ELSEVIER

Journal of Chromatography A, 771 (1997) 71–79

JOURNAL OF CHROMATOGRAPHY A

Preparative separation of bitter acids from hop extracts by centrifugal partition chromatography

A.C.J. Hermans-Lokkerbol*, A.C. Hoek, R. Verpoorte

Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Received 19 September 1996; revised 23 December 1996; accepted 27 January 1997

Abstract

Centrifugal partition chromatography was used for preparative separation of α- and β- bitter acids from a crude supercritical carbon dioxide extract of hop cones. A one-step gradient was applied; after elution of the α-acids in the system toluene–0.1 *M* triethanolamine-HCl pH 8.4 in water the aqueous mobile phase was changed to 0.2 *M* diethanolamine-PO_4 pH 9.75 in water–methanol (4:1, v/v) for elution of the β-acids. For the α-acids the yield/cost ratio was optimized through sample size and injection volume. The role of pK_a values and hydrophobicity in the separation mechanism for the α-acids is discussed. The elution order differs from the order predicted after measurement of the partition coefficients.

Keywords: Centrifugal partition chromatography; Hop extracts; *Humulus lupulus*; Preparative chromatography; Bitter acids; Humulones; Lupulones

1. Introduction

Extracts of hop cones, the female flowers of *Humulus lupulus* L., are used in the beer brewing process. The main components of hop extracts are the two groups of bitter acids: α-acids or humulones and β-acids or lupulones. Each group contains several analogues of which cohumulone, humulone, adhumulone and colupulone, lupulone and adlupulone are the major compounds; the post- and pre-analogues are the minor compounds (Fig. 1). During the brewing process the α-acids of the hop extract are converted into the iso-α-acids, which give the typical bitter taste to beer. Hop extracts, as delivered by the supply industry, vary not only in their total bitter acid content, but also in the ratio

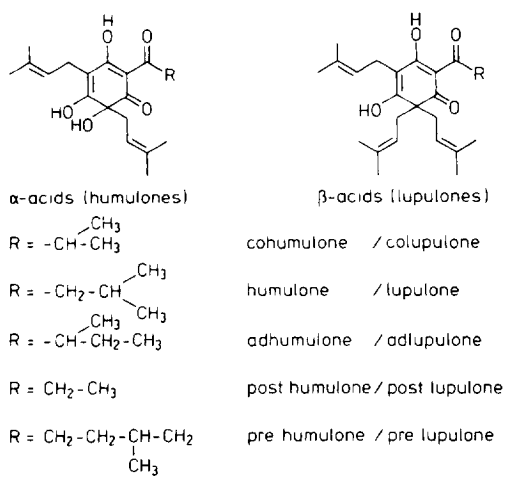

Fig. 1. Chemical structures of the main hop bitter acids.

*Corresponding author.

PII S0021-9673(97)00123-4

α:β-acids as well as in the relative amounts of each of the homologues. As the pure bitter acids are not commercially available, different hop extracts are used as calibration standards, giving rise to different qualifications of hop extracts [1]. This indirect way could be shortened if the pure bitter acids were available in relatively large amounts. Also, for a study of the contribution of each (individual) bitter acid to the final taste of the beer the pure compounds should be available.

In our laboratory we study the biosynthesis of both α- and β-acids in hops [2] for which we need the pure compounds. We recently described a method for the preparative separation and isolation of the three main α-acids from hop extract, using centrifugal partition chromatography (CPC) [3]. Respectively, 250 and 350 mg of pure cohumulone and humulone can thus be isolated in one run of 8 h. Furthermore, a large-scale CPC-apparatus is commercially available, the use of which makes gram-scale yields possible.

Here we report the preparative separation and isolation of the three major β-acids from hop extract, using centrifugal partition chromatography. The method is related to that used for the α-acids and can be performed immediately after the isolation of the α-acids in the same chromatographic run. In addition, the optimization of the α-acids separation in the small-scale CPC with regards to sample volume and concentration will be described and new insights in the mechanism of the separation will be discussed.

2. Experimental

2.1. Hop extract

Two carbon dioxide extracts of hops were obtained from Mr. L.C. Verhagen (Heineken Brewery, Zoeterwoude, Netherlands); one contained 45.5% α-acids and 21.2% β-acids; the other was a supercritical carbon dioxide extract containing 55% α-acids and 30% β-acids.

2.2. 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 an LKB 17000 Minirac fraction collector. In all experiments 6 cartridges (total internal volume 125 ml) were used. The pressure was limited to 60 bar.

2.3. Preparation of two-phase solvent systems for CPC

The two-phase system used for the α-acids was described before [3]. For the system for the β-acids 0.2 *M* diethanolamine (Merck, Darmstadt, Germany) in water–MeOH (4:1, v/v), adjusted to pH 9.75 with phosphoric acid (85%), was saturated with toluene. The volume ratio, aqueous solution–toluene, was 2:1; the toluene layer was used as the stationary phase.

2.4. HPLC

For the quantitative analysis of the α- and β-acids the HPLC system was described before [4].

2.5. Determination of partition coefficients, CPC sample preparation and extraction of the bitter acids from the aqueous phase after CPC separation

The methods as described before [3] were used. The isolated bitter acids were stored in MeOH at −20 °C in the dark.

2.6. NMR

All NMR experiments were performed on a Bruker DPX 300 machine at 300 MHz. All samples were dissolved in C^2HCl_3.

3. Optimization of a two-phase system for separation of the β-acids

3.1. Results and discussion

We developed a two-phase system for the separation and isolation of the three major α-acids from hop extract by means of CPC [3]. The stationary phase of this two-phase system is toluene; the mobile phase is 0.1 *M* triethanolamine in water, brought to pH 8.4 with HCl. The triethanolamine acts as an ion-pairing reagent for the partly dissociated α-acids. The β-acids are weaker acids than the α-acids and are not, or are to a very low extent, dissociated at pH 8.4. As a consequence they are strongly retained in the stationary phase. For their elution and separation the two-phase system for the α-acids has to be modified. The difference in pK_a values of α- and β-acids is about 2; this means that a mobile aqueous phase with pH about 10.4 would suffice. As β-acids decompose at pH>10, the pH of the aqueous phase was chosen to be ca. 9.5. Addition of an alcohol to this aqueous phase proved to be necessary to decrease its polarity.

Partition coefficients of the bitter acids in several two-phase systems, with pH between 9.5 and 10, were determined. The results are given in Table 1.

These systems are not suited for the α-acids; partition coefficients are very low because of the high pH and this results in elution immediately after the injection front peak. System 1 and several modifications with regard to methanol concentration, triethanolamine molarity and pH were tested for their CPC performance for β-acid separation with disappointing outcomes. The ion-pairing as well as the buffer capacity of triethanolamine (pK_a=7.8) is low at the high pH needed for β-acid elution, resulting in low chromatographic efficiency. Systems 6 and 7 were both tested in CPC runs. For system 7 the pH had to be raised slightly in order to achieve elution within a reasonable time. After some optimization experiments, best results were obtained with the two-phase system 0.2 *M* diethanolamine in 20% (v/v) methanol in water, phosphoric acid added to pH 9.75-toluene. The results of two CPC experiments are shown in Fig. 2 and in Table 2, Exp. 1 and 2. In both experiments the humulones eluted first with the aqueous mobile phase for the α-acids;

Table 1
Partition coefficients of the bitter acids in various two-phase systems with buffer capacity at pH 7.8 and 9.5

System number	Basic compound	pK_a of the basic compound	pH of aq. phase	Partition coefficients						Remarks
				Cohumulone	Humulone	Adhumulone	Colupulone	Lupulone	Adlupulone	
1	Triethanolamine	7.8	9.6	0.08	0.19	0.15	9.8	23.9	6.3	
2	Triethanolamine + LiCl	7.8	9.6	0.49	1.4	1.0	88.9	>100	>100	Li^+ causes strong retention
3	Borax + LiCl in water	9.2	9.9		0.33	0.24	78.5	>100	>100	Strong retention despite high pH
4	Borax + LiCl in water–methanol (4:1)	9.2	9.9		0.19	0.15	14.2	14.4	14.2	No resolution of β-acids any more
5	Ephedrine	10.0	9.6	>100	>100	>100	>100	>100	>100	Ephedrine retains excessively
6	Diethanolamine		9.5	0.05	0.15	0.10	6.07	7.95	3.19	Promising for β-acids
7	Ethanolamine	9.5	9.5	0.06	0.19	0.13	11.1	24.9	5.9	Promising for β-acids

Org. phase: toluene; aq. phase: 0.1 *M* basic compound in water–MeOH 75:25 (v/v).

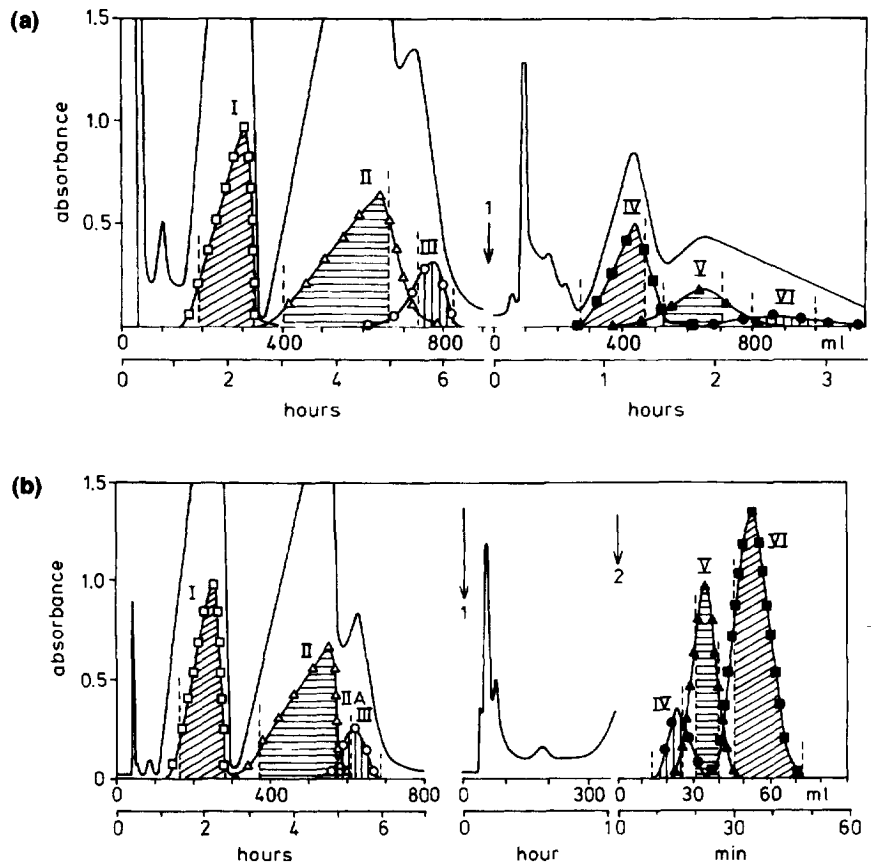

Fig. 2. CPC separation of hop extract. (a) Two-phase systems for the α- and β-acids as described in Section 2. Both are run in descending mode. Arrow 1 indicates the step from the α-acid eluent to the β-acid eluent. Plain line: UV absorbance of the CPC eluate (aqueous phase). CPC separation was monitored by HPLC analysis of the fractions; □=cohumulone, △=humulone, ○=adhumulone, ■=colupulone, ▲=lupulone, ●=adlupulone. (b) As (a) until arrow 2; from then on ascending mode. For more details about sample size, yield and purity of the indicated fractions I–VI and purity see Table 2.

subsequently the β-acids were eluted, using the more basic diethanolamine containing aqueous phase. In the first experiment this was continued until the last β-acid, adlupulone, was eluted (Fig. 2a). In the second experiment the mode was reversed just before elution of the first β-acid (colupulone). Mode reversion causes the mobile phase to become stationary and vice versa. As a consequence the elution order was reversed; adlupulone came out first, followed by lupulone and colupulone was eluted as the last compound (Fig. 2b). For both α- and β-acids there is a discrepancy between partition coefficients as given in Table 1 (e.g. for systems 1, 6 and 7) and the elution order and volume as given in Table 2 and Fig. 2. This will be discussed below, under the discussion of the optimization for the α-acids.

For routine preparations of the β-acids we now inject the hop extract and elute with the mobile phase for the β-acids without the pre-elution with the mobile phase for the α-acids; the α-acids come out together as one peak shortly after the solvent front peak. This fraction can be used as an injection sample for another run for separation and isolation of the individual α-acids. The result of a routine injection is given in Table 2, exp. 3. It should be noted that we did not yet optimize the separation method for sample size, injection volume and molarity of the ion-pairing reagent.

Table 2
Yield and purity of the bitter acids after CPC separation

Experiment and total run time[a]	Mobile phase and elution volume[b]	Fraction	Yield bitter acid (mg)	Composition of fraction (%)					
				Cohu-mulone	Humu-lone	Adhu-mulone	Colu-pulone	Lupu-lone	Adlu-pulone
1 (Fig. 2a)	A (850 ml)	I	115	100					
10 h		II	169	1	99				
		III	28		9	91			
	B (1050 ml)	IV	77				96	4	
		V	53				2	92	6
		VI	39					5	95
2 (Fig. 2b)	A (800 ml)	I	148	100					
11 h		II	188		100				
		IIA	83		69	31			
		III	32			100			
	C (430 ml)	IV	15					9	91
		V	37				6	91	3
		VI	88				99	1	
3	D (2000 ml)	I	±2000	±40	±50	±10			
18 h		II	456				99	1	
		IIA	271				72	28	
		III	148				1	99	
		IIIA	107					65	35
		IV	49					2	98

[a] Amounts injected: Experiment 1: 1.0 g hop CO_2 extract (45% α- and 21% β-acids); inj. volume: 3 ml (stationary phase); Experiment 2: 1.0 g hop supercritical CO_2 extract (55% α- and 30% β-acids); inj. volume 2 ml (stationary phase); Experiment 3: 4.0 g hop supercritical CO_2 extract (55% α- and 30% β-acids); inj. volume 10 ml (stationary phase).

[b] Mobile phases: (A) Aqueous phase as described for the separation of the α-acids; (B) Aqueous phase as described for the separation of the β-acids; (C) 350 ml aqueous phase as described for the separation of the β-acids; after that organic phase (toluene) in reversed mode; (D) As (B) but 0.4 *M* diethanolamine instead of 0.2 *M*.

The identity of the isolated compounds was confirmed by UV, HPLC and ^{1}H NMR. From the NMR experiments it was clear that no NMR detectable compounds (impurities) were present in the isolated products. The relative amount of each of the bitter acids in the isolated fractions was determined by means of HPLC, assuming that the three α-acids have the same molar extinction and that the same is valid for the β-acids.

4. Chemical stability

The β-acids are known to be unstable compounds. However, decomposition of the β-acids could not be detected after storage in the mobile phase for 24 h at 20 °C, if kept in the dark. At temperatures ≥30 °C a rapid decomposition of the β-acids was noticed, both in mobile phase and in methanol solution. We also have strong indications that UV and direct sunlight should be avoided to prevent decomposition; this is under further investigation. As stated above, the β-acids decompose if kept in solutions of pH>10. They seem to be quite stable if kept in methanol solution at −20 °C.

For the α-acids we found a good stability if kept in methanol solution at −20 °C; no decomposition could be detected after 1 year. This is in accordance with the results as given by Wärtgen [5].

5. Optimization of sample size and injection volume to improve the yield/cost ratio for the α-acids

5.1. Results

After the development of the mobile phase for the

β-acids, we sought to optimize the yield/cost ratio for the isolation of the α-acids. Our experiments were not only designed to improve the yield per run for our laboratory-scale CPC (total volume 125 ml), but also to predict the performance in a large-scale CPC (total volume 5 l). We varied quantity, concentration and volume of the sample injected and evaluated the results with regard to yield and purity of the α-acids, as well as resolution and run time.

An overview of the most informative (characteristic) CPC runs is given in Fig. 3. In all cases cohumulone, humulone and adhumulone eluted in that order. At low injection volumes (5.0 ml) and low amounts of hop extract injected (1.0 g) resolution and purity are high; 150 mg of cohumulone, 190 mg of humulone and 30 mg of adhumulone were obtained at over 99% purity. Of the three peaks only the adhumulone peak has a Gaussian shape; the other two have a characteristic sharp fall-down at the moment the next compound starts to elute. Increasing the amount of hop extract injected (from top to bottom in Fig. 3) results in tailing and an increase in fronting; the elution profiles are more Gaussian, and the maxima are flattened. Retention of humulone and adhumulone increases and therefore the run time also increases.

Larger injection volumes (from left to right in Fig. 3) result in broader peaks and more fronting and tailing; no effect on retention or run time was observed.

Very poor resolution was obtained after injection of 20.5 g hop extract in a large injection volume (40 ml stationary phase). As the total volume of stationary phase is 80 ml, in this case half of the column volume is replaced by the sample solution after loading. The fronting of humulone and adhumulone is extensive, as is the tailing of cohumulone. The top of all peaks is flat and the peak shape is almost rectangular.

5.2. Discussion

The partition coefficient of an undissociated α-acid is determined by the hydrophobicity of the α-acid and is defined as

$$K = \frac{[HA_{org}]}{[HA_{aq}]}$$

The actual partition behaviour of the acids is regulated by the apparent partition coefficient K_{app}:

$$K_{app} = \frac{[HA_{org}]}{[HA_{aq}] + [A^-]}$$

where $[HA_{org}]$ and $[HA_{aq}]$ are the concentrations of the undissociated acid in the organic and the aqueous phase, respectively, and $[A^-]$ is the concentration of the dissociated hop acid in the aqueous phase. It is assumed that there are no acid anions in the organic phase. $[A^-]$ is determined by the pH and the pK_a value of the α-acid. The pK_a values of the α-acids are 4.7, 5.5 and 5.7 for cohumulone, humulone and adhumulone, respectively [6]. For the water–toluene system the K value of the α-acids is very large (>1000); the more polar α-acid ions have a low partition coefficient. As a result of this the apparent partition coefficients of the α-acids show a sharp decrease between pH 6.5 and 9 (Fig. 3A in Ref. [3]). During a CPC run the availability of the positively charged proton-accepting triethanol amine counterions is determining the degree of ionization of the α-acids and thus the overall apparent partition coefficients.

Two mechanisms are active in this CPC separation, partition of the non-ionized acids (regulated by hydrophobicity) and dissociation (regulated by pK_a). From measurements of apparent partition coefficients of both α- and β-acids in several two-phase systems we conclude that the order of hydrophobicity of these compounds differs from the pK_a order. In many cases the partition coefficient order was cohumulone<adhumulone<humulone for the α-acids and colupulone<adlupulone<lupulone or adlupulone<colupulone<lupulone for the β-acids [7]. From this we conclude that adhumulone is less hydrophobic than humulone and may be less hydrophobic than cohumulone; for the β-acids an analogous order will be valid. In all our CPC experiments however, the order of elution was cohumulone, humulone, adhumulone and colupulone, lupulone, adlupulone (that is in the sequence of their pK_a values). Partition coefficients were measured in a two-phase system with a fixed pH of the aqueous layer; whereas during the CPC run the pH of the mobile phase varies from inlet to outlet, the apparent partition coefficient of each individual bitter acid has

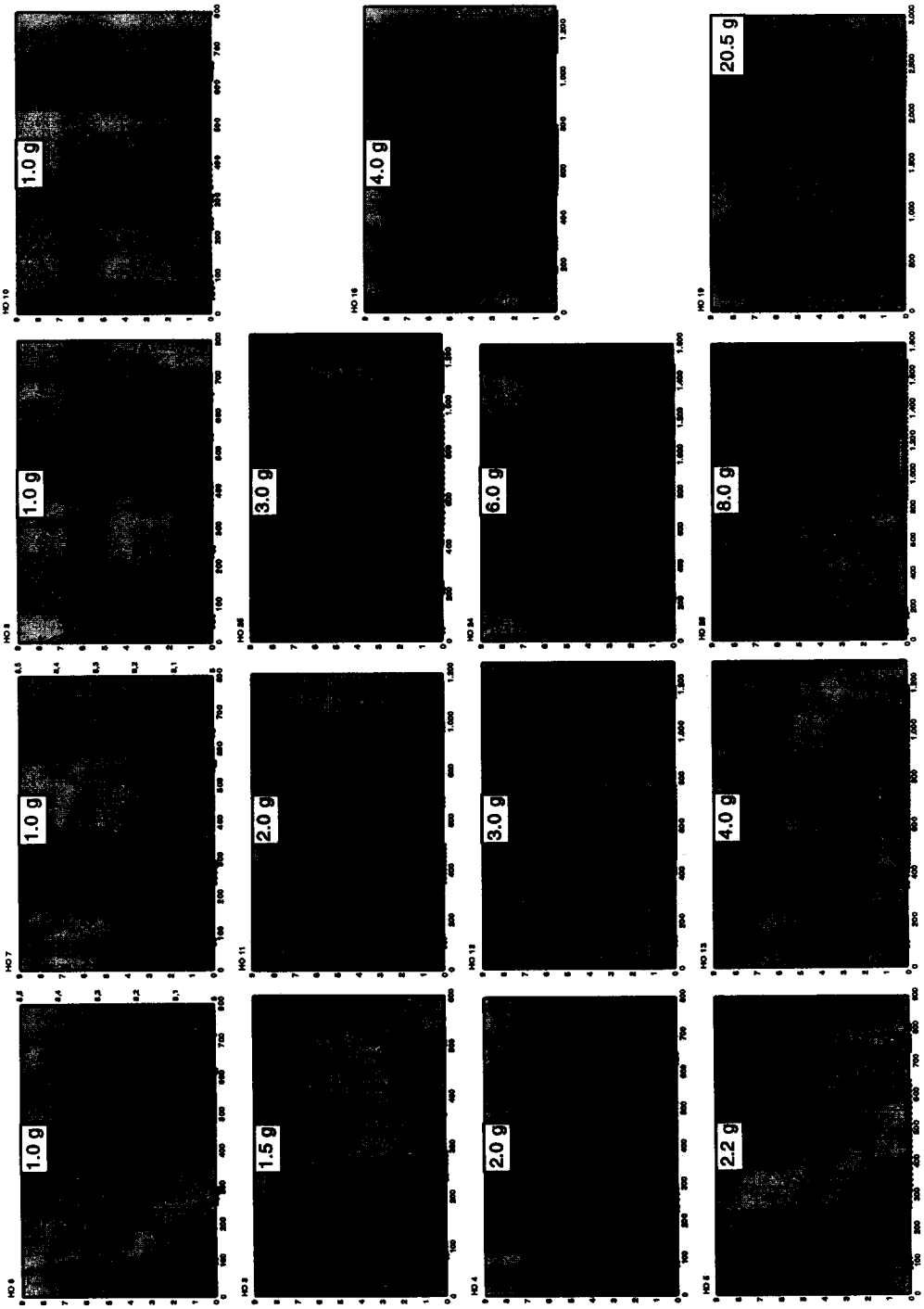

Fig. 3. Overview of CPC runs with variations in injection volume and amount injected. Hop supercritical carbon dioxide extract was dissolved in stationary phase; the injection volume is indicated on top of each column and the amount injected is given in each diagram. Horizontal axes: elution volume (ml). Vertical axes: left = concentration of the bitter acid in arbitrary units and right = pH of the eluate. CPC separation was monitored by HPLC analysis of the fractions; x = cohumulone, * = humulone, ■ = adhumulone, □ = pH of the fractions. Two-phase system: toluene–0.1 *M* triethanolamine-HCl pH 8.4 in water; descending mode; experimental conditions: rotation speed 1100–1300 rpm; flow 2 ml min^{-1}.

not got the same value in the subsequent ducts of the CPC. A confirmation is seen in the order of elution in HPLC at various pH values (Fig. 4 in Ref. [4]). This order is reversed for humulone and adhumulone at pH 5.5 and for lupulone and adlupulone at pH 8. At the lower pH, with the acids in the non-dissociated form, the influence of pK_a on retention decreases and hydrophobicity becomes determinant.

If we thus look at the dynamic process, at the start of a CPC run (after injection of the sample, dissolved in stationary phase) the α-acids are partitioned over the two phases according to their respective, individual partition coefficients at pH 8.4. Consequently the pH of the mobile phase decreases and thus the ionization of the weak acids; the acids are then partly replaced in the organic stationary phase. This replacement affects cohumulone less than humulone and adhumulone because of the sequence of the pK_a values: cohumulone<humulone<adhumulone. This self-regulating process is repeated in the next channels of the CPC and results in the resolution of the α-acids: the strongest acid moving fastest. This replacement mechanism differs from pH zone refining and displacement chromatography [8] in that no external displacer is used; it also explains the increase of the retention of humulone and adhumulone in the case where larger amounts of hop extract are injected (see Fig. 3). Injection of 1.0 g in 10 ml resulted in elution of humulone between 250 and 550 ml, with the maximum at 500 ml; when 3.0 g in 10 ml was injected the elution of humulone started at 400 ml, reached a maximum at 1000 ml and ended at 1100 ml. We also injected a sample containing only humulone and adhumulone; humulone elution started at 120 ml as no cohumulone was present to take precedence. In the same way humulone in its turn takes precedence over adhumulone (results not shown).

A skewed peak form can be explained by non-linear isotherms. However, the K_{app} of the α-acids is linear over the concentration range used in our CPC experiments. We measured the K_{app} between pH 7 and 9 at four different concentrations over three orders of magnitude; the highest concentration (127 mM for humulone, 86 mM for cohumulone and 26 mM for adhumulone) matches the injection sample of the CPC run (bottom-right in Fig. 3). From these experiments there was no proof for a non-linear isotherm: the deviations were within the standard error. First efforts to model the separation, using the parameters of the present system, make clear that the separation is pH controlled and that the skewed shape could be a result of the limited availability of non-protonated triethanol amine; the sharp decline at the end of the peaks is a result of the logarithmic relation between K_{app} and pH [9]. In accordance with this the pH profile of the effluent followed the elution profile of the α-acids (Fig. 3, first diagram).

When higher amounts are injected (3 g or more, see Fig. 3 lower part) both cohumulone and humulone give significant tailing; this can be explained by the assumption that in the first channels of the CPC the stationary phase is saturated with the α-acids. At these high concentrations the α-acids probably tend to stick to the wall and detach only at lower concentrations with some delay. At higher concentrations the α-acids probably form complexes with each other, which results in co-elution (lower right part of Fig. 3). These conditions are not useful for the isolation of α-acids of high purity; however, they are acceptable for use as a pre-separation; fractions can subsequently be injected in a second run.

The mechanism of the chromatographic behaviour of the hop bitter acids is still under further investigation.

6. Conclusions

We have shown that measurement of partition coefficients does not always result in a correct prediction of the selectivity of a system. Partition coefficients are determined in a static system and are subsequently used to predict the performance in a dynamic system, in which absolute and relative concentrations of the solutes change along the column, as well as the pH value and probably also the concentration of organic solvent in water and vice versa (the composition of the two-phase system).

The six main bitter acids, cohumulone, humulone, adhumulone, colupulone, lupulone and adlupulone, can be obtained pure from hop carbon dioxide extracts by means of centrifugal partition chromatography. For the humulones the two-phase system is

toluene–0.1 *M* triethanolamine-HCl pH 8.4 in water (descending mode). Subsequently the lupulones can be eluted with 0.2 *M* diethanolamine-PO_4 pH 9.75 in water–methanol (4:1, v/v). When only the lupulones are to be isolated, the first step can be omitted. In the case of a high sample amount an increase of triethanol amine concentration is advised. Thus from 4.0 g hop supercritical carbon dioxide extract 450 mg colupulone, 150 mg lupulone and 50 mg adlupulone (all ≥98%) can be obtained, using 0.4 *M* diethanolamine-PO_4 pH 9.75 in water–methanol (4:1, v/v) as the mobile phase and toluene as the stationary phase.

In the separation mechanism both dissociation and hydrophobicity play a role; at the pH values that exist in the CPC separations, the pK_a value dependent dissociation prevails over the hydrophobicity.

Acknowledgments

The authors wish to thank Mr. L.C. Verhagen (Heineken Brewery, Zoeterwoude, Netherlands) for the kind gift of the hop extracts.

References

[1] P. Anderegg, H. Pfenniger, Cerevinia Biotechnol. 19 (1994) 17.
[2] S.-Y. Fung, J. Brussee, R.A.M. van der Hoeven, W.M.A. Niessen, J.J.C. Scheffer, R. Verpoorte, J. Nat. Prod. 57 (1994) 452.
[3] A.C.J. Hermans-Lokkerbol, R. Verpoorte, J. Chromatogr. A 664 (1994) 45.
[4] A.C.J. Hermans-Lokkerbol, R. Verpoorte, J. Chromatogr. A 669 (1994) 65.
[5] K. Wärtgen, Ph.D. Thesis, Über den Einfluss äusserer Faktoren auf die Stabilität der Hopfenbitter-stoffe, Philipps-Universität Marburg/Lahn Germany, 1990.
[6] J.S. Hough, D.E. Briggs, R. Stevens, T.W. Young, Malting and Brewing science, Vol. II, Chapman and Hall, London, 1982.
[7] A.C.J. Hermans-Lokkerbol, unpublished results.
[8] Y. Ito, in Y. Ito and W.D. Conway (Editors), Chemical Analysis, Vol. 132, High-Speed Countercurrent Chromatography, Wiley, New York, 1996, Ch. 6, pp. 121–175.
[9] M.J. van Buel, J.L. den Hollander, A.C. Hoek, A.C.J. Hermans-Lokkerbol, M.T. Gude, L.A.M. van der Wielen, R. Verpoorte and K.Ch.A.M. Luyben, unpublished results.