was 0.05 *M* triethanolamine and 0.01 *M* sodium phosphate in water–acetonitrile (5:3, v/v). The pH was set at 11.1. A chromatogram obtained from the injection of the hop extract, using this eluent, is shown in Fig. 2. A qualitatively good separation was obtained; however the baseline

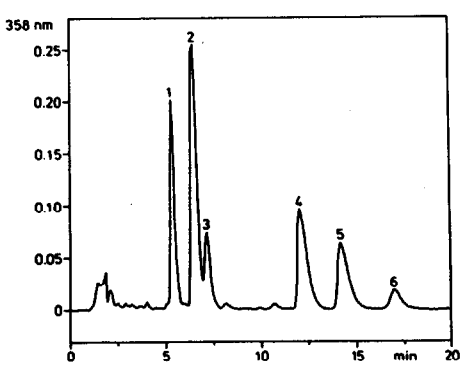

Fig. 2. Chromatogram of hop extract. Column: Polymer PL RP-S, pore type 100 Å, 150 × 4.6 mm. Eluent: 0.05 *M* triethanolamine and 0.01 *M* Na_2HPO_4 in water–acetonitrile (5:3, v/v), pH 11.1. Flow-rate 1.00 ml/min. Peaks: 1 = cohumulone; 2 = humulone; 3 = adhumulone; 4 = colupulone; 5 = lupulone; 6 = adlupulone.

separation of humulone and adhumulone could not be achieved under isocratic conditions.

The other five columns tested were all of the silica-based C_{18} type. Of these the Macherey–Nagel column was taken in the test only during the end phase of the experiments (see section 3.7). No eluent optimization experiments were done with this column. Apart from the Chromsep Microspher C_{18} column, which is specially recommended for bitter acid analysis, two other standard reversed phase columns (Shandon Hypersil ODS and Phenomenex Hypersil C_{18}) were tested. The extra endcapped Phenomenex Ultracarb ODS, which has a high carbon load, was also tested. For each of these four columns the eluent parameters (methanol–water ratio, pH, triethanolamine molarity) were optimized. While testing these columns only the α-acids were injected.

Good resolution, similar performance and selectivity were obtained with the Chromsep Microspher C_{18}, Shandon Hypersil ODS and Phenomenex Hypersil C_{18} columns. The (optimized) eluent parameters were 0.05 *M* triethanolamine and pH 6.85 while the methanol–water ratio was 56:44 (v/v) for the Chromsep column and 65:35 (v/v) for the other two. The use of the Phenomenex Ultracarb resulted in high backpressure. Consequently, the flow-rate had to be lowered resulting in an increase of the retention times. In addition, in none of our optimization experiments with this column, a resolution between adhumulone and humulone could be obtained which could compete with the resolution achieved with the other three C_{18} columns.

Complete resolution for all six bitter acids and a run time of less than 25 min was obtained using the Phenomenex Hypersil C_{18} column and the eluent optimized for the separation of the α-acids [0.05 *M* triethanolamine in methanol–water (65:35, v/v)], brought to pH 6.85 with 85% H_3PO_4). The resulting chromatogram of the hop CO_2 extract and the UV photodiode array spectra of the bitter acids are presented in Fig. 3A and B. Similar separation of all six bitter acids was achieved with the Shandon Hypersil ODS column. The Macherey–Nagel column, specially treated and recommended for hop bitter acid analysis, gave a high backpressure; consequently the flow-rate had to be lowered.

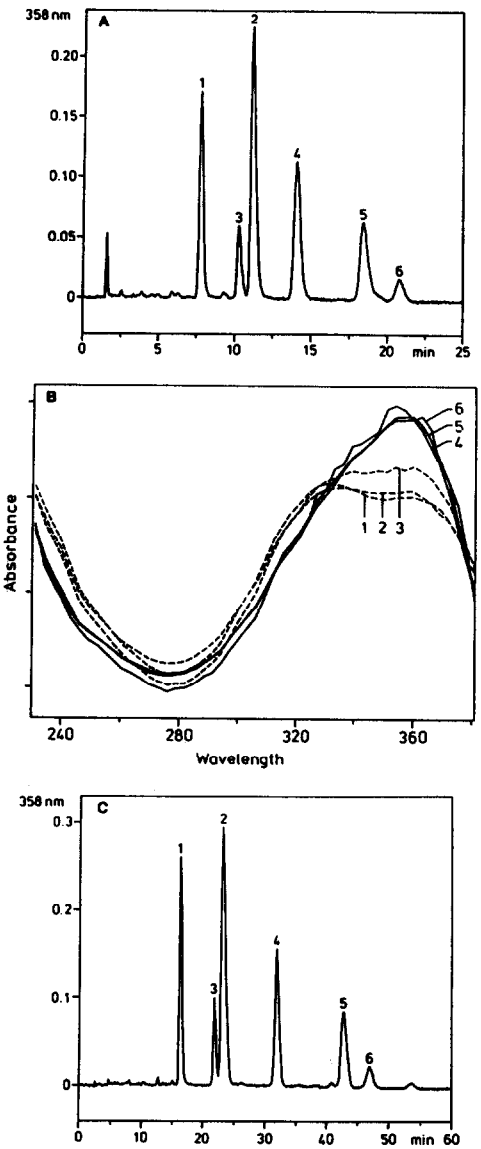

Fig. 3. (A) Chromatogram of hop extract. Column: Phenomenex Hypersil 5 C_{18}, 250 × 4.6 mm; eluent: 0.05 *M* triethanolamine in methanol–water (65:35, v/v), pH brought to 6.85 with H_3PO_4; flow-rate 1.75 ml/min; peak numbers as in Fig. 2. (B) UV photodiode array spectra (wavelength in nm) of peaks 1–6 of (A). (C) Chromatogram of hop extract. Column: Macherey–Nagel 5 C_{18} Hop, 250 × 4 mm; eluent and peak numbers as in (A); flow-rate 1.00 ml/min.

Moreover the capacity factors for this column are higher. This resulted in a run time of at least 55 min. The chromatogram obtained with this column is presented in Fig. 3C.

The resolution between adhumulone and humulone is less than with the Phenomenex and Shandon ODS columns; on the other hand the distance between the last α-acid and the first β-acid peak is such that a slightly lower methanol–water ratio, combined with a slightly higher pH of the eluent would probably solve this problem. (See the following paragraphs for the influence of eluent parameters on retention). The chromatographic parameters (capacity factors, resolution, asymmetry and plate numbers) of the Macherey–Nagel column and the Phenomenex column are discussed in the paragraph dealing with these parameters.

There proved to be no significant difference in the ratio peak area: amount of sample injected for the three columns (Phenomenex C_{18}, Shandon ODS and Macherey–Nagel C_{18} Hop). Bitter acids form ligands with Fe ions and the Macherey–Nagel column material is specially treated to remove these ions. When the eluent described above is used, there seems to be no need for the use of special treated columns, as Fe ions are masked by triethanolamine. No evidence was found for absorption of bitter acids to the Phenomenex column.

3.2. Influence of the eluent parameters

The contribution of each of the components of the eluent to resolution and retention was investigated. The Phenomenex Hypersil C_{18} column, an arbitrary choice out of the three C_{18} columns, was used for this study. For these experiments hop extract solutions, containing α- and β-acids were injected.

3.3. Organic solvent

A notable difference in elution pattern was obtained using methanol and acetonitrile. Using acetonitrile the sequence is co-, normal-, ad- for both α- and β-acids; however, with methanol humulone and adhumulone interchange, while lupulone still elutes before adlupulone. For further discussion about the selectivity see below, under pH. Using acetonitrile, adhumulone, which is present in lower concentrations in the extracts, elutes immediately after humulone, hampering its detection.

Fig. 4 shows the relation between methanol concentration and capacity factors. As expected, the higher concentrations of organic solvent in the eluent resulted in shorter retention times. However, these conditions resulted in less separation between the normal- and ad-isomers. The concentration of methanol in the eluent strongly influenced the retention times. Complete resolution (≥ 1.5) between adhumulone and humulone was obtained at methanol–water ratios $\leq 65{:}35$.

3.4. Triethanolamine concentration

The relation between triethanolamine concentration and capacity factors is presented in Fig.

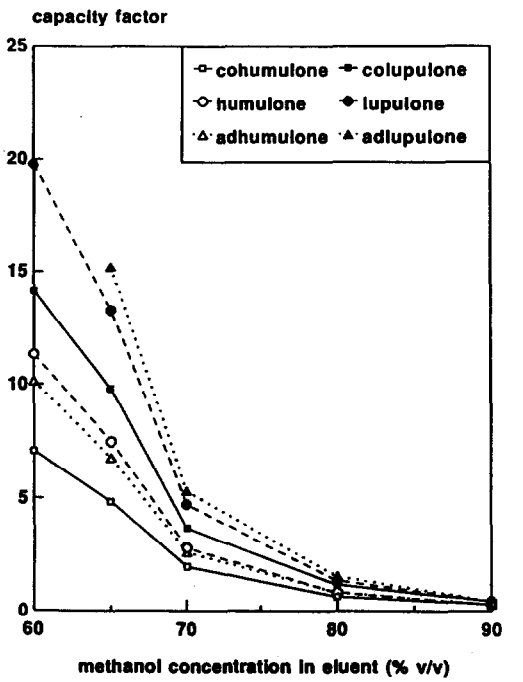

Fig. 4. Capacity factors as a function of methanol concentration in the eluent. Column: Phenomenex Hypersil 5 C_{18}. Eluent: 0.05 *M* triethanolamine in methanol–water, pH brought to 6.8 with H_3PO_4.

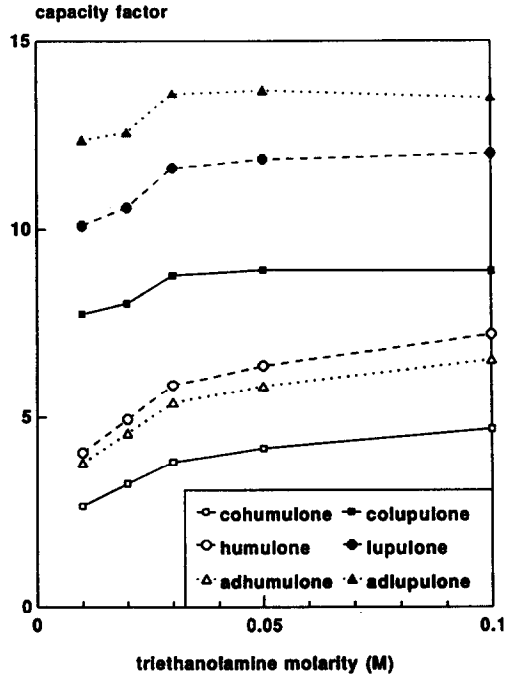

Fig. 5. Capacity factors as a function of triethanolamine concentration in the eluent. Column: Phenomenex Hypersil 5 C_{18}. Eluent: triethanolamine in methanol–water (65:35, v/v), pH brought to 6.85 with 85% (v/v) H_3PO_4.

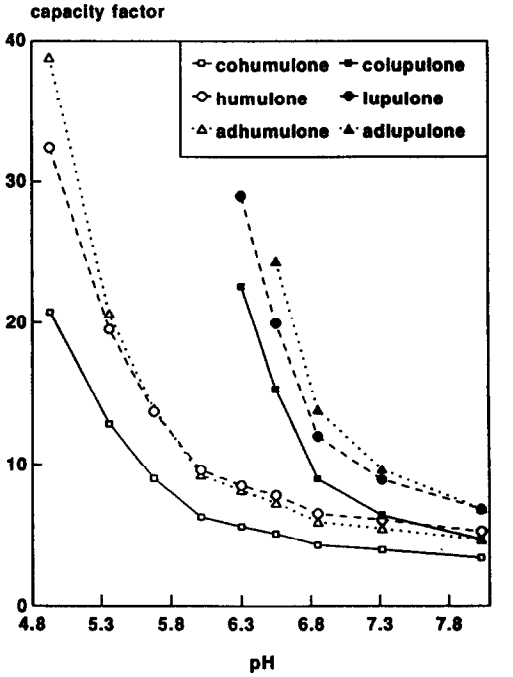

Fig. 6. Capacity factors as a function of pH of the eluent. Column: Phenomenex Hypersil 5 C_{18}. Eluent: 0.05 *M* triethanolamine in methanol–water (65:35, v/v), pH set with 85% (v/v) H_3PO_4; for pH values below 6.1 the eluent was buffered with 0.02 *M* sodium phosphate.

5. The shape of the curve suggests an ion-pair mechanism. Resolution between adhumulone and humulone is ≥ 1.4 at triethanolamine concentrations ≥ 0.05 *M*.

3.5. Influence of pH value

Fig. 6 shows the relation between capacity factors and eluent pH in the range 4.9–8.0. The bend in the graph at pH ± 6.0 for the α-acids and pH 7.2 for the β-acids probably indicates the transition of adsorption chromatography at lower pH to ion-pair retention mechanism at higher pH. It is clear that the retention of the bitter acids by adsorption is stronger than by the ion-pair mechanism. Noteworthy is the loss of resolution between adhumulone and humulone at pH 6.0 and the inversion of elution sequence at pH lower than 5.5. The ion-pairing mechanism, predominant at pH values above or near the pK_a value of an acid compound, seems to retain humulone more than adhumulone. At lower pH values, retention will mostly be regulated by adsorption chromatography, resulting in elution of adhumulone after humulone. At pH values higher than 8.0 the same inversion for ad-lupulone and lupulone would probably have occurred. This was not tested in order to avoid deterioration of the column material. As stated before the Polymer PL RP-S column can be used until pH 13, but with this column the inversion phenomenon could not be studied. The reason is that, using this column, methanol is not suitable for the analysis of bitter acids because of its lower eluting force while with acetonitrile both ad-isomers elute after the normal-isomer.

3.6. Sodium phosphate addition for higher buffer capacity

The buffer capacity of triethanolamine is low at pH 6.85. Hence, sodium phosphate was added to the eluent in concentrations up to 0.1 *M*

phosphate at pH 6.85 to increase the buffer capacity. For all compounds retention increased, although the effect on the α-acids was stronger than on the β-acids. This resulted in incomplete resolution between the last α-acid (humulone) and the first β-acid (lupulone) at phosphate concentrations >0.02 *M*. The increase of ionic strength of the eluent through addition of phosphate buffer apparently changes the selectivity of α- and β-acids. Peak broadening or sharpening resulting from the higher ionic strength, as can be measured through the peak height/area ratio, could not be detected.

Therefore the regular pH control of the eluent is the preferred method over pH stabilization by means of sodium phosphate buffer. If necessary the pH can be readjusted to the desired value by means of H_3PO_4 or triethanolamine.

3.7. Chromatographic parameters, detection limits and linearity

The HPLC systems, as described in Fig. 3A and C, were used for the determination of chromatographic parameters, detection limits and linearity for quantitative analysis.

For both the Phenomenex and the Macherey–Nagel column the chromatographic parameters: capacity factors, resolution, asymmetry factors and plate numbers were measured; the data are presented in Table 1. Selectivity and signal to noise ratio are nearly the same for both columns. Despite the lower asymmetry factor and the higher plate number of the Macherey–Nagel column, the gain of time achieved with the Phenomenex is such that this column is preferred. It can be expected that any standard RP C_{18} column will give results, similar to those obtained with the Phenomenex C_{18}.

Detection limits were determined on the Phenomenex column after injection of a known amount of hop extract of which the total concentrations of α-acids and β-acids were known. Assuming that the three α-acids have identical molar extinction coefficients and that the same is valid for the three β-acids, the absolute amounts present in the injection volume were calculated. The detection limit was defined as the amount giving a peak height of two times the noise level,

Table 1
Chromatographic parameters for two columns and the HPLC eluent, described in the text

	Capacity factor[a]	Resolution	Asymmetry	Plate number[b]
Using the Phenomenex C_{18} column				
Cohumulone	3.96	>2	1.64	4700
Adhumulone	5.55	1.42	1.50	5700
Humulone	6.09	>2	2.05	4700
Colupulone	7.93	>2	1.37	5800
Lupulone	10.70	2.28	1.66	6200
Adlupulone	12.18		1.26	6900
Using the Macherey–Nagel column				
Cohumulone	5.63	>2	2.16	7400
Adhumulone	7.88	1.09	1.46	10300
Humulone	8.39	>2	2.58	5200
Colupulone	11.97	>2	1.29	8900
Lupulone	16.39	2.18	1.25	9200
Adlupulone	18.06		1.15	9700

[a] Calculated as $(t_R - t_0)/t_0$ in which t_R is the retention time of the compound and t_0 the time of the first "peak" appearing in each chromatogram; t_0 was 1.60 min at flow-rate 1.75 ml/min for the Phenomenex column and 2.46 min at flow-rate 1.00 ml/min for the Macherey–Nagel column.

[b] Plate number calculated as $N = 4(t_R/2\sigma)^2$.

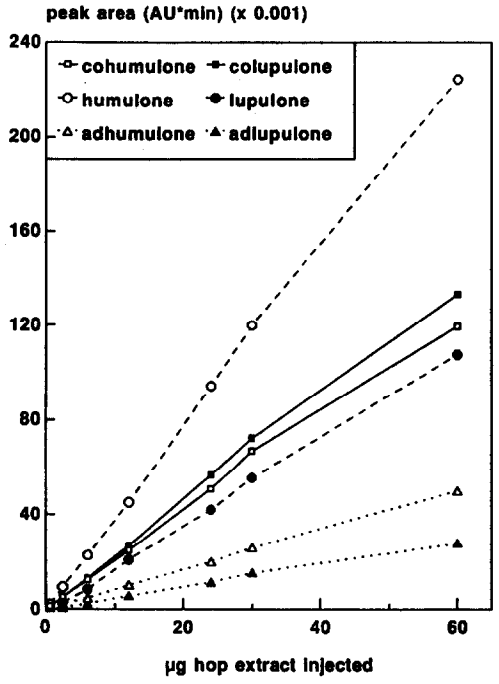

Fig. 7. Peak areas as a function of injected amount of hop extract. Column and eluent as in Fig. 3A. Detection wavelength 358 nm.

measured at the absorbance maximum (325 nm for the α-acids; 358 nm for the β-acids). Detection limits of 25, 20 and 24 ng were measured for humulone, cohumulone and adhumulone respectively, and 46, 33 and 41 ng for the corresponding lupulones.

The relationship between peak area and amount injected was investigated by injecting 20 μl hop extract solutions. The amounts injected were in the range of 600 ng–60 μg of hop extract. All concentrations were injected in triplicate. As is clear from the data in Fig. 7 the relation can be considered to be linear over the investigated x range.

3.8. Comparison of the bitter acid profile of two hop extracts

Apart from the hop carbon dioxide extract, as used for all experiments, a dichloromethane extract of a wild variety of *Humulus lupulus* L., as grown in our own garden, was analyzed. The bitter acid profile of both extracts are compared in Table 2. The table illustrates the obvious variation between hop extracts of different origin. Though the difference in extraction method may account for part of the difference in the α-acid/β-acid ratios, it is not to be expected that this is also valid for the variation between the co-/normal- and ad-/normal- ratios of both α-acids and β-acids.

4. Conclusions

Fast and routinely quantitative analysis of the six main bitter acids in hop extract is possible with the isocratic HPLC system: 0.05 *M* triethanolamine in methanol–water (65:35, v/v), brought to pH 6.85 by means of H_3PO_4, combined with a C_{18} column, such as the Phenomenex Hypersil 5 C_{18} and the Shandon ODS.

5. Acknowledgements

We wish to thank Mr. L.C. Verhagen for the kind gift of hop extracts, Polymer Laboratories for the supply of the polymeric column during

Table 2
Relative amounts of the main bitter acids in hop extracts as determined by HPLC peak area (358 nm) ratios

	Ratio cohumulone/ humulone	Ratio adhumulone/ humulone	Ratio colupulone/ lupulone	Ratio adlupulone/ lupulone	Ratio α-acids/ β-acids
Hop CO_2 extract	0.55	0.22	1.34	0.27	1.5
Wild variety	0.31	0.18	0.80	0.18	0.63

the test period and Dr. J. Carrier for reading the manuscript.

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