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Preparative separation and isolation of three α bitter acids from hop, *Humulus lupulus* L., by centrifugal partition chromatography

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Abstract

Centrifugal partition chromatography was used for the preparative separation of bitter acids from a crude supercritical carbon dioxide extract of hop cones. The main α-acids, humulone, cohumulone and adhumulone were obtained pure in one chromatographic run with the system toluene–0.1 *M* triethanolamine · HCl pH 8.4 in water. The two-phase system was optimized for pH and the effect of ethylene glycol on the separation was investigated.

1. Introduction

The bitter taste of beer is caused by the presence of iso-α-acids, which during the brewing process are formed from the α-acids, present in the hop extracts. The extracts of hop cones, the female inflorescence of *Humulus lupulus* L., also contain the closely related β-acids. The main α-acids are humulone, cohumulone and adhumulone; the corresponding main β-acids are lupulone, colupulone and adlupulone (Fig. 1). In our studies on the biosynthesis of bitter acids in hop [1], we need relatively large amounts of the pure compounds. Also for brewery experiments on the taste of beer, the pure compounds are useful, in order to estimate the individual contribution of each bitter acid to final taste and foam formation. Despite the obvious need for these compounds they are not commercially available. This is caused on the one hand by their instability, on the other by the difficulty to separate these compounds.

For the isolation and purification of natural

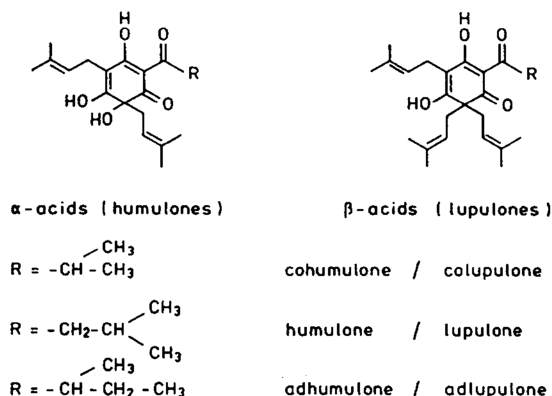

Fig. 1. Chemical structures of main hop bitter acids.

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products liquid–liquid chromatography is often the method of choice; it is a gentle method without the risk of decomposition or irreversible adsorption of the often unstable compounds by a solid stationary phase. Particularly in recent years counter-current chromatography (CCC) has been shown to be such an efficient liquid–liquid chromatographic method for the purification of natural products from complex plant extracts [2–4].

For the isolation of hop bitter acids two solvent systems for CCC have been described. Fischer *et al.* [5] used the non-aqueous system hexane–*tert.*-butyl methyl ether–acetonitrile (10:1:10) for small-scale separation (circa 24 mg) of the bitter principles from a crude carbon dioxide hop extract in an Ito multi-layer coil separator extractor. The bitter acids eluted as three peaks: the ascending side of the first peak contained cohumulone, while humulone and adhumulone eluted as the rest of the peak. The second and third peak contained the corresponding β-acids. In their experiments, replacement of the ether modifier with dichloromethane (13:3:7) increased the number of theoretical plates for four test compounds.

The second system described in literature for the separation of the three major α-acids consisted of benzene as upper phase and an aqueous buffer solution pH 8.4 (triethanolamine, hydrochloric acid and 25% ethylene glycol) as lower phase [6]. The separation was achieved by classical counter-current distribution, a tedious and time-consuming method.

In our attempts to isolate and purify the hop bitter acids we used centrifugal partition chromatography (CPC). In case of CPC the stationary phase is retained in the column by a centrifugal force, while the driving force for the movement of the mobile phase is a pump, allowing much shorter run times than in the more classic CCC methods such as the Craig type of apparatus and droplet CCC, in which the stationary phase is retained by gravity. The risk of decomposition of labile compounds is thus reduced considerably.

In this paper we report the successful purification of the main α-acids from a crude supercritical carbon dioxide hop extract. Based on the two-phase solvent systems which have been reported in literature for the counter-current separation of the hop bitter acids [5,6] systems were developed suited for a rapid separation of the bitter acids by CPC. Special attention was paid to the physical and chemical properties of the solvent systems and their influence on selectivity and efficiency of CCC.

2. Experimental

2.1. Hop extract

Crude supercritical carbon dioxide hop extract was obtained from Mr. L.C. Verhagen (Heineken Brewery, Zoeterwoude, Netherlands)

2.2. HPLC

System A

System A consisted of the following. Pump: type 2150 LKB (Bromma, Sweden); injector: Rheodyne, type 7125, equipped with a 10-μl loop; precolumn: 20×2 mm I.D., Uptight C-130B (Upchurch Scientific, Oak Harbor, WA, USA), packed with LiChrosorb RP-18, particle size 5 μm (Merck, Darmstadt, Germany); column: 250×4.6 mm I.D., LiChroma Rosil $C_{18}D$, particle size 5 μm (Alltech, Breda, Netherlands) (the column was always used at room temperature); detector: variable-wavelength monitor, type 2151 LKB; integrator: Shimadzu type CR-501; eluent: methanol–water–85% phosphoric acid (85:17:0.25, v/v/v), filtered over a 0.45-μm nylon membrane filter (type NL17, Schleicher & Schüll, Dassel, Germany) and degassed by means of helium.

With flow-rate 1.50 ml min^{-1} the pressure was 180 bar and the retention times were 7.6 min (cohumulone), 9.2 min (humulone and adhumulone), 12.7 min (colupulone) and 15.8 min (lupulone and adlupulone).

System B

System B is a modification of a gradient system described by David *et al.* [7] and enables separation of humulone and adhumulone. Pump:

type 2150 LKB; precolumn: 20 × 2 mm I.D., Uptight C-130B (Upchurch Scientific), packed with LiChrosorb RP-18, particle size 5 μm (Merck); column: 100 × 4.6 mm I.D., Chromsep microspher C_{18}, particle size 3 μm (Chrompack, Bergen op Zoom, Netherlands) (the column was always used at room temperature); detector and integrator: photodiode array detector, type 990 (Waters); injector: autosampler, type Wisp 710B (Waters); eluent: 5 m*M* cetyltrimethylammonium bromide (CTAB, Merck, for synthesis) in acetonitrile–water–85% phosphoric acid (6:4:0.5, v/v/v), filtered over a 0.45-μm nylon membrane filter (type NL17, Schleicher & Schüll) and degassed by vacuum.

With flow-rate 1.00 ml min^{-1} pressure was 165 bar and retention times were 7.8, 9.8 and 10.3 min for cohumulone, humulone and adhumulone, respectively.

2.3. CPC apparatus

For all experiments a modular Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLN) was used. It consists of a power supply (Model SPL), a centrifuge (Model NMF), a loop sample injector plus flow director (Model FCU-V), equipped with a 3.4-ml loop, and a triple-head constant-flow pump (Model LBP-V). To a UVIS 200 detector (Linear Instruments, Reno, NV, USA) a Panasonic Pen-recorder (Model VP-67222A) was connected. Fractions were collected by means of a LKB 17000 Minirac fraction collector. In all experiments six cartridges (total internal volume 125 ml) were used. The pressure was limited to 60 bar.

2.4. Preparation of two-phase solvent systems for CPC

The 0.1 *M* triethanolamine · HCl pH 8.4 in 25% ethylene glycol in water was prepared by dissolving 15.0 g triethanolamine (Sigma, St. Louis, MO, USA) in water. The pH was adjusted to 8.4 with hydrochloric acid and the volume was brought to 730 ml. To this solution 250 ml ethylene glycol were added and the total volume was brought to 1000 ml.

The solvents of the two-phase systems were mixed thoroughly at room temperature for 1 h; the two phases were separated in a separation funnel before use, leaving some ml of the lower layer in the upper layer and *vice versa*, thus guaranteeing saturation.

2.5. Determination of partition coefficients

The partition coefficient (k) is defined as c_{org}/c_{aq} (or $c_{non\text{-}polar}/c_{polar}$) for two-phase systems. Hop carbon dioxide extract (2–10 mg) was added to 10 ml of each of both phases of the solvent system. The mixture was shaken vigorously, kept in the dark at room temperature for 1 h, put in an ultrasonic bath for 2 min and set aside for the layers to separate. The concentration of the compounds in each of the layers was measured: part of the layer was evaporated until dryness, taken up in methanol, centrifuged 2 min at 15 500 *g* (14 000 rpm) and injected in the HPLC system. In case the layer was aqueous, the sample was not evaporated, but diluted with methanol, centrifuged and injected as such.

2.6. Determination of settling times

Volumes of 10 ml of both phases of an already saturated system were shaken by hand in a stoppered graduated cylinder during 10 s. The cylinder was put in an upright position and time was measured until complete separation of the phases. The mean of three measurements was taken. Settling times with hop extract were determined in the same manner by addition of *ca.* 10 mg of the extract. In case no separation was obtained within 3 min, the qualification "emulsion" was given.

2.7. Determination of polarity

In each of the two phases of a solvent system a small amount (*ca.* 0.02 mg ml^{-1}) of Reichardt's dye (Aldrich, Steinheim, Germany) was dissolved. The absorption maximum between 350 and 900 nm (λ_{max}) was determined. Polarity, expressed as E_tN, was calculated according to the method used by Gluck and Wingeier [8]:

$E_tN = (28\,590\lambda_{max}{}^{-1} - 30.7)(32.4)^{-1}$.

Non-polar liquids have an E_tN close to zero and E_tN of polar liquids is close to 1.

2.8. Determination of the volume of the stationary and the mobile phase in the cartridges (V_s and V_m, respectively)

The cartridges of the CPC were filled with the stationary phase. Under experimental conditions (flow, centrifugal speed, flow direction) mobile phase was pumped through the cartridges. The effluent was led into a graduated cylinder. Pumping was continued about 30 min after the first mobile phase was eluted. The volume of the stationary phase in the cylinder was corrected for volume of inlet and outlet tubing to give V_m; the volume of the stationary phase in the cartridges, V_s, is $(125 - V_m)$ ml.

2.9. CPC sample preparation and operation

A weighed amount of the hop extract was dissolved in 3 ml stationary phase and centrifuged 2 min at 15 500 *g* (14 000 rpm) in an Eppendorf centrifuge; the supernatant was injected in the CPC system.

2.10. Extraction of the bitter acids from the aqueous phase after CPC separation

CPC fractions were combined, according to the HPLC analysis results (yielding a volume of V_{fr} ml), acidified with 2 *M* hydrochloric acid until pH 2.5–3 and extracted twice with 0.5 V_{fr} ml chloroform. The combined chloroform layers were extracted once with V_{fr} ml slightly acidified water and filtered over a paper filter, wetted with chloroform. After addition of some methanol the solvents were evaporated under vacuum; if necessary traces of toluene were removed by addition of methanol and repeated vacuum evaporation. Especially the β-, but also the α-acids are unstable compounds. Decomposition occurred, even after storage under nitrogen in the dark at −15°C. Stability was observed to be enhanced when the bitter acids were stored in methanol at −20°C. It is suspected that light has an adverse effect on the stability of the compounds. No decomposition was observed in the crude carbon dioxide extract, kept in the dark at 4°C.

3. Results and discussion

CPC was first developed by Murayama *et al.* [9] and has been studied in detail by Berthod and co-workers [10–15]. Many two-phase solvent systems for CCC have been described for all types of compounds. Reviews of solvent system applications as well as of instrumentation are published regularly [2–4]. A wide choice of two-phase systems is available; nevertheless the search for an optimal or even satisfactory solvent system for a special separation problem can be tedious and time consuming, as many factors influence the ultimate performance of the chromatography.

As each CPC run is quite time consuming, methods are needed which can be used to predict the behaviour of a particular solvent system for the desired separation. Measuring partition coefficients of the solutes and determining settling times of the two-phase systems are such methods.

By measurement of the partition coefficients of the solutes, the selectivity of a solvent system can easily be investigated. As the bitter acids are not available as pure compounds, the partitioning between the two phases has to be measured with crude extracts and the concentration of the individual compounds in the two phases has to be determined by means of HPLC analysis.

By measuring settling times [16] information is obtained about viscosity and interfacial tension which are important parameters of the solvent systems, because they affect the efficiency and separation capacity of the column. Low viscosity enables fast mass transfer of the solutes within the phases; low interfacial tension favours fast mass transfer between the phases. However, due to the formation of emulsions, it can also be the cause of flooding, which not only results in a noisy detector signal but also in a considerable loss of efficiency, capacity and reproducibility of

the chromatographic system. As already reported by Foucault and Nakanishi [17] for proteins, also hop extracts can cause quite large changes in settling time and even cause the formation of emulsions (see below). This results in temporary flooding during the elution of high concentrations of the bitter acids. The interfacial tension lowering properties of the bitter acids is well known, as they are also connected with the stability of the foam of beer.

Thus for a series of two-phase systems we determined the partition coefficients and separation factors of the bitter acids. The solvent systems tested were among others based on the systems reported by Fischer *et al.* [5], as well as some commonly applied systems. Settling times and polarities of the phases were measured. The results are presented in Table 1. Of the water-containing systems neither the highly polar butanol-containing systems 6 and 7, nor the less polar chloroform-containing 8 were promising for the separation of the bitter acids, because of small separation factors. No further attempts were made to improve selectivity by means of other modifiers.

Based on the results of the tests of the selectivity and the settling times, systems 1, 2 and 3 were selected for further studies in CPC for the separation of the bitter acids. All systems were suitable for use in CPC, no problems with flooding were encountered. The selectivity was in accordance with the data for the partition coefficients, given in Table 1. Best results were obtained with system 3, the system used by Fischer *et al.* [5]. This system was tested in both descending and ascending mode. In Fig. 2 the chromatograms of both modes are given. Analysis of fractions of the experiment in ascending mode (Fig. 2B) in HPLC system B (allowing the separation between adhumulone and humulone), showed that the first α-acid-containing fractions contained only adhumulone, which could not be detected in later fractions. From this it is concluded that there was some resolution between humulone and adhumulone. But no complete resolution of all three α-acids was achieved.

Therefore further studies were made with the solvent system as reported by Verzele and De Keukeleire [6]. In this slightly basic aqueous system (benzene–pH 8.4 aqueous buffer containing triethanolamine, hydrochloric acid and 25% ethylene glycol) the partition coefficients were reported as: 0.74 for cohumulone, 1.35 for humulone and 2.00 for adhumulone. To avoid the use of the carcinogenic benzene, we tested a system containing toluene. The triethanolamine concentration in the aqueous layer was 0.1 *M*, with a pH of 8.4, set by means of hydrochloric acid. The solvent system showed quite large differences in the partitioning coefficients for the three α-acids. The settling times were quite high and with hop extracts emulsions were formed (Table 2). Because of the high selectivity the system was tested in the CPC. In descending mode with the aqueous phase as mobile phase, nearly baseline separation was achieved between cohumulone and humulone, while adhumulone eluted in the end of the second, humulone-containing, peak. The β-acids were eluted after mode reversion with only little resolution. The pH of the aqueous phase of this system was optimized for the α-acids separation; partition at pH values between 6.5 and 9.0 is visualized in Fig. 3A. Partition coefficient values should preferably be between 0.2 and 5 to ensure a good balance between resolution and elution volume (analysis time); this corresponds with a percentage between 17 and 83% in the upper layer. For toluene as the stationary phase, best results are thus expected for pH values between 8.0 and 8.6 (Fig. 3A). Partition coefficients are strongly pH dependent, indicating that the retention mechanism mainly works by ion-pair partitioning.

Chromatographic performance of system 10 was examined at a slightly elevated temperature (30°C); V_s and pressure were the same as at room temperature, the retention volumes were *ca.* 30% lower; however, the selectivity was not changed.

In order to investigate the role of ethylene glycol in the separation of the α-acids, settling times and partition coefficients were determined for systems containing 0–25% ethylene glycol. As expected settling times increase with increasing ethylene glycol percentage (see Table 2). Partition coefficients decrease with increasing

Table 1
Some physico-chemical parameters of two-phase systems tested for the separation of α bitter acids

No.	System	Partition coefficient				Separation factor			Polarity (E_tN)		Settling times (s)	
		cohum k_1	(ad)hum k_2	colup k_3	(ad)lup k_4	k_2/k_1	k_3/k_2	k_4/k_3	Upper phase	Lower phase	Without hop extract	With hop extract
1	Hexane–dichloromethane–acetonitrile (13:3:7)	0.407	0.450	0.616	0.714	1.11	1.37	1.16	0.348	0.418	–[c]	–[c]
2	Hexane–dioxane–acetonitrile (13:3:7)	0.318	0.366	0.523	0.602	1.15	1.43	1.15	0.309	0.418	–[c]	–[c]
3	Hexane–*tert.*-butyl methyl ether–acetonitrile (10:1:10)	0.363	0.437	0.628	0.795	1.20	1.44	1.27	–[a]	0.444	–[c]	–[c]
4	Hexane–acetonitrile	0.231	0.278	0.568	0.717	1.20	2.04	1.26	–[a]	0.451	5	7
5	Hexane–methanol–water (100:99:1)	0.220	0.252	0.315	0.343	1.15	1.25	1.09	0.648	0.724	11	13
6	*n*-Butanol–water	>250	>250	>250	>250				0.657	–[a]	28	emulsion
7	*n*-Butanol–acetic acid–water (4:1:5)	>250	>250	>250	>250				–[b]	–[b]	43	47
8	Chloroform–methanol–water (5:6:4)	>250	>250	>250	>250				0.742	0.592	22	emulsion

Abbreviations: cohum = cohumulone; (ad)hum = humulone and adhumulone; colup = colupulone; (ad)lup = lupulone and adlupulone.
[a] The solubility of Reichardt's dye is too low to be able to measure the absorption maximum.
[b] The method is not applicable to acidic solutions.
[c] Not determined.

Table 2
Correlation between physico-chemical parameters and ethylene glycol concentration in the two-phase system toluene–0.1 M triethanolamine · HCl pH 8.4 in water

System No.	Glycol in aqueous layer (%, v/v)	Partition coefficient c_{org}/c_{aq}			Separation factor		Polarity (E_tN)		Settling times (s)	
		cohumulone k_1	humulone k_2	adhumulone k_3	k_2/k_1	k_3/k_2	Upper phase	Lower phase	Without hop extraction	With hop extraction
9	25	0.64	1.57	1.88	2.47	1.20	0.214	0.918	136[b]	emulsion
10	20.3	0.83	1.93	2.36	2.32	1.22	–[a]	–[a]	82[b]	emulsion
11	12.2	1.33	3.29	3.81	2.47	1.16	–[a]	–[a]	60[b]	emulsion
12	4.1	1.77	4.47	6.40	2.52	1.43	–[a]	–[a]	35[b]	emulsion
13	0	2.45	6.32	10.95	2.58	1.73	0.129	[c]	33[b]	emulsion

[a] Not determined.
[b] The aqueous layer stayed turbid after separation of layers.
[c] The solubility of Reichardt's dye is too low to be able to measure the absorption maximum.

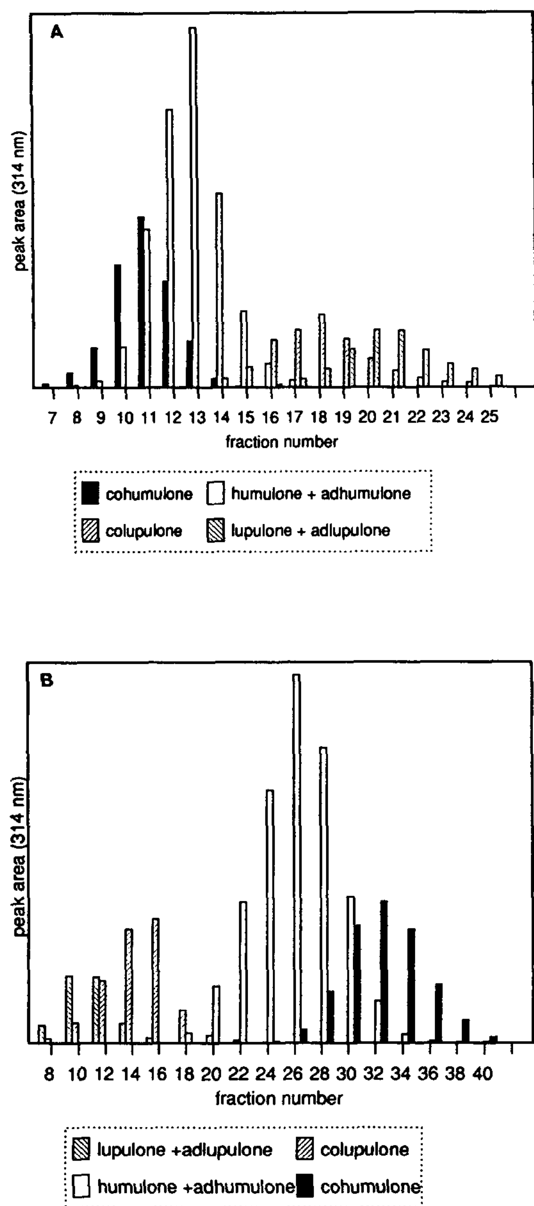

Fig. 2. Composition of fractions after CPC separation of a crude supercritical carbon dioxide hop extract as analyzed by HPLC (system A). Two-phase system: hexane–*tert.*-butyl methyl ether–acetonitrile (10:1:10, v/v/v). (A) Descending mode; rotation speed 1000 rpm; flow-rate 2.75 ml min^{-1}; pressure 29 bar; amount injected 40 mg; fraction size 3.2 ml; total elution volume after 25 fractions is 130 ml; total run time 48 min. (B) Ascending mode; rotation speed 1200 rpm; flow-rate 2.8 ml min^{-1}; V_s 80 ml; pressure 44 bar; amount injected 50 mg; fraction size 3.9 ml; total elution volume after 41 fractions is 250 ml; total run time 54 min.

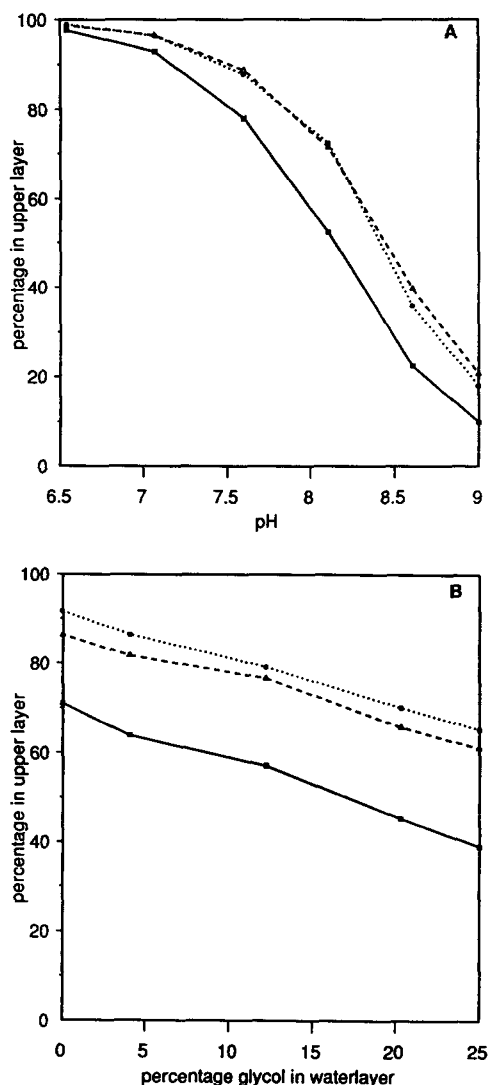

Fig. 3. Partitioning of α-acids in different two-phase systems. The percentage in the upper layer is calculated from the partition coefficients. (A) Two-phase systems: toluene–0.1 *M* triethanolamine in 25% ethylene glycol in water; the pH of the aqueous phase is set with hydrochloric acid; the pH values of the aqueous layers after addition of the hop extract are used in the graph. (B) Two-phase systems: toluene–0.1 *M* triethanolamine · HCl pH 8.4 in solutions of the indicated percentage (v/v) ethylene glycol in water. ■ = Cohumulone; △ = humulone; ● = adhumulone.

ethylene glycol concentration, resulting in larger retention volumes and thus longer chromatographic runs (see Table 2 and Fig. 3B). On the other hand the separation factors increase, espe-

cially for adhumulone/humulone. Moreover, absence of ethylene glycol in the solvent system is also advantageous, considering isolation and further purification of the α-acids from the aqueous layer. In the ethylene glycol-free system higher pH values decreased the partition coefficients (and so diminished elution volumes), but there was also a slightly lower separation factor between adhumulone and humulone. On the other hand no improvement of the separation can be expected at pH <8.4 because of the increase of partition coefficient values of the α-acids at lower pH values. So pH 8.4 is regarded optimal for the preparative isolation of the main α-acids from hop carbon dioxide extract, both in a system with and without ethylene glycol. Results of the separation of the α-acids from hop carbon dioxide extracts by means of CPC with both systems (Nos. 9 and 13 in Table 2) are shown in Fig. 4. Using system 13 from 500 mg hop extract (total content α-acids 45.4%) 59 mg cohumulone, 82 mg humulone and 18 mg adhumulone were obtained within 5 h. Identity and purity (estimated ≥95%) were confirmed by NMR, UV spectra and HPLC (system A).

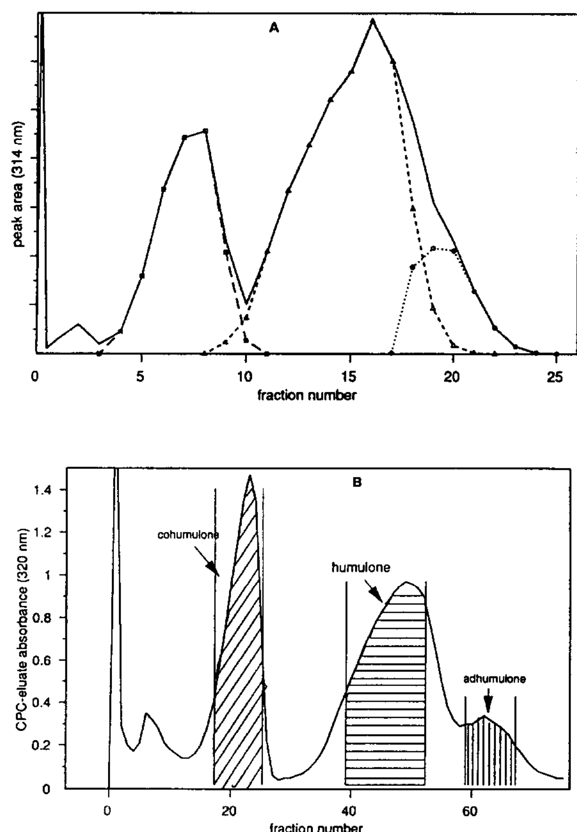

Fig. 4. CPC separations of α-acids from a crude supercritical carbon dioxide hop extract. (A) Two-phase system: toluene–0.1 M triethanolamine · HCl pH 8.4 in 25% ethylene glycol in water; descending mode; experimental conditions: rotation speed 800 rpm, flow-rate 2.17 ml min^{-1}, V_s 81 ml, amount injected 480 mg, fraction size 8.7 ml; total elution volume after 26 fractions is 260 ml, total run time 2 h; CPC separation was monitored by HPLC analysis (system B) of the fractions; dotted line (○) = adhumulone; broken lines: □ = cohumulone, △ = humulone; solid line = total peak area. (B) Two-phase system: toluene–0.1 M triethanolamine · HCl pH 8.4 in water; descending mode; experimental conditions: rotation speed 1000 rpm, flow 2.6 ml min^{-1}, V_s 81 ml, pressure 47 bar, amount injected 500 mg, fraction size 10.1 ml, total elution volume after 79 fractions is 850 ml, total run time 5 h; isolation of the bitter acids from the indicated fractions resulted in 59 mg cohumulone, 89 mg humulone and 18 mg adhumulone.

4. Conclusions

The three main α-acids, humulone, cohumulone and adhumulone, can be obtained pure from hop carbon dioxide extracts after separation by CPC, using the two-phase system toluene–0.1 M triethanolamine · HCl pH 8.4 in water. For analytical purposes an aqueous layer containing 0.1 M triethanolamine · HCl pH 8.4 in 2% (v/v) ethylene glycol in water can be used; in this system separation factors are somewhat lower but run times are shorter, retention volumes smaller and detection limits lower.

5. Acknowledgements

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6. References

[1] S.-Y. Fung, J. Brussee, R.A.M. van der Hoeven, W.M.A. Niessen, J.C. Scheffer and R. Verpoorte, *J. Nat. Prod.*, in press.

[2] J.B. McAlpine and J.E. Hochlowski, in G.H. Wagman and R. Cooper (Editors), *Natural Products Isolation* (*Journal of Chromatography Library*, Vol. 43), Elsevier, Amsterdam, 1989, p. 1.
[3] A. Marston, I. Slacanin and K. Hostettmann, *Phytochem. Anal.*, 1 (1990) 3.
[4] D.E. Schaufelberger, *J. Chromatogr.*, 538 (1991) 45.
[5] N. Fischer, B. Weinreich, S. Nitz and F. Drawert, *J. Chromatogr.*, 538 (1991) 193.
[6] M. Verzele and D. De Keukeleire, *Chemistry and Analysis of Hop Bitter Acids* (*Developments in Food Science*, Vol. 27), Elsevier, Amsterdam, 1991, p. 33.
[7] F. David, P. Sandra, W.S. Pipkin and J. Smith, *Hewlett-Packard Application Note 228-115*, Hewlett-Packard, May 1990.
[8] S. Gluck and M. Wingeier, *J. Chromatogr.*, 547 (1991) 69.
[9] W. Murayama, T. Kobayashi, Y. Kosuge, H. Yano, Y. Nunogaki and K. Nunogaki, *J. Chromatogr.*, 239 (1982) 643.
[10] A. Berthod and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 547.
[11] A. Berthod and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 567.
[12] A. Berthod, J.D. Duncan and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 1171.
[13] A. Berthod and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 1187.
[14] A. Berthod, Y.I. Han and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 1441.
[15] A. Berthod and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 1457.
[16] Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
[17] A. Foucault and K. Nakanishi, *J. Liq. Chromatogr.*, 11 (1988) 2455.